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Original Article

Metabolic Effects of Avocado/Soy Unsaponifiables on Articular Chondrocytes

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Avocado/soy unsaponifiable (ASU) components are reported to have a chondroprotective effect by virtue of anti-inflammatory and proanabolic effects on articular chondrocytes. The identity of the active component(s) remains unknown. In general, sterols, the major component of unsaponifiable plant material have been demonstrated to be anti-inflammatory *in vitro* and in animal models. These studies were designed to clarify whether the sterol content of ASU preparations were the primary contributors to biological activity in articular chondrocytes. ASU samples were analyzed by high pressure liquid chromatography (HPLC) and GC mass spectrometry. The sterol content was normalized between diverse samples prior to *in vitro* testing on bovine chondrocytes. Anabolic activity was monitored by uptake of 35-sulfate into proteoglycans and quantitation of labeled hydroxyproline and proline content after incubation with labeled proline. Anti-inflammatory activity was assayed by measuring reduction of interleukin-1 (IL-1)-induced synthesis of PGE2 and metalloproteases and release of label from tissue prelabeled with S-35.All ASU samples exerted a similar time-dependent up-regulation of 35-sulfate uptake in bovine cells reaching a maximum of greater than 100% after 72 h at sterol doses of 1–10 μg/ml. Non-collagenous protein (NCP) and collagen synthesis were similarly up-regulated. All ASU were equally effective in dose dependently inhibiting IL-1-induced MMP-3 activity (23–37%), labeled sulfate release (15–23%) and PGE2 synthesis (45–58%). Up-regulation of glycosaminoglycan and collagen synthesis and reduction of IL-1 effects in cartilage are consistent with chondroprotective activity. The similarity of activity of ASU from diverse sources when tested at equal sterol levels suggests sterols are important for biologic effects in articular chondrocytes.

Keywords: arthritis avocado unsaponifiables – cartilage – sterols

Introduction

There is a great deal of interest in the use of botanical material in osteoarthritic and rheumatoid arthritic disorders (1). For example, bromelain, an extract from the pineapple plant, demonstrates anti-inflammatory and analgesic properties in clinical osteoarthritis trials (2). Anti-inflammatory activity is also present in the sesquiterpenes from turmeric species (3) and avocado and soybean oils contain a class of biologically active compounds classified as unsaponifiable lipids [avocado/soy unsaponifiables (ASU)] (4). The major components of ASU by weight are the phytosterols beta-sitosterol, campesterol and stigmasterol.

Early studies indicate the primary beneficial action of phytosterols was their ability to inhibit cholesterol absorption and interfere with endogenous cholesterol biosynthesis (5). Phytosterols in general, and beta-sitosterol in particular, are now considered potent anti-inflammatory agents with antioxidant and analgesic activity (6–8). Sterol extracts from different plant sources indicate their widespread distribution, and animal tests show their anti-inflammatory potency (9, 10).

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ASU has also been recommended for treating arthritis with published clinical trials in animals and humans (10–13). For example, oral administration of ASU to meniscectomized sheep for 3–6 months elicited a protective effect on articular cartilage (12). The authors suggested ASU may be considered a symptomatic disease-modifying osteoarthritic compound.

ASUs stimulate aggrecan synthesis while inhibiting stromelysin activity in osteoarthritic chondrocytes (14). The *in vitro* cartilage model generally used to test ASU is based on monitoring reduction of interleukin-1 (IL-1)-induced metalloprotease activity, nitric oxide or eicosanoid synthesis (all agents promoting cartilage degradation and tissue inflammation) (15). The validity of the model generally is accepted, as elevated levels of IL-1 are believed to be involved in initiating cartilage degradation (16). *In vitro* testing of ASU on articular cartilage showed a 2 : 1 soy/avocado combination was more effective than the unsaponifiable fraction of avocado or soy used separately (17). Fibroblasts also appear responsive to ASU as the metalloproteases MMP-2 and MMP-3 are inhibited at low doses, while the tissue inhibitors of metalloproteases are increased at higher doses (14). Other indices tested relate to a possible increased capacity for repair and regeneration of articular cartilage. For example, the anticatabolic activity of ASU is paired with a direct effect of ASU on stimulating collagen and proteoglycan production, possibly by increasing transforming growth factor beta synthesis (18). ASU is generally a complex mixture of many compounds including fat-soluble vitamins, sterols, triterpene alcohols and possibly furan fatty acids. Therefore, the identity of the active agent(s) remains uncertain.

