

5-1-2011

**Antibiotics induce prostaglandin e2 production and cytotoxicity in equine chondrocytes that can be inhibited by avocado soybean unsaponifiables, glucosamine, and chondroitin sulfate**

Cathleen Ann Mochaling

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ANTIBIOTICS INDUCE PROSTAGLANDIN E2 PRODUCTION AND  
CYTOTOXICITY IN EQUINE CHONDROCYTES THAT CAN BE  
INHIBITED BY AVOCADO SOYBEAN UNSAPONIFIABLES,  
GLUCOSAMINE, AND CHONDROITIN SULFATE

By

Cathleen Ann Mochal-King

A Thesis  
Submitted to the Faculty of  
Mississippi State University  
in Partial Fulfillment of the Requirements  
for the Degree of Master of Science  
in Veterinary Clinical Sciences  
in the Department of Veterinary Clinical Science

Mississippi State, Mississippi

April 2011

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Pages in Study: 35

Candidate for Degree of Master of Science

Amikacin (AK) and enrofloxacin (EF) concentrations consistent with intra-articular and regional limb perfusion were evaluated for their effects on equine chondrocytes. We evaluated the production of prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) by equine chondrocytes in response to AK and EF administration, and if the combination of avocado soybean unsaponifiables (ASU), glucosamine (GLU), and chondroitin sulfate (CS) could reduce the production of PGE<sub>2</sub>. Monolayer cell cultures of equine chondrocytes were treated with clinically relevant concentrations of AK and EF plus combinations of ASU, GLU, and CS. AK and EF generated a dose dependent cytotoxicity. The induction of PGE<sub>2</sub> following EF administration was significantly greater than PGE<sub>2</sub> levels induced by positive controls. Induction of PGE<sub>2</sub> by EF was significantly reduced in chondrocytes pretreated with ASU, GLU, and CS. We have demonstrated for the first time that EF can induce production of PGE<sub>2</sub> in equine chondrocytes and that this effect can be attenuated with the combination of ASU, GLU, and CS.

## ACKNOWLEDGEMENTS

The author expresses her sincere gratitude to the research and development staff of Nutramax Laboratories, Inc. To Dr. Frondoza and all of your staff: Thank you for helping make this research experience fun and enjoyable. The time and financial support generated from Nutramax Laboratories is much appreciated. Thank you also to Reinhard Grzanna, PhD for the immunohistochemical experiments, microscopic analysis, and for editing and constructive critique of the manuscript. Additionally I would thank all the staff at Mississippi State University for making this manuscript possible.

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## CHAPTER I

### INTRODUCTION

Degenerative joint disease is a common cause of lameness and joint pain in the horse. Destruction of the articular cartilage and disruption of the subchondral bone is the result of inflammation within the synovial structure. This destruction originates from multiple different modalities. Most commonly, osteoarthritis in the horse is the result of chronic inflammation from repetitive use or traumatic injury. However, on more rare occasions, osteoarthritis can develop from non-traumatic, septic origins. Ultimately the goals of managing degenerative changes within the joint are to modulate the destructive inflammatory response.

Septic arthritis is a debilitating orthopedic condition. In adult horses, septic arthritis most often results from synovial bacterial contamination via wounds, or iatrogenic induction following arthroscopy or intra-articular injections.<sup>1-4</sup> Septic arthritis is a common consequence of bacteremia and septicemia in foals.<sup>1,2,5</sup> In one study, septic arthritis was identified as the cause of death in 12.5% of foals aged 8 to 31 days.<sup>5</sup> Septic arthritis is bacterial in origin. *Streptococcus species* and *Staphylococcus aureus* are the most common isolates in adult horses.<sup>1,2</sup> The most frequent bacterial isolates cultured in foals are *Actinobacillus*, *E.coli*, *Klebsiella*, *Pseudomonas*, and *Salmonella*.<sup>5</sup>

Septic arthritis causes such profound inflammation within the joint that articular cartilage and subchondral bone are destroyed, often resulting in persistent lameness and loss of athletic potential. The management goals for septic arthritis are immediate

identification and elimination of the infection as well as prompt reduction in inflammation.<sup>1-3</sup> Treatment includes joint lavage with a balanced electrolyte solution, intra-articular antibiotics and systemic antimicrobials.<sup>1-3</sup> Outcome of treatments are variable with 25% to 55% returning to athletic soundness in one report.<sup>4</sup> The goals of treatment are to improve performance outcomes through aggressive antimicrobial therapy delivered systemically, intra-articularly, or via regional limb perfusion. Although the hallmark of managing septic arthritis is intra-articular antimicrobial drugs, minimal information is available about the effects of these drugs on equine chondrocytes.

This project was undertaken to identify the potential harmful effects of two intra-articular anti-microbial drugs and determine if these negative effects could be attenuated by the use of nutraceuticals as discussed in Chapter 3. Traditionally the harmful effects of intra-articular antimicrobial drug have been evaluated with the observation of lameness following administration, as well as macroscopic changes on post mortem examination. The goal of our study is to evaluate the potential harmful effects on a more sensitive scale by measuring the production of PGE<sub>2</sub>, an inflammatory mediator and the cytotoxic effects of these antibiotics on equine chondrocytes.

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## CHAPTER II

### ANTIMICROBIAL MANAGEMENT IN SEPTIC ARTHRITIS

Septic arthritis is a very debilitating and overwhelming disease. Intra-articular antibiotics are utilized not only to treat septic arthritis, but in some cases to prevent the development of a septic joint following arthroscopy or intra-articular medications. Only a few antibiotics have been evaluated for their bioavailability, pharmacokinetics, and effects on the equine joint. To date, information about the use of antibiotics in equine joints has been published in regard to the following medications: gentamicin<sup>1-7</sup>, amikacin<sup>8-10</sup>, ampicillin<sup>11</sup>, enrofloxacin<sup>12-15</sup>, ceftiofur<sup>16</sup>, trimethoprim sulfadiazine<sup>17</sup> and cefotaxime<sup>18</sup>.

