EVALUATION OF THE PHARMACOKINETICS, PHARMACODYNAMICS, AND SAFETY OF ENROFLOXACIN ADMINISTRATION TO PREGNANT MARES

BY

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DISSERTATION

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ABSTRACT

Antimicrobial therapy is necessary in pregnant mares affected by severe bacterial infections such as cellulitis, bronchopneumonia, or placentitis. Despite the many antimicrobials available for human use, very few antimicrobials are cost-effective and approved for use in the horse. Enrofloxacin is a fluoroquinolone antibiotic that is available in both oral and intravenous formulations and is commonly used in veterinary medicine. This fluoroquinolone acts by inhibiting bacterial DNA synthesis. Previous work suggests that the neonatal animal is sensitive to fluoroquinolones, and that exposure during development will affect cartilage in the neonate. However, fluoroquinolones are used in some pregnant species without obvious effects on the fetus. To understand potential toxic effects on the fetus, the pharmacokinetics and pharmacodynamics of the enrofloxacin in both the intravenous form and a newer oral form were evaluated in the pregnant mare. After determining the optimal dose, and a higher dose that might be used to treat intermediately susceptible bacteria, mares were treated with intravenous enrofloxacin at 260 days gestation. Both enrofloxacin and its active metabolite ciprofloxacin crossed the placenta, and reached therapeutic levels in the fetus and fetal fluids, without apparent effects on the fetus. Next, to explore long-term effects of enrofloxacin on fetal development, mares were treated for 14 days at 280 days gestation, and allowed to foal. Foal cartilage and tendon strength were then evaluated at 30 days of age, and no effects of treatment were observed on gross or histologic examination. In contrast, foals treated with enrofloxacin at 14 days postnatally developed clinical lameness and moderate to severe lesions on histopathology. All foals were then subjected to additional testing to determine if normal veterinary assessments were insufficient to detect long term effects of mild *in utero* toxicity. Structural MRI, qMRI and RT-PCR analysis failed to detect changes in cartilage properties in the *in utero*-exposed animals. Overall, it is proposed that enrofloxacin and its active metabolite ciprofloxacin cross the equine placenta and reach the equine fetus at therapeutic concentrations. At therapeutic doses, exposure to these fluoroquinolones *in utero* during the first or third trimesters does not appear to cause either acute, or chronic cartilaginous lesions in the developing fetus.

For my father, Dr. Roy August Ellerbrock, who passed away shortly after I began my residency in Illinois

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CHAPTER 1: OVERVIEW

Antimicrobial therapy is necessary in broodmare practice to treat a variety of systemic diseases, from cellulitis to peritonitis and placentitis. Placentitis alone is a significant cause of pregnancy loss in the horse, and affects 3-5% of all equine pregnancies [1–4]. Despite the many antimicrobials available for human use, very few antibiotics are cost effective and approved for use in the horse, and none are approved for use in pregnant mares. Fluoroquinolones would be an excellent choice as a second-line antibiotic in pregnant mares; however, they are believed to be toxic to the fetus based upon questionable reports in other species and are mostly avoided during pregnancy [5–7]. To date, only a few studies have assessed *in vivo* effects of enrofloxacin in the horse, often using supratherapeutic doses to attain maximum toxicity [8]. No studies have assessed the safety of enrofloxacin administration to pregnant mares, therefore the use of enrofloxacin is generally avoided in pregnant mares due to the assumption it crosses the placenta and will cause cartilage lesions in the fetus.

Traditionally, toxicity studies have determined adverse effects based on clinical signs, blood work, and histological evaluations of tissues of interest. Given the athletic demands of the horse, toxicity studies should ensure that enrofloxacin does not affect tendon tensile strength or cartilage strength in treated animals. Additionally, given the significant pressure to reduce the number of animals used in research, there is a critical need to validate noninvasive means, such as magnetic resonance imaging (MRI), for monitoring drug toxicity to cartilage and tendons.

This dissertation details administration of enrofloxacin to the pregnant mare in the first and third trimester, and the subsequent evaluation of the pharmacokinetics, pharmacodynamics, and potential drug toxicity in the developing fetus and resulting foal. The dissertation is divided into eight chapters, and each chapter stands alone, with the figures, tables and references included at the end of each chapter. The appendix that follows the end of the dissertation details four unrelated studies completed during my graduate studies that were carried out to improve our understanding of male subfertility and infertility in the horse.

Chapter 2 provides a brief background on antimicrobial use in the horse, with special detail paid to known effects on reproduction, and known effects of pregnancy on drug distribution and metabolism. In addition, background is provided on the advanced imaging techniques and biomechanical tests used in subsequent chapters. Chapters 3-7 are composed of the five

publications derived from this dissertation; four have been submitted for publication at time of dissertation submission, two of which have been accepted at this time. Chapter 3 details the evaluation of enrofloxacin pharmacokinetics and pharmacodynamics in pregnant mares. Chapter 4 confirms the transplacental diffusion of enrofloxacin and ciprofloxacin in the horse, and investigates potential effects on fetal histology. Chapter 5 is a follow-up to Chapter 4, where mares were allowed to foal to determine the long-term effects of *in utero* fluoroquinolone exposure. Chapter 6 explores the use of advanced imaging and biomechanical testing to detect subtle differences in cartilage strength and biomechanics in the horse. This is followed by the study in Chapter 7 investigating potential effects of *in utero* exposure to enrofloxacin during the first trimester of equine pregnancy. Our project conclusions and future directions for research are outlined in the final chapter, Chapter 8.

While it is important to note that failure to demonstrate toxicity in a small number of healthy animals does not promise safety, this dissertation details studies exploring enrofloxacin's potential effects on the developing equine fetus and foal, and provides critical information for practitioners faced with resistant infections in pregnant horses.

CHAPTER 2: INTRODUCTION AND REVIEW OF PERTINENT LITERATURE

ANTIMICROBIAL USE IN THE HORSE

Antimicrobial therapy is necessary in pregnant mares when treating a variety of severe bacterial diseases such as placentitis, bronchopneumonia, pleuropneumonia, cellulitis, or septic arthritis. Despite the many antimicrobials available for human use, very few antibiotics are cost effective and approved for use in the horse, and none are approved for use in pregnant mares. Additionally, the assumption of antimicrobial safety in the mare is based almost entirely on anecdotal clinical experience, rather than prospective or retrospective analysis.

It is assumed that β-lactams, potentiated sulfonamides, and metronidazole are safe in pregnant mares based both on reports in other species and the widespread use of these antimicrobials in equine clinical practice [7,9,10]. Interestingly, while trimethoprim sulfa is widely used throughout the entire equine gestation, sometimes even in a prophylactic manner to prevent placentitis, human retrospective studies suggest an increased risk of cardiovascular defects and neural tube defects in infants when mothers are treated with trimethoprim in the first trimester [9,11,12]. A similar association has not been described in the horse, and trimethoprim sulfas are commonly prescribed in the horse from pre-conception to treat endometritis, all the way through late gestation to treat placentitis [1,7,12,13], in part because trimethoprim sulfa has good uterine penetration and is able to cross the third trimester equine placenta [13]. Unfortunately, and perhaps in part due to trimethoprim sulfa's widespread use in equine practice, there is an increasing resistance among common equine pathogens, especially *Escherichia coli,* to trimethoprim sulfa [14,15].

Penicillin and gentamicin are commonly prescribed in combination for broad-spectrum antimicrobial coverage in horses with more severe systemic infections that are resistant to trimethoprim sulfa and require parental antibiotics. Both penicillin and gentamicin are believed to be safe for use throughout gestation, with penicillin considered to have no known negative effects on human pregnancy [16]. Gentamicin was classified as FDA class D due to isolated reports of potential risks of otic toxicity [17], however large retrospectives have failed to demonstrate risks to the human fetus, and gentamicin in often prescribed during human pregnancy [9,18,19].

Penicillin has previously been shown to cross the equine placenta and reach effective concentrations in the fetal fluids [20], however there are conflicting reports on gentamicin. After one dose of gentamicin was administered within 2 hours of parturition, gentamicin was not detected in the resulting foals (n=3) or amniotic fluid (n=1). In another study using microdialysis, gentamicin was detected in the fetal fluids at low concentrations (3.9-8.5 ug/ml). These lower concentrations may indicate that a combination of gentamicin and penicillin is not adequate for placental or fetal infections with Gram-negative bacteria. The other disadvantage to penicillin and gentamicin administration is the need for intravenous administration (gentamicin) and frequent administration (penicillin).

Cephalosporins are beta lactam antibiotics with broader spectrum activity than penicillin and are excellent antimicrobials frequently used in large animal species. Besides increased resistance to beta-lactamases, cephalosporins are bactericidal, effectively penetrate bacterial cell walls, and are generally considered less likely to cause side effects seen with the administration of other antimicrobials such as renal or hepatic toxicity or colitis [21]. The main disadvantages are that cephalosporins must be administered parentally, and that cephalosporins are also considered antimicrobials of importance in human medicine, and thus, like enrofloxacin, should be reserved for second line treatment due to both cost and appropriate antimicrobial stewardship. 3rd generation cephalosporins such as ceftiofur are considered safe for pregnant animals due to anecdotal data in horses, and retrospective studies in humans [9]. Unfortunately, while ceftiofur reaches therapeutic concentrations in the non-gravid equine uterus [22], it does not cross the equine placenta and thus is not useful for reproductive related diseases such as placentitis [23].

Tetracyclines are bacteriostatic in nature, and act by reversibly binding the 30S subunit of the bacterial ribosome to inhibit protein synthesis. Oxytetracycline, doxycycline, and occasionally minocycline are used in equine practice to treat systemic infections from *Borrelia burgdorferi* to *Neorickettsia risticii.* Oxytetracycline is often the drug of choice for fevers of unknown origin in the horse. While there is no data on toxicity or transplacental diffusion in equine species, oxytetracycline is also commonly administered in the final trimester of ruminant pregnancy to prevent abortion in herds or flocks at risk of aborting due to *Chlamydia* infection [24]. Despite widespread use without consequence in ruminants, tetracyclines are avoided in human pregnancy given the reported effects on tooth discoloration in neonates and young children [25]. Interestingly, given the effectiveness of doxycline against fevers of unknown origin, and in tropical diseases

such as typhus in humans, attitudes towards doxycline use during pregnancy may be changing in human medicine [26]. More recent reports have suggested that short courses of doxycline treatment during gestation have no negative effects on the resulting child's teeth, or overall health [27]. Regardless, aside from anecdotal clinical experience, the pharmacokinetics, pharmacodynamics, and side effects of tetracycline use in the pregnant horse are still unknown.

In summary, there are limited options for gram negative placental or fetal infections in the horse that are resistant to trimethoprim sulfa. Other than trimethoprim sulfas and the tetracyclines, enrofloxacin is the only other widely available broad-spectrum antibiotic affordable and safe to use in horses. In addition, enrofloxacin, doxycycline, minocycline, and the trimethoprim sulfas are the only commonly prescribed oral antibiotics available for use in the horse. Oral antibiotics have the benefit of increased ease of treatment on farm by owners, and increased owner compliance. Enrofloxacin is the sole oral antibiotic that can be administered once a day in the horse, making enrofloxacin an attractive choice if it can be safely administered during pregnancy.

Enrofloxacin is a lipophilic fluoroquinolone with activity against both Gram-negative and Gram-positive pathogens. Fluoroquinolones inhibit topoisomerase I (DNA Gyrase) and topoisomerase IV, which results in inhibition of DNA synthesis and separation in dividing bacteria [28,29]. Fluoroquinolones are attractive antimicrobials because they act rapidly in a dose dependent manner, and provide a post-antibiotic effect [28,30]. Enrofloxacin is also converted *in vivo* into ciprofloxacin, which is an active metabolite effective against many common pathogens. While ciprofloxacin cannot be safely and effectively administered to horses [31,32], enrofloxacin is commonly safely administered to horse either orally or intravenously [8,33,34]. Oral availability, rapid onset of action, and relative affordability in equine sized doses makes enrofloxacin an excellent choice when first-line antibiotics such as sulfonamides and β-lactams fail.

Fluoroquinolones are believed to be toxic to the fetus based upon questionable reports in other species and are mostly avoided in pregnant mares [5,35–37]. According to FDA guidelines for risk assessment during human pregnancy, a product is teratogenic if the product has *teratogenic potential at clinical doses used in humans*. Most studies demonstrating fluroquinolone toxicity in neonates used doses far exceeding clinical doses, and thus to do not meet the clinical dose criteria for a teratogen [38]. Additionally, the assumption that fluoroquinolones are teratogenic is based

on effects in the juvenile animal, and so far the teratogenicity assumption has not been validated by the few existing animal studies [16], or by human retrospective studies [9,39] .

FLUOROQUINOLONE TOXICITY

Fluoroquinolones are avoided in pregnant animals because *in vitro* and *in vivo* studies have provided evidence that administration of enrofloxacin can cause tendonitis and arthropathies, especially in young, growing animals [5,6,37,40]. To date, only a few studies with small sample sizes have assessed *in vivo* effects of enrofloxacin in the horse, often using supratherapeutic doses to attain maximum toxicity. When enrofloxacin was administered at 3 to 5 times the recommended therapeutic dose for 21 days, 3 of 12 horses developed musculoskeletal complications such as lameness, cellulitis, tendinitis and tarsal sheath effusion [8]. Horses receiving the recommended therapeutic dose (5 mg/kg q 24h) did not develop these complications, suggesting that prolonged administration of enrofloxacin at a therapeutic dose is safe in adult non-pregnant horses. When four two-week old foals were treated with twice the recommended dose (10 mg/kg, IV, q 24 hours) for eight days, three foals became moderately to severely lame and had tibial-tarsal joint effusion with roughening of the articular cartilage, suggesting that foals might be more sensitive to cartilage damage [6]. However, this increased dosage is generally not used and no studies have assessed the effects of the recommended dose on foals.

No studies have assessed the safety of enrofloxacin administration to pregnant mares. Use of enrofloxacin is generally avoided in pregnant mares under the assumption it crosses the placenta and will cause cartilage lesions in the fetus. In pregnant rabbits, both enrofloxacin and ciprofloxacin diffuse through the placenta, with enrofloxacin achieving much higher diffusion rates than ciprofloxacin [41]. However, rabbits have a hemochorial placenta, and it is possible that enrofloxacin does not cross the thicker epitheliochorial equine placenta. As studies with ceftiofur have shown, transplacental diffusion can differ between species [23]. Additionally the degree of fluoroquinolone toxicity depends on the specific antibiotic and species [42], and it is possible that enrofloxacin administration does not have the same effects on fetal cartilage that it does on neonatal animals.

Quinolones are believed to induce arthropathies by chelating divalent cations such as magnesium (Mg+2) [43–46]. Cartilage and tendon are poorly vascularized tissues, and a decrease in available magnesium impairs fibronectin signaling in the extracellular matrix (ECM) [44,47].

This impairment disturbs cell adhesion, proliferation, and reduces proteoglycan synthesis, which is the main component of ECM [44,47]. Disturbances in cell adhesion and proliferation can lead to severe changes in cartilage architecture, as seen in studies where supratherapeutic doses of enrofloxacin were administered to chickens and puppies [5,37]. *In vitro* studies have demonstrated enrofloxacin exposure has a greater detrimental effect on chondrocyte glycosaminoglycan production in neonatal cartilage than in adult cartilage [46,48]). *In vitro* work by Davenport *et al* suggests that concentrations of enrofloxacin as low as 25 μg/ml will have a detrimental effect on neonatal equine cartilage; in comparison, in no effect was seen on adult cartilage proteoglycan concentrations until media enrofloxacin concentrations reached 1000 μg/ml [46].

While *in vivo* studies of enrofloxacin's effects on matrix metalloprotease and cartilage related gene mRNA expression are lacking, *in vitro* studies have shown conflicting results [46,49] .When neonatal and adult equine cartilage explants and chondrocyte cultures were exposed to varying concentrations of enrofloxacin or magnesium deficient cultures, a decrease in glycosaminoglycan synthesis was seen, but no differences were seen in mRNA expression of aggrecan, *Coll II*, biglycan, decorin, link protein, or *MMPs* 1,3, and 13 in the cultured chondrocytes[46]. In contract, when canine chondrocytes were cultured with an unknown concentration of enrofloxacin, chondrocytes produced less s-GAG, and increased expression of *IL-1B*, *TNF*, and *MMP3* [42]. In the same study, marbofloxacin had a more severe effect on canine chondrocytes, highlighting the importance of caution when assuming similar toxicities between fluoroquinolones. Additionally, it is likely that *in vitro* systems do not adequately reflect the dynamics of cartilage gene expression, and data on gene expression in fluoroquinolone-treated cartilage is lacking.

CARTILAGE BIOLOGY

Articular cartilage is a viscoelastic tissue comprised of chondrocytes and extracellular matrix, and healthy articular cartilage is vital to the performance animal. Normal adult cartilage consists of a superficial, middle, and deep zone, and each zone is composed of its own arrangement of chondrocytes and extracellular matrix. Equine cartilage extracellular matrix is approximately 70% water, 15 % collagen, and 10% proteoglycans, and 5% other lipids and minerals [50,51]. The superficial zone consists of flat cells oriented along the axis of joint movement, and chondrocytes in this zone both produce lubricating molecules that aid in joint movement, and resist shear stress

on the joint. Extracellular matrix is dense in the superficial zone. The middle and deep zones contain larger, round chondrocytes organized in vertical rows, perpendicular to the joint surface. These chondrocytes produce collagen II, aggrecan, and other cartilage matrix molecules, and provide articular cartilage with biomechanical resilience. Collagen II is organized in fibrils providing tensile strength, and aggrecan provides the necessary elasticity. The deep zone contains the largest chondrocytes, which both produce extracellular matrix and interact with the underlying subchondral bone.

Neonatal articular cartilage is less organized than adult cartilage, and at birth, is compact, highly cellular, and contains relatively less matrix [52]. Equine neonatal articular cartilage is thicker than adult cartilage[53], contains high levels of collagen II, and expresses a high degree of matrix adaptation [52,54,55]. After birth, articular cartilage thins as chondrocytes hypertrophy, matrix accumulates, and zonal organization begins [53,56]. Proteoglycan and collagen content vary in the cartilage based on age, joint of interest, and region within the joint [54,57]. Normally, glycosaminoglycan chains in the proteoglycan molecules carry a negative charge, which attracts sodium, and draws water into the cartilage by osmotic pressure. When cartilage is compressed, some water is expelled, but returns as soon as compression, or loading ceases. When cartilage begins to degrade and glycosaminoglycans are lost, less water is retained in the tissue.

Cartilage that forms after injury is described as fibrocartilage, and usually contains more collagen type I, and fewer proteoglycans than normal articular cartilage [58]. This repair tissue is also less strongly bound to the surrounding healthy cartilage, and often detaches or separates from surrounding cartilage when injured, or as seen in damaged cartilage exposed to high concentrations of enrofloxacin [5,37].

While there is an extensive body of research surrounding cartilage injury and repair, there is still a relatively poor understanding of embryonic cartilage formation and development throughout gestation. The early embryonic skeleton forms as continuous uninterrupted cartilage masses [59], and then “interzones” form at future joint sites [60]. Cells present in the interzone of the forming embryonic joint are critical for proper joint formation [60,61], and these interzones are composed of compacted and flat mesenchymal cells that originate from de-differentiated chondrocytes after they stop expressing Col 2a1 [62–64]. Cell lineage tracing experiments have demonstrated that progenitor cells of the interzone contribute to the formation of the articular cartilage, synovial lining, and joint ligaments [62,65–68]. Articular chondrocytes descend from

progenitor cells present in the interzone, and signals affecting these cells could compromise articular cartilage in the resulting foal.

Normal limb bud development begins around 20 days gestation in the horse, and is quickly followed by footpad and elbow joint formation (days 30 to 40). Next the femorotibial joint develops, from initial cavitation at 45 days gestation, to complete joint formation by 65 days [61]. Around day 40, the equine fetus first begins intrinsic muscular activity such as head movements [69]. Fetal movement is vital to proper joint formation, and avian and murine studies that prevented embryo limb movement demonstrated a failure in normal joint development [70–72]. Any insult resulting in failure in proper interzone formation, or initiation of fetal movement could have severe effects on fetal development. Collectively, 45-60 days of gestation is a critical time-point in equine synovial joint development, making fluoroquinolone exposure potentially harmful during this time period.

Post-natally, articular cartilage thickens, matrix accumulates, and zonal organization occurs that is vital to joint biomechanical function[52,56]. While relatively little is known about postnatal cartilage growth, a few equine studies have compared gene expression in neonatal cartilage to mature repair cartilage, and to stem cells. Mienaltowski *et al* demonstrated that equine neonatal cartilage gene expression was consistent with cellular growth and expansion, while mature cartilage was functionally focused on withstanding the stress associated with weight bearing and locomotion [73]. While the exact time frame is not known for the organization of the articular cartilage into three zones, murine and bovine studies have demonstrated differential gene expression between cartilage zones [64,74], with deeper cartilage similar to the resting growth plate, and superficial cartilage similar to the hypertrophic zone of the growth plate. Collectively, it is possible that fluoroquinolone exposure during this time of intense reorganization postnatally may have more drastic effects than if exposure occurs in utero, before zonal reorganization occurs.

DRUG PHARMACOKINETICS AND PHARMACODYNAMICS

Given the potentially narrow margin of safety of enrofloxacin administration to pregnant mares, it was essential to determine enrofloxacin pharmacokinetics and pharmacodynamics in the pregnant mare. While very little is known about how pregnancy affects pharmacodynamics in the mare, pregnancy can affect maternal drug absorption, distribution, and elimination in other species. Plasma volume, total body water, and renal blood flow increase during and can have an effect on

pharmacokinetics and pharmacodynamics. Pregnant humans can experience delayed gastric emptying and decreased gastrointestinal motility, which is believed to be progesterone mediated [75–78]. Progesterone can also induce changes in hepatic microsomal enzyme activity [78,79]. These physiologic changes can have profound effects on the bioavailability, distribution, metabolism, and excretion of drugs [79,80]. In addition to maternal physiological changes, the fetal-placental unit can affect drug distribution and elimination if the placenta or fetus is involved in drug metabolism, or if the fetal fluids affect volume of drug distribution[78]. As a result, it may be necessary to adjust doses and dosing intervals of an antibiotic during pregnancy to optimize maternal and fetal health.

Traditionally, serum drug concentrations and bacterial minimum inhibitory concentrations (MIC) have been used to determine optimal antimicrobial dosage. For fluoroquinolones, pathogens with an MIC<0.5 μg/ml are considered susceptible to enrofloxacin according 2008 CLSI breakpoints[81]. However, this single parameter is not sufficient to predict cure rate in many cases, and other efficacy parameters are important to consider. The ratio of AUC_{24}: MIC has been used to predict antimicrobial efficacy, and a study assessing fluoroquinolone efficacy for gram-negative infections in mice suggested that a AUC_{24}: MIC > 100 is optimal for bacterial cure [82]. However, the AUC_{24}:MIC ratio needed for clinical cure varies, and in animals with competent immune systems, ratios of 30 to 60 may be sufficient for treatment some infections [83,84].

One limitation of using AUC_{24}: MIC ratios as a marker of clinical efficacy is the fact that ciprofloxacin is an active metabolite of enrofloxacin. If enrofloxacin and ciprofloxacin have an additive effect in bacterial infections, then the AUC_{24}/MIC ratio may be calculated by adding enrofloxacin and ciprofloxacin AUC_{24}, as it was calculated in previous poultry and bovine studies [85,86]. However, if enrofloxacin and ciprofloxacin act synergistically, or in cases where ciprofloxacin MIC_{90} and enrofloxacin MIC_{90} are vastly different (*Strep. zooepidemicus* for example), then addition of the two AUC_{24} is likely not appropriate [87]. In such cases, it may be preferable to evaluate only the enrofloxacin AUC_{24}: MIC_{90} ratios.

The AUC_{24}: MIC ratio cut-offs for clinical cure and bacterial resistance are not only antibiotic and species specific, but pathogen specific as well. While there is no *in vivo* data for enrofloxacin, or for equine infections, *in vitro* studies with ciprofloxacin and human dosing regimens have suggested that AUC/MIC ratios > 45 h may be sufficient for bacterial and clinical cure of certain gram-positive bacteria such as *Streptococcus pneumonia* [84,88,89]. The

recommended AUC_{24}:MIC ratios for a cure rate are based on free plasma concentrations. While local tissue concentrations are important to consider for ocular or central nervous system infections, fluoroquinolones are lipophilic drugs that are moderately protein bound in the plasma and penetrate most tissues well in the horse [90]. Reported endometrial enrofloxacin concentrations after systemic administration range from 1.689 µg/g after an single dose [91] to 10.19 µg/g (n=2) two hours after the 6th dose of enrofloxacin [90], and it has recently been shown that both enrofloxacin and ciprofloxacin cross the equine placenta [92]. Similarly, enrofloxacin and ciprofloxacin reach synovial fluid concentrations similar to the plasma in horses [90] and reach interstitial fluid similar to plasma concentrations in calves [86] and pigs [83], suggesting that plasma AUC_{24}:MIC ratios are sufficient to estimate local interstitial fluid concentrations at the site of infection.

Appropriate antimicrobial stewardship necessitates the selection of the optimal drug for a given microorganism without compromising patient health. Regardless of *in vitro* sensitivity patterns, antimicrobial efficacy depends on multiple factors, including the pathogen present, the infected tissue, the potential synergistic effects of ciprofloxacin and enrofloxacin, systemic concentrations after multiple doses, and the known post-antibiotic effect observed with fluoroquinolones [28]. Pregnancy can affect drug metabolism and excretion, and these changes are especially important to consider for medications with apparently narrow therapeutic windows, or a large volume of distribution that may encompass both foetal tissues and fluids.

FETAL FLUID ANALYSIS

While amniocentesis is a commonly employed practice in human medicine, few studies have attempted fetal fluid analysis in the pregnant horse. Options include transabdominal amnio- and allocentesis [93], fluid collection at parturition [94], or placement of microdialysis probes for continuous monitoring [20]. Transabdominal ultrasound-guided fluid collection allows for differentiation between amniotic and allantoic fluids, and enables the collection of larger volumes of fluid, but also results in large bore puncture of the fetal membranes, and potentially poses an increased risk of bacterial infection, or initiation of parturition. Fluid collection at abortion or parturition only allows for the collection of one sample, and the fluid may be contaminated by vaginal secretions at time of collection. Microdialysis probes have the benefit of continuous analysis without multiple punctures, but only measure the unbound drug fractions, and thus may

greatly underestimate, or fail to detect, highly protein bound drugs [20]. Additionally, microdialysis probes may not be optimal for long term monitoring. Direct puncture of fetal membranes was chosen in the following studies to allow for collection of both allantoic and amniotic fluid, and to allow for collection over a period of ten or more days.

TENDON EVALUATION

Traditionally, toxicity studies have determined adverse effects based on clinical signs, blood work, and histological evaluations of tissues of interest. Given the athletic demands of the horse, it was vital to confirm that enrofloxacin did not affect tendon tensile strength or cartilage strength in treated animals. Flexor tendon tensile strength has been evaluated in equine studies investigating regenerative techniques [95], and may be one method to detect small weaknesses in tendon strength that would predispose foals to tendon rupture as is occasionally seen in humans with Achilles tendon rupture [96].

ADVANCED IMAGING

At a time when there is a significant pressure to reduce the use of animals in research, there is a critical need to develop noninvasive means, such as magnetic resonance imaging (MRI), for monitoring toxicity to cartilage and tendons over time. Traditionally, radiographs and ultrasound have been used to evaluate equine musculoskeletal disease, however both methods are relatively insensitive to articular surface changes. Most subtle early bony changes are not detectable on radiographs, and only severe joint effusion is visible. Ultrasonography is excellent for evaluating tendon and ligament lesions, but is only capable of detecting small areas of cartilage[97]. In addition, cartilage thickness and appearance is very easily influenced by operator error or ultrasound beam angle [57,98].

As technology improved, and allowed for imaging of larger body segments, MRI became available for use in equine medicine. MRI has the advantage that it provides a non-invasive means of evaluating cross sectional and three dimensional views of joints and articular cartilage[99]. MRI is particularly well suited for analyzing cartilage because it images water and fat within the animal, and equine cartilage is roughly 70% water [50]. Many different MRI imaging sequences exist, and they are classified by the effect the radiofrequency pulse has on tissue magnetization.

T2 relaxation time, or transverse relaxation time, is dependent on cartilage hydration. Tissue relaxation times are usually determined using a gradient echo sequences, where a series of images are used to determine the relaxation time. A series of images composed of different T2-weighted relaxation times can t be fitted to an equation $S= S*0^{exp}(-1/T2)$ to create a single image of T2 relaxation time. Articular T2 relaxation time is affected by the collagen fibril network in the cartilage. Normal mature cartilage has a laminar appearance in T2 relaxation time maps [100], which is attributed to the properties of collagen fibrils causing a magic angle effect [101,102]. The histological zones of cartilage are determined by organization of cartilage, and appear distinct on T2 maps.

Quantitative MRI (qMRI) can be used to evaluate proteoglycan content (T1p maps) and collagen structure (T2 maps) and has been used to evaluate cartilage damage, cartilage necrosis, and juvenile articular cartilage development. Recently, Martel and colleagues used qMRI to evaluate proteoglycan and collagen content as well as structure of neonatal equine epiphyseal cartilage with q3T MRI [103]. T2 relaxation time is affected by cartilage health, and in general increases in degenerating cartilage[104,105]. The expected pathologic changes in T2 relaxation times depend on species, chronicity of the lesion, and animal maturity. Fibrous repairs of osteochondral defects are expected to have shorter T2 relaxation times, whereas hyaline-like repairs may exhibit prolonged T2 relaxation times [100,106]. Experimental enzymatic digestion of cartilage has demonstrated that T2 maps reflect the degree of collagen fibril network integrity [107]. Similar changes in T2 relaxation time can be seen in proteoglycan depleted cartilage [108]. On the contrary, some fibrous cartilage has decreased relaxation times compared to controls [109,110].

While qMRI has been employed to monitor repair of experimentally induced lesions over time, one goal of the following dissertation is to validate the use of qMRI in toxicity studies. By comparing qMRI to traditional histology and radiographs, we hope to validate the use of qMRI for lesion detection in future toxicity studies.

Anecdotal reports suggest that enrofloxacin can be administered to pregnant mares without apparent major musculoskeletal complications to the resulting foal. Given the current ambiguity surrounding the safety of enrofloxacin use in pregnant animals and its potential adverse effects on the fetus, there is a critical need to evaluate the relative safety of enrofloxacin to newborn foals when pregnant mares are treated with enrofloxacin in late gestation. Identifying a potential

negative impact of *in utero* fluoroquinolone exposure on the fetus and resulting foal is essential before recommending use of the drug in the veterinary clinical setting. Information from this dissertation will provides critical information for practitioners faced with resistant infections in pregnant animals.

In summary, this dissertation will address the following questions:

DISSERTATION AIMS

To determine if fluoroquinolones cross the equine placenta and if they accumulate in the fetus or fetal fluids without affecting the developing fetus. In addition, to explore advanced imaging and biomechanical testing as methods for evaluating subtle toxicities and to allow for future long-term studies that reduce animal use.

SPECIFIC AIM 1: Determine pharmacokinetics of enrofloxacin administered orally and intravenously to pregnant and non-pregnant stage using a cross-over design.

1a) Does pregnancy affect the bioavailability, distribution, plasma concentrations and excretion of enrofloxacin?

1b) Is oral enrofloxacin absorbed well in the horse, and is once a day dosing appropriate?

SPECIFIC AIM 2: Evaluate the articular cartilage of long bones and the structure of tendons of fetuses from mares treated with intravenous enrofloxacin.

2a) Do enrofloxacin and ciprofloxacin cross the equine placenta and reach MIC for common bacteria in fetal fluids and the fetus?

2b) Are morphologic lesions detectable in long bone articular cartilage of the fetus after enrofloxacin administration to the mare?

2c) Does exposure to enrofloxacin in utero cause detectable differences in fetal tendon morphology?

SPECIFIC AIM 3: Evaluate articular cartilage of long bones and tendons in 35-day-old foals from mares treated with enrofloxacin during late pregnancy to determine if toxicity is load-dependent.

3a). Does enrofloxacin administration to late term pregnant mares cause changes in the long bone articular cartilage and tendon histology of foals at 35 days of age?

3b). Are lesions detectable on radiographs in 35-day-old foals exposed to enrofloxacin in utero?

3c). Can qMRI be used to detect lesions in foal tendons in cartilage, and do these findings correlate with changes seen on radiographs or histological evaluation?

3d). Does enrofloxacin exposure in utero affect tendon and cartilage strength at 35 days of age?

3f). Does enrofloxacin exposure in utero result in differences in chondrocyte mRNA expression or collagen production in the resulting 35-day-old foals?

SPECIFIC AIM 4: Determine the effects of enrofloxacin exposure in on the first trimester equine fetus.

3a). Determine fluoroquinolone concentrations in the fetal fluids in the early equine pregnancy.

3b). Evaluate gross and histologic morphology of the 60-day-old equine fetus.

REFERENCES

1. Canisso, I., Ball, B.A., Erol, E., Ms, D.V.M., Squires, E.L. and Troedsson, M.H.T. (2015) Comprehensive Review on Equine Placentitis. **61**.
2. Hong, C.B., Donahue, J.M., Giles, R.C., Poonacha, K.B., Roberts, A.W., Smith, B.J., Tramontin, R.R., Tuttle, P.A. and Swerczek, T.W. (1993) Etiology and pathology of equine placentitis. *J. Vet. Diagnostic Investig.* **5**, 56–63.
3. Hong, C.B., Donahue, J.M., Giles, R.C., Poonacha, K.B., Roberts, A.W., Smith, B.J., Tramontin, R.R., Tuttle, P.A. and Swerczek, T.W. (1993) Equine abortion and stillbirth in central Kentucky during 1988 and 1989 foaling seasons. *J. Vet. Diagnostic Investig.* **5**, 560–566.
4. Giles, R.C., Donahue, J.M., Hong, C.B., Tuttle, P.A., Petrites-Murphy, M.B., Poonacha, K.B., Roberts, A.W., Tramontin, R.R., Smith, B. and Swerczek, T.W. (1993) Causes of abortion, stillbirth, and perinatal death in horses: 3,527 cases (1986-1991). *J. Am. Vet. Med. Assoc.* **203**, 1170–1175.
5. Stahlmann, R., Kuhner, S., Shakibaei, M., Schwabe, R., Flores, J., Evander, S.A. and Sickle, D.C. van (2000) Chondrotoxicity of ciprofloxacin in immature beagle dogs: immunohistochemistry, electron microscopy and drug plasma concentrations. *Arch Toxicol* **73**, 564–572.
6. Vivrette, S., Bostian, A., Bermingham, E. and Papich, M. (2001) Quinolone-induced arthropathy in neonatal foals. *AAEP Proc.* **47**, 376–377. publication_detail.
7. LeBlanc, M.M. (2009) The current status of antibiotic use in equine reproduction. *Equine Vet. Educ.* **21**, 156–167.
8. Bertone, A.L., Tremaine, W.H., Macoris, D.G., Simmons, E.J., Ewert, K.M., Herr, L.G. and Weisbrode, S.E. (2000) Effect of long-term administration of an injectable enrofloxacin solution on physical and musculoskeletal variables in adult horses. *J. Am. Vet. Med. Assoc.* **217**, 1514–21.
9. Crider, K.S., Berry, R.J., Hobbs, C.A. and Hu, D.J. (2009) Antibacterial medication use during pregnancy and risk of birth defects. *Arch. Pediatr. Adolesc. Med.* **163**, 978–985.
10. Mehta, N. and Larson, L. (2011) Pharmacotherapy in pregnancy and lactation. *Clin. Chest Med.* **32**, 43–52. http://dx.doi.org/10.1016/j.ccm.2010.11.002.
11. Hernandez-Diaz, S., Werler, M., Walker, A. and Mitchell, A. (2000) Folic acid antagonists during pregnancy and the risk of birth defects. *N. Engl. J. Med.* 1608–1614.
12. Bailey, C.S., Macpherson, M.L., Pozor, M.A., Troedsson, M.H.T., Benson, S., Giguere, S., Sanchez, L.C., LeBlanc, M.M. and Vickroy, T.W. (2010) Treatment efficacy of trimethoprim sulfamethoxazole , pentoxifylline and altrenogest in experimentally induced

equine placentitis. *Theriogenology* **74**, 402–412.
13. Rebello, S.A., Macpherson, M.L., Murchie, T.A., LeBlanc, M.M. and Vickrow, T.W. (2006) Placental transfer of trimethoprim sulfamethoxazole and pentoxifylline in pony mares. *Anim. Reprod. Sci.* **94**, 432–433.
14. Ferrer, M.S. and Palomares, R. (2017) Aerobic uterine isolates and antimicrobial susceptibility in mares with post-partum metritis. *Equine Vet. J.* **50**, 202–207.
15. Davis, H. a, Stanton, M.B., Thungrat, K., Boothe, D.M. and D, P. (2013) Uterine bacterial isolates from mares and their resistance to antimicrobials: 8,296 cases (2003–2008) Heather. *J. Am. Vet. Med. Assoc.* **242**, 977–983.
16. Nahum, G.G., Uhl, K. and Kennedy, D. (2006) Antibiotic use in pregnancy and lactation: what is and is not known about teratogenic and toxic risks. *Obstet. Gynecol.* **107**, 1120–1138.
17. Kirkwood, A. (2007) Is Gentamicin Ototoxic to the Fetus? *J. Obstet. Gynaecol. Canada* **29**, 140.
18. Martingano, D., Guan, X., Renson, A., Singh, S., Kesavan, M., Kim, J. and Carey, J. (2018) Daily dosing of gentamicin using ideal body weight for the treatment of intrapartum chorioamnionitis : a pilot study. *J. Matern. Neonatal Med.* **31**, 1194–1197. https://doi.org/10.1080/14767058.2017.1311861.
19. Chean, R., Garland, S.M. and Leung, L. (2017) Gentamicin in pregnancy: seeing past the drug categorisation in pregnancy. *Intern. Med. J.* 124–125.
20. Murchie, T.A., Macpherson, M.L., LeBlanc, M.M., Luznar, S. and Vickroy, T.W. (2006) Continuous monitoring of penicillin G and gentamicin in allantoic fluid of pregnant pony mares by in vivo microdialysis. *Equine Vet. J.* **38**, 520–525.
21. Prescott, J.F. (2013) Beta-lactam antibiotics : Cephalosporins. In: *Antimicrobial therapy in veterinary medicine*, 5th ed., Eds: S. Giguere, J. Prescott and P. Dowling, John Wiley & Sons, Hoboken. pp 153–173.
22. Scofield, D., Black, J., Wittenburg, L., Gustafson, D., Ferris, R. and Hatzel, J. (2014) Endometrial tissue and blood plasma concentration of ceftiofur and metabolites following intramuscular administration of ceftiofur crystalline free acid to mares. *Equine Vet. J.* **46**, 606–610.
23. Macpherson, M.L., Giguère, S., Hatzel, J.N., Pozor, M., Benson, S., Diaw, M., Sanchez, L.C., Vickroy, T.W., Tell, L., Wetzlich, S. and Sims, J. (2013) Disposition of desfuroylceftiofur acetamide in serum, placental tissue, fetal fluids, and fetal tissues after administration of ceftiofur crystalline free acid (CCFA) to pony mares with placentitis. *J. Vet. Pharmacol. Ther.* **36**, 59–67.
24. Nietfeld, J.C. (2001) Chlamydial infections in small ruminants. *Vet. Clin. North Am. Food Anim. Pract.* **17**, 301–314. http://dx.doi.org/10.1016/S0749-0720(15)30030-X.
25. Conchie, J., Munroe, J. and Anderson, D. (1970) The incidence of staining of permanent teeth by the tetracyclines. *C.M.A. J.* **103**, 351–356.
26. Cross, R., Ling, C., Day, N.P.J., Mcgready, R., Paris, D.H., Cross, R., Ling, C., Day, N.P.J., Mcgready, R. and Paris, D.H. (2016) Revisiting doxycycline in pregnancy and early childhood – time to rebuild its reputation ? *Expert Opin. Drug Saf.* **15**, 367–382. http://dx.doi.org/10.1517/14740338.2016.1133584.
27. Reports, B. (1998) Doxycycline and staining of permanent teeth. *Pediatr. Infect. Dis. J.* **17**, 429–431.
28. Giguere, S. and Dowling, P. (2013) Fluoroquinolones. In: *Antimicrobial therapy in*

veterinary medicine. pp 295–314.
29. Vancutsem, P.M., Babish, J.G. and Schwark, W.S. (1990) The fluoroquinolone antimicrobials: structure, antimicrobial activity, pharmacokinetics, clinical use in domestic animals and toxicity. *Cornell Vet.* **80**, 173–186.
30. Mitchell, M.A. (2006) Enrofloxacin. *J. Exot. Pet Med.* **15**, 66–69.
31. Yamarik, T.A., Wilson, W.D., Wiebe, V.J., Pusterla, N., Edman, J., Papich, M.G., Pharmacokinetics, M.G. and Yamarik, T.A. (2010) Pharmacokinetics and toxicity of ciprofloxacin in adult horses. *J. Vet. Pharmacol. Ther.* **33**, 587–594.
32. Dowling, P.M., Wilson, R.C., Tyler, J.W. and Duran, S.H. (1995) Pharmacokinetics of ciprofloxacin in ponies. *J. Vet. Pharmacol. Ther.* **18**, 7–12. https://doi.org/10.1111/j.1365-2885.1995.tb00543.x.
33. Steinman, A., Britzi, M., Levi, O., Lavy, E., Lichter, A. and Soback, S. (2006) Lack of effect of diet on the pharmacokinetics of enrofloxacin in horses. *J. Vet. Pharmacol. Ther.* .
34. Nieuwoudt, C., Epstein, K., Cohen, N., Boothe, D. and Chandler, J. (2004) Pharmacokinetics and stability of an enrofloxacin oral gel formulation in horses. *Proc. Am. Assoc. Equine Pract.* .
35. Ebrahim Babaahmady (2011) Toxicology of baytril (enrofloxacin). *African J. Plant Sci.* .
36. Stahlmann, R. (2002) Clinical toxicological aspects of fluoroquinolones. *Toxicol. Lett.* **127**, 269–277.
37. Maślanka, T. and Jaroszewski, J.J. (2009) Effect of long-term treatment with therapeutic doses of enrofloxacin on chicken articular cartilage. *Pol. J. Vet. Sci.* **12**, 21–33.
38. Food and Drug Administration (2005) Reviewer Guidance: Evaluating the risks of drug exposure in human pregnancies.
39. Yefet, E., Schwartz, N., Chazan, B., Salim, R., Romano, S. and Nachum, Z. (2018) The safety of quinolones and fluoroquinolones in pregnancy : a meta-analysis. *BJOG An Int. J. Obstet. Gynaecol.* **125**, 1069–1076.
40. Khazaeil, K., Mazaheri, Y., Hashemitabar, M., Najafzadeh, H., Morovvati, H. and Ghadrdan, A.R. (2012) Enrofloxacin effect on histomorphologic and histomorphometric structure of lamb articular cartilage. *Glob. Vet.* **9**, 447–453.
41. Aramayona, J.J., Garcia, M.A., Fraile, L.J., Abadía, A.R. and Bregante, M.A. (1994) Placental transfer of enrofloxacin and ciprofloxacin in rabbits. *Am. J. Vet. Res.* **55**, 1313—1318. http://europepmc.org/abstract/MED/7802401.
42. Siengdee, P., Euppayo, T., Buddhachat, K., Chomdej, S. and Nganvongpanit, K. (2016) Two fluoroquinolones and their combinations with hyaluronan: comparison of effects on canine chondrocyte culture. *J. Vet. Pharmacol. Ther.* **39**, 439–451.
43. Egerbacher, M., Wolfesberger, B. and Gabler, C. (2001) In vitro evidence for effects of magnesium supplementation on quinolone-treated horse and dog chondrocytes. *Vet. Pathol.* **38**, 143–148.
44. Egerbacher, M., Edinger, J. and Tschulenk, M. (2001) Effects of enrofloxacin and ciprofloxacin hydrochloride on canine and equine chondrocytes in culture. *Am. J. Vet. Res.* **62**, 704–708.
45. Egerbacher, M., Wolfesberger, B., Walter, I. and Seirberl, G. (1999) Integrins mediate the effects of quinolones and magnesium deficiency on cultured rat chondrocytes. *Eur J Cell Biol* **78**, 391–397.
46. Davenport, C., Boston, R. and Richardson, D. (2001) Effects of enrofloxacin and magnesium deficiency of matrix metabolism in equine articular cartilage. *Am. J. Vet. Res.*

62, 160–166.
47. Sendzik, J., Lode, H. and Stahlmann, R. (2009) Quinolone-induced arthropathy: an update focusing on new mechanistic and clinical data. *Int. J. Antimicrob. Agents* **33**, 194–200.
48. Hildebrand, H., Kempka, G., Schlüter, G. and Schmidt, M. (1993) Chondrotoxicity of quinolones in vivo and in vitro. *Arch. Toxicol.* **67**, 411–415.
49. Lim, S., Hossain, M.A., Park, J., Choi, S.H. and Kim, G. (2008) The effects of enrofloxacin on canine tendon cells and chondrocytes proliferation in vitro. *Vet. Res. Commun.* **32**, 243–253.
50. Brama, P.A.J., Bank, R.A., Karssenberg, D., Barneveld, A. and Weeren, V. (2000) Topographical mapping of biochemical properties of articular cartilage in the equine fetlock joint. *Equine Vet. J.* **32**, 19–26.
51. Todhunter, R. (1996) Anatomy and physiology of synovial joints. In: *Joint Disease in the Horse*, Eds: C. McIlwraith and G. Trotter, Saunders, Philadelphia.
52. Brama, P. a J., TeKoppele, J.M., Bank, R. a, Barneveld, A. and Weeren, P.R. van (2002) Development of biochemical heterogeneity of articular cartilage: influences of age and exercise. *Equine Vet. J.* **34**, 265–269.
53. Brommer, H., Brama, P.A.J., Laasanen, M.S., Helminen, H.J., Weeren, P.R. Van and Jurvelin, J.S. (2005) Functional adaptation of articular cartilage from birth to maturity under the influence of loading : a biomechanical analysis. *Equine Vet. J.* **37**, 148–154.
54. Brama, P.A.J., Tekoppele, J.M., Bank, R.A., Barneveld, A. and Weeren, P.R. van (2000) Functional adaptation of equine articular cartilage: the formation of regional biochemical characteristics up to age one year. *Equine Vet. J.* **32**, 217–221.
55. Mienaltowski, M.J., Huang, L., Stromberg, A.J. and Macleod, J.N. (2008) Differential gene expression associated with postnatal equine articular cartilage maturation. *Musculoskelet. Disord.* **9**, 1–14.
56. Cluzel, C., Blond, L., Fontaine, P., Olive, J. and Laverty, S. (2013) Foetal and postnatal equine articular cartilage development: Magnetic resonance imaging and polarised light microscopy. *Eur. Cells Mater.* **26**, 33–48.
57. Barthez, P., Bais, R. and Vernooij, J. (2007) Effect of ultrasound beam angle on equine articular cartilage thickness measurement. *Vet. Radiol. Ultrasound* **48**, 457–459.
58. Riddle, W. (1970) Healing of articular cartilage in the horse. *J. Am. Vet. Med. Assoc.* **157**, 1471–1479.
59. Holder, N. (1977) An experimental investigation into the early development of the chick elbow joint. *J. Embryol. Exp. Morphol.* **39**, 115–127.
60. Holder, N. (1977) An experimental investigation into the early development of the chick elbow joint. *J. Embryol.* **39**, 115–127.
61. Jenner, F., Osch, G. van, Weninger, W., Geyer, S., Stout, T., Weeren, R. van and Brama, P. (2015) The embryogenesis of the equine femorotibial joint : The equine interzone. *Equine Vet. J.* **47**, 620–622.
62. Hyde, G., Boot-H, Andford, R.P. and Wallis, G.A. (2008) Col2a1 lineage tracing reveals that the meniscus of the knee joint has a complex cellular origin. *J. Anat.* 531–538.
63. Soeda, T., Deng, J.M., Crombrugghe, B. De, Behringer, R.R., Nakamura, T. and Akiyama, H. (2010) Sox9-expressing precursors are the cellular origin of the cruciate ligament of the knee joint and the limb tendons. *Genesis* 635–644.
64. Zhang, Q., Cigan, A.D., Marrero, L., Lopreore, C., Liu, S., Ge, D., Savoie, F.H. and You, Z. (2011) Expression of doublecortin reveals articular chondrocyte lineage in mouse

embryonic limbs. *Genesis* 75–82.
65. Decker, R.S., Koyama, E. and Pacifici, M. (2014) Genesis and morphogenesis of limb synovial joints and articular cartilage. *Matrix Biol.* **39**, 5–10.
66. Rountree, R.B., Schoor, M., Chen, H., Marks, M.E., Harley, V., Mishina, Y. and Kingsley, D.M. (2004) BMP Receptor signaling is required for postnatal maintenance of articular cartilage. *PloS Biol.* **2**, 1815–1827.
67. Koyama, E., Shibukawa, Y., Nagayama, M., Sugito, H., Young, B., Yuasa, T., Okabe, T., Ochiai, T., Kamiya, N., Rountree, R.B., Kingsley, D.M., Iwamoto, M., Enomoto-iwamoto, M. and Pacifici, M. (2008) A distinct cohort of progenitor cells participates in synovial joint and articular cartilage formation during mouse limb skeletogenesis. *Dev. Biol.* **316**, 62–73.
68. Hyde, G., Dover, S., Aszodi, A., Wallis, G.A. and Boot-handford, R.P. (2007) Lineage tracing using matrilin-1 gene expression reveals that articular chondrocytes exist as the joint interzone forms. *Dev. Biol.* **304**, 825–833.
69. Ginther, O. (1992) *Reproductive biology of the mare: basic and applied aspects*, 2nd ed., Equiservices Publishing, Cross Plains.
70. Fell, H. and Canti, R. (1934) Experiments on the development in vitro of the avian knee-joint. *Proc. R. Soc. London* **116**, 316–351.
71. Persson, M. (1983) The role of movements in the development of sutural and diarthrodial joints tested by long-term paralysis of chick embryos. *J. Anat.* **137**, 591–599.
72. Kahn, J., Shwartz, Y., Blitz, E., Krief, S., Sharir, A., Breitel, D.A., Rattenbach, R., Relaix, F., Maire, P., Rountree, R.B., Kingsley, D.M. and Zelzer, E. (2009) Muscle Contraction Is Necessary to Maintain Joint Progenitor Cell Fate. *Dev. Cell* **16**, 734–743. http://dx.doi.org/10.1016/j.devcel.2009.04.013.
73. Mienaltowski, M.J., Huang, L., Bathke, A.C., Stromberg, A.J. and Macleod, J.N. (2010) Transcriptional comparisons between equine articular repair tissue , neonatal cartilage , cultured chondrocytes and mesenchymal stromal cells. *Brief. Funct. Genomics* **9**, 238–250.
74. Amanatullah, D.F., Yamane, S. and Reddi, A.H. (2014) Distinct patterns of gene expression in the super fi cial , middle and deep zones of bovine articular cartilage. *J. Tissue Eng. Regen. Med.* **8**, 505–514.
75. Costantine, M.M. (2014) Physiologic and pharmacokinetic changes in pregnancy. *Front. Pharmacol.* **5 APR**, 1–5.
76. Lawson, M., Kern, F. and Everson, G. (1985) Gastrointestinal Transit Time in Human Pregnancy : Prolongation in the Second and Third Trimesters Followed by Postpartum Normalization. *Gastroenterology* **89**, 996–999.
77. Chiloiro, M., Darconza, G., Piccioli, E., Carne, M. De, Clemente, C. and Riezzo, G. (2001) Gastric emptying and orocecal transit time in pregnancy. *J. Gastroenterol.* **36**, 538–543.
78. Loebstein, R., Lalkin, A. and Koren, G. (1997) Pharmacokinetic changes during pregnancy and their clinical relevance. *Clin. Pharmacokinet.* **33**, 328–43.
79. Koren, G. and Pariente, G. (2018) Pregnancy- associated changes in pharmacokinetics and their clinical implications. *Pharmacol. Res.* 35–61.
80. Bengtsson, B., Jacobsson, S.-O., Luthman, J. and Franklin, A. (1997) Pharmacokinetics of penicillin-G in ewes and cows in late pregnancy and in early lactation. *J. Vet. Pharmacol. Ther.* **20**, 258–261.