The objectives of this investigation were (i) to identify the sterol composition of unsaponifiable material in ASU from diverse sources; (ii) test the influence these materials have on up-regulation of glycosaminoglycan and collagen synthesis of bovine chondrocytes *in vitro* and (iii) similarly test this material for anticatabolic/anti-inflammatory activity in an IL-1-induced *in vitro* model of articular cartilage breakdown. Prior to testing, all ASU material was normalized to identical sterol content. This approach allowed testing of the hypothesis that the biological activities of ASU from diverse sources are comparable and may be dependent on sterol content.

Methods

Assays of anabolic and anticatabolic activity in chondrocytes exposed to various 2 : 1 soy/avocado ASU formulations. We standardized the sterol content of each sample before testing and in some studies, compared the data with that for purified beta-sitosterol. Before testing, the compositional profile of each preparation was analyzed by high pressure liquid chromatography (HPLC) (19) and gas chromatography (GC) mass spectrometry.

We tested 2 : 1 formulations of soy/avocado ASU obtained from several sources. Formula 1 was NMX-1000™ obtained from Nutramax Laboratories (Edgewood, MD). Formula 2 consisted of two-part soy unsaponifiables (Cargill, Minneapolis, MN) and one-part avocado unsaponifiables (Croda, Yorkshire, England). Additional materials tested include a commercially available researched preparation (Laboratories Pharmascience, Courbevoie, France) and a sample of beta-sitosterol (95% pure; Sigma-Aldrich, St Louis, MO). All samples were used after 100% ethanol extraction/dissolution of the product using continuous shaking at 50°C for 60 min and dilution to a final sterol concentration of 10 mg/ml.

Chromatographic Procedures

HPLC separation was achieved by applying 600 µg total weight of material onto a Luna C-18, 250 × 4.6 mm, 5 µm reverse-phase column (Phenomenex, Torrance, CA) with samples at 40°C and the column at room temperature. The flow rate was 1 ml/min with acetonitrile/methanol (50 : 50, v/v) containing 3% water as the mobile phase. Peaks were monitored at 210 nm and ASU components were identified by comparison with authentic standards when possible. The concentrations of the components stigmasterol, beta-sitosterol and campesterol were determined by calculating peak area ratios against an internal standard with known concentrations of the analytes.

GC analysis was performed on an Agilent 6890 System (Palo Alto, CA) equipped with a split/splitless injector port and a flame ionization detector. The separation of phytosterols was accomplished on a Restek XTI-5 column (30 m × 0.32 mm × 0.25 µm) with the injector and detector temperatures maintained at 330°C; column temperature program was started at 250°C and increased to 330°C at a rate of 10°/min and held at 330°C for 5 min. We injected 1 µl with a split ratio of 15 : 1. Positive identification of the analytes was confirmed by mass spectroscopy and comparison of retention times with authentic samples.

In vitro Culture Studies

For *in vitro* metabolic synthesis studies, articular chondrocytes obtained by collagenase digestion of the metacarpal joint of retired, aged Holstein cows (viability by trypan blue ≥95%) were plated into 48 well multicultured plates at 100 000 cells/well and incubated in DMEM/F-12 (Cambrex, East Rutherford, NJ.) plus 10% fetal calf serum, 50 µg/ml ascorbic acid, penicillin G (100 000 U/l), streptomycin (100 000 µg/l) and amphotericin B (250 µg/l) for 7–10 days to acclimate the cells. Since chondrocyte synthetic activity is influenced by the glucose content of the medium and normal culture