The most commonly evaluated antibiotics in equine literature are the aminoglycosides amikacin and gentamicin. These antibiotics are the most popular for their apparent systemic tolerance, gram-negative spectrum of activity, and effective concentration against most equine pathogens. Both amikacin and gentamicin irreversibly bind to the 30S ribosomal unit of bacteria preventing protein synthesis.<sup>19</sup> They are water soluble and have a high volume of distribution into the extracellular fluid. This is a favorable trait for drugs used within synovial fluid. Aminoglycosides are minimally protein bound, which decreases the likelihood that they would be inhibited by plasma proteins. The bactericidal effects of aminoglycosides are concentration dependent.<sup>19</sup> In the treatment of septic arthritis multiple days of systemic dosing to maintain concentrations of aminoglycosides great enough to be effective can have undesirable

systemic effects. The most clinically significant toxicity is renal tubular necrosis from repetitive elimination through the kidney.<sup>19</sup> In attempts to maintain higher local concentrations without systemic accumulation, these drugs are administered intra-articularly. Administration of gentamicin within the joint has been associated with mild transient synovitis. Twenty-four hours following administration, significant increases in synovial white blood cells and a remarkable drop in synovial fluid pH have been documented.<sup>1,2</sup> Gentamicin has been evaluated for its effects on equine joints following five days of continuous intra-articular administration. At the end of this period, the articular cartilage and synovial membranes were (tested or examined) for loss of glycosaminoglycan content and synovial membrane inflammation.<sup>4</sup> There was no difference between the gentamicin treated animals and the control animals. Therefore, gentamicin is perceived to be minimally inflammatory. Most pharmacokinetic studies of gentamicin indicated that not only synovial fluid concentrations remain around the minimal inhibitory concentration (MIC), but that the subchondral bone concentrations do as well.<sup>3-7</sup> These concentrations are difficult to achieve with systemic administration of gentamicin.

Amikacin is the most commonly administered intra-articular aminoglycoside in equine practice due to the perceived tolerance as well as its convenient packaging -in a 2ml sterile vial and relatively low cost. These traits make it the initial antibiotic of choice in prevention and treatment of septic arthritis. Amikacin has been evaluated in several studies for its pharmacokinetics and effects in the equine joint.<sup>8-10</sup> The therapeutic concentrations are targeted at the MIC of the selected bacteria. The MIC is the concentration of drug required to inhibit growth of a specific bacteria. Most equine publications critically evaluating the effectiveness of an antibiotic evaluate the MIC of

*Pseudomonas species*.<sup>20</sup> This is because of *Pseudomonas*'s fairly resistant nature to many antibiotics and its likelihood of creating persistent infections. Amikacin has been shown to maintain levels above the MIC for *Pseudomonas sp.* of 4 µg/ml for 24 hours following a single intra-articular injection.<sup>8,9</sup> Administration of 500mg of amikacin into a normal joint has been demonstrated to maintained concentrations greater then 4 ug/ml for up to 72 hours with no indications of inflammation, such as elevation in synovial fluid cell counts or protein or onset of lameness.<sup>9</sup> Although amikacin appears to be non-inflammatory to the equine joint, all of these studies have evaluated only gross pathological changes and overt lameness. One significant limitation to amikacin is its gram negative spectrum, preventing successful treatment of the gram positive species of organisms like *Streptococcal* and *Staphylococcal species* that commonly plague the equine joint.<sup>20</sup>

Another antibiotic that would be favorable for orthopedic infections due to its antimicrobial spectrum and large volume of distribution is enrofloxacin. Enrofloxacin is a fluoroquinolone that inhibits DNA-gyrase.<sup>21</sup> It prevents super-coiling of the DNA molecules to prevent replication of bacteria.<sup>21</sup> Enrofloxacin is highly lipid soluble which allows for a large volume of distribution and bone penetration. This makes it a favorable drug for orthopedic infection. The pharmacokinetics of enrofloxacin after oral and intravenous administration has been reported to achieve concentrations of 1-5ug/ml in peripheral tissues.<sup>13, 14, 22</sup> The use of enrofloxacin for regional limb perfusions has been investigated in which the greatest synovial fluid concentrations were detected immediately after administration: values ranged from 7- 216 ug/ml.<sup>22</sup> Concentrations within the synovial fluid exceeded MIC for approximately 24 hours.<sup>22</sup>

Enrofloxacin appears to be a favorable candidate for use in the treatment of septic arthritis; however, its effect on chondrocytes after intra-articular administration is unknown. Fluoroquinolones have demonstrated that at various concentrations they may cause inflammation, cell death, and proteoglycan loss in chondrocytes and synovial structures.<sup>14,15</sup> These adverse effects have been observed on articular cartilage in rats, dogs and foals.<sup>12-15, 21, 22</sup> Beluche demonstrated that concentrations of enrofloxacin consistent with systemic (intravenous) doses did not suppress chondrocyte growth, but higher concentrations were toxic to chondrocytes.<sup>14</sup> Concentrations of enrofloxacin greater than 1000ug/ml eliminated proteoglycan synthesis regardless of animal age. Additionally, these elevated concentrations (1000ug/ml) resulted in a significant number of pyknotic nuclei within the chondrocytes, indicating cell death.<sup>14</sup>

High concentrations of antibiotics in joints have been associated with the development of lameness and elevated synovial fluid cell counts.<sup>1-3, 12</sup> *In vitro* studies have indicated that antibiotics can induce proteoglycan loss and cytotoxicity in chondrocytes.<sup>14, 15</sup> Proteoglycan loss, which is indicative of extracellular matrix (ECM) breakdown, has been attributed to production of pro-inflammatory mediators capable of inducing degradative enzymes. Chondrocytes, the only cell constituent of cartilage, synthesize pro-inflammatory mediators such as prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) and other ECM degradative enzymes. Excessive production of these mediators and enzymes lead to articular cartilage breakdown.

Evaluation of the ability of antibiotics to induce inflammatory mediators would allow for the development of strategies to minimize antibiotics potential adverse effects on equine joints. Therefore, the objective of this study is to clarify the role of amikacin and enrofloxacin in inducing inflammation. Amikacin and enrofloxacin concentrations



consistent with intravenous, intra-articular and regional limb perfusions were evaluated for their effect on equine chondrocytes. We tested the hypothesis that chondrocytes treated with these particular concentrations of amikacin and enrofloxacin would produce PGE<sub>2</sub>, a pro-inflammatory marker, in response to the antibiotic.

