Mitigation of Antibiotic-Induced Toxicity in Equine Chondrocytes by Soybean/Glucosamine/Chondroitin Combination

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Abstract

Objectives The aim of this study was to evaluate the effect of amikacin (AK) and enrofloxacin (EF) at concentrations consistent with those obtained by intra-articular and intravenous regional limb perfusion on both cytotoxicity and prostaglandin E2 (PGE2) production by equine chondrocytes. This study also determines if PGE2 production could be reduced by avocado/soybean unsaponifiables (ASU), glucosamine (GLU) and chondroitin sulphate (CS).

Study Design Chondrocytes were grown in monolayer from the articular cartilage of 12 horses and treated with clinically relevant concentrations of AK and EF, with or without the combination of ASU + GLU + CS. Positive controls consisted of chondrocytes that were activated with lipopolysaccharide (LPS). Chondrocyte response was evaluated using both MTT cytotoxicity assay and immunoassay for PGE2 production.

Results Amikacin and EF generated a dose-dependent cytotoxicity. Amikacin induced 90% cell death at a concentration of 25 mg/mL. Enrofloxacin induced 90% cell death at 1.0 mg/mL and 98% cell death at 10 mg/mL (p < 0.05). Amikacin failed to induce PGE2 production at any of the concentrations studied. In contrast, EF and the positive control (LPS) induced PGE2 production at all concentrations. Induction of PGE2 by EF at all concentrations was significantly reduced (p < 0.05) by pre-treatment with ASU + GLU + CS.

Conclusions and Clinical Relevance Horses receiving commonly used dosages of AK and EF may benefit from administration of ASU + GLU + CS.
Introduction

Horses often receive antibiotic medications via intra-articular administration and intravenous regional limb perfusion (IVRLP) for the prevention and treatment of septic arthritis. The effect of the higher antibiotic concentrations achieved by these routes has been associated with chondrocyte death. Chondrocyte death from antibiotic medication use in cartilage explants has been reduced with simultaneous corticosteroid administration. The use of nutraceuticals to minimize the negative impact of antibiotic medications at these higher concentrations has not been studied. Nutraceuticals offer an advantage over corticosteroids in the treatment of sepsis by virtue of their immunomodulatory and anti-inflammatory effects without potentiating sepsis or causing chondrocyte cell death.

Aminoglycosides, most commonly gentamicin and amikacin (AK), are routinely used in horses for the treatment and prevention of joint sepsis. The bactericidal effects of aminoglycosides are concentration dependent, with higher bactericidal effects occurring at higher peak concentrations. Higher antibiotic concentrations are particularly important when treating infections caused by less susceptible pathogens. Taintor and colleagues demonstrated that 500 mg of AK administered into a normal joint (~50 mg AK per mL joint fluid at onset) maintains the AK concentration above the minimum inhibitory concentration (MIC) for most equine pathogens (i.e. 4 µg/mL) for 72 hours, but when the same dose is administered into an inflamed joint, the concentration is maintained above MIC for only 48 hours. Interestingly, IVRLP for inflamed radiocarpal joints resulted in a higher maximal cAK concentration (Cmax) (144.48 ± 43.17 µg/mL) than in normal radiocarpal joints (60.02 ± 28.81 µg/mL; p = 0.0301). Finally, Cmax appears to be not only dependent upon which joints and what technique of regional limb perfusion are being evaluated but also on individual horses. One study demonstrated a median Cmax for AK by IVRLP of the distal interphalangeal joint of 600 µg/mL with a range of 37 to 2,400 µg/mL. Although AK appears to have no gross inflammatory effect when administered via regional limb perfusion, this is not true when the AK is repetitively administered via multiple intra-articular injections. Also, treatment of cartilage explants with AK results in an increase in chondrocyte death.

