

Differential anti-inflammatory and chondroprotective effects of simulated digests of indomethacin and an herbal composite (Mobility™) in a cartilage explant model of articular inflammation

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Herbs are an increasingly popular treatment option for horses with cartilage inflammation, despite a relative paucity of research demonstrating efficacy. The research objective was to evaluate the differential anti-inflammatory and chondroprotective efficacy of a simulated digest of indomethacin and a commercially available herbal product in a cartilage model of osteoarthritis. Cartilage explant was integrated with simulated digestion of indomethacin and the herbal product in order to account, at least in part, for the actions of major digestive enzymes and pH. The resulting digests were ultrafiltrated (50 kDa), to account for absorption from the GI tract and movement into the cartilage matrix. We hypothesized that (i) a simulated digest of indomethacin would block interleukin 1 β -(IL-1) dependent formation of prostaglandin E₂ (PGE₂) and nitric oxide (NO) without protecting cartilage against IL-1-induced glycosaminoglycan (GAG) release, and (ii) the herbal product would reduce PGE₂ and NO in IL-1-stimulated explants, and inhibit release of GAG, in IL-1-stimulated explants. Results showed that indomethacin is an effective anti-inflammatory, evidenced by strong inhibition of IL-1-induced PGE₂ and NO from cartilage explants. However, indomethacin provided no protection against IL-1-induced GAG release. Simulated digest of the herbal extract significantly inhibited IL-1-induced NO production and GAG release, while having a slight increase in PGE₂. These data provide evidence for the anti-inflammatory effect of indomethacin on IL-1-stimulated cartilage explants, and the herbal product Mobility™ may be a useful adjunct in arthritis because of its chondroprotective properties in IL-1-stimulated cartilage.

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INTRODUCTION

Since the isolation of salicylic acid from the bark of the white willow tree (*Salix alba*) in 1828, and subsequent manufacture of aspirin, herbs have been recognized as one of the world's richest sources of anti-inflammatory drugs. Persuasive epidemiological and experimental evidence has accumulated for such anti-inflammatory herbs as devil's claw (*Harpagophytum procumbens*) (Andersen *et al.*, 2004; Huang *et al.*, 2006), comfrey (*Symphytum officinale*) (Koll *et al.*, 2004; Grube *et al.*, 2007), white willow (Chrubasik *et al.*, 2001), skullcap (*Scutellaria* sp.) (Martin & Dusek, 2002), stinging nettle (*Urtica dioica*) (Schulze-Tanzil *et al.*, 2002) and burdock (*Marrubium vulgare*) (Stulzer *et al.*, 2006).

Although scientific evidence is mounting for the effectiveness of herbs in improving clinical outcomes in inflammatory disorders, nonsteroidal anti-inflammatory drugs (NSAIDs) remain the treatment of choice for most cases of equine lameness. This is due in large part to the undisputable analgesic effect of many of these drugs; indeed it is arguably unethical to withhold this type of treatment from horses suffering from acute pain. NSAIDs exert their pronounced anti-inflammatory activity by inhibiting the formation of pro-inflammatory prostaglandins (PGs), particularly prostaglandin E₂ (PGE₂). They do this by blunting the catalytic activity of the cyclo-oxygenase (Cox) enzymes which catalyze formation of PGE₂. Some NSAIDs nonselectively block both major isoforms of Cox (Cox1 and

Cox2) via acetylation of the peroxidase active site. This prevents the abstraction of hydrogen from arachidonic acid (AA) by sterically preventing binding of AA to the active site (Rowlinson *et al.*, 2000). Others, however, selectively block Cox2 such that AA is oxidized but the Cox activity is inhibited and AA oxidation yields 15-R-HETE (Rowlinson *et al.*, 2000), a noninflammatory oxidation product. Selective inhibition of Cox2 is considered a safer form of drug therapy, associated with fewer gastric side-effects, than drugs that also inhibit Cox1 (Patrignani *et al.*, 2003; Mamdani *et al.*, 2004). However, recent concerns regarding the safety of a common selective Cox2 inhibitor 'Rofecoxib' has resulted in the withdrawal of this drug from the marketplace (Couzin, 2004; Dieppe *et al.*, 2004). The most common NSAIDs used in management of equine musculoskeletal pain are phenylbutazone (PBZ), flunixin (banamine), ketoprofen, naproxen and carprofen (Goodrich & Nixon, 2006). Each of these drugs have varying degrees of Cox selectivity and cytokine inhibition, and differing analgesic vs. anti-inflammatory effects; their applications, efficacy and toxicity in equine arthritis have recently been reviewed (Goodrich & Nixon, 2006).