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## CHAPTER III

### NUTRICEUTICAL AND THE CONTROL OF OSTEOARTHRITIS

We have demonstrated that osteoarthritis is an important cause of lameness in horses. Although a number of factors can initiate the disease process, the hallmark of the disease is the degeneration of the articular cartilage and its matrix. This degeneration is attributed to the excess production of proinflammatory cytokines like PGE<sub>2</sub> and interleukin-1 $\beta$  (IL-1 $\beta$ ). Over production of these cytokines stimulates cartilage matrix degradation by inhibiting the production of proteoglycan and type II collagen while up regulating aggrecanase, which perpetuates matrix destruction.<sup>1</sup> Because cartilage degeneration is directly linked to levels of inflammatory mediators, evaluating compounds that alter these proinflammatory mediators is a promising treatment strategy in managing the progression of osteoarthritis (OA). PGE<sub>2</sub> is an important mediator in the pathologic process of arthritis and cultured chondrocytes respond to interleukin-1 (IL-1) with enhanced expression and activity of cyclooxygenase-2.<sup>2</sup> Palliative relief in affected horses is attributable in part to the reduction of PGE<sub>2</sub> synthesis.<sup>2</sup>

Oral administration of nutraceuticals containing glucosamine and chondroitin sulfate has received considerable attention as a palliative treatment of humans with OA. Ongoing research in oral administration of nutraceuticals indicates that both glucosamine and chondroitin sulfate have chondroprotective and articular sparing properties. Glucosamine acts as a substrate for the production of proteoglycans and glycosaminoglycans. It has been demonstrated that synovial concentrations achieved

via oral administration of glucosamine significantly reduced the production of the inflammatory mediators IL-1 $\beta$  and aggrecanase-1 in equine chondrocytes.<sup>3</sup> Although chondroitin sulfate has a less explicit mode of action it has been associated with glucosamine in its ability to minimize matrix degradation. This combined ability to alter the catabolism of the ECM had been demonstrated in several studies by reducing inflammatory mediators.<sup>2-4</sup> Chan demonstrated that glucosamine and chondroitin sulfate altered the production of PGE<sub>2</sub>, COX-2, and aggrecanase in long term bovine chondrocyte explant cultures.<sup>4</sup> Furthermore glucosamine and chondroitin sulfate have also been observed to down regulate the expression of recombinant equine IL-1 $\beta$  in monolayer chondrocyte cultures.<sup>3</sup>

Within the last twenty years another oral nutraceutical, avocado soybean unsaponifiables(ASU) has been evaluated for its potential role in the prevention and management of OA. Although currently the mechanism of action is not thoroughly understood, the avocado soybean unsaponifiables were initially evaluated as a potential chondroprotective agent. These chondroprotective effects were demonstrated in a human study where ASU preserved the glycosaminoglycan and hydroxyproline content of the ECM.<sup>5</sup> Several human dietary trials began utilizing ASU as a chondroprotective and preventative to OA.

In 1997 Blotman performed a double blind placebo controlled study evaluating the effects of ASU on humans with osteoarthritis of the knee.<sup>6</sup> The initial hypothesis of this study was that the ASU would promote repair of the cartilage ECM. However, the key finding in Blotman's study was that all patients on the ASU had a significant reduction in the daily use of non-steroidal anti-inflammatories. This finding seemed to demonstrate that ASU may not only have chondroprotective effects but also appeared to



have significant anti-inflammatory effects.<sup>6</sup> Later ASU began to be evaluated directly for its role in modulating inflammation. Nutramax Laboratories demonstrated the ability of ASU to alter PGE<sub>2</sub> productions by 40% in chondrocyte monolayer cell cultures as well as suppressing TNF- $\alpha$ , IL1- $\beta$ , and COX-2. The same study showed a reduction in COX-2 expression by 50%.<sup>7</sup> ASU has exhibited a remarkable ability to reduce several markers of inflammation and key components of OA. The combined anti-inflammatory effects of ASU with the chondroprotective characteristics of glucosamine and chondroitin sulfate are the basis for the modulation of inflammation in our study. Our hypothesis is that the addition of ASU and glucosamine and chondroitin sulfate to equine chondrocyte cultures will minimize the inflammation induced by the antibiotics.

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## CHAPTER IV

### METHODOLOGY

The use of antibiotics within synovial structures is paramount for the treatment and prevention of septic arthritis. As discussed in previous chapters, minimal information is available about the potential deleterious effects of antibiotics on equine chondrocytes. Our first goal in this study is to evaluate the effects of amikacin and enrofloxacin at concentrations consistent with intra-articular and regional limb perfusion on equine chondrocytes. We will determine if there are concentrations of these drugs that are cytotoxic to chondrocytes. Our second goal is to assess the role of gentamicin and enrofloxacin in inflammation by measuring the production of PGE<sub>2</sub> by equine chondrocytes treated with these antibiotics and determine if the production of PGE<sub>2</sub> could be reduced by the combination of avocado soybean unsaponifiables (ASU), glucosamine (GLU), and chondroitin sulfate (CS).

#### **Materials and Methods**

##### Cell Culture

Equine cartilage was aseptically diced into <5 mm pieces and digested in type II collagenase (110 U/ml) for 12-18 hours at 37°C, 5% CO<sub>2</sub>. Chondrocytes were filtered through a wire mesh screen to remove debris and rinsed four times with Hank's Balanced Salt Solution (HBSS) (ATCC; Manassas, VA, USA). Cells were counted and assessed for viability using the Trypan-blue exclusion method. Chondrocytes were propagated in

monolayer culture until confluency in media composed of Dulbeccos' Modified Eagle's basal medium (Sigma; St. Louis, MO, USA) supplemented with 10% fetal bovine serum (Gemini Bio-Products; Woodland, CA, USA), 300 µg/ml L-glutamine (Sigma), and 3.7 g/L sodium bicarbonate (Sigma). When confluent, chondrocytes were propagated in monolayer culture until ready for use.

