A dietary nutraceutical reduces synovial fluid PGE₂ in horses with osteoarthritis: a double-blind randomized trial

Summary

Reasons for performing study: A dietary nutraceutical product (Sasha’s EQ™: DN) has previously demonstrated prophylactic PGE₂-inhibitory effects in IL-1-stimulated cartilage explants and in horses with experimentally-induced articular inflammation. The current study was undertaken in order to evaluate the therapeutic effect of including DN in the diet of horses with articular inflammation and/or cartilage damage secondary to osteochondral fragmentation of the carpal or metacarpal joints.

Hypothesis or Objectives: It was hypothesized that inclusion of DN in the diet of horses immediately following surgical removal of osteochondral fragment would reduce synovial fluid prostaglandin E₂ (PGE₂), nitric oxide (NO) and glycosaminoglycan (GAG), while improving clinical signs of articular inflammation in these horses.

Methods: Fifteen horses presenting at an equine hospital for surgical removal of an osteochondral fragment of the carpal or metacarpal joints were included. Horses received DN (0 or 21 g/day) for 42 days beginning immediately after surgery. Synovial fluid pre- and post-supplementation was analyzed for PGE₂, GAG, and NO. Radiographs and lameness assessments were also obtained.

Results: Synovial fluid PGE₂ was significantly reduced from baseline in horses receiving DN, but not in those horses treated with surgery alone. There was no difference between treatments on GAG, NO, radiographs or lameness grade.

Conclusions: These data support previously published experimental evidence of an inhibitory effect of DN on synovial fluid PGE₂. In the current study, DN effectively reduced synovial fluid PGE₂ in horses with surgical removal of osteochondral fragments.

Potential relevance: DN may be a useful post-surgical treatment for elevated PGE₂ in horses with surgical removal of osteochondral fragments.
Introduction
Sasha’s EQ\textsuperscript{a} (DN) is a dietary nutraceutical product composed of New Zealand green lipped mussel [NZGLM (Perna canaliculus)], abalone [AB (Haliotis sp.)], marine cartilage [MKC (Galorhinus galeus)], and a proprietary lipid extract of Biota [BO (Biota orientalis)]. This product has been extensively studied with respect to its anti-inflammatory activity in a cartilage model of inflammation and has demonstrated an inhibitory effect on interleukin 1β (IL-1)-induced prostaglandin E\textsubscript{2} (PGE\textsubscript{2}), and nitric oxide and loss of cartilage glycosaminoglycan (GAG) \cite{1, 2}. In subsequent studies, DN provided as part of the diet inhibited synovial fluid PGE\textsubscript{2} and GAG produced in response to 110ng intra-articular IL-1 \cite{3}. Dietary DN did not produce adverse effects in biochemistry or hematology screens at doses up to 75g/horse/day \cite{3}.

The previous in vitro and in vivo studies provide rationale for the use of the product in clinical situations that may benefit from reduction of elevated synovial fluid PGE\textsubscript{2}. Thus, the objective of the current study was to evaluate the post-surgical effect of DN in horses that have undergone osteochondral fragment removal from the intercarpal, metacarpophalangeal and/or radiocarpal joints.

Methods
All reagents were purchased from Sigma Aldrich\textsuperscript{b} unless otherwise noted.

Inclusion criteria
Informed consent of horse owners; males or non-pregnant females; between 3-15 years old; body weight between 450 – 550 kg; osteochondral fragment localized to an intercarpal, radiocarpal and/or metacarpophalangeal joint removed by arthroscopic surgery; not receiving routine non-steroidal anti-inflammatory drugs or corticosteroids (systemic or intra-articular) within 2 weeks of entering the trial.

Exclusion criteria
Lameness grade over 3 (AAEP scale); synovial fluid PGE\textsubscript{2} concentration < 200 pg/mL; horse owner not willing to eliminate routine non-steroidal anti-inflammatory drugs.

Inclusion assessment
Upon receiving informed consent of owners of horses meeting inclusion criteria, an inclusion assessment was performed. Lameness grade and flexion test were assessed by the attending veterinarian (NC). Radiographs of the affected joint were taken from at least 2 perspectives.