81. Papich, M.G. (2013) Antimicrobials, susceptibility testing, and minimum inhibitory concentrations (MIC) in veterinary infection treatment. *Vet. Clin. North Am. Small Anim. Pract.* **43**, 1079–1089.
82. Craig, W.A. (1998) State of the art clinical article: pharmacokinetic/pharmacodynamic parameters: rationale for antibacterial dosing of mice and men. *Clin. Infect. Dis.* **26**, 1–10.
83. Messenger, K.M., Papich, M.G. and Blikslager, A.T. (2011) Distribution of enrofloxacin and its active metabolite , using an in vivo ultrafiltration sampling technique after the injection of enrofloxacin to pigs. *J Vet Pharmacol Ther* 452–459.
84. Wright, D.H., Brown, G.H., Peterson, M.L. and Rotschafer, J.C. (2000) Application of fluoroquinolone pharmacodynamics. *J. Antimicrob. Chemother.* **46**, 669–683.
85. Dimitrova, D.J., Lashev, L.D., Yanev, S.G. and Pandova, B. (2007) Pharmacokinetics of enrofloxacin in turkeys. *Res. Vet. Sci.* **82**, 392–397.
86. Davis, J.L., Foster, D.M. and Papich, M.G. (2007) Pharmacokinetics and tissue distribution of enrofloxacin and its active metabolite ciprofloxacin in calves. *J Vet Pharmacol Ther* **30**, 564–571.
87. Peyrou, M., Laroute, V., Vrins, A. and Doucet, M.Y. (2006) Enrofloxacin and marbofloxacin in horses: comparison of pharmacokinetic parameters, use of urinary and metabolite data to estimate first-pass effect and absorbed fraction. *J Vet Pharmacol Ther* **29**, 337–344.
88. Lister, P.D. and Sanders, C.C. (1999) Pharmacodynamics of trovafloxacin , ofloxacin, and ciprofloxacin against Streptococcus pneumoniae in an in vitro pharmacokinetic model. *Antimicrob. Agents Chemother.* **43**, 1118–1123.
89. Lacy, M.K., Lu, W., Xu, X., Tessier, P.R., Nicolau, D.P., Quintiliani, R. and Nightingale, C.H. (1999) Pharmacodynamic comparisons of levofloxacin , ciprofloxacin , and ampicillin against streptococcus pneumoniae in an in vitro model of infection. *Antimicrob. Agents Chemother.* **43**, 672–677.
90. Giguere, S., Sweeney, R.W. and Belanger, M. (1996) Pharmacokinetics of enrofloxacin in adult horses and and concentration of the drug in serum, body fluids, and endometrial tissues after repeated intragastrically administered doses. *Am. J. Vet. Res.* **57**.
91. Papich, M.G., Camp, S.D. Van, Cole, J.A. and Whitacre, M.D. (2002) Pharmacokinetics and endometrial tissue concentrations of enrofloxacin and the metabolite ciprofloxacin after i.v. administration of enrofloxacin to mares. *J. Vet. Pharmacol. Ther.* **25**, 343–350.
92. Ellerbrock, R.E., Canisso, I.F., Roady, P., Curcio, B., Lima, F.S. and Li, Z. (2018) Enrofloxacin and its active metabolite ciprofloxacin cross the equine placenta and did not cause lesions in the articular surface of the fetuses and foals. *J. Equine Vet. Sci.* **66**, 232.
93. Canisso, I.F., Ball, B.A., Squires, E.L. and Troedsson, M.H. (2014) How to perform transabdominal ultrasound-guided fetal fluid sampling in mares. *J. Equine Vet. Sci.* **34**, 1143–1147. http://dx.doi.org/10.1016/j.jevs.2014.06.013.
94. Santschi, E.M. and Papich, M.G. (2000) Pharmacokinetics of gentamicin in mares in late pregnancy and early lactation. *J. Vet. Pharmacol. Ther.* **23**, 359–363.
95. Durgam, S.S., Stewart, A.A., Sivaguru, M., Wagoner Johnson, A.J. and Stewart, M.C. (2016) Tendon-derived progenitor cells improve healing of collagenase-induced flexor tendinitis. *J. Orthop. Res.* **34**, 2162–2171.
96. Khaliq, Y. and Zhanel, G.G. (2005) Musculoskeletal injury associated with fluoroquinolone antibiotics. *Clin. Plast. Surg.* **32**, 495–502.
97. Barrett, M.F., Frisbie, D.D., Mcilwraith, C.W. and Werpy, N.M. (2011) The arthroscopic

and ultrasonographic boundaries of the equine femorotibial joints. *Equine Vet. J.* **44**, 57–63.
98. Kaleva, E., Saarakkala, S., Jurvelin, J.S., Virén, T. and Töyräs, J. (2009) Effects of ultrasound beam angle and surface roughness on the quantitative ultrasound parameters of articular cartilage. *Ultrasound Med. Biol.* **35**, 1344–1351. http://www.sciencedirect.com/science/article/pii/S0301562909001094.
99. Nelson, B.B., Kawcak, C.E., Barrett, M.F., Mcilwraith, C.W., Grinstaff, M. and Goodrich, L. (2018) Recent advances in articular cartilage evaluation using computed tomography and magnetic resonance imaging. *Equine Vet. J.* **50**, 564–579.
100. Nieminen, M.T., Nissi, M.J., Mattila, L. and Kiviranta, I. (2012) Evaluation of chondral repair using quantitative MRI. *Osteoarthr. Cartil.* **36**, 1287–1299.
101. Rubenstein, J.D., Kim, J.K., Morova-Protzner, I., Stanchev, P.L. and Henkelman, R.M. (1993) Effects of collagen orientation on MR imaging characteristics of bovine articular cartilage. *Radiology* **188**, 219–226.
102. Mlynarik, V., Degrassi, A., Toffanin, R., Jarh, O. and Vittur, F. (1996) A method for generating magnetic resonance microimaging T2 maps with low sensitivity to diffusion. *Magn. Reson. Med.* **35**, 423–425.
103. Martel, G., Couture, C.A., Gilbert, G., Bancelin, S., Richard, H., Moser, T., Kiss, S., Légaré, F. and Laverty, S. (2016) Femoral epiphyseal cartilage matrix changes at predilection sites of equine osteochondrosis: Quantitative MRI, second-harmonic microscopy, and histological findings. *J. Orthop. Res.* **34**, 1743–1752.
104. Nieminen, M.T., Rieppo, J., To, J., Hakuma, J.M., Silvennoinen, J., Hyttinen, M.M., Helminen, H.J. and Jurvelin, J.S. (2001) T 2 Relaxation reveals spatial collagen architecture in articular cartilage : A comparative quantitative MRI and polarized light microscopic study. *Magn. Reson. Med.* **46**, 487–493.
105. Xia, Y., Moody, J.B. and Lust, G. (2001) Quantitative in situ correlation between microscopic MRI and polarized light microscopy studies of articular cartilage. *Osteoarthr. Cartil.* **9**, 393–406.
106. Watanabe, A., Boesch, C., Anderson, S.E., Brehm, W. and Varlet, P.M. (2009) Ability of dGEMRIC and T2 mapping to evaluate cartilage repair after microfracture : a goat study. *Osteoarthr. Cartil.* **17**, 1341–1349. http://dx.doi.org/10.1016/j.joca.2009.03.022.
107. Nieminen, M.T., To, J., Rieppo, J., Hakuma, J.M., Silvennoinen, J., Helminen, H.J. and Jurvelin, J.S. (2000) Quantitative MR Microscopy of Enzymatically Degraded Articular Cartilage. *Magn. Reson. Med.* **43**, 676–681.
108. Watrin-Pinzano, A., Ruaud, J.-P., Olivier, P., Grossin, L., Gonord, P., Blum, A., Netter, P., Guillot, G., Gillet, P. and Loeuille, D. (2005) Effect of Proteoglycan Depletion on T2 Mapping in Rat Patellar Cartilage. *Radiology* **234**, 162–170. https://doi.org/10.1148/radiol.2341030394.
109. Nissi, M., Toyras, J., Laasanen, M., Rieppo, J., Saarakkala, S., Lappalainen, R., Jurvelin, J. and Nieminen, M. (2004) Proteoglycan and collagen sensitive MRI evaluation of normal and degenerated articular cartilage. *J. Orthop. Res.* **22**, 557–564.
110. David-Vaudey, E., Ghosh, S., Ries, M. and Majumdar, S. (2004) T2 relaxation time measurements in osteoarthritis. *Magn. Reson. Imaging* **22**, 673–682.

CHAPTER 3: THE PHARMACOKINETICS OF INTRAVENOUS AND ORAL ADMINISTRATION OF ENROFLOXACIN TO LATE-TERM PREGNANT AND POST-PARTURIENT MARES

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Running title: Pharmacokinetics of enrofloxacin in late-term pregnant and non-pregnant mares

ABSTRACT

Enrofloxacin, a fluoroquinolone antimicrobial with rapid bactericidal activity against gram-negative and gram-positive pathogens, may be an alternative to overcome unresponsive select cases of severe bacterial infections in pregnant mares. As pregnancy may affect drug bioavailability, distribution, metabolism, and excretion, dose adjustment might be necessary for pregnant mares. The objectives of this study were to determine the disposition of orally and intravenously administered enrofloxacin in pregnant and non-pregnant mares. Six light-breed, healthy pregnant mares (260 d gestation) were randomized in a crossover design for administration of single dose of either intravenous (5mg/kg) or oral compounded (7.5mg/kg) enrofloxacin with the opposite dose administered after a 7d washout period. The protocol was repeated 45-60 days

post-partum, 14-30 days after foals were weaned. Plasma samples were obtained via venipuncture at 0, 5, 10, 20, 30, 45, 60, 90 min, and 2, 3, 4, 6, 8, 12, 24, 36, 48 and 72h after enrofloxacin administration. Enrofloxacin and ciprofloxacin concentrations were measured by LC-MS/MS. Concentration versus time data were analyzed based on non-compartmental pharmacokinetics. Enrofloxacin $AUC_{0-\infty}$ was significantly higher in pregnant mares than non-pregnant mares after PO administration and tended to be higher after IV administration. Ciprofloxacin maximum plasma concentration (C_{max}) and concentration at 24 h (C_{24h}) were higher, and half-life of the terminal phase ($t_{½\lambda z}$) was longer in pregnant mares than non-pregnant mares after oral administration. Similarly, ciprofloxacin C_{24h} was higher in pregnant mares with intravenous administration. Oral bioavailability did not differ based on pregnancy status. Limitations of the study were that only six healthy mares were assessed, and disease may affect the endpoints evaluated. Other breed types and horse populations may have different results. A lack of established enrofloxacin AUC/MIC targets for equine pathogens limits pharmacokinetic-pharmacodynamic conclusions. In conclusion, the oral form of enrofloxacin used in this study was well absorbed, and oral bioavailability was comparable to previous studies. While differences in enrofloxacin and ciprofloxacin pharmacokinetics were seen between pregnant and non-pregnant mares, the recommended drug dose and dose intervals are appropriate for MIC < 0.25 μg/ml. Dosages may need to be adjusted for bacteria with a MIC > 0.25 μg/ml.
Keywords: fluoroquinolone, ciprofloxacin, pregnancy, LC-MS/MS, horse

INTRODUCTION

Antimicrobial therapy is necessary in equine practice to treat bacterial infections during pregnancy; however, the pharmacokinetics and pharmacodynamics of many antimicrobials during pregnancy are not known. β-Lactam antibiotics and aminoglycosides cross the equine placenta [1] and are commonly used in pregnant mares [2]. Cephalosporin antibiotics are also commonly and safely used during equine pregnancy but do not cross the equine placenta [3]. Potentiated sulphonamides cross the equine placenta [4] and are frequently used to treat infections in late-pregnant mares [2,5,6].

Fluoroquinolones act rapidly in a dose-dependent fashion and provide a post-antibiotic effect [7]. This class of drug inhibits topoisomerases II and IV, thus inhibiting DNA synthesis and separation in dividing bacteria [8]. Enrofloxacin is a fluoroquinolone antimicrobial with

bactericidal activity against gram-negative and gram-positive pathogens, and is metabolized *in vivo* into ciprofloxacin, an antimicrobial also effective against many pathogens [7,9].

Pregnancy induces physiologic changes in humans such as increases in plasma volume, total body water, and renal blood flow, while gastric emptying can be delayed [10]. Additionally, hormonal fluctuations during pregnancy can induce changes in hepatic microsomal enzyme activity [10,11]. These changes may affect bioavailability, distribution, metabolism, and excretion of many medications [10,12]. As a result, adjustment of dose or dosing interval during pregnancy may be needed to optimize maternal and fetal health [13]. Enrofloxacin is toxic in a dose-dependent manner, thus making dose optimization important, particularly in the pregnant mare [14,15].

The suggested therapeutic dose of enrofloxacin in horses is 5 mg/kg q24h for intravenous administration or 7.5 mg/kg q24h for oral administration [16,17]. The intravenous enrofloxacin formulation is used extra-label in horses, and can be irritating when administered intravascular or orally [18,19]. A recently available compounded liquid oral formulation is more practical and may have fewer side effects than intravenous administration; however, absorption and pharmacokinetics have not been evaluated.

The present study was designed to assess the pharmacokinetics of enrofloxacin administration to pregnant mares. The objectives were to determine the pharmacokinetics of oral and intravenous enrofloxacin administered to mares during both late gestation and postpartum. We hypothesized that the pharmacokinetics of enrofloxacin, and its active metabolite ciprofloxacin, differ between pregnant mares and non-pregnant mares.

MATERIALS AND METHODS

The study protocol was approved by the University of Illinois Institutional Animal Care and Use Committee (protocols, #14243 and #17245). and was carried out between 2016 and 2017.

Six healthy light-breed (four Standardbred, one Thoroughbred, and one cross) pregnant mares were enrolled at 260 days gestation and housed at the Veterinary Medicine Research Farm at the University of Illinois in Urbana, Illinois. Immediately before enrollment, physical examination and transrectal placental ultrasonography of pregnant mares revealed no abnormalities, and all mares went on to foal normally after the conclusion of the study. The animals were kept on pasture and supplemented with grass hay and trace minerals.

Mares were kept on pasture and were not fasted before antibiotic administration; however, supplemental hay was not provided until sixty minutes after antibiotic administration. The mares were randomly treated in a cross-over design with a single dose of either intravenous (5mg/kg bwt, Baytril®)[a] or compounded oral (7.5mg/kg bwt)[b] enrofloxacin. The oral formulation is an oil-based suspension (200 mg/ml) and was administered orally using a 35 ml syringe. This compounded suspension is proprietary but consisted of two vehicles, enrofloxacin, paraben-preserved water, and flavor. Three different batches were used in the study, and all batches were used within 90 days of compounding. Stability information provided by the compounder from a third-party reference laboratory demonstrated drug strength consistent with the labeled dose (200 mg/ml), and no change in drug concentration for 210 days after compounding.

Plasma samples were obtained via jugular venipuncture at 0, 5, 10, 20, 30, 45, 60, 90 min, and 2, 3, 4, 6, 8, 12, 24, 36, 48 and 72 h after enrofloxacin administration (IV or PO). The opposite route of administration was performed following a seven-day washout period with the entire protocol repeated 45-60 days post-partum. Foals were weaned at 30 days post-partum for another study, and the protocol was repeated 14-30 days after weaning. Thus, at the time of post-partum enrofloxacin administration, none of the mares were lactating or pregnant. Blood samples were collected in heparinized tubes and centrifuged at 600g/x10 minutes and plasma samples stored at -80^{o}C until analysis.

LC-MS assessments and validation

Concentrations of total plasma enrofloxacin and ciprofloxacin were measured by liquid chromatography-tandem mass spectrophotometry (LC-MS) in the Metabolomics Laboratory of the Roy J. Carver Biotechnology Center, University of Illinois Urbana-Champaign. Samples were prepared by mixing 150 µL plasma with 600 µL acetonitrile and 6 µL 5 µg/mL ofloxacin. After vortex, the mixture was centrifuged (5 min, 8,000 rpm), and the supernatant was used for instrument injection. Samples were analyzed with the 5500 QTRAP LC/MS/MS system[c]. Software Analyst 1.6.2 was used for data acquisition and analysis. The 1200 series HPLC system[d] includes a degasser, an autosampler, and a binary pump. The LC separation was performed on an Agilent SB-Aq column[d] (4.6 x 50 mm, 5 µm) with mobile phase A (0.1 % formic acid in water) and mobile phase B (0.1 % formic acid in acetonitrile). The flow rate was 0.3 mL/min. The linear gradient was as follows: 0-2 min, 95 % A; 10-13 min, 2 % A; 13.1-17 min, 95 % A. The

autosampler was set at 15°C. The injection volume was 1 µL. Mass spectra were acquired under electrospray ionization (ESI) with the ion spray voltage of +5000 V. The source temperature was 450 °C. The curtain gas, ion source gas 1, and ion source gas 2 were 33, 50, and 60 psi, respectively. Multiple reaction monitoring (MRM) was used for quantitation with MRM transition time and retention time (min) of m/z 360.1 --> m/z 316.1; 9.4 min (ciprofloxacin), m/z 332.1 --> m/z 288.1; 9.6 min (enrofloxacin), and m/z 362.1 --> m/z 318.1; 9.3 min (ofloxacin, internal standard).

The limit of detection was 0.3 ng/ml, lowest limit of quantitation was 1 ng/ml, and linearity was 1 - 1500 ng/mL for both enrofloxacin and ciprofloxacin. Overall, all standard curves have correlation coefficient values larger than 0.998 (the weighting factor is 1/x).

The recovery was calculated by the signal ratio of spiked standard into the blank plasma to spiked standard into 60 % acetonitrile for 6 s. Enrofloxacin recovery ranged from 94.4 ± 3.5 % for 5 ng/ml to 97.0 ± 3.8 % for 500 ng/ml (mean ± SD). Ciprofloxacin recovery ranged from 91.4 ± 2.2 % for 500 ng/ml to 93.7 ± 2.3 % for 5 ng/ml. Ofloxacin recovery was 97.3 ± 2.7%. The matrix effect was checked with constant post-column infusion experiment. There was no notable signal up and down, which indicates the absence of matrix effect.

Between-run and within-run accuracy and precision were calculated by measuring six samples of enrofloxacin and ciprofloxacin at concentrations of 5, 50, and 500 ng/ml, and measurements were repeated on three different days. Enrofloxacin had a within-run accuracy of 95.6 ± 2.1% to 99.1 ± 0.6%, and a between- run accuracy of 97.3 ± 2.0% to 98.4 ± 1.0%, and ciprofloxacin had a mean within-run accuracy of 95.4 ± 2.3 to 99.6 % ± 1.2 %, and between- run accuracy of 97.0 ± 2.4% to 99.2 ± 0.9%. Coefficients of variations between-run and within-run for enrofloxacin ranged from 0.9-1.6% and 1.1-3.5%, respectively, whereas coefficients of variations for ciprofloxacin between- run and within-run ranged from 0.9- 2.4% and 1.0- 5.4 %, respectively.

Plasma stability data was calculated at room temperature and at -75°C (n = 6). Samples stored in the autosampler for 24 h at 15 °C had bias of 3.2-4.8% and samples stored at -75 °C for 2 months had a bias of 1.3-3.4%.

Pharmacokinetics analyses

For each horse, plasma enrofloxacin and ciprofloxacin concentration versus time data were analyzed based on non-compartmental pharmacokinetics using a commercially available

software[e]. Maximum plasma concentrations (C_{max}) and times to achieve maximum plasma concentrations (T_{max}) were obtained directly from the plasma concentration data. The rate constant of the terminal phase (λ_z) was determined by linear regression of the logarithmic plasma concentration versus time curve using a minimum of four data points. The half-life of the terminal phase ($t_{½\lambda z}$) was calculated as ln 2 divided by λ_z. The area under the concentration-time curve (AUC) and the area under the first moment of the concentration-time curve (AUMC) were calculated using the trapezoidal rule, with extrapolation to infinity using C_{48h}/λ_z, where C_{48h} is the plasma concentration at the 48h sampling time. Mean residence time (MRT) was calculated as AUMC/AUC. Bioavailability was calculated as (AUC_{PO} /AUC_{IV}) x ($dose_{IV}$/$dose_{PO}$). Apparent volume of distribution based on the AUC (Vd_{area}) was calculated as IV dose /AUC• λ_z, apparent volume of distribution at steady state (Vd_{ss}) was calculated as (IV dose/AUC)/(AUMC/AUC), and systemic clearance (CL) was calculated from IV dose/AUC. The AUC/MIC ratios were calculated as AUC_{24}/MIC, and ratios were calculated in three different ways using the enrofloxacin AUC_{24}, ciprofloxacin AUC_{24}, or an AUC_{24} that combined both the enrofloxacin AUC_{24} and ciprofloxacin AUC_{24}.

Statistical analysis

Normality of the data was assessed based on histograms of differences in means, normal quantiles plots of the residuals, and the Shapiro-Wilk test. Constant variance of the data was assessed with Levene's test. Comparison of each pharmacokinetic variable between pregnant and non-pregnant mares was performed using the paired t-test (parametric data), or the Wilcoxon signed rank test (C_{max}, T_{max}). Significance was set at $p<0.05$, and tendency was defined as $0.1>p>0.05$.

RESULTS

There were no adverse effects noted after administration of oral or intravenous doses of enrofloxacin to the mares or resulting foals. Profile plots for enrofloxacin and ciprofloxacin concentrations are depicted in Figures 1 and 2. After IV injection, there was no difference in enrofloxacin elimination half-life ($t_{½\lambda z}$), volume of distribution (Vd_{area}), mean residence time (MRT), or C_{24h} between pregnant or non-pregnant mares (Table 3.1). There was a tendency for systemic clearance (CL) to be lower and area under the plasma concentration versus time curve

($AUC_{0-\infty}$) to be higher in pregnant mares compared to non-pregnant mares after administration of a single intravenous dose of enrofloxacin. No difference in ciprofloxacin C_{max} or T_{max} was seen after IV enrofloxacin administration to pregnant and non-pregnant mares. Plasma ciprofloxacin concentrations 24 h after IV enrofloxacin administration were higher in pregnant than in non-pregnant mares, and there was a tendency for ciprofloxacin $AUC_{0-\infty}$ to be higher, and $t_{½\lambda z}$ to be longer in pregnant than non-pregnant mares. One pregnant mare in the IV administration group had a $t_{½\lambda z}$ double that of the other five mares, contributing to the larger variability seen in the $t_{½\lambda z}$ and V_d in the pregnant IV group than the other three groups.

For oral administration of enrofloxacin, there was a greater enrofloxacin area under the curve concentration vs. time curve ($AUC_{0-\infty}$) in pregnant mares than non-pregnant mares (Table 2), and there was a tendency for T_{max} to be longer, and C_{24h} to be higher in pregnant mares than non-pregnant mares. Ciprofloxacin C_{max} after oral enrofloxacin administration was lower in pregnant mares than in non-pregnant mares, ciprofloxacin $t_{½\lambda z}$ was longer in pregnant mares, and ciprofloxacin C_{24h} was higher in pregnant mares than non-pregnant mares (Table 3.2). There was a tendency for time to maximum plasma ciprofloxacin concentration to be longer in pregnant mares than non-pregnant mares. Ciprofloxacin $AUC_{0-\infty}$ were not different between pregnant and non-pregnant mares after oral enrofloxacin administration. Oral bioavailability was not significantly different between the groups (pregnant mares 67.15 ± 11.36 %; non-pregnant mares 52.56 ± 16.14 %).

When oral and intravenous enrofloxacin administration were compared in pregnant mares, ciprofloxacin C_{24h} showed a tendency to be higher after IV administration than oral (Table 3). In non-pregnant mares, enrofloxacin $AUC_{0-\infty}$ tended to be greater after IV administration, and enrofloxacin C_{24h} was significantly higher after IV administration than PO. In non-pregnant mares, ciprofloxacin C_{max}, $AUC_{0-\infty}$, and C_{24h} were lower after IV enrofloxacin administration and after oral enrofloxacin administration (Table 3.3). When enrofloxacin or ciprofloxacin AUC_{24} was used to calculate AUC:MIC ratios, the AUCs obtained in this study were adequate for pathogens with an enrofloxacin MIC of < 0.25 µg/ml (Table 3.4).

DISCUSSION

This is first report to compare pharmacokinetic parameters in pregnant mares during late-gestation and non-pregnant mares. Despite minor variations in the endpoints assessed in the

present study, the recommended enrofloxacin dose of 5 mg/kg intravenously, or 7.5 mg/kg orally, is appropriate for treatment of susceptible organisms (MIC< 0.25 μg/mL) in both categories. Our findings suggest that the oral preparation of enrofloxacin used herein is well-absorbed, and bioavailability in the current study was similar to previous studies administering 5 mg/kg of crushed enrofloxacin tablets intragastrically (62.53 ± 19.65%) [20], or administering a 10% enrofloxacin poultry formulation orally (65.6% ± 14.9%) to non-pregnant mares [21]. No oral lesions were seen in any of the mares, in contrast to the lesions previously reported with oral administration of the intravenous product [19]. This suggests that mucosal irritation was likely due to n-butyl alcohol or benzyl alcohol present in the intravenous preparation, rather than enrofloxacin itself. Given the lack of oral lesions, good drug stability, and the satisfactory bioavailability, the oral formulation used in this study seems to be a suitable product to treat infections with enrofloxacin susceptible microorganisms.

Enrofloxacin $t_{½\lambda z}$ of the terminal phase after both intravenous and oral administration was slightly longer in both pregnant and non-pregnant mares than reported in previous studies [20,22,23] while shorter than the $t_{½\lambda z}$ reported in foals (~17h) [16]. Moreover, $t_{½\lambda z}$ was remarkably longer (~8h) in the current study than the reported $t_{½\lambda z}$ of 1.4 to 2.5 h in rabbits, calves and dogs [24,25]. There were no differences in ciprofloxacin T_{max} or C_{max} between pregnant and non-pregnant mares after intravenous enrofloxacin administration.

While there was no difference in enrofloxacin C_{max} after oral enrofloxacin administration, ciprofloxacin C_{max} was lower in pregnant mares than non-pregnant mares. Despite this difference in ciprofloxacin C_{max} after oral enrofloxacin administration, there was no difference in the C_{24} ciprofloxacin concentrations. Ciprofloxacin AUC_{24} tended to be greater in pregnant than non-pregnant mares; however, AUC_{0-t} and $AUC_{0-\infty}$ were not different. Since pregnancy is associated with delayed gastric emptying in humans [10], and both enrofloxacin and ciprofloxacin T_{max} tended to be longer in pregnant than non-pregnant mares, it is possible that the initial decrease in $C_{max,}$ and AUC_{24} could be due to delayed gastric emptying, although effects of pregnancy on gastrointestinal transit time are unknown in the horse. Delayed transit time in the small intestine may also explain the tendency for increased enrofloxacin bioavailability if delayed transit allowed for increased absorption.

Finally, it is possible that a first pass effect contributed to the larger enrofloxacin $AUC_{0-\infty}$ in pregnant mares than non-pregnant mares. Ciprofloxacin accounted for 17.2% (pregnant) or

16.0% (non-pregnant) of the enrofloxacin $AUC_{0-\infty}$ after intravenous enrofloxacin administration, and 20.6% (pregnant) or 38.2 % (non-pregnant) of the enrofloxacin $AUC_{0-\infty}$ after oral administration. The larger ciprofloxacin: enrofloxacin $AUC_{0-\infty}$ ratio after oral administration supports a first pass effect previously described after enrofloxacin administration to horses [26]. Additionally, the differences observed in oral enrofloxacin $AUC_{0-\infty,}$ and in ciprofloxacin: enrofloxacin ratios suggest that the first pass effect may be less pronounced in pregnant mares than non-pregnant mares. It remains to be determined if this difference is due to pregnancy associated changes in hepatic blood flow or hepatic enzyme activity. Enrofloxacin is metabolized by cytochrome p450 enzymes, and hepatic enzyme activity during pregnancy appears to be species specific. The activity of many hepatic cytochrome p450 enzymes decreases in pregnant rats and mice [27,28], while only cytochrome p450 3A decreases in dairy cattle [29], and pregnancy associated changes are unknown in the horse. Studies in poultry and carp have shown that iatrogenic changes in cytochrome p450 enzyme expression affect enrofloxacin pharmacokinetics [30,31], suggesting it is possible that pregnancy associated changes in enzymatic activity could have an effect on enrofloxacin metabolism in the horse.

Early enrofloxacin pharmacokinetic studies used an agar well microbiological diffusion assay, which did not allow investigators to differentiate between ciprofloxacin and enrofloxacin [17,20,23]. More recent studies used reverse phase high-performance liquid chromatography, which allows for differentiation between fluoroquinolones [22,32,33]. The analytical approach used in the present study enabled the precise measurement of pharmacokinetic curves of both enrofloxacin and its metabolite ciprofloxacin, as LC-MS combines high-performance liquid chromatography with mass spectrometry to reduce experimental error and improve accuracy. Precise determination of enrofloxacin and ciprofloxacin concentrations is valuable when assessing drug toxicity and drug dosages based on pathogen MIC.

Ciprofloxacin $t_{½\lambda z}$ tended to be longer in pregnant than the non-pregnant mares when enrofloxacin was administered intravenously. In addition, the $t_{½\lambda z}$ of this metabolite was significantly longer in pregnant mares than non-pregnant mares when enrofloxacin was administered orally. While early studies assessing enrofloxacin pharmacokinetics were unable to assess ciprofloxacin concentrations due to the bioassay used, ciprofloxacin $t_{½\lambda z}$ reported by Boeckh and collaborators were similar to this study [21]. Given that ciprofloxacin has known adverse effects such as colitis and laminitis in the horse when administered intravenously [34],

further research is needed to determine plasma ciprofloxacin concentrations after prolonged enrofloxacin administration to pregnant mares, as prolonged high plasma concentrations could potentially cause serious complications.

As fluoroquinolones are concentration-dependent antimicrobials, the absolute concentrations are more important than time exceeding MIC for maximum efficacy. In the present study, the $AUC_{0-\infty}$ for enrofloxacin was greater in late-gestation mares than non-pregnant mares after oral administration, and tended to be greater after intravenous administration, which is consistent with the $AUC_{0-\infty}$ reported elsewhere [21,22]. Enrofloxacin $AUC_{0-\infty}$ for late-gestation mares was comparable to a previous study using a bioassay reporting the combined $AUC_{0-\infty}$ of enrofloxacin and ciprofloxacin [23]. In contrast, the $AUC_{0-\infty}$ for non-pregnant mares was lower, but similar to the values reported in another study using liquid chromatography to measure drug concentrations [21]. These findings are important for determining whether dosing adjustments are needed when faced with bacterial infections with intermediate susceptibilities.

The ratio of AUC_{24}: MIC has been used to predict antimicrobial efficacy, and a study assessing fluoroquinolone efficacy for gram-negative infections in mice suggested that a AUC_{24}: MIC > 100 is optimal for bacterial cure [35]. At the doses given in the current study, and using published MICs for common equine bacterial isolates, these criteria are met consistently only for for *E .coli* isolates (Table 4). However, the AUC_{24}:MIC ratio needed for clinical cure varies, and in animals with competent immune systems it has been suggested that ratios of 30 to 60 may be sufficient for some infections [36,37]. Using these guidelines, the enrofloxacin AUCs obtained in the present study would be appropriate for bacterial isolates with an MIC of <0.25µg/mL, and questionable for isolates that fall in the intermediate range of 0.25 to 1.025µg/mL. Pathogens with an MIC<0.5 µg/ml are considered susceptible according to 2008 CLSI breakpoints[38]; however, pathogens with an MIC> 0.25µg/mL should be deemed of intermediate susceptibility at the dosages used in this study.

One limitation of using AUC_{24}: MIC ratios as a marker of clinical efficacy is the fact that ciprofloxacin is an active metabolite of enrofloxacin. If enrofloxacin and ciprofloxacin have an additive effect in bacterial infections, then the AUC_{24}/MIC ratio may be calculated by adding enrofloxacin and ciprofloxacin AUC_{24}, as was calculated in previous poultry and bovine studies [38,39]. However, if enrofloxacin and ciprofloxacin act synergistically, or in cases where ciprofloxacin MIC_{90} and enrofloxacin MIC_{90} are vastly different (*Strep. Zooepidemicus,* for

example), then addition of the two AUC_{24} is likely not appropriate [26]. In such cases, it may be preferable to evaluate only the enrofloxacin AUC_{24}: MIC_{90} ratios, as Peyrou and collaborators did in an equine study [26]. In the present study, combining both AUC_{24} would change only the interpretation of susceptibility for intravenous enrofloxacin administration to non-pregnant animals infected with pathogens with an MIC of 0.25 µg/ml (Table 3.4).

The AUC_{24}: MIC ratio cut-offs for clinical cure and bacterial resistance are not only antibiotic and species specific, but pathogen specific as well. While there is no *in vivo* data for enrofloxacin, or for equine infections, *in vitro* studies with ciprofloxacin and human dosing regimens have suggested that AUC/MIC ratios > 45 h may be sufficient for bacterial and clinical cure of certain gram-positive bacteria such as *Streptococcus pneumoniae* [37,40,41]. A cut-off ratio of 50 h was chosen for the current study based on published ratios for *in vivo* cut-offs for human gram-positive pathogens (>40 h), > 46 h for bactericidal action with Mannheimia haemolytica in calves [42], and > 60 h used in a previous equine fluoroquinolone study [43]. Other authors have chosen separate cut-offs for gram-positive (>50 h) and gram-negative (>100 h) organisms in enrofloxacin-treated pigs [36], or for low likelihood (>60 h) and high likelihood (>125 h) of successful clinical outcomes after marbofloxacin administration in horses [43]. Thus, while the dosages used in this study are appropriate for most bacterial pathogens with an MIC < 0.25 µg/ml, dose may need to adjusted for gram-negative pathogens with an MIC>0.06 µg/ml (non-pregnant) or MIC >0.12 µg/ml (pregnant) horses to reach an AUC_{24}: MIC predictive of a high chance of a successful clinical outcome.

The recommended AUC_{24}:MIC ratios for a cure rate are based on free plasma concentrations. However, previous work in dogs, calves, and pigs demonstrated that for antimicrobials with moderate protein binding, such as enrofloxacin and ciprofloxacin, total plasma AUCs are similar to the tissue AUCs [36,38,44]. While local tissue concentrations were not measured in the current study, fluoroquinolones are also moderately protein bound in the plasma and penetrate most tissues well in the horse [20]. Reported endometrial enrofloxacin concentrations after systemic administration range from 1.689 µg/g after an single dose [33] to 10.19 µg/g (n=2) two hours after the 6^{th} dose of enrofloxacin [20], and recently it has been shown that both enrofloxacin and ciprofloxacin cross the equine placenta [45]. Similarly, enrofloxacin and ciprofloxacin reach synovial fluid concentrations similar to the plasma in horses [20] and reach interstitial fluid similar to plasma concentrations in calves [38] and pigs [36], suggesting that total

plasma AUC_{24}:MIC ratios are sufficient to estimate local interstitial fluid concentrations at the site of infection [26,44].

Appropriate antimicrobial stewardship necessitates the selection of the optimal drug for a given microorganism without compromising patient health. Regardless of *in vitro* sensitivity patterns, antimicrobial efficacy depends on multiple factors, including the pathogen present, the infected tissue, the potential synergistic effects of ciprofloxacin and enrofloxacin, systemic concentrations after multiple doses, and the known post-antibiotic effect observed with fluoroquinolones [7]. Pregnancy can affect drug metabolism and excretion, and these changes are especially important to consider for medications with apparently narrow therapeutic windows, or a large volume of distribution that may encompass both fetal tissues and fluids. Given the few differences in pharmacokinetic variables in this study, both 5 mg/kg IV or 7.5 mg/kg PO enrofloxacin appear to be the appropriate doses to treat susceptible equine pathogens (MIC < 0.25 μg/ml) in the late-gestation mare. However, if enrofloxacin is used to treat pathogens with intermediate susceptibility to enrofloxacin (4> MIC> 0.25 μg/ml), dosages may need to be adjusted, and further studies exploring the safety and efficacy of increased dosages are warranted.

Acknowledgments & Author Contributions

We thank Jailson Honorato and Jordan Grossman for assistance with sample collection. IFC and SG designed the study. REE, JH, and BC carried out animal work. ZL performed LC-MS/MS evaluations. SG and REE did the pharmacokinetics analyses. REE and IFC wrote the manuscript. All authors reviewed the final draft.

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TABLES AND FIGURES

Table 3.1**:** Pharmacokinetic variables (mean ± SD) for enrofloxacin and its main metabolite ciprofloxacin after IV administration of a single dose of enrofloxacin (5 mg/kg of body weight) to 6 healthy mares. Mares were dosed during late gestation (260-275 days) and again 45 to 60 days post-partum, 15- 30 days after foals were weaned.

Variable	Status		*P* value
	Pregnant	Non-pregnant	
Enrofloxacin			
$t_{½\lambda z}$ (h)	9.2 ± 5.5	8.1 ± 0.5	0.611
$C_{initial}$ (µg/mL)	8.126 ± 4.145	5.853 ± 1.809	0.074
Vd_{area} (L/kg)	5.0 ± 4.5	5.0 ± 1.1	0.988
Vd_{ss} (L/kg)	2.6 ± 0.9	2.7 ± 1.3	0.795
CL (ml/h/kg)	338 ± 100	429 ± 89	0.078
AUC_{24} (µg • h/mL)	14.71 ± 3.99	11.42 ± 2.48	0.062
AUC_{0-t} (µg • h/mL)	15.67 ±4.26	11.94 ± 2.32	0.067
$AUC_{0-\infty}$ (µg • h/mL)	15.81 ± 4.18	12.03 ± 2.32	0.061
MRT (h)	7.56 ± 1.72	5.98 ± 1.77	0.139
C_{24h} (µg/mL)	0.086 ± 0.042	0.050 ± 0.032	0.224
Ciprofloxacin			
C_{max} (µg/mL)	0.272 ± 0.127	0.240 ± 0.064	0.535
T_{max} (h)*	0.6 (0.5 – 6.0)	0.6 (0.5 – 0.75)	0.313
AUC_{24} (µg • h/mL)	2.25 ± 0.67	1.71 ± 0.32	0.112
AUC_{0-t} (µg • h/mL)	2.63 ± 0.73	1.89 ± 0.39	0.078
$AUC_{0-\infty}$ (µg • h/mL)	2.72 ± 0.7	1.93 ± 0.41	0.065
C_{24h} (µg/mL)	0.032 ± 0.011	0.017 ± 0.005	0.041
$t_{½\lambda z}$ (h)	11.4 ± 4.0	8.6 ± 1.5	0.085

*Median and range

$t_{½\lambda z}$ = half-life of the terminal phase. $C_{initial}$ = Initial measured plasma concentration (at 30 min). Vd_{area} = Apparent volume of distribution based on AUC. Vd_{ss} = Apparent volume of distribution at steady state. CL = Systemic clearance. AUC_{24}= Area under the plasma concentration versus time curve from 0 to 24h. AUC_{0-t} = Area under the plasma concentration versus time curve to limit of quantification. $AUC_{0-\infty}$ = Area under the plasma concentration versus time curve extrapolated to infinity. MRT = Mean residence time. C_{max} = Maximum plasma concentration (observed). T_{max} = Time to maximum plasma concentration (observed). C_{24h} = Plasma concentrations 24h after administration.

Table 3.2: Pharmacokinetic variables (mean ± SD) for enrofloxacin and its main metabolite ciprofloxacin after oral administration of a single dose of enrofloxacin (7.5 mg/kg of body weight) to 6 healthy mares. Mares were dosed during late gestation (260-275 days) and again 45 to 60 days post-partum, 15- 30 days after foals were weaned.

Variable	Status		*P* value
	Pregnant	Non-pregnant	
Enrofloxacin			
C_{max} (µg/mL)	1.636 ± 0.587	1.777 ± 1.432	0.862
T_{max} (h)*	3.5 (0.5 – 6.0)	1.25 (0.1 – 4.0)	0.063
AUC_{24} (µg • h/mL)	14.02 ± 4.04	8.39 ± 1.86	0.046
AUC_{0-t} (µg • h/mL)	15.62 ± 4.73	9.06 ± 2.11	0.044
$AUC_{0-\infty}$ (µg • h/mL)	15.74 ± 4.68	9.20 ± 2.17	0.046
C_{24h} (µg/mL)	0.153 ± 0.079	0.062 ± 0.039	0.055
$t_{½\lambda z}$ (h)	7.5 ± 0.8	8.0 ± 2.2	0.540
F (%)	67.1 ± 11.4	52.6 ± 16.1	0.108
Ciprofloxacin			
C_{max} (µg/mL)	0.245 ± 0.089	0.416 ± 0.200	0.048
T_{max} (h)*	3.0 (1.5 – 5.0)	1.3 (0.8 – 4)	0.094
AUC_{24} (µg • h/mL)	2.56 ± 0.99	3.07 ± 0.79	0.091
AUC_{0-t} (µg • h/mL)	3.17 ± 1.20	3.51 ± 0.85	0.305
$AUC_{0-\infty}$ (µg • h/mL)	3.24 ± 1.19	3.61 ± 0.88	0.273
C_{24h} (µg/mL)	0.048 ± 0.014	0.037 ± 0.008	0.029
$t_{½\lambda z}$ (h)	10.6 ± 1.0	9.1 ± 0.9	0.040

*Median and range

$t_{½\lambda z}$ = half-life of the terminal phase. AUC_{0-24h} = Area under the plasma concentration versus time curve from time 0 to 24 h. AUC_{0-t} = Area under the plasma concentration versus time curve to limit of quantification. $AUC_{0-\infty}$ = Area under the plasma concentration versus time curve extrapolated to infinity. C_{max} = Maximum plasma concentration (observed). T_{max} = Time to maximum plasma concentration (observed). C_{24h} = Plasma concentrations 24 h after administration. F = Oral bioavailability.

Table 3.3: Pharmacokinetic variables (mean ± SD) for enrofloxacin and its main metabolite ciprofloxacin after IV (5mg/kg) or oral (7.5 mg/kg) administration of a single dose of enrofloxacin to six healthy mares. Mares were dosed during late gestation (260-275 days) and again 45 to 60 days post-partum, 15- 30 days after foals were weaned.

Variable	Pregnant		*P* value
	IV	PO	
Enrofloxacin			
$t_{½\lambda z}$ (h)	9.2 ± 5.5	7.5 ± 0.8	0.449
AUC_{24} (µg • h/mL)	14.71 ± 3.99	14.02 ± 4.04	0.447
AUC_{0-t} (µg • h/mL)	15.67 ±4.26	15.62 ± 4.73	0.970
$AUC_{0-\infty}$ (µg • h/mL)	15.81 ± 4.26	15.74 ± 4.68	0.955
C_{24h} (µg/mL)	0.086 ± 0.042	0.153 ± 0.079	0.120
Ciprofloxacin			
C_{max} (µg/mL)	0.272 ± 0.127	0.245 ± 0.089	0.583
T_{max} (h)*	0.6 (0.5 – 6.0)	3.0 (1.5 – 5.0)	0.177
AUC_{24} (µg • h/mL)	2.25 ± 0.67	2.56 ± 0.99	0.265
AUC_{0-t}(µg • h/mL)	2.63 ± 0.73	3.17 ± 1.20	0.171
$AUC_{0-\infty}$ (µg • h/mL)	2.72 ± 0.71	3.24 ± 1.19	0.177
C_{24h} (µg/mL)	0.032 ± 0.011	0.048 ± 0.014	0.052
Variable	Non-pregnant		*P* value
	IV	PO	
Enrofloxacin			
$t_{½\lambda z}$ (h)	8.1 ± 0.5	8.0 ± 2.2	0.969
AUC_{24} (µg • h/mL)	11.42 ± 2.48	8.39 ± 1.86	0.047
AUC_{0-t} (µg • h/mL)	11.94 ± 2.33	9.06 ± 2.11	0.055
$AUC_{0-\infty}$ (µg • h/mL)	12.03 ± 2.32	9.20 ± 2.17	0.066
C_{24h} (µg/mL)	0.050 ± 0.032	0.062 ± 0.039	0.001
Ciprofloxacin			
C_{max} (µg/mL)	0.272 ± 0.127	0.416 ± 0.200	0.033
T_{max} (h)*	0.6 (0.5 – 6.0)	1.3 (0.8 – 4)	0.091
AUC_{24} (µg • h/mL)	1.71 ± 0.32	3.07 ± 0.79	0.001
AUC_{0-t} (µg • h/mL)	1.89 ± 0.39	3.51 ± 0.85	0.001
$AUC_{0-\infty}$ (µg • h/mL)	1.94 ± 0.41	3.61 ± 0.88	0.001
C_{24h} (µg/mL)	0.032 ± 0.011	0.037 ± 0.008	0.002

Table 3.4a: MIC_{50} and MIC_{90} for commonly isolated equine pathogens. MIC data obtained from [§]Trundell et al 2017 [131] , [⌘]Ferrer and Palomares 2018 [14],[*]Estell et al 2016[132].

Common equine pathogens				
Pathogen	Origin	Enrofloxacin MIC_{50}	Enrofloxacin MIC_{90}	Ciprofloxacin MIC_{90}
Escherichia coli	Endometrial	0.25⌘	0.5⌘	0.031§
Strep. zooepidemicus	Endometrial	1.0⌘	1.0⌘	4.0§
Enterobacter spp	Endometrial	0.5⌘	2.0⌘	
Klebsiella spp	Endometrial	0.5⌘	0.5 ⌘	1.0§
	Lower airway	≤0.25*	≤0.5*	
Enterococcus spp	Endometrial	2.0⌘	2.0⌘	
Pseudomonas aeruginosa	Endometrial			2.0§

Table 3.4b: Mean AUC_{24} enrofloxacin and ciprofloxacin after IV (5 mg/kg) or oral (7.5 mg/kg) administration of a single dose of enrofloxacin (n=6). Mares were dosed during late gestation (260-275d) and again 45 to 60d post-partum, 15- 30 days after weaning. MIC values where AUC_{24}:MIC are > 50 in bold. * AUC= AUC_{24} (µg • h/mL) after a single dose.