#### Phenotype Analysis by Immunohistochemistry and Western Blot Analysis

Chondrocytes were plated on microscope slides and fixed with 10% paraformaldehyde. Slides were then incubated with goat anti-type I collagen, anti-type II collagen, or anti-aggrecan antibodies (Southern Biotechnology Associates; Birmingham, AL, USA). The slides were next washed in buffer three times and incubated with FITC labeled anti-goat antibodies. Immunostaining was visualized using a Nikon Eclipse epifluorescent microscope TE200. To identify secreted collagen and aggrecan, spent culture media were electrophoresed on 4-15% sodium dodecyl sulfate-polyacrylamide gels. Following electrophoresis, the gels were electrophoretically transferred to PolyVinylidene DiFluoride (PVDF) membranes (Bio-Rad Laboratories, Hercules, CA, USA) in Tris-glycine buffer, pH 8.5, containing 20% methanol. Blotted PVDF membranes were washed twice with deionized water and stained using a chromogenic Western blot immunodetection kit (Gibco Invitrogen, Carlsbad, CA, USA). To block non-specific staining, membranes were treated with a blocking solution provided with the immunodetection kit following the instructions of the manufacturer. PVDF membranes were then processed for immunostaining using goat anti-collagen type II, type I antibodies, or anti-aggrecan (Southern Biotechnology Associates) in combination with an

alkaline phosphatase labeled rabbit anti-goat antibody with 5-bromo-4-chloro-3-indolylphosphate-nitroblue tetrazolium (BCIP/NTB) (Gibco Invitrogen) as the substrate.

### Experimental Design

ASU (ASU-NMX<sup>®</sup> 1000; Nutramax Laboratories, Inc., Edgewood, MD, USA) was dissolved in 100% ethanol (Sigma) and diluted with HBSS (GIBCO) to achieve the required final concentration. The concentration used in this study was previously shown to exert significant anti-inflammatory effect (Au et al, 2007).<sup>1</sup> Glucosamine (FCHG49<sup>®</sup>, Nutramax Laboratories Inc.) and chondroitin sulfate (TRH122<sup>®</sup>, Nutramax Laboratories Inc.) were dissolved in HBSS (GIBCO) to achieve the working concentrations.

Chondrocytes harvested from monolayer cultures were first seeded into 6 well ( $5 \times 10^5$ ) and 24 well ( $1 \times 10^5$ ) plates for 24 hours. The monolayer cultures were next incubated with: control media alone or ASU (8.3  $\mu\text{g/ml}$ ), Glu (11  $\mu\text{g/ml}$ ), and CS (20  $\mu\text{g/ml}$ ) for another 24hrs. Following pretreatment, lipopolysaccharide (LPS, 20ng/ml; Sigma-Aldrich) or IL-1 $\beta$  (10ng/ml) and TNF $\alpha$  (1ng/ml) was added as a positive control. Enrofloxacin (0.1mg/ml-10mg/ml) (Sigma) or amikacin (0.25mg/ml-25mg/ml) (Sigma) was added with or without the combination of ASU, Glu, and CS for another 24 hrs to measure secreted PGE<sub>2</sub> levels by immunoassay or determine cytotoxicity through MTT cell proliferation assay. 10% EtOH was added in triplicates to the 24 well plates as a positive control of cell death.

### MTT Cell Proliferation

To determine whether or not enrofloxacin or amikacin caused cell death, a MTT cell proliferation assay kit (Cayman Chemical) was used to measure cytotoxicity. The cellular supernatant was aspirated off from the 24 well plates. 1ml of sterile HBSS and

100µl of reconstituted MTT were then added to each well. Plates were incubated at 37°C, 5% CO<sub>2</sub> for 3hrs. After 3hrs 1ml of MTT solubilization solution was added and thoroughly mixed. Plates were allowed to sit in room temperature for at least 15 minutes to allow crystal dissolution. The contents of the well were again mixed thoroughly and 200µl aliquots were plated in triplicates into a 96 well plate. Absorbance was measured using a Spectramax Plus (Molecular Devices, Sunnyvale, CA, USA) at a double wavelength of 570nm and 690nm.

#### PGE<sub>2</sub> High Sensitivity Immunoassay

A commercial PGE<sub>2</sub> immunoassay (R&D Systems, Minneapolis, MN USA) was used to quantify secreted PGE<sub>2</sub> levels in the cellular supernatant, according to the manufacturer's instructions. A PGE<sub>2</sub> standard was run in parallel to the supernatant samples. Briefly, 100 µl of each supernatant sample was assayed in triplicates on a 96-well microplate coated with a goat anti-mouse polyclonal antibody. 50 µl of PGE<sub>2</sub> HS conjugate was added to each sample well. Next, 50 µl of PGE<sub>2</sub> antibody solution was added to each sample well. The microplate was incubated for 18-24 hrs at 2-8°C. After the incubation period, the microplate wells were aspirated and washed with PGE<sub>2</sub> wash buffer for a total of three washes. After the last wash, 200 µl of pNPP substrate was added to the microplate wells. After incubation for 20min at room temperature, 50 µl of Stop Solution was added to the sample wells. Optical density was measured immediately using the SpectraMAX 340 microplate reader (Molecular Devices, Sunnyvale, CA, USA) at 405 nm with wavelength correction set between 570 nm and 590 nm.

### Statistical Analysis

Data is presented as the mean  $\pm$  1 SD. Pair-wise multiple comparisons were carried out using Student-Neuman-Keuls one-way ANOVA and Tukey post-hoc using SigmaStat statistical software (Windows Version 3.11) where  $p < 0.05$  was considered statistically 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 three to five days. Chondrocytes propagated on monolayer cultures at passage 3 showed elongated, spindle-shaped morphology (Figure 4.1). Immunohistochemical analysis confirmed that chondrocyte cultures continued to produce the ECM components aggrecan and type II collagen (Figure 4.2 and 4.3, respectively). Production of type II collagen was further verified by Western blot. 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.

