

Simulated digest of a glucosamine-based equine nutraceutical modifies effect of IL-1 in a cartilage explant model of inflammation

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We have recently described an *in vitro* cartilage explant model which combines cartilage explant with simulated digestion and ultrafiltration of putative anti-inflammatories (Pearson *et al.*, 2007a,b). This model is an improvement upon the conventional cartilage explant model because it accounts for the effects of gastric and intestinal enzymes and pH on the bioactivity of dietary products *in vitro*, while removing molecules that are too large to have a reasonable chance of moving from the GI tract and into the synovial space. We have evaluated the effects of simulated digest of indomethacin in this model (Pearson *et al.*, 2007b), as well as a commercial herbal anti-inflammatory product 'Mobility' (Pearson *et al.*, 2007b) and shark cartilage, perna mussel and abalone from a commercial nutraceutical 'Sasha's Blend' (Pearson *et al.*, 2007a). Due to their relative low cost and short time-to-result, these experiments they can provide important preliminary information on the potential bioactivity and/or cytotoxicity of putative anti-inflammatory nutraceuticals thus improving the scientific basis for dietary anti-inflammatory nutraceuticals.

The purpose of the current study was to obtain pilot, proof-of-principle data on a dietary nutraceutical Hyalcare (HC: Selected Bioproducts Ltd., Guelph, Canada). HC is a glucosamine-based composite, intended for use in horses, containing glucosamine hydrochloride (GI-H), methylsulfonylmethane (MSM), shark cartilage, *Boswellia serrata*, hyaluronic acid and flaxseed oil. GI-H is a hexosamine salt, and is among the most popular non-allopathic treatments for arthritis in horses (Trumble, 2005). It has inhibited nitric oxide and PGE₂ production (Walsh *et al.*, 2007) and activity of matrix metalloproteinases (MMPs) (Uitterlinden *et al.*, 2006) in osteoarthritic cartilage explants. It is also the primary constituent of a number of commercial products showing some evidence for clinical anti-inflammatory and/or cartilage-sparing effect in equine *in vivo* trials (Forsyth *et al.*, 2006; Hanson *et al.*, 1997). The mode of action of GI-H is considered to be global modulation of the genetic response of cells to stimulation with interleukin-1 (Gouze *et al.*, 2006). In addition, oral MSM – 500 mg three times daily for 12 weeks (Usha & Naidu, 2004) and 3 g twice daily for 12 weeks (Kim *et al.*, 2006) – has been shown to inhibit pain and swelling associated with arthritis of the knee in human patients.

The null hypotheses tested in the current experiment were, compared with stimulated controls: 1) HC does not reduce IL-1-induced production of PGE₂; 2) HC does not reduce IL-1-induced production of nitrite; 3) HC does not reduce IL-1-induced [GAG] release; 4) HC reduces cell viability at up to 10× the recommended dose.

Simulated digest of HC (HC_{sim}) was prepared using 1 g of HC as previously described (Pearson *et al.*, 2007a), with consideration that the manufacturer's recommended dose for a 500 kg horse (10 g daily) was suspended within the relative fluid volumes of the stomach and intestine (Marciani *et al.*, 2005). The simulated digest was centrifuged at 3000 *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, Canada). The low molecular weight fraction was diluted to a concentration consistent within the total volume of equine body water (300 L) (Forro *et al.*, 2000). This approach assumes complete distribution of bioactive constituents into the body water compartment, and provides a single dose concentration of 0.03 mg/mL. A blank digest (i.e. no product included) was prepared simultaneously using identical methodology. The dosages used in the experiments were representative of one (0.03 mg/mL), five (0.15 mg/mL) and 10 times (0.30 mg/mL) the manufacturer's recommended dose.

Cartilage explants (4 mm diameter) from the intercarpal joints of six market-weight pigs were aseptically prepared as previously described (Pearson *et al.*, 2007a,b). They were placed two explants per well in columns (two columns per animal per treatment) of a 24-well tissue culture plate. Wells of the tissue culture plate contained Dubeloco's Modified Eagle Medium supplemented with amino acids, sodium selenite, manganese sulfate, NaHCO₃ and ascorbic acid (TCM – tissue culture medium) such that tissue from each animal was exposed to each treatment. Plates were incubated at 37 °C, 7% CO₂ in a humidified atmosphere for 120 h.

