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Research Article

Anti-inflammatory and chondroprotective effects of nutraceuticals from Sasha's Blend in a cartilage explant model of inflammation

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New Zealand green lipped mussel (NZGLM), abalone (AB), and shark cartilage (SC) are extensively used for treatment of and/or as preventatives for arthritis, despite a relative paucity of scientific evidence for efficacy. This research integrated a simulated digestion protocol with ultrafiltration and cartilage explants to generate new information on the anti-inflammatory and chondroprotective properties of NZGLM, SC, and AB. Each nutraceutical was artificially digested using simulated gastric and intestinal fluids, and the crude digest was ultrafiltered (50 kDa). Each filtrate was applied individually to cartilage explants before the explants were stimulated with IL-1 to induce an acute inflammatory response. Media were collected daily for 48 h and analyzed for prostaglandin E_2 (PGE_2), glycosaminoglycan (GAG), and nitric oxide (NO), and cartilage tissue was differentially stained to determine the relative proportion of live and dead cells. SC and NZGLM significantly inhibited IL-1-induced PGE_2 synthesis and IL-1-induced GAG release, and AB was an effective inhibitor of IL-1-induced NO production. The three test nutraceuticals affect at least three major pathways involved in the catabolic cycle of arthritis and may prove important treatments and/or preventatives for the pain and degradation associated with this condition. The methodology and results describe a useful model for evaluating dietary nutraceuticals *in vitro*.

Keywords: Arthritis / Nutraceuticals / Perna mussel / Shark cartilage / Simulated digest

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1 Introduction

Nutraceuticals such as New Zealand green lipped mussel (NZGLM), abalone (AB), various fish oils, and shark cartilage (SC) are extensively used as nutritional interventions for arthritic conditions [1, 2], despite a paucity of scientific evidence for efficacy. The suppositional success of these nutraceuticals is based on their very rich content of omega-3 fatty acids [3, 4], glycosaminoglycans (GAGs) [5], and proteins [6]. Provision of these dietary components has been associated with reduced clinical signs of arthritis [7, 8], together with improvement of the underlying pathology of the disease [9].

Culturing cartilage explants is a well-established tool for studying inflammatory processes [10], and has been used as a model to test antiarthritis treatments. Although the explant approach is valuable for testing products that do not undergo extensive modifications through digestion and absorption, its utility is substantively limited for testing dietary products that would be susceptible to these modifications. These limitations restrict interpretations of efficacy and/or toxicity of these products when they are tested in cartilage explant models.

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Abbreviations: AB, abalone; **C-AM,** calcein-AM; **EthD-1,** ethidium homodimer 1; **GAG,** glycosaminoglycan; **NZGLM,** New Zealand green lipped mussel; **PGE_2,** prostaglandin E_2; **SC,** shark cartilage; **TCM,** tissue culture media

The main purposes of these experiments were: (i) to integrate simulated digestion, cartilage explant, and ultrafiltration procedures in order to develop a unique *in vitro* model of inflammation; (ii) to evaluate the effect of simulated digests of three nutraceuticals ingredients of Sasha's Blend (Interpath, Melbourne, Australia) (NZGLM, AB, and SC) on prostaglandin E_2 (PGE_2), GAG, and nitric oxide (NO) produced by IL-1-stimulated cartilage explants; and (iii) to determine the effect of these nutraceuticals on cell viability.

2 Methods

2.1 Nutraceuticals

Dehydrated powder of NZGLM, AB, and SC were obtained from Interpath Pty (Melbourne, Australia). Chemical analysis of the three products was undertaken prior to digestion (see below) at a commercial analytical laboratory (Tables 1a–c).

2.2 Simulated digestion

NZGLM, SC, and AB each 0.85 g, and indomethacin; 0.71 g (reference anti-inflammatory drug; nonselective cycloxygenase inhibitor) were suspended individually in 35 mL of simulated gastric fluid (37 mM NaCl, 0.03 M HCl, 3.2 mg/mL pepsin) and shaken at 37°C for 2 h [11]. At 2 h, acidity was neutralized by adding 1.15 mL of 2.2 M NaOH and 36.15 mL of simulated intestinal fluid (30 mM K_2HPO_4, 160 mM NaH_2PO_4, 20 mg/mL pancreatin; pH adjusted to 7.4). The mixture was then shaken in a 37°C incubator for a further 2 h, centrifuged at 3000 $\times g$ for 25 min at 4°C, warmed to room temperature and filtered (0.22 μm), and then fractioned using a size-exclusion ultrafiltration centrifuge unit (50 kDa; AmiconUltra; Millipore, ON). A blank digest (*i.e.*, no product included) was prepared simultaneously using identical methodology.

2.3 Explant culture

Using aseptic technique, the intercarpal joint of market-weight pigs was opened and the cartilage surfaces exposed. A 4 mm dermal biopsy punch and scalpel were used to take explants (~0.5 mm thickness; ~15 mg/explant) of healthy cartilage from the weight-bearing region of both articulating surfaces. Cartilage discs were washed three times in DMEM supplemented with $NaHCO_3$. Two cartilage discs were placed into each well of 24-well tissue culture plates containing tissue culture medium (TCM) comprised of DMEM supplemented with amino acids, sodium selenite, manganese sulfate, $NaHCO_3$, and ascorbic acid [12]. Plates were incubated at 37°C, in a humidified atmosphere with 7% CO_2 for up to 144 h. Every 24 h TCM was completely aspirated and transferred to 1 mL microcentrifuge tubes containing indomethacin (10 μg in DMSO). Indomethacin

Table 1a. Chemical analysis of shark cartilage[a)]

Constituent	g/100 g
protein	52.1
carbohydrates	5.3
Chondroitin sulfate	13.8
glucosamine	14.9
fat	1.4
minerals	
calcium	16.5
phosphorus	9.8
moisture	2.1

a) Food Laboratories (Aust.) Pty Ltd. 2/1G Marine Parade, Abbotsford 3067 Australia.