In cases of more chronic pain wherein the equine patient requires ongoing, daily pharmaceutical support to maintain functionality and quality of life, the usefulness of NSAIDs is offset by the potential for adverse effects. The most well-known adverse effect of chronic NSAID use is the development of gastric ulceration (Meschter *et al.*, 1990; Reed *et al.*, 2006). NSAIDs produce gastropathy via a number of mechanisms, including induction of reactive oxygen species (ROS), inhibition of PGE₂ synthesis, and induction of inflammatory cell infiltration. ROS are highly reactive molecules, the production of which is induced during NSAID administration (Chattopadhyay *et al.*, 2006; Ganguly *et al.*, 2006; Olaleye & Farombi, 2006). Induction of ROS by many NSAIDs is associated with concomitant decline in protective endogenous antioxidant enzyme systems (Chattopadhyay *et al.*, 2006), leading to compromised extracellular matrix integrity and impaired remodeling capacity of gastric tissue (Ganguly *et al.*, 2006). At the same time, an NSAID-induced decline in gastric tissue [PGE₂] (Shimamoto *et al.*, 2006) retards healing of ulcers already present (Poonam *et al.*, 2005) and inhibits optimal mucous production by goblet cells and Brunner's glands (Akiba *et al.*, 2000). The gastric mucosa is thus functionally and structurally compromised, and vulnerable to accumulation of activated neutrophils which promote localized catabolic inflammatory responses (Wallace *et al.*, 1990). In addition to toxic erosions of gastric tissue, cartilage exposure to NSAIDs (including PBZ and indomethacin) has been associated with advanced release of proteoglycan from cartilage (Freen *et al.*, 2002) and suppression of normal proteoglycan synthesis in horses (Beluche *et al.*, 2001). So while NSAIDs may effectively reduce synovial [PGE₂] (Morton *et al.*, 2005), and thus the pain associated with articular inflammation (Owens *et al.*, 1996), these data suggest that NSAIDs may further shift the metabolic balance of arthritis to one of advanced catabolism.

The toxicological implications of chronic NSAID use are well known within the equine industries, and are likely contributors to the growing use of 'alternative' treatments for chronic

inflammatory disorders in horses (Trumble, 2005). Unfortunately, there is a lack of experimental evidence for efficacy and safety of anti-inflammatory herbs from which recommendation can be made for their use in horses. Many *in vitro* experiments are conducted almost exclusively on plant extracts (Choi *et al.*, 2006; Wu *et al.*, 2006) or isolated phytochemicals (Liacini *et al.*, 2005; Nizamutdinova *et al.*, 2007), and do not account for the effects of digestion, absorption and/or distribution. This is an important limitation, as some anti-inflammatory herbs (e.g. *H. procumbens*) are rendered inactive by the actions of stomach acid (Lanthers *et al.*, 1992; Catelan *et al.*, 2006), and beneficial effects seen *in vitro* may not be repeatable *in vivo*. Furthermore, absorption, distribution and pharmacokinetics of many bioactive compounds are markedly different in horses compared with small laboratory animals (Kararli, 1995; Court, 2001), making direct correlations across species questionable.

The experimental product in the current study is 'MobilityTM' (MB; Selected Bioproducts Ltd., Guelph, ON, Canada), a poly-herbal composite formulated for horses. Its composition is based on the reported anti-inflammatory properties of *S. officinale* (Koll *et al.*, 2004; Grube *et al.*, 2007), *H. procumbens* (Andersen *et al.*, 2004; Huang *et al.*, 2006), *U. dioica* (Schulze-Tanzil *et al.*, 2002) and *M. vulgare* (Stulzer *et al.*, 2006). The research objective was to evaluate the efficacy of MB in a cartilage model of osteoarthritis, while simulating the impact of upper gastrointestinal digestion and absorption of the herbal product.

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 (Dechant *et al.*, 2005; Uitterlinden *et al.*, 2006). Some limitations to this conventional approach may influence interpretation of the data include: (i) the experimental product does not undergo digestion-dependent 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 (Coleman *et al.*, 1997) but permeability of cartilage matrix is increased up to approximately four times in osteoarthritis, allowing molecules of larger molecular weight to diffuse in (Alexopoulos *et al.*, 2005). 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 (Quinn *et al.*, 2001). 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 chondrocyte metabolism.

In order to confirm that the simulated digestion/ultrafiltration protocol did not mask known anti-inflammatory activity, a reference anti-inflammatory was run as a positive control in the

cartilage explant culture experiments. When selecting a reference anti-inflammatory drug it was necessary to choose one which has shown significant effect on joint pain *in vivo* (i.e. established efficacy), in addition to having been evaluated in a cartilage explant model *in the absence of simulated digestion*. This is important because the bioactivity of the reference anti-inflammatory drug (indomethacin) in the absence of simulated digestion would provide a benchmark against which the drug could be compared after it had been put through simulated digestion/ultrafiltration. Indomethacin, a nonselective Cox inhibitor, was chosen as a reference anti-inflammatory drug due, in part, to its well-characterized effect on cartilage explants in the absence of simulated digestion (Arner *et al.*, 1998; Frean *et al.*, 2002) and its demonstrated anti-inflammatory effect *in vivo* (Rainsford *et al.*, 1999).