Explants were acclimated in TCM for 24 h post-dissection, after which time they were conditioned with 0, 0.03 (HC_{sim}-1), 0.15 (HC_{sim}-5) or 0.30 mg/mL (HC_{sim}-10) of HC_{sim} (conditioned TCM) for the duration of the experiment. Explants were

stimulated with human recombinant IL-1β (10 ng/mL; Biosource, Camarillo, CA, USA) 72 h after dissection, and continuing for an additional 48 h. This protocol allowed for 48 h of pre-conditioning of explants before IL-1 was introduced, and was designed to detect any preventive effect of HC against IL-1-induced inflammation. TCM was collected every 24 h and replaced with TCM containing IL-1 for total culture duration of 120 h. The collected TCM was transferred to 1.8 mL microcentrifuge tubes containing indomethacin [10 µg in dimethyl sulfoxide (DMSO)]. 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 until analyzed for PGE₂ (ELISA; Pearson *et al.*, 2007a), GAG (DMMB; Pearson *et al.*, 2007a), and nitrite (Griess Reaction; Pearson *et al.*, 2007a). Upon termination of the experiment, explants were collected and immediately stained for viability of chondrocytes using ethidium homodimer-1 and calcein-AM as described previously (Pearson *et al.*, 2007a). Viability data are presented as a unit-less ratio of calcein-AM fluorescence (live cells) to ethidium homodimer-1 fluorescence (dead cells).

Data are presented as mean ± SE. A best-fit third order (PGE₂) or linear (GAG, nitrite) standard curve was developed for each microtitre plate ($R^2 \geq 0.99$), and these equations were used to calculate concentrations for samples from each plate. Repeated measures 1-way ANOVA was used to detect time-dependent changes within treatments; repeated measures 2-way ANOVA was used to compare treatments with controls over time. When a significant F-ratio was obtained, the Holm-Sidak post-hoc test was used to identify significant differences between treatments. Viability data were analyzed using Student's t-test comparing each treatment with controls. Significance was accepted when $P \leq 0.05$.

Results for PGE₂, GAG, nitrite and viability are presented in Table 1. There was no change in media [PGE₂] from unstimulated control explants over the 48 h experimental period. Stimulation with IL-1 (10 ng/mL) resulted in significant increases in media [PGE₂] from pre-stimulation levels, and these were significantly greater than [PGE₂] of unstimulated explants. Conditioning of IL-1-stimulated explants with HC_{sim} did not prevent IL-1-induced PGE₂ production. Media PGE₂ was significantly higher in IL-1-stimulated explants conditioned with 0.03 and 0.3 mg/mL HC_{sim} than in stimulated controls at 24 h.

There was a significant decline in media [GAG] from unstimulated control explants over the 48 h experimental period ($P < 0.001$) (Fig. 1). Stimulation with IL-1 significantly increased media [GAG] ($P = 0.05$) relative to 0 h. [GAG] was significantly greater in stimulated than in unstimulated controls ($P = 0.004$). Conditioning of explants with HC_{sim} (0.03 mg/mL) did not prevent IL-1-induced increase in media [GAG] compared with pre-stimulation concentrations. Media [GAG] was significantly lower in explants conditioned with HC_{sim} (0.03 mg/mL) than in stimulated controls after 48 h of exposure to IL-1 ($P = 0.04$). Media [GAG] in HC_{sim}-conditioned explants (0.03 mg/mL) was not significantly different from unstimulated controls at this time point. IL-1 did not result in significant

Table 1. Response of cartilage explants to stimulation with IL-1 (10 ng/mL) over 48 h in presence of HC_{sim} (0, 0.03, 0.15 and 0.3 mg/mL). BOLD denotes values significantly different from stimulated control

