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# BROMEACT ARTHRIDOL

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# BROMEACT

ΦΟΡΜΟΥΛΑ

**αντιφλεγμονώδης**

για την αντιμετώπιση
του πόνου

30 κάψουλες των 350mg

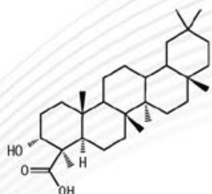
***Bromelain* | *Boswellia* | *Cumarin***

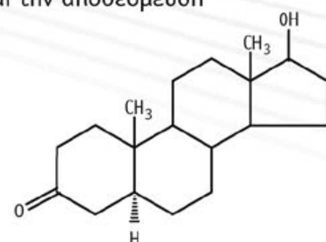
*ενισχύει τα αρθρικά υγρά του σώματος*
*για την καλύτερη λειτουργία των αρθρώσεων*
*και εμποδίζει τη δημιουργία οιδημάτων*

# Αντιφλεγμονώδης δράση

**Bromelain** (Βρομελαΐνη)
- Αυξάνει την παραγωγή προσταγλαδινών με αντιφλεγμονώδη δράση.
- Αναστέλλει την μετατροπή του κινινογόνου σε κινίνη.
- Διεγείρει την PGE 1: Αναστέλλει επιλεκτικά την συσσώρευση αιμοπεταλίων και συνεπώς την πήξη του αίματος.

**Boswellic Acid:** (Πεντακυκλικό Τριτερπένιο)
- Αναστέλλει επιλεκτικά την 5-λιποξυγενάση, εμποδίζοντας τη σύνθεση των:
  1) **Λευκοτριένιο B4**: Υποδοχέας της φάσης χρόνιας φλεγμονής.
  2) **Λευκοτριένια C4, D4, E4**: Υποδοχείς της φάσης οξείας φλεγμονής.
- Δεν αναστέλλει τη σύνθεση γαστροπροστατευτικών προσταγλαδινών όπως συμβαίνει με τα NSAIDS (μη στεροειδή αντιφλεγμονώδη).
- Αναστέλλει τη μετατόπιση των πολυμορφοπύρηνων λευκοκυττάρων και την αποδέσμευση πρωτεολυτικών ενζύμων.

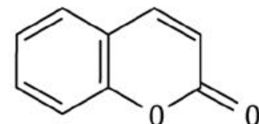



# Αποιδηματική δράση

**Bromelain:** (Βρομελαΐνη)
- Σε αλληλεπίδραση με τη λιποξυγενάση αναπτύσσεται μια ινωδολυτική δράση μέσω του πλασμινογόνου που αυξάνει την δημιουργία πλασμίνης η οποία διασπά θρόμβους ινικής (ινωδόλυση) εμποδίζοντας έτσι τη δημιουργία τοπικού οιδήματος.

**Coumarin:** (Κουμαρίνη)
- Η κουμαρίνη δρα στο λεμφικό σύστημα χάρη στην ικανότητα να αυξάνει την πρωτεολυτική δράση των μακροφάγων.
- Ελαττώνει τον καταβολισμό των κατεχολαμινών (αδρεναλίνη) σε αγγειακό επίπεδο και συνεπώς συντελεί στη βελτίωση της ικανότητας συστολής των αγγείων και τη μείωση του οιδήματος και του πόνου.

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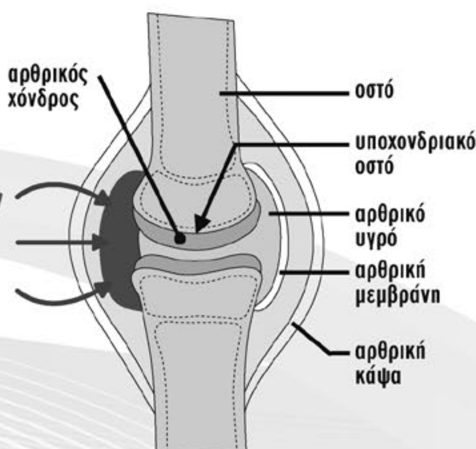


| ΔΙΑΤΡΟΦΙΚΕΣ ΠΛΗΡΟΦΟΡΙΕΣ | ανά 100g | ανά ημερήσια δόση 1 κάψουλα |
|---|---|---|
| Βρομελίνη 2500 GDU από βλαστό του Ananas comosus | 28,57g | 100mg |
| Βοσουέλια (λιβάνι) ξηρό εκχύλισμα | 28,57g | 100mg |
| Ίσο με τα βοσουελικά οξέα | 18,57g | 65mg |
| Αγριοτρίφυλλο ξηρό εκχύλισμα | 14,29g | 50mg |
| Ίσο με την κουμαρίνη | 2,86g | 10mg |

## ΣΥΝΙΣΤΑΤΑΙ ΣΕ ΠΕΡΙΠΤΩΣΕΙΣ
- **Οιδημάτων**
- **Φλεγμονών**
- **Πόνου αρθρώσεων**

## ΔΟΣΟΛΟΓΙΑ
- Μία κάψουλα ημερησίως.

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**CMLS Cellular and Molecular Life Sciences**

## Review

# Bromelain: biochemistry, pharmacology and medical use

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**Abstract.** Bromelain is a crude extract from the pineapple that contains, among other components, various closely related proteinases, demonstrating, in vitro and in vivo, antiedematous, antiinflammatory, antithrombotic and fibrinolytic activities. The active factors involved are biochemically characterized only in part. Due to its efficacy after oral administration, its safety and lack of undesired side effects, bromelain has earned growing acceptance and compliance among patients as a phytotherapeutical drug. A wide range of therapeutic benefits has been claimed for bromelain, such as reversible inhibition of platelet aggregation, angina pectoris, bronchitis, sinusitis, surgical traumas, thrombophlebitis, pyelonephritis and enhanced absorption of drugs, particularly of antibiotics. Biochemical experiments indicate that these pharmacological properties depend on the proteolytic activity only partly, suggesting the presence of nonprotein factors in bromelain. Recent results from preclinical and pharmacological studies recommend bromelain as an orally given drug for complementary tumor therapy: bromelain acts as an immunomodulator by raising the impaired immunocytotoxicity of monocytes against tumor cells from patients and by inducing the production of distinct cytokines such as tumor necrosis factor-$\alpha$, interleukin (Il)-1$\beta$, Il-6, and Il-8. In a recent clinical study with mammary tumor patients, these findings could be partially confirmed. Especially promising are reports on animal experiments claiming an antimetastatic efficacy and inhibition of metastasis-associated platelet aggregation as well as inhibition of growth and invasiveness of tumor cells. Apparently, the antiinvasive activity does not depend on the proteolytic activity. This is also true for bromelain effects on the modulation of immune functions, its potential to eliminate burn debris and to accelerate wound healing. Whether bromelain will gain wide acceptance as a drug that inhibits platelet aggregation, is antimetastatic and facilitates skin debridement, among other indications, will be determined by further clinical trials. The claim that bromelain cannot be effective after oral administration is definitely refuted at this time.

**Key words.** Bromelain; proteinases; antiinflammation; edema prevention and destruction; fibrinolysis; thrombosis and metastasis prophylaxis; immunomodulation; skin debridement; complementary tumor therapy.

## Introduction

### The role of proteolytic enzymes for therapeutic use

Bromelain belongs to a group of proteolytic enzymes which are used as drugs for the oral systemic treatment of inflammatory, blood-coagulation-related and malignant diseases. Apart from the plant cysteine-proteinases bromelain and papain, the group comprises proteinases from animal organs such as trypsin and chymotrypsin. These enzymes offer a wide spectrum of therapeutic efficacies: they demonstrate, in vitro and in vivo, antiedematous, antiinflammatory, antithrombotic and fibrinolytic activities. They modulate the functions of adhesion molecules on blood and endothelial cells, and also regulate and activate various immune cells and their cytokine production. Indeed, these enzymes are used in the United States and Europe as an alternative or complementary

medication to glucocorticoids, nonsteroidal antirheumatics and immunomodulatory agents. Their very low toxicity makes them suitable tools for controlling chronic inflammatory diseases.

For the therapy of inflammatory and malignant disorders, these proteinases are employed as

- additives for chemotherapy (to reduce side effects of drugs and to improve quality of life);
- additives for radiotherapy (to reduce inflammation and edema);
- additives in surgery (to reduce edema and to improve wound healing);
- additives to prevent lymphedema by reducing lympho-congestion, detritus, viscosity of the exsudate and stimulation of phagocytosis of associated leukocytes.

It should be added that clinical studies support these recommended indications only to a limited extent. Yet the large body of preclinical, pharmacological and daily experience offers an important and worthwhile field for well-designed clinical studies in order to evaluate evidence-based medical indications.

## Biochemistry of bromelain

Bromelain is a crude, aqueous extract from the stems and immature fruits of pineapples (*Ananas comosus* Merr., mainly var. Cayenne from the family of bromeliaceae), constituting an unusually complex mixture of different thiol-endopeptidases and other not yet completely characterized components such as phosphatases, glucosidases, peroxidases, cellulases, glycoproteins and carbohydrates, among others [1, 2]. In addition, bromelain contains several proteinase inhibitors [3, 4]. Stem-bromelain (EC. 3.4.22.32) is distinguished from fruit-bromelain (EC. 3.4.22.33), previously called bromelin [2]. Today bromelain is prepared from cooled pineapple juice by centrifugation, ultrafiltration and lyophilization. The process yields a yellowish powder, the enzyme activity of which is determined with different substrates such as casein (FIP units), gelatine (gelatine digestion units) or chromogenic tripeptides [1, 5, 6]. In aqueous solution, bromelain rapidly deteriorates through self-digestion. The addition of serum containing $\alpha_2$-macroglobulin will prevent self-digestion (see below). By high-resolution fast protein liquid chromatography (FPLC) and other biochemical methods, basic (stem bromelain, ananain, comosain) and acidic thiol-proteinases have been isolated from crude bromelain, partially or fully sequenced and characterized in more detail [6–8]. They mainly comprise glycosylated multiple enzyme species of the papain superfamily with different proteolytic activities, molecular masses between 20 and 31 kDa, and isoelectric points between >10 and 4.8. Two major basic proteinases, F4 and F5, were further characterized and showed molecular masses of 24,397 and 24,472 Da, respectively [6] (Table 1). In addition, numerous, different protein fractions were obtained by means of various biochemical methods [SDS-polyacrylamide gel electrophoresis (PAGE), isoelectric focusing (IEF), multicathodal-PAGE]. Among the basic proteinases, one fraction (F9, ananain) reveals the highest specific proteinase activity, is not glycosylated and has a molecular mass of 23,427 Da [6, 8, 9]. The enzymatic activities comprise a wide spectrum with pH optima between 5.5 and 8.0 [10]. The substrate spectrum is similarly broad, extending from synthetic low molecular mass amides and dipeptides up to high molecular substrates such as fibrin, albumin, casein, angiotensin II, bradykinin. Bromelain preferentially cleaves glycyl, alanyl and leucyl bonds.

Commercial bromelain preparations are evaluated according to their proteolytic activity. The platelet aggrega-

Table 1. Cysteine proteinases (bromelains) from pineapples (*Ananas comosus*).

| Name (EC number) accordimg to [2, 8] | Abbreviation according to [6, 7] | Molecular mass (Dalton) | Isoelectric point | Sequences | Glycosylation | Reference |
|---|---|---|---|---|---|---|
| *From pineapple stems:* | | | | | | |
| Stem bromelain (EC 3.4.22.32) | F4 and F5 | 23,800 (sequence + sugar) | > 10 | completely sequenced (212 amino acids) | glycosylated | 6 |
| Ananain (EC 3.4.22.31) | F9 | 23,464 (sequence) | > 10 | completely sequenced (216 amino acids) | not glycosylated | 6, 9 |
| Comosain | F9/b | 24,509 and 23,569 (esms) | > 10 | N-term. sequence | glycosylated | 6, 8 |
| | SBA/a and SBA/b | 23,550 and 23,560 (esms) | 4.8 and 4.9 | N-term. sequence | highly glycosylated | 7 |
| *From pineapple fruits:* | | | | | | |
| Fruit bromelain (EC 3.4.22.33) | | 23,000 | 4,6 | N-term. sequence | not glycosylated | 1 |

tion inhibitory and antiinflammatory action seem to be related to the protease activity. However, other effects such as inhibition of tumor cell growth and metastasis as well as debridement of burns are associated with other nonproteinolytic components contained in bromelain. Thus, the determination of the proteolytic activity alone may not be sufficient to completely characterize the pharmacological properties of bromelain [11].

## Pharmacology of bromelain: preclinical studies

### Pharmacodynamics of bromelain

From a variety of in vitro and animal experiments, mainly with rodents, as well as from clinical observations, based on uncontrolled and controlled studies, the general properties of bromelain may be summarized as follows [11–14] Bromelain

- prevents edema formation and reduces existing edemas
- reduces the blood level of fibrinogen
- supports fibrinolysis
- activates plasmin
- prolongs the prothrombin and partial thromboplastin time (after relatively high doses)
- prevents aggregation of blood platelets
- prevents adhesion of platelets to endothelial cells of blood vessels
- reduces the blood level of plasmakinins
- reduces the level of prostaglandine $E_2$ and of thromboxane $A_2$ in exsudates during acute inflammation
- acts as an antiinflammatory agent
- induces the secretion of interleukin (Il)-1, Il-6, Il-8 and tumor necrosis factor (TNF)-$\alpha$ from blood monocytes and granulocytes
- supports the oxidative burst and the cytotoxicity of granulocytes against tumor cells
- increases the tissue permeability of antibiotic drugs
- prevents metastases in a mouse model
- supports skin debridement of burns

Some effects of bromelain may result from its capacity to alter and modulate distinct cell surface structures by cleaving off peptides [15]. Thus, the bromelain-mediated modification of adhesion molecules on platelets and on other normal and malignant tumor cells may inhibit their aggregation. The dissolution of cell membrane constituents and the effects on components of hemostatic processes may explain antiedemateous and fibrinolytic phenomena.

### Bromelain prevents edema formation and reduces existing edema

Several groups have provided significant evidence for both the edema-protective and edema-reducing efficacy of bromelain in a variety of classical animal experiments [16–20]. Among them, the data by Netti et al. [17] are particularly interesting, since papain, another cysteine proteinase, was ineffective in all experimental models, whereas bromelain induced 41% (carrageenin) and 45% (dextran) inhibition of edema formation. In addition, bromelain showed the strongest edema-protective efficacy of all drugs tested, such as indometacin, acetylsalicylic acid, aescin, oxyphenbutazon, and so on [18]. Moreover, both intraperitoneal (i.p.) and orally applied bromelain proved significantly capable of reducing edema (induced by cotton tissue, carrageenin, croton oil) in various animal models [20]. It was concluded that bromelain increases tissue permeability by fibrinolysis and promotes reabsorption of edema fluid into blood circulation. Uhlig and Seifert [18] compared enteral and i.p. application of bromelain and demonstrated a highly significant edema reduction (by 50%) 12 h after oral application, whereas i.p. administration was effective during the first hours only.

### Bromelain promotes the absorption of antibiotic drugs

It has been known for a number of years that bromelain is capable of enhancing the tissue permeability of penicillins and tetracyclins after oral administration. This increases absorption and leads to an improved diffusion after subcutaneous and intramuscular application of the antibiotics. Higher serum and tissue levels are obtained, and side effects are reduced [21–23].

Among others, Neubauer evaluated the combined bromelain and antibiotic therapy of 53 hospitalized patients with pneumonia, bronchitis, staphylococcus infections, thrombophlebitis, pyelonephritis and rectal abscesses [24]. Twenty-three of the patients had been on antibiotic therapy without success. Twenty-two of these patients responded favorably to the combined treatment of bromelain and antibiotics. In every disease state significant reduction in morbidity was noted as opposed to antibiotics alone. Similarly, Ryan concluded from his double-blind clinical study on acute sinusitis that of the patients receiving bromelain, 83% showed complete resolution of nasal mucosal inflammation versus 52% in the placebo group [25].

### Bromelain affects blood coagulation and fibrinolysis

The data on the edema-protective and -reducing efficacy of bromelain suggest that hemostatic processes are involved, such as prolongation of the prothrombin time, partial thromboplastin time and decrease of the fibrinogen blood level.

In the inflammatory animal models of Pirotta et al. [26], bromelain increased the fibrinolytic activity in a dose-dependent manner. Livio et al. [27] found an increase of prothrombin and partial thromboplastin time as well as a decrease of ADP-induced platelet aggregation. All these effects were clearly dose dependent and related to the

proteolytic activity of bromelain, since inactivation of the enzyme abolished the effects [28].

**Bromelain prevents platelet aggregation**

In 1972 Heinicke et al. [29] observed that oral administration of bromelain to healthy persons, particularly those with high platelet counts, significantly lowers the ADP-induced aggregation of platelets. Morita et al. [28] attempted to isolate and characterize platelet aggregation inhibitory factors from bromelain. At about the same time, other authors reported on fibrinolytic activities of bromelain and experiments to isolate fractions by means of biochemical methods [26, 30].

Aggregation and adhesion of platelets to endothelial cells have recently been studied in more detail [31]. When the platelets were incubated with bromelain prior to activation with thrombin, aggregation was completely prevented. Papain was less effective. Bromelain reduced, in vitro, the adhesion of thrombin-activated, fluorescent-labelled platelets onto bovine aorta endothelial cells. Using an in vivo laser thrombosis model, oral administration of bromelain to rats could significantly decrease the thrombus formation in mesenterial arterioles by 11%, in venols by 6%.

**Bromelain effects on plasminogen, Quick- and partial thromboplastin time**

In vitro, bromelain was able to activate plasminogen to yield plasmin, which is known to cleave fibrin [32]. This property is shared with streptokinase. In addition, bromelain inhibited the thrombin-induced formation of blood plasma fibrin in vitro; the other cysteineproteinase papain was less effective in this respect. In contrast to streptokinase, bromelain was not able to dissolve fibrin aggregates. After oral administration [3000 Fédération International Pharmaceutique (FIP) units daily for 10 days] to healthy persons, no significant influence was determined as to thromboplastin (Quick) time and plasmin formation, yet a moderate increase of the partial thromboplastin time (a PTT) still within normal range [33].

Table 2 summarizes bromelain effects on blood coagulation, fibrinolysis and platelet functions.

**Mechanism of action of bromelain, apparently due to its proteolytic activity**

The antiedematous, antiinflammatory, antithrombotic and fibrinolytic efficacy of bromelain reported so far suggests that several mechanisms of action are involved on different levels: The blood clotting, complement and kinin systems interconnect and mutually influence each other. Generally, they regulate cascade systems of proteinase-mediated reactions.

Besides interactions with plasmakinins, prostaglandins and the fibrinogen/fibrin system the reaction with the plasmaprotein $\alpha_2$-macroglobulin plays a significant role (see below). Like most endoproteinases, bromelain circulates in blood, bound to this high molecular mass proteinase inhibitor [35]. However, binding does not completely inactivate the enzyme; the capacity to hydrolyze small substrates is still preserved [36].

Bromelain is an effective fibrinolytic agent in vitro and in vivo. However, this property is more evident in purified fibrinogen solutions than in plasma, probably due to proteinase inhibitors present in plasma. Despite this limitation, an increase of the fibrinolytic activity was observed

Table 2. Bromelain effects, in vitro (BP, F4, F9) and in vivo (POS), on blood coagulation, fibrinolysis and platelet functions.

| Protease | Parameter | Effect | Signifiance | Ref. |
|---|---|---|---|---|
| Bromelain POS<br>Bromelain F9 | extrinsic blood coagulation<br>(Quick-test) | =<br>= | blood coagulation, dependent on factor II, V<br>VII, X and fibrinogen | 31<br>33 |
| Bromelain POS<br>Bromelain F9 | intrinsic blood coagulation<br>(PTT-Test) | ↓<br>= | blood coagulation, dependent on plasmatic<br>factors except factor VII | 31<br>33 |
| Bromelain POS<br>Bromelain BP<br>Bromelain F9 | activation of plasminogen<br>to plasmin | =<br>↑ | activation causes fibrin degradation<br>(fibrinolysis) | 31<br>33<br>26, 27 |
| Bromelain BP<br>Bromelain F4, F9 | thrombin-dependent fibrin<br>formation | ↓ | reduction of blood clotting | 34 |
| Bromelain BP | thrombin-stimulated platelet<br>aggregation | ↓ | reduction of thrombus formation | 31 |
| Bromelain BP | thrombin-stimulated platelet<br>adhesion onto endothelial cells | ↓ | reduction of thrombus formation | 31 |
| Bromelain BP | in vivo thrombus formation in<br>arterioles and venoles after oral<br>administration | ↓ | reduction of thrombus formation | 31 |

Bromelain BP (base powder), commercial crude extract; POS, BP given orally; F4, F9, bromelain fractions.

after bromelain administration [26]. Besides direct cleavage of fibrin, activation of fibrinolytic factors was discussed [37], e.g. by increasing the plasmin concentration [20]. The fibrinolytic activity of bromelain has been attributed to the enhanced conversion of plasminogen to plasmin, which limits the spread of the coagulation reaction by degrading fibrin [38].

By means of these reactions the vascular permeability may be enhanced and edematous fluid may again be absorbed by tissues. This is in agreemant with clinical findings in that bromelain administration may lead to an increased concentration of concomitantly given antibiotics in body fluids as well as in tissues (see above).

Plasmakinins and prostaglandins play important roles as mediators of pain and vascular phenomena associated with acute inflammation. Animal experiments demonstrated that bromelain lowers the plasmakinin level [39]. Similarly, bromelain injections caused a dose- dependent decrease of bradykinin levels at inflammatory sites and a parallel decrease of the prekallikrein levels in sera [40]. Studies of prostaglandin metabolism during acute inflammation showed that orally administered bromelain reduces the level of both $PGE_2$ and of thromboxane $B_2$ dose-dependently [41].

Nonsteroidal antiinflammatory drugs inhibit the enzyme cyclooxigenase, resulting in a decrease of both pro- and antiinflammatory prostaglandins. In contrast, bromelain may, according to Taussig, selectively inhibit the proinflammatory thromboxane generation and shift the ratio of thromboxane/prostacyclin ($PGI_2$ ) in favor of the antiinflammatory prostacyclin. The mechanism of action of the recently introduced 'superaspirins' and of bromelain were suggested to be identical [11].

## Effects of bromelain on malignant growth

### First observations on the effects of bromelain on cancer patients

Gerard in 1972 [42] and Nieper in 1976 [43] reported on beneficial effects following oral administration of bromelain to cancer patients. After treatments with relatively high doses for several weeks and months, respectively, they noted remarkable remissions of malignant tumors with negligible side effects. However, these reports must be considered to be anectodal by and large.

### Bromelain inhibits tumor cell growth in vitro

Bromelain inhibits the proliferation of different tumor cells in vitro. The inhibitory activity can be traced neither to the proteolytic nor to the peroxidase activity or to the platelet aggregation-inhibitory activity [44]. Later, Garbin et al. [45] as well as Grabowska et al. [46] confirmed the concentration-dependent inhibitory activity of bromelain crude extract and bromelain fractions on various tumor cells in vitro. Maurer et al. [47] found that bromelain may induce differentiation of leukemic cells in vitro and proposed this phenomenon as a possible mechanism of action. Apoptosis of tumor cells may result from induction of differentiation, a process by which many cytostatic drugs may eliminate tumor cells.

### Relationships between risk of thrombosis and risk of metastases

An association between venous thromboembolism (VTE) and cancer has been recognized since at least 1865 [48]. Patients with clinically evident malignant diseases or occult cancer have an increased risk of VTE, and necropsy studies document an increased prevalence of thrombosis among patients with visceral cancer. Conversely, two recent studies have shown that the risk of revealing malignancy one year after a VTE is increased three to four times [49, 50]. We know by now that upon contact with platelets, tumor cells release a variety of factors (growth factors such as platelet-derived growth factor (PDGF), transforming growth factor-$\beta$ (TGF-$\beta$), thrombin, thrombospondin, prostaglandins, cathepsins, among others) that promote platelet aggregate formation. They are also capable of damaging the vascular wall, thereby inducing congestion, which in turn causes coagulation. Platelets may form aggregates with tumor cells that can adhere to the endothelium and stimulate initial processes of metastases [51–55].

Taussig et al. [11] deserve the credit for recognizing the significance of bromelain as an anticoagulant and as a potential antimetastatic drug. In 1988, he and co-workers [56] reported that bromelain fed to C57bl/6 mice would dramatically lower the take of subcutaneous (s.c.)-injected Lewis lung tumor cells, leading to 77–98% reduction, and that this effect was due neither to the proteolytical anticoagulant nor to the peroxidase activity of bromelain. Similarly, Grabowska et al. [46] found that B16F10 mouse melanoma cells, preincubated in vitro with bromelain, significantly reduced lung metastatic tumor weight about three times. However, no survival benefit was seen. Furthermore, bromelain diminished the capacity of these cells to migrate through an extracellular matrix layer in an in vitro invasion assay and inhibited the growth of the tumor cells in a concentration-dependent manner, whereas the antiproliferation effect did not correlate with the proteolytic activity. Finally, human platelets pretreated in vitro with bromelain lost their capacity to stimulate the invasiveness of several metastatic tumor cells in the in vitro invasion assay.

Table 3 summarizes the effects of bromelain on tumor cell growth and metastasis, whereas figure 1 depicts, in more detail, a number of reactions and targets with which bromelain may interfere when it meets interacting plate-

Table 3. Bromelain effects, in vitro (BP, F4, F5, F9) and in vivo (POS), on tumor cell growth and metastasis.

| Protease | Parameter | Effect | Signifiance | Ref. |
|---|---|---|---|---|
| Bromelain BP<br>Bromelain F9 | tumor cell proliferation | ↓ | reduction of tumor cell growth | 44, 45<br>46 |
| Bromelain BP<br>Bromelain F4, F9<br>Bromelain F5 | tumor cell invasion through extracellular matrix | ↓<br>= | reduction of the invasive potential of tumor cells | 46 |
| Bromelain POS | growth of lung metastases in mice | ↓ | reduction of the metastatic potential of tumor cells | 44 |
| Bromelain BP<br>Bromelain F9 | CD44 expression on metastatic cells | ↓ | reduction of tumor cell adhesion to endothelial cells | 46, 57<br>58 |
| Bromelain BP | growth of lung metastasis in mice | ↓ | reduction of the metastatic potential of tumor cells | 46 |
| Bromelain BP | survival time of mice bearing lung metastases (in vitro → in vivo) | = | mouse survival time | 46 |

Bromelain BP (base powder), commercial crude extract; POS, BP given orally; F4, F9, Bromelain fractions.

Figure 1. Young pineapple fruits between leaves suggested to secrete bromelain.

lets, tumor and endothelial cells in blood vessels. Metastasized tumor cells, while migrating through the vessels, carry CD44 adhesion molecules on their surface, by which they adhere to endothelial cells via the ligand hyaluron. Bromelain preferentially cleaves off CD44 molecules by virtue of its proteolytic activity, thus inhibiting one of the first steps of the metastatic process (shown at the upper right).

In addition, metastasized tumor cells carry the receptor (uPAR) for the urokinase plasminogen activator (uPA), which generates plasmin from plasminogen. Plasmin degrades the extracellular matrix (ECM), composed of collagen type IV, laminin and fibronectin. Tumor cells also secrete matrix metalloproteinases (MMPs), enabling the malignant cells to invade through the ECM. Bromelain diminishes uPAR expression and uPA activity, thus inhibiting the invasion step of metastasis (shown at the upper left).

Recently, relevant interactions between tumor cells and platelets were elucidated in more detail. They take place on different levels: intravasal distribution, adhesion on endothelial cells, invasion and extravasation. Platelets directly bind to tumor cells, a process promoted by the release of factors such as platelet factor 4, thrombospondin, thrombin and gelatinase A from platelets, which facilitate thrombus formation.

TGF-$\beta$, produced by both platelets and tumor cells, plays an important role: it induces the synthesis of ECM proteins and stimulates the activity of uPA, MMPs and angiogenesis. Thus, disturbance of the blood coagulation system may lead to the formation of thrombi by aggregating platelets and tumor cells. Bromelain is capable of inhibiting both platelet aggregation in vitro and in vivo, as well as platelet-stimulated invasiveness of tumor cells (shown at the lower endothelium).

### Bromelain modulates the function of cell adhesion molecules

In 1992, Hale and Haynes [15] reported that in vitro treatment of T lymphocytes with bromelain removes distinct surface molecules, thereby enhancing CD2-mediated T cell stimulation. The adhesion molecule CD44 has attracted particular interest since it was recognized as a marker for circulating cells, especially metastasizing tumor cells [59]. Harrach et al. [57] showed that bromelain diminished CD44 expression on Molt 4/8 leukemia and SK-Mel 28 melanoma cells, the effect being most pronounced with bromelain fraction F9, with its high proteolytical activity. Yet the proteinase activity of the bromelain fractions tested did not correlate with the manner in which CD44 expression could be modulated. Bromelain was more effective than chymotrypsin, papain and trypsin. Treatment of human lymphocytes with bromelain F9 reduced the expression of CD44, yet did not affect CD11a (LFA-1) molecules; the adhesion of lymphocytes onto umbilical vein endothelial cells was also lowered [60].
In addition, selective influences of bromelain on the expression of other surface molecules on lymphocytes were observed by Kleef et al. [61]. It is still an open question whether the modulation of CD44 molecules is a prerequisite for the inhibition of metastasis by bromelain. Oral administration of bromelain (3000 FIP units daily for 10 days) did cause a moderate reduction of CD44 expression on the lymphocytes from mammary tumor patients. Conversely, the expression of CD11a and CD62L molecules was weakly increased; CD16 molecules remained unchanged [33].

### Bromelain modulates functions of immune cells

Bromelain and papain stimulate, in vitro, mononuclear blood leukocytes to produce considerable quantities of TNF-$\alpha$, Il-1$\beta$ and Il-6, particularly in monocytes [62, 63]. The production of these cytokines by leukocytes could also be demonstrated after oral administration of a polyenzyme drug containing bromelain [64]. Moreover, granulocytes reacted to the same drug by forming reactive oxygen radicals known to exert antimicrobial and antitumor inhibitory effects [65]. Garbin et al. [45] found that purified bromelain fraction F9 augments, in vitro, at suboptimal concentrations of Il-2, the lymphocyte-mediated inhibition of proliferation of various tumor cells. It remains to be shown in clinical studies whether bromelain can be used for unspecific immunostimulation to treat distinct disorders.
Mynott et al. recently suggested that bromelain may be a novel inhibitor of T cell signal transduction [66]. However, since bromelain contains different biological activities, these findings need confirmation with purified factors.
In a study with 15 healthy donors and 15 mammary tumor patients, orally given bromelain (3000 FIP units daily for 10 days) doubled the capacity of patient's blood monocytes to kill tumor cells in vitro [33]. Among both patients and healthy donors, individual monocyte responses varied to a great extent. Bromelain responders ($>10\%$ increase in immunocytotoxicity) showed a significantly weaker cytotoxicity than those grouped as nonresponders. This weakness could be overcome by bromelain. The effects were reversible. NK- and LAK-cell activities dropped during bromelain administration, but normalized again afterwards.
Table 4 summarizes immune-cell-mediated effects of bromelain against tumor cells.

### Bromelain achieves debridement of burns

Rapid debridement of third-degree burns considerably reduces the morbidity and mortality of severely burned patients. It permits early skin grafting and lessens the problem of sepsis, thus abbreviating the convalescence period. Chemical debridement as opposed to surgical debridement selectively removes only the burned dena-

Table 4. Immune cell mediated bromelain effects, in vitro (by bromelain F9) and in vivo (by bromelain POS), against tumor cells.

| Protease | Parameter | Effect | Significance | Ref. |
|---|---|---|---|---|
| Bromelain F9 | immunocytotoxicity of lymphocytes against tumor cells | ↑ | increase of cellular immune response | 45 |
| Bromelain F9 | secretion of TNF$\alpha$, Il-2 | ↑ | stimulation of lymphocytes | 45 |
| Bromelain POS | immunocytotoxicity of lymphocytes, monocytes from tumor patients against tumor cells in vivo | = ↑ | cellular immune response in vivo | 39 |
| Bromelain POS | secretion of Il-1$\beta$ from human monocytes | ↑ | reduction of tumor cell adhesion to endothelial cells | 33 |
| Bromelain POS | expression of cell surface markers on lymphocytes from tumor patients CD44 | ↓ | regulation of cell adhesion and signal transduction of lymphocytes | 33 |

tured skin. Topical bromelain (35% in a lipid base) has achieved complete debridement on experimental burns in rats in about 2 days, as compared with collagenase, which required about 10 days, with no side effects or damage to adjacent burned tissue [67]. A debridement agent apparently free of proteolytic activity was extracted from commercial bromelain; it was called escharase [68]. Morever, the use of topical bromelain for frostbite eschar removal was investigated: no debridement other than that of the superficial eschar layers was noted; after two topical applications of bromelain, frostbite injuries remained unaffected [69].

## Pharmacokinetics of bromelain

### Is bromelain absorbed following oral application?

This frequently asked question can now be answered in the affirmative. In 1992, Smyth et al. [20] showed that bromelain given orally to rabbits increases the plasmin serum level and prolongs the prothrombin and antithrombin times. Seifert et al. [70] found that up to 40% of $^{125}$J-labelled bromelain is absorbed from the intestine in high molecular form. Later, by means of different methods, a large body of direct and indirect evidence supported the conclusion that bromelain is absorbed from the intestine [36,71]. Similarly, other enzymes, such as kallikrein [72], known to lower arterial blood pressure, and several cytokines such as, IL-2, -5 and -6 [73] reveal pharmacological effects following oral application, thus strongly suggesting intestinal absorption of these proteins. However, one question still remains to be answered: How much bromelain is absorbed and in which form does it circulate in the blood? Proteinases similar to bromelain are rapidly complexed with antiproteinases, mainly with $\alpha_2$-macroglobulin (AMG) and $\alpha_1$-antitrypsin. This fact creates difficulties for the quantitative determination of bromelain in serum. Consequently, the recovery of bromelain considerably varied depending on the analytical method used. In any case, the protective AMG molecule leaves the proteolytical activity of bromelain intact but reduced [74]. Three days after oral administration of 8.6 g of bromelain, Castell [71] determined a mean half-life of 6–9 h and a plasma concentration (AUC) of 2.5–4 ng/ml.

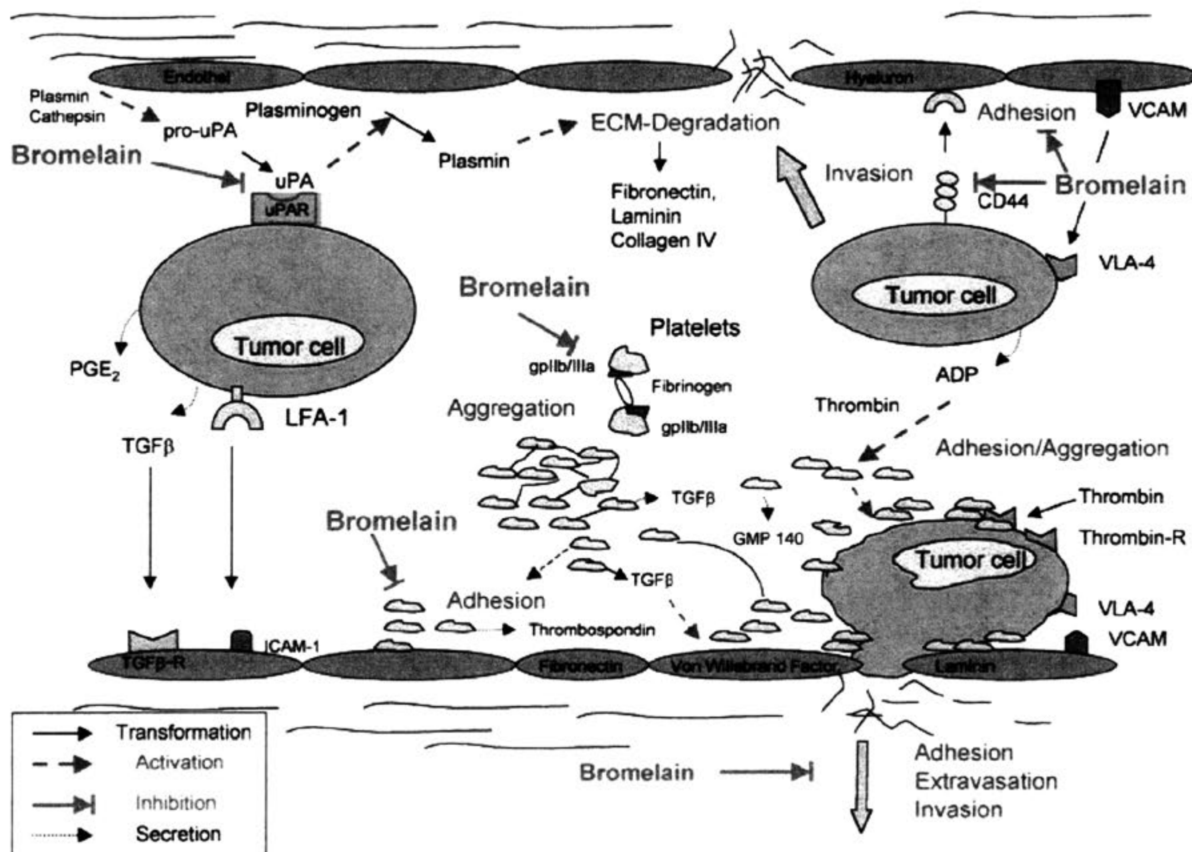


Figure 2. Effects of bromelain on the interactions of platelets, tumor and endothelial cells inside a blood vessel. ECM, extracellular matrix; Il-1$\beta$, interleukin-1$\beta$; PGE$_2$, prostaglandin E$_2$; TGF$\beta$, transforming growth factor $\beta$; TGF$\beta$-R, transforming growth factor $\beta$ receptor; uPA, urokinase plasminogen activator; uPAR, urokinase plasminogen activator receptor; LFA-1, ICAM-1, VCAM, VLA-4 are cell surface adhesion molecules and their corresponding ligands; GMP 140 and Gp IIb/IIIa (fibrinogen receptor) are platelet products.

AMG, the main complexing agent for bromelain in blood, is secreted by macrophages and consists of four identical subunits (or two identical halves) in the so-called slow form [36]. After entrapping the bromelain molecule, the slow form will undergo a conformational change in order to yield the AMG fast form, causing an increased affinity to LDL or other AMG receptors, yet retaining proteolytic activity. The plasma half-life of the AMG slow form is reduced from its original 8 days to 10–30 min in the fast form. The latter can still interact with additional cytokines (Il-1$\beta$, Il-6, IFN$\gamma$, TNF$\alpha$, TGF$\beta$, PDGF etc.) and hormones and is known as activated AMG. The immunogenic determinants of the entrapped enzyme molecules are covered by AMG, which prevents determination using antibodies.

In a remarkable clinical study meeting all requirements of good clinical practice (randomized, double-blind, cross-over-design), the bioavailability of a polyenzyme drug (combining bromelain, trypsin, and rutosid) was examined in 21 healthy males [75–78]. Following oral administration of 400-mg and 800-mg tablets (corresponding to 1.94 and 3.88 × $10^4$ FIP units) four times daily up to 4 days, the specific activities of bromelain and trypsin were determined in plasma. The activities and AUC values proportionally correlated with the respective dosage. In addition, quantitative studies by means of enzyme immunoassays and Western blot analyses confirmed these findings. Moreover, plasma concentrations of trypsin and specific proteinase activities correlated as well. These results support the notion that the enzymes are absorbed from the gastrointestinal tract in a functionally intact form. The relatively high doses were well tolerated, with few side effects such as pasty faeces, flatulence, and fullness. This is consistent with observations in athletes taking as much as 1.2 × $10^4$ FIP units of bromelain daily.

## Toxicology of bromelain

### Acute toxicology

According to Moss et al. [16] no $LD_{50}$ could be determined with oral doses up to 10 g/kg in mice, rats or rabbits. Lethal doses ($LD_{50}$) after i.p. administration: mice 37 mg/kg, rats 85 mg/kg; after intravenous (i.v.) administration: mice 30 mg/ kg, rabbits 20 mg/kg. No immediate toxic reactions were seen. These relatively high doses exceed those normally given to human by far.

### Chronic toxicology

Five hundred milligrams of bromelain per kilogram per day given orally to rats did not provoke any alteration in food intake, growth, histology of the heart, kidney and spleen, or hematological parameters [16]. Normal doses of 3000 FIP units/day given to human over a period of 10 days did not significantly affect blood coagulation parameters [33].

### Drug safety

In 12 placebo-controlled studies, very few side effects were observed; one study noted a 1.8% incidence of diarrhea, nausea, occasional gastric disorders and allergic reactions. One company registered only eight cases of side effects such as exanthema and urticaria out of >3.5 million bromelain tablets (500 FIP units each) sold over 7 years [79]. Bromelain is considered to be nontoxic and without side effects; therefore it can be used without concern in daily doses from 200 up to 2000 mg (500–5000 FIP units) for prolonged periods of time [13]. Bromelain has shown therapeutic benefit in doses as small as 160 mg/day, but the best results occur when starting at a dose of 750 mg/day. It is generally recommended that bromelain be taken at least 1 h before meals. To minimize trauma from sporting activities or tooth extractions, administration should begin 48 h prior to event. Tablets must be coated in such a way that they resist stomach digestion.

## Clinical efficacy of bromelain

Table 5 presents a selection of controlled clinical studies, demonstrating, in human, several of the pharmacological effects reported above from in vitro and animal experiments.

It should be noted that the medical use of drugs containing bromelain and other proteinases such as papain, trypsin and chymotrypsin (to mention the most frequently used) is a matter of dispute: Many medical doctors are still sceptical about findings that proteinases are absorbed from the gastrointestinal tract in a functionally intact form, and consequently deny any efficacy of orally applied enzymes. On the other hand, a remarkable list of clinical studies, conforming to GCP rules and mainly performed with polyenzyme drugs, clearly makes a case for evidence-based pharmacological efficacy of proteinases [85].

## Arguments for the use of bromelain for medical indications

- Bromelain belongs to a group of proteolytical enzymes that have found a wide range of applications for the indications mentioned above.
- Bromelain is a phytotherapeutic drug that can be given orally and reveals few toxic side effects, thus favoring acceptance and compliance by patients.
- Bromelain is orally absorbed and generates various pharmacological systemic effects: prevention and re-

Table 5. Selection of controlled clinical studies with bromelain.

| Diagnosis | Design of study | *n* | Drug, daily dosage | Critical parameters, results, observations | Ref. |
|---|---|---|---|---|---|
| Acute sinusitis | r, db, Pl | V : 23<br>Pl : 25 | 4 × 40 mg Br | inflammation, secretion, breathing, disturbance, pain. V significantly better than Pl | 25 |
| Face and head trauma | db, Pl | V : 20<br>Pl : 21 | 4 × 40 mg Br | edema, ecchymoses; reduction by V highly significant | 19 |
| Trauma of lower extremity | r, b, Cd | V : 18 | 3 × 40 mg Br<br>3 × 1000 mg Cd | pain, edema, hematoma. V significantly better than oxyphenbutazone (Cd) | 80 |
| Posttraumatic inflammation and swelling | r, b, Cd | V : 60<br>Cd : 60 | 3 × 40 mg Br<br>3 × 1000 mg Cd | hematoma, edema, flexibility, pain; equivalence of V and oxyphenbutazone (Cd) | 81 |
| Postoperative tumefactions | r, db, Pl | V : 50<br>Pl : 50 | 3 × 80 mg Br | girth of ball of forefoot, smallest girth of forefoot pain intensity; significant improvement of all parameters by V | 82 |
| Mediolateral episiotomy | r, db, Pl | V : 80<br>Pl : 80 | 4 × 40 mg Br | edema, inflammation, pain; V significantly better than Pl | 83 |
| Oral surgery (teeth extraction) | r, db, cr | 16 | 4 × 40 mg Br | swelling, pain; less inflammation and pain by V | 84 |

*n*, number of patients; r, randomized; db, double-blind; cr, cross-over; Pl, placebo; Cd, control drug; Br, bromelain; V, verum.

duction of edema antiinflammation, stimulation of monocytes to secrete cytokines such as Il-1$\beta$ and TNF-$\alpha$, induction of phagocytosis and cytotoxicity by granulocytes, inhibition of platelet aggregation and stimulation of fibrinolysis, immunomodulatory effects promoting antigen-unspecific tumor cytotoxicity, among others.
- In vitro and in vivo data suggest that bromelain may act as a prophylactic drug to prevent metastases. However, clinical data to support this suggestion are still lacking.
- There are satisfactory prescriptions for quality control and production of a standardized pharmaceutical drug.

These arguments may recommend bromelain as a suitable model substance for further scientific evaluation of the class of proteinases to which bromelain belongs. The reversible platelet aggregation inhibitory property of bromelain may attract interest in cardiovascular surgery. Bromelain's potential for debridement of skin burns may be beneficial for early skin grafting. For applications in oncology, further preclinical and well-designed clinical studies are undoubtedly required. Bromelain effects on lymphedema in mammary tumor patients, and the prophylaxis of metastases and antiangiogenic phenomena with respect to tumor growth should be worthwhile subjects for further, more detailed investigations.

## Finally: Why do pineapple plants produce and need bromelain?

The significance of bromelain proteinases for pineapples has been a mystery for a long time. The most compelling hypothesis is based on the well-known fact that carnivorous plants derive their supply of nitrogen and phosphorus from degradation of organic material (foliage, insects, microbes) by means of highly active proteinases and other digesting enzymes. In the tropical jungle, the pineapple plant is an epiphytic bromeliad, growing on other plants which offer hardly any nutrients. The rosette-like arrangement of the pineapple plant's leaves forms funnel-type rainwater reservoirs, so-called phytotelmata, that are always filled with water, as well as with nitrogen and phosphorus suppliers. This hypothesis is supported by recent findings that leaves react to mechanical stimuli of only 2 s by producing proteinkinases [86]. Moreover, the carnivorous pitcher plant Sarracenia purpurea was shown to respond to various chemical signals (nucleic acids, proteins, ammonia) by secreting hydrolytic enzymes [87]. In order to digest as many proteins from insects and microorganisms as possible, enzyme 'families' with a broad spectrum of pH optima, such as the 'papain superfamily', have evolved.


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# BROMELAIN, THE ENZYME COMPLEX OF PINEAPPLE (*ANANAS COMOSUS*) AND ITS CLINICAL APPLICATION. AN UPDATE

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## Summary

After a short description of the uses of pineapple as folk medicine by the natives of the tropics, the more important new pharmaceutical applications of bromelain, reported between 1975 and 1978, are presented. Although the exact chemical structure of all active components of bromelain is not fully determined, this substance has shown distinct pharmacological promise. Its properties include: (1) interference with growth of malignant cells; (2) inhibition of platelet aggregation; (3) fibrinolytic activity; (4) anti-inflammatory action; (5) skin debridement properties. These biological functions of bromelain, a non-toxic compound, have therapeutic values in modulating: (a) tumor growth; (b) blood coagulation; (c) inflammatory changes; (d) debridement of third degree burns; (e) enhancement of absorption of drugs. The mechanism of action of bromelain affecting these varied biological effects relates in part to its modulation of the arachidonate cascade.

## Introduction

Bromelain obtained from the stem of the pineapple plant (*Ananas comosus* Merr.) contains all soluble components of the pineapple stem in their original, unaltered form. It has diverse biological properties which may involve malignant cell growth, circulation and inflammation among others (Cohen, 1964). It is non-toxic and compatible with most drugs (Renzini, 1972; Tinozzi, 1978).

The history of bromelain is linked to that of the pineapple. Pineapple was found by Columbus in 1493 in Gouadeloupe. It was used as a folk medicine to cure different ailments by the aboriginal inhabitants of Central and South America, uses reported by explorers in the 17th century, who visited the New World after its discovery by Columbus.

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Thévet (1558) writes: "the fruit of which the natives of America make the greatest medicinal use is called nana" (ananas, pineapple). Rochefort (1605) reported that the juice of pineapple "admirably recreates and exhilarates the Spirits and comfort the Heart; it also fortifies the Stomack, cureth queasiness and causeth Appetite; it gives present ease to such as are troubled with the Stone or stoppage of Urine; 'nay it destroys the force of Poyson. If the Fruit is not procurable the root will have the same Effects". This information had no practical significance at that time, since pineapple could not be shipped without spoiling. Even later, after introduction of canning, the pharmaceutically active ingredients in pineapple were destroyed in the canning process.

The first uses of pineapple by the natives is now becoming justified by the realization of an important ingredient of the pineapple, namely: bromelain.

Peckold et al. (1876) found in the Brazilian pineapple a proteolytic enzyme and Chittenden (1891) isolated bromelain from its juice. Not until Heinecke (1957) found that the stem of the pineapple contains more bromelain than the expensive fruit, was it produced on a commercial scale.

Pineapple is widely grown today around the globe in tropical and subtropical regions. As a pharmaceutical product, bromelain was first manufactured in 1956 in Hawaii. Later, small amounts were produced temporarily in Puerto Rico and Brazil. Today, the main bromelain producer is Taiwan. Since the mid 1950s when bromelain first became commercially available, at least 800 scientific papers appeared on its chemistry, pharmacology, industrial and clinical applications. The last reviews on bromelain were published in the mid 1970s by Dupaigne (1975), Taussig (1975) and Cooreman (1976). In the past 12 years, several important clinical applications on bromelain have been published.

The purpose of the present paper is to highlight some relevant contributions regarding bromelain's therapeutic applications which have not been reported in previous reviews. The main topics will include: cancer, circulation, inflammation and third-degree burns.

## Cancer

Gérard (1972) was the first to use oral bromelain by itself or combined with conventional chemotherapeutic agents on cancer patients. He treated 12 patients with different tumours with 600 mg of oral bromelain daily from 6 months to several years and reported resolution of cancerous masses of ovarian carcinoma, a marked decrease of most breast cancers and metastases. However, the bromelain effect in this small number of patients is only suggestive and not statistically significant. Nieper (1974) uses high doses of oral bromelain with eventually subtoxic doses of chemotherapeutic agents such as 5-FU and vincristine. Over a period of over 4 years, Nieper (1976) reported tumor regressions. Like Gérard, he found that not all

bromelain preparations had the same effect; that doses less than 1000 mg of active bromelain per day are inadequate and suggested the use of up to 2.4 g per day. Nieper attributed the beneficial action of bromelain to its "deshielding" of the tumor cell's fibrin coat by fribrinolysis. The immune system may now have a more ready access to the tumor cells. The fibrinolytic activity of bromelain has been further detailed by Ako (1981). The reports of Dvorak et al. (1979, 1981) of a fibrin gel forming around many tumors, acting in part as a physical, immunological barrier, support Nieper's deshielding hypothesis. The possibility of fibrin enhancing angiogenesis of solid tumors was also considered. Metastasis has also been linked to the increased fibrin coagulation and platelet activity in tumors. Anticoagulant and/or antiplatelet therpapy has therefore been considered, and various findings have been reported (Marx, 1982; Honn and Marnett, 1984; O'Donnell, 1985; Rubio, 1986; Tsung-Hsien, 1983).

Prostaglandins have been implicated in several biological processes. These include platelet aggregation, coagulability, normal hemostasis and cancer development and progression (Karmali, 1983; Bockman, 1983; Honn et al., 1984; Honn et al., 1981; Bennett, 1981). The antagonistic effects of prostaglandins (thromboxane A2 (TxA2) and prostacyclin (PGi2)) having both positive and negative effects on platelet aggregation and c-AMP, related to the properties of bromelain (Honn et al., 1982). Since bromelain can affect generation of various prostaglandins and influence tumor growth its use may be feasible. Bromelain with its fibrinolytic and antiplatelet aggregation activities may thus be a relevant compound to consider interfering with the fibrin and coagulation features of tumor cells. It may also increase the host's iummune cancer defenses and decrease metastasis by enhancing prostacyclin levels. In general, with its increased levels of prostaglandins, the tumor growth may be altered by changed prostacyclin/thromboxane ratio. Better prognosis relates to increased prostacyclin tumor tissue levels and vice versa (Honn-Dunn et al., 1982; Honn-Cicone et al., 1981; Fantone et al., 1982). The varying results noted using anticoagulants and anti-inflammatory drugs as addition agents in cancer therapy may be due to their effect on the arachidonate cascade. However, those compounds do not have a selective action on prostaglandins. Bromelain also acts on the prostaglandin cycle but does so in a selective manner. It decreases thromboxane and changes the ratio thromboxane/prostacyclin in favor of the later and implicitly increasing an important cell growth modulating compound, cyclic-3,5-adenosine monphosphate (c-AMP). The reports of Gérard, Nieper, and others were based on clinical observations without proper controls and could be considered as anecdotal evidence at best. During the past 10 years a number of controlled studies have been carried out in vitro and in vivo with the purpose of testing the validity of the empirical observations of clinicians. The studies were carried out either on cancer cells grown in cell culture or on animals. All studies show a close correlation between bromelain dose and retardation of tumor growth.

It should be noted, however, that bromelain is more effective in the early stages of tumor initiation and development (Taussig and Goldstein 1976).

*Studies in cell culture*

Taussig et al. (1985) studied three cell lines in vitro: Lewis lung carcinoma, YC-lymphoma and MCA-1 ascitic tumor. Bromelain causes an up to 90% dose-dependent growth retardation of all three tumors. It was equally effective when its proteolytic activity was destroyed by heating at 70 °C for 30 min, which suggests that the protease in bromelain is not the critical factor responsible for its anticancer effect. Oishi et al. (1985) found that the human gastric carcinoma Kato III exposed either to bromelain alone or to bromelain combined with chemotherapeutic agents (5-FU, mitomycin-C, adriamycin or cis-platinum) decreased cell growth by 80%. DNA studies on Kato III indicates that there appears to be a significant DNA-perturbation by bromelain alone and enhanced perturbation when bromelain was used in combination with chemotherapeutic drugs.

*Controlled animal studies*

Two long term studies were carried out with hairless mice. The animals were irradiated with ultraviolet light and the development of skin cancer studied. Goldstein et al. (1975) found that bromelain-feeding enhanced the resistance of mice to the harmful effect of UV irradiation. It took twice as long for the bromelain fed group to develop precancerous lesions as compared to the control group. The tests were carried for one whole year and the animals were irradiated during the entire period. Taussig et al. (1976) repeated the test with identical results. In another study with C57BL/6 mice (Batkin et al., 1986), feeding bromelain to Lewis lung carcinoma bearing mice, prevented development of lung metastases to a significant degree; feeding mice with 0.3% bromelain containing lab chow decreased the number of metastic lesions by over 90%. Thus the clinical observations of Nieper and Gérard have been partly confirmed and were probably not placebo effects. The use of bromelain as a non-toxic adjuvant in cancer therapy should now definitely be considered.

## Circulation

This is the most recently discovered use of bromelain and might also be of clinical importance. A major cause of deaths in western industrial nations is due to heart attack and stroke. The first conclusive evidence that bromelain prevents aggregation of blood platelets was reported by Heinecke et al. (1972). Bromelain inhibits dose-dependently ADP-induced platelet aggregation. Heinecke's tests were carried out with human volunteers with a history of heart attack or stroke or with people having high aggregation

values, as well as with healthy subjects. Bromelain (160—1000 mg per day) administered orally decreased aggregation of blood platelets in all the subjects. In their search for the factor responsible for this effect, Morita et al. (1979) separated bromelain into three fractions. They concluded that the platelet aggregation inhibitory effect is connected with the proteolytic activity and suggest that this action of bromelain may involve the prostaglandin synthetic pathway. Nieper (1978) treated 14 angina pectoris patients with oral bromelain. All responded to the treatment; the symptoms resolved while under treatment reappeared after bromelain administration was discontinued. Oral doses were 1000—1400 mg bromelain per day for several months. The possibility of a placebo effect still, however, remains to be considered. Gutfreund et al. (1987) studied the effect of bromelain on blood pressure and heart rate of hypertensive patients. They studied 19 patients of both sexes between 33 and 73 years of age (average age 56) having blood pressure between 215/105 and 140/95 (ave. 180/104). They administered one dose per day ranging from 230 mg bromelain (in form of Anavit F3, a commercial product containing 230 mg bromelain/capsule) up to 8 times the normal dose. The purpose of the test was to investigate the effect of large doses of bromelain. At normally recommended dose of 1—2 capsules no change in blood pressure or heart rate could be detected. Increasing the dose up to 8 times the normal dose, the heart rate of all patients increased proportionately with up to 80% its original value. The blood pressure remained, however, practically unchanged. The authors hypothesized that the failure of the tachycardia to be accompanied by hypertension in attributed to the balanced effects of increased stroke volume and decreased peripheral resistance due to the effect of bromelain to modulate the biosynthesis of prostaglandins (see Mechanism of Action). This hypothesis was confirmed by Carlson et al. (1969), who found that infusion of prostaglandin E1 (PGE1) into healthy volunteers increased both heart rate and stroke volume with minimal change in arterial or venous blood pressure still within the normal range. Other evidence for the correctness of the hypothesis was determined by O'Grady et al. (1980), who, by infusing only prostacyclin (PGI2) intravenously (another prostaglandin) produced inhibition of platelet aggregation, tachycardia and dose-related hypotension. It is interesting to note, that the concentration-dependent increase of heart rate by prostacyclin and orally administered bromelain as described by Gutfreund et al. (1978) are identical.

Several studies published in the late 1970s on the effect of bromelain on circulation, attribute its usefulness to activation of human plasminogen and subsequent increased fibrinolytic activity of the serum via prostaglandin modulation. Pirotta et al. (1978) found that oral bromelain besides its potent anti-inflammatory activity, strongly stimulated serum fibrinolytic activity in Sprague-Dawley rats. De Giuli-Morghen et al. (1978) attributes the increase of serum fibrinolytic activity to activation of plasminogen by bromelain. Miyatani et al. (1975a) found that bromelain lyses fibrin the same way as

plasmin and converts plasminogen to plasmin as urokinase. Likewise, Livio et al. (1978), found bromelain to be an effective fibrinogenolytic agent both in vitro and in vivo, impairing coagulation of blood in rats. They suggest that the fibrinogen digestion products are responsible for this effect. Bromelain also activates Hageman Factor (Factor XII) and plasma prekallikrein. By intravenous injection of bromelain into rats it produced hypotension (Oh-Ishi et al., 1979).

Another important effect of bromelain has been reported by Chen (1975) who studied total blood cholesterol in rabbit serum. He reported that cholesterol is released from the protein binding by bromelain, which can act on the plaque itself or on the lipoprotein in the serum prior to being deposited on the arterial wall. Chen carried out experiments both in vitro and in vivo. He postulates this result due to direct protelolysis. However, more recent reports suggest that the process might be more complex. Hajjar et al. (1982) found that PGI2 increases acid cholesteryl ester hydrolytic activity four times.

Summarizing these findings, administration of oral bromelain might reduce the danger of clot-related problems such as heart attack, or stroke by: (1) decreasing prostaglandin E2 (PGE2) and/or thromboxane (TxA2), thus increasing the relative concentration of prostacyclin (PGI2) so that clot formation is prevented; (2) hydrolyzing the cholesterol plaque it cleans the arterial walls and minimizes the probability of a blood vessel being obstructed by a circulating clot.

**Inflammation**

Bromelain has been used as an anti-inflammatory agent since the time it became available as a drug. Its various applications have been described in a previous review (Taussig et al., 1975). These include rheumatoid arthritis, thrombophlebitis, hematomas, oral inflammations, diabetic ulcers, rectal and perirectal inflammations, athletic injuries, general, oral and plastic surgery. In all cases oral bromelain caused a significant reduction of swelling and pain, the duration of healing being reduced to half the time needed by conventional treatment. Most papers published in the past 10 years on these subjects are controlled studies and deal with the mechanism of action of bromelain as an anti-inflammatory agent.

Uhlig (1981) compared the anti-inflammatory effect of several commonly used drugs on experimentally induced edemas in rats. He found that bromelain was the most potent among all nine tested drugs, including aspirin. Vellini et al. (1986) found a direct relationship between bromelain dose and inhibition of biosynthesis of prostaglandin E2 in rats. They also found that reduction of inflammation and platelet aggregation are related. They obtained the same anti-inflammatory effect with the corticosteroid, prednisone, but at 10 times higher dose, an important advantage of bromelain, considering the severe side effects of steroids.

Other studies on previously reported uses were published: Barbarino et al. (1982) and Seiffert et al. (1979) describe the effect of bromelain on digestion, Rimoldi et al. (1978), Takahashi (1978) and Tinozzi et al. (1978) indicate that bromelain enhances absorption of antibiotics.

### Debridement of third-degree burns

This is the only known topical use of bromelain. Significant advances have been made in this field in the past 10 years. The importance of chemical debridement as opposed to surgical debridement is that only the burned, denatured skin is selectively removed and grafting can be performed as early as 24 h after the accident.

Klaue et al. (1979a) using 35% bromelain in a lipid base achieved complete debridement on experimental burns on rats with no side effects or damage to adjacent unburned tissue. Concomitant use of local antimicrobial agents did not interfere with the action of bromelain. They also (Klaue et al., 1979b) studied the effect of bromelain concentration on the dynamics of burn debridement: complete debridement has been achieved with a 35% suspension in 1.9 days, as compared to 10.6 days, if collagenase was used instead of bromelain. Klein et al. (1977, 1980) patented a debridement agent extracted from commercial bromelain. Houck et al. (1983) investigated this substance. It has a molecular weight of 45,000, is a potent debriding agent and is free of proteolytic activity. The authors call it "escharase" and claim that some bromelains contain considerably less escharase than others. Its mechanism of action is not known, but could be attributed at least partly to prostaglandins (Lord et al., 1980).

Rapid debridement of third-degree burns would considerably reduce the morbidity and mortality of severely burned patients. It permits early skin grafting and lessens the problem of sepsis reducing the convalescence period considerably. Levenson et al. (1974) combined bromelain treatment with chemotherapeutic agents, without either interfering with the action of the other.

### Mechanism of bromelain action

Since bromelain affects such a wide variety of disease it is obvious that its mechanism of action cannot be explained by one mechanism only. Although no single compound responsible for all its pharmacological effects has been identified, it is probable that bromelain contains more than one active factor. The platelet aggregation inhibition and the anit-inflammatory effect are likely linked to its proteolytic activity (Morita et al., 1979; Pirotta et al., 1978) however, its anticancer effect as well as burn debridement are definitely independent of the proteolytic activity. The pharmacological actions of bromelain are due probably, at least partly, to its property to selectively modulate the biosynthesis of two groups of prostaglandins with

opposite actions and ultimately to the modulation of c-AMP. In order to facilitate the description of the mechanism of action, those prostaglandins which stimulate inflammation, platelet aggregation and vasoconstriction, will be referred to as "pro-inflammatory PGs". Such as prostaglandin E2 (PGE2), and thromboxane A2 (TxA2). Those prostaglandins with opposite effects, such as prostaglandin E1 (PGE1) or prostacyclin (PGI2) will be referred to as "anti-inflammatory PGs". Originally it was suspected that bromelain might act similarly like aspirin (Taussig et al., 1975). Vane (1974) confirmed that although bromelain, like aspirin is a PGE2-inhibitor, its action is much weaker than that of aspirin. This might explain why, although bromelain prevents platelet aggregation, it does not promote bleeding. Ako (1981) further strengthened this view; he found that bromelain is a fibrinolysis enzyme activator, Felton (1980) and Taussig (1980) indicated that bromelain activates human plasminogen. Further, Storms et al. (1982) found that bromelain-feeding produces an increase in the level of PGI2 in Sprague – Dawley rats and a tendency towards decrease in pro-inflammatory PGs. Vellini et al. (1986) studying the anti-inflammatory action of bromelain found a decrease of prostaglandin levels in the exudate and concluded that the effect of bromelain might be due to its interference with the arachidonate cascade. Miyatani et al. (1975, 1977) states that bromelain is a potent plasminogen activator.

The mechanism of action of bromelain might be the following: as a plasminogen activator it stimulates production of the serum protease plasmin. Plasmin splits off small active peptides from fibrin, which are inhibitors of pro-inflammatory PGs. By decreasing the concentration of the pro-inflammatory prostaglandins in the serum, the ratio between the pro- and anti-inflammatory PGs is changed in favor of the anti-inflammatory PGs. Being in a higher relative concentration, they attach to the PG receptors on the cell membrane (McDonald et al., 1974) and activate adenyl cyclase which stimulates production of c-AMP in the cell. The interaction between adenyl cyclase and c-AMP is amply documented, as is the effect of prostaglandins on cancer (Jaffe 1977) and their regulatory role on the immune response (Goodwin et al., 1980). Pro-inflammatory PGs decrease, while anti-inflammatory PGs increase the c-AMP level responsible partly for the control of cell proliferation. Cancer patients have an increased level of pro-inflammatory PGs and a proportionate decrease of c-AMP and a depressed cell-mediated immunity (Hokama et al., 1981).

It has been suggested recently that coagulation may be general host response which contributes to the pathology of heart disease, cancer and inflammation. Coagulation is not only a necessary defense to prevent blood loss following injury, but activation of the clotting system is part of the immune response. Recent studies show that the clotting pathways may also be involved in the pathology of inflammation and deposition of fibrin around cancer cells (Dvorak, 1981) shielding them from being recognized by the immune system (Nieper, 1976). Taussig (1984) shows the relevance of

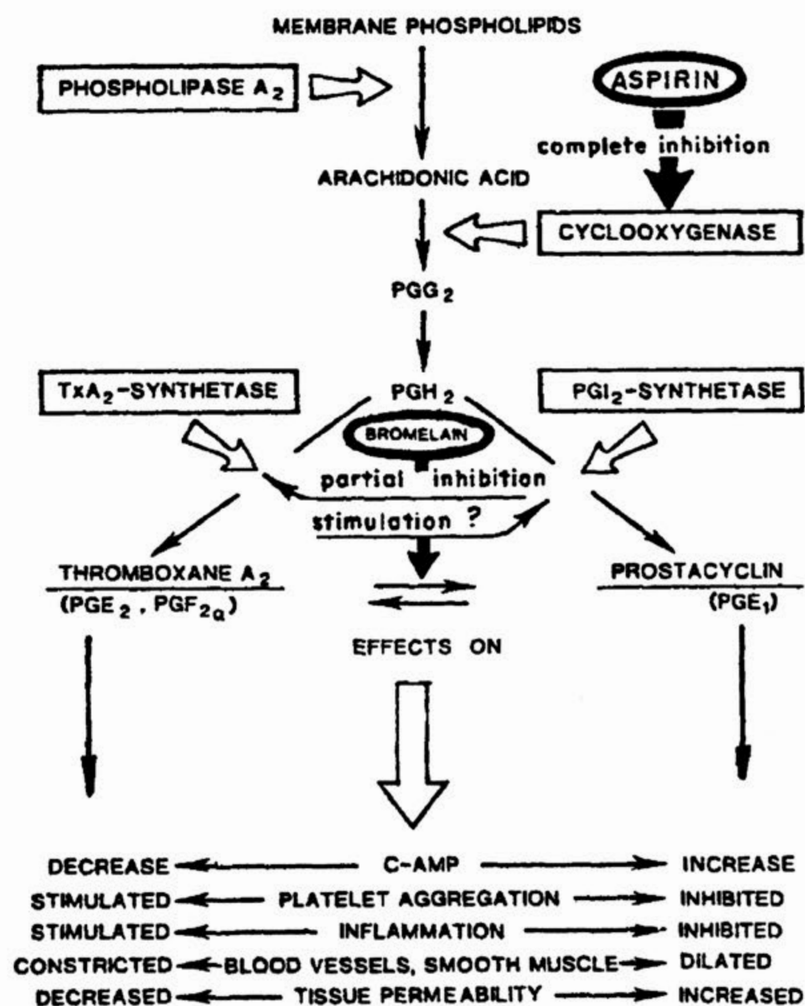


Fig. 1. Biosynthesis of prostaglandins and their effects (Taussig, 1984). The sites where aspirin and bromelain interfere in the arachidonate cascade are indicated.

anticoagulant properties of bromelain to its antimetastatic effect. Figure 1 shows how aspirin and bromelain act on different levels of the arachidonic acid cascade. While the apsirin-type non-steroidal anti-inflammatory drugs act on cyclooxygenase and thus prevent formation of both the pro- and anti-inflammatory prostaglandins, bromelain selectively inhibits only the biosynthesis of pro-inflammatory prostaglandins. Thus, the ratio between the two types of prostaglandins with opposite effects is shifted in favor of the beneficial anti-inflammatory PGs and all their effects shown in Fig. 1. Although this hypothesis as a whole is based partly on circumstantial evidence, each step in the sequence is well documented and included in the references.

## Conclusions

Bromelain, being a plant extract, contains a number of active ingredients

whose ratio to each other might vary according to soil composition, climatic conditions during plant growth, variety of pineapple and also the manufacturing process. These factors might contribute to the variations of bromelain's pharmacological activities.

Commerical bromelains are evaluated according to their proteolytic activity assayed on gelatin, casein, or other protein substrate. The platelet aggregation inhibition and anti-inflammatory action seem to be related to the proteolytic activity; however, inhibition of cancer cell growth and metastasis, as well as the debridement of burns are not linked to the proteolytic activity of bromelain (Batkin, 1987; Gerard, 1972; Houck, 1983). Thus, the proteolytic activity alone is not sufficient to characterize bromelain. Other factors, such as its fibrinolytic activity and increase of c-AMP may also be considered as important anti-cancer properties.

With the varied actions of bromelain, a common denominator of its actions on tumor cell growth, circulation and vascular problems and inflammatory responses may be its modulation of coagulation, platelet aggregation inhibition via fibrinolysis, proteolysis, increased c-AMP production and decreasing tumor angiogenesis. A complete chemical study of bromelain may now be of importance to fully assess its pharmacological value.

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# Placebo-controlled Randomized Clinical Trial on the Immunomodulating Activities of Low- and High-Dose Bromelain after Oral Administration – New Evidence on the Antiinflammatory Mode of Action of Bromelain

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**Bromelain has been used for treatment of inflammatory diseases for decades. However, the exact mechanism of action remains poorly understood. While *in vitro* investigations have shown conflicting effects on the release of various cytokines, no *in vivo* data were available. In this study, the effects on inflammation-related cytokines of two doses of bromelain were tested in a single dose placebo-controlled 3 × crossover randomized clinical trial. Cytokine circadian profiles were used to investigate the effects of bromelain on the human immune system by using stimulated whole-blood leukocytes. The effects seen in these cultures demonstrated a significant shift in the circadian profiles of the Th1 cell mediator interferon gamma (IFNγ; $p < 0.043$) after bromelain 3000 FIP (Fédération Internationale Pharmaceutique) units, and trends in those of the Th2-type cytokine IL-5 as well as the immunosuppressive cytokine interleukin (IL)-10. This suggests a general effect on the antigen-specific (T cell) compartment of the human immune system. This is the first time that bromelain has been shown to modulate the cellular responses of lymphocyte after oral use. It is postulated that the immunomodulating effect of bromelain observed in this trial is part of its known antiinflammatory activities. Further investigations will be necessary to verify the relevance of these findings to a diseased immune system.** **Copyright © 2012 John Wiley & Sons, Ltd.**

*Keywords:* bromelain; cytokine; human; inflammation; immune system; clinical trial.

## INTRODUCTION

The use of pineapple bromelain in human medicine has been known for a long time (Taussig and Batkin, 1988). One of the major indications for this enzyme is inflammatory diseases (Secor *et al.*, 2005; Brien *et al.*, 2006), including those of autoimmune origin (Targoni *et al.*, 1999; Roep *et al.*, 2002). Currently, bromelain is used for the treatment of postoperative states, sports injuries and acute inflammation (Yuan *et al.*, 2006). It has been suggested that these effects may be the result of this protease acting either on soluble proteins (such as α-2-macroglobulin; Lauer *et al.*, 2001) or on proteins at the cell surface level (Mynott *et al.*, 1999; Engwerda *et al.*, 2001; Barth *et al.*, 2005). *In vitro* investigations using isolated lipopolysaccharide (LPS)-stimulated peripheral blood mononuclear cells (PBMC) from healthy volunteers and patients revealed conflicting results after treatment of the cells with bromelain: *in vitro*, Barth *et al.* (2005) found an increase in interleukin (IL)-6, TNFα and interferon gamma (INFγ), while IL-4 and IL-5 were not induced. Rose *et al.* (2005) also report a dose-dependent induction of IL-6 production, while there was no effect on the production of IFNγ and IL-10. On the other hand the investigations by Huang *et al.* (2008) revealed a reduced TNFα, IL-1 beta and IL-6 production. However, only little reliable information is available on the effects bromelain triggers *in vivo* in humans, especially after oral ingestion. Therefore, the aim of the study presented was to investigate the effects of bromelain on immune cell activities after oral ingestion by healthy volunteers.

The following cytokines and chemokines relevant to judge inflammatory processes were selected as relevant endpoints. Interleukin-2 and IFNγ both pertain to the Th1-type of immune response (Schroder *et al.*, 2004), whereas IL-5 and IL-13 mark the Th2-driven reactions (O'Byrne, 2006). These two T helper cell types are seen mutual counter-regulators (Santoni *et al.*, 2008). Under the conditions chosen in these experiments, IL-6 and IL-10 are produced mainly by the cells of the native immune system (as a product of zymosan stimulation)

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and also can be seen as mediators that have opposite effects in the activation of immune cells [IL-6 being mostly of pro-inflammatory activity (Kishimoto, 2006), while one of the major functions of IL-10 is its inhibitory effect on cytokine synthesis (Mosser and Zhang, 2008)]. Interleukin-8 and monocyte chemotactic protein (MCP)-1 belong to the chemokines and can be seen as the most prominent representatives of both the major subclasses, the α- (or CXC) and the β-chemokines (CC) (Viola and Luster, 2008).

## METHODS

**Study design and volunteers.** The trial was performed as a monocentric, partially placebo-controlled, double-blind, randomized, 3 × cross-over study at the Center for Pharmacology and Toxicology, Medical Faculty, University of Rostock. It was performed according to the German Drug Law (Arzneimittelgesetz), the GCP guideline CPMP/ICH/135/95, as well as the guideline 2001/20/EG, and in compliance with the Declaration of Helsinki (EudraCT-Nr 2005-002302-28). After approval of the study by the ethics committee of the University of Rostock, 15 healthy male, non-smoking volunteers with an age of 18–50 yr and a body mass index $> 17.5\,kg/m^2$ to $< 30\,mg/m^2$ were included in the study. Volunteers were determined to be healthy on the basis of medical history, physical examination, routine clinical chemistry laboratory (including negative virus screen on hepatitis B, C and HIV and a negative drug screening) and electrocardiogram. Volunteers were excluded from participation if they had known allergy to pineapple ingredients, used any drugs either presently or up to 4 weeks prior to study start, had an acute infection either presently or up to 2 weeks before the start of the study, had undergone surgery or had serious injuries up to 12 weeks before the study, had a vaccination within 4 weeks before the start of the study or had any chronic or inflammatory disease. Furthermore any self-medication with drugs or food supplements, excessive exercise, intake of pineapple or papaya (fruit and juice) and alcohol were prohibited throughout the study period.

The study period consisted of three identical 1-week periods in which the study medication was provided in a randomized manner. Randomization was performed in two steps: in a first step, 12 instances of treatment orders for the different treatments were created. In the next step these 12 instances were randomized, resulting in a randomization list for 12 cases. Three identical lists and medication sets were prepared to replace any drop-outs correspondingly, if necessary. The trial preparations (in the following scheme called 'X1, X2, …') were applied orally once a week with 1 week of wash-out in between. The order of intake of the trial preparations bromelain 3000 FIP (Fédération Internationale Pharmaceutique) units, bromelain 1000 FIP units and placebo were determined by a randomization scheme. TruCulture® (TC) blood sampling was done according to the scheme in Table 1.

On the day of trial drug intake the first blood sample was drawn before breakfast, for each blood sample a new puncture was performed. No venous catheter was inserted and no blood sample was drawn through a venous catheter. Blood donors who missed more than one time point or developed an infection were excluded from this evaluation.

**Trial medication.** The trial drug medication was manufactured at Ursapharm Arzneimittel GmbH (Saarbrücken, Germany). These were placebo tablets (lot No. 004035), as well as two different concentrations of bromelain tablets: 500 FIP units (lot No. 018035) or 1000 FIP units (lot No. 005025): those containing 500 FIP units were the commercial product Bromelain-POS®, as marketed by Ursapharm Arzneimittel GmbH, the medication containing the higher dose, as well as the placebo tablets, were produced separately as trial medication. The placebo tablets were identical to the bromelain tablets containing 1000 FIP units with regard to appearance and composition of the excipients, but did not contain bromelain.

The daily dose of 1000 FIP units was achieved by applying two tablets of the marketed Bromelain-POS® (open label). For the dose of 3000 FIP units 3 tablets with 1000 FIP units were given. Three tablets of placebo were given to achieve blinding of trial participants, physicians involved in the study and staff assessing the primary outcome of the study with regard to the therapies bromelain 3000 FIP units and placebo.