A simulated digest of indomethacin should inhibit PGE₂ and nitric oxide (NO) production in the presence of pro-inflammatory interleukin 1 β (IL-1) (Panico *et al.*, 2006), but not reduce IL-1-induced glycosaminoglycan (GAG) release (Frean *et al.*, 2002). This would be similar to the effect on cartilage explant cultures reported for undigested indomethacin in dimethyl sulfoxide (DMSO) (Arsenis & McDonnell, 1989; Frean *et al.*, 2002; Bica *et al.*, 2007). We hypothesize that simulated digest of MB will blunt cartilage production of PGE₂ and NO while inhibiting release of GAG by IL-1-stimulated cartilage explants.

MATERIALS AND METHODS

Simulated digestion

MobilityTM is a dried composite of five herbs (Table 1). One gram of MB and 0.71 g indomethacin were suspended individually in 35 mL of simulated gastric fluid (37 mM NaCl, 0.03 N HCl, 3.2 mg/mL pepsin) and shaken at 37 °C for 2 h (Rininger *et al.*, 2000). At 2 h, acidity was neutralized by adding 1.15 mL of 2.2 N NaOH and 36.15 mL of simulated intestinal fluid (30 mM K₂HPO₄, 160 mM NaH₂PO₄, 20 mg/mL pancreatin; pH adjusted to 7.4) was added, then shaken in a 37 °C incubator for a further 2 h. The resultant mixture was centrifuged at 3000 \times g for 25 min at 4 °C, warmed to room temperature and filtered (0.22 μ m), and then fractionated using a size-exclusion ultrafiltration centrifuge unit (50 kDa; AmiconUltra; Millipore, ON,

Canada). A blank digest (i.e. no product included) was prepared simultaneously using identical methodology.

Explant culture

Cartilage tissue from pigs was used for the experiments. Tissue from horses was not readily available, and equine tissue that was accessible was from animals of different ages, breeds, and health status, and information on their source and background was often lacking. This would have introduced a large amount of uncontrolled variability into our experiments. We selected pigs as donor animals because of their breed, age and health consistency from the slaughterhouse, their relative small size which facilitates dissection, and because we expected a similar porcine response to IL-1 as that observed in equine cartilage (Rainsford *et al.*, 1997; Frean *et al.*, 2002).

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 (*c.* 0.5 mm thickness; *c.* 15 mg/explant) of healthy cartilage from the weight-bearing region of both articulating surfaces. Cartilage discs were washed 3 times in DMEM supplemented with NaHCO₃. Two cartilage discs were placed into each well of 24-well tissue culture plates containing DMEM supplemented with amino acids, sodium selenite, manganese sulfate, NaHCO₃ and ascorbic acid (tissue culture medium – TCM). Plates were incubated at 37 °C, 7% CO₂ in a humidified atmosphere for up to 144 h. Every 24 h TCM was completely aspirated and transferred to 1 mL microcentrifuge tubes containing indomethacin (10 μ g in DMSO). This indomethacin was added to the collected tissue culture media after it was removed from the cartilage explants in order to prevent further formation of PGE₂ during storage, and was added to all collected TCM samples. 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.

Effect of indomethacin and MB on IL-1-induced inflammation

A single dose of MB digest was determined by calculating the concentration of the manufacturer recommended dose for a 500 kg horse (165 g twice daily) suspended within the relative fluid volumes of the stomach and intestine (Marciani *et al.*, 2005), with the assumption that this dose was dispersed 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 1.1 mg/mL.

Explants from six pigs were prepared as previously described (Pearson *et al.*, 2007) and arranged nonrandomly into 24-well tissue culture plates such that explants from each animal were exposed to each treatment. Explants were acclimated in TCM for 24 h, after which time they were conditioned with 0, 1.1 (MB-1)

Table 1. Composition of MobilityTM

Botanical name	Common name	Bioactive phytochemical(s) ^a
<i>Harpagophytum procumbens</i>	Devils Claw	Iridoid glycosides (harpagoside)
<i>Urtica dioica</i>	Stinging nettle	Polysaccharides, Phenolics, sterols
<i>Taraxacum officinale</i>	Dandelion	triterpenoids
<i>Symphytum officinale</i>	Comfrey	Allantoin, polysaccharides
<i>Arctium lappa</i>	Burdock	Inulin, phenolics

^aBruneton (1999).

or 3.3 mg/mL (MB-3) of MB digest, or 0.02 mg/mL indomethacin digest (indo) (conditioned TCM) for the duration of the experiment. Explants were stimulated with IL-1 (15 ng/mL) beginning 72 h after dissection and continuing through the duration of the experiment. This protocol allowed for a 48 h preconditioning of explants before IL-1 was introduced, and was designed to detect a prophylactic effect of indomethacin and/or MB against IL-1-induced inflammation. TCM was collected every 24 h and replaced with TCM containing IL-1 (0 or 15 ng/mL) for total culture duration of 120 h. Collected samples were immediately frozen at -80°C until analyzed for PGE₂, GAG, and NO.