Table 1b. Chemical analysis of New Zealand Green Lipped Mussel[a)]

Constituent	% wet weight
Ash	9
carbohydrate	17
Lipids and fat	13.5
Cholesterol	2
Sodium (Na)	2
Potassium (K)	1
Magnesium (Mg)	0.2
Calcium (Ca)	0.3
Glycosaminoglycans	> 2
betaine	4.2 g/100 g
Protein	**55**
Amino acid[b)]	**% total protein[c)]**
Cysteic acid	7
Aspartic acid	11
Threonine	5
Serine	4
Glutamic acid	14
Proline	5
Glycine	9
Alanine	5
Valine	4
Cystine	1
Methionine	2
Iso-leucine	4
Leucine	6
Tyrosine	3
Phenylalanine	4
Lysine	7
Histidine	2
Arginine	8

a) Food Laboratories (Aust.) Pty Ltd. 2/1G Marine Parade. Abbotsford 3067 Australia.
b) Typical analysis based on NZGLM (NutriZeal, New Zealand) from whom Interpath Pty Ltd. purchases their NZGLM powder. Analysis performed by Cawthron Institute.
c) Food Laboratories (Aust.) Pty. Ltd. 2/1G Marine Parade Abbotsford 3067 Australia.

Table 1c. Chemical analysis of abalone[a)]

Constituent		
Chondroitin sulfate	1.4 g/100g	
glucosamine	3.4 g/100g	
Total fat	**18.9 g/100g**	
Omega3 fat	2 g/100g	
Fatty acid	**% Fatty acids**	**g/100 total fat**
C14:0 Myristic acid	13.9	2.6
C16:0 Palmitic acid	29.5	5.6
C18:0 Stearic acid	2.4	0.5
C18:1 *cis* oleic acid	23.4	4.4
C18:1 *trans* oleic acid	0.7	0.1
C18:2 *cis* linoleic acid	1.5	0.3
C18:3 a linolenic acid	2.4	0.5
C18:4 octadecatetraenoic acid	2.1	0.4
C20:0 arachidic acid	0.2	≤0.1
C20:1 gadoleic acid	0.3	0.1
C20:5 (EPA) eicosapentaenoic acid	4.2	0.8
C22:5 docosapentaenoic acid	4.3	0.8
C22:6 (DHA) docosahexaenoic acid	1.6	0.3

a) Food Laboratories (Aust.) Pty. Ltd. 2/1G Marine Parade Abbotsford 3067 Australia.

was added to collected samples in order to prevent further formation of PGE_2 upon storage. Once collected, TCM from each well was immediately replaced with control, conditioned and/or stimulated TCM (described below) before the plate was returned to the incubator. Collected TCM was stored at –80°C for subsequent analysis. Cartilage was harvested at the end of each experiment with one explant *per* well stained for cytotoxicity (see cell viability staining below).

2.4 Effect of nutraceuticals on IL-1-induced inflammation

A single dose of each nutraceutical digest was determined by calculating the concentration of the manufacturer recommended dose for a 500 kg horse (17 g twice daily) suspended within the relative fluid volumes of the stomach and intestine [13], and then dispersed within the total volume of equine body water (300 L) [14]. This approach assumes complete distribution of bioactive constituents into the body water compartment, and provides a single dose concentration of 0.06 mg/mL.

Explants from six to nine pigs were prepared as previously described and arranged nonrandomly into 24-well tissue culture plates such that explants from each animal were exposed to each treatment. Timeline of acclimation, conditioning, and stimulation is provided in Fig. 1.

Explants were acclimated in TCM for 24 h, after which time they were conditioned with 0, 0.06, or 0.18 mg/mL of NZGLM, AB, or SC, or 0.02 mg/mL indomethacin (conditioned TCM) for the duration of the experiment. Explants were stimulated with IL-1 (10 ng/mL) beginning 72 h after dissection and continuing through the duration of the experiment. TCM was collected and refreshed every 24 h for total culture duration of 144 h. Collected samples were immediately frozen at –80°C until analyzed for PGE_2, GAG, and NO.

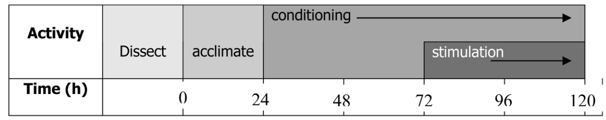

Figure 1. Timeline of acclimation, conditioning, and IL-1 stimulation of cartilage explants.

2.5 Sample analyses

2.5.1 PGE_2

PGE_2 concentrations in media were determined using a PGE_2 ELISA kit (Amersham, Baie D'Urfé, Québec). Plates were read using a Victor 3 microplate reader (Perkin-Elmer, Woodbridge, ON) with absorbance set at 450 nm. A best-fit third-order polynomial standard curve was developed for each plate ($R^2 \geq 0.99$), and these equations were used to calculate PGE_2 concentrations for samples from each plate.

2.5.2 GAG

Media GAG concentration was determined using a 1,9-DMB spectrophotometric assay as described by Chandrasekhar *et al.* [15]. Samples were added to 96-well plates at 50% dilution, and serially diluted 1:2 up to a final dilution of 1:64. Guanidine hydrochloride (275 mg/mL) was added to each well followed immediately by addition of 150 µL of dimethyl methylene blue (DMB) reagent. Plates were incubated in the dark for 10 min and absorbance was measured using a Victor 3 microplate reader at 530 nm. Sample absorbance was compared to that of a bovine chondroitin sulfate standard (Sigma, Oakville ON). A best-fit linear standard curve was developed for each plate ($R^2 \geq 0.99$), and these equations used to calculate GAG concentrations for samples on each plate.

2.5.3 NO

Nitrite (NO^{2-}), a stable oxidation product of NO, was analyzed by the Griess reaction [16]. Undiluted TCM samples were added to 96 well plates. Sulfanilamide (0.01 g/mL) and *N*-(1)-napthylethylene diamine hydrochloride (1 mg/mL) dissolved in phosphoric acid (0.085 g/L) were added to all wells, and absorbance was read within 5 min on a Victor 3 microplate reader at 530 nm. Sample absorbance was compared to a sodium nitrite standard. A best-fit linear standard curve was developed for each plate ($R^2 \geq 0.99$), and these equations were used to calculate nitrite concentrations for samples from each plate.