Treatment	PGE ₂ ng/mL (SE)			GAG µg/mL (SE)			Nitrite µg/mL (SE)			Viability - (SE)
	Baseline	24 h	48 h	Baseline	24 h	48 h	Baseline	24 h	48 h	
Stimulated control	338.2 (48.6)	803.3* (66.6)	807.7* (68.9)	152.5 (11.1)	218.3* (36.8)	202.3* (33.8)	0.21 (0.04)	1.32* (0.15)	0.86* (0.08)	536.1 (32.3)
Unstimulated control	406.2 (90.8)	380.1 (85.7)	378.1a (105.7)	173.1 (22.5)	138.7 (11.8)	78.8* (7.5)	0.21 (0.05)	0.27 (0.08)	0.18 (0.10)	584.1 (37.7)
HC _{sim} (0.03 mg/mL)	436.6 (94.8)	1139.5* (141.7)	734.8 (122.9)	156.8 (15.5)	237.7* (35.2)	139.6 (9.3)	0.09 (0.09)	0.99* (0.14)	0.73* (0.05)	574.2 (25.5)
HC _{sim} (0.15 mg/mL)	470.5 (155.8)	1153.1 (380.1)	933.7 (238.5)	148.6 (13.6)	191.4 (15.7)	153.9 (29.0)	0.11 (0.09)	0.87* (0.09)	0.72* (0.06)	574.9 (41.5)
HC _{sim} (0.3 mg/mL)	517.5 (138.4)	1414.7* (158.8)	1166.5* (221.8)	168.7 (17.0)	193.9 (14.3)	155.6 (26.1)	0.08 (0.08)	0.93* (0.15)	0.65* (0.12)	561.5 (27.3)

*Denotes significant change from baseline within treatment ($P < 0.05$).

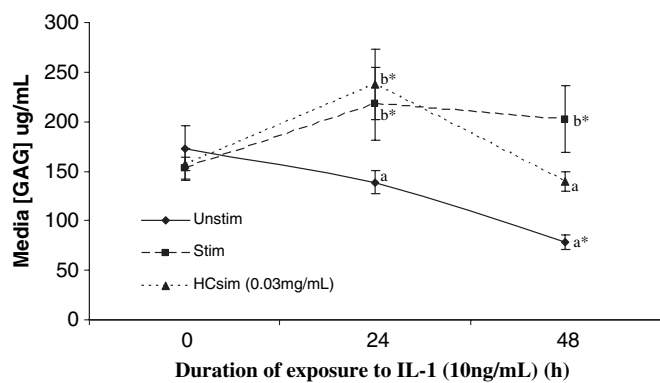


Fig. 1. GAG release from cartilage explants conditioned in the presence or absence of HC_{sim} (0.03 mg/mL). Porcine articular cartilage explants were acclimated in tissue culture media for 24 h prior to conditioning with simulated digest of HyalCare (HC_{sim}) (0, 0.03, 0.15 or 0.3 mg/mL) for a further 48 h. Explants were then stimulated with IL-1 for an additional 48 h. Graphs shows data from unstimulated (unstim) and stimulated (stim) controls, and those conditioned with HC_{sim} (0.03 mg/mL). *Denotes significant change from baseline within treatments. Letters denote significant differences between at given time points. ($P < 0.05$).

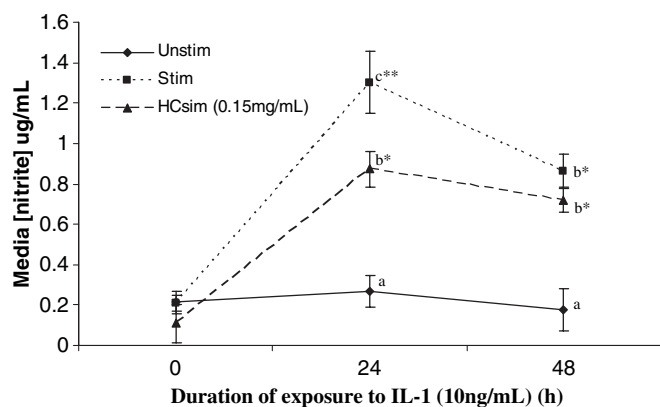


Fig. 2. Nitrite production from explants conditioned in the presence or absence of HC_{sim} (0.15 mg/mL). Porcine articular cartilage explants were acclimated in tissue culture media for 24 h prior to conditioning with simulated digest of HyalCare (HC_{sim}) (0, 0.03, 0.15 or 0.3 mg/mL) for a further 48 h. Explants were then stimulated with IL-1 for an additional 48 h. Graphs shows data from unstimulated (unstim) and stimulated (stim) controls, and those conditioned with HC_{sim} (0.15 mg/mL). *Denotes significant change from baseline within treatments. Letters denote significant differences between treatments at given time points. ($P < 0.05$).

increase in media [GAG] in explants conditioned with HC_{sim}-5 or HC_{sim}-10.