ENROFLOXACIN			MIC values (µg/ml)								
	Dose	AUC*	**0.03**	**0.06**	**0.12**	**0.25**	0.5	1	2	4	8
Pregnant	5 mg/kg IV	14.7	**490**	**245**	**123**	**59**	29	15	7.4	3.7	1.8
	7.5 mg/kg PO	14.0	**467**	**233**	**117**	**56**	28	14	7	3.5	1.8
Postpartum	5 mg/kg IV	11.4	**380**	**190**	**95**	46	23	11	5.7	2.9	1.4
	7.5 mg/kg PO	8.4	**280**	**140**	**70**	33	17	8.4	4.2	2.1	1.1
CIPROFLOXACIN			MIC values (µg/ml)								
	Dose	AUC*	**0.03**	**0.06**	0.12	0.25	0.5	1	2	4	8
Pregnant	5 mg/kg IV	2.25	**75**	37.5	18.8	9	4.5	2.3	1.1	0.6	0.3
	7.5 mg/kg PO	2.56	**85**	43	21.3	10.2	5.1	2.6	1.3	0.6	0.3
	5 mg/kg IV	1.71	**57**	29	14	6.8	3.4	1.7	0.9	0.4	0.2
Postpartum	7.5 mg/kg PO	3.07	**102**	**51**	26	12	6.1	3.1	1.5	0.8	0.4
CIPROFLOXACIN + ENROFLOXACIN			MIC values (µg/ml)								
	Dose	AUC*	**0.03**	**0.06**	**0.12**	**0.25**	0.5	1	2	4	8
Pregnant	5 mg/kg IV	16.95	**565**	**282**	**141**	**68**	34	17	8.4	4.2	2.1
	7.5 mg/kg PO	16.56	**552**	**276**	**138**	**66**	33	17	8.2	4.1	2.1
Postpartum	5 mg/kg IV	13.11	**447**	**218**	**109**	**52**	26	13	6.7	3.3	1.6
	7.5 mg/kg PO	11.47	**382**	**191**	**96**	46	23	11	5.7	2.9	1.4

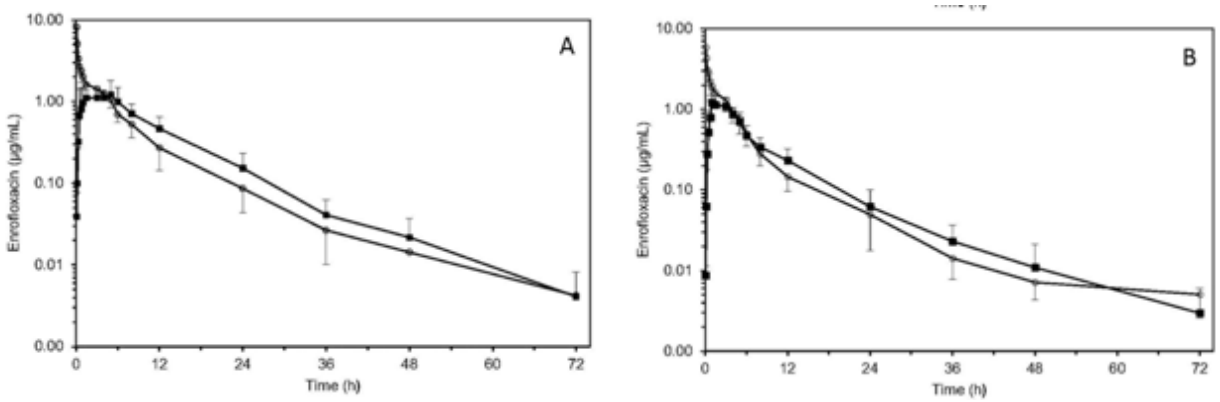

Figure 3.1: Mean ± s.d. plasma enrofloxacin concentrations following administration of a single enrofloxacin dose intravenously (5 mg/kg bwt, open dots) or orally (7.5 mg/kg bwt, solid squares) to healthy mares at 260 days gestation (A) or to non-lactating mares 45-60 days post-partum (B).

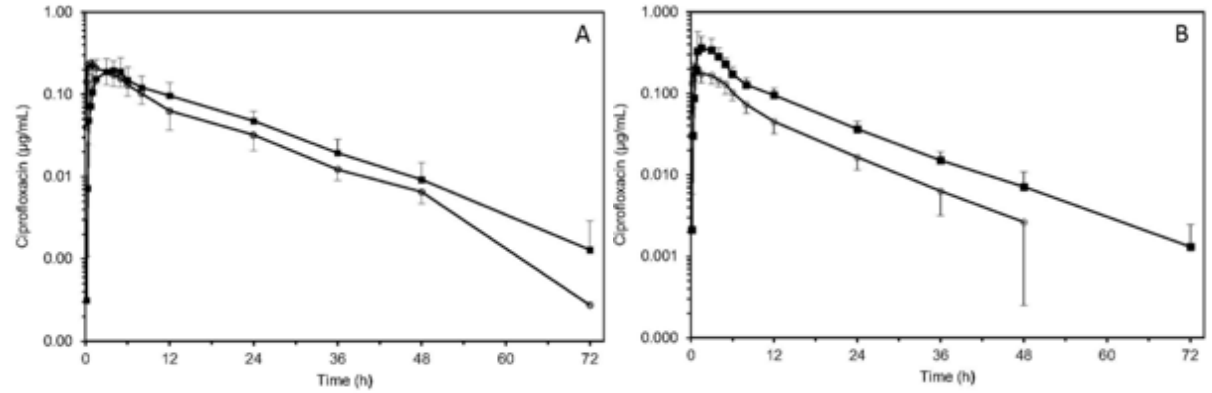

Figure 3.2: Mean ± s.d. plasma ciprofloxacin concentrations following administration of a single enrofloxacin dose intravenously (5 mg/kg bwt, open dots) or orally (7.5 mg/kg bwt, solid squares) to healthy mares at 260 days gestation (A) or non-lactating mares 45-60 days post-partum (B).

REFERENCES

1. Murchie, T.A., Macpherson, M.L., LeBlanc, M.M., Luznar, S. and Vickroy, T.W. (2006) Continuous monitoring of penicillin G and gentamicin in allantoic fluid of pregnant pony mares by in vivo microdialysis. *Equine Vet. J.* **38**, 520–525.
2. LeBlanc, M.M. (2009) The current status of antibiotic use in equine reproduction. *Equine Vet. Educ.* **21**, 156–167.
3. Macpherson, M.L., Giguère, S., Hatzel, J.N., Pozor, M., Benson, S., Diaw, M., Sanchez, L.C., Vickroy, T.W., Tell, L., Wetzlich, S. and Sims, J. (2013) Disposition of desfuroylceftiofur acetamide in serum, placental tissue, fetal fluids, and fetal tissues after administration of ceftiofur crystalline free acid (CCFA) to pony mares with placentitis. *J. Vet. Pharmacol. Ther.* **36**, 59–67.
4. Rebello, S.A., Macpherson, M.L., Murchie, T.A., LeBlanc, M.M. and Vickrow, T.W. (2006) Placental transfer of trimethoprim sulfamethoxazole and pentoxifylline in pony

mares. *Anim. Reprod. Sci.* **94**, 432–433.
5. Prescott, J.F. (2013) Sulfonamides, diaminopyrimidines, and their combinations. In: *Antimicrobial therapy in veterinary medicine*, 5th ed., Eds: J.F. Prescott, S. Giguère and P.M. Dowling, John Wiley & Sons, Hoboken. pp 279–294.
6. Bailey, C.S., Macpherson, M.L., Pozor, M.A., Troedsson, M.H.T., Benson, S., Giguere, S., Sanchez, L.C., LeBlanc, M.M. and Vickroy, T.W. (2010) Treatment efficacy of trimethoprim sulfamethoxazole , pentoxifylline and altrenogest in experimentally induced equine placentitis. *Theriogenology* **74**, 402–412.
7. Giguere, S. and Dowling, P. (2013) Fluoroquinolones. In: *Antimicrobial therapy in veterinary medicine*. pp 295–314.
8. Martinez, M., McDermott, P. and Walker, R. (2006) Pharmacology of the fluoroquinolones: A perspective for the use in domestic animals. *Vet. J.* **172**, 10–28.
9. Brown, S.A. (1996) Fluoroquinolones in animal health. *J Vet Pharmacol Ther* **19**, 1–14.
10. Loebstein, R., Lalkin, A. and Koren, G. (1997) Pharmacokinetic changes during pregnancy and their clinical relevance. *Clin. Pharmacokinet.* **33**, 328–43.
11. Koren, G. and Pariente, G. (2018) Pregnancy- associated changes in pharmacokinetics and their clinical implications. *Pharmacol. Res.* 35–61.
12. Bengtsson, B., Jacobsson, S.-O., Luthman, J. and Franklin, A. (1997) Pharmacokinetics of penicillin-G in ewes and cows in late pregnancy and in early lactation. *J. Vet. Pharmacol. Ther.* **20**, 258–261.
13. Heikkila, A. and Erkkola, R. (1994) Review of beta-lactam antibiotics in pregnancy. The need for adjustment of dosage schedules. *Clin. Pharmacokinet.* **27**, 49–62.
14. Maślanka, T. and Jaroszewski, J.J. (2009) Effect of long-term treatment with therapeutic doses of enrofloxacin on chicken articular cartilage. *Pol. J. Vet. Sci.* **12**, 21–33.
15. Bertone, A.L., Tremaine, W.H., Macoris, D.G., Simmons, E.J., Ewert, K.M., Herr, L.G. and Weisbrode, S.E. (2000) Effect of long-term administration of an injectable enrofloxacin solution on physical and musculoskeletal variables in adult horses. *J. Am. Vet. Med. Assoc.* **217**, 1514–21.
16. Bermingham, E.C., Papich, M.G. and Vivrette, S.L. (2000) Pharmacokinetics of enrofloxacin administered intravenously and orally to foals. *Am. J. Vet. Res.* **61**, 706–709.
17. Langston, V.C., Sedrish, S. and Boothe, D.M. (1996) Disposition of single-dose oral enrofloxacin in the horse. *J. Vet. Pharmacol. Ther.* **19**, 316–319.
18. Nieuwoudt, C., Epstein, K., Cohen, N., Boothe, D. and Chandler, J. (2004) Pharmacokinetics and stability of an enrofloxacin oral gel formulation in horses. *Proc. Am. Assoc. Equine Pract.* .
19. K. Gary Magdesian (2009) Update on Common Antimicrobials. In: *Current Therapy in Equine Medicine*, 6th ed., Eds: E.N. Robinson and K.A. Sprayberry, Saunders Elsevier, St Louis. pp 12–14.
20. Giguere, S., Sweeney, R.W. and Belanger, M. (1996) Pharmacokinetics of enrofloxacin in adult horses and and concentration of the drug in serum, body fluids, and endometrial tissues after repeated intragastrically administered doses. *Am. J. Vet. Res.* **57**.
21. Boeckh, S., Buchanan, C., Boeckh, A., Wilkie, S., Davis, C., Buchanan, T. and Boothe, D. (2001) Pharmacokinetics of the bovine formulation of enrofloxacin (Baytril 100) in horses. *Vet. Ther.* **2**, 129–34.
22. Kaartinen, L., Panu, S. and Pyörälä, S. (1997) Pharmacokinetics of enrofloxacin in horses after single intravenous and intramuscular administration. *Equine Vet. J.* **29**, 378–381.

23. Haines, G.R., Brown, M.P., Gronwall, R.R. and Merritt, K.A. (2000) Serum concentrations and pharmacokinetics of enrofloxacin after intravenous and intragastric administration to mares. *Can. J. Vet. Res.* **64**, 171–177.
24. Vancutsem, P.M., Babish, J.G. and Schwark, W.S. (1990) The fluoroquinolone antimicrobials: structure, antimicrobial activity, pharmacokinetics, clinical use in domestic animals and toxicity. *Cornell Vet.* **80**, 173–186.
25. Broome, R.L., Brooks, D.L., Babish, J.G., Copeland, D.D. and Conzelman, G.M. (1991) Pharmacokinetic properties of enrofloxacin in rabbits. *Am. J. Vet. Res.* **52**, 1835–1841.
26. Peyrou, M., Laroute, V., Vrins, A. and Doucet, M.Y. (2006) Enrofloxacin and marbofloxacin in horses: comparison of pharmacokinetic parameters, use of urinary and metabolite data to estimate first-pass effect and absorbed fraction. *J Vet Pharmacol Ther* **29**, 337–344.
27. He, X.J., Ejiri, N., Nakayama, H. and Doi, K. (2005) Effects of pregnancy on CYPs protein expression in rat liver. *Exp. Mol. Pathol.* **78**, 64–70.
28. Koh, K.H., Xie, H., Yu, A.-M. and Jeong, H. (2011) Altered cytochrome P450 expression in mice during pregnancy. *Drug Metab. Dispos.* **39**, 165–169.
29. Hart, C.G., Camacho, L.E., Swanson, K.C., Vonnahme, K.A. and Lemley, C.O. (2014) Hepatic steroid metabolizing enzyme activity during early , mid , and late bovine pregnancy. *Domest. Anim. Endocrinol.* **49**, 31–38.
30. Antonissen, G., Devreese, M., Baere, S. De, Martel, A., Immerseel, F. Van and Croubels, S. (2017) Impact of Fusarium mycotoxins on hepatic and intestinal mRNA expression of cytochrome P450 enzymes and drug transporters , and on the pharmacokinetics of oral enrofloxacin in broiler chickens. *Food Chem. Toxicol.* **101**, 75–83.
31. Fu, G., Zhou, C., Wang, Y., Fang, W., Zhou, J. and Zhao, S. (2016) Effects of inducers of cytochrome P450s on enrofloxacin N -deethylation in crucian carp Carassius auratus gibelio. *Environ. Toxicol. Pharmacol.* **46**, 188–193. http://dx.doi.org/10.1016/j.etap.2016.07.015.
32. Bermingham, E.C., Papich, M.G. and Vivrette, S.L. (2000) Pharmacokinetics of enrofloxacin administered intravenously and orally to foals. *Am. J. Vet. Res.* **61**, 706–709.
33. Papich, M.G., Camp, S.D. Van, Cole, J.A. and Whitacre, M.D. (2002) Pharmacokinetics and endometrial tissue concentrations of enrofloxacin and the metabolite ciprofloxacin after i.v. administration of enrofloxacin to mares. *J. Vet. Pharmacol. Ther.* **25**, 343–350.
34. Yamarik, T.A., Wilson, W.D., Wiebe, V.J., Pusterla, N., Edman, J., Papich, M.G., Pharmacokinetics, M.G. and Yamarik, T.A. (2010) Pharmacokinetics and toxicity of ciprofloxacin in adult horses. *J. Vet. Pharmacol. Ther.* **33**, 587–594.
35. Craig, W.A. (1998) State of the art clinical article: pharmacokinetic/pharmacodynamic parameters: rationale for antibacterial dosing of mice and men. *Clin. Infect. Dis.* **26**, 1–10.
36. Messenger, K.M., Papich, M.G. and Blikslager, A.T. (2011) Distribution of enrofloxacin and its active metabolite , using an in vivo ultrafiltration sampling technique after the injection of enrofloxacin to pigs. *J Vet Pharmacol Ther* 452–459.
37. Wright, D.H., Brown, G.H., Peterson, M.L. and Rotschafer, J.C. (2000) Application of fluoroquinolone pharmacodynamics. *J. Antimicrob. Chemother.* **46**, 669–683.
38. Papich, M.G. (2013) Antimicrobials, susceptibility testing, and minimum inhibitory concentrations (MIC) in veterinary infection treatment. *Vet. Clin. North Am. Small Anim. Pract.* **43**, 1079–1089.
39. Davis, J.L., Foster, D.M. and Papich, M.G. (2007) Pharmacokinetics and tissue

distribution of enrofloxacin and its active metabolite ciprofloxacin in calves. *J Vet Pharmacol Ther* **30**, 564–571.
40. Dimitrova, D.J., Lashev, L.D., Yanev, S.G. and Pandova, B. (2007) Pharmacokinetics of enrofloxacin in turkeys. *Res. Vet. Sci.* **82**, 392–397.
41. Lacy, M.K., Lu, W., Xu, X., Tessier, P.R., Nicolau, D.P., Quintiliani, R. and Nightingale, C.H. (1999) Pharmacodynamic comparisons of levofloxacin , ciprofloxacin , and ampicillin against streptococcus pneumoniae in an in vitro model of infection. *Antimicrob. Agents Chemother.* **43**, 672–677.
42. Lister, P.D. and Sanders, C.C. (1999) Pharmacodynamics of trovafloxacin , ofloxacin, and ciprofloxacin against Streptococcus pneumoniae in an in vitro pharmacokinetic model. *Antimicrob. Agents Chemother.* **43**, 1118–1123.
43. Aliabadi, F.S. and Lees, P. (2002) Pharmacokinetics and pharmacokinetic / pharmacodynamic integration of marbofloxacin in calf serum , exudate and transudate. *J. Vet. Pharmacol. Ther.* **25**, 161–174.
44. Peyrou, M., Doucet, M.Y., Vrins, A., Concordet, D., Schneider, M. and Bousquet-Melou, A. (2004) Population pharmacokinetics of marbofloxacin in horses: preliminary analysis. *J Vet Pharmacol Ther* **27**, 283–288.
45. Bidgood, T.L. and Papich, M.G. (2005) Plasma and interstitial fluid pharmacokinetics of enrofloxacin , its metabolite ciprofloxacin , and marbofloxacin after oral administration and a constant rate intravenous infusion in dogs. *J. Vet. Pharmacol. Ther.* **28**, 329–341.
46. Ellerbrock, R.E., Canisso, I.F., Roady, P., Curcio, B., Lima, F.S. and Li, Z. (2018) Enrofloxacin and its active metabolite ciprofloxacin cross the equine placenta and did not cause lesions in the articular surface of the fetuses and foals. *J. Equine Vet. Sci.* **66**, 232.
47. Trundell, D.A., Ferris, R.A., Hennet, M.R., Wittenburg, L.A., Gustafson, D.L., Borlee, B.R. and McCue, P.M. (2017) Pharmacokinetics of intrauterine ciprofloxacin in the mare and establishment of minimum inhibitory concentrations for equine uterine bacterial isolates. *J. Equine Vet. Sci.* **54**, 54–59.
48. Ferrer, M.S. and Palomares, R. (2017) Aerobic uterine isolates and antimicrobial susceptibility in mares with post-partum metritis. *Equine Vet. J.* **50**, 202–207.
49. Estell, K.E., Young, A., Kozikowski, T., Swain, E.A., Byrne, B.A., Reilly, C.M., Kass, P.H. and Aleman, M. (2016) Pneumonia Caused by Klebsiella spp. in 46 Horses. *J. Vet. Intern. Med.* **30**, 314–321.

CHAPTER 4: DIFFUSION OF ENROFLOXACIN TO PREGNANCY FLUIDS AND EFFECTS ON FETAL CARTILAGE AFTER INTRAVENOUS ADMINSTRATION TO LATE PREGNANT MARES

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ABSTRACT

In select cases, enrofloxacin may be an alternative antibacterial agent to treat unresponsive infections in pregnant mares. Supratherapeutic doses of enrofloxacin are toxic to adult horses and also to newborn foals; however, it is unknown if enrofloxacin crosses the equine placenta or if it is toxic to the fetus. The objectives of this study were to assess the diffusion of enrofloxacin and its metabolite to fetal fluids and its effects on fetal cartilage when administered to pregnant mares. Healthy mares at 260 d gestation were allocated into three groups: untreated (n=3), therapeutic treatment (5 mg/kg enrofloxacin, IV, n=7), or supratherapeutic treatment (10 mg/kg, IV, n=6) for

11 days. Fetal fluids were collected on days 1, 5, and 11 of treatment. Premature delivery was induced on day 11 with oxytocin, and fetal plasma was collected at delivery. Plasma and fetal fluid enrofloxacin and ciprofloxacin concentrations were measured by liquid chromatography-mass spectrometry. Fetal articular cartilage was examined macroscopically and histologically for lesions. Enrofloxacin and ciprofloxacin reached the minimum inhibitory concentrations for common pathogens in all fluids. Ciprofloxacin did not increase with the double enrofloxacin dose in maternal plasma, but allantoic fluid showed a 10-fold increase relative to fetal trough plasma concentrations. Administration of enrofloxacin at recommended doses did not result in cartilaginous lesions in fetuses. It remains to be determined if enrofloxacin shows toxicity at other stages of pregnancy, after a longer duration of treatment, or once the foals are delivered and articular surfaces are weight-bearing. Short-term administration of enrofloxacin to late gestation mares resulted in detectable enrofloxacin and ciprofloxacin concentrations in fetal fluids, and did not result in macroscopic or microscopic lesions in the fetus. While further research is needed to address long-term foal outcomes, enrofloxacin may be useful for select bacterial infections in pregnant mares.
Keywords: fluoroquinolone, ciprofloxacin, LC-MS/MS, fetal toxicity

INTRODUCTION

Antimicrobial therapy is necessary in equine practice to treat bacterial infections during pregnancy, however the pharmacodynamics and toxicity of many antimicrobials in pregnant animals are not known. β-Lactam antibiotics and aminoglycosides cross the equine placenta [1] and are commonly used in pregnant mares [2,3]. Cephalosporin antibiotics are also commonly and safely used during equine pregnancy, but do not cross the equine placenta due to differences in molecular structure [4]. Potentiated sulphonamides cross the equine placenta [5] and are frequently used to treat infections in late-pregnant mares [2,6,7] despite knowledge that sulphonamides have been associated with an increased risk of birth defects in the first trimester in humans [8] and rarely in the horse [9]. Conversely, fluoroquinolones are believed to be chondrotoxic to the foetus given the known side effects in growing animals, and fluoroquinolones are generally avoided in pregnant animals [10–12].

Enrofloxacin is a lipophilic fluoroquinolone antimicrobial that inhibits topoisomerase I (DNA Gyrase) and topoisomerase IV, and has bactericidal activity against Gram-negative and some Gram-positive pathogens [13]. Enrofloxacin either undergoes hepatic metabolism into ciprofloxacin, or is excreted unchanged in the urine, and both enrofloxacin and ciprofloxacin are effective against many common equine pathogens [13,14]. Quinolones are believed to induce arthropathies by chelating divalent cations such as magnesium (Mg^{2+}) [15–19] , and *in vitro* and *in vivo* studies have provided evidence that administration of enrofloxacin can cause tendonitis and arthropathies, especially in young, growing animals [10–12].

To date, only two studies have assessed *in vivo* effects of enrofloxacin in the horse [6, 15]. When enrofloxacin was administered at 3 to 5 times the recommended therapeutic dose for 21 days, 3 of 12 horses developed musculoskeletal complications [20]. Horses receiving the recommended therapeutic dose (5 mg/kg q 24h) did not develop complications. When four two-week-old foals were treated with twice the recommended dose (10 mg/kg, PO, q24 hours) for 8 days, three foals became moderately to severely lame and developed tibial-tarsal joint effusion with roughening of the articular cartilage [11]. However, this increased dosage is generally not used in practice, and no studies have assessed the effects of the recommended dose on foals. Additionally, no studies have assessed the effects of fluoroquinolones on the equine foetus, and there is a clear need to determine potential detrimental effects of fluoroquinolone use in pregnancy.

The objectives of this study were (i) to determine enrofloxacin and ciprofloxacin concentrations in the maternal plasma, foetal plasma, and pregnancy fluids when enrofloxacin is administered during late-term pregnancy, and (ii) to evaluate the articular cartilage of long bones in foetuses from those mares. We hypothesized that enrofloxacin and its active metabolite (ciprofloxacin) cross the equine placenta and that enrofloxacin administration during late-term pregnancy is associated with cartilaginous lesions in the foetus.

MATERIALS AND METHODS

The study protocol was approved by the University of Illinois Institutional Animal Care and Use Committee (protocols, #14243 and #17245). The present study was carried out between 2014 and 2017. Sixteen healthy, light-breed pregnant mares (six Quarter Horses, three Standardbreds, three grade horses, one Paint, one Arabian, one Tennessee Walking Horse, and one Thoroughbred were enrolled in the study at 260 days gestation. The mares were housed on pasture

at the Veterinary Medicine Research Farm at the University of Illinois in Urbana, Illinois, and were provided free choice supplemental minerals.

Study Design

Immediately before enrollment, physical examinations and ultrasonography of the caudal placental pole were performed on all mares, and both were within normal limits. Mares were randomly allocated into three groups: control (n=3, no treatment), enrofloxacin [Baytril®][a] 5 mg/kg IV (n=7), and enrofloxacin [Baytril®][a] 10 mg/kg IV, (n=6). Enrofloxacin was administrated intravenously every 24 hours for 11 days to the treated mares (5 mg/kg and 10 mg/kg groups). A second treatment group (10 mg/kg) was included to determine if slight dosing errors, such as those that might occur if a weight was inaccurately estimated, or if two doses were accidentally given in a day, would induce lesions in the fetus. Heparinized blood samples were collected via jugular venipuncture daily, samples were centrifuged at 600g/x10 minutes, and plasma samples were harvested and stored at -80^{o}C until analysis.

Fetal fluid sampling was performed on days 1, 5 and 11 after treatment onset as previously described [21]. Mares were sedated with xylazine hydrochloride [Anased[b]] 0.4 mg/kg IV. and butorphanol tartrate [Torbogesic[c]] 0.004 mg/kg IV, placed in stocks, and their ventral abdomens were clipped and aseptically prepped with betadine scrub. After regional analgesia with 10 mL 2% lidocaine[d], was administered, fetal fluids were collected aseptically with transabdominal ultrasound guidance using a SonoScape S8 Exp with convex probe[e], and an echotip spinal needle (18Gx6"; 30o short bevel, Chiba-Type spinal needle, with stylet)[f]. Fluid was only collected and saved if a pure white colour (amniotic) fluid was obtained. A minimum of 5 mL and maximum of 12 mL was collected, and the needle was removed. A new, sterile needle was then used to collect a sample of allantoic fluid, and fluid (5-12 mL) was collected only if an amber fluid consistent with allantoic fluid was obtained [21].

Abortion was induced by manual cervical dilation on day 11. Oxytocin[g] (10 IU IV) was administered after cervical dilation was at least 15 cm to assist with delivery. Mares were sedated with xylazine hydrochloride[b] (0.4 mg/kg- 0.8 mg/kg IV), and fetuses were delivered vaginally. Fetuses were euthanised with pentobarbital sodium and phenytoin sodium [Euthasol[h]], 900 mg/kg IV and 115 mg/kg, respectively, immediately following delivery, and plasma samples were collected. All placentas were examined macroscopically, and fetuses were necropsied.

Blood assessments

Mare plasma magnesium was measured on Day 0 and on Day 11. Full chemistry panels were run on fetal plasma collected at time of abortion. Concentrations of enrofloxacin and ciprofloxacin were measured by liquid chromatography-tandem mass spectrophotomy (LC-MS/MS) in the Metabolomics Laboratory of the Roy J. Carver Biotechnology Center, University of Illinois Urbana-Champaign. Validation and detailed description of LC-MS/MS methods are reported in a parallel study [22]. Between-run and within-run accuracy and precision were calculated by measuring six samples of enrofloxacin and ciprofloxacin at concentrations of 5, 50, and 500 ng/ml, and measurements were repeated on three different days. Enrofloxacin had a within-run accuracy of 95.6 ± 2.1 % to 99.1 ± 0.6%, and a between- run accuracy of 97.3 ± 2.0% to 98.4 ± 1.0%, and ciprofloxacin had a mean within-run accuracy of 95.4 ± 2.3 to 99.6 % ± 1.2 %, and between- run accuracy of 97.0 ± 2.4 % to 99.2 ± 0.9%. Coefficients of variations between-run and within-run for enrofloxacin ranged from 0.9-1.6% and 1.1-3.5%, respectively, whereas, coefficients of variations for ciprofloxacin between- run and within-run ranged from 0.9- 2.4% and 1.0- 5.4 %, respectively. Plasma stability data was calculated under various conditions (n= 6). Samples stored in the autosampler for 24 h at 15 °C had a bias of 3.2-4.8% and samples stored at -75 °C for 2 months had a bias of 1.3-3.4%.

Pathology

Fetal limbs were disarticulated, and all joint surfaces were inspected for gross lesions by a board-certified veterinary pathologist. Sections of the proximal articular cartilage of the distal scapula, proximal and distal humerus, proximal radius, proximal and distal femur, and proximal and distal tibia were collected from all four limbs using a bone saw. All slices were preserved in 10% neutral buffered formalin immediately after collection. The fixed tissues were then routinely processed and embedded in paraffin in an automated tissue processor. Slides were stained with haematoxylin & eosin and toluidine blue for analysis, as described in Supplementary table 4.1. Slides stained with haematoxylin & eosin were used to assess structural and cellular features, whereas slides stained with toluidine blue were used to evaluate the extracellular-matrix. To determine if enrofloxacin or ciprofloxacin had detrimental effects on other organ systems, fixed tissue sections of fetal liver, fetal kidney, and placenta were also evaluated after staining with haematoxylin & eosin.

Statistical analysis

MedCalc software v16.4[i] was used to determine the *a priori* number of animals required to detect a statistical difference between groups using the Monte Carlo's estimation, with an alpha = 0.05, a power of 0.8, and an expected difference between groups of 30% or greater. While seven animals were to be used per group, when no lesions were seen in treated fetal cartilage, the study was terminated before all seven control mares were aborted to decrease the number of animals sacrificed for the study. Continuous variables were assessed for normality with histograms and normal quantile plots. The association between fetal chemistry values and fetal plasma fluorquinolones concentrations were analyzed by one way ANOVA. The association between mare trough plasma concentrations and fetal fluid concentrations at each time point were analyzed by ANOVA repeated measures. To determine whether mare plasma magnesium levels differed on Day 0 and Day 11, plasma magnesium levels were compared with a paired t-test. The histomorphometric analyses were evaluated using a non-parametric Kruskal-Wallis one-way analysis of variance and any changes were noted on evaluation by the pathologist. Significance was set at $p < 0.05$. Data were analysed using R libraries (R version 3.3.3)[j].

RESULTS

Transient loose manure or defecation immediately after drug administration was observed in three mares administered 10 mg/kg enrofloxacin. Two mares had mild vascular irritation by Day 11 of intravenous enrofloxacin administration. No other side effects were observed, and vital parameters remained within normal limits for the duration of the study. Four mares suffered varying degrees of cervical lacerations at time of abortion. One mare experienced moderate vaginal haemorrhage post abortion and was treated with a 20 L IV bolus Lactated Ringers' solution[k], flunixin meglumine[l] [Banamine®] 1.1 mg/kg IV BID, for two days, and trimethoprim sulfamethoxazole[m] (30 mg/kg PO BID) for seven days.

Pathology

No lesions were noted on gross inspection of any fetal articular surface, and representative images are shown in Figure 4.1. Four foetuses had clustering of cells in one or more articular surface. Clustering was noted in 3/8 joints (proximal right and left ulna, and proximal tibia, 10 mg/kg), 2/8 joints (proximal left ulna, proximal right ulna, 5mg/kg), and 1/8 joints in two foals (proximal right tibia, control, and proximal left tibia, 5mg/kg). No difference was seen in cell

clustering between treatment groups (p=0.62). No other abnormal findings were observed in any joint from any foal on histological evaluation.

All placentas were grossly normal. All fetal liver samples were unremarkable. One mare from the 5 mg/kg group had mild mineralization of the amnion, and one mare from the 10 mg/kg group had marked hemorrhage noted in the allantois. One fetus from the 10 mg/kg group had moderately swollen and vacuolated proximal convoluted tubule epithelial cells on renal histology. Remaining histological evaluations were unremarkable.

Enrofloxacin and ciprofloxacin

Both enrofloxacin and ciprofloxacin reached fetal circulation, as indicated by Day 11 fetal plasma concentrations (Figure 4.3). Enrofloxacin and ciprofloxacin crossed the equine placenta and accumulated in the allantoic fluid over time (Figure 4.4). Mean ciprofloxacin levels increased from 123 ± 94 ng/ml in the allantoic fluid on Day 1, to 876 ± 325 ng/ml (5 mg/kg group) by Day 11 ($p<0.0001$). Mean allantoic enrofloxacin concentrations showed a tendency to increase ($p=0.068$), from 195 ± 128 ng/ml on day 1, to 333 ± 228 ng/ml on day 11 (5 mg/kg group). Amniotic enrofloxacin concentrations were lower in the 5mg/kg group than in the 10 mg/kg group ($p=0.02$), and increased by day 11 in the 5 mg/kg group ($p=0.01$) but did not increase in the 10 mg/kg group ($p=0.65$). No differences were seen in amniotic ciprofloxacin concentrations between groups ($p>0.18$), although there was a tendency for ciprofloxacin concentrations to increase from day 1 to 11 in the 5 mg/kg group ($p=0.06$) but not the 10 mg/kg group ($p=0.11$). While trough plasma enrofloxacin levels were not consistently higher in the 10mg/kg group than the 5 mg/kg group ($p>0.05$), mares receiving the higher dose of enrofloxacin had consistently higher trough ciprofloxacin concentrations ($p<0.05$) (Figure 4.5).

Blood assessments

While mean fetal plasma creatinine, phosphorus, potassium, alkaline phosphatase (Alk Phos), bilirubin, creatinine phosphokinase (CPK), and cholesterol were all higher than reference ranges for adult horses, no differences were seen in values between control and treatment groups (Table 4.2) ($p<0.05$). Calcium plasma concentrations were approximately 25% higher than published ranges for 24 hour-old foals, although no differences were seen between treated and control foetuses (Table 4.2). Mean fetal plasma total protein, globulin, chloride, glucose, and aspartate amino transferase (AST) were lower than adult laboratory references range, but there was no difference between fetal groups (Table 4.2). Mare plasma magnesium concentrations were

not different at the beginning (2.0 ± 0.1 mg/dL (control), 2.0 ± 0.2 mg/dL (5 mg/kg), 1.9 ± 0.2 mg/dL (10 mg/kg)), or at the end of the study (1.75=± 0.4 mg/dL (control), 2.0 ± 0.1 mg/dL (5 mg/kg), 1.9 ± 0.2 mg/dL (10 mg/kg)), and were within the laboratory reference range (1.5- 2.1 mg/dL) for both time points.

DISCUSSION

This study investigated whether or not enrofloxacin and its active metabolite distribute to the fetoplacental unit, and whether potential toxic effects of fluoroquinolone exposure on the foetus are evident. Administration of intravenous enrofloxacin to late pregnant mares did not induce any systemic side effects in the mares, even at twice the recommended clinical dose. Both enrofloxacin and its active metabolite ciprofloxacin cross the equine placenta and reach detectable concentrations in both the allantoic and amniotic fluid. Interestingly, while enrofloxacin concentrations were similar between amniotic and allantoic fluid, concentrations of ciprofloxacin were higher in the allantoic than in the amniotic fluid. While both ciprofloxacin and enrofloxacin would be excreted preferentially into the allantoic cavity over the amniotic cavity, it is possible that enrofloxacin more readily diffuses back into maternal circulation than ciprofloxacin for maternal excretion. It is also possible that there is placental metabolism of enrofloxacin into ciprofloxacin in the late-pregnant mare. However, neither of these hypotheses has been critically evaluated.

The high concentrations of ciprofloxacin noted by Day 11 were significant, and indicate that enrofloxacin might be beneficial against select infections affecting the foetal membranes. While study design did not allow for continuous monitoring of antibiotic concentrations in the allantoic fluid, trough allantoic fluid ciprofloxacin concentrations remained above the MIC_{90} of common equine uterine *E. coli* isolates by Day 11 [23]. Given that the pregnancies were terminated on the final day of treatment, it is unknown how long ciprofloxacin and enrofloxacin remain in the fetal fluids after the last intravenous dose of enrofloxacin. Additionally, it is unknown how placental infections will affect drug distribution and transfer across the placenta, so additional studies are warranted.

The demonstration that enrofloxacin and ciprofloxacin cross the equine placenta and reach detectable levels in the fetal plasma necessitated evaluation of potential effects on the fetus. No lesions were detected on gross examination of the fetal cartilage, despite eleven days of

antimicrobial treatment. Previous studies describing fluoroquinolone toxicity observed lesions within 24 hours at supratherapeutic doses in broiler chickens [24], or by eight days in foals [11], suggesting that lack of gross lesions in this study was not due to insufficient treatment duration. Histological evaluation of fetal cartilage also failed to detect evidence of extracellular matrix changes, and no fissures or shrunken or pyknotic nuclei were noted in chondrocytes. Chondrocyte clustering, or random chondrocyte arrangement was noted in seven out of 104 treated joints in the late term fetuses; however, previous studies assessing equine cartilage development describe random distribution of proliferative chondrocytes as normal in the developing fetus and foal [25].

While no significant differences were noted in fetal chemistries between treatment groups, some parameters were different from published ranges for neonatal foals [26-28]. The observed fetal calcium concentrations were consistent with previously published observations that foals have plasma calcium concentrations up to 30% higher than adults, a finding that has been attributed to active placental transport [27]. While alkaline phosphatase concentrations were elevated in the fetuses compared to neonatal reference ranges, alkaline phosphatase is also elevated in late pregnant mares, and is known to vary widely in neonatal foals. The increases seen in fetal plasma cholesterol and creatinine were hypothesized to be due to the method of abortion, with placental separation and moderate dystocia occurring before fetal delivery and blood sampling.

Although no differences were noted in fetal plasma creatinine concentrations between treatment groups, the plasma creatinine for one fetus in the 10 mg/kg group was 13.7 mg/dL (1211 μmol/L), which was twice the creatinine concentration of any other fetuses. This fetus was also had renal pathology noted on histopathology. While we cannot rule out enrofloxacin as a cause of the changes affecting the proximal convoluted tubules and high plasma creatinine, fluoroquinolones have not previously been shown to be nephrotoxic. In fact, fluoroquinolones are frequently prescribed to patients with urinary tract infections or compromised renal function [29,30]. All other hepatic and renal samples from treated and control fetuses were normal. The changes noted in the chorioallantois and amnion membranes of two mares were presumed to be associated with needle puncture during amnion and allocentesis.

It has been previously demonstrated that magnesium deficiency causes similar lesions to fluoroquinolones in rat cartilage, and in *in vitro* cultures of equine and canine tendon cells [17,18,31,32]. All mares in this study had normal plasma magnesium levels at the beginning of the study, and no change was seen in plasma magnesium by the end of the study. Similarly, while

no reference ranges were found for fetal magnesium plasma concentrations, fetal plasma magnesium was within normal limits for neonatal foals [28] at the end of the study. While it is not possible to state if lesions would have been observed in animals that were deficient in magnesium, it may be prudent to check plasma magnesium concentrations before instituting fluoroquinolone treatment in horses.

It is important to note that orthopedic disease might not be evident until an animal becomes weight bearing. While it appears that enrofloxacin administration to late pregnant mares does not cause lesions in the fetus, further studies assessing long term effects of *in utero* exposure are warranted. Similarly, this study focused on potential side effects in the third trimester, and safety of fluoroquinolone administration in the first and second trimester is still unknown.

In conclusion, we confirmed our hypothesis that enrofloxacin and ciprofloxacin are detectable in the fetal fluids and fetus after enrofloxacin administration to late-term mares. However, contrary to expectations, exposure to fluoroquinolones *in utero* did not have detectable toxic effects on the cartilage, kidney, or liver of the fetus. Appropriate antimicrobial stewardship necessitates the selection of the optimal antimicrobial for a given microorganism without compromising patient health. Enrofloxacin administration to pregnant mares should be limited to cases of life-threatening infections determined by a microbial culture indicating a resistance to first line antimicrobials and a sensitivity to enrofloxacin. Clients should be informed of the risks of fluoroquinolone administration and the unknown effects of fluoroquinolone exposure on the developing fetus. While it appears that enrofloxacin could be an ideal choice for severe infections susceptible to enrofloxacin, cases should be selected carefully, as this study does not rule out the possibility of a low incidence of toxicity and does not demonstrate safety of administration during the first or second trimester of equine pregnancy, nor does it rule out long term complications to the foal that was exposed to fluoroquinolones *in utero*.

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TABLES AND FIGURES

Table 4.1. Relative mean enrofloxacin and ciprofloxacin concentrations in the fetal fluids and plasma of mares and fetuses at time of abortion. Mares (261 ± 4 days gestation) were administered either 5 mg/kg (n= 7) or 10 mg/kg (n= 6) enrofloxacin intravenously once a day for 11 days before abortion.

Drugs	Fluid ratios	5 mg/kg	10 mg/kg
Enrofloxacin	Maternal: Fetal plasma	1.4	1.6
	Fetal plasma: allantois	0.5	0.4
	Fetal plasma: amniotic	0.7	0.9
	Allantoic: amniotic	1.5	2.1
Ciprofloxacin	Maternal: Fetal plasma	4	4.9
	Fetal plasma: allantois	0.02	0.02
	Fetal plasma: amniotic	0.1	0.2
	Allantoic: amniotic	5.7	9.1

Table 4.2. Fetal serum chemistries at time of abortion (272 ± 4 days gestation). Mares were untreated (Control, n=3), or treated with 5mg/kg (n=7) or 10mg/kg (n=6) enrofloxacin intravenously once a day for 11 days before abortion. Adult range was provided by the reference laboratory. All other values are mean ± SD (range). No significant differences were seen between treatment groups for any value ($p<0.05$). Neonatal reference ranges from Axon and Palmer (2008) within an hour of birth, (§) Bauer (1990) at less than 12 hours old, or (*)from Southwood (2013) at 24 hours of age.

Value	Adult Range	Neonatal range	Control	5 mg/kg	10 mg/kg	p value
Creatinine (μmol/L)	70-159	13.3-32.7	460 ± 80 (389-548)	398 ± 124 (150-539)	574 ± 318 (309-1211)	0.39
BUN (mmol/L)	3.9-8.2	4.3-9.6	6.9 ± 1.6 (5.4-8.6)	7.9 ± 1.6 (6.1-10.0)	7.0 ± 1.1 (5.4-8.2)	0.47
TP (g/L)	55-73		36 ± 5 (31-40)	39 ± 14 (32-70)	36 ± 4 (31-40)	0.80
Albumin (g/L)	27-37	20-50*	27 ± 02 (25-29)	27 ± 3 (6-33)	26 ± 2 (23-29)	0.75
Globulin (g/L)	20-46		9 ± 3 (6-11)	12 ± 11 (6-37)	10 ± 2 (8-13)	0.81
Calcium (mmol/L)	2.6-3.2	2.4-3.4*	4.0 ± 1.3 (3.2-5.4)	3.6 ± 0.5 (3.0-4.2)	3.7 ± 0.4 (3.3-4.1)	0.69
Phosphorus (mmol/L)	0.5-1.3	1.2-2.4*	1.9 ± 0.3 (1.6- 2.1)	1.9 ± 0.7 (0.8-2.6)	2.1 ± 0.2 (1.8-2.3)	0.81
Sodium (mmol/L)	133-142	148 ± 15§	134.0 ± 2.0 (132-136)	133.1 ± 2.5 (130-137)	132 ± 3.1 (128-135)	0.56
Potassium (mmol/L)	2.5-5	4.4 ± 1.0§	5.4 ± 0.9 (4.5-6.2)	5.7 ± 1.0 (4.1 -6.8)	6.4 ± 1.3 (5.2-6.8)	0.44
Chloride (mmol/L)	98-105	105 ± 12§	99.0 ± 2.0 (97-101)	97.6 ± 1.8 (96-101)	95.2 ± 2.4 (92-99)	0.05
Glucose (mmol/L)	3.9-6.2	5.9-6.1§	3.3 ± 0.3 (3.1-3.7)	3.6 ± 1.1 (2.3-5.2)	3.1 ± 0.9 (1.6-4.1)	0.71
Alk Phos (U/L)	41-137	152-2835	2060 ± 1199 (1318-3444)	1525 ± 1002 (198-3445)	1501 ± 930 (16-2733)	0.71

Table 4.2 (cont.). Fetal serum chemistries at time of abortion (272 ± 4 days gestation). Mares were untreated (Control, n=3), or treated with 5mg/kg (n=7) or 10mg/kg (n=6) enrofloxacin intravenously once a day for 11 days before abortion. Adult range was provided by the reference laboratory. All other values are mean ± SD (range). No significant differences were seen between treatment groups for any value ($p<0.05$). Neonatal reference ranges from Axon and Palmer (2008) within an hour of birth, (§) Bauer (1990) at less than 12 hours old, or (*)from Southwood (2013) at 24 hours of age.

Value	Adult Range	Neonatal range	Control	5 mg/kg	10 mg/kg	p value
AST (U/L)	150-294	97-315	68.0 ± 16.1 (51-83)	100.6 ± 61.1 (67-238)	81.2 ± 13.0 (71-99)	0.39
GGT (U/L)	4-20	8-33	8.7 ± 2.1 (7-11)	8.6 ± 3.8 (5-15)	9.8 ± 2.9 (6-13)	0.51
Bilirubin (μmol/L)	8.6-39.3	34.2-51.3	34.2± 10.3 (23.9-42.8)	47.9 ± 8.6 (35.9-58.1)	41.0 ± 10.3 (34.2-59.9)	0.77
CPK (U/L)	71-300	65-380	262 ± 209 (131-503)	360 ± 226 (157-759)	282 ± 69 (219-401)	0.13
Cholesterol (mmol/L)	1.55-4.45	0.31-1.11	7.58 ± 1.99 (6.08-9.83)	6.88 ± 3.52 (1.66-10.29)	9.54 ± 1.86 (7.45- 11.82)	0.25
GLDH (U/L)	1-5		5.2 ± 3.7 (1.7-9.1)	2.6 ± 0.8 (1.5-3.9)	2.2 ± 0.8 (1.2-3.4)	0.06
TCO2 (mmol/L)	24-33	21-34*	27.0 ± 2.6 (24-29)	26.1 ± 1.3 (24-28)	24.8 ± 3.4 (19-28)	0.46
Mg+ (mmol/L)	0.6-0.9	0.3-1.7*	0.9 ± 0.1 (0.9-0.95)	0.8 ± 0.1 (0.6-1.0	0.8 ± 0.4 (0.1-1.4	0.94
Triglycerides (mmol/L)	0.12-0.63	0.05-1.74*	0.31 ± 0.08 (0.21-0.37)	0.39 ± 0.12 (0.26-0.52)	0.41 ± 0.09 (0.25-0.58)	0.40

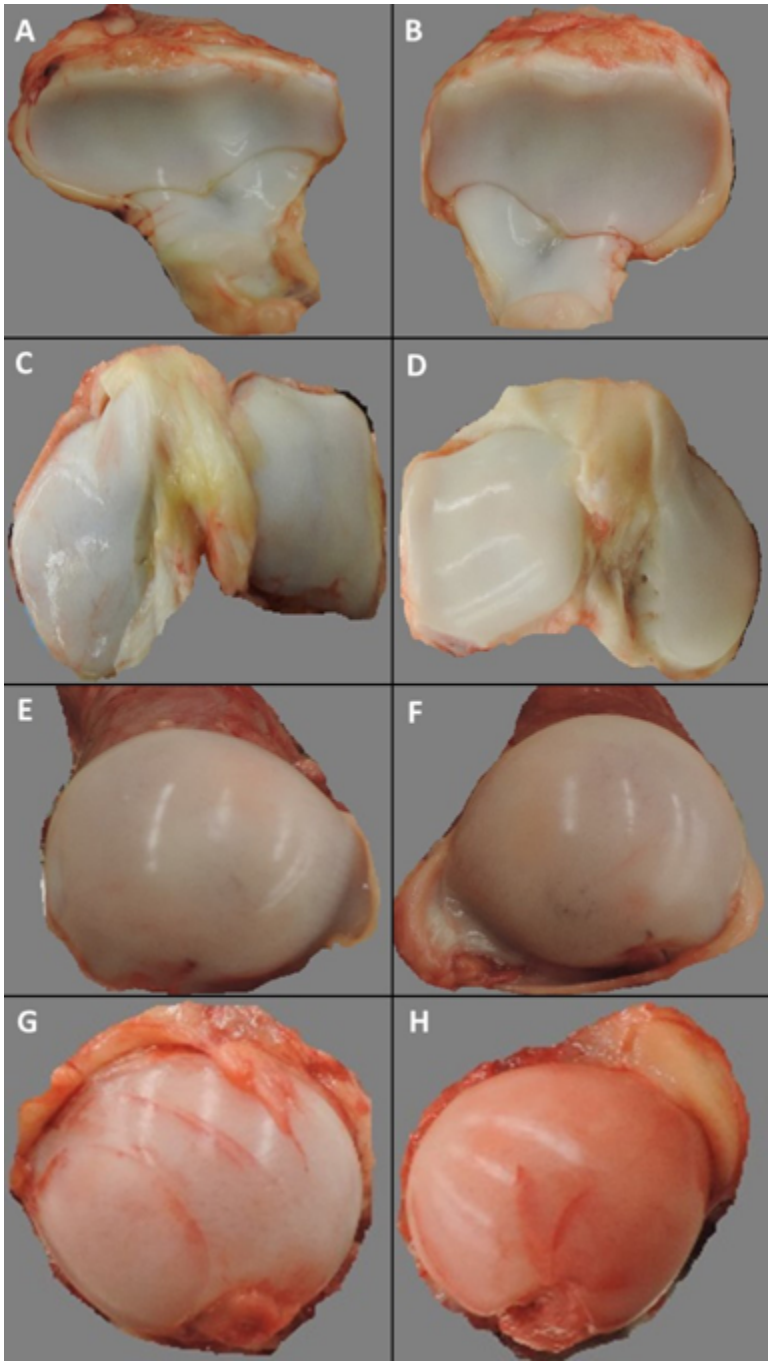

Figure 4.1. Representative images of the proximal articular surfaces of the humerus, radius, femur, and tibia in a fetus (272d gestation) from a mare treated with enrofloxacin (10 mg/kg) for 11 consecutive days. (A) Left radius, B) right radius, C) left humerus, D) right humerus, E) left tibia, F) right tibia, G) left femur, H) right femur. No fissures or erosions in the articular surface were observed in any of the foals.

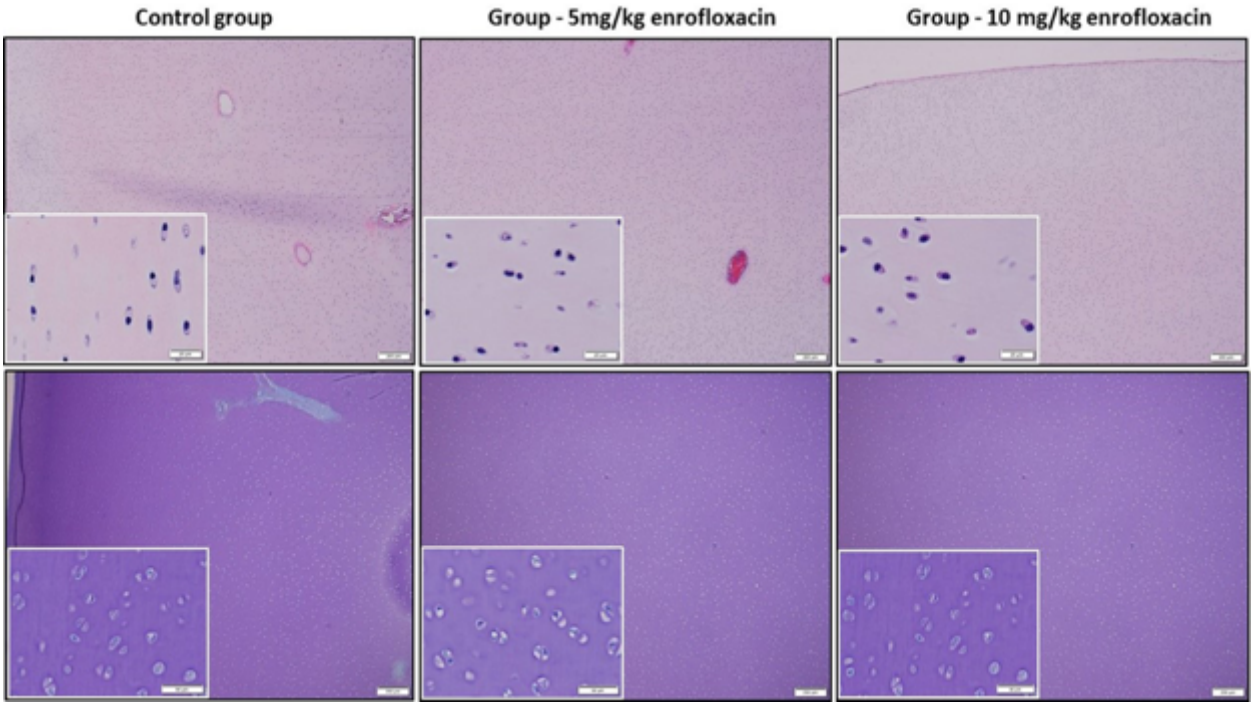

Figure 4.2. Representative images of the proximal femur cartilage of fetuses from a control mare and mares treated with 5 mg/kg or 10 mg/kg of enrofloxacin. Slides in the top row were stained with standard H&E, whereas slides in the bottom row were stained with Toluidine Blue. Magnification 4x (insets 40x).