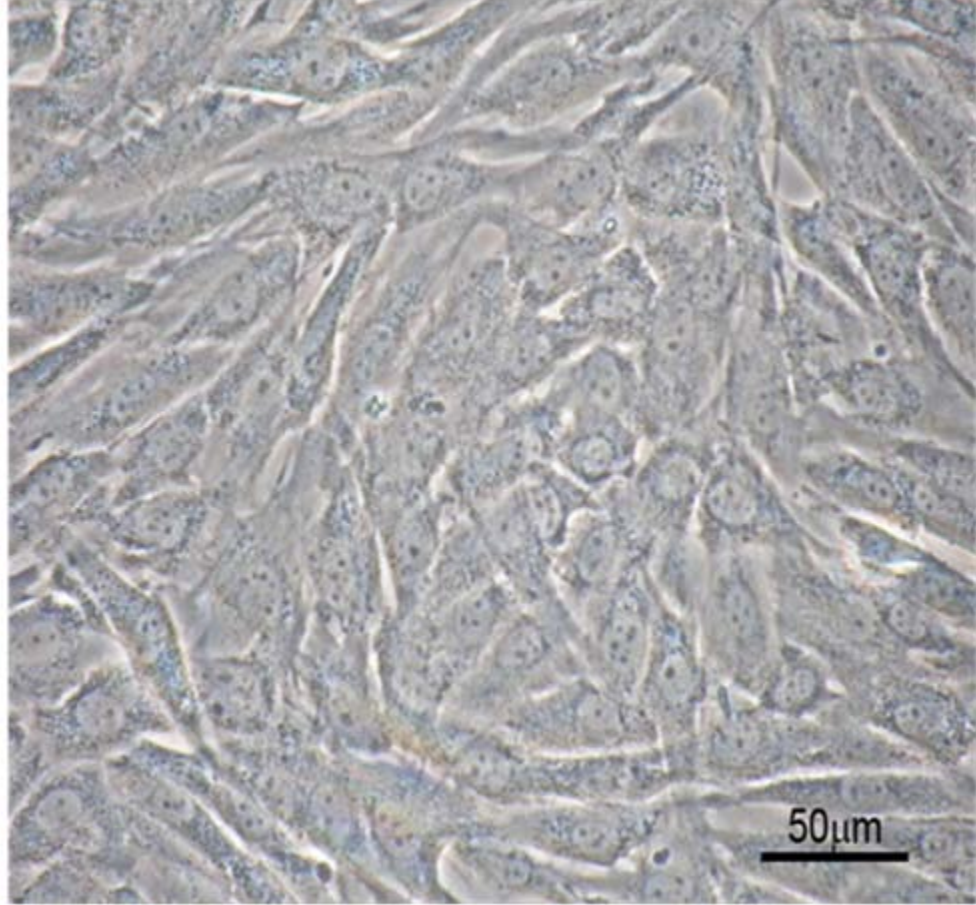


Figure 4.1 Phase-contrast photomicrograph of equine chondrocyte culture



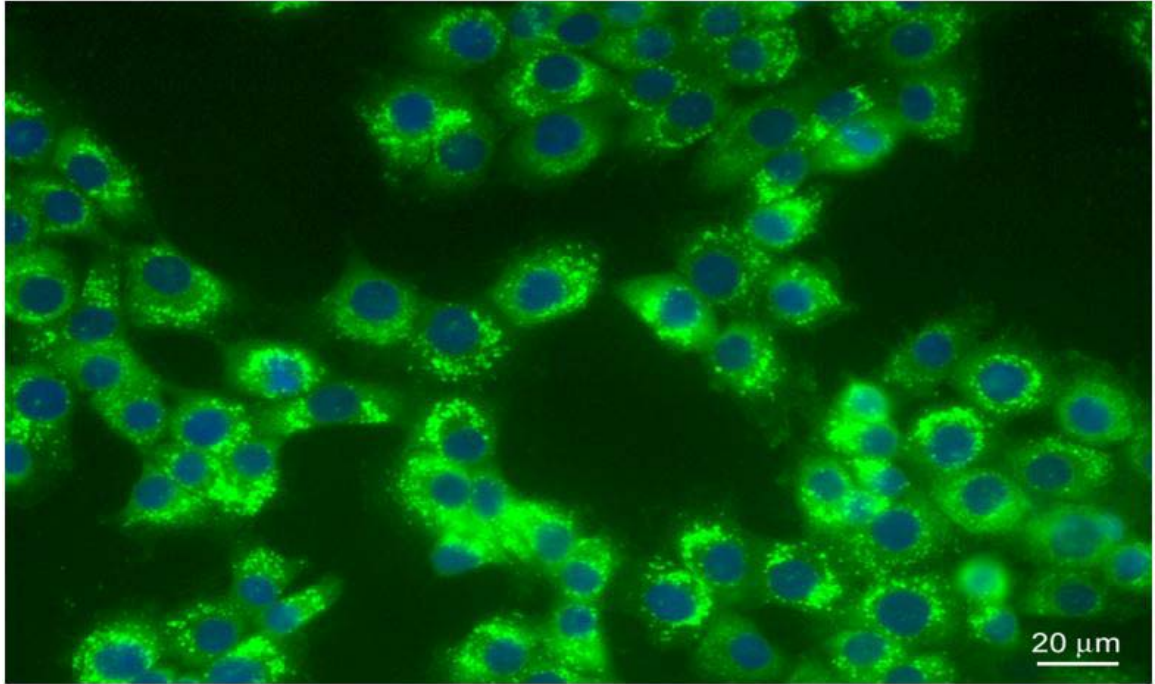


Figure 4.2 Immunostaining for type II collagen

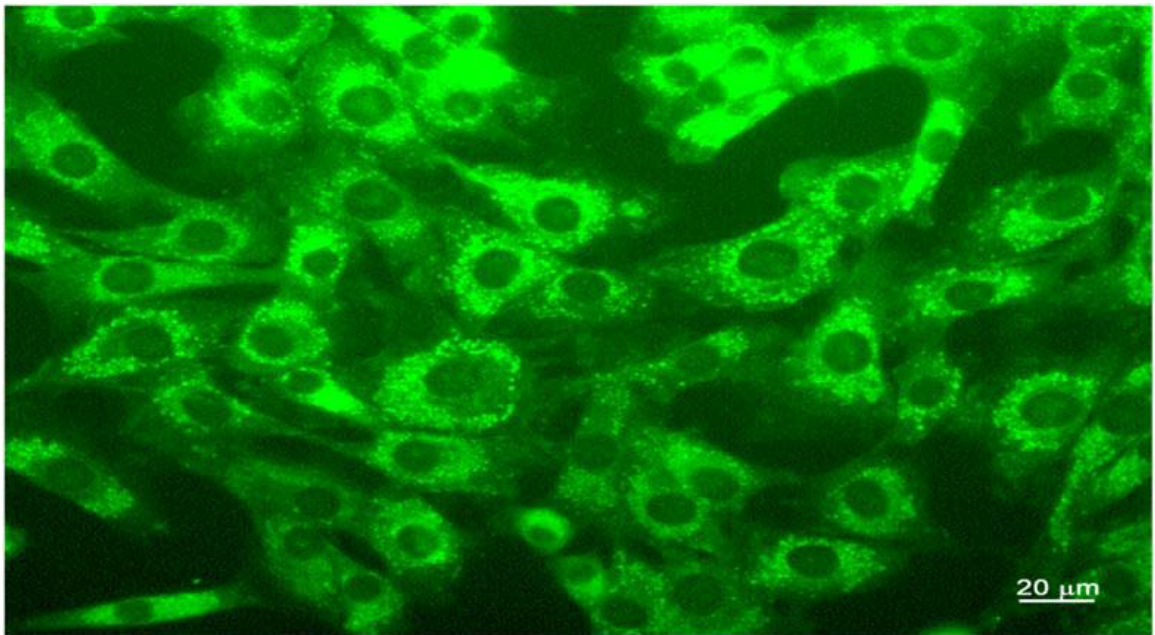


Figure 4.3 Immunostaining for aggrecan

## The Cytotoxic Effect of Amikacin and Enrofloxacin on Equine Chondrocytes

Amikacin and enrofloxacin both have dose dependent cytotoxic effects on equine chondrocytes. Optical density measurements for amikacin demonstrated no difference from the control non-activated cells at the concentration of 0.25 mg/ml. Chondrocytes treated with 2.5mg/ml concentrations of amikacin demonstrated an increase in