medium glucose can be as high as 17 mmol/l, we formulated DMEM/F-12 medium to contain 5 mmol/l glucose to mimic *in vivo* serum concentrations (20). The rationale for this was the low synovial fluid glucose levels in a canine model of osteoarthritis (21) and the association with inflammatory joint disease (22). To more closely mimic *in vivo* conditions, all cell cultures subsequently were acclimated for 24 h to a modified DMEM/F-12 medium containing physiologic levels of glucose (5 mmol/l) plus 1% fetal calf serum. All testing was performed in this same medium. For comparison purposes, all ASU preparations were diluted to yield identical sterol concentrations (each containing $< 0.1\%$ ethanol). Cultures were exposed for 24 or 72 h to the various unsaponifiable preparations at doses of 0.1–10 and 0.1–25 μg/ml, respectively, based on sterol content. Radiolabeled sulfate at 5 μCi/ml was added during the terminal 6 h of incubation. For the long-term exposure study and for the collagen analysis, we tested only the commercial preparation and Formula 1. To assess the long-term effects, replicate cultures were similarly exposed to ASU for 10 days with media changes every 2 days. Increases in GAG (glycosaminoglycan) synthetic activity was determined by assaying GAG using 1,9-Dimethylmethylene blue reagent (23) and monitoring CPM 35-sulfate uptake/culture. No attempt was made to characterize the distribution of label into the various classes of proteoglycans and the data was analyzed with one way analysis of variance (ANOVA).

Collagen Synthesis

We analyzed collagen synthesis by comparing the effect of the commercial ASU and Formula 1 ASU normalized to contain equal sterol levels. Collagenase-sensitive material was assayed according using the method of Diegelmann *et al.* (24). Briefly, chondrocytes cultured in a 24 well plate at a density of 200 000 cells/well with DMEM-F-12 containing 5 mmol/l glucose and 1% fetal calf serum were exposed to 1, 5 and 10 μg/ml of each preparation for 72 h. Media were changed daily and 5 μCi/ml tritiated proline was added for the last 24 h. Cultures were terminated by addition of concentrated trichloroacetic acid (TCA) to 10% at 5°C. The precipitate was repeatedly washed with 10% TCA with a last rinse of a 1 : 1 solution of diethylether and methanol. Collagen in TCA-precipitated, air-dried plates was digested by adding an incubation cocktail containing 25 μg purified collagenase (CLSPA; Worthington Biochemical Corp, Lakewood, NJ) in 200 μl 0.05 mol/l Tris (pH 7.6) and 0.005 mol/l $CaCl_2$. The plates were incubated for 3–6 h at 37°C. Radioactivity in the supernatant (collagen fraction) and TCA-precipitated pellet (NCP) were counted in multiplate wells after adding 300 μl scintillant (Hewlett-Packard, Houston, TX) to 100 μl sample.

Anticatabolic Studies

All ASU preparations were tested for anticatabolic activity at sterol doses of 10, 5, 1 and 0.1 μg/ml. Chondrocytes plated into 48 well multicultured plates (100 000 cells/well) were prelabeled with 35-sulfate for 72 h in DMEM/F-12 plus 10% fetal calf serum. Unincorporated label was washed out by preincubation for 24 h while the cells were acclimated to DMEM/F-12 containing 1% fetal calf serum and 5 mmol/l glucose before adding the agents. All agents were added in a total volume of 0.5 ml and released activity monitored after 24 h. Additionally, 100 μl aliquots of media were removed for analysis of general metalloprotease activity using the OmniMMP™ fluorescence substrate (Mca-PLGDpaAR) (Biomol International, Plymouth Meeting, PA). Total enzyme activity was assayed after activating latent enzymes with trypsin and reading the reaction in 96 well culture wells in kinetic mode on a Molecular Devices Spectra Max Fluorescence reader (Sunnyvale, CA). In effect, the sulfate release assay was a measure of active enzyme activity, while the fluorescence MMP assay reflected the total enzyme content.

Anti-inflammatory Assays

Six millimeter diameter bovine cartilage explants from normal metacarpal joints of young steers (15 months) were conditioned to a metabolic steady state in DMEM/F-12–10% fetal bovine serum and penicillin/streptomycin for 6–10 days before use. Cartilage explants were transferred to a 48 well culture plate with two explants per well. Each well contained 1 ml of DMEM-F12 + 10% fetal bovine serum with penicillin/streptomycin and 0, 0.001, 0.01, 0.1 and 1 μg/ml of Formula 1 or the commercially available ASU for 72 h before adding 5 ng/ml of recombinant human IL-1 beta (R&D Systems, Minneapolis, MN) and monitoring prostaglandin E_2 (PGE_2) levels after 24 h. The PGE_2 levels from the media samples were analyzed using the R&D Systems PGE_2 kit. We performed all experiments in duplicate using different animals. An estimation of the toxic effect of a 5-day exposure of chondrocytes to ASU also was assessed using Molecular Bioprobes Fluorescent Live/Dead Cell Kit (Invitrogen, Chicago, IL).