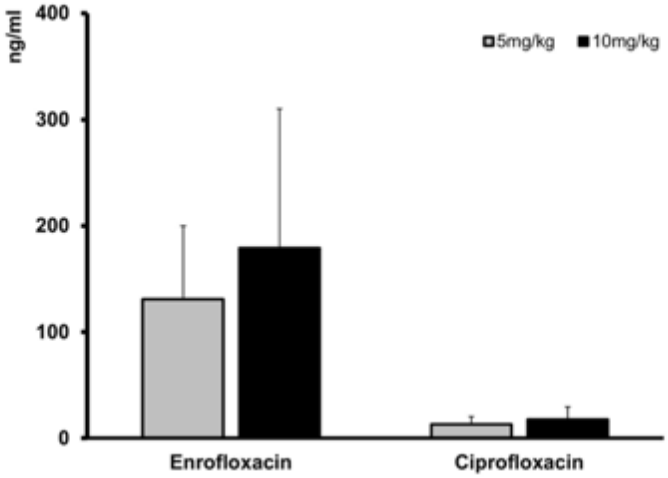

Figure 4.3. Mean (± SD) enrofloxacin and ciprofloxacin concentrations in the plasma of fetuses at time of abortion. Mares (261 ± 4 days gestation) were administered 5 mg/kg (n=7) or 10 mg/kg enrofloxacin (n=6) intravenously once a day for 11 days, or were administered no drug (control, n= 3). Differences between treatment group plasma concentrations were not significant (p> 0.05).

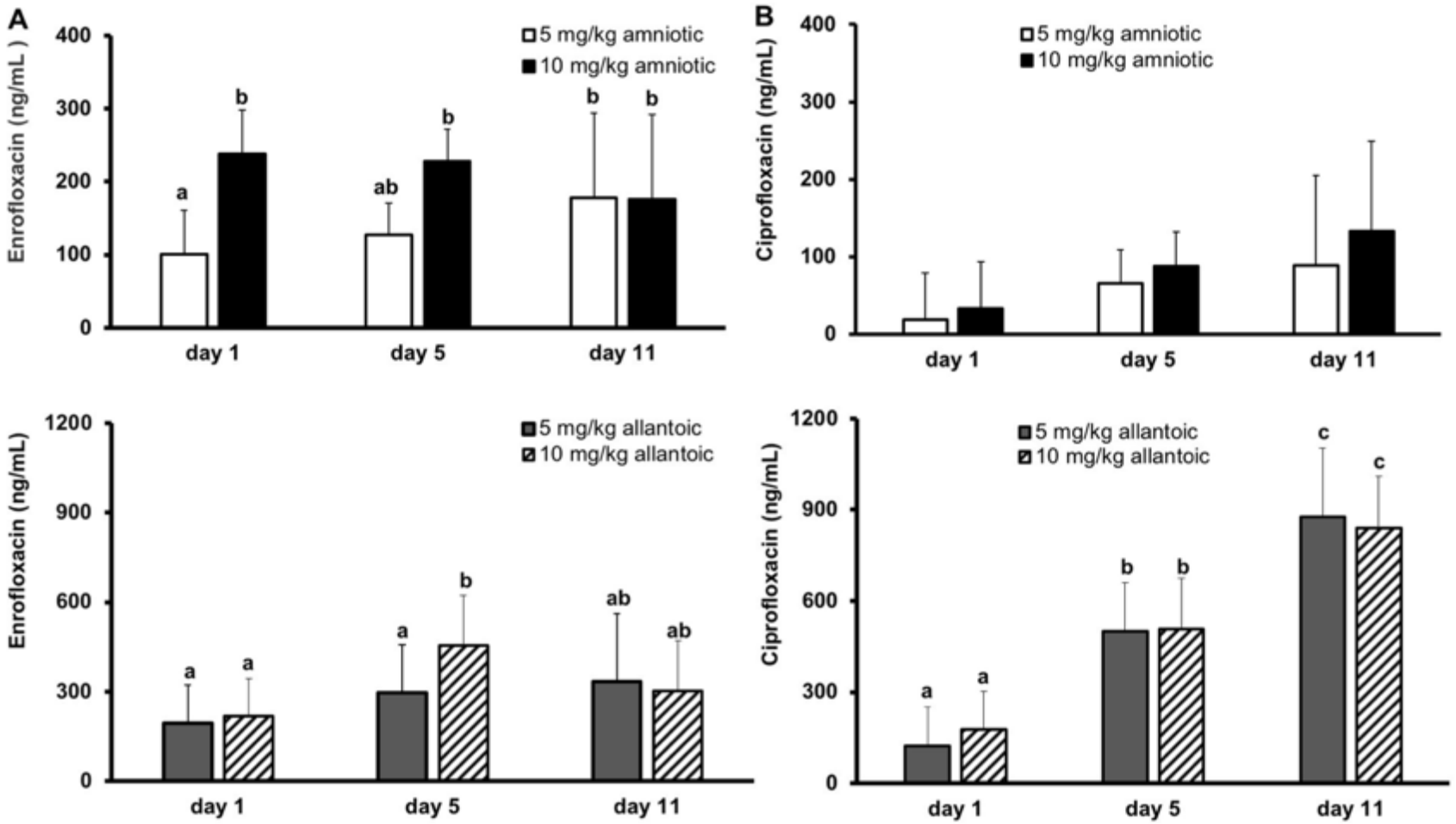

Figure 4.4. Mean (± SD) enrofloxacin (A,C) and ciprofloxacin (B,D) concentrations in the amniotic (A,B) and allantoic (C,D) fluid of pregnant mares. Mares (261 ± 4 days gestation) were administered 5 mg/kg (n=7) or 10 mg/kg (n=6) enrofloxacin intravenously once a day for 11 days, or were administered no drug (control, n= 3). Foetal fluids were sampled on days 1, 5, and 11. Superscripts denote significant differences ($p<0.05$).

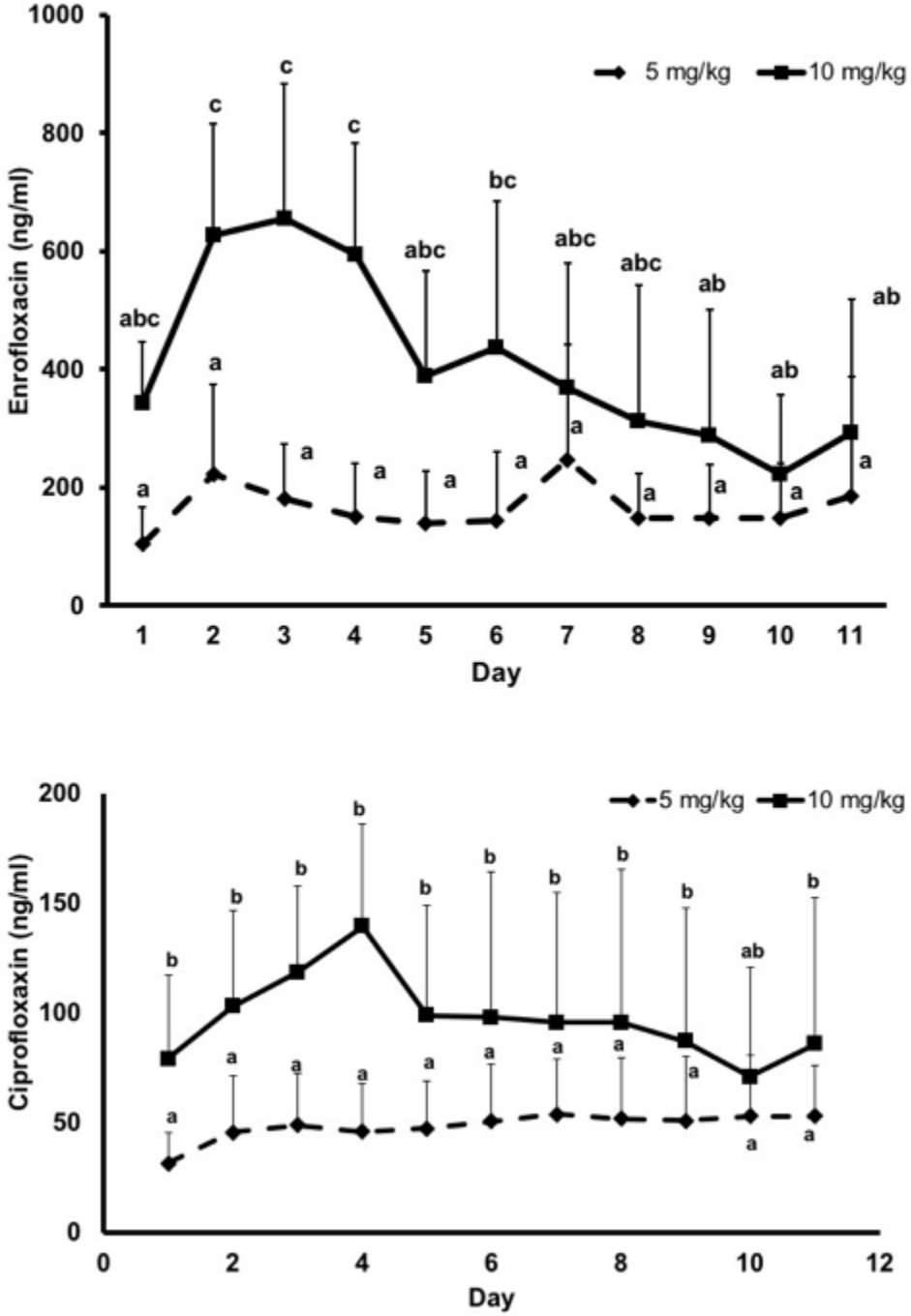

Figure 4.5. Mean (± SD) trough enrofloxacin (top) and ciprofloxacin (bottom) concentrations in the plasma of mares. Mares (261 ± 4 days gestation) were administered either 5 mg/kg (n=7) or 10 mg/kg enrofloxacin (n=6) intravenously once a day for 11 days, or were administered no drug (control, n= 3). Superscripts denote significant differences ($p<0.05$).

Supplementary Table 4.1. Scoring system used for histologic evaluations of the foal cartilage. Adapted from Maslanka and collaborators [37].

Category	Criteria	Evaluation	Score
Structural changes	Fissure intermediate zone	Absent	0
		Present	2
Cellular changes	Cellularity	Normal	0
		Hypocellularity	2
	Chondrocytes clusters	Absent	0
		Present	2
	Shrunken cytoplasm and pyknotic nuclei	Not numerous	0
		Moderately	1
		Numerous	2
		Very numerous	3
	Necrotic chondrocytes	Not numerous	0
		Moderately numerous	1
		Numerous	2
		Very numerous	3
	Spindle-shaped cells	Absent	0
		Present	1
		Surrounding cartilage canals	
Extracellular matrix changes	Uptake of toluidine blue stain	Normal	0
		Lack uptake in matrix in cartilage canals	0.5
		Slight reduction	1
		Moderate reduction	2
		Severe reduction	3.5
		No dye	4.5

REFERENCES

1. Murchie, T.A., Macpherson, M.L., LeBlanc, M.M., Luznar, S. and Vickroy, T.W. (2006) Continuous monitoring of penicillin G and gentamicin in allantoic fluid of pregnant pony mares by in vivo microdialysis. *Equine Vet. J.* **38**, 520–525.
2. LeBlanc, M.M. (2009) The current status of antibiotic use in equine reproduction. *Equine Vet. Educ.* **21**, 156–167.
3. Prescott, J.F. (2013) Beta-lactam antibiotics : Cephalosporins. In: *Antimicrobial therapy in veterinary medicine*, 5th ed., Eds: S. Giguere, J. Prescott and P. Dowling, John Wiley & Sons, Hoboken. pp 153–173.
4. Macpherson, M.L., Giguère, S., Hatzel, J.N., Pozor, M., Benson, S., Diaw, M., Sanchez, L.C., Vickroy, T.W., Tell, L., Wetzlich, S. and Sims, J. (2013) Disposition of desfuroylceftiofur acetamide in serum, placental tissue, fetal fluids, and fetal tissues after administration of ceftiofur crystalline free acid (CCFA) to pony mares with placentitis. *J. Vet. Pharmacol. Ther.* **36**, 59–67.
5. Rebello, S.A., Macpherson, M.L., Murchie, T.A., LeBlanc, M.M. and Vickrow, T.W. (2006) Placental transfer of trimethoprim sulfamethoxazole and pentoxifylline in pony mares . *Anim. Reprod. Sci.* **94**, 432–433.
6. Prescott, J.F. (2013) Sulfonamides, diaminopyrimidines, and their combinations. In: *Antimicrobial therapy in veterinary medicine*, 5th ed., Eds: J.F. Prescott, S. Giguère and P.M. Dowling, John Wiley & Sons, Hoboken. pp 279–294.
7. Bailey, C.S., Macpherson, M.L., Pozor, M.A., Troedsson, M.H.T., Benson, S., Giguere, S., Sanchez, L.C., LeBlanc, M.M. and Vickroy, T.W. (2010) Treatment efficacy of trimethoprim sulfamethoxazole , pentoxifylline and altrenogest in experimentally induced equine placentitis. *Theriogenology* **74**, 402–412.
8. Crider, K.S., Berry, R.J., Hobbs, C.A. and Hu, D.J. (2009) Antibacterial medication use during pregnancy and risk of birth defects. *Arch. Pediatr. Adolesc. Med.* **163**, 978–985.
9. Toribio, R., Bain, F., Mrad, D., Messer, N., Sellers, R. and Hinchcliff, K. (1998) Congenital defects in newborn foals of mares treated for equine protozoal myeloencephalitis during pregnancy. *J. Am. Vet. Med. Assoc.* **212**, 697–701.
10. Stahlmann, R., Kuhner, S., Shakibaei, M., Schwabe, R., Flores, J., Evander, S.A. and Sickle, D.C. van (2000) Chondrotoxicity of ciprofloxacin in immature beagle dogs: immunohistochemistry, electron microscopy and drug plasma concentrations. *Arch Toxicol* **73**, 564–572.
11. Vivrette, S., Bostian, A., Bermingham, E. and Papich, M. (2001) Quinolone-induced arthropathy in neonatal foals. *AAEP Proc.* **47**, 376–377.
12. Khazaeil, K., Mazaheri, Y., Hashemitabar, M., Najafzadeh, H., Morovvati, H. and Ghadrdan, A.R. (2012) Enrofloxacin effect on histomorphologic and histomorphometric structure of lamb articular cartilage. *Glob. Vet.* **9**, 447–453.
13. Giguere, S. and Dowling, P. (2013) Fluoroquinolones. In: *Antimicrobial therapy in veterinary medicine*. pp 295–314.
14. Martinez, M., McDermott, P. and Walker, R. (2006) Pharmacology of the fluoroquinolones: A perspective for the use in domestic animals. *Vet. J.* **172**, 10–28.
15. Sendzik, J., Lode, H. and Stahlmann, R. (2009) Quinolone-induced arthropathy: an update focusing on new mechanistic and clinical data. *Int. J. Antimicrob. Agents* **33**, 194–200.

16. Egerbacher, M., Edinger, J. and Tschulenk, M. (2001) Effects of enrofloxacin and ciprofloxacin hydrochloride on canine and equine chondrocytes in culture. *Am. J. Vet. Res.* **62**, 704–708.
17. Egerbacher, M., Wolfesberger, B., Walter, I. and Seirberl, G. (1999) Integrins mediate the effects of quinolones and magnesium deficiency on cultured rat chondrocytes. *Eur J Cell Biol* **78**, 391–397.
18. Davenport, C.L.M., Boston, R.C. and Richardson, D.W. (2001) Effects of enrofloxacin and magnesium deficiency on matrix metabolism in equine articular cartilage. *Am. J. Vet. Res.* **62**, 160–166.
19. Lim, S., Hossain, M.A., Park, J., Choi, S.H. and Kim, G. (2008) The effects of enrofloxacin on canine tendon cells and chondrocytes proliferation in vitro. *Vet. Res. Commun.* **32**, 243–253. http://dx.doi.org/10.1007/s11259-007-9024-8.
20. Bertone, A.L., Tremaine, W.H., Macoris, D.G., Simmons, E.J., Ewert, K.M., Herr, L.G. and Weisbrode, S.E. (2000) Effect of long-term administration of an injectable enrofloxacin solution on physical and musculoskeletal variables in adult horses. *J. Am. Vet. Med. Assoc.* **217**, 1514–21.
21. Canisso, I.F., Ball, B.A., Squires, E.L. and Troedsson, M.H. (2014) How to perform transabdominal ultrasound-guided fetal fluid sampling in mares. *J. Equine Vet. Sci.* **34**,
22. Curcio, B., Giguère, S., Li, Z., Honorato, J., Ellerbrock, R.E., Lima, F.S. and Canisso, I.F. (2017) Pharmacokinetics of enrofloxacin and its active metabolite (ciprofloxacin) in late-term pregnant and post-parturient mares. *Clin. Theriogenology* **9**, 466
23. Trundell, D.A., Ferris, R.A., Hennet, M.R., Wittenburg, L.A., Gustafson, D.L., Borlee, B.R. and McCue, P.M. (2017) Pharmacokinetics of intrauterine ciprofloxacin in the mare and establishment of minimum inhibitory concentrations for equine uterine bacterial isolates. *J. Equine Vet. Sci.* **54**, 54–59.
24. Maślanka, T. and Jaroszewski, J.J. (2009) Effect of long-term treatment with therapeutic doses of enrofloxacin on chicken articular cartilage. *Pol. J. Vet. Sci.* **12**, 21–33.
25. Henson, F.M., Davies, M.E. and Leffcott, L.B. (1997) Equine dyschondroplasia (osteochondrosis) - histological findings and type VI collagen localization. *Vet. J.* **154**, 53–62.
26. Southwood, L.L. (2013) Normal ranges for hematology and plasma chemistry and conversion table for units. In: *Practical Guide to Equine Colic*, 1st ed., Ed: L.L. Southwood, John Wiley & Sons, Hoboken. pp 339–342.
27. Axon, J.E. and Palmer, J.E. (2008) Clinical pathology of the foal. *Vet. Clin. North Am. - Equine Pract.* **24**, 357–385.
28. Bauer, J. (1990) Normal blood chemistry. In: *Equine Clinical Neonatology*, Eds: A. Koterba, W. Drummond and P. Kosch, Lea & Febiger, Philadelphia. p 607.
29. Muzi, F., Gravante, G., Tati, E. and Tati, G. (2007) Fluoroquinolones-induced tendinitis and tendon rupture in kidney transplant recipients: 2 Cases and a review of the literature. *Transplant. Proc.* **39**, 1673–1675.
30. Brown, S.A. (1996) Fluoroquinolones in animal health. *J Vet Pharmacol Ther* **19**, 1–14.
31. Stahlmann, R., Forster, C., Shakibaei, M., Vormann, J., Gunther, T. and Merker, H.-J. (1995) Magnesium deficiency induces joint cartilage lesions in juvenile rats which are identical to quinolone-induced arthropathy. *Antimicrob. Agents Chemother.* **39**, 2013–2018.
32. Egerbacher, M., Wolfesberger, B. and Gabler, C. (2001) In vitro evidence for effects of magnesium supplementation on quinolone-treated horse and dog chondrocytes. *Vet. Pathol.* **38**, 143–148.

CHAPTER 5: ADMINISTRATION OF ENROFLOXACIN DURING LATE PREGNANCY FAILED TO INDUCE LESIONS IN THE RESULTING NEWBORN FOALS

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ABSTRACT

Enrofloxacin may be an alternative antibacterial agent to treat select severe infections in pregnant mares. A recent study has shown that enrofloxacin and ciprofloxacin cross the equine placenta without causing gross cartilage or tendon lesions in the 9-mo fetus; however, long-term effects of *in utero* fluoroquinolone exposure are unknown. The objective of the following study was to assess effects of fetal exposure to enrofloxacin on the resulting foal's cartilage and tendon strength. Healthy mares at 280 d gestation were allocated into four groups: untreated (n=5), therapeutic

treatment (7.5 mg/kg enrofloxacin, PO x 14 days, n=6), supratherapeutic treatment (15 mg/kg, PO x 14 days, n=6), or treatment of the foals postpartum (n=2). Mares were allowed to carry pregnancy to term, and foals were maintained on pasture for five weeks. After that foals were euthanized, and articular cartilage and extensor and flexor tendons were examined macroscopically and histologically for lesions. Tendon strength was tested by loading until failure. Administration of enrofloxacin at recommended doses in late gestation did not result in cartilaginous lesions or clinical lameness in any foal by 5-wks-old. Tensile strength was greater in hind tendons than front tendons, but no difference was found between foals born from treated and control mares. Expectedly, osteochondral changes were present both in foals born from enrofloxacin-treated mares and in negative control foals with no apparent association with fluoroquinolone treatment during pregnancy. Short-term administration of enrofloxacin to late gestation mares did not result in macroscopic or microscopic lesions in the resulting foal by five weeks of age. While further research is needed to assess other stages of gestation and long-term foal outcomes, enrofloxacin may be useful for select bacterial infections in pregnant mares.

Keywords: fluoroquinolone, ciprofloxacin, pregnancy, fetal toxicity

INTRODUCTION

Antimicrobial therapy is necessary for pregnant mares with bacterial infections, however the toxicity of many antimicrobials is not known. Aminoglycosides and β-Lactam antibiotics cross the equine placenta [1] and are commonly used in pregnant mares [2,3]. Cephalosporin antibiotics are also commonly and safely used during equine pregnancy, but do not cross the equine placenta [4]. Potentiated sulphonamides cross the equine placenta [5] and are frequently used to treat infections in late-pregnant mares [2,6,7] despite knowledge that sulphonamides have been associated with an increased risk of birth defects in the first trimester in humans [8] but rarely in the horse [9]. Conversely, fluoroquinolones are believed to be chondrotoxic to the foetus and are avoided in pregnant animals [10–12].

Enrofloxacin is a lipophilic fluoroquinolone antimicrobial that inhibits topoisomerase I (DNA Gyrase) and topoisomerase IV, and has bactericidal activity against Gram-negative and some Gram-positive pathogens [13]. Enrofloxacin either undergoes hepatic metabolism into ciprofloxacin, or is excreted unchanged in the urine, and both enrofloxacin and ciprofloxacin are

effective against many common equine pathogens [13,14]. Quinolones are believed to induce arthropathies by chelating divalent cations such as magnesium (Mg^{2+}) [15–19]. *In vitro* and *in vivo* studies have provided evidence that administration of enrofloxacin can cause tendonitis and arthropathies, especially in young, growing animals [10–12].

While the limited previous research might suggest that enrofloxacin should be avoided in pregnant mares, enrofloxacin is reportedly safe to administer during canine pregnancy according to manufacturer label. Two studies have assessed *in vivo* effects of enrofloxacin in the horse, using supratherapeutic doses to attain maximum toxicity [11,20]. In adult horses, long-term (i.e., 21d) treatment with supratherapeutic doses (i.e., 15 mg/kg and 25 mg/kg q 24 hours) resulted in musculoskeletal complications in 3 of 12 animals, while no complications were seen with long term treatment with the recommended dose (5 mg/kg q 24 hours)[20]. When four two-week old foals were treated with twice the recommended dose (10 mg/kg, IV, q 24 hours) for 8 days, three foals became moderately to severely lame and had tibial-tarsal joint effusion with roughening of the articular cartilage, suggesting that foals might be more sensitive to cartilage damage [11]. However, this increased dosage is generally not used and to date no studies have assessed the effects of the recommended dose on foals.

Despite concerns about foetal toxicity, practitioners occasionally treat pregnant mares with enrofloxacin when faced with multi-drug resistant infections. Saving the mare in these cases is more important than potential effects on the foetus or might be the only way to prevent pregnancy loss. Additionally, a recent study showed that enrofloxacin and ciprofloxacin cross the equine placenta, and that when enrofloxacin is administered intravenously for 11 days in late pregnancy, no lesions were detectable in the foetus [21]. However, it is possible that side effects might not be apparent until after the resulting foal becomes weight bearing. Traditionally, toxicity studies have determined adverse effects based on clinical signs, blood work, and histological evaluations of tissues of interest. Given the athletic demands of the horse, and reports of tendon rupture in humans after long-term fluoroquinolone use [22,23], it is vital to investigate if enrofloxacin affects tendon tensile strength or cartilage strength in foals born from enrofloxacin-treated mares.

The major objectives of this study were to determine: (i) the clinical outcomes of enrofloxacin administration at therapeutic doses during late-term pregnancy on the resulting foal, (ii) if exposure to enrofloxacin and ciprofloxacin *in utero* results in cartilage or tendon lesions in

5-wks-old foals. The minor objectives of this study were to assess enrofloxacin and ciprofloxacin concentrations in mares' plasma at the beginning and in the middle of the treatment, and in the foetal fluids close to foaling. We hypothesized that enrofloxacin administration during late gestation would not affect foal health or soundness by five weeks of age.

MATERIALS AND METHODS

The study protocol was approved by the University of Illinois Institutional Animal Care and Use Committee (protocols, #14243 and #17245). The present study was carried out between 2015 and 2018. Nineteen healthy, light-breed pregnant mares were enrolled in the study at 280 days gestation. Mares were kept at the Veterinary Medicine Research Farm at the University of Illinois in Urbana, Illinois, where they were housed on pasture and supplemented with grass hay and trace minerals.

Animal husbandry and study design

Clinically healthy multiparous mares (14 ±4 yrs-old) were included in the present study if ultrasonography of the caudal placental pole was within normal limits. Mares were randomly allocated to four groups: recommended dose of enrofloxacin[a] (7.5 mg/kg, PO, n=6), double therapeutic dose of enrofloxacin[a] (15 mg/kg, n=6), negative control (n=5, no enrofloxacin), or positive control (n=2, no enrofloxacin administration during pregnancy; foals were treated with enrofloxacin 2-3 wks. after birth). Mares were treated for fourteen days. Heparinized blood samples were collected via jugular venipuncture on days 1 and 8 of treatment. Blood was collected starting at t=0 and then repeated at 2, 3, 4, 6, 8, and 24 hours after enrofloxacin administration. The samples were centrifuged at 600 x g for 10 minutes, and plasma samples were harvested and stored at -80^{o}C until analysis. Mare plasma magnesium concentrations were measured with an automated quantitative analyzer[b] before enrofloxacin administration on Day 1, and again on Day 14, immediately before the last dose was administered. The compounded commercial oral formulation of enrofloxacin used in this study has been shown previously to be well absorbed by pregnant mares [135].

Fetal fluid sampling

In a subset of mares (7.5 mg/kg n= 3 and 15 mg/kg n=2), fetal fluid sampling was performed 30-40 days after last enrofloxacin dose, as previously described [25]. Mares were sedated with xylazine [Anased[c]] 0.4 mg/kg/IV, and butorphanol [Torbogesic[d]] 0.004 mg/kg/IV, placed in stocks, and their ventral abdomens were clipped, and aseptically prepped with betadine scrub. After regional analgesia with 10 mL 2% lidocaine[e], foetal fluids were collected aseptically with transabdominal ultrasound guidance using a SonoScape S8 EXP with convex probe[f], and an echotip spinal needle[g]. Fluid was collected and saved only if a pure white color (amniotic) fluid was obtained. A minimum of 5 mL and maximum of 12 mL was collected, and the needle was removed. A new, sterile needle was then used to collect a sample of allantoic fluid, and fluid (5-12 mL) was collected only if an amber fluid consistent with allantoic fluid was obtained [25].

Foaling management and neonatal care

All mares carried their foals to term. Mares had pre-foaling mammary gland secretions pH assessed daily in the evening to determine the night of foaling as previously described [26,27]. Mares were kept in foaling stalls (6 x 4 m) and monitored with an internal video camera system. Once signs of stage two of labour were detected, technicians contacted the investigators to supervise all parturition. If needed, mares received oxytocin[e] (10 units/IM q30min) to hasten release of the fetal membranes. Thereafter, the fetal membranes from all mares were examined after parturition for completeness, meconium staining, and signs of hemorrhage and infection. Colostrum was administered by nasogastric tube if the foal did not nurse well within three hours of birth. Foals' umbilical navels were submerged in dilute chlorhexidine gluconate[e] twice a day for five days. Full physical examinations were performed on mares and foals immediately after delivery and then at 12-24 hours intervals. Complete blood counts and plasma IgG[h] were evaluated at 12 hours of age, and foals with IgGs < 800 mg/dL were administered 1 L hyperimmune plasma[i]. Foals with total white cell counts < 5,000 x 10^3 cells/µl were treated with ceftiofur sodium [Naxcel[d]] (4.4 mg/kg IV) for three to five days. Mares and foals were maintained on pasture after foaling.

The two foals in the positive control groups were administered 10 mg/kg enrofloxacin PO once a day for seven days starting at 2-wks-old (foal 1) or once a day for 10 days starting at 3-wks-old (foal 2). At 5-wks-old, foals were euthanized with pentobarbital sodium and phenytoin

sodium [Euthasol[j]], 900 mg/kg IV and 115 mg/kg, respectively. Blood samples were collected in heparinized tubes and immediately centrifuged at 600x g for 10 minutes. Plasma samples were stored at -80°C until analysis.

Determination of enrofloxacin and ciprofloxacin concentrations

Concentrations of enrofloxacin and ciprofloxacin were measured by liquid chromatography-tandem mass spectrophotomy (LC-MS/MS) in the Metabolomics Laboratory of the Roy J. Carver Biotechnology Center, University of Illinois Urbana-Champaign. Validation and detailed description of LC-MS/MS methods are reported elsewhere [24]. Between-run and within-run accuracy and precision were calculated by measuring six samples of enrofloxacin and ciprofloxacin at concentrations of 5, 50, and 500 ng/ml, and measurements were repeated on three different days. Enrofloxacin had a within-run accuracy of 95.6 ± 2.1 % to 99.1 ± 0.6%, and a between-run accuracy of 97.3 ± 2.0% to 98.4 ± 1.0%, and ciprofloxacin had a mean within-run accuracy of 95.4 ± 2.3 to 99.6 % ± 1.2 %, and between-run accuracy of 97.0 ± 2.4 % to 99.2 ± 0.9%. Coefficients of variations between-run and within-run for enrofloxacin ranged from 0.9-1.6% and 1.1-3.5%, respectively, whereas, coefficients of variations for ciprofloxacin between-run and within-run ranged from 0.9- 2.4% and 1.0-5.4 %, respectively. Plasma stability data was calculated under various conditions (n= 6). Samples stored in the auto-sampler for 24 h at 15 °C had a bias of 3.2-4.8% and samples stored at -75 °C for 2 months had a bias of 1.3-3.4%.

Pathology

After euthanasia, foal limbs were disarticulated, and all joint surfaces were inspected for gross lesions by a board-certified veterinary pathologist blinded to foal treatment group. The extensor tendons were collected mid metacarpus and mid metatarsus from all limbs for histological evaluation. Superficial flexor tendons were removed intact, wrapped in saline soaked towels and saran wrap and frozen at -80° C for biomechanical testing. The joint surfaces collected for histology were selected based on the most affected joints in the positive control foals. Sections of articular cartilage of the distal scapula, distal humerus, distal radius, distal femur, distal tibia, and talus were collected from all four limbs using a bone saw. All slices were preserved in 10% neutral buffered formalin immediately after collection. The fixed tissues were then routinely decalcified, processed and embedded in paraffin in an automated tissue processor. Slides were stained with

haematoxylin & eosin for analysis and histological changes graded (Supplemental Table 5.1) as previously published elsewhere [39].

Biomechanical testing

The right front and hind limb superficial flexor tendons were thawed at room temperature, and divided in half axially in the longitudinal plane. The four hemi-tendons were placed in parallel, and all four hemi-tendons were imaged by computed tomography[k]. Images were acquired with a 16-slice helical CT scanner[k] using a Bone+ algorithm (17mAs, 80KV, 5 x 10mm). Mean cross sectional area of each hemi-tendon was calculated by averaging the cross sectional area in 10 fields using imaging software[l]. For biomechanical testing, each hemi-tendon was tested individually and the two values averaged to provide a single value per limb per foal. For testing, the tendon was centred in a hydraulic testing device[m] and the tendon ends were secured using custom designed cryogrips (Figure 5.1). The grips were serrated and kept cold with dry ice throughout the experiment.

Testing procedure was slightly modified from previous studies [28,29]. The tendon was centered so the distance between grips was 10 cm at the start of testing. Tendons were loaded at 10N and cycled at 2.5% strain for 5 cycles prior to tensile testing. For testing, tendons were then loaded to failure in tension. Displacement was measured as change in grip-to-grip distance from the start of testing until yield load was obtained. Yield stress and maximum stress ($N/mm^2 = MPa$) were calculated by dividing the yield load (N) and maximum load (N) by mean cross sectional area obtained from the CT scan (mm^2). The elastic modulus was calculated by the yield stress/strain.

Statistical analysis

MedCalc software v16.4[n] was used to determine the *a priori* number of animals required to detect a statistical difference between groups, with an alpha = 0.05, a power of 0.8, and an expected difference between groups of 30% or greater. While six animals were to be allocated per group, only nineteen mares were available at 260 days gestation. The decision was made to use five control (untreated) mares to minimize euthanasia of healthy foals. Similarly, only two foals were treated postpartum to minimize animal suffering once it was determined that lesions were detectable in these foals. To determine whether mare plasma magnesium levels differed on Day 0

and Day 14, plasma magnesium levels were compared with a paired t-test. Histomorphometric analyses were evaluated using a non-parametric Kruskal-Wallis one-way analysis of variance. Tendon tensile strength, mare gestation length, foal age, and time from treatment to foaling were compared between treatment groups and controls using a one-way ANOVA, and a Tukey HSD was used for post-hoc analysis. Day 1 and Day 8 enrofloxacin and ciprofloxacin concentrations were compared using paired t tests. Significance was set at $p < 0.05$. Data were analysed using R libraries (R version 3.3.3)[p].

RESULTS

Clinical outcomes and magnesium concentration

All mares had normal physical examinations for the 14-day treatment period, and no adverse effects were seen from oral administration of enrofloxacin, or from transabdominal ultrasound-guided fetal fluid sampling. No oral lesions were noted at any point in the study. Mares foaled at 339 ± 11 days of gestation, and there was no difference between gestation length and treatment group (335 ±7 days (control), 339 ±16 days (7.5 mg/kg), 344 ±12 days (15 mg/kg)). Mean time from last enrofloxacin treatment to foaling was 45 ±17 days, and time to foaling did not differ between treatment groups (42 ±17 days (7.5 mg/kg), 48 ± 20 days (15 mg/kg)).

Three of the Standardbred mares experienced dystocias (elbow lock, easily corrected) (Control), bilateral carpal flexion (7.5 mg/kg mare), and unilateral shoulder flexion, uterine artery haemorrhage (15 mg/kg mare) (Table 5.1). None of the mares retained their fetal membranes beyond three hours post-delivery. Upon inspection post-partum, there were no gross abnormalities in the fetal membranes of any mare. The foal from the mare with the uterine artery hemorrhage required significant traction for foal delivery and had bilateral fetlock effusion for five days after birth. One negative control foal, and one treated foal (7.5 mg/kg), had transient effusion of the tibiotarsal and fetlock joints respectively, both of which resolved by one week of age, and no lameness was noted in either foal at the end of the study. One foal in the 7.5 mg/kg group had leukopenia and an IgG of 780 at 24 hours of age and was administered 1L hyperimmune plasma[i] (Table 5.1). Foals were 35 ±3 days of age at time of euthanasia, and age did not differ between groups (35 ±3 days (control), 35 ±2 days (7.5 mg/kg), 34 ±3 days (15 mg/kg)). The two positive control foals were younger at time of euthanasia (26 ±4 days) than the rest of the foals.

Mare plasma magnesium concentrations were not different between the control or treated groups at the start of the study (0.88 ± 0.16 mmol/L (control), 0.78 ± 0.04 mmol/L (7.5 mg/kg), 0.78 ± 0.04 mmol/L (15mg/kg)), or after 14 days of enrofloxacin administration (0.82 ± 0.04 mmol/L (control), 0.78 ± 0.04 mmol/L (7.5 mg/kg), 0.78 ± 0.04 mmol/L (15 mg/kg)).

Enrofloxacin and ciprofloxacin concentrations

Peak plasma concentrations were 1040 ± 228 ng/mL (enrofloxacin) and 210 ± 25 ng/mL (ciprofloxacin) for the 7.5 mg/kg group and 1638 ± 246 ng/mL (enrofloxacin) and 327 ± 21 ng/mL (ciprofloxacin) for the 15 mg/kg group on Day 1. Day 8 peak plasma concentrations were 1160 ± 230 ng/mL (enrofloxacin) and 244 ± 52 ng/mL (ciprofloxacin) for the 7.5 mg/kg group, and 2602 ± 1060 ng/mL (enrofloxacin) and 384 ± 87 ng/mL (ciprofloxacin) for the 15 mg/kg group (Figure 2). Peak plasma enrofloxacin ($p=0.085$) and ciprofloxacin ($p=0.066$) concentrations tended to be higher on Day 8 than Day 1 in the 7.5 mg/kg group, but were not significantly higher by Day 8 in the 15 mg/kg group. Both enrofloxacin and ciprofloxacin were still detectable in the amniotic (7.4 ± 4.7 ng/mL of enrofloxacin and 15.3 ±12.4 ng/mL of ciprofloxacin) and allantoic (11.2 ±1.0 ng/mL of enrofloxacin and 148.3 ± 61.1 ng/mL of ciprofloxacin)) fluids thirty to forty days after administration of the last dose of oral enrofloxacin.

Pathology

After examining all joints from each foal, no gross lesions were noted in any joint of foals in the negative control, 7.5 mg/kg or 15mg/kg groups (Figures 5.3 and 5.4). Expectedly, gross lesions were noted in 21 of 34 joints examined in one positive control foal and 3 of 34 joints examined in the other positive control foal. Lesions noted were superficial and deep erosions in the articular surface, with mild to severe joint effusion, fibrin, and synovitis. Distal joint surfaces were more severely affected than proximal surfaces, with the exception of the talus, which had severe deep erosions (Figure 5.4). Histological evaluation of the extensor tendons were unremarkable.

A total of 239 joints were assessed histologically from the 19 foals (Table 5.2). No differences were seen between the negative control, 7.5 mg/kg and 15 mg/kg treatment groups. Fissures, spindle cells, and shrunken/pyknotic nuclei were the most commonly observed changes (Table 5.2, Figure 5.5). The fissures noted in the deep zone were consistent with chondrifying cartilage canals (Figure 5.6). In addition to the spindle cells and shrunken/pyknotic nuclei noticed in the other three treatment groups, the two positive control foals had marked changes noted on

histology. Extensive, deep erosions were noted in the distal humerus, distal scapula, proximal tibia and talus, and superficial erosions were noted in the distal tibia, proximal and distal ulna, and proximal metacarpus of the foal treated at 14 days of age. Deep erosions of the articular cartilage of this foal can be seen grossly in figure 4 and on histology in figure 5.5. Less severe erosion of the distal metacarpus and rare chondrocyte necrosis in the distal humerus and proximal femur were noted in the foal treated at 21 days of age.

Biomechanical testing

All but four tendon samples failed at mid-tendon via longitudinal extension of the interfaces between the collagen bundles. Two tendon samples failed by sharp separation at the grip (one for the control group and other from the 15 mg/kg group), and two failed by sharp separation mid-tendon (one 7.5 mg/kg, one 15 mg/kg). No differences were seen in the elastic modulus, max stress, or yield stress of tendons from control or treated foals. Tendon yield stress was similar between treatment groups (Control = 7.6 ± 1.0 MPa), (7.5mg/kg = 8.6 ± 1.5 MPa) and (15 mg/kg = 9.2 ± 1.9 MPa). Interestingly, the front limb tendons had a lower elastic modulus than hind limb tendons. Similarly, yield stress, max stress, and stiffness were lower in the front limb tendons than hind limb tendons (Table 5.3).

DISCUSSION

Administration of enrofloxacin to late pregnant mares resulted in diffusion of enrofloxacin and ciprofloxacin into the fetus and pregnancy fluids, but did not result in apparently chondrotoxic lesions in the fetuses [21]. However, it is possible that adverse effects of *in utero* exposure to enrofloxacin may not be apparent until after the resulting foal becomes weight bearing. In the current study, administration of enrofloxacin to late pregnant mares failed to cause musculoskeletal disease (lameness, tendinopathies, erosions of articular cartilage) in the resulting foals from birth to 30 days of age, while foals treated after birth (positive controls) did develop clinical signs and pathology similar to those described in a previous report involving administration of enrofloxacin to newborn foals [11].

Cellular changes were noted in the cartilage of foals from all treatment and negative control groups consistent with normal cartilaginous changes seen in foals at 30 days of age [30]. Defects classified as fissures were consistent with cartilage canals normally noted in developing foals [30]. The degenerating cartilage canal vessels and occasional adjacent areas of necrotic cartilage are

typical findings, and are interpreted as either normal loss of cartilage canals that occurs during development, or early osteochondrosis lesions. Interestingly, the fissures or cartilage canals were seen most frequently in the distal scapula, and not in the joints where osteochondrosis lesions typically develop, or where lesions were seen in treated foals [11, 31-33]. Superficial and deep erosions were seen only in the foals treated with enrofloxacin postpartum.

No differences were seen in flexor tendon tensile strength after enrofloxacin administration to pregnant mares. Flexor tendon CSA was 1/3 of the previously reported CSA for older foals, and mean yield stress was approximately 2/3 to ¾ lower than yield stress reported for older foals [29]. Interestingly, while no changes were seen in any tensile parameters between treatment groups, a clear difference was noted between the front and hind tendons. This difference between forelimb and hindlimb flexor tendons has not been detected previously in adult horses. While fluoroquinolone exposure during pregnancy did not affect flexor tendon tensile strength, it is important to acknowledge that mares enrolled in this study were healthy. In humans, risk factors for tendonitis or Achilles rupture with fluoroquinolone treatment include concurrent corticosteroid use, renal disease, and age (higher in elderly people) [22,23]. The risk of fluoroquinolone administration to comprised or older mares remains to be determined.

Magnesium deficiency causes similar lesions to fluoroquinolones in rat cartilage, and in *in vitro* cultures of equine and canine tendon cells [17,18,34,35]. All mares in this study had normal plasma magnesium levels at the beginning of the study, and no change was seen in plasma magnesium by the end of the study. While it is not possible to say if lesions would have been observed in animals that were deficient in magnesium, it may be prudent to check plasma magnesium concentrations before instituting fluoroquinolone treatment in pregnant mares.

The severe clinical signs seen in one of the two positive control foals demonstrated that the enrofloxacin formulation used in this study induced previously described adverse effects in foals [11] if administered during a susceptible window in foal development. The adverse effects were much more severe in the younger, heavier foal (2-wks-old) when compared to the older but lighter foal (3-wks-old), which suggests that there might be a very specific window when foals are most susceptible to cartilage damage. All mares in the current study foaled at least 30 days after the last dose of enrofloxacin; thus, it remains to be determined if administering a fluoroquinolone close to expected foaling date causes lesions in the resulting foal.

Incidence of dystocia in this study was 15%, which is similar to previously reported incidences of on-farm equine dystocia [36,37]. Two of the three mares that experienced dystocias at time of foaling were treated with enrofloxacin during late gestation, and those two dystocias resulted in the loss of the foal in one group, and the loss of the mare in the other group. Given the small number of foaling mares herein, and the fact that dystocia rates were comparable to other Standardbred breeding farms in the area, it is not possible to establish an association between enrofloxacin administration during late pregnancy and dystocia.

A previous study assessing equine fetal toxicity after fluoroquinolone exposure during late pregnancy demonstrated that ciprofloxacin accumulates at high concentrations in the allantoic fluid by the 11th day of drug administration, and it was not determined how long ciprofloxacin would be detectable in the allantoic fluid after the last dose [21]. While determining the rate of ciprofloxacin elimination was not an objective of the current study, allocentesis 30-40 days after the last dose of enrofloxacin clearly demonstrated residual ciprofloxacin in the allantoic fluid. Concentrations in the amniotic fluid were lower, or not detectable in one mare. This suggests that enrofloxacin might be an alternative antimicrobial for severe placental infections due to the high concentrations initially, and the high levels maintained potentially for weeks after the last dose is administered parentally. However, since the study was conducted in healthy mares, it is still unknown how concurrent disease might affect placental drug transfer.

Collectively, these findings suggest that enrofloxacin administration to the late pregnant mare reaches therapeutic drug concentrations in the fetus and fetal fluids without apparent detrimental effects on the cartilage and tendons of the resulting foal. Additionally, ciprofloxacin concentrations in the allantoic fluid may remain elevated for many days after administration of the last enrofloxacin dose. This suggests that enrofloxacin may be a useful antibiotic option for a select group of late pregnant mares suffering severe bacterial infections. In order to comply with appropriate antimicrobial stewardship, enrofloxacin administration should be limited to life-threatening infections where a microbial culture indicates microbial resistance to first line antimicrobials and a sensitivity to enrofloxacin. If enrofloxacin is administered to late pregnant mares, clients should be informed of the potential risks of fluoroquinolone administration, as this study does not rule out the possibility of a low incidence of toxicity. Additionally, practitioners should be aware that both enrofloxacin and ciprofloxacin cross the equine placenta, and that while no lesions were detected in the 30 day-old foal, enrofloxacin was administered for only 14 days,

and that antibiotics were discontinued at least a month before the mare foaled. Additional studies are warranted to investigate longer-term effects on foals after intrauterine exposure to fluoroquinolones, as well as exposure in the first or second trimester, or when the mare is treated for longer than fourteen days.

Manufacturers

[a]Enrofloxacin Oral Suspension, Rood and Riddle Pharmacy, Lexington, KY
[b]Beckman Coulter, Pasadena, CA, USA
[c]Akorn Inc, Decatur, Illinois, USA
[d]Zoetis, Parsipanny, NJ, USA.
[e]VetOne, Boise, Idaho, USA.
[f]SonoScape, Shenzhen, China
[g]Havel's, Cincinnati, Ohio, USA
[h]Animal Reproduction Systems, Chino, CA, USA
[i]Lake Immunogenics, Ontario, NY, USA
[j]Virbac Animal Health, Fort Worth, Texas, USA
[k]GE Healthcare, Chalfont St Giles, Buckinghamshire, England
[l]Carestream, Rochester, NY, USA
[m]Bionix 858, MTS Corp., Eden Prairie, MN, USA
[n]MedCalc Software bvba, Ostend, Belgium
[p]R Foundation for Statistical Computing, Vienna, Austria

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Authorship

I Canisso contributed to study design, study execution, data analysis, interpretation, and preparation of manuscript. R. Ellerbrock contributed to study execution, data analysis, interpretation, and preparation of manuscript. P. Roady contributed to study execution, data analysis, and preparation of manuscript. G. Podico contributed to study execution. S. Durgam, A. Litsky, and L. Zhong contributed to study execution and data analysis. F. Lima contributed to study design and preparation of manuscript.

FIGURES

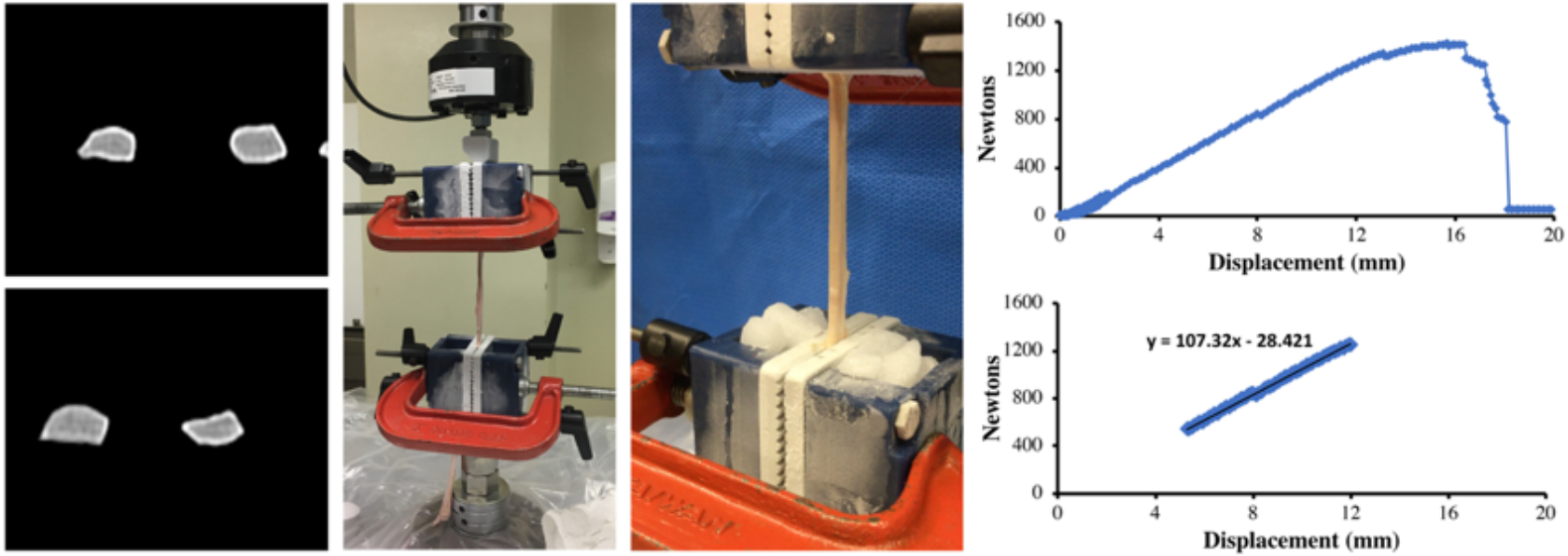

Figure 5.1: Representative images of computed tomography of the flexor tendons (left), the tendon tensile testing apparatus (middle), and of a tendon stress/strain curve and stiffness curve (right).