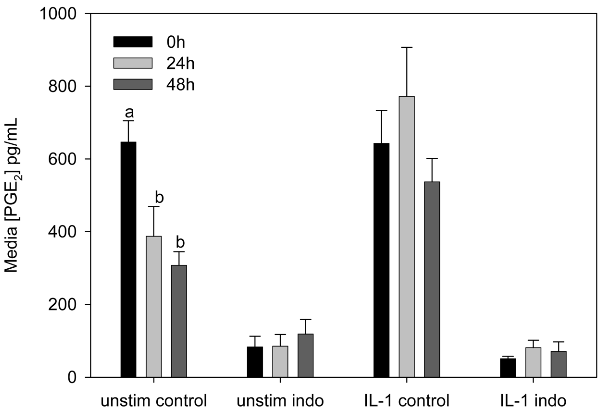

Figure 2. PGE_2 production by unconditioned cartilage explants, and explants conditioned with indomethacin. In the absence of IL-1, media [PGE_2] significantly declined between 0 and 24 h in unstimulated control explants. Indomethacin completely blocked PGE_2 production in both unstimulated and IL-1-stimulated explants. Letters denote significant difference ($p \leq 0.05$) from baseline.

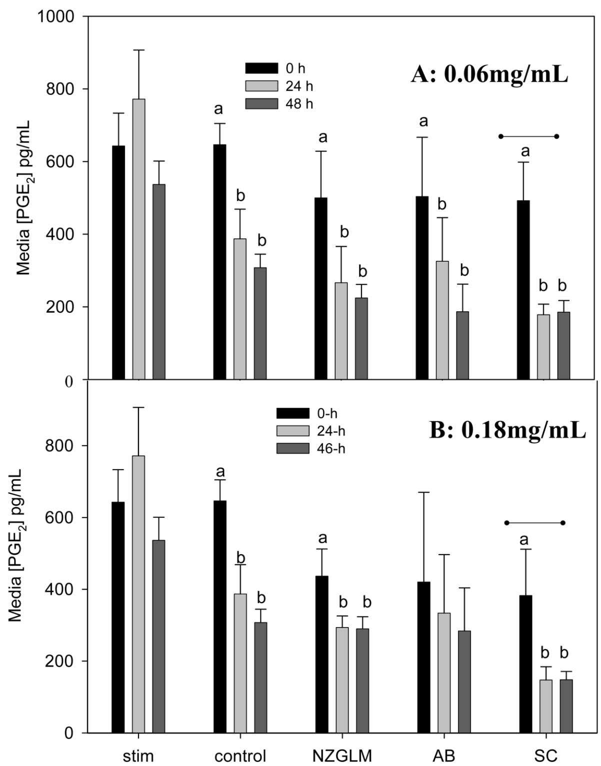

Figure 3. PGE_2 produced by unstimulated cartilage explants conditioned with 0.06 mg/mL (A) or 0.18 mg/mL (B) of SC, NZGLM, or AB. SC-conditioning (0.06 and 0.18 mg/mL) resulted in significantly reduced [PGE_2] compared with control. Letters denote significant time- or IL-1-dependent changes; ●–● denotes significant difference from control.

2.5.4 Cell viability

Calcein-AM (C-AM) stock was prepared by diluting 10 μL of 4 mM C-AM (Molecular Probes) in 9.99 mL PBS to a final concentration of 4 nmol/mL. Ethidium homodimer 1 (EthD-1) stock was prepared by diluting 100 μL 2 mM EthD-1 (Molecular Probes) in 9.9 mL PBS to a final concentration of 20 nmol/mL.

2.6 Cell viability

2.6.1 Assay development

This assay was developed according to recommendations for optimizing dye concentrations to tissue type (Molecular Probes Live/Dead Cytotoxicity Kit, Product information, revised Dec. 21, 2006). One porcine joint was dissected as described previously, and 48 explants were placed into TCM (live explants). Forty-eight explants previously frozen in liquid nitrogen were thawed to room temperature and incubated for 30 min in 100 μL 70% isopropyl alcohol to kill any viable chondrocytes in the explants (killed explants). All explants were washed three times in PBS and placed into a 96-well plate. Both live and killed explants were treated with increasing concentrations of EthD-1 (0, 2, 4, 6, 8, and 10 μM) and C-AM (0, 0.1, 0.4, 1, and 4 μM). Measures of fluorescence were obtained every 10 min, beginning at 5 min postexposure to the dye, for a total of 70 min. The reader was set to scan each well, beginning at the bottom, using ten horizontal steps at each of three vertical displacements set 0.1 mm apart. C-AM and EthD-1 fluorescence in live and killed explants were obtained using excitation/emission filters of 485/530 and 530/685 nm, respectively.

Results from this experiment demonstrated that [C-AM] and [EthD-1] of 4 and 8 μM, respectively, with incubation duration of 40 min, were most appropriate for fluorometric separation of live and dead cells (see Results). Subsequently, all viability assessments of explants used these parameters.

2.6.2 Data analysis

One-way repeated measures ANOVA with respect to time was used to detect changes in IL-1-dependent and time-dependent media concentrations of GAG, NO, and PGE_2. A two-way repeated measures ANOVA was used to compare treatments over time. When a significant F-ratio was obtained, the Holm–Sidak *post hoc* test was used to identify significant differences between treatments. One-way ANOVA without repeated measures was used to compare treatments against controls in cell viability analyses. Significance was accepted at $p \leq 0.05$. All data are presented as mean ± SEM, unless otherwise stated.