**TruCulture® blood collection and whole-blood culture system.** The TruCulture® blood collection and operation of the whole-blood culture device followed the manufacturer's instruction manual (EDI GmbH). In brief, the TruCulture® syringe-tubes, containing a proprietary nutrient solution together with a combination of stimulants, were sent to the trial site at −40°C on dry ice and stored at −20°C at the trial site until use. The blood leukocytes in this trial were stimulated by (i) zymosan, a β-glucan from yeast (Sigma-Aldrich, Deisenhofen, Germany), used at a final concentration of 300 µg/mL; (ii) *Staphylococcus* enterotoxin B (SEB, from the Bernhard Nocht Institute, Hamburg, Germany; final concentration 200 ng/mL); as well as (iii) anti-CD28 antibodies (Beckmann-Coulter, Frankfurt, Germany, final concentration 1 µg/mL). This combination of stimuli was used to activate simultaneously the phagocytes (granulocytes and monocytes) by zymosan, as well as the T-cells by SEB plus anti-CD28, giving a very physiological pattern of activation. The TruCulture® syringe front end is a screw cap into which a male-type connector with a rubber septum is inserted.

**Table 1. Drug application and sampling scheme**

| Sampling time | Mon | Tue | Wed | Thu | Fri | Sat | Sun | Mon | Tue | Wed | etc. |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Drug | | X1 | | | | | | | X2 | | |
| 8:00 | TC | TC | TC | – | – | – | – | TC | TC | TC | |
| 10:00 | | TC | | | | | | | TC | | |
| 11:00 | | TC | | | | | | | TC | | |
| 13:00 | | TC | | | | | | | TC | | |
| 17:00 | | TC | | | | | | | TC | | |
| 19:00 | | TC | | | | | | | TC | | |

TC, TrueCulture blood sampling.
X1: drug 1.
X2: drug 2.

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Together with a special butterfly needle system (21G) attached to a female-type connector that punctures this rubber septum during the blood draw (Sarstedt, Nümbrecht, Germany), this ensures a closed state of these syringe-tubes, before as well as after the blood draw, thereby preventing the contamination of the cultures.

After filling the TruCulture® tubes with blood they were immediately transferred into a desktop dry-block incubator (VLM Vogler, Bielefeld, Germany), where they were incubated for 24 h at 37°C. At the end of this incubation the supernatants were separated by insertion of a valve separator and the tubes were frozen at −20°C until measuring the cytokine contents.

**Mediator quantification.** Cytokine and chemokine concentrations in the supernatants were determined by sandwich-type enzyme-linked immune-sorbent assays (ELISAs). These were performed strictly according to the procedures suggested by the manufacturer using the matched antibody pairs for IL-2, IL-5, IL-6, IL-8, IL-10, IL-13, IFNγ and MCP-1, but also the cytokine standards from R&D Systems (Wiesbaden, Germany).

**Statistical methods.** Only few data about circadian rhythms of the cytokines have been published (Petrovsky *et al.*, 1998) and there are no data on the effects of bromelain or its variability. The sample size of $n = 12$ was therefore a matter of agreement in this study that addressed completely new questions. The statistical analysis was performed at SCIRM, Fürth, and Ohm-University of Applied Sciences, Nuremberg.

**Statistical analysis.** A thorough exploratory analysis of the data revealed that the considerable variability in the data of each cytokine was due to extreme differences between individual subjects (up to a factor of 70), while the within-subject variability was very small and caused mainly by the circadian rhythm (publication in preparation). Thus it was decided to $z$-transform the cytokine data individually for each subject for each cytokine. The resulting data were then adjusted to zero at 8 am of the medication day. For the analysis, a linear mixed model with 'group' and 'time' as fixed effects and an interaction term 'group*time' was used to compare the effects of the medication and the changes during daytime and to identify significant changes over the day. All $p$ values given below are Bonferroni-adjusted for multiple comparisons.

## RESULTS

Twelve volunteers with a mean age of $27.1 \pm 4.3$ yr, a mean height of $183.9 \pm 5.8$ cm, a mean weight of $77.3 \pm 10.3$ kg and a mean body mass index of $22.8 \pm 2.8\,\text{kg/m}^2$ finished the study and were available for evaluation. Three volunteers were withdrawn from the study because of acute respiratory infection during the study period. For all cytokines investigated in the study, significant changes in the circadian profile have been established, with IFN-γ, IL-8 and IL-13 showing a profound diurnal increase ($p < 0.001$, $p = 0.008$ and $p < 0.001$, respectively), and IL-10 as well as MCP-1 display a diurnal decrease ($p < 0.001$ for both). Interleukin-6 has a morning and an afternoon peak ($p = 0.001$) and IL-5 a steady increase towards the evening hours ($p < 0.001$) (Schmolz *et al.*, submitted).

### Interleukins 2, 6, 8 and 13 and MCP-1

The release of the cytokines IL-2, IL-6 and IL-13, as well as the two chemokines IL-8 and MCP-1, tested in this clinical trial did not show statistically significant drug effects in this trial (data not shown).

### Interleukin-10 diurnal profile modulation after bromelain 3000 FIP

The daily profile of the release of IL-10 after treatment with bromelain 3000 FIP units showed a statistically significant difference to bromelain 1000 FIP units ($p = 0.028$) and a borderline significance versus placebo ($p = 0.069$) (Fig. 1). After placebo and Bromelain 1000 FIP units, IL-10 decreased during morning hours and returned to baseline in the evening, but after Bromelain 3000 FIP units this decrease in the morning hours was only marginal. Thus Bromelain 3000 FIP units prolonged IL-10 production as compared with both other treatments. For the rest of the day, all groups showed similar courses.

### Diurnal profile modulation of IFNγ after bromelain

For IFNγ the placebo group demonstrated a peak in cell activity around noon, with a slow decrease towards the evening hours until evening (see Fig. 2). Both bromelain treatments did exhibit differences compared with placebo, but significance was found only with the higher dose ($p = 0.043$), which was apparently caused by a faster rise in cell activity in the morning and an increased secretion upon stimulation towards the

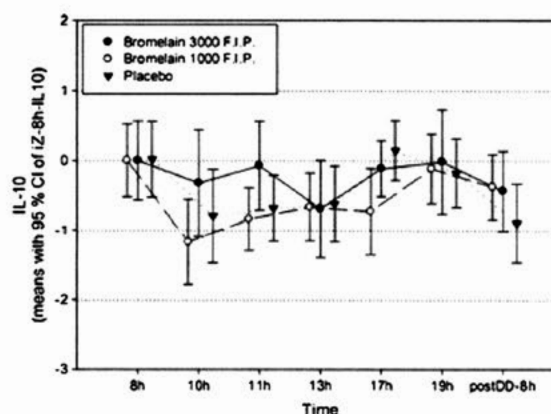


**Figure 1.** Means with 95% confidence intervals in the circadian profile of leukocyte activity of $n = 12$ healthy volunteers regarding the experimentally stimulated release of IL-10 for placebo, bromelain 1000 and 3000 FIP units. After bromelain 3000 FIP units, the levels of IL-10 are statistically significantly elevated compared with bromelain 1000 FIP ($p = 0.028$), but not to placebo ($p = 0.069$) due to the inhibition of the decrease between 10 am and 11 am (all $p$ values are Bonferroni adjusted).

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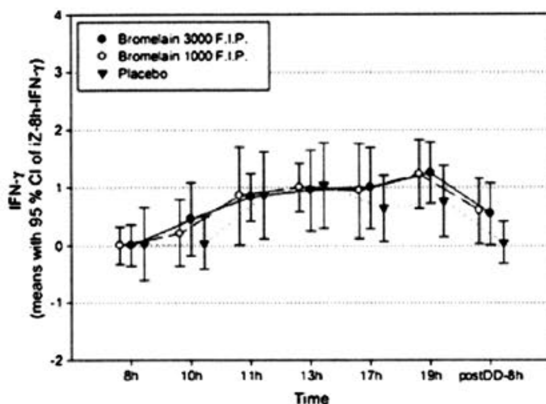


**Figure 2.** Means with 95% confidence intervals in the circadian profile of leukocyte activity of $n = 12$ healthy volunteers; test of the experimentally stimulated release of IFN-γ for placebo, bromelain 1000 and 3000 FIP units. After bromelain 3000 FIP units, levels of IFN-γ were increased, especially in the afternoon hours ($p = 0.043$; Bonferroni adjusted).

evening. This latter activity seems to be prolonged even until the next morning.

### Possible IL-5 diurnal profile modulation after bromelain 3000 FIP units

Bromelain at 3000 FIP units also seems to affect the production upon stimulation of IL-5, also a T-cell cytokine, although produced by Th2 lymphocytes. However, due to the high variation significance was not established ($p = 0.146$). The reason why this increase in IL-5 secretion did not lead to a significant result seems to be the particularly large data scatter in the placebo group at time point 11 hours (see Fig. 3).

### Tolerability of the study medication

The study medication was well tolerated. During the study no serious adverse events and no adverse drug effects related to study medication were observed. The three volunteers that were withdrawn from the study developed an acute respiratory infection, which was an exclusion criterion because it would have affected the cytokine release. The respiratory infection episodes were not regarded as being related to the medication.

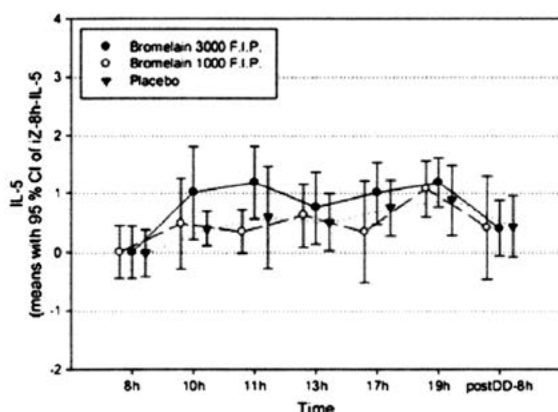


**Figure 3.** Means with 95% confidence intervals in the circadian profile of leukocyte activity of $n = 12$ healthy volunteers regarding the experimentally stimulated release of IL-5 for placebo, 1000 and 3000 FIP units bromelain, respectively. The differences between bromelain 3000 FIP units and placebo were statistically not significant ($p = 0.146$).

## DISCUSSION

Bromelain is a crude extract from the pineapple plant that contains, in addition to low molecular weight compounds and other proteins, various closely related cysteine proteases. It has been used medicinally for decades in the treatment of various conditions, such as injuries, postoperative states, sinusitis and inflammatory diseases (Maurer, 2001; Braun *et al.*, 2005; Inchingolo *et al.*, 2010; Yuan *et al.*, 2006). The treatment of inflammatory diseases especially remains an important research area because the available therapeutic drugs are often not sufficiently efficient or side effects are intolerable, all the more in cases of long-time therapy (Trelle *et al.*, 2011). Therefore this study aimed, for the first time, to characterize the effects of orally taken bromelain on immunomodulation with regard to the cytokine secreting capacities of peripheral blood leukocytes.

One of the largest obstacles in the characterization of pharmacological effects on cells of the human immune system is the well-known interindividual variability in leukocyte activities (Yaqoob *et al.*, 1999; Wurfel *et al.*, 2005). By means of the highly standardized test system we used (TruCulture®), the variability of the cell cultivation procedure also could be reduced to a minimum (Schmolz *et al.*, submitted). An additional source of increasing the data scatter of investigations especially looking at short-term kinetics (over 24 h) is the circadian changes in the reactivity of the cells of the human immune system. There are only a few reports on such rhythmic changes in immune cell functions so far (Petrovsky *et al.*, 1998). We controlled this factor by tight measurement intervals during the day and comparing the same individuals after application of the two study drugs and placebo in randomized order. Finally, we analysed the remaining variability, which was still considerable. It turned out that the intraindividual levels were of remarkable stability over the whole study period of 4 weeks for each volunteer, and that the interindividual variability is obviously a consequence of the fact that low, middle and high 'producers' exist, who differed by up to a factor of 70 depending on the cytokine analysed.

Based on this finding of individual corridors of cytokine production levels within each subject, *z*-standardization of each individual's cytokine data was the chosen means of transforming the original data into a dataset with a mean of zero and a standard deviation of 1 in order to make them comparable. Additionally, the data were adjusted to have their mean at 8 am. Thus, the circadian profile is expressed in terms of deviation from the 8 am value.

Results obtained within this study showed that all cytokines exhibited significant diurnal changes with $p$-values $< 0.02$ (Schmolz *et al.*, submitted). Although there are investigations on circadian rhythms regarding leukocyte activities, it is difficult to compare their data

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to ours, as they were using different cell culture systems and/or other activators (Petrovsky and Harrison, 1998). However, when investigating effects of drugs on cytokine production, circadian changes have to be taken into account; this is one of the important general results of the study.

The goal of the current trial was to describe any type of systemic effect that can be related to the administration of bromelain on the cells of the human immune system, irrespective of the underlying mechanism. The trial revealed a significant modulation in the release of IFN$\gamma$, seen as an increase in the afternoon hours ($p < 0.043$ vs. placebo) after bromelain 3000 FIP units. For IL-10 the group effect was significant ($p = 0.019$), but the Bonferroni-adjusted $p$ value versus placebo was not significant ($p = 0.069$). With respect to IL-5 ($p = 0.146$), the profiles obviously differed between bromelain 3000 FIP units and the other groups in the morning hours, but significance was not established ($p = 0.07$ for the group effect and $p = 0.146$ vs. placebo). Larger group sizes are necessary to find out if this was a random or a systematic difference.

Only at the higher dose of bromelain (3000 FIP units) were significant changes of the diurnal profiles seen; they seem to affect both types of T-helper cells, Th1 (IFN$\gamma$), as well as Th2 (IL-5). This suggests a general effect of this enzyme preparation on T lymphocytes, regardless of the different subpopulations of these cells existing in the human body. The third mediator, IL-10, is rather known for its suppressive effects on cytokine synthesis (Mosser and Zhang, 2008) and – according to the type of leukocyte activation that was chosen in these cultures – can be considered to be secreted by monocytes rather than by Th2 lymphocytes. As this effect was most pronounced in the morning hours (while that on IFN$\gamma$ had an additional late component in the evening hours), one might assume that at least at its onset the bromelain-mediated modulation of immune cell activities touches a broad spectrum of leukocyte subtypes, while the prolonged effect might affect mainly the T cells.

The effects observed during bromelain treatment in this trial were detected in cultures that involved a physiologic pro-inflammatory activation of immune cells, very similar to what occurs during acute or chronic inflammation *in vivo*. It cannot be speculated how these effects of bromelain would modulate the course of any type of inflammatory disease, at least because the treatment would usually involve repeated application of the medication. Despite that, two major findings can be derived from these results:

(1) Even after a single oral dose of bromelain significant changes in the response of physiologically stimulated immune cells were found. This demonstrates that the enzyme is able either to reach the blood circulation in an intact, biologically active form [as was suggested earlier by others (Castell, 1996; Desser *et al.*, 2001)], or it acts indirectly, e.g. on the class of protease-activated receptors (PARs), which was shown for bromelain recently (Reddy and Lerner, 2010; Borrelli *et al.*, 2011), thereby possibly stimulating intestinal epithelial cells that, in turn, could then change the activities of close-by cells of the mucosal immune system of the gut. In the latter case, one further step in this reaction cascade would have to be postulated, which could be a recirculation of these cells, or a local secretion of systemically active mediators that consequently modulate the activities of leukocytes in the blood circulation (giving then rise to the changes seen in the whole-blood cultures of this clinical trial).

(2) The fact that all of the effects reaching significant $p$ levels or showing clear trends were observed after applying 3000 FIP units can be regarded as an indicator of the concentration-dependency of these bromelain activities.

## CONCLUSIONS

The immunomodulating effect of bromelain 3000 FIP units demonstrated in this study is an initial step towards an understanding of this natural compound, as it could be demonstrated that effects on the immune system are present after a single application. Further studies should analyse repeated applications and effects in inflammatory conditions to close the gap between *in vitro* results and empirical use.

In a remarkable set of animal experiments, Baez and coworkers (Baez *et al.*, 2007) have demonstrated, *in vivo*, convincing antitumor effects of the substance. It is a serious and valuable task to elaborate the mechanisms behind these results in order to lay the grounds of their transfer to human cancer treatment and other immune system related diseases, where bromelain has a more or less empirical role as an adjunct therapy.

Taken together, the benefits of the medicinal product bromelain are – and might become even more – a valuable constituent of a modern (supportive) treatment of inflammatory diseases. The results of these experiments support the idea of bromelain having a 'multiple-target activity profile', similar to what is claimed for several other herbal preparations. This is postulated to have advantages over the inhibition of single targets, especially in the treatment of complex diseases, such as chronic inflammation. Further investigations will be necessary to evaluate the pharmacodynamics of repeated bromelain application and the spectrum of effects under disease situations.

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### Conflict of Interest

The authors have declared that there is no conflict of interest.

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ORIGINAL RESEARCH

# Bromelain Limits Airway Inflammation in an Ovalbumin-induced Murine Model of Established Asthma

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**ABSTRACT**

**Context** • Allergic asthma continues to increase despite new pharmacological advances for both acute treatment and chronic-disease management. Asthma is a multifactorial disease process with genetic, allergic, infectious, environmental, and dietary origins. Researchers are investigating the benefits of lifestyle changes and alternative asthma treatments, including the ability of bromelain to inhibit inflammation. Bromelain is a commonly used, proteolytically active pineapple extract.

**Objective** • The present study intended to determine the ability of bromelain to reduce the inflammation of preexisting asthma via an ovalbumin (OVA)-induced murine model of allergic airway disease (AAD).

**Design** • The research team designed a study examining the effects of bromelain in a control group of mice that received phosphate buffered saline (PBS) only and in an intervention group that received bromelain in PBS.

**Setting** • The study took place in the Department of Immunology at the University of Connecticut's School of Medicine, Farmington.

**Intervention** • The research team sensitized female C57BL/6J mice with intraperitoneal OVA/alum and then challenged them with OVA aerosolization for 10 consecutive days. On day 4, the team began administering daily doses of PBS to the control group (n = 10) and bromelain (6mg/kg) in PBS to the bromelain (intervention) group (n = 10).

**Outcome Measures** • The primary measures included bronchoalveolar lavage (BAL) cellular differential, cellular phenotype via flow cytometry, and lung histology. Additional outcomes included testing for serum cytokines and immunoglobulin.

**Results** • Bromelain treatment of AAD mice (bromelain group) resulted in significant anti-inflammatory activity as indicated by reduced BAL total leukocytes ($P < .05$), eosinophils ($P < .05$), and cellular infiltrates via lung pathology ($P < .005$), as compared to the control group. In addition, bromelain significantly reduced BAL $CD4^+$ and $CD8^+$ T cells without affecting cell numbers in the spleen or hilar lymph node. The study found decreased interleukins IL-4, IL-12, IL-17, as well as IFN-α in the serum of bromelain-treated animals.

**Conclusions** • The results suggest that bromelain has a therapeutic effect in established AAD, which may translate into an effective adjunctive therapy in patients with similar conditions, such as allergic asthma, who have chosen to initiate treatment after the onset of symptoms. (*Altern Ther Health Med.* 2012;18(5):9-17.)

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Allergic asthma continues to increase despite new pharmacological advances for both acute treatment and chronic-disease management. The keystones of asthma therapy include (1) avoidance of triggers, (2) relief of symptoms with inhaled β-adrenergic agonists, and (3) control of airway inflammation with inhaled and oral corticosteroids, inhaled chromones, and oral leukotriene antagonists.

Asthma is a multifactorial disease process with genetic, allergic, infectious, environmental, and dietary origins.[1] Researchers are investigating the benefits of lifestyle changes and alternative asthma treatments.[2] They seek evidenced-based treatments that might become safe and efficacious therapies adjunctive to the current treatment formulary.



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According to the 2007 National Health Interview Survey, roughly 40% of adults in the US population use some form of complementary or alternative medicine (CAM).[3] Several studies have examined the use of CAM by patients with asthma, with use estimates ranging from 4% to 79% in adults and from 33% to 89% in children.[4-7] Among the most commonly used CAMs are breathing techniques, herbal products, homeopathy, and acupuncture.[4,8] Nevertheless, researchers have not found strong evidence for the effectiveness of these modalities.[9] Lack of evidence has been attributed to clinical trials with small sample sizes, poor methodology, and a paucity of mechanistic data generated in well-characterized preclinical models.[10,11]

Researchers have established several murine models of asthma or allergic airway disease with the aim of understanding the immunological mechanisms that underlie this complex disease.[12-15] These models share several features that include (1) a sensitization phase with an adjuvant (alum) and a model antigen ovalbumin (OVA), (2) an antigen challenge phase via tracheal instillation or nose-only aerosolization, and (3) the generation of lung inflammation characterized by infiltration of white blood cells (eosinophils), lung pathology, and inflammatory Th2-skewed cytokines (interleukins IL-4, IL-5, IL-3).[12-14,16,17]

Modeling allows for investigation of the effectiveness of numerous, novel, antiasthma therapeutics that have a rich history of traditional use, such as propolis[18]; *Camellia sinensis*[19]; Chinese-patent botanical formulas[20]; *Boswellia serrata* (frankincense), *Glycyrrhiza glabra* (licorice), and *Curcuma longa* (turmeric)[21]; *Mimosa pudica*[22]; and *Ananas comosus* (common pineapple).[12-14] A critical distinction exists, however, between the models and the actual use of the therapies (ie, the schedule at which practitioners deliver the therapy). This schedule includes the periods before or during sensitization, after sensitization, before the acute antigen challenge, or after the development of inflammatory disease.

Several investigators have been interested in the anti-inflammatory mechanisms of specific extracts of *A comosus*, such as the commonly used, proteolytically active pineapple-derived bromelain. Researchers are evaluating the extracts in a variety of both in vivo and in vitro models. Bromelain is a combination of sulfur-containing cysteine endopeptidases that have broad specificity for the cleavage of proteins. Some researchers have proposed one mechanism of action to account for bromelain's therapeutic activity: the cleavage of lymphocyte-cell surface receptors, such as CD4, CD8, CD44, and CD62L. Receptor cleavage can result in altered cell communication, cell trafficking, and cell signaling pathways, leading to the modulation of pro- and anti-inflammatory cytokines such as IL-2, IL-4, IL-6, and TNF-α[23-27]. This modulation makes it a unique choice for modulating allergic asthma.

Researchers have shown that bromelain has potential beneficial effects in treating infections, cancer, cardiovascular disease, musculoskeletal injuries, and arthritis.[28] The research team for the current study has shown previously that bromelain, injected intraperitoneally and administered orally, can reduce airway inflammation in a mouse model of asthma,[29,30] at least in part through enzymatic cleavage of CD25 from activated T cells.[31] The current team notes, however, that its previous studies addressed the ability of bromelain to attenuate the development of asthma when given to sensitized mice prior to and during exposure to an aerosol antigen. While this model may have merit for preventing the development of asthma in previously sensitized allergic individuals, it did not assess whether bromelain could modulate existing asthma when practitioners apply treatment after onset. The outcomes for a trial studying the second circumstance hold more relevance in a clinical setting, and as the goal of its present study, the research team has chosen to examine that second circumstance.

## METHODS

### Animals

The research team purchased female C57BL/6J mice, 3 to 6 months of age with weights from 17 g to 20 g (Jackson Laboratory, Bar Harbor, Maine), and housed them conventionally in plastic cages with corncob bedding. The team maintained the mouse room at 22°C to 24°C with a daily light/dark cycle (light from 0600 to 1800 hours). The study supplied food and water *ad libitum*. The Animal Care Committee at the University of Connecticut Health Center approved all protocols for mouse use.

### Interventions

**Ovalbumin Sensitization and Aerosol Exposure Protocol.** The research team immunized mice with three weekly intraperitoneal injections of a suspension containing 25 μg of OVA (grade V; Sigma Chemical, St Louis, Missouri) and 2 mg of aluminum hydroxide (alum) in 0.5 mL of 0.9% sodium chloride (pH 5.5, 308 mOsmol/L; Baxter Healthcare, Deerfield, Illinois). One week after the last injection, the research team exposed the mice to aerosolized 1% OVA in normal saline, 1 hour per day for 10 days.[29,30] A BANG nebulizer (CH Technologies, Westwood, New Jersey) generated the aerosols into a 7.6-L inhalation-exposure chamber with attached restraint tubes. The mass-median aerodynamic diameter and geometric standard deviations were 1.4 μm and 1.6 μm, respectively. The estimated daily inhaled OVA dose approximated 30 μg to 40 μg per mouse.

**Control and Bromelain Treatment.** The research team made a stock solution of stem bromelain, Lot no. 2890 (Vital Nutrients, Middletown, Connecticut), using 60 mg of bromelain dissolved in 250 mL of phosphate buffered saline (PBS). For the bromelain group (n = 10), the research team administered 6 mg/kg of bromelain in 0.5 mL of PBS. The control group (n = 10) received 0.5 mL of PBS only. The team delivered the treatments intraperitoneally twice daily for 7 consecutive days, beginning on day 4 of the OVA aerosol challenge (Figure 1). The research team optimized the bromelain dose based on previous in vivo dose-response studies that its laboratory had performed.[29,30] As previously report-



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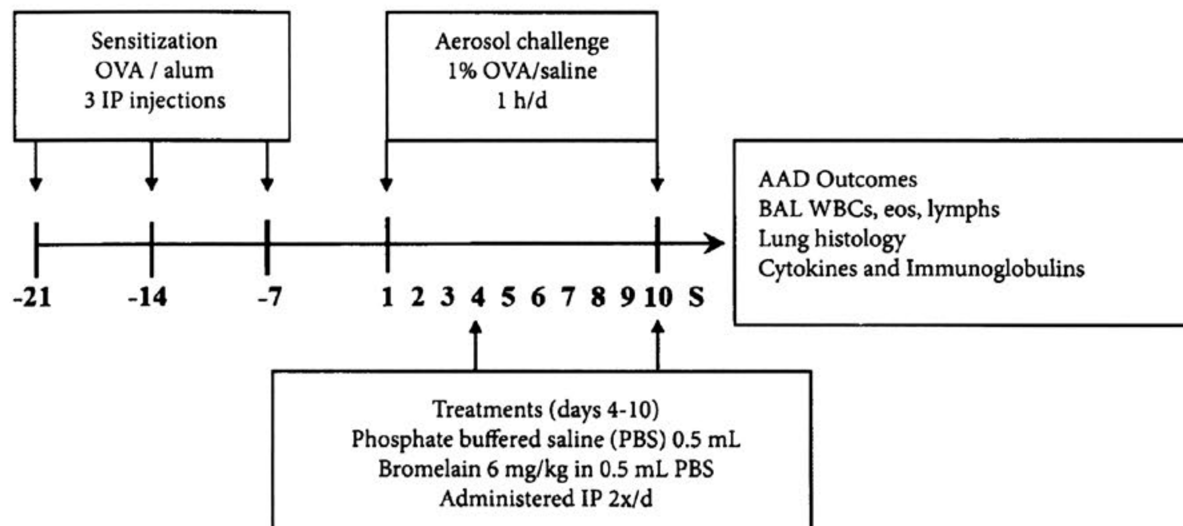
**Figure 1.** Bromelain Treatment Protocol
The research team sensitized mice (n = 20) with three ovalbumin-alum intraperitoneal injections 1 week apart (day −21, day −14, day −7). From days 1 to 10, animals underwent aerosol challenge with 1% OVA in saline for 1 hour daily. From days 4 to 10, animals received treatment with either bromelain (6 mg/kg in 0.5 mL phosphate buffered saline [PBS]) or PBS only (0.5 mL) twice daily. The research team sacrificed the animals 24 h after the last treatment and harvested bronchoalveolar lavage, lung tissue, hilar nodes, and spleen from each mouse to assess the outcomes of allergic airway disease.




**Abbreviations:** AAD, allergic airway disease; BAL, bronchoalveolar lavage; eo, eosinophil; IP, intraperitoneal injections; lymph, lymphocyte; OVA, ovalbumin; PBS, phosphate buffered saline; S, sacrificed; WBC, white blood cell (leukocyte).

ed, the research team had tested bromelain independently for authenticity, potency (2400-2660 GDU/g), microbial contamination, residual solvents, heavy metals, aflatoxins, and endotoxin (Vital Nutrients, Middletown, Connecticut; ChromaDex, Clearwater, Florida; and Pharmline, Florida, New York).[31] Twenty-four hours after the final aerosol exposure and the final bromelain treatment, the research team sacrificed the mice for tissue analysis.

## OUTCOME MEASURES

### Bronchoalveolar Lavage and Tissue Processing

At sacrifice, the research team harvested bronchoalveolar lavage (BAL) fluid, hilar lymph nodes (HLN), and spleens and processed them for the isolation and enumeration of leukocytes. For collection of BAL, the team lavaged the lungs *in situ*, with five 1.0-mL aliquots of sterile saline. The team determined the total protein concentrations in BAL fluid using the Pierce Bicinchoninic Acid Protein Assay Kit (Thermo Scientific Pierce, Rockford, Illinois) with OVA as the standard. The team harvested the lymph nodes and spleens and mechanically disrupted them into single-cell suspensions, using lysis of splenic erythrocytes Tris ammonium chloride (TAC) lysis buffer (9 parts 0.83% w/v $NH_4Cl$; 1 part 2.57% w/v Tris, pH 7.0; TAC solution). For all tissue samples, the team obtained counts of the total nucleated cells using a hemocytometer with nigrosin dye exclusion as a measure of viability.

### Flow Cytometry

Post centrifugation (1500 rpm x 10 min) the research team resuspended cell pellets via manual disruption in PBS containing 0.2% bovine serum albumin (BSA) and 0.1% $NaN_3$ at a concentration of 1 x $10^5$ to 1 x $10^6$ white blood cells/mL. The team then incubated 100 μL of the cells with 100 μL mAb (diluted per manufacturers' recommendations for 30 min at 4°C). After staining the cells, the team washed them twice with PBS-0.2% BSA-0.1% $NaN_3$ solution and measured their relative fluorescence intensities on a 4-decade log scale. For the measurement, the research team used flow-cytometric analysis with a LSR II (Becton-Dickinson, San Diego, California) and BD FACSDiva Software v 4.1 (Becton Dickinson, San Jose, California). The team analyzed the results with FlowJo 7.6.1 (Tree Star, Ashland, Oregon) and used the following fluorescence-labeled monoclonal antibodies: CD4-PacOrange (RM4-5); CD8a-APC-780 (53-6.7); CD19-PE (1D3); CD25-AF-700 (PC61.5); F4/80-PECy7 (BM8); CDllc-APC (N418); and MHCII-FITC (2G-9) (Pharmingen, San Jose, California, or eBioscience, San Diego, California).



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**Lung Histology**

For animals that did not undergo BAL (n=4 per group), the research team fixed the removed lungs with a 10% buffered formalin and processed them in a standard manner. The team stained tissue sections with hematoxylin and eosin and evaluated all specimens with a microscope-mounted Nikon Eclipse 400camera (Tokyo, Japan). The team created digital images using Spot RT Slider Software (Sterling Heights, Michigan) and evaluated them in Microsoft Photo Editor (Redmond, Washington). Five separate individuals graded the degree of cellular infiltration (0-5) in a blinded manner.

**Serum Immunoglobulin and Cytokine Measurements**

The research team thawed all serum samples and vortexed them prior to immunoglobulin and cytokine measurement. The team determined immunoglobulin concentrations using Milliplex Mouse Immunoglobulin Isotype Kit Panels (Cat#MGAM-300; Millipore, Billerica, Massachusetts) following the manufacturer's instructions. Assay sensitivities (minimum detectable concentrations) were IgM 0.3 ng/mL; IgG1 0.3 ng/mL; IgG3 0.4 ng/mL; IgG2a 0.4 ng/mL; IgG2b 0.4 ng/mL; and IgA 0.7 ng/mL. For assessment of cytokines and chemokines, the team processed samples with a Milliplex Mouse Cytokine/Chemokine kit (Cat #MPXMCYTO70KPMX22; Millipore, Billerica, Massachusetts) following manufacturer's instructions. Assay sensitivities were IL-1α 5.1 pg/mL; IL-1β 2.0 pg/mL; IL-2 0.8 pg/mL; IL-4 0.4 pg/mL; IL-5 0.7 pg/mL; IL-6 1.8 pg/mL; IL-12(p40) 4.9 pg/mL; IL-13 6.3 pg/mL; IL-15 6.5 pg/mL; IL-17 0.5 pg/mL; and TNFα 1.0 pg/mL.

**Statistical Analysis**

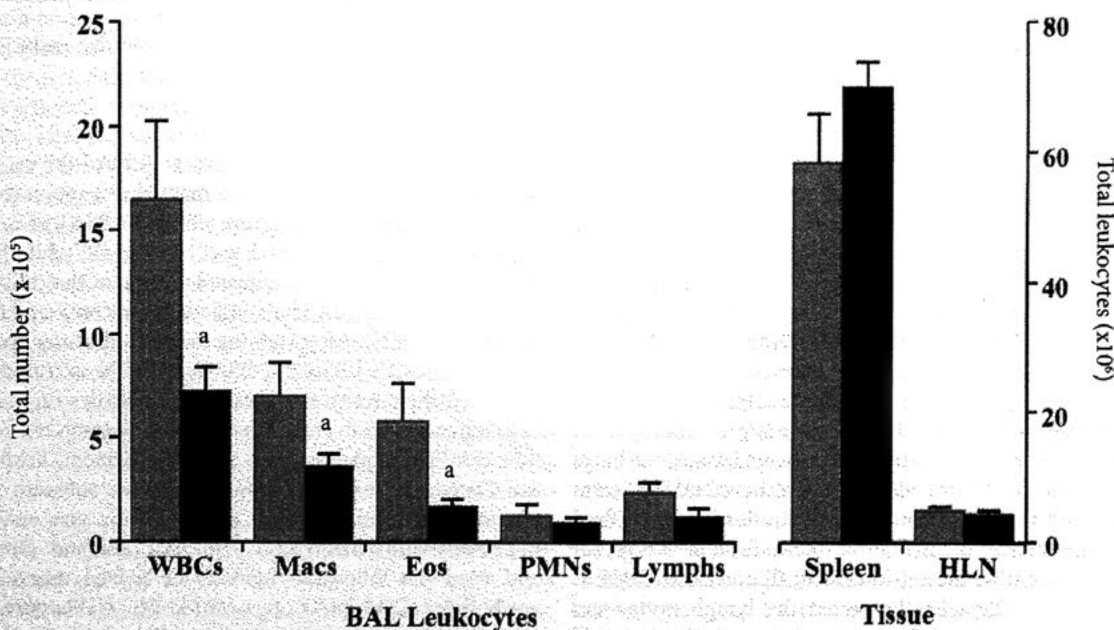
The research team made statistical comparisons between groups with analysis of variance and unpaired *t* tests using JMP Software (SAS Institute, Cary, North Carolina). Serum cytokine levels were not normally distributed, and the team log-transformed them for statistical analysis. The team expressed all data as means ± standard error of the mean and considered differences to be significant at $P \leq .05$.

## RESULTS

**Bromelain Treatment Was Nontoxic**

As the research team has reported previously,[29] intraperitoneal bromelain was nontoxic, as assessed by body weight and BAL protein concentration. The team noted no

**Figure 2.** Effect of Bromelain Treatment on Total Leukocytes
For mice with allergic airway disease, bromelain significantly reduced total bronchoalveolar (BAL) lavage leukocytes and eosinophils in the bromelain group (black bars) as compared to the control group (gray bars) without change in BAL macrophages, neutrophils, or lymphocytes. The research team saw no significant reduction in leukocytes in the tissue of the spleen or hilar lymph nodes. The data represent mean ± standard error of the mean values.




**Abbreviations:** BAL, bronchoalveolar lavage; eo, eosinophil; HLN, hilar lymph node; lymph, lymphocyte; mac, macrophage; PMN, neutrophil; WBC, white blood cell (leukocyte).

[a]Indicates $P < .05$ for the bromelain vs the control group; n = 10 animals per group.



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difference in body weight in bromelain-treated mice as compared to PBS-treated control animals (19.1 ± 0.26 g vs 18.8 ± 0.35 g; $P = .54$). Also, no significant change in BAL total protein concentrations occurred between bromelain-treated mice and PBS controls (143 ± 10 µg/mL vs 174 ± 17 µg/mL; $P = .18$; n = 10 animals per group).

**Bromelain Decreased Pulmonary Eosinophilia in Established Asthma**

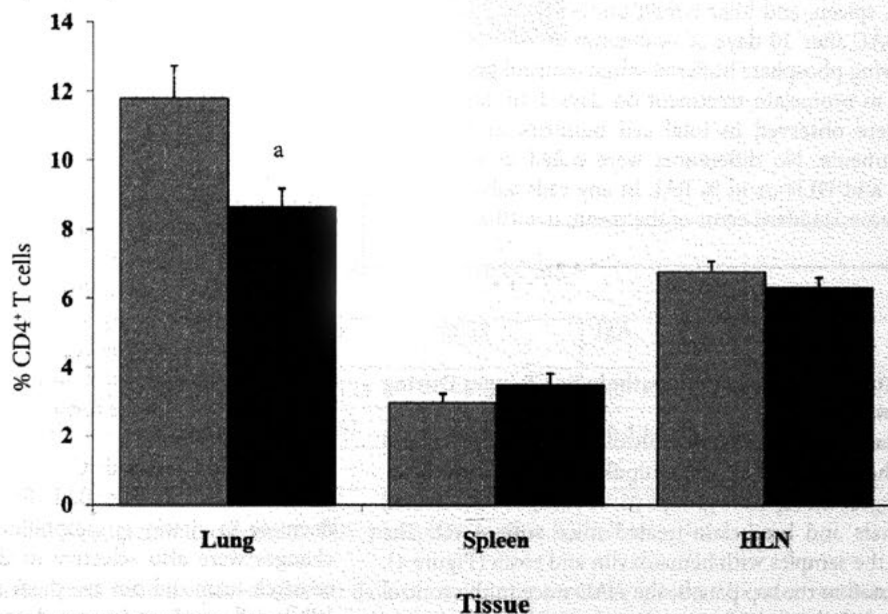
Exposure of OVA-sensitized mice to daily OVA aerosols resulted in AAD, characterized by robust increases in BAL leukocytes and eosinophils (Figure 2). Treatment with bromelain significantly inhibited OVA-induced increases in BAL leukocytes (7.22 ± 1.20 vs 16.41 ± 3.84 x $10^5$ cells in control mice; $P = .035$; n = 10 each) and eosinophils (1.65 ± 0.40 vs 5.80 ± 1.87 x $10^5$ cells; $P = .044$), without change in other cell types. In contrast to leukocyte numbers in BAL fluid, bromelain did not change those numbers in the spleens and HLNs of the sensitized and challenged mice (Figure 2).

**Bromelain Selectively Decreased $CD4^+$ T and $CD8^+$ T Cells in Bronchoalveolar Lavage From Mice With Asthma**

Although the bromelain treatment did not significantly reduce the total number of BAL lymphocytes (Figure 2; control [2.3x$10^6$ ± 0.6]; bromelain [1.2x$10^6$ ± 0.4, $P = .13$]), bromelain did reduce the distribution of BAL $CD4^+$ T cells and $CD8^+$ T cells in the treated animals of the intervention group (Table 1). BAL $CD19^+$ B cells were trended toward significance between the two groups $P = .05$. Nevertheless, bromelain did not affect the relative percentages of (1) $CD4^+$ (control [10.3 ± 1.8%], bromelain [7.6 ± 1.4%], $P = .26$); (2) $CD8^+$ (control [10.8 ± 2.7%], bromelain [7.8 ± 1.4%], $P = .35$); and (3) $CD19^+$ lymphocytes (control [9.3 ± 2.3%], bromelain [11.3 ± 2.7%], $P = .57$) (Table 1). Similarly, bromelain exerted no effects on the number or percentages of splenic or HLN lymphocytes (Table 1). In contrast, both the absolute numbers and percentages of BAL $CD4^+$ T cells that expressed the surface activation marker $CD25^+$ were lower in the bromelain group compared to control animals ($P = .012$; Figure 3). The percentages of $CD4^+$ T cells expressing CD25 were unchanged in the spleen ($P = .27$) and HLNs ($P = .33$; Figure

**Figure 3.** Effect of Bromelain Treatment on $CD4^+CD25^+$ T Lymphocytes

In bronchoalveolar lavage fluid, bromelain treatment significantly decreased the percentage of $CD4^+CD25^+$ T cells (black bars) as compared to phosphate buffered saline–treated control mice (gray bars). In contrast, bromelain had no effect on $CD4^+CD25^+$ T cell percentages in the spleens or hilar lymph nodes. The data represent mean ± standard error of the mean values; n = 10 animals per group.




**Abbreviation:** HLN, hilar lymph node.

[a]Indicates $P < .05$ for the bromelain vs the control group.



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**Table 1.** Lymphocyte Distributions in Tissues From Bromelain-treated and Control Mice[a]

| BAL (total cells x $10^6$) | Control | Bromelain | *P*-value |
|---|---|---|---|
| $CD4^+$ T cell | 2.41 ± 0.6 | 0.83 ± 0.16 | .028 |
| $CD8^+$ T cell | 2.29 ± 0.6 | 0.87 ± 0.19 | .046 |
| $CD19^+$ B cell | 2.81 ± 0.8 | 1.41 ± 0.44 | .050 |
| Spleen (total cells x$10^6$) | | | |
| $CD4^+$ T cell | 602.1 ± 53 | 578.8 ± 36.2 | .98 |
| $CD8^+$ T cell | 425.3 ± 34 | 428.4 ± 28.0 | .99 |
| $CD19^+$ B cell | 3964 ± 1150 | 4989 ± 1170 | .29 |
| HLN (total cells x$10^6$) | | | |
| $CD4^+$ T cell | 48.8 ± 7.1 | 43.2 ± 5.8 | .55 |
| $CD8^+$ T cell | 42.5 ± 6.2 | 42.8 ± 6.0 | .98 |
| $CD19^+$ B cell | 42.5 ± 7.1 | 43.5 ± 7.3 | .98 |
| BAL (%) | | | |
| $CD4^+$ T cell | 10.3 ± 1.8% | 7.6 ± 1.4% | .26 |
| $CD8^+$ T cell | 10.8 ± 2.7% | 7.8 ± 1.4% | .35 |
| $CD19^+$ B cell | 9.3 ± 2.3% | 11.3 ± 2.7% | .57 |

**Abbreviations:** BAL, bronchoalveolar lavage; HLN, hilar lymph node.

[a]The distribution of lymphocyte subsets (CD4+ and CD8+ T cells and CD19+ B cells) was measured in the bronchoalveolar lavage (BAL), spleen, and hilar lymph nodes (HLNs) and % of cells in the BAL after 10 days of ovalbumin aerosol exposures in mice receiving phosphate buffered saline (control group) and as compared to bromelain treatment on days 4-10. Significant reductions were observed in total cell numbers in the BAL between treatments. No differences were noted in total cells in the spleen and HLN or in % BAL in any cells subsets. Data represent mean ± standard error of the mean; n = 10 animals in each group.

3).

**Bromelain Attenuated Lung Histopathologic Changes During Allergic Airway Disease**

The research team performed histological evaluations and obtained pathology scores on unmanipulated, uninflated, formalin-fixed lungs from separate groups (n = 4 each) of PBS-treated control animals and bromelain-treated mice with AAD. The team stained the samples with hematoxylin and eosin (Figure 4). As demonstrated in the top panels, the AAD mice in the control group (Figure 4A) showed substantial peribronchial and perivascular inflammation comprising lymphocytes, plasma cells, and eosinophils. Bromelain-treated AAD mice had less histological injury (Figure 4B). Statistical comparison of pathological scoring by five blinded reviewers demonstrated significantly less pathology in the bromelain-treated mice than in the control animals (mean score 1.40 ± 0.27 vs 2.35 ± 0.31; $P = .0077$; Figure 4C).

**Bromelain Did Not Affect Serum Immunoglobulin Levels but Decreased Selective Serum Cytokines**

Bromelain treatment did not elicit generalized immune suppression, as evidenced by lack of a direct effect on serum immunoglobulin levels (Table 2) or generalized effect on serum cytokines. As seen in Figure 5, however, bromelain-treated animals did demonstrate selective decreases in key cytokines, including IL-4 (0.33 vs 1.38 pg/mL; $P = .0002$), IL-12 (8.67 vs 36.8 pg/mL; $P = .003$), IL-17 (0.54 vs 3.88 pg/mL; $P = .0004$), IFN-g (1.56 vs 86.5 pg/mL; $P = .016$). In contrast, the chemokine interferon, gamma-induced protein (IP-10) increased in bromelain-treated animals (61.6 vs 39.2 pg/mL; $P = .02$).

**DISCUSSION**

The current study is a continued characterization of the use of bromelain, a cysteine protease extracted from pineapple, in asthma. The research team has previously shown that intraperitoneal and oral administration of bromelain can prevent the development of asthma in sensitized mice when given prior to aerosolized antigen exposure.[29,30] Since most people taking bromelain for asthma, however, would have an established disease already and may be experiencing symptoms, a more relevant question is whether bromelain can attenuate existing asthma. To the research team's knowledge, few reports exist that study the effects of botanicals in models of established or chronic asthma.[20] The present study demonstrated a beneficial effect of bromelain when administered 3 days after consecutive OVA aerosol challenge, a time when eosinophilic airway inflammation and airway hyperresponsiveness were well established.[17,29,31] At that point, bromelain was capable of attenuating the airway leukocytosis and eosinophilia and reducing the histopathological changes in the lung that occur in asthma. This anti-inflammatory effect appeared to be tissue specific, as no changes occurred in the spleen or HLN leukocyte populations or in serum immunoglobulin levels, even though the research team gave the bromelain systemically.

Broad decreases in $CD4^+$ and $CD8^+$ T cells and $CD19^+$ B cells in the BAL fluid accompanied the decrease in airway eosinophilic inflammation. Such changes were also selective to the airways, and the research team did not see them in HLNs or spleens. While cell numbers decreased significantly, no changes occurred in the relative percentages of major lymphocyte subpopulations in BAL for either the control or the bromelain-treated mice. This finding differed from what the research team had observed when it gave bromelain before the initiation of the OVA aero-



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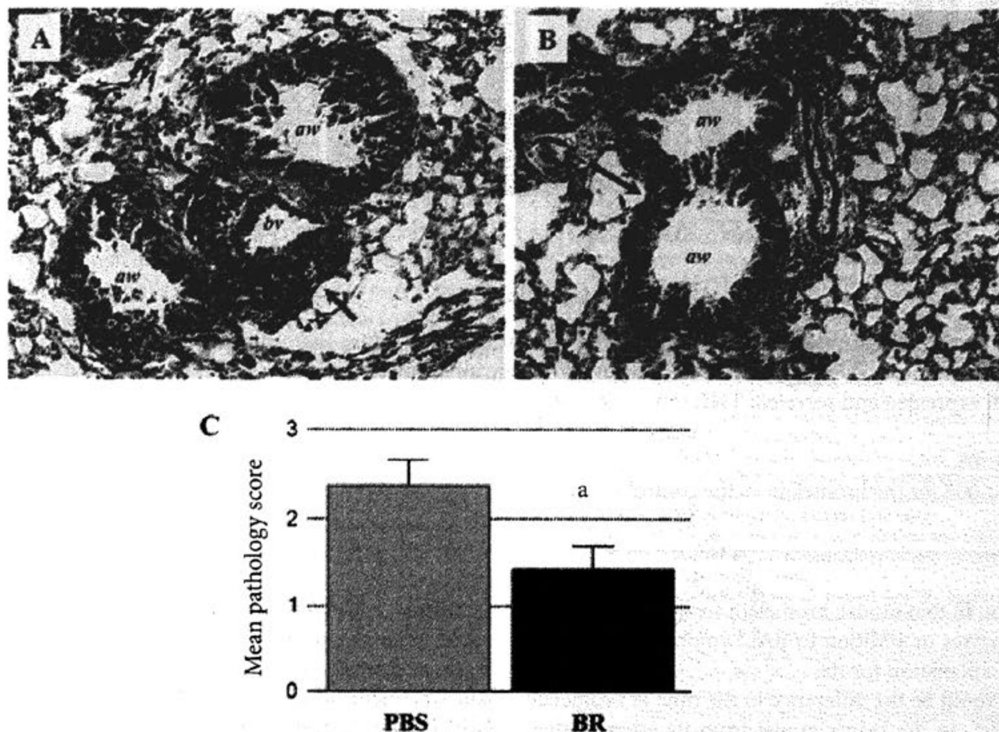
**Figure 4.** Bromelain Treatment Reduces Lung Pathology
The research team stained lung sections with hematoxylin and eosin, and five individuals scored the samples blindly on a scale from 0 to 5, based on the level of pathology. Panels A and B illustrate representative pathology from a control, phosphate buffered saline-treated mouse (A) and a bromelain-treated mouse (B). The inflammation (arrows) surrounding airways (aw) and blood vessels (bv) appeared significantly reduced in the bromelain (B) animal as compared to the control animal (A). Figures are at 10X magnification. Panel C demonstrates mean ± standard error of the mean pathology scores for each group (n = 4 each).




**Abbreviations:** BR, bromelain; PBS, phosphate buffered saline.

[a]Indicates $P < .05$ between groups.

**Table 2.** Serum Immunoglobulin Levels in Control and Bromelain-treated Mice[a]

| | IgG1 | IgG2[a] | IgG2b | IgG3 | IgA | IgM |
|---|---|---|---|---|---|---|
| PBS | 1836 ± 427 | 15.8 ± 5.1 | 359 ± 128 | 135 ± 49 | 16.7 ± 3.3 | 622 ± 161 |
| BR | 2167 ± 240 | 15.5 ± 2.4 | 380 ± 87 | 106 ± 46 | 16.4 ± 5.4 | 616 ± 214 |
| *P*-value | .57 | .96 | .89 | .48 | .96 | .98 |

**Abbreviations:** BR, bromelain; Ig, immunoglobulin; PBS, phosphate buffered saline.

[a]Serum immunoglobulin levels were measured after 10 days of ovalbumin aerosol exposures in mice receiving phosphate buffered saline (control) and receiving bromelain (intervention) on days 4-10. The team saw no significant differences in any immunoglobulin class. Data represent mean ± standard error of the mean; n = 4 animals in each group.



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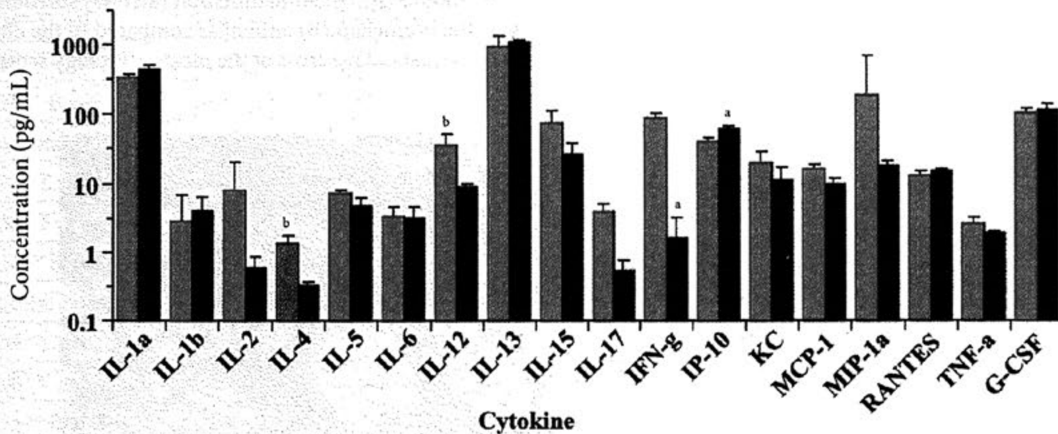
**Figure 5.** Bromelain Reduces Selected Cytokine Levels
Bromelain treatment selectively decreased specific cytokine levels in serum of mice with allergic airway disease. The data represent mean±standard error of the mean values; n=4-10 samples per group.




**Abbreviations:** G-CSF, granulocyte colony-stimulating factor; IL, interleukin; IFN, interferon; KC, keratinocyte chemoattractant; MCP, monocyte chemotactic protein; MIP, macrophage inflammatory protein; RANTES, regulated upon activation, normal T-cell expressed and secreted; TNF, tumor necrosis factor.

[a]Indicates $P<.05$.
[b]Indicates $P<.005$ for the bromelain vs the control group.

sol challenges. In that model, bromelain treatment decreased total lymphocytes in addition to BAL lymphocyte subsets.[30] A potential explanation for the discrepancy could be the difference in the time of bromelain administration. In the team's model from its other studies, significant lymphocyte recruitment to the airways occurred by day 3 of OVA exposures.[29,30]

Carson et al found from day 3 to day 10 that the predominant Th2-skewed lymphocyte found in the BAL during AAD was the CD4$^+$CD25$^+$, activated T effector cells.[14] In the research team's other studies, treatment with bromelain prior to aerosol challenge may have altered the pattern of initial CD4+ T-cell recruitment whereas, in the current study, later administration of bromelain (from day 3 to 10), occurred after asthma was well established (eg, CD4 T cells are prominent). While total CD4$^+$ T cells were unchanged in the current study, bromelain treatment significantly reduced the percentage of CD4$^+$ T cells expressing the activation marker CD25. Again, this finding was specific to BAL, and the research team did not see it in the spleen or HLN. The general inhibition of airway inflammation exerted by bromelain may explain the regional reduction in CD4$^+$CD25$^+$ T cells and hence the existence of fewer cells that have an activated (CD25$^+$) phenotype. It may also reflect selected removal of CD25 (the high affinity IL2-Rα) from T cells by the bromelain treatment, which the research team previously has reported occurs with treatment.[31] Reduction of CD25 may limit IL-2 from binding, which corresponds to reduced cell expansion and differentiation. Amelioration of disease also could occur by selective expansion of regulatory T cells. The research team, however, has shown previously that bromelain treatment also reduced CD25 from regulatory T cells (which constitutively express CD25) while having no effect on expression of Foxp3.

Bromelain did not affect serum immunoglobulin levels, further suggesting a lack of generalized immunomodulatory effect. The bromelain treatment, however, selectively decreased some serum cytokines—specifically, interleukins 4, 12, and 17 as well as IFN-γ. The treatment did not affect other Th2 cytokines, such as IL-5 and IL-13. The role of IL-4 in asthma is well established. The differentiation of naïve T lymphocytes into Th2 cells in the presence of an allergen requires this cytokine, and it is the principal stimulus for B-cell isotype switching to IgE.[32] The research team would expect inhibition of this key Th2 cytokine to attenuate the AAD response. IL-17 expression in the airways is also upregulated after sensitization and challenge with antigen, and it is associated particularly with neutrophil recruitment and more severe asthma.[33] Of interest, Th17 cells are resistant to steroids in vitro, and a recent study indicated that airways hyperreactivity induced by transferred Th17 cells in a mouse model is steroid-resistant.[34] Zhao et al have found that some human participants with steroid-resistant asthma have elevated levels of Th17 cells compared to healthy controls.[35] That bromelain decreased IL-17 in AAD mice raises the intriguing speculation that it could be useful in some



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humans with refractory, Th17-driven asthma. Additionally, bromelain significantly increased serum levels of interferon γ-inducible protein (IP-10). Researchers have found that IP-10 expression occurs in bronchial epithelial cells and believe that the effect is associated with human immune defense against pathogens.[36] It is unclear, however, if increasing levels of IP-10 in a nonviral, allergic, airway-disease model would provide added support. Likewise, researchers would not expect bromelain's inhibition of IFN-γ and IL-12 to be a therapeutic target in asthma and that inhibition may demonstrate some broader immunomodulatory effects of the agent.

## CONCLUSION

In summary, administration of bromelain after the onset of AAD (asthma) inhibited progressive airway eosinophilia by ~70%. This effect was similar to the ~55% reduction in airway eosinophils seen when the current research team gave bromelain to sensitized mice before initiation of OVA aerosol challenges in a prior study.[29,30] Thus, bromelain may be as effective in treating existing asthma as it is in preventing the development of allergic airway inflammation. The attenuated airway eosinophilia was associated with fewer airway $CD4^+$ and $CD8^+$ T cells, decreased numbers of activated $CD4^+CD25^+$ T cells, lower levels of Th2 cytokines, and reduced lung histopathology. This modulatory role of bromelain appeared to be specific to the tissue or site of inflammation, as treatment did not affect lymphocyte numbers in the spleen or HLN. These observations suggest that bromelain may be effective in human asthmatics with existing disease, perhaps particularly in those with more steroid-resistant asthma.


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# Gum resin of *Boswellia serrata* inhibited human monocytic (THP-1) cell activation and platelet aggregation

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ABSTRACT

*Ethnopharmacological relevance:* Stem bark gum resin extract of *Boswellia serrata* is traditionally used in India for its hemostatic, antiinflammatory and cardiovascular health effects and it is named as Śallakī in Ayurvedic medicine.
*Aim of the study:* This study was conducted to evaluate the antioxidative and antithrombotic properties of stem bark gum resin extracts of *Boswellia serrata* (BS).
*Materials and methods:* The inhibitory activity of the BSWE and BSAE on $FeCl_3$ induced lipid peroxidation (*in vitro*) in rat liver and heart homogenates was measured spectrophotometrically. Their effect on $H_2O_2$ induced reactive oxygen species (ROS) generation in human monocytic (THP-1) cells was investigated by tracking intensity of a cell permeable fluorescent dye, $H_2DCFDA$ and subjecting the cell samples to confocal microscopy. Further, the effect of BSAE and BSWE on ADP-induced platelet aggregation was assessed using a multimode detection plate reader, plasma coagulation times using an automated blood coagulation analyzer and on human blood clotting factors Xa and XIa using chromogenic substrate. Phytomarker analysis of the water (BSWE) and hydroalcoholic (BSAE) extracts of BS-gum resin was done through HPLC using a standard compound AKβBA.
*Results:* BSAE and BSWE inhibited, to varied extents, the lipid peroxidation in liver (80%) and heart (50%) tissue homogenates of male Wistar rats. Further, BSAE (30 μg dwt/mL) and BSWE (300 μg dwt/mL) attenuated ≥60% of $H_2O_2$ mediated ROS generation in THP-1 cells. In case of standard compounds, ascorbate (20 μg dwt/mL) and butylated hydroxytoluene (BHT) (10 μg dwt/mL) completely scavenged ROS in the cells. BSAE and BSWE at 3 mg dwt/mL completely inhibited ADP induced platelet aggregation and activities were comparable to 20 μg/mL of heparin. The extracts also showed very high activity in prolonging coagulation time periods. Both types of extracts extended prothrombin time (PT) from ~13 to >60 s and activated partial thromboplastin time (APTT) from ~32 s to >90 s. BSAE inhibited clotting factors Xa and XIa remarkably at 6 μg of dwt where as BSWE did not show much effect on FXa and showed 30% inhibition on FXIa at 120 μg. 10 μg of heparin was required to inhibit about 30% activity of the above factors. HPLC analyses suggested that BSAE and BSWE had AKβBA of 9% (w/w) and 7.8% (w/w) respectively.
*Conclusion:* Present study demonstrated antioxidant and antithrombotic anticoagulant activities of water and hydroalcoholic extracts of *Boswellia serrata*'s gum resin. We suggest that BS-gum resin as a good source for lead/therapeutic compounds possessing antioxidant, antiplatelet and anticoagulant activities.

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*Abbreviations:* AKβBA, 3-acetyl-11-keto-β-boswellic acid; APTT, partial thromboplastin time; BHT, butylated hydroxytoluene; BSAE, hydroalcoholic extract of *Boswellia serrata*'s gum resin; BSWE, water extract of *Boswellia serrata*'s gum resin; $H_2DCFDA$, 2′,7′-dichlorofluorescin diacetate; PPP, platelet poor plasma; PRP, platelet rich plasma; PT, prothrombin time; ROS, reactive oxygen species; THP-1, human acute monocytic leukemia cell line.

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## 1. Introduction

Extracts of *Boswellia serrata*'s stem bark gum resin (Śallakī or Salai guggal or Frankincense) are traditionally used in India (Ayurvedic/ethnomedicine) to treat various types of blood disorders, inflammatory health ailments, pain and cardiac debility (Paranjpe, 2001). There are several scientific reports on pharmacological activities of BS-gum resin. Gummy exudates of *Boswellia serrata*'s stem bark or its major constituents, boswellic acids have anti-inflammatory (Ammon, 2006; Shen and Lou, 2008), anticancerous (Aman et al., 2009) and anti-ulcerous (Singh et al., 2008) activities. Molecular targets for *Boswellia* extract in the inflammation pathway are identified and these include leukotrienes (inflammatory chemical messengers), 5-lipoxygenase, human leukocyte elastase, toposiomerase I and II, as well as IκB kinases (Poeckel and Werz, 2006).Clinical trials with *Boswellia serrata*'s gum resin extracts indicated its non-toxic nature (Arieh et al., 2010). A patented polyherbal formulation BHUx, containing gum resin of *Boswellia serrata* as one of the herbal components is formulated for treating atherosclerosis (Tripathi, 2009), a cardiovascular disease (CVD). Lately, CVD is recognized to develop as a result of complexed interactions between the processes of inflammation, oxidative stress and thrombosis (Ross, 1999; Boos and Lip, 2006). The objective of the present study is to investigate, if BS-gum resin extract can interrupt these complexed interactions and thus offer cardioprotective effect. To the best of our knowledge, there are no reports on antithrombotic properties of *Boswellia serrata*'s gum resin. Inhibition of platelet function represents a promising way for the prevention of thrombosis. Drugs with anticoagulant and antithrombotic effects, e.g. heparin, are among the primary drugs of choice, for the prevention of thromboembolic disorders. However, alternative drugs for heparin are in high demand due to the long-term side effects of heparin. Therefore, the objective of the present study is to evaluate antioxidant and antithrombotic activities using *in vitro* and cell based assays and to provide scientific basis for the traditional use of *Boswellia serrata*'s gum resin.

## 2. Materials and methods

### 2.1. Chemicals

Ascorbic acid, butylated hydroxytoulene (BHT) ferric chloride, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrezolium bromide (MTT), sodium acetate trihydrate, 2,4,6-tripyridyl-s-triazine (TPTZ) were from Hi-media, India. 3-Acetyl-11-keto-β-boswellic acid (AKβBA), 2,7-dichlorofluorescin diacetate ($H_2DCFDA$), ursolic acid, 2-thiobarbituric acid (TBA), and trypan blue were purchased from Sigma–Aldrich (Germany). RPMI 1640 medium, L-glutamine, and fetal bovine serum (FBS) were purchased from Invitrogen. Hydrogen peroxide ($H_2O_2$) obtained from Merck. Heparin purchased from Samarth Life Sciences (Mumbai). Human blood clotting factors Xa, XIa purchased from American Diagnostic Inc., Stanford. All other reagents used were of analytical grade.

### 2.2. Plant material and animal tissues

The voucher specimen (# 428) was identified by Dr. K. Madhava Chetty and deposited at Sri. Venkateshwara University Herbarium, Tirupati. Male Wistar rats were housed in ventilated cages and fed with pellet diet and water. Liver and heart tissues were kindly provided by Prof. P. Prakash Babu, Department of Biotechnology, University of Hyderabad.

### 2.3. Preparation of medicinal plant extracts.

Hydroalcoholic extract (BSAE) of gum resin was prepared by soaking dried gum powder in 80% ethanol followed by continuous stirring at 40 °C for 5–6 h. Water extract (BSWE) was prepared by soaking gum powder in water for overnight at room temperature. The extracts were centrifuged at 10,000 rpm for 10 min and subjected to various studies. BSAE or BSWE, obtained from 1 g of gum resin, was subjected to complete dryness under vacuum. Dry weight of the extract was determined to calculate the yield.

### 2.4. Phytochemical analysis of plant extracts

Preliminary phytochemical screening of the extracts showed positive reaction for triterpenoids, polyphenols and flavonoids. Using standard compound, i.e. one of the major boswellic acids, 3-acetyl-11-keto-β-boswellic acid (AKβBA) of 95% purity (Sigma), quantity of AKβBA in BSAE and BSWE has been quantitated by subjecting the extracts to HPLC analyses. The analysis was outsourced to Pharmatrain, Kukatpally, Hyderabad, methodology in brief is as follows. The instrument used for this analysis was Waters HPLC 2487 dual λ absorbance detector and 2695 separation module. The separation was performed on $C_{18}$ 100 Å (250 mm × 4.6 mm, make: Waters) reverse phase analytical column. Compound was eluted using a mobile phase consisted of water (A) and acetonitrile (B), used in 35A and 65B in an isocratic mode at a flow-rate of 0.6 mL/min for 9 min. Absorption spectra and retention times were recorded at 210 with a UV detector connected to the HPLC system.

### 2.5. Cell culture

THP-1 human monocytes were purchased from National Centre for Cell Science (NCCS Pune, India) and cultured in RPMI 1640 medium (10 mM HEPES, 1 mM sodium pyruvate, 4.5 g/L glucose, 1.5 g/L sodium bicarbonate) supplemented with 2 mM L-glutamine and 10% fetal bovine serum. Cells were maintained in 5% $CO_2$ and 95% air at 37 °C during growth and treatments.

Treatments with BSAE, BSWE or standard compounds were conducted at a cell density of ~$0.5 \times 10^6$ cells/mL for 16–18 h. Vehicle (alcohol or water) concentration was limited to <1% of the cell culture volume. Cell viability was monitored after all the treatments using trypan blue exclusion and cell count was performed using an inverted microscope (Leica DMR) with the aid of a haemocytometer. MTT cell viability assay was performed according to manufacturer's protocol. Briefly, THP-1 cells were seeded in 24-well plates ($2 \times 10^5$ cell per well). Cells were pre-treated with and without plant extracts or BHT for 24 h or with ascorbate for 1 h. After respective time periods of incubation, cells were washed twice with medium in order to remove trace amounts of extracts or standard compounds. Similarly, wells with media without cells were processed. MTT (5 mg/mL) was added to all the wells with or without cells and incubated for 3 h. The converted MTT dye was solubilised with 0.04 N HCl in isopropanol. Absorbance of the dye was measured at a wavelength of 570 nm with background subtraction at 690 nm. Media without cells with respective concentrations of extracts or standard compounds were taken as blanks.

### 2.6. Lipid peroxidation assay

#### 2.6.1. Liver tissue homogenate lipid peroxidation

The peroxide formation was monitored according to the method of Gaurav et al. (2007) by measuring the colour of thiobarbituric acid reactive substances (TBARS) formed at the end of the reaction. The reaction mixture contained rat liver homogenate, 1 mM ferric chloride and various concentrations of BSAE/BSWE. Lipid peroxidation was initiated by adding 100 μl of 1 mM fer-

ric chloride and incubated for 30 min at 37 °C. The reaction was terminated by addition of 2 mL of ice cold HCl (0.25 N) containing 15% trichloroacetic acid (TCA), 0.38% TBA to the reaction mixture followed by heating at 80 °C for 60 min. The samples were then cooled and centrifuged at 5000 × g for 15 min, and the absorbance of supernatants was measured at 532 nm against the blank. Identical experiments were performed to determine the normal and induced lipid peroxidation. The protective effects of different extracts against lipid peroxidation were calculated as follows: % Inhibition = (control – sample/control) × 100. Ascorbic acid was used as a reference compound.

### 2.6.2. Heart tissue homogenate lipid peroxidation

This assay was performed according to the method reported by Meera et al. (2009). The reaction volume composed of 50 μL heart homogenate, 10 mM ferric chloride, and various concentrations of BSAE/BSWE. Buffer blank was prepared w/o ferric chloride. All the tubes were incubated at 37 °C for 1 h. After incubation, 500 μL of 70% ethanol was added to all the tubes to arrest the reaction. TBA (1%, 1 mL) was added to all the tubes followed by boiling in water bath for 20 min. After cooling to room temperature the tubes were centrifuged to clear the solution and the supernatants collected. To the supernatants, 50 μL of acetone was added. TBARS were measured at 532 nm using a spectrophotometer. An assay medium corresponding to 100% oxidation was considered by adding tissue homogenate, ferric chloride, without BSAE/BSWE. Ascorbic acid was used as a reference compound.

### 2.7. Measurement of ROS generated by $H_2O_2$ in THP-1 monocyte cell line using $H_2DCFDA$

Intracellular ROS were measured by using cell permeable fluorescent dye, $H_2DCFDA$ according to Evgeniy et al. (2010). THP-1 cells were seeded in 24-well plates and pre-treated with or without different concentrations of BSAE/BSWE for overnight at 37 °C in a humidified atmosphere containing 5% $CO_2$. After incubation, wells were loaded with 5 μM $H_2DCFDA$ and incubated for 30 min at 37 °C. Then the cells were washed twice with the growth medium to ensure the removal of unbound dye as well as plant extract in the medium containing the cells. After washing, cells were exposed to 10 μM $H_2O_2$ for 10 min. Images were obtained by subjecting the cells to confocal laser-scanning electron microscopy using excitation and emission wavelengths at 488 nm and 525 nm respectively. The quantitation of fluorescence intensities of the cell samples was done by spectrofluorimetry.

### 2.8. Assay of ROS scavenging potential in THP-1 cells

ROS scavenging assays were done in THP-1 cell lysates after the treatments done as detailed in Section 2.5. Cells were washed twice prior to exposure with $H_2O_2$ to avoid direct interaction between plant extracts and $H_2O_2$. After two washes, cells were exposed to $H_2O_2$ and then sonicated, thus obtained cell lysates were centrifuged at 10,000 rpm for 2 min. Supernatants were subjected to the following assays.

### 2.8.1. Catalase assay

Catalase (CAT) enzyme assay was done according to the method of Prasenjit et al. (2007). 7.5 mM $H_2O_2$ was added to cell-lysates and the decrease in absorbance at 240 nm was monitored at 37 °C using a multimode microplate reader for about 5 min and absorbance was recorded at an interval of 10 s. CAT activity was expressed in fold change which reduced 1 μmol of $H_2O_2$ per min at 25 °C.

### 2.8.2. Ferric reducing/antioxidant power (FRAP) assay

The FRAP assay was done according to the method of Prasenjit et al. (2007), with cell lysates by measuring the changes in absorbance at 593 nm. 1.5 mL of freshly prepared and prewarmed (37 °C) FRAP reagent (300 mM acetate buffer, pH 3.6, 10 mM TPTZ in 40 mM HCl and 20 mM $FeCl_3 \cdot 6H_2O$ in the ratio of 10:1:1) was added to THP-1 cell lysates and incubated at 37 °C for 10 min. Blue colored $Fe^{II}$-tripyridyltriazine compound formed from the colorless oxidized $Fe^{III}$ form by the action of electron donating antioxidants was assayed. The absorbance of the sample was read against reagent blank (1.5 mL FRAP reagent + 50 μL distilled water) at 593 nm.

### 2.9. Antiplatelet aggregation assay

Platelet aggregation activity was determined according to Mary et al. (2003). 10 mL of blood was obtained from normal healthy volunteers. Platelet rich plasma (PRP) was collected after centrifugation of blood at 200 × g for 5 min. 1.5 mL of acid citrate dextrose (ACD) was used as anticoagulant for every 8.5 mL of blood. Platelet poor plasma (PPP) collected by centrifugation (1500 × g for 5 min) was kept as reference. PRP with or without different concentrations of BSAE or BSWE or heparin were added to microwell plate. Aggregation of platelets in PRP was initiated by adding ADP to a final concentration of 20 μM. Absorbance of the samples was recorded at 600 nm using multimode detection plate reader preset at 37 °C for a time period of 12 min at an interval of 2 min. Commercial heparin (20 μg/mL) was used as reference compound.

### 2.10. Assay plasma clotting times

Prothrombin time (PT) and activated partial thromboplastin time (APTT) assays belong to the class of diagnostic tests were done to assess the function of coagulation system. APTT measures contact activation (intrinsic) pathway and PT measures tissue factor (extrinsic) pathway and initiated by release of tissue factor. To assay the effect of BSAE/BSWE on extrinsic and intrinsic pathways of coagulation, PT and APTT time periods of normal human plasma were compared in the presence of BSAE/BSWE. These reactions were carried out using kit UNIPLASTIN obtained from Tulip Diagnostics (Goa, India) by using the instrument MC100 single channel coagulation analyzer. Samples were outsourced commercially to analyse at Regional Diagnostic Centre, Hyderabad. The protocol followed was briefly as follows: 0.9 mL of blood sample was transferred into a 0.109 M trisodium citrate (1:9, v/v) and then centrifuged at 1800 × g for 10 min to obtain plasma. 100 μL of the plasma were mixed with different concentrations of BSAE/BSWE and the coagulation was started by addition of $CaCl_2$, 100 μL of thromboplastin and thrombin added to the incubated plasma for PT and APTT assays individually.

### 2.11. Clotting factor assays

Human clotting factors were assayed according to Robert et al. (2010). Incubations were performed in 96-well plates. The final concentration of the reactants included 70 ng of factor Xa or 100 ng of factor XIa and different concentrations of test sample in 100 μL of 50 mM Tris, pH 8.3 containing 5 mM calcium chloride and 0.2 mM sodium chloride. Factor Xa/XIa was added last to initiate the reaction. After 60-s of incubation at room temperature, 0.8 mM of chromogenic peptide substrate (CH3OCO-D-CHA-Gly-Arg-pNA-AcOH) for the above factor was added, and the absorbance at 405 nm was recorded for 5 min. Heparin was used as positive drug control.

*2.12. Statistical analysis*

All data obtained were analyzed by one way analysis of variance (ANOVA) test using Statistical Package for the Life Sciences (SPLS version 11). All results were expressed as mean ± standard deviation of mean (S.D.). $p < 0.001$ was considered to be statistically highly significant.

## 3. Results

*3.1. Plant extracts*

Hydroalcoholic extracts yielded 4.8% (w/w) and water extracts yielded 5.2% (w/w) based on their starting material of dried gum resin. The phytochemical screening of gum resin showed positive reaction for polyphenols, flavonoids and triterpenoids. BSAE contained 0.647 ± 0.09 UAE mg/mg dwt; 22.5 ± 1.6 GAE μg/mg dwt and 2.29 ± 1.4 QE μg/mg dwt. Whereas BSWE contained 0.271 ± 0.026 UAE mg/mg dwt, 79.2 ± 2.8 GAE μg/mg dwt and 6.35 ± 3 QE μg/mg dwt. In summary hydroalcoholic extracts had higher amounts of total triterpenoids and water extracts had higher amounts of TPC and flavonoids.

*3.2. Quantititation of AKβBA*

Under the experimental conditions, retention time of standard compound AKβBA was 4.784 min (Fig. 1B). HPLC analyses with BSAE and BSWE showed peaks corresponding to standard AKβBA with retention times of 4.623 and 4.616 respectively (Fig. 1C). Calculations with corresponding peak areas determined that AKβBA content of BSAE to 9% (w/w) and BSWE to 7.8% (w/w) of their extracts' dwt.

To test the linearity of the compound, we used standard solutions of AKβBA in the range from 10 μg/mL to 120 μg/mL. 20 μL of these standard solutions were injected for each assay, which correspond to 200–2400 ng. Least amount of detection and quantification under the experimental conditions was 2.69 (±0.17) μg/mL and 9.08 (±0.58) μg/mL respectively. Each sample was measured in triplicate (Fig. 1A).

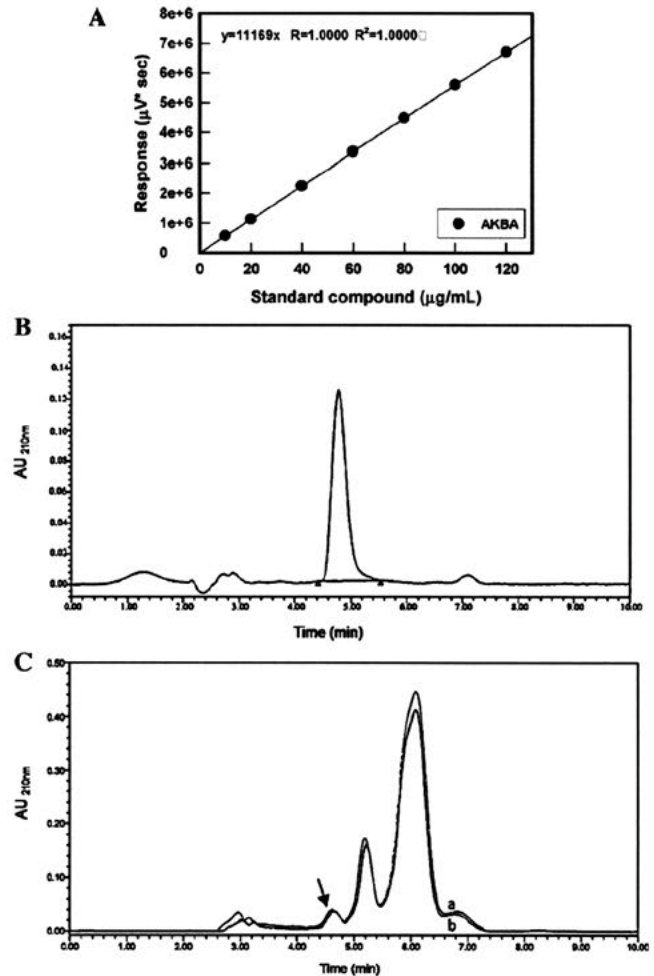


**Fig. 1.** HPLC analysis for quantification of AKβBA in plant extracts. (A) Regression plot with varied concentrations of AKβBA ranging from 20 to 120 μg/mL. LOD and LOQ of AKβBA are 2.69 ± 0.17 μg/mL and 9.08 ± 0.58 μg/mL. (B) An example chromatogram of standard compound AKβBA showing a peak with the retention time period of 4.784 min. (C) Example chromatograms of BSAE (a) and BSWE (b), with marked peak, corresponding to standard AKβBA with retention times of 4.623 min and 4.616 min respectively. Peak areas were used to determine the AKβBA content in the extracts.