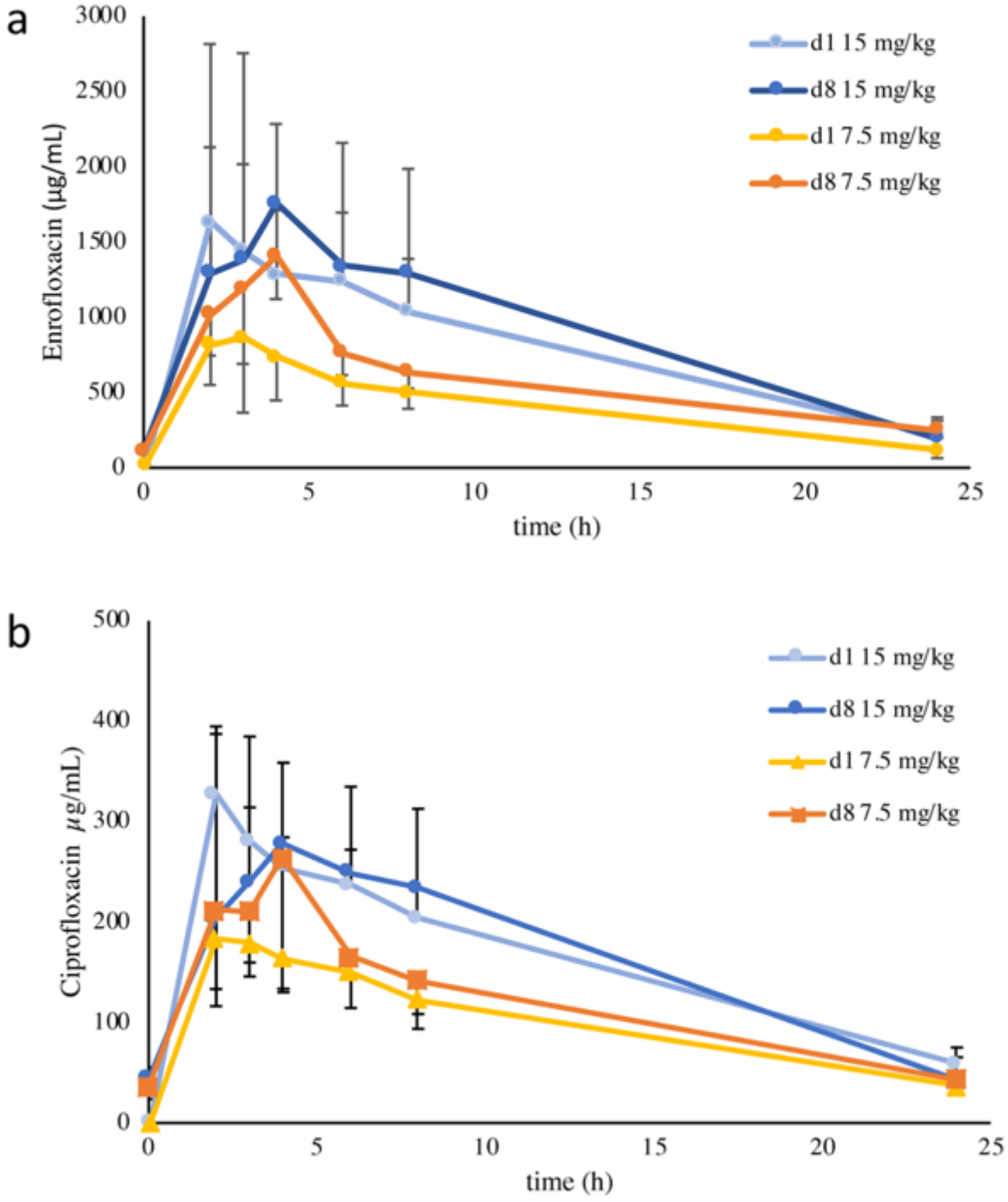

Figure 5.2**.** Mean (± SD) enrofloxacin (a) and ciprofloxacin (b) concentrations in the plasma of mares on day 1 and day 8 of oral enrofloxacin administration. Mares (280 ± 4 days gestation) were administered 7.5 mg/kg (n=4) or 15 mg/kg enrofloxacin (n=4) orally once a day for 14 days.

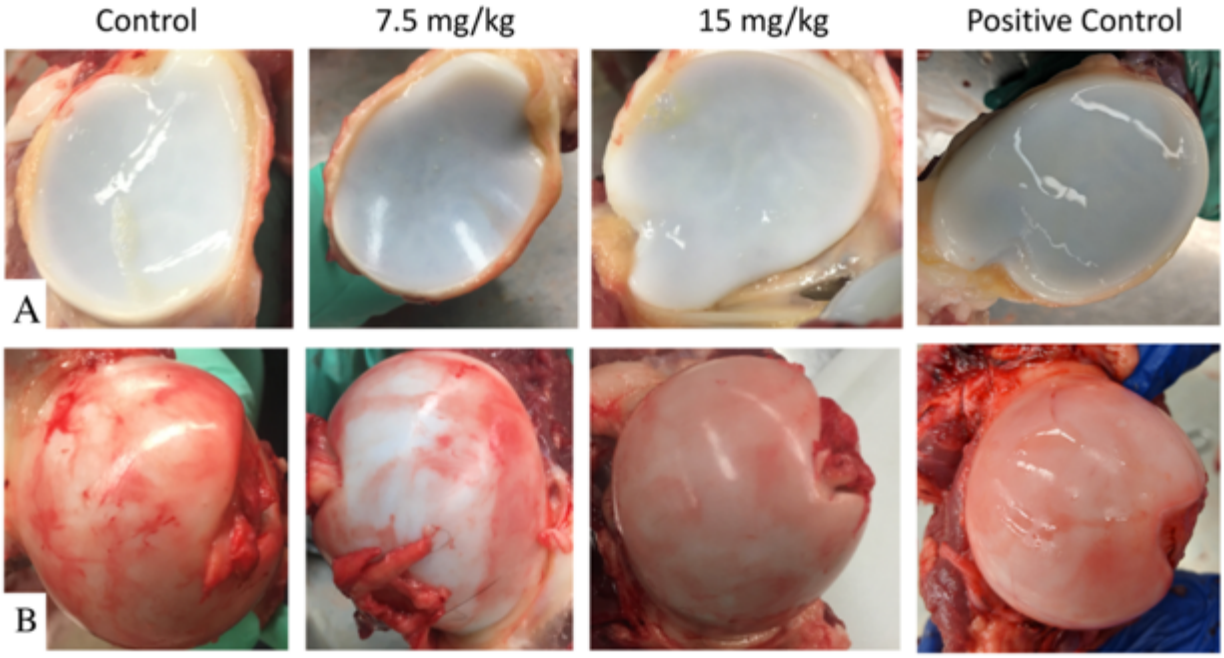

Figure 5.3: Representative gross images of the articular surfaces of the scapula (a) and proximal femur (b) of 5-wk-old foals at euthanasia. Foals were born from mares that were either untreated (Negative Control), treated with 7.5 mg/kg enrofloxacin orally SID x 14d at 280 days gestation (7.5mg/kg), treated with 15 mg/kg enrofloxacin SID x 14d at 280 days gestation (15 mg/kg). The two positive control foals were not treated during pregnancy, but foals were treated with 10 mg/kg enrofloxacin PO SID either for 7 d beginning at 2-wks-old, or for 10 d starting at 3-wks-old.

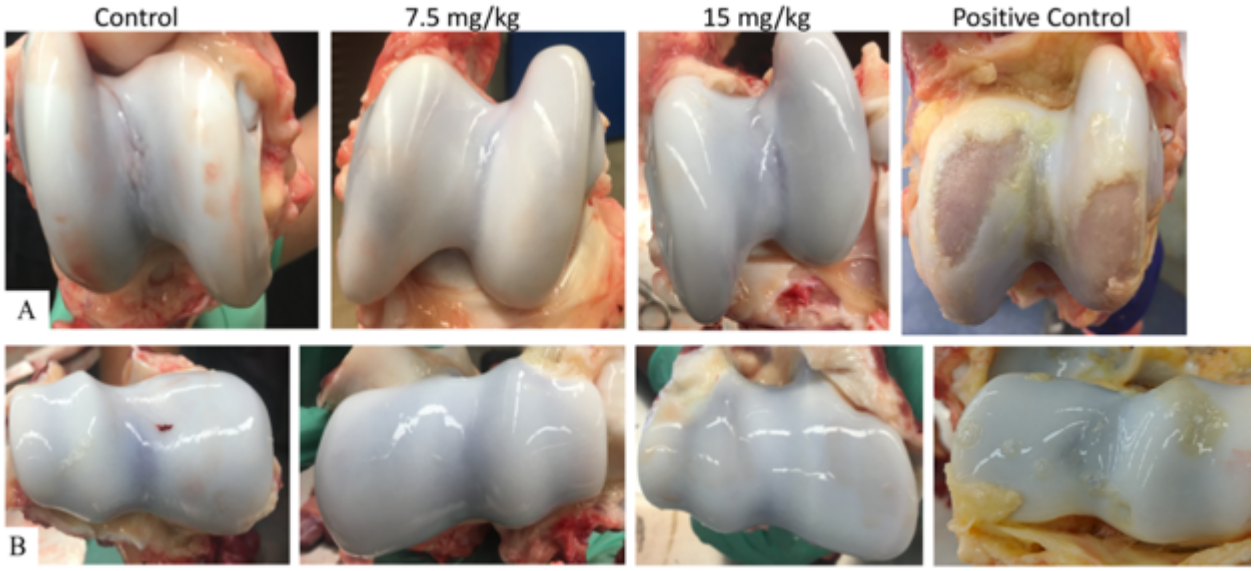

Figure 5.4: Representative gross images of the articular surfaces the tarsus (a) and distal humerus (b) of 5-wk-old foals at time of euthanasia. Foals were born from mares that were either untreated (Negative Control), treated with 7.5 mg/kg enrofloxacin orally SID x 14d at 280 days gestation (7.5mg/kg), treated with 15 mg/kg enrofloxacin orally SID x 14d at 280 days gestation (15 mg/kg). The two positive control foals were not treated during pregnancy, but the foals were treated with 10 mg/kg enrofloxacin PO once day either for 7 days beginning at 2-wks-old, or for 10 days starting at 3-wks-old.

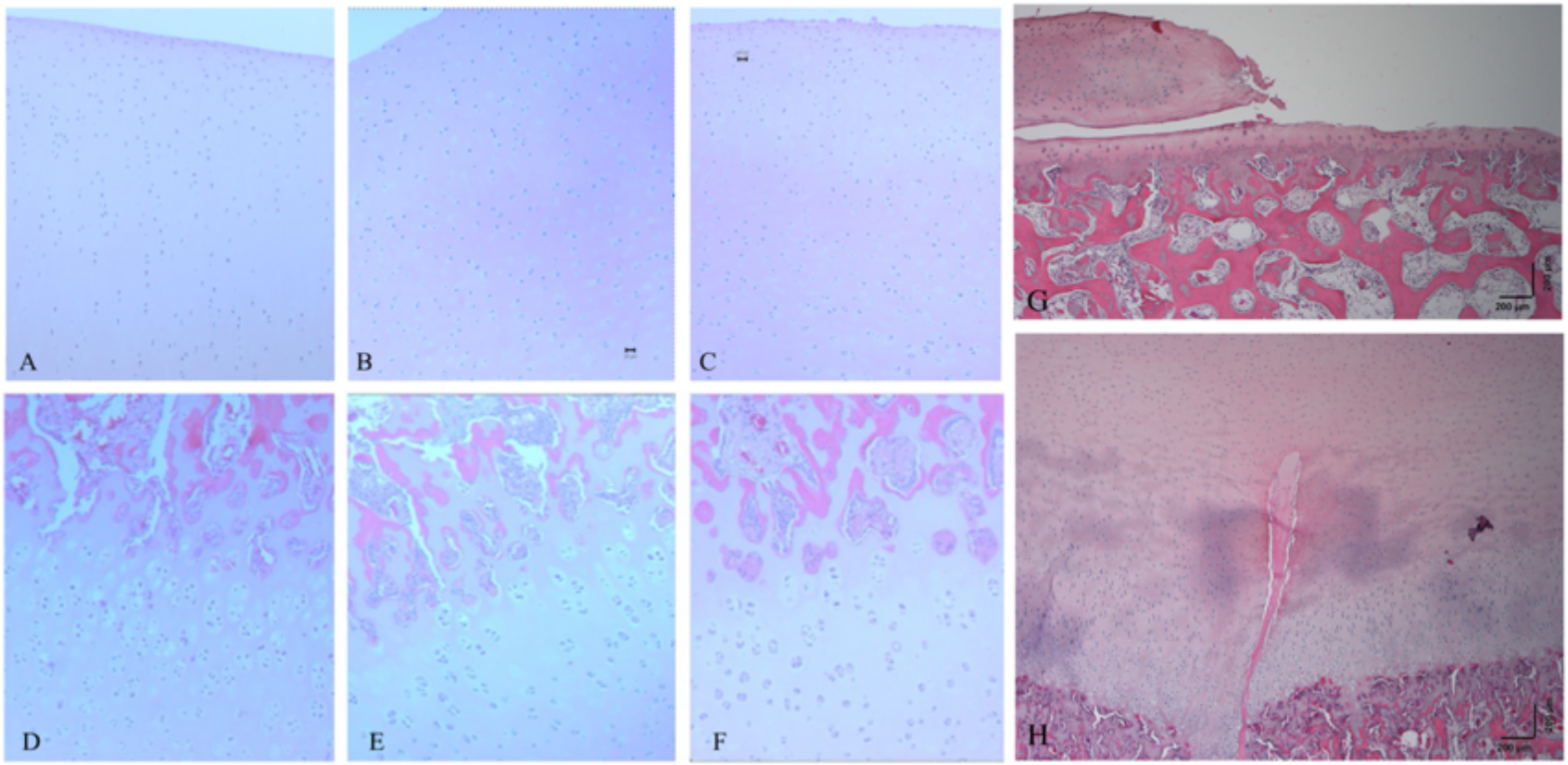

Figure 5.5. Representative histological images (A-C,G,H 10x, D-F 40x) of articular cartilage of the left talus of 5-wk-old foals from mares that were either untreated (control, A,D), or treated with 7.5 mg/kg enrofloxacin orally once a day for 14 days (B,E), or 15 mg/kg enrofloxacin orally once a day for 14 days (C,F). Image (G) shows the deep erosion present in the talus of a positive control foal (treated with 10 mg/kg enrofloxacin x 7d at 14 days of age), compared to the normal cartilage thickness of the distal scapula of the same foal (H). Haematoxylin & eosin stained slides.

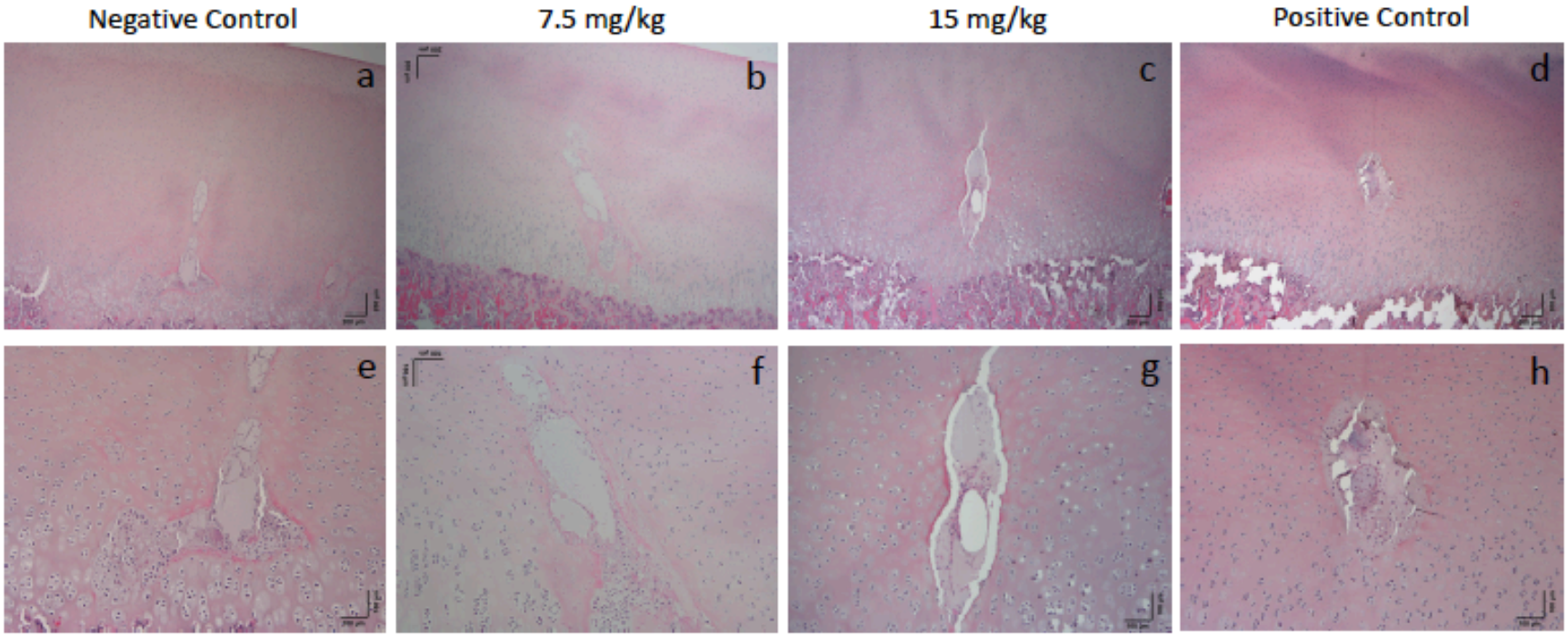

Figure 5.6. Representative histological images (a-c 10x, d-f 40x magnification) of articular cartilage of 5-wk-old foals demonstrating cartilage canals and associated changes observed in foals from all treatment groups. Mares were either untreated (control), or treated with 7.5 mg/kg enrofloxacin orally once a day for 14 days (7.5 mg/kg), 15 mg/kg enrofloxacin orally once a day for 14 days (15 mg/kg), or the mares were not treated during gestation and the foal was treated with 10 mg/kg enrofloxacin orally for 7 days starting at 14 days of age (positive control). Haematoxylin & eosin stained slides.

REFERENCES

1. Murchie, T.A., Macpherson, M.L., LeBlanc, M.M., Luznar, S. and Vickroy, T.W. (2006) Continuous monitoring of penicillin G and gentamicin in allantoic fluid of pregnant pony mares by in vivo microdialysis. *Equine Vet. J.* **38**, 520–525.
2. LeBlanc, M.M. (2009) The current status of antibiotic use in equine reproduction. *Equine Vet. Educ.* **21**, 156–167.
3. Prescott, J.F. (2013) Beta-lactam antibiotics : Cephalosporins. In: *Antimicrobial therapy in veterinary medicine*, 5th ed., Eds: S. Giguere, J. Prescott and P. Dowling, John Wiley & Sons, Hoboken. pp 153–173.
4. Macpherson, M.L., Giguère, S., Hatzel, J.N., Pozor, M., Benson, S., Diaw, M., Sanchez, L.C., Vickroy, T.W., Tell, L., Wetzlich, S. and Sims, J. (2013) Disposition of desfuroylceftiofur acetamide in serum, placental tissue, fetal fluids, and fetal tissues after administration of ceftiofur crystalline free acid (CCFA) to pony mares with placentitis. *J. Vet. Pharmacol. Ther.* **36**, 59–67.
5. Rebello, S.A., Macpherson, M.L., Murchie, T.A., LeBlanc, M.M. and Vickrow, T.W. (2006) Placental transfer of trimethoprim sulfamethoxazole and pentoxifylline in pony mares. *Anim. Reprod. Sci.* **94**, 432–433.
6. Prescott, J.F. (2013) Sulfonamides, diaminopyrimidines, and their combinations. In: *Antimicrobial therapy in veterinary medicine*, 5th ed., Eds: J.F. Prescott, S. Giguère and P.M. Dowling, John Wiley & Sons, Hoboken. pp 279–294.
7. Bailey, C.S., Macpherson, M.L., Pozor, M.A., Troedsson, M.H.T., Benson, S., Giguere, S., Sanchez, L.C., LeBlanc, M.M. and Vickroy, T.W. (2010) Treatment efficacy of trimethoprim sulfamethoxazole , pentoxifylline and altrenogest in experimentally induced equine placentitis. *Theriogenology* **74**, 402–412.
8. Crider, K.S., Berry, R.J., Hobbs, C.A. and Hu, D.J. (2009) Antibacterial medication use during pregnancy and risk of birth defects. *Arch. Pediatr. Adolesc. Med.* **163**, 978–985.
9. Toribio, R., Bain, F., Mrad, D., Messer, N., Sellers, R. and Hinchcliff, K. (1998) Congenital defects in newborn foals of mares treated for equine protozoal myeloencephalitis during pregnancy. *J. Am. Vet. Med. Assoc.* **212**, 697–701.
10. Stahlmann, R., Kuhner, S., Shakibaei, M., Schwabe, R., Flores, J., Evander, S.A. and Sickle, D.C. van (2000) Chondrotoxicity of ciprofloxacin in immature beagle dogs: immunohistochemistry, electron microscopy and drug plasma concentrations. *Arch Toxicol* **73**, 564–572.
11. Vivrette, S., Bostian, A., Bermingham, E. and Papich, M. (2001) Quinolone-induced arthropathy in neonatal foals. *AAEP Proc.* **47**, 376–377.
12. Khazaeil, K., Mazaheri, Y., Hashemitabar, M., Najafzadeh, H., Morovvati, H. and Ghadrdan, A.R. (2012) Enrofloxacin effect on histomorphologic and histomorphometric structure of lamb articular cartilage. *Glob. Vet.* **9**, 447–453.
13. Giguere, S. and Dowling, P. (2013) Fluoroquinolones. In: *Antimicrobial therapy in veterinary medicine*. pp 295–314.
14. Martinez, M., McDermott, P. and Walker, R. (2006) Pharmacology of the fluoroquinolones: A perspective for the use in domestic animals. *Vet. J.* **172**, 10–28.
15. Sendzik, J., Lode, H. and Stahlmann, R. (2009) Quinolone-induced arthropathy: an update focusing on new mechanistic and clinical data. *Int. J. Antimicrob. Agents* **33**, 194–200.
16. Egerbacher, M., Edinger, J. and Tschulenk, M. (2001) Effects of enrofloxacin and

ciprofloxacin hydrochloride on canine and equine chondrocytes in culture. *Am. J. Vet. Res.* **62**, 704–708.
17. Egerbacher, M., Wolfesberger, B., Walter, I. and Seirberl, G. (1999) Integrins mediate the effects of quinolones and magnesium deficiency on cultured rat chondrocytes. *Eur J Cell Biol* **78**, 391–397.
18. Davenport, C.L.M., Boston, R.C. and Richardson, D.W. (2001) Effects of enrofloxacin and magnesium deficiency on matrix metabolism in equine articular cartilage. *Am. J. Vet. Res.* **62**, 160–166.
19. Lim, S., Hossain, M.A., Park, J., Choi, S.H. and Kim, G. (2008) The effects of enrofloxacin on canine tendon cells and chondrocytes proliferation in vitro. *Vet. Res. Commun.* **32**, 243–253.
20. Bertone, A.L., Tremaine, W.H., Macoris, D.G., Simmons, E.J., Ewert, K.M., Herr, L.G. and Weisbrode, S.E. (2000) Effect of long-term administration of an injectable enrofloxacin solution on physical and musculoskeletal variables in adult horses. *J. Am. Vet. Med. Assoc.* **217**, 1514–21.
21. Ellerbrock, R.E., Canisso, I.F., Roady, P.J., Rothrock, L.T., Zhong, L., Wilkins, P., Dirikolu, L., Lima, F.S. and Honoroto, J. (2018) Diffusion of enrofloxacin to pregnancy fluids and effects on fetal cartilage after intravenous administration to late pregnant mares. *Equine Vet. J.* . https://doi.org/10.1111/evj.13044.
22. Muzi, F., Gravante, G., Tati, E. and Tati, G. (2007) Fluoroquinolones-Induced Tendinitis and Tendon Rupture in Kidney Transplant Recipients: 2 Cases and a Review of the Literature. *Transplant. Proc.* **39**, 1673–1675.
23. Khaliq, Y. and Zhanel, G.G. (2005) Musculoskeletal injury associated with fluoroquinolone antibiotics. *Clin. Plast. Surg.* **32**, 495–502.
24. Curcio, B., Giguère, S., Li, Z., Honorato, J., Ellerbrock, R.E., Lima, F.S. and Canisso, I.F. (2017) Pharmacokinetics of enrofloxacin and its active metabolite (ciprofloxacin) in late-term pregnant and post-parturient mares. *Clin. Theriogenology* **9**, 466.
25. Canisso, I.F., Ball, B.A., Squires, E.L. and Troedsson, M.H. (2014) How to perform transabdominal ultrasound-guided fetal fluid sampling in mares. *J. Equine Vet. Sci.* **34**, 1143–1147.
26. Ellerbrock, R.E. and Canisso, I.F. (2016) How to interpret pH profiles of mammary gland secretions to predict imminent parturition in mares. *Proc. Am. Assoc. Equine Pract. Annu. Conf.* 187–192.
27. Canisso, I.F., Ball, B.A., Troedsson, M.H., Silva, E.S.M. and Davolli, G.M. (2013) Decreasing pH of mammary gland secretions is associated with parturition and is correlated with electrolyte concentrations in prefoaling mares. *Vet. Rec.* **173**, 218.
28. Durgam, S.S., Stewart, A.A., Sivaguru, M., Wagoner Johnson, A.J. and Stewart, M.C. (2016) Tendon-derived progenitor cells improve healing of collagenase-induced flexor tendinitis. *J. Orthop. Res.* **34**, 2162–2171.
29. Cherdchutham, W., Meershoek, L.S., Weeren, P.. Van and Barneveld, A. (2001) Effects of exercise on biomechanical properties of the superficial digital flexor tendon in foals. *Am. J. Vet. Res.* **62**, 1859–1864.
30. Hellings, I.R., Ekman, S., Hultenby, K., Dolvik, N.I. and Olstad, K. (2016) Discontinuities in the endothelium of epiphyseal cartilage canals and relevance to joint disease in foals. *J. Anat.* 162–175.
31. Martel, G., Couture, C.A., Gilbert, G., Bancelin, S., Richard, H., Moser, T., Kiss, S.,

Légaré, F. and Laverty, S. (2016) Femoral epiphyseal cartilage matrix changes at predilection sites of equine osteochondrosis: Quantitative MRI, second-harmonic microscopy, and histological findings. *J. Orthop. Res.* **34**, 1743–1752.
32. Jeffcott, L.B. and Henson, F.M.D. (1998) Studies on growth cartilage in the horse and their application to aetiopathogenesis of dyschondroplasia (osteochondrosis). *Vet. J.* **156**, 177–192.
33. Henson, F.M., Davies, M.E. and Leffcott, L.B. (1997) Equine dyschondroplasia (osteochondrosis) - histological findings and type VI collagen localization. *Vet. J.* **154**, 53–62.
34. Stahlmann, R., Forster, C., Shakibaei, M., Vormann, J., Gunther, T. and Merker, H.-J. (1995) Magnesium Deficiency Induces Joint Cartilage Lesions in Juvenile Rats Which Are Identical to Quinolone-Induced Arthropathy. *Antimicrob. Agents Chemother.* **39**, 2013–2018.
35. Egerbacher, M., Wolfesberger, B. and Gabler, C. (2001) In vitro evidence for effects of magnesium supplementation on quinolone-treated horse and dog chondrocytes. *Vet. Pathol.* **38**, 143–148.
36. Ginther, O.J. and Williams, D. (1996) On-the-farm incidence and nature of equine dystocias. *J. Equine Vet. Sci.* **16**, 159–164.
37. Squires, E.L., Hughes, S.E., Ball, B.A., Troedsson, M.H.T. and Stowe, J. (2013) Effect of season and reproductive status on the incidence of equine dystocia. *J. Equine Vet. Sci.* **33**, 375.
38. Maślanka, T. and Jaroszewski, J.J. (2009) Effect of long-term treatment with therapeutic doses of enrofloxacin on chicken articular cartilage. *Pol. J. Vet. Sci.* **12**, 21–33.

CHAPTER 6: ADVANCED IMAGING OF 30-DAY OLD EQUINE FOALS FOLLOWING *IN UTERO* FLUOROQUINOLONE EXPOSURE

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ABSTRACT

Enrofloxacin may be a suitable antibacterial agent to treat select unresponsive infections in pregnant mares. Recent work has shown that enrofloxacin and ciprofloxacin cross the equine placenta without causing gross lesions in the third trimester fetus. However, long-term effects on the cartilage of *in utero* exposure to fluoroquinolones are unknown. The aim of this study was to utilize quantitative magnetic resonance imaging (qMRI) and biomechanical testing to evaluate the limbs of foals exposed to enrofloxacin during the third trimester of pregnancy. Healthy mares at 280d gestation were allocated into three groups: untreated (n=5), therapeutic treatment (7.5 mg/kg enrofloxacin, PO, n=5), or supratherapeutic treatment (15 mg/kg, PO, n=6) for 14 days. Mares were allowed to foal, and foals were maintained on pasture for 30 days. At 30 days, foal stifles,

hocks, elbows, and shoulders were radiographed, foals were euthanized, and foal limbs were analyzed by quantitative MRI, structural MRI, and biomechanical testing. Osteochondral lesions were detected in both treated and control foals on both radiographs and structural MRI. Severe cartilage erosions, synovitis, and joint capsular thickening was identified on foals treated with enrofloxacin post-partum. Median cartilage T2 relaxation times differed between joints, but did not differ between treatment groups. While further research is needed to address long-term foal outcomes, no differences were seen in advanced imaging or biomechanical testing at 30 days of age, and enrofloxacin may be useful for select bacterial infections in pregnant mares.

Keywords: fluoroquinolone, ciprofloxacin, pregnancy, fetal toxicity, qMRI

INTRODUCTION

Antimicrobial therapy is necessary for pregnant mares with bacterial infections; however, the toxicity of many antimicrobials is not known. Fluoroquinolones are believed to be chondrotoxic to the fetus given the known side effects in growing animals, and fluoroquinolones are generally avoided in pregnant animals [1-3]. Quinolones such as enrofloxacin are believed to induce arthropathies by chelating divalent cations such as magnesium (Mg^{2+}) [4-8], and *in vitro* and *in vivo* studies indicate that administration of enrofloxacin may cause tendonitis and arthropathies, especially in young, growing animals [2,9,10]. While limited previous research in neonates of other species might suggest that enrofloxacin should be avoided in pregnant mares, enrofloxacin is reportedly safe to administer during canine pregnancy according to the manufacturer label.

Recent work has shown that enrofloxacin and ciprofloxacin cross the equine placenta without causing detectable lesions in the fetus, when enrofloxacin is administered intravenously for 11 days in late pregnancy [11]. However, orthopedic lesions such as osteochondral dissecans do not become apparent until after birth in the horse [12,13], and it is possible side effects might not be apparent until after the resulting foal becomes weight bearing. Traditionally, toxicity studies have determined adverse effects based on clinical signs, blood work, and histological evaluations of tissues of interest. Given the athletic demands of the horse, and reports of tendon rupture in humans after long-term use [14,15] it is vital to confirm that enrofloxacin does not affect the long-term cartilage health of treated animals. Additionally, given the dynamic nature of osteochondral

lesions in the growing foal, long-term studies need additional ways to evaluate pathology over time.

Currently, magnetic resonance imaging (MRI) is the best non-invasive modality to evaluate cartilage, and there are a number of sequences that have been used to evaluate articular and physeal cartilage in foals [16-18]. Quantitative MRI (qMRI) can be used to evaluate proteoglycan content (T1 mapping) and collagen structure (T2 mapping), and has been used recently to evaluate osteochondrosis in the foal stifle [16,17]. T2 mapping has been used to evaluated normal cartilage development in humans [19,20], and to evaluate cartilage necrosis after surgically induced femoral lesions in goat kids [21], and cartilage repair after surgically induced lesions in rabbits [22]. Validation of qMRI for lesion detection in equine toxicology studies would enable long-term follow up and potentially limit, or eliminate, the need for terminal studies to determine lesion incidence and severity.

The objectives of this study were (i) to determine if exposure to enrofloxacin and ciprofloxacin *in utero* results in radiographic lesions at 30 days of age, (ii) to determine if qMRI enabled the detection of minor lesions not noted on gross or histopathological analysis, (iii) to determine if enrofloxacin administration to pregnant mares affected biomechanical properties of the 30-day-old equine stifle, and (iv) to determine if *in utero* enrofloxacin affected cartilage gene expression at 30 days of age. We hypothesized that enrofloxacin administration during late gestation would not affect foal health or soundness by 30 days of age.

MATERIALS AND METHODS

The study protocol was approved by the University of Illinois Urbana-Champaign Institutional Animal Care and Use Committee (protocols, #14243 and #17245). The present study was carried out between 2015 and 2018. Nineteen healthy, light-breed pregnant mares (10 Standardbreds, two Quarter Horses, two Thoroughbreds, two ponies, two grade horses, and one Tennessee Walking Horse) were enrolled in the study at 280 days gestation. The mares were kept at the Veterinary Medicine Research Farm at the University of Illinois in Urbana, Illinois, where they were housed on pasture and supplemented with grass hay and trace minerals.

Mares were included in the present study if physical examinations and ultrasonography of the caudal placental pole were within normal limits. Mares were randomly allocated to three groups: recommended dose of enrofloxacin[a] (7.5mg/kg, PO, n=6), double therapeutic dose of

enrofloxacin[a] (15 mg/kg, n=6), no enrofloxacin (n=5), or positive control (n=2). Mares in the no enrofloxacin and positive control groups were not treated during pregnancy. The oral formulation of enrofloxacin used in this study has previously been shown to be well absorbed by pregnant mares [23].

All mares carried their foals to term and mare and foal treatments and outcomes were previously described [24]. The two foals allocated to the positive control group were administered 7.5 mg/kg enrofloxacin PO once a day for seven days starting at 14 days of age (foal 1) or once a day for 10 days starting at 21 days of age (foal 2). At 30 days of age, foals were euthanized with pentobarbital sodium and phenytoin sodium [Euthasol[b]], 900 mg/kg IV and 115 mg/kg, respectively.

Radiographs

Prior to euthanasia, standard four-view radiographs were taken of the carpus, hock, and stifle, and two views were taken of the elbow and shoulder. Additionally, two-view radiographs were taken of any effusive or swollen fetlock or pastern joint. All radiographs were reviewed by a board-certified veterinary radiologist (Childs).

Pathology

After euthanasia, foal limbs were removed from the foal, and the left limbs were wrapped for immediate MRI. After MRI, the left stifle was further dissected, wrapped in saline-soaked paper towels and plastic wrap, and frozen at -20ºC until biomechanical testing. The remainder of the limbs were then disarticulated, and all joint surfaces were evaluated by a board-certified pathologist.

Magnetic Resonance Imaging

The left front and hind limbs were orientated parallel to the main magnetic field, and imaged with a Prisma 3T MRI scanner[c]. The imaging protocol included include structural imaging for high resolution delineation of cartilage, and quantitative T_2 mapping of four joints: the scapula-humeral, humerus-radioulnar, femoral-tibial, and tibo-tarsal. Structural imaging included the entire joint, and structural scans were then used to place two slice groups of spin-echo sequences in the center of each condyle, as shown in Fig. 6.1. Because the scapula-humeral

joint does not include two condyles, one slice group consisting of five slices was placed in the center of the joint (scan time: 5’54’’).

Structural imaging was performed with a 3D Double Echo Steady State (DESS) sequence with a sagittal orientation (Repetition Time: 14.84 ms, Echo Time: 5.04 ms, flip angle: 25^0, Field of view 150 x 140 mm, matrix size: 256, 160 slices, slice thickness: 0.6 mm, GeneRalized Autocalibrating Partial Parallel Acquisition (GRAPPA) acceleration factor: 2, phase partial Fourier: 7/8, slice partial Fourier: 6/8, scan time 3’6’’). T_2 mapping was performed using a 2D multi-echo spin-echo sequence (Repetition Time: 2800 ms, 32 echo times linearly spaced from 14 ms to 448 ms, flip angle: 150^0, Field of view: 140 x 140 mm, matrix size 256, two slice groups with three slices each, slice thickness: 3 mm, slice gap: 0.6 mm, GRAPPA acceleration factor: 2, scan time: 6’36’’). All structural MRIs were evaluated by a board-certified equine surgeon (Garrett) for cartilage integrity.

All joints included in the MRI portion of the study were evaluated by a scoring system adapted from Smith *et al* 2016 [25]. Images were scored ordinally based on nine articular cartilage or bony lesions. Cartilage signal abnormalities were scored based on changes in signal and morphology on the DESS images, and were graded as: 0 = no abnormalities, 1= single lesions <5mm, 2= single lesion 5- 10mm, or multiple lesions < 5 mm, 3= single lesion > 10 mm, or multiple lesions < 10 mm. High signal bone lesions were defined as poorly-marginated areas of increased signal intensity in normally hypo-intense trabecular or subchondral bone, and were evaluated by the size of high signal bone lesion based on regional involvement. A score of 0 indicated no abnormalities, 1 indicated <25% involved, 2 indicated 25-50% involved, and 3 indicated > 50% involved. The intensity of a lesion was graded on severity of signal increase compared to surrounding subchondral/trabecular bone, and a grade of 0 indicated no abnormalities, 1 indicated mild, 2 indicated moderate, or 3 indicated marked signal changes. Subchondral bone irregularities were also scored on severity, with 0 defined as a smooth and regular chondro-osseous junction, 1= mild subchondral plate irregularity, 2= marked subchondral plate irregularity extending to trabecular bone, 3= bone irregularity extending to trabecular bone with some normal bone trabecular pattern, and a 4 = cyst like formation. Ligaments were defined as normal (0), or abnormal (1). Synovial thickening was evaluated as normal, or abnormal based on intensity and distribution of synovium. A score of 0 indicated no abnormalities, indicated mild, 2 indicated moderate, and 3 indicated marked distension. Joint

effusion was graded as normal (0), <33% max distension (1), >33 and <66 % max distension (2), or >66 % max distension (3). Osteochondral lesions were identified by number of fragments, with 0 = no abnormalities, 1= 1 fragment, 2= 2 fragments, or 3 indicating >2 fragments. Osteochondral lesion size was measured, and classified as 0 (no lesions), 1 (< 250 mm^2 lesion), 2 (250-500 mm^2 lesion), or 3 (>500 mm^2 lesion).

Statistical analysis

Data were analyzed using R libraries (R version 3.3.3)[d]. Scores were not normally distributed and were reported as median (minimum, maximum). The Wilcoxon Signed Rank Test was used to test the null hypothesis that median scores for pathological changes was not different between the control, 7.5 mg/kg, 15 mg/kg, and Positive control groups. Mean T_2-relaxation times, and biomechanical testing results were compared by one-way ANOVA, and a Tukey HSD was used for post-hoc analysis. Significance was set a $p < 0.05$.

T_2- data processing

T_2-maps were generated from the spin-echo sequence using specialized Matlab ®[e] codes written for the study (Supplement 6.1). This code calculated a T_2 relaxation time for each voxel by fitting the signal, *S*, obtained at each echo time to the equation $S = c_1 \exp(-TE / T_2)$. Before performing the fit, signals from the lowest echo times, 14, 28 and 52 ms were discarded. Low signals obtained at longest echo times were also discarded. To obtain representative T_2 values from various regions within the cartilage, a line was drawn across the cartilage, and a seed T2 time identified from the line generated (Fig. 6.2). This seed T2 time was then used to create a map of the cartilage, and a line was drawn down the middle of the cartilage. Mean and standard deviation cartilage relaxation time was then calculated (Fig 6.3). This was repeated for five different locations in the femoro-tibial joint, and one location in the tibiotarsal, scapula-humeral, and humeral-radius joint. Mean relaxation time was calculated for the six different slices through the joint.

Cartilage pressure testing

Cartilage pressure testing was conducted in the laboratory of Dr. Mariana Kersh, in the Department of Mechanical Engineering. One tibiofemoral joint per foal (the same limb used for

qMRI) was frozen at -80C immediately after quantitave MRI until analysis. After thawing, the stifle was mounted in a custom fixture (Fig. 6.4), which allowed the tibiofemoral joint to be placed in a physiologically relevant position for pressure testing using a hydraulic testing device[f]. Cartilage pressure was measured by a dynamic measurement sensor[g], and a dynamic pressure map was created that is used to determine average pressure during stifle compression.

Cartilage gene expression

Full-thickness articular cartilage was harvested from the coxo-femoral joint immediately after foal euthanasia, and was snap frozen in liquid nitrogen until processing. RNA isolation was done as previously described [26], cartilage was pulverized while still frozen, and then homogenized on ice in a buffer of 4M guanidinium isothiocyanate, 0.1 M Tris-HCL, 25 mM EDTA and 1% (v/v) 2-mercaptoethanol. Total RNA was isolated by phenol/chloroform extraction and precipitated using isopropanol. RNA samples were purified using a QIAGEN RNeasy kit[f] according to manufacturer instructions. For RT-PCR, one microgram of total RNA was reverse transcribed into cDNA using oligo-dT primers. RT-qPCR was performed in duplicate using the 7900 HT Fast Real-Time PCR Systems[h], TaqMan Gene Expression Master Mix[h] and equine primer/probes listed in Table 1. Elongation factor-1alpha and GAPDPH were used as reference genes. Gene-specific primers were designed for previous studies using sequences available in Genbank. Relative expression of genes of interest were normalized to control genes. Equine synovial RNA from osteoarthritic joints was used as an additional positive control. For each gene of interest, mean fold change from control foals was calculated for each treatment group by comparing mean expression for each treatment group using the 2-ΔΔCT method [27]. Statistical significance of qPCR results was determined using the Mann-Whitney-Wilcoxon rank sum test.

RESULTS

Radiographs

Osteochondral lesions were detected in seven of the eighteen foals (Table 6.2), with lesions present in 2/5 Control foals, 3/5 foals in the 7.5 mg/kg group, 2/6 foals in the 15 mg/kg group, and neither of the foals in the Positive control group. Moderate to severe joint effusion was seen in all imaged joints of one positive control foal, and mild to moderate effusion was seen in the tibiotarsal

joints of the other positive foal, and moderate intracapsular effusion of the tibiotarsal joint of one Control foal, associated with an osteochondral defect in the medial malleolus.

Structural MRI

Osteochondral lesions were detected in seven of the eighteen foals, as seen on radiographs. Moderate to severe joint effusion was seen in the femoro-tibial and tibiotarsal joints of one positive control foal, and mild to moderate effusion was seen in the tibiotarsal joints of the other positive foal, and moderate intracapsular effusion of the tibiotarsal joint of one Control foal, associated with an osteochondral defect in the medial malleolus. In addition, severe cartilage erosion was noted on the medial and lateral trochlea of one positive control foal, and in addition to the joint effusion noted on radiographs, moderate intracapsular effusion was noted in the scapula-humeral and humero-radial joints (Fig. 6.5). The punctate lucencies seen in the medial trochlea of one foal from the High group were also noted on structural MRI. Median, minimum and maximum MRI scores were significantly different between positive control foals and the other three groups as shown in Table 6.3, but no differences were seen between control and *in utero* exposed foals for any category.

Quantitative MRI

Median cartilage T2 relaxation times differed between joints and but did not differ between treatment groups, or within joints, as can be seen in Fig. 6.6.

Biomechanical testing

No differences were seen between treatment groups in cartilage stiffness (Control 65 ± 12.5 N/mm, 7.5 mg/kg 49 ± 15 N/mm, 15 mg/kg, 71 ± 17 N/mm) or peak load at failure (Control 600 ± 175 N, 7.5 mg/kg 700 ± 215 N, 15 mg/kg, 560 ± 310 N).

Cartilage mRNA expression

Quantitative PCR analysis revealed that expression of most cartilage genes did not differ after *in utero* exposure to enrofloxacin (Fig. 6.7). No significant differences were seen in *ADAMST4*, *MMP2*, *MMP3*, or *MMP9* expression between groups, and expression of *MMP13* was only significantly increased in the osteoarthric positive control sample. Similarly, no differences were

seen in *Col IIA*, *Col X*, AGG, or *Sox 9* expression. There was a 10-fold increase in *Alk Phos* expression in the positive control foals.

DISCUSSION

This study was initiated in an effort to enhance the ability to detect potential significant cartilaginous changes after *in utero* fluoroquinolone exposure. Enrofloxacin administration to late pregnant mares failed to induce clinical or subclinical disease in the foals in this study, and gross and histological observations were similar to those described on advanced imaging. Radiographs were sufficient to pick of osteochondral lesions at a rate similar to lesion discovery on MRI. Osteochondrosis was noted in both treated and untreated foals, and there were insufficient numbers in this study to determine if treatment increases incidence of lesions. Additionally, many osteochondrosis lesions resolve before a year of age in foals, so it is possible that if the study duration had been longer, lesions detected before 30 days of age would have resolved.

Unsurprisingly, MRI detected subtle increases in joint effusion that were not noted on physical examination, and joint effusion was seen in both foals treated with enrofloxacin postpartum, as well as a control foal with an osteochondral lesion that was not noted at time of euthanasia. Additionally, MRI detected mild cartilaginous changes that were previously described on gross histology [24], in addition to mild changes in the subchondral bone. It appears that MRI would be a feasible alternative to evaluate ongoing changes in long-term toxicology studies to reduce or eliminate the number of animals required for a terminal study. No differences were noted in articular cartilage T2 relaxation times between any of the control or treated groups, which suggests that enrofloxacin exposure does not affect cartilage in a significant way to affect the water content in the cartilage. The failure to see a difference in relaxation times in the positive control animals may be in part due to the fact that the severe cartilage erosion the affected joints prevented generation of the same global cartilage map in those joints. Irrespective of the positive control foals, the similar T2 relaxation times between *in utero* exposed foals and control foals suggest that cartilage structure was similar between groups.

The mechanism for the severe cartilage erosions in one foal treated at 14 days of age is still unclear. *In vitro* studies with equine cartilage did not show an increase in GAG degradation in cartilage explants exposed to enrofloxacin [28], and it is possible that either lesions are due to decreased GAG production in treated animals, or failures in integrin function and cell adhesion.

The failure to detect a difference in mRNA expression in the foals is perhaps not surprising given previous findings *in vitro* at physiological enrofloxacin doses [29]. *In vitro* work by Davenport et al suggested that enrofloxacin concentrations reached 25 μg/ml before having an effect on chondrocyte GAG production[28], and work by Beluche *et al* confirmed that no effects were seen in equine explant cultures at enrofloxacin doses of 10 μg/ml, but were seen at 100 μg/ml [29]. Previous work demonstrated mean synovial enrofloxacin concentrations of 4.71 μg/ml (5 mg/kg enrofloxacin PO BID for 4 days) in adult horses [30], which suggests that systemically administered enrofloxacin does not reach toxic doses in the synovial fluid when administered at recommended dosages in adult horse. Findings in the current study would suggest that similarly to adult horses, systemically administered enrofloxacin does not reach toxic concentration in the fetal synovial fluid, and that short-term exposure *in utero* exposure at physiologically relevant doses may not have long term effects on the resulting foal. In contrast, a toxic dose may be achieved if administered to the neonate as previously shown [10].

Minimal changes were seen in mRNA expression even in our positive control foals. One explanation is that only two animals were available for this study. A second explanation is the joint from which the cartilage was harvested. The acetabulum was chosen to allow for cartilage collection immediately after euthanasia without interfering with MRI or histological analyses. However, this joint was not affected grossly even in the positive control foals. In hindsight, given the significant differences in cartilage lesions observed in the positive control animals, it is possible differences would have been seen in mRNA expression with cartilage harvest from the tarsus or scapula-humeral joint. Unfortunately, the positive control animals were the final animals selected in the study, at which point cartilage had already been selected from the *in utero* exposed and control foals. Additionally, cartilage was collected at termination of the experiment, not during, or immediately after enrofloxacin exposure. It is possible that differences would have been noted if cartilage had been collected *in utero*; however, the objectives of this study were to determine longer-term consequences to *in utero* exposure, thus, small changes in expression *in utero* might not have a significant effect on the cartilage of the neonatal foal. The only significant increase in mRNA expression was alkaline phosphatase expression in the positive control foals treated post-partum. While this has not been described previously in enrofloxacin treated animals, it is perhaps not surprising given a reported increase in bone alkaline phosphatase in the synovial fluid of horses with active osteoarthritis.

In a parallel study looking at foal outcomes, we noted that mare serum magnesium levels did not change throughout the course of the study, and that neonatal foal magnesium levels were normal [24]. Given that *in vitro* enrofloxacin studies have shown that media type effects the toxic enrofloxacin dose [28], it is also important to consider that fetal effects may be different in systemically compromised mares, or mares on poor diets. One limitation of this study was that it was conducted in healthy mares, and it is unknown how placentitis would affect placental drug transfer. Additionally, foals were only monitored until one month of age, and it is possible that either tendon or cartilage damage may take longer to appear, or that the OC lesions seen here would resolve by a year of age, as they do in up to 50% of foals that demonstrate lesions at one moth of age (personal communications with Dr. Annette McCoy).

Further studies assessing long-term effects of *in utero* exposure are warranted. Similarly, this study focused on potential side effects in the third trimester, and safety of fluoroquinolone administration in the first two trimesters is still unknown. Practitioners should advise clients of the current research investigating long-term effects on the foal, and ensure that they are not administering supratherapeutic doses. Collectively, these findings suggest that enrofloxacin administration to the late pregnant mare does not have apparent detrimental effects on the cartilage of the resulting foal. This suggests that enrofloxacin may be a useful antibiotic option for a select group of late pregnant mares suffering severe bacterial infections.