3 Results

3.1 PGE_2-control explants

Exposure of explants to IL-1 (10 ng/mL) resulted in a non-significant increase in media [PGE_2] that was significantly

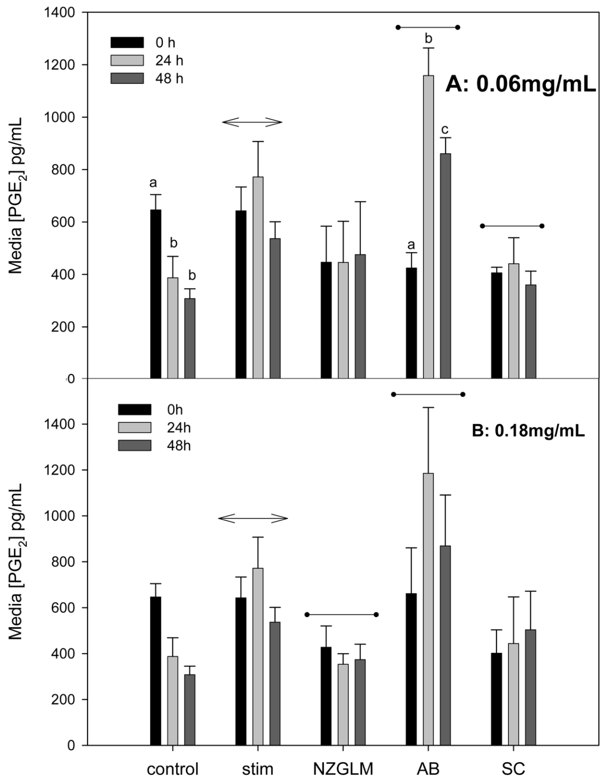

Figure 4. PGE_2 release from unstimulated cartilage explants ($n = 6$) conditioned with 0.06 (A) or 0.18 mg/mL (B) SC and NZGLM, and explants conditioned with indomethacin (0.02 mg/mL). Indomethacin completely inhibited IL-1-dependent PGE_2 production. PGE_2 was significantly lower in explants conditioned with SC (0.06 mg/mL) and NZGLM (0.18 mg/mL) than in stimulated controls. AB significantly increased PGE_2 production compared with stimulated controls. Letters denote significant IL-1- or time-dependent increases in PGE_2; ↔ denotes significant difference from unstimulated controls. ●–● denotes significant differences from stimulated controls.

higher than that of unstimulated controls. The significant difference in media [PGE_2] between IL-1-stimulated and unstimulated explants resulted primarily from a significant decline in media [PGE_2] from unstimulated control explants. Conditioning of explants with indomethacin for 48 h prior to IL-1 stimulation resulted in almost complete blockade of PGE_2 production at 0 h (83.0 ± 29.6 pg/mL) compared with unstimulated (646.4 ± 58.5 pg/mL) and stimulated controls (642.7 ± 90.8 pg/mL) at the same time. Conditioning of IL-1-stimulated explants with simulated digest of indomethacin completely inhibited IL-1-dependent PGE_2 production (Fig. 2).

3.2 PGE_2-unstimulated conditioned explants

Compared with unconditioned unstimulated explants, conditioning with SC (0.06 and 0.18 mg/mL) significantly reduced media [PGE_2]. There was also a trend ($p = 0.07$) to decreased media [PGE_2] in AB-conditioned (0.18 mg/mL)

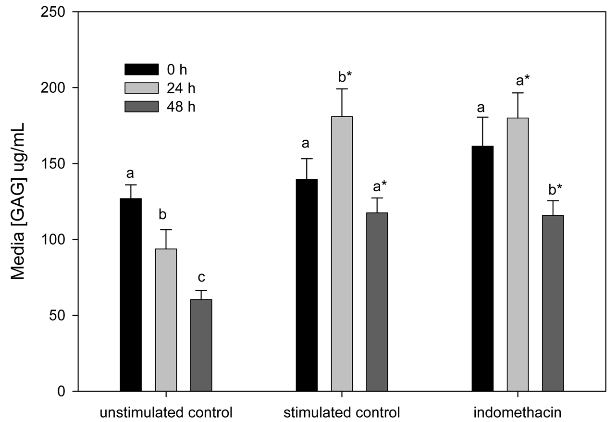

Figure 5. GAG release (μg/mL) from unconditioned cartilage explants, and explants conditioned with indomethacin. IL-1 stimulation resulted in significant increase in media [GAG] in stimulated control and indomethacin-conditioned explants. In the absence of IL-1, media [GAG] significantly declined in unstimulated control explants. Letters denote significant time- or IL-1-dependent changes; * denotes significant difference from unstimulated controls.

explants compared with unconditioned, unstimulated controls. There was no significant effect of conditioning with NZGLM or AB in unstimulated explant (Fig. 3).

3.3 PGE_2-IL-1-stimulated conditioned explants

Conditioning with SC (0.06 mg/mL) and NZGLM (0.18 mg/mL) resulted in significantly lower media [PGE_2] than IL-1-stimulated, unconditioned controls. There was a trend for inhibition of PGE_2 production from explants conditioned with SC (0.18 mg/mL) ($p = 0.08$) and NZGLM ($p = 0.09$) (Fig. 4). Conditioning with AB (0.06 and 0.18 mg/mL) resulted in significantly increased IL-1-dependent PGE_2 production compared with IL-1-stimulated controls.

3.4 GAG-control explants

Exposure of unconditioned control explants to IL-1 (10 ng/mL) resulted in a significant increase in media [GAG] between 0 (139.4 ± 13.7 μg/mL) and 24 h (180.8 ± 18.3 μg/mL). In contrast, there was a significant decline in GAG release from unstimulated controls between 0 (126.9 ± 9.0 μg/mL) and 24 h (93.8 ± 12.6 μg/mL), and a further decline between 24 and 48 h (60.4 ± 6.0 μg/mL) (Fig. 5). Stimulated and unstimulated controls were significantly different from each other. Conditioning of IL-1-stimulated explants with simulated digest of indomethacin also resulted in significant increase in media [GAG].