There was no change in media [nitrite] in unstimulated control explants (Fig. 2). Stimulation of control explants with IL-1 increased [nitrite] ($P < 0.001$). Conditioning of explants with HC_{sim} (all doses) did not prevent significant increases in [nitrite] relative to 0 h. Media [nitrite] was significantly lower in explants stimulated with HC_{sim} (0.15 mg/mL) at 24 h relative to stimulated controls ($P = 0.05$). Media nitrite showed a tendency

to be lower in HC_{sim} (0.03 and 0.3 mg/mL) compared with stimulated controls ($P < 0.1$).

Simulated digest of HC did not reduce viability of chondrocytes within explants at any concentration, up to 0.3 mg/mL which is equivalent to 10 times the manufacturer's recommended dose.

The mechanism(s) by which the effects of HC are exerted are not known. Global inhibition of inflammatory genes resulting from IL-1 stimulation is unlikely, as has been reported for glucosamine (Gouze *et al.*, 2006), because PGE₂ production was not inhibited by the product. The observed reduction of IL-1-induced nitrite may result from at least four of its constituents [Gl-H (Walsh *et al.*, 2007), *Boswellia* (Pandey *et al.*, 2005), α -linolenic acid (Ren & Chung, 2007) and glutamine (Brasse-Lagnel *et al.*, 2007)], all of which have demonstrated effects on nitric oxide or genes associated with its formation. An HC-dependent reduction in IL-1-induced GAG release is consistent with the effects of simulated digest of shark cartilage on porcine cartilage explants (0.06 and 0.18 mg/mL; Pearson *et al.*, 2007a), Gl-H on equine cartilage explants (25 mg/mL; Fenton *et al.*, 2000) and hyaluronic acid in early human osteoarthritic cultures of chondral, meniscal, and synovial explants (various doses and molecular weights; Hsieh *et al.*, 2007). The combination of nitric oxide reduction and prevention of GAG release is suggestive of a direct peroxynitrite scavenging effect; scavenging of reactive nitrogen species is coupled with protective effects on cartilage homeostasis, whereas direct inhibition of nitric oxide mediates other indicators of inflammation, but does not protect structural integrity of cartilage (Bezerra *et al.*, 2004). Further research is needed to characterize the mechanism(s) of HC on nitric oxide production and GAG release. The combined effect of decreased nitrite and decreased GAG loss subsequent to cartilage exposure to IL-1 suggests that this product may be a useful agent for protecting cartilage integrity during times of inflammatory stress. The reduction of nitric oxide production by HC may also be associated with attenuation of pain transmission *in vivo*, as inducible nitric oxide synthase (Bujalska *et al.*, 2007) and nitric oxide (Wei *et al.*, 2007) have been implicated in the neuronal transmission of nociceptive stimuli.

The dose-dependent stimulatory effect of HC_{sim} on PGE₂ production was not expected given the pain relieving properties of MSM (Usha & Naidu, 2004) and *Boswellia* (Kimmattkar *et al.*, 2003). Furthermore, Gl-H has significantly inhibited IL-1 induced PGE₂ production *in vitro* (Walsh *et al.*, 2007). It is not known why the PGE₂ response to conditioning with HC_{sim} differed from that expected from many of the individual constituents. Given the known variability in quality and consistency of some glucosamine-based equine nutraceutical products (Oke *et al.*, 2006), it appears that individual *in vitro* assessment of each of the ingredients of HC in a cartilage explant model is necessary to rule out possible uncharacteristic behaviour of the individual constituents which may indicate poor product quality or contamination. This would provide insight into whether the PGE₂-stimulatory effect of HC_{sim} results from unique behaviour of the combination of ingredients or from contaminants and/or poor quality of one or more of the constituents.

The major findings of this pilot study are that HC_{sim} (0.03 mg/mL) reduces media [GAG] in IL-1-stimulated explants,

and HC_{sim} (0.15 mg/mL) reduces media [nitrite] compared with stimulated controls. HC_{sim} was not cytotoxic *in vitro* at any of the doses tested, but significantly increased PGE₂. The results obtained with this pilot project provide some evidence for potential biological activity of the product in modulating response of cartilage to stimulation with IL-1. The reduction in GAG loss and nitrite production subsequent to IL-1 stimulation suggests a potential cartilage-sparing role of this product that should be investigated in further research. The PGE₂-stimulatory activity of the product is of concern and if similar results are obtained *in vitro* then the product may not be indicated in cases of arthritis without concurrent NSAID treatment. *In vivo* bioavailability and pharmacokinetic studies are important to establish the amount of individual ingredients which actually reaches cartilage tissues, and will help to more fully characterize a biologically active and safe dose of the product.

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