We calculated the mean and standard error of the mean for each sample group. For the isotopic uptake studies and collagen analysis, means were compared using the unpaired Student's *t*-test. For assays of GAG-specific activity, one-way analysis of variance (ANOVA) and the student Newman-Keul's multiple comparison test for significance was used. Statistical significance was accepted at $P < 0.05$. All experiments were performed in triplicate and representative data included in the manuscript.

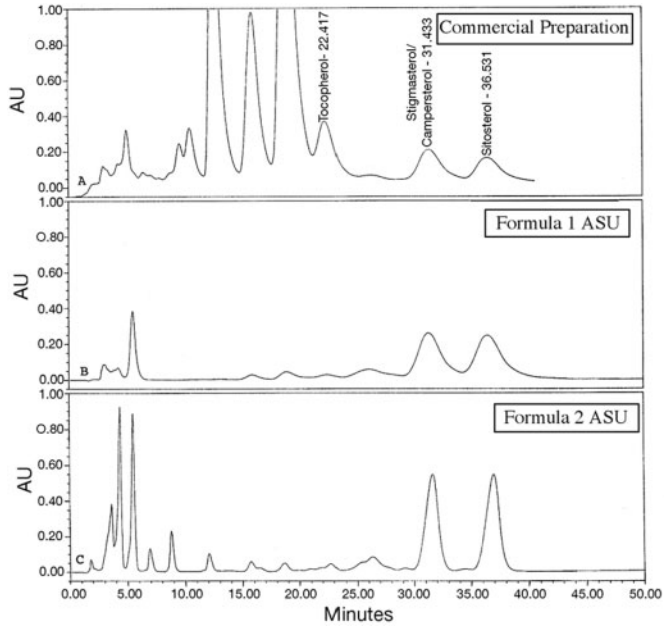

Figure 1. (**A**) The HPLC chromatogram of the commercial preparation is characterized by the presence of multiple unidentified peaks not present in the other preparations. GC mass spectrophotometry analysis was suggestive only of long-chain hydrocarbons. (**B**) The chromatogram of Formula 1 shows predominantly known sterols. (**C**) The chromatogram of Formula 2 also shows predominantly known sterols with trace amounts of unidentified compounds corresponding to the commercial preparation.

Results

Chromatographic Analysis

Slightly varying levels of the three primary sterols were present in all preparations (Fig. 1) with the commercial preparation containing three additional major unidentified peaks (Fig. 1A). Based on area, Formulas 1 and 2 ASU had similar HPLC profiles (Fig. 1B and C, respectively), but only 1.5% of the total chromatographic area corresponded to the three unknown peaks compared to the 80% seen in the commercial preparation.

Gas chromatographic analysis revealed the total sterol content of the commercial preparation, Formula 1, and components of Formula 2 were 34%, 30%, 60% (soy) and 70.8% (avocado) by weight, respectively (Fig. 2). Mass spectra data confirmed the sterol identity in all samples and additionally identified several major and some minor components of each sample (Table 1).

Metabolic Analysis

Short-term Exposure

Twenty-four hour exposure of chondrocytes to ASU resulted in a stimulation ($P = 0.05$) at all doses tested (Fig. 3) with no pronounced dose response or any difference between the different ASU preparations.

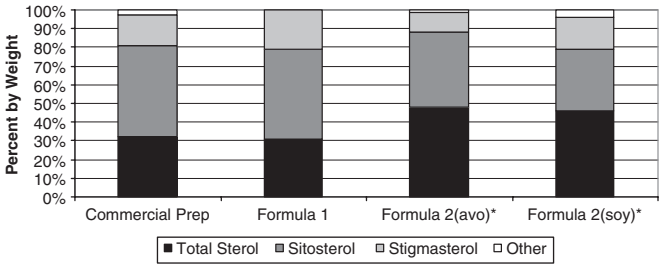

Figure 2. Gas chromatograph of major sterol components of tested samples. Asterisk indicates values supplied by manufacturer.