3.5 GAG-unstimulated conditioned explants

In unstimulated explants, conditioning with SC (0.06 and 0.18 mg/mL) resulted in a significant decline in [GAG]

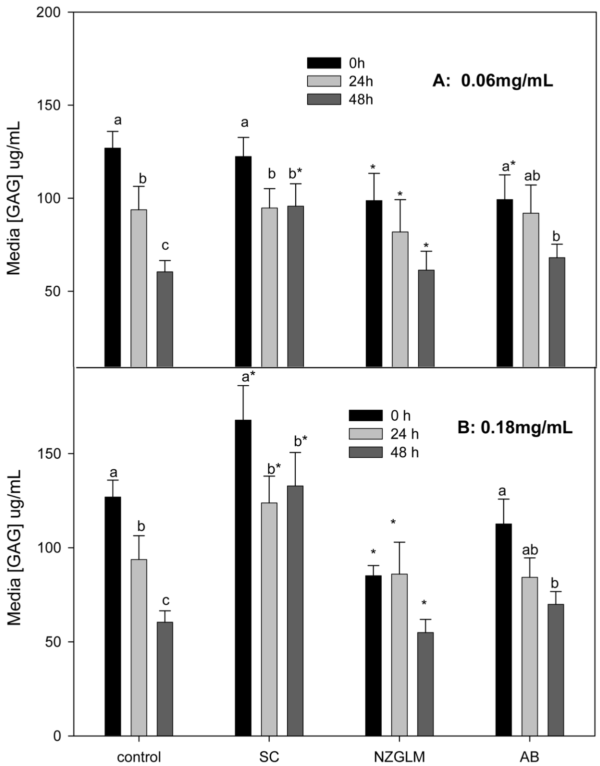

Figure 6. GAG release (μg/mL) from unstimulated (no IL-1) cartilage explants conditioned with 0.06 mg/mL (A) or 0.18 mg/mL (B) of SC, NZGLM, and AB. SC-conditioning (0.06 and 0.18 mg/mL) resulted in significantly elevated [GAG] compared with unstimulated control, while AB (0.06 mg/mL) and NZGLM (0.18 mg/mL) significantly reduced media [GAG] compared with unstimulated controls. Letters denote significant time- or IL-1-dependent changes; * denotes significant difference from unstimulated controls.

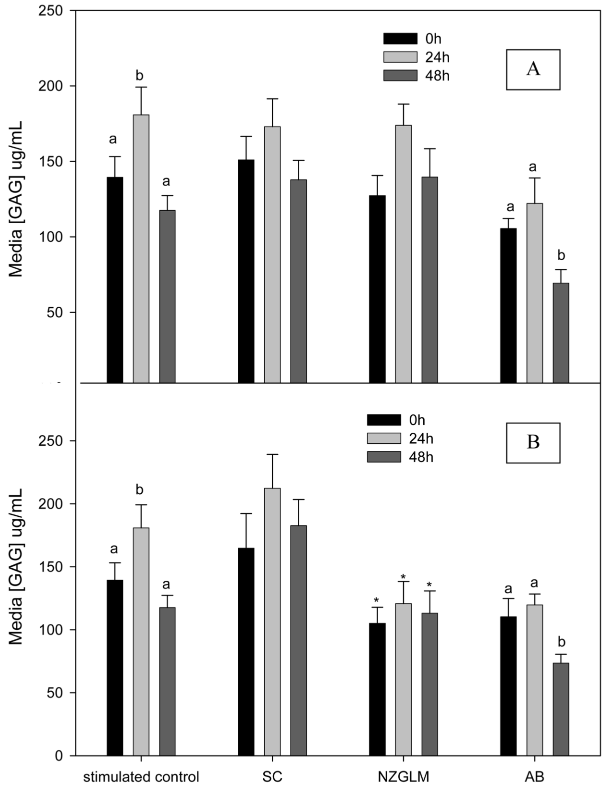

Figure 7. GAG release from IL-1 (10 ng/mL)-stimulated cartilage explants conditioned with 0.06 mg/mL (A) or 0.18 mg/mL (B) of SC, NZGLM, and AB. Conditioning with SC, NZGLM, and AB (0.06 and 0.18 mg/mL) prevented a significant IL-1-induced increase in media [GAG]. AB (0.06 and 0.18 mg/mL) significantly reduced media [GAG] after 48 h of IL-1 exposure. NZGLM (0.18 mg/mL) resulted in media [GAG] that was significantly lower than stimulated controls. Letters denote significant time- or IL-1-dependent changes; * denotes significant difference from stimulated controls.

between 0 and 24 h, but there was no further decline at 48 h (Figs. 6 A and B). There was a significant increase in media [GAG] from SC-conditioned explants compared with unconditioned controls. Conditioning with NZGLM (0.06 and 0.18 mg/mL) resulted in no time-dependent change in media [GAG]. However, media [GAG] from NZGLM (0.18 mg/mL)-conditioned explants was significantly lower than in control explants (Fig. 6B). Conditioning of unstimulated explants with AB (0.06 and 0.18 mg/mL) resulted in significant decline in media [GAG] over time, and media [GAG] was significantly lower in those explants conditioned with AB (0.06 mg/mL) compared with controls (Figs. 6 A and B).

3.6 GAG-IL-1-stimulated conditioned explants

Conditioning with SC or NZGLM (0.06 and 0.18 mg/mL), prevented an IL-1-dependent increase in media [GAG] (Figs. 7A and B). Media [GAG] was significantly lower in explants conditioned with NZGLM (0.18 mg/mL) than that of stimulated controls. Conditioning of IL-1-stimulated explants with AB (0.06 mg/mL) resulted in significant decrease in media [GAG] between 0 (105.5 ± 6.6 μg/mL) and 48 h (69.4 ± 8.8 μg/mL) with no IL-1-induced increase in media [GAG] at 24 h poststimulation (Fig. 7A). There was no significant effect of SC or AB on [GAG] when compared with IL-1-stimulated controls.

3.7 NO-control explants

Exposure of unconditioned control explants to IL-1 resulted in significant increases in media [NO] between 0 (0.47 ± 0.09 μg/mL) and 24 h (1.19 ± 0.13 μg/mL); media [NO] from unstimulated controls declined steadily over the 48 h experimental period (Fig. 8). Media [NO] from IL-1-stimulated explants was significantly higher than that of unstimulated control explants. Simulated digest of indomethacin had no effect on IL-1-dependent NO production by explants.