Enrofloxacin (EF), a fluoroquinolone, has an appropriate antimicrobial spectrum and a large volume of distribution that is advantageous for the treatment of orthopaedic infections. Pharmacokinetic studies of EF after oral and intravenous administration have produced concentrations of 1 to 5 µg/mL in peripheral tissues. An early study on the use of EF for regional limb perfusions demonstrated synovial fluid concentrations for EF ranging from 7 to 216 µg/mL. Beluche and colleagues showed that concentrations of EF consistent with systemic (intravenous) doses did not suppress equine chondrocyte growth, but higher concentrations (>1,000 µg/mL) were toxic to chondrocytes, could eliminate proteoglycan synthesis and cause significant cell death. Adverse effects of EF have been observed on articular cartilage in a variety of species, which are likely the result of irregular integrin signalling and subsequent cellular changes. In vitro studies have demonstrated that antibiotic medications can alter proteoglycan synthesis and contribute to proteoglycan loss in chondrocytes, potentially through pro-inflammatory mediators. For example, excessive production of prostaglandin E2 (PGE2) can lead to reduced proteoglycan synthesis and may indirectly contribute to proteoglycan loss by inducing interleukin-1 (IL-1) expression. Prostaglandin E2 is an important mediator in the pathological process of arthritis and cultured chondrocytes respond to IL-1 with enhanced expression and activity of cyclooxygenase-2 (COX-2). Palliative relief in affected horses is attributable in part to the reduction in PGE2 synthesis. The production of PGE2 has previously been used as an inflammatory biomarker and a negative outcome variable in vivo studies of nutraceuticals on experimentally induced synovitis. Au and colleagues demonstrated the ability of avocado/soybean unsaponifiables (ASU) to alter chondrocyte PGE2 production and suppress TNF-α, IL-β and COX-2. Therefore, ASU has the ability to reduce several markers of inflammation and key components of osteoarthritis (OA). The combined anti-inflammatory effects of ASU with the chondroprotective characteristics of glucosamine (GLU) and chondroitin sulphate (CS) are the basis for the modulators of inflammation for our study. This combination is one of the few products available that is commonly used in the horse for the prevention or treatment of OA that has no known adverse immunological effects (unlike corticosteroids or polysulphated glycosaminoglycan). Despite aggressive treatment, horses that have had septic arthritis often develop a secondary OA that precludes their use as performance animals. The destruction of articular structures by the septic process has long been acknowledged; however, the extent to which antibiotic medications can contribute to joint degradation in a septic or osteoarthritic joint has not been thoroughly evaluated. Evaluating the ability of AK and EF to induce PGE2 and cytotoxicity in chondrocyte monolayer culture serves as a sensitive means to screen these antibiotic medication’s potential adverse effects on equine joints. Therefore, the objectives of this study were to clarify the role of AK and EF in inducing cell death and inflammation and to see if these adverse events could be reversed with ASU + GLU + CS.

Materials and Methods

Cell Culture

Carpal joints were collected from 12 horses which included nine quarter horses, two thoroughbreds and one Tennessee walking horse. Ages ranged from 2 to 23 years of age. All horses were donated for reasons unrelated to carpal lameness. Chondrocytes were harvested from joints that were clinically normal and had normal synovium, joint fluid, articular cartilage and surrounding structures at the time of collection. All procedures were approved by the Animal Care and Use Committee of Mississippi State University (Protocol# 08–402). Retrieved cartilage from equine (n = 12) radiocarpal and middle carpal joints were aseptically diced into <5 mm pieces and digested in type II collagenase (110 U/mL) for 12 to 18 hours at 37°C, 5%
CO₂. The combined chondrocytes were next filtered through a wire mesh screen, rinsed four times with Hank’s Balanced Salt Solution (ATCC; Manassas, Virginia, United States) and viability was assessed using the Trypan-blue exclusion method. Chondrocytes were propagated in monolayer culture until ready for use as previously described.²³

**Phenotype Analysis by Immunohistochemistry and Western Blot Analysis**

Chondrocytes on microscope slides fixed with 10% paraformaldehyde were incubated with goat anti-type I collagen, anti-type II collagen or anti-aggrecan antibodies (Southern Biotechnology Associates; Birmingham, Alabama, United States) as previously described.²³ The slides were washed in buffer three times and incubated with fluorescein isothiocyanate (FITC) labelled anti-goat antibodies to visualize immunostaining using a Nikon Eclipse epifluorescent microscope (TE200). Secreted collagen and aggrecan in spent culture media were electrophoresed on 4 to 15% sodium dodecyl sulphate-polyacrylamide gels then electrophoretically transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, California, United States) in tris-glycine buffer, pH 8.5, containing 20% methanol. Polyvinylidene difluoride membranes were then processed for immunostaining using goat anti-collagen type II, type I or anti-aggrecan antibodies (Southern Biotechnology Associates) in combination with an alkaline phosphatase labelled rabbit anti-goat antibody with 5-bromo-4-chloro-3-indolylphosphate-nitroblue tetrazolium (Gibco Invitrogen Carlsbad, CA, United States) as the substrate.