*3.3. Lipid peroxidation inhibition activity of BSAE/BSWE*

BSWE inhibited $FeCl_3$ induced peroxidation in liver homogenates by 70% where as BSAE showed maximum inhibition of only 40% (Fig. 2A). In case of heart homogenate, both types of extracts inhibited maximum of 50% lipid peroxidation (Fig. 2B). The concentration of the BSWE needed for 50% inhibition in liver homogenate was 34 μg dwt/mL. In case of heart homogenate, $IC_{50}$ of BSAE and BSWE were 129 μg dwt/mL and 88 μg dwt/mL respectively. Extracts showed lower effect in inhibiting peroxidation in heart tissue homogenates from 200 μg dwt/mL onwards. $IC_{50}$ values for standard compound ascorbate were 38 μg/mL and 153 μg/mL for lipid peroxidation in liver and heart tissues respectively. Ascorbate showed maximum inhibition of 80% lipid peroxidation in liver tissue but only 55% inhibition with heart tissue. Thus our results indicated that water extracts of BS-gum resin were more potential than standard compound ascorbate in suppressing lipid peroxidation of both rat's liver and heart homogenates.

*3.4. Cell viability*

Dosage of plant extracts or standard compounds for THP-1 cell treatments were determined based on cell viability experiments (Fig. 3). At the used concentrations of plant extracts or standard compounds for the present study, no cell death was found, >95% cells were alive (Fig. 3). In case of standard compounds, there was no cell death found after treatments with ascorbate up to 50 μg/mL for 1 h time period (Fig. 3A) with BHT up to 10 μg/mL for 24 h time period (Fig. 3B). Cell death was not found after treatments with BSAE up to 40 μg dwt/mL (Fig. 3C) and up to 400 μg dwt/mL of BSWE (Fig. 3D). Therefore, we restricted our dosage of standard compounds and plant extracts to the maximum of above cited concentrations.

*3.5. Attenuation of $H_2O_2$ induced ROS by BSAE/BSWE in THP-1 Cells*

We evaluated the potential of BS-extracts in scavenging intra cellular ROS in human monocytic cell line using a cell permeable ROS sensitive fluorescent marker $H_2DCFDA$. THP-1 cells treated with 10 μM $H_2O_2$ for 10 min showed much brighter fluorescence compared to cells not treated with $H_2O_2$ (Fig. 4A). THP-1 cells treated with 10 μM $H_2O_2$ for 10 min showed 4.5 folds greater fluorescence compared to cells not treated with $H_2O_2$ (Fig. 4B). Pretreatment of cells with BSAE (30–40 μg dwt/mL) or BSWE (300–400 μg dwt/mL) markedly attenuated $H_2O_2$-dependent flu-

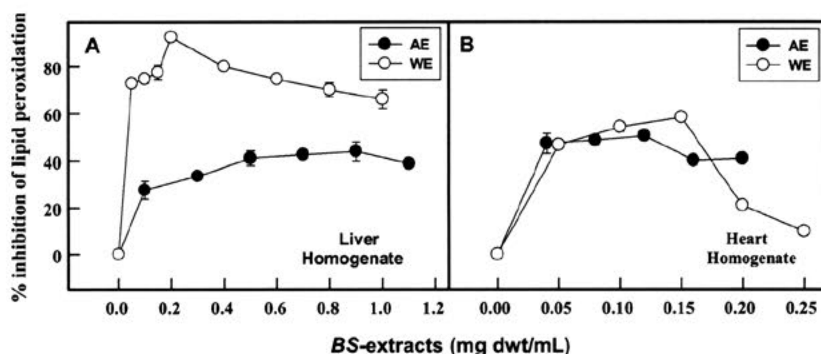


**Fig. 2.** Effect of BS-gum resin on ferric chloride induced lipid peroxidation on (A) liver and (B) heart tissue homogenates of rat. Data presented are mean ± S.D., $n = 6$. S.D. not seen where S.D. values are within the symbol of data point.

orescence increase with statistical significance of $p$ value <0.001. $IC_{50}$ of BSAE (0.011 mg dwt/mL) is much lower than $IC_{50}$ of BSWE (0.08 mg dwt/mL), which reflects that alcoholic extract was highly potential in scavenging intra cellular ROS. Washing $H_2$DCFDA dyed cells twice with medium prior to the exposure of cells to $H_2O_2$ ensured not only the removal of excess fluorescent dye, but also removal of BS-extracts from the medium, otherwise there is a possibility of extracts scavenging $H_2O_2$ extracellularly in the medium. Positive drug controls, ascorbic acid at 20 μg/mL and BHT at 10 μg/mL completely scavenged intracellular ROS (Fig. 4B).

### 3.6. *Effect of BSAE/BSWE on scavenging mechanisms of cellular ROS*

To elucidate the mechanism offered by plant extracts in scavenging intracellular ROS, effect of BSAE/BSWE treatments (concentrations, from Fig. 4B, at which the extracts were effective in scavenging intracellular ROS), cells were treated similarly and subjected to cellular catalase enzyme and total reducing power assays.

On exposure of cells to $H_2O_2$ for a short period, catalase activity was increased by 40% (Fig. 5A) in THP-1 cells. Whereas, catalase activity was increased by two fold in the cells treated with BSWE at 300 μg dwt/mL, with a statistical significance of $p$ value <0.001. BSAE treated cells showed an increase in the enzyme activity by only 40%. Ascorbate (20 μg/mL) increased catalase activity by two folds but BHT (10 μg/mL) inhibited the enzyme by 50% (Fig. 5A).

Total reducing power of the THP-1 cells was rapidly declined by 70% upon treatment with $H_2O_2$ (Fig. 5B). Plant extracts or standard compound treated cells maintained cellular reducing power even after treatment with $H_2O_2$ (Fig. 5B). BSAE at 30 μg dwt/mL sustained cellular reducing power by 80%, BSWE at 300 μg dwt/mL by 70%, standard compounds, ascorbate (20 μg/mL) by 60% and BHT

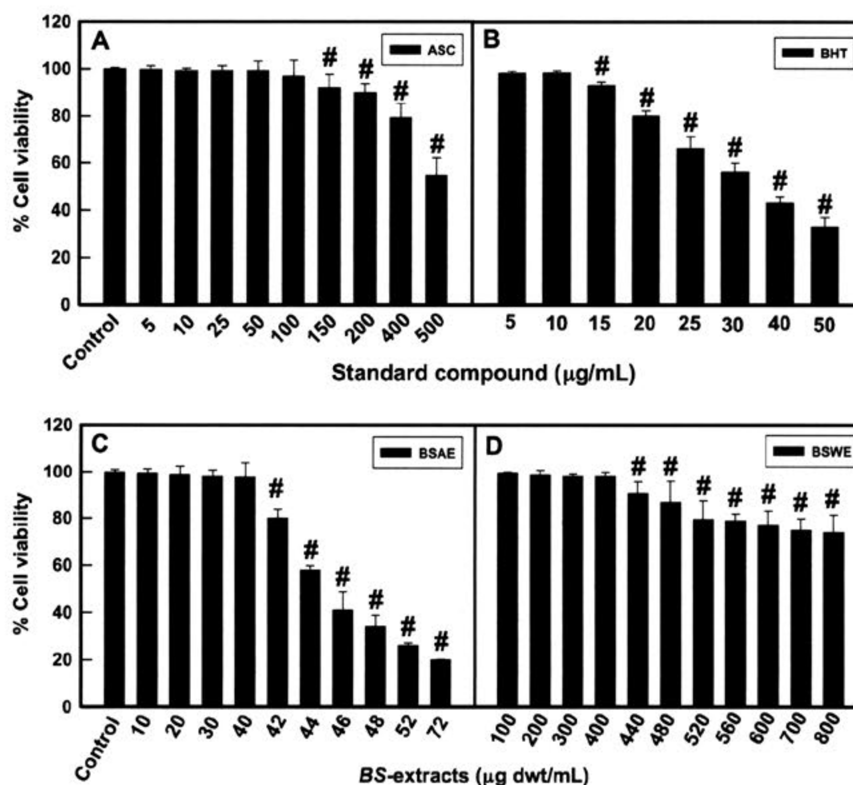


**Fig. 3.** Cell viability was checked by MTT assay after treatment with (A) ascorbate for 1 h, (B) BHT for 24 h, (C) BSAE for 24 h, (D) BSWE for 24 h, at 37 °C and 5% $CO_2$. Media without cells but with respective concentrations of standard compounds or plant extracts were used as appropriate blanks. Data presented are mean ± S.D., $n = 6$.

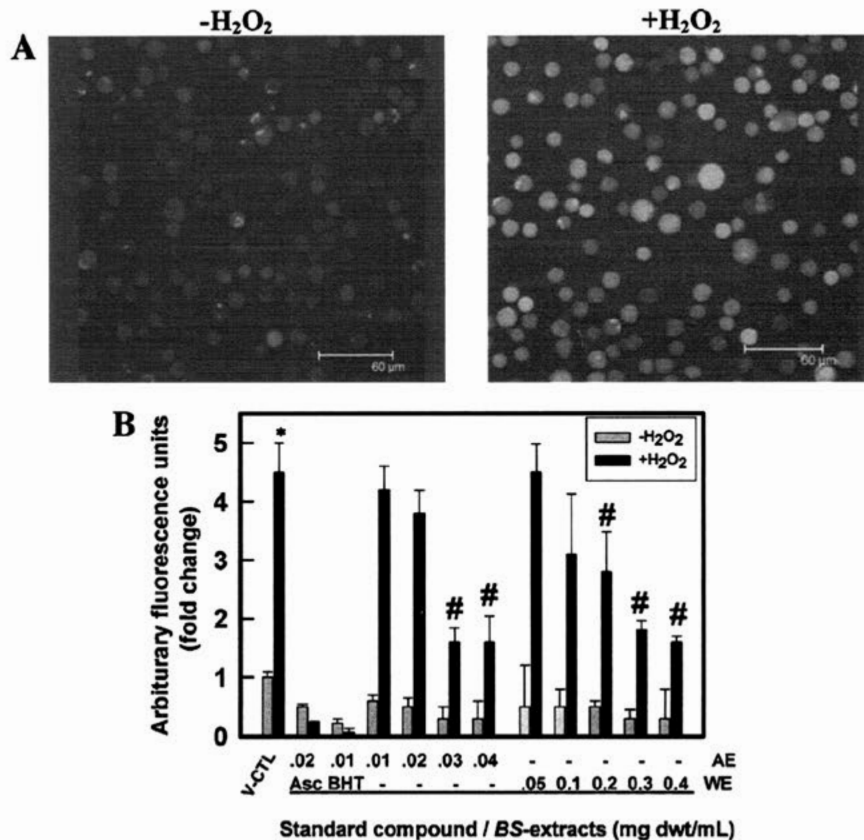


**Fig. 4.** Effect of BS-gum resin extracts on $H_2O_2$ induced ROS generation in THP-1 cells. (A) Confocal images: left panel – control cells – $H_2O_2$; right panel – cells + $H_2O_2$. ROS species were detected by cell permeable fluorescent dye, $H_2$DCFDA, and cells were observed with a fluorescence microscope. (B) Fluorescence intensity of $H_2$DCFDA taken by the cells treated as above was measured by spectrofluorimetry at excitation wavelength of 488 nm and emission wavelength of 525 nm. One-way ANOVA test was performed between the experimental groups represent mean ± S.D. ($n = 4$). *statistical significance of $p < 0.001$ within the control groups i.e. cells + $H_2O_2$ vs cells – $H_2O_2$, #statistical significance of $p < 0.001$ for groups of cells treated with $H_2O_2$ in the presence of BS-extracts vs in the absence of BS-extracts.

(10 µg/mL) by 100% that of $H_2O_2$ untreated cells. These results suggest that the selected plant extracts help the cells in maintaining their total reducing power under oxidative stress conditions and thus help in scavenging excess ROS.

### 3.7. Antiplatelet aggregation and anticoagulant activity of BSAE/BSWE

Addition of 20 µM ADP to human platelets caused the decrease in absorbance units indicating the aggregation of platelets (Fig. 6). In the present study heparin was used as positive drug control, which inhibited ADP-induced platelet aggregation as shown in Fig. 6. BSAE and BSWE markedly inhibited the platelet aggregation in a concentration dependent manner (Fig. 6). Both types of extracts BSAE and BSWE (3 mg dwt/mL) showed high antiplatelet aggregatory activity with p value <0.001 (Fig. 6). Activity of the extracts was comparable to heparin. Thus our results demonstrated antiplatelet aggregatory effect of BS gum resin.

Blood coagulation system, not only initiated by complex coagulation pathways but also involves the interactions between platelets and plasma factors. In this regard, APTT is used to evaluate intrinsic clotting index and PT for extrinsic index, these tests used to identify the coagulation risk factors.

The concentration-dependent effects of BSAE and BSWE on the PT and APTT clot times of human plasma are shown in Fig. 7. Both BSAE and BSWE showed very potent activity in prolongation of clot times. BSAE and BSWE prolonged APTT time period by 2.6 fold and PT time period by 4 folds. Thus our data on prolongation of both PT and APTT clotting times by both types of BS extracts clearly demonstrate anticoagulant property of BS-gum resin.

In order to know target sites of BSAE or BSWE in clotting cascade pathway, we tested the effect of extracts on human clotting factors Xa and XIa. BSAE inhibited both the factors significantly ($p < 0.001$). BSAE at 6 and 8 µg dwt/mL inhibited 70% of FXa (Fig. 8A) and 55% of FXIa (Fig. 8B). BSWE did not show inhibitory effect on FXa and showed about 25% inhibition on FXIa. Heparin showed about 35% on both the factors (Fig. 8A and B).

## 4. Discussion

The interest in natural plant products is on a steep rise due to their increased use of traditional medicine to treat chronic metabolic diseases (Mukherjee et al., 2010). *Boswellia serrata*'s gum resin is one such plant used in Indian Ayurvedic and folk medicine to treat blood disorders, curtail inflammatory diseases like rheumatoid arthritis and to promote cardiac health (Clarisse et al., 2008; Arieh et al., 2010). Present study is designed to investigate the mechanism by which BS-gum resin offers cardiac health. The link between the oxidative stress, inflammation and thrombosis leading to cardiovascular disease is well established (Ross, 1999; Libby, 2002; Boos and Lip, 2006). Therefore, antioxidant and antithrombotic properties of BS-gum resin have been investigated.

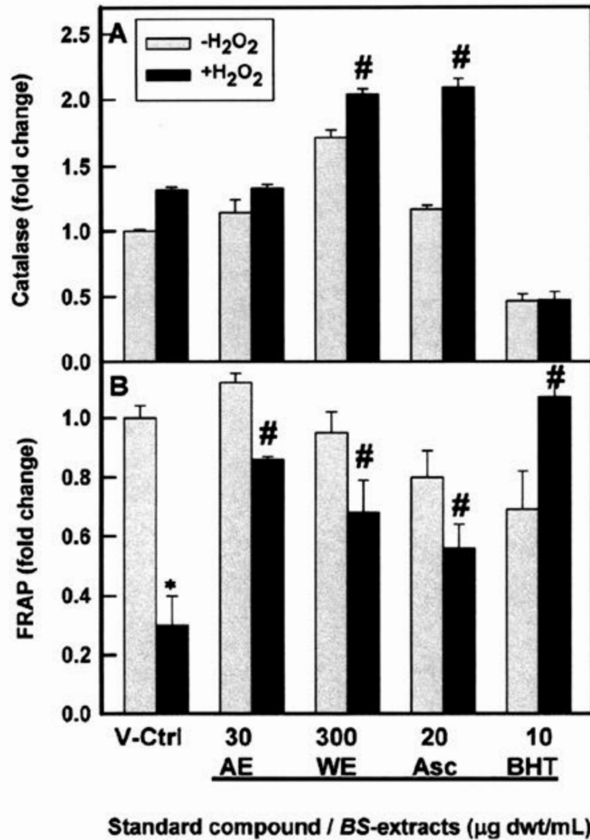


**Fig. 5.** (A) Effect of BS-extracts on catalase enzyme activity in THP-1 cells exposed to $H_2O_2$. Ascorbic acid and BHT serve as positive drug control. (B) FRAP assay performed with cells pretreated with BS-gum resin and induced with $H_2O_2$. Data represented mean ± S.D., $n = 4$; *statistical significance of $p < 0.001$ within the control groups i.e. cells + $H_2O_2$ *vs* cells − $H_2O_2$. #statistical significance of $p < 0.001$ for groups of cells treated with $H_2O_2$ in the presence of BS-extracts *vs* in the absence of BS-extracts.

### 4.1. Antioxidant activity of BS-gum resin

The enrichment of BS-gum resin with triterpenoids (Assimopoulou et al., 2005; Bushra et al., 2007; Magesh et al., 2008) implies their antioxidant activity based on its chemical composition but direct line of evidences for such antioxidant activity was not available. Investigating antioxidant activity of the BS-gum resin during our experiments, BSAE and BSWE showed anti-lipid peroxidation activity when checked with liver and heart

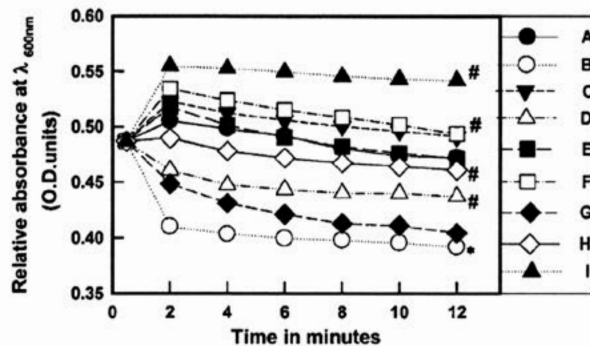


**Fig. 6.** Effect of BS-extracts on ADP induced aggregation of human platelets. (A) PRP; (B) PRP + ADP (20 µM); (C) PRP + Hep (20 µg/mL) + ADP; (D) PRP + BSAE (1 mg dwt/mL) + ADP; (E) PRP + BSAE (2 mg dwt/mL) + ADP; (F) PRP + BSAE (3 mg dwt/mL) + ADP; (G) PRP + BSWE (1 mg dwt/mL) + ADP; (H) PRP + BSWE (2 mg dwt/mL) + ADP; (I) PRP + BSWE (3 mg dwt/mL) + ADP. Data presented are mean ± S.D., $n = 3$. S.D. not seen where S.D. values are within the symbol of data point. #$p < 0.001$ between platelets treated with BS extracts and ADP *vs* treated with ADP alone, *$p < 0.001$ indicates comparison between PRP + ADP *vs* PRP alone.

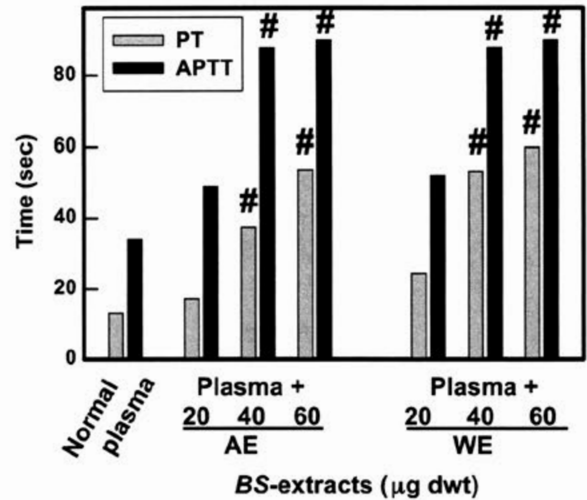


**Fig. 7.** Effects of BS-gum resin on clotting PT and APTT time periods. Data presented are mean ± S.D., $n = 3$. S.D not seen where S.D. values are within the symbol of data point. Statistically significant values are indicated by # with $p < 0.001$.

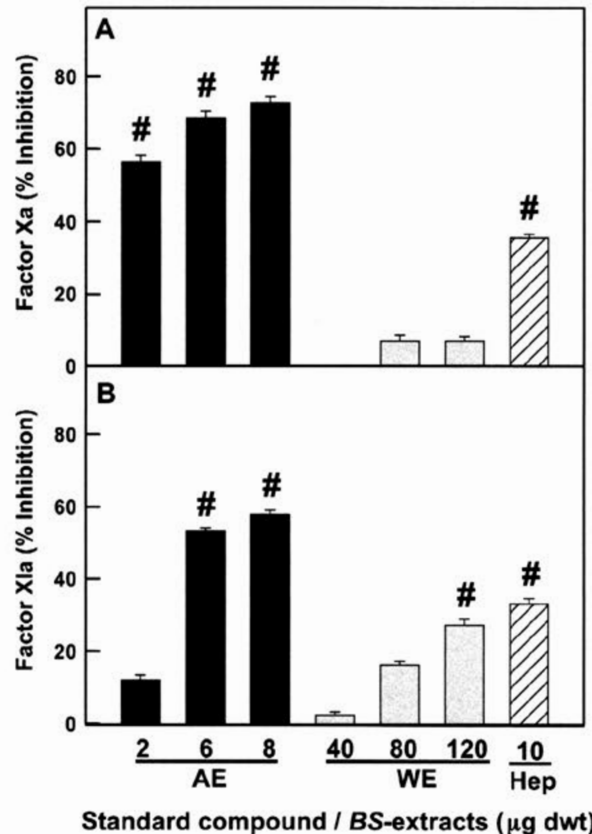


**Fig. 8.** % Inhibitory effect of BS-extracts or heparin on (A) factor Xa and (B) factor XIa. Clotting assays were conducted by monitoring decrease in the absorbance of chromogenic substrate using microplate reader. Values in the bar graphs represent mean ±S.D. ($n = 4$). #$p < 0.001$ highly significant compared with control.

homogenates. These extracts were also capable of scavenging ROS in human monocytic (THP-1) cells (Fig. 4B). Since activated monocytes promote inflammatory cascade of events (Ross, 1999) leading to atherosclerosis. The cellular ROS scavenging activity offered by BSAE and BSWE may help in attenuating the above outlined cascade of events.

BSAE did not show significant effect on catalase where as BSWE significantly increased cellular catalase activity in Thp-1 cells (Fig. 5A). BSAE and BSWE helped Thp-1 cells in sustaining their cellular reducing power to great extent, even on exposure to $H_2O_2$ (Fig. 5B). Therefore, we think that BSAE and BSWE scavenge excess ROS generated by oxidative stress by modulating either cellular reducing power or catalase enzyme activity.

The reasons for higher potential of BSAE ($IC_{50}$: 0.011 mg dwt/mL), that of BSWE ($IC_{50}$: 0.08 mg dwt/mL) in terms of ROS scavenging, cannot be explained based on the analyzed phytomarker, AKβBA. BSAE and BSWE contained AKβBA of 9% (w/w) and 7.8% (w/W) respectively. Detailed chemical composition analyses of these extracts may help in knowing other active metabolites.

### 4.2. Antithrombotic potential of BS-extracts

To further elucidate antiatherogenic potential of BS-gum resin, antiplatelet aggregation and anticoagulant activities of the extracts were examined. Platelets of the blood play an important role in the process of hemostasis. Platelet activation is essential to perform many of their functions, however, activated platelets also has tendency to stick to each other and form aggregates leading to thrombosis and clot formation in the vessel and thus contributes to cardiovascular disease and stroke (Ross, 1999; Boos and Lip, 2006). Inhibition of platelet aggregation and enhancing coagulation time can help to a great extent in the management of atherosclerosis (Ross, 1999). Alternative drugs to heparin are of great interest in view of its limitations and allergic problems (Henry et al., 2009). Therefore, efforts on identifying factor Xa inhibitors (Pinto Donald et al., 2010) and screening herbal resources possessing significant antithrombotic activity with minimal side effects are highly essential (Winston, 1999; Kim et al., 2010). To the best of our knowledge, there are no scientific studies reported on antithrombotic effect of BS-gum resin. In our present study, both BSAE and BSWE inhibited platelet aggregation (Fig. 6). We used ADP to induce platelet aggregation since it is an important endogenous aggregating agent involved in thrombus formation (Park et al., 2004). BSAE and BSWE at 3 mg dwt/mL showed stronger effect in inhibiting platelet aggregation and are as potential as positive drug control, heparin.

Both BSAE and BSWE also enhanced PT and APTT coagulation time periods (Fig. 7). Blood coagulation is not only the result of a complex process initiated by the intrinsic system or the extrinsic system and or/a common pathway, but also a highly regulated process involving interaction between platelets, plasma coagulation factors, and the vessel wall. Anticoagulant drugs are screened using PT and APTT tests (Arif et al., 2007). BSAE at 57 μg dwt and BSWE at 40 μg dwt exhibited maximum of 2.65 fold increase in clotting time period of APTT. The same concentrations increased clotting time periods of PT by four fold. The above concentrations of BSAE significantly inhibited FXa and FXIa and this could be the possible mechanism by which it enhanced the clotting time of both PT and APTT. Where as BSWE did not inhibit FXa but inhibited FXIa by 25%. Mechanism of enhancing PT time period by BSWE needs to be elucidated. Detailed analysis of these extracts on various factors and enzymes involved in clotting cascade may reveal the mechanism of action.

## 5. Concluding remarks

Conclusively, our results indicated that both water and hydroalcoholic extracts of *Boswellia serrata*'s gum resin contain high amounts of AKβBA and other boswellic acids, also contain significant levels of phenolic compounds, which may be responsible for its exhibited high antioxidant and antithrombotic activity. BSAE and BSWE showed cellular ROS scavenging, antiplatelet aggregation and anticoagulation activities. With all these shown wide spectrum of activities of phytomedicine, BS-gum resin can be considered as an effective antiatherogenic resource for preventing coronary artery diseases and may serve as a good source for isolating lead compounds of antiplatelet and anticoagulant therapeutics.

## Acknowledgements

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# *Boswellia serrata*

## An Overall Assessment of *In Vitro*, Preclinical, Pharmacokinetic and Clinical Data

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## Abstract

Non-steroidal anti-inflammatory drug (NSAID) intake is associated with high prevalence of gastrointestinal or cardiovascular adverse effects. All efforts to develop NSAIDs that spare the gastrointestinal tract and the cardiovasculature are still far from achieving a breakthrough. In the last two decades, preparations of the gum resin of *Boswellia serrata* (a traditional ayurvedic medicine) and of other *Boswellia* species have experienced increasing popularity in Western countries. Animal studies and pilot clinical trials

support the potential of *B. serrata* gum resin extract (BSE) for the treatment of a variety of inflammatory diseases like inflammatory bowel disease, rheumatoid arthritis, osteoarthritis and asthma. Moreover, in 2002 the European Medicines Agency classified BSE as an 'orphan drug' for the treatment of peritumoral brain oedema. Compared to NSAIDs, it is expected that the administration of BSE is associated with better tolerability, which needs to be confirmed in further clinical trials.

Until recently, the pharmacological effects of BSE were mainly attributed to suppression of leukotriene formation via inhibition of 5-lipoxygenase (5-LO) by two boswellic acids, 11-keto-β-boswellic acid (KBA) and acetyl-11-keto-β-boswellic acid (AKBA). These two boswellic acids have also been chosen in the monograph of Indian frankincense in European Pharmacopoiea 6.0 as markers to ensure the quality of the air-dried gum resin exudate of *B. serrata*. Furthermore, several dietary supplements advertise the enriched content of KBA and AKBA. However, boswellic acids failed to inhibit leukotriene formation in human whole blood, and pharmacokinetic data revealed very low concentrations of AKBA and KBA in plasma, being far below the effective concentrations for bioactivity *in vitro*. Moreover, permeability studies suggest poor absorption of AKBA following oral administration. In view of these results, the previously assumed mode of action – that is, 5-LO inhibition – is questionable. On the other hand, 100-fold higher plasma concentrations have been determined for β-boswellic acid, which inhibits microsomal prostaglandin E synthase-1 and the serine protease cathepsin G. Thus, these two enzymes might be reasonable molecular targets related to the anti-inflammatory properties of BSE. In view of the results of clinical trials and the experimental data from *in vitro* studies of BSE, and the available pharmacokinetic and metabolic data on boswellic acids, this review presents different perspectives and gives a differentiated insight into the possible mechanisms of action of BSE in humans. It underlines BSE as a promising alternative to NSAIDs, which warrants investigation in further pharmacological studies and clinical trials.

One-third of patients permanently taking non-steroidal anti-inflammatory drugs (NSAIDs) suffer from gastric or duodenal ulcers.[1] In the US, NSAID-induced ulcers and gastrointestinal bleeding in arthritic patients are linked to over 100 000 hospitalizations and 16 500 deaths per year.[2] Furthermore, the annual costs of direct and indirect NSAID-related adverse effects are estimated to exceed $US7 billion in the US.[3] In recent years, highly selective cyclo-oxygenase (COX)-2 inhibitors with an improved gastric tolerability profile have been developed.[4] However, severe cardiovascular adverse reactions and market withdrawals of some coxibs have reduced the initial enthusiasm for this new class of anti-inflammatory drugs.[5,6]

Despite the enormous efforts made in order to develop NSAIDs that spare the gastrointestinal tract, we are still far from achieving a real breakthrough in the discovery of absolutely safe NSAIDs.[7] As a consequence, the interest in alternative, well tolerated anti-inflammatory herbal remedies has re-emerged. Gum resin extracts of *Boswellia serrata* (BSE; Indian frankincense) have been found to represent a promising anti-inflammatory herbal remedy. For centuries, BSE has been traditionally applied in folk medicine to treat various topical and systemic inflammatory diseases. Recent experimental data from animal and human studies support the potential of BSE for the treatment of a variety of inflammatory disorders like inflammatory bowel disease (IBD), rheumatoid arthritis (RA), osteoarthritis (OA) and asthma.[8] In addition, BSE has been found to reduce peritumoral brain oedema accompanying glioma.[9] This is of particular importance, since corticosteroids, which up to now have been indispensable for the control of cerebral oedema, are associated with severe adverse effects. In 2002, the European Medicines Agency classified BSE as an 'orphan drug' for the treatment of peritumoral brain oedema.

In comparison to NSAIDs, it is expected that the administration of BSE is associated with better tolerability, which needs to be confirmed in further clinical trials.[10] Moreover, such extracts are devoid of the typical adverse effects associated with corticosteroids. Based on this background, BSE and preparations from the gum resin of other *Boswellia* species have attracted increasing popularity in Western countries in the last decade.

The pharmacological effects of BSE have been mainly attributed to boswellic acids, especially 11-keto-β-boswellic acid (KBA) and acetyl-11-keto-β-boswellic acid (AKBA), which were proposed as selective 5-lipoxygenase (5-LO) inhibitors.[11] These two boswellic acids have been also chosen in the monograph of Indian frankincense in European Pharmacopoiea 6.0 as markers to ensure the quality of air-dried BSE. However, results from recent studies have questioned the pharmacological relevance of 5-LO inhibition by 11-keto boswellic acids,

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since neither any boswellic acid nor BSE caused significant inhibition of 5-LO in a physiologically relevant assay (i.e. the human whole-blood assay), and oral intake of BSE failed to reduce leukotriene-$B_4$ levels in human subjects.[12]

One of the most important prerequisites for the anti-inflammatory effects of BSE *in vivo* is sufficient availability of the active ingredients. However, following oral administration of BSE, only very low steady-state concentrations of KBA and AKBA in plasma (<0.33 and <0.1 µmol/L, respectively) were observed, whereas 100-fold higher levels were evident for β-boswellic acid.[13] In view of the results of clinical trials and animal studies of BSE, as well as experimental data from *in vitro* studies, and the available pharmacokinetic and metabolic data on boswellic acids, this review presents different perspectives and gives a differentiated insight into the possible mechanisms of action of BSE. There are strong indications that a paradigmatic change in the previous assumption regarding the active principles, as well as the mode of action of boswellic acids and BSE, should be taken into consideration. Thus, instead of 5-LO inhibition by AKBA, inhibition of cathepsin G (catG) and microsomal prostaglandin E synthase (mPGES)-1 by β-boswellic acid might represent the principal mode of action of BSE.

## 1. Composition of *Boswellia serrata* Gum Resin Extract (BSE) and Recommended Dosage

The gum resin is obtained by incision of the stem or branches of *B. serrata*. Following air-drying, the gum resin exudate consists of translucent, roundish or irregularly shaped, variable-size pieces of up to 3 cm. The main components are volatile oils (5–15%), pure resin (55–66%) and mucus (12–23%).[14] The gum resin typically contains 30% boswellic acids.[10]

Boswellic acids are pentacyclic triterpenes, which may exist in an α-configuration (geminal methyl groups at C-20) or a β-configuration (vicinal methyl groups at C-19/C-20). Furthermore, boswellic acids may contain an oxo-moiety at C-11 and an acetyl moiety at the C-3 OH group. Various pharmacological studies indicate that β-configurated derivatives exert stronger bioactivities as compared to the respective α-isomers.[8] The structures of the different boswellic acids are illustrated in figure 1. According to European Pharmacopoiea 6.0, the air-dried gum resin exudate should contain a minimum of 1.0% KBA and AKBA each.

The gum resins from different *Boswellia* species vary in their boswellic acid composition. Whereas the gum resin of Indian frankincense (*B. serrata*) contains quite similar amounts of KBA (3–4.7%) and AKBA (2.2–2.9%), the gum resin of African frankincense (*Boswellia carterii* Birdw.) contains less KBA (0.5%) than AKBA (3.3%). Medicinal products of *B. serrata* were first marketed in India under the trade name Sallaki™ and were later imported into Switzerland under the trade name H15 Gufic™. Sallaki™ or H15 Gufic™ contains 400 mg of *B. serrata* dry extract, which is prepared by extraction of the air-dried gum resin exudate of *B. serrata* with chloroform/methanol, according to a standardized procedure. The obtained dry extract consists of a light-brown or yellowish-brown powder or easily pulverizable yellowish-brown irregular pieces with glassy fissures. As KBA and AKBA were considered to represent the active ingredients of *B. serrata*, they were chosen as markers for the standardization of *B. serrata* dry extract and of H15 Gufic™. Ennet et al.[15] reported that H15 Gufic™ contains, on average, 2.6% KBA and 2.8% AKBA. Other sources report a composition of 3.5% or 2.8% KBA and 1.9% or 2.2% AKBA, as well as 8% acetyl-β-boswellic acid (AβBA), for the standardized products Sallaki™ and H15 Gufic™.[16,17]

Many different *B. serrata* products with differing composition have been subject to clinical investigations. Frequently, the methods used to obtain the gum resin extracts, as well as the

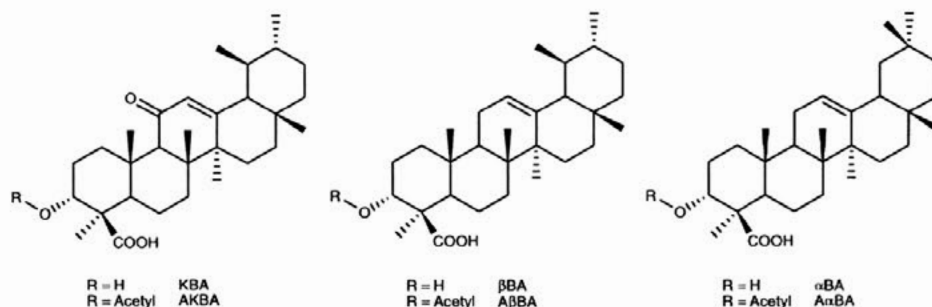


**Fig. 1.** Structures of different boswellic acids. **αBA** = α-boswellic acid; **βBA** = β-boswellic acid; **AαBA** = acetyl-α-boswellic acid; **AβBA** = acetyl-β-boswellic acid; **AKBA** = acetyl-11-keto-β-boswellic acid; **KBA** = 11-keto-β-boswellic acid.

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composition of the extracts, were not disclosed. But wherever the composition was retrievable, we have mentioned it in this review. For example, the test preparation BSE-018 with 262 mg *B. serrata* dry extract/capsule was reported to contain 3.7% AKBA, 10.5% AβBA, 6.1% KBA, 18.2% βBA, 13.2% αBA and 3.3% acetyl α-boswellic acid (AαBA).[18] On the other hand, S. Compound™, another standardized BSE product, contains only 0.63% KBA, 0.7% AKBA and 1.5% AβBA/βBA.[19] In addition, several BSE products are marketed as dietary supplements for patients with arthritis or other inflammation-related disorders. Many of them advertise the enriched content of KBA and AKBA.

In the package insert for H15 Gufic™ it is recommended to take two 400 mg tablets of *B. serrata* dry extract three times daily for the treatment of severe complaints and at the beginning of therapy, otherwise one tablet three times daily. For the treatment of acute complaints, a short-term intake of 3600 mg daily (three tablets three times daily) is advised. For children under 10 years, half the above-mentioned dose is recommended.

Depending on the *Boswellia* products used and the various manufacturer's dosing recommendations, the daily intake of total boswellic acids may vary up to 6-fold (from 18.49 to 109.62 mg/day).[20]

## 2. Pharmacokinetics of Boswellic Acids

In spite of the widespread use of preparations from the gum resin of *Boswellia* species, there are only limited data available on the pharmacokinetic properties of boswellic acids. Nevertheless, the sparse studies clearly indicate that the plasma concentrations of boswellic acids vary markedly between subjects and depend on the pharmaceutical preparation and the conditions of intake. The plasma concentrations obtained for boswellic acids in humans after oral administration are summarized in table I.[13,18,21-23]

Following single oral administration of a relatively low dose of BSE, no αBA, AαBA or AβBA were detected in plasma analysed by high-performance liquid chromatography with UV detection. The lower limit of quantification was about 80 ng/mL, corresponding to 0.16 μmol/L.[18] Only after repeated administration of a relatively high dose of BSE could αBA, AαBA and AβBA be detected in a concentration range of 2.4–4.0 μmol/L. Although these data originate from one patient only, they are an indication of possible accumulation of acetylated boswellic acids lacking the 11-oxo moiety and αBA in plasma. In contrast, much higher plasma concentrations were observed for βBA at steady state in one patient, reaching

**Table I.** Overview of the maximum plasma concentration ($C_{max}$) of boswellic acids (BAs) determined in different studies

| Dosage | Condition | $C_{max}$ of BAs determined in human plasma (μmol/L) | | | | | |
|---|---|---|---|---|---|---|---|
| | | AKBA | KBA | βBA | AβBA | αBA | AαBA |
| BSE 1600 mg PO sd (n = 1)[21] | | NA | 1.9 | NA | NA | NA | NA |
| BSE 4 × 786 mg/day (containing αBA 137.8 mg/g, AαBA 33.7 mg/g, βBA 192.2 mg/g, AβBA 100.4 mg/g, KBA 66.6 mg/g and AKBA 38.1 mg/g) PO for 10 d (n = 1)[13] | Steady state | 0.1 | 0.34 | 10.1 | 2.4 | 3.5 | 4.0 |
| WokVel™ capsule (containing BSE 333 mg) PO sd after a meal (n = 12)[22] a | | ND | 2.72 ± 0.18 | NA | NA | NA | NA |
| Three BSE 800 mg capsules PO od for 4 wk (n = 3)[23] a | Steady state | 0.04 ± 0.01 | 0.33 ± 0.1 | 6.35 ± 1.0 | 4.9 ± 0.5 | NA | NA |
| Three BSE-018 dry extract 282 mg capsules (in total: αBA 103.71 mg, AαBA 26.25 mg, βBA 143.4 mg, AβBA 82.71 mg, KBA 48.12 mg, AKBA 28.71 mg) PO sd in the fasted state and after a high-fat meal (n = 12)[18] b | Fasted state | 0.01 [0.002–0.08] | 0.17 [0.05–0.52] | 0.4 [0.10–3.9] | ND | ND | ND |
| | Fed state | 0.06 [0.03–0.52] | 0.48 [0.21–0.9] | 2.5 [0.91–4.7] | Only a few subjects had measurable plasma concentrations | 0.69 [0.1–2.9] | 0.24 [0.09–0.8] |

a Values are expressed as mean ± standard deviation.

b Values are expressed as mean [range].

**αBA** = α-boswellic acid; **βBA** = β-boswellic acid; **AαBA** = acetyl-α-boswellic acid; **AβBA** = acetyl-β-boswellic acid; **AKBA** = acetyl-11-keto-β-boswellic acid; **BSE** = *Boswellia serrata* gum resin extract; **KBA** = 11-keto-β-boswellic acid; **NA** = not analysed, **ND** = not detected; **od** = once daily; **PO** = orally; **sd** = single dose.

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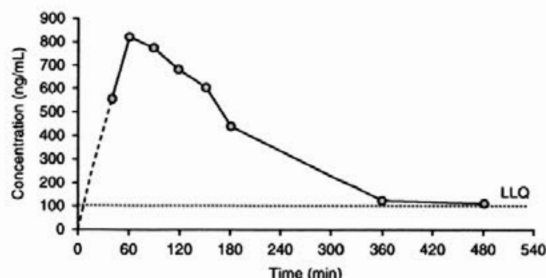


**Fig. 2.** Concentration-time curve of 11-keto-β-boswellic acid (KBA) in human plasma after administration of 1600 mg of commercial *Boswellia serrata* gum resin extracts. **LLQ** = lower limit of quantification.

up to 10.1 µmol/L compared with single administration.[13] The mean concentrations of KBA varied considerably with different formulations, ranging between 0.17 and 2.72 µmol/L. A representative plot of plasma KBA concentration versus time following the single administration of 1600 mg of a commercial BSE product is presented in figure 2.[21]

Recently, a more detailed analysis was performed in a phase IIa trial. Treatment with BSE 800 mg three times daily for 4 weeks revealed average steady-state plasma concentrations of boswellic acids in three patients as follows: βBA 6.35 µmol/L, AβBA 4.9 µmol/L, KBA 0.33 µmol/L and AKBA 0.04 µmol/L.[23] Sterk et al.[18] studied the effect of food intake on the bioavailability of boswellic acids in healthy subjects following the intake of 786 mg BSE. Of note, a 3-fold increase in the maximum plasma concentration ($C_{max}$) of KBA could be achieved when BSE was administered together with a high-fat meal. In the case of AKBA and βBA, a 6-fold increase in the $C_{max}$ was observed. In addition, the area under the plasma concentration time-curve (AUC) under high-fat meal conditions compared with fasted conditions was increased by 272%, 414% and 369% for KBA, AKBA and βBA, respectively.[18] A detailed overview of the pharmacokinetics of KBA, AKBA and βBA is given in table II. We conclude that concomitant food intake together with the administration of BSE is an important aspect that should be considered in daily therapy. In all cases, however, the determined plasma concentrations of KBA and AKBA were found to be far below the effective concentrations required for bioactivity *in vitro* and in animal studies. After 10 days or 4 weeks of treatment with BSE 786 mg four times daily or BSE 800 mg three times daily, the concentrations of AKBA in human plasma were only 0.1 and 0.04 µmol/L, respectively.[13,23]

For a long time, it was assumed that deacetylation of AKBA to KBA is the major cause of the low plasma concentrations of AKBA obtained after oral administration of BSE.[21,22,24] However, recent studies with rat hepatocytes and rats fed with AKBA revealed that deacetylation of AKBA occurs only to a minor extent, not exceeding 2%,[25] excluding this event as the factor responsible for the low plasma concentrations of AKBA. Further attempts to explain the low plasma concentrations of AKBA suggested accumulation in lipophilic tissues because of the high lipophilicity of the compound.[24] In fact, higher brain penetration was observed for AKBA as compared with the less

**Table II.** Pharmacokinetics of 11-keto-β-boswellic acid (KBA), acetyl-11-keto-β-boswellic acid (AKBA) and β-boswellic acid (βBA) after administration of three dry *Boswellia serrata* gum resin extract (BSE) 786 mg capsules as an oral single dose under fasted and fed conditions[18,24]

| Parameter | Values (geometric mean [range]) | |
|---|---|---|
| | fasted state (n = 12) | fed state (n = 12) |
| **KBA** | | |
| $AUC_{\infty}$ (ng • h/mL) | 1660.72 [840.3–3778.1] | 3037.15 [1481.9–6583.1] |
| $AUC_{last}$ (ng • h/mL) | 658.4 [137.0–2447.3] | 2451.8 [1085.0–6125.4] |
| $C_{max}$ (ng/mL) | 83.8 [24.9–243.8] | 227.1 [101.0–418.1] |
| $C_{max}$ (µmol/L) | 0.18 [0.05–0.52] | 0.48 [0.21–0.89] |
| $t_{max}$ (h)[a] | 3.5 [2.0–4.0] | 4.0 [3.0–8.0] |
| $k_e$ ($h^{-1}$) | 0.017 | 0.027 |
| $t_{½}$ (h) | 40.8 | 25.7 |
| **AKBA** | | |
| $AUC_{\infty}$ (ng • h/mL) | 153.6 [59.2–647.9] | 748.9 [271.4–5316.8] |
| $AUC_{last}$ (ng • h/mL) | 47.4 [8.0–232.0] | 243.7 [53.0–3528.0] |
| $C_{max}$ (ng/mL) | 6.0 [0.9–45.7] | 28.8 [13.0–264.5] |
| $C_{max}$ (µmol/L) | 0.01 [0.002–0.09] | 0.06 [0.03–0.52] |
| $t_{max}$ (h)[a] | 2.0 [0.0–24.0] | 3.0 [0.5–60.0] |
| $k_e$ ($h^{-1}$) | 0.066 | 0.046 |
| $t_{½}$ (h) | 10.5 | 15.0 |
| **βBA** | | |
| $AUC_{\infty}$ (ng • h/mL) | 6697.1 [3117.7–190 009.9] | 23 316.7 [6920.7 –57 674.8] |
| $AUC_{last}$ (ng • h/mL) | 4566.3 [857.6–18 640.9] | 21 421.3 [6302.2–51 928.8] |
| $C_{max}$ (ng/mL) | 188.2 [44.0–1810.7] | 1120.1 [413.2–2134.8] |
| $C_{max}$ (µmol/L) | 0.41 [0.10–4.0] | 2.46 [0.91–4.7] |
| $t_{max}$ (h)[a] | 4.0 [0.0–12.0] | 8.0 [8.0–12.0] |
| $k_e$ ($h^{-1}$) | 0.009 | 0.01 |
| $t_{½}$ (h) | 77.0 | 69.3 |

a Median [range].

**AUC** = area under the plasma concentration-time curve; $\mathbf{AUC_{last}}$ = AUC from time zero to last quantifiable concentration; $\mathbf{AUC_{\infty}}$ = AUC from time zero to infinity; $\mathbf{C_{max}}$ = maximum plasma concentration; $\mathbf{k_e}$ = overall elimination rate constant; $\mathbf{t_{½}}$ = terminal elimination half-life; $\mathbf{t_{max}}$ = time to reach $C_{max}$.

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lipophilic KBA, and despite its lower plasma concentrations, the brain-to-plasma ratio for AKBA was determined to be 0.8 compared with 0.5 for KBA. This corresponds to concentrations of 95 and 99 ng/g brain for AKBA and KBA, respectively, following the administration of a single dose of 240 mg/kg of BSE to Wistar rats.[26] Nevertheless, with regard to the high administered dose, the concentrations of AKBA and KBA found in rat brain are still rather low. Beyond the above-mentioned determinations in plasma and rat brain, no detailed compartmental analyses of the distribution of boswellic acids have been performed to date.

Among many factors affecting bioavailability, poor absorption and/or extensive metabolism may play a crucial role in limiting the systemic availability of drugs. Studies on the contribution of extensive first-pass metabolism to the low plasma concentrations of AKBA and KBA revealed that KBA is extensively metabolized in rat liver microsomes (RLMs) and human liver microsomes (HLMs). After only 15 minutes, more than 80% and 60% of the initial KBA concentration were converted to hydroxylated derivatives in RLMs and HLMs, respectively. On the other hand, AKBA was found to be more stable than KBA, with more than 80% of the initial AKBA concentration remaining at the end of the incubation period with RLMs and HLMs, as can be seen in figure 3.[25] In the course of permeability studies carried out using Caco-2 cells (human colon carcinoma cell line), which is a well accepted model to simulate human intestinal absorption *in vitro*, KBA was found to be moderately permeable, whereas AKBA showed poor permeability.[27] On the basis of these results, it may be concluded that the extensive metabolism of KBA strongly contributes to the observed low plasma concentrations and that AKBA is poorly absorbed after oral administration. These results determined *in vitro* were also verified in daily practice. The analysis of plasma samples from patients with glioma taking different BSEs in the course of their treatment revealed steady-state concentrations not exceeding 1.6 μmol/L for KBA and 0.45 μmol/L for AKBA (unpublished observations).

In view of the observed low permeability of the proposed major active principle AKBA, one might not expect any therapeutic efficacy of BSE. However, various clinical trials indicate promising effects of BSE which are apparently unrelated to AKBA but instead might be attributable to the high concentrations of boswellic acids lacking the 11-oxo moiety.

In the following sections, we review the common uses of BSE, taking into consideration the underlying results of clinical trials and animal studies, as well as the experimental data from *in vitro* studies.

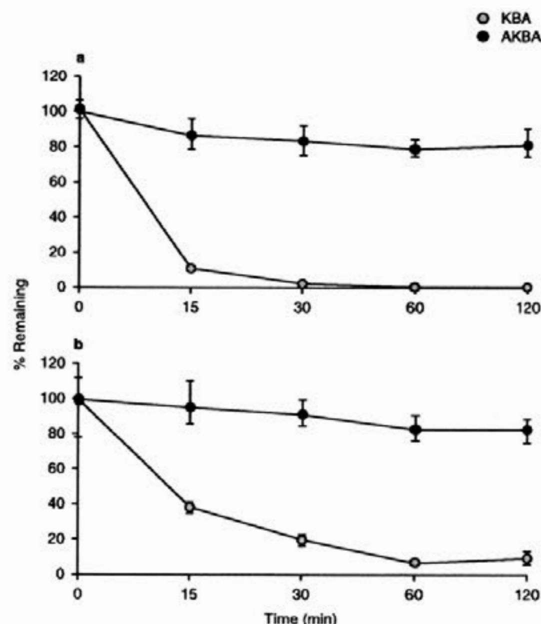


**Fig. 3.** Metabolic stability of 11-keto-β-boswellic acid (KBA) and acetyl-11-keto-β-boswellic acid (AKBA) in (**a**) rat liver microsomes and (**b**) human liver microsomes. The data are presented as mean ± standard deviation.

## 3. Inflammatory Bowel Disease (IBD)

### 3.1 Effects of BSE in Clinical Trials

Based on the anti-inflammatory properties observed in animal models and *in vitro* studies, BSE has been suggested for the treatment of IBD. A number of preliminary clinical trials have been carried out.[16,28-30] An overview of the outcome of these studies is given in table III.

The greatest number of patients were included in the study of Gerhardt et al.,[28] and the results of this trial suggests comparable efficacy of BSE and mesalazine in the treatment of Crohn's disease without statistically significant differences in the mean scores of both groups, using the validated Crohn's Disease Activity Index as a measure. Although no power calculation was conducted, the large sample size may be regarded as sufficient to guarantee actual equivalence between both therapies. Another clinical trial with relatively small numbers of patients suggests promising effects of BSE in the treatment of collagenous colitis.[30] On the other hand, no firm conclusion can be drawn regarding the efficacy of BSE in ulcerative colitis, due to the methodological weakness of the poorly controlled studies, which lacked blinding and randomization, thus increasing the possibility of bias.[16,29]

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As all of these studies were rather explorative, further studies are necessary to confirm the observations made.

### 3.2 Effects of BSE in Animal Models

The observed anti-inflammatory effects of BSE in clinical trials have been also demonstrated in several animal models of IBD, the results of which are summarized in table IV.[17,31,32] Thus, the study applying the indometacin-induced rat model of chronic ileitis suggests that BSE and AKBA at high doses attenuate leukocyte-endothelial cell adhesive interactions.[17] The anti-inflammatory effects were comparable to those achieved by benchmark drugs applied in the treatment of IBD, such as prednisolone and sulfasalazine.[33] Interestingly, an effect of AKBA was only observed at relatively high concentrations equivalent to the total content of all boswellic acids (20%) rather than at test doses equivalent to the concentration of AKBA (2.2%) in the extract.[17]

Also, in dextran sulfate sodium (DSS)-induced colitis, marked protection against colonic inflammation was obtained with semi-synthetic AKBA (sAKBA) after intraperitoneal administration, similar to that observed with H15 Gufic™ in indometacin-induced ileitis.[31] Other studies, however, have been unable to demonstrate any benefit in DSS-induced colitis (responding to mucosal damage) and in trinitrobenzene sulfonic acid (TNBS) colitis (T-cell-mediated reaction) in mice administered dietary supplements with either n-hexane or methanolic BSE.[32] The reasons for these divergent anti-inflammatory responses have not been further studied; however, the different routes of administration resulting in unequal bioavailability might be responsible for these controversial effects. Moreover, the exact dose of the administered BSE is often difficult to retrace.

### 3.3 Effects of BSE on Inflammatory Mediators Relevant for IBD

There are several mechanisms of action that may contribute to the observed effects of BSE in the clinical trials and animal models of IBD. Before addressing these mechanisms of action, the effects of BSE on inflammatory mediators that may play a role in IBD are illustrated.

#### *3.3.1 Cytokines*

The nuclear transcription factor (NF)-κB is a key player in the development and progression of chronic inflammatory diseases, such as IBD, because it plays a central role in the regulation of a wide range of cytokines such as tumour necrosis factor (TNF)-α and interleukin (IL)-1β. In Caco-2 cells transiently transfected with an NF-κB reporter construct, αBA, AβBA, KBA and AKBA at a concentration of 50 μmol/L each increased the activity of NF-κB. Kiela et al.[32] ascribed this effect to the possible involvement of oxidative stress. Moreover, AKBA at a concentration of 50 μmol/L and 100 μmol/L reversed the obviously NF-κB independent proliferative effect of IL-1β, suggesting that AKBA may impair the epithelial restitution in the course of colitis. Note that the applied concentrations of boswellic acids in this *in vitro* study were higher than the respective concentrations of boswellic acids obtained after administration of the highest recommended single dose of 1600 mg BSE.

**Table III.** Overview of the effects of *Boswellia serrata* gum resin extract (BSE) in clinical trials addressing inflammatory bowel disease

| Condition | Study design | Dosage | n | Comments | Reference |
|---|---|---|---|---|---|
| Crohn's disease | 8 wk randomized, double-blind, equivalence study | H15 Gufic™ 1200 mg or mesalazine 1.5 g tid | 102 | 19% drop out. No difference between H15 Gufic™ and mesalazine | 28 |
| Ulcerative colitis | 6 wk non-randomized, open-label, equivalence study | BSE capsule 350 mg or sulfasalazine 1 g tid | 34 and 8 | Improved signs and symptoms in the BSE group vs the sulfasalazine group. No power calculation. Therefore, it is unclear whether lack of significance was due to actual equivalence or an inadequate sample size | 16 |
| | 6 wk non-randomized, open-label, equivalence study | S. Compound™ 300 mg or sulfasalazine 1 g tid | 30 | Improved signs and symptoms in the BSE group vs the sulfasalazine group. No power calculation. Therefore, it is unclear whether lack of significance was due to actual equivalence or an inadequate sample size | 29 |
| Collagenous colitis | 6 wk randomized, double-blind, placebo-controlled study | BSE 400 mg or placebo tid | 31 | Remission higher in the BSE group but no effect of BSE on histology and quality of life | 30 |

tid = three times daily.

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**Table IV.** Overview of the effects of *Boswellia serrata* gum resin extract (BSE) in animal models of inflammatory bowel disease

| Extract/dose | *In vivo* model | Observed anti-inflammatory effects | Reference |
|---|---|---|---|
| BSE as H15 Gufic™ 17.1 and 34.2 mg/kg; AKBA 3.4 and 5.1 mg/kg (corresponding to 20% BAs found in H15 Gufic™) [doses equivalent to those used in human diseases] PO | Indometacin-induced rat model of chronic ileitis | Dose-dependent decrease in number of rolling leukocytes (up to 90%) and adherent leukocytes (up to 98%); attenuation of inflammation at higher dose of BSE and AKBA | 17 |
| sAKBA 5 mg/kg/d IP on d26–d30 | DSS-induced colitis in mice | Significant reduction of leukocyte adherence by 90% and platelet adherence by 92.2%. Effect comparable to 1 mg/kg dexamethasone. In addition, sAKBA prevented P-selectin upregulation associated with DSS-induced colitis | 31 |
| Mouse diet supplemented with 0.1% and 1% hexane (containing 29.9% BAs), and 0.1% and 1% methanol (containing 34.4% BAs) | DSS- and TNBS-induced colitis in mice | Further exacerbation of symptoms and increased histopathology score in the acute phase of DSS-induced colitis and no improvement in the recovery phase. Diet with 1% methanol extract led to pronounced hepatomegaly. Steatosis in HepG2 cells was observed at high doses (50 and 100 μg/mL) of hexane and methanol extracts | 32 |

**AKBA** = acetyl-11-keto-β-BA; **BA** = boswellic acid; **dx** = day x; **DSS** = dextran sulfate sodium; **IP** = intraperitoneally; **PO** = orally; **sAKBA** = semi-synthetic AKBA; **TNBS** = trinitrobenzene sulfonic acid.

On the other hand, lower concentrations of AKBA (3 μmol/L and 10 μmol/L) and AαBA (10 μmol/L) inhibited lipopolysaccharide (LPS)-induced expression of TNFα in monocytes.[34] Also, in human embryonic kidney (HEK)-293 cells, 10 μmol/L AKBA was found to be the most effective inhibitor of NF-κB activation, leading to a 76.9% reduction compared with 40.9% inhibition obtained with 10 μmol/L AαBA.[34] The effect could be mediated by direct inhibitory action on IκBα kinases, which enable NF-κB to enter the nucleus, where it activates TNFα expression.[34] Furthermore, Ammon[24] mentioned inhibition of IL-1β and TNFα production in human monocytes in a concentration range of 5–20 μmol/L. By studying the basis of the anti-inflammatory effects of BSE containing 30% AKBA (25 μg/mL) in human microvascular endothelial cells, it was found that BSE prevented the TNFα-induced expression of matrix metalloproteinases (MMPs). Moreover, the TNFα-induced expression of endothelial vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) was completely abrogated upon pre-exposition to BSE.[35] The anti-inflammatory effects of BSE were further confirmed by Chevrier et al.[36] in murine splenocytes. Thus, gum resin extracts from *B. carterii* in sesame oil at concentrations of 10–200 μg/mL simultaneously inhibited the pro-inflammatory T helper ($T_h$)-1 cytokines IL-2 and interferon (IFN)-γ, and promoted the anti-inflammatory $T_h$2 cytokines IL-4 and IL-10. However, a very small quantity of $T_h$2 cytokines was produced by an extract with a concentration of 200 μg/mL. Together, the interference of BSE with certain cytokines that play critical roles in inflammation (i.e. IL-1β and TNFα, which are targets for therapeutic antibodies in inflammatory diseases) may contribute to the anti-inflammatory actions.

Recently, incensol acetate, a cembrane-type diterpene, was isolated from the gum resin of *B. carterii*. It was found that incensol acetate also attenuated the expression of TNFα, IL-1β, IL-6 and prostaglandin (PG)-$E_2$ in a concentration-dependent manner in human peripheral monocytes exposed to LPS. Furthermore, incensol acetate (50 mg/kg) inhibited the messenger RNA (mRNA) expression of TNFα and IL-1β at 3 hours post-trauma in the mouse model.[37] In contrast, BOS 2000, representing a water-soluble extract from *B. serrata* free from boswellic acids, implied rather immunostimulatory effects. BOS 2000 is obtained by aqueous extraction of the marc remaining after extracting the *B. serrata* gum resin with organic solvents. This aqueous extract contains mainly polysaccharides and is called the biopolymeric fraction. It is composed of 26.9% glucose, 37% arabinose and 33.1% galactose. When applied at a concentration of 10 mg/kg orally to mice it increased the concentration of cytokines (IL-4, IFN-γ and TNFα).[38] Therefore it was suggested as a promising adjuvant for vaccine applications.[39]

#### 3.3.2 5-Lipoxygenase

The enzyme 5-LO catalyses the synthesis of leukotrienes from arachidonic acid (figure 4), which are involved in the pathogenesis of certain inflammatory and allergic disorders.[40] In particular, $LTB_4$, a potent chemotactic factor for neutrophils, has been postulated to play an important role in chronic conditions like IBD.

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5-LO has been proposed as a target for boswellic acids.[24,41] Thus, an ethanolic extract of the gum resin of *B. serrata* in a concentration range between 10 µg/mL and 80 µg/mL inhibited the generation of $LTB_4$ in rat polymorphonuclear leukocytes (PMNLs) after stimulation with the calcium ionophore A23187. Subsequent studies confirmed the inhibition of leukotriene biosynthesis in isolated cells by isolated boswellic acids,[11,42] and AKBA and KBA were most effective, with drug concentration producing 50% inhibition ($IC_{50}$) values of 1.5 µmol/L and 3 µmol/L, respectively. Other boswellic acids like βBA and AβBA revealed only partial inhibition of 5-LO. However, much higher boswellic acid concentrations were required to suppress the activity of crude 5-LO activity in cell-free assays as compared to intact cells. For example, the $IC_{50}$ values for AKBA in the soluble fraction of broken rat PMNLs and for isolated human 5-LO were determined at 8 µmol/L and 16 µmol/L, respectively.[43]

Using the cytosolic fraction of differentiated human promyelocetic leukaemia (HL-60) cells and monocytic Mono Mac 6 cells, the $IC_{50}$ for AKBA was 50 µmol/L.[44,45] The higher potency in cell-based assays as compared to cell-free experiments suggests that inhibition of 5-LO product formation might be due to the interference with additional events regulating 5-LO in the cell.[8] On the one hand, 5-LO requires a certain level of peroxides in order to enter the catalytic cycle, but on the other hand, it is sensitive to oxidants at higher concentrations.[46] In fact, 11-keto-boswellic acids elicited mobilization of $Ca^{2+}$ in PMNLs and activated extracellular signal-regulated kinases and p38 mitogen-activated protein kinase[47,48] and this was associated with the formation of

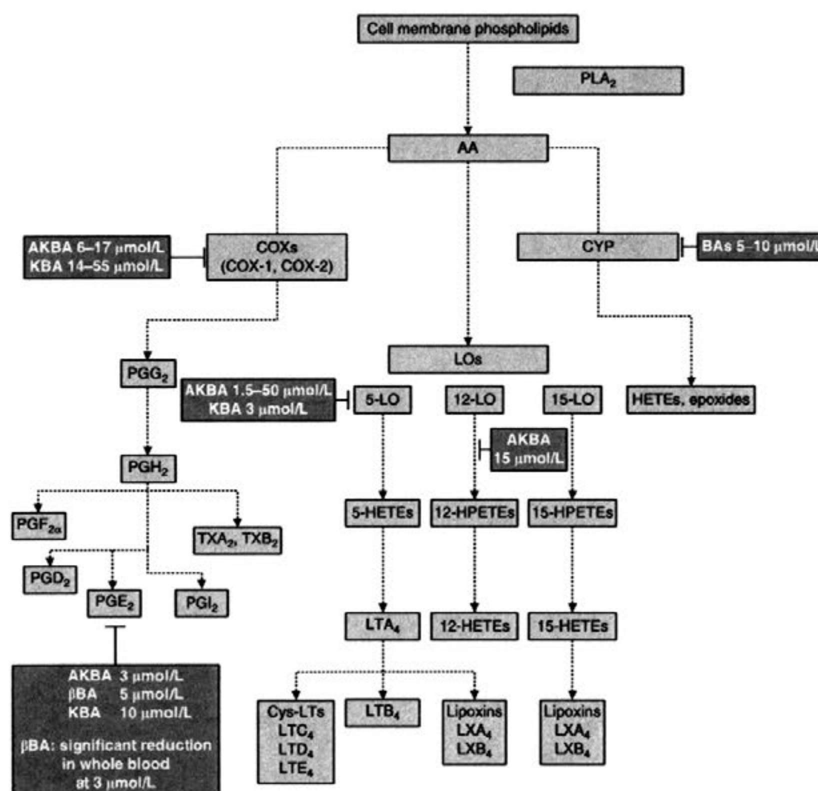


**Fig. 4.** Effects of *Boswellia serrata* gum resin extracts (BSE) on different enzymes of the eicosanoid biosynthetic pathway. 5-Lipoxygenase (5-LO) is the key enzyme in leukotriene (LT) formation, 12-LO catalyses the formation of 12(S)-hydroperoxyeicosatetraenoic acid (12-HPETE) and 15-LO is involved in lipoxin (LX) biosynthesis. Cyclo-oxygenase (COX)-1 and COX-2 catalyse the formation of prostaglandin (PG)-$H_2$, which is converted by cell-specific PG synthase enzymes to biologically active products, including PGs ($PGE_2$, $PGD_2$, $PGF_{2\alpha}$), prostacyclin ($PGI_2$) and thromboxane (TX)-$A_2$. The cytochrome P450 (CYP) oxygenase pathway leads to epoxides and dihydroxyeicosatetraenoic acids (DiHETEs). **βBA** = β-boswellic acid; **AA** = arachidonic acid; **AKBA** = acetyl-11-keto-β-boswellic acid; **BA** = boswellic acid; **KBA** = 11-keto-β-boswellic acid; **$PLA_2$** = phospholipase $A_2$.

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substantial amounts of reactive oxygen species (ROS).[48] As high levels of ROS can irreversibly inactivate the redox-sensitive 5-LO enzyme, Altmann et al.[48] hypothesized that pronounced induction of ROS by 11-keto-boswellic acids may contribute to suppression of 5-LO in intact cells.

A recent study evaluated the interference of boswellic acids with 5-LO, as well as the pharmacological relevance, in more detail.[12] It was found that factors influencing 5-LO activity (i.e. $Ca^{2+}$, phospholipids, substrate concentration) modulate the potency of 11-keto-boswellic acids to inhibit 5-LO, in addition to the effect of ROS.

In conclusion, KBA and AKBA are direct (but relatively poor) inhibitors of 5-LO and additional cellular events evoked by these 11-keto-boswellic acids synergize to more efficiently block 5-LO product formation in the cell.

Interestingly, low concentrations (less than 10 μg/mL) of a gum resin extract from *B. carterii* and *B. serrata* resulted in stimulation of leukotriene formation, with maximum values of up to 218% and 196%, respectively, at 5 μg/mL after stimulation of PMNLs with $Ca^{2+}$ ionophore.[49,50] This agonistic action was attributed to the tetracyclic triterpene 3-oxo-tirucallic acid, which reached its maximum at 10 μmol/L. In contrast, 20 μmol/L 3-oxo-tirucallic acid revealed inhibitory effects.[51] Also, when PMNLs were exposed to 11-keto-boswellic acids and immediately provided with exogenous arachidonic acid, formation of 5-LO products was increased due to induction of an oxidative state in the PMNLs.[48]

### 3.4 Overall Assessment of *In Vitro*, Preclinical, Pharmacokinetic and Clinical Data on IBD

Several studies have suggested that leukotrienes act as mediators of inflammation in IBD.[52-55] Nevertheless, other studies in human IBD have shown that despite a successful decrease in leukotriene levels, disease symptoms were not improved.[56,57]

Concerning the animal models used to assess the anti-inflammatory effects of BSE in IBD, the complete pathogenesis underlying the indometacin-induced ileitis in rats and the DSS-induced colitis in mice has not been completely elucidated. The role of leukotrienes in IBD is particularly controversial. However, there is growing recognition that endothelial activation is an early and rate-determining component of the inflammatory response. An important consequence of endothelial activation is increased expression of glycoproteins that regulate the adhesion of blood cells to the endothelial cell surface and consequently regulate the recruitment of leukocytes into inflamed tissue. VCAM-1, ICAM-1 and P-selectin were found to be dramatically upregulated on the endothelial cells during experimental IBD.[31] The importance of these endothelial cellular adhesion molecules in the pathogenesis of intestinal inflammation is evidenced by reports demonstrating reduced disease activity and tissue injury in mice that are genetically deficient in specific cellular adhesion molecules.

According to Krieglstein et al.[17] the effects observed with BSE in indometacin-induced ileitis may be primarily attributed to the inhibition of leukocyte activation and/or downregulation of endothelial cell adhesion, rather than to leukotriene inhibition. This assumption is further supported by the fact that TNFα-induced expression of endothelial VCAM-1 and ICAM-1 was completely abrogated upon pre-exposition to BSE in human microvascular endothelial cells. This is an interesting finding, as endothelial VCAM-1 is a central player in recruiting leukocytes to sites of inflammation.[35] This is also in line with the observed reduction in leukocyte adherence upon intraperitoneal administration of semi-synthetic AKBA in DSS-induced colitis.[31]

In general, the mode of action of anti-inflammatory drugs in IBD is believed to be based on topical relief in the intestine. Hence, the absorption and systemic availability of boswellic acids may play a secondary role in IBD. Also, sulfasalazine and its metabolite 5-aminosalicylic acid, representing the most prescribed drugs in Morbus Crohn and Colitis ulcerosa, are effective although they are poorly absorbed, with an estimated bioavailability of only 3–12%.[58] In general, it may be assumed that the local concentrations of boswellic acids in the intestine following oral administration substantially exceed those found in plasma. Accordingly, boswellic acids, especially AKBA and KBA, should be available in sufficiently high concentrations in the intestine and might act as 5-LO inhibitors, inhibiting the synthesis of $LTB_4$. However, although early studies demonstrated elevated leukotriene levels in the colonic mucosa of patients with IBD, inhibition of endogenous leukotriene synthesis by selective 5-LO inhibitors did not lead to a therapeutic benefit.[59] Against this background, 5-LO inhibition may not represent the major mechanism of action in humans, leading to therapeutic effects of BSE that are comparable to those of sulfasalazine or mesalazine in clinical trials.

Substantial *in vitro* data suggest that activation of NF-κB is a critical proximal step in the pathogenesis of IBD,[60] and increased NF-κB activity was observed in patients with Crohn's disease compared with controls.[61] Increased NF-κB activity was also associated with high levels of pro-inflammatory cytokines such as IL-1, IL-6 and TNFα in macrophages in IBD.[60,62] AKBA and AαBA inhibited NF-κB in isolated monocytes and HEK cells at a concentration range of 10 μmol/L, which may be readily achieved in the intestinal lu-

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men.[24,34] Moreover, BSE (25 µg/mL ≅ 10 µmol/L) completely abrogated the TNFα-induced expression of VCAM-1 and ICAM-1 in human microvascular endothelial cells.[35] This is another important finding, as upregulation of ICAM-1 has been observed in actively inflamed IBD mucosa, which was in line with elevated serum levels of ICAM-1 and VCAM-1 in patients with active disease.[63] ICAM-1 causes leukocytes to adhere and to migrate beyond the endothelial vessel walls.[64] Animal models[63,64] and a human intervention study[65] have shown that blocking ICAM-1 inhibited intestinal inflammation. Likewise, NF-κB blockade in animal models abolished experimental colitis.[66] Attenuation of IBD symptoms associated with a decrease in rolling and adherent leukocytes was also observed in rats with chronic ileitis upon oral administration of H15 Gufic™ at doses equivalent to those used in human diseases.[17] Thus, it may be concluded that inhibition of leukocyte activation and/or downregulation of ICAM-1 may represent an important mechanism of action of BSE in the treatment of IBD in humans, with AKBA showing the greatest inhibitory activity. Also, dexamethasone, which revealed comparable effects to sAKBA in the DSS-induced colitis model in mice, is known to interact with inflammatory transcription factors like NF-κB, similar to other corticosteroids.[61,67]

In addition, catG may constitute a pharmacological target for BSE in the treatment of IBD diseases (the effects of boswellic acids on catG are described below). CatG is a serine protease stored in azurophilic granules of neutrophils and is released upon degranulation where it cleaves extracellular matrix proteins, implying a role in local destruction of connective tissue at sites of injury. CatG also functions as a chemoattractant for T cells and other leukocytes. In fact, it was shown that luminal catG is involved in the alteration of the colonic epithelial barrier in ulcerative colitis and that the prevalence of anti-catG antibodies in the sera of patients with ulcerative colitis was significantly higher during the active than the inactive phase of the disease.[68,69] However, there are also indications that there is no remarkable association of anti-neutrophil cytoplasmic antibodies with target antigens like catG in IBD.[70] Therefore, the role of catG in generating and/or amplifying relapses in ulcerative colitis and its importance as a relevant target of BSE in the treatment of IBD remains to be investigated. Based on the current knowledge about effects of boswellic acids *in vitro*, the reduction in leukocyte adherence in several animal studies, and the positive therapeutic effects comparable to sulfasalazine and mesalazine in clinical trials, inhibition of NF-κB and ICAM-1 by boswellic acids likely underlies the beneficial effects of BSE in the treatment of IBD.

## 4. Arthritic Diseases

### 4.1 Effects of BSE in Clinical Trials

In traditional Indian ayurvedic medicine, preparations of gum resins from *B. serrata* have been used for centuries to treat arthritic diseases. Several pilot clinical trials have been carried out.[71-74] An overview of the outcome of these studies is given in table V. In the case of RA, contradicting reports are available. No measurable efficacy was observed in the study of Sander et al.[71] Because no power calculation was performed, it is not clear whether the sample size of this study was chosen adequately to identify true differences. Moreover, the study suffers from the drawback that only the effect of BSE without comparison to standard therapy was tested. Therefore, the results cannot be considered definitive. On the other hand, cumulative findings of 11 unpublished studies reported by Etzel[75] suggest that H15 Gufic™ is effective in improving chronic symptoms like joint swelling and stiffness but not in relieving pain. These studies, ranging from 1 to 6 months, were conducted between 1985 and 1990 and included more than 260 patients in total. The designs varied from direct observation (most common) to placebo-controlled and double-blind studies.[10,75] Unfortunately, the primary data from each and every study are not available and the majority of the evidence was derived from non-controlled studies and is therefore only of limited value.

Two randomized, double-blind, placebo-controlled clinical studies were carried out with ayurvedic combination products that include BSE, revealing no statistical difference compared with placebo for RA-1® but promising effects for Articulin-F®.[76,77] However, in these combination products, the effects of BSE itself cannot be assessed.

In contrast, the effects of BSE observed in clinical trials in OA patients are more definite. Statistically significant improvements in pain and physical function were observed in BSE-treated patients compared with placebo or baseline. Moreover, a recent study with 5-Loxin®, a BSE product enriched with 30% AKBA, was found to inhibit the cartilage-degrading enzyme MMP-3 in the synovial fluid of OA patients. As a result of the trial, 5-Loxin® reduced pain and improved physical functioning significantly in OA patients, and may improve joint health by reducing the enzymatic degradation of cartilage.[74]

Altogether, despite the poor-quality design of some arthritic studies, clinical data favour the therapeutic efficacy of BSE in treatment of RA and OA. Hence, further trials should be carried out to assess the potential of BSE in the treatment of arthritic diseases in more detail. In view of these considerations,

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**Table V.** Overview of the effects of *Boswellia serrata* gum resin extract (BSE) in clinical trials addressing rheumatoid arthritis (RA) and osteoarthritis (OA)

| Condition | Study design | Dosage | n | Comments | Reference |
|---|---|---|---|---|---|
| RA | 12 wk double-blind, placebo-controlled, pilot study in outpatients with active RA | Nine tablets of H15 Gufic™ 400 mg/d (total 3600 mg/d) or placebo in addition to previous therapy. Doses of NSAID could be adjusted on demand | 37 | No measurable efficacy of H15 Gufic™. Dose reduction of NSAID in 5.8% of the H15 Gufic™ group and in 3.1% of the placebo group. Reduction of NSAID dose was statistically non-significant. No effect on pain. Methods of randomization and blinding were not well described. Results cannot be considered definitive. Effect of BSE alone vs standard therapy was not studied | 71 |
| OA (knee) | 8 wk randomized, double-blind, placebo-controlled, crossover study | Three WokVel™ (BSE 333 mg) capsules tid or placebo. After wash-out, alternate treatment | 30 | Improved pain, flexion, walking distance after 8 wk in the BSE group ($p < 0.001$) | 72 |
| | 6 mo randomized, open-label, equivalence study | Three WokVel™ (BSE 333 mg) capsules tid or valdecoxib 10 mg od after meals | 66 | In the BSE group, significant difference in WOMAC score (pain, stiffness, difficulties in performing daily activity) vs baseline only after 2 mo of therapy ($p < 0.001$), which persisted 1 mo after discontinuation of therapy. In the valdecoxib group, significant difference in WOMAC score after 1 mo ($p < 0.001$) of therapy but benefit deteriorated after discontinuation. At the end of 7 mo the WOMAC scores with BSE were significantly lower ($p < 0.001$) than with valdecoxib for all three parameters | 73 |
| | 90 d randomized, double-blind, placebo-controlled study | 5-Loxin® (BSE enriched with 30% AKBA) 100 mg/d or 250 mg/d, or placebo | 75 | Both doses of 5-Loxin® potentially improved VAS, LFI, WOMAC pain, WOMAC stiffness and WOMAC functional ability scores addressing pain, joint stiffness and physical function in OA patients vs placebo ($p < 0.0001$). Concentration of the proteolytic enzyme MMP-3 in synovial fluid ($p < 0.001$) and VAS pain score ($p = 0.01$) were significantly lower in the high-dose group vs the low-dose group. Improvement was observed 7 d after starting treatment | 74 |

**AKBA** = acetyl-11-keto-β-boswellic acid; **LFI** = limb function index; **MMP** = matrix metalloproteinase; **NSAID** = non-steroidal anti-inflammatory drug; **od** = once daily; **tid** = three times daily; **VAS** = visual analogue scale; **WOMAC** = Western Ontario McMaster Osteoarthritis Index.

the effects of BSE in diverse animal models of inflammation and arthritis should be explored as well.