Randomization and blinding
Fifteen identical containers containing placebo (6 containers) or DN (9 containers) were labelled A-1, A-2, A-3, B-1, B-2, B-3, C-1, C-2, C-3, D-1, D-2, D-3, E-1, E-2, or E-3 by a person not involved in assessment of horses, treatment of horses, analysis of samples or analysis of data. The key was written down and sealed in an envelope which was not opened until after analysis of Day 42 data. Containers were placed in random order into a box; horses successfully completing the inclusion assessment were randomly assigned to receive either DN (21g of finished product/day for 42 days;
n=10) or an inactive placebo (Rice bran with raspberry and molasses flavours; 21g of finished product/day for 42 days; n=5).

Administration of the assigned supplement to the trial horses was the responsibility of the horse owners. If a single dose was forgotten, owners were permitted to double the dose on the next consecutive day. If they missed 2 consecutive doses, they were permitted to triple the dose on the next consecutive day. If they missed 4 doses, the horse was to be removed from the trial.

**Concurrent medications**
Treatment of lameness in trial horses with non-steroidal anti-inflammatory drugs (NSAIDs) was permitted, but only on the recommendation of the attending veterinarian. Details of all medications administered to horses were recorded. Treatment of illness or injury not related to the trial was permitted, but only on the recommendation of the attending veterinarian. Provided there were no anti-inflammatory properties of the drugs used, there was no limit to duration of treatment.

**Study protocol**
The number of animals was calculated using the Power of Analysis Test using a standard deviation for PGE$_2$ of 141 pg/mL [4]. PGE$_2$ was selected as the primary outcome variable from amongst inflammation markers because we have reported significant inhibition of PGE$_2$ in our previous *in vitro* and *in vivo* studies with DN. Use of PGE$_2$ as a primary dependent variable allows for detection of $\geq 250$ pg/mL minimum difference between means at $p \leq 0.05$ with a power of 0.8. A minimum of 6 animals per group was required. For this study we recruited 6 horses into the placebo control group, and 9 horses into the DN treatment group for a total of 15 horses. We increased the number of horses in the treatment group in order to buffer an unknown variability in response to the treatment.

Fifteen horses successfully completing the inclusion assessment (see INCLUSION ASSESSMENT above) were recruited from clients of an established equine hospital (Milton Equine Hospital, Campbellville ON Canada). Nine (9) horses received DN (21 g/day; see Table 1 for composition) once daily and 6 horses received an inactive placebo (21 g/day; see Table 1 for composition) for 42 days (see RANDOMIZATION AND BLINDING above). Horses were identified throughout the trial by their given name. Names were converted to numbers for the purpose of reporting in order to preserve confidentiality of participants.

*Baseline data collection:* upon successful completion of the inclusion assessment, detailed information was collected on each horse. This included diet (concentrate and hay; amounts and analyses), daily exercise regimen, housing and turnout, any allopathic medications currently administered, vitamins, mineral supplements, any herbal products or nutraceutical products. Lameness at the trot was scored utilizing the American Association of Equine Practitioners - protocol (AAEP) and flexion test was conducted. Joint circumference was measured with a flexible tape.

During surgical removal of the chip fracture under general anaesthesia, synovial fluid was aseptically aspirated and placed into a heparin-coated vacutainer and immediately refrigerated (4°C). Synovial
Fluid was then centrifuged (1500 x g; 15 minutes) to remove cellular debris. The cell pellet was removed, and the remaining supernatant was stored at -80°C until analysis for PGE2 (ELISA), nitric oxide (NO; Griess Reaction) and glycosaminoglycan (GAG; dimethyl methylene blue spectrophotometric assay).

**Follow-up data collection**

On day 42, radiographs were taken of the affected joint from at least 2 perspectives. Lameness at the trot (AAEP) and after flexion test was graded, and joint circumference was measured using a flexible tape. The surface of the affected joint was aseptically prepared and a sterile 22 gauge needle was inserted. A sterile 3cc syringe was attached to the needle hub and approximately 1 mL of synovial fluid was aspirated and collected in a sterile heparin-coated vacutainer tube. The vacutainer was placed on ice. Subsequently, synovial fluid was centrifuged and stored at -80°C until analysis.

**Synovial fluid biomarker analysis**

PGE2
Synovial fluid was diluted 1:5 with dilution buffer provided as part of the ELISA kit. PGE2 was then quantified according to kit protocol. Microtitre plates from the ELISA kit were coated with sheep anti-mouse PGE2 antibody. There are no known differences in arachidonic acid derivative structure between species, so the assay can be appropriately applied to equine samples. Plates were read on a microplate reader with absorbance set at 450nm. A best-fit 3rd order polynomial standard curve was developed for each plate ($R^2 \geq 0.99$), and the equations used to calculate PGE2 concentrations for samples from each plate.