**Experimental Design**

Antibiotic concentrations consistent with physiological concentrations following intra-articular administration (25 mg/mL at onset for AK and 10 mg/mL at onset of administration for EF) were used as the highest concentrations tested. Because studies differ in the achievable concentrations obtained by regional limb perfusion methods,⁸ the presence or absence of joint inflammation⁷ and the specific joints perfused,⁸ ⁹ we elected to perform two 10-fold dilutions of the above concentrations to incorporate a range of concentrations achieved by IVRLP. Therefore, AK was tested at 25, 2.5 and 0.25 mg/mL and EF was tested at 10, 1 and 0.1 mg/mL.

**MTT Cytotoxicity-Cell Proliferation Assay**

MTT ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)) assay kit (Cayman Chemical; Ann Arbor, Michigan, United States) was used to determine whether EF or AK induced cytotoxicity. Chondrocytes harvested from monolayer cultures were seeded into 24-well (1 × 10⁵) plates and were incubated for 24 hours. Cells were next treated with control media alone (Sigma-Aldrich), EF (0.1–10 mg/mL, Sigma) or AK (0.25–25 mg/mL, Sigma). All plates were incubated for an additional 24 hours to determine cytotoxicity using the MTT assay. Ethanol solution (10%) was added to triplicate chondrocyte-seeded wells as a positive control of cell death. The cellular supernatant was aspirated and Hanks balanced salt solution (HBSS) (1 mL) and MTT (100 μL) were then added to each well. Plates were incubated at 37°C, 5% CO₂ for 3 hours and then 1 mL of MTT solubilization solution was added and thoroughly mixed at room temperature for 15 minutes. A 200 μL aliquot was plated in triplicate into a 96-well plate and absorbance was measured using a Spectramax Plus (Molecular Devices; Sunnyvale, California, United States) at a double wavelength of 570 and 690 nm.

**PGE₂ High Sensitivity Immunoassay**

Chondrocytes were seeded into six-well (5 × 10⁵) tissue culture plates for 24 hours and were treated with control media alone, positive control of lipopolysaccharide (LPS, 20 ng/mL; Sigma-Aldrich),¹⁹ EF (0.1–10 mg/mL, Sigma) or AK (0.25–25 mg/mL, Sigma). All plates were incubated for another 24 hours to measure secreted PGE₂ levels by commercial immunoassay kit (R&D Systems; Minneapolis, Minnesota, United States) according to manufacturer’s instructions. A PGE₂ standard was run in parallel to the supernatant samples. Optical density was measured immediately using the SpectraMAX 340 microplate reader (Molecular Devices) at 405 nm with wavelength correction set between 570 and 590 nm.

**Inflammation Mitigation**

Additional monolayer cultures were treated with control media alone, positive control of LPS (20 mg/mL; Sigma-Aldrich), EF (0.1–10 mg/mL, Sigma) and a combination of ASU + GLU + CS NMX1000 ASU, GLU (FCHG49 GLU and CS (TRH122 CS, Nutramax Laboratories, Inc. Edgewood, Maryland, United States). The concentration of ASU used in the present study was (8.3 μg/mL) and was selected from earlier in vitro work showing significant anti-inflammatory effects. The concentrations of the nutraceuticals used in this study were previously shown to exert significant biological effects primarily as anti-inflammatory agents.²¹,²³–²⁹

**Statistical Analysis**

Data are presented as the mean ± 1 standard deviation. Pair-wise multiple comparisons were performed using Student–Newman–Keuls one-way analysis of variance and Tukey post-hoc using SigmaStat statistical software where p < 0.05 was considered significant.