cell death by approximately 40%. Cells treated with 25 mg/ml concentrations of amikacin demonstrated profound cytotoxicity, with 99% cell death (Figure 4.4). Low dose concentrations of enrofloxacin (0.1mg/ml) demonstrated an increase in cell death by 40%. Concentrations of enrofloxacin at (1.0mg/ml) increased cytotoxicity by 82% and concentrations of enrofloxacin at 10 mg/ml were severely cytotoxic with 99% cell death. (Figure 4.5).

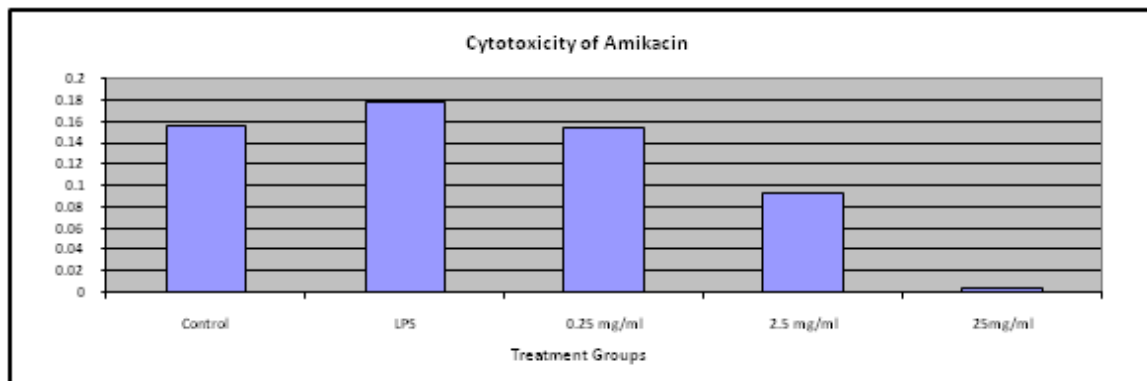


Figure 4.4 Effect of Amikacin (0.25, 2.5 and 25 mg/ml) on chondrocyte cytotoxicity

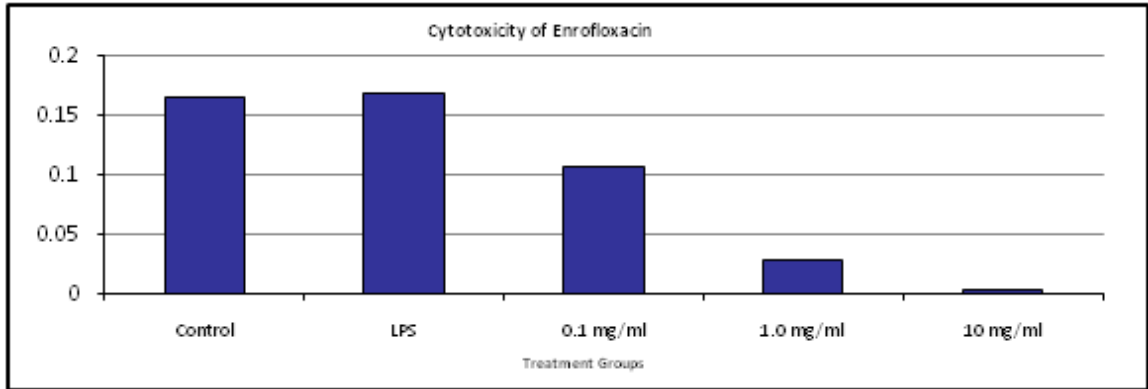


Figure 4.5 Effect of Enrofloxacin (0.1, 1.0 and 10 mg/ml) on chondrocyte cytotoxicity