In the one instance where tested, beta-sitosterol activity was slightly less than the other preparations. However, exposure to ASU for 72 h showed a trend toward an increase ($P < 0.01$) in positive dose response (Fig. 4). There was little difference between preparations except at greater than 6.25 μg/ml sterol doses where the increase in synthetic activity varied slightly between samples.

Long-term Exposure

Insulin-like growth factor (IGF-1) was used as a positive control since it exerts a stimulatory effect on chondrocytes (25). Continuous exposure of cells to 50 ng/ml (IGF-1) or 1 μg/ml Formula 1 for 10 days had a chronic stimulatory effect on glycosaminoglycan synthesis. When measured as CPM 35-sulfate/culture, the level of synthesis of GAG over the time of exposure to isotope, increases of 233% (±22%) and 148% (±32%) ($P < 0.05$) for IGF-1 and Formula 1, respectively were seen compared to controls (data not graphed).

Collagen Synthesis

All ASU agents' up-regulated collagen and NCP synthesis with a dose-dependent stimulation apparent between doses of 1 and 10 μg/ml (Fig. 5). The effect on NCP synthesis was greater than ($P < 0.05$) collagen synthesis by at least 2-fold. At 10 μg/ml, the commercial ASU stimulated NCP by 58% and collagen by 30% ($P < 0.05$). NCP and collagen synthesis were equally up-regulated by 82% with 10 μg/ml Formula 1 ASU ($P < 0.01$) (Fig. 3).

Anti-inflammatory Activity

Assays of anti-inflammatory/anticatabolic activity including a fluorogenic assay of MMP levels (after trypsin activation) varied although there was a little evidence of a dose-dependent response with any preparation at the doses tested. The commercial preparation and Formula 1 had a maximum inhibition of IL-1-induced MMP of 35–37% associated with a dose of 10 μg/ml and a 18–39% reduction at 0.1 μg/ml, the lowest dose tested. In contrast, Formula 2 was one-third as active (Table 2). The MMP assay may reflect total enzyme content after IL-1 exposure (depending on whether

Table 1. Positive mass spectra identification of unsaponifiable lipid preparations

Components	Commercial preparation	Formula 1	Formula 2 (soy unsaponifiables)	Formula 2 (avocado unsaponifiables)
Major components				
	C20H30O2 (?)	Sitosterol	Sitosterol	Sitosterol
	C20H28O2 (?)	Stigmasterol	Stigmasterol	Stigmasterol
	Sitosterol	Campesterol	Campesterol	Campesterol
	Stigmasterol		Nonacosane	
	Campesterol		Ergost-5-en-3-ol	
	Squalene			
	Beta tocopherol			
	Des-methyl tocopherol			
Minor components				
	Oleic Acid	Ergostenol	Oleic acid	na
	Docosane	Hexadecanoic Acid	Stigmast-4-en-3-one	
	Alpha Amyrin	Heptacosane		
	Cholesterol	Nonacosane		
		Tocopherol		
		Stigmast-7-en-3-ol		

na, analysis not performed.

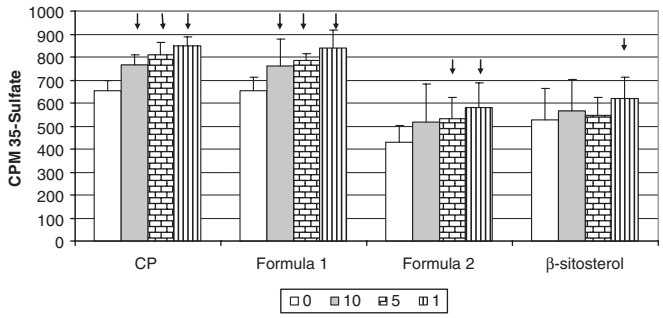

Figure 3. Comparison of 35-sulfate incorporation into glycosaminoglycans of bovine chondrocytes exposed to various ASU preparations for 24 h under conditions of equal sterol content. Values given as mean CPM (±standard deviation). Arrows indicate doses which differed significantly from control at $P < 0.05$ or better.