In vitro sample analyses

PGE₂

Prostaglandin E₂ concentrations in media were determined using a PGE₂ ELISA kit (Amersham, Baie D'Urfé, Québec, Canada). Plates were read using a Victor 3 microplate reader (Perkin Elmer, Woodbridge, ON, Canada) with absorbance set at 450 nm.

GAG

Media GAG concentration was determined using a 1,9-DMB spectrophotometric assay (Chandrasekhar *et al.*, 1987). 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 g/L) was added to each well followed immediately by addition of 150 μL DMB reagent. Plates were incubated in the dark for 10 min and absorbance measured using a Victor 3 microplate reader at 530 nm. Sample absorbance was compared with that of a bovine chondroitin sulfate standard (Sigma, Oakville, ON, Canada).

NO

Nitrite (NO^{2-}), a stable oxidation product of NO, was analyzed by the Griess reaction (Fenton *et al.*, 2002). Undiluted TCM samples were added to 96 well plates. Sulfanilamide (0.01 g/mL) and *N*-(1)-Naphthylethylene diamine hydrochloride (1 mg/mL) dissolved in phosphoric acid (0.085 g/L) was 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.

Data analysis

A best-fit third order (PGE₂) or linear (GAG, NO) 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 one-way ANOVA was used to detect time-dependent changes within treatments; repeated measures two-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. Significance was accepted when $P \leq 0.05$.

RESULTS

For all figures with two panels, panel a represents data from explants not stimulated with IL-1, and panel b represents data from explants stimulated with IL-1 (15 ng/mL). Stimulated controls (stim) and unstimulated controls (unstim) are the same data for each of panels a and b within each figure, and are shown on each panel for comparison. 'Conditioning' refers to treatment of explants with simulated digests of either MB (1.1 or 3.3 mg/mL) or indomethacin (0.02 mg/mL), and 'stimulation' refers to treatment with IL-1.

PGE₂

Unstimulated, unconditioned explants demonstrated a significant decline in PGE₂ production over the 48-h experimental period (Fig. 1a), resulting from recovery from the inflammation caused by dissection (Gruber *et al.*, 2004). Stimulation with IL-1 (15 ng/mL) resulted in a nonsignificant increase in media [PGE₂] from prestimulation levels, which was significantly greater than [PGE₂] of unstimulated explants (Fig. 1b). Conditioning with simulated digest of indomethacin significantly blunted PGE₂ production in both unstimulated and stimulated explants.

Conditioning of unstimulated explants with MB-1 resulted in a trend to increased PGE₂ relative to unstimulated controls ($P = 0.08$); conditioning of unstimulated explants with MB-3 caused a significant increase in media [PGE₂] compared with both unstimulated and stimulated controls (Fig. 1).

Conditioning of stimulated explants with MB-1 and MB-3 did not prevent IL-1-induced PGE₂ production, and mean [PGE₂] was significantly higher in media from MB-3-conditioned explants than in media from IL-1-stimulated unconditioned controls.

GAG

Unstimulated, unconditioned explants (Fig. 2a) demonstrated a significant decline in GAG release over the 48-h experimental period, consistent with a gradual decline in IL-1-induced GAG release resulting from dissection and explantation (Gruber *et al.*, 2004). Stimulation with IL-1 (15 ng/mL) resulted in a nonsignificant increase in media [GAG] from prestimulation levels, which was significantly greater than [GAG] of unstimulated explants (Fig. 2b). There was no significant decline in media [GAG] from unstimulated explants conditioned with simulated digest of indomethacin, but concentrations were not significantly different from those of unconditioned unstimulated controls. Conditioning with simulated digest of indomethacin did not prevent IL-1-induced increase in GAG release.

There was no significant effect of MB-1 or MB-3 on GAG release by unstimulated cartilage explants. However, MB-1 conditioning resulted in significantly decreased GAG release in IL-1-stimulated explant compared with IL-1 stimulated unconditioned controls. This protective effect was not seen with MB-3 conditioning.