Manufacturers

[a]Enrofloxacin Oral Suspension, Rood and Riddle Pharmacy, Lexington, KY
[b]Virbac Animal Health, Fort Worth, Texas, USA
[c]Siemens Medical Solutions, Malvern, PA, USA
[d]R Foundation for Statistical Computing, Vienna, Austria
[e]MatLab, MathWorks, Natick, Massachusetts, USA
[f]Bionix 858, MTS Corp., Eden Prairie, MN, USA
[g]Tekscan, South Boston, MA, USA
[g]Qiagen, Germantown, MD, USA
[h]Applied Biosystems, Foster City, CA, USA

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Authorship

R. Ellerbrock contributed to study design, study execution, data analysis, interpretation, and preparation of manuscript. I Canisso contributed to study design, study execution, data analysis, interpretation, and preparation of manuscript. P. Larsen contributed to study design, execution, and data analysis. G. Podico, S. Mosage, B. Childs, K Herzog, and K Garrett contributed to study execution. M. Kersh contributed to study design. F. Lima contributed to study design and preparation of manuscript.

TABLES AND FIGURES

Table 6.1. Primers used for qPCR reactions.

Gene	BP	S/AA	Primer
EF1a [32]	328	S	CCCGGACACAGAGACTTCAT
		A	AGCATGTTGTCACCATTCCA
Coll II [33]		S	TTCTGGAGACCAAGGTGCTT
		A	CATTCAGGGTGGCAGAGTTT
Aggrecan [32]	202	S	5'GACGCCGAGAGCAGGTGT
		A	5'AAGAAGTTGTCGGGCTGGTT
MMP3 [33]		S	5' GTTGGACAGATCTGCAAGGG
		A	5' ATGTAAGTGGAGTCACCTCC
MMP9		S	5’ GACATCGTCATCCAGTTTGG
		A	5' GTAGAGTCTCTCGCTGGGGC
TNFa		S	5’ AGCCCATGTTGTAGCAAACC
		A	5’ GAGACAGCTAAGCGGCTGAT
MMP13		S	5’ GGTGCAAGCTCATCTGTCAA
		A	5’ GTGGCTCCAGTTGGAATTGT
ADAMTS4		S	5' ATGGCTATGGGCACTGTCTC
		A	5' ATTGAGATGGCCAGAACACC
MMP2		S	5' TACGATGACGACCGAAAGTG
		A	5' CCCGTAGAGCTCTTGAATGC
Coll X [32]	244	S	5' TGCCAACCCAGGGTGTAACAG
		A	5' ACATTACTGGGGTGCCGTTC
Sox9 [32]	304	S	5' GAACGCACATCAAGACGGAG
		A	5' CTGGTGGTCTGTGTAGTCGT
ALP [32]	260	S	5' CCACGTCTTCACATTTGGTG
		A	5' AGACTGCGCCTGGTAGTT

Table 6.2. Foal signalment and radiographic findings at 30 days of age. Foals were from mares that were either untreated (control, n=5), or treated with 7.5 mg/kg enrofloxacin orally once a day for 14 days (7.5 mg/kg, n=5), or 15 mg/kg enrofloxacin orally once a day for 14 days (15 mg/kg, n=6) starting at 280 days gestation. Positive control foals (n=2) were not treated during pregnancy, but were treated with 10 mg/kg enrofloxacin PO once day for either 7 days beginning at 14 days gestation, or for 10 days starting at 21 days gestation. OC= Osteochondral

Mare signalment	Group	Sex	Lameness	Radiographic findings
Grade mare, 15 yr	15 mg/kg	colt	normal	None
Quarter Horse, 17 yr	15 mg/kg	colt	normal	None
Standardbred, 14 yr	15 mg/kg	filly	normal	OC defect distal intermediate ridge tibia
Standardbred, 21 yr	15 mg/kg	filly	normal	None
Thoroughbred, 12yr	15 mg/kg	filly	normal	L stifle: OC defect proximal margin medial trochlea L tarsus: OC defect medial trochlear ridge R tarsus: Heterogenous trabecular bone & punctate lucenies medial trochlea
Grade mare, 14 yr	15 mg/kg	colt	normal	None
Morgan/Arab, 7 yr	7.5mg/kg	colt	normal	None
Standardbred, 14yr	7.5mg/kg	colt	normal	L Stifle: OC defect proximal margin medial trochlea
Standardbred, 11 yr	7.5mg/kg	colt	normal	OC lesion medial trochlear ridge left tarsus
Standardbred, 20 yr	7.5mg/kg	filly	Bilateral carpal flexion, stillborn foal	N/A
Tennessee Walker	7.5mg/kg	colt	normal	None
Standardbred, 20 yr	7.5mg/kg	filly	Dystocia; orphan foal, windswept, swollen LF fetlock resolved bandaging & Naxcel. Bucket fed 10 days; nurse mare for 25 days.	R tarsus: OC medial malleolus L tarsus: OC lesion distal margin lateral trochlear ridge L & R carpi: thin lucent line distal metaphysis of radius
Grade mare, 14 yr	Control	filly	normal	None
Quarter Horse, 8 yr	Control	filly	Left hock effusion at 3 days old, treated with flunixin meglumine (1.1 mg/kg IV SID x 3 days)	LH tarsus- moderate intracapsular effusion osseous fragment medial malleollus distal tibia extracapsular swelling plantarolateral fourth tarsal bone
Standardbred, 12 yr	Control	filly	Normal	None
Standardbred, 15 yr	Control	filly	Normal	None
Standardbred, 23 yr	Control	filly	extreme flexor tendon laxity for first 10 days life	RH tarsus - irregular subchondral bone at the calcaneus R&L elbow- irregular subchondral bone of the proximal radial epiphysis medially
Thoroughbred, 14yr	Positive control	filly	Normal foaling; mildly lame & bilateral hock effusion with enrofloxacin treatment	
Standardbred, 16yr	Positive Control	colt	Normal foaling; foal severely lame with joint effusion in all fetlocks, carpi, hocks, stifle, and elbows after enrofloxacin administration	R&L tarsus: severe intracapsular effusion R& L elbow, stifle, fetlocks, carpus moderate intracapsular effusion, no osseous defects

Table 6.3. Foal cartilage and joint scores based on structural MRI. Scoring system was adopted from Smith *et al* 2016 [157]. Scores are reported as median (minimum, maximum) Foals were from mares that were either untreated (control, n=5), or treated with 7.5 mg/kg enrofloxacin orally once a day for 14 days (7.5 mg/kg, n=5), or 15 mg/kg enrofloxacin orally once a day for 14 days (15 mg/kg, n=6) starting at 280 days gestation. Positive control foals (n=2) were not treated during pregnancy, but were treated with 10 mg/kg enrofloxacin PO once day for either 7 days beginning at 14 days gestation, or for 10 days starting at 21 days gestation. * Denotes significantly different from the other groups, $p < 0.05$.

	Control	7.5 mg/kg	15 mg/kg	Positive
Cartilage signal (0-3)	0 (0-2)	0 (0-1)	0 (0-1)	2 (0-3)*
Bone Lesions (0-3)	0 (0-1)	0 (0-1)	0 (0-1)	1 (0-2)
Subchondral bone (0-3)	0 (0-0)	0 (0-0)	0 (0-2)	1 (0-1)
Synovium (0-3)	0 (0-1)	0 (0-0)	0 (0-0)	2 (0-3) *
Joint effusion (0-3)	0 (0-2)	0 (0-1)	0 (0-1)	2 (1-3) *
Osteochondral lesion (0-3)	0 (0-1)	1 (0-1)	0 (0-2)	0 (0-2)
Ligaments (0-1)	0 (0-0)	0 (0-0)	0 (0-0)	1 (0-1)

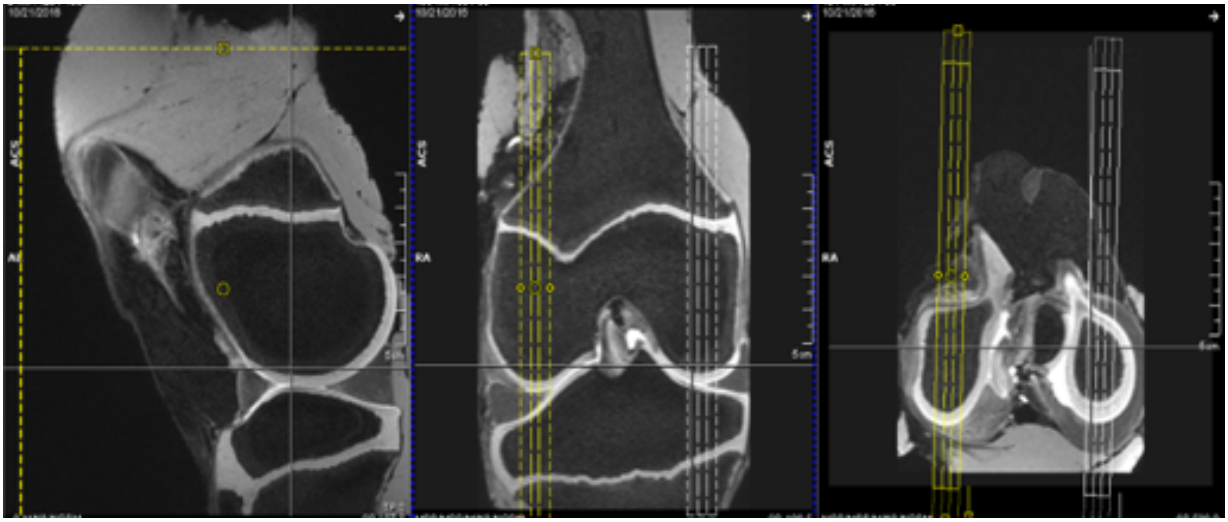

Figure 6.1. Structural (DESS) image of the femoro-tibial joint for placement of two slice groups (yellow lines) of spin-echo sequences in the center of each condyle in sagittal orientation for T2 spin-echo sequences. Because the scapula-humeral joint does not include two condyles, one slice group consisting of 5 slices was placed in the center of the scapula-humeral joint.

.

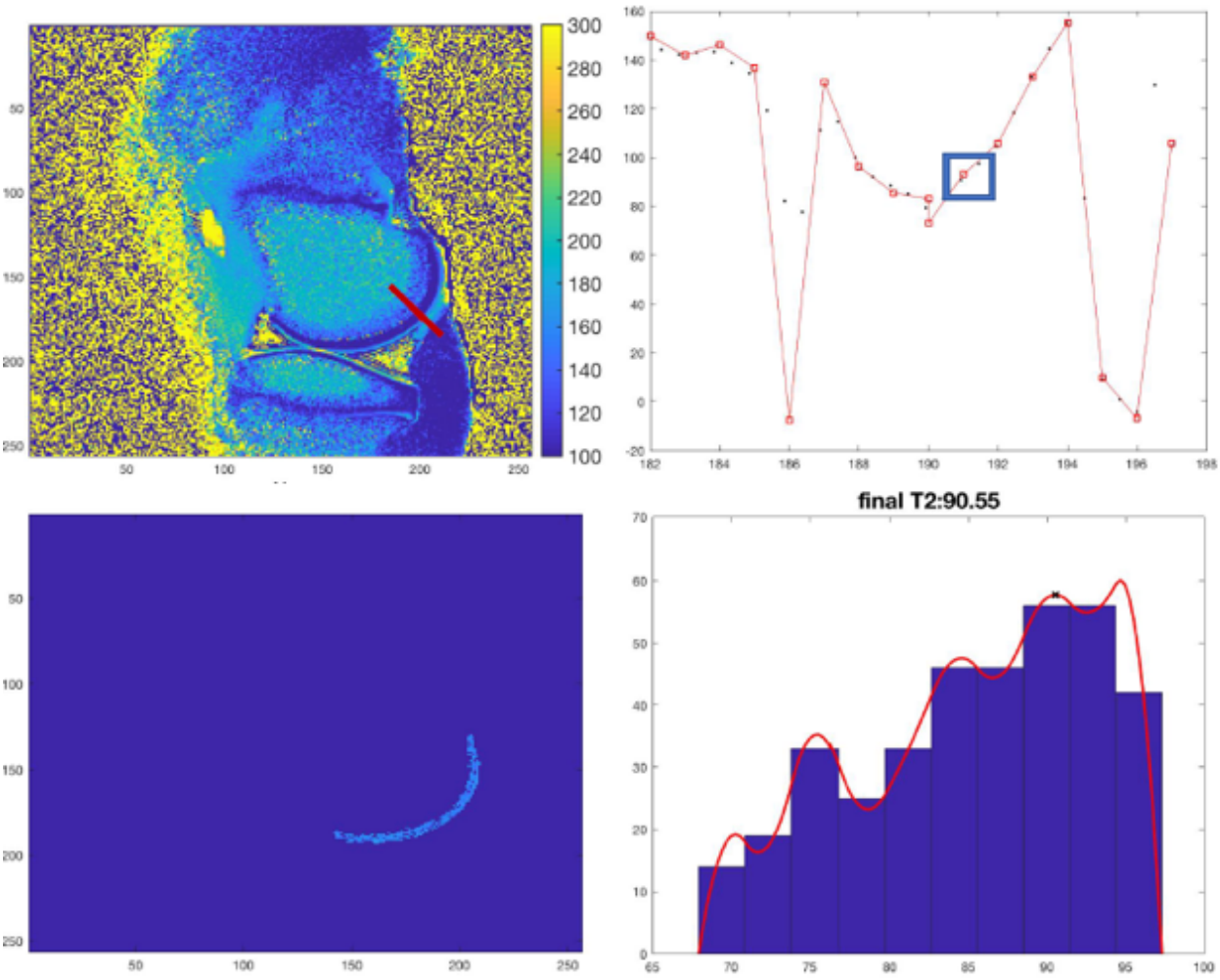

Figure 6.2**.** Median T2 relaxation time calculation for a foal stifle (clockwise from upper left). Quantitative map of T_2 values with the region of interest highlighted with a red line. T_2 values in the cartilage are approximately 90 ms, and in the synovial fluid are approximately 180 ms. (Upper right) T2 relaxation times across the highlighted region of interest. Seed T2 time is highlighted by the blue square. The corresponding segment of cartilage (lower left), and a histogram of T2 relaxation times within the region of interest (lower right).

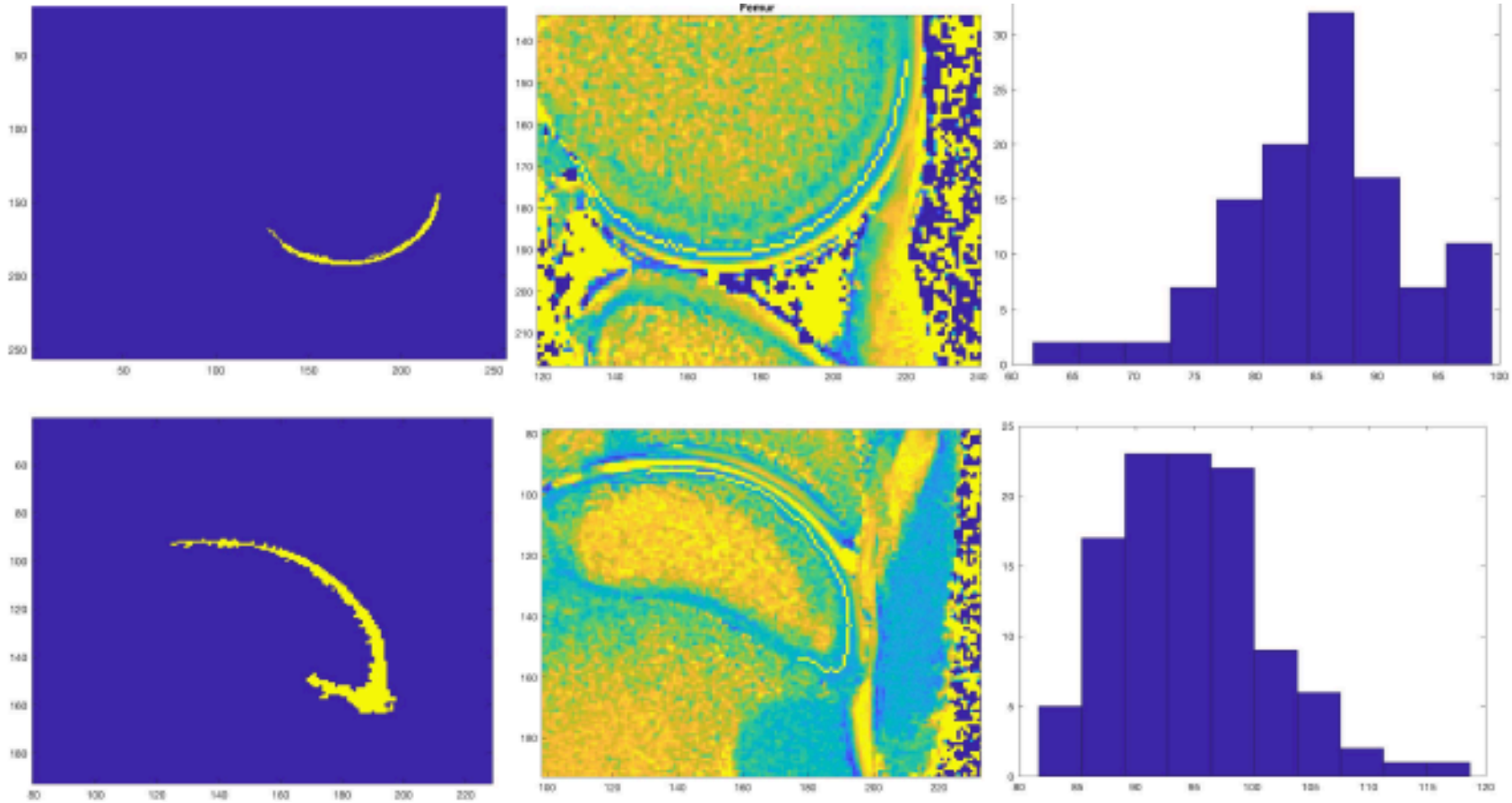

Figure 6.3. Median T2 relaxation time calculation for a foal distal femur (top row) and foal proximal humerus. The seed time selected in figure 6.2 was used to generate the cartilage maps on the left. The corresponding line drawn across the cartilage is shown in the center, and the histogram of cartilage T2 values shown on the right.

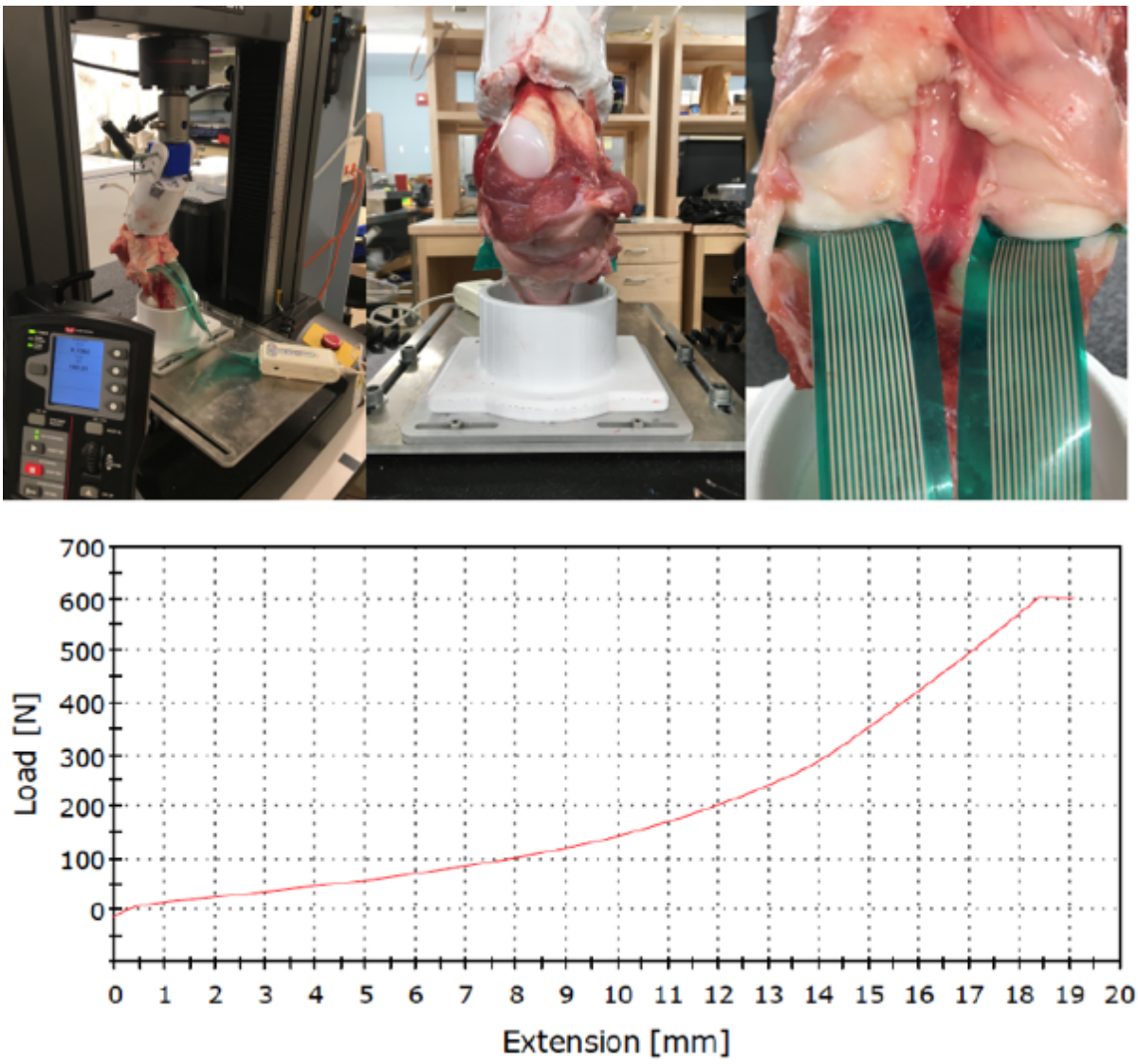

Figure 6.4. Stifle cartilage stress-relaxation testing. Top images are of the stifle of a 33-day old foal, loaded into the custom designed pot and mounted in the Instron machine. The Tek-scan placement is shown in the upper right image, and the resulting load-extension curve is depicted below.

Figure 6.5. Structural MRI of the tibiotarsal joints (A-D) and femorotibial joints (E-H) of 35 ± 3 day old foals. Limbs were imaged immediately after euthanasia. Foal A&E was never treated with fluoroquinolones, Foals B,C,F&G were born to mares treated with fluoroquinolones for 14 days at 300 days gestation (B& F, 7.5 mg/kg PO and C&G, 15 mg/kg PO). Foal D&H was treated with 10 mg/kg enrofloxacin orally for 7 days at 14 days of age. The red rectangle highlights the severe joint effusion and pericapsular thickening seen in the tibiotarsal joint and scapulohumeral joint of this foal.

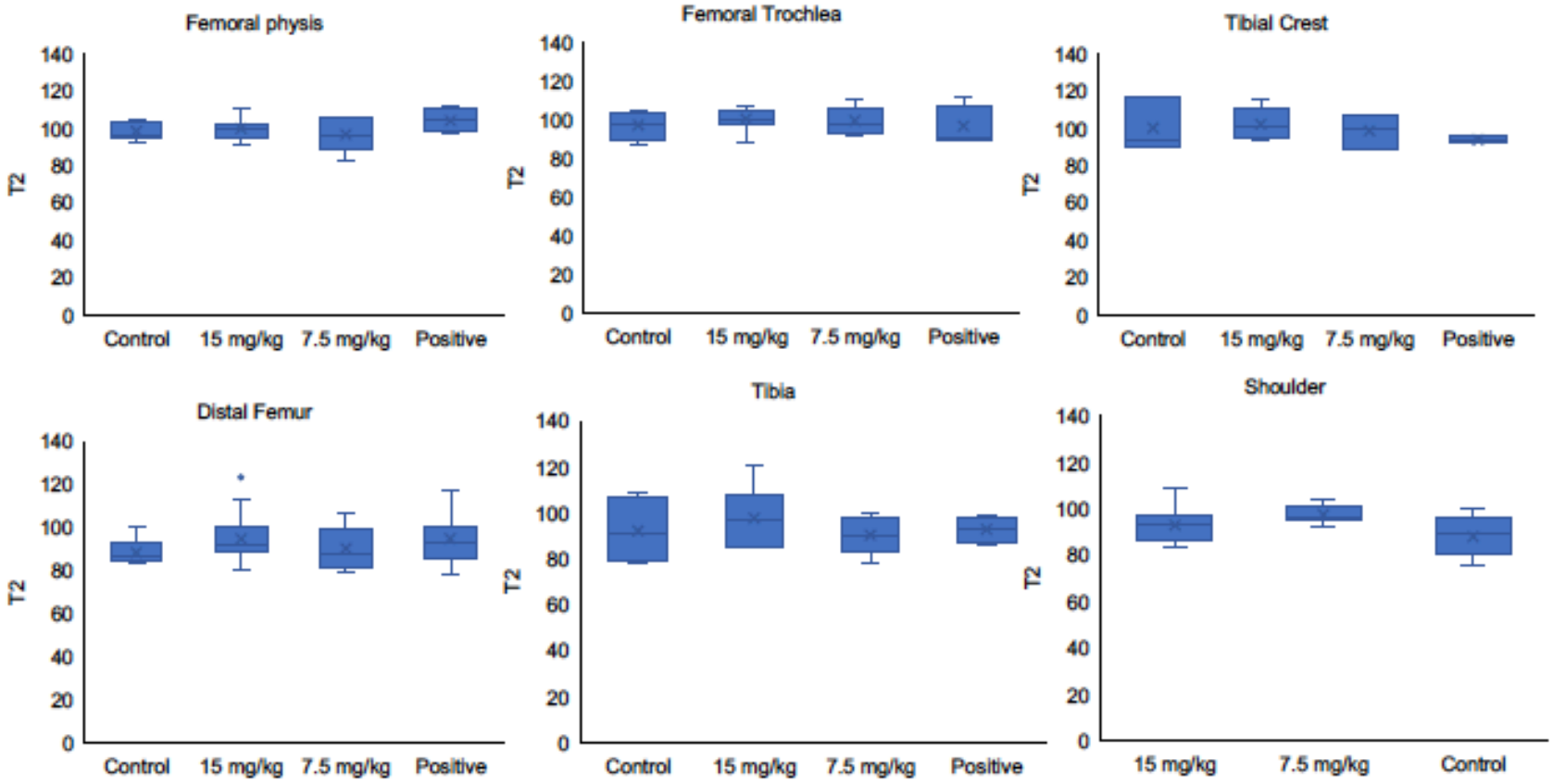

Figure 6.6. Mean T2 relaxation times for articular cartilage of the femur, tibia, shoulder, and femoral physis of 35 ± 3 day old foals. Foals were born to mares treated with either 7.5 mg/kg oral enrofloxacin (n=5) or 15 mg/kg oral enrofloxacin (n=6) for 14 days during the third trimester, from foals never exposed to enrofloxacin (Control, n=4), or from foals treated with 10 mg/kg oral enrofloxacin for 7-10 days at 14 days of age (Positive, n=2). Mean relaxation times were calculated in from in five joint locations using MatLab.

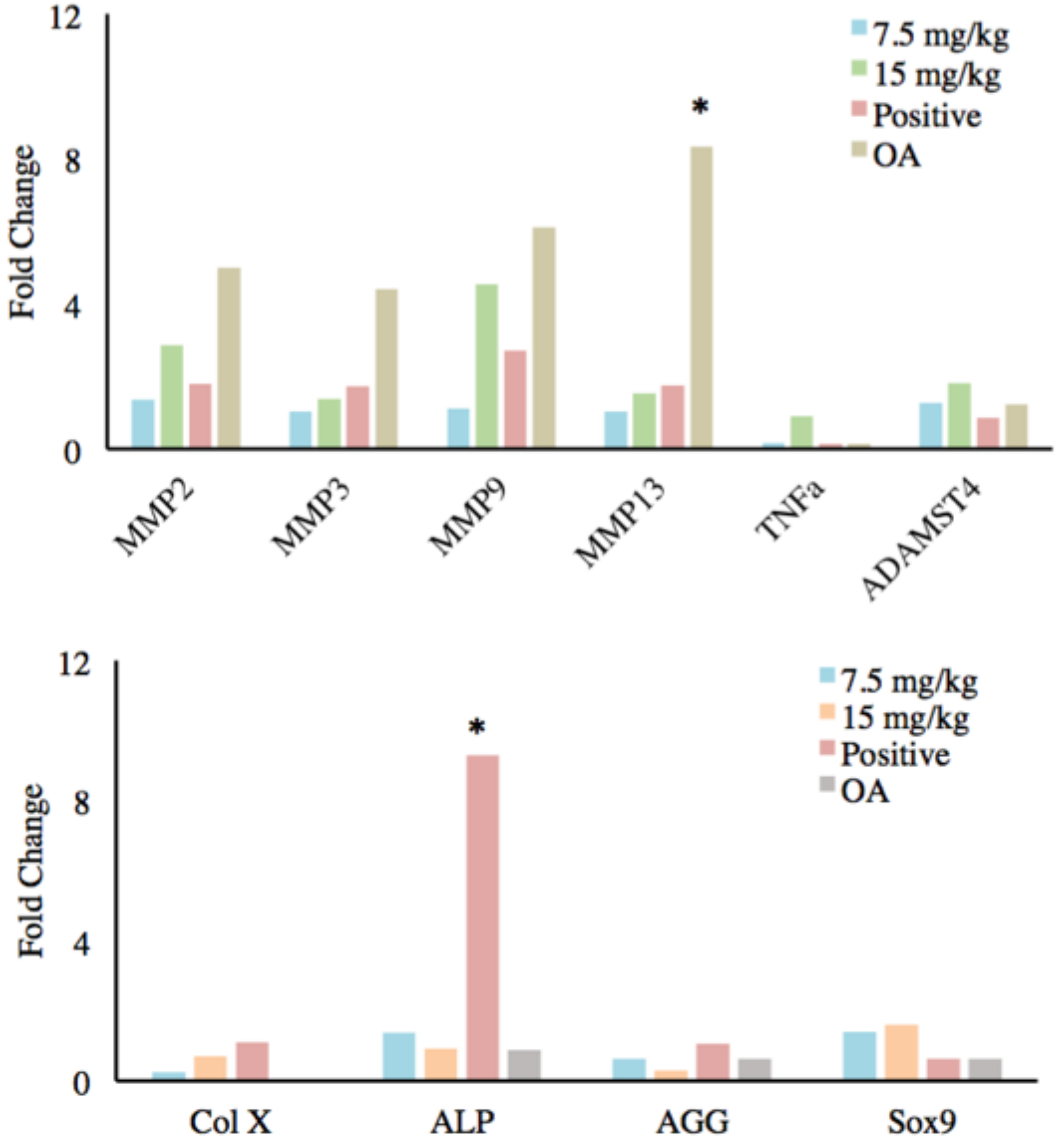

Figure 6.7**.** Relative mRNA expression in articular cartilage of the acetabulum of foals from mares treated with either 7.5 mg/kg oral enrofloxacin (n=5) or 15 mg/kg oral enrofloxacin (n=6) for 14 days during the third trimester, or from foals treated with 10 mg/kg oral enrofloxacin for 7-10 days at 14 days of age (Positive) compared to foals never exposed to enrofloxacin (Control, n=4). OA is a positive control for mRNA expression from the synovium of a mature horse with osteoarthritis. Error bars indicate standard deviation, and * denote significant differences in fold gene expression between treatment groups for a gene.

REFERENCES

1. LeBlanc, M.M. (2009) The current status of antibiotic use in equine reproduction. *Equine Vet. Educ.* **21**, 156–167.
2. Stahlmann, R., Kuhner, S., Shakibaei, M., Schwabe, R., Flores, J., Evander, S.A. and Sickle, D.C. van (2000) Chondrotoxicity of ciprofloxacin in immature beagle dogs: immunohistochemistry, electron microscopy and drug plasma concentrations. *Arch Toxicol* **73**, 564–572.
3. Maślanka, T. and Jaroszewski, J.J. (2009) Effect of long-term treatment with therapeutic doses of enrofloxacin on chicken articular cartilage. *Pol. J. Vet. Sci.* **12**, 21–33.
4. Sendzik, J., Lode, H. and Stahlmann, R. (2009) Quinolone-induced arthropathy: an update focusing on new mechanistic and clinical data. *Int. J. Antimicrob. Agents* **33**, 194–200.
5. Egerbacher, M., Edinger, J. and Tschulenk, M. (2001) Effects of enrofloxacin and ciprofloxacin hydrochloride on canine and equine chondrocytes in culture. *Am. J. Vet. Res.* **62**, 704–708.
6. Egerbacher, M., Wolfesberger, B., Walter, I. and Seirberl, G. (1999) Integrins mediate the effects of quinolones and magnesium deficiency on cultured rat chondrocytes. *Eur J Cell Biol* **78**, 391–397.
7. Davenport, C.L.M., Boston, R.C. and Richardson, D.W. (2001) Effects of enrofloxacin and magnesium deficiency on matrix metabolism in equine articular cartilage. *Am. J. Vet. Res.* **62**, 160–166.
8. Lim, S., Hossain, M.A., Park, J., Choi, S.H. and Kim, G. (2008) The effects of enrofloxacin on canine tendon cells and chondrocytes proliferation in vitro. *Vet. Res. Commun.* **32**, 243–253.
9. Khazaeil, K., Mazaheri, Y., Hashemitabar, M., Najafzadeh, H., Morovvati, H. and Ghadrdan, A.R. (2012) Enrofloxacin effect on histomorphologic and histomorphometric structure of lamb articular cartilage. *Glob. Vet.* **9**, 447–453.
10. Vivrette, S., Bostian, A., Bermingham, E. and Papich, M. (2001) Quinolone-induced arthropathy in neonatal foals. *AAEP Proc.* **47**, 376–377. publication_detail.
11. Ellerbrock, R.E., Canisso, I.F., Roady, P.J., Rothrock, L.T., Zhong, L., Wilkins, P., Dirikolu, L., Lima, F.S. and Honoroto, J. (2018) Diffusion of enrofloxacin to pregnancy fluids and effects on fetal cartilage after intravenous administration to late pregnant mares. *Equine Vet. J.* . https://doi.org/10.1111/evj.13044.
12. Mccoy, A.M., Toth, F., Dolvik, N.I., Ekman, S., Ellermann, J., Olstad, K., Ytrehus, B. and Carlson, C.S. (2013) Articular osteochondrosis : a comparison of naturally-occurring human and animal disease. *Osteoarthr. Cartil.* **21**, 1638–1647. http://dx.doi.org/10.1016/j.joca.2013.08.011.
13. Semevolos, S.A. (2017) Osteochondritis dissecans development. *Vet. Clin. NA Equine Pract.* **33**, 367–378. http://dx.doi.org/10.1016/j.cveq.2017.03.009.
14. Muzi, F., Gravante, G., Tati, E. and Tati, G. (2007) Fluoroquinolones-Induced Tendinitis and Tendon Rupture in Kidney Transplant Recipients: 2 Cases and a Review of the Literature. *Transplant. Proc.* **39**, 1673–1675.
15. Khaliq, Y. and Zhanel, G.G. (2005) Musculoskeletal injury associated with fluoroquinolone antibiotics. *Clin. Plast. Surg.* **32**, 495–502.
16. Martel, G., Couture, C.A., Gilbert, G., Bancelin, S., Richard, H., Moser, T., Kiss, S., Légaré, F. and Laverty, S. (2016) Femoral epiphyseal cartilage matrix changes at

predilection sites of equine osteochondrosis: Quantitative MRI, second-harmonic microscopy, and histological findings. *J. Orthop. Res.* **34**, 1743–1752.
17. Martel, G., Kiss, S., Gilbert, G. and Anne-archard, N. (2016) Differences in the vascular tree of the femoral trochlear growth cartilage at osteochondrosis-susceptible sites in foals revealed by SWI 3T MRI. *J. Orthop. Res.* 1539–1546.
18. Cluzel, C., Blond, L., Fontaine, P., Olive, J. and Laverty, S. (2013) Foetal and postnatal equine articular cartilage development: Magnetic resonance imaging and polarised light microscopy. *Eur. Cells Mater.* **26**, 33–48.
19. Morgan, P., Spiridonov, S., Goebel, R., Nissi, M., Frei, R. and Ellermann, J. (2014) MR Imaging with T2 * - mapping for improved acetabular cartilage assessment in FAI – a case report with arthroscopic correlation. *Orthop. Traumatol. Surg. Res.* **100**, 971–973. http://dx.doi.org/10.1016/j.otsr.2014.09.014.
20. Nissi, M., Rieppo, J., Toyras, J., Laasanen, M., Kiviranta, I., Jurvelin, J. and Nieminen, M. (2006) T2 relaxation time mapping reveals age- and species-related diversity of collagen network architecture in articular cartilage. *Osteoarthr. Cartil.* **14**, 1265–1271.
21. Toth, F., Nissi, M.J., Wang, L., Ellermann, J.M. and Carlson, C.S. (2015) Surgical induction, histological evaluation, and MRI identification of cartilage necrosis in the distal femur in goats to model early lesions of osteochondrosis. *Osteoarthr. Cartil.* **23**, 300–307.
22. Endo, J., Watanabe, A., Sasho, T., Yamaguchi, S., Saito, M., Akagi, R., Muramatsu, Y., Mukoyama, S., Katsuragi, J., Akatsu, Y., Fukawa, T., Okubo, T., Osone, F. and Takahashi, K. (2015) Utility of T2 mapping and dGEMRIC for evaluation of cartilage repair after allograft chondrocyte implantation in a rabbit model. *Osteoarthr. Cartil.* **23**, 280–288.
23. Curcio, B., Giguère, S., Li, Z., Honorato, J., Ellerbrock, R.E., Lima, F.S. and Canisso, I.F. (2017) Pharmacokinetics of enrofloxacin and its active metabolite (ciprofloxacin) in late-term pregnant and post-parturient mares. *Clin. Theriogenology* **9**, 466.
24. Ellerbrock, R.E., Canisso, I.F., Roady, P., Curcio, B., Lima, F.S. and Li, Z. (2018) Enrofloxacin and its active metabolite ciprofloxacin cross the equine placenta and did not cause lesions in the articular surface of the fetuses and foals. *J. Equine Vet. Sci.* **66**, 232.
25. Smith, A., Morton, A., Winter, M., Colahan, P., Ghivizzani, S., Brown, M., Hernandez, J. and Nickerson, D. (2016) Magnetic resonance image scoring of an experimental model of post-traumatic osteoarthritis in the equine cartilage. *Vet. Radiol. Ultrasound* **57**, 502–514.
26. Stewart, M.C., Saunders, K.M., Burton-Wurster, N. and Macleod, J.N. (2000) Phenotypic stability of articular chondrocytes in vitro : the effects of culture models, bone morphogenic protein 2, and serum supplementation. *J. Bone Miner. Res.* **15**, 166–174.
27. Livak, K.J. and Schmittgen, T.D. (2001) Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT Method. *Methods* **25**, 402–408. http://www.sciencedirect.com/science/article/pii/S1046202301912629.
28. Davenport, C., Boston, R. and Richardson, D. (2001) Effects of enrofloxacin and magnesium deficiency of matrix metabolism in equine articular cartilage. *Am. J. Vet. Res.* **62**, 160–166.
29. Beluche, L.A., Bertone, A.L., Anderson, D.E., Kohn, C.W. and Weisbrode, S.E. (1999) In vitro dose-dependent effects of enrofloxacin on equine articular cartilage. *Am. J. Vet. Res.* **60**, 577–582.
30. Giguere, S., Sweeney, R.W. and Belanger, M. (1996) Pharmacokinetics of enrofloxacin in adult horses and and concentration of the drug in serum, body fluids, and endometrial

tissues after repeated intragastrically administered doses. *Am. J. Vet. Res.* **57**.
31. Bertone, A.L., Tremaine, W.H., Macoris, D.G., Simmons, E.J., Ewert, K.M., Herr, L.G. and Weisbrode, S.E. (2000) Effect of long-term administration of an injectable enrofloxacin solution on physical and musculoskeletal variables in adult horses. *J. Am. Vet. Med. Assoc.* **217**, 1514–21.
32. Chen, Y., Bianchessi, M., Pondenis, H. and Stewart, M. (2016) Phenotypic characterization of equine synovial fluid- derived chondroprogenitor cells. **3**, 1–10.
33. Carlson, E., Stewart, A., Carlson, K., Durgam, S. and Pondenis, H. (2013) Effects of serum and autologous conditioned serum on equine articular chondrocytes treated with interleukin-1 β. *Am. J. Vet. Res.* **74**, 700–705.

Supplemental material 6.1: MatLab code for generation of T2 relaxation maps, and for analysis of median cartilage relaxation times. gq is the slice number and is adjusted for the desired slice of the desired condyle for analysis.

```
%first view the dicom using the command: dicomread.  Use the pointer tool
%to find the center of the vial of interest.

samp = 'A';
vial_num = [1 2 3 4 5 6];
myxs = [167 145 109 167 145 109];
myys = [72 230 139 167 145 109];

samp_mark = 'ks-';

npts = 256;
crad = 1; %this is the radius around center point for making a mask. The mask
tells which voxels to average.

for gq = 2:2

    ycor = myys(gq);
    xcor = myxs(gq);

    %set up the meshgrid:
    %the dist matrix tells you the distance between each voxel and the center
    %voxel that you previously chose.
    xvect = [1:npts];
    [xx yy] = meshgrid(xvect,xvect);
    dist = sqrt((xx - xcor).^2 + (yy - ycor).^2);

    masterdir =
'/Users/ree44767/Desktop/Original_Data/Foal_15_A2/scans/4.stifel/DICOM';
    %TEs = [12 50 70 90 110 130 150 200 250 280 300];
    cd(masterdir)
    TEs = [14 28 42 56 70 84 98 112 126 140 154 168 182 196 210 224 238 252
266 280 294 308 322 336 350 364 378 392 406 420 434 448];
    fmd = dir(strcat('IM*'));
    datl = zeros(size(TEs,2),npts,npts);
    for pt = 1:size(TEs,2) %loop through files
        pt2 = (pt-1)*6+gq;
```

```
        dat = dicomread(fmd(pt2).name);
        d2 = dicominfo(fmd(pt2).name);
        TEs_new(pt) = d2.EchoTime
        figure(1)
        imshow(dat)
        imagesc(dat)
        %sig(pt) = dat(ycor,xcor);

        mask = zeros(npts,npts);
        mask(find(dist<crad)) = 1; %define the mask

        maski = ones(npts,npts);
        maski(find(dist<crad)) = 0; %define the mask
        %                 db = double(d2.RescaleIntercept);
        %          dm = d2.RescaleSlope;
        dat2 = double(dat);
        %           dat2 = dat2.*dm + db;
        datl(pt,:,:) = dat2;

        modi = dat2.*mask;
        figure(22)
        imagesc(dat2.*maski)
        %pause(0.5)
        [r, p, v] = find(modi);
        sig(pt) = mean(v);  %average the intensity of all the points in the
mask.
        pt
    end
    datl = datl(3:23,:,:);
    sig = sig(3:23);
    TEs = TEs(3:23);
    figure(2)
    semilogy(TEs,sig,'ks','linewidth',2,'markersize',8)
    set(gca,'YMinorTick','on')
    set(gca,'XMinorTick','on')
    hold on;
    for i1 = 1:npts
        i1
        for i2 = 1:npts
            yfit = squeeze(datl(1:size(TEs,2),i1,i2))';
            [r, c,v] = find(yfit==0);
            if size(c,2) ~= 0
                yfit = yfit(1:c(1)-1);
                xfit = TEs(1:c(1)-1);
            else
                xfit = TEs;
            end
            yfit = log(yfit);
%             if (i1 == 193) && (i2 == 151)
%                 asdf=5
%             end
            [ps] = polyfit(xfit,yfit,1);
            T2m(i1,i2) = 1/ps(1);
        end
    end
    % %1/ps(1) is T2, which is 2333 ms.
    %ylim([800 1200])
```

```
    T2_measurement = 1/ps(1)

    TEsa = [0:.01:400];
    Ssa = exp(ps(1)*TEsa + ps(2));
    plot(TEsa,Ssa,'k-','linewidth',2)

    set(gca,'TickLength',[0.03 0.03])
    set(gca,'Fontsize',26','linewidth',3)
    xlabel('TE (ms)','fontsize',26)
    ylabel('Signal a.u','fontsize',26)
    xlim([0 500])

    T1s_meas(gq) = -T2_measurement;

cd(strcat('/Users/ree44767/Desktop/Original_Data/Foal_15_A2/scans/4.stifel/DI
COM'));
    print('-f22', '-
dtiff',strcat('vial_pick_',samp,'_',num2str(vial_num(gq)),'.tif'));
    print('-f2', '-
dtiff',strcat('T2_',samp,'_',num2str(vial_num(gq)),'.tif'));

    close all
end

T2m(isnan(T2m)) = 0;
T2m = -T2m;
figure(33)
imagesc(T2m,[100 300])

set(gca,'xticklabel',{' '});
set(gca,'yticklabel',{' '});
colorbar('fontsize',16,'linewidth',1)
%
% T1_A = T1s_meas(1:7);
% figure(55)
% plot(T1_A,samp_mark,'linewidth',2)
% hold on;

axis equal

save(fullfile('/Users/ree44767/Desktop/Original_Data/scripts','T2m'),'T2m')

%%%%%%now the image processing file %%%%%%
close all
clear

%%%%%%%save(fullfile('T:\Ryan_Larsen\Foal\Original_Data\scripts','T2m'),'T2m'
)
load(fullfile('/Users/ree44767/Desktop/Original_Data/scripts','T2m'),'T2m')
T2m(find(T2m == -Inf)) = 0;

%load(fullfile('T:\Ryan_Larsen\Foal\Original_Data\scripts','dat154'),'dat154'
)
%T2m = double(dat154);
```

```
figure(33)
imagesc(T2m,[100 300])
%imagesc(T2m)

xlabel('X','fontsize',16)
ylabel('Y','fontsize',16)
% set(gca,'xticklabel',{' '});
% set(gca,'yticklabel',{' '});
colorbar('fontsize',16,'linewidth',1)
hold on;

%%%% make_line_trace %%%%%%%
figure(34)
close(34)
figure(33)

[x,y] =ginput(2)

%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
%use interpolation:
lleng = sqrt(range(x)^2+range(y)^2);
Npts = round(lleng/0.5);

ax = linspace(x(1),x(2),Npts);
ay = linspace(y(1),y(2),Npts);

plot(ax,ay,'r-','linewidth',3)

[X,Y] = meshgrid(1:256);

T2q = interp2(X,Y,T2m,ax,ay);
T2q(isinf(T2q)) = 1E4;
T2q(isoutlier(T2q)) = median(T2q);

if range(ay) > range (ax)
    mycoor = ay;
    mycoor_vox = round(ay);
    myc_label = 'Y';
elseif range(ay) <= range (ax)
    mycoor = ax;
    mycoor_vox = round(ax);
    myc_label = 'X';
end
figure(34)
plot(mycoor,T2q,'k.')
hold on;
xlabel(myc_label,'fontsize',16)
%%%%%%%%%%%%%%%%%%
%work at single voxel level:

rax = round(ax);
ray = round(ay);

clear bm
for ii = 1:Npts
    bm(ii) = T2m(rax(ii),ray(ii))
```

```
end
 T2vox = interp2(X,Y,T2m,rax,ray);
T2vox(isinf(T2vox)) = 1E4;
T2vox(isoutlier(T2vox)) = median(T2vox);

plot(mycoor_vox,T2vox,'rs-')
% hold on
% plot(mycoor_vox,bm,'bd-')

[mycor_seed,yseed] = ginput(1)

if range(ay) > range (ax)
    yseed = round(mycor_seed);
    xseed = rax(find(ray==yseed));
elseif range(ay) <= range (ax)
    xseed = round(mycor_seed);
    yseed = ray(find(rax==xseed));
end

if length(xseed)~=1
    xseed = xseed(1);
end
if length(yseed)~=1
    yseed = yseed(1);
end
disp(strcat('Seed T2: ',num2str(T2m(yseed,xseed))))

seedT2 = T2m(yseed,xseed);

%%%%%% now the make region file %%%%%%

figure(33)
 bw = grayconnected(T2m,yseed,xseed,15);

figure(35)
imagesc(T2m.*bw,[20 250])

allT2s = T2m(find(T2m.*bw))

figure(38)
close(38)
figure(38)

hist(allT2s)
hold on;
%smooth the histogram:

[heights,centers] = hist(allT2s);
% hold on
% ax = gca;
% ax.XTickLabel = [];
n = length(centers);
w = centers(2)-centers(1);
t = linspace(centers(1)-w/2,centers(end)+w/2,n+1);
p = fix(n/2);
% fill(t([p p p+1 p+1]),[0 heights([p p]),0],'w')
```

```
% plot(centers([p p]),[0 heights(p)],'r:')
% h = text(centers(p)-.2,heights(p)/2,'   h');
% dep = -70;
% tL = text(t(p),dep,'L');
% tR = text(t(p+1),dep,'R');
%hold off

dt = diff(t);
Fvals = cumsum([0,heights.*dt]);

F = spline(t, [0, Fvals, 0]);

DF = fnder(F);  % computes its first derivative
% h.String = 'h(i)';
% tL.String = 't(i)';
% tR.String = 't(i+1)';
hold on
fnplt(DF, 'r', 2)
hold off
ylims = ylim;
ylim([0,ylims(2)]);

%now find the peak:

t2a = median(allT2s)+[-iqr(allT2s)/2:0.01:iqr(allT2s/2)]

%t2a = min(ab):0.01:max(ab)
bs = fnval(DF,t2a);

[Y ind] =max(bs);
averaget2 = t2a(ind)

hold on;
plot(averaget2,Y,'kx','markerfacecolor','k','linewidth',2)

seedT2
title(strcat('final T2: ',num2str(round(averaget2,2))),'fontsize',18)
```

CHAPTER 7: DIFFUSION OF FLUOROQUINOLONES TO THE FETAL FLUIDS DID NOT RESULT IN LESIONS IN THE FIRST TRIMESTER FETUS

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ABSTRACT

Enrofloxacin may be an alternative antibacterial agent to treat severe infections in pregnant mares. While enrofloxacin and ciprofloxacin diffuse to the fetoplacental unit without causing obvious lesions in the 9-mo fetus or resulting foal, the effects of fluoroquinolone exposure in the first trimester are unknown. Since four to eight weeks of gestation is a critical time for skeletal development in the horse, it is possible that fluoroquinolones are more chondrotoxic to the fetus in early gestation. The objectives of the following study were to assess effects of 2-wks-exposure to enrofloxacin on the equine fetus between 6-8 wks gestation. Healthy mares at 46d gestation were allocated into two groups: untreated (n=7), or treatment (7.5 mg/kg enrofloxacin, PO x 14 days, n=6). Prostaglandin was used to induce abortion 24 hours after the last enrofloxacin dose, or on the equivalent day of gestation for untreated mares. Pregnancy fluids from treated mares were

analysed for enrofloxacin and ciprofloxacin concentrations. Fetal organs (heart, lungs, spleen, kidney, and liver) and limbs were examined histopathologically. Enrofloxacin and ciprofloxacin diffused to the fetal fluids during early gestation and did not result in detectable abnormalities in the fetus after 14 days of treatment. Enrolled mares were healthy during treatment, and antibiotic effects may be different in ill animals. It is also possible that histological evaluations were not sensitive enough to detect subtle changes or long-term consequences. Short-term administration of enrofloxacin to early gestation mares did not result in pathologic lesions in the fetus. While further research is needed to assess other stages of gestation and to determine if enrofloxacin administration during early pregnancy potentiates osteochondral changes later in life, enrofloxacin may be useful for select bacterial infections in pregnant mares.
Keywords: fluoroquinolone, ciprofloxacin, pregnancy, horse, fetal toxicity

INTRODUCTION

During pregnancy, antimicrobial therapy is necessary to treat bacterial diseases ranging from pneumonia or septic arthritis to peritonitis and placentitis. Enrofloxacin, a fluoroquinolone antimicrobial with documented bactericidal activity against Gram-negative and some Gram-positive equine pathogens [1], would be useful if safe to administer to pregnant mares. Fluoroquinolone antibiotics are thought to be chondrotoxic to the fetus, and this drug class is generally avoided in pregnant mares. Studies conducted *in vitro* and *in vivo* have demonstrated enrofloxacin exposure can cause tendonitis and arthropathies, especially in young, growing animals [2-4]. Quinolone-induced arthropathy is mediated via chelation of divalent cations such as magnesium (Mg^{2+}), which affects fibronectin signalling and integrin function, leading to disturbances in cell adhesion, proliferation, and proteoglycan synthesis, and ultimately changes in cartilage architecture [5-9].