**Results**

**Phenotype Characterization of Chondrocyte Monolayer Culture**

Equine chondrocytes proliferated with ease in monolayer culture with 100% viability. The doubling time for monolayer cultures was 3 to 5 days chondrocytes propagated on monolayer cultures at passage 3 showed heterogeneity in morphology from elongated, spindle-shaped to more rounded shapes, and the cells produced the extracellular matrix components aggrecan and type II collagen (►Fig. 1A and 1B respectively). Production of type II collagen was further verified by Western blot (►Fig. 1C). Chondrocyte cultures showed negligible production of type I collagen. The high molecular weight aggrecan protein did not enter the gel and could not be visualized on Western blot.
The Cytotoxic Effect of Amikacin and Enrofloxacin on Equine Chondrocytes
Both AK and EF have a dose-dependent cytotoxic effect on equine chondrocytes (► Fig. 2). Amikacin failed to induce chondrocyte death at the 0.25 mg/mL concentration; however, chondrocytes treated with 2.5 mg/mL of AK demonstrated 40% cell death, and those treated with 25 mg/mL of AK demonstrated 90% chondrocyte death (p < 0.05). In comparison, the lowest concentration of EF tested (0.1 mg/mL) failed to induce chondrocyte death. However, EF at 1.0 mg/mL demonstrated 90% cell death and 10 mg/mL produced greater than 95% chondrocyte death (p < 0.05).

The Effect of AK and EF on PGE2 Production by Equine Chondrocytes
Chondrocytes treated with AK at 0.25, 2.5 and 25 mg/mL failed to induce PGE2 production when compared with the control chondrocytes during the 24 hours of incubation (► Fig. 3). In contrast, EF dramatically induced the production of PGE2 in a dose-dependent fashion with concentrations of 0.1, 1.0 and 10 mg/mL (p < 0.001, ► Fig. 4). Prostaglandin E2 production following exposure of chondrocytes to EF was significantly greater than PGE2 levels induced by the positive controls (LPS). Treatment of chondrocytes with EF concentration of 0.1, 1.0 and 10 mg/mL resulted in the production of PGE2 at 28,000, 48,000 and 160,000 pg/mL respectively. The response of the different chondrocyte lines to the pro-inflammatory LPS (20 ng/mL) positive control showed variability (► Figs. 3–6). Chondrocytes line 2 PGE2 production was less robust as compared with both line 3 (► Fig. 3) and line 4 (► Figs. 4–6).

Induction of PGE2 synthesis by EF at 0.1 and 1.0 mg/mL (► Fig. 5A and 5B) was significantly (p < 0.05) reduced when chondrocytes were pre-treated with the combination of ASU + GLU + CS. Pre-treatment with ASU + GLU + CS failed to reduce PGE2 production induced by 10 mg/mL of EF (► Fig. 6).

Discussion
Amikacin and EF induced a dose-dependent cytotoxicity and cell death (p < 0.05) at clinically relevant concentrations. The AK concentration of 25 mg/mL, which resulted in cell death, is compatible with injecting 250 mg of AK into the radiocarpal joint of a neonatal foal or the metacarpal phalangeal joint of an

Fig. 1  Representative immunofluorescent staining of collagen and aggrecan in equine chondrocytes. Passage 3, chondrocyte line 1 was stained green for type II collagen and for aggrecan. Note the heterogeneity in morphology from rounded to spindle shape. (A) Immunostaining for aggrecan, (B) immunostaining for type II collagen and (C) Western blot for type II collagen.
Fig. 2  Cytotoxicity of amikacin (AK) and enrofloroxacin (EF) in equine chondrocytes. Both AK and EF have a dose-dependent cytotoxic effect on chondrocytes. Equine chondrocyte line 2, passage 3 was treated with different concentrations of AK or EF as described in Materials Methods section.

Fig. 3  Prostaglandin E2 (PGE2) production (pg/mL) in equine chondrocytes. Cell line 2, passage 3 was incubated with amikacin (AK) at concentrations of 0.25, 2.5 and 25 mg/mL as described in Materials and Methods section. Amikacin failed to induce PGE2 production when compared with the control chondrocytes during 24 hours of incubation. LPS, lipopolysaccharide.