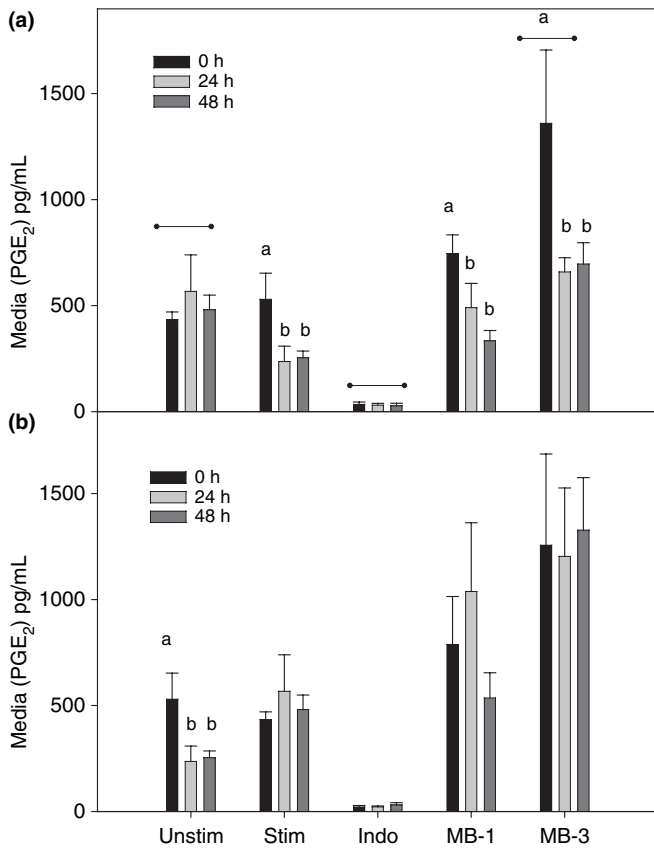


Fig. 1. Effect of simulated digest of indomethacin (indo: 0.02 mg/mL) or Mobility (MB-1: 1.1 mg/mL; MB-3: 3.3 mg/mL) on prostaglandin E₂ (PGE₂) production by cartilage explants. Panel a describes results from explant *not exposed to IL-1* (i.e. 0 ng/mL), while panel b describes results from explants that were *stimulated with IL-1* (15 ng/mL). ‘Unstim’ and ‘stim’ refer to unstimulated and stimulated controls, respectively; these data are the same for panels a and b, and are presented for comparison of treatment effects. *n* = 6 for all treatments. Explants were preconditioned for 48 h with MB or indomethacin, after which time they received MB (1.1 or 3.3 mg/mL) or indomethacin (0.02 mg/mL) concurrently with IL-1 (0 or 15 ng/mL). Zero hour represents media PGE₂ immediately prior to addition of IL-1 [0 ng/mL – panel a; 15 ng/mL – panel b], while 24 and 48 h show [PGE₂] after addition of IL-1. Letters denote significant changes in PGE₂ within treatments in response to conditioning and time (panel a), or conditioning, time and IL-1 (panel b). ●—● denotes significant treatment effect compared with unstimulated controls (panel a) or stimulated controls (panel b). Changes are significant when *P* ≤ 0.05.

NO

Unstimulated, unconditioned explants demonstrated a significant decline in NO production over the 48-h experimental period. IL-1 (15 ng/mL) resulted in a significant increase in media [NO] at 24 h poststimulation, which was significantly greater than [NO] of unstimulated explants (Fig. 3a). Simulated digest of indomethacin did not alter NO production of unstimulated explants, but significantly protected explants against IL-1-induced increase in NO production.

Conditioning of unstimulated explants with MB-1 resulted in a small but significant increase in NO compared with unstimulated controls (*P* = 0.05). This effect was not seen in unstimulated