#### The Effect of Amikacin and Enrofloxacin on PGE<sub>2</sub> Production by Equine Chondrocytes

Chondrocytes treated with various concentrations of amikacin (0.25, 2.5 and 25mg/ml) did not induce significant production in PGE<sub>2</sub> when compared to the control non-treated chondrocytes during the 24 hours of incubation. In contrast, enrofloxacin dramatically induced PGE<sub>2</sub> production in a dose dependent fashion. Chondrocytes responded to enrofloxacin at concentrations of 0.1, 1.0 and 10 mg/ml with a significant increase in PGE<sub>2</sub> production ( $p < 0.001$ , Figure 4.6). The synthesis of PGE<sub>2</sub> following exposure of chondrocytes to enrofloxacin was significantly greater than PGE<sub>2</sub> levels induced by LPS (< 20 fold) or cytokines IL-1 $\beta$  and TNF- $\alpha$  (< 5 fold). Treatment of chondrocytes with 0.1mg/ml of EF resulted in the production of PGE<sub>2</sub> at 28,000 pg/ml. Concentrations of 1.0mg/ml resulted in the production of 48,000 pg/ml PGE<sub>2</sub>. Most significant production of PGE<sub>2</sub> was at 160,000 pg/ml from the 10 mg/ml concentration of enrofloxacin.

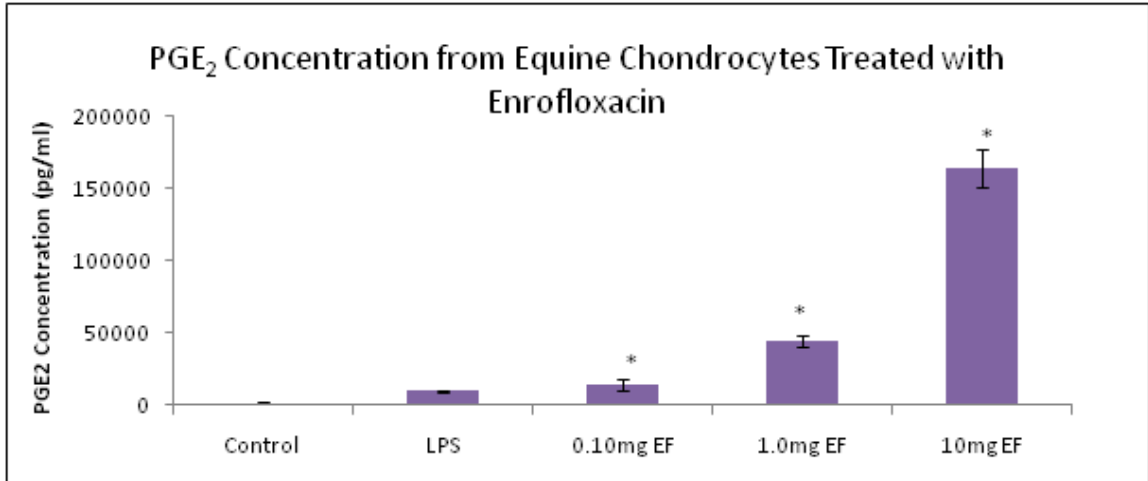


Figure 4.6 PGE<sub>2</sub> production in response to enrofloxacin at concentrations of 0.1, 1.0 and 10 mg/ml with a significant increase in ( $p < 0.001$ )

Induction of PGE<sub>2</sub> synthesis by enrofloxacin was significantly ( $p < 0.05$ ) down-regulated when chondrocytes were pre-treated with the combination of ASU, GLU, and CS. Pre-treatment with ASU (8.3  $\mu\text{g/ml}$ ) and glucosamine and chondroitin sulfate at concentrations ranging from 4 to 400 ng/ml significantly decreased PGE<sub>2</sub> production at 0.1 mg/ml and 1.0 mg/ml concentrations of enrofloxacin ( $p > 0.05$ , Figure). The combination of ASU at 8.3  $\mu\text{g/ml}$  with EGCG at concentrations 4 to 400 ng/ml significantly reduced PGE<sub>2</sub> production relative to the activated control IL-1 $\beta$  and TNF- $\alpha$  ( $p < 0.05$ , Figure 4). The combination of ASU at 4  $\mu\text{g/ml}$  and EGCG at 40 ng/ml to the 10 mg/ml concentrations of enrofloxacin did not reduce PGE<sub>2</sub> production.

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## CHAPTER V

### CONCLUSIONS

Our study demonstrated a significant increase in PGE<sub>2</sub> following administration of all concentrations of enrofloxacin. The expectation had been that the elevated levels of enrofloxacin would induce an inflammatory response. Enrofloxacin was chosen as one of the antimicrobials to be utilized in this study due to the spectrum of activity, the volume of distribution, and the previous association with harmful effects on chondrocytes. Bertone, et al. (2000) studied the effects of long term administration of systemic enrofloxacin on lameness and the response of articular cartilage in the horse. Within her study, mild swelling was observed in 1 or more joints in 9 horses after administration of enrofloxacin, and lameness developed in 3 treated animals and then resolved before the treatment period ended.<sup>1</sup> Arthroscopic evaluation of affected joints was normal on gross examination.<sup>1</sup> However, the evidence of mild joint swelling within the study population suggested that even though there were no macroscopic changes, mild inflammation had occurred. The goal of our study was to demonstrate evidence of antibiotic-induced inflammation through more sensitive measures.

The manufacturer's recommended dosage of enrofloxacin is 5.0mg/kg administered intravenously which generates a concentration of 2 to 10 ug/ml within the peripheral tissues.<sup>2,3</sup> The same dosing regime was observed to generated peak serum concentration of 4-7 ug/ml and peak synovial concentrations of 1-4ug/ml 8 to 24 hours

post administration.<sup>2</sup> Synovial fluid concentrations of enrofloxacin achievable by regional limb perfusions range from 7- 216 ug/ml.<sup>4</sup>

One study demonstrated that concentrations of enrofloxacin greater than 10 ug/ml decreased chondrocytes' ability to adhere to culture dishes.<sup>5</sup> Chondrocyte cell size was reduced and the actin cytoskeleton decreased with increasing concentrations of enrofloxacin, as well as a reduction in type-2 collagen.<sup>5</sup> These repeatable negative effects of enrofloxacin demonstrate that synovial concentrations consistent with parental administration of enrofloxacin elicit inflammatory and cytotoxic responses in chondrocytes. Based on these previous clinical findings and the findings of our study, enrofloxacin does induce inflammation that is harmful to chondrocytes. We were successful at stimulating the production of PGE<sub>2</sub> at all of our treatment concentrations of enrofloxacin. Additionally at concentrations greater than 0.1mg/ml, cytotoxicity was also observed.