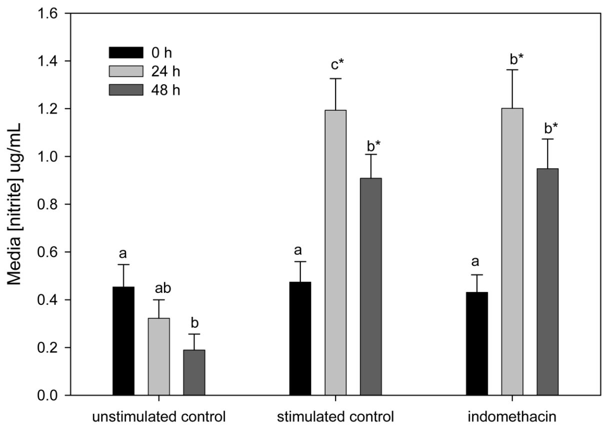

Figure 8. NO production by unconditioned cartilage explants, and explants conditioned with indomethacin. IL-1 stimulation resulted in significant increase in media [NO] in stimulated control and indomethacin-conditioned explants. In the absence of IL-1, media [NO] significantly declined in unstimulated control explants. Letters denote significant time- or IL-1-dependent changes; * denotes significant difference from unstimulated controls.

3.8 NO-unstimulated conditioned explants

Conditioning of unstimulated explants with NZGLM, SC (0.06 mg/mL), or AB (0.06 and 0.18 mg/mL) had no effect on time-dependent change in media [NO] (Fig. 9). However, NO from explants conditioned with NZGLM (0.06 and 0.18 mg/mL) was lower compared with unconditioned controls. NZGLM- and SC-conditioning (0.18 mg/mL) of unstimulated explants both resulted in a significant decline in [NO] over time, but media [NO] was significantly higher in unstimulated explants conditioned with SC (0.18 mg/mL) compared with unconditioned controls.

3.9 NO-IL-1-stimulated conditioned explants

Conditioning with NZGLM, AB, and SC did not prevent IL-1-induced increase in media [NO] (Fig. 10). However, 48 h media [NO] from AB-conditioned (0.06 and 0.18 mg/mL) explants (0.4 ± 0.07 µg/mL) was not significantly different from prestimulation concentration (0.2 ± 0.07 µg/mL), while control explants and those conditioned with NZGLM and SC remained significantly elevated at 48 h. AB conditioning (0.06 mg/mL) resulted in a significantly reduced media [NO] compared with IL-1-stimulated controls.

3.10 Cell viability

Stimulation of explants with IL-1 for 48 h had no significant effect on cell viability (Fig. 11). None of the nutraceutical treatments had any effect on cell viability in unstimulated explants (Fig. 11). However, the presence of IL-1

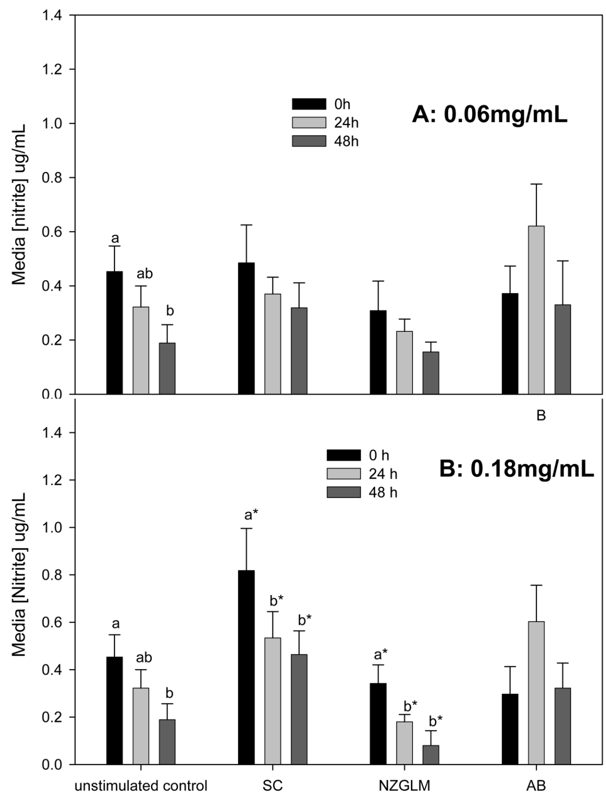

Figure 9. NO production by unstimulated cartilage explants conditioned with 0.06 mg/mL (A) or 0.18 mg/mL (B) of SC, NZGLM, and AB. Conditioning with SC, NZGLM, and AB (0.06 mg/mL) resulted in no change in media [NO], while unstimulated control explants had a significant decline in NO over the duration of the experiment. SC at the higher dose (0.18 mg/mL) significantly increased media [NO] compared with unstimulated controls, while NZGLM (0.18 mg/mL) significantly decreased media [NO] compared with unstimulated controls. Letters denote significant time- or IL-1-dependent changes; * denotes significant difference from unstimulated controls.

NZGLM (0.06 and 018 mg/mL) and SC (0.06 mg/mL) slightly but significantly increased cell viability compared with IL-1 stimulated control explants. Simulated digest of indomethacin had no effect on cell viability in the presence or absence of IL-1.