GAG
GAG concentration of synovial fluid was determined directly using a 1,9-Dimethyl Methylene Blue (DMB) spectrophotometric assay [3]. 1,9-DMB (4mg) was dissolved in 100% ethanol (1.25 mL), combined with sodium formate (0.5 g) and formic acid (0.5 mL), then made up to 250 mL with double distilled water (ddH2O) (pH 6.5; DMB Reagent). Samples were diluted 1:3 with dilution buffer (4.1 mg sodium acetate and 0.5µL Tween 20 per mL ddH2O) and placed into a 96-well microtitre plate. Guanidine hydrochloride (275 g/L in ddH2O) was added to each well followed immediately by addition of 150 µL DMB reagent. Plates were incubated in the dark for 10 minutes, and absorbance read at 530 nm. Sample absorbance was compared to that of a bovine chondroitin sulfate standard. A best-fit linear standard curve was generated for each plate ($R^2 \geq 0.99$), and the equations used to calculate GAG concentrations for samples on each plate.

NO
Nitrite (NO²), a stable oxidation product of NO, was analyzed by the Griess reaction [3]. Undiluted synovial fluid 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) was
added to all wells, and absorbance was read within 5 minutes at 530 nm. Sample absorbance was compared to a sodium nitrite standard.

Statistics
The experimental unit was affected joints. Thus, when a horse presented with arthritis in multiple joints, samples from each joint were analyzed and treated as discrete units. Data are presented as mean ± SEM. Statistical analysis of data was conducted using a 2-way repeated measures ANOVA (with respect to time and treatment), and significance was set at p<0.05. The study is statistically powered (0.8) to detect a minimum difference of 250 pg/mL PGE₂, assuming a population standard deviation of 141 pg/mL.

Results

Experimental cohort
The trial ran from Nov 2008 through Jan 2010. Thirteen horses completed the trial (n=7 DN; n=6 CON). Table 2 provides a description of the experimental horses. Eleven were racing American Quarter Horses (n=6 DN; n=5 CON) and 2 were racing Standardbreds (n=1 DN; n=1 CON). Data from horses lost to follow-up were not included in the analysis. Analysis was performed on 7 DN horses and 6 control horses.

Concurrent medications
None of the trial horses received any systemic or intra-articular medications at any time during the trial, or in the 2 weeks prior to inclusion in the trial. No ‘other drugs’ or nutraceutical/herbal supplements were fed during the trial or in the 2 weeks prior to the trial.

Exercise
Prior to surgery, 7 horses were on paddock rest (n=4 DN; n=3 CON), 5 horses maintained their normal training schedule (n=3 DN; n=2 CON), and one horse (n=1 CON) was on stall rest. After surgery, all horses were on stall rest for 12 days followed by 4-6 weeks of hand-walking.

Management and diet
Ten of the 13 horses completing the trial were presented by 2 trainers. Trainer A provided 3 horses (n=2 DN, n=1 CON) and Trainer B provided 7 horses (n=4 DN; n=3 CON). This assisted in standardizing the conditions under which the horses were managed. Feed for these horses were consistent within their respective training yards, with the exception that 2 horses from Trainer B (n=1 DN; n=1 CON) received only hay without any grain ration.

Synovial fluid biomarkers

PGE₂
PGE₂ concentrations between CON and DN horses were not different at baseline (CON: 563.2 ± 58.6 pg/mL; DN: 647.7 ± 81.9 pg/mL; p=0.4). At Day 42, PGE₂ in CON horses was unchanged (480.3 ±
66.9 pg/mL; (p=0.3), while DN horses showed a significant decrease in PGE$_2$ (418.0 ± 31.9 pg/mL; p=0.03) (Figure 1).

**GAG**

Synovial fluid GAG was significantly higher in CON horses (26.2 ± 1.4 µg/mL) than in DN horses (DN: 22.7 ± 1.0 µg/mL) at baseline (p=0.05). 2-way analysis detected a significant decline in synovial fluid GAG by Day 42 when diet was removed from the model (p=0.02) but the two treatment groups were not different from each other (p=0.3; Figure 2).

**Nitric oxide (NO):**

NO concentrations between CON and DN horses were not different at baseline (CON: 0.60 ± 0.07 µg/mL; DN: 0.61 ± 0.12 µg/mL; p=0.9). There was no significant decline in NO in either treatment group (Figure 3).