### 4.2 Effects of BSE in Animal Models

The anti-inflammatory effects of BSE have been tested in a variety of acute and chronic animal models of inflammation and arthritis.[24,78-88] The results are summarized in table VI.

It was shown that alcoholic extracts of the gum resin of *B. serrata* revealed considerable anti-inflammatory effects in acute test models like carrageenan- and dextran-induced rat- and mouse-paw oedema, as well as in chronic test models of arthritis like adjuvant-induced polyarthritis in rats and bovine serum albumin (BSA)-induced arthritis in rabbits.[24,78,79] Drugs that are active in these models include corticosteroids and NSAIDs. However, since the anti-inflammatory effect of BSE was also observed in adrenalectomized rats, liberation of glucocorticoids as a mode of action was excluded. This is further supported by the fact that BSE shows only weak inhibitory activity in the cotton pellet-induced granuloma test in rats, which is considered to be more sensitive to corticosteroid-type drugs.[78] Compared to NSAIDs, BSE did not exert analgesic or antipyretic effects.[79] Moreover, the positive effects of BSE observed in Freund's adjuvant-induced rat-paw oedema suggest that at least part of the anti-inflammatory effects could be linked to a favourable modulation of the immune system.[24,80]

In contrast, no anti-inflammatory effects were observed with an aqueous extract of gum resin from *B. carterii* Birdw. in carrageenan-induced rat-paw oedema.[89] On the other hand, the acetone extract of the same species was effective in a Freund's adjuvant-induced rat-paw oedema model.[80] This observation was attributed to the seemingly poor water solubility of the active principles.

Also, in animal models assessing anti-arthritic activity, such as formaldehyde- and adjuvant-induced arthritis in rats,[78,81] and in BSA-induced arthritis in rats and rabbits,[78,86] BSE revealed positive effects, reducing the total leukocyte count in synovial fluids and resolving symptoms like lameness, local pain and stiff gait in dogs with arthritis.[88]

Unfortunately, plasma concentrations of boswellic acids have been never determined in connection with these animal

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studies, and thus no correlation can be drawn between the observed effects of the extracts and the plasma concentrations of the individual boswellic acids. Only one pharmacokinetic study in Wistar rats depicted (very low) maximal plasma concentrations in the range of 0.3–0.5 μmol/L and 0.18–0.22 μmol/L for KBA and AKBA, respectively, following the single administration of 240 mg/kg BSE.[26] As no compartmental analysis of the distribution of boswellic acids has been carried out in animal models, no data exist to date on the availability of boswellic acids in different compartments.

**Table VI.** Overview of the effects of *Boswellia serrata* gum resin extract (BSE) in animal models of inflammation and arthritis

| Extract/dosage | *In vivo* model | Observed anti-inflammatory effects | References |
|---|---|---|---|
| Methanolic BSE 50–200 mg/kg PO od | Carrageenan-induced oedema in rats and mice | Inhibition of paw volume by 26–43% in rats and 20–34% in mice | 24,78,79 |
| | Dextran-induced oedema in rats | Inhibition of oedema by 21–51% | |
| | Cotton pellet-induced granuloma test in rats | Weak inhibitory action | |
| | Formaldeyde arthritis in rats | Inhibition of paw volume by 23–44% | |
| | Gouty arthritis in dogs | Reduction of total leukocyte count by 16–42% in synovial fluid; moderate to marked improvement in gait | |
| Drug treatment started on d14 and terminated on d28 | Adjuvant-induced established arthritis in rats | 32–50% inhibition on d14 with marked inhibition of secondary lesions | |
| | Adjuvant-induced developing arthritis in rats | 34–52% inhibition on d28 | |
| Methanolic BSE 25–100 mg/kg PO od | BSA-induced arthritis in rabbits | Reduction of leukocyte count by 18–48% in synovial fluid | |
| Methanolic BSE 50–200 mg/kg PO od | Carrageenan-induced pleurisy in rats | Reduction of exudate volume by 19–25% and total leukocyte count by 36–44% | |
| 70% aqueous acetone extract of *Boswellia carterii* 0.45–0.9 g/kg/d PO (IG) for 7 d | Freund's adjuvant-induced rat-paw oedema | Significant reduction in paw oedema vs control, in addition to lengthening paw withdrawal latency | 80 |
| Aqueous acetone extract of *B. carterii* 0.9 g/kg PO (IG) for 10 d | Adjuvant arthritis in Lewis rats | Significant reduction of arthritic scores, paw oedema and local TNFα and IL-1β vs control | 81 |
| Mixture of BAs 100 mg/kg PO | Papaya latex-induced rat-paw inflammation | Inhibition of inflammation by 41% (3 h) vs control | 82 |
| Mixture of BAs 50–150 mg/kg PO | Papaya latex-induced rat-paw inflammation | Inhibition of inflammation by 19.9% with 50 mg/kg dose, 26.7% with 100 mg/kg dose and 29.7% with 150 mg/kg dose, vs control | 83 |
| BAs, lupeolic acids, tirucallane-type acids isolated from methanolic extract of *B. carterii* | TPA-induced ear inflammation in mice | $ID_{50}$ of all tested compounds: 0.05–0.49 mg/ear | 84 |
| Alcoholic BSE 50–200 mg/kg | Carrageenan-induced pleurisy in rats | Significant reduction of volume of pleural exudate vs control and inhibition of infiltration of PMNLs into the pleural cavity | 85 |
| Alcoholic BSE 25–100 mg/kg PO and local injection (5–20 mg/knee) | BSA-injected knee of rat | Significant reduction of total leukocyte count at 50 and 100 mg/kg oral doses and after local injection | 86 |
| Acetyl BA mixture (50% AβBA, 37% AKBA, 5% AαBA, 5% other terpinoids) 20 mg/kg IP od for 21 d | Experimental autoimmune encephalomyelitis in guinea pigs | Reduction of experimental symptoms between d11 and d21 | 87 |
| BSB108 400 mg/10 kg PO od for 6 wk | Dogs with OA and degenerative conditions | Resolution of intermittent lameness, local pain, stiff gait | 88 |

**AαBA** = acetyl-α-BA; **AβBA** = acetyl-β-BA; **AKBA** = acetyl-11-keto-β-BA; **BA** = boswellic acid; **BSA** = bovine serum albumin; **dx** = day *x*; **$ID_{50}$** = dose that produces 50% inhibition; **IG** = intragastrically; **IL** = interleukin; **IP** = intraperitoneally; **OA** = osteoarthritis; **od** = once daily; **PMNL** = polymorphonuclear leukocyte; **PO** = orally; **TNF** = tumour necrosis factor; **TPA** = 12-*O*-tetradecanoylphorbol-13-acetate.

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### 4.3 Effects of BSE on Inflammatory Mediators Relevant for Inflammation and Arthritis

Besides NF-κB (a key player in the development and progression of chronic inflammatory diseases, such as RA and atherosclerosis), PGs and leukotrienes represent important inflammatory mediators that mediate inflammatory symptoms. Since the effects of boswellic acids on cytokines and 5-LO have been discussed in detail before, in the following section, the focus is placed on other lipid mediators that have been analysed as possible targets for the action of boswellic acids with regard to their anti-inflammatory properties.

#### *4.3.1 Lipid Mediators*

Eicosanoids including PGs, leukotrienes and lipoxins are lipid mediators derived from arachidonic acid via the COX, LO or cytochrome P450 (CYP) epoxygenase pathway, as illustrated in figure 4. The COX and 5-LO pathways are of particular importance during inflammation, because their products are involved in the pathogenesis of many inflammatory diseases.

As mentioned before, 5-LO was initially proposed to be the main target for boswellic acids, explaining the anti-inflammatory effects of BSE. However, all boswellic acids (50 μmol/L each), as well as BSE, failed to inhibit 5-LO product formation in a human whole-blood assay.[12] In the presence of albumin (10 mg/mL), 5-LO inhibition by AKBA 30 μmol/L in neutrophils was abolished, apparently due to strong albumin binding (>95%) of AKBA. Finally, a single dose of BSE (800 mg, administered orally) failed to suppress $LTB_4$ plasma levels in human healthy subjects.[12] Hence, in view of these findings, along with the low plasma concentrations of 11-keto-boswellic acids (submicromolar) obtained after oral administration of BSE, the pharmacological relevance of interference with 5-LO *in vivo* seems questionable.[12]

For a long time, it was considered that there is no interference of AKBA with other arachidonic acid metabolizing enzymes like 12-LO or COX. However, it was shown recently that AKBA suppressed platelet-type p12-LO product formation with an $IC_{50}$ of 15 μmol/L in cell-free assays, whereas βBA and AβBA stimulated p12-LO activity. In intact platelets, βBA and AβBA also stimulated the formation of the 12-LO product 12(S)-hydroperoxyeicosatetraenoic acid [12-HPETE] in a concentration-dependent manner, in contrast to keto-boswellic acids, which were hardly effective.[50]

Recently, studies with washed human platelets revealed a concentration-dependent inhibitory action on COX-1 product synthesis by AKBA (with $IC_{50}$ values in the range of 6–17 μmol/L) and by KBA (with $IC_{50}$ values of 14–55 μmol/L), depending on the stimulus used. In contrast, boswellic acids without the 11-oxo moiety were less potent COX-1 inhibitors, with $IC_{50}$ values >100 μmol/L. COX-2 was less efficiently inhibited by all of these boswellic acids.[90] Moreover, boswellic acids were shown to inhibit the activity of various CYP enzymes like CYP2C8/2C9/3A4 ($IC_{50}$ 5–10 μmol/L) that also metabolize arachidonic acid, leading to epoxyeicosatrienoic acids.[91,92]

Taken together, these results showed that AKBA is a non-selective inhibitor of a variety of enzymes that metabolize arachidonic acid, and there is still no proof of inhibition of 5-LO *in vivo*.

#### *4.3.2 Cathepsin G and Human Leukocyte Elastase*

Human leukocyte elastase (HLE) is a serine protease released from PMNL upon activation in a variety of inflammatory and hypersensitivity-based human diseases. It was found that AKBA decreased the activity of HLE *in vitro*, with an $IC_{50}$ of about 15 μmol/L.[93] Substantial inhibition was also observed with βBA 20 μmol/L. In another study, HLE inhibition by AKBA was shown to be only moderate, with $IC_{50}$ values >30 μmol/L.[23]

CatG inhibitors are proposed to exhibit potential in treating certain inflammatory disorders such as asthma, chronic obstructive pulmonary disease (COPD), emphysema, reperfusion injury, psoriasis and RA. Using a boswellic acid-affinity Sepharose matrix, catG was identified as a high-affinity boswellic acid-binding protein. In rigid automated molecular docking experiments, boswellic acids tightly bound to the active centre of catG, occupying the same part of the binding site as a synthetic catG inhibitor. Boswellic acids potently suppressed the proteolytic activity of catG (the $IC_{50}$ for AKBA was 0.6 μmol/L) in a competitive and reversible manner.[23] Note that βBA and AβBA also inhibited catG, with $IC_{50}$ values of 0.8 and 1.2 μmol/L, respectively. KBA inhibited catG, with an $IC_{50}$ of 3.7 μmol/L. Moreover, boswellic acids inhibited catG-mediated chemoinvasion of challenged neutrophils and suppressed catG-induced $Ca^{2+}$ mobilization in humans. Finally, oral administration of defined BSE significantly reduced catG activities in human blood *ex vivo* versus placebo.[23] In conclusion, catG is a functional and pharmacologically relevant target of boswellic acids, and interference with catG could explain at least some of the anti-inflammatory properties of BSE.

### 4.4 Overall Assessment of *In Vitro*, Preclinical, Pharmacokinetic and Clinical Data on Rheumatoid Arthritis and Osteoarthritis

In the frame of various *in vitro* tests, boswellic acids exerted inhibitory activity on cytokines (e.g. TNFα, IL-1β) and pro-

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inflammatory enzymes (catG, 5-LO, p12LO, COX-1, CYP-2C8/2C9/3A4) in a concentration range between 0.6 and 55 μmol/L. The most pronounced inhibitory effects were determined for AKBA on catG, with an $IC_{50}$ of 0.6 μmol/L.

A prerequisite for any desired pharmacological effect *in vivo* is sufficient availability. However, the plasma concentrations, ranging between 0.002 and 0.52 μmol/L for AKBA, and 0.05 and 2.72 μmol/L for KBA, were revealed to be very low with respect to the concentrations needed to efficiently inhibit the proposed targets. Of course, it cannot be excluded that KBA and AKBA, being lipophilic substances, may be present in higher concentrations in other intra- and extracellular compartments, where they may reach high local concentrations (intracellular) or from which they are then continuously and slowly released (extracellular) into the blood circulation. In fact, a high volume of distribution (142.97 L)[24] and a long terminal elimination half-life ($t_{1/2}$) of 40.8 hours[18] were reported for KBA, indicating possible accumulation in lipophilic tissues. This, besides the extensive metabolism of KBA, may result in a reduced availability of KBA in plasma. For AKBA, high protein binding (>95%)[12] and a $t_{1/2}$ of 10.5 hours[18] have been reported. Moreover, the poor permeability of AKBA determined in the Caco-2 model suggests poor absorption following oral administration. Unfortunately, the compartmental distribution of KBA and AKBA has not yet been determined, making it difficult to estimate the local availability of boswellic acids for their targets in inflammatory tissues or synovial fluids. However, the extensive metabolism and possible accumulation of KBA in fat tissues, high protein binding and poor absorption of AKBA suggest reduced availability of KBA and AKBA for pharmacological targets like 5-LO, COX-1, p12-LO and NF-κB.

On the other hand, the $IC_{50}$ values for catG inhibition of 0.8 μmol/L for βBA and 1.2 μmol/L for AβBA determined *in vitro* are easily achieved after oral administration of BSE, with steady-state plasma concentrations ranging between 6.35 and 10.1 μmol/L for βBA, and between 2.4 and 4.9 μmol/L for AβBA. Although no data on the metabolism, permeability and compartmental distribution of βBA and AβBA exist yet, preliminary pharmacokinetic data indicate several-fold higher $C_{max}$ and AUC values of βBA compared to AKBA, suggesting that catG may be a functional target of boswellic acids with pharmacological relevance.

CatG is also released by macrophages during RA-associated inflammatory and angiogenic events.[94] Furthermore, considerable catG monocyte chemotactic activity has been reported in the synovial fluid of RA patients, which was partially decreased by treatment with inhibitors of catG, indicating a role of catG in the pathogenesis of RA synovial fluid inflammation.[95] Moreover, catG was found to induce leukocyte migration and infiltration into inflamed tissues. Accordingly, the interference of boswellic acids with catG could explain some of the anti-inflammatory properties of BSE and the observed reduction of leukocyte counts in several arthritic animal models. However, the importance and value of catG as a therapeutic target in arthritic diseases has not been entirely assessed to date and needs to be further investigated.[23]

Of note, results from clinical studies using 5-LO inhibitors (i.e. zileuton) and animal studies on 5-LO-deficient mice or 5-LO-activating protein-deficient mice excluded a role of leukotrienes in RA.[46,96] These results contradict the long-lasting assumption that leukotrienes are important mediators of inflammation in RA.[97]

On the other hand, $PGE_2$, synthesized in the joints of arthritic patients, is a prostanoid with a pivotal role in inflammation and the beneficial anti-inflammatory effects of NSAIDs are essentially related to suppression of $PGE_2$ formation.[98] $PGE_2$ is synthesized from $PGH_2$ by PGE synthase (PGES), enzymes which act downstream of COX enzymes. Several isoforms of PGES were characterized, among which mPGES-1 has received the greatest attention, because this enzyme is highly upregulated by pro-inflammatory stimuli, with concomitantly increased expression of COX-2. Thus, upregulation of mPGES-1 mRNA and protein expression was observed in rat paws in acute (carrageenan-induced) and chronic (adjuvant-induced) arthritis models, which was also associated with elevated $PGE_2$ levels. Consistent with these findings, increased mPGES-1 expression is also seen in the synovial tissue and serum of patients with RA and OA.[98-100]

We recently found that boswellic acids suppressed the transformation of $PGH_2$ to $PGE_2$ mediated by inducible mPGES-1 in microsomal membranes of A549 cells in a reversible manner and essentially independent of the substrate concentration.[101] AKBA, βBA and KBA were most efficient, with $IC_{50}$ values of 3, 5 and 10 μmol/L, respectively, thus being almost equipotent to MK-886. Also, in intact A549 cells, AKBA, βBA and KBA suppressed $PGE_2$ synthesis in a concentration-dependent manner (the $IC_{50}$ was approximately 10–30 μmol/L) while AβBA was hardly effective. Moreover, βBA 3 μmol/L was shown to significantly reduce LPS-induced $PGE_2$ formation in whole-blood experiments. In particular, βBA – the major boswellic acid present in BSE, reaching the highest plasma concentrations (up to 10 μmol/L) among the boswellic acids in humans – was associated with the most potent $PGE_2$ suppression. Finally, intraperitoneal and oral administration of βBA (1 mg/kg) to rats reduced the development of carrageenan-induced pleurisy, accompanied by reduced $PGE_2$ levels in respective inflammatory exudates *in vivo*.[101]

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Based on these findings, one may speculate that the beneficial effects of BSE observed in anti-inflammatory and arthritic animal models, as well as in clinical studies of OA and RA, are related to the interference with $PGE_2$ synthesis due to direct inhibition of mPGES-1. Such speculations are favoured by the close correlation between steady-state plasma concentrations of βBA in humans obtained after oral administration of BSE and the effective mPGES-1 inhibitory concentration determined for βBA *in vitro*. In fact, it was shown that mPGES–/– mice exhibit reduced inflammatory responses in collagen-induced arthritis compared with wild-type mice.[98] Also, animals receiving 2B5, a $PGE_2$-neutralizing monoclonal antibody, developed less arthritis. In carrageenan-induced paw inflammation, pretreatment of rats with 2B5 substantially prevented the development of tissue oedema and hyperalgesia in affected paws.[102] In each model, the anti-inflammatory effects afforded by selective $PGE_2$ neutralization were equivalent to those obtained in animals treated with indometacin, supporting the pivotal role of $PGE_2$. By inhibiting mPGES-1, and thus specifically blocking the $PGE_2$ synthesis, BSE should not interfere with the synthesis of other COX-derived eicosanoids *in vitro* and *in vivo* that may have important physiological ($PGI_2$, thromboxane) or anti-inflammatory ($PGD_2$) functions.[101]

Based on the above-mentioned data it seems reasonable to suggest that CatG and mPGES-1 may serve as the major pharmacological targets for boswellic acids, especially for βBA. If this assumption proves true in future studies, then BSE may represent a promising candidate for the anti-inflammatory treatment of OA and RA, without exerting the severe adverse effects associated with NSAIDs or corticosteroids.

## 5. Asthma

### 5.1 Effects of BSE in Clinical Trials

The data currently available on the action of BSE in asthmatic diseases are scarce. Only one explorative clinical trial, where BSE revealed significant improvement compared with placebo, has been reported.[19] In this 6-week, double-blind, placebo-controlled study, 80 patients were randomized to receive either S. Compound™ 300 mg or lactose (placebo) 300 mg three times daily. Whereas improvement was observed in only 27% of the patients in the placebo group, 70% of the patients in the BSE group showed improvement of the disease. This was manifested by a statistically significant increase in the forced expiratory volume in 1 second ($FEV_1$), forced vital capacity (FVC) and peak expiratory flow (PEFR) in the *Boswellia* group compared to the placebo group ($p=0.0001$), suggesting a definite role of BSE in the treatment of bronchial asthma. However, future studies should assess the long-term efficacy of BSE and compare it with standard therapies.

### 5.2 Effects of BSE in Animal Studies

To date, BSE has only been tested on general models of acute inflammation – such as carrageenan-and dextran-induced oedema in rats, cotton pellet-induced granuloma in rats, carrageenan-induced pleurisy in rats[78] and papaya latex-induced rat-paw inflammation[82,83] – and models of chronic inflammation, such as Freund's adjuvant-induced rat-paw oedema.[80] In all of these models, BSE revealed promising anti-inflammatory properties. However, BSE has not been tested yet on specific animal models of asthma, thus making the evaluation of specific anti-asthmatic effects in animal models difficult. Since the inflammation caused by carrageenan is attributed to the release of histamine besides PGs, and since the allergic response plays a major role in the progression of asthma, the effect of BSE on histamine has been investigated.[103]

### 5.3 Effects of BSE on Histamine

Histamine is released by mast cells and is a well known mediator in type 1 allergic reactions.[24] It was shown that a BSE containing 60% AKBA revealed promising mast cell-stabilizing activity in rats. Whereas the extract administered orally at a dose of 20 mg/kg failed to offer any protection, 40 and 80 mg/kg doses exhibited 20% and 30% protection, respectively, compared with 62% protection obtained with 50 mg/kg disodium cromoglycate administered intraperitoneally. Also, dose-dependent inhibition of passive paw anaphylaxis was observed, which, however, turned out to be much weaker than oral dexamethasone 0.27 mg/kg.[103]

In addition to histamine, leukotrienes and CatG may play a role in the progression of asthma. The effects of BSE on these inflammatory mediators have been discussed in detail above.

### 5.4 Overall Assessment of *In Vitro*, Preclinical, Pharmacokinetic and Clinical Data on Asthma

In contrast to IBD and RA, the role of leukotrienes as important inflammatory mediators in asthma has been well established.[59,104] Clinical studies have demonstrated the therapeutic value of anti-leukotriene therapy with CysLT receptor antagonists and a 5-LO inhibitor.[59] Nevertheless, this mechanism of action is unlikely to be attributed to BSE, as discussed above. Also, the mechanism of mPGES-1 inhibition, which seems plausible in arthritic diseases, plays rather a minor role in asthma.

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**Table VII.** Overview of the effects of *Boswellia serrata* gum resin extract (BSE) in clinical trials addressing peritumoral brain oedema

| Condition | Study design | Dose | n | Comments | Reference |
|---|---|---|---|---|---|
| Peritumoral brain oedema in glioma patients | Prospective, open-label study of H15 Gufic™ | 14 patients received 1200 mg tid, 9 patients received 800 mg tid and 5 patients received 400 mg tid for 7 d. No dexamethasone was allowed during the study | 28 | Most pronounced reduction in oedema (33.61%) in the highest-dose group. No effect in the lowest-dose group. No antiproliferative effect | 106 |
| Intracranial tumours | Median 9 mo palliative therapy with H15 Gufic™ | Maximum dose 126 mg/kg/d | 19 | Anti-oedemateous effect in 1 patient. 5 of 19 children reported improvement in general health status. 3 of 17 patients with malignant tumours showed a mainly transient improvement of neurological symptoms. Increased muscle strength in 3 further patients. No antiproliferative effect | 107 |
| | Up to 6 mo therapy with H15 Gufic™ | 1200 mg tid | 12 | Reduction of oedema in 2 of 7 patients with glioblastoma and in 3 of 5 patients with radiochemotherapy-related leukoencephalopathy | 108 |

**tid** = three times daily.

In the lung, as opposed to many other parts of the body, $PGE_2$ is involved in limiting the immune-inflammatory response and exerting protective activity in lung inflammation.[105]

It is known that bronchoconstriction in asthma is mainly ascribed to $PGD_2$ and histamine, which are released predominantly by mast cells.[104] Therefore, mast cell stabilizers are assigned a great role in reducing asthma symptoms. *In vitro* BSE at a dose of 40 mg/kg, which is equivalent to dosages in humans, revealed potent mast cell-stabilizing effects.[103] This may be a reasonable mechanism by which BSE might exert its positive effects in asthmatic disease.

Moreover, catG is also thought to contribute to asthma and especially to COPD, by triggering an array of harmful reactions, including matrix degradation, leukocyte recruitment and tissue remodelling. In fact, catG inhibitors were suggested for treatment of asthma, for which BSEs were proposed to have therapeutic benefits. However, since data from clinical trials with catG inhibitors are still missing, it is difficult to evaluate the contribution of catG inhibition to the observed anti-inflammatory effects observed with BSE.[12] In summary, more studies are needed before a definitive conclusion can be drawn with regard to an adequate mechanism of action of BSE in asthma.

## 6. Peritumoral Brain Oedema

### 6.1 Effects of BSE in Clinical Trials

Based on the positive effect of BSE in the prospective study of Böker and Winking,[106] the European Medicines Agency classified BSE as an orphan drug for the treatment of peritumoral brain oedema. In this explorative study a dose-dependent reduction of peritumoral brain oedema was observed in glioblastoma patients. Furthermore, two more studies confirmed this observation, suggesting positive effects of BSE in the treatment of peritumoral brain oedema.[107,108] The results are summarized in table VII. However further studies are necessary to adequately evaluate the potential of BSE in reducing peritumoral brain oedema and to prove the observed effects.

### 6.2 Effects of BSE in Animal Models

In order to elucidate the effects of boswellic acids, Wistar rats were treated with BSE for 14 days after the inoculation of C6 tumour cells into the caudate nucleus. BSE at 240 mg/kg was able to inhibit tumour progression. Furthermore, the highest dose of 240 mg/kg doubled the survival time of rats with glioma compared with controls.[9]

### 6.3 Effects of BSE on Inflammatory Mediators Relevant for Peritumoral Brain Oedema

The effect of BSE on 5-LO, which has been suggested as a relevant pathophysiological mechanism for peritumoral brain oedema formation,[9] has been discussed in detail above.

### 6.4 Overall Assessment of *In Vitro*, Preclinical, Pharmacokinetic and Clinical Data on Peritumoral Brain Oedema

Until recently, conventional malignant glioma therapy (surgery, radiation therapy and chemotherapy) did not yield satisfying results.[9] Adjuvant therapy is necessary to improve

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quality of life and to reduce neurological disability and morbidity resulting from peritumoral brain oedema. In a clinical trial on glioblastoma patients, BSE at a dosage of 1200 mg three times daily showed a considerable reduction of peritumoral brain oedema, which was associated with improvement of the clinical conditions of the patients.[106] The positive effects of BSE were suggested to be due to inhibition of leukotrienes, without providing a clear proof of principle. However, the concentrations of AKBA, the most potent 5-LO inhibitor, were found to be very low in the brain.[26] Therefore, inhibition of 5-LO by boswellic acids might be regarded as questionable.

PGs – in particular, $PGE_2$ – have also been shown to play a role in the pathogenesis of a number of biological processes, such as inflammation, autoimmune diseases and oncogenesis. It was found that microglia (the macrophages of the CNS), but not glioma cells, produced high levels of $PGE_2$.[109] In an attempt to test whether the observed COX-2-dependent formation of $PGE_2$ in microglia played a role in brain oedema formation in gliomas, tumour-bearing rats were treated with rofecoxib, a selective COX-2 inhibitor.[109] Rofecoxib inhibited $PGE_2$ production and abolished the diffusion of contrast material from the tumour into brain parenchyma, suggesting a reduction in the blood-tumour barrier (BTB) permeability. This decrease in BTB permeability was similar to that achieved by dexamethasone, which is clinically used to reduce brain oedema in patients with malignant brain tumours. These findings may provide an explanation for the reduction of oedema in patients with glioblastoma and radiochemotherapy-related leukoencephalopathy, as well as for the improvement in neurological symptoms in the above-mentioned clinical trials on intracranial tumours.[107,108] Certainly, interference with $PGE_2$ formation by boswellic acids as an underlying mechanism of action of BSE in reduction of oedema is a vague assumption at the moment and needs further investigations *in vitro*, in animal studies and in clinical studies.

## 7. Safety of *Boswellia serrata*

Currently, no approved medicinal product is available on the market which might provide pharmacovigilance data. Furthermore, no data from phase III clinical trials are available to adequately evaluate the safety of BSE. However, based on observations made in the frame of the above-mentioned clinical trials, adverse effects of BSE were consistently rare and were judged as not causally related to the treatment and not markedly different from those noted in the placebo group. Thus, in the included trials, no serious, long-term or irreversible adverse effects were noted. Only diarrhoea, abdominal pain and nausea were reported in more than one study.[74,110] Moreover, it was shown in animal models that boswellic acids possess a dose-dependent anti-ulcer effect against different experimental models. Thus, the ulcer score against various ulcer-inducing agents was inhibited by 31–51% at 250 mg/kg.[111] Of course, the observed low incidence of adverse effects has to be confirmed in further clinical trials with greater numbers of patients. However, the previous insights suggest BSE to be well tolerated with fewer adverse effects as compared with NSAIDs.

## 8. Conclusions

The anti-inflammatory effects of BSE have been tested in a variety of *in vitro* studies, animal models and clinical trials. The obtained data suggest promising beneficial effects in the treatment of inflammatory diseases like IBD, RA, OA and asthma, as well as in the reduction of peritumoral oedema. Even if no medicinal product is approved yet, preparations containing BSE marketed as dietary supplements are often used by patients in the frame of the above-mentioned inflammatory diseases. For many of these products, the enriched content of KBA and AKBA is advertised, and these boswellic acids are also mentioned in the European Pharmacopoieia as markers to ensure the quality of BSE.

At present, various data exist on the obtained plasma concentrations of boswellic acids and the CNS availability of KBA and AKBA after oral administration of BSE, which in turn are helpful to understand the mechanism of action of BSE. Unfortunately, data regarding the distribution of boswellic acids in various compartments are lacking, probably because of the difficult experimental approaches to obtain such values.

For a long time the anti-inflammatory effects were attributed to the inhibition of 5-LO by AKBA and KBA. However, detailed studies applying pharmacologically relevant test systems excluded this mechanism and pharmacokinetic data revealed no clear correlation between the plasma concentrations of AKBA and KBA and the effective concentrations needed to inhibit 5-LO. Thus, AKBA, which is considered to be the most potent 5-LO inhibitor, is very low in plasma following oral administration, mainly because of its poor absorption. On the other hand, much higher steady-state plasma concentrations were determined for βBA in humans (6.35–10.1 μmol/L) treated with BSE, which are clearly in the range of the concentrations effective on catG and mPGES-1 *in vitro* ($IC_{50}$ values of 0.8 and 4 μmol/L, respectively). Based on these interrelations and additional results, interference with catG and/or mPGES-1 might thus represent a reasonable molecular mechanism related to the anti-inflammatory properties of BSE. Acting downstream of

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COX enzymes as a selective producer of $PGE_2$, mPGES-1 is gaining more and more importance as a potential new therapeutic target for anti-inflammatory treatment. The novel insights into the mPGES-1 and catG inhibitory activities of boswellic acids draws the attention of further research activities to βBA which, in contrast to previous assumptions, might constitute the active principle of BSE.

Based on the current data, BSE might be regarded as a promising alternative to NSAIDs, with multiple targets and modes of action, and warrants further investigation in clinical trials and pharmacological studies. In view of the previously available results of clinical trials, the experimental data from *in vitro* studies, and the pharmacokinetic and metabolic data on boswellic acids, novel thoughts have emerged that may be of importance for future studies to confirm the therapeutic efficacy of BSE on a large scale and to answer the important question as to which actions are central and essential for the specific anti-inflammatory effects of boswellic acids in various inflammatory diseases.

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# Coumarins: Old Compounds with Novel Promising Therapeutic Perspectives

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**Abstract:** Natural as well as synthetic coumarins have recently drawn much attention due to its broad pharmacological activities. Many coumarins and their derivatives exert anti-coagulant, anti-tumor, anti-viral, anti-inflammatory and anti-oxidant effects, as well as anti-microbial and enzyme inhibition properties. The recognition of key structural features within coumarin family is crucial for the design and development of new analogues with improved activity and for the characterization of their mechanism of action and potential side effects. The different substituents in the coumarin nucleus strongly influence the biological activity of the resulting derivatives. Although some coumarins have been already characterized to evoke a particular biological activity, the challenge would be the design and synthesis of new derivatives with high specific activity for other pharmacological targets and define their mechanism of action to achieve new therapeutic drugs. The present review highligts the current progress in the development of coumarin scaffolds for drug discovery as novel anti-cancer agents. The major challenges about coumarins include the translation of current knowledge into new potential lead compounds and the repositioning of known compounds for the treatment of cancer.

**Keywords:** Coumarins, structure-activity relationship, lead compound, drug development.

## BACKGROUND

Over a century ago, Crum-Brown and Frasser proposed that the physiological action of a substance was linked to its chemical composition and constitution [1]. In the last decades considerable progress has been made regarding the isolation, synthesis, pharmacokinetics, pharmacology and toxicology of coumarins. As most studies are unrelated, a comprehensive review of current literature would be a valuable contribution towards the discovery, development or resurgence of biologically active coumarin derivatives with application in diverse human diseases. The present review summarizes the key structural features of this family and its related properties, with particular emphasis on cancer.

From a chemical standpoint, coumarin (2*H*-1-benzopyran-2-one) is the parent compound of the coumarin family, a large class of naturally occurring phenolic compounds. Coumarin could be considered like the resulting fusion of benzene and a 2-pyrone ring. In nature, the heterocyclic ring is oxygenated at C-7 and less frequently at C-5, C-6 and C-8. These extra phenolic hydroxyls groups are sometimes derivatized as glycosides. The oxygenation patterns mentioned above are typical for benzenoid rings of C6-C3 units derived from the shikimic acid pathway. Compared with alkaloids synthesized through shikimic acid, there is a remarkably large number of compounds in which the nucleus is alkylated by one or more isoprenoid units [2]. In general, it can be established that this family of compound obey Lipinski's rule of five and exhibit cell membrane permeability, which are common characteristics found in most available drugs today [3].

Based on the substitution pattern, coumarins show anti-coagulant, anti-tumor or antiviral properties whereas other derivatives behave as enzyme inhibitors or display anti-oxidant or anti-inflammatory properties. Although the coumarin system can be considered as one of the most important classes of heterocyclic compounds, based on *in vivo* experiments in rats, coumarin was banned from the market by the Food and Drug Administration in 1952. Since then a dispute over its toxicity has been raised [4]. Several reports point out that the toxicity of coumarin is metabolism and species dependent [5, 6]. Therefore, the evaluation of coumarin cytotoxicity in humans based on studies performed in rabbits or rats seems rather inapropiate. Several authors reported that coumarin compounds show no evidence of initiating tumors in different animal models [7]. Furthermore, coumarin and its derivatives are not mutagenic in the AMES or micronucleus tests [8, 9], and fail to exibit teratogenic properties [4].

Over the last 50 years coumarin compounds have been widely used as anti-coagulant, anti-microbial and anti-inflammatory agents supported by different clinical studies. Nevertheless, these compounds or their analogues have also emerged as promising drugs for cancer. In the present review selected examples will be discussed to illustrate the progress made in the development of natural and synthetic coumarins as potential anti-tumor agents.

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## WARFARIN, FROM AN ANTI-COAGULANT TOWARDS AN ANTI-TUMORAL AGENT

Oral anticoagulants of the 4-hydroxycoumarin class, such as warfarin (Fig. **1A**), represent the most commonly prescribed drug for therapy and prevention of thromboembolic conditions for over 50 years. It was designed followed the identification of dicoumarol as the causal agent of a haemorrhagic disorder affecting cattle after consuming spoiled sweet clover hay [10]. Although the anticoagulants of the 4-hydroxycoumarin class exhibit high efficacy and are rather inexpensive, their narrow therapeutic index may sometimes complicate patient management [11].

4-Hydroxycoumarins inhibit vitamin K epoxide reductase (VKORC1) [12], leading to deficiency of vitamin K and subsequent deficiency of vitamin K-dependent proteins, including those involved in thrombus formation. The minimal structural requirements for the anticoagulant activity of the 4-hydroxycoumarin class, represented by warfarin, are an intact 4-hydroxycoumarin residue and a carbon chain in position 3 [13]. Recently, Gebauer *et al.*, demonstrated that the *in vitro* inhibition of VKORC1 requires deprotonation of the 4-hydroxycoumarin moiety whereas the substituent on carbon 3 modulates the inhibition, being more potent those derivatives with an isoprenyl side chain. Thus 4-hydroxycoumarins would bind to the active site of the enzyme mimicking a transition state [14].

Recent studies point to warfarin as a promising drug for cancer treatment. However, a few randomized trials have addressed the therapeutic efficacy of these anticoagulant agents in cancer [15]. Therefore, it is not possible to determine whether the possible benefit of anticoagulation results from an effect on the clotting system, a direct cytotoxic activity of the anticoagulant, or a change in the pharmacokinetics of the cytotoxic drugs caused by the anticoagulant. A study by McCulloch *et al.*, suggests that warfarin may inhibit tumour metastasis without affecting growth rate of tumour cells *in vitro* at concentrations below 1 mM [16]. In accordance, Velasco-Velazquez *et al.*, reported that 4-hydroxycoumarin, which lacks anticoagulant activity since it is unsubstituted on carbon 3, selectively disorganizes the actin cytoskeleton in a highly invasive melanoma cell line [17, 18]. These findings indicate that 4-hydroxycoumarin might be useful in metastasis and melanoma therapy. Furthermore, it highlights the fact that different molecular shapes are responsible for the anti-coagulant and the anti-tumoral activity.

Other studies suggest that dicoumarol [an anticoagulant coumarin; 3,3'-methylenebis(4-hydroxycoumarin)] (Fig. **1B**) and its analogues may inhibit cell proliferation by interfering with the spindle microtubule dynamics [19]. There is growing interest to design combinations of antimitotic coumarins and chemotherapeutic agents to improve efficacy and lower toxicity, such as taxol and dicoumarol, which results in a synergistic inhibition of cell division [20]. However, recent data published by Buey *et al.*, showed that dicoumarol fail to stabilize microtubule in carcinoma cells [21].

Nowadays, there is renewed interest in determining whether anticoagulation therapy may improve the survival of oncology patients. In addition, the pharmacomodulation of anticoagulant coumarins have led to the development of novel analogues which inhibit the formation of experimental metastases.

## NOVOBIOCIN, FROM GRAM-POSITIVE BACTERIA TOWARDS CANCER CELLS

Although most of the natural coumarins have been isolated from plants, the aminocoumarin antibiotics novobiocin, chlorobiocin and coumermycin A1 were isolated from diverse *Streptomyces spp* and exhibit a potent activity against Gram-positive bacteria. These compounds target the bacterial enzyme DNA gyrase and inhibit the enzyme-catalyzed hydrolysis of ATP [22].

Novobiocin bears a carbamoylated sugar residue, a 3-amino-8-methyl-4,7-dihydroxycoumarin moiety (ring B) and an isopentenyl-substituted hydroxybenzoyl moiety (ring A) (Fig. **2**). Both, the ring B and the sugar residue are involved in ATPase inhibition at the B-subunit of DNA gyrase. Examination of the binding site of novobiocin reveals an extensive hydrogen bonding network, involving especially the novobiose sugar. It appears that the coumarin ring is crucial in directing the sugar moiety to the appropriate site whereas the ring A moiety would influence the uptake of the compound into bacterial cells [23-25]. Their poor oral absorption as well as their ability to develop resistance limit the use of aminocoumarins. In the past years several studies were conducted to design effective orally bioavailable coumarin antibiotic inhibitors of bacterial DNA gyrase [26-28].

Lately, the heat shock protein 90 (Hsp90) emerged as a promising target for cancer therapy [29] and novobiocin analogues gathered the attention of researchers since structure-activity relationship (SAR) studies showed that these coumarins bind to the Hsp90 C-terminal ATP binding site and induce degradation of Hsp90 client proteins [30-32].

In order to establish coumarin compounds that could differentiate between the C-terminus of Hsp90 and DNA gy-

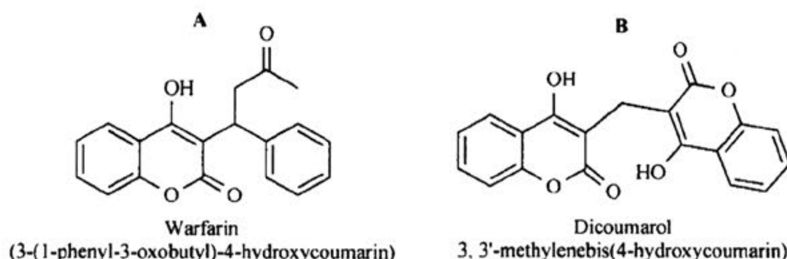


**Fig. (1).** Warfarin and dicoumarol, the parent compounds of the anticoagulant coumarins.

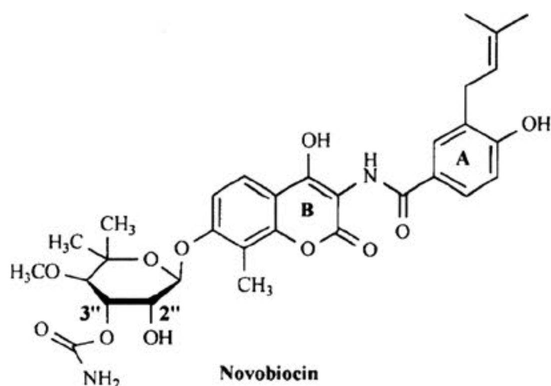


**Fig. (2).** Novobiocin, an aminocoumarin antibiotic.

rase, a library of novobiocin analogues was designed to convert a well-established gyrase inhibitor into a selective Hsp90 inhibitor. Studies show that the elimination of the 4-hydroxy group of the coumarin ring and the 3''-carbamate of the novobiose residue are necessary to achieve derivatives with a higher selective activity for the Hsp90 protein. These findings suggest that the 2'',3''-diol of the novobiose appendage, the novobiose moiety to the 7-position and an amide linker at the 3-position of the coumarin ring play a critical role for anti-Hsp90 activity [33, 34]. Donnely *et al.*, conducted an extensive work to further explore novobiocin derivatives with variations in the coumarin scaffold. These coumarin-derived motifs possess hydrogen bond acceptors placed at positions 5-, 6- and 8- of the coumarin ring and analogues bearing modification of the coumarin lactone. The authors showed that the secondary amide at the position 3 is required for the antiproliferative activity and substituents as *o*-propoxy and methoxy at 6- and 8-position, respectively, lead to an increased activity. The lactone moiety of coumarins may provide beneficial hydrogen bonding interactions with the binding pocket. However, these interactions may not be required to manifest antiproliferative activity, suggesting that the coumarin scaffold just acts like a connecting structure between the sugar and the benzamide motifs [35]. On the other hand, another studies demonstrated that the removal of the novobiose moiety in novobiocin together with the introduction of a tosyl substituent at C-4 or C-7 of the coumarins provides novel lead structures with a 1000-fold increase in activity and enhanced rates of cell death, by stimulation of the extrinsic apoptosis pathway due to the activation of caspases 7, 8 and cleavage of the poly-(ADP-ribose)-polymerase (PARP) [36, 37].

These are the first set of coumarins designed to target the Hsp90 protein for cancer treatment. However, further studies are needed to achieve improved analogues in order to confirm whether the coumarin structure is essential as scaffold for Hsp90 inhibition in cancer cells.

## OLD AND NEW COUMARIN COMPOUNDS AS POTENTIAL ANTI-CANCER DRUGS

Cancer therapy depends on the type of tumor, its location and extension. Radiation and chemotherapy (e.g. apoptosis induction) are the most conventional therapeutic modalities used but they are frequently associated with the development of drug resistance and systemic toxicity. In the last decades alternative cancer therapies like differentiation therapy, angiotenesis inhibition and hormone or tyrosine kinase inhibition were developed.

### Apoptosis-Inducer Agents

Chemotherapy is the treatment of cancer with anticancer drugs, and its main purpose is to eliminate cancer cells. Necrosis and apoptosis are two experimentally distinguishable mechanisms of cell death whereas the term cytotoxicity simply refers to the cell-killing property of a chemical compound without defining a specific cellular death mechanism. In the literature, a considerable number of reports show that diverse simple coumarins exert cytotoxicity in various cancer cell lines and experimental animal models of cancer. However, the mechanism through which most of these compounds induce cell death in these models remains to be established.

Among the simple coumarins with pro-apoptotic properties, esculetin (**1**) exhibits anti-proliferative effect by inducing apoptosis in human leukemic cells [38] or in 3T3-L1 adipocytes in a time-dependent manner [39]. Moreover, it enhances taxol-induced apoptosis in human hepatocellular carcinoma cells (HepG2) [40]. The treatment with 6-nitro-7-hydroxycoumarin (**2**), 8-nitro-7-hydroxycoumarin (**3**) or 3,6,8-trinitro-7-hydroxycoumarin (**4**) exerts a cytotoxic effect leading to cell death by apoptosis in different human cell lines [8, 9, 41, 42]. Scopoletin (**5**) causes apoptosis in HL-60 promyelocytic cells [43] and in human prostate tumor cells [44]. 7,8-dihydroxy-4-methylcoumarin (DHMC, **6**) induces apoptosis in A549 human non-small cell lung carcinoma cells and leukemic cell lines (U-937 and HL-60) in a dose-dependent and time-dependent manner, although in those cell lines different signal transduction systems would be activated [45, 46]. Despite the differences among the cell lines, the relationship between the structure and the activity is clear. Kolodziej *et al.*, reported that the high citotoxicity of coumarins depends on the existence of at least two polar aromatic functional groups [47]. These findings were further confirmed by other authors who showed that at least two polar groups in the benzene ring, particularly phenolic groups at positions 6,7 or 7,8, are essential to induce apoptosis in tumor cell lines, whereas coumarin derivatives bearing the *ortho*-dihydroxy substitution exert a higher cytotoxicity effect in those cells [48, 49]. Likewise, the presence of two neighboring hydroxyl groups at positions 5, 6, 7 and 8 of the aromatic nucleus is necessary for the anti-inflammatory effect of hydroxycoumarins [50-53]. In addition, the relative position of the *ortho* cathecol moiety in the benzenoid ring of the coumarin is an important feature for the anti-oxidant activity [54, 55].

Ishihara *et al.*, performed a quantitative structure-cytotoxicity relationship analysis of twenty coumarin analogues in the human squamous cell carcinoma line (HSC-2) [56]. Different hydroxycoumarins with the *ortho*-cathecol arrangement which exerted the highest cytotoxicity effect in this cell line were studied. The authors found a highly significant correlation between the cytotoxicity concentration 50 values and the following descriptors: absolute hardness, ionization potential and highest occupied molecular orbital (HOMO) energy. This finding shows that the cytotoxicity of

certain hydroxycoumarins depends on the electronic properties of the molecule. Hardness and softness properties are important factors to estimate the cytotoxic activity of coumarin derivatives.

Most plant-derived polyphenolic anti-oxidants may under certain conditions act as pro-oxidants and generate ROS thus behaving as cytotoxic and pro-apoptotic agents [57-59]. In this sense, flavonoids with the *ortho*-dihydroxy moiety are able to inhibit lipid peroxidation and scavenge superoxide but they also behave as pro-oxidant agents [60]. Studies by Paya *et al.*, showed that the dihydroxylated coumarins fraxetin (**7**), esculetin (**1**), 4-methylesculetin (**8**), daphnetin (**9**) and DHMC (**6**), are not only effective inhibitors of $Fe^{3+}$-ascorbate-dependent microsomal lipid peroxidation and aqueous alkylperoxyl radicals, but also scavengers of superoxide anion radicals [61, 62]. However, coumarins with *ortho*-dihydroxylation enhance hydroxyl radical generation in the $Fe^{3+}$–EDTA–$H_2O_2$ deoxyribose system, but decrease it in the $Fe^{3+}$–ascorbate–$H_2O_2$ deoxyribose system, supporting that they can chelate iron ions and also donate electrons, promoting a Fenton type reaction. These findings support that hydroxylated coumarins may either behave as ROS scavengers or pro-oxidant compounds depending on factors such as excess of free transition metal ions, metal reducing potential, metal chelating behavior, pH or solubility. It was reported that 7,8-dihydroxylated coumarins fail to act as cytotoxic agents but behave as scavengers of superoxide anion radicals. However, in the presence of free ferric ions they may exert potentially damaging pro-oxidant actions, including cytotoxicity. Conversely, 5,7-dihydroxycoumarin-4-methylcoumarin (**10**) inhibits lipid peroxidation and scavenges alkylperoxyl radicals but fails to display pro-oxidant activity [61, 62].

HL-60 cells exposed to scopoletin (**5**) undergo apoptosis that is prevented by an anti-oxidant suggesting that ROS generation is involved in scopoletin-induced apoptosis [43]. In agreement we reported a close relationship between the ability of hydroxycoumarins to induce apoptosis in leukemic cells (U-937 and HL-60 cells) and ROS generation. In terms of SAR, the existence of two adjacent phenolic hydroxyl grups is the most relevant factor, whereas the position of the *o*-dihydroxyl groups in the aromatic nucleus has little effect [49]. Similar SAR results were previously reported for hydroxycoumarins as inducers of $Cu^{2+}$-dependent DNA strand breakage [63]. We further reported that the methylation of the 6-OH group reduces the pro-apoptotic activity, being the reduction higher for monohydroxy-coumarins. Derivatives where the phenolic hydroxyl group is replaced by an amino, methoxy or methyl group fail to exhibit pro-apoptotic activity in U-937 cells. The presence of a methyl or hydrogen group at position 4 of the coumarin ring in most of the derivatives does not influence their pro-apoptotic activity. The presence of a hydroxyl group at position 3 in the pyrone ring does not display pro-apoptotic activity in leukemic cells [49]. Our findings support that DHMC (**6**) increases ROS and generates a phenoxyl radical as measured by ESR spectroscopy in U-937 cells, indicating that the increased oxidative stress induced by DHMC (**6**) causes cell death. Furthermore, in U-937 cells pretreated with the radical scavenger N-acetyl-L-cysteine (NAC), DHMC (**6**) fails to induce DNA fragmentation and to trigger apoptosis.

In agreement with our results supporting the dual role of hydroxycoumarins as pro-oxidant and anti-oxidant agents, other polyphenolic compounds bearing free phenol groups, such as curcumin, resveratrol or epigallocatechin-3-gallate (EGCG) were shown to act as antioxidants at lower doses and pro-oxidants at higher doses under certain circumstances [64]. These molecules can participate in electron transfer reactions. It has been described that they may reduce ferric iron to ferrous iron, which can catalyze Fenton reactions and lead to the generation of the highly reactive hydroxyl radical. These chemical reactions disrupt mitochondrial redox homeostasis and induce mitochondrial-mediated apoptosis in various tumor cell types [65-68]. Similar results have been described for 2-methoxy-4-(2-propenyl)-phenol (eugenol) [69] or 2-(3,4- dihydroxyphenyl)-3,5,7-trihydroxy-4*H*-chromen-4-one (quercetin) [57]. Moridani *et al.*, reported a correlation between polyphenol toxicity and their lipophilicity (log *P*) in addition to phenoxy radical formation marked by the electronic Hammett parameter ($\sigma$+) and the OH homolytic bond dissociation energy [70]. Current evidence points to the coumarin ring as a modulator of the free hydroxyl groups to induce pro-oxidant effects.

Despite the numerous studies, the mechanisms underlying the beneficial effect of coumarins in cancer still remain to be fully elucidated. Our studies provided a clear relationship between ROS generation and the pro-apoptotic activity of hydroxycoumarins [49]. It is well established that ROS may stimulate and inhibit distinct signaling pathways, being the net result of ROS generation highly dependent upon the nature of the oxidative stressor and its cellular location [71]. Several researchers focused on different intracellular pathways in an attempt to elucidate the mechanisms triggered by coumarins in cancer cells. It is possible that exposure to selected coumarins might bring about significant cellular stress, resulting in a modulation of different intracellular pathways leading to cell death. However, whether other mechanisms are involved in the response it is presently unknown.

We recently reported that following 24 h treatment, DHMC (**6**) induces selective apoptosis in leukemic cells through the activation of the JNKs pathway and inhibition of the ERK1/2 pathway without contribution of the p38-MAP kinase cascade, members of the mitogen-activated protein kinases superfamily (MAPK). In addition cells exposed to DHMC for 18 h showed inhibition of the PI3K/Akt pathway, an important survival pathway in leukemic cells. Furthermore, down-regulation of c-myc protooncogene and induction of the cell cycle inhibitor $p21^{WAF1/CIP1}$ through a p53 independent mechanism was also observed. In these cells NAC pre-treatment delayed c-Myc and $p21^{WAF1/CIP1}$ expression, suggesting that these cellular pathways may be regulated by DHMC-induced oxidative stress [46]. Nevertheless, Goel *et al.*, showed that DHMC (**6**) caused apoptosis in human non-small cell lung carcinoma cells providing evidence that DHMC (**6**) induces apoptosis through a ROS independent mechanism by downregulation of Bcl-xl, Bax, p21, p53, Cox-2, ERK/MAPK and upregulation of c-Myc [45].

A recent study indicates that esculetin (**1**) enhances arsenic trioxide-induced apoptosis in U-937 promonocytic leukemia cells, but the response is reduced by NAC pre-

treatment. The authors propose that esculetin modulates MEK/ERK and JNK pathways and decreases intracellular reduced-glutathione levels, leading to a higher oxidative stress which would enhance arsenic trioxide-induced apoptosis [72]. A similar mechanism was observed in 3T3-L1 adipocytes [39]. On the other hand, in human renal carcinoma cells 6-nitro-7-hydroxycoumarin (**2**) induces apoptosis by sustained activation of p38-MAPK whereas 7-hydroxycoumarin (**11**) by activation of ERK1/2 without affecting p38-MAPK or JNK cascades [41, 73].

In the present review we described several studies where coumarin compounds bearing polar groups modulate members of the mitogen-activated protein kinase family. The underlying mechanism of coumarin-induced changes in the activation of MAPK cascades remains presently unknown but it is likely that coumarins may act upstream MAPK cascades. In this sense, it was reported that the anti-inflammatory and anti-cancer properties displayed by various coumarin derivatives result from an allosteric MEK1 inhibition, blocking ERK1/2 phosphorylation with no changes in total ERK1/2 levels. These novel MEK1 inhibitors are 7-aminocarbonyloxy-coumarins (named **G8935** and **GC63**) (Fig. **3**). Those coumarins were successfully docked into the allosteric site of the MEK1 structure, showing that **G8935** overlaps with PD3180088, a known MEK inhibitor. The carbamate moiety at C7 position, the carbonyl oxygen from the coumarin ring and the benzyl group at C3 seem to be essential requirements for the activity of these coumarins as MEK1 inhibitors [74]. As the coumarins bearing an *ortho* cathecol group mentioned as inducers of apoptosis in the present review, do not share the structural requirements described by Han *et al.*, they are likely to cause oxidative stress leading to the inhibition of survival cascades such as ERKs and PI3K/Akt, as previously described for other cellular types [75, 76].

In addition, coumarins are also involved in the inhibition of other protein kinases, Yang *et al.*, studied the effect of five mono- and di- hydroxycoumarins [77] and found that only daphnetin (**9**) inhibits the activity of serine/threonine-specific protein kinases, such as EGF receptor tyrosine kinase, protein kinase C (PKC) and cAMP-dependent protein kinase (PKA), which are implicated in cell proliferation, differentiation and death. In an attempt to establish a relationship between the structure and the inhibitory activity, it was concluded that the hydroxylation at C8 would be a structural requirement for daphnetin to act as a protein kinase inhibitor.

Other studies indicate that dihydroxycoumarins, such esculetin (**1**) and DHMC (**6**), or mono-hydroxycoumarins, such as scopoletin (**5**) and 7-hydroxycoumarin (**11**) inhibit cell cycle progression in different cell lines by inducing arrest in the G1 phase caused by an up-regulation of G1 associated cyclin-dependant kinase inhibitor $p21^{WAF1/CIP1}$, a downregulation of cyclin D1, an up-regulation of p27 and hypophosphorylation of retinoblastoma protein [46, 73, 78-81]. These findings support that blockade of G1 phase occurs following hydroxycoumarin-treatment, which ultimately is necessary for cell death. Considering that most used anti-neoplastic drugs induces cell cycle blockade in the S or G2/M phase, cancer therapy would be improved by combination of these drugs with coumarins that block the G1 phase. In addition, it has been reported that 7-hydroxycoumarin and coumarin itself cause a reversible inhibition of ras- and myc-induced neoplastic properties in transformed fibroblasts and in the MTV-EJras cell line [82, 83].

The selective tumor cell-specific cytotoxicity of coumarins has also been well documented [8, 42, 46, 48]. Finn *et al.*, showed the selective cytotoxicity of 6-nitro-7-hydroxycoumarin (**2**) and daphnetin (**9**) in human renal carcinoma cells, relative to non-carcinoma proximal tubular cells [8]. Other studies demonstrate that 6-nitro-7-hydroxycoumarin (**2**) and 3,6,8-trinitro-7-hydroxycoumarin (**4**) exibit high cytotoxicity in a melanoma cell line and reduce cytotoxicity in a normal fibroblastic skin cell line [42]. In accordance, we reported that DHMC (**6**) exerts significant less cytotoxic effect in normal mononuclear cells after 24 h treatment than in leukemic cells [46]. 7-Hydroxycoumarin (**11**) displays anti-proliferative effects in malignant cell lines but not in human peripheral blood mononuclear cells and human bone marrow progenitor stem cells at concentrations lower than to 200 μg/ml [84]. Kawase *et al.*, proposed that the tumor-specific cytotoxicity of esculetin (**1**) can be further enhanced by proper substitutions at 3- and/or 4-position(s) of the molecule [48]. However, the underlying mechanisms of the tumor-selectivity of coumarins are not well understood yet. Interestingly, cell malignization is often accompanied by a decrease in activity of antioxidant enzymes (superoxide dismutase, catalase, glutathione peroxidase), which increases the cell sensitivity to pro-oxidant compounds [85, 86].

In the last years the potential application of coumarins with metal complexes as cyto-selective therapeutic agents for cancer therapy gained growing interest. Complexes of coumarins with lanthanum(III), zirconium(IV) or cerium(III) represent interesting metalorganic compounds with antitumor activity in different cell lines [19, 87-90]. The cytotoxicity of the lanthanum complex of bis-coumarins in the chronic myeloid leukemia cell line is partly mediated by the stimulation of programmed cell death whereas the inorganic salt exerts a very weak cytotoxic effect [90]. Thati *et al.*, demon-

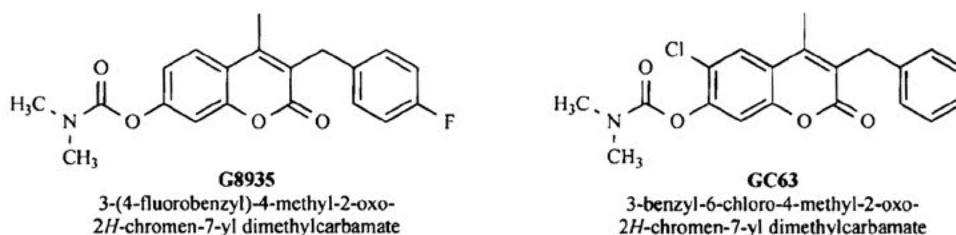


**Fig. (3).** Coumarin derivatives with MEK1 inhibitor activity.

strated the anti-proliferative effects of a series of silver(I) complexes of coumarin-3-carboxylic acid derivatives using human-derived carcinoma cell lines. The authors concluded that hydroxylation particularly at position 6 and complexation with silver are structural requirements for the execution of apoptotic cell death [91, 92].

### Differentiation Inducer Agents

Another potentially less toxic approach to treat cancer employs certain chemicals to induce differentiation of neoplastic cells. This approach fostered the concept of treating tumors by forcing malignant cells to undergo terminal differentiation instead of being killed through cytotoxicity. It is based on the assumption that many neoplastic cell types exhibit reversible defects in differentiation, which upon appropriate treatment, resulting in tumor reprogramming and a concomitant loss in proliferative capacity and induction of terminal differentiation [93, 94].

Several coumarin derivatives induce differentiation of human neoplastic cells. Daphnetin (**9**) exerts potent anti-proliferative and differentiation effects in a human renal cell carcinoma line [95]. Furthermore, esculetin (**1**) and 4-methylesculetin (**8**) differenciate HL-60 cells to mature monocyte/macrophage cells [96]. It was also shown that esculetin (**1**) significantly enhanced retinoic acid or DMSO-induced differentiation in HL-60 cells [97].

We reported that two pure trioxygenated coumarins, 5-methoxy-6,7-methylenedioxycoumarin (**C-1**) and 5-(3-methyl-2-butenyloxy)-6,7-methylenedioxy coumarin (**C-2**) isolated from *Pterocaulon polystachyum*, have anti-proliferative and differentiation properties in U-937 cells (Fig. **4**) [98]. These promising findings prompted us to investigate the anti-leukemia activity of a broader range of related polyoxygenated coumarins. Thus related natural and synthetic coumarins, including a variety of 5-substituted-6,7-methylenedioxycoumarins easily obtained by newly developed synthetic methods, were evaluated to identify the key structural requirements to induce differentiation in leukemic cells [99-102]. We found that the treatment with 5-(2-hydroxy-3-methoxy-3-methylbutoxy)-6,7-methylenedioxycoumarin (**D-2**) and 5-(2,3-dihydroxy-3-methylbutoxy)-6,7-methylenedioxycoumarin (**D-3**) inhibit cell growth and induce the differentiation of U-937 cells after 48 h treatment (Fig. **4**). These results provide fruther insights into the correlation between some structural properties of polyoxygenated coumarins and their *in vitro* leukemic differentiation activity, showing that only 5-substituted-6,7-methylenedioxycoumarins display anti-proliferative and differentiation activity. Derivatives lacking an alkoxy group at position 5 or the 6,7-methylenedioxy arrangement fail to induce U-937 cell differentiation [102]. It is important to note that if the methylendioxy substituent is replaced by a furan group, the resulting 3,2-g-furanocoumarin loses the differentiation activity on leukemia cells and exhibits relevant applications in photochemotherapy for the treatment of psoriasis and other dermatological diseases [103-105].

The mechanisms underlying the effect of 6,7-methylenedioxycoumarins in leukemic cell proliferation and differentiation are presently unknown. Coumarins such as esculetin (**1**) act as a differentiation agent by modulating 5-lipoxygenase metabolism [97]. Finn *et al.*, showed that p38-MAPK mediates the effect of daphnetin (**9**) in human renal cell carcinoma [95], although it has been described that daphnetin (**9**) can also inhibit EGF receptor tyrosine kinase, PKC and PKA activities, which have a relevant role in the control of cell proliferation, differentiation and metabolism [77]. The key molecular target of this group of compounds has to be identified in order to facilitate the development of new pharmacological tools with potential differentiation activity for the management of cancer. This may be useful to improve combined therapies, especially because they often have few side effects.

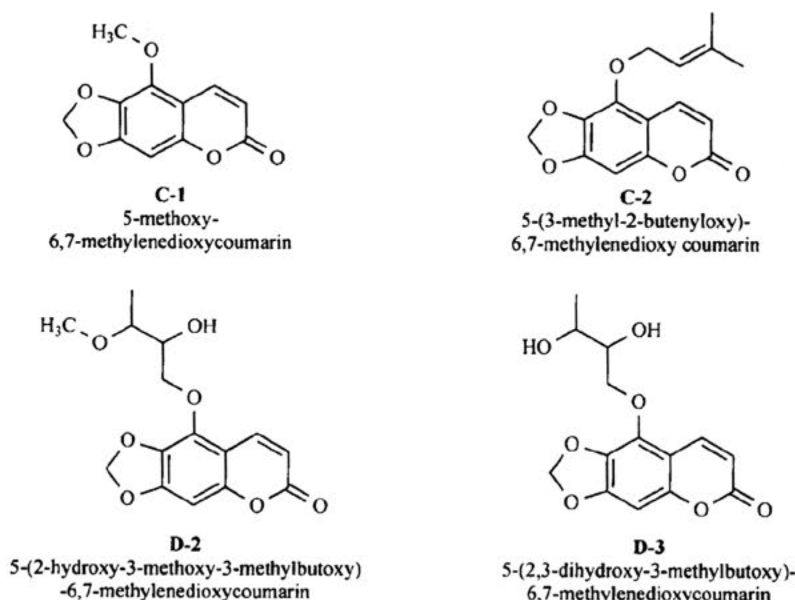


**Fig. (4).** 5-oxygenated-6,7-methylenedioxycoumarins with differentiation activity in human leukemic cells.

### Hormone-Dependent Tumor Inhibitors

Briefly, we will mention a group of tricyclic coumarins designed as part of a programme to identify potent non-estrogenic steroid sulfatase inhibitors. Comprehensive reviews on steroid sulphatase inhibitors have been recently published [106, 107]. The development of inhibitors for the production of 5-androstenediol and estrone from sulfated precursors represents a new therapeutic approach for the treatment of hormone-dependent breast cancer. Studies with diverse tricyclic coumarin sulfamates tested for their ability to inhibit estrone sulfatase activity (E1-STS) showed that COUMATE (4-methylcoumarin-7-O-sulfamate) acts an E1-STS inhibitor in MCF-7 cells. 667 COUMATE (6-oxo-8,9,10,11-tetrahydro-7H-cyclohepta-[c] [1] benzopyran-3-O-sulphamate), not only inhibits STS activity but it also inhibits carbonic anhydrase II activity and behaves as a weaker aromatase inhibitor [108-111]. Encouraging results from a phase I trial show that 667 COUMATE (STX64) is a potent and well-tolerated STS inhibitor. It inhibits STS activity in peripheral blood lymphocytes and breast tumor cells, leading to a significant decrease in the serum concentration of steroids with estrogenic properties [112]. It has been suggested that STS inhibitors may also have a role in the treatment of other hormone-dependent cancers including those of the endometrium, ovary and prostate [106]. *In vivo* studies showed that coumarin itself strongly inhibits the growth of prostate tumours and DMBA-induced mammary carcinomas in rat. In addition, it also reduces the number of lung and lymph node metastases formed by the R3327-MatLu prostate tumor [113-115]. Nevertheless, the mechanisms of the antineoplastic and antimetastatic effects of coumarins *in vivo* have not been fully elucidated.

### Multidrug Resistance Reversal Agents

Multidrug resistance (MDR) is a major complication in cancer therapy. One of the main causes of failure in cancer chemotherapy is the over-expression of P-glycoprotein (Pgp), an ATP-driven membrane exporter of hydrophobic xenobiotics, including anticancer agents. Therefore, modulation of Pgp has gained a great interest lately in cancer research [116, 117].

Several furanocoumarins, such as bergamottin (5-[(3,7-dimethyl-2,6-octadienyl)oxy]-furanocoumarin) and their derivatives have been reported as inhibitors of Pgp activity [118, 119].

Furthermore, (±)-praeruptorin A (**PA**) [(±)-3′-angeloyl-4'-acetoxy-cis-khellactone], a naturally occurring 7,8-pyranocoumarin abundantly found in *Peucedanum praeruptorum* Dunn., suppresses Pgp expression and reverses Pgp-MDR in KB V1 cells [120]. In an attempt to develop novel Pgp inhibitors, a number of PA derivatives were synthesized and a SAR study performed. **DMDCK** [(+/-)-3 '-O,4 '-O-bis(3,4-dimethoxycinnamoyl)-cis-khellactone)] (Fig. **5A**), bearing two 3',4'-dimethoxycinnamoyl groups, resulted the most effective Pgp inhibitor of the series. **DMDCK** is not a transport substrate of Pgp but it is an effective inhibitor of Pgp-mediated transport, suggesting a non-competitive mode of inhibition [121]. A pharmacophore group search was performed using the verapamil-based template as a model for Pgp substrates or inhibitors. This model involves two essential hydrophobic planes, three optional hydrogen bond (HB) acceptor points and one optional HB donor point. Both stereoisomers of **DMDCK** had four functional groups (two hydrophobic points and two HB acceptor points) simultaneously involved in the interaction with Pgp, implying a higher binding affinity and Pgp modulating activity. Results of the pharmacophore search provide an explanation on structural bases for MDR reversing activity of these pyranocoumarins derivatives [121, 122]. Furthermore, pyranocoumarins are as effective as verapamil, a calcium voltage channel blocker, in enhancing doxorubicin accumulation. **PA** was also reported to act as a calcium channel blocker, but further studies are needed to gain insight into the mechanism of pyranocoumarins [123, 124].

A 3D-quantitative structure-activity relationship was performed to evaluate the ability of a series of natural and synthetic coumarins to reduce the Pgp-mediated drug efflux of daunorubicin in human leukemic cells (K562/R7) overexpressing Pgp. The inhibitory activity was enchanced by the substitution at position 4 with a phenyl group, as supported by a 3D-QSAR analysis showing that a hydrophobic bulk group is favorable in that position of the nucleus. The importance of some substituents particularly dihydrofuranic moieties at positions C7–C8, which confers favorable electrostatic and steric effects for the activity, was also demonstrated. Acyclic substituents (i.e., acyl, prenyl and 2-hydroxy-3-methylbut-3-enyl residues) at position 6 or 8 only produce slight variations in the inhibitory activity of Pgp [125]. In other SAR study using 10 analogues of 4-phenyl coumarin, the authors confirmed the structural requirement in the aromatic ring of the [α-(hydroxyisopropyl) dihydrofuran] substructure with a positive effect due to steric considerations. They further described that the presence of methoxy groups at positions 5 and 7 also impacts on the Pgp inhibition (Fig. **5C**) [126]. In addition, the substitution of the lactonic ring by a hydrophobic moiety, like a 3-α,α-dimethylallyl group, also increases the inhibitory activity [127]. This SAR study confirms previous results reported for cnidiadin (Fig. **5B**), a furanocoumarin with a [α,β-di(hydroxyisopropyl)-dihydrofuran] group at positions C7–C8, which exhibits an anti-MDR activity in the MDCK-MDR1 cell line [128]. It should be noted that cnidiadin was evaluated with umbelliferone (**11**), esculin (**12**), esculetin (**1**), angelicin (**13**) and psoralen (**14**) and it was the only tested coumarin to competitiverly inhibit the binding and efflux of drugs by Pgp in the MDCK-MDR1 cell line.

A SAR study with 44 coumarin compounds was carried out by Kawase *et al.*, to identify the basic features of coumarin structures responsible for the MDR reversal activity. The most active compound was 6-hydroxy-3-(2-hydroxyethyl)-4-methyl-7-methoxycoumarin which was equally potent as the MDR modulator verapamil but failed to display toxicity in normal cells, suggesting that the presence of the 2-hydroxyethyl group is favorable for the activity [129].

Preliminary observations suggest that the activity is largely influenced by modifications of the substitution pattern, particularly by the presence of hydrophobic bulk residues in the coumarin nucleus. Coumarin derivatives may become novel MDR reversal agents given their ability to

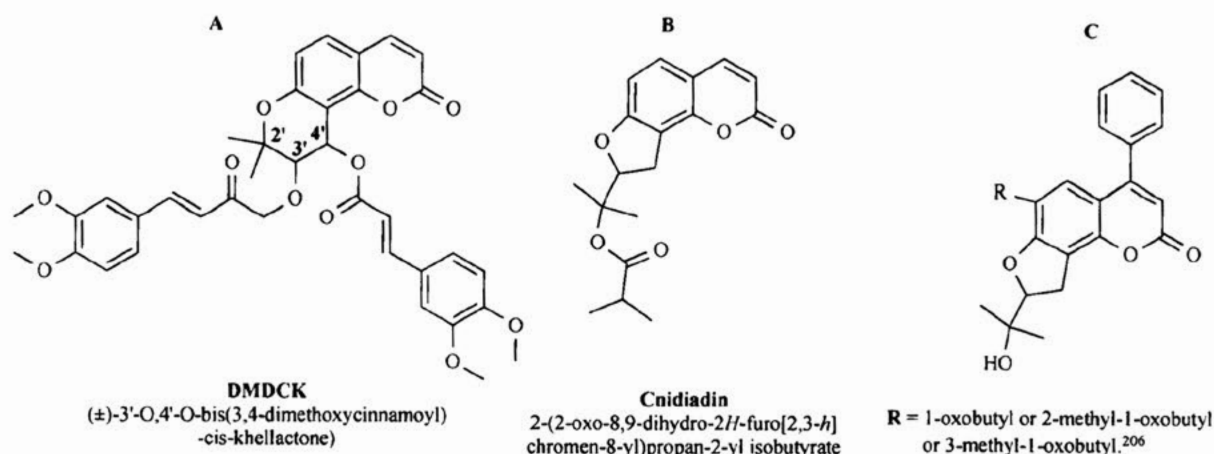


**Fig. (5).** Coumarin derivatives with multidrug resistance reversal activity.

**Table 1. Simple Coumarins Mentioned in this Update**

| Number | Name | Structure | Number | Name | Structure |
|---|---|---|---|---|---|
| 1 | **Esculetin** (6,7-dihydroxy coumarin) | | 8 | **4-methylesculetin** (6,7-dihydroxy-4-methylcoumarin) | |
| 2 | 6-nitro-7-hydroxycoumarin | | 9 | **Daphnetin** (7,8-dihydroxycoumarin) | |
| 3 | 8-nitro-7-hydroxycoumarin | | 10 | 5,7-dihydroxy-4-methylcoumarin | |
| 4 | 3,6,8-trinitro-7-hydroxycoumarin | | 11 | **Umbelliferone** (7-hydroxy coumarin) | |
| 5 | **Scopoletin** (6-methoxy-7-hydroxycoumarin) | | 12 | **Esculin** (7-hydroxy-2-oxo-2H-chromen-6-yl beta-D-glucopyranoside) | |
| 6 | **DHMC** (7,8-dihydroxy-4-methylcoumarin) | | 13 | **Angelicin** (furo[2,3-h]chromen-2-one) | |
| 7 | **Fraxetin** (6-methoxy-7,8-dihydroxycoumarin) | | 14 | **Psoralen** (7H-furo[3,2-g]chromen-7-one) | |

specifically inhibit Pgp in the absence of toxicity in normal cells.

## DEFINING THE CLINICAL COURSE OF COUMARIN COMPOUNDS

There are several drugs in the market belonging to the coumarin family, mainly oral anticoagulants used for more than 50 years in the treatment of thromboembolic diseases [130, 131]. Other marketed coumarins include novobiocin, licensed for the treatment of human infections as supported by several clinical trials [132-134] and Venalot® Depot (Shaper & Brummer; Germany) that is used for the therapy of severe non-organic venous complaints [61].

Over two decades ago *in vivo* studies about the potential use of coumarins in cancer treatment were initiated. The treatment of patients suffering from locally advanced or metastatic renal cell carcinoma with coumarin (100 mg orally) and cimetidine induce a 6-33% of response rate (complete or partial remissions) according to the different schedules in clinical trials [135-137]. Patients showed no symptomatic organ dysfunction or toxicity. Other pilot studies were designed to evaluate the effect of coumarin and cimetidine in patients with melanoma, but unfortunately these drugs failed to exhibit any beneficial effect in this population. However a multicentre prospectively randomized double blind placebo-controlled trial showed that a daily oral dose of 50 mg coumarin prevented early recurrence of malignant melanoma. A significant reduction in the recurrence values without toxic effects associated with coumarin treatment was observed in these patients [138-140]. A multicenter trial including patients with metastatic hormone naive or hormone refractory prostatic carcinoma that received 3 g coumarin daily showed that partial responses occurred in 8% of the patients and toxicity was limited to asymptomatic hepatic transaminases elevation in three patients and nausea and vomiting in four patients [141].

In a phase I trial, a tricylic coumarin-based sulfamate (667 COUMATE), that irreversibly inhibits steroid sulfatase (STS) activity was evaluated in postmenopausal women with breast cancer. Four patients showed evidence of stable disease for 2 to 7 months and decreased serum concentration of estrone, estradiol, androstenediol, and DHEA. The drug was well tolerated with only minor adverse effects [112]. It was shown that the coumarin antibiotic novobiocin potentiates the activity of etoposide (VP-16) *in vitro* by increasing intracellular accumulation of VP-16. Therefore, a clinical trial was carried out in patients with refractary cancer treated with VP-16 combined with novobiocin. Novobiocin (7 g/m$^2$/day) failed to augment the toxicity of VP-16 and the dose-limiting toxicities consisted of neutropenic fever and reversible hyperbilirubinemia. Nausea, which was a limiting side effect in other trials using novobiocin, was well controlled by the administration of serotonergic antiemetics. Diarrhea was common but mild in most patients [142].