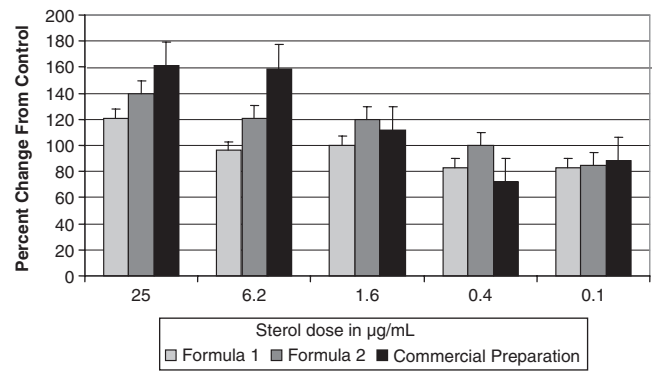

Figure 4. Up-regulation of 35-sulfate incorporation in bovine chondrocytes exposed to ASU for 72 h. Data are plotted as the percent change from control ± SEM. All values are statistically different from control at $P < 0.05$.

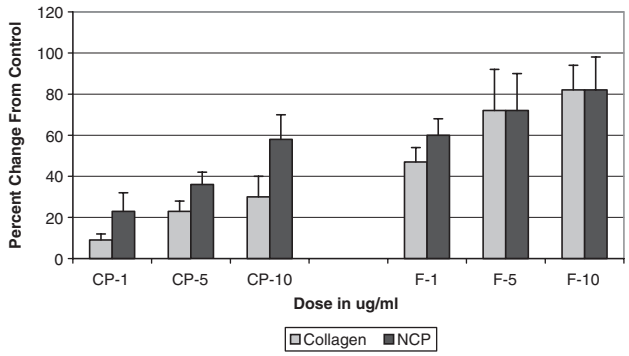

Figure 5. Graph demonstrating the dose-dependent effect of ASU preparations (µg/ml sterol) on collagen and NCP synthesis by bovine chondrocytes. Data are plotted as percent change from control ± SEM. CP, commercial product; F, Formula 1. All values are significantly different from control at $P < 0.05$ with CP effect on NCP 2-fold greater than CP effect on collagen ($P < 0.05$).

IL-1-treated cultures release aggrecan fragments as well as intact molecules). The release of 35-sulfate from prelabeled cultures more accurately reflected enzyme activity. There was no dose response, but all three preparations of ASU-inhibited sulfate release at doses of 1–10 µg/ml, but not at 0.1 µg/ml. At the time period tested, (24 h) prostaglandin E_2 synthesis, elevated by 860% after IL-1 exposure, was inhibited by 45 to 54% with the commercial ASU and Formula 1 ASU at doses of 0.01, 0.1 and 1 µg/ml, but not at 0.001 µg/ml, the lowest dose tested. Formula 2 was not tested. There was no apparent dose response in this assay.

Table 2. Dose-dependent antiinflammatory/anticatabolic effects of ASU on IL-1 treated chondrocytes: comparative analysis of ASU preparations

Sample Dose (μg/ml)	MMP activity	% of Total CPM 35-Sulfate released 24 h after IL-1 exposure	24 h PGE_2 synthesis	Change from IL-1 (%)
Commercial preparation				
Control	51.3 (3.6)	15% (4%)	104 (12)	
IL-1	110.9 (5.1)	25% (8%)[b]	999 (240)	
10	72.1 (3.4)[a]	14% (9%)	na	na
5	82.1 (3.2)[a]	12% (9%)	na	na
1	86.7 (2.2)[a]	16% (8%)	512 (76)[a]	−49
0.1	92.1 (4)[a]	27% (8%)[b]	547 (70)[a]	−45
0.01	–	–	472 (49)[a]	−53
0.001	–	–	849 (76)	−15
Formula 1 (ASU)				
Control	48 (1.4)	10% (3%)	104 (12)	
IL-1	105.3 (5.4)[a]	23% (9%)[b]	999 (240)	
10	66.3 (2.1)[a]	7% (3%)	na	na
5	66.8 (2.1)[a]	12% (7%)	na	na
1	70.8 (0.5)[a]	14% (6%)	459 (67)[a]	−54
0.1	64.7 (3)[a]	26% (9%)[b]	507 (76)[a]	−50
0.01	–	–	417 (26)[a]	−58
0.001	–	–	725 (79)	−37
Formula 2 (ASU)				
Control	38.5 (2.5)	16% (5%)	na	
IL-1	98 (3)	30% (11%)[b]	na	
10	88.2 (5.2)	14% (5%)	na	
5	88 (2)[a]	16% (8%)	na	
1	82.7 (3.2)[a]	15% (7%)	na	
0.1	85.5 (5.8)	26% (5%)[b]	na	

Values are the means ± SEM; ASU, avocado/soy unsaponifiables; [a]significant reduction of IL-1 induced increase in MMP or PGE2 expressed as pM/min/ml substrate conversion ($n = 4$) and pg PGE2/10 mg tissue ($n = 8$), respectively; SO_4 release expressed as% of total CPM incorporated released over 24 h ($n = 8$); [b]values significantly different from control (minus IL-1) cultures; na, not analyzed.