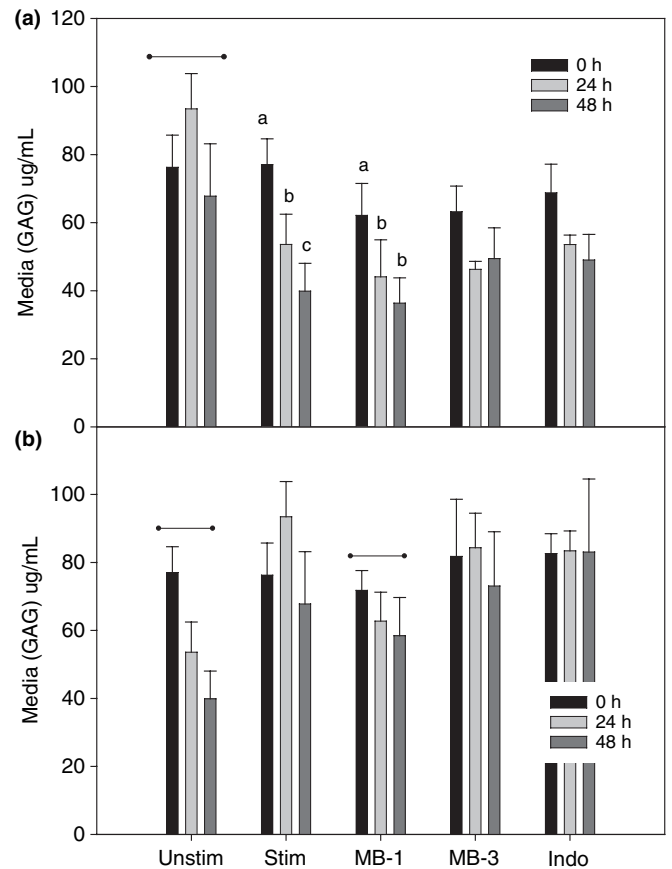


Fig. 2. Effect of simulated digest of indomethacin (indo: 0.02 mg/mL) or Mobility (MB-1: 1.1 mg/mL; MB-3: 3.3 mg/mL) on glycosaminoglycan (GAG) release by cartilage explants. Panel a describes results from explant *not exposed to IL-1* (i.e. 0 ng/mL), while panel b describes results from explants that were *stimulated with IL-1* (15 ng/mL). ‘Unstim’ and ‘stim’ refer to unstimulated and stimulated controls, respectively; these data are the same for panels a and b, and are presented for comparison of treatment effects. *n* = 6 for all treatments. Explants were preconditioned for 48 h with MB or indomethacin, after which time they received MB (1.1 or 3.3 mg/mL) or indomethacin (0.02 mg/mL) concurrently with IL-1 (0 or 15 ng/mL). Zero hour represents media GAG immediately prior to addition of IL-1 [0 ng/mL – panel a; 15 ng/mL – panel b], while 24 and 48 h show [GAG] after addition of IL-1. Letters denote significant changes in GAG within treatments in response to conditioning and time (panel a), or conditioning, time and IL-1 (panel b). ●—● denotes significant treatment effect compared with unstimulated controls (panel a) or stimulated controls (panel b). Changes are significant when *P* ≤ 0.05.

explants conditioned with MB-3. MB-1 significantly protected explants from an IL-1-induced increase in media [NO] (Fig. 3b). There was no effect of MB-3 on IL-1-induced NO production.

DISCUSSION

The major findings of this study were that simulated digest of indomethacin significantly inhibited PGE₂ and NO production by IL-1-stimulated cartilage explants. However, despite pronounced inhibition of PGE₂ and NO in cartilage explants, simulated digest

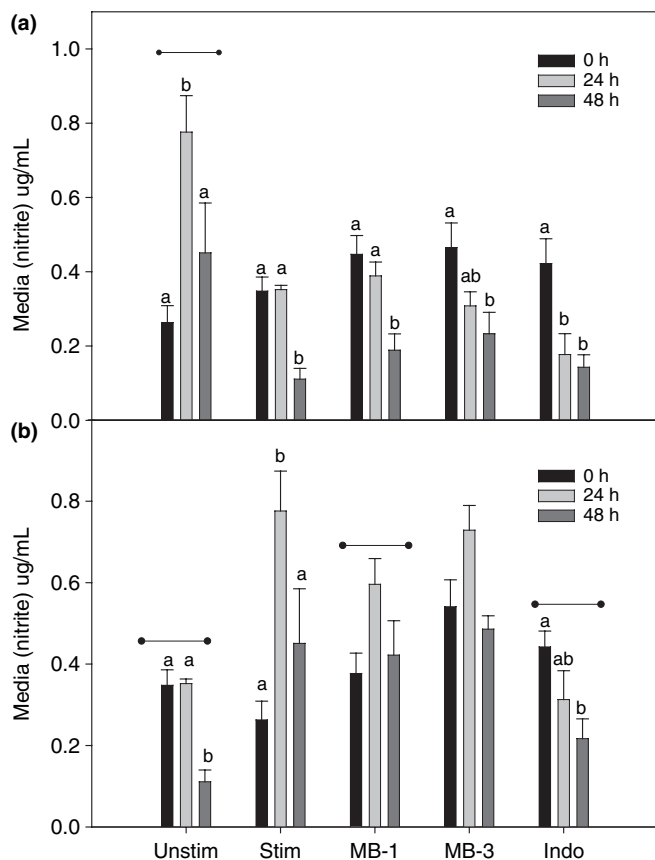


Fig. 3. Effect of simulated digest of indomethacin (indo: 0.02 mg/mL) or Mobility (MB-1: 1.1 mg/mL; MB-3: 3.3 mg/mL) on nitric oxide (NO) production by cartilage explants. Panel a describes results from explant not exposed to IL-1 (i.e. 0 ng/mL), while panel b describes results from explants that were stimulated with IL-1 (15 ng/mL). 'Unstim' and 'stim' refer to unstimulated and stimulated controls, respectively; these data are the same for panels a and b, and are presented for comparison of treatment effects. $n = 6$ for all treatments. Explants were preconditioned for 48 h with MB or indomethacin, after which time they received MB (1.1 or 3.3 mg/mL) or indomethacin (0.02 mg/mL) concurrently with IL-1 (0 or 15 ng/mL). Zero hour represents media [NO] immediately prior to addition of IL-1 [0 ng/mL – panel a; 15 ng/mL – panel b], while 24 and 48 h show [NO] after addition of IL-1. Letters denote significant changes in NO within treatments in response to conditioning and time (panel a), or conditioning, time and IL-1 (panel b). —•— denotes significant treatment effect compared with unstimulated controls (panel a) or stimulated controls (panel b). Changes are significant when $P \leq 0.05$.

of indomethacin did not prevent GAG release from stimulated explants. These results provide evidence for the drug's anti-inflammatory activity, and are consistent with the effects of *nondigested* indomethacin in isolated chondrocyte preparations (Panico *et al.*, 2006), and cartilage explants (Arsenis & McDonnell, 1989). The present results also support previous reports that indomethacin does not prevent significant proteoglycan degradation in inflammatory conditions (Rainsford *et al.*, 1999). In addition to providing support for previously reported effects of indomethacin on cartilage metabolism, the data also demonstrate that the simulated digestion/ultrafiltration protocol

does not mute anti-inflammatory potential of treatments. Thus, the methodology can be applied to other dietary anti-inflammatories to evaluate their effectiveness in modulating the inflammatory response to IL-1.