Fig. 4  Prostaglandin E2 (PGE2) production (pg/mL) in equine chondrocytes following the administration of enrofloroxacin (EF) at concentrations of 0.1, 1.0 and 10 mg/mL (* denotes p < 0.05). Enrofloroxacin induced the production of PGE2 in a dose-dependent fashion in chondrocyte line 3, passage 3.
adult horse. Although the 2.5 mg/mL dose, which also resulted in significant cell death, is ~2.5 times the concentration achieved by isolated limb infusion (1 mg/mL), the concentration achievable in an inflamed joint is likely to be greater than double this amount (over 2mg/mL). This indicates that AK should be used judiciously.

Cell death was elicited by EF at a concentration of 10 and 1.0 mg/mL, which is compatible with intra-articular administration. These findings further support that EF should not be administered by the intraarticular route.

None of the AK concentrations evaluated (0.25, 2.5 and 25 mg/mL) induced significant PGE2 production compared with controls. This suggests that the chondrocyte death observed in the cytotoxicity aspect of this study occurred via other mechanisms and either occurred so quickly that PGE2 could not be produced, or that AK simply did not activate these inflammatory pathways and thus could not induce PGE2 production.

Enrofloxacin induced a significant increase in PGE2 at all concentrations tested. The lowest of these concentrations was 0.1 mg/mL which is approximately one half that achieved by IVRLP. Prostaglandin E2 was used in this study as a well-defined marker of inflammation and as a key participant in the pathogenesis of OA. It stimulates the production of degradative enzymes and inhibits the synthesis of cartilage components particularly proteoglycans. It perpetuates the inflammatory response and damage to cartilage by inducing production of other pro-inflammatory mediators. Significant production of PGE2 after exposure to EF demonstrates its ability to induce inflammation and is potentially part of the mechanism by which animals develop arthropathy when EF is administered.

This study also demonstrated that addition of ASU + Glu + CS to cell cultures resulted in a significant reduction in PGE2 concentrations in EF treated chondrocytes. The effects of AK and ASU + Glu + CS treated chondrocytes...
were not evaluated due to the failure of AK to produce PGE2 in this study. The potential benefits of reducing PGE2 production with the addition of ASU + GLU + CS are the preservation of the articular cartilage in the septic joint through reduction of inflammation.

The combination of ASU + GLU + CS was utilized in this study to demonstrate an anti-inflammatory effect by minimizing the production of PGE2. Ongoing research evaluating oral administration of nutraceuticals ASU, GLU and CS individually or in combination indicates that they may have chondroprotective and articular-sparing properties.\textsuperscript{19,20,23–25,34} Avocado/soybean unsaponifiables, GLU and CS have been documented to reduce inflammation in vitro and in vivo.\textsuperscript{19,20,23–25,34} Avocado/soybean unsaponifiables, GLU and CS have also been reported to be beneficial in the management of OA in man and animals with minimal adverse side effects.\textsuperscript{24,25,35} The concentrations of GLU, CS and ASU used in the present study were drawn on previous bioavailability studies and on the in vitro as well as in vivo studies. Bioavailability was earlier studied in horses.\textsuperscript{36} Glucosamine HCl (20 mg/kg) administered by nasogastric intubation (NG) resulted in 6 µM (1 µg/mL), and by intravenous injection (IV) in 300 µM (50 µg/mL). Synovial fluid concentrations reached 9 to 15 µM with IV dosing and 0.3 to 0.7 µM with NG dosing, persisting at 0.1 to 0.7 µM in most animals. Studies in dogs, using the same formulations as in the present study, showed mean bioavailabilities for GLU HCl of between 12 and 28%, following oral administration of 1,500 and 2,000 mg. Detectable plasma levels were 7 to 12 µg/mL with mean bioavailabilities for CS of approximately 4.8 to 5% following oral 1,200 and 1,600 mg doses respectively. Detectable plasma levels were 19 to 21 µg/mL and indicate that GLU and CS are absorbed orally.\textsuperscript{37} Detectable plasma levels of GLU and CS suggest that they may also localize in the joint. The concentrations comparable to bioavailable levels were reported to be effective in relieving pain, discomfort and improving joint tissue structural changes.\textsuperscript{24,25} Although bioavailability of ASU is not known, oral doses used in man and horse have exhibited anti-inflammatory, disease modifying effects.\textsuperscript{24,35} The concentration of ASU (8.3 µg/mL) used in the present study was selected from earlier in vitro work showing significant anti-inflammatory effects.\textsuperscript{21,23,28} Of the three compounds, ASU has more recently been studied for its anti-inflammatory, anti-catabolic and anabolic effects on cartilage metabolism. It inhibits the expression and production of cytokines, chemokines, PGE\textsubscript{2}, nitric oxide and matrix metalloproteinases as well as enhances the synthesis of cartilage matrix components such as collagen and proteoglycans.\textsuperscript{21,23,24,26,28} Glucosamine and CS have been observed to downregulate the inflammatory gene expression in monolayer chondrocyte cultures.\textsuperscript{28,34} The combined anti-inflammatory and chondroprotective effects of ASU, GLU and CS may account for the modulation of inflammation and decreased production of PGE\textsubscript{2} in our study.