In summary, most pharmacological studies involve mainly coumarin itself as an anti-neoplastic drug. In some trials, a positive outcome following coumarin treatment was observed. However, it is important to point out that treatments were generally well tolerated over a wide range of oral coumarin doses, from 50 mg to 7 g daily according to the protocol design. Self-limited side effects included insomnia, nausea, vomiting, diarrhea, and asymptomatic abnormal elevations of serum hepatic transaminases. These side effects disappeared when coumarin therapy was stopped and there was no record of significant hepatic, hematologic or renal toxicity during the trials [143].

As coumarin compounds are relatively non-toxic and they can be combined with other chemotherapeutic or biological agents to improve their efficacy, further investigations with coumarin derivatives are important to eventually develop new drugs for the treatment of cancer.

## FUTURE PERSPECTIVES

In a very interesting review, Dueñas-González *et al.* arise the metaphor of drug discovery and development process as the tale *The Prince and the Pauper* by Mark Twain [144]. In accordance, we support the idea that it is not just princely, (interpreting as high cost) new drugs that can help to treat diseases that maybe that pauper (interpreting as low cost) drugs developed, could bear the same potential for efficacy. Classical drug discovery involves target discovery and validation, lead identification by high-throughput screening, and lead optimization by medicinal chemistry. Pre-clinical follow-up evaluation includes analysis in animal models of compound efficacy, pharmacology, toxicology, specificity and drug interaction studies, hence, the majority of the newer drug lead are simply cost-prohibitive by researchers at non-profit academic organizations [145]. This relevant issue led to reflect upon alternatives for drug development strategy, as named drug *repositioning, drug repurposing*, or *indication switch*. The *repositioning* term refers to the exploitation of established drugs that have already been approved for the treatment of certain diseases and expand their therapeutical indication to other human pathologies.

Based on the pharmacovigilance data of the prescribed coumarin derivatives, their presence in the diet and herbal medicines, their low toxicity against normal cells and selectively for neoplastic cells, we firmly believe that the potential of coumarin compounds as chemotherapeutic agents needs to be further investigated. Although some coumarin compounds seem to be privileged structures for at least some biological activities, there remains the challenge to design and synthesize molecules with high specific affinity for other pharmacologically important targets or to characterize their mechanism of action to become available therapeutics drugs. This review highlights the progress that has been made in the development of coumarin scaffolds for anti-cancer drug discovery.

Several molecules with a coumarin framework were reported to have multiple biological activities (Fig. 6). These studies strongly support that the biological activity and therapeutic applications of coumarins rely on their chemical structure, namely, the pattern of substitution on the aromatic ring.

We would like to convey the concept of crosstalk from biology to the drug design process in medicinal chemistry. At least for coumarin molecules, some pharmacophoric groups can bring about several biological effects. Current

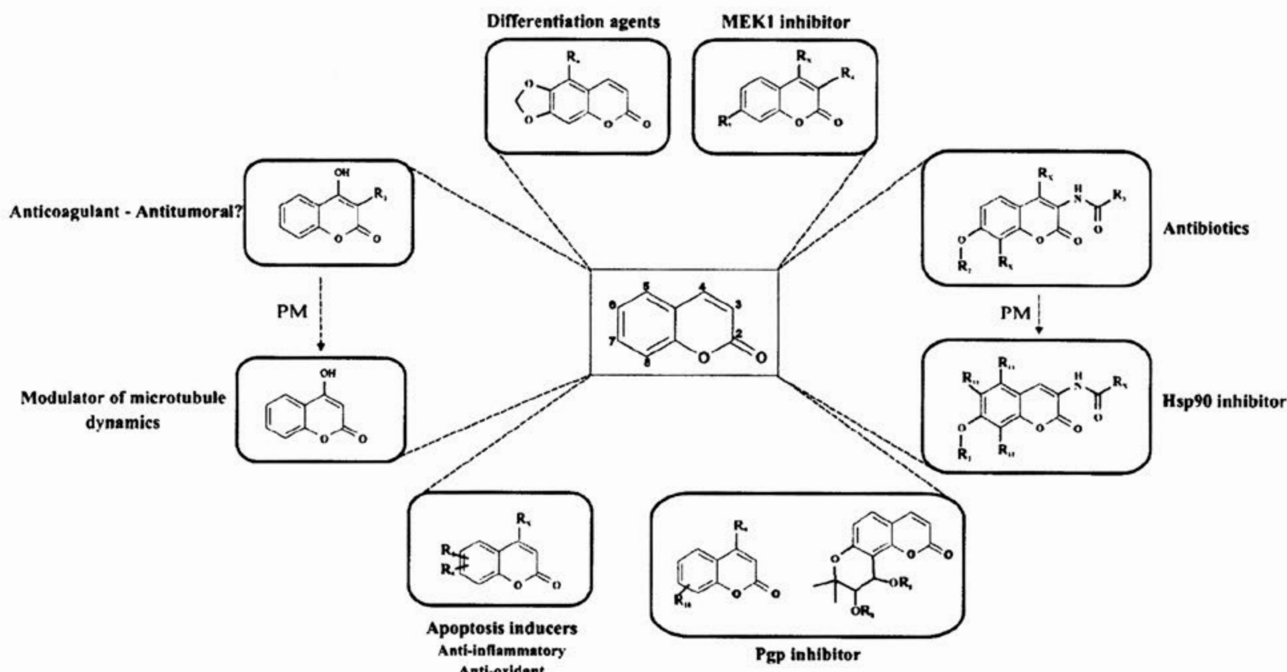


**Fig. (6).** Coumarin framework and lead compounds with diverse biological activities.

$R_1$: Carbon chain residue. $R_2$: H-bond donor residue (noviose moiety). $R_3$: Bulky residue. $R_4$: Hydrophobic residue. $R_5$: Polar groups (hidroxyl; methoxy; acetoxy). $R_6$: Alkoxy residue. $R_7$: Carbamate residue. $R_8$: Hydrophobic and H-bond aceptor residue. $R_9$: Hydrophobic bulky residue. $R_{10}$: H-bond donor and bulky residue. $R_{11}$: H-bond acceptor. $R_x$: Not essential group. ROS: reactive oxygen species; Pgp: P-glycoprotein; PM: Pharmacomodulation.

findings suggest that certain structural features, such as the neighboring dihydroxy functionality in simple coumarins, are not only important for their promoting ROS scavenging action but also for their anti-inflammatory and pro-apoptotic activity in cancer cells. It is clear that the redox properties of these molecules may lead to several effects *in vivo* and in some situations turn into side effects. As it was previously discussed, certain substituents at position 4 and 3 in the coumarin nucleus are structural requirements for the anticoagulant activity, so this should be considered when introducing modifications in the pyrone ring to avoid side-effects.

This comprehensive review focused on the current literature on the structure-activity relationship of coumarin derivatives. This knowledge is crucial for the understanding of their pharmacological properties, mechanism of action and potential future therapeutic applications of these compounds as anti-cancer agents. Further studies will certainly reveal new aspects of coumarins that may eventually result in the design and development of promising coumarin clinical candidates in the near future.

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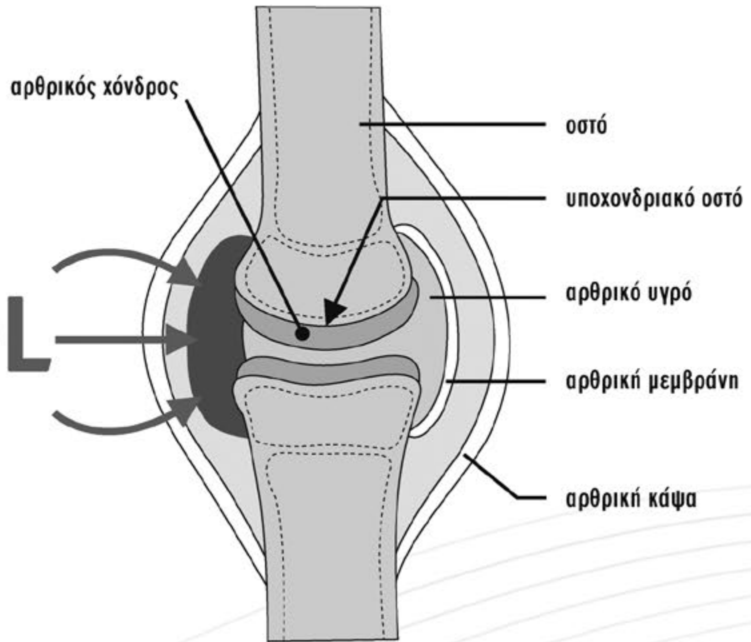
# ARTHRIDOL

ΦΟΡΜΟΥΛΑ
**αρθρώσεων**
για την επαναφορά
της λειτουργικότητας των αρθρώσεων

60 δισκία των 1200mg



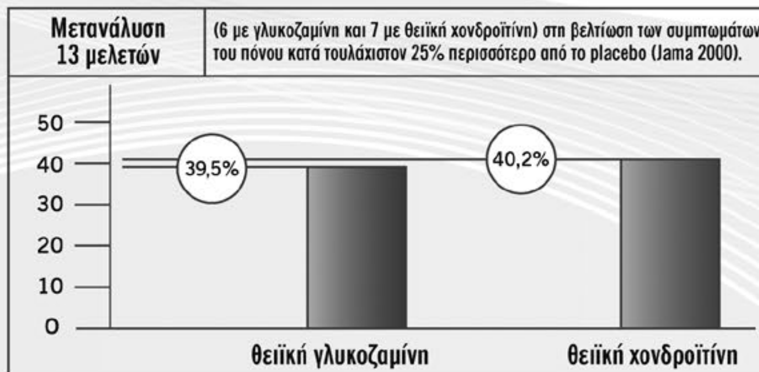

ARTHRIDOL

***Σε αντίθεση με τα NSAIDS (μη στεροειδή αντιφλεγμονώδη) δεν προκαλεί γαστρικά προβλήματα και εκφύλιση των χόνδρων***

# Προστασία του χόνδρου:

**Θειϊκή Γλυκοζαμίνη: Χαμηλό Μοριακό Βάρος (179)**
- Αυξημένη βιοδιαθεσιμότητα.
- Γρήγορη ενσωμάτωση με τον αρθρικό χόνδρο.

- Μόριο βάσης για τη σύνθεση των γλυκοζαμινογλυκανών (GAG).
- Αναστέλλει την απελευθέρωση των πρωτεολυτικών ενζύμων (Pharmacy, 2000).
- Εμποδίζει τη συρρίκνωση του ενδοαρθρικού χώρου (Lancet, 2001).

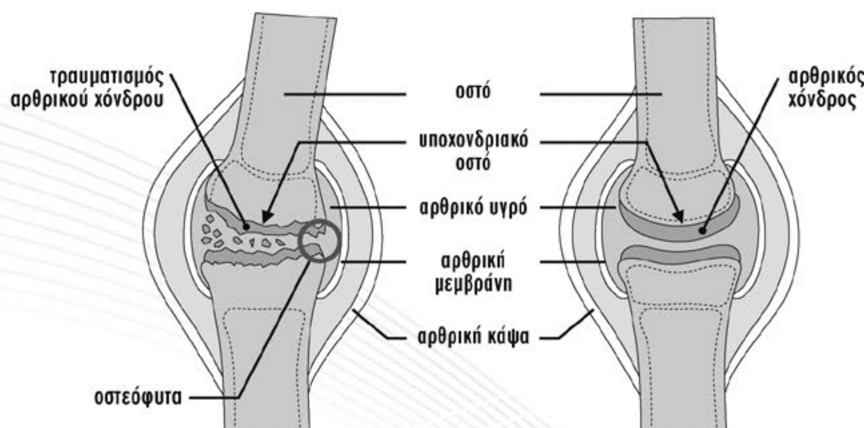



**Η συγκέντρωση της γλυκοζαμίνης στο Arthridol, δεν προκαλεί αντίσταση στην ινσουλίνη και βάρος στο στομάχι**

**Θειϊκή χονδροϊτινη:**
- Είναι η σημαντικότερη ουσία του χόνδρου των αρθρώσεων.
- Αυξάνει την περιεκτικότητα του υαλουρονικού οξέως μέσα στο αρθρικό υγρό, βελτιώνοντας το ιξώδες των αρθρώσεων.

| | | |
|---|---|---|
| **Χόνδρος από καρχαρία:** | ▪ | Αποτελείται από εστέρα θειϊκού άλατος χονδροϊτίνης και κολλαγόνο (κύριο συστατικό του αρθρικού υγρού). Έχει **λιπαντική δράση**. |
| **Κολλαγόνο τύπου ΙΙ:** | ▪ | Αυξημένη βιοδιαθεσιμότητα που το βοηθά να εισέλθει στον χόνδρο. |
| **MSM:** | ▪ | Αντιφλεγμονώδη δράση. |

# Αποκατάσταση του χόνδρου




## *Μια νέα πολυκεντρική προσέγγιση στην αντιμετώπιση των χρονίων προβλημάτων των αρθρώσεων*

### ΣΥΝΙΣΤΑΤΑΙ ΣΕ ΠΕΡΙΠΤΩΣΕΙΣ

- **Μετατραυματικών πόνων**
- **Ρευματισμών**
- **Εκφύλισης των αρθρώσεων**
- **Αρθρίτιδας**

### ΔΟΣΟΛΟΓΙΑ

- Σε έξαρση των συμπτωμάτων:
  Δύο δισκία, 1-2 φορές την ημέρα.
- Σε συντήρηση:
  Ένα δισκίο, 1-2 φορές την ημέρα.

| ΔΙΑΤΡΟΦΙΚΕΣ ΠΛΗΡΟΦΟΡΙΕΣ | ανά 100g | ανά ημερήσια δόση 2 δισκία |
|---|---|---|
| Θειϊκή γλυκοζαμίνη | 35,20g | 845mg |
| Ίση με γλυκοζαμίνη | 20,83g | 500mg |
| Θειϊκή χονδροϊτίνη | 20,83g | 500mg |
| Μεθυλο σουλφονικό μεθάνιο | 16,67g | 400mg |
| Χόνδρος του Καρχαρία | 8,33g | 200mg |
| Γηγενές Κολλαγόνο τύπου ΙΙ | 0,00167mg | 0,04mg |

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*Review Article*

# Effects of Glucosamine and Chondroitin Sulfate on Cartilage Metabolism in OA: Outlook on Other Nutrient Partners Especially Omega-3 Fatty Acids

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Osteoarthritis (OA) is a degenerative joint disease that is characterized by increasing loss of cartilage, remodeling of the periarticular bone, and inflammation of the synovial membrane. Besides the common OA therapy with nonsteroidal anti-inflammatory drugs (NSAIDs), the treatment with chondroprotectives, such as glucosamine sulfate, chondroitin sulfate, hyaluronic acid, collagen hydrolysate, or nutrients, such as antioxidants and omega-3 fatty acids is a promising therapeutic approach. Numerous clinical studies have demonstrated that the targeted administration of selected micronutrients leads to a more effective reduction of OA symptoms, with less adverse events. Their chondroprotective action can be explained by a dual mechanism: (1) as basic components of cartilage and synovial fluid, they stimulate the anabolic process of the cartilage metabolism; (2) their anti-inflammatory action can delay many inflammation-induced catabolic processes in the cartilage. These two mechanisms are able to slow the progression of cartilage destruction and may help to regenerate the joint structure, leading to reduced pain and increased mobility of the affected joint.

## 1. Introduction

Osteoarthritis (OA), the most common type of arthritis, is characterized by gradual wear and loss of cartilage in the joints resulting in friction between the bones, which leads to pain and swelling. It was long thought that only the cartilage is affected. However, it is now known that the underlying bone, as well as the synovium, also undergoes changes [1–3]. The periarticular bone reacts with osteophyte formation which causes additional restriction in joint movement. It can occur in any joint, but predominates in weight-bearing joints, such as the knee and hip. In Germany, the prevalence of diagnosed osteoarthritis (all age groups combined) in at least one joint is 27%, and more than 50% of the population over 60 suffer from OA in at least one joint [4]. In the United States of America, OA is responsible for total joint replacement in half a million Americans each year [5], indicating that OA is not only a burden to the patients, but also a financial burden on society.

Common OA therapy focuses mainly on the treatment of symptoms, such as pain reduction, but does not treat the cause. However, the main goal of OA therapy should be to delay cartilage degeneration and even help to regenerate the cartilage structure. One approach in this direction is the treatment with chondroprotectives, differentiated in symptomatic slow-acting drugs in OA (SYSADOA) or structure-modifying OA drugs (SMOAD).

This paper will focus on the ability of such chondroprotectives to retard the degenerative process of cartilage destruction and will discuss the evidence of symptomatic and structure-modifying effects of this nutritional approach. Furthermore, the role of inflammation and especially obesity in the process of osteoarthritis and how this process could be addressed will be discussed.

## 2. Common Risk Factors for the Development of Osteoarthritis

There are still questions concerning the *causal factors of OA*. The nature of the initiating event is often unknown, although many processes involved in the progression of

OA are known. Due to disruption of the cartilage collagen matrix, the water content of the cartilage increases. Together with the progressive loss of proteoglycans, the elasticity of the cartilage diminishes. This is followed by a progressive loss of cartilage and the formation of osteophytes and calcium deposits. Osteophytes further limit flexibility of the joint. OA progression is associated with synovial inflammation, joint swelling, stiffness and pain, leading to progressive functional impairment [5, 6].

There are several known *risk factors*. One of the primary risk factor for OA is *age* [3, 7]. During aging, the articular cartilage softens. The ability to remodel and repair the cartilage extracellular matrix (ECM) decreases with age [8]. Furthermore, changes are due to the structural organization of the ECM [9, 10]. During aging, cross-linking of collagen fibers is enhanced which results in increased cartilage stiffness [11]. Aging also leads to reduced muscle mass and strength, which in turn reduces joint stability and leads to misalignment. This can cause abnormal mechanical stress on the joint and thus cartilage degeneration [12].

Another commonly accepted risk factor is *overweight and obesity*. A recent meta-analysis addressed the incidence of comorbidity related to overweight and obesity. It was able to show that overweight and obesity lead to a significantly higher OA risk [13]. The mechanisms by which obesity contribute to OA development are described below.

A number of studies demonstrated that there are strong *genetic determinants* for OA (for review see [14, 15]). A classic twin study in which twins were radiologically screened for OA, showed a clear genetic influence on hand and knee OA in women. Hence the genetic influence was calculated to be 39–65% [16]. Several genetic abnormalities have been identified that are responsible for the onset and progression of OA. These gene variations result in defects or variability of cartilage and ECM composition and metabolism [14, 17, 18].

Patients with *developmental dysplasia* of joints, such as hip dysplasia, develop OA much earlier than normal individuals. *Misalignment* leads to a reduced contact area within the joint resulting in locally elevated pressure on the cartilage [19]. This is related to the progression and onset of OA [20, 21]. *Injuries* involving the joint surface, injured ligaments, or meniscectomy, are also associated with the development of OA. Injuries can often cause joint movement beyond the physiological range which leads to *uneven load distribution* in the joint.

Despite the difference in the primary causes of OA, they all lead to similar clinical symptoms, cartilage destruction, bone remodeling, osteophyte formation, inflammation of the synovial membrane, pain, and immobility.

## 3. Regeneration of the Cartilage Structure

*3.1. Basic Structure and Turnover of Normal Joint Cartilage.* To understand the structure-modifying effect of different nutrients and how they can support the process of cartilage regeneration, it is important to know the composition of cartilage and the metabolic mechanisms involved in normal turnover.

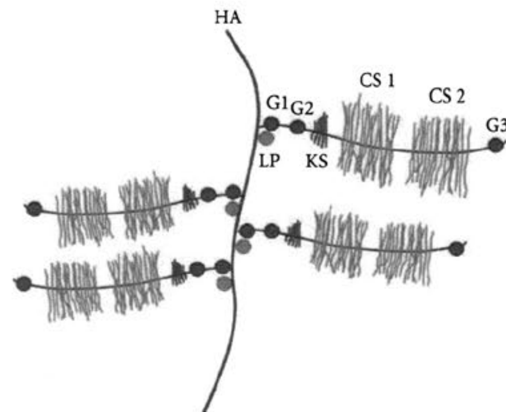


FIGURE 1: Schematic representation of the aggrecan structure (HA: hyaluronic acid, CS 1, CS 2: chondroitin sulfate domains 1 and 2; KS: keratan sulfate; G1, G2, G3: globular domains; LP: link protein).

Cartilage is classified into three different types, based on the collagen type used and the relative amount of the main components, that is, elastic cartilage, hyaline cartilage, and fibrocartilage. Unlike other tissue it is not innervated and does not contain blood vessels or lymphatic structures. There are only a small number of chondrocytes within the cartilage and they only account for 1–5% of the cartilage volume. The chondrocytes are responsible for maintaining the composition and organization of the matrix. They produce this extracellular matrix composed of collagen and elastin fibers, as well as proteoglycans.

*Hyaline cartilage*, found in joints, is characterized by its high elasticity and pressure resistance. In contrast to bone and muscle, it does not increase its tissue mass postnatally due to mechanical stimulation. The morphology of cartilage seems to be strongly related to genetic factors [22]. It is composed of four different zones: the superficial tangential zone, the middle or transitional zone, the deep or radial zone, and the calcified cartilage zone [23, 24]. The collagen network of the joint cartilage consists mainly of type II collagen fibrils. Collagen fibers are important for the response to tensile forces within the joint.

*Proteoglycans* are intertwined with the collagen network. Due to the net negative charge of the proteoglycans, a large amount of water is enclosed in the cartilage. The water content is important for the resilience and elasticity of the tissue, as well as for lubrication of the joint system. The proteoglycans of the articular cartilage are large supramolecular complexes, composed of a central hyaluronic acid (HA) filament, to which aggrecan molecules composed of chondroitin sulfate and keratan sulfate are attached by a link protein in a brush-like configuration (see Figure 1). The amino sugar glucosamine is a necessary component for the synthesis of many of these proteoglycans, which include hyaluronic acid, heparan sulfate, and keratan sulfate. The production of glucosamine is one of the rate-limiting steps in proteoglycan production.

The ability of the *articular cartilage* to regenerate or adapt to mechanical changes is very limited. It has been

postulated that this inability to adapt to mechanical changes is related to its inability to repair after mechanical or other damage [25]. One reason is the avascular nature of this tissue, which makes it difficult to move progenitor cells to lesion sites. In *in vivo* models of rabbits and goats, it has been shown that lesions smaller than 3 mm in diameter can heal (chondral or subchondral zone) while defects larger than 6 mm in diameter rarely if ever heal and lead to progressive degeneration (for review see [26]).

Due to the lack of blood vessels, the chondrocytes within the cartilage receive nutrients only by diffusion from the surrounding tissue. Therefore, a large amount of basic components should be available in that tissue.

The viscous *synovial fluid* is composed of hyaluronic acid (hyaluronan), lubricin (a large, water-soluble glycoprotein), glucose, and water. Hyaluronan is synthesized by the synovial membrane and released into the joint cavity.

*3.2. Chondroprotectives.* As shown above, glucosamine, hyaluronic acid, and chondroitin sulfate are important basic natural components of cartilage and synovial fluid. They are naturally formed by the body, but can also be provided in the diet.

Supplementation of such basic components may be beneficial, especially when there is a disturbed balance between catabolic and anabolic processes, such as in osteoarthritis. During OA progression, the chondrocytes are no longer able to fully compensate for the loss of collagen type II fibers and proteoglycans, even at increased synthesis rates [24].

It has been shown in many *in vitro* and *in vivo* trials and in numerous clinical studies that these SMOAD can modify, stabilize, retard, or even reverse the pathology of OA.

*3.2.1. Glucosamine Salts.* Glucosamine or 2-amino-2-deoxy-D-glucose ($C_6H_{13}NO_5$) is an amino monosaccharide. It is synthesized from glucose in almost every human tissue and is most abundant in connective tissue and cartilage. Glucosamine can be extracted from chitin, found primarily in the exoskeleton of crustaceans (crabs, prawns, and lobsters), as well as in the cell membranes of mushrooms. It is an important precursor of the glycoprotein and glycosaminoglycan (GAG) synthesis. Within cartilage, it is most important for the formation of hyaluronic acid, chondroitin sulfate as well as keratan sulfate, which are—aside from the collagen fibers—the most important components of the extracellular matrix of the articular cartilage and the synovial fluid (for review see [6, 44, 45]). Glucosamine production is the rate-limiting step in GAG synthesis, and glucosamine supplementation may overcome this bottleneck.

Due to its basic role in cartilage and synovial fluid synthesis, glucosamine—administered as glucosamine sulfate (GlcN·S) or hydrochloride (GlcN·HCl)—has been tested in numerous clinical OA trials and the effects have been summarized in reviews and meta-analyses [6, 33, 34, 37, 38, 44–50].

A recent comprehensive review published in 2010 [51], summarized, on the basis of peer-reviewed publications, the currently available chemical and pharmacokinetic data of GlcN salts, and their role in the treatment of clinical OA. An important aspect of GlcN is the structure of various oral GlcN compounds: regardless of the nature of the salt, GlcN·HCl or GlcN·S, the organic component glucosamine is structurally identical. GlcN·HCl dissociates completely in the stomach to GlcN and HCl, and GlcN·S dissociates to GlcN, HCl, sodium sulfate, and sulfuric acid. Investigators have claimed in favor of the GlcN sulfate salt that the sulfate anion would stimulate the chondroitin sulfate synthesis, however, to achieve this serum concentrations of 50 times the serum sulfate concentration would be necessary [51].

In horse studies (see e.g., [52]) $C_{max}$ was about 10 $\mu$M at 2 h, and here also, the sulfate and chloride salts of GlcN were essentially identical. In human volunteers $C_{max}$ was determined to be between 1 and 4 hours after ingestion of a dose of 20 mg GlcN·S per kg body weight (for a typical adult with a body weight of 75 kg, this corresponds to a daily dose of 1500 mg). In four pharmacokinetic studies in humans, maximum serum levels were between 9 and 11 $\mu$m, and in one group of OA patients, mean $C_{max}$ was 7 $\mu$M. Laverty et al. [52] were the first to demonstrate that free GlcN can be detected in synovial fluid after administration (cited in [53]). They found that the synovial fluid concentrations of GlcN remained elevated in most animals even at 12 h after administration. This is in contrast to the nearly complete clearance of GlcN in serum 6 hours after dosing.

*In Vitro Studies. In vitro* studies on isolated chondrocytes, or cartilage explants from healthy or OA patients, provide much evidence for the proposed mechanisms regarding how glucosamine supports joint health. It has been shown that glucosamine enhances the production of cartilage matrix components in chondrocyte culture, such as aggrecan and collagen type II [54, 55]. Glucosamine increases hyaluronic acid production in synovium explants [56]. Further experiments have shown that glucosamine prevents collagen degeneration in chondrocytes by inhibiting lipoxidation reactions and protein oxidation [57]. MMPs (matrix metalloproteinases) and aggrecanases are the predominant cleavage enzymes in the cartilage. These enzymes are responsible for cleavage preferentially in the interglobular domain of the aggrecan molecule, which leads to loss of aggrecan function [24]. Glucosamine is able to inhibit the MMP synthesis, and further proteoglycan degeneration is therefore prevented [58, 59]. Glucosamine also inhibits aggrecanase by suppression of glycosylphosphatidylinositol-linked proteins [60]. Inflammatory processes, which are also responsible for degeneration of the cartilage, are inhibited by glucosamine. These mechanisms will be explained in Section 4.

*Selected Clinical Trials.* The summarized data of major clinical trials (RCTs) between 2001 and 2007 with form of glucosamine used, active reference agents, patient characteristics, outcome measure, and results are listed in Table 1.

The positive effects of glucosamine on the progression of knee OA was not shown in patients suffering from hip OA. In a recent clinical trial, GlcN·S (1500 mg/day) was not able to show superiority over placebo [61], even when a subgroup

TABLE 1: Characteristics and results of selected placebo-controlled trials.

| Author(s), year | Agent/Doses | Duration | Pts ($n$) | Outcome measure | Results and conclusion |
|---|---|---|---|---|---|
| Bruyere et al. 2008 (follow-up of two RCTs 2001/02-see below) [27] | GlcN·S/1500 mg | Formerly: 3 yrs Now: mean 5 yrs after trial termination | 340∗(275 = 81% retrieved) GlcN·S: 144 Plac: 131∗ at least 12-month treatment | Incidence of total knee replacement | *Formerly*: knee OA progression reduced, structure- and symptom-modifying effects *Now*: 6.3% of GlcN·S pts underwent total knee replacement surgery versus 14.5% of the plac. pts $P = 0.026$ Risk of total knee replacement could be reduced 5 yrs after drug discontinuation. |
| Clegg et al. 2006 (GAIT, Glucosamine/Chondroitin Arthritis Intervention Trial) [28] | GlcN·HCl/1500 mg CS/1200 mg Celecoxib 200 mg | 6 months | 1583 | 20% reduction of knee pain | GlcN·HCl + CS: 66.4% of pts. had 20% pain reduction versus Plac: 61.1% of pts. $P = 0.09$—n.s. |
| | | | 354 (subgroup with moderate-to-severe pain) | 20% reduction of knee pain | GlcN·HCl + CS: 79,2% versus Plac. 54.3% $P = 0.002$ Combination of GlcN·HCl + CS was effective in reducing moderate-to-severe pain in knee OA. |
| Herrero-Beaumont et al. 2007 (GUIDE, Glucosamine Unum In Die Efficacy) [29] | GlcN·S/1500 mg Acetaminophen 3000 mg | 6 months | 318 GlcN·S 106 Acet. 108 | OARSI-A responder (relative change WOMAC pain subscale of at least 55%) | GlcN·S: 39.6% versus plac. 21.2% $P = 0.004$ Acet.: n.s. GlcN·S was superior to plac. in the treatment of knee OA |
| Reginster et al. 2001 [30] | GlcN·S/1500 mg | 3 yrs | 212: GlcN·S 106 | Radiographs of the knee: joint spacenarrowing; Lequ. index, WOMAC score | GlcN·S: no significant joint space loss, WOMAC score reduction Plac.: progressive joint space narrowing, WOMAC score increased: Structure- and symptom-modifying effects |
| Pavelka et al. 2002 [31] | GlcN·S/1500 mg | 3 yrs | 202: GlcN·S 101 | Radiographs of the knee: joint space narrowing; Lequ. index, WOMAC score | GlcN·S: no significant joint space loss Plac.: progressive joint space narrowing $P = 0.001$ GlcN·S: Lequ. and WOMAC scores improved by 20% to 25%; Structure- and symptom-modifying effects |
| Bruyere et al. 2004 [32] | GlcN·S 1500 mg | 3 yrs | 319 postmenopausal women (of 414 pts of two RCTs, see above) | Radiographs of the knee: joint space narrowing; WOMAC score | GlcN·S: no significant joint space loss Plac.: progressive joint space narrowin $P < 0.0001$ WOMAC score reduction: "Pain" ($P < 0.02$) and "Function" ($P < 0.004$) |

analysis of the available data was made [62]. The reason why GlcN·S is effective for knee OA, but not for hip OA, is unclear.

Furthermore, it is not understood why many trials stated that there was a significant superiority of GlcN·S over placebo or NSAIDs (e.g., Qiu et al. [63]), whereas others did not. Other trials failed to achieve significance due to a high placebo effect. The heterogeneity of the subjects was also a possible reason, as well as bias due to industry funding. The opinions on this differ and have recently caused much debate [35, 64, 65].

*Selected Reviews and Meta-Analyses.* The quality of evidence was recently evaluated by comparing data from clinical studies, meta-analyses, and reviews (published between 1950 and 2007) on the effect of SYSADOA, including glucosamine sulfate [33]. Using a specialized rating method (GRADE), 5 meta-analyses and one comprehensive review were identified which were included in the evaluation of glucosamine sulfate. Based on this data, it was concluded that glucosamine sulfate, among others, has "demonstrated pain reduction and physical function improvement with very low toxicity, with moderate to high quality evidence" [33]. The results of the Cochrane review by Towheed et al. [34] were included in their evaluation.

The summarized data of selected systematic reviews/ meta-analyses, published between 2005 and 2008 with their conclusions are listed in Table 2.

In most trials, dosages of 1500 mg/day were used; the dose was as safe as placebo and was tolerated better than NSAIDs.

From the clinical trials, it can be concluded that long-term treatment with glucosamine:

(i) reduces pain,

(ii) improves function/mobility of the joint,

(iii) reduces OA progression,

(iv) reduces risk of total joint replacement.

The European League Against Rheumatism (EULAR) came to similar conclusions and rated GlcN·S in their guidelines for knee OA with the highest level of evidence, 1A, and recommended its use with an A [66].

The results of all these studies demonstrate that glucosamine has many favorable effects on cartilage. First, it has shown an anabolic stimulating effect on cartilage synthesis. Furthermore, it inhibits by means of several anti-inflammatory and antioxidant mechanisms, the catabolic cartilage degenerating reactions observed in OA (see Section 4). This can delay cartilage degeneration in OA which leads to a reduction in pain and swelling as well as to increased mobility of the affected joint.

*3.2.2. Chondroitin Sulfate.* Chondroitin sulfate (CS) is one of the natural glycosaminglycans (GAG) composed of the alternating sugars D-glucuronic acid (GlcA) and N-acetyl-D-galactosamine (GalNAc). It is an important component of the extracellular matrix (ECM). CS is the most frequent GAG in the aggrecan molecule of the cartilage. Due to the negative charge of CS, it is responsible for the water retention of the cartilage, which is important for pressure resistance. It can be extracted from the cartilaginous tissue of cows, pigs, birds, and fish (sharks) and is ingested in the diet.

In the European League Against Rheumatism (EULAR) recommendation concerning knee OA, they gave CS both the highest evidence grade and the highest recommendation strength, 1A and A, respectively [66]. CS is one of the SYSADOA. The first effects of SYSADOA treatments, other than analgesics and NSAIDs, become noticeable after 2 to 3 weeks of regular intake and has a prolonged effect that remains for up to several months. CS influences the symptoms of OA such as pain and inflammation, but also acts as a structure-modifying drug in OA (SMOAD). It may retard OA progression and could modify the course of OA (for review see [39]; details from this systematic review on the clinical use of oral CS in OA is provided in Table 3).

The ability of CS to slow down the development of OA has been demonstrated in several clinical trials [43, 67, 68]. These results were confirmed in a recent long-term study (see also Table 3 for trial data; [42]). With this study, the authors were able to confirm the results of a study performed previously (see Table 3; [43]).

The positive impact of CS on OA was also confirmed by meta-analyses, which all showed a significant favorable effect of CS over placebo [33, 40, 41]. Another comprehensive review of CS was written by the Natural Standard Monograph team. These authors listed 39 clinical studies or meta-analyses in which CS was used to treat OA. Most of these studies came to the conclusion that CS has a significant positive effect on OA patients [69].

One of the studies without a significant effect was the GAIT study [28] (see Table 1 for further details). In that study, intake of CS resulted in only a 5.3% higher responder rate than placebo, which was not statistically significant. However, treatment with CS led to a statistically significant improvement in knee joint swelling [28]. The statistical nonsuperiority of CS in pain reduction can probably be explained by the unexpectedly high placebo effect in this study (61% responder). All of the studies and meta-analyses [37, 40, 41, 70] gave CS an excellent safety profile, therefore there are no safety concerns for long-term use [71].

Similar to the GAIT study, many clinical studies tested chondroitin sulfate together with glucosamine [6, 47, 72–74]. The results suggest that both components may enhance each other's efficacy. This synergistic effect was also proposed by various *in vivo* and *in vitro* studies [55, 75–78].

CS increases the hyaluronan production by human synovial cells, which has a beneficial effect on maintaining viscosity in the synovial fluid [79]. It has been shown that CS stimulates the chondrocyte metabolism, leading to the synthesis of collagen and proteoglycan, the basic components of new cartilage. Furthermore, CS inhibits the enzymes leukocyte elastase and hyaluronidase, which are found in high concentration in the synovial fluid of patients with rheumatic diseases. CS also increases the production of hyaluronic acid by synovial cells, which subsequently improves the viscosity and the synovial fluid levels.

Table 2: Characteristics and results of selected reviews/meta-analyses-glucosamine.

| Author(s), year | Analyzed publications | Trial details | Conclusions |
|---|---|---|---|
| Bruyere et al. 2008 [33] | (i) Towheed et al., Cochrane Review 2005 [34] | 20 RCTs: GlcN·S superior to Plac. with a 28% improvement in pain and a 21% improvement in function (Lequ. index). | Significantly superior to placebo in terms of its ability to reduce levels of pain. |
| | (ii) Vlad et al. 2007 [35] | 15 RCTs Summary effect sizes ranged: 0.05 to 0.16 in trials without industry involvement, but 0.47 to 0.55 in trials with industry involvement. | Heterogeneity among trials of glucosamine is larger than would be expected by chance. Glucosamine hydrochloride is not effective. |
| | (iii) Reginster 2007 [36] (update following Richy et al. 2007, [37]) | 3 pivotal RCTs: WOMAC pain and function subscores: significant beneficial effect of GlcN·S versus Plac. | The effect size was consistent across the parameters, and it was approx. 0.30 or slightly higher. This effect is small to medium, but it is clinically valid (>0.20), and especially, it is of the same magnitude as that commonly encountered with other OA treatments, including NSAIDs. |
| Poolsup et al. 2005 [38] | | 14 RCTs: GlcN·S: Risk of disease progression was reduced by 54% ($P = 0.0011$). Pooled effect sizes for pain reduction and improvement in physical function were 0.41 ($P < 0.0001$) and 0.46 ($P < 0.0001$), respectively. | GlcN·S may be effective and safe in delaying the progression and improving the symptoms of knee OA. |

In general, CS inhibits cartilage destruction processes and stimulates the anabolic processes involved in new cartilage formation (for review see [6, 69]). In addition, CS, when added to chondrocyte cultures, produces a dose-dependent increase in cell proliferation.

Several mechanisms are discussed which lead to the positive impact of CS on OA patients. Pharmacokinetic studies were able to show that orally ingested chondroitin sulfate is absorbed as a high molecular mass polysaccharide and can be detected in plasma, together with derivatives, resulting from partial depolymerization and/or desulfation [80]. A pharmacokinetic study (1990) in rats and dogs [81] tested the distribution of tritiated CS orally and intramuscularly. More than 70% of the orally administered radioactivity was absorbed. Independently of the route of administration, radioactivity was mainly excreted through the urine. Plasma levels showed a rapid increase after oral administration, followed by a large plateau with a maximum after 14 or 28 hours in rats and dogs, respectively.

In the years after the publication of the GAIT study, using a combination of GlcN·HCl and CS, new pharmacokinetic data in humans, for both chondroprotectives became available. Jackson et al. [82] tried to assess the pharmacokinetic behavior of oral GlcN and CSeither separately or combined. First they found that the basal levels of GlcN in plasma were at any time below the detection limit, while with CS plasma levels were approximately. 20 $\mu$g/mL and did not show any circadian variation. In a second trial phase, they examined the pharmacokinetics of 1500 mg of GlcN·HCl, 1200 mg CS, or a combination of both substances. In a third phase, they selected a group of patients with symptomatic knee OA (as part of GAIT) who had already received 1500 mg GlcN·HCl, 1200 mg CS, or a combination of both for more than 3 months every day. The main finding was that none of the experimental procedures led to alterations in the endogenous plasma CS concentration. The basal GlcN levels in plasma which had not been detectable before increased, but with combined administration together with CS were significantly reduced.

The authors concluded that the clinical improvement of OA symptoms which was obvious in the numerous clinical trials (also for a subgroup of the GAIT patient population, [28]) is not caused by a synergistic effect of both agents during intestinal absorption, but that there may be indirect effects of these two agents on joint health. They hypothesize that the favorable clinical effects of both compounds may result from "changes in cellular activities in the gut lining or in the liver, where concentrations of ingested CS, or its breakdown products, could be substantially elevated following oral ingestion" [82].

In summary, all the information from these *in vitro* and *in vivo* studies, the clinical trials, as well as meta-analyses lead to the conclusion that there is sufficient data to support the use of oral CS in OA. The findings show that CS reduces pain, improves function/mobility of the joint, and reduces the progression of OA by its structure-modifying effects.

*3.2.3. Other Compounds.* In addition to the combination GlcN·S + CS, other related substances, for example,

Table 3: Characteristics and results of selected meta-analyses and RCTs-chondroitin sulfate.

(a)

| Author(s), year | Analyzed publications | Trial details | Conclusions |
|---|---|---|---|
| Uebelhart 2008 [39] | Meta-analysis | 3 RCTs with CS in knee OA: 462 pts., 2 × 3 mo. 800 mg for 1 yr; 800 mg daily and continuously for 12 and 24 months. 2 RCTs with CS in finger joint OA: 284 pts., 3 × 400 mg CS for 3 yrs. CS decreased the number of pts. with new erosive OA finger joints. | CS influences the symptoms of OA such as pain and inflammation, but also acts as a structure-modifying drug in OA (SMOAD). CS may retard OA progression and could modify the course of OA. |
| Lee et al. 2010 [40] | Meta-analysis | 2 RCTs with GlcN·S + 4 RCTs with CS (800 mg daily) in OA: 1502 pts. CS: Small, but significant protective effect on minimum joint space narrowing after 2 years ($P < 0.001$). | CS may delay radiological progression of OA of the knee after daily administration for over 2 years. |
| Hochberg et al. 2008 [41] | Meta-analysis | 3 RCTs with CS in knee OA: Small significant effect on the reduction in rate of decline in minimum joint space width of 0.07 mm/year. The effect size is 0.26 ($P < 0.0001$). | CS is effective for reducing the rate of decline in minimum joint space width in OA of the knee; CS may have a role as a structure-modifying agent in the management of patients with knee OA. |

(b)

| Author(s), year | CS/Dose | Duration | Pts. ($n$) | Outcome measure | Results and conclusion |
|---|---|---|---|---|---|
| Kahan et al. 2009 (STOPP: Study on Osteoarthritis Progression Prevention) [42] | CS/800 mg | 2 yrs | 622 (knee OA) CS: 309 | X-ray images, tibiofemoral joint: joint space narrowing | Progression of joint space narrowing was significantly reduced versus plac. (28% CS pts. versus 41% Plac. pts. showed progressive joint space narrowing, $P < 0.0005$) Combined structure- and symptom-modifying effects of CS suggest that it could be a disease-modifying agent in patients with knee OA. |
| Michel et al. 2005 [43] | CS/800 mg | 2 yrs | 300 (knee OA) CS: 150 | X-ray images, tibiofemoral joint: joint space narrowing | CS: no significant joint space loss, $P = 0.04$ versus Plac. Plac.: significant joint space narrowing ($P = 0.001$ versus baseline) CS: no significant symptomatic effect, but halts structural changes in OA for over 2 yrs. |

*hyaluronic acid* (HA, hyaluronan) and collagen hydrolysate, have been used in OA patients.

Regarding therapeutical use of HA, the backbone of a proteoglycan aggregate within the ECM, not all clinical trials reported the same positive result. It seems that higher-molecular-weight hyaluronic acid may be more effective than lower molecular-weight HA. Intra-articular treatment with HA has been accepted and is widely used as OA therapy. However, there is a controversy over the efficacy of orally administered HA.

Based on basic pharmacokinetic research it has been found that orally administered high-molecular-weight HA also reached the joint [83], which provides a rationale for the oral supplementation of HA. Authors of a clinical pilot study [84] concluded that HA enhances several aspects of quality of life in adults with knee OA. A larger sample size would be necessary to confirm this result.

In a recent review in which the SYSADOA treatment was analyzed using the GRADE system [33], experts came to the conclusion that—in addition to chondroitin sulfate or glucosamine sulfate—also hyaluronic acid has "demonstrated pain reduction and physical function improvement with very low toxicity, with moderate to high quality evidence" [33]. In summary, the described effects justify the use of these three cartilage components in patients suffering from OA.

For *collagen hydrolysate*, from the available *in vitro* and *in vivo* studies as well as clinical trials [85, 86], it may be concluded that collagen hydrolysate is absorbed by

the gastrointestinal tract and incorporated into the joint cartilage. It may lead to increased mobility and physical function with a significant pain relief.

## 4. Anti-Inflammatory and Antioxidant Effects of Nutrients

*4.1. Inflammation and Reactive Oxygen Species: New Metabolic Approaches to Osteoarthritis.* While OA is not synonymous with inflammatory arthropathy, new results indicate that inflammation is not only a secondary event, it is involved in the development of OA from the very beginning [87–89]. Many inflammatory mediators are expressed in the cartilage and synovial tissue in early OA stages. The findings of Benito [89] indicate that inflammatory mediators and nuclear transcription factors involved in the inflammatory cascade are significantly higher in early-stage OA patients, when compared to late-stage OA. Additionally, reactive oxygen species (ROS) increase during OA [90–93]. The various inflammatory and oxidative processes in OA are summarized in Figure 2.

Many studies have identified *overweight* (BMI 25–29.9 kg/m$^2$) and *obesity* (BMI >29.9 kg/m$^2$) [94–96] as major OA risk factors. Hart and Spector [97] showed that a BMI increase of 2 units will increase the risk of knee OA manifestation by 36%. This is not only due to the additional weight and mechanical stress on the joints, as nonweight-bearing joints—such as the hands—are significantly more affected in patients with high BMI [89], due to metabolic reactions. These include increased inflammation, induced by leptin and other adipocytokines, and dietary lipids or lipid peroxidation, which can lead to cartilage destruction. Therefore, OA is not induced by biomechanical factors and age alone, and several metabolic factors are also involved [98–106].

*Leptin is overexpressed in obese patients* and is present in the synovial fluid, as well as articular chondrocytes [104]. Chondrocytes in joint cartilage also express leptin receptors [107]. Under physiological conditions, leptin stimulates the synthesis of insulin-like growth factor 1 (IGF-1) and transforming growth factor beta (TGFb-1), two mediators important for proliferation of chondrocyte and extracellular matrix synthesis, by binding to the leptin receptor [103, 104]. These two factors appear to have a positive anabolic impact on the joint by increasing the cartilage matrix production. Excessive and pathological concentrations of leptin, however, like those found in obese patients, have an opposite effect on chondrocytes, cartilage, and bone, leading to osteophyte formation and cartilage degeneration [108]. Osteophytes in the joints usually limit joint movement and thus provoke pain.

*In vitro* experiments have elucidated several mechanisms by which excessive amounts of adipokines lead to the destruction of articular joints. In cartilage derived from human OA patients, leptin enhances the synthesis of several proinflammatory mediators, such as NO, $PGE_2$, IL-6, and IL-8, via inducible nitric oxide synthase (iNOS) pathways. By inhibiting the iNOS activity, NO synthesis was nearly completely blocked. This reduction of NO reduces the production of $PGE_2$, IL-6, and IL-8 [109]. Furthermore, membrane bound prostaglandin E synthase 1 (mPGES-1) and COX-2 enzyme are overexpressed in the cartilage of such patients. COX-2 further increases the production of prostaglandins. This overexpression can be induced by IL-1 and TNF-alpha, factors released by adipose tissue. mPGES-1 mediates the production of $PGE_2$ [110]. $PGE_2$ overproduction enhances NO-induced cell death of OA chondrocytes [111]. When IL-1 acts together with leptin, they can activate nitric oxide synthase type II, which increases NO production in chondrocytes [112]. Elevated NO levels lead to various catabolic processes in the cartilage, such as the loss of chondrocyte phenotype, thereby reducing production of ECM, and to chondrocyte apoptosis, and ECM degradation [113, 114].

Leptin induces the synthesis of matrix metalloproteinases (MMP), especially MMP9 and MMP13 [115–117], via IL-1 and TNF-alpha. MMPs are a large family of enzymes that degrade different components of collagen and proteoglycans [118]. Both MMP9 (gelatinase) and MMP13 (collagenase) are involved in cartilage damage [116, 117]. MMP13 is produced by chondrocytes and cleaves collagen type II (the main collagen type in articular cartilage) and the proteoglycan molecule aggrecan, leading to structural damage of the cartilage tissue [115]. These experiments clearly show that obesity, mediated by leptin, exerts a proinflammatory and catabolic effect on cartilage, leading to apoptosis of chondrocytes and the degradation of the extracellular matrix.

Leptin is not the only adipokine associated with inflammatory actions. Resitin and visfatin, together with leptin, increase the inflammatory status by means of various mechanisms, which together with mechanical overload leads to phenotype loss and apoptosis of chondrocytes, as well as cartilage matrix degeneration [99, 101].

Thus, overweight and obesity play an important role in the genesis of knee and hip joint OA not only as a result of mechanical overload but also by the complex combined action of genetic, metabolic, neuroendocrine, and biomechanical factors and represent a *significant modifiable risk factor* [102] not least for this reason.

Inflammation is also induced by *overloading* the joints. Various mechanoreceptors are expressed on the surface of chondrocytes. It has been reported that mechanical compression significantly increases $PGE_2$ release in chondrocyte explants. It was shown that mechanical stress induced COX-2 expression and that mPGES-1 mRNA (PGE synthase 1) and protein are increased in cartilage explants. mPGES-1 is involved in $PGE_2$ synthesis during inflammation. $PGE_2$ is most likely a key regulator of cartilage degeneration in OA [119]. mPGES-1 and COX-2 have also been found to be stimulated by IL-1 in chondrocytes [110].

*Traumatic injury* to the joints results in activation of many genes, including inflammatory mediators, cartilage degrading proteinases, and stress response factors [3]. Degeneration of the cartilage leads to fibronectin fragments (FN-f). Fibronectin and fibronectin fragments are found in the synovial fluid after traumatic injuries. Investigators were able to show that these fragments stimulate the expression

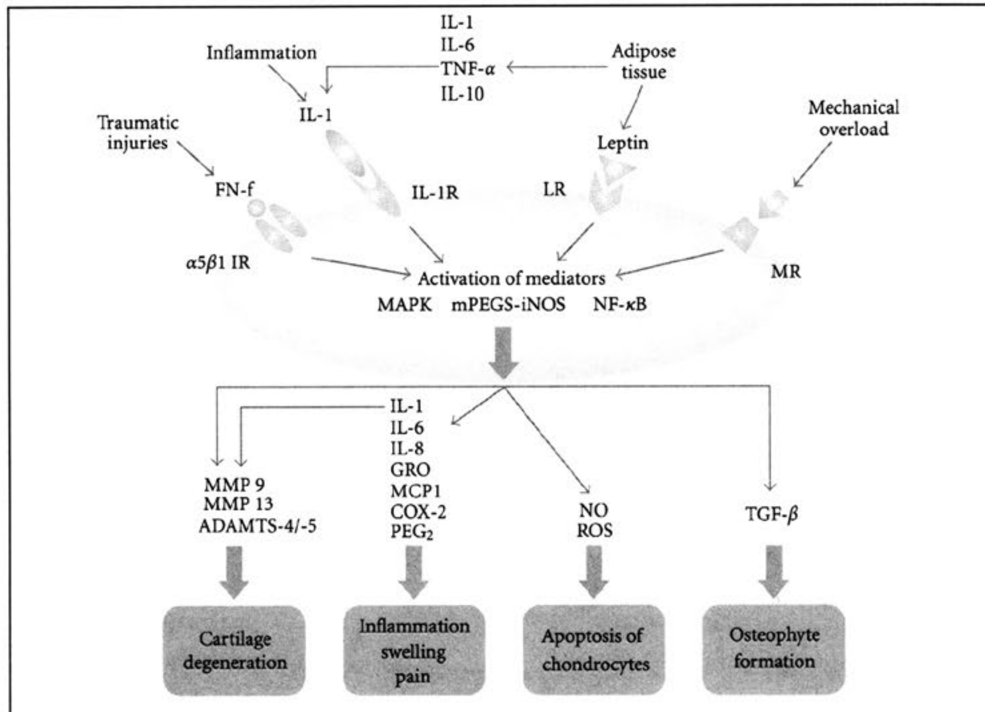


FIGURE 2: Inflammatory and oxidative processes involved in OA; FN-f: fibronectin fragment; IL-1 R: interleukin receptor; IR: integrin receptor; LR: leptin receptor; MR: mechanoreceptor.

of inflammatory cytokines and chemokines, such as IL-8, IL6, and IL-1, indicating that cartilage damage can result in further progressive cartilage degradation. The stimulation of the cytokines by FN-f is mediated by the NF-κB pathway [120]. It was further shown that FN-f stimulates MMPs in chondrocytes, which breaks down the cartilage [121, 122]. MMP13, for example, destroys type II collagen, the main collagen component of the hyaline cartilage [123, 124].

Regardless of the source, increased concentrations of inflammatory mediators activate specific aggrecanases (ADAMTS-4/-5), which cleave the aggrecan molecule in a specific region and thereby destroy the activity of this important cartilage structure molecule [125].

Inflammation and oxidative stress are prominent mechanisms which lead to progression of OA. Thus, therapy must also address this aspect.

*4.2. How Can Nutrients Modulate Inflammation Processes and Oxidative Stress Involved in Osteoarthritis?* The complex relationship between obesity and OA shows that overweight certainly represents the most significant modifiable risk factor for avoiding knee or hip joint OA. Weight reduction and weight stabilization on the basis of a balanced diet with low energy density is crucial in manifest OA [127]. But also the metabolic processes can be influenced by a dietary therapy which mainly includes chondroprotectives, such as glucosamine and chondroitin sulfate or omega-3 fatty acids.

An alternative treatment to the common NSAID therapy for OA is the use of so-called nutraceuticals, such as glucosamine, chondroitin sulfate, hyaluronic acid, hydrolyzed collagen, and omega-3 fatty acids and various vitamins and minerals. In addition to cartilage metabolism stimulation and thereby cartilage regeneration, many of them possess mechanisms which modulate the inflammatory events and oxidative processes involved in OA. As they are components of natural foods, they have far fewer adverse effects in long-term use than NSAIDs or COX-2 inhibitors, as shown in many clinical trials (see above).

They interfere with the inflammatory scenario, illustrated above, at various points (see also Figure 2).

The glucosamine and chondroitin sulfate combination suppresses IL-1-induced gene expression of iNOS, COX-2, mPGEs, and NF-κB in cartilage explants. This leads to reduced production of NO and $PGE_2$, two mediators responsible for the cell death of chondrocytes and inflammatory reactions [128, 129]. There are several ways by which glucosamine or chondroitin sulfate reduce synthesis of the COX-2 enzyme. Inhibition of the IL-1 beta induced NF-κB pathway by glucosamine sulfate results in reduced synthesis of the COX-2 enzyme [130–133]. Another manner in which glucosamine hydrochloride inhibits COX-2 activity is the prevention of COX-2 co-translational N-glycosylation and the facilitation of COX-2 protein turnover [134]. CS alone diminishes the nuclear translocation of NF-κB, which reduces the formation of proinflammatory cytokines IL-1beta and TNF-alpha and proinflammatory enzymes such

as cyclooxygenase 2 (COX-2) and nitric oxide synthase-2 (NOS-2) (for review see [135]).

The anti-inflammatory capability of CS was also tested in a rabbit atherosclerosis model. In that model, CS reduced the proinflammatory molecules C-reactive protein and IL-6 in serum, as well as the expression of MCP-1 and COX-2 in the peripheral blood mononuclear cells. It also influenced NF-$\kappa$B [136] that is responsible for the induction of inflammatory processes.

Additionally, inflammation mediators activate various cartilage degenerating enzymes. The mRNA expression of such enzymes (MMP-13 and aggrecanases (ADAMTS-5)) was reduced in cartilage explants incubated with GlcN·S and CS. In the same study, the tissue inhibitor of metalloproteinase-3 (TIMP-3), a potent inhibitor of ADAMTS, was upregulated [128]. Glucosamine sulfate alone was shown to inhibit the activation process of MMP-2 and MMP-9 expression, via downregulation of the NF-$\kappa$B pathway [137].

Inflammatory mediators are responsible for reduced biosynthesis of cartilage material. Experiments with rat chondrocytes have shown that IL-1$\beta$ inhibits the expression of the enzyme galactose-$\beta$-1,3-glucuronosyltransferase I (GlcAT-I), a key enzyme in the biosynthesis of cartilage GAG chains. Dose-dependently glucosamine was able to reduce this inhibition [132].

In addition to their anti-inflammatory action, glucosamine and chondroitin sulfate exhibit an antioxidant action which leads to a significant reduction in iNOS expression and activity [138, 139]. This is one explanation why glucosamine and chondroitin reduce the otherwise NO-induced cell death of chondrocytes. In comparison to glucosamine and CS, hyaluronic acid exerted a very minor anti-inflammatory and antiapoptotic effect, while it significantly reduced NO levels [139].

*Vitamins and Minerals.* Many vitamins are known for their antioxidant capacity. Under physiological conditions, the reactive oxygen species (ROS), produced by the body are neutralized by the body's antioxidant defense system, such as peroxidase, superoxide dismutase, or catalase. Under disease conditions, however, the increased amount of ROS can no longer be managed by the natural defense system. Arthropathies such as osteoarthritis and rheumatoid arthritis are characterized by the increased formation of free radicals [98, 102, 140]. ROS, which are extensively expressed during OA [92, 93, 141, 142], are involved in matrix and cartilage degeneration, inhibition of matrix synthesis, cell death, and apoptosis of chondrocytes. *In vitro* experiments confirmed that mechanical shear stress increases the production of oxidants in cartilage explants [90].

In a study, serum samples of 29 patients with knee OA and 26 healthy controls were analyzed for their oxidative status [92]. Total antioxidant capacity (TAC) and, in addition, the oxidative stress index (OSI Index) were determined as antioxidant parameters. The oxidative stress was measured based on total peroxide (TP) content and lipid hydroperoxide and the OSI Index was calculated from the TP/TAC ratio. Compared with the healthy controls, the OA patients had a significantly higher OSI Index, whereas all the markers for antioxidant activity were lower. Prolidase activity (collagen synthesis marker) was also significantly lower in the OA patients. Moreover, the enzyme activity correlated positively with the antioxidant concentration (TAC) and negatively with oxidative stress (OS). Hence, the higher the antioxidant concentration, the better the cartilage metabolism process. Conversely, oxidative stress was associated with impaired cartilage metabolism [92].

A working group showed that OA patients have a significantly reduced concentration of antioxidants (vitamins C and E) and increased oxidative stress. Oxidative stress was measured on the basis of the malondialdehyde (MDA) concentration [93].

Therefore, OA treatment should not only focus on regeneration and anti-inflammatory processes but also on the reduction of oxidative stress in these patients. Positive effects on OA have been found for a number of vitamins and minerals (for review see [143, 144]).

*Vitamin C*, for example, stimulates collagen synthesis, and to a lesser extent the synthesis of aggrecan. Proteoglycan synthesis is increased in chondrocyte cultures [145] (for review see [143, 146]). An animal study showed that vitamin C has a protective effect on knee cartilage [147]. The effect of chondrocyte protection could be mediated by its antioxidant capacity. Similar results were reported for vitamin E, which is known for its strong antioxidant effects, its protection against ROS, and enhancement of chondrocyte growth [143]. The positive effects of vitamin E were demonstrated in clinical trials. Patients treated with vitamin E displayed a significant reduction in pain when compared to placebo, and comparable effects to diclofenac (for review see [143]).

*Selenium, zinc, and copper* are minerals under discussion as supporting OA treatment. They exhibit antioxidant characteristics and are part of antioxidant enzymes. Rats fed with a low selenium diet showed a decrease in sulfotransferase activity. This enzyme is involved in the process of glycosaminoglycan synthesis, which is important for the cartilage matrix [148]. In a double-blind, placebo-controlled study, the combination of *selenium and vitamins A, C, and E*, had a positive but nonsignificant effect that tends to improve pain and stiffness in OA patients, compared to placebo [149]. *Manganese* is a component of glycosal and xylosyltransferase enzyme which are responsible for the glycosidic binding and thus for the glycosaminoglycan synthesis. Manganese is also involved in the cross-linking of collagen fibrils and inhibits elastin-degrading elastases [150]. Copper, an essential component of lysyl oxidase, contributes to the cross-linking of collagen and elastin in cartilage and bone tissue, and molybdenum is a cofactor of sulfitoxidase enzyme producing sulfates which are important for proteoglycan synthesis.

*Synergistic Action of Chondroprotectives, Omega-3 Fatty Acids, and Other Nutrients. Omega-3 polyunsaturated fatty acids* (PUFAs), such as linolenic acid and eicosapentaenoic acid (EPA), are found in walnut, flaxseed, and fish oils. They are known for their anti-inflammatory actions, which has

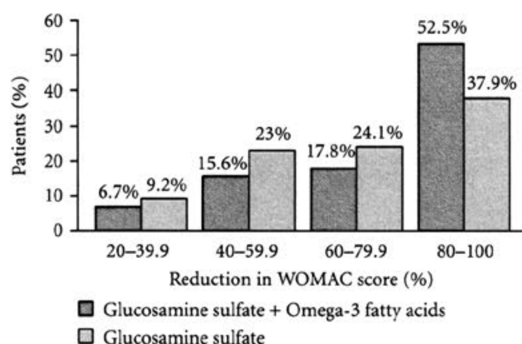


FIGURE 3: A greater proportion of patients with combination therapy GlcN·S + O-3 FA showed the highest WOMAC improvements of 80–100% [126].

been shown in several studies (see [144, 147, 151]). They have been successfully used in clinical trials, mainly to treat rheumatoid arthritis [152–154]. *In vitro* studies showed that omega-3 fatty acids increase collagen synthesis and decrease the inflammation mediator $PGE_2$ [155]. EPA, when oxygenated, results in the bioactive product resolving El (RvE1). By activation of a specific receptor, ChemR23, RvE1 dramatically reduces inflammatory processes by inhibiting the NF-$\kappa$B pathway that is responsible for many of these processes [156]. Omega-3 fatty acids decrease IL-1-induced aggrecanase and collagenase activity and reduce mRNA expression of ADAMTS-4, COX-2, IL-1$\alpha$, and TNF-$\alpha$. Furthermore, they decrease the protein levels of several MMPs [157] (for review see [144]).

*PUFAs* are important components of a dietary OA therapy. Oxygen radicals are eliminated through the supplementation of antioxidants. They are generated to an increased extent in OA and are involved in cartilage degeneration (99,152), but also promote inflammatory processes in the body quite generally. Numerous studies have dealt with the anti-inflammatory effects of the polyunsaturated fatty acids *eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)* and their role in cartilage metabolism [157].

A recent study was able to demonstrate that the combined administration of EPA and DHA in a glucosamine therapy markedly alleviated the discomfort of knee and hip joint OA patients [126]. In this randomized study, 177 patients suffering from moderate to severe OA of the knee or hip joint were subdivided in two groups. One group took a combination of 1,500 mg of glucosamine sulfate plus the omega-3 fatty acids EPA and DHA as well as vitamins A, D, and E every day for 26 weeks. The other group was given a preparation without EPA and DHA. At baseline and at weeks 13 and 26 the subjects were examined and their complaints were documented based on the Western Ontario and McMaster Universities Osteoarthritis-Index (WOMAC). Both groups showed an improvement as a result of the therapy demonstrated by a reduction in the WOMAC pain score of 20% or more. If the criterion of therapy success was greater, for example, 80%, a significantly greater number of patients in the combination group (52.2%) reached this aim as compared to the group taking the preparation without EPA and DHA (37.9%; $P = 0.044$; Figure 3). In addition, typical OA symptoms such as joint stiffness or joint pain had already decreased at week 13 and towards the end of the trial continued to decrease by 48.5% to 55.5% in the EPA and DHA group as compared to 41.7% to 55.3% in the control group.

The results of these *in vivo* and *in vitro* experiments clearly demonstrate an anti-inflammatory action for glucosamine, chondroitin sulfate, hyaluronic acid, and omega-3 fatty acids. Due to these abilities, it is plausible that such nutrients can reduce collagen degradation [157] in osteoarthritis.

## 5. Conclusion

Based on the preclinical and clinical data, it is obvious that chondroprotectives such as glucosamine, chondroitin sulfate, and other nutrients, such as antioxidants and PUFAs, can modulate osteoarthritis. In long-term use they exhibit, in contrast to NSAIDs, an excellent safety profile, with as few adverse events as placebo.

The chondroprotectives are essential components of the cartilage metabolism and stimulate important cartilage regeneration processes, thereby adjusting the imbalance of catabolic and anabolic processes in osteoarthritis.

Newer data point out that inflammation and oxidative stress are characteristics of all stages of the disease. Chondroprotectives are able to inhibit many of these processes. They defend chondrocytes against oxidative stress-induced apoptosis, reduce the inflammatory mediator-induced joint cartilage degeneration, and reactivate the inflammation-reduced anabolic processes of extracellular matrix components. This leads to reduced inflammation, swelling, and pain, and to an increased mobility of the affected joints. Especially when used in combination with other nutrients, such as antioxidants and omega-3 fatty acids, these substances are able to exert synergistic effects on the osteoarthritic joints.

Recently new study results were published that demonstrate promising effects of further food substances or phytochemicals, such as contained in ginger extracts, showing various antiosteoarthritic actions and, for example, even intra-articular resveratrol showing chondroprotective effects in a rat animal model.

In summary, future "nutraceutical" approaches to OA most likely will have to be more complex and should include glucosamine sulfate (and/or chondroitin sulfate) together with hyaluronic acid, collagen hydrolysate, and several other nutrients which were shown to have promising actions on joint cartilage, synovial fluid, and overall clinical outcome in OA patients.

## Abbreviations

CS: Chondroitin sulfate
COX: Cyclooxygenase
ECM: Extracellular matrix

| | |
|---|---|
| EULAR: | The European League Against Rheumatism |
| FN-f: | Fibronectin fragment |
| GAG: | Glycosaminoglycan |
| GlcN: | Glucosamine |
| GlcN·HCl | Glucosamine hydrochloride |
| GlcN·S: | Glucosamine sulfate |
| IL: | Interleukin |
| IR: | Integrin receptor |
| JSW: | Joint space width |
| LR: | Leptin receptor |
| MMP: | Matrix metalloproteinase |
| MR: | Mechanoreceptor |
| NF-κB: | Nuclear factor-κB |
| iNOS: | Inducible nitric oxide synthase |
| NSAIDs: | Nonsteroidal anti-inflammatory drugs |
| OA: | Osteoarthritis |
| $PGE_2$: | Prostaglandin $E_2$ |
| ROS: | Reactive oxygen species |
| SMOAD: | Structure-modifying OA drug |
| SYSADOA: | Symptomatic slow-acting drug in osteoarthritis |
| WOMAC: | Western Ontario and McMaster Universities. |

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# Efficacy of methylsulfonylmethane (MSM) in osteoarthritis pain of the knee: a pilot clinical trial[1,2]

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## Summary

*Objective*: Osteoarthritis (OA) is the most common form of arthritis and the second most common cause of long-term disability among middle-aged and older adults in the United States. Methylsulfonylmethane (MSM) is a popular dietary supplement used as a single agent and in combination with other nutrients, and purported to be beneficial for arthritis. However, there is paucity of evidence to support the use of MSM.

*Methods*: A randomized, double-blind, placebo-controlled trial was conducted. Fifty men and women, 40–76 years of age with knee OA pain were enrolled in an outpatient medical center. Intervention was MSM 3 g or placebo twice a day for 12 weeks (6 g/day total). Outcomes included the Western Ontario and McMaster University Osteoarthritis Index visual analogue scale (WOMAC), patient and physician global assessments (disease status, response to therapy), and SF-36 (overall health-related quality of life).

*Results*: Compared to placebo, MSM produced significant decreases in WOMAC pain and physical function impairment ($P < 0.05$). No notable changes were found in WOMAC stiffness and aggregated total symptoms scores. MSM also produced improvement in performing activities of daily living when compared to placebo on the SF-36 evaluation ($P < 0.05$).

*Conclusion*: MSM (3 g twice a day) improved symptoms of pain and physical function during the short intervention without major adverse events. The benefits and safety of MSM in managing OA and long-term use cannot be confirmed from this pilot trial, but its potential clinical application is examined. Underlying mechanisms of action and need for further investigation of MSM are discussed.
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*Key words*: Osteoarthritis, Knee arthritis, Arthritis pain, Methylsulfonylmethane, Randomized controlled trial.

## Introduction

Osteoarthritis (OA) is the leading cause of disability, limiting everyday activities of more than 7 million Americans[1], and is associated with restrictions on quality of life[2]. The demand for arthritis pain control has resulted in the widespread use of palliative drugs, e.g., nonsteroidal anti-inflammatory drugs (NSAIDs), acetaminophen, and cyclooxygenase-2 (COX-2) inhibitors[3,4], surgical interventions[5], and in recent years, the use of complementary and alternative medicine (CAM)[6]. Additional treatment options are being actively sought out by patients as discontinuation of COX-2 drugs rises due to concerns of safety.

A dietary supplement with increasing use is methylsulfonylmethane (MSM) often in combination with glucosamine and chondroitin sulfate, which as opposed to MSM has numerous efficacy trials supporting its use in OA[7–9]. MSM is popularly used for arthritic and rheumatic pain; in 2003, the retail sales of MSM as a single ingredient were $115 million[10]. MSM is a naturally occurring organosulfur molecule and a putative methyl donor. MSM is the first oxidized metabolite of dimethylsulfoxide (DMSO). In the troposphere, DMSO is a byproduct of phytoplankton and algae decay. In commercial production, MSM is synthesized by reacting DMSO and hydrogen peroxide, which yields

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MSM and water. In the body, approximately 15% of orally ingested DMSO is metabolized into MSM[11]. A recent study showed that MSM was found in human cerebrospinal fluid and plasma at 0–25 μmol/l concentrations[12]. Because of MSM's sulfur content, it is used by the body to maintain normal connective tissues. MSM may have anti-inflammatory activities, chemopreventive properties, prostacyclin ($PGI_2$) synthesis inhibition, anti-atherosclerotic action, salutary effect on eicosanoid metabolism, and free radical scavenging activity[13–15]. In murine models, MSM was shown to effect inflammatory conditions such as rheumatoid arthritis and lupus[16,17]. One randomized controlled trial of MSM and OA has been published[18]. In Usha and Naidu's[18] 12-week trial ($n = 118$), patients with knee OA received either 1.5 g MSM, 1.5 g glucosamine sulfate, 1.5 g MSM plus glucosamine sulfate, or placebo; significant decreases in the Lequesne Index were reported with MSM, glucosamine sulfate, and their combination ($P < 0.05$). The authors reported a 33% decrease in pain in the MSM group; joint mobility, swelling, global evaluation, and walking time also improved.

MSM safety and toxicity clinical studies have not been published. Acute and subchronic animal toxicity studies using single dose of 2 g/kg and daily doses of 1.5 g/kg MSM for 90 days showed no adverse events, organ pathology or mortality[19]. These doses are considered five to seven times the maximum dose used in humans. MSM is generally considered safe, and listed on *The Arthritis Foundation's Guide to Alternative Therapies* for OA with a cautionary note on lack of research[20]. There have been unconfirmed reports of mild adverse effects from oral use of MSM including gastrointestinal (GI) symptoms, headaches, amplified effects of blood thinning drugs resulting in easy bruising and blood in stool, increased blood pressure, increased hepatic enzymes, and insomnia if taken at bedtime[21]. However, there are no clinical studies on adverse effects, changes in blood chemistry, safety monitoring data or possible subclinical neurotoxicity symptoms of MSM. MSM is currently sold in over 52 different products as a single agent in capsule, caplet, lotion and cream forms, and in more than 30 different products in combination with other dietary supplements (glucosamine and chondroitin sulfate being the most common). MSM is readily available at health food stores and on the Internet with alarmingly little guidance on safety and how to take the supplement. Investigation is needed on MSM efficacy and safety in the dosages commonly used by practitioners and consumers alike to treat OA, which are higher than the dosage used in Usha's study. Equally significant is the public service provided by testing a highly prevalent dietary supplement to contribute to the scientific repertoire of this new supplement that is not regulated by the Food and Drug Administration (FDA). Considering the popularity of MSM and purported improvements in OA pain, additional efficacy and safety trial of MSM will thus be valuable in advising practitioners and patients in the appropriate use, if any, of MSM for arthritis pain management. Although a murine model reported decreasing joint degeneration[22], due to the preliminary design and short intervention period, treatment responses were limited to OA symptoms, and did not include radiographic changes of the joints following intervention.

## Methods

### PARTICIPANTS

The study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Review Board at Southwest College of Naturopathic Medicine. Written consents were obtained prior to enrollment. Knee OA was selected as opposed to hip or hands to evaluate a single joint for a preliminary efficacy clinical trial. Study inclusion criteria included men and women >40 years diagnosed with knee OA according to modified criteria of the American College of Rheumatology (ACR)[23,24]; ACR functional class I, II or III[25]; radiographic confirmed Kellgren–Lawrence grades 2–3 (mild to moderate osteophytes and joint space narrowing, previous 3 years)[26]; regular arthritis pain (arthritis pain in most days) for 3 months or more; >40 mm arthritis pain rating of target knee (100 mm visual analogue scale (VAS)); and >2 rating on patient global assessment (GA) of overall arthritis disease status (five-point Likert scale). Patients were not required to be asymptomatic in the other joints. Study exclusion criteria included any other type of arthritis; rheumatoid or inflammatory arthritis; fibromyalgia or other chronic pain syndrome; arthroscopy or intra-articular corticosteroids/hyaluronic acid injections in the previous 3 months; concurrent anti-coagulant/anti-platelet drugs, corticosteroids or narcotic pain killers use; history of epilepsy or bleeding disorders; gastric ulcers; renal or hepatic disease; uncontrolled hypertension, or body mass index (BMI) >45 kg/m$^2$. A washout period of 7 days was required for NSAIDs users. Discontinuing the use of common CAM therapies for arthritis (e.g., glucosamine, chondroitin sulfate, bromelain, DMSO, acupuncture) was required for 7 days prior to enrollment.

### ENROLLMENT AND RANDOMIZATION PROCEDURES

Patients were recruited from the Phoenix metropolitan area using newspaper advertisements, flyers at local clinics, and press releases. Initial screening was conducted over the phone or in person. Qualified patients ($n = 50$) were assigned to MSM ($n = 25$) or placebo ($n = 25$) in a 12-week randomized, double-blind, placebo-controlled trial using computer-generated random numbers (Fig. 1). The generation of numbers and assignments were provided by different research staff not involved with patient contacts or data collection. Rescue analgesic, 325 mg acetaminophen tablets (100 tablets), was provided with instructions for use with intolerable pain and to not exceed taking 2.6 g/day. To monitor compliance and adverse events, weekly and biweekly phone calls to patients were made during the 12 weeks by the research staff.

### MSM DOSAGE AND PREPARATION

A dosage of 6 g/day was selected based on common clinical and over-the-counter uses of MSM. A 1-week, stepwise approach to the full dose was undertaken. Week 1, started with 2 g/day in two divided doses for 3 days, and then increased to 4 g/day for 4 days. Week 2, increased to 6 g/day. Patients were instructed to take with food, and to avoid taking them at bedtime. Distilled MSM microprill (OptiMSM®, Cardinal Nutrition, Vancouver, WA) in 1 g caps was used. Purity of MSM was confirmed to be 99.9% by high-resolution gas chromatography. DMSO content was <0.05%. The placebo consisted of inert ingredients and was indistinguishable in color, size and taste compared to the MSM. Test materials were certified to be free of microbiological contamination. Heavy metal analysis by graphite furnace atomic absorption spectrophotometry and cold vapor analysis verified no quantifiable lead, arsenic, cadmium, aluminum, or mercury. The analytical tests were validated for

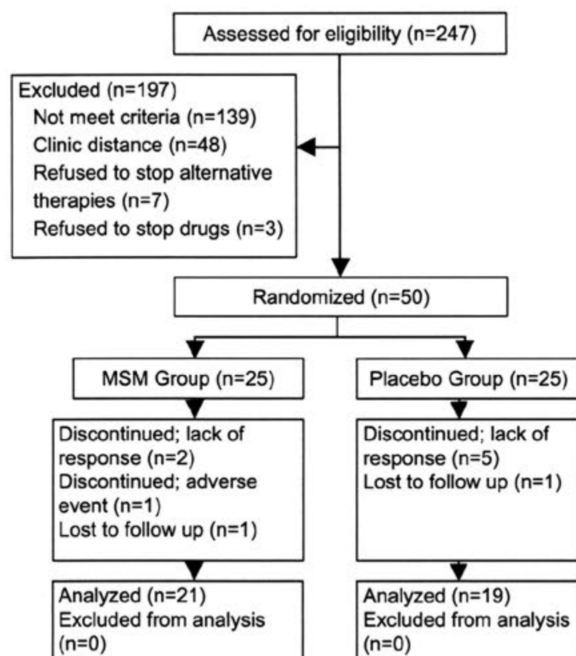


Fig. 1. Patient enrollment and follow-up diagram.

the sample matrix and reported on a signed certificate of analysis from the manufacturer. These assays were performed by an independent, third-party laboratory as part of the standard quality control in the manufacture of the raw ingredient MSM.