Once inflammation was successfully induced the next objective of our study was to modify these inflammatory responses. Several modulators of inflammation have been identified in osteoarthritis. Avocado-soybean unsaponifiables, glucosamine, and chondroitin sulfate are just a few currently available. Previous evaluation of avocado-soybean unsaponifiables at concentrations of 8.3 µg/ml added to bovine monolayer cell cultures reduced TNF $\alpha$ , IL-1 $\beta$ , COX-2 and iNOS expression in LPS-activated chondrocytes to levels similar to non-activated control levels. The suppression of COX-2 and iNOS expression was paralleled by a significant reduction in PGE<sub>2</sub>.<sup>6</sup>

Glucosamine, at concentrations of 10 ug/ml, significantly reduces expression of MMP13 , aggrecanase-1, and cytokine-induced expression of nitric oxide and cyclooxygenase-2.<sup>7</sup> In our study we treated cells with glucosamine and chondroitin

sulfate at concentrations ranging from 4 to 400 ng/ml. Synovial fluid concentrations of glucosamine at 10 ug/ml are obtainable through oral administration.<sup>6</sup> Others have demonstrated that cartilaginous disks treated with glucosamine and chondroitin sulfate decrease nitric oxide release and reduce nitric oxide synthase, cyclooxygenase-2, and PGE synthase production.<sup>8</sup> This down regulation of inflammatory mediators driven by glucosamine and chondroitin sulfate are helpful in the management of synovial inflammation.

Our study demonstrated a significant reduction of chondrocyte PGE<sub>2</sub> expression in the presence of enrofloxacin concentrations equal to and less than 1.0mg/ml following the addition of ASU, GLU, and CS to cell cultures. PGE<sub>2</sub> concentrations were reduced to baseline levels of less than 10,000pg/ml. Only at 10 mg/ml concentrations of enrofloxacin were we unable to attenuate the deleterious effects. The potential benefits of reducing PGE<sub>2</sub> production at concentrations of enrofloxacin less than 1.0mg/ml with the additions of ASU, GLU, and CS are the reduction in inflammation and the availability of these products. Furthermore, they may be added to concurrent septic arthritis treatment plans regardless of the antimicrobial used.

One potential downfall of this study is the treatment time period of the chondrocyte cell cultures. All cultures were treated for 24 hours. Most literature of regional limb perfusions indicated that peak synovial concentrations are obtained approximately 60 minutes post administration and then begin to taper dramatically in the next 24 hours. Our cells were exposed to enrofloxacin for a longer duration than regional limb perfusions. This may account for the profound induction of PGE<sub>2</sub>. However, if this is the case, then regardless of the exposure period still successfully reduced the



expression of PGE<sub>2</sub> in the face of significant inflammation with the additions of ASU, GLU, and CS.

Amikacin was the next drug of choice due to its success in treating orthopedic infections. A study of bacterial species isolated from septic arthritis, tenosynovitis and osteomyelitis in 233 horses yielded Enterbacteriaceae, beta-haemolytic streptococci and coagulase-negative staphylococci as the predominant species isolated.<sup>9</sup> Within this study amikacin was the most effective antibiotic against the wide range of bacteria isolated.<sup>9</sup> Amikacin was highly effective against coagulase-positive staphylococci, Enterobacteriaceae and *Psuedomonas*. Gentamicin was not highly effective against any bacterial group.<sup>9</sup> Isolated limb retrograde venous injection yielded synovial concentrations of amikacin ten times the minimum inhibitory concentration for 90% of isolates (80 ug/ml) and ten times the MIC breakpoint (160 ug/ml) of amikacin-susceptible bacteria reported to cause septic arthritis in horses.<sup>10</sup> This technique achieved synovial fluid concentrations of amikacin consistent with concentration dependent killing of bacteria commonly encountered in horses with septic arthritis.<sup>10</sup>

Our study demonstrated that amikacin did not induce the production of PGE<sub>2</sub> levels/concentrations that were greater than the levels/concentrations of the control non-treated cells at any of the study concentrations. Additionally cytotoxicity was only observed at the greater concentrations of amikacin. These findings indicates that the use of amikacin for regional limb perfusion and intra-articular injections is relatively safe and unlikely to induce inflammation.<sup>11</sup> Solidifying amikacin as the gold standard of antimicrobials for the treatment of septic arthritis.

Treatment of septic arthritis in horses can be a difficult and expensive undertaking. Unresolved infection and inflammation leading to joint damage can end a

horses' athletic career or even its life. Successful treatment depends on many factors including the efficacy of the chosen antimicrobial and tissue concentrations of the antimicrobial administration. Resolution of infections requires delivery of an antimicrobial to the target tissues in concentrations greater than the MIC for the bacteria<sup>4</sup>. In equine practice, local delivery of antimicrobials for the treatment of septic arthritis is used to supplement or replace systemic administration. Local delivery techniques are favored because they are capable of achieving high tissue concentrations of antimicrobials at the site of infections while reducing undesirable systemic effects. The ability to deliver high tissue concentrations of drug for an appropriate time interval results in maximum bacterial killing and reduced bacterial resistance.<sup>10</sup>

In the present study, we have shown for the first time that enrofloxacin can induce production of PGE<sub>2</sub> in equine chondrocytes. This induction of PGE<sub>2</sub> synthesis can be attenuated with the combination of ASU, GLU, and CS. Our observation that the combination of ASU, GLU, and CS modulates the pro-inflammatory response in equine chondrocytes suggests the potential utility of this agent for down regulating the adverse effects of antibiotics. The combination of ASU, GLU, and CS may alleviate inflammation in equine joints following the use of enrofloxacin and amikacin.

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