The endpoints we selected to evaluate the effectiveness of indomethacin and MB against IL-1-induced inflammation were GAG, NO and PGE₂, all of which are elevated by stimulation of chondrocytes by IL-1. GAGs are released from cartilage explants as a result of IL-1 stimulation (Frean *et al.*, 2002), because of IL-1-dependent induction of proteases involved in matrix catabolism by chondrocytes (McGuire-Goldring *et al.*, 1984; Schnyder *et al.*, 1987; Inoue *et al.*, 2005). An increase in media [GAG] in cartilage explant models reflects a net increase in proteoglycan breakdown.

Prostaglandin E₂ and NO are closely linked in the biological processes of inflammation and pain. PGE₂ was first described in 1935 as a potent vasodilator and stimulant of smooth muscle contraction (Von Euler, 1935). Since then it has been implicated in a wide range of pathophysiological actions within articular tissues including inhibition of collagen synthesis through interactions with IL-1 (Rapala *et al.*, 1997), stimulation of matrix metalloproteinase (MMP) expression and activity (Zahner *et al.*, 1997), amplification of IL-1 expression through stimulation of cAMP (Lorenz *et al.*, 1995), induction of chondrocyte apoptosis (Miwa *et al.*, 2000) and initiation and propagation of pain. Pain responses to PGE₂ are believed to be predominantly elicited through the interaction of PGE₂ with EP1 and EP3, two of its four cellular receptors (Minami *et al.*, 2001; Ueno *et al.*, 2001). Interactions of PGE₂ with EP1 increases intracellular Ca²⁺ (Nakayama *et al.*, 2004), which leads to activation of inducible nitric oxide synthase (iNOS) and formation of NO (Matsumura *et al.*, 2005). Additionally, interaction of PGE₂ with EP3 causes a translocation of iNOS to the membrane of the cell, which also increases formation of NO (Matsumura *et al.*, 2005). NO is a ROS which plays a fundamental role in the pathogenesis of cartilage inflammation. NO has a number of pro-inflammatory consequences in arthritic cartilage including inhibition of aggrecan and type II collagen synthesis (Taskiran *et al.*, 1994; Cao *et al.*, 1997), stimulation of MMP activity (Sasaki *et al.*, 1998), induction of chondrocyte apoptosis (Surendran *et al.*, 2006) and enhancement of nociceptive pain transmission (Levy & Zochodne, 2004). Inhibition of NO in cartilage inflammation has been associated with reduced pain and inflammation in humans (Reddy *et al.*, 2005) and animals (Sakaguchi *et al.*, 2004) with articular pain (Cuzzocrea, 2006).

Like simulated digest of indomethacin, MB significantly inhibited IL-1-induced NO production, which would have similar anti-inflammatory implications to those described for indomethacin. However, unlike indomethacin, MB did prevent IL-1-induced increase in GAG release and did not inhibit IL-1-induced PGE₂ production. Indeed, there was dose-dependent increase in media PGE₂ from both unstimulated and IL-1-stimulated explants exposed to MB, which was significant at the higher dose. This suggests that MB does not elicit a PGE₂-mediated suppression of inflammation, and cartilage metabolism is affected along PGE-independent pathways. The observed ability of a

simulated digest of MB to reduce IL-1-dependent NO production is consistent with the ability of several of its principle anti-inflammatory constituents to inhibit iNOS and/or NO production, including *H. procumbens* (Kaszkin *et al.*, 2004; Huang *et al.*, 2006), *Marrubium* sp. (Rigano *et al.*, 2006), *U. dioica* (Harpur *et al.*, 2005), and *S. officinale* (Hu & Kitts, 2005).

Nitric oxide inhibition alone could account for the concurrent inhibition of GAG release observed in the current study, as one of the biological actions of NO is upregulation of MMP activity (Sasaki *et al.*, 1998). However, other mechanisms may be activated which account for the protective effect of MB on proteoglycan catabolism. Extracts of *U. dioica* (Schulze-Tanzil *et al.*, 2002) and *H. procumbens* (Schulze-Tanzil *et al.*, 2004) inhibit MMP-1, 3 and 9 production by human chondrocytes, which may contribute to the observed inhibition of IL-1-induced GAG release.