EFFICACY EVALUATIONS

The knee with the worst arthritis pain (target joint) at screening was the joint evaluated for efficacy. Primary endpoints were the composite subscales in the Western Ontario and McMaster University Osteoarthritis Index VAS (WOMAC version 3.1) on pain (five questions), stiffness (two questions), physical function (17 questions), and aggregated total symptoms (24 questions)[27,28]. The WOMAC was scored from 0 mm to 100 mm (0 = no pain, 100 = worst pain), and collected at baseline (following the washout period of 7 days), 2, 4, 8 and 12 weeks. Secondary endpoints were the patient GA, physician GA, and SF-36 (version 2) for the overall health-related quality of life, collected at baseline and 12 weeks. The patient GA and physician GA were scored on a five-point Likert scale for overall arthritis disease status (0 = very well, 1 = well, 2 = moderate, 3 = poor, 4 = very poor) and response to therapy (0 = excellent response, 1 = good response, 2 = moderate response, 3 = slight response, 4 = no response). SF-36 was chosen for its previous application in a variety of diseases including OA efficacy studies[29–31]. Responses to the 36 items are categorized into nine domains: physical functioning, role physical, bodily pain, general health, vitality, social functioning, role emotional, mental health, and reported health transition. Scores ranged from 0 to 100 with higher scores indicating better state of health and quality of life.

To investigate MSM's potential activity, although not commonly evaluated in OA studies, serum homocysteine, high sensitive C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), and urine malondialdehyde (MDA) were measured at baseline and 12 weeks. Hyperhomocysteinemia is associated with ischemic cardiovascular conditions; and reducing homocysteine with micronutrients has been demonstrated to decrease heart disease[32]. Homocysteine is metabolized by a remethylation pathway (transfer of methyl by methylcobalamin or betaine [trimethylglycine]) generating methionine, and a transsulfuration pathway which degrades homocysteine into cysteine and then taurine[33]. Since MSM is a putative methyl donor, its activity as a co-factor in reducing homocysteine levels was tested. To monitor any anti-inflammatory activities of MSM, as suggested from empirical and published literature, CRP and ESR were tested. The MDA was measured to examine the possible antioxidant effect of MSM, which has also been suggested[15]. Thus, these lab tests were included in the pilot clinical trial to explore MSM's mechanisms of action. Other endpoints included the use of rescue analgesics and compliance with MSM regimen.

ADVERSE EVENTS EVALUATIONS

Laboratory tests, questionnaires, blood pressure, weight, BMI, and other vitals were collected at baseline and 12 weeks. The laboratory tests included hematology (complete blood counts and differential white blood cells), clinical chemistry (renal and hepatic functions), fasting lipid profile, urinalysis, and stool occult blood test. The questionnaires included the standard GI symptoms and modified neurotoxic symptoms using a four-point Likert scale ranging from 0 to 3 (0 = no, 1 = mild, 2 = moderate, 3 = severe). Questions related to changes in blood clotting were also included. Modifications were made on neurotoxicity questionnaires used in drug trials for application in our study[34–36]: cognitive function (fatigue, concentration, slowing, memory, motor coordination and language), peripheral neurological symptoms (sensory disturbance and muscle weakness), and other symptoms (insomnia, headache and blurred vision).

STATISTICAL ANALYSIS

The intent-to-treat analysis was performed using SPSS (version 11.0) software. The changes from baseline to 12 weeks between treatment and placebo groups were considered significant for Student's *t* test $P$ values $< 0.05$ at the 95% confidence level. Estimated sample size was calculated using 80% power with a two-sided (tailed) test, alpha of 0.05 to detect a 25% improvement in VAS arthritis pain score from baseline to 12 weeks in the MSM treated group, indicating that 22 patients were required[37,38]. The estimated variance and power calculation were based on previous knee OA pilot trial publications[39,40]. With an anticipated 10% attrition rate, 25 patients per group were adequate to meet the sample size requirement.

## Results

DEMOGRAPHIC PROFILE

About 90% of patients were in the ACR classes I and II, and 5% in class III (Table I). Average arthritis duration was about 6 years. The mean pain level was 55 mm in the placebo group and 58 mm in the MSM group. Thirty-seven percent and 38% of patients in the placebo group and MSM group, respectively, used some type of NSAIDs,

Table I
*Demographic profile of patients and arthritis characteristics*

| | MSM (*n* = 21) | Placebo (*n* = 19) |
|---|---|---|
| Sex (%) | | |
| Men | 42.9 | 31.6 |
| Women | 57.1 | 68.4 |
| Age, mean (years) | 56.6 (SD = 8.6) | 55.6 (SD = 8.7) |
| Ethnicity (%) | | |
| White/non-Hispanic | 100 | 89.5 |
| Asian/Pacific islander | 0 | 10.5 |
| NSAID use (%) | 38.1 | 36.8 |
| MSM use (%) | 28.6 | 26.3 |
| DMSO use (%) | 9.5 | 5.3 |
| Glucosamine plus chondroitin sulfate use (%) | 4.8 | 10.5 |
| ACR functional capacity classification (%) | | |
| I | 23.8 | 26.3 |
| II | 71.4 | 68.4 |
| III | 4.8 | 5.3 |
| Kellgren–Lawrence grade (%) | | |
| 2 | 61.9 | 57.9 |
| 3 | 38.1 | 42.1 |
| Arthritis duration, mean (years) | 5.8 (SD = 5.5) | 5.9 (SD = 5.2) |
| Pain VAS, mean ± S.E.M. (0–100 mm, VAS) | 58.0 ± 5.5 | 55.1 ± 5.8 |
| Patient GA of disease status, mean ± S.E.M. (0–4, Likert) | 3.0 ± 0.1 | 2.8 ± 0.2 |
| Physician GA of disease status, mean ± S.E.M. (0–4, Likert) | 2.8 ± 0.2 | 2.5 ± 0.1 |

and 26% and 29%, respectively, may have used MSM-containing products. The patients with history of MSM intake used less than 1 g/day, were inconsistent in daily use and /or used MSM predominantly in the form of combination dietary supplement products rather than as monotherapy, and the quality of MSM was not determined. Also, one patient in the MSM group and two patients in the placebo group were using glucosamine plus chondroitin sulfate prior to study enrollment. Expectation bias and confounding variables of participating in a study testing MSM were therefore likely to be minimal (patients could not have discerned the individual effects of MSM when taken in products containing many other active ingredients, e.g., glucosamine, chondroitin sulfate, herbs, vitamins, and minerals). No major differences in the arthritis disease status and other characteristics were found between the MSM and placebo groups at enrollment. The baseline patient profiles suggest that any changes in response to the intervention were not due to variability of patients in the two groups. Compliance with pill taking and other study instructions were obtained from the majority of patients. In the MSM group 89.5% and in the placebo group 90.5% took at least five pills a day. The study bottles were returned at the end of treatment, and the number of pills remaining was counted.

#### EFFICACY RESULTS

The results of WOMAC are listed in Table II. The primary endpoint pain changes at 12 weeks in the MSM group were significantly greater than in the placebo group, $P = 0.041$. The changes in the physical function in the MSM group were also greater than in the placebo group at 12 weeks, $P = 0.045$. The pain and physical function mean decreases from baseline to 4, 8 and 12 weeks in the MSM group were greater compared to the placebo group (Figs. 2 and 3). The changes in stiffness and aggregated total symptoms after 12 weeks of treatment were not significant between the two groups, $P > 0.05$. There were changes found in the placebo group. The differences between the MSM and placebo groups were relatively small in the WOMAC subscales. In the MSM group pain decreased by 14.6 mm (25.1%), and in placebo it decreased by 7.3 mm (13.2%) at 12 weeks. The difference in pain improvement was 7.3 mm (12%) between the MSM and placebo groups. For physical function, stiffness and total symptoms, the decreases in MSM group were 15.7 mm (30.4%), 10.1 mm (19.7%), and 13.4 mm (25.1%) and the decreases in placebo group 8.8 mm (16.7%), 6.5 mm (11.7%), and 7.5 mm (13.8%), respectively. The differences between the groups were 6.8 mm (13.7%), 3.6 mm (8.0%), and 5.9 mm (11.3%) at 12 weeks, respectively. The patient GA and physician GA of overall arthritis disease status changes at 12 weeks in the MSM group and placebo group were not significant, $P > 0.05$ (Table II). However, the changes in disease status suggest a trend toward improvement in the treatment group. The patient GA and physician GA of response to therapy also showed no major differences. In the SF-36 quality of life results, of the nine domains, only the role physical domain at 12 weeks in the MSM group was significant with a mean change of 16.45 (SD = 20.84), $P = 0.021$. While in the placebo group, a mean change of 12.48 (SD = 23.17) was observed on the role physical domain, $P = 0.175$. No notable changes were found in the other eight domains, $P > 0.05$. There were no appreciable differences in the use of rescue analgesics; the mean use was 37.9 (SD = 25.7) tablets over 12 weeks in the placebo group compared to 27.4 tablets (SD = 21.2) in the MSM group.

#### LAB MONITORING

Hematology, clinical chemistry and urinalysis did not have any abnormal changes from baseline to 12 weeks. There were no major changes in the complete blood counts, differential white blood cell counts, hepatic and renal functions, lipid profiles, BMI, vitals, stool occult test, swelling or tenderness of the target knee joints. Three patients did have positive hemoccult tests at 12 weeks, two in the placebo group and one in the MSM group. The hemoccult was repeated 2 weeks later; the results were negative. Homocysteine ($P = 0.004$) and urine MDA ($P = 0.010$) were the only two laboratory markers with significant differences at 12 weeks between the MSM and placebo groups (Table III).

#### ADVERSE EVENTS

The incidences of GI and other side effects included bloating, constipation, indigestion, fatigue, concentration issues, insomnia, and headache (Table IV). These symptoms were minor, without complications, and did not interfere with daily activity or require treatment. Patients in the MSM and placebo groups reported the symptoms in comparable frequency. Of the 50 patients enrolled, 40 completed the study: 21 (84%) in the MSM group and 19 (76%) in the placebo group. The majority of patient withdrawals were reportedly due to lack of perceived response to therapy, two in the

Table II
*WOMAC, patient and physician GAs*

| | MSM ($n=21$) | | | Placebo ($n=19$) | | | Between group difference at 12 weeks *P* values |
|---|---|---|---|---|---|---|---|
| | Baseline mean ± S.E.M. | 12 weeks mean ± S.E.M. | Change ± S.E.M. | Baseline mean ± S.E.M. | 12 weeks mean ± S.E.M. | Change ± S.E.M. | |
| WOMAC (0–100 mm, VAS) | | | | | | | |
| Pain | 58.0 ± 5.5 | 43.4 ± 4.6 | −14.6 ± 1.3 | 55.1 ± 5.8 | 47.9 ± 4.8 | −7.3 ± 3.3 | 0.041* |
| Stiffness | 51.2 ± 5.4 | 41.1 ± 4.8 | −10.1 ± 2.6 | 55.2 ± 6.2 | 48.7 ± 6.8 | −6.5 ± 2.4 | 0.320 |
| Physical function | 51.5 ± 4.5 | 35.8 ± 3.2 | −15.7 ± 2.0 | 52.9 ± 5.9 | 44.1 ± 5.1 | −8.8 ± 2.7 | 0.045* |
| Total symptoms | 53.6 ± 4.9 | 40.1 ± 3.9 | −13.4 ± 1.7 | 54.4 ± 5.6 | 46.9 ± 5.2 | −7.5 ± 2.5 | 0.054 |
| Patient GA (0–4, Likert) | | | | | | | |
| Disease status | 3.0 ± 0.1 | 2.5 ± 0.2 | −0.5 ± 0.2 | 2.8 ± 0.2 | 2.5 ± 0.2 | −0.3 ± 0.2 | 0.549 |
| Physician GA (0–4, Likert) | | | | | | | |
| Disease status | 2.8 ± 0.2 | 2.5 ± 0.1 | −0.3 ± 0.1 | 2.5 ± 0.1 | 2.3 ± 0.2 | −0.2 ± 0.2 | 0.447 |

*Between group differences in the MSM and placebo evaluated using the Student's *t* test. The changes were considered significant for $P < 0.05$. The changes in the primary endpoints WOMAC pain and physical function at 12 weeks were significant between the MSM and placebo groups.

MSM group and five in the placebo. One patient dropped out in each group due to loss to study follow-up. One patient in the MSM group discontinued prematurely due to an adverse event in the first 2 weeks. The patient reported having neck and back pain that were similar in symptoms to a previous kidney infection. Clinical examination and lab tests of the patient showed that there was no infection or other major health problems. The patient also reported worsening of arthritis pain and joint swelling.

## Discussion

In this trial, MSM at 3 g twice a day for 12 weeks produced improvement in two of the three WOMAC subscales, pain and physical function, $P < 0.05$. Comparable efficacy results have been reported in the literature but for different MSM dosages. The dosage we used was four times the dosage of Usha's study, but in our study arthritis pain decreased by 25.1% compared to 33% decrease seen in Usha's study[18]. The small sample size with variations in arthritis pain and other patient characteristics confounding the data may have contributed to the smaller decrease observed in our study compared to Usha's study. An unpublished trial ($n = 16$) of MSM for arthritis pain, 2.25 g, also reported some improvement[41]. The changes in WOMAC subscales, pain, physical function, stiffness, and total symptoms in the placebo group by 7.3 mm (13.2%), 8.8 mm (16.7%), 6.5 mm (11.7%), and 7.5 mm (13.8%), respectively, at 12 weeks are worthy of discussion. The changes in the placebo group and the small differences between the two groups indicate that the effect of MSM was modest. Thus, while improvements in pain and physical function were shown to be statistically significant, the clinical significance of these symptoms improvements remains uncertain. The overall trend in WOMAC subset decreases does show benefits of MSM, and further evaluation for practical application is justified. Another noteworthy finding is that the WOMAC subsets continued to decline at 12 weeks, suggesting that the full effects of MSM were not captured during the relatively short intervention; a longer study is needed to determine if and when the effects of MSM would plateau. The patient GA and physician GA trends correlated with those observed with the WOMAC in the MSM group. However, efficacy changes in previously published COX-2 drug trials are greater, e.g., celecoxib decreased WOMAC pain,

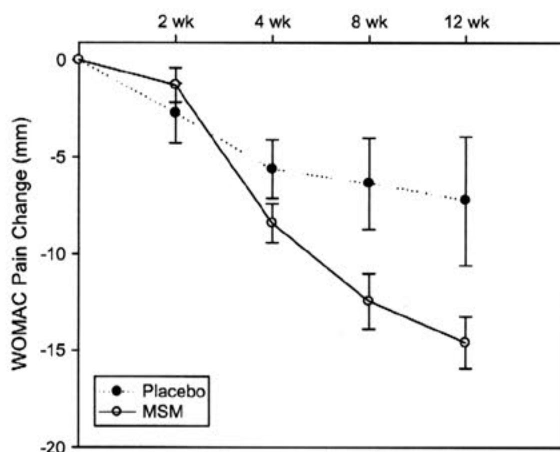


Fig. 2. WOMAC pain changes from baseline to 2, 4, 8 and 12 weeks.

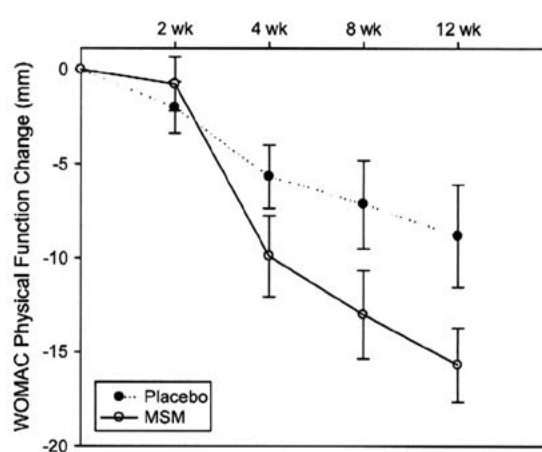


Fig. 3. WOMAC physical function changes from baseline to 2, 4, 8 and 12 weeks.

Table III
*Lab markers: total cholesterol, homocysteine, CRP, ESR and urine MDA*

| | MSM ($n = 21$) | | | Placebo ($n = 19$) | | | Between group difference at 12 weeks *P* values |
|---|---|---|---|---|---|---|---|
| | Baseline mean ± S.E.M. | 12 weeks mean ± S.E.M. | Change ± S.E.M. | Baseline mean ± S.E.M. | 12 weeks mean ± S.E.M. | Change ± S.E.M. | |
| Total cholesterol (mg/dL) | 213.5 ± 10.4 | 203.5 ± 8.5 | −10.0 ± 3.2 | 201.5 ± 9.8 | 193.7 ± 9.9 | −7.8 ± 2.7 | 0.607 |
| Homocysteine (μmol/L) | 8.0 ± 0.4 | 7.2 ± 0.4 | −0.8 ± 0.2 | 8.3 ± 0.4 | 8.6 ± 0.5 | 0.4 ± 0.3 | 0.004* |
| CRP (mg/L) | 1.6 ± 0.3 | 1.5 ± 0.2 | −0.1 ± 0.2 | 2.3 ± 0.4 | 2.3 ± 0.4 | 0.1 ± 0.2 | 0.540 |
| ESR (mm/h) | 6.4 ± 1.2 | 5.8 ± 0.9 | −0.6 ± 0.7 | 5.7 ± 1.3 | 6.2 ± 1.1 | 0.4 ± 0.6 | 0.324 |
| Urine MDA (μmol/L) | 16.7 ± 1.0 | 14.3 ± 0.8 | −2.4 ± 0.9 | 15.0 ± 1.0 | 16.3 ± 0.9 | 1.3 ± 1.0 | 0.010* |

*Between group differences in the MSM and placebo evaluated using the Student's *t* test. The changes were considered significant for $P < 0.05$. The changes in homocysteine and urine MDA at 12 weeks were significant between the MSM and placebo groups.

stiffness and physical function by 28.6 mm, 27.9 mm, and 24.9 mm, respectively[3], and etoricoxib decreased by 22.29 mm, 19.01 mm, and 22.87 mm[4], compared to our MSM trial, which decreased by 14.6 mm, 10.1 mm, and 15.7 mm, respectively. The differences in OA disease characteristics should be noted for these COX-2 studies, where the patients typically had more severe arthritis compared to the patients enrolled in our study. Symptoms improved in WOMAC subsets, particularly pain from 58.0 mm to 43.3 mm at 12 weeks, these values indicate that the patients were experiencing pain.

MSM has been shown to reduce seasonal allergic rhinitis symptoms[42]. MSM's anti-inflammatory activities were sought by testing CRP and ESR. These markers are used to predict chronic conditions such as cardiovascular disease (CVD) and to monitor inflammatory disease processes[43,44]. In our study, the high sensitive CRP and ESR showed no changes. The Centers for Disease Control and the American Heart Association guidelines on CRP levels define three categories of CVD risks: low risk <1.0 mg/L, average risk 1.0–3.0 mg/L, and high risk >3.0 mg/L[44]. The baseline mean CRP was 1.6 mg/L in the MSM group and 2.3 mg/L in the placebo group, indicating average risks for CVD and no acute inflammation. The difference in CRP values between the two groups at baseline was not statistically significant ($P > 0.05$). However, there were observational clinical differences in the baseline values between the two groups. This may be due to the small sample size. Baseline mean ESR was 6.4 mm/h in the MSM group and 5.7 mm/h in the placebo group, indicating no acute inflammation since ESR < 30 mm/h is considered normal for our patient age group[45]. Thus, the effects of MSM as an anti-inflammatory agent were not determined.

Table IV
*Incidence of patients reporting adverse events based on the GI and modified neurotoxic symptoms questionnaires*

| | Number (%) | |
|---|---|---|
| | MSM ($n = 21$) | Placebo ($n = 19$) |
| GI symptoms | | |
| Bloating | 3 (25) | 2 (18) |
| Constipation | 2 (17) | 2 (18) |
| Indigestion | 1 (8) | 1 (9) |
| Loose stool | 0 | 0 |
| Gas | 0 | 0 |
| Diarrhea | 0 | 0 |
| Stomach pain | 0 | 0 |
| Acid reflux | 0 | 0 |
| Heartburn | 0 | 0 |
| Vomiting | 0 | 0 |
| Nausea | 0 | 0 |
| Blood clotting | | |
| Bruise easily | 0 | 0 |
| Nose bleeding | 0 | 0 |
| Bleeding (clotting) time longer | 0 | 0 |
| Modified neurotoxic symptoms | | |
| Cognitive function | | |
| Fatigue | 2 (17) | 3 (27) |
| Concentration | 1 (8) | 1 (9) |
| Slowing | 0 | 0 |
| Memory | 0 | 0 |
| Motor coordination | 0 | 0 |
| Language | 0 | 0 |
| Peripheral neurological symptoms | | |
| Sensory disturbance | 0 | 0 |
| Muscle weakness | 0 | 0 |
| Others | | |
| Insomnia | 2 (17) | 1 (9) |
| Headache | 1 (8) | 1 (9) |
| Blurred vision | 0 | 0 |

Reduction of lipid peroxidation signified by MDA levels has been suggested to be beneficial in patients with long-term inflammatory conditions[46]. MDA has been evaluated for mutagenic activity and lipid peroxidation-linked DNA damages, carcinogenicity and genotoxicity[47]. The decrease in urine MDA levels in the MSM group was significantly different from placebo, suggesting changes in oxidative stress with MSM. Although the baseline homocysteine levels were not elevated, the levels did decrease significantly in the MSM group. The decrease in homocysteine may be due to the donation of MSM's two methyl groups. Folic acid and B vitamins are known to reduce hyperhomocysteinemia through similar mechanisms[48]. The decreases in homocysteine and MDA suggest potential role of MSM in supporting metabolic processes requiring methylation, such as antioxidant capacities. The high total cholesterol observed in our study population (Table III) is not uncommon in OA patients[49,50], and hypercholesterolemia and increased homocysteine concentrations have been reported in chronic diseases such as CVD[51,52]. The correlation between oxidative damage and cartilage degeneration in OA has recently been demonstrated[53]. Pro-coagulant factors have been shown to compromise subchondral vasculature, and may thus accelerate joint damage[54]. MSM's effect on homocysteine and MDA could potentially exert favorable effects on hypercoagulation and articular inflammation. The role of oxidative stress, hypercholesterolemia and other dyslipidemia in contributing to joint degeneration and pain control should be explored, and this may provide new treatment possibilities to hasten progression of cartilage degeneration and other articular deformities and develop new palliative

treatment options in an integrative approach to OA. For oxidative damage, cellular damage and low-grade inflammation found in chronic conditions such as OA and increasingly evident as we age, diets that are low in simple carbohydrates, sugar, dairy, and saturated fats shown to be effective in reducing metabolic syndrome and CVD may also be helpful in joint protection and pain control in OA[55]. Such integrative method of reducing oxidative damage and inflammation, improving antioxidative capacity and metabolic markers specific for OA (e.g., hyperlipidemia, elevated inflammatory markers, and compromised vasculature), effective palliative controls, and diet modifications could address the underlying complex pathophysiology of OA.

Considering the risks associated with COX-2 inhibitor drugs[56,57] and the prevalence of coronary risk factors often found in patients with OA (e.g., hypercholesterolemia, dyslipidemia, postmenopausal women, and older age population), providing safe treatment options without life threatening CV events should receive serious consideration. Preparing guidelines for clinical application of MSM at this time is difficult, and to make suggestions that MSM, after only 12 weeks of intervention, is safer than COX-2 drugs would be inappropriate. However, in lieu of controversies surrounding the drugs for OA, and the low incidence of major adverse events and some improvements in pain reported, the possible use of MSM in managing OA symptoms warrants discussion.

Our trial did not find adverse events such as high blood pressure, changes in blood chemistry, increased bruising, or bleeding time. However, since patients taking concurrent anti-coagulant/anti-platelet drugs were excluded from our study, the effects of MSM interfering with these medications need further testing. Our study weaknesses include small sample size and short duration of treatment (12 weeks) resulting in limitations in extrapolating to the target population. Because of the single enrollment site, majority of participants were those nearby the clinic which may have further decreased patient pool size and external validity. Prior history of MSM intake of unknown MSM quality and dosing regimen by patients in both the MSM and placebo groups should be noted in interpreting the outcomes of this preliminary trial for possible influence of such prior use. Other factors to consider are narrowed interpretation of toxicity and adverse events, e.g., inclusion of patients with high blood pressure and heart disease, which are typically found in the age group with OA, may have resulted in more incidences of side effects. Also the adverse events reported by few of the patients including the one patient who prematurely discontinued with worsening of joint pain and swelling, call for further safety studies to identify possible at risk patient populations contraindicated to take MSM. Our study findings are only preliminary, and no dose–response guidance can be determined, e.g., the positive changes at varying daily dosages, 1.5–6 g, need clarification for optimum dosage appropriate for symptoms control in OA. Based on our results and previous studies, future research direction for MSM must include long-term treatments, dose–response trials, larger sample sizes, study design with greater extrinsic value, and preclinical and clinical studies to elucidate bioactivities of MSM to better understand mechanisms of action. Equally critical are MSM-drug interaction studies for safety and toxicity, since the group benefiting most from OA palliative drugs is the elderly with co-morbid conditions taking many different drugs. Future research should also consider MSM's possible antioxidant activity (like folic acid and B vitamins) which may be actually beneficial in this population who has high cholesterol and other CVD risks.

Another possibility to explore is combination treatments for palliative control in arthritis pain, such as recent practice of NSAID plus acetaminophen[58]. MSM appears to be less effective than COX-2 drugs, but its use as an adjuvant with other treatments for OA could be considered. Critical appraisal of MSM is complicated by the fact that the standard drug discovery, safety and efficacy studies from preclinical to phase I, II and III clinical trials are not being followed for MSM. This is commonly the case for CAM therapies currently in the U.S. Without first conducting human safety, pharmacokinetic and pharmacodynamics studies, dietary supplements typically undergo phase II efficacy clinical trials because biological agents under CAM do not require FDA's Investigational New Drug Application or the New Drug Application. Since MSM is generally regarded as safe with no reported serious risks, the animal toxicity study[19], previously published randomized trial[18], one unpublished trial[41], and other publications combined with findings of our trial are encouraging for MSM indication in OA. Our results support short-term intervention with MSM when NSAIDs and COX-2 drugs are contraindicated or when other treatments are ineffective. An approach based on the literature is to start at 1.5 g/day, then to increase up to 6 g/day in divided doses, and to discontinue use if no improvements in arthritis pain are noted in 4 weeks. Thus, while large, long-term, dose–response studies in a more diverse patient population are warranted, MSM should be considered in certain OA patient populations.

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Research Paper

# Safety and efficacy of undenatured type II collagen in the treatment of osteoarthritis of the knee: a clinical trial

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**Abstract**

Previous studies have shown that undenatured type II collagen (UC-II) is effective in the treatment of rheumatoid arthritis, and preliminary human and animal trials have shown it to be effective in treating osteoarthritis (OA). The present clinical trial evaluated the safety and efficacy of UC-II as compared to a combination of glucosamine and chondroitin (G+C) in the treatment of OA of the knee. The results indicate that UC-II treatment was more efficacious resulting in a significant reduction in all assessments from the baseline at 90 days; whereas, this effect was not observed in G+C treatment group. Specifically, although both treatments reduced the Western Ontario McMaster Osteoarthritis Index (WOMAC) score, treatment with UC-II reduced the WOMAC score by 33% as compared to 14% in G+C treated group after 90 days. Similar results were obtained for visual analog scale (VAS) scores. Although both the treatments reduced the VAS score, UC-II treatment decreased VAS score by 40% after 90 days as compared to 15.4% in G+C treated group. The Lequesne's functional index was used to determine the effect of different treatments on pain during daily activities. Treatment with UC-II reduced Lequesne's functional index score by 20% as compared to 6% in G+C treated group at the end of 90-day treatment. Thus, UC-II treated subjects showed significant enhancement in daily activities suggesting an improvement in their quality of life.

Key words: undenatured type II collagen, osteoarthritis, glucosamine, chondroitin, WOMAC, visual analog scale, Lequesne's Functional Index

## INTRODUCTION

Arthritis afflicts approximately 43 million Americans or approximately 16.6% of the US population. The two most common types of arthritis are osteoarthritis (OA) and rheumatoid arthritis (RA). OA of the knee and hip is a growing health concern and is the most common forms of arthritis (1-3). Pain and disease can range from very mild to very severe (3). Patients with OA have pain that typically worsens with weight bearing, including walking and standing, and improves with rest (4). Other symptoms include morning stiffness and gelling of the involved joint after periods of inactivity. Currently, OA affects

nearly 21 million people in the United States, accounting for 25% of visits to primary care physicians, and half of all Non-Steroidal Anti-Inflammatory Drugs (NSAID) prescriptions. The diverse clinical patterns of OA are observed in approximately 10% of people older than 60 years thus compromising the quality of life of millions of Americans. In addition, OA costs the North American economy approximately $60 billion per year.

Current treatment of OA includes exercise, heat/cold therapy, joint protection, weight loss, physiotherapy/occupational therapy and medications (3-5). The most common medications include acetaminophen and NSAIDs. Although these drugs are effective for reducing pain associated with OA, they do not reverse the disease. In addition, there are considerable side effects associated with the use of these drugs. As a result, OA sufferers have turned to natural nutraceuticals to ease their pain and discomfort. These products are commonly used because they are well tolerated and considered safe. Nutraceuticals are defined as functional foods, natural products, or parts of food that provide medicinal, therapeutic, or health benefits, including the prevention or treatment of disease. Currently, glucosamine and chondroitin are the two most commonly used nutraceuticals in humans as well as in animals to alleviate pain associated with arthritis (6). However, recent randomized controlled trials and meta-analysis of these supplements have shown only small-to-moderate symptomatic efficacy in human OA (7). An emerging novel nutraceutical ingredient known as UC-II has received considerable attention in the treatment of OA. UC-II is a novel undenatured type II collagen derived from chicken sternum cartilage. Previous studies have shown that undenatured type II collagen is effective in the treatment of RA (8-11), and preliminary human (12) and animal (13) trials have shown it to be effective in treating OA. Obese-arthritic dogs given 4 mg or 40 mg daily dose of UC-II for 90 days showed significant declines in overall pain, pain during limb manipulation and lameness after physical exertion (14). Greater improvement was observed with the 40 mg dose. No adverse effects or significant changes in serum chemistry were noted. Following UC-II withdrawal for a period of 30 days, all dogs experienced a relapse of overall pain, exercise-associated lameness and pain upon limb manipulation. Studies have also shown that small doses of orally administered undenatured type II chicken collagen inhibit killer T-cell attack (15). The present clinical trial evaluated the safety and efficacy of UC-II in the treatment of the knee in OA patients.

## Materials and Methods

### Study Design

This clinical trial (Human Clinical Trial Approval #06UOHI) was managed by KGK Synergize Inc. (London, ON, Canada). The study was conducted at two sites: 1) KGK Synergize Inc., and 2) Corunna Medical Research (Corunna, ON, Canada). Figure 1 illustrates the study design while Table 1 lists the procedures and observations at each time point.

Briefly, at screening (Visit 1) the consent form was discussed, signed and a complete physical examination was performed. Activity level, diet history, medication/supplement use and medical history were recorded. The VAS score, the WOMAC Index and Lequesne scores were obtained. Urine was collected for a pregnancy test for women of childbearing potential. A blood sample was taken for determination of uric acid, CBC count and differentiation, albumin, total protein, sodium, potassium, chloride, BUN, creatinine, ALT, AST, bilirubin, erythrocyte sedimentation rate (ESR) and rheumatoid factor. Upon review of blood test results, eligible subjects were instructed to get an X-ray of the affected knees to confirm diagnosis. A total of 52 subjects were recruited using the inclusion and exclusion criteria outlined in Table 2. At the first treatment visit (Visit 2), selected subjects were randomly assigned to receive UC-II (n = 26) or glucosamine HCl plus chondroitin sulfate (n = 26, G+C). On each test day (day 0, 30, 60, 90), subjects were required to come to the clinic for clinical assessment. The clinical assessments included WOMAC, Lequesne's functional index and 100-mm VAS pain scores. A subject treatment diary was completed by each patient throughout the study period to determine side effects, medication use, and product compliance.

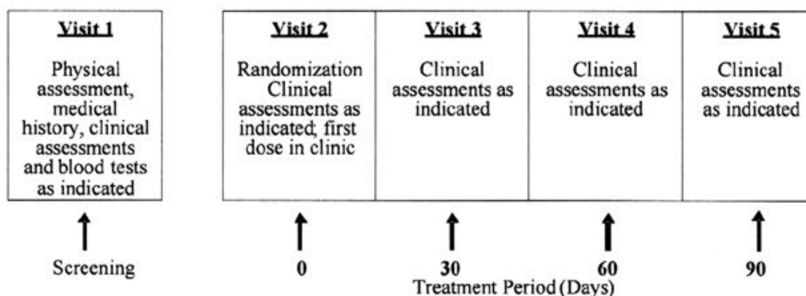


**Figure 1.** UC-II clinical study design. The study was a two-site, randomized, double-blind study conducted in London, Ontario and Corunna, Ontario, Canada.

**Table 1**. Schedule of observations and procedures

| Procedure | Visit 1 Screening | Visit 2 Day 0 | Visit 3 Day 30 | Visit 4 Day 60 | Visit 5 Day 90 |
|---|---|---|---|---|---|
| Informed consent | X | | | | |
| Review inclusion/exclusion | X | X | X | X | X |
| Medical history including activity level and diet history | X | | | | |
| Physical examination | X | | | | |
| Biometric measurements: Weight, height*, heart rate and blood pressure. | X | X | X | X | X |
| Urine pregnancy test | X | | | | |
| Concomitant medications | X | X | X | X | X |
| Blood samples: Uric acid, CBC count and differentiation, albumin, total protein, sodium, potassium, chloride, BUN, creatinine, ALT, AST, bilirubin, ESR, rheumatoid factor | X | | | | X |
| WOMAC, VAS and Lequesne scores | X | X | X | X | X |
| X-ray | X | | | | |
| Randomization | | X | | | |
| Blood sample: ALT, AST, bilirubin, albumin. | | | X† | X† | |
| Knee flexion, Time to walk 50m, Swelling in the knee joint, Time for climbing 10 steps | | X | X | X | X |
| Physician's Global Assessment | | X | X | X | X |
| Subject's Global Assessment | | X | X | X | X |
| Investigational Product dispensed | | X | X | X | |
| Subject Treatment Diary dispensed | | X | X | X | |
| Investigational Product returned Compliance calculated | | | X | X | X |
| Subject Treatment Diary returned | | | X | X | X |
| Adverse Events | | | X | X | X |

* height was only measured at visit 1

† If acetaminophen use was greater than 2 g/day for more than 7 days

**Table 2**. Inclusion and exclusion criteria

| Inclusion Criteria |
|---|
| Males and females 40-75 years old |
| Females of childbearing potential must agree to use a medically approved form of birth control and have a negative urine pregnancy test result |
| Unilateral or bilateral OA of the knee for greater than 3 months (American College of Rheumatology criteria) confirmed by radiologist's report, i.e. X-rays showing osteophytes, joint space narrowing or subchondral bone sclerosis (eburnation) |
| Erythrocyte sedimentation rate (ESR) < 40 mm/hr |
| Moderate OA as indicated by Lequesne's functional index score of 4.5-7.5 after 7 day withdrawal of usual medications |
| Able to walk |
| Availability for duration of study period (3-4 months) |
| Subject using other therapies for OA, such as exercise, heat/cold therapy, joint protection and physiotherapy/occupational therapy agrees to continue these therapies as normal avoiding changes in frequency or intensity and to record therapies in the study diary |
| Subject agrees not to start any new therapies for OA during the course of the study |
| Able to give informed consent |
| **Exclusion Criteria** |
| History of underlying inflammatory arthropathy; septic arthritis; inflammatory joint disease; gout; pseudogout; Paget's disease; joint fracture; acromegaly; fibromyalgia; Wilson's disease; ochronosis; haemochromatosis; heritable arthritic disorder or collagen gene mutations or rheumatoid arthritis |
| History of asthma, history of diabetes (Type I or Type II) |
| Hyperuricemia (urate, males > 480 umol/L, females > 450 umol/L) |
| Expectation of surgery in the next 4 months |
| Recent injury in the area affected by OA of the knee, i.e. meniscal tear (past 4 months) |
| Cartilage reconstruction procedure in the target knee |
| Severe OA as indicated by Lequesne's functional index score of 8 or greater, after 7 day withdrawal of usual medications |
| Intra-articular corticosteroid injections in the target knee within the last 3 months |
| Viscous injections in the target knee within the last 6 months |
| Hypersensitivity to NSAIDs |
| Abnormal liver or kidney function tests (ALT or AST > 2 times the upper limit of normal; elevated creatinine, males > 125 umol/L, females > 110 umol/L) |

| |
|---|
| Abnormal findings on complete blood count |
| History of coagulopathies, history of peptic ulceration and upper GI hemorrhage |
| Uncontrolled hypertension |
| History of congestive heart failure, history of allergic reaction to chicken and/or eggs |
| History of allergic reaction to local anesthetic or to any ingredients in the test product including shellfish |
| Hyperkalemia (potassium > 6.2 mmol/L) |
| Anticipated problems with product consumption |
| History of cancer as well as gastrointestinal, renal, hepatic, cardiovascular, hematological, or neurological disorders |
| High alcohol intake (>2 standard drinks per day) |
| Pregnant, breastfeeding or planning to become pregnant during the study |
| History of psychiatric disorder that may impair the ability of subjects to provide written informed consent |
| Use of other natural health products, including glucosamine and chondroitin, one month prior to study and during the study, other than multivitamin and mineral supplements containing vitamins and minerals as the sole medicinal ingredients |
| Use of concomitant prohibited medication (narcotics, oral NSAIDs, topical NSAIDs) within four weeks of randomization |
| Use of acetaminophen or ibuprofen within 7 days of randomization |
| Subject is unwilling to stop taking pain medication other than the study medication (for arthritis or other types of pain) or is unwilling to stop taking other medications for the treatment of OA |
| Any other condition that, in the opinion of the investigator, would adversely affect the subject's ability to complete the study or its measures |

## Supplements

Each UC-II (InterHealth Nutraceuticals, Inc., Benicia, CA) capsule contained 20 mg UC-II standardized to 5 mg of bioactive undenatured type II collagen. Subjects in the UC-II group were instructed to take two "sugar pills" in the morning to protect blinding and two UC-II capsules in the evening accounting for a daily dose of 40 mg UC-II containing 10 mg of bioactive undenatured type II collagen.

Each G+C capsule contains 375 mg of glucosamine HCl (USP Grade) and 300 mg of chondroitin sulfate (USP Grade). The subjects were instructed to take two G+C capsules in the morning and two in the evening for a daily dose of 1500 mg glucosamine and 1200 mg chondroitin.

## Removal of Patients from Therapy or Assessment

The criteria for removal of patients from the study included:

### Adverse events

For any adverse event, patients were examined and appropriately managed or the patients would be referred to another medical professional for proper evaluation and treatment. If medical problems were attributed to the trial compounds, then the trial drugs were discontinued and the toxicities were reported.

### Personal reasons

As stated in the Consent Form, subjects were able to withdraw from the study for any reason at any time.

### Clinical judgment of physician

Subjects were withdrawn from the study (without penalty) if, in the opinion of the treating physician, it was not in the patient's best interest to continue. For instance, if during the course of the study a patient became pregnant, she would be withdrawn from the study because it was not known how the study compounds/medications might affect an unborn child.

### Protocol violation

Any subject found to have entered this study in violation of the protocol or failed to follow the study protocol were discontinued from the study at the discretion of the Principal Investigator. Subjects were withdrawn for protocol non-compliance if they adhered to the dosing schedule less than 75% of the time.

### Method of assigning patients to treatment groups

Patients were assigned to treatment groups (order of treatments) using computer-generated randomization tables. Patients were not stratified or assigned using any other specific method and were not randomized after stratification or blocking procedures.

### Selection of doses in the study

The justification for the daily dose of 40 mg UC-II in capsules (providing 10 mg of undenatured collagen II) is based on efficacy demonstrated in earlier studies (8,9).

### Blinding

In order to protect blinding, subjects were given bottles containing product labeled with "AM" or "PM" to distinguish the time in which treatment was to be taken. Each bottle contained descriptions of all potential products to ensure blinding was protected. Additionally, each bottle was labeled with a randomization number. In the event that an adverse effect was considered serious and related to the investigational product, the blind would be broken for

that individual subject.

Neither the patient, nor investigator, nor research staff, were aware which test compound the subject was assigned. Interim analysis was performed in order to write a preliminary report and thus preliminary unblinding occurred by an individual unrelated to the study conduct. Personnel related to analysis, statistics, and report writing remained blinded.

Prior and concomitant therapy

Uses of medications such as narcotics, oral NSAIDS, topical NSAIDS within four weeks of randomization and during the study, were not allowed.

Treatment compliance

Compliance was assessed by capsule count at visits 3, 4, and 5 and review of subject diary.

### Efficacy and Safety Variables

Efficacy and safety measurements assessed

*Adverse events*

During the study, subjects recorded adverse effects in their subject diary. At each visit, the subjects were asked if they experienced problems or difficulties. Any adverse events were documented and recorded in the study record and was classified according to the description, duration, severity, frequency, and outcome. The investigator assessed the adverse events and decided causality. Classifications were as per the Coding Symbol Thesaurus of Adverse Reaction Terms (COSTART) U.S. Food and Drug Administration (16).

*Blood tests*

Blood samples were taken from all subjects during screening (visit 1) and at end of study (visit 5). Blood samples (approximately 15 ml) were taken from subjects at day 30 and day 60 (visits 3 and 4) for the determination of ALT, AST, bilirubin, and albumin if the subjects had been taking acetaminophen greater than 2 g/day for more than 7 days. All blood samples were analyzed by MDS Laboratory Services (London, Ontario, Canada).

### Appropriateness of Measurements

The efficacy and safety assessments used in this study were standard for OA and are widely used and recognized as reliable, accurate, and relevant.

WOMAC scores were determined, at screening, and baseline, as well as at days 30, 60 and 90 as described in Bellamy et al (17). Other objectives also performed at days 0, 30, 60 and 90 included determination of Lequesne's functional index, VAS pain scores, knee flexion, time to walk 50 m, time to climb 10 steps, physician's and subject's global assessment. The Lequesne's functional index is described in Lequesne et al. (18).

### Statistical Methods

Sample size of 25 subjects per group was based on the subject number used in Braham et al. (1). To compare UC-II with G+C group, a linear contrast was included in the analysis of variance. Data missing subsequent to 30 days were imputed using the last-observation-carried forward technique. Furthermore, comparisons between the UC-II and G+C groups were made at each visit using analysis of variance, using the baseline visit as a covariate. SAS version 9.1 has been used to perform the statistical analysis. Probability values less than 0.05 were considered statistically significant for between-group comparisons.

## Results

### Baseline Statistics and Compliance of Trial Subjects

Demographic and baseline characteristics of patients are summarized in Table 3. Overall, the patient profiles with respect to age, sex, height, weight, blood pressure, heart beat and target knee were similar between both treatment groups. Table 4 shows treatment compliance of the trial patients. There were no significant interaction terms or between-group differences for compliances. When compliances were compared at each visit, there were no overall between-group differences among the two treatment groups.

**Table 3.** Demographic and baseline characteristics of the trial subjects

| | UC-II (N=26) | G + C (N=26) |
|---|---|---|
| Age (years) | 58.9 ± 9.79 | 58.7 ± 10.3 |
| Sex: male/female (%) | 13/26 (50%) | 17/26 (65%) |
| Height (cm) | 167.7 ± 9.90 | 167.0 ± 8.73 |
| Weight (kg) | 84.3 ± 17.4 | 86.6 ± 21.0 |
| Systolic Blood Pressure (mm) | 128.2 ± 9.36 | 126.3 ± 12.5 |
| Diastolic Blood Pressure (mm) | 81.9 ± 7.43 | 79.7 ± 8.60 |
| Heart Rate (bpm) | 68.2 ± 7.72 | 67.4 ± 8.47 |
| Target knee | | |
| Left; n (%) | 16 (61.5%) | 13 (50%) |
| Right; n (%) | 10 (38.5%) | 13 (50%) |

Where applicable, values are expressed as mean ± SD

**Table 4.** Treatment compliance as assessed during specified visits

| Visit | Treatment Group | |
|---|---|---|
| | UC-II | G + C |
| AM Capsule Compliance | | |
| Visit 3 | [25] 90.5 ± 19.2 | [25] 93.6 ± 11.5 |
| Visit 4 | [24] 93.2 ± 9.66 | [26] 94.5 ± 11.8 |
| Visit 5 | [23] 98.5 ± 5.15 | [26] 93.3 ± 11.0 |
| PM Capsule Compliance | | |
| Visit 3 | [25] 88.1 ± 18.7 | [25] 92.5 ± 12.5 |
| Visit 4 | [24] 92.8 ± 8.97 | [26] 91.6 ± 12.3 |
| Visit 5 | [22] 95.3 ± 9.92 | [26] 89.7 ± 12.6 |

There were no significant interaction terms and between-group differences for compliances. When compliances were compared at each visit, there were no overall between-group differences among the five treatment groups. Values are expressed as [n] mean ± SD.

## WOMAC Score

The interaction between visit and treatment was significant in UC-II treated group for "pain walking on flat surface" ($p=0.034$), "difficulty walking on flat surface" ($p=0.038$) and "performing heavy domestic duties" ($p=0.031$) as compared to G+C treated group. There was evidence that UC-II treatment has a significant effect for "ascending stairs" ($p=0.013$) as compared to G+C treatment. Additionally, when groups were compared at each visit, UC-II was significantly better than G+C for "ascending stairs at 30 days and 60 days" ($p=0.019$ & $0.040$ respectively), "at night while in bed" ($p=0.015$) at 60 days and difficulty walking on flat surface at 90 days ($p=0.035$). There were no further statistically significant differences for any other individual WOMAC components or summary scores. Treatment with UC-II was most effective and reduced the WOMAC scores by 33% compared to 14% in (G+C)-treated groups after 90 days. Within-group analysis indicated that treatment with UC-II for 90 days significantly ($p<0.05$) improved WOMAC scores at all treatment time points measured. In contrast, subjects received G+C did not show any statistical significant change in WOMAC scores at Day 90 of treatment (Fig. 2).

## VAS Score

The interaction between visit and treatment was non-significant for all VAS components and summary scores. However there was evidence that UC-II treatment had a significant effect for "pain during climbing up and down stairs", "night pain" and "resting pain" ($p=0.035$, $0.030$ and $0.024$ respectively). When groups were compared at each visit, UC-II was significantly better than G+C for "night pain" ($p=0.040$) and "resting pain" ($p=0.020$) at 60 days and "pain during climbing up and down stairs" ($p=0.014$) and "resting pain" at 90 days ($p=0.034$). There were no between-group differences for any of the VAS components or summary scores. Although both the treatments reduced the VAS score, UC-II was found to be more effective with a 40% decrease after 90 days of treatment compared to a 15% decrease in G+C treated groups.

Within-group analysis indicated that subjects on UC-II showed a significant reduction in total VAS scores at Day 60 and Day 90 as compared to baseline. However, subjects on G+C showed a significant reduction in total VAS scores at Day 30 and no significant difference was observed at either Day 60 or Day 90 as compared to baseline (Fig. 3).

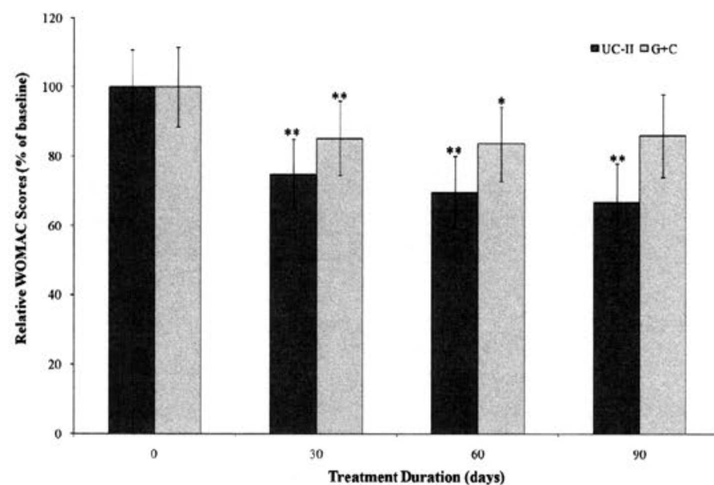


**Figure 2.** Changes in WOMAC scores at Day 90 from baseline. WOMAC scores from each treatment group were compared to baseline value at specified time points. Each bar presents mean ± SEM. *$p<0.05$, **$p<0.005$ indicate significantly different from baseline.

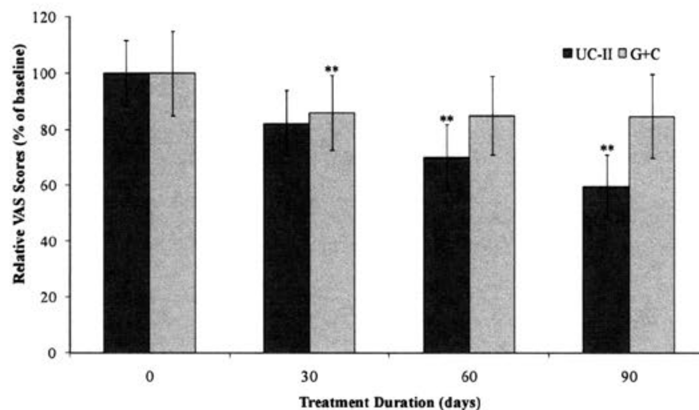


**Figure 3**. Changes in VAS score at Day 90 from baseline. VAS scores from each treatment group were compared to baseline value at specified time points. Each bar presents mean ± SEM. **$p<0.05$ indicates significantly different from baseline.

## Lequesne Score

The Lequesne's functional index was used to determine the effect of different treatments on pain during daily activities. The interaction between visit and treatment was non-significant for all Lequesne's components and summary scores. Furthermore, there were no between-group differences for any of the Lequesne's components or summary scores. However there was evidence that visit has a significant effect in UC-II treated group for "pain while up from sitting" and "maximum distance walked" ($p=0.036$ and 0.002 respectively) as compared to G+C treated group.
There was as a strong trend toward UC-II efficacy. UC-II treatment effectively reduced Lequesne's functional index score by 20.1% as compared to 5.9 % by G+C treatment.

Within-group analysis suggested that subjects on UC-II demonstrated a significant reduction in total Lequesne's index of severity score from baseline to Day 90, whereas no significant difference from baseline was observed for subjects on G+C at any treatment time points evaluated (Fig. 4).

## Adverse Events

Adverse effects that occurred during the 90-day trial period are summarized in Table 5. Overall, there were 58 adverse events noted in the subjects receiving G+C treatment, whereas, only 35 adverse events were observed in UC-II group. In terms of severity, 60% of mild and 38% of moderate adverse events were experienced by subjects on G+C in comparison to 43% and 54% by subjects on UC-II. In relationship to test product a higher number of subjects (23%) on G+C demonstrated adverse events possibly related to product as compared to 11.4% of subjects on UC-II. For UC-II the possible adverse events related to products were constipation and headaches (intermittently). For G+C the possible adverse events related to products were bloating, stomach pain, rash, water retention (edema around eyes and scars), hives on face and chest, and headache. However, there was no significant difference in the occurrence of adverse effects between the two treatment groups.

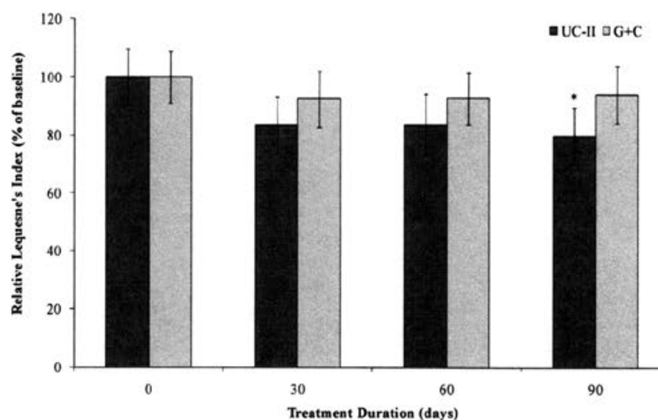


**Figure 4**. Changes in Lequesne's functional index at Day 90 from baseline. Lequesne's functional index from each treatment group was compared to baseline value at specified time points. Each bar presents mean ± SEM. *$p<0.05$ indicates significantly different from baseline.

### Rescue Medication

A greater percentage of subjects used rescue medication while on G+C as compared to UC-II at every time point assessed. From baseline to Day 30 a total of 8 subjects (33.3%) on UC-II used rescue medication as compared to 23 subjects (88.5%) on G+C. From Day 30 to Day 60, 13 subjects (54.2%) on UC-II used rescue medication as compared to 21 subjects (80.8%) on G+C. Fourteen subjects (63.6%) on UC-II used rescue medication as compared to 19 subjects (79.2%) on G+C from Day 60 to Day 90.

**Table 5.** Summary of analysis of adverse events in all subjects

| | Treatment Group | |
|---|---|---|
| | UC-II (n=26) | G + C (n=26) |
| **Severity (n)** | | |
| Mild | 15 | 35 |
| Moderate | 19 | 22 |
| Severe | 1 | 1 |
| **Relationship to Test Article (n)** | | |
| Not related | 17 | 20 |
| Unlikely | 14 | 30 |
| Possible | 4 | 8 |
| Probable | 0 | 0 |
| Most Probable | 0 | 0 |
| **Body System (n)** | | |
| Pain | 10 | 17 |
| Gastrointestinal | 5 | 15 |
| Musculoskeletal/Soft Tissue | 7 | 5 |
| Neurology | 0 | 2 |
| Pulmonary / Upper Respiratory | 2 | 1 |
| Hemorrhage/Bleeding | 2 | 1 |
| Blood/Bone Marrow | 2 | 1 |
| Dermatology/Skin | 2 | 3 |
| Allergy / Immunology | 0 | 1 |
| Infection | 1 | 3 |
| Lymphatics | 0 | 1 |
| Hepatobilary / Pancreatic | 0 | 0 |
| Renal / Genitoruinary | 0 | 0 |
| Constitutional Symptoms | 2 | 3 |
| Syndromes | 1 | 1 |
| Auditory/Ear | 0 | 1 |
| Ocular / Visual | 0 | 1 |
| Metabolic / Laboratory | 1 | 2 |
| **Total Number of Adverse Events Experienced During Study (n)** | 35 | 58 |
| **Total Number of Subjects Experiencing Adverse Events: n (%)** | 16/26 (61.5%) | 20/26 (76.9%) |

## Discussion

OA is the most common form of arthritis, and it is often associated with significant disability and an impaired quality of life. Clinical and radiographic surveys have found that the prevalence of OA increases with age from 1% in people <30 years to 10% in those <40 years to more than 50% in individuals >60 years of age (19). Although there are no curative therapies currently available for OA, individualized treatment programs are available to help relieve pain and stiffness, and to maintain and/or improve functional status.

In the last few years, various nutritional supplements including chondroitin, glucosamine, avocado/soybean unsaponifiables and diacerein have emerged as new treatment options for osteoarthritis (20). In this study, the efficacy of UC-II was studied in patients identified with moderate to severe OA. The objective of this study was to determine the effect of UC-II on disease specific measures and blood measures of OA of the knee compared to G+C. It was hypothesized that UC-II would reduce symptoms of OA of the knee to a greater extent than G+C.

A meta-analysis of 20 randomized control studies (2570 patients) comparing the effects of glucosamine (glucosamine sulphate, GS or glucosamine HCl, GH) vs. placebo was done. Of these only eight studies met the required controlled conditions for adequate

allocation concealment and received a quality score of 4 or higher (rated on the JADAD scale). These studies failed to show the benefit of glucosamine (GS or GH) for pain and WOMAC function. When all 20 studies were included in the meta-analysis, the results favored glucosamine with improvement in pain and functionality; however, the results were not uniformly positive and the parameters for WOMAC pain, daily function and stiffness did not reach statistical significance. Combinations of glucosamine and chondroitin have been studied in the "GAIT" study. These authors reported that glucosamine HCl and chondroitin sulphate alone or in combination did not reduce pain significantly in patients with OA of the knee. However in a subgroup of patients with moderate to severe knee pain the combination of compounds were found to be effective. Limitations to this study included a high rate of response to placebo (60.1%) and the fact that 78% of the participants were in the mild pain subgroup (21).

Previous studies have shown that UC-II is effective in the treatment of RA (8-11), and preliminary human (12) and animal (13-15) trials have shown it to be effective in treating OA. In obese-arthritic dogs given 4 mg or 40 mg per day UC-II for 90 days, significant declines in overall pain, pain during limb manipulation and lameness after physical exertion were noted (15). Greater improvement was observed with the 40 mg dose. No adverse effects or significant changes in serum chemistry (creatinine, blood urea nitrogen, alanine aminotransferase, and aspartate aminotransferase) were noted. Following UC-II withdrawal for a period of 30 days, all dogs experienced a relapse of overall pain, exercise-associated lameness and pain upon limb manipulation.

In a recent investigation, efficacy of UC-II was evaluated in arthritic horses (22). In this study, groups of horses were orally administered with a daily dose of placebo, UC-II at 320, 480 or 640 mg, or a combination of glucosamine (5.4 g) and chondroitin (1.8 g) for 150 days. Horses receiving placebo did not show any improvement in arthritic condition, while those receiving a daily dose of 320, 480 or 640 mg of UC-II exhibited significant reduction in arthritic pain. Although G+C treated group showed significant reduction in pain compared to baseline values, the efficacy was less as compared to that observed with UC-II treatment. In fact, UC-II at 480 or 640 mg/day was found to be more effective than G+C in treatment of arthritic pain in horses. Clinical conditions (body weight, body temperature, respiration rate, and pulse rate), and liver (bilirubin, GGT, and ALP) and kidney (BUN and creatinine) functions were not affected by UC-II treatment, suggesting that UC-II is well tolerated and does not cause any adverse effects (22).

In a preliminary trial of subjects with OA, taking a single oral daily dose of 40 mg UC-II on an empty stomach prior to bedtime for 42 consecutive days, an average of 26% reduction of pain was noted in four of five subjects in the study. No side effects were associated with treatment (12). The precise biochemical mechanism involved in UC-II induced pharmacological anti-arthritic effects in humans, dogs or horses is not clearly established. Type II collagen is the primary form of collagen contained in cartilage. Type II collagen extracts contain the amino acids found in the framework of human cartilage. In addition, these amino acids are required for the synthesis and repair of connective tissue throughout the body. These products reportedly aid in reducing the destruction of collagen within the body, may provide anti-inflammatory activity, and may improve joint flexibility (8-12).

The current study indicated that both treatments reduced the WOMAC scores, which measures the difficulty in physical function, stiffness and pain in the knee. However, treatment with UC-II was found to be more effective in reducing the WOMAC scores by 33% as compared to 14% in G+C treated groups after 90 days. Similar results were observed for VAS scores. Although both the treatments reduced the VAS score, UC-II was found to be more effective with 40% decrease after 90 days of treatment as compared to 15.4% in G+C treated groups. The Lequesne's functional index was used to determine the effect of different treatments on pain during daily activities. Treatment with UC-II reduced Lequesne's functional index by 20.1% as compared to 5.9 % in G+C treated groups. Thus, UC-II supplementation showed improvement in daily activities suggesting an improvement in overall quality of life in the patients receiving UC-II.

## Acknowledgement

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## Conflict of Interest

The authors have declared that no conflict of interest exists.

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# Anti-arthritic Action Mechanisms of Natural Chondroitin Sulfate in Human Articular Chondrocytes and Synovial Fibroblasts

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**To clarify the exact anti-arthritic action mechanisms of chondroitin sulfate (CS), we evaluated the effects of CS derived from shark cartilage (CS-SC) composed mainly of chondroitin-6-sulfate and porcine trachea cartilage (CS-PC) composed mostly of chondrotin-4-sulfate on the functions of human articular chondrocytes and synovial fibroblasts. Both CS-SC and CS-PC (from 1 to 100 μg/ml) effectively suppressed the interleukin (IL)-1β (10 ng/ml)-enhanced gene expression of aggrecanase-1/a disintegrin and metalloproteinase with thrombospondin-like motifs (ADAMTS)-4 and aggrecanase-2/ADAMTS-5 in articular chondrocytes embedded in alginate beads and synovial fibroblasts. In addition, CS-SC and CS-PC overcame the IL-1β-mediated suppression of the aggrecan core protein mRNA, and suppressed the IL-1β-enhanced collagenase-3/matrix metalloproteinase (MMP)-13 gene expression in chondrocytes. CS-PC, but not CS-SC effectively recovered the IL-1β-reduced gene expression of tissue inhibitor of metalloproteinases (TIMP)-3 in chondrocytes, and enhanced the production of TIMP-1 in synovial fibroblasts. It is noteworthy that CS is able to modulate the function of synovial fibroblasts as well as that of chondrocytes. Therefore, CS is very likely to be multifunctional chondroprotective material for degenerative arthritic diseases.**

**Key words** chondroitin sulfate; tissue inhibitor of metalloproteinase; aggrecanase; cartilage; chondrocyte; synovial fibroblast

Osteoarthritis (OA) is the most common form of arthritis, and is characterized by cartilage destruction with loss of compressive and tensile strength properties.[1] Cartilage is a specialized connective tissue that consists mainly of extracellular matrix (ECM) including type II collagen, hyaluronan, and aggrecan, and a small amount of chondrocytes.[2] Aggrecan, a large aggregating proteoglycan, interacts with hyaluronan and link protein to form large aggregates that are immobilized in the cartilage by type II collagen, resulting in a dense network of collagen fibrils and drawing of water into cartilage.[3] These ECM components are enzymatically degraded in OA. The loss of aggrecan is considered to be a critical early event of OA that is followed by the degradation of collagen fibrils.[4]

Aggrecanases that specifically cleave the $^{373}$Glu-$^{374}$Ala bond of aggrecan core protein are thought to be the most critical enzymes for the cartilage ECM degradation under physiological and pathological conditions.[4] The products of this cleavage have been found in synovial fluid of patients with OA, suggesting that aggrecanase exclusively participates in the catabolism of cartilage matrices in OA.[5] Among the several aggrecanases, aggrecanase-1/a disintegrin and metalloproteinase with thrombospondin-like motifs (ADAMTS)-4 and aggrecanase-2/ADAMTS-5 have been characterized as the critical enzymes for pathological cartilage destruction.[4] In particular, ADAMTS-5 has been shown to play crucial roles in cartilage destruction in experimental osteoarthritis.[6] Therefore, aggrecanases are the most important molecular targets for the treatment of OA. On the other hand, matrix metalloproteinases (MMPs), especially collagenase-3/MMP-13 that prefers to degrade type II collagen, have also been shown to play a significant role in cartilage destruction.[7] Active MMPs are inhibited by tissue inhibitors of metalloproteinases (TIMPs)-1, -2, and -3.[8] Thus, the enzymic activity of MMPs is critically regulated by a quantitative balance between MMPs and TIMPs. Besides, TIMP-3 is also known as a potent inhibitor of ADAMTS-4 and -5, and thus it closely participates in the control of aggrecanase activity.[9]

The various medicines are applied for the treatment of rheumatoid arthritis (RA), currently available medical therapies primarily address only the treatment of joint pain in OA including non-steroidal anti-inflammatory drugs (NSAIDs). Natural chondroitin sulfates are widely used for the treatment of joint pain in the world.[10] Chondroitin sulfate (CS) is a glycosaminoglycan with polysaccharide chains composed of an alternate sequence of D-glucuronic acid and D-*N*-acetyl galactosamine linked by $\beta(1\rightarrow3)$ and $\beta(1\rightarrow4)$ bonds,[11] and it is widely distributed in various connective tissues; it is particularly abundant in cartilage, skin, corpus vitreum, and blood vessels. Most CS exists as the sugar chains of aggrecan in the cartilage, and its high water retaining capacity ensures proper cartilage hydration.[11] Thus, CS contributes to the visco-elastic property of the cartilage, and a reduced level of CS in the cartilage has been reported to be a risk factor for arthritic diseases in elderly people.[12] CS is well recognized as a symptomatic slow-acting drug for OA (SySADOA), the same as diacerhein and avocado unsaponifiables, in a few European countries.[13] In addition, CS is popular as a dietary supplement expected to alleviate joint pain in North America.[14] In Japan, CS is available as an over-the-counter drug for knee joint pain. However, the therapeutic action mechanisms of CS remain unclear.

In the present study, we provide novel evidence of the chondroprotective actions of CS derived from shark and porcine trachea cartilages on human articular chondrocytes and synovial fibroblasts: *i.e.*, they effectively interfere with interleukin (IL)-1β-stimulated ADAMTS-4 and -5 gene expression in human articular chondrocytes and synovial fi-

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broblasts in addition to the recovery of IL-1$\beta$-mediated suppression of aggrecan core protein mRNA. We also demonstrate that porcine cartilage-derived CS specifically enhances TIMP-1 production in human synovial fibroblasts and recovered with the IL-1$\beta$-mediated suppression of TIMP-3 mRNA in human articular chondrocytes.

## MATERIALS AND METHODS

**Materials** Chondroitin sulfate SC (CS-SC) with a molecular weight of 113 kDa was isolated from shark cartilage, and it contains mainly chondroitin-6-sulfate (C6S) (69.3%) and chondroitin-4-sulfate (C4S) (17.8%), respectively (Table 1). Chondroitin sulfate PC (CS-PC) with a molecular weight of 37 kDa was isolated from porcine tracheal cartilage, and it was made up of C6S (20.7%) and C4S (72.8%), respectively (Table 1). The analysis for the property of those CS was performed as previously described.[15]

**Cell Culture** Normal human articular chondrocytes were purchased from Cambrex Bio Science Walkersville, Inc. (Walkersville, MD, U.S.A.). Chondrocytes were encapsulated in alginate beads and cultured in Dulbecco's modified Eagle's medium (DMEM)-F12 (Invitrogen, Co., Carlsbad, CA, U.S.A.)/10% fetal bovine serum (FBS) (Thermo ELECTRON Co., Melbourne, Australia) as previously described.[16] Briefly, chondrocytes were suspended in 1.2% alginate/ 0.15 M NaCl at the density of $4\times10^6$ cells/ml, and the suspension was dropped into 102 mM $CaCl_2$ solution under gentle stirring to form the alginate beads. The beads (approximately $4\times10^4$ cells/bead) were washed several times in 0.15 M NaCl, and then equilibrated with DMEM-F12/10% FBS. The cells embedded in alginate beads were cultured in DMEM-F12/10% FBS with 50 $\mu$M ascorbic acid-2-phosphate in 24-well plates (4 beads/well) for one week, and then cells were treated with IL-1$\beta$ (10 ng/ml) and CS-SC or CS-PC (1, 10, 100 $\mu$g/ml) in DMEM-F12/0.2% lactalbumin hydrolysate (LAH) (Sigma Chemical Co., St. Louis, MO, U.S.A.) with 50 $\mu$M ascorbic acid-2-phosphate for 6 d. The culture medium was changed every 3 d.

Normal human synovial fibroblasts (Cell Systems Biotechnologie Vertrieb GmbH, St. Katharinen, Germany) were cultured in DMEM (Invitrogen Co.)/10% FBS until confluence. Synovial fibroblasts up to the 10th passage were seeded in 60-mm diameter culture dishes or 12-well plates. After confluence, cells were treated with IL-1$\beta$ (10 ng/ml) and CS-SC or CS-PC (1, 10, 100 $\mu$g/ml) in DMEM/0.2% LAH. The harvested culture medium was stored at −20 °C until use.

**RNA Extraction and Quantitative Real-Time RT-PCR** Total RNA was extracted from the cells using Isogen (Nippon Gene Co., Ltd., Tokyo, Japan) according to the manufacturer's instructions. Total RNA (1 $\mu$g) was subjected to reverse transcription (RT) using QuantiTect Reverse Transcription Kit (Qiagen K.K., Tokyo, Japan) according to the manufacturer's instructions. A portion (equivalent to 2.5 ng of total RNA) of the products of RT was subjected to real-time polymerase chain reaction (PCR) using QuantiTect SYBR Green PCR Kit (Qiagen K.K.) and QuantiTect Primer Assays [Cat No. QT00032949 for human ADAMTS-4, Cat No. QT00011088 for human ADAMTS-5, Cat No. QT00001764 for human MMP-13, Cat No. QT00084168 for human TIMP-1, QT00046382 for human TIMP-3, and QT00079247 for human glyceraldehyde 3-phosphate dehydrogenase (GAPDH), Qiagen K.K.]. PCR was performed using ABI PRISM 7000 sequence detection system (Applied Biosystems Japan, Ltd., Tokyo, Japan) under the following conditions, denaturation for 15 s, annealing for 30 s, and extension for 30 s.

**Western Blot Analysis** The protein level of TIMP-1 in culture medium was determined by Western blot analysis as described previously.[17] Briefly, the proteins separated by SDS-PAGE were electro-transferred onto a nitrocellulose membrane filter, and the filter was reacted with sheep anti-(human TIMP-1)antiserum (kindly provided by Prof. Hideaki Nagase, Kennedy Institute of Rheumatology, Imperial College London) that was then complexed with horseradish peroxidase-conjugated goat anti-(sheep IgG)IgG (Sigma Chemical Co.). Immunoreactive TIMP-1 bands were detected by enhanced chemiluminescence (ECL)-Western blotting detection reagents (GE Healthcare UK Ltd., Buckinghamshire, U.K.) and LAS-1000 plus (Fujifilm Co., Tokyo, Japan). Detected bands were quantitated using computer software Image Gauge version 3.41 (Fujifilm Co.).

**Statistical Analysis** One-way ANOVA was performed using computer software StatView version 5.0 (SAS Institute, Inc., SAS Campus Drive Cary, NC, U.S.A.) for the data analysis. Independent Student's *t*-test was applied for pair comparisons, and Fisher's PLSD *post-hoc* test was performed for multiple comparisons. The level of statistically significant difference was set at $p<0.05$.

## RESULTS

**CS Interfered with IL-1$\beta$-Mediated ADAMTS-4 and -5 Gene Expression and Augmented the Expression of Aggrecan Core Protein mRNA in Human Articular Chondrocytes** We examined the effects of CS-SC and CS-PC on the gene expression of ADAMTS-4 and -5 in cultured human articular chondrocytes. After one-week subculture chondrocytes in alginate beads were treated with IL-1$\beta$ and CS-SC or CS-PC for 6 d. Results are shown in Fig. 1. Although both CS-SC and CS-PC suppressed IL-1$\beta$-enhanced expression of ADAMTS-4 and -5 mRNA in human articular chondrocytes,

Table 1. Disaccharide Composition of CS-SC and CS-PC

| | Unsaturated disaccharide (%) | | | | | | |
|---|---|---|---|---|---|---|---|
| | ΔDi-0S | ΔDi-4S | ΔDi-6S | ΔDi-diS$_E$ | ΔDi-diS$_B$ | ΔDi-diS$_D$ | ΔDi-triS |
| CS-SC | 1.1 | 17.8 | 69.3 | 1.5 | N.D. | 10.0 | N.D. |
| CS-PC | 5.3 | 72.8 | 20.7 | 0.4 | N.D. | 0.5 | N.D. |

ΔDi-0S, chondroitin; ΔDi-4S, chondroitin-4-sulfate (C4S); ΔDi-6S, chondroitin-6-sulfate (C6S); ΔDi-diS$_E$, chondroitin-4,6-disulfate; ΔDi-diS$_B$, chondroitin-2,4-disulfate, ΔDi-diS$_D$, chondroitin-2,6-disulfate; ΔDi-triS, chondroitin-2,4,6-trisulfate; and N.D., not detected.

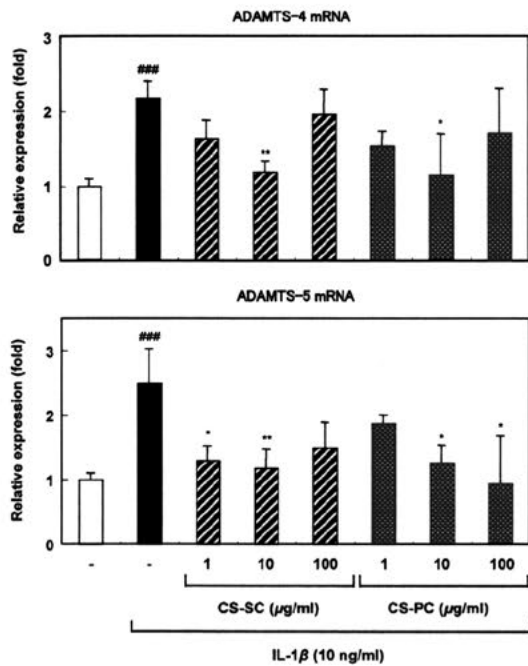


Fig. 1. Chondroitin Sulfate Interfered with IL-1$\beta$-Mediated Expression of ADAMTS-4 and -5 in Human Articular Chondrocytes Embedded in Alginate Beads

Human articular chondrocytes embedded in alginate beads ($4\times10^6$ cells/ml) were treated with or without IL-1$\beta$ and shark cartilage-derived chondroitin sulfate (CS-SC) or porcine cartilage-derived CS (CS-PC) at the indicated concentrations for 6 d. Total RNA was subjected to quantitative real-time RT-PCR for ADAMTS-4 and -5, and GAPDH mRNA as described in the text. Experiments were conducted in triplicate and data are represented as the mean±S.E.M. of three independent experiments. ### $p<0.001$ *versus* untreated control cells, and * $p<0.05$ and ** $p<0.01$ *versus* cells treated with IL-1$\beta$.

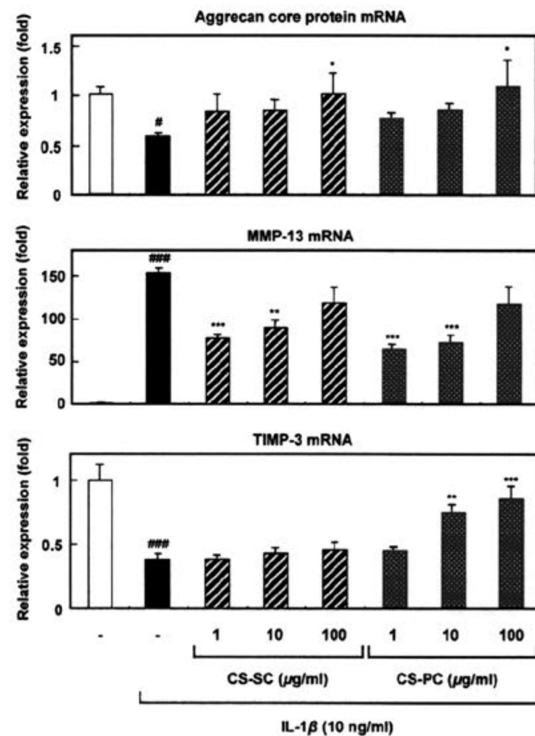


Fig. 2. Effects of Chondroitin Sulfate on the Expression of Aggrecan Core Protein, MMP-13, and TIMP-3 in Human Articular Chondrocytes Embedded in Alginate Beads

Human articular chondrocytes embedded in alginate beads ($4\times10^6$ cells/ml) were treated with or without IL-1$\beta$ and shark cartilage-derived chondroitin sulfate (CS-SC) or porcine cartilage-derived CS (CS-PC) at the indicated concentrations for 6 d. Aggrecan core protein, MMP-13, TIMP-3, and GAPDH mRNA were determined by real-time RT-PCR as described in the text. Experiments were conducted in triplicate and data are represented as the mean±S.E.M. of three independent experiments. # $p<0.05$ and ### $p<0.001$ *versus* untreated control cells, and * $p<0.05$, ** $p<0.01$, and *** $p<0.001$ *versus* cells treated with IL-1$\beta$.

no obvious dose-dependent suppression was seen with either CS; the most effective concentration was 10 $\mu$g/ml for both CS. In contrast, CS-SC and CS-PC recovered the IL-1$\beta$-mediated suppression of aggrecan core protein mRNA in a dose-dependent manner (Fig. 2). While expression of interstitial collagenase/MMP-1 and stromelysin-1/MMP-3 was not modulated by CS-SC and CS-PC (data not shown), both CS interfered with IL-1$\beta$-mediated MMP-13 gene expression at the doses of 1 and 10 $\mu$g/ml (Fig. 2).

CS-SC and CS-PC modulated the expression of TIMPs in human articular chondrocytes, which constitutively expressed mRNA of TIMPs-2 and -3, and also slightly expressed TIMP-1. CS-SC and CS-PC did not affect the expression of TIMPs-1 and -2 (data not shown). CS-PC, but not CS-SC effectively recovered the IL-1$\beta$-mediated suppression of TIMP-3 mRNA in a dose-dependent manner (Fig. 2).