Despite concerns about toxicity, clinicians occasionally select enrofloxacin to treat multi-drug resistant infections when mare survival outweighs potential risks to the fetus. Two recent studies suggest enrofloxacin administration in late pregnancy may not affect the fetus or subsequent foal [10,11]. However, even if no side effects are seen with fluoroquinolone exposure in late pregnancy, it is possible that toxicity would occur in the first trimester, when organogenesis and limb development begin [12,13].

Normal limb bud development begins around 20 days gestation in the horse, and is quickly followed by footpad and elbow joint formation (days 30 to 40). Around this time, the equine fetus first begins intrinsic muscular activity such as head movements [14]. Next the femorotibial joint develops, from initial cavitation at 45 days gestation, to complete joint formation by 65 days [15,16]. Cells present in the interzone of the forming embryonic joint are critical for proper joint formation [17,18], as these cells form the articular cartilage, ligaments associated with the joint, and synovial lining [19,20,21]. Articular chondrocytes descend from progenitor cells present in the interzone, and signals affecting these cells could compromise articular cartilage in the resulting foal. Collectively, 45-60 days of gestation is a critical time-point in equine synovial joint development, making fluoroquinolone exposure potentially harmful during this time period.

The objectives of this study were to determine: (i) if enrofloxacin or its metabolite ciprofloxacin crosses the early equine placenta (ii) if exposure to enrofloxacin and ciprofloxacin early in pregnancy causes developmental changes in the fetal limbs. We hypothesized that enrofloxacin administration during early gestation would affect cartilage development in the fetus.

MATERIALS AND METHODS

The study protocol was approved by the University of Illinois Urbana-Champaign Institutional Animal Care and Use Committee (protocol #16129), and the present study was carried out between November 2017 and August 2018. Nine healthy, light-breed pregnant mares were enrolled in the study at five weeks (45 days) of gestation. Mares were kept at the Veterinary Medicine Research Farm at the University of Illinois in Urbana, Illinois, where they were housed on pasture and supplemented with grass hay and trace minerals.

Animal husbandry and study design

Nine clinically healthy, light-breed multiparous mares were enrolled in the study. Four of nine mares were bred for a second gestation to serve as their own treatment or control (Fig 1). The mares were 13 ± 4.5 yrs-old and were a mix of breeds (two Standardbreds, three Quarter Horses, one Tennessee Walking Horse, one POA, and two mixed breeds). Mares were monitored via transrectal ultrasound three times per week, and when a CL was detected, prostaglandin F2 alpha[b]

[Lutalyse®] (5 mg/mare IM) was administered to return the mare to estrus. When at least a pre-ovulatory follicle (follicles >35mm, and moderate endometrial edema) was detected, the mare was artificially inseminated with fresh semen, containing a minimum of 500 x 10^6 progressively motile sperm from a Quarter Horse stallion housed at the same facility. Mares were scanned every 24 - 48 hours to detect the ovulation. When present, post-breeding intrauterine fluid was treated with oxytocin[c] (20 IU IM q12h) and uterine lavage as needed.

Pregnancy was confirmed at 14 days post ovulation and recheck ultrasound exams were repeated at 28 and 45 days. Mares were included in the present study if ultrasonography of the pregnant uterus was within normal limits at 45 days gestation (singleton pregnancy with normal fetal heart beat, normal fetal fluid echogenicity, and normal cervical tone and appearance). Mares were blocked into groups based on date of pregnancy determination, and then randomly allocated to two groups: recommended dose of enrofloxacin[d] (7.5 mg/kg, PO, n=6), or no treatment (n= 7). Mares were treated orally once a day for fourteen days. The compounded commercial oral formulation of enrofloxacin used in this study has previously been shown to be well absorbed by pregnant mares [22].

Ultrasound measurements

Starting on Day 10 of treatment, B mode ultrasonography [EVO Ibex[e]], fetuses were assessed daily for fetal heartbeat and fetal mobility. Fetal mobility was assessed over a set a period of 5 minutes, with the number of fetal movements recorded for further comparisons between groups and over time. Fetal movements were classified as major (rotation around the axis, or fetal movement in the dorso-ventral or cranio-caudal direction), or minor (head nods, limb flexion or extension), similar to a previous study in older fetuses [23]. Fetal heart rate (FHR) was evaluated at the beginning of the ultrasound and at the end, and the two readings were averaged for one daily FHR.

Induction of Abortion and Fetal Fluid Sampling

At 60 days, abortion was induced as part of a parallel study. Abortion was induced by either one uterine infusion of 500 µg cloprostenol sodium[f] [Estrumate®] diluted in 8ml of saline (n=6), or with an intramuscular injection of 250 µg cloprostenol sodium, q12 h until abortion (n=7). In transcervically treated mares, a standard equine AI pipette[g] was passed through the cervix, and the

solution (sodium chloride-cloprostenol) was placed caudally to the allantochorion membrane [24,25]. Fetal heart rate was monitored every six hours by transrectal ultrasound, and at the first time point when a fetal heart rate was no longer detected, the mare's perineum was aseptically scrubbed, and the fetus and fetal membranes were manually recovered via transvaginal manipulation. After removal, the fetus and membranes were examined, and fluid was aspirated from the allantoic and amniotic cavities with a 22-gauge needle and 3 ml syringe. Fetal fluids were stored at -80°C until analysis. Full chemistry panels were run on all fetal fluids harvested.

Determination of enrofloxacin and ciprofloxacin concentrations

Concentrations of enrofloxacin and ciprofloxacin were measured by liquid chromatography-tandem mass spectrophotometry (LC-MS/MS) in the Metabolomics Laboratory of the Roy J. Carver Biotechnology Center, University of Illinois Urbana-Champaign. A detailed description of LC-MS/MS methods and validation are reported elsewhere [10,22]. Between-run and within-run accuracy and precision were calculated by measuring six samples of enrofloxacin and ciprofloxacin at concentrations of 5, 50, and 500 ng/ml. Measurements were repeated on three different days. Enrofloxacin had a within-run accuracy of 95.6 ± 2.1 % to 99.1 ± 0.6%, and a between-run accuracy of 97.3 ± 2.0% to 98.4 ± 1.0%. Ciprofloxacin had a mean within-run accuracy of 95.4 ± 2.3 to 99.6 % ± 1.2 %, and between-run accuracy of 97.0 ± 2.4 % to 99.2 ± 0.9%. Coefficients of variations between-run and within-run for enrofloxacin ranged from 0.9-1.6% and 1.1-3.5%, respectively. Coefficients of variations for ciprofloxacin between-run and within-run ranged from 0.9- 2.4% and 1.0-5.4 %, respectively. Plasma stability data was calculated under various conditions (n = 6). Samples stored in the auto-sampler for 24 h at 15°C had a bias of 3.2-4.8% and samples stored at -75°C for two months had a bias of 1.3-3.4%.

Fetal Pathology

After the abortion, fetuses were grossly inspected for any defects or lesions (Fig 7.2). Fetal limbs were disarticulated from the axial skeleton, and left front and hind limbs were preserved in 10% neutral buffered formalin immediately after collection. Samples of fetal liver, lung, kidney, and heart were also preserved in formalin for analysis. Fixed tissues were then routinely processed and embedded in paraffin in an automated tissue processor. Fetal limbs were sectioned as a whole limb in the sagittal plane, and three slides were stained with either haematoxylin & eosin, Mason's

Trichrome, or toluidine blue for analysis. All haematoxylin & eosin stained slides were evaluated for structural and cellular changes by two board-certified veterinary pathologists, who analysed the tissues blinded as to treatment groups. One pathologist also evaluated the slides stained with Mason's Trichrome and toluidine blue.

Statistical analysis

G* Power software v 3.1.9.3[h] was used to determine the *a priori* sample size, with an alpha = 0.05, a power of 0.8, and the expectation that 2/3 of the treated fetuses would be affected. All statistical analyses were completed using R libraries (R version 3.3.3)[i]. Associations between fetal chemistry values and treatment group were analyzed by one way ANOVA. Fetal age at abortion, time from treatment to abortion, and average fetal heart rate were compared between groups using a one-way ANOVA. Significance was set at $p < 0.05$, and tendency was defined as $0.1 > p > 0.05$.

RESULTS

Clinical outcomes

All mares had normal physical examinations for the 14-day treatment period, and no side effects were seen from oral administration of enrofloxacin. Mean fetal heart rate before abortion was not different between the untreated (147± 12 bpm) and treated (146 ± 7 bpm) mares. No differences in fetal movements were observed in the five days leading up to abortion; major movements varied from 0 to 2 per 5 minutes, and minor movements varied from 1 to 3 movements during the five minutes' assessment.

One untreated mare showed signs of colic after the first intramuscular prostaglandin administration. The mare was treated with a 10 L bolus of Lactated Ringers' solution[j] and flunixin meglumine[f] [Banamine®] (1.1 mg/kg q12h). Prostaglandin treatment was not repeated, and the fetus and its membranes were extracted via manual dilation of the cervix. Mares aborted at 62 ± 0.5 days of gestation, and there was no difference in gestation length between treated and control mares. The mean time from the first prostaglandin administration to abortion was 37.3 ±10.6 hours, with no difference between treated and control mares.

Fetal fluid analyses

One treated fetus was expelled before loss of fetal heartbeat was detected, and when the fetus was discovered, no allantoic or amniotic fluid was recovered. In two fetuses recovered from the

vagina, only amniotic fluid was obtained. Both enrofloxacin and ciprofloxacin were detectable in the amniotic and allantoic fluids at time of abortion, 24 to 48 hours after the last dose of enrofloxacin (Table 7.1). The ratio of enrofloxacin: ciprofloxacin concentration was 3.6:1 in the amniotic fluid, and 2.1:1 in the allantoic fluid. There were no differences in allantoic fluid chemistries between groups. There was a tendency for bilirubin to be decreased ($p = 0.10$) in the amniotic fluid of treated mares, but no other differences were seen (Table 7.2). One mare in the untreated group had elevated allantoic CPK, AST, and ALK Phos concentrations, resulting in the large standard deviations noted (Table 7.3).

Fetal Pathology

There were no differences noted in the histological features of the front and hind limbs, heart, kidney, lung, liver, or spleen of fetuses in the control and enrofloxacin exposed experimental groups (Fig 7.3). No differences were seen in proteoglycan content of fetal limbs stained with toluidine blue (Fig 7.4), or in collagen or muscle fibers stained with Masson's trichrome (Fig 7.5). The stage of development and ossification of the bones of the limbs was comparable among the groups.

DISCUSSION

The current study hypothesized that *in utero* exposure to enrofloxacin would have chondrotoxic effects on the fetus if exposure occurred at the time of organogenesis or initial cartilage development. However, enrofloxacin administration to early pregnant mares failed to induce detectable lesions after two weeks of *in utero* exposure. These findings are consistent with two previous studies showing that administration of enrofloxacin to late pregnant mares did not result in apparent abnormalities in the fetal limbs [10] or resulting 30 day old foals [11].

Previous studies assessing fluoroquinolone exposure during late equine pregnancy demonstrated that ciprofloxacin accumulates in the allantoic fluid after 11 days of enrofloxacin administration [10], and that low levels of both enrofloxacin and ciprofloxacin were detectable in the allantoic fluid long after last treatment [11]. This accumulation was attributed to excretion in the fetal urine or diffusion into the allantoic fluid. While the present study design only determined fetal fluid fluoroquinolone concentrations 24 to 48 hours after the last dose of enrofloxacin, placental drug transfer or diffusion of fluoroquinolones into the fetal fluids occurred in the early equine pregnancy as well. Fluoroquinolones concentrations were lower in the 60-day fetal fluids

than concentrations reported in fetal fluids of late pregnant mares [11]; these discrepancies may be due to increased blood flow to the fetoplacental unit in the late pregnant mare, increased renal excretion in 3rd trimester fetuses, or differences in fetal fluid composition or pH allowing for drug accumulation. In 60-day fetal fluids, fluoroquinolone concentrations were similar between the allantoic fluid and amniotic fluid, unlike in the late pregnant mare, where ciprofloxacin accumulates in the allantoic fluid over time [10]. This supports the hypothesis that ciprofloxacin accumulates in the allantoic fluid due to fetal urine excretion in late gestation. In the first trimester, fetal kidneys are not fully functional; instead, drug transport is likely due to passive diffusion. The study was conducted in healthy mares, and it is unknown how concurrent disease might affect placental drug transfer or diffusion.

Fetal fluid chemistry parameters were similar to those previously reported [26,27]. Total protein in the amniotic and allantoic fluid was higher than previously reported [26,27], possibly due to method and timing of fetal fluid collection. In the current study, fluids were collected after induction of abortion, likely causing fetal stress and an inflammatory response. In previous studies fluids were collected at time of mare death or slaughter and may not have resulted in similar inflammation. While amniotic bilirubin concentrations tended to be lower in treated fetuses than untreated fetuses, all were within normal limits for the adult equine, suggesting that this difference is not clinically relevant. Allantoic fluid concentrations were more variable in this study for many parameters, and it is hypothesized that this was due to fluid loss, and potential cross contamination as fetal membranes were expelled. No allantoic membranes were recovered intact, and mixing of vaginal secretions and allantoic fluid at the time of conceptus recovery was likely.

One reason no evidence of cartilage toxicity was found in the 60-day old fetuses may be due to the very early stage of articular cartilage development at this time period. Previous studies of quinolone-induced arthropathy have suggested that there is a window of susceptibility in young animals. Rats are susceptible between three to six weeks of age but not at eight weeks, while dogs are susceptible at three months but not at 18 months. Lesions occurred in the weight-bearing joints, which implies mechanical forces may also be critical to lesion development. These findings and others indicate that the stage of cartilage development is an important factor in quinolone toxicity and that the metabolically active chondrocytes facilitating rapid growth are the most susceptible. Therefore, it is likely that the mostly undifferentiated articular chondrocytes of the fetuses in this study were not negatively affected by enrofloxacin. In addition, there did not appear to be any

effects on ossification of the long bone cartilage as it appeared comparable to that in the untreated controls (Fig 7.4).

Collectively, these findings suggest that enrofloxacin administration to the early pregnant mare may not result in gross or histologic lesions in the fetus. Enrofloxacin may be a useful antibiotic for select pregnant mares suffering life-threatening bacterial infections. Enrofloxacin administration should be limited to severe infections where microbial culture indicates microbial resistance to first line antimicrobials and a sensitivity to enrofloxacin. Practitioners should be aware that both enrofloxacin and ciprofloxacin cross the equine placenta, and that while no lesions were detected in the fetus immediately after drug administration, no long-term follow up studies were conducted to investigate survival to term and musculoskeletal soundness after fluoroquinolone exposure in the first trimester. If enrofloxacin is administered to pregnant mares, clients should be informed of the potential risks, as this study does not rule out a low incidence of toxicity, or long-term effects of early fetal exposure. Additional studies are warranted to investigate longer-term effects on foals after intrauterine exposure to fluoroquinolones.

Manufacturers

[a]Bayer HealthCare, Shawnee Mission, Kansas, USA
[b]Zoetis, Parsipanny, NJ, USA.
[c]VetOne, Boise, Idaho, USA
[d]Enrofloxacin Oral Suspension, Rood and Riddle Pharmacy, Lexington, KY
[e]E.I Medical Imaging, Loveland, Colorado, USA
[f]Merck Animal Health, Summit, New Jersey, USA
[g]Nasco, Fort Atkinson, Wisconsin, USA
[h]Universität Düsseldorf, Düsseldorf, Germany
[i]R Foundation for Statistical Computing, Vienna, Austria
[j]Abbott Laboratories, North Chicago, Illinois, USA

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Authorship

R. Ellerbrock contributed to study design, execution, data analysis, interpretation, and preparation of manuscript. I Canisso contributed to study design, execution, data analysis, interpretation, and preparation of manuscript. G. Podico contributed to study design and execution. E. Uhl and P. Roady contributed to study execution.

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TABLES AND FIGURES

Table 7.1. Concentrations of enrofloxacin and ciprofloxacin in the foetal fluids (ng/ml) at time of fetal abortion (62 ± 0.5 days gestation). Mares were treated with 7.5mg/kg enrofloxacin orally once a day for 14 days before abortion. Values are mean ± SD, n=5 (amniotic fluid), n=4 (allantoic fluid).

	Amniotic fluid	**Allantoic fluid**
Enrofloxacin	228.4 ± 223	178.1 ± 184.1
Ciprofloxacin	54.4 ± 47.3	75.2 ± 62.8

Table 7.2. Amniotic fluid chemistries at time of abortion (62 ±0.5 days gestation). Mares were untreated (n=7), or treated with 7.5mg/kg (n=5) enrofloxacin orally once a day for 14 days before abortion. Values are mean ± SD (range). No differences were seen between treatment groups for any value ($p<0.05$). Reported ranges published elsewhere [x][29] or [y][28].

Analyte	Overall mean	Reported ranges	Untreated	Treated	p value
Creatinine (μmol/L)	<17	20.8-25.8[y]	<17 (<17 -35)	<17	
BUN (mmol/L)	6.9 ±1.7	6.4-8.5 [y]	7.8 ±2.4 (5.0- 9.7)	6.3 ±0.8 (5.4- 7.1)	0.5
TP (g/L)	1.3 ±0.5	0.35 ± .01[x]	1.6 ±0.4 (1.3-2.0)	1.1 ±0.5 (0.5-1.6)	0.1
Albumin (g/L)	<0.02		<0.02	<0.02	
Calcium (mmol/L)	2.07 ±1.14	1.35 ± 0.52[x]	2.47 ±1.01 (1.60-3.58)	1.76 ±1.27 (0.90- 3.75)	0.6
Phosphorus (mmol/L)	1.23 ±0.62		1.52 ±0.37 (1.16-1.91)	1.03 ±0.73 (0.48- 2.07)	0.5
Sodium (mmol/L)	122 ±10	123- 128[y]	119 ±11 (107-129)	124 ±10 (109-132)	0.8
Potassium (mmol/L)	6.6 ±2.3	8.1 ± 5.2[x] 2.75- 4.13[y]	8.4 ±2.2 (5.9-9.9)	5.2 ±1.2 (4.1-6.9)	0.5
Chloride (mmol/L)	98 ±10	19.5 ± 4.42[x] 95.0- 99.8[y]	97 ± 11(86-108)	100 ±11 (84-107)	0.8
Glucose (mmol/L)	0.68 ±0.42	0.42 ± 0.33[x]	0.93 ±0.60 (0.50-1.61)	0.50 ±0.14 (0.39- 0.67)	0.1
Alk Phos (U/L)	11 ±10	69.6 ± 44.4	19 ±2 (12-33)	5 ±3 (3-10)	0.5
AST (U/L)	70 ±69		132 ±51 (73-163)	23 ±31 (3- 69)	0.9
GGT (U/L)	4 ±3		7.0 ±2.7 (5-10)	1.8 ±0.5 (1-2)	0.5
Bilirubin (μmol/L)	3.2 ±1.2		3.9 ±1.0 (3.4-5.1)	2.6 ±1.0 (1.7-3.4)	0.06
CPK (U/L)	44 ±53		65 ±73 (<10-146)	29 ±38 (<10-86)	0.6
Cholesterol (mmol/L)	< 0.6		< 0.6	< 0.6	
GLDH (U/L)	3.7 ±2.4		5.2 ±3.1 (1.6-7.0)	2.6 ±1.0) (1.5-3.9)	0.6
TCO2 (mmol/L)	14.9 ±6.7		11.0 ±8.9 (4-21)	17.8 ±3.5 (14-22)	0.6
Mg+ (mmol/L)	0.68 ±0.22		0.71 ±0.22 (0.45-0.86)	0.66 ±0.25 (0.41-0.95)	0.8
Triglycerides (mmol/L)	0.12±0.07		0.15 ±0.10 (0.06-0.25)	0.10 ±0.02 (0.07- 0.11)	0.8

Table 7.3. Allantoic fluid chemistries at time of abortion (62 ± 0.5 days gestation). Mares were untreated (Control, n=6), or treated with 7.5mg/kg (n=4) enrofloxacin orally once a day for 14 days before abortion. Values are mean ± SD. No differences were seen between treatment groups for any value ($p<0.05$). Reported ranges published elsewhere [x][29] or [y][28].

Analyte	Reported range	Overall mean	Control	Treated	p value
Creatinine (μmol/L)	24.3-30.3[y]	19.4 ± 8.4	23.6 ± 13.3	22.1 ± 6.2	0.5
BUN (mmol/L)	10.4-14.8[y]	9.73 ± 1.63	8.75 ± 0.74	11.07 ± 0.5	0.2
TP (g/L)	0.11 ± 0.09[x]	3.08 ± 3.4	2.7± 3.08	2.5 ± 2.9	0.6
Albumin (g/L)		<0.02	<0.02	<0.02	
Calcium (mmol/L)	1.03 ± 0.75[x]	1.17 ± 0.14	1.56 ± 0.5	1.14 ± 0.09	0.8
Phosphorus (mmol/L)		0.96 ± 0.33	1.32 ± 0.49	0.83 ± 0.13	0.5
Sodium (mmol/L)	18.1 ± 10.4[x]	30 ± 20	37 ± 30	22 ±10	0.5
Potassium (mmol/L)	7.6 ± 5.6[x]	10.0 ± 7.9	15.4 ± 8.0	4.6 ± 2.6	0.4
Chloride (mmol/L)	7.5 ± 3.3[x]	28.0 ± 15.8	33.5 ± 23.3	22.5 ± 9.2	0.5
Glucose (mmol/L)		1.83 ± 2.0	2.55 ± 2.94	1.0 ± 0.61	0.5
Alk Phos (U/L)	18.7 ± 13.3[x]	86 ± 101	110 ± 148	61 ± 79	0.5
AST (U/L)		106 ± 141	163 ± 217	50 ± 20	0.6
GGT (U/L)		33 ± 30	47 ± 38	21 ± 25	0.2
Bilirubin (μmol/L)		2.2 ± 4.3	4.2 ± 6.0	0	0.5
CPK (U/L)		468 ± 803	857 ± 1150	80 ± 63	0.5
Cholesterol (mmol/L)		< 0.6	< 0.6- 0.9	< 0.6	
GLDH (U/L)		2.3 ± 1.3	1.6 ± 1.7	2.0 ± 0.4	0.5
TCO2 (mmol/L)		6.0 ± 5.2	7.5 ± 6.4	3 ± 2	0.5
Mg+ (mmol/L)		0.49 ± 0.15	0.51 ± 0.20	0.47 ± 0.14	0.5
Triglycerides (mmol/L)		<0.11	<0.11	<0.11	

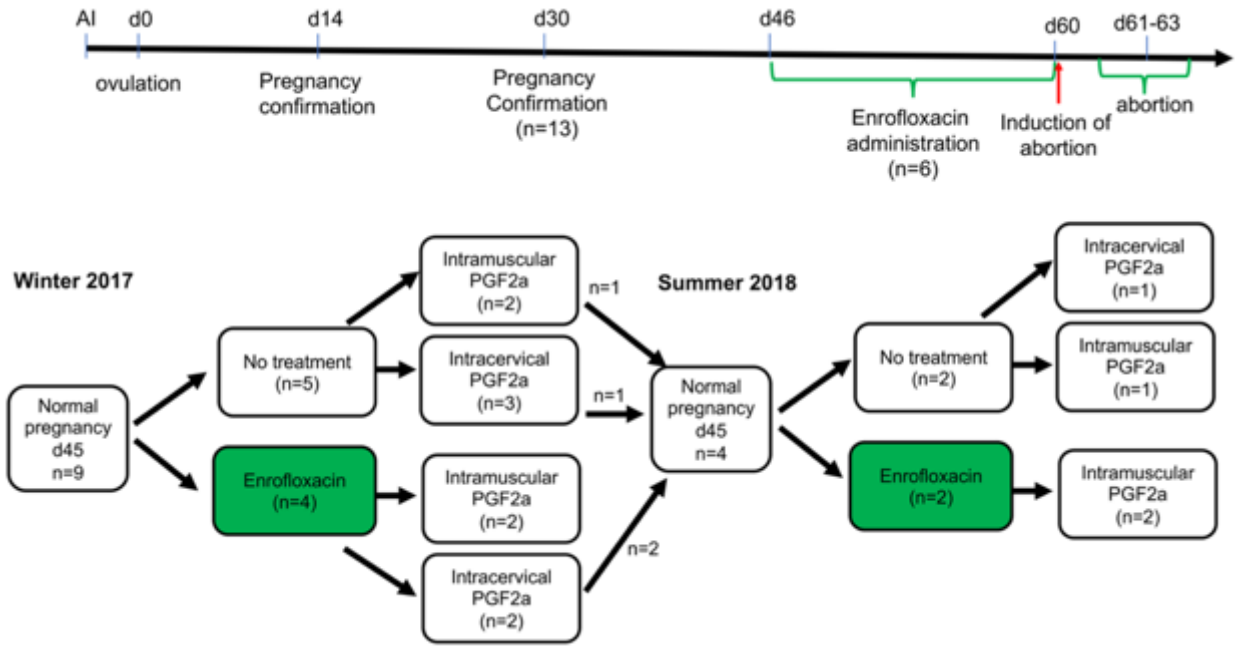

Figure 7.1: Timeline and flowchart of mare enrollment and progression through the study. Only 6 mares were available for a second breeding, 4 of which conceived and carried a second pregnancy.

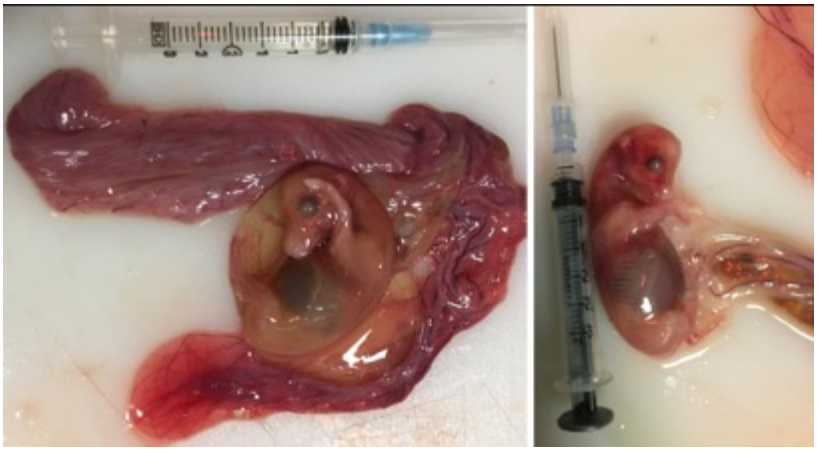

Figure 7.2**:** Representative images of 62-day gestation equine fetuses immediately after retrieval at abortion. Untreated fetus (left), and a fetus from a treated mare (right) that was administered 7.5 mg/kg enrofloxacin PO SID for 14 days, starting at 46 days gestation.

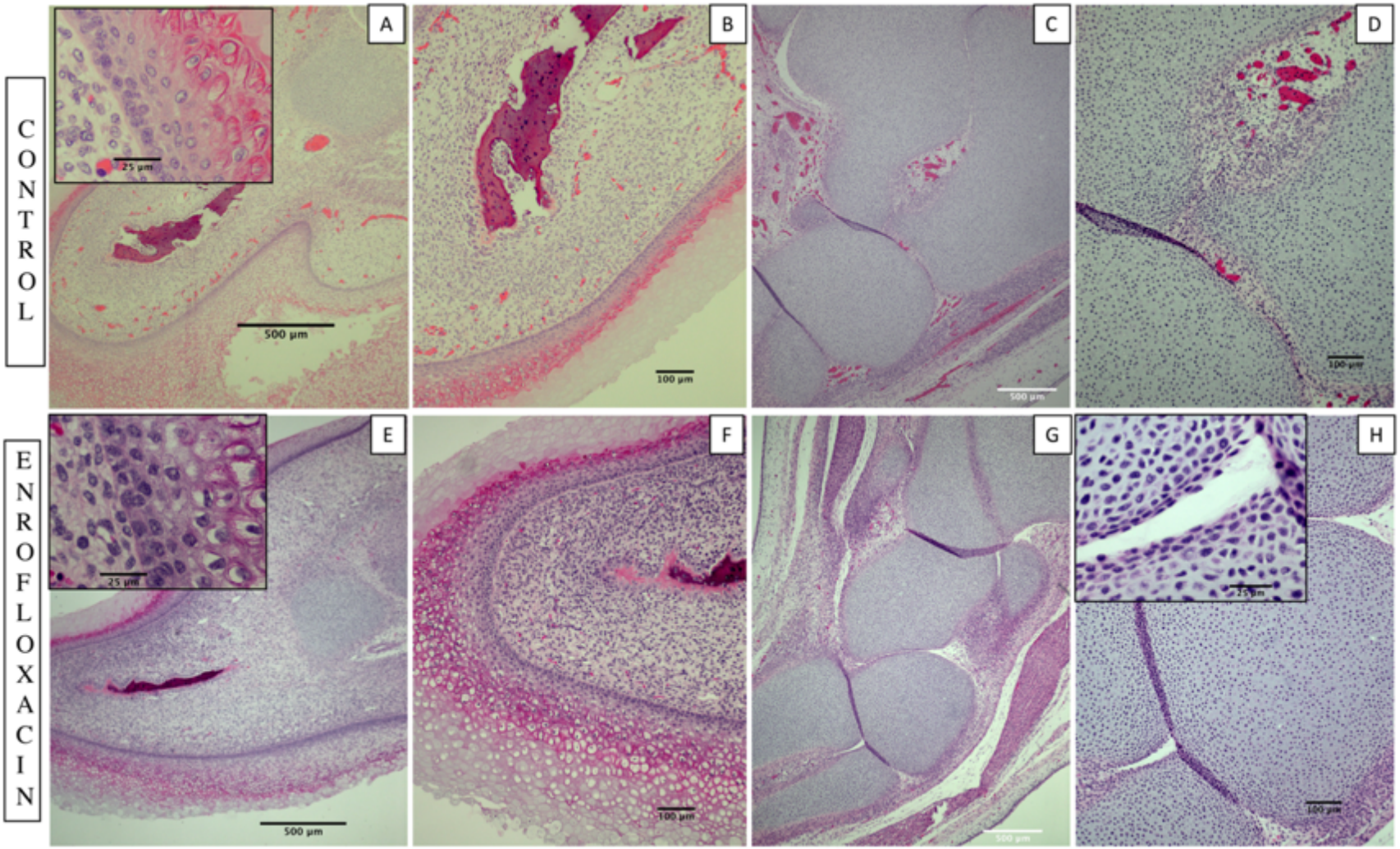

Figure 7.3**.** Sagittal section of the left front hoof (A, B, E, F) and tarsocrural joint (C, D, G, H) of an untreated (A-D) and treated (E-H) 62-day gestation fetus. Treated fetuses were obtained from mares administered 7.5 mg/kg enrofloxacin PO SID for 14 days, starting at 46 days gestation. (A, C, E, G: 4x; B, D, F, H: 10x; insets 40x). Haematoxylin & eosin stained slides.

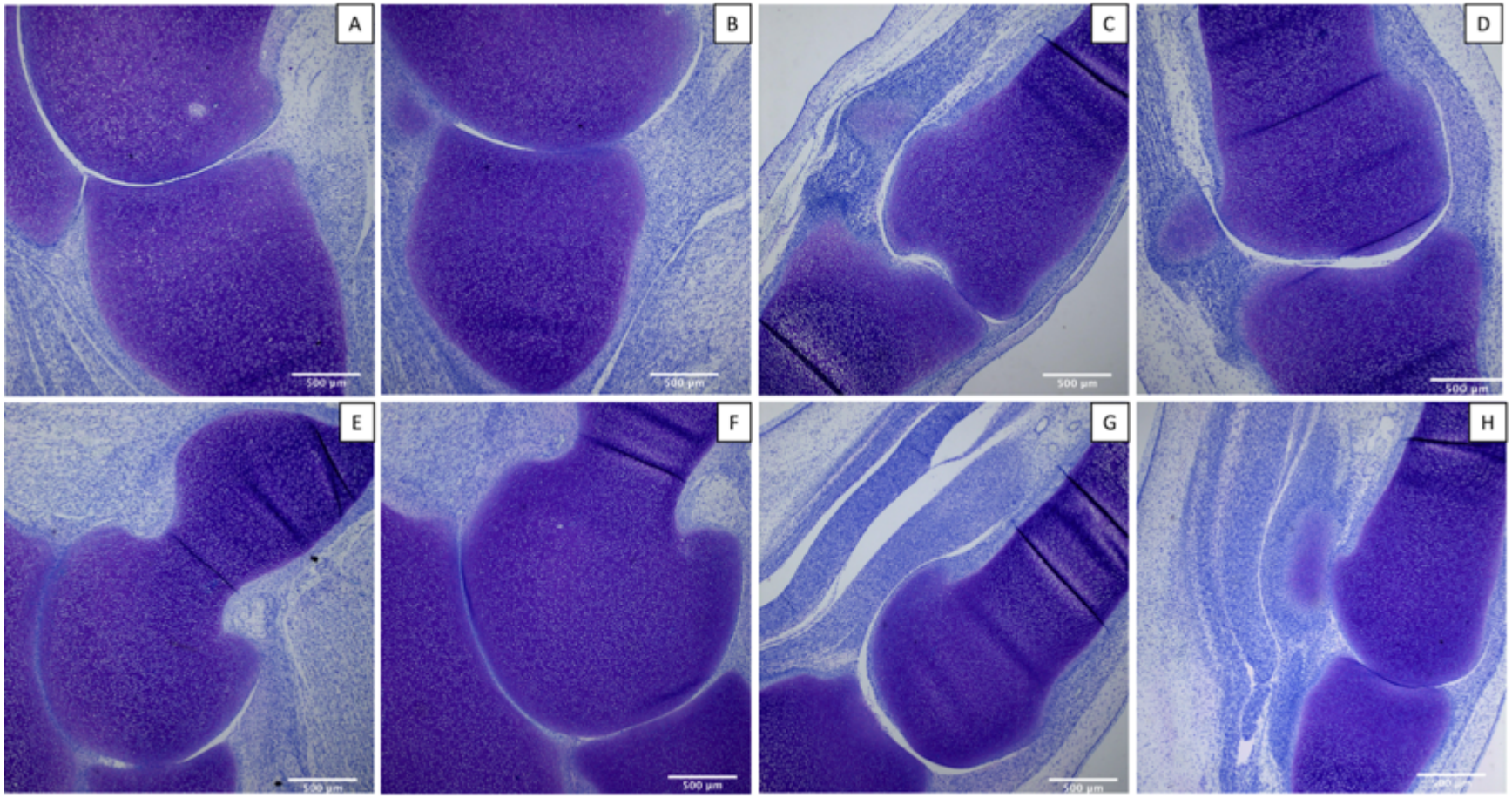

Figure 7.4: Sagittal section of the left front humeroradial (A, B, E, F) and fetlock (C, D, G, H) joint of an untreated (A-D) and treated (E-H) 62-day gestation fetus. Treated fetuses were obtained from mares that were administered 7.5 mg/kg enrofloxacin PO SID for 14 days, starting at 46 days gestation. All images 4x magnification, toluidine blue stained slides.

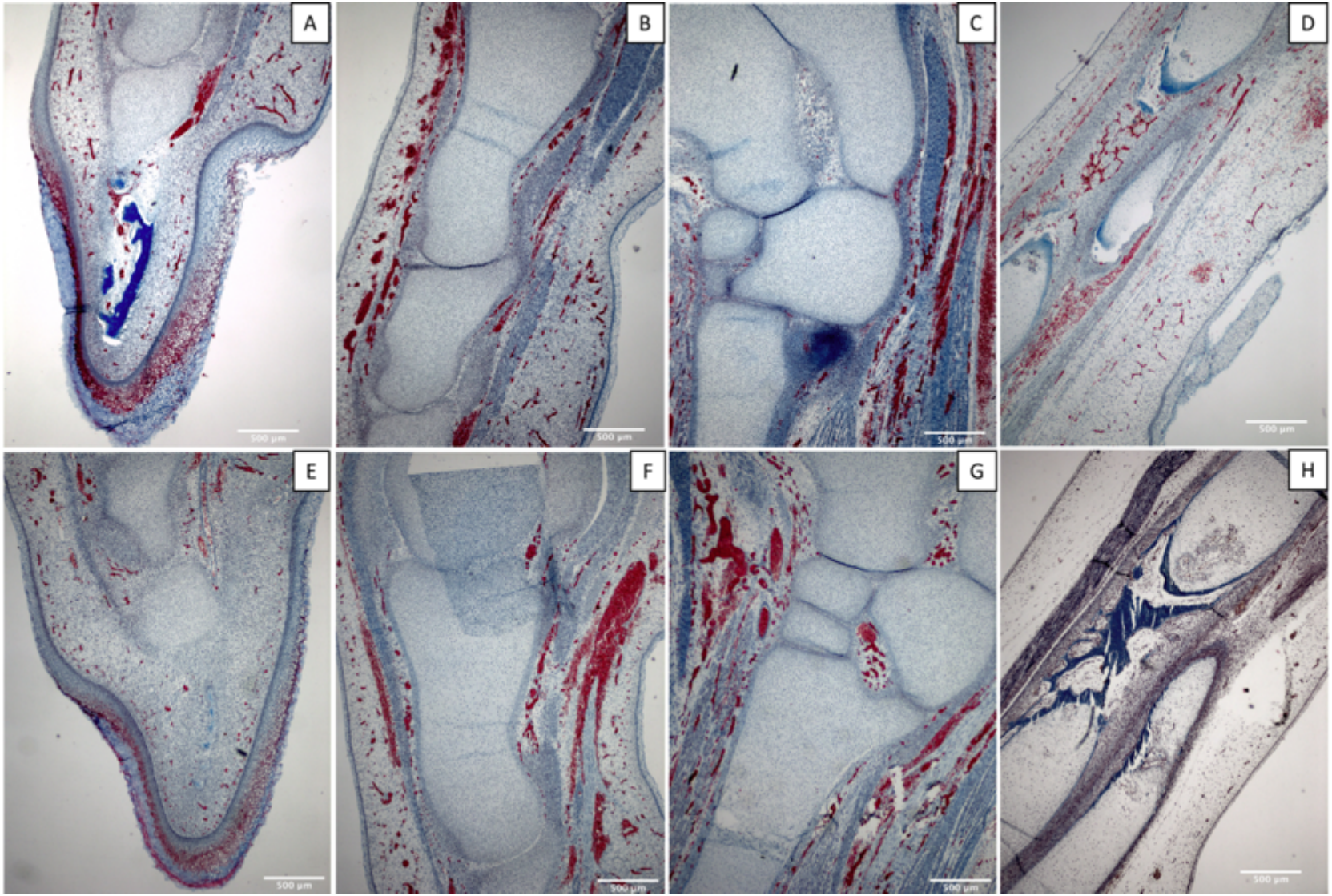

Figure 7.5: Sagittal sections of the left hind hoof (A, B, E, F) and tarsocrural joint (C, D, G, H) of an untreated (A-D) and treated (E-H) 62-day gestation fetus. Treated fetuses were obtained from mares that were administered 7.5 mg/kg enrofloxacin PO SID for 14 days, starting at 46 days gestation. All images 4x magnification, Mason's Trichrome stained slides.

REFERENCES

1. Giguere, S. and Dowling, P. (2013) Fluoroquinolones. In: *Antimicrobial therapy in veterinary medicine*. pp 295–314.
2. Stahlmann, R., Kuhner, S., Shakibaei, M., Schwabe, R., Flores, J., Evander, S.A. and Sickle, D.C. van (2000) Chondrotoxicity of ciprofloxacin in immature beagle dogs: immunohistochemistry, electron microscopy and drug plasma concentrations. *Arch Toxicol* 73, 564–572.
3. Vivrette, S., Bostian, A., Bermingham, E. and Papich, M. (2001) Quinolone-induced arthropathy in neonatal foals. *AAEP Proc*. 47, 376–377. publication_detail.
4. Khazaeil, K., Mazaheri, Y., Hashemitabar, M., Najafzadeh, H., Morovvati, H. and Ghadrdan, A.R. (2012) Enrofloxacin effect on histomorphologic and histomorphometric structure of lamb articular cartilage. *Glob. Vet*. 9, 447–453.
5. Sendzik, J., Lode, H. and Stahlmann, R. (2009) Quinolone-induced arthropathy: an update focusing on new mechanistic and clinical data. *Int. J. Antimicrob. Agents* 33, 194–200.
6. Egerbacher, M., Edinger, J. and Tschulenk, M. (2001) Effects of enrofloxacin and ciprofloxacin hydrochloride on canine and equine chondrocytes in culture. *Am. J. Vet. Res*. 62, 704–708.
7. Egerbacher, M., Wolfesberger, B., Walter, I. and Seirberl, G. (1999) Integrins mediate the effects of quinolones and magnesium deficiency on cultured rat chondrocytes. *Eur J Cell Biol* 78, 391–397.
8. Davenport, C.L.M., Boston, R.C. and Richardson, D.W. (2001) Effects of enrofloxacin and magnesium deficiency on matrix metabolism in equine articular cartilage. *Am. J. Vet. Res*. 62, 160–166.
9. Lim, S., Hossain, M.A., Park, J., Choi, S.H. and Kim, G. (2008) The effects of enrofloxacin on canine tendon cells and chondrocytes proliferation in vitro. *Vet. Res. Commun*. 32, 243–253.
10. Ellerbrock, R.E., Canisso, I.F., Roady, P., Curcio, B., Lima, F.S. and Li, Z. (2018) Enrofloxacin and its active metabolite ciprofloxacin cross the equine placenta and did not cause lesions in the articular surface of the fetuses and foals. *J. Equine Vet. Sci*. 66, 232.
11. Ellerbrock, R.E., Canisso, I.F., Roady, P.J., Rothrock, L.T., Zhong, L., Wilkins, P., Dirikolu, L., Lima, F.S. and Honoroto, J. (2018) Diffusion of enrofloxacin to pregnancy fluids and effects on fetal cartilage after intravenous administration to late pregnant mares. *Equine Vet. J.* . https://doi.org/10.1111/evj.13044.
12. Acker, D.A., Curran, S., Bersu, E.T. and Ginther, O.J. (2001) Morphologic stages of the equine embryo proper on days 17 to 40 after ovulation. *Am. J. Vet. Res*. 62, 1358–1364.
13. Luis, A., Franciolli, R., Mascaro, B., Toledo, E., Nogueira, M., Alberto, C., Sarmento, P., Eduardo, C., Flavia, A., Carvalho, D., Angélica, M. and Andrade, L. (2011) Characteristics of the equine embryo and fetus from days 15 to 107 of pregnancy. *Theriogenology* 76, 819–832.
14. Ginther, O. (1992) *Reproductive biology of the mare: basic and applied aspects*, 2nd ed., Equiservices Publishing, Cross Plains.
15. Jenner, F., Närväinen, J., Stout, T.A.E., Weeren, P.R. van and Brama, P. (2014) Magnetic resonance microscopy atlas of equine embryonic development. *Equine Vet. J.* 46, 210–215.
16. Jenner, F., Osch, G. van, Weninger, W., Geyer, S., Stout, T., Weeren, R. van and Brama,

P. (2015) The embryogenesis of the equine femorotibial joint : The equine interzone. *Equine Vet. J.* 47, 620–622.
17. Holder, N. (1977) An experimental investigation into the early development of the chick elbow joint. *J. Embryol.* 39, 115–127.
18. Koyama, E., Ochiai, T., Rountree, R.B., Kingsley, D.M., Enomoto-iwamoto, M. and Pacifici, M. (2007) Synovial joint formation during mouse limb skeletogenesis: roles of indian hedgehog signaling. *Ann. N. Y. Acad. Sci.* 1116, 100–112.
19. Hyde, G., Boot-handford, R.P. and Wallis, G.A. (2008) Col2a1 lineage tracing reveals that the meniscus of the knee joint has a complex cellular origin. *J. Anat.* 531–538.
20. Hyde, G., Dover, S., Aszodi, A., Wallis, G.A. and Boot-handford, R.P. (2007) Lineage tracing using matrilin-1 gene expression reveals that articular chondrocytes exist as the joint interzone forms. *Dev. Biol.* 304, 825–833.
21. Decker, R.S., Koyama, E. and Pacifici, M. (2014) Genesis and morphogenesis of limb synovial joints and articular cartilage. *Matrix Biol.* 39, 5–10.
22. Curcio, B., Giguère, S., Li, Z., Honorato, J., Ellerbrock, R.E., Lima, F.S. and Canisso, I.F. (2017) Pharmacokinetics of enrofloxacin and its active metabolite (ciprofloxacin) in late-term pregnant and post-parturient mares. *Clin. Theriogenology* 9, 466.
23. Bucca, S., Fogarty, U., Collins, A. and Small, V. (2005) Assessment of feto-placental well-being in the mare from mid-gestation to term: Transrectal and transabdominal ultrasonographic features. *Theriogenology* 64, 542–557.
24. Cuervo-Arango, J., Aguilar, J.J., Vettorazzi, M.L. and Martínez-Boví, R. (2015) eCG concentrations, luteal structures, return to cyclicity, and postabortion fertility in embryo transfer recipient mares. *Theriogenology* 84, 1003–1013.
25. Aguilar, J., Luzuriaga, I., Pardo Chavero, V., Leaniz, J., Casale, P., Carboneti, L. and Marino, V. (2012) Interruption of gestation and reuse of recipient mares in a commercial embryo transfer program. *J. Equine Vet. Sci.* 32, 412–413.
26. Holdstock, N.B., Mcgladdery, A.J., Ousey, J.C., Rossdale, P.D. and Stables, B.C. (1995) Assessing Methods of Collection and Changes of Selected Biochemical Constituents in Amniotic and Allantoic Fluid throughout Equine Pregnancy. *Biol. Reprod.* 38, 21–38.
27. Zanella, L., Takahira, R., Melo e Ona, C., Magalhaes, L. and Prestes, N. (2014) Biochemical Profile of Amniotic and Allantoic Fluid During Different Gestational Phases in Mares. *J. Equine Vet. Sci.* 34, 403–406.

CHAPTER 8: CONCLUSIONS AND FUTURE DIRECTIONS

The work described within this dissertation investigated the potential consequences of enrofloxacin exposure *in utero* on the resulting foal. Collectively the works failed to demonstrate any postnatal effects of *in utero* exposure to enrofloxacin. Chapter 3 demonstrated that the recommended oral and intravenous enrofloxacin doses are also appropriate for pregnant mares with susceptible bacterial infections. This work also highlighted the need to consider bacterial MIC when choosing not only an appropriate antimicrobial, but also appropriate dose, and suggest the need for further exploration of potential differences in drug absorption, excretion, and p450 metabolism in pregnant mares.

Intravenous administration of enrofloxacin to third trimester mares in chapter 4 demonstrated that that both enrofloxacin and its active metabolite ciprofloxacin cross the equine placenta, and that ciprofloxacin accumulates in the allantoic fluid over time without causing gross or histological lesions in the fetus. Unfortunately, many orthopedic lesions in the horse do not develop until the post-natal period, when the animal becomes weight-bearing, and with this in mind, Chapter 5 details the treatment of third trimester mares with an oral enrofloxacin, and the subsequent evaluation of their foals at 30 days postpartum. This study demonstrated that ciprofloxacin remained detectable in the fetal fluids long after cessation of enrofloxacin treatment, and that foals from enrofloxacin-treated mares did not develop orthopedic lesions at a faster rate than their controls, nor did they demonstrate any difference in tendon morphology or tendon strength post-partum. While these results were encouraging, it is possible that normal necropsy procedures are not adequate to detect subtle changes in foal cartilage composition.

Multiple modalities were then used to evaluate the 30-day old foals in Chapter 6 to determine if more sensitive techniques would detect differences in foal cartilage development. Radiographs demonstrated normal foal orthopedic lesions commonly found in the breeds included in the study. Structural qMRI described differences in foal cartilage for the first time, and demonstrated differences in positive control animals treated postpartum, but no differences in foals exposed *in utero*. Similarly, no difference was seen in expression of multiple cartilage associated genes in the resulting foals.