It is unknown which herb, or phytochemical within the herb, is responsible for the inhibitory effect on NO production and GAG release in the current study. *Harpagophytum procumbens* has been the subject of recent scientific inquiry (Chrubasik *et al.*, 2004; Gagnier *et al.*, 2004; Huang *et al.*, 2006), with early research pointing to 'harpagoside' as the putative active phytochemical (Eichler & Koch, 1970). It is recognized that the anti-inflammatory activity of *H. procumbens* cannot be entirely accounted for by the actions of harpagoside (Lanhers *et al.*, 1992; Kaszkin *et al.*, 2004). However, stomach acid mutes the bioactivity of *H. procumbens* (Lanhers *et al.*, 1992; Soulimani *et al.*, 1994). If indeed *H. procumbens* is contributing to the observed bioactivity of MB, it can only be via a phytochemical that is resistant to, or activated by, interactions with digestive enzymes and/or pH. It is likely that the observed effect of MB resulted from combined, or perhaps synergistic activities of the other herbal ingredients. Preliminary studies in our lab have shown that MB without *S. officinale* did not show the same anti-inflammatory activity as MB with *S. officinale* (W. Pearson, M. W. Orth and M. I. Lindinger, unpublished data), providing support for an important role of this herb in the bioactivity of MB. This raises questions about the safety of MB when fed to livestock or small animals, because of the well-known hepatotoxicity associated with ingestion of *S. officinale* (Mei *et al.*, 2005, 2006). MB utilizes the leaf portion of the *S. officinale* plant, which contains significantly lower concentrations of the hepatotoxic pyrrolizidine alkaloids per gram of plant tissue than the root portion (Betz *et al.*, 1994). For a 500 kg horse receiving a daily maintenance dose of MB, the daily intake of *S. officinale* would be approximately 11 g (Betz *et al.*, 1994), with an approximate daily intake of pyrrolizidine alkaloids of 0.1 µg/g (1.1 g; 2.2 mg/kg). A 10-year history of MB use in horses without a single adverse report associated with liver dysfunction suggests this is not a sufficient dose to induce acute toxicity. It is recommended that acute and chronic safety studies be undertaken with MB, with plasma gamma glutamyl-transferase as a primary endpoint (Curran *et al.*, 1996) to establish safety of MB in horses.

Product consistency and quality is a major concern in the nutraceutical industries (Oke *et al.*, 2006; Van Breemen *et al.*, 2007). MB is produced in a facility which is Hazard Analysis and

Critical Control Point and Good Manufacturing Practices certified, providing assurance of the botanical ingredients within MB. However, given that botanical composition can be sensitive to a number of factors including time of harvest (Hudaib *et al.*, 2002), and drying and storage conditions (Yang, 2002), the phytochemical profile of MB may differ from batch to batch. Thus, it is recommended that a marker bioactive compound be selected (e.g. harpagoside) and used as a measure of product standardization.

LIMITATIONS OF THIS STUDY

Although data obtained using our simulated digestion/ultrafiltration/explant model may more accurately represent the *in vivo* situation than traditional cartilage explant alone, they should still be viewed with some caution. Some persisting limitations of our methodology include:

- The digestible portion of the product is assumed to be 100% bioavailable in the animal. There is currently no information on the absorption of the various constituents of MB in the horse. Pharmacokinetic information on a few candidate bioactive phytochemicals within MB would be very useful in reducing the impact of this limitation.
- Species differences in digestion, gastrointestinal absorption and/or distribution is assumed to be of negligible importance in the bioactivity of the plant material. Our simulated digestion protocol provides a basic pH and enzyme profile of the human upper gastrointestinal tract. As such, there may be differences in how plant material is assimilated in different species. An interesting possibility for further research would be to extract gastric juice from the stomach and upper intestine from the horse and use it directly to digest the plant material *ex vivo*, and observe any differences in the bioactivity of the simulated digest in the cartilage explant model.
- The active constituents of the product are assumed to be substantively unaltered by biotransformation in the liver. This limitation could be addressed by introducing an S9 fraction of liver microsomes into the model, which may provide additional information on biotransformation products of the plant material.
- The digested and absorbed product is assumed to be evenly distributed throughout the total body water compartment of the animal, and is not preferentially sequestered into any particular tissue or cell-type. Substantial phytochemical characterization of the ingredients of MB would be required, followed by pharmacokinetic studies in horses, before this limitation could be addressed.
- 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.

Although 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.

CONCLUSIONS

The data generated from this experiment show that simulated digest of indomethacin is an effective inhibitor of PGE₂ and NO, but does not protect against IL-1-induced GAG release. MB has demonstrated a significant protective effect against IL-1-induced GAG release and NO production, suggesting it may be a useful adjunct to the pain-relieving properties of indomethacin or possibly other NSAIDs which are lacking in chondroprotective activity. However, the significant increase in PGE₂ in unstimulated, MB-exposed explants suggest that this product is not indicated for use as a prophylactic in healthy animals, and there is a narrow safety margin with respect to PGE₂ production – animals should not be provided with MB doses in excess of recommended amounts. Substantially more research is required to further define the role that MB can play in modulating the inflammatory response in arthritis. As a minimum, acute and chronic safety must be demonstrated in horses, followed by either a clinical cohort study or laboratory *in vivo* cartilage inflammation model in horses as a measure of clinical efficacy. It is also important to conduct pharmacokinetic and pharmacodynamic studies on the major phytochemical constituents of MB, which would increase information available for standardizing and optimizing dose.

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