**CS Also Interfered with the Expression of IL-1$\beta$-Mediated ADAMTS-4 and -5 mRNA in Human Synovial Fibroblasts** Since synovial fibroblasts produce ADAMTS-4 and -5, the same as articular chondrocytes, we examined the effects of CS on the gene expression of these enzymes in cultured human synovial fibroblasts. The synovial fibroblasts were co-treated with IL-1$\beta$ and CS-SC or CS-PC for 24 h, and the expression of ADAMTS-4 and -5 was evaluated by real-time RT-PCR. The expression of both aggrecanases was significantly increased by IL-1$\beta$ (Fig. 3). Both CS-SC and CS-PC effectively interfered with IL-1$\beta$-enhanced ADAMTS-4 and -5 gene expression in human synovial fibroblasts already demonstrated in articular chondrocytes (Fig. 3), whereas MMPs-1 and -3 expression was not affected by these CS (data not shown).

Unlike articular chondrocytes, human synovial fibroblasts predominantly secrete TIMP-1, which controls MMP activity. When human synovial fibroblasts were treated with CS-PC for 4 d, TIMP-1 in the culture medium was increased in a dose-dependent manner (Figs. 4A, B). CS-SC also enhanced TIMP-1 production but the increase was not statistically significant (Figs. 4A, B). In contrast, neither CS-SC nor CS-PC modulated TIMP-1 mRNA in human synovial fibroblasts at 24 h (data not shown) nor at 4 d (Fig. 4C). These results suggest that both CS-SC and CS-PC promote the production of TIMP-1 in human synovial fibroblasts at a post-transcriptional level.

## DISCUSSION

Although natural CS of different origins is used worldwide to treat arthritic diseases such as OA, its anti-arthritic action mechanisms have not been clarified. CS has been reported to decrease IL-1$\beta$-induced gene expression of MMPs-1, -3, and -13, and ADAMTS-4 and -5 in bovine chondrocytes cultured in alginate beads under hypoxic (5% $O_2$) conditions.[18] While

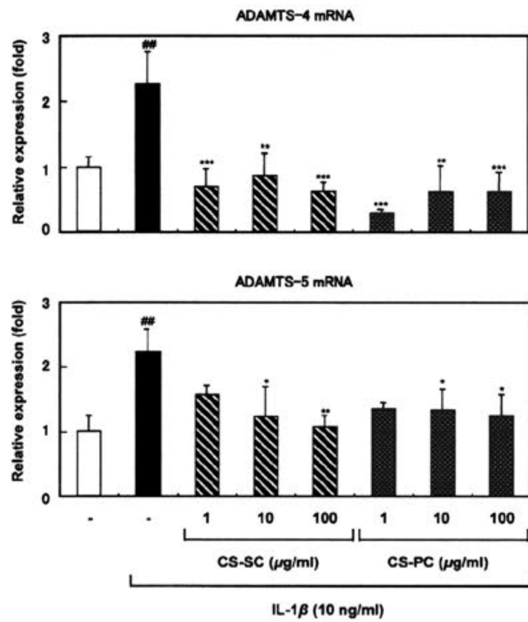


Fig. 3. Effects of Chondroitin Sulfate on the Expression of ADAMTS-4 and -5 in Human Synovial Fibroblasts

Human synovial fibroblasts were treated with or without IL-1$\beta$ and shark cartilage-derived chondroitin sulfate (CS-SC) or porcine cartilage-derived CS (CS-PC) at the indicated concentrations for 24 h. Total RNA extracted from the cells was subjected to quantitative real-time RT-PCR for ADAMTS-4 and -5, and GAPDH mRNA as described in the text. Experiments were conducted in triplicate and data are represented as the mean±S.E.M. of three independent experiments. ## $p<0.01$ *versus* untreated control cells, and * $p<0.05$, ** $p<0.01$, and *** $p<0.001$ *versus* cells treated with IL-1$\beta$.

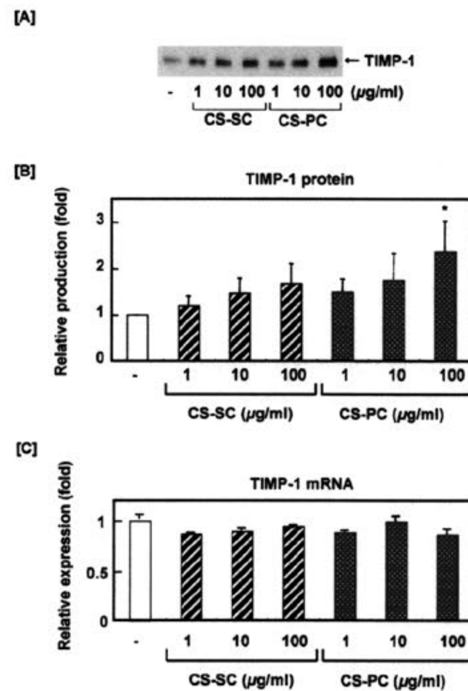


Fig. 4. Chondroitin Sulfate Effectively Augmented the Production of TIMP-1 Protein in Human Synovial Fibroblasts

Human synovial fibroblasts were treated with or without shark cartilage-derived chondroitin sulfate (CS-SC) or porcine cartilage-derived CS (CS-PC) at the indicated concentrations for 4 d. [A] Western blot analysis for TIMP-1. Collected culture medium was subjected to Western blot analysis for TIMP-1. Each experiment was repeated three times, and typical data are shown. [B] Quantitative analysis of Western blot. Data are represented as the mean±S.E.M. of three independent experiments. [C] Real-time RT-PCR analysis for TIMP-1 mRNA. Total RNA was subjected to quantitative real-time RT-PCR for TIMP-1 and GAPDH mRNA as described in the text. Data are represented as the mean±S.E.M. of three independent experiments. * $p<0.05$ *versus* untreated control cells.

Tahiri *et al.*[19] have reported that CS suppressed the IL-1$\beta$-stimulated ADAMTS-5 gene expression, but not that of ADAMTS-4 in cultured rabbit articular chondrocytes. Besides, these authors found that CS did not exert any effect on the IL-1$\beta$-mediated MMP-1 gene expression.[18] Thus, the effects of CS on the expression of MMPs and aggrecanases vary widely from report to report,[18—20] probably due to the cell species used, culture conditions, and the origin of CS.

Since CS was first applied to recover articular function, most attention has been paid on how it exerts its effects on articular chondrocytes. In the present study, we first examined the effects of CS-SC and CS-PC on the expression of aggrecanases in human articular chondrocytes and synovial fibroblasts. IL-1$\beta$ was used as a stimulant in ADAMTS-4 and -5 mRNA expression, because IL-1$\beta$ is one of the most prominent and potent catabolic factors in OA cartilage.[21,22] In addition, CS has been reported not to bind to IL-1$\beta$.[23] Both CS-SC and CS-PC effectively interfered with the IL-1$\beta$-enhanced gene expression of ADAMTS-4 and -5 in human articular chondrocytes and synovial fibroblasts. Messenger RNA levels of ADAMTS-4 and -5 well correlate with their enzymatic activities,[24,25] therefore CS-SC and CS-PC are likely to attenuate the aggrecanase-mediated destruction of articular cartilage in OA. However, no dose-dependent suppression was observed, and at a low concentration CS-SC and CS-PC suppressed ADAMTS-4 and -5 mRNA expression more effectively than at the highest concentration used in this study. Similar effects were observed with MMP-13 expression, and thus the optimal concentration of CS-SC and CS-PC to suppress gene expression of ADAMTS-4 and -5, and MMP-13 was about 10 $\mu$g/ml. On the other hand, the expression of aggrecan core protein, which is suppressed by IL-1$\beta$, was significantly recovered by 100 $\mu$g/ml of CS-SC and CS-PC. Taken together, these results suggest that at the low concentration (*ca.* 10 $\mu$g/ml) CS interfered with enzymatic cartilage degradation, while at the highest concentration (*ca.* 100 $\mu$g/ml) it accelerated ECM anabolism in the articular cartilage. In contrast, we could not identify the precise mechanisms of the suppression of CS-SC and CS-PC on gene expression and the production of MMPs-1 and -3, unlike as previously reported.[18,20] There might be less or no effect of CS-SC and CS-PC on MMPs-1 and -3 production in human articular chondrocytes and synovial fibroblasts.

Furthermore, we have provided novel evidence that IL-1$\beta$-reduced expression of TIMP-3 mRNA was recovered by CS-PC, but not by CS-SC in articular chondrocytes. Since TIMP-3 is characterized as the only endogenous inhibitor of aggrecanases,[9] CS-PC probably suppressed the aggrecanase-mediated aggrecan degradation through the recovery of TIMP-3 expression in addition to the inhibition of ADAMTS-4 and -5 expression. TIMP-3 is also reported to inhibit tumor necrosis factor (TNF) $\alpha$ converting enzyme/ADAM-17[26] and to interact with vascular endothelial growth factor receptor 2, resulting in an interference of angiogenesis.[27] Therefore, the enhancement of TIMP-3 by CS-PC is likely to result in multi-functional anti-inflammatory

actions in the articular cartilage. Moreover, CS-PC significantly increased TIMP-1 production without affecting its mRNA expression in human synovial fibroblasts, suggesting that CS-PC up-regulates the production of TIMP-1 at a post-transcriptional level. CS-SC also increased TIMP-1 production but to a lesser extent. Since CS-PC mainly consists of C4S (72.8%) and CS-SC mostly contains C6S (69.3%), the compositional distinction is considered to make a difference of their functions. Regarding the different actions of CS isoforms, Campo *et al.*[28] reported that intradermally injected C4S suppressed nuclear factor (NF)-$\kappa$B activation in mice with collagen-induced arthritis. Furthermore, the transfection of TIMP-3 has been reported to inhibit TNF$\alpha$-induced activation of NF-$\kappa$B in synovial fibroblasts[29]; therefore, the increase of TIMP-3 expression by C4S may suppress NF-$\kappa$B activation in arthritic mice. On the other hand, C6S has been reported to exert anti-arthritic action in mice with collagen-induced arthritis[30] and in rats with bradykinin-induced proteoglycan depletion.[31] Thus, both C6S and C4S would exert an anti-arthritic action through mutual and individual mechanisms.

Recent meta-analyses of the treatment of osteoarthritis by CS revealed that CS has slight to moderate efficacy or non-existent efficacy against pain due to OA.[32,33] During the preparation of this paper, it was reported that long-term application of C4S and C6S improve symptoms and delay joint structure degradation, and they serve as disease-modifying agents in patients with knee joint OA.[34] Moreover, application of CS has an excellent safety profile, allowing a long-term treatment. Thus, moderate efficacy with extremely lower side effects characterizes the preventive action of CS on cartilage destruction.

In conclusion, both CS-SC and CS-PC prevent cartilage destruction due to the suppression of gene expression of ADAMTS-4 and -5, and MMP-13, and promote cartilage remodeling through the up-regulation of aggrecan core protein expression. In addition, CS-PC increases the TIMP-1 production and TIMP-3 mRNA, resulting in further inhibitory effect on MMPs and aggrecanase activities. Furthermore, we provide the first evidence that some of the above actions of CS are also exerted in synovial fibroblasts. Therefore, CS probably modulates functions of both chondrocytes and synovial fibroblasts, and thereby exerts multifunctional chondroprotective action in degenerative arthritic diseases including OA.

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# Clinical review of chondroitin sulfate in osteoarthritis

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## Summary

Symptomatic slow-acting drugs for the treatment of osteoarthritis (SYSADOA; OA) are compounds which are prescribed as drugs in European countries since many years, whereas they are sold as nutraceuticals in USA. In Europe, the publication of the EULAR Recommendations for the Treatment of Knee OA in 2003 has listed oral chondroitin sulfate (CS) as evidence 1A and strength of recommendation A which represents the highest level for a therapeutic strategy.

Symptomatic slow-acting drugs are intended to be used as ground therapy for OA; these compounds are not rapidly acting agents such as Non Steroidal Anti-Inflammatory Drugs (NSAIDs), and their clinical efficacy on algo-functional symptoms can only be demonstrated after a couple of weeks of regular intake. Interestingly, once the administration is stopped, they do show carry-over effects of various durations, from about 3 months with the oral formulations to 6–9 months with intra-articular formulations. The main rationale behind the use of the SYSADOA therapeutic class is the reduction of NSAIDs in the overall drug management of OA disease and therefore consequently to limit the very significant risks of upper Gastro-intestinal (GI) tract erosions, ulcers with bleeding and/or deleterious renal effects in elderly patients.

The evidence for clinical efficacy of oral CS as a drug able to significantly improve the algo-functional symptoms of OA disease does come from a set of randomized clinical studies published a couple of years ago. Indeed, it was demonstrated that the drug was effective in knee and finger OA, whereas previous data suggested that hip OA patients could also benefit from it. In addition, oral CS supported the comparison with NSAIDs such as diclofenac sodium in a medium/long-term clinical study in patients with knee OA. A dose-finding study in patients with knee OA did provide strong data supporting the administration of 800 mg of CS orally which had nearly the same effects as 1200 mg/day, whereas the use of a sequential 3 months administration mode, twice a year was also shown to provide the same results as a continuous treatment. The good tolerability and safety aspects of oral CS were largely documented in these CTs. Taking these important points into account, we definitively have enough clinical data available supporting the view that oral CS is a valuable and safe symptomatic treatment for OA disease. More recent data based on a couple of previous trials and two pivotal studies do provide further evidence that oral CS does also have structure-modifying effects in knee OA patients.

A couple of other compounds such as hyaluronan, diacerein, avocado and soya unsaponifiables, doxycycline have also been tested with respect to their potential disease-modifying effects. Additional compounds including receptor activator of NF-κB (RANK) ligand inhibitors, cathepsin K inhibitors, bisphosphonates are further assessed regarding their potential structure-modifying effect.
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*Key words*: Chondroitin sulfate, osteoarthritis, SYSADOA, DMOADS.

## Introduction

Symptomatic slow-acting drugs for the treatment of osteoarthritis (SYSADOA; OA) are compounds which are prescribed as drugs in European countries since many years, whereas they are sold as nutraceuticals in USA. In Europe, the publication of the EULAR Recommendations for the Treatment of Knee OA in 2003 has listed oral chondroitin sulfate (CS) as evidence 1A and strength of recommendation A which represents the highest level for a therapeutic strategy.

Symptomatic slow-acting drugs are intended to be used as ground therapy for OA; these compounds are not rapidly acting agents such as Non Steroidal Anti-Inflammatory Drugs (NSAIDS) and their clinical efficacy on algo-functional symptoms can only be demonstrated after a couple of weeks of regular intake. Interestingly, once the administration is stopped, they do show carry-over effects of various durations, from about 3 months with the oral formulations to 6–9 months with intra-articular formulations. The main rationale behind the use of the SYSADOA therapeutic class is the reduction of NSAIDs in the overall drug management of OA disease and therefore consequently to limit the very significant risks of upper Gastro-intestinal (GI) tract erosions, ulcers with bleeding and/or deleterious renal effects in elderly patients.

## Oral CS as a SYSADOA

CS belongs to the oral SYSADOA and the substance does have a delayed mode of action in OA which means that the first effects on pain and mobility can only be assessed after a couple of weeks of therapy (2–3 weeks), in sharp contrast with analgesics and NSAIDs, which do act more rapidly (1–3 days). Importantly, when stopped after 3 months of continuous daily administration, CS will present in most cases with a remanent effect which can last for a couple of months in some cases, a feature which is never observed with analgesics and NSAIDs, substances which need to be continuously administered in order to provide relief from pain and increased mobility in OA patients.

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An important consideration to be made at this stage is that oral CS is registered as a drug in many European countries, whereas it is sold since many years as a prescription-free and over the counter (OTC) substance in all drugstores in the USA. The differences between both markets are obvious. The oral CS sold and used on the European market has been fully registered as a drug, which means that it had to fulfill severe criteria of quality and safety and was fully analyzed regarding its pharmacotoxicologic characteristics and industrial processing. These requirements are not applied to the CS sold as OTC on the American market. There is no doubt that the actual content of active substance and its quality in various brands of CS sold on the US market are not directly comparable with the CS at disposal in the European countries, which might also explain why the results of the clinical trials might differ significantly between both North America and Europe.

We did recently perform a survey of the available randomized clinical trials (RCTs) to assess the clinical efficacy and tolerability of oral CS and did choose to review the evidence on the basis of the published literature which was critically analyzed. The results of this review were published in 2006[1]. Briefly, the authors did assess the effects of oral CS on OA of the knee using the available outcome criteria such as the Lequesne's Algo-functional Index (AFI), the Huskisson Visual Analog Scale for Pain, the Walking Time, the Western Ontario MacMaster score (WOMAC) Score as well as analyzing the safety and tolerability data.

Two independent reviewers did assess the methodological quality of the studies according to the Delphi Criteria List[2], which consists of a set of nine criteria for quality assessment from 1 (use of randomization) to 9 (use of an intention-to-treat analysis). For each quality criterion, three rating categories were available: yes, met criteria, no, did not meet the criteria, and does not know. In addition, percentage agreement and Cohen's Kappa statistic were calculated with GRAPHPAD Software (Version 2002) and were interpreted with Landis and Koch's benchmarks for assessing the agreement between the two raters[3].

The literature search yielded 11 reports that met the basic eligibility criteria of being an RCT which assessed the effects of oral CS on knee OA. A total of 1443 patients were included originating from France: Mazières *et al.*[4]; Bourgeois *et al.*[5]; Conrozier, 1998[6]; and L'Hirondel, 1992[7]; Switzerland: Michel *et al.*[8]; Uebelhart *et al.*[9]; Uebelhart *et al.*[10], and Uebelhart *et al.*[11]; Belgium: Malaise *et al.*[12]; Hungary: Bucsi and Poor[13]; Tschech Republic: Pavelka *et al.*[14]. Several varieties of CS were used (bovine, shark, and avian) which also differed in dosage (500–1200 mg/day), treatment time (3–24 months) and mode of administration, daily continuously (3–24 months) or intermittently ($2 \times 3$ months).

The results of this survey exclusively taking RCTs into account, 10 published in peer-reviewed journals and one study not yet published, having the highest methodological quality, can be qualified overall as very positive for CS as an oral SYSADOA for the treatment of knee OA. Indeed, they did prove that the long-term administration of oral CS is safe, well tolerated and fully indicated to control the symptoms of pain and increase the overall mobility of knee OA patients.

## Oral CS as a disease or structure-modifying drug in osteoarthritis (SMOAD)

Oral CS was also checked by humans as a disease or structure-modifying drug (SMOAD) in the treatment of OA. Using the same analytic method described above for the RCTs concerning the symptomatic effects of oral CS, we were able to identify a total of six published studies in which the SMOAD effect was assessed as primary or secondary evaluation outcome. These RCTs were performed in patients suffering from knee and finger OA. The main idea behind the potential SMOAD effect of oral CS is that the drug might be able to modify the course of the OA disease, may stop its progression and not only positively act on symptoms and mobility of the OA patients.

### SMOAD EFFECT OF CS IN KNEE OA

A total of three RCTs originating from Switzerland and which did evaluate the chondroprotective aspects of oral CS as primary or secondary outcome were available including 462 knee OA patients. One study was a one-center study[8], whereas the two other studies[9,10] were multicentric and included OA patients from various countries (France, Italy, and Belgium) as well. In addition, two treatment modalities were used which differed in their administration sequence. One study reported an intermittent treatment schedule of oral CS of $2 \times 3$ months daily oral CS 800 mg during 1 year[9], whereas the two other studies used a treatment schedule of oral CS 800 mg daily given continuously for a total of 12[10] vs 24[8] consecutive months.

Importantly, only one[8] out of these three studies had disease-modifying effect as a primary outcome, whereas in the two other ones this was a secondary outcome. The radiological progression was assessed in all three studies using the medial femoro-tibial joint space narrowing (JSN) measured with a digitalized analysis of high-quality knee X-rays. The results of the digitized X-rays analysis of the JSN were identical in these studies, originally shown in the two earlier and confirmed in the third one.

The newest Study on Osteoarthritis Progression Prevention (STOPP) multicentric international study was designed as a RCT with the aim to test the SMOAD effect of oral CS 800 mg daily vs placebo during a 2-year survey in a total of 600 symptomatic knee OA patients. The recruitment of the OA patients who had to be symptomatic was performed on the basis of a blinded knee X-rays analysis performed in the Lyon-Schuss view. Only those patients who complied with the very strict inclusion criteria were enrolled and followed for 2 years after having been randomized to both CS or placebo (PBO) treatment groups. The results were already partially presented in some international meetings, but the publication is still in preparation. The main outcome of the STOPP study was the evolution of the medial femoro-tibial JSN over 2 years of treatment with oral CS as compared to PBO and did confirm that oral CS was able to significantly stabilize the JSN whereas PBO did not. A recent survey of the available RCTs to assess the structure-modifying effect of oral was published in 2006[15].

### SMOAD EFFECT OF CS IN FINGER JOINT OA

A total of two RCTs could be identified including a total of 284 patients. Both studies were performed at the Department of Rheumatology of the University of Ghent, Belgium. A first study was based on the use of a numerical scoring system for the anatomical evolution of finger joint OA developed by Verbruggen and Veys[16]. A total of 119 patients suffering from finger OA were included in this RCT with primary outcome being SMOAD effect of oral CS given $3 \times 400$ mg/day over a period of 3 years

assessed on standard PA X-rays of the interphalangeal joints. The results of this study showed that in the CS treated group of patients, there was a significant decrease in the number of patients with new erosive OA finger joints[17,18]. Two additional studies were conducted by Verbruggen *et al.*[19] to assess the progression of finger OA using different Disease Modyfying Anti-Osteoarthritic Drugs (DMOADs) (polysulfated chondroitin sulfate (CPS) and CS) including oral CS 3 × 400 mg daily for a total duration of 3 years. In this study, the 34 patients treated with oral CS presenting with the classical OA anatomical lesions presented with less progressive evolution with both CPS and oral CS. In addition, fewer patients of both CPS and oral CS treated groups developed erosive OA of the finger joints. Even if additional studies are needed, the SMOAD effect of oral CS in finger OA was clearly demonstrated in these studies.

## Meta-analyses on CS in OA

Before the last meta-analysis of Reichenbach *et al.*[20] was published, two other ones were available. Based on data originating from published studies available at the time of the publication, both Leeb *et al.*[21] and McAlindon *et al.*[22] did provide some positive effects (moderate to large) of oral CS on the relief of painful symptoms in OA patients.

The latest published meta-analysis by Reichenbach *et al.*[20] did provide some more critical insights in the effects of oral CS in OA pain. The authors did analyze 20 trials including a total of 3846 patients originating from randomized and quasi-randomized published studies focusing on pain relief in patients suffering from knee and hip OA as well. The authors did find for CS a pooled effect size of −0.75 (95% confidence interval [CI], −0.99–0.50) corresponding to a large symptomatic effect of the substance. This very positive result definitively goes along with the previous published meta-analyses, but the final interpretation of the authors was surprisingly negative about a symptomatic effect of the substance. It is worth noting that this meta-analysis presented with numerous bias and methodological problems which were also addressed as sound critics by some experts in the field of OA therapy who wrote letters to the Editor.

The only way to solve the problem raised by this controversial publication and to become a clear picture of the effects of oral CS in OA patients would be to perform a high-quality new meta-analysis based on the raw data of the RCTs on CS and not based on the extracted data from the publications which are always incomplete.

## Conclusion

Based upon published peer-reviewed RCTs and all available meta-analyses, oral CS has proven efficacy in symptomatic knee OA and structural-modifying effects in finger and knee OA. In addition, the tolerability and safety profile of the substance were found to be very favorable in all available RCTs performed. Additional work related to both the symptomatic and the structure-modifying effects of oral CS in OA patients is currently ongoing and should help to further clarify the situation of the substance in this indication.

## Conflict of interest

The author declares that he is responsible for its design and content and the corrections of the proofs and approval of its final version.

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Review

# Current role of glucosamine in the treatment of osteoarthritis

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**Objectives.** To evaluate the interest of using the various preparations of glucosamine for symptomatic and structural management of osteoarthritis (OA).
**Methods.** A critical analysis of the literature based on an exhaustive search (Medline, PubMed and manual search within the bibliography of retrieved manuscripts) from 1980 to 2005.
**Results.** Despite multiple controlled clinical trials of the use of glucosamine in OA (mainly of the knee), controversy on efficacy related to symptomatic improvement continues. Differences in results originate from the differences in products, study design and study populations. Symptomatic efficacy described in multiple studies performed with glucosamine sulphate (GS) support continued consideration in the OA therapeutic armamentarium. The most compelling evidence of a potential for inhibiting the progression of OA is also obtain with GS.
**Conclusions.** GS has shown positive effects on symptomatic and structural outcomes of knee OA. These results should not be extrapolated to other glucosamine salts [hydrochloride or preparations (over-the-counter or food supplements)] in which no warranty exists about content, pharmacokinetics and pharmacodynamics of the tablets.

KEY WORDS: glucosamine, osteoarthritis, treatment, symptoms, structure.

## Introduction

Osteoarthritis (OA), the most common form of arthritis, is a public health problem throughout the world. The prevalence of OA of the knee in Western Europe has been estimated as 18–25% in men and 24–40% in women between ages 60–79 in Holland [1] and 28–34% in Spain [2]. There are estimates of 100 million people with OA in the European Union. The estimated direct cost of OA in France in 2001 was 1.64 billion Euros [3]. In the United States, the burden of arthritis is 69.9 million people in 2001 [4, 5].

Glucosamine is an aminosaccharide, acting as a preferred substrate for the biosynthesis of glycosaminoglycan chains and, subsequently, for the production of aggrecan and other proteoglycans of cartilage [6]. Because of the essential role aggrecans play in giving the cartilage its hydrophilicity, compounds enhancing synthesis of aggrecans may be beneficial in cases of OA, a disorder characterized by an increase in matrix structural protein turnover, with catabolism being predominant over synthesis [7].

*In vitro*, glucosamine sulphate (GS) has been demonstrated to reduce prostaglandin E2 (PGE2) production and interfere with nuclear factor kappa B (NF$\kappa$B) DNA binding in chondrocytes and synovial cells [8, 9].

Glucosamine inhibits gene expression of OA cartilage *in vitro* [10]. It was suggested that since glucosamine inhibits both anabolic and catabolic genes, the therapeutic effects of glucosamine might be due to anti-catabolic activities, rather than due to anabolic activities. GS is a stronger inhibitor of gene expression than glucosamine hydrochloride [11].

## Methods

We included meta-analyses or randomized controlled trials (RCTs) comparing glucosamine for the management of OA with a placebo or an active comparator. The results had to be reported with a follow-up of at least 4 weeks for symptomatic interventions and 1 yr for the assessment of structure efficacy. The following outcomes were considered relevant: pain, Western Ontario and MacMaster University index (WOMAC), Lequesne index, function or stiffness for symptoms and joint space narrowing or osteophytes progression for structure. We searched Medline from 1980 to 2005 and databases such as the Cochrane Controlled Register, for citations of relevant articles. After this extensive search of the literature, a critical appraisal of the data was obtained through a meeting of the authors.

### *Symptomatic effects in OA*

Efficacy and safety of GS were tested in several RCTs that included patients with OA, predominantly of the knee or spine. In OA of the knee, intramuscular GS (400 mg twice/week for six weeks) was compared with a placebo ($n = 155$). At the end of the treatment and two weeks after drug discontinuation, a significant difference in the decrease of the Lequesne's index (an index assessing pain and function and initially developed to identify patients in the need for surgical joint replacement) was observed for the GS group compared with the placebo. A positive rate (responders were those patients with at least a three-point reduction in the Lequesne's index) was significantly higher in the GS group when considering evaluable patients (55% *vs* 33%) or by intention-to-treat analysis (51% *vs* 30%) [12]. In humans, pharmacokinetic studies have shown that after oral administration, almost 90% of GS was absorbed. The pharmacokinetic patterns of $^{14}C$ revealed that oral administration achieved only 26% bioavailability of intravenous or intramuscular administration [13].

To optimize the long-term compliance of osteoarthritic patients with OA, glucosamine was administered predominantly orally in subsequent clinical trials. In 252 out-patients with OA of the knee [stage I, III], those treated with 1500 mg/day GS for four weeks had a significantly higher decrease in the Lequesne's index than those receiving a placebo. The response rates were within the same range as those observed with the intramuscular formulation (55% *vs* 38% evaluable patients; 52% *vs* 37% patients in an intention-to-treat analysis) [14]. These results were confirmed by a 16-week, randomized, double-blind placebo-controlled crossover trial of a combination of glucosamine hydrochloride (1500 mg/day), chondroitin sulphate (1200 mg/day) and manganese ascorbate (228 mg/day),

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performed in 34 males from the US Navy diving and special warfare community with chronic pain and radiographic degenerative joint diseases of the knee or low back. While the study did not demonstrate, or exclude, a benefit for the spine, knee OA symptoms were relieved, as evidenced by the changes observed in a summary disease score, incorporating results of pain and functional questionnaire, physical examination score and running time [15].

In a 3-yr trial including 319 patients randomized to 1500 mg/day of GS or a placebo, preliminary results suggested that GS significantly improved the long-term symptomatic evolution of knee OA assessed by Lequesne's Algo-Functional index [16]. However, it was observed that glucosamine hydrochloride does not induce symptomatic relief in knee OA to the same extent that GS does. In an 8-week double-blind, placebo-controlled study, followed by 8 weeks off-treatment observation, glucosamine hydrochloride yielded only beneficial results in response to a daily diary pain questionnaire with no effects on the primary end-point (WOMAC questionnaire) [17]. This questions the importance of sulphate and its contribution to the overall effects of glucosamine.

GS (1500 mg/day) was also compared with placebo in 160 outpatients with spinal OA (68 with cervical, 57 with lumbar and 37 with thoracic localizations) and induced a significant improvement of pain and function parameters (visual analogue scale) at both localizations. The improvement with glucosamine lasted up to 4 weeks after drug discontinuation [18].

The symptomatic action of GS was also compared with that of non-steroidal anti-inflammatory drugs. GS (1500 mg orally) and ibuprofen (1200 mg) had the same success rate (48% for GS *vs* 52% for ibuprofen) after 4 weeks in 200 hospitalized patients with OA of the knee. The effect of ibuprofen tended to occur sooner than that of GS (48% ibuprofen *vs* 28% GS after the first week of treatment). However, significantly fewer patients reported adverse effects (mainly of gastrointestinal origin) with GS (6%) than with ibuprofen (35%) and the number of adverse events-related dropouts differed between the two groups (7% ibuprofen *vs* 1% GS) [19]. These results were perfectly duplicated in another study that included 68 Chinese patients with a non-significant difference between ibuprofen and GS (in favour of GS) in the reduction of the symptoms of OA, but GS was better tolerated (6% of patients with adverse reactions and 0% of drug-related dropouts) than ibuprofen (16% of adverse reactions and 0% of drug-related dropouts) [20]. A total of 319 patients with symptomatic OA of the knee received GS (1500 mg/day), piroxicam (20 mg/day), both drugs, or a placebo for 12 weeks followed by eight weeks without treatment. In the GS group, the Lequesne's index decreased by 4.8 points during treatment, for a decrease of 2.9 and 0.7 points, in the piroxicam and placebo groups, respectively ($P < 0.001$). The association did not differ from GS alone. GS did not differ in safety (14.8% incidence of adverse events during treatment) from placebo (23.7%) but was significantly better tolerated than piroxicam (40.9%) or the association (35%). The improvement in GS-treated patients persisted during the 8-week follow-up period, whereas the improvement with piroxicam did not [21].

In 45 adult subjects diagnosed with temporomandibular joint (TMJ) OA, GS (1500 mg/day) and ibuprofen (1200 mg/day), given for 90 days, both induced significant improvement in TMJ pain with function and pain-free and voluntary maximum mouth opening. Between-groups comparison revealed that patients taking GS have a significant greater decrease in TMJ pain with function and used less acetaminophen (chosen as rescue medication) during the 30-day period following the treatment [22].

Few investigations have tested alternative routes of administration for GS. No head-to-head comparison between the oral and topical routes is currently available. However, a topical application of a preparation containing GS, chondroitin sulphate and shark cartilage reduced, within 4 weeks, pain related to knee OA to a significantly greater extend than a placebo cream [23].

Studies with less stringent methodology did not, however, systematically replicate these positive results. In a study of pragmatic design, including 80 patients with a wide range of pain severity from knee OA, the administration of GS (1500 mg/day for 6 months) did not provide a significant pain relief compared with the administration of calcium carbonate (CC). It should be emphasized, however, that the GS preparation used in this trial was an over-the-counter (OTC) formulation containing a mixture of GS, vitamin C and CC [24]. Similarly, when using another OTC preparation of GS, Rindone and colleagues [25] where unable to detect an analgesic effect of 1500 mg of GS daily over two months, compared with placebo, in 98 patients with OA of the knee. Both studies were performed with GS preparations purchased from global suppliers and packaged and sold OTC as nutritional supplements. They are not regulated as drugs and might have important variations in content [26, 27]. Noteworthy is that both above referenced trials [24, 25] were conducted without performing any quality control assays for GS [26]. In a prototypical double-blind, randomized, placebo trial of GS (1500 mg/day) among subjects recruited and followed entirely over the Internet, no differences between treatment and control groups were observed, over 12 weeks concerning pain, stiffness, or function on total WOMAC scores. In this trial, the initial GS (OTC) provider declined to supply placebo capsules during the course of the study and the patients were subsequently treated with a glucosamine hydrochloride formulation, manufactured to pharmaceutical grade purity [28].

The symptomatic efficacy of glucosamine in OA has been analysed through high-quality quantitative systematic reviews [29–32]. The most recent of these meta-analysis [31], incorporating the results of two long-term studies [33, 34], demonstrated the highly significant efficacy of glucosamine on OA-related symptoms (Lequesne Index, WOMAC, or visual analogue scales) with a minimal time reported for the onset of significant action being 2 weeks [31].

Despite multiple double-blind, controlled clinical trials on the use of glucosamine in OA of the knee, controversy on efficacy related to symptomatic improvement continues [11]. Indeed, meta-analyses have produced conflicting results [31, 35].

The most recent update of the Cochrane Database of Systematic Reviews on glucosamine was realized on May 15 2006 [35]. The authors concluded that this 2006 update included 20 studies with 2570 patients. Pooled results from studies using a non-Rotta preparation or adequate allocation concealment failed to show benefit in pain and WOMAC function, while those studies evaluating the Rotta preparation show that glucosamine was superior to placebo in the treatment of pain and functional impairment resulting from symptomatic OA. Glucosamine was found to be superior for pain (SMD −1.31, 95% CI −1.99, −0.64) and function using the Lequesne index (SMD −0.51, 95% CI −0.96, −0.05). WOMAC outcomes of pain, stiffness and function did not show a superiority of glucosamine over placebo for both Rotta and non-Rotta preparations of glucosamine. Glucosamine was considered as safe as placebo, in terms of the number of subjects reporting adverse reactions (RR = 0.97, 95% CI 0.88, 1.08) [35].

Two recent studies, add further information regarding glucosamine clinical status [36, 37].

A National Institutes of Health sponsored study labelled the Glucosamine/chondroitin Arthritis Intervention Trial (GAIT), examined placebo *vs* glucosamine hydrochloride (500 mg three times daily) *vs* chondroitin sulphate (400 mg three times daily) *vs* the combination of glucosamine and chondroitin *vs* celecoxib (200 mg/day) in a parallel, blinded 6-month multicentre study of response in knee OA [36]. The primary efficacy variable was a 20% improvement in knee pain from baseline to 24 weeks. Overall, glucosamine hydrochloride and chondroitin sulphate were not significantly better than placebo in reducing knee pain by 20%. However, for patients with moderate-to-severe pain at baseline, the rate of response (OMERACT-OARSI criteria) was

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significantly higher with combined therapy than with placebo (79.2% *vs* 54.3%, $P = 0.002$).

The Glucosamine Unum In Die [once-a-day] Efficacy (GUIDE) trial, a 6-month double-blind, multicentre trial in Spain and Portugal examining placebo *vs* GS (1500 mg once daily) *vs* acetaminophen (3000 mg/day) has also recently been presented [11, 37]. The primary efficacy variable was a change in the Lequesne Algo-Functional index. Although there was a numeric difference in improvement in the Lequesne Algo-Functional index between acetaminophen and placebo, only the improvement in the Lequesne Algo-Functional index for GS *vs* placebo was significant ($P = 0.032$). Secondary analyses, including the OARSI responder indices were significant for glucosamine ($P = 0.004$).

There are several potential confounders than may have relevance when trying to interpret the seemingly contradictory results of the clinical trials, such as the GAIT and GUIDE.

(i) In North America, glucosamine hydrochloride or sulphate and chondroitin sulphate are considered nutraceuticals, whereas in most European countries these are marketed as pharmaceuticals. Therefore, production and marketing of glucosamine are more closely monitored in Europe. In North America, varying quantities of glucosamine have been noted in a survey of several nutraceuticals [38].
(ii) Most of the negative clinical trials were performed with glucosamine hydrochloride 500 mg three times daily, whereas most of the positive trials were performed with the GS powder for oral solution at the dose of 1500 mg once daily. This obviously raises the question, so far unanswered, of the importance of sulphate and of its contribution to the overall effects of glucosamine. Although the sulphate is readily hydrolysed from the glucosamine in the gastrointestinal tract, there are suggestions that sulphate is in itself clinically relevant [39, 40].
(iii) Interestingly, the most clinically relevant results in GAIT were seen when sodium chondroitin sulphate was taken with glucosamine hydrochloride; whether this may be explained by an increase in the bioavailablity of sulphates together with glucosamine requires further study. It is of note that several of the glucosamine preparations contain other salts that could potentially influence uptake and utilization of glucosamine [41].
(iv) The placebo response for many clinical trials with oral agents in treatment of knee OA has traditionally been around 30% [42] and these usual figures were replicated in the GUIDE study. The high placebo response in the GAIT (60.1%) is of unknown significance.

From these studies, we have learned that OA of the knee continues to be difficult to study and that our instruments that measure change are good, but could be better. Indeed, what seems to be minor differences in protocols often result in differing and confusing information.

Although there has been a public comment that the differences in the trials are due to corporate *vs* non-corporate sponsorship, there have been no data produced to support such allegation. Indeed, one could argue that the differences in results were more from the differences in product, study design and study populations. Although, unfortunately, the controversy continues, symptomatic efficacy describes in multiple studies performed with GS support continued consideration in the OA therapeutic armamentarium [11].

## Structural effects in OA

To test the long-term effects of GS on the progression of OA joints structural changes and symptoms, two parallel studies including, respectively, 212 and 202 patients with knee OA were designed. Patients were randomly assigned in a double-blind fashion to a continuous treatment with GS (1500 mg once/day) or placebo for 3 yrs. Weight-bearing, antero-posterior radiographs of each knee were taken at enrollment and after 1 and 3 yrs, standardizing patients' positioning and radiographic procedures. Total mean joint space width of the medial compartment of the tibio-femoral joint was assessed by digital image analysis by a validated computerized algorithm, with the narrowest joint space at enrollment being taken for the primary evaluation (signal joint). Symptoms were scored at each 4-month visit by a total WOMAC index or Lequesne's Algo-Functional Index.

In the first trial, the 106 patients on placebo had progressive joint-space narrowing, with a mean joint-space loss after 3 yrs of −0.31 mm (95% = −0.48 to −0.13). There was no significant joint-space loss in the 106 patients on GS −0.06 mm (−0.22 to 0.09). Similar results were reported with minimum joint-space narrowing. As assessed by WOMAC scores, symptoms worsened slightly in patients on placebo compared with the improvement observed after treatment with GS. There were no differences in safety or reasons for early withdrawal between the treatment and placebo groups [43].

In the second trial, progressive joint space narrowing with placebo use was −0.19 mm (95% CI, −0.29 to −0.09 mm) after 3 yrs. Conversely, there was no average change with GS use (0.04 mm; 95% CI, −0.06 to 0.14 mm), with a significant difference between groups ($P = 0.001$). Fewer patients treated with GS experienced predefined severe narrowing (>0.5 mm): 5% *vs* 14% ($P = 0.05$). Symptoms improved modestly with placebo use but as much as 20–25% with GS use, with significant final differences on the Lequesne index and the WOMAC total index and pain, function, and stiffness subscales. Safety was good and without differences between groups [33].

Additional *post-hoc* analyses were performed in order to identify patients who would be particularly responsive to GS as a symptom or structure-modifying drug.

At baseline, in the overall population, mean joint space width and narrowest joint space point were not significantly correlated with the scores recorded for the WOMAC global index or its pain, stiffness, or function subscales. A statistically significant correlation was observed between the joint space narrowing over 3 yrs and stiffness or function subscale of the WOMAC during the same period. The 3-yr changes in the global WOMAC index in patients within the lowest and highest quartiles of mean joint space width at baseline showed, in both cases, a statistically ($P < 0.05$) significant favourable difference between patients treated with GS and those having received a placebo [34].

In the placebo group, baseline joint space width was significantly and negatively correlated with the joint space narrowing observed after 3 yrs ($r = 0.34$, $P = 0.003$). In the lowest quartile of baseline mean joint space width (<4.5 mm), the joint space width increased after 3 yrs by a mean of 3.8% (S.D. 23.8) in the placebo group and 6.2% (S.D. 17.5) in the GS group. The difference between the two groups of patients' with severe OA at baseline was not statistically significant ($P = 0.70$). In the highest quartile of baseline mean joint space width (>6.2 mm), a joint space narrowing of 14.9% (S.D. 17.9) occurred in the placebo group after 3 yrs while patients from the GS group only experienced a narrowing of 6.0% (S.D. 15.1). Patients with the most severe OA at baseline had an RR of 0.42 (0.17–1.01) to experience a 0.5 mm joint space narrowing over 3 yrs, compared with those with the less affected joint. In patients with mild OA, (i.e. in the highest quartile of baseline mean joint space width) GS use was associated with a trend ($P = 0.10$) toward a significant reduction in joint space narrowing [44].

These results were further supported by the demonstration that patients with the highest cartilage turnover at baseline, presented a decrease in collagen type II degradation (CTX-II) after 12 months of GS therapy and that these changes in CTX-II were correlated with the changes in average joint space width observed after 36 months [45].

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These results suggest that patients with a less severe radiographic knee OA will be particularly responsive to GS as a structure-modifying drug. However, GS provides long-term relief of symptoms independently of baseline joint space width in patients with mild to moderate OA of the knee.

These studies were, however, challenged for the potential systematic error that might have been introduced by the major effect observed—the significant improvement of symptoms in the GS-treated patients compared with placebo-treated patients. It has been hypothesized that the concomitant reduction in pain seen in the GS arm, relative to placebo, altered the positioning of the knee (in particular favouring a better knee full extension), resulting in a change in joint space width that might have confounded the estimate of joint space narrowing and exaggerated the difference between treatment groups [46]. This hypothesis, however, was demonstrated to be wrong when it was shown that patients from the placebo group, with a major clinical improvement, observed over 3 yrs, did actually present with a joint space narrowing while patients with a similar significant symptomatic response, in the GS group, did not experience this structural progression. Patients completing the 3-yr treatment course were selected based on a WOMAC pain decrease at least equal to the mean improvement in the GS arms in either of the original studies, irrespective of treatment with GS or placebo (drug responders or placebo responders). In a second approach, 3-yr completers were selected if their baseline standing knee pain was 'severe' or 'extreme' and improved by any degree at the end of the trials. In both cases, changes in minimum joint space width were compared between treatment groups. The placebo subsets in both studies underwent an evident mean (S.D.) joint space narrowing, which was not observed with glucosamine sulphate. Similar results were found in the smaller subsets with greater than or equally severe baseline standing knee pain that improved after 3 yrs, with a joint space narrowing with placebo not observed with GS [47].

A 5-yr follow-up evaluation of patients from this trial was performed to assess long-term outcomes of disease progression after the end of the study [48]. The primary end-point of this follow-up study was the occurrence of OA-related joint surgery. Out of the 177 patients participating in this follow-up evaluation, 26 (14.7%) underwent OA-related lower limb surgery during the follow-up. There were twice as many patients from the former placebo group that underwent any of these surgeries, with a 48% decrease in risk with GS that was borderline statistically significant ($P = 0.06$). The time-to-event analysis confirmed the results of the crude primary outcome, indicating a decreased ($P = 0.05$) cumulative incidence in OA-related lower limb surgeries for the patients formerly on GS. When only total hip and/or knee replacements were considered the trend was similar, with over 40% reduction in risk after GS, but the level of probability was lower and only showed a trend towards the significance threshold ($P < 0.2$).

The structure-modifying effect of GS was confirmed by a similar trial in a population of 202 subjects from both sexes with a slightly worse degree of knee OA [33]. In this trial, the effect of 1500 mg/day GS on the rate of progression of the disease was statistically significant as early as the first year and remained so until the end of the 3-yr follow-up. The authors also described a significant ($P = 0.03$) reduction in the proportion of patients worsening their osteophyte score at the endpoint (20% in the placebo *vs* 6% in the GS group).

## Tolerance

The safety profile of GS was evaluated in a systematic review of 12 RCTs and was deemed excellent, with 7 of 1486 patients randomized to GS who were withdrawn for GS-related toxicity and only 48 having reported any GS-related adverse reactions [32].

Furthermore, an open study carried out by 252 physicians throughout Portugal evaluated the tolerability of GS in 1208 patients. Patients were given, 500 mg GS orally, 3 times a day, for a mean period of 50.3 days (range 13–99 days). Most patients (88%) reported no side effects. In the remaining 12% of the study population, the reported adverse effects were generally mild and predominantly affected the gastrointestinal tract (e.g. epigastric pain, heartburn, and diarrhoea). All the reported complaints were reversible with discontinuation of GS [49]. While some questions were raised regarding the role of glucosamine in glucose metabolism [50] and the possibility of increased insulin resistance, a detailed review of scientific studies performed with GS ruled out this possibility and re-emphasized the safety of short- and long-term use of GS [51].

While, in Europe, GS is regarded as a medication and is thus subject to the usual quality controls, this is not so in Canada and the US. In Canada, it is widely available as a nutritional supplement and is not subject to even rudimentary checks on purity. GS is very hygroscopic and unstable. Hence, during manufacturing, varying amounts of potassium or sodium chloride are added to improve stability. Because of concerns that the labelling description may not always be valid, 14 commercially available capsules or tablets of GS were analysed in a coughed, blind manner, with a high performance liquid chromatography system. The amount of free base varied from 41% to 108% of the milligram content stated on the label; the amount of glucosamine varied from 59% to 138% even when expressed as sulphate [38]. Therefore, the results obtained with one single preparation of GS, registered as a drug in Europe, cannot be extrapolated to the vast majority of OTC preparations sold without the appropriate quality controls. In conclusion, however, there is a high degree of consistency in the literature to consider that when a quality product free of impurities is used, GS has an excellent profile of safety [49, 52, 53], including no induction of glucose intolerance in healthy adults [43, 54].

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**Rheumatology key messages**

- The therapeutic effect of glucosamine might be due to anti-catabolic rather than to anabolic activities.
- Symptomatic efficacy described in multiple studies performed with GS support continued consideration in the OA therapeutic armamentarium.
- Compelling evidence exists that GS may reduce the progression of knee osteoarthritis.
- Results obtained with GS may not be extrapolated to other salts (hydrochloride) or formulations (OTC or food supplements) in which no warranty exists about content, pharmacokinetics and pharmacodynamics of the tablets.

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# Importance of synovitis in osteoarthritis: Evidence for the use of glycosaminoglycans against synovial inflammation

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ABSTRACT

*Objectives:* After detailing the different aspects of synovial inflammation (i.e., cellular, biochemical, and vascular) and based on the current knowledge, the aim of this review was to collect the available in vitro and in vivo data regarding the potency of some glycosaminoglycan (GAG) compounds to target synovial inflammation, an important aspect of osteoarthritis.

*Methods:* The first part of the review corresponds to a qualitative review of the inflammatory status of OA synovial membrane. The second part corresponds to a systematic review of the literature regarding the potential effects of some GAGs on the previously described phenomenon.

*Results:* The synovial aspect of the inflammatory status of OA has been detailed. Chondroitin sulfate has demonstrated to control the three aspects of synovial membrane inflammation: cell infiltration and activity, biochemical mediators release, and angiogenesis. Glucosamine is also active on both cellular and molecular aspects of the inflammatory reaction. Hyaluronic acid seems to be anti-inflammatory in its native form, while products of degradation are reported to be pro-angiogenic.

*Conclusion:* Much evidence suggests that some of the studied GAG compounds could target different aspects of synovitis. Some of them could be considered in combination therapy since they exhibit complementary properties. Most of the studies have concentrated on articular cartilage and chondrocytes. In order to achieve a structure modification, one may now consider all joint tissues and investigate the drug potency on all of them. Potent treatment should trigger the most important features of OA: cartilage degradation, subchondral bone sclerosis, and all aspects of synovial inflammation.

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## Introduction

Osteoarthritis (OA), one of the most disabling arthritic affection, is now clearly defined as the disease of an organ, the joint [1]. It is acknowledged that cartilage is not the sole tissue affected by OA, but that the subchondral bone and the synovial membrane (SM) undergo metabolic and structural modifications related to the disease [2]. SM is indeed the scene of inflammation, resulting in joint swelling due to effusion in the joint cavity and stiffness [3,4]. Synovitis has been shown to be correlated with the severity and the progression of the disease [5,6]. It is the main cause of pain in OA patients.

The main recommendations for the management of OA [7–12] consist in the control of symptoms, i.e., inflammation and pain. Synovitis is classically treated by the intra-articular injection of corticosteroids and oral or topical NSAIDS in order to relieve pain [13,14]. Some of the recommendations agree with the use of glycosaminoglycan (GAG) compounds like chondroitin sulfate (CS), glucosamine (GlcN) sulfate, and hyaluronic acid (HA) [7,8,10–12]. They are characterized by a delayed, but significant effect on pain and function in knee OA. GlcN and CS have also been described with disease-modifying effects in knee OA.

Based on the current knowledge of the different aspects of synovial inflammation in OA, this review documents how selected GAGs, i.e., chondroitin sulfate, glucosamine, and hyaluronic acid could be potent interventions against synovitis in OA.

## Methods

The first part of the review corresponds to a qualitative review of the inflammatory status of OA and synovitis. A search was performed on PubMed in order to provide an overview of this

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aspect of OA. The used keywords were "osteoarthritis" and "inflammation" or "synovitis" or "synovial inflammation."

The second part of the review corresponds to a systematic review of the literature regarding the potential effects of the most studied GAGs on the previously described phenomenon. A thorough search has been performed on PubMed through March 2013 using the following keywords: "osteoarthritis," "arthritis"; "synovial," "synoviocytes"; "synovial fibroblasts"; "synovitis" or "inflammation"; "chondroitin"; "glucosamine"; "hyaluronic acid," "hyaluronate" or "hyaluronan" or "glycosaminoglycans." The inclusion criteria for this search were the following: in vitro and in vivo evidences related to inflammation and synovial tissues; results directly linked to OA (in vivo or in vitro and ex vivo); results obtained in related models (i.e., arthritis); results obtained in synovial tissue, synovial cells, and/or inflammatory cells; results with a direct link to the events described in the first part of the review; and only articles in English language. The exclusion criteria were the following: results reported in other inflammatory conditions, results with no direct link to inflammation, results obtained in other OA tissues (i.e., cartilage and subchondral bone), results obtained in other cell types than the included ones, and results obtained with other compounds than with the specified ones.

## Results

### Overview of the current knowledge on OA inflammation and synovitis

The inflammatory status of OA remained controversial for a long time. There was indeed no sign of systemic manifestation, and neutrophils in the synovial fluid of OA patients were way less important than in gout or rheumatoid arthritis (RA). However, inflammation in OA includes local inflammation that has been described in both early and late stages [15]. The inflammatory status in OA patients is undoubtedly less important than in RA patients but it is different from normal SM [15]. The importance of synovitis in OA has been largely reported. The use of new imaging techniques such as ultrasound (US), magnetic resonance imaging (MRI), or scintigraphy has revealed the high prevalence of synovitis in OA. Thus, synovitis was found in 95% of OA patients with effusion and also in 70% of patients without effusion [16]. Furthermore, synovitis is an indicator of pathology and a predictor of disease progression [17]. The prevalence and the severity of synovitis increase with advancing stage of OA.

The origin of synovitis [18,19] is strongly related to cartilage and meniscus degradation [20]. It is described as part of a vicious circle perpetuating OA. In addition, synovial tissue could contribute to the bone remodeling observed in OA by the differentiation of synovial macrophages into functional osteoclasts [21,22]. Taken together, these evidences position the SM at the center of the OA pathophysiological process and reveal the potential target, i.e., synovial inflammation for OA treatment. It would target both symptoms and structure modifications.

Synovial inflammation is classically revealed by histology. It is characterized by synovial thickening (hypertrophy and hyperplasia), cell infiltrate (macrophages and lymphocytes infiltration), and angiogenesis. These events imply the involvement of various mediators (inflammatory, immune, etc.) (Fig. 1).

#### *Cellular aspect of synovitis*

The increased number of lining cells consists in synovial layers made of synovial fibroblasts and in a mixed inflammatory infiltrate containing macrophages, T cells, and B cells [23–27]. Macrophages drive inflammation and destructive response. They are responsible for the production of the major inflammatory mediators [25]. They are also involved in osteophytes formation and synovial fibrosis through, at least partially, the local secretion of TGF-β [28]. Higher levels of mononuclear cell infiltration are found in synovial tissue of early OA patients than of late OA patients [26].

Innate immunity has been described as an early event of the OA synovitis [29,30]. The T cell infiltrate in OA SM is the site of Th1 differentiation and activation to produce Th1 cytokines, such as interferon γ (IFNγ) [31]. The local chronic T cell activation results in an angiogenic organization, especially in the perivascular areas [31] (Fig. 1). They express both leukocyte and endothelial adhesion

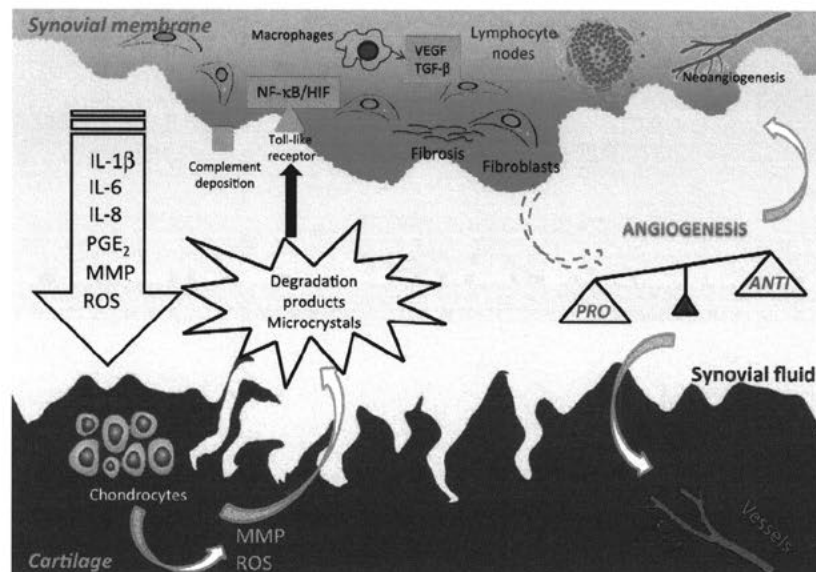


**Fig. 1.** The complex interaction between articular cartilage and synovial membrane during OA. The degradation products and microcrystals generated by cartilage activate the synovial membrane. In turn, it produces pro-inflammatory cytokines (IL-1β and TNFα), prostanoids, reactive oxygen species (ROS), and matrix metalloproteinase (MMP) that are freed in the synovial fluid and activate and contribute to cartilage degradation. The Toll-like receptors (TLR) present at the surface of synovial cells are activated and the complement is requested. The production of angiogenic factors is imbalanced in favor of the pro-angiogenic ones, resulting in the development of neo-vascularization in both cartilage and synovial membrane.

molecules. The T cell response is directed against auto-antigens. As proposed decades ago, antigens could come from products of cartilage breakdown [32]. Results remain conflicting, but a protein derived from cartilage, chitinase-3-like protein 2 (YKL-39), and type II collagen derivatives have been proposed as potential antigens [31,33,34].

B cell infiltration is found in half of OA patients [35]. Even if rarely present, they are in an activated state. Moreover, the presence of CXC-chemokine ligand (CXCL) 13, a potent chemo-attractant of B cells, has been shown in OA synovial lymphoid aggregates [36].

*Biochemical aspect of synovial inflammation (involved mediators)*

The inflamed SM is the site of an excessive production of mediators, cytokines, prostanoids, and growth factors involved in inflammation, catabolism, immunity, and oxidative stress.

*Inflammatory mediators*

The major ones, interleukin (IL)-1β and tumor necrosis factor (TNF) α, are produced by activated synoviocytes and mononuclear cells in the SM, in addition to articular cartilage. The increased expression of IL-1β and TNFα by synovial tissue explants from moderate to severe OA patients is correlated with the increased number of lining cells [37]. They contribute to synovial inflammation and cartilage degradation and are mainly produced by macrophages. In OA SM, macrophages potently regulate the production of several important fibroblast-produced cytokines (IL-6 and IL-8), leukocyte-inhibitory factor (LIF), monocyte chemo-attractant protein 1 (MCP-1), matrix metalloproteinases (MMP-1, 2, 3, 9, and 13), and aggrecanases (ADAMTS-4 and -5) via a combined effect of TNFα and IL-1β [25]. The cytokines and mediators diffuse through the synovial fluid to act therefore on cartilage and chondrocytes. Some other mediators secreted by OA synovial cells, such as IL-17, nitric oxide (NO), prostaglandin (PG) $E_2$ and leukotriene (LT) $B_4$, granulocyte macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-8, MCP-1, vascular cell adhesion molecule (VCAM)-1, and intercellular cell adhesion molecule (ICAM)-1 [37–40] have been shown to contribute to the progression of chondropathy [41].

Different profiles of released cytokines and mediators can be designed according to the stage of the disease. They are related to the differences in mononuclear cell infiltration. Early OA patients, characterized by knee pain, normal radiographs, and arthroscopic signs of OA, have more IL-1β and TNFα in synovial fluid than that in late OA patients [26]. IL-21 and IL-15 are the most present cytokines in the synovial tissue in the early stage of OA.

Key intermediates in the cell signaling, i.e., nuclear factor kappa B (NF-κB), c-Jun, and p38, have been described in the inflamed synovial tissue to be involved in the expression of most of the inflammatory mediators [42]. These observations have yielded the conclusion that NF-κB could be a therapeutic target for the treatment of OA [43]. However, recent papers suggest that NF-κB down-regulation has a different effect on IL-1β and TNFα production by RA or OA SM cells. It was observed that although TNFα and IL-1β were very strongly dependent of NF-κB activation in RA SM, adenoviral transfer of IκBα did not affect IL-1β and had only a partial effect on TNFα in OA synovial cells [44]. These results suggest that IL-1β and TNFα production regulatory pathways at least partially differ in RA and OA.

Finally, the synovial fibroblasts produce anti-inflammatory cytokines. IL-13, IL-14, IL-1Ra, and IL-10 can be found in OA synovial fluid [3,45].

*Synovial cells activation in OA*

The involvement of innate immunity has been described in synovitis. Toll-like receptors (TLRs) [46] and TLR ligands [47,48] are expressed by OA synovium. TLR-2 and TLR-4 are involved in the activation of the synovium and inflammation. They might recognize the hyaluronan and fibronectin present in the OA cartilage matrix [19]. IL-15Ra is present in the lining synovial layers and endothelium. It is involved in MMP production and recruitment and survival of CD8+ T cells. IL-2, a cytokine involved with Th1 cell response, is present in activated T cells in the joint of OA patients with chronic inflammation [31,49]. IL-12 produced by macrophages induces the production of cytokine by Th1 [50].

Furthermore, the expression and activation of complement are now known to have a key role in OA pathogenesis [51]. It is clearly involved in the activation of synovial cells. The C3a, C3b, and C5 fractions promote inflammation and phagocytosis. They could be activated by fibromodulin, COMP, and osteoadherin. The terminal complement protein complex, the membrane attack complex (MAC), can lyse target cell membrane and extra-cellular matrix proteins.

Finally, OA SM, like RA SM is hypoxic. Hypoxia is responsible for cell activation and recruitment [52,53]. It provokes the synthesis of hypoxia-inducible factors (HIF), also induced by pro-inflammatory mediators [54]. HIF-1 is a transcription factor that is constitutively expressed in SM cells. It gains transcriptional activity in hypoxic cells, leading to the expression of genes involved in angiogenesis, inflammation, and tissue degradation. HIF-1 promotes cartilage degradation and induces angiogenesis [52,55].

*Catabolic mediators*

The synovial membrane is, as cartilage, the site of the production of large quantities of MMPs (MMP-1, MMP-3, MMP-9, and MMP-13) [39,56]. MMP-1, MMP-3, MMP-13, and cysteine cathepsin B and S can be detected in synovial fluid [57]. Tissue inhibitor of metalloproteinase (TIMP)-1 is also produced by synovial tissue [26].

The reactive oxygen species (ROS) have been described as key factors involved in cartilage pathogenesis of OA [58,59]. They are also produced by the synovial tissue [60]. SM inflammation is favored by external cellular mechanisms that lead to the increased production of oxidated proteins and increased concentration of ROS. The oxidative damage contributes to inflammation. This fact constitutes a vicious circle between cartilage degradation and synovial inflammation.

*Neuropeptides and mediators of pain*

The inflamed SM is the source of pain in the OA joint. There are changes in nociception, sensitization by inflammatory mediators, and production of specific neuropeptides [61]. Bradykinin is a mediator involved in vasodilatation and also an inflammatory peptide. It is involved in the initiation and maintenance of inflammation. It is responsible for the excitation and sensitization of the sensory nerve fibers. It acts alone or in synergy with pro-inflammatory cytokines [62]. Substance P is another neuropeptide found in the subintimal portion of the OA SM. It is also present in areas of SM close to osteophyte or cartilage lesion [41]. It mediates pro-inflammatory signals, vasodilatation, and contributes to pain. It is involved in $PGE_2$ and collagenase production by synovial cells. It is also involved in the proliferation of synoviocytes themselves [63]. The opioid receptors (μ and δ) are found at the surface of immune cells and sensory nerve endings in RA as well as in OA [64]. Corticotropin-releasing factor (CRF), urocortin, and vasoactive intestinal peptide (VIP) have a role in OA pain, perpetuation of synovial inflammation, and cartilage degradation by exerting both pro- and anti-inflammatory potencies [41]. Finally, nerve growth factor (NGF), whose production is stimulated by IL-1β and TNFα, is expressed by OA synovial tissue. Synovial cells not only express NGF receptor but are also sensitive to this growth factor [65].

It stimulates the production of pro-inflammatory cytokines (IL-1β, IL-2, IL-6, IL-8, and TNFα) and participates in the activation and migration of leukocytes. It is additionally involved in synovial cells proliferation.

*Other lipid mediators and adipokines*

The underlining cells in SM are adipocytes. They are responsible for the release of several factors [66–68]: adiponectin, visfatin, and leptin. Indeed, the synovial tissue is the main source of adipokines in the joint. They are involved in both inflammation and cartilage degradation.

Mast cells and macrophages in the inflamed SM also produce pro-angiogenic factors, such as vascular endothelial growth factor (VEGF) [69]. It is present in abundance during chronic inflammation in the OA synovial tissue [38].

*Vascular aspect of synovial inflammation*

The vascular aspect of synovial inflammation is crucial. Indeed, angiogenesis is not only an important physiological event but it has also been clearly identified in joint tissues during OA [70]. Increased angiogenesis has been described in OA synovial tissue [38] as in other OA tissues (i.e., cartilage and menisci). It is of clinical relevance in OA patients [25,37]. Angiogenesis is associated with chronic synovitis but occurs at all stages of OA [71]. It could contribute to the transition from acute to chronic inflammation [38]. Inflammation stimulates angiogenesis that in turn perpetuates the latter by the transport of inflammatory cells and mediators [72]. It has been linked to the increase of macrophage infiltration and to the histological grade of inflammation [71,73]. Angiogenesis and inflammation are closely integrated processes and may affect disease progression and pain [74]. In addition, adhesion molecules found in new vessels, such as E-selectin, may facilitate cell infiltration [75]. In turn, cell infiltration contributes to the self-increase of angiogenesis [72].

This is noteworthy that angiogenesis observed in OA is comparable to angiogenesis observed in RA patients [76]. Moreover, there is a link between vascular growth and pain in OA.

Angiogenesis during OA results from an imbalance between pro-angiogenic (VEGF and IL-8) and anti-angiogenic factors (troponin I and thrombospondin). Indeed, our group has recently showed that the expression patterns of inflammatory and angiogenic mediators are different between inflamed and normal/reactive areas of the OA SM and that the inflamed area expressed a pro-angiogenic phenotype characterized by an increase of VEGF and a decrease of TSP-1.

## Effects of some GAG compounds on OA synovitis

*Chondroitin sulfate*

Chondroitin sulfate (CS) is a natural component of cartilage extra-cellular matrix. It was first supplied to OA patients in order to restore the damaged cartilage matrix. Since then, the anti-inflammatory effects of CS have been detailed in chondrocytes and extensively reviewed [77,78]. In addition, CS has been shown to affect in different ways the synovial inflammatory phenomenon. It was indeed shown to be potent on the three main tissues of the joint (Fig. 2).

Several evidences have been gathered from in vivo studies. CS reduced synovitis histological parameters (i.e., cell infiltration, fibrosis, and proliferation of lining cells) in collagen-induced arthritis (CIA) in mouse [79]. It was also able to inhibit the oxidative stress and the plasma level of TNFα in rat CIA [80]. In addition, it reduced the levels of inflammatory cytokines: IL-6 in

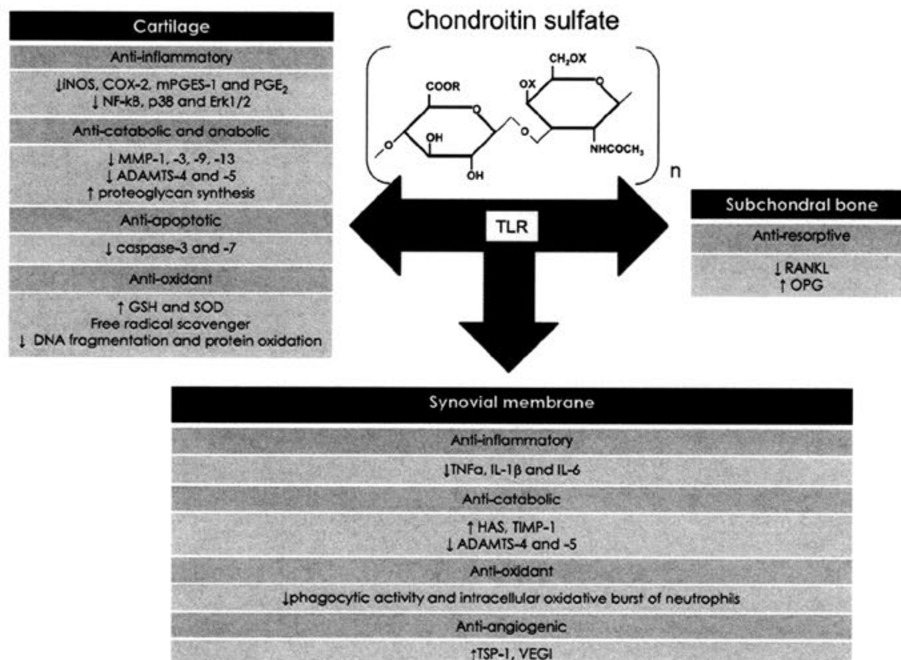


**Fig. 2.** Summary of the biological activities of chondroitin sulfate on the three main tissues of the joint. The chemical structure of chondroitin sulfate corresponds to a succession of the presented motif where R is Na or H and X is SO3R or H. The effects on articular cartilage and subchondral bone have been adapted from studies by Henrotin et al. [77] and Hochberg et al. [114]. The effects on the synovial membrane are detailed in the present review. These effects might occur through the activation of the Toll-like receptors (TLR). iNOS: inducible nitric oxide synthase; COX: cyclo-oxygenase; mPGES: microsomal prostaglandin E synthase; $PGE_2$: prostaglandin E2; NF-κB: nuclear factor kappa B; MMP: matrix metalloproteinase; TIMP: tissue inhibitor of metalloproteinase; ADAMTS: a disintegrin and metalloproteinase with thrombospondin motifs; GSH: glutathione; SOD: superoxide dismutase; TNF: tumor necrosis factor; IL: interleukin; HAS: hyaluronic acid synthase; TIMP: tissue inhibitor of metalloproteinase; RANKL: receptor activator of nuclear factor kappa B ligand; OPG: osteoprotegerin.

mice CIA [81] and IL-1β in rat adjuvant arthritis (AA) [82]. More recently, CS was shown to reduce the plasmatic levels of inflammatory cytokines (IL-1β and IL-6) in rats suffering arthritis [83]. Moreover, the same study demonstrated that CS inhibited the phagocytic activity and the intracellular oxidative burst of neutrophils.

In vitro experiments provide additional information regarding the potencies of CS to affect synovial inflammation in OA. CS was shown to inhibit the oxidative stress in vitro in human skin fibroblasts [84]. Additionally CS inhibited the TLR-mediated IL-6 secretion by macrophage-like cells [85]. This effect was shown to be dependent on CS structure, i.e., sulfation sites and size. Finally, CS was able to affect the metabolism of synovial fibroblasts by the regulation of hyaluronate synthase [86], the suppression of ADAMTS-4 and -5, and the recovery of TIMP-1 expression [87].

Using the microarray technique, we have investigated the effect of CS on the expression of a gene coding for pro- and anti-angiogenic factors (personal unpublished data). In a first set of experiments, we have compared gene expression pattern of synovial cells coming from inflammatory (I) area of OA SM cultured for 7 days with or without highly purified bovine CS (200 μg/ml; Bioiberica SA, Barcelona, Spain) and in low glucose condition. A total of 219 genes were identified as differentially expressed between I and I-CS conditions. Among them, we identified a number of genes implicated in angiogenesis and cell migration pathways. The endothelial cell-specific molecule-1 (ESM-1), the transmembrane-4-L-six-family-1 (TM4SF1), the 5′-ectonucleotidase (NT5E), and the growth arrest-specific gene 6 (GAS6) were down-regulated by CS. In a second set of experiments, we have compared the effect of CS on IL-1β-treated synovial fibroblast cells coming from OA SM. A total of 3308 genes were identified as differentially expressed genes between control and IL-1β conditions. The most up-regulated pro-angiogenic gene was stanniocalcin 1 (STC1). Interestingly, CS tended to decrease STC1 gene expression. Moreover, we also demonstrated the modulatory effects of CS on lysyl oxidase-like 4 (LOXL4) and claudin 11 (CLD11), two genes that negatively regulate cell invasion (personal unpublished data). Using real-time RT-PCR, a more sensitive and gene-targeted method, we have investigated the effect of CS (200 mg/ml) on the expression of selected pro- and anti-angiogenic genes by IL-1β-treated synoviocytes [88]. The stimulating effect of IL-1β on VEGF, bFGF, NGF, Ang-1, and MMP-2 was unaffected by CS. After 24 h treatment, CS counteracted the inhibitory effect of IL-1β on VEGI and TSP-1. This effect was confirmed at the protein level by immunoassays. As an inhibitor of angiogenesis, TSP-1 overexpression decreased inflammation and blood vessel density in SM. It also reduced cartilage lesion in rats where OA is induced by anterior cruciate ligament transection.

Besides its beneficial effect on cartilage, CS may also reduce synovitis in animal models, probably in part by not only inhibiting the production of pro-inflammatory cytokines and ROS but also stimulating anti-angiogenic factors. The gathered evidences regarding CS effect on synovial inflammation are promising. However, they need to be further investigating in human clinical trials using biochemical, histological, and imaging markers to confirm and go deeper in the understanding of the mechanism of action on synovitis.

*Glucosamine*

Glucosamine (GlcN), as a constituent of the natural glycosaminoglycan, can be provided to OA patients under several forms, GlcN sulfate (GlcN-S), GlcN hydrochloride (GlcN-HCl), and N-acetyl-GlcN. It was first thought to be efficient by providing building blocks necessary for the repair of the cartilage extracellular matrix. Nevertheless, anti-inflammatory and anti-catabolic effects have been described in cartilage [89] (Fig. 3).

However, few evidences were found on the effect of GlcN on synovial inflammation. Several studies have reported the effect of GlcN on OA progression and pain in vivo [89]. GlcN, under its native form or as a synthetic analog, reduced the incidence of CIA in rat [90]. The same study showed the potency of GlcN to reduce

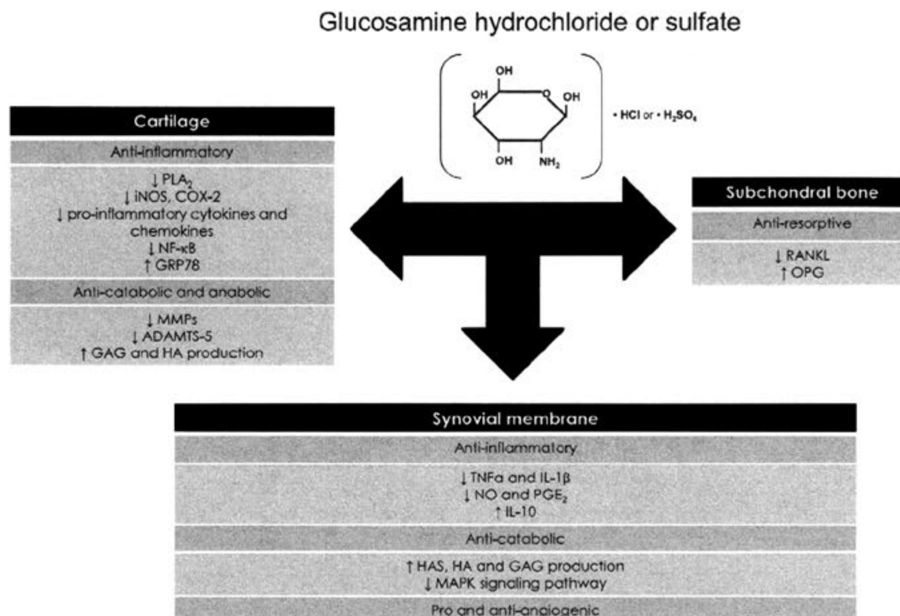


**Fig. 3.** Summary of the biological activities of glucosamine sulfate (• $H_2SO_4$) or hydrochloride (• HCl). The effects on cartilage and subchondral bone were adapted from a study by Henrotin et al. [89]. The effects on the synovial membrane are described in the present review. PLA2: phospholipase A2; iNOS: inducible nitric oxide synthase; NO: nitric oxide; $PGE_2$: prostaglandin E2; COX: cyclo-oxygenase; NF-κB: nuclear factor kappa B; GRP: chaperone protein; MMP: matrix metalloproteinase; TIMP: tissue inhibitor of metalloproteinase; ADAMTS: a disintegrin and metalloproteinase with thrombospondin motifs; TNF: tumor necrosis factor; IL: interleukin; GAG: glycosaminoglycan; HA: hyaluronic acid; HAS: hyaluronic acid synthase; TIMP: tissue inhibitor of metalloproteinase; MAPK: mitogen-activated protein kinase; RANKL: receptor activator of nuclear factor kappa B ligand; OPG: osteoprotegerin.

synovial inflammation, including cell infiltration and hyperplasia. Interestingly, GlcN inhibited IL-6 production, whereas it up-regulated IL-10 in SM of mouse CIA [91].

The anabolic properties of GlcN were demonstrated in human OA synovial explants where it increased HA production [92] or in human synovial cells where it stimulated HA synthase activity and both HA and GAG production [93]. The latest study showed the same potency in both chondrocytes and synoviocytes. GlcN-HCl inhibited $PGE_2$, NO, and MMP in synoviocytes and chondrocytes [94]. In addition, GlcN-S exhibits a slight inhibitory potency on IL-1β, TNFα, and $PGE_2$ in macrophages, RAW 264.7 cells [95].

Interestingly, there is a body of evidence supporting the anti-inflammatory effects of GlcN in animal models and in vitro. GlcN seems to be active on the cellular and biochemical component of the inflammatory reaction. In contrast to CS, GlcN has not been studied either on synovial angiogenesis or on angiogenic factors production by synovial cells. This point should be added on the future research agenda. Again, we lack evidence supporting the anti-inflammatory action of GlcN in human OA SM.

*Hyaluronic acid*

Hyaluronic acid (HA) is basically used through intra-articular injection to restore synovial fluid viscoelasticity in order to consequently improve joint functionality and symptoms. HA effect has been studied so far on cartilage and SM (Fig. 4).