Finally, there was no decline in viability after exposing cells to 1 or 10 μg/ml with any ASU preparation.

Discussion

Three different ASU preparations equalized to contain equivalent sterol content had similar metabolic effects on articular chondrocytes. Chondrocyte response was monitored *in vitro* under conditions of physiologic glucose levels to define the role of ASU as an asymptomatic disease-modifying osteoarthritic agent. There are limitations of our study with regard to interpreting the effects of ASU on osteoarthritic joints. One is the lack of definitive identification of the various minor components of ASU. Although sterols constitute a major fraction of ASU, other components including alpha- and beta-amyrin also are considered to have anti-inflammatory and possible anabolic effects on tissue metabolism (9,26). We did not assay for these components and although our data suggest sterols as the major component with biologic activity, the difference between preparations with regard to MMP content suggest these other agents may also be active. When beta-sitosterol was tested at similar doses, slightly lesser activity was noted, suggesting perhaps an interaction between the various ASU components that promotes greater responses. The available clinical evidence suggests symptomatic relief in patients with osteoarthritis which relates more to ASU's anti-inflammatory activity rather than the up-regulation of cartilage matrix synthesis (11).

Our results mimic those of a previously published report on ASU (15). However, our doses are expressed as μg/ml sterol rather than total weight ASU. In that report, Henrotin *et al.* (15). used doses of ASU by weight, ranging from 0.1 to 40 μg/ml, with greater activity seen at the higher concentrations. Considering their preparation contained approximately 34% sterol content (based on our analysis of the commercial product), the actual doses of sterol were 0.03–13.3 μg/ml, which was within the range of our tests. Also, their data applied to cells cultured in an alginate system and measurement of accumulated matrix for a 12-day exposure period. Our data on short-term 24 h exposure did not show a pronounced dose response, but longer-term exposure of 72 h showed a positive direct-dose response with maximum activity at 25 μg/ml sterol (75 μg/ml ASU), the highest dose tested. Our extended 10-day exposure also resulted in a significant increase of glycosaminoglycan synthesis.

Our findings suggest a relationship between ASU content and biologic activity at 0.1–25 μg/ml sterol levels, with some variation in chondrocyte synthetic response depending on time of exposure. Regarding anti-inflammatory indices, we observed maximum inhibition of prostaglandin synthesis, metalloprotease activity and release of radiolabeled sulfate from prelabeled cartilage, at all the intermediate doses tested.

An important question is whether the major peaks in the commercial ASU preparation, which were almost absent in the other preparations, contributed to ASU biologic activity. Based on our experimental design, where all preparations were tested after equalization to contain identical sterol content, we observed no additional effects that could be attributed to other components.

Evaluation of collagen synthesis suggests greater stimulation at the higher doses. Similar results were found by Mauviel *et al.* (27) and Werman *et al.* (28) However, variation was seen between commercial product and Formula 1 preparations in the degree

of stimulation. A 3- to 5-fold greater dose-dependent stimulation occurred in collagen and NCP synthesis with Formula 1. The reason for these differences nor the significance is not known, as both preparations contained equal sterol concentrations.

Several mechanisms could influence chondrocyte metabolism. For example, ASU enhances synthesis of transforming growth factor beta (18), inhibits metalloprotease activity and eicosanoid synthesis (17). Sterols are also rapidly incorporated into cells causing an increase in cellular antioxidant status (29). Sterols also inhibit synthesis and release of PGE_2, a potent proinflammatory eicosanoid (30). previously shown to have an inhibitory effect on cartilage metabolism (31). Whatever the mechanism, there is substantial evidence that ASU containing sterols are anti-inflammatory and provide protection against cartilage degeneration.

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