We used equine chondrocytes in two-dimensional (2D) monolayer culture since they display phenotypic changes similar to those of cells in inflamed joints, as observed in osteoarthritic cartilage.\textsuperscript{30} Chondrocytes shift from producing the articular cartilage phenotype marker type II collagen to the fibroblastic–osteoblastic type I collagen in monolayer culture. These chondrocytes also readily responded to IL-1β stimulation resulting in significantly increased PGE\textsubscript{2} production. As in other 2D cultures, monolayer culture conditions are limited with the lack of biomechanical forces, constraint in space and nutrients. In contrast, 3D cultures where chondrocytes are seeded in scaffolds or microcarriers, cells can be exposed to dynamic mechanical forces simulating those in the normal joint. For the present study, we felt that the 2D monolayer culture system, which is routinely used for screening a wide variety of cell effects, would facilitate adequate assessment of chondrocyte response to test agents with ease.\textsuperscript{23,26,28}

One limitation of this study is the treatment period of the chondrocyte cell cultures (24 hours). Most RILP literature
indicating that peak synovial concentrations occur ~60 minutes post administration and taper dramatically in the next 24 hours. Our cells maintained their exposure to the concentrations of AK and EF for a longer duration than most RILPs which is likely responsible for a more profound cytotoxicity and EF’s induction of PGE₂ production. However, when techniques such as insertion of antibiotic medication-impregnated gels or gel sponges, constant rate infusion of antibiotic medications and using IVRILPs concurrently with the administration of intra-articular antibiotic medications are taken into consideration, the time frame remains clinically relevant. Regardless of the exposure period, expression of PGE₂ and the ensuing inflammation was reduced with the addition of ASU + GLU + CS.

Treatment of septic arthritis in horses can be a difficult and expensive undertaking and still result in significant morbidity and mortality in affected horses. Successful treatment depends on many factors including the efficacy and concentration of the chosen antimicrobial agent against the causative organism. Resolution of infection requires delivery of an antimicrobial to the target tissues in concentrations greater than the MIC for the bacteria. In equine practice, intra-articular and IRLP are commonly used to locally deliver high concentrations of antimicrobials to treat septic arthritis and supplement systemic administration. While reducing undesirable systemic effects, the use of these techniques results in maximum bacterial killing and reduced bacterial resistance.

In the present study, significant chondrocyte cell death occurred with clinically relevant dosing of both AK and EF. Furthermore, we have shown for the first time that EF can induce production of PGE₂ in equine chondrocytes. Additionally, the induction of PGE₂ synthesis by EF was inhibited by the combination of ASU + GLU + CS. Our observation that the combination of ASU + GLU + CS modulates the pro-inflammatory response in equine chondrocytes suggests the potential utility of this agent for downregulating the adverse effects of select antibiotic medications.

Author Contribution
The idea for the project was originated by Ann Rashmir. Experimental design was conceived by Carmelita Frondoza, Ann Rashmir and Cathleen Mochal. Acquisition of the data and data analysis were performed by Cathleen A. Mochal-King, Angela Au, Lowella Fortuno, Mark Granna, Carmelita Frondoza and Jillian Dougherty, Cathleen Mochal, Carmelita Frondoza and Ann Rashmir interpreted the data and prepared the manuscript. All authors reviewed the manuscript.

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Conflict of Interest
Dr. Rashmir reports that the primary author and one co-author were present at Nutramax Laboratories, Inc. throughout the duration of the project and performed the research with assistance from Nutramax Laboratories, Inc. personnel (four of the co-authors were employed at Nutramax which produces the nutraceutical being tested).

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