4 Discussion

4.1 Importance of simulated digestion and ultrafiltration

The cartilage explant model has often been utilized for the purpose of generating information on the effect of dietary nutraceuticals on cartilage health and metabolism [17, 18]. However, there are some limitations to this conventional approach which may influence interpretation of the data: (i) the experimental products undergo no digestion-dependent

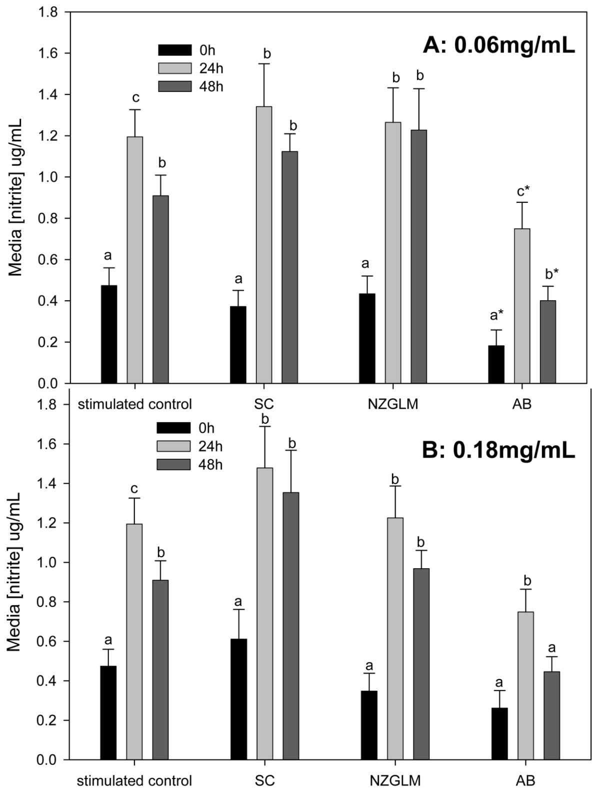

Figure 10. NO release from IL-1 (10 ng/mL)-stimulated cartilage explants conditioned with 0.06 mg/mL (A) or 0.18 mg/mL (B) of SC, NZGLM, and AB after a 72 h acclimation period. IL-1 stimulation resulted in significant increase in media [NO] in stimulated control and in explants conditioned with SC, NZGLM, and AB (0.06 and 0.18 mg/mL). AB (0.06 mg/mL) resulted in media [NO] that was significantly lower than stimulated controls. Letters denote significant time- or IL-1-dependent changes; * denotes significant difference from stimulated controls.

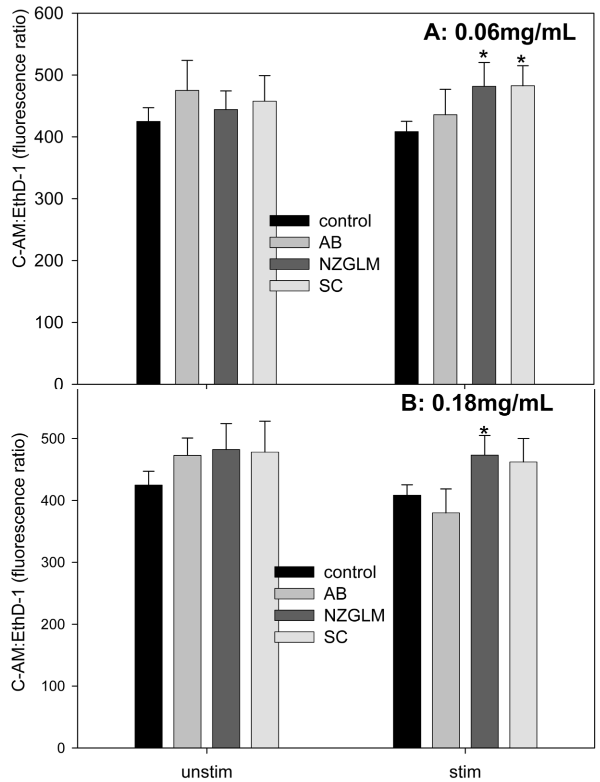

Figure 11. Cell viability in explants conditioned with AB, NZGLM or SC (0.06 or 0.18 mg/mL) in the presence of IL-1 (0 or 10 ng/mL). Histogram shows viability after 48 h exposure to IL-1 (0 or 10 ng/mL) and conditioning (0.06 mg/mL, A; 0.18 mg/mL, B). There was no effect of any conditioning on unstimulated explants. Conditioning of IL-1-stimulated explants with NZGLM (0.06 or 0.18 mg/mL) and SC (0.06 mg/mL) slightly, but significantly increased cell viability compared with IL-1-stimulated controls.

modifications, as they would *in vivo*, that may substantively alter bioactivity; and (ii) experiments have been designed such that all components of the experimental product are applied to the cartilage matrix irrespective of molecular size or structure. For these reasons, we integrated a simulated digestion step to impose digestion-dependent modifications on the products, followed by ultrafiltration to remove molecules with molecular weight greater than 50 kDa, *i. e.*, molecules whose movement into the joint capsule and cartilage matrix would be prohibited *in vivo*. Molecules of 10 kDa molecular weight readily diffuse into cartilage matrix [19] but permeability of cartilage matrix is increased up to ∼four times in osteoarthritis, allowing molecules of larger molecular weight to diffuse [20]. Furthermore, free-swelling, noncompression conditions, as would be found in nonweight-bearing joints (and in a cartilage explant system), allow molecules of 40 kDa to readily diffuse into the matrix [21]. Therefore, the 50 kDa fraction tests all of those low-molecular weight constituents of the simulated digest that have a reasonable chance of diffusing into the cartilage matrix and altering pathophysiological processes of chondrocytes. Our simulated digestion/ultrafiltration approach still requires consideration of some limitations: (i) the digestible portion of the product is assumed to be 100% bioavailable in the animal, (ii) the active constituents of the product are assumed to be substantively unaltered by biotransformation in the liver, (iii) the digested and absorbed product is assumed to be evenly dispersed throughout the total body water compartment of the animal, and is not preferentially sequestered into any particular tissue or cell-type, and (iv) it is assumed that there are no constituents in the 50 kDa fraction that would undergo extensive physiological regulation *in vivo* (*e.g.*, blood glucose), that results in very rapid removal from the extracellular fluids,

Though many of these assumptions would only partially hold true *in vivo*, our simulated digestion procedure at the least accounts for the actions of major digestive enzymes, lipid emulsification and changes in pH on the bioactivity of nutraceutical products. Furthermore, the ultrafiltration step removes molecules that are not likely to exert direct effects on the cartilage *in vivo*, but which may confound *in vitro* results.