The third trimester studies were followed by a pilot study described in Chapter 7, which investigated the effects of *in utero* fluoroquinolone exposure during the first trimester. This study looked at 14 days exposure during organogenesis, and did not detect gross or histological defects.

While the results of the dissertation are encouraging, it is important to acknowledge that it is possible even with the advanced imaging and molecular techniques employed in Chapter 6, there is still the possibility of a failure of test sensitivity to detect minor effects of treatment that may affect cartilage quality or tendon strength over time. Additionally, it is possible that the work failed to identify the critical time in gestation where fluoroquinolones may affect cartilage development, that an effect may only be seen in diseased or nutritionally compromised animals, or that lesions would develop in the mares were treated until parturition. Further work should be completed to determine the effect of placental disease on drug transfer, and also the effects of systemic illness on fluoroquinolone pharmacokinetics and pharmacodynamics.

Collectively the works did not demonstrate any postnatal effects of *in utero* exposure to enrofloxacin. While these findings do not rule out a potential low incidence of toxicity or longer-term consequences when this fluoroquinolone is administered during an equine pregnancy, the findings described above are useful for risk assessment when veterinarians are faced with severe disease, and is thus invaluable information for veterinarians in broodmare practice.

APPENDIX A: ENDOCRINE AND METABOLIC PROFILE OF PERIPUBERTAL STANDARDBRED COLTS

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ABSTRACT

While the onset of puberty and its association with somatic growth and testicular development is well described in production animals, similar studies are lacking in peripubertal colts. The objectives of this study were to determine the concentrations of reproductive and metabolic hormones during the peripubertal period and to assess their relationship with testicular development and body fat deposition. Blood samples were collected from 23 healthy Standardbred colts via jugular venipuncture between 8:30 and 10:00 am every four weeks for twelve months, and plasma samples were stored at -80°C until analysis. The colts were weighed monthly, and percent of body fat and testicular volume were estimated by B-mode ultrasound measurements

using previously published equations. Onset of puberty was determined as the month when testosterone was two standard deviations above the previous mean. Plasma FSH, LH, leptin, estradiol-17β, androstenedione, IGF-1, insulin, inhibin-A, and inhibin-B were analyzed for a peripubertal period of seven months (-3, -2, -1, 0, +1, +2, +3; 0= month of puberty onset). Spring born Standardbred colts underwent puberty at 13 months of age; onset of puberty coincided with exponential testicular growth but did not coincide with an increase in cutaneous body fat deposition or leptin. Plasma Inhibin B concentrations were significantly increased in the postpubertal period, but no increase was seen in Inhibin A, androstenedione, FSH, LH, or estradiol-17β. In conclusion, the rise in testosterone and subsequent onset of puberty coincides with rapid testicular growth but is not correlated with an increase in gonadotropins, IGF-1, cutaneous body fat or leptin in the horse.
Keyword: puberty, reproductive hormones, testicular development, somatic development, metabolic hormones.

INTRODUCTION

The peripubertal period is poorly documented in horses, particularly when compared to other farm animals. Assessment of semen features (i.e., the first ejaculate containing ≥ 50 million total spermatozoa with ≥ 10 % motile sperm) has been used to define the onset of puberty in bulls[1-4] and by one study in colts[5]. In ruminants, semen can be obtained via electroejaculation regardless of libido or physical ability to mount. However, this method is inefficient in horses and requires general anesthesia[6]. Thus, using semen features to determine the onset of puberty requires that the colt is mature enough to express good libido and mount a mare or phantom to collect semen in an artificial vagina. Training colts to collect semen can be a daunting task and might not be successful until weeks or months after puberty has occurred.

Given these challenges, studies have used changes in gonadotropins and sex steroids to assess puberty in colts, and as an alternative method to study puberty in calves[7-9]. In colts, the onset of puberty occurs from 11 to 20 months, depending on breed, geographical location, year, foaling season (spring vs. fall), and individual variations[5,9-11].

The association of metabolism, growth, and development is well studied in peripubertal ruminants [2,3,12-15], but not well defined in horses[16,17]. Nutrition influences not only the onset of puberty but also long-term testicular function in rams and bulls [12,13,15]. Metabolic

regulators (e.g., insulin, leptin, and IGF-1) are thought to modulate gonadotropin and steroid production in peripubertal bulls [2,14]. Studies in bulls have shown that not only testosterone but also plasma leptin and IGF-1 increase during the peripubertal period, without a corresponding increase in gonadotropins[3]. In contrast to females, leptin in both boys and bull calves decreases once puberty is reached[18,19]; it is likely that a similar finding may occur in colts. Previous studies assessing hormonal changes and weight gain in prepubertal colts over time did not look for changes in body fat, leptin, or testicular development[9,17]. Measurement of body fat in conjunction with peripheral metabolic regulators is useful to assess energy status [20,21], and measuring testicular volume is useful for estimating potential daily sperm output [22].

Inhibin (subtypes A & B) is produced by the Sertoli cells and is thought to regulate steroid secretions via inhibition of FSH [23]. In colts, concentrations of inhibin increase in peripubertal animals and may decrease in subfertile or infertile stallions [8,23,24]. While those studies quantified total immunoreactive inhibin, other studies have recently shown differences in inhibin B and inhibin A in males. Inhibin A predominates over inhibin B in males in other species such as the boar, and it is possible that such differences will also be present in colts[25].

To date, the associations between testicular development and metabolic and reproductive hormones in peripubertal colts are still unclear. Therefore, the objectives of this study were to determine the concentrations of sex steroids, gonadotropins, inhibin-A and –B, and metabolic hormones during the peripubertal period, and evaluate their relationship with testicular development and body fat deposition. We hypothesized that an increase in total body fat corresponds with an increase in sex steroids and the onset of puberty in the colt. We also hypothesized that onset of puberty, as defined by an increase in testosterone, would precede an increase in body fat and exponential testicular growth, and that onset of puberty is associated with an increase in gonadotropins, testicular and metabolic hormones in colts.

MATERIALS AND METHODS

Animals and experimental design

Twenty three Standardbred colts (pre-pubertal male horses) were housed on pasture, at farm one (n=9) and farm two (n=14), in central Illinois according to industry standards. Colts enrolled in the study had monthly blood samplings, and assessments (rump fat, testicular volume, and body weight) carried out from October 2014 to August 2015. Two colts were sold in May and

June, and did not complete the study. All experimental procedures were performed in compliance with the Guide for the Care and Use of Agriculture Animals in Research and Training and approved by the Institutional Animal Care and Use Committee of the University of Illinois at Urbana-Champaign (Protocol # 14174).

Blood sampling and body weight

Blood samples were collected via jugular venipuncture into heparinized tubes once a month between 08:30 and 10:00 am. Blood tubes were kept on ice until centrifugation for 10 min at 600g within 2h of sample collection. Plasma was harvested and stored at -80^{o}C until analysis. Body weight was estimated with a commercial weight tape (Purina Mills, Inc).

Body fat and testicular volume

Rump fat was measured using B-mode ultrasound approximately 5 cm lateral to the midline at the center of the pelvis, and the left and right side were averaged. Percent total body fat was calculated using an equation derived by Westervelt et al., [26], where body fat is estimated by the equation 3.83 + 5.58x, where x= cutaneous rump fat in centimeters. Testicular volume was calculated according to the equation derived by Love et al.[22], where testicular volume = 0.5233 x height x length x width (cm). The volume of both testes was combined for analysis.

Hormonal analyses

Androstenedione

Plasma androstenedione was measured by competitive binding ELISA according to manufacturer's directions (RCAN-AD-208R, BioVendor, Modrice, Czech Republic). The assay uses a rabbit anti-androstendione polyclonal antibody, and androstendione-horseradish peroxidase conjugate, and has a reported 100% cross-reactivity with androstenedione, 1.8% with DHEA, 0.2% with testosterone, and <0.1 % with estradiol, progesterone, or 5α-DHT. Reported sensitivity is 0.05 ng/ml. The ELISA demonstrated an intra-assay CV of 3.4% and interassay CV of 10.1%.

Estradiol-17β

Concentrations of estradiol-17β were determined by a competitive binding chemiluminescent enzyme immunoassay (Estradiol, Immulite 1000, Siemens Healthcare, Malvern, PA) previously used in equine research[127,28]. The assay employs a polyclonal rabbit anti-estradiol antibody, and an alkaline phosphatase bound estradiol conjugate. The assay has a

reported range of 20 to 2,000 pg/mL and sensitivity of 15pg/mL. Reported cross-reactivity for this assay is 2.09% for estrone, 0.032% for 17α-estradiol, 0.535% for estriol, and not detectable for other adrenal and reproductive steroids. Intra- and inter-assay coefficient of variations are less than 4%.

Insulin-like growth factor-1

Concentrations of IGF-1 were analyzed via ELISA (EIA-4140, DRG Instruments GmBH, Marburg, Germany), similarly to previous studies.[29-31] The assay uses a monoclonal antibody, has a reported cross-reactivity of 100% with IGF-1, 1.02% with IGF-2, and 3.3% with insulin, and a sensitivity of 9.75ng/ml, with a range of 9.75-600 ng/ml. The intra-assay CV was 13%, and the interassay CV was 2.8%.

Insulin

Insulin was analyzed via ELISA (10-1205-01, Mercodia AB, Uppsala, Sweden, reported sensitivity 0.91 μg/ml, range 0.02-1.5 μg/ml. This ELISA uses a mouse monoclonal anti-insulin primary and secondary antibody, has no cross-reactivity to C-peptide or proinsulin and has been validated for use in the horse[32,33]. The intra-assay CV was 6.2%, and the interassay CV was 3.9%.

Testosterone, FSH, LH, and leptin

Concentrations of FSH, LH, testosterone, and leptin were analyzed via radioimmunoassay at Louisiana State University, and the assays were previously validated for use in the horse [34-37]. Testosterone was extracted from plasma and quantified by radioimmune assay as previously described [37]. The testosterone assay used an antibody to testosterone-11β-bovine antibody, and a

testosterone-11 α -tyrosine methyl ester as the radioiodinated antigen. The assay has reported cross reactivity of 3.4% with 5α-DHT, 2.2% with 5α-androstane-3β,17β-diol, 2% with 11-oxotestosterone, 0.95% with 6β-hydroxytestosterone, 0.63% with 5β-DHT, 0.56% with androstenedione, 0.20 with epiandrosterone, and less than 0.01% with other steroids. The assay had intraassay and interassay CVs of 5.9% and 1.8% respectively, and a sensitivity of 0.006 ng/ml, consistent with the established ranges for the assay.

The leptin assay used commercially available reagents (Multispecies leptin kit, Linco Research Inc, St Charles, MO), has a reported cross-reactivity of less than 0.01% with eLH, eFSH, equine GH, or equine prolactin, and was previously validated in the horse [36]. 125I-human leptin

standards provided by the kit, human recombinant leptin, and three horse plasma pools were used to produce parallel inhibition curves. For analysis, equal amounts of sample and guinea pig anti-multi species leptin antibody were incubated at 5 C for 48 h. One hundred mL 125I-human leptin was then added to all tubes, vortexed, and tubes were again incubated 24 h at 5 C. On day 3, 200 mL of anti-rabbit gamma globulin serum was added to all tubes except total count tubes, tubes were vortexed and incubated for 24 h at 5 C. Tubes centrifuged at 1200 x g for 30 min to separate antibody-bound leptin from free leptin and the supernatant was decanted. The pellet was washed with 1.0mL PBS and samples were again centrifuged. Radioactivity was assessed by solid scintillation counting for 1 min. The assay for leptin had a sensitivity of 0.2 ng/ mL, 6% intra-assay CV and 4% inter-assay CV, consistent with the established ranges.

The FSH radioimmunoassay was performed as previously described, using a primary anti-human FSH antiserum prepared in rabbits, and a secondary anti-rabbit gamma globulin prepared in sheep [34,38]. Partially purified eFSH (LER-1686-2) was used as a standard. Samples were run in duplicate, intra-assay and inter-assay CVs were 7% and 11% respectively, and the assay has a sensitivity of 1.4 ng/mL, these results are within the established ranges. The assay has a reported cross reactivity of 3.5% with LH, 2.0% with TSH, 0.02% with eCG, and <0.1% with all other pituitary hormones.

LH was measured by radioimmunoassay, as previously described, and used a rabbit anti-eCG and radioiodinated ovine LH [34]. LER-958-1 was used as a standard. The assay has a reported cross-reactivity of 2.4% with eFSH, 3.8% with eTSH, and 28.7% with eCG. Samples were run in duplicate, and the LH immunoassay had intraassay and interassay CVs of 6% and 9% respectively, and a sensitivity of 0.2 ng/mL. These results are within the established ranges.

Inhibin A & B

Concentrations of inhibin A and B were analyzed via ELISA using commercial assays (Equine/Canine/Rodent Inhibin A (AL-161) and B (AL-163) Ansh Labs, Webster, TX). Both assays are quantitative immunoassays and use mouse monoclonal antibodies against βA- (inhibin A assay) and βB- (inhibin B assay) subunits of inhibin for capture, and a monoclonal antibody specific to the inhibin α subunit for detection. Assays were run at the Endocrinology Laboratory of the University of California-Davis, where the assays were previously validated for use in the horse[39]. The inhibin A assay had an intraassay and interassay CVs of 5.9% and 3.2%

respectively, and a sensitivity of 2.3 pg/ml. Inhibin B had a sensitivity of 2.3 pg/mL, 5.5% intra-assay CV and 3.8% inter-assay CV.

Data analysis

Puberty was determined by the month that plasma testosterone first reached two standard deviations above the previous mean plasma concentrations[9]. The peripuberty period was selected (-3, -1, -1, 0 (month of puberty), +1, +2, and +3 months) according to Brito et al., [174] and all other hormones were analyzed accordingly. Data are also presented according to the equivalent sampling age (months) not adjusted for peripuberty (supplementary tables). Data were analyzed using RStudio v 0.99.489 (RStudio Team, Boston, MA). Hormone data not normally distributed were log-transformed prior to analyses. Variables were analyzed with mixed models, with month relative to puberty as the fixed factor, and colt and location as random factors. Tukey HSD test was used for posthoc analysis. Pearson correlations were performed between variables and presented as defined as strong ($r \geq 0.7$), moderate ($0.3 < r \leq 0.7$), or weak ($r < 0.3$) correlations. Data are expressed as mean ± SEM. Statistical significance was set at $p < 0.05$.

RESULTS

At the onset of puberty, colts were 13 ± 0.2 months of age, weighed 338.4 ± 6.7 kg, with a body fat of 6.3 ±0.2%, and a testicular volume of 53.5 ± 7.8 cm^3 (Table A.1). Weight and testicular volume increased with age, and pre-pubertal measurements were significantly lower than post-pubertal values (Table A.1). Body fat was significantly lower at puberty than three months before, or three months following the onset of puberty (Table A.1). As expected, age was correlated with weight, testosterone, and testicular volume (Table A.2).

Steroid hormones

The month of puberty, plasma testosterone concentrations were 0.5 ± 0.06 ng/ml, and estradiol-17β were 46 ± 3.7 pg/ml (Table A.3). Testosterone was moderately correlated with testicular volume and body weight (Table A.2), and pre-pubertal testosterone concentrations two months before the onset of puberty were significantly lower than post-pubertal concentrations (Table A.3). Plasma androstenedione was 0.6 ng/ml at the month of puberty, and significantly higher than three months before the onset of puberty, but not different than any other month (Table

A.3). Androstenedione was weakly correlated with testosterone but not with other variables (Table A.2).

Gonadotropins

The month of puberty, LH was 0.9 ± 0.4 ng/ml, and FSH was 9.9 ± 1 ng/ml (Table A.3). Concentrations of FSH were not significantly different at the onset of puberty and were not correlated with age (Table A.2,3). The LH concentrations were moderately correlated with testosterone and FSH (Table A.2); however, no significant differences were seen in LH concentrations by month of sampling. No significant difference was observed in inhibin-A over time, whereas concentrations of inhibin-B were significantly higher by two months post puberty compared to concentrations two months pre-puberty (Table A.3). Inhibin-B was moderately correlated with testicular volume, weight, and age, and weakly correlated with FSH, body fat, and testosterone concentrations (Table A.2).

Metabolic hormones

Plasma leptin concentration was 1.4 ng/dl ± 0.31 at onset of puberty, but no significant differences in leptin were seen during the peri-pubertal period (Table A.3), and a weak correlation was seen with plasma leptin and percent body fat (Table A.2). Mean insulin concentration at the onset of puberty was 0.15 ± 0.04 μg/ml, and was significantly higher than 3 months pre-puberty, and lower than 3 months post-puberty (Table A.3). Weak correlations were observed between insulin concentrations and body fat and testicular volume, and insulin concentrations were moderately correlated with age (Table A.2).

DISCUSSION

Puberty in colts is associated with changes in serum testosterone and occurs during a time of exponential growth. Given the known associations between body mass and the onset of puberty in other species, this study evaluated the relationship between body fat, reproductive and metabolic hormones, and the onset of puberty in Standardbred colts. Understanding such relationships is critical for improving how prospective sires are raised for maximum fertility.

Onset of puberty (i.e., 13 month) for Standardbred colts was consistent with previous studies involving other horse breeds raised in other parts of the world[8,9,11]. Body weight at puberty onset was also similar to those values obtained in spring-born Thoroughbred colts raised

in Japan [8] and New Zealand[9]. This finding suggests that onset of puberty is similar between these two breeds when animals are raised under standard management conditions.

Body fat deposits and leptin concentrations were shown to be positively correlated in Standardbred horses[20,21]. It is unclear why body fat deposits were not correlated with leptin concentrations in the present study. It is possible that the equation developed by Westervelt and collaborators [23] does not provide an accurate prediction of body fat in colts as it does for adult horses. Additionally, peripubertal colts have very small deposits of subcutaneous fat, and it is possible that ultrasound was not sensitive enough to detect small changes in body fat during this period. Of interest, colt plasma leptin concentrations at puberty (1.4 ng/ml) were similar to fit Standardbred racehorses (1.02 ng/ml), but calculated % body fat in colts was lower than the same racehorses (6.2 ± 0.2 % vs. 11.9 ± 0.3)[21].

No correlation was found between plasma leptin and insulin concentrations, which is similar to findings comparing fit and unfit Standardbred racehorses[21]. Studies in humans have shown that insulin and leptin concentrations are correlated in fasted people, but not in postprandial people, and that is one possible explanation for the lack of correlation in the present study [40,41]. While the colts were always sampled at the same time every morning, they had access to pasture and hay overnight and were not fasted at time of sampling. Plasma insulin concentrations were increased three months post-puberty, when compared to three months pre-puberty, and this increase was seen at the same time that a concentrated diet was introduced to the colts in preparation for sales. While this additional grain supplementation may represent a confounding factor, all colts were offered the same diet, and the animals were kept under practical commercial conditions, with minimal interference from the investigators.

Growth hormone (GH) is released in a pulsatile manner, and has a short half-life (<15 min), so frequent sampling is required for proper assessment[42,43]. While such frequent sampling is not feasible in on private farm studies, IGF-1 is more stable in the plasma and has been used as an indirect method to measure GH, and as a proxy to assess metabolic energy status[44]. Insulin-like growth factor-1 circulates in the blood bound to binding proteins (e.g., IGFB3), which increases the half-life of IGF, and modulates IGF-I activity[44-46]. Of interest, both IGF-1 and IGFB3 are regulated by GH [47]. Due to GH's short half-life, and the similarities between IGF-1 and IGFB3 expression seen in peripubertal colts[44], IGF-1 was chosen as the marker of interest for assessing growth hormone concentrations during the peripubertal period. Previous studies have shown that

plasma IGF-1 concentrations are highest in pubertal colts[29,44], and then decrease post onset of puberty. We did not detect a significant difference in plasma IGF-1 concentrations in our sampling period, possibly because the decrease in IGF-1 occurs before 12 months of age. Additionally, immunoassays are perhaps not the best choice for IGF-1 analysis as poor correlations have been shown between laboratories analyzing human samples, even when automated assays are used[48]. In this study we were most interested in changes over time, and not in absolute values, making this less of a concern. In addition, the while the assay used in the present study has been used by previous studies [30,31], it is unclear whether this assay has been validated for the horse.

Plasma gonadotropins and estradiol did not increase significantly from the pre- to post-pubertal period, which may be attributed to pulsatile release and timing of sample collection in this study. These findings are similar to Wesson and Ginther[11], and Dhakal et al[8] who found that FSH and LH concentrations did not increase significantly in the months preceding the onset of puberty. While Mizukami et al detected an increase in LH from the fall to spring in yearling colts, however those colts were older than the colts in the current study. A previous study assessing gonadotropins in prepubertal colts did not see an increase in LH and FSH until after 70 weeks of age[5], and it is possible that these changes occur later in the post pubertal period.

Aromatase, the enzyme responsible for converting androgens into estrogens, has apparently increased expression in the testicles of post-pubertal colts[49] and estrogen has been shown to be increased in six post-pubertal Thoroughbred colts[8], however we did not see an increase in plasma estrogens in the three months of the post-pubertal period in this study. This discrepancy could be due to differences in the assays used between the different studies, differences between testicular and plasma concentrations, or diet.

Inhibin is produced by the Sertoli cells in response to FSH and its concentration in plasma has previously been shown to decrease in subfertile stallions and aging men [24,50]. Inhibin concentrations decrease at puberty in the rat and ram, while concentrations increase in monkeys and man at puberty[51-54]. Developments in immunoassays have made possible to differentiate between inhibin-A and inhibin B, and this is the first report of the differences in subtypes in the peripubertal colt. While no change was seen in colt plasma Inhibin A concentrations during the peripubertal period, the increase in plasma Inhibin B seen in the post-puberty may indicate testicular maturation and an increase in Sertoli cell function. In boars, it appears that inhibin A predominates over inhibin B at puberty[25], however in colts, we found that inhibin B

concentrations predominate over inhibin A at puberty, and inhibin B increases in the post-pubertal period. Inhibin is thought to play a role in germinal epithelium maturation, nonetheless, the physiological role of different inhibin types in peripubertal colts remains to be determined.

Testicular growth is regulated by many factors, as demonstrated by studies in ruminants [2,3,14]. Restricting nutritional support in young bull calves has been demonstrated to decrease LH pulse frequency and result in decreased testicular size at time of puberty[14]. While we did not detect a relationship between metabolic status, gonadotropins, and testicular development, this study was not designed to assess differences in onset of puberty based on diet, and it is possible that differences would be observed in colts fed on different planes of nutrition. Additionally, colts were not assessed in the early postnatal period (i.e., less than six months of age), when nutritional influences might have played a bigger role on gonadotropin secretion and eventual testicular development.

Onset of puberty, as characterized by increased peripheral testosterone concentrations, corresponded with the beginning of exponential testicular growth, an increase in body weight, and in increase in plasma Inhibin-B. Contrary to our hypotheses, the onset of puberty did not correspond with an increase in body fat, IGF-1, or plasma leptin, and suggests that body fat may play a less significant role in the onset of puberty in the horse than other species.

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TABLES

Table A.1. Peripubertal Standardbred colt somatic parameters relative to puberty onset. Month indicates the month relative to the onset of puberty as determined by increase in plasma testosterone. Age indicates the mean colt age each month. Percent body fat was measured according the equation 3.83 + 5.58x, where x= cutaneous rump fat in centimeters, and testicular volume was calculated as 0.5233 x length x width x height in cm. All data shown as mean ± SEM.

Time	n	Age (mo)	Body fat (%)	Testicular volume (cm^3)	Weight (kg)
-3	23	10 ± 0.2	6.9 ± 0.2^{a}	19.2 ± 3.7^{ab}	325.9 ± 6.4^{a}
-2	23	11 ± 0.2	6.7 ± 0.3^{ab}	25.6 ± 4.3^{b}	325.9 ± 7.1^{a}
-1	23	12 ± 0.2	6.3 ± 0.2^{b}	36.1 ± 5.9^{bc}	331.3 ± 6.5^{a}
0	23	13 ± 0.2	6.3 ± 0.2^{b}	53.5 ± 7.8^{c}	338.4 ± 6.7^{ab}
1	21	14 ± 0.2	6.3 ± 0.2^{ab}	79.1 ± 12.0^{d}	346.5 ± 7.6^{b}
2	20	15 ± 0.2	6.2 ± 0.3^{b}	85.4 ± 9.0^{d}	359.6 ± 8.4^{c}
3	16	15.9 ± 0.2	6.9 ± 0.3^{a}	93.8 ± 11.9^{d}	380.1 ± 8.1^{d}

Different superscripts within column denote differences ($p<0.05$).

Table A.2. Pearson's coefficient of correlations for reported measurements. Testosterone, androstenedione, FSH, LH, estradiol-17β, leptin, insulin, IGF-1, inhibin A, and inhibin B concentrations in Standardbred colts (n=23). Significance set at $p<0.05$ for inclusion in table.

	Testosterone	Weight	Body fat	Testicular volume	IGF-1	Age	FSH	Estradiol-17β	Inhibin-B
Body fat	-0.21								
Testicular Volume	0.46	0.65			-0.24				0.41
Leptin	-0.18	-0.34		-0.22		-0.17			-0.24
LH	0.34	0.17	0.22	0.16			-0.31	0.16	0.36
FSH	0.35							-0.28	-0.29
Age	0.53	0.68	-0.17	0.68					0.45
Weight	0.38								
Androstenedione	0.26		-0.14						
Insulin			0.26	0.21		0.31			
Inhibin-A								-0.22	0.31
Inhibin-B	0.24	0.34	0.22	0.41		0.45	-0.29		

Table A.3. Mean (± SEM) plasma concentrations of testosterone, androstenedione (AND), FSH, LH, estradiol-17β (E2), leptin, insulin, IGF-1, inhibin A (IN-A), and inhibin B (IN-B) concentrations in peripubertal Standardbred colts. Time indicates the month relative to the onset of puberty as determined by an increase in plasma testosterone two standard deviations above the previous mean.

Time	n	Testosterone (ng/mL)	E2 (pg/mL)	AND (ng/mL)	FSH (ng/mL)	LH (ng/mL)	leptin (ng/mL)	insulin (μg/mL)	IGF-1 (ng/mL)	IN-A (pg/mL)	IN-B (pg/mL)
-3	23	0.1 ± 0.01^{d}	43.3 ± 3.1	0.1 ± 0.04^{a}	7.3 ± 0.8	0.7 ± 0.3	3.3 ± 0.2	0.06 ± 0.01^{b}	140 ± 17	10.3 ± 2.8	48.5 ± 8.8^{b}
-2	23	0.1 ± 0.02^{ad}	45.7 ± 2.7	0.2 ± 0.08^{ab}	8.0 ± 0.8	0.6 ± 0.15	1.8 ± 0.3	0.09 ± 0.01^{ab}	116 ± 9	11.7 ± 1.8	54.6 ± 9.5^{b}
-1	23	0.2 ± 0.03^{ab}	43.8 ± 3.0	0.3 ± 0.06^{ab}	7.7 ± 0.9	0.6 ± 0.3	1.6 ± 0.4	0.10 ± 0.02^{ab}	113 ± 9	13.5 ± 1.7	80.3 ± 11.5^{ab}
0	23	0.5 ± 0.06^{c}	46.0 ± 3.7	0.6 ± 0.26^{b}	9.9 ± 1.0	0.9 ± 0.4	1.4 ± 0.3	0.15 ± 0.04^{ab}	130 ± 10	11.9 ± 1.2	91.2 ± 11.1^{ab}
1	21	0.2 ± 0.03^{bc}	46.3 ± 2.9	$0.3 \pm 0.10^{ab*}$	8.9 ± 1.0	0.7 ± 0.2	1.5 ± 0.3	0.09 ± 0.02^{ab}	120 ± 11	14.8 ± 2.4	89.1 ± 8.5^{ab}
2	20	0.3 ± 0.05^{bc}	46.6 ± 3.3	0.2 ± 0.06^{ab}	7.3 ± 0.8	0.7 ± 0.3	1.4 ± 0.3	0.14 ± 0.02^{ab}	103 ± 9	13.2 ± 1.6	109.1 ± 15.3^{a}
3	16	0.3 ± 0.03^{bc}	45.2 ± 3.3	0.2 ± 0.08^{ab}	8.1 ± 0.8	0.5 ± 0.05	1.3 ± 0.4	0.23 ± 0.03^{a}	101 ± 12	13.1 ± 1.8	106.8 ± 10.8^{a}

Different superscripts within columns denote differences ($p<0.05$).

Supplemental Table A.1. Standardbred colt somatic parameters from January (Month 1) to August (Month 8) 2015. Percent body fat was measured according the equation 3.83 + 5.58x, where x= cutaneous rump fat in centimeters, and testicular volume was calculated as 0.5233 x length x width x height in cm. All data shown as mean ± SEM.

Month	n	Age (mo)	Body fat (%)	Testicular volume (cm^3)	Weight (kg)
1	23	10 ± 0.2	6.9 ± 0.2[a]	19.2 ±3.7[ab]	325.9 ±6.4[a]
2	23	11 ± 0.2	6.7 ± 0.3[ab]	25.6 ± 4.3[b]	325.9 ±7.1[a]
3	23	12 ± 0.2	6.3 ± 0.2[b]	36.1 ± 5.9[bc]	331.3 ± 6.5[a]
4	23	13 ± 0.2	6.3 ± 0.2[b]	53.5 ± 7.8[c]	338.4 ± 6.7[ab]
5	21	14 ± 0.2	6.3 ± 0.2[ab]	79.1 ± 12.0[d]	346.5 ±7.6[b]
6	20	15 ± 0.2	6.2 ± 0.3[b]	85.4 ± 9.0 [d]	359.6 ± 8.4[c]
7	16	15.9 ± 0.2	6.9 ± 0.3[a]	93.8 ± 11.9 [d]	380.1 ± 8.1[d]

Superscripts denote significantly different parameters ($p<0.01$).

Supplemental Table A.2. Hormone concentrations in peripubertal Standardbred colts**.** Mean (± SEM) plasma testosterone (T4, ng/ml), androstenedione (AND, ng/ml), FSH (ng/ml), LH (ng/ml), estradiol (E2, pg/ml), leptin (LEPT, ng/ml), insulin (INS, µg/ml), IGF-1 (ng/ml), inhibin A (INH-A, pg/ml), and inhibin B (INH-B, pg/ml) concentrations in Standardbred colts from January (Month 1) to August (Month 8) 2015. Superscripts denote significantly different parameters ($p<0.01$).

Mo.	T4	AND	FSH	LH	E2	LEPT	INS	IGF-1	INH-A	INH-B
1	0.03 ± 0.01[a]	0.02 ± .01[a]	10 ± 1.6[a]	0.4 ± 0.06[ab]	35 ± 2.0	1.3 ± 0.4	0.1 ± 0.02[abc]	102 ± 22	NA	45.0 ± 9.8[bc]
2	0.06 ± 0.01[a]	0.1 ± 0.02[ab]	8 ± 0.6[a]	0.4 ± 0.03[ab]	41 ± 2.8	1.5 ± 0.2	0.10 ± 0.01[a]	131 ± 15	11.1± 2.2	42.2 ± 7.6[c]
3	0.09 ± 0.02[ae]	0.3 ± 0.10[ab]	7.7 ± 0.8[ab]	0.4 ± 0.03[ab]	51 ± 3.2	1.7 ± 0.3	0.08 ±.01[a]	170 ± 50	11.0 ± 1.8	56.2 ± 8.4[bc]
4	0.26 ± 0.04[b]	0.2 ± 0.10[ab]	7.7 ± 1.0[ab]	0.3 ± 0.06[a]	47 ± 3.6	1.6 ± 0.4	0.10 ± 0.04[ab]	109 ± 8	13.2 ± 1.7	77.2 ± 8.2[abc]
5	0.48 ± 0.26[c]	0.6 ± 0.3[ab]	10.4 ± 0.8[b]	0.5 ± 0.06[b]	42 ± 3.0	1.5 ± 0.3	0.14 ± 0.03[ab]	134 ± 10	13.1 ± 1.4	94.1 ± 8.9[ab]
6	0.24 ± 0.16[b]	0.3 ± 0.10[ab]	9.1 ± 1.0[a]	0.4 ± 0.03[ab]	46 ± 3.0	1.4 ± 0.3	0.05 ± 0.01[a]	166 ± 50	15.0 ± 2.3	92.1 ± 10.4[ab]
7	0.29 ± 0.22[b]	0.2 ± 0.06[ab]	8.1 ± 0.7[a]	0.4 ± 0.04[ab]	46 ± 3.1	1.4 ± 0.3	0.18 ± 0.02[bc]	103 ± 9	12.2 ± 1.7	101.8 ± 8.8[ab]
8	0.21 ± 0.14[bc]	0.2 ± 0.07[ab]	7.8 ± 0.8[a]	0.5 ± 0.04[ab]	44 ± 3.5	1.5 ± 0.4	0.23 ± 0.03[c]	100 ± 11	13.3 ±1.6	110.8 ± 18.4[a]

LH only 5-4 interaction is significant ($p<0.004$) and 8-4 $p=0.05$
n= 23 for months 1-4, n=21 month 5, and n=16 months 6-8.

REFERENCES

1. Wolf FR, Almquist JO, Hale EB. Prepuberal Behavior and Puberal Characteristics of Beef Bulls on High. J Anim Sci. 1965;24:761–765. PMID: 14313741
2. Brito LFC, Barth AD, Rawlings NC, Wilde RE, Crews DH, Mir PS, Kastelic JP. Effect of improved nutrition during calfhood on serum metabolic hormones, gonadotropins, and testosterone concentrations, and on testicular development in bulls. Domest Anim Endocrinol. 2007;33(4):460–469. PMID: 17029677
3. Brito LFC, Barth AD, Rawlings NC, Wilde RE, Crews DH, Mir PS, Kastelic JP. Circulating metabolic hormones during the peripubertal period and their association with testicular development in bulls. Reprod Domest Anim. 2007;42(5):502–508. PMID: 17845606
4. Casas E, Lunstra DD, Cundiff L V., Ford JJ. Growth and pubertal development of F1 bulls from Hereford, Angus, Norwegian Red, Swedish Red and White, Friesian, and Wagyu sires. J Anim Sci. 2007;85(11):2904–2909. PMID: 17591712
5. Naden J, Amann RP, Squires EL. Testicular growth, hormone concentrations, seminal characteristics and sexual behaviour in stallions. J Reprod Fertil [Internet]. 1990;88(1):167–176.
6. Cary JA, Madill S, Farnsworth K, Hayna JT, Duoos L, Fahning ML. A comparison of electroejaculation and epididymal sperm collection techniques in stallions. Can Vet J. 2004;45(1):35–41. PMID: 14992252
7. Aravindakshan J, Honaramooz A, Bartlewski P, Beard A, Pierson R, Rawlings N. Pattern of gonadotropin secretion and ultrasonographic evaluation of developmental changes in the testis of early and late maturing bull calves. Theriogenology. 2000;54:339–354.
8. Brown-Douglas CG, Parkinson TJ, Firth EC, Fennessy PF. Bodyweights and growth rates of spring- and autumn-born Thoroughbred horses raised on pasture. N Z Vet J. 2005;53(5):326–331. PMID: 16220125
9. Dhakal P, Hirama A, Nambo Y, Harada T, Sato F, Nagaoka K, Watanabe G, Taya K. Circulating pituitary and gonadal hormones in spring-born thoroughbred fillies and colts from birth to puberty. J Reprod Dev. 2012;58(5):522–530.
10. Skinner J, Bowen J. Puberty in the Welsh Stallion. 1967;(December):8–10.
11. Wesson J, Ginther OJ. Puberty in the male pony: plasma gonadotropin concentrations a n d the effects of castration. Anim Reprod Sci. 1981;4:165–175.
12. Brown BW. A review of nutritional influences on reproduction in boars, bulls and rams. Reprod Nutr Dev. 1994;34(2):89–114. PMID: 8179818
13. Renaville R, Van Eenaeme C, Breier BH, Vleurick L, Bertozzi C, Gengler N, Hornick JL, Parmentier I, Istasse L, Haezebroeck V, Massart S, Portetelle D. Feed restriction in young bulls alters the onset of puberty in relationship with plasma insulin-like growth factor-I (IGF-I) and IGF-binding proteins. Domest Anim Endocrinol. 2000;18(2):165–176. PMID: 10764973
14. Brito LFC, Barth AD, Rawlings NC, Wilde RE, Crews DH, Boisclair Y, Ehrhardt R, Kastelic JP. Effect of feed restriction during calfhood on serum concentrations of metabolic hormones, gonadotropings, testosterone, and on sexual development in bulls. Domest Anim Endocrinol. 2007;134:171–181. PMID: 17029677
15. Dance A, Thundathil J, Wilde R, Blondin P, Kastelic J. Enhanced early-life nutrition promotes hormone production and reproductive development in Holstein bulls. J Dairy Sci. Elsevier; 2015;98(2):987–998.

16. Mizukami H, Suzuki T, Nambo Y, Ishimaru M, Naito H, Korosue K, Akiyama K, Miyata K, Yamanobe A, Nagaoka K, Watanabe G, Taya K. Comparison of growth and endocrine changes in Thoroughbred colts and fillies reared under different climate conditions. J Equine Sci. 2015;26(2):49–56.
17. Tena-Sempere M, Barreiro ML. Leptin in male reproduction: The testis paradigm. Mol Cell Endocrinol. 2002;188(1–2):9–13. PMID: 11911940
18. Gholami H, Towhidi A, Zare Shahneh A, Dirandeh E. The relationship of plasma leptin concentration and puberty in Holstein bull calves (Bos taurus). J Anim Physiol Anim Nutr (Berl). 2010;94(6):797–802. PMID: 20455963
19. Kearns CF, McKeever KH, Roegner V, Brady SM, Malinowski K. Adiponectin and leptin are related to fat mass in horses. Vet J. 2006;172(3):460–465. PMID: 15996495
20. Gordon ME, McKeever KH, Betros CL, Manso Filho HC. Plasma leptin, ghrelin and adiponectin concentrations in young fit racehorses versus mature unfit standardbreds. Vet J. 2007;173(1):91–100. PMID: 16377220
21. Roser JF, McCue PM, Hoye E. Inhibin activity in the mare and stallion. Domest Anim Endocrinol. 1994;11(1):87–100.
22. Ohnuma K, Kaneko H, Noguchi J, Kikuchi K, Ozawa M, Hasegawa Y. Production of inhibin A and inhibin B in boars: Changes in testicular and circulating levels of dimeric inhibins and characterization of inhibin forms during testis growth. Domest Anim Endocrinol. 2007;33(4):410–421. PMID: 17010559
23. Westervelt R, Stouffer J, Hintz H, Schryver H. Estimating fatness in horses and ponies. J Anim Sci. 1976;43(4):781–785.
24. Love CC, Garcia MC, Riera FR, Kenney RM. Evaluation of measures taken by ultrasonography and caliper to estimate testicular volume and predict daily sperm output in the stallion. J Reprod Fertil Suppl. England; 1991;44:99–105. PMID: 1795307
25. Esteller-Vico A, Ball BA, Troedsson MHT, Squires EL. Endocrine changes, fetal growth, and uterine artery hemodynamics after chronic estrogen suppression during the last trimester of equine pregnancy. Biol Reprod. 2017;96(2):414–423.
26. Canisso IF, Ball BA, Esteller-Vico A, Williams NM, Squires EL, Troedsson MH. Changes in maternal androgens and oestrogens in mares with experimentally-induced ascending placentitis. Equine Vet J. 2017;49(2):244–249. PMID: 26729310
27. Munoz A, Trigo P, Riber C, Malonda V, Castejon F. A study of serum insulin-like growth factor type 1 (IGF-1) concentrations in resting untrained Andalusian horses: Influence of age and gender. Vet Med (Praha). 2011;56(5):231–242.
28. Abo El-Maaty AM, Mohamed AH, Abu-Aita NA, Morgan HM. Markers for Predicting Overweight or Obesity of Broodmares. J Equine Vet Sci [Internet]. Elsevier Ltd; 2017;56:9–18.
29. El-Badry DA, El Sisy GA, Abo El-Maaty AM. Seminal plasma hormonal profile of Arabian stallions that are classified “good” or “poor” for semen freezing. Asian Pacific J Reprod [Internet]. Hainan Medical College; 2016;5(6):453–458.
30. Öberg J, Bröjer J, Wattle O, Lilliehöök I. Evaluation of an equine-optimized enzyme-linked immunosorbent assay for serum insulin measurement and stability study of equine serum insulin. Comp Clin Path [Internet]. 2012;21(6):1291–1300.
31. Tinworth KD, Wynn PC, Boston RC, Harris PA, Sillence MN, Thevis M, Thomas A, Noble GK. Evaluation of commercially available assays for the measurement of equine insulin. Domest Anim Endocrinol [Internet]. Elsevier Inc.; 2011;41(2):81–90.

32. Thompson DL, Godke RA, Squires EL. Testosterone effects on mares during synchronization with altrenogest: FSH, LH, estrous duration and pregnancy rate. J Anim Sci. 1983;56(3):678–686.
33. Thompson DLJ, Reville-Moroz SI, Derrick DJ, Walker MP. Effects of testosterone, dihydrotestosterone and estradiol on gonadotropin release after gonadotropin releasing hormone administration in cyclic mares. Biol Reprod. United States; 1983 Nov;29(4):970–976.
34. Cartmill J, Thompson D, Gentry L, Storer W, Huff N. Endocrine responses in mares and geldings with high body condition scores grouped by high vs. low resting leptin concentrations. Am Soc Anim Sci. 2003;81:2311–2321.
35. Conley AJ, Scholtz EL, Dujovne G, Cotterman RF, Legacki EL, Uliani RC, Alvarenga MA, Ball BA, Kalra B, Savjani G V., Kumar A. Inhibin-A and inhibin-B in cyclic and pregnant mares, and mares with granulosa-theca cell tumors: Physiological and diagnostic implications. Theriogenology [Internet]. Elsevier Ltd; 2018;108:192–200.
36. Brown-Douglas CG, Firth EC, Parkinson TJ, Fennessy PF. The pituitary and testicular responses to GnRH challenge between 4 and 14 months of age in thoroughbred colts born in spring and autumn. Anim Reprod Sci. 2005;88(3–4):287–298. PMID: 16143218
37. Brown-Douglas CG, Firth EC, Parkinson TJ, Fennessy PF. Onset of puberty in pasture-raised Thoroughbreds born in southern hemisphere spring and autumn. Equine Vet J [Internet]. 2004;36(6):499–504.
38. Dagogo-Jack S, Fanelli C, Paramore D, Brothers J, Landt M. Plasma leptin and insulin relationships in obese and nonobese humans. Diabetes. 1996;45(5):695–698. PMID: 8621026
39. Considine R, Sinha M, Heiman M, Kriauciunas A, Stephens T, Nyce M, Ohannesian J, Marco C, McKee L, Bauer T, Caro J. Serum Immunoreactive-Leptin Concentrations in Normal-Weight and Obese Humans. Endocrinologist [Internet]. 1996;6(4):349.
40. Thomas MG, Keisler DH, Loch WE. Insulin-Like Growth Factor-I in Prepuberal Quarter Horses and Ponies. J Equine Vet Sci. 1998;18(1):52–55.
41. Thompson DL, Rahmanian CL, DePew CL, Burleigh DW, DeSouza CJ, Colborn DR. Growth Hormone in Mares and Stallions: Pulsatile Secretion, Response to Growth, Hormone-Releasing Hormone, and Effects of Exercise, Sexual Stimulation, and Pharmacological Agents. J Anim Sci. 1992;70:1201–1207.
42. Fortier LA, Kornatowski MA, Mohammed HO, Jordan M, O'Cain L, Stevens W. Age related changes in serum insulin-like growth factor-I, insulin-like growth factor binding protein-3, and articular cartilage structure in Thoroughbred horses. Equine Vet J. 2005;37(1):37–42.
43. Firth SM, Baxter RC. Cellular actions of the insulin-like growth factor binding proteins. Endocr Rev. 2002;23(6):824–854. PMID: 12466191
44. Duan C, Xu Q. Roles of insulin-like growth factor (IGF) binding proteins in regulating IGF actions. Gen Comp Endocrinol. 2005;142(1–2 SPEC. ISS.):44–52.
45. Le Roith D, Bondy C, Yakar S, Liu JL, Butler A. The somatomedin hypothesis: 2001. Endocr Rev. 2001;22(1):53–74. PMID: 11159816
46. Junnila RK, Strasburger CJ, Bidlingmaier M. Pitfalls of Insulin-like Growth Factor-I and Growth Hormone Assays. Endocrinol Metab Clin North Am. 2015;44(1):27–34.
47. Almeida J, Conley AJ, Mathewson L, Ball BA. Expression of steroidogenic enzymes during equine testicular development. Reproduction. 2011;141(6):841–848.

48. Stewart BL, Roser JF. Effects of age, season, and fertility status on plasma and intratesticular immunoreactive (Ir) inhibin concentrations in stallions. Domest Anim Endocrinol. 1998;15(2):129–139. PMID: 9532426
49. Tenover JS, McLachlan RI, Dahl KD, Burger HG, de Kretser DM, Bremner WJ. Decreased serum inhibin levels in normal elderly men: evidence for a decline in Sertoli cell function with aging. J Clin Endocrinol Metab. 1988;67(3):455–459. PMID: 3137240
50. Rivier C, Cajander S, Vaughan J, Hsueh AJW, Vale W. Age-dependent changes in physiological action, content, and immunostaining of inhibin in male rats. Endocrinology. 1988;123(1):120–126.
51. Schanbacher B. Radioimmunoassay of inhibin: Serum responses to unilateral and bilateral orchidectomy. Endocrinology. 1988;123(5):2323–2330.
52. Keeping HS, Winters SJ, Attardi B, Troen P. Developmental changes in testicular inhibin and androgen-binding protein during sexual maturation in the cynomolgus monkey, Macaca fascicularis. Endocrinology. 1990;126(6):2858–2867. PMID: 2351098
53. Burger HG, Mclachlan RI, Bangah M, Quigg H, Findlay JK, Robertson DM, Kretser DMDE, Warne GL, Werther GA, Hudson IL, Cook JJ, Fiedler R, Greco S, Yong ABW, Smith P. Serum Inhibin Concentrations Rise Throughout Normal Male and Female Puberty. J Clin Endocrinol Metab. 1988;67(4):689–694.

APPENDIX B: DIAGNOSIS AND EFFECTS OF URINE CONTAMINATION IN COOLED-EXTENDED STALLION SEMEN

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ABSTRACT

Urospermia is known to affect semen quality in many mammals, including stallions. Determinations of semen pH and creatinine and urea concentrations have been used to diagnose urine contamination in raw stallion semen. Unfortunately, practitioners suspecting urine contamination in cooled-shipped samples have no proven means to confirm the presence of urine. Therefore, the objectives of this study were (1) to assess the effects of urine contamination on sperm motility of extended fresh and cooled-stored stallion semen, (2) to evaluate the usefulness of semen color, odor, pH, and creatinine and urea concentrations for urospermia diagnosis, and (3) to evaluate the accuracy of a commercial blood urea nitrogen test strip in diagnosing urine contamination in extended-cooled stallion semen. Thirty-seven ejaculates were obtained from 11 stallions with no history of urospermia before division into 5 mL aliquots, and contamination with stallion urine. Each resulting sample was assessed for sperm motility, color, odor, pH, creatinine, and urea nitrogen concentration using both a semiquantitative test strip (Azostix), and a quantitative automated analyzer before and aftercooling for 24 hour. Sperm motility parameters,

pH, and creatinine and urea concentrations were analyzed using mixed models. Urine contamination decreased total and progressive motility in all samples before and after cooling ($P < 0.05$). Mean control total motility was 80% at 0 hour and 67% at 24 hours, whereas urine-contaminated samples ranged from 30% to 71% at 0 hour and 27% to 61% at 24 hours. Control mean urea (29 mg/dL) and creatinine (0.6 mg/dL) concentrations were significantly different ($P < 0.05$) from all urine-contaminated samples (158 mg/dL and 11.6 mg/dL, respectively) at 0 hour. Similarly, control mean urea (8 mg/dL) and creatinine (0.9 mg/dL) concentrations were significantly different than all urine-contaminated samples at 24 hours. Odor assessment presented moderate sensitivity (65%) and high specificity (100%), while color assessment presented low sensitivity (47%) and moderate specificity (79%) for urine in extended semen. Azostix strips were highly sensitive (95%) and specific (97%). Assessment of color, odor, and pH are not reliable methods to diagnose urine in experimentally contaminated cooled-stored stallion semen. Sperm motility parameters (in raw and cooled semen) are significantly reduced by the presence of urine in a concentration dependent. The results of the present study indicated that determination of urea and creatinine concentrations can be used to diagnose urospermia and that Azostix can be used as a point of care method for diagnosing urine contamination in extended cooled stallion semen.
Keywords: urospermia, stallion, semen, urea, creatinine

INTRODUCTION

Contamination of semen with urine (urospermia) is a well-recognized ejaculatory dysfunction that affects the quality of raw semen and may affect fertility in stallions [1-3]. The effects of urine on semen quality appear to be mediated by an increase in semen pH and osmolarity [4]. In one report at a referral hospital, urine contamination was the second most common ejaculatory dysfunction in stallions [5] Contamination of the semen with urine can occur at any time during ejaculation and can manifest as a continual or intermittent problem with an unpredictable pattern [6-9]. Urospermia has been associated with many conditions ranging from neoplasia or fractures that interfere with normal lumbosacral neurological pathways, to osteomyelitis, herpes-virus 1, sorghum toxicosis, cystitis, hyperkalaemic periodic paralysis, or idiopathic causes [1,6,7,10]. Overall, idiopathic causes appear to be the most prevalent [3,8].

Different management practices have been attempted to manage stallions presenting with recurrent urospermia, from encouraging stallions to urinate before collection or breeding, to

urinary bladder catheterization prior to collection, fractionated semen collection with open artificial vagina, or pharmacological intervention with drugs such as imipramine hydrochloride to improve bladder sphincter control [1-3,6,9]. However, the multifaceted and unpredictable occurrence of urospermia makes management and treatment difficult [2,7], and pharmacological interventions such as bethanechol, imipramine or furosemide are not always effective [11