In vivo, HA was demonstrated to reduce synovitis in rat anterior cruciate ligament transection (ACLT) model of OA [96]. HA was also proven to reduce synovial hyperplasia and inflammation in meniscectomy model in sheep [97,98]. HA treatment reduced the synovial score with a significant reduction of the vascularity score and the aggregate score. It reduced the depth of fibrosis and the vessel number in sheep after bilateral meniscectomy [97]. HA was recently shown to modulate inflammation in rat synovium in AA model by the reduction of HIF, NO, and MMP-3 [99]. In contrast, HA was demonstrated to increase angiogenesis in ACLT rabbit after a single intra-articular injection [100]. Moreover, high-molecular-weight HA decreases TLR-2 and -4 cartilage expression in mouse CIA [101].

The anti-inflammatory properties of HA consisted in the inhibition of many cellular aspects of synovitis, including the inhibition of leukocyte migration, leukocyte phagocytosis, lymphocyte proliferation, and several mediators such as prostaglandins, pro-inflammatory cytokines, and ROS [102]. In addition, HA was proven to reduce NO in synovial fluid of OA patients when concomitantly improving WOMAC pain and physical function scores [103]. Moreover, ICAM-1 and VCAM-1 were decreased in the synovial fluid of patients treated with HA [104]. These adhesion molecules are increased by IL-1β, TNFα, and IFN [105].

HA exhibited different potencies on the gene expression of cytokines and enzymes in fibroblast-like synoviocytes extracted from the synovial fluid of early OA patients [106]. It was able to down-regulate IL-8 and iNOS when not stimulated with IL-1β and ADAMTS-5 and TNFα when stimulated with IL-1β. HA was shown to produce a significant decrease in the number of lining cells, macrophages, lymphocytes, mast cells, and fibrin [107] when examining synovial biopsies from OA patients 6 months after the last injection of HA. On the contrary, Schumacher et al. [108] showed no difference between synovitis and synovial fluid from patients treated with or not treated with HA, even if the treatment was proven to be beneficial on walking pain. This difference may be due to the short time between the examination and the last injection. Indeed, HA is known to have a long onset of action.

Anti-catabolic properties of HA have been shown in vitro in SM. HA suppressed ADAMTS-4 expression through the down-regulation of p38 MAPK and JNK protein phosphorylation in human fibroblast-like synoviocytes in culture [109]. It was also proven to possess anti-inflammatory properties through the up-regulation of IL-10 in synovial fibroblasts after tibial plateau fracture [110]. The same study showed that HA reduced synovial cells proliferation. This effect was previously observed in rabbit synovial cells in culture [111].

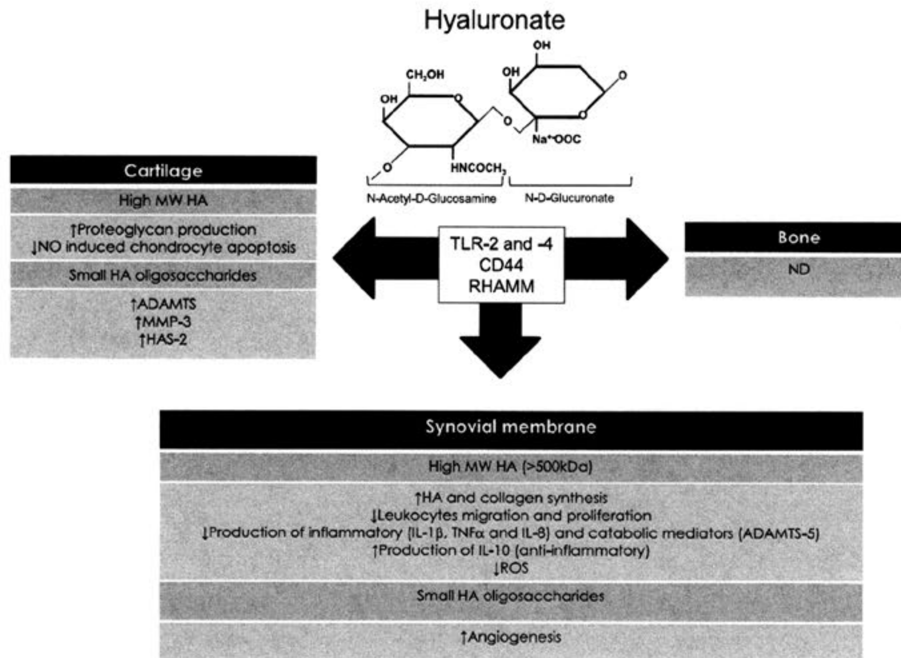


**Fig. 4.** Summary of the biological activities of hyaluronic acid (HA) on the three main tissues of the joint. The distinction was made between high-molecular-weight (MW) HA and small HA oligosaccharides. These effects might occur through the activation of the Toll-like receptors (TLR), CD44, and/or receptor for hyaluronic acid-mediated mobility (RHAMM). NO: nitric oxide; MMP: matrix metalloproteinase; ADAMTS: a disintegrin and metalloproteinase with thrombospondin motifs; ROS: reactive oxygen species; TNF: tumor necrosis factor; IL: interleukin; HAS: hyaluronic acid synthase.

The pro-angiogenic effect of HA was recently confirmed in HUVECs [112]. This increase of angiogenesis was shown to be independent of VEGF. This should be related with the recent discovery that HA degradation produced by either ROS or hyaluronidase generated small oligosaccharides that were able to increase pro-inflammatory (IL-1β and TNFα) and pro-angiogenic cytokines (IL-18) production by synovial fibroblasts obtained from mice subjected to CIA [113]. This effect was mediated by the activation of both CD44 and TLR-4. The stimulation of CD44 and TLR-4 in turn activated NF-κB that induced the production of these cytokines.

Intra-articular injection of HA exhibits anti-inflammatory activity by limiting inflammatory cells invasion in SM and by inhibiting the production of pro-inflammatory mediators like cytokines, ROS, or prostanoids by synovial cells. However, the effects of HA fragments on angiogenesis are intriguing. In vitro, they promote angiogenesis, suggesting that when HA is degraded enzymatically or oxidated, it could promote chronic inflammation. Therefore, one may wonder about the pro-angiogenic effect of injected HA, since it could be degraded in vivo by enzymes into smaller HA.

This hypothesis needs to be further explored in vivo, even if so far no worsening of inflammation after HA injection has been observed in clinical trials.

## Discussion and conclusion

There is no additional proof to provide to demonstrate how important synovitis is in the OA process. It is closely involved by all its aspects in the pathophysiology of the disease. It is a potent target for the treatment of OA to produce not only a symptomatic effect but also a structure modification. Specific targets (i.e., control of inflammation and angiogenesis) should be clearly defined for the treatment of OA synovitis.

Chondroitin sulfate has demonstrated to control the three aspects of SM inflammation: cell infiltration and action, biochemical mediators release, and angiogenesis. Glucosamine is also active on both cellular and molecular aspects of the inflammatory reaction. It would be interesting to compare the effects of glucosamine and chondroitin sulfate on angiogenesis. This could give a rationale to the use of a combination of glucosamine and chondroitin sulfate to treat synovitis. One major concern in the interpretation of the anti-inflammatory activity of these compounds is that they have been tested in an acute inflammatory model. Indeed, the most commonly used model to test the anti-inflammatory properties of these compounds was collagen-induced arthritis (CIA). This model is characterized by a strong immune reaction, which is absent from the OA synovitis. Therefore, the extrapolation of CIA observation to OA needs to be done with caution.

Hyaluronic acid seems to be anti-inflammatory in its native form, while products of degradation are reported to be pro-angiogenic in vitro. This observation is particularly interesting because it gets the perspective on the use of a modified formulation, i.e., with mannitol, which could protect HA against enzymatic or oxidative degradation.

Much evidence suggests that some of the studied GAG compounds could target different aspects of synovitis. Some of them could be considered in combination therapy since they exhibit complementary properties.

Most of the studies have concentrated on articular cartilage and chondrocytes. In order to achieve a structure modification, one may now consider all joint tissues and investigate the drug potency on all of them. Potent treatment should trigger the most important features of OA: cartilage degradation, subchondral bone sclerosis, and all aspects of synovial inflammation.

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**RESEARCH ARTICLE**

# Effects of shark cartilage polysaccharides on the secretion of IL-6 and IL-12 in rheumatoid arthritis

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**Abstract**

*Context*: Rheumatoid arthritis (RA) is a chronic inflammatory disease caused by inflammation of the synovial membrane, leading to articular cartilage destruction. Shark cartilage polysaccharide (SCP) is a biodegradable, biocompatible, nontoxic, non-immunogenic and non-inflammatory polysaccharide that may be used in treating RA.

*Objective*: The anti-RA activities of SCP given orally to rats are investigated here for the first time.

*Materials and methods*: SCP treatment group were administered with SCP-1, SCP-2 in the dosage of 9 mg/day for 24 days. The effect of SCP-1 and SCP-2 on the body weight, paw swelling, morphological changes of ankle and IL-6, IL-12 secretion in RA rats are examined.

*Results*: On day 24, there were no obvious differences in BMI between positive, SCP-1 and SCP-2 group. The swelling degree of SCP-1 and SCP-2 group was less serious than model group. X-ray revealed that SCP-1, SCP-2 group owned normal joint alignment and more smooth and tidy articular surface. The SCP-1 and SCP-2 have inhibitory effect on IL-6 (430.3 ± 25.6 pg/mL, 439.1 ± 35.9 pg/mL) and IL-12 (63.9 ± 20.1 pg/mL, 64.9 ± 14.1 pg/mL) secretion, which showed significant differences with model group (612 ± 72.3 pg/mL, 230.1 ± 29.2 pg/mL), but still higher than normal group (361.8 ± 47.1 pg/mL, 34.2 ± 15.1 pg/mL) and lower than positive group (418.1 ± 42.5 pg/mL, 90.2 ± 17.8 pg/mL). Especially, when the concentration of SCP was 125 μg/mL, the contents of IL-6 (431.1 ± 43.3 pg/mL, 401.7 ± 55.7 pg/mL) and IL-12 (63.2 ± 12.3 pg/mL, 52.3 ± 8.7 pg/mL) were lowest.

*Discussion and conclusion*: These findings demonstrate that SCP have excellent anti-RA activities and thus have great potential as a drug for treating RA diseases.

**Keywords:** Glycosaminoglycans, autoimmune disease, lymphokine, inflammatory

## Introduction

Rheumatoid arthritis (RA) is a common systemic autoimmune disease, which is characterized by chronic polyarthritis symptom (Walsmith et al., 2002). According to the RA pathogenesis studies, anti-inflammatory therapy has become one important treatment (Wilson et al., 2000, 2003). Cytokines are extracellular protein/peptide messengers that play a critical role in cell-to-cell communication in RA. They are the primary mediators by which communication occurs between leukocytes such as lymphocytes and macrophages, and between other immune and non-immune cells and, thus, contribute significantly to regulating and controlling immune responses (Romagnani, 2004; Merly et al., 2007). In the pathogenesis of RA, IL-6 (McNiff et al., 1995; Jikko et al., 1998; Ishihara & Hirano, 2002; Nishimoto & Kishimoto, 2004; Wong et al., 2006) and IL-12 are two very important inflammatory mediators. IL-6 and IL-12 can stimulate the release of inflammatory cytokines and attract inflammatory cells into the joint cavity, which result in aggravating the pathogenesis of RA (Taylor, 2003; Maruotti et al., 2006). It is reported that glycosaminoglycans (GAGs) such as shark cartilage extract has anti-inflammatory effects and fibrinolytic activities (Chen et al., 2000; Ratel et al., 2005), the effects of shark cartilage extract on the attachment and spreading properties and the focal

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adhesion structure of cultured bovine pulmonary artery endothelial cells were examined, treatment with cartilage extract resulted in cell detachment from the substratum, and the inhibitory effects of cartilage extract on cell attachment and spreading are mediated by modification of the organization of focal adhesion proteins.

Shark cartilage contains proteins, polysaccharides, fat, minerals, calcium, phosphorus and moisture (Pearson W et al., 2007). Shark cartilage polysaccharides (SCPs) are composed of GAGs including keratan sulfate (KS), chondroitin sulfate A (CSA), chondroitin sulfate C (CSC) and hyaluronic acid (HA). The major disaccharides of CSA and CSC are glucuronic acid and sulfated *N*-acetylgalactosamine (GalNAc), which are connected by β1-3 glucosidic bond, and the disaccharide units are connected by β1-4 glucosidic bond to each other, forming the liner chain polysaccharides. Shark cartilage is a promising source of CS (Ping Wang & Junmin Tang, 2009). CS is an anionic linear polysaccharide which consists of alternating disaccharide units of glucuronic acid (GlcUA) and GalNAc. Over the past few years, through many scientific studies about the use of GAGs and chondroitin by millions of people, experiments have proved that these substances orally can help people with joint problems, most significantly people with osteoarthritis. These polysaccharides can relieve joint pain, and can actually help rebuild cartilage and slow the progression of osteoarthritis. Other than its antineuralgia, antihemicrania and antitumor activities, SCPs is speculated to have anti-rheumatoid arthritis activity.

The purpose of the research conducted in our lab was to evaluate the immuno-effectiveness and bioactivity of commercial shark cartilage that is available as a RA therapy agent. In this study, the peritoneal macrophages were extracted from RA rat, the secretion of IL-6 and IL-12 from peritoneal macrophages culture supernatant was researched in SCP-treated group and model group, and the influence of different concentrations of SCP on the secretion of IL-6 and IL-12 were further studied.

## Materials and methods

### Materials

Shark cartilage polysaccharide SCP-1 (molecular weight: 60 kDa, determined by HPLC after enzymatic degradation of SCP-2) and SCP-2 (molecular weight: 100 kDa, determined by HPLC, C4384) were purchased from Sigma, sulfur ≥ 4%, 6-sulfate:4-sulfate ratio by HPLC ≥ 0.33:1. Freund's complete adjuvant (CFA), Sigma, F5881. Methotrexate (MTX), Sigma, M4010. Calf serum, Hangzhou, Four Seasons Green Engineering Co., Ltd. PBS, Gibco Co, USA. Trypan blue, Sigma. RPMI-1640, GIBCO, USA. Trypsin, Amresco, USA. EDTA, Shanghai Biotechnology Co., Ltd. LPS, Sigma. Rat-IL-6 ELISA KIT, Bender Medsystems, USA. Rat-IL-12 ELISA KIT, Biosource Medsystems, USA. LG-10 centrifuge, Beijing Medical Centrifuge Factory. Inverted optical microscope, LEICA, Germany. $CO_2$-incubator, HERAEUS, Germany. Multiskan-MK3 microplate reader Leibo, Thermosystems, Canada. Ultra-low temperature refrigerator, SANYO, Japan.

### Laboratory animal and animal grouping

Wistar rats (male), 8–10 weeks, weighing 120–180 g, were supplied by the Experimental Animal Center of Shandong Province, and carried out in accordance to the "NIH Guidelines for the Care and Use of Laboratory Animals". Male Wistar rats (25) were randomly divided into five groups ($n = 5$), which were: A. model group, B. positive control group, C. normal control group, D. SCP-1 treatment group, E. SCP-2 treatment group. The mice were maintained in a specific pathogen-free environment that was temperature controlled (23 ± 2°C) and humidity controlled (60 ± 10%), under a 12 h light/dark cycle.

### Rheumatoid arthritis model in rats

After a few days feeding, each rat was injected with 0.1 mL complete Freund's adjuvant (CFA) (10 mg/mL) in the right rear paw subcutaneous except the normal group. In the same day, SCP-1 treatment group and SCP-2 treatment group were administered with SCP-1, SCP-2 in the dosage of 9 mg/day for 24 days. Positive group was administered once a week with methotrexate (MTX) 0.55 mg/kg for 24 days. Normal group was given 2 mL normal saline each day, for 24 days.

### The changes of body weight and paw swelling index

The weight and the paw swelling index of rat was measured on day 1, 3, 6, 12, 18 and 24 after administration (Liu M et al., 2011). The thickness of paw was measured before and after proinflammatory and paw swelling index was calculated by the following formula:

Paw swelling index = (thickness after proinflammatory − thickness before proinflammatory)/thickness before proinflammatory × 100%.

### X-ray evaluation

X-ray photograph of the hind legs were taken after the rats were killed (60 kV, 15 s). The alteration of ossicular skeleton by X-ray method could assess the degree of arthritis.

### Preparation of the peritoneal macrophage suspension

Rats from different groups were killed by cervical dislocation. Then, the rats were dissected to expose the peritoneal wall and 75% alcohol was used to scrub peritoneal wall. Ice-cold PBS (20 mL) was injected into the abdominal cavity and abdomen was gently pressed (about 2 min). Peritoneal exudate cells were harvested by flushing peritoneal cavities with ice-cold PBS. Cells were plated in a 24-well plate at an initial density of $1 \times 10^6$ cells/mL in a 500 mL volume of RPMI 1640, supplemented with 10% FBS. Two hours later, the wells were washed to remove all nonadherent cells and



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500 mL of fresh medium was added together with LPS (1.0 mg/mL/well).

### Determination of cytokines

After a 48-h incubation, the supernatants from $1 \times 10^6$ peritoneal macrophage were collected and IL-6 ELISA was performed using a standard kit from Sigma. The same kind of test was used for IL-12 measurement.

### Statistical analysis

All data were expressed as mean ± SEM. Significant differences among groups were analyzed by one-way analysis of variance (ANOVA). Differences were considered statistically significant at $p < 0.05$.

## Results

### Body weight and paw swelling index changes

The weight of rat was measured on days 1, 3, 6, 12, 18, and 24 after administration. Measurement results were shown in Figure 1. From Figure 1, it was found that the body weight of rats from different groups gradually increased in 24 days. However, the body weight of rats in model group grew slowly, and this trend continued to day 24. There were no significant differences between each group from day 1 to day 12. But there were obvious differences in body weight between model group and normal group, and the differences increased significantly until day 24, which indicated that the RA model was successfully established. The body weight of rats from SCP-1 and SCP-2 group did not significantly higher than that of model group until on day 18 ($p < 0.05$), but there were no obvious differences in body weight between positive group and SCP-2 group, which indicated that SCP-2 has similar therapeutic effect compared with positive control. On day 24, there were no obvious differences in body weight between positive group, SCP-1 group and SCP-2 group, which indicated that both SCP-1 and SCP-2 have similar therapeutic effect in RA therapy.

### Changes in rat paw swelling index

The paw swelling index of rat was measured on days 1, 3, 6, 12, 18 and 24 after administration. From Figure 2, it can be concluded that at first few days, all groups except normal group, the joints swelling of entire foot and ankle of the right hind leg was apparent, which was significantly higher than that of normal group. MTX (positive control) has the most benefit effect on paw swelling index, and the paw swelling index of positive control was closely to model group. The rats paw swelling reached to the peak on day 21 and day 24. There were no significant differences between the model group and treatment groups until day 18. Besides, the swelling degree of rats from SCP-1 and SCP-2 group were less serious than that of model group on day 18 and day 24 ($p < 0.05$). However, there were no significant differences between the two treatment groups.

### X-ray evaluation

Rats of model group had significantly narrow knee gap, irregular joint edges and low-density areas along the joint edge caused by multiple erosions (Figure 3). Compared with the normal group, it could be seen the rheumatoid arthritis model was successfully established. X-ray revealed that rats of SCP-1 and SCP-2 groups had normal joint alignment and more smooth and well organized articular surfaces. In addition, a narrow gap was not obvious and knee lesions were less serious than that of the model group.

### The determination of IL-6 and IL-12

The concentration of rat IL-6 and IL-12 was determined at 450 nm, and the standard curves were fit by Origin Pro7.0. The regression equation of IL-6 and IL-12 were as follows, $y = 0.0015x + 0.0689$, $R^2 = 0.9975$, $y = 0.0032x + 0.1646$, $R^2 = 0.9927$.

The contents of IL-6 and IL-12 from rat peritoneal macrophages of different experimental groups are shown in Figure 4. The results showed the content of IL-6 from peritoneal macrophage culture supernatant of the model group was 611.93 ± 72.3 pg/mL, which was higher than that of the normal group (361.84 ± 47.1 pg/mL) ($p < 0.05$). The content of IL-6 in the positive group was 418.11 ± 42.5 pg/mL, which was significantly lower than that of the model group. In two treatment groups the rat IL-6 levels were also lower than that of the model group but still higher than that of the positive group. It can be seen from Figure 3 that two SCPs have an inhibitory effect on IL-6 secretion, which showed significant differences with the model group and the normal control ($p < 0.05$), but showed no significant differences with the positive

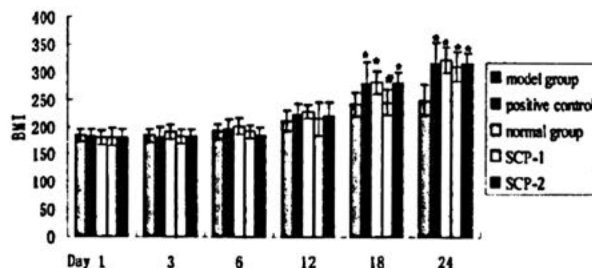


Figure 1. Rat body mass index changes in 24 days (*: vs model group, $p < 0.05$. *: vs positive control, $p < 0.05$.

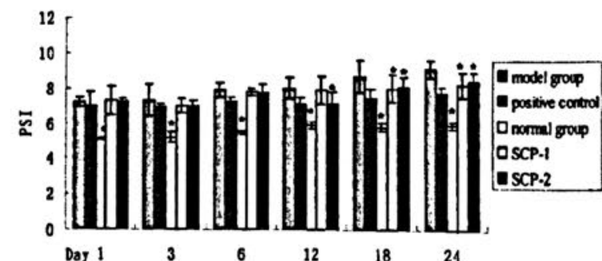


Figure 2. Rat paw swelling index changes in 24 days (*: vs model group, $p < 0.05$.

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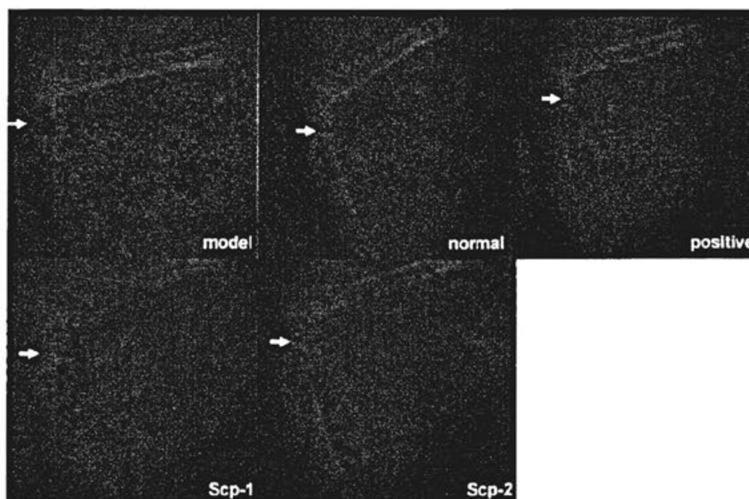


Figure 3. X-ray evaluation of all the groups. Model group: joint space narrowing, joint edges irregular, not sharpness, patella forward displacement, low-density area of joint edge caused by multiple erosion. Positive control: Normal joint alignment, articular surface smooth, gap narrow not obvious, small amount of proliferation of the articular surface. Normal control: normal joint alignment, articular surface smooth, normal bone structure. SCP-1: normal joint alignment, articular surface smooth, gap narrow not obvious, small amount of proliferation of the articular surface. SCP-2: normal joint alignment, articular surface smooth, gap narrow not obvious.

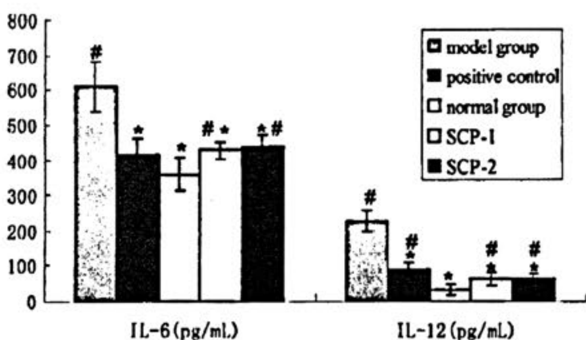


Figure 4. Content of rat IL-6 and IL-12 in all the groups (*: vs model group, $p < 0.05$, #: vs normal group, $p < 0.05$).

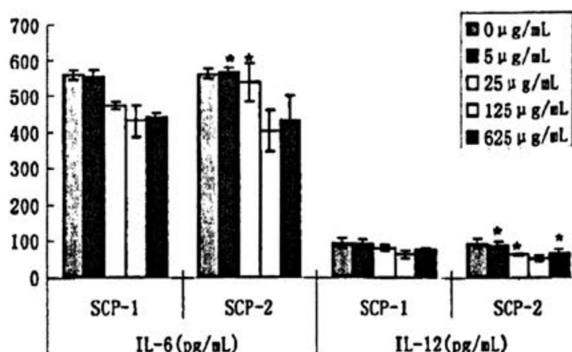


Figure 5. Content of rat IL-6 and IL-12 under SCPs with different concentrations (*: vs SCP-1group, $p < 0.05$).

group. There was no significant difference between the treatment groups.

The content of IL-12 from peritoneal macrophage supernatants of the model group was 230.13 ± 29.2 pg/mL, which was significant higher than that of the normal group (34.19 ± 15.1 pg/mL) ($p$ <0.05).The content of IL-12 in the positive group was only 90.19 ± 17.78 pg/mL. Similarly, the rat IL-12 levels in two treatment groups were also lower than that of the model group and the positive group but still higher than that of the normal group. Two SCPs have an inhibitory effect on IL-12 secretion, which showed significant differences with the positive group and the normal control ($p < 0.05$) but showed no significant difference with the model group. There was no significant difference between the treatment groups.

### The effect of different concentrations of SCPs on the contents of IL-6 and IL-12

It can be seen that as the concentration of SCP increased, IL-6 and IL-12 secreted by macrophages decreased (Figure 5). When the concentration of SCPs was 125 μg/mL, the contents of IL-6 and IL-12 were lowest, and there were no significant differences between the SCP-1 and SCP-2 groups ($p > 0.05$). On the contrary, SCP-2 showed a better therapeutic effect than SCP-1 ($p < 0.05$) in other concentrations (0, 5, 25, and 625 μg/mL).

## Discussion

In this experiment, the RA model was successfully established and the weight of the rats and paw swelling during the experiment were observed. In addition, IL-6 and IL-12 levels of culture supernatants of peritoneal macrophages were determined by ELISA after SCPs administration (Joe et al., 1999; Joe & Wilder, 1999). In the rat RA model, the rat right hind foot swelled, and the inflammatory reaction spread to another hind foot and even the front foot after a period of time. In addition, the expression levels of rat IL-6 and IL-12 rose



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continuously. This study explored the influence of two SCPs on expression levels of rat IL-6 and IL-12, which further play an important role in the RA inflammation process including autoantibody generation and inflammatory cells in joint cavities and synovia. Taking the dosage (Shark Cartilage Capsules US time, 720 mg mucopolysaccharides/d) of shark cartilage capsules in human as the reference, the conversion factor between human and rat was 6.3, the dosage of the rat was calculated as follow: 720 mg/d ÷ 60 kg × 6.3 × 0.12 kg = 9 mg/d. The results showed that SCP-1 and SCP-2 could significantly reduce paw edema in rats and attenuate weight loss caused by inflammatory response in rats. In addition, SCP-1 and SCP-2 could significantly inhibit the expression of IL-6 and IL-12 in the RA model.

IL-6 is a glycoprotein with a molecular weight of 19-28 kD and activated monocytes are the mainly source of IL-6 in the blood. Interestingly, the ability of monocytes to synthetise IL-6 decreases when they differentiate into macrophages (Huang et al., 2003). In RA pathogenesis, IL-6 can stimulate B cells to proliferate, differentiate into plasma cells and secrete Ig and rheumatoid factor and other autoantibodies. In addition, IL-6 can activate endothelial cells to secrete IL-1, activate fibroblasts to express TH and MHC molecules, and induce inflammatory cell aggregation, infiltration, involved in inflammatory and immune responses, which enhance the role of IL-6 to induce synovitis. Furthermore, IL-6 can induce liver cells to synthesize acute phase proteins, activate synovial macrophages and STAT proteins, induce IL-1, IFN and TNF increasing, thus causing acute synovitis response and pain. IL-6 can activate various cells, such as APC, T cells, B cells, synovial and cartilage cells, and can present the antigen to T cells to start the inflammatory and immune responses. IL-6 can recruit inflammatory cells into the joint cavity and synovial,and promote the production and secretion of inflammatory mediators, resulting in immune-mediated synovial inflammation (Ishihara & Hirano, 2002). Therefore, investigation of the effects of IL-6 on RA from the perspective of molecular immunology may provide a new target for the drug treatment. In this study, the effects of two kinds of SCP on the expression levels of IL-6 of peritoneal macrophage culture medium in the rat model of RA were studied. The results showed that, the expression of IL-6 of peritoneal macrophage supernatant in the model group was significantly improved. Two kinds of SCPs both exhibited an inhibitory effect on the expression of IL-6 in rat peritoneal macrophages culture supernatant with different degrees. Different concentrations of SCP-1 and SCP-2, SCP-2 exhibited a stronger inhibitory effect, suggesting SCP-2 play a better role in the treatment of RA. Meanwhile, the inhibitory effect was dose-dependent, when the drug concentration was 125 μg/mL, the two shark cartilage polysaccharides showed the strongest inhibition.

IL-12 is a heterodimeric glycoprotein with the molecular weight of 75 kD, which is mainly activated by macrophages, dendritic cells and neutrophils and has proinflammatory and immunoregulatory effects (Ozenci et al., 2000). In RA pathogenesis, IL-12 can induce T cells (CD4+) proliferation, differentiation and activate T cells, which can secrete more cytokines to induce an inflammatory response. IL-12 has the ability of attracting and recruiting inflammatory cells into the joint cavity and synovium, promoting the production and secretion of various inflammatory mediators and leading to the degradation and synthesis decreased of cartilage proteoglycan. IL-12 can reduce apoptosis and promote the proliferation of synovial cells. Furthermore, it can promote the degradation and destruction of cartilage cells. In addition, IL-12 can induce Th0 cells to differentiate into Th1 cells, but also promote the proliferation of the CTL and activate its cytotoxicity (Sakkas et al., 1998; Watford et al., 2003). Hence, inhibiting the activity of IL-12 is conducive to treatment of RA. In this study, the effects of two kinds of SCP on the expression levels of IL-12 of peritoneal macrophage culture supernatant in the rat model of RA were investigated. It can be seen from the results that the expression of IL-12 of peritoneal macrophage supernatant in the RA model rats was significantly increased. SCP-1 and SCP-2 could inhibit the expression of IL-12 with different degrees. Different concentrations of SCP-1 and SCP-2, SCP-2 exhibited a stronger inhibitory effect, suggesting that SCP-2 play a better role in the treatment of RA. Meanwhile, the inhibitory effect was dose-dependent, when the drug concentration was 125 μg/mL, SCPs showed the strongest inhibition on the expression of IL-12. When the drug concentration is higher or lower than the drug concentration, the inhibition decreased, which was consistent with the inhibitory effect of shark cartilage polysaccharides on the expression of IL-6.

## Conclusions

The current study demonstrated that oral administration of SCP effectively blocked the process of RA by inhibiting joint inflammation and joint damage progression, improving radiologic change of bone, increasing weight loss and inhibiting swelling index of paw. The inflammatory process in RA rats without treatment was shown to lead to substantial increases in the levels of the proinflammatory cytokines IL-6 and IL-12, meanwhile SCP showed significant inhibition of the over-production of IL-6 and IL-12. In sum, we examined the effect of the two kinds of shark cartilage polysaccharides on paw swelling and the secretion of IL-6 and IL-12 in the rat RA model. Studies have shown that SCP-1 and SCP-2 can relieve paw swelling and inhibit the expression of IL-6 and IL-12 of RA rats. SCP-2 showed stronger effect than SCP-1 in inhibiting the expression of IL-6 and IL-12, and the effect was dose-dependent. When the drug concentration was 125 μg/mL, the inhibition was strongest.

## Declaration of interest

The authors declared no conflict of interest.

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RESEARCH ARTICLE **Open Access**

# Undenatured type II collagen (UC-II®) for joint support: a randomized, double-blind, placebo-controlled study in healthy volunteers

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**Abstract**

**Background:** UC-II contains a patented form of undenatured type II collagen derived from chicken sternum. Previous preclinical and clinical studies support the safety and efficacy of UC-II in modulating joint discomfort in osteoarthritis and rheumatoid arthritis. The purpose of this study was to assess the efficacy and tolerability of UC-II in moderating joint function and joint pain due to strenuous exercise in healthy subjects.

**Methods:** This randomized, double-blind, placebo-controlled study was conducted in healthy subjects who had no prior history of arthritic disease or joint pain at rest but experienced joint discomfort with physical activity. Fifty-five subjects who reported knee pain after participating in a standardized stepmill performance test were randomized to receive placebo (n = 28) or the UC-II (40 mg daily, n = 27) product for 120 days. Joint function was assessed by changes in degree of knee flexion and knee extension as well as measuring the time to experiencing and recovering from joint pain following strenuous stepmill exertion.

**Results:** After 120 days of supplementation, subjects in the UC-II group exhibited a statistically significant improvement in average knee extension compared to placebo (81.0 ± 1.3° vs 74.0 ± 2.2°; p = 0.011) and to baseline (81.0 ± 1.3° vs 73.2 ± 1.9°; p = 0.002). The UC-II cohort also demonstrated a statistically significant change in average knee extension at day 90 (78.8 ± 1.9° vs 73.2 ± 1.9°; p = 0.045) versus baseline. No significant change in knee extension was observed in the placebo group at any time. It was also noted that the UC-II group exercised longer before experiencing any initial joint discomfort at day 120 (2.8 ± 0.5 min, p = 0.019), compared to baseline (1.4 ± 0.2 min). By contrast, no significant changes were seen in the placebo group. No product related adverse events were observed during the study. At study conclusion, five individuals in the UC-II cohort reported no pain during or after the stepmill protocol (p = 0.031, within visit) as compared to one subject in the placebo group.

**Conclusions:** Daily supplementation with 40 mg of UC-II was well tolerated and led to improved knee joint extension in healthy subjects. UC-II also demonstrated the potential to lengthen the period of pain free strenuous exertion and alleviate the joint pain that occasionally arises from such activities.

**Keywords:** UC-II, Undenatured type II collagen, Joint function, Knee extension, Stepmill, Joint pain

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## Introduction

The impact of strenuous exercise on knee joints may cause localized pain and stiffness, which are hallmark features of pathologic inflammatory disease [1]. It has been shown that when dogs undergo a strenuous running regimen, significant losses in articular cartilage and glycosaminoglycans occur [2]. Such studies suggest that strenuous exercise may activate some of the same physiological processes that occur in arthritic disease [2-4]. In fact, *in vitro* studies have shown that many of the cytokines implicated in the onset and progression of both rheumatoid arthritis (RA) and osteoarthritis (OA) also appear to regulate the remodeling of the normal knee extracellular matrix (ECM) following strenuous exertion [5].

When normal chondrocytes undergo strenuous mechanical stimulation under static conditions, their physiology shifts towards ECM breakdown, as indicated by the upregulation of several metalloproteinases (MMPs), such as MMP-13 as well as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, and various aggrecanases [5,6]. This *in vitro* catabolic response is mediated by changes in the phosphorylation, expression, or translocation of several transcription factors to the cell nucleus such as NF-κB, p38 MAPK, Akt, and ERK [7,8]. By contrast, normal chondrocytes produce the anti-inflammatory cytokine IL-4 when mechanically stimulated under moderate and dynamic conditions [9]. The secretion of this autocrine molecule not only helps in shifting chondrocyte metabolism towards the synthesis of aggrecan and type II collagen, it also downregulates production of nitric oxide (NO) and various MMPs and aggrecanases [10-12]. This conclusion is corroborated by the finding that pretreatment of strenuously compressed normal chondrocytes with IL-4 attenuates their catabolic response [11]. This suggests that IL-4 plays a key role in downregulating remodeling functions, restoring articular cartilage homeostasis, as well as decreasing chondrocyte apoptosis following strenuous mechanical loading [12,13].

Mechanically stressed chondrocytes also produce a number of other molecules known to participate in inflammatory responses, including prostoglandin E2, NO, and vascular endothelial growth factor [14]. These are proinflammatory molecules that, in conjunction with TNF-α, IL-6 and IL-1β, result in a localized, and transitory inflammatory-like response that is part of the normal repair process occurring in knee joints, serves to moderate remodeling events [3]. Ostrowski et al. [15] showed that healthy individuals express up to 27-fold greater concentrations of the anti-inflammatory cytokine IL-10 in blood following a marathon run when compared to IL-10 blood levels at rest. This finding is not surprising given that these same individuals also show marked increases in the proinflammatory cytokines TNF-α, IL-1β, and IL-6. It therefore appears that in healthy subjects undergoing strenuous exertion, the induction of proinflammatory cytokines is offset by the synthesis of anti-inflammatory agents as part of the recovery process. This view is supported by the observation that IL-10 reduces the catabolic impact of IL-1β and TNF-α on cartilage explants from healthy volunteers, and this effect is enhanced by combining IL-10 with IL-4 [13].

Another protein released by dynamically compressed chondrocytes is transforming growth factor (TGF)-β [16-18]. This factor is secreted by many cell types and is known to interfere with the cell cycle and arrest differentiation [19]. With regard to chondrocytes, TGF-β induces cell proliferation *in vitro* and slows terminal differentiation into hypertrophic cells [20]. Numerous studies have shown that TGF-β reverses the *in vitro* catabolic effect of various proinflammatory cytokines on normal chondrocytes as well as chondrocytes harvested from RA and OA donors [21-23].

The overall findings discussed above point to a new, unifying view of joint physiology. It suggests that many of the biological processes occurring in knee joints affected by RA and OA also participate in the maintenance of healthy knees [1,4,5]. It therefore seems appropriate to test the efficacy of natural supplements or ingredients, which have been shown to moderate joint pain in RA and OA, as possible candidates for treating the joint discomfort that occasionally results from strenuous exercise in healthy individuals.

UC-II is a natural ingredient which contains a glycosylated, undenatured type-II collagen [24]. Previous studies have shown that small doses of UC-II modulate joint health in both OA and RA [24-26]. Tong et al. [27], using an *in vivo* model of collagen induced arthritis (CIA), demonstrated that ingesting microgram quantities of undenatured type II collagen significantly reduces circulating levels of inflammatory cytokines, potentially serving to decrease both the incidence and the severity of arthritis [28]. The ability to alter immunity via the ingestion of a food, or an antigen, is called oral tolerance. This is an ongoing normal physiological process that protects the alimentary tract against untoward immunological damage [29,30]. Research into its mechanism of action has revealed that several distinct types of T regulator cells mediate this phenomenon by releasing IL-10 and TGF-β [30]. It has also been shown that this effect is transitory in nature requiring that the food, or antigen, be consumed continuously in order to maintain the tolerogenic state [30]. Given these findings, plus our current understanding of the role of various cytokines in normal joint physiology, it was hypothesized that supplementation with UC-II might relieve joint discomfort and restore joint function in healthy subjects.

The aim of this randomized, double blind, placebo-controlled study was to assess the impact of UC-II on knee function in otherwise healthy subjects with no prior history of arthritic disease who experienced knee pain upon strenuous physical exertion. The primary efficacy variable for assessing knee function included measurements of flexibility using range of motion (ROM) goniometry.

## Methods

### Investigational product

The investigational study product UC-II is derived from chicken sternum. It is manufactured using a patented, low-temperature process to preserve its native structure. For the clinical study, 40 mg of UC-II material (Lot 1109006), which provides 10.4 ± 1.3 mg of native type-II collagen, was encapsulated in an opaque capsule with excipients. Placebo was dispensed in an identical capsule containing only excipients (microcrystalline cellulose, magnesium stearate and silicon dioxide). Both study materials were prepared in a good manufacturing practice (GMP)-certified facility and provided by InterHealth Nutraceuticals, Inc. (Benicia, CA). Subjects were instructed to take one capsule daily with water before bedtime.

### Recruitment of subjects

One hundred and six subjects were screened for eligibility using the inclusion–exclusion criteria defined in Table 1. Only healthy adults who presented with no knee joint pain at rest and no diagnosable markers indicative of active arthritic disease, as outlined by the American College of Rheumatology (ACR) guidelines [31,32], were admitted into the study. To accomplish this, all potential subjects were screened by a board certified clinician. Subjects presenting with any knee pain at rest and at least 3 of 6 clinical classification criteria, which included age greater than 50 years, morning stiffness in the joint lasting 30 minutes or less, crepitus on knee joint manipulation, body tenderness, bony enlargements, knee swelling or presence of excess fluid, and palpable warmth, were excluded. Potential subjects reporting the occasional use of NSAIDs, other pain relief medication, or anti-inflammatory supplements underwent a 2-week washout period before randomization.

Subjects were required to undergo a 10 minute period of performance testing using a standardized stepmill test developed and validated by Medicus Research (Udani JK, unpublished observation). It involved exercising at level 4 on a StepMill® model 7000PT (StairMaster® Health & Fitness Products, Inc., Kirkland, WA) until one or both knees achieved a discomfort level of 5 on an 11 point (0–10) Likert scale [33]. This pain threshold had to be achieved within a 10 minute period otherwise the subject was excluded. Once the requisite pain level was achieved the subject was asked to continue stepping for an additional two minutes in order to record the maximum pain level achieved before disembarking from the stepmill. The following knee discomfort measures were recorded from the start of the stepmill test: (1) time to onset of initial joint pain; (2) time to onset of maximum joint pain; (3) time to initial improvement in knee joint pain; (4) time to complete recovery from knee joint pain. Subjects who experienced a pain score of 5 (or greater) within one minute of starting the stress test were excluded. Out of 106 screened candidates, 55 subjects were enrolled in the study. Each subject voluntarily signed the IRB-approved informed consent form. After enrollment, the subjects were randomly assigned to either the placebo or the UC-II group.

### Study design and trial site

This randomized, double blind, placebo-controlled study was conducted at the Staywell Research clinical site located in Northridge, CA. Medicus Research (Agoura Hills, CA) was the contract research organization (CRO) of record. The study protocol was approved by Copernicus Group IRB (Cary, NC) on April 25th 2012. The study followed the principles outlined in the Declaration of Helsinki (version 1996).

### Randomization and blinding

Simple randomization was employed using a software algorithm based on the atmospheric noise method (www.random.org). Sequential assignment was used to determine group allocation. Once allocated, the assignment was documented and placed in individually numbered envelopes to maintain blinding. Subjects, clinical staff, plus data analysis and management staff remained blinded throughout the study.

### Study schedule

The study duration was 17 weeks with a total of 7 visits that included screening, baseline, days 7, 30, 60, 90 and 120 (final visit). Table 2 summarizes the study visits and activities. Figure 1 depicts the sequence of study procedures that subjects underwent during each visit. All subjects completed a medical history questionnaire at baseline and compliance reports during follow-up evaluations at 7, 30, 60, 90 and 120 days. Subjects were assessed for anthropometric measures, vital signs, knee range of motion (flexion and extension), six-minute timed walk, as well as the onset and recovery from pain using the Udani Stepmill Procedure. A Fitbit (San Francisco, CA) device was used to measure daily distance walked, steps taken and an average step length for study participants. Subjects were also asked to complete the KOOS survey as well as the Stanford exercise scales.

**Table 1 Inclusion–exclusion criteria**

**Inclusion**

- Subject must be ≥30 and ≤65 years of age
- Body mass index (BMI) must be ≥18 and ≤35 kg/m$^2$
- Knee joint criteria: (1) no knee joint discomfort at rest; (2) must achieve a knee joint discomfort score of at least 5 on an 11-point Likert scale within 10 minutes of initiating the stepmill protocol
- Maintain existing food and physical activity patterns throughout the study period
- Judged by Investigator to be in general good health on the basis of medical history
- Subject understands the study procedures and provides signed informed consent to participate in the study and authorizes the release of relevant health information to the study investigator
- Females of child bearing age must agree to use approved birth control methods during the study

**Exclusion**

- Subjects with any indicators of arthritis, joint disorders, or history of immune system or autoimmune disorders
- Daily use of NSAIDs; however, daily use of 81 mg of aspirin for cardioprotection is allowed
- Daily use of anti-inflammatory or omega-3-fatty acid dietary supplements or using supplements to maintain joint health 30 days prior to screening
- Subjects with a history of knee or hip joint replacement surgery, or any hip or back pain which interferes with ambulation
- Use of any immunosuppressive drugs in the last 12 months (including steroids or biologics)
- Glucocorticoid injection or hyaluronic acid injection in affected knee within 3 months prior to enrollment
- History of surgery or significant injury to the target joint within 6 months prior to study enrollment, or an anticipated need for surgical or invasive procedure that will be performed during the study
- Subjects with a chronic pain syndrome and in the judgment of the Investigator is unlikely to respond to any therapy
- Participation in a clinical study with exposure to any non-registered drug product within 30 days prior
- Subjects who have any physical disability which could interfere with their ability to perform the functional performance measures included in this protocol
- Any significant GI condition that would potentially interfere with the evaluation of the study product
- Clinically significant renal, hepatic, endocrine (including diabetes mellitus), cardiac, pulmonary, pancreatic, neurologic, hematologic, or biliary disorder
- Subjects with vascular condition which interferes with ambulation
- Known allergy or sensitivity to herbal products, soy or eggs
- Vegetarian or Vegan
- History or presence of cancer in the prior two years, except for non-melanoma skin cancer.
- Individual has a condition the Investigator believes would interfere with his or her ability to provide informed consent, comply with the study protocol, which might confound the interpretation of the study results or put the person at undue risk

**Table 1 Inclusion–exclusion criteria** *(Continued)*

- Untreated or unstable hypothyroidism, an active eating disorder, or evidence of any neurological disorders
- Recent history of (within 12 months) or strong potential for alcohol or substance abuse
- Females who are pregnant, lactating, or unwilling to use adequate contraception during the study

### Knee range of motion measurements

Knee extension was measured by goniometry. Briefly, subjects were instructed to sit in an upright position on a table edge with their backs straight (knee position defined as 90°). The axis of a goniometer was placed at the intersection of the thigh and shank at the knee joint. Subjects were asked to bring their knees to full extension without changing the position of the pelvis and lumbar spine. The extended knee joint angle was measured and recorded. For knee flexion measurement, subjects were asked to actively flex their knees while lying in a prone position with their shins off the end of the table. The range of knee flexion motion was then measured and documented.

### Timed joint discomfort measurements

Briefly, a stopwatch was started when subjects began climbing the stepmill. Time to onset of pain was recorded at the first sign of pain in the target knee. The baselines at each time point were normalized to account for dropouts. Percent change in time to complete recovery from pain was measured as follows: a new stopwatch was started when the subjects disembarked from the stepmill and the time to complete recovery from pain was recorded. The baselines at each time point were normalized to account for dropouts then compared against the reference interval which was defined as the percentage change between the study baseline and day 7.

### KOOS knee survey & Stanford exercise scales

The KOOS survey is a validated instrument consisting of 42 questions that are classified into sub-scales such as symptoms, stiffness, pain, daily activities, recreational activities and quality of life [34]. It measures the subjects' opinion about their knees and their ability to perform daily activities during the past week. The Stanford exercise behavior scale comprises 6 questions designed to assess exercise behaviors during the previous week [35].

### Six minute timed walk

Subjects were instructed to walk up and down a hallway for 6 minutes as rapidly as possible without causing any pain. A measuring wheel (RoadRunner Wheel, Keson Industries, Aurora, IL) was used to measure distance travelled in 6 minutes.

**Table 2 Protocol summary**

| Protocol activities | V1 Day −7 Screen | V2 Day 0 Baseline | V3 Day 7 | V4 Day 30 | V5 Day 60 | V6 Day 90 | V7 Day 120 End |
|---|---|---|---|---|---|---|---|
| Informed consent | x | | | | | | |
| Inclusion/Exclusion | x | | | | | | |
| Medical history and physical exam | x | | | | | | |
| Vital signs/anthropometric measures | x | x | x | x | x | x | x |
| Urine pregnancy test | x | x | | | | | |
| Administer and review scales/questionnaires/diaries | x | x | x | x | x | x | x |
| Stressor (Udani Stepmill protocol) | x | x | x | x | x | x | x |
| Functional measures (6-min timed walk) | x | x | x | x | x | x | x |
| Goniometry (range of motion) | | x | x | x | x | x | x |
| Review concomitant therapies | x | x | x | x | x | x | x |
| Intercurrent medical issues review | | x | x | x | x | x | x |
| Compliance assessment (including phone calls) | | x | x | x | x | x | x |
| Randomization | | x | | | | | |
| Study supplement preparation & dispensing | | x | x | x | x | x | |

### Rescue medication

No rescue medications were allowed during the course of the study. At all study visits, subjects were given a list of the 43 prohibited medications and supplements (Table 3). Changes in overall medication history, or the use of these substances, were then recorded by the study coordinator. Subjects found to have used any of these prohibited substances were excluded from further participation in the study as per protocol.

### Statistics

Outcome variables were assessed for conformance to the normal distribution and transformed as required. Within group significance was analyzed by non-parametric Sign

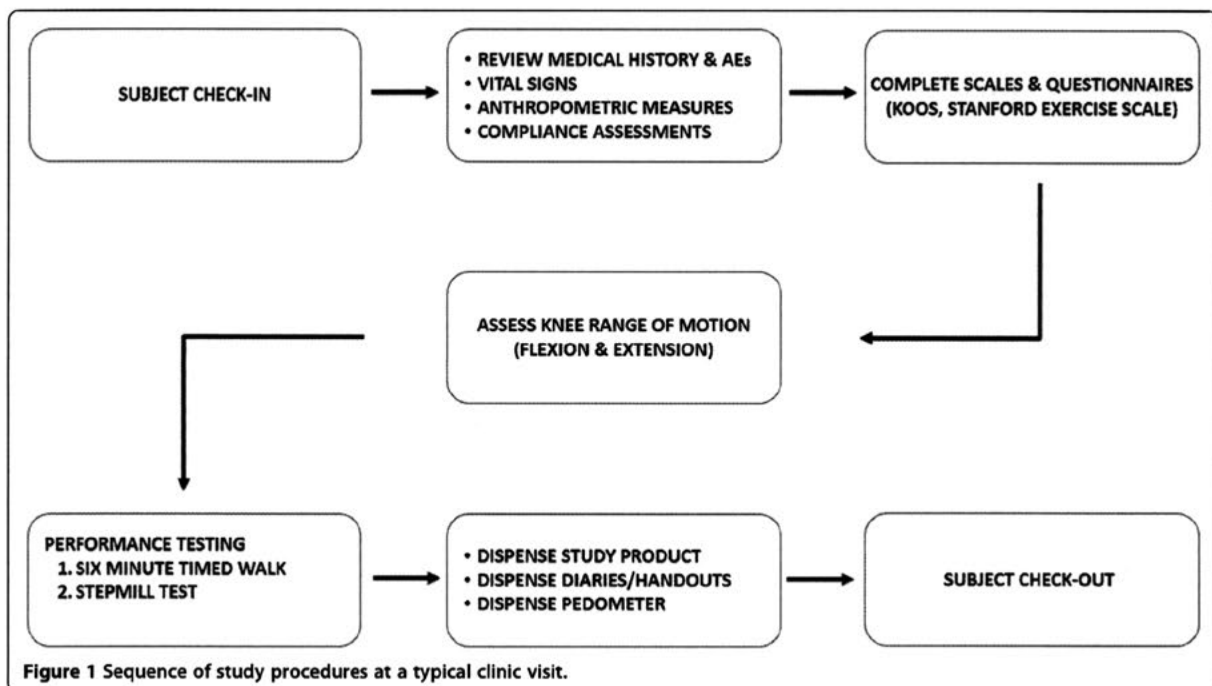


**Figure 1 Sequence of study procedures at a typical clinic visit.**

**Table 3 Representative list of prohibited medications* by category**

| Category | Medications |
|---|---|
| Joint supplements (Omega-3, Omega-6 plus others) | Alpha-Linolenic acid |
| | Docosapentaenoic acid |
| | Docosahexaenoic acid |
| | Eicosatrienoic acid |
| | Eicosatetraenoic acid |
| | Eicosapentaenoic acid |
| | Hexadecatrienoic acid |
| | Heneicosapentaenoic acid |
| | Stearidonic acid |
| | Tetracosapentaenoic acid |
| | Tetracosahexaenoic acid |
| | Glucosamine (all forms) |
| | Chondroitin (all forms) |
| | Other herbal ingredients |
| NSAIDs (OTC and prescription) | Aspirin |
| | Diflunisal |
| | Diclofenac |
| | Celecoxib |
| | Etodolac |
| | Fenoprofen |
| | Flurbiprofen |
| | Ibuprofen |
| | Indomethacin |
| | Ketoprofen |
| | Meclofenamate |
| | Mefenamic acid |
| | Meloxicam |
| | Nabumetone |
| | Naproxen |
| | Oxaprozin |
| | Piroxicam |
| | Rofecoxib |
| | Sulindac |
| | Tolmetin |
| | Valdecoxib |

*Selected from a list of 43 prohibited medications and supplements.

test or by non-parametric Wilcoxon Signed Rank test, while Wilcoxon Mann–Whitney test was used to analyze between groups significance. The Fisher Exact test was used to evaluate the complete loss of pain between study cohorts whereas the binomial test was used to assess the likelihood of complete loss of pain at each visit. P-values equal to or less than 0.05 were considered statistically significant. All analyses were done on a per protocol basis using SPSS, v19 (IBM, Armonk, NY). Results were presented as mean ± SEM.

## Results

### Baseline demographics

A total of 55 individuals met the eligibility criteria and were randomized to the placebo (n = 28) or to the UC-II (n = 27) group. Baseline demographic characteristics for subjects in both groups were similar with respect to age, gender, height, weight and BMI (Table 4). A total of nine subjects, three in UC-II group and six in placebo group, were lost to follow-up. The results presented herein encompass 46 total subjects, 22 subjects in the placebo group plus 24 subjects in the UC-II group. It should be noted that the average age of the study participants was approximately 46 years which is about 16 years younger than the average age observed in many OA studies [36-38].

### Knee extension and flexion

Figure 2 summarizes the average knee extension changes over time for subjects supplemented with either UC-II or placebo. The UC-II supplemented cohort presented with a statistically significant greater increase in the ability to extend the knee at day 120 as compared to the placebo group (81.0 ± 1.3° vs 74.0 ± 2.2°, $p = 0.011$) and to baseline (81.0 ± 1.3° vs 73.2 ± 1.9°, $p = 0.002$). The UC-II group also demonstrated a significant increase in knee extension at day 90 (78.8 ± 1.9° vs 73.2 ± 1.9°, $p = 0.045$) compared to baseline only. An intent to treat (ITT) analysis of these data also demonstrated a statistically significant net increase in knee extension at day 120 versus placebo (80.0 ± 1.3° vs 73.7 ± 1.8°, $p = 0.006$). No statistically significant changes were observed in the placebo group at any time during this study. With respect to knee flexion, no significant changes were noted in either study group ($p > 0.05$). The power associated with the former per protocol statistical analyses was 80%.

### Time to onset of initial joint pain

Supplementation with UC-II resulted in statistically significant increases in the time to onset of initial joint pain at day 90 (2.75 ± 0.5 min, $p = 0.041$) and at day 120 (2.8 ± 0.5 min, $p = 0.019$) versus a baseline of 1.4 min for each visit. No statistically significant differences were noted for either the placebo group or between groups (Figure 3).

Five individuals in the UC-II group and one in the placebo group reported no onset of pain by the end of study (see below and Table 5). Given this unexpected finding, an additional analysis was undertaken which included these individuals in the time to onset of initial pain analysis. The 10 minute limit of the stepmill procedure was used as the lower limit to pain onset. Under

**Table 4 Demographic and baseline characteristics of enrolled subjects**

| Characteristics | UC-II | Placebo |
|---|---|---|
| Total number of subjects | 27 | 28 |
| Number of males | 11 | 12 |
| Number of females | 16 | 16 |
| Age (years) | 46.1 ± 1.5 | 46.6 ± 1.8 |
| Weight (kg) | 75.5 ± 2.9 | 77.5 ± 3.1 |
| Height (cm) | 167.1 ± 2.0 | 168.4 ± 2.0 |
| BMI (kg/m$^2$) | 26.8 ± 0.8 | 27.1 ± 0.7 |

Values are expressed as Mean ± SEM.

these conservative assumptions, supplementation with UC-II yielded statistically significant increases in time to onset of pain at day 90 (3.65 ± 0.7 min, $p = 0.011$) and day 120 (4.31 ± 0.7 min, $p = 0.002$) versus a baseline of 1.4 min for each visit. The between-group comparison at day 120 approached the statistical level of significance favoring the UC-II cohort ($p = 0.051$).

#### Time to onset of maximum joint pain

A statistically significant difference between groups was noted at day 60 (6.39 ± 0.5 min vs 4.78 ± 0.5 min; $p = 0.025$) favoring the UC-II cohort. This significance did not persist during the remainder of the study suggesting that this was a random occurrence.

#### Time to initial improvement in knee joint pain

The time to offset of joint pain was recorded immediately upon the subject stepping off the stepmill. Both groups began to recover from pain with the same rate resulting in no significant differences between groups in the time to initial offset of joint pain ($p > 0.05$).

#### Time to complete recovery from knee joint pain

The time to complete recovery from joint pain showed significant reductions at days 60, 90 and 120 compared to baseline for both the UC-II group as well as the placebo group (Figure 4). Percent changes in times were calculated after normalizing the baselines against the reference range of baseline to day 7. The UC-II group exhibited average reductions of 31.9 ± 11.7% ($p = 0.041$), 51.1 ± 6.1% ($p = 0.004$) and 51.9 ± 6.0% ($p = 0.011$) at days 60, 90 and 120, respectively. By contrast, the reductions for the same time points for the placebo cohort, 21.9 ± 10.2% ($p = 0.017$), 22.2 ± 15.5% ($p = 0.007$) and 30.0 ± 11.8% ($p = 0.012$), were of lower magnitude but nonetheless statistically significant versus baseline. None of these between group differences achieved statistical significance.

#### Time to complete loss of knee joint pain

During the course of this study it was noted that a number of subjects in both the placebo and the supplemented cohorts no longer reported any pain during the stepmill protocol. For the UC-II group, 5 subjects (21%) no longer reported pain by day 120, whereas only 1 subject (5%) in placebo group reported complete loss of pain (Table 5). This effect did not reach statistical significance between groups but there was an evident trend in the data towards a greater number of subjects losing pain in the UC-II cohort ($p = 0.126$). A binomial analysis for complete loss of pain at each visit demonstrated a statistical significance for the UC-II group by day 120 ($p = 0.031$). It is important to note that the complete loss of knee pain was not a random event. The pattern among the subjects indicates that loss of knee pain appeared to be a persistent phenomenon that spanned multiple visits (Table 5). A detailed review of the clinical report forms showed that none of these individuals consumed pain relief medication prior to their visits.

#### Six-minute timed walk & Daily number of steps

No significant differences were observed between the study groups for the six-minute time walk or the daily number of steps taken ($p > 0.05$). The distance walked in six-minutes by the UC-II (range = 505 to 522 meters) and the placebo (range = 461 to 502 meters) groups were within the reference range previously reported [39] for healthy adults (399 to 778 meters, males; 310 to 664 meters, females). Similarly, the average step length calculated from Fitbit data for both study groups (0.69 to 0.71 meters) also agreed with previously published results for normal adults [40].

#### KOOS knee survey & Stanford exercise scales

No significant differences were seen between the study groups for either the KOOS survey or the Stanford exercise scale ($p > 0.05$).

#### Use of analgesics and NSAIDs

Review of the clinical report forms showed that no subject in either study cohort consumed any of the 43 prohibited medicines or supplements during the study.

#### Safety assessments

A total of eight adverse events, equally dispersed between both groups, were noted (Table 6). None of the adverse events was considered to be associated with UC-II supplementation. All events resolved spontaneously without the need for further intervention. No subject withdrew from the study due to an adverse event. Finally, no differences were observed in vital signs after

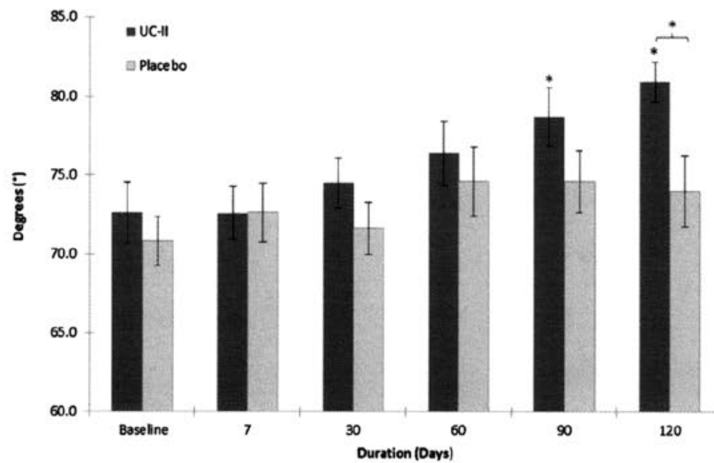


**Figure 2 Knee extension as measured by goniometry.** Values are presented as Mean ± SEM. *p ≤ 0.05 indicates a statistically significant difference versus baseline or placebo. Number of completers: n = 24 in UC-II group (n = 3 dropouts); n = 20 in placebo group (n = 6 dropouts; n = 2 did not participate in ROM assessment).

seventeen weeks of supplementation, and no serious adverse events were reported in this study.

## Discussion

In this study, the UC-II supplement, consisting of undenatured type II collagen, was investigated for its ability to improve joint function in healthy subjects who develop joint pain while undergoing strenuous exercise. The rationale behind this approach centered on the hypothesis that strenuous exercise might uncover transient joint changes due to daily physical activities that are not attributable to a diagnosable disease. In the same way that nominally elevated blood levels of lipids, glucose plus high blood pressure and obesity can be predictive of future progression to diabetes and heart disease [41], the development of joint pain upon strenuous exercise may be indicative of possible future joint problems.

At study conclusion, we found that subjects ingesting the UC-II supplement experienced a significantly greater forward ROM in their knees versus baseline and placebo

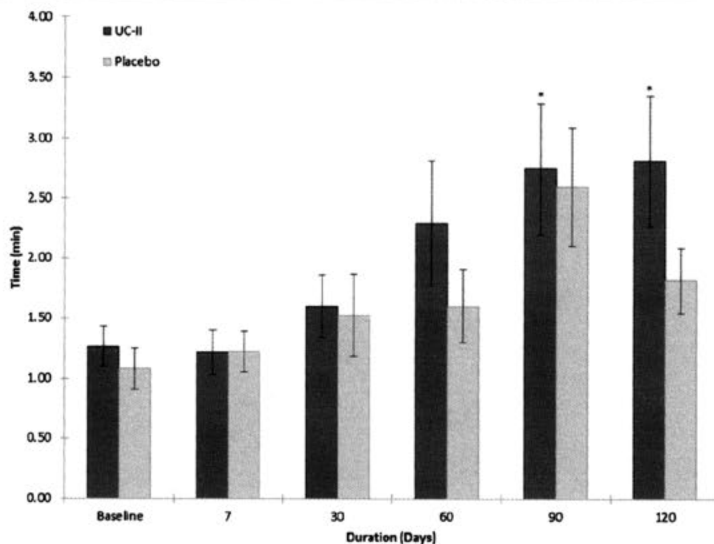


**Figure 3 Impact of stepmill procedure on the onset of pain.** Values are presented as Mean ± SEM. *p ≤ 0.05 indicates a statistically significant difference from baseline. Number of completers: n = 19 in UC-II group (n = 3 dropouts; n = 5 did not have pain); n = 20 in placebo group (n = 6 dropouts; n = 1 did not have pain; n = 1 did not use stepmill).

**Table 5 Subjects reporting complete loss of knee pain on stepmill test**

| Visit | UC-II | | | Placebo | | |
|---|---|---|---|---|---|---|
| | No. of pain free subjects (%) | Continuity of pain loss[#] | P value (Binomial test) | No. of pain free subjects (%) | Continuity of pain loss[#] | P value (Binomial test) |
| Baseline | 0.0 (0) | 0 | NA | 0.0 (0) | 0 | NA |
| Day 7 | 0.0 (0) | 0 | NA | 0.0 (0) | 0 | NA |
| Day 30 | 1.0 (4) | 1N | 0.5 | 0.0 (0) | 0 | NA |
| Day 60 | 3.0 (13) | 1R, 2N | 0.125 | 0.0 (0) | 0 | NA |
| Day 90 | 3.0 (13) | 2R, 1N | 0.125 | 1 (5) | 1N | 0.5 |
| Day 120 | 5.0 (21) | 3R, 2N | 0.031[†] | 1 (5) | 1R | 0.5 |

Values denote number of subjects while parenthesis provides the percent of total subjects who did not have any pain on stepmill. Continuity indicates the number of subjects in whom the absence of pain was maintained across visits. [†]Significant at $p \leq 0.05$ based on independent binomial testing of each visit using the null hypothesis that the probability of a subject experiencing no joint pain is equal to zero. There was no statistical difference between groups. [#]*R* Repeat subject (i.e. same subject who reported no pain in previous visit), *N* New subject who reports no pain for the first time.

as measured by knee extension goniometry. Knee extension is necessary for daily function and sport activities. Loss of knee extension has been shown to negatively impact the function of the lower extremity [42,43]. For example, loss of knee extension can cause altered gait patterns affecting ankles and the hip which could result in difficulty with running and jumping [42,43]. Studies have further shown that a permanent loss of 3-5° of extension can significantly impact patient satisfaction and the development of early arthritis [44].

By contrast, when knee flexion, another measure of knee function, was assessed via goniometry, no differences in clinical outcomes were observed between the two study cohorts. From a structure-function perspective this outcome is not surprising. During the earliest characterized phases of OA there is an apparent preferential loss of knee extension over knee flexion, and this loss has been shown to correlate with WOMAC pain scores [45,46]. In addition, MRI imaging of the early osteoarthritic knee has shown that initial changes in knee structure appear to center on articular cartilage erosions (fibrillations) about the patella and other weight bearing regions of the knee [47]. Such changes might favor a loss in knee ROM that preferentially affects extension over flexion. The pathophysiology of the early osteoarthritic knee, we believe, provides insight regarding the effect of daily physical activities on the healthy knee insofar as it helps explain the discordance in clinical outcomes between knee extension and flexion.

Both the time to onset of initial joint pain as well as time to full recovery were measured in this study. For

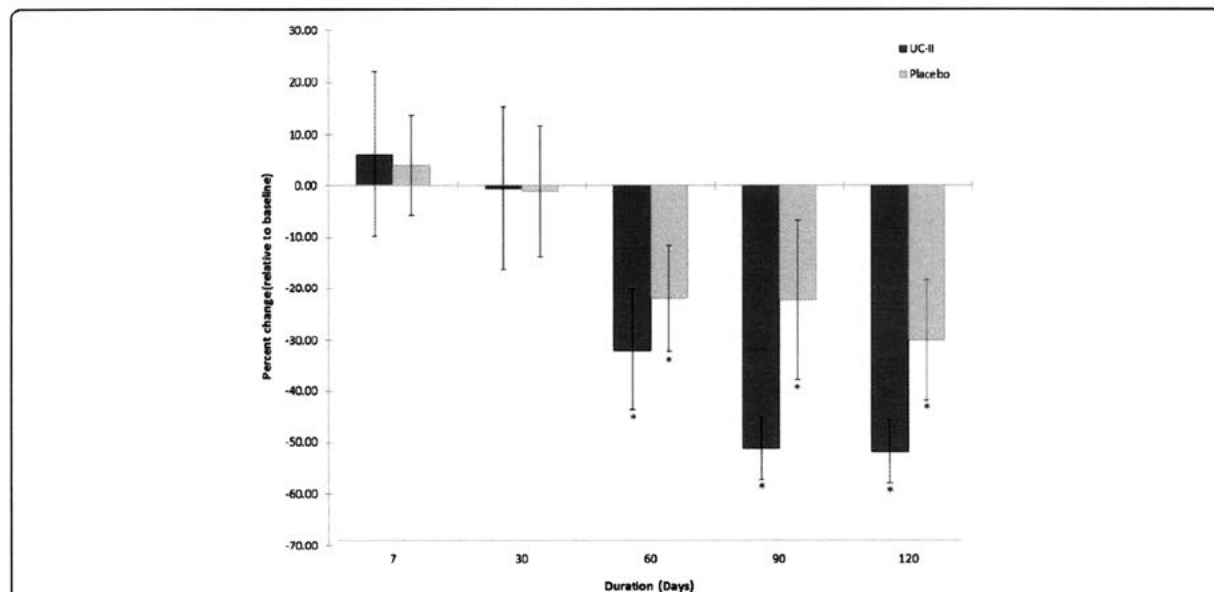


**Figure 4 Percent change in time to complete recovery from pain.** Values are presented as Mean ± SEM. $^{*}p \leq 0.05$ indicates a statistically significant difference from baseline. Number of completers: n = 18 in UC-II group (n = 3 dropouts; n = 5 did not have pain; n = 1 time to complete recovery from pain was not achieved); n = 20 in placebo group (n = 6 dropouts; n = 1 did not have pain; n = 1 did not use stepmill).

**Table 6 Summary of analysis of adverse events (AEs) in all subjects**

| Study groups | Adverse event (Body system) | Number of AEs |
|---|---|---|
| UC-II | Upper respiratory infection (Pulmonary) | 3 |
| | Food poisoning (Gastrointestinal) | 1 |
| **Total number of AEs** | | **4** |
| **Total number of subjects reporting AEs: n** | | **4/27** |
| Placebo | Bilateral ankle edema (Musculoskeletal) | 1 |
| | Right ankle fracture (Musculoskeletal) | 1 |
| | Sinusitis (Ears/Nose/Throat) | 1 |
| | Skin infection right ankle (Dermatological) | 1 |
| **Total number of AEs** | | **4** |
| **Total number of subjects reporting AEs: n** | | **2/28** |

each of these measures the clinical outcomes favored the UC-II supplemented cohort versus their baseline status. The ability of UC-II to modulate knee extension may relate to its ability to moderate knee joint pain. Crowley et al. [26] and Trentham et al. [25] demonstrated that UC-II effectively enhances joint comfort and flexibility thereby improving the quality of life (QoL) in both OA and RA subjects, respectively. This effect may be attributable to the finding that microgram quantities of undenatured type II collagen moderate CIA in both the rat and the mouse via the induction of T regulator cells [27,28,48]. The induction of these T regulators takes place within gut associated lymphatic tissues (GALT), including mesenteric lymph nodes, in response to the consumption of undenatured type II collagen [27]. Studies have shown that these regulatory cells produce IL-10 and TGF-β [30,49]. A special class of $CD103^+$ dendritic cells, found almost exclusively in the GALT, facilitates this process [48,50]. Once activated, T regulator cells appear to downregulate a wide range of immunologic and proinflammatory activities resulting in the moderation of the arthritic response initiated by undenatured type II collagen [27]. The phenomenon of oral tolerance has also been demonstrated in humans, and appears to involve a similar set of T regulators [30,51-53].

The above description of how UC-II might modulate joint function is most easily understood in the context of RA given that the CIA animal model resembles this disease most closely [27,28,54]. However, the case for T regulators and immune cytokines having a moderating effect on healthy or OA knee joint function appears less apparent. This view has changed in recent years due to a growing body of evidence suggesting that both OA and normal chondrocyte biology appears to be regulated by some of the same cytokines and chemokines that regulate inflammation [5,6,55]. For example, Mannelli and coworkers [56] recently reported that feeding microgram amounts of native type II collagen (porcine) prevents monoiodoacetate-induced articular cartilage damage in this rat model of osteoarthritis, as measured by pain thresholds and by circulating levels of cross linked c-telopeptides derived from type II collagen. This finding corroborates the efficacy of undenatured type II collagen in improving joint comfort in osteoarthritic conditions [26].

In the present study, we show for the first time that UC-II can improve joint function in healthy subjects undergoing strenuous physical exercise. This observation, when considered in context with normal chondrocyte physiology, suggests that activated T regulator cells, specific for undenatured type II collagen, home to an overstressed knee joint where their release of the anti-inflammatory cytokines, IL-10 and TGF-β reverse the catabolic changes caused by strenuous exertion [13,21,57]. In addition, the IL-10 and TGF-β produced by these T regulators may tilt the $T_H$ balance in the knee joint towards $T_H2$ [30,58] responses which preferentially result in IL-4 production further fostering a shift in chondrocyte metabolism towards ECM replenishment.

Several additional tests were used in this study to assess overall joint function, QoL, and physical activity. The additional parameters and tests measured included a six minute timed walk plus the Stanford exercise scale and KOOS survey. With respect to the KOOS survey, both cohorts were statistically significant versus baseline for symptoms, pain, daily function, recreational activities and QoL but were not significant from each other. This is not an unexpected finding given that this study was carried out with healthy subjects who do not present with any joint issues at rest. It is only when the knee is stressed via the stepmill do subjects report any joint discomfort. Under these conditions, and as indicated above, the UC-II group appears to experience less joint discomfort and greater joint flexibility. No difference in clinical outcomes between groups was seen in the six minute timed walk, the daily distance walked, or the Stanford exercise scale questionnaire. Once again we are not surprised by these results given that these tests and questionnaires are designed and clinically validated to assess the severity of arthritic disease in unhealthy populations.

No clinical biomarkers associated with arthritic diseases were assessed in this study. Healthy subjects would not be expected to present with significant alterations in their inflammatory biomarker profile as they lack clinical disease [59]. In addition, it should be noted that the joint discomfort measured in this study is acute pain induced by a stressor rather than due to an ongoing inflammatory event. Therefore, any elevation in inflammation markers that might occur in these healthy subjects may simply be due to the physiological impact of strenuous exercise.

There are two study limitations to consider when reviewing these results. The first, time to onset of initial pain, was limited to a 10-minute interval. The current study design did not address the possibility that subjects might cease to experience pain on the stepmill. Future studies should allow for an extension of the exertion interval in order to gauge how much longer a subject can exercise before reporting pain. In this way better defined parameters can be placed upon the degree to which UC-II supplementation results in the cessation of joint pain due to strenuous exercise in healthy subjects.

The second limitation that merits consideration is the possibility that study subjects may have early signs of arthritis that do not meet the ACR criteria. This possible limitation was addressed by performing an extensive medical examination for signs and symptoms of OA and by excluding volunteers who experienced pain levels of 5 or greater within one minute of using the stepmill.

UC-II is a unique ingredient that supports healthy joints. Previous studies have focused on the efficacy of this ingredient in OA subjects. By including healthy subjects in this study, and using non-disease endpoints as a measure of efficacy, it is believed that the benefits that derive from UC-II usage now extends to include healthy individuals. Further, this ingredient appears to be safe for human consumption based on an extensive series of *in vivo* and *in vitro* toxicological studies as well as the absence of any adverse events in this and in previous human studies [24,26,60]. In conclusion, daily supplementation with 40 mg of UC-II supports joint function and flexibility in healthy subjects as demonstrated by greater knee extension and has the potential both to alleviate the joint pain that occasionally arises from strenuous exercise as well as to lengthen periods of pain free exertion.

#### Abbreviations

RA: Rheumatoid arthritis; OA: Osteoarthritis; ECM: Extracellular matrix; TNF-α: Tumor necrosis factor-alpha; IL-1β: Interleukin-1 beta; IL-6: Interleukin-6; IL-4: Interleukin 4; IL-10: Interleukin-10; MMP: Matrix metalloproteinase; NF-κB: Nuclear factor-kappa-light-chain-enhancer of activated B cells; MAPK: Mitogen activated protein kinase; ERK: Extracellular receptor kinase; NO: Nitric oxide; TGF-β: Transforming growth factor-beta; CIA: Collagen induced arthritis; KOOS: Knee injury and osteoarthritis outcome score; ROM: Range of motion; MRI: Magnetic resonance imaging; GALT: Gut associated lymphatic tissue; QoL: Quality of life; MIP-1β: Macrophage inflammatory protein-1 beta; IP-10: Interferon gamma-induced protein 10; $T_H$: T helper cell; WOMAC: Western Ontario and McMaster universities osteoarthritis index; ACR: American College of Rheumatology.

#### Competing interests

Medicus Research received research grants from InterHealth Nutraceuticals, Inc., Benicia, California. Dr. Udani has provided consulting services to InterHealth Nutraceuticals, Inc. Drs. JPL, ZMS, and FCL are employees of InterHealth Nutraceuticals, Inc. Medicus Research does not endorse any brand or product nor does it have any financial interests with any supplement manufacturer or distributor.

#### Authors' contributions

JKU was the principal investigator and together with JPL, JKU, ZMS, FCL JPM, MNP and ANS contributed to the writing, data analyses and data interpretation that are a part of this manuscript. All the authors read and approved the final draft of the manuscript.

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-σημειώσεις-

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