4.2 SC

The hallmark effects of SC digest within our explant model were significant inhibition of IL-1-induced PGE_2, and significant increase of IL-1-independent media [GAG]. The active constituent(s) within SC are not known, but there is considerable evidence for the bioactivity of glucosamine and chondroitin sulfate. These two molecules comprise approximately 30% of the dry weight of our experimental SC material. While the mechanism of SC-induced PGE_2 inhibition is not known, our data suggest a pathway that is independent of NO production. Reduced transcription of Cox2 and mPGEs1 [22] could account for the inhibition of IL-1-induced PGE_2 in our study. However, given the reported inhibition of iNOS [12], we would expect to see reduced NO concurrent with reduced PGE_2, but this was not observed in our study. Indeed, SC slightly increased NO production in our study, and this increase was significant in unstimulated explants. The dose of glucosamine/chondroitin (30 mg/mL) used by Chan *et al.* [12] was higher than in the current study (approximately 20 µg/mL, given a 30% GAG content); the IL-1 stimulus (15 ng/mL) was also higher, and the GAGs used were isolated from bovine cartilage rather than from SC. Furthermore, these authors utilized explant tissue from cows rather than pigs. Also, other constituents of the heterogeneous SC (*i.e.*, constituents other than glucosamine and chondroitin sulfate) used in our study may have altered the NO-inhibiting effect of glucosamine/chondroitin alone.

Conditioning of unstimulated cartilage explants with simulated digest of SC resulted in significant dose-dependent increases in media [GAG] relative to unconditioned controls. It is not likely that SC caused an IL-1-independent increase in proteoglycan degradation because SC [23], glucosamine, and chondroitin sulfate [17] inhibit matrix metalloproteinases, and up-regulated expression of tissue inhibitors of metalloproteinases [22]. Therefore, the increase in media [GAG] is more likely attributable to a direct increase in media [GAG] from the SC digest. Indeed, there was a positive, dose-dependent increase in media [GAG] in unstimulated explants which was not seen in IL-1 stimulated explants, providing evidence that SC-conditioning may inhibit IL-1-induced degradation of proteoglycan, consistent with findings of other authors [17, 22, 23].

4.3 NZGLM

Simulated digest of NZGLM demonstrated effective inhibition of IL-1-induced PGE_2 production and GAG release in our explant model. While the active constituent(s) of NZGLM responsible for its anti-inflammatory effect is unknown, the activity is blunted by destruction of protein [6]. The most abundant amino acid in our NZGLM was glutamate, from which glutamine is formed through the action of glutamine synthetase. Glutamine is considered a "conditionally essential amino acid", as in pathophysiological conditions it can become essential and rate limiting [24]. It is a precursor to proline [24] which, together with hydroxyproline, make up 22% of the amino acids in fibrillar collagen molecules [25]. These collagen molecules provide the scaffold upon which cartilage matrix is supported [25]. Furthermore, glutamine (and glutamate mutants) plays a critical role in the function of 15-hydroxyprostaglandin dehydrogenase, the enzyme which oxidizes the C-15 hydroxyl group of prostaglandins and lipoxins to produce 15-keto metabolites which exhibit greatly reduced biological activities [26]. Thus the provision of exogenous glutamate, and possibly other as-yet unknown additional actives, from NZGLM may contribute to the observed effects of decreased GAG release and PGE_2 production by IL-1-stimulated cartilage explants.

4.4 AB

Conditioning with simulated digest of AB protected cartilage explants from IL-1-induced catabolism in a manner different from either SC or NZGLM, by inhibiting the production of IL-1-induced NO. NO is a reactive oxygen species which augments cytokine-dependent susceptibility of chondrocytes to oxidant injury, and contributes to chondrocyte death and progressive cartilage destruction [27]. Furthermore, NO is increasingly implicated in pain sensitization due to interactions between NO and PGE_2 [28]. The mechanisms by which AB exerts an inhibitory effect on NO are not known. However, given the high fat content of our experimental AB (~20%), it is likely that the omega 3 portion of fat (about 10% of total fat) at least in part accounts for this effect, as omega 3 fats are known to be inhibitory to NO production [29], possibly by inhibiting iNOS [30].

4.5 Cell viability

Given the inverse relationship between synovial fluid [PGE_2] and [NO] with cell viability [31, 32], any agent capable of reducing IL-1-induced PGE_2 and/or NO should also increase cell viability relative to unconditioned, stimulated controls. This hypothesis was supported by the fact that simulated digests of both NZGLM and SC slightly but significantly increased cell viability in IL-1 stimulated cartilage explants. AB, the most effective NO inhibitor that we tested, did not increase cell viability, perhaps due to its augmenting effect on PGE_2 [32]. It is noteworthy, however, that despite very strong inhibition of IL-1-induced PGE_2 production by indomethacin, the NSAID did not significantly increase cell viability over stimulated controls. This likely resulted from the antiproliferative effect of indomethacin on some cell lines *via* an unknown mechanism that is independent of its inhibitory activity on Cox [33]. This antiproliferative effect is also seen in osteoblasts [34], and therapeutic concentrations of indomethacin have antiproliferative and apoptotic effects on cultured chondrocytes [35].

4.6 Summary

These data provide evidence for anti-inflammatory and chondroprotective role of SC and NZGLM in inflamed cartilage. Both nutraceuticals were capable of inhibiting IL-1-induced PGE_2 production, and modulating IL-1-induced GAG release. The stimulatory effect of SC on IL-1-independent NO is probably not of physiological significance as the increase was significantly less than that induced by IL-1, and the augmentation was not seen in IL-1-stimulated explants. Similarly, though green lipped mussel significantly inhibited NO in unstimulated explants, it is not likely to be an important pathway through which it exerts anti-inflammatory effect as the effect was not seen in IL-1-stimulated explants. AB may also play a role in modulating cartilage inflammation through its inhibitory effect on IL-1-induced NO release, but its augmenting activity on PGE_2 production could counteract any positive physiological effect. Using these ingredients in combination may blunt this augmenting effect, which should be tested through further research. Future studies should also be conducted to determine bioavailability and bioactivity of these nutraceuticals *in vivo*, and to identify potential synergisms when they are used in combination.

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