**Other outcome measures**

All 13 horses recruited were diagnosed with osteochondral fragment off a radial, intercarpal or metacarpophalangeal joint surface either by radiography or surgery or both. Surgical and radiographic findings at baseline and follow up are provided in Table 3.

Mean joint circumference was not different between CON (28.7 ± 1.26 cm) and DN horses (28.1 ± 1.37 cm) at baseline. Joint circumference was significantly increased in both CON (29.7 ± 1.31 cm) and DN (30.5 ± 0.83 cm) horses at Day 42 compared to baseline, but the groups were not different from each other (p=0.9).

Two (2) horses scored a positive flexion test at follow-up (DN: n=2).

**Discussion**

The experimental cohort recruited into this study was standardized as much as possible with respect to breed, training regimen, feed and management and localization of the injury. Potentially confounding factors such as diet, exercise and breed were controlled for as much as possible, allowing the CON and DN groups to be reasonably balanced.

This trial has provided further support for the ability of DN to modulate inflammatory biomarkers in horses with osteoarthritis. Previous *in vitro* [1, 2] and *in vivo* [3] studies have focused on a preventive and therapeutic effect of DN on IL-1-induced PGE$_2$ production, and the current study demonstrates that DN also has a therapeutic effect on pre-existing elevation of synovial fluid PGE$_2$.

PGE$_2$ is a pro-inflammatory eicosanoid that is substantively up-regulated in inflammatory conditions such as arthritis [5]. PGE$_2$ plays a central regulatory role in initiation and progression of arthritis [4] and continues to be the primary pharmacological target for treatment of pain and inflammation associated with pre-existing arthritis [6]. The mechanisms by which PGE$_2$ is produced during initiation of inflammation and during progression of existing inflammation are multifaceted but
highly conserved, which offers a possible explanation for apparent inflammatory remission followed by flare-ups [7]. Thus, products such as DN that offer protection from initiation of inflammation via inhibition of synovial fluid PGE$_2$ can be predicted to provide a therapeutic effect for pre-existing inflammation through the same process. This hypothesis has been supported by the current study, as demonstrated by a significant reduction in synovial fluid PGE$_2$ 42 days after surgical removal of an osteochondral fragment in horses which were treated with DN; a reduction that was not seen in control horses which were treated with surgery alone.

Synovial fluid NO was not reduced in the current study, neither by surgery alone nor by surgery followed by supplementation with DN. This is not consistent with previous *in vitro* research that clearly demonstrated an inhibitory effect of DN on IL-1-induced NO production. This may be because peak NO concentration *in vitro* (~1.3 µg/mL) were more than double peak NO concentrations (~0.6 µg/mL) in the current study. DN reduced IL-1-induced NO to approximately 0.6 µg/mL *in vitro*, similar to peak NO in the current study.

**Conclusions**

It is concluded from the present study that DN significantly inhibits post-surgical synovial fluid PGE$_2$ in horses that have recently undergone osteochondral fragment excision. These data support the use of the product as a post-surgical strategy for reducing inflammatory and degenerative consequences of aberrantly elevated intra-articular PGE$_2$.

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**Manufacturers Addresses**

a) Interpath Pty Ltd. PO Box 723, Ballarat, Victoria Australia 3350
b) Sigma Aldrich Canada Ltd. 2149 Winston Park Dr. Oakville, Ontario L6H 6J8 Canada
c) R&D Systems, Cedarlane Laboratories, Burlington Ontario Canada
d) ELX 800 Universal Microplate Reader. Biotech Instruments Inc., Winooski, VT)
**Figure 1:** Synovial fluid PGE$_2$ (pg/mL) in Control (n=6) and Sasha’s EQ (DN; n=9) horses. Horses underwent surgical removal of an osteochondral fragment on Day 0 and received their allocated dietary supplement for 42 days following surgery. * denotes significant change from baseline.

**Figure 2:** Synovial fluid GAG (µg/mL) in Control (n=6) and Sasha’s EQ (DN; n=9) horses. Horses underwent surgical removal of an osteochondral fragment on Day 0 and received their allocated dietary supplement for 42 days following surgery.
**Figure 3:** Synovial fluid NO (µg/mL) in Control (n=6) and Sasha’s EQ (DN; n=9) horses. Horses underwent surgical removal of an osteochondral fragment on Day 0 and received their allocated dietary supplement for 42 days following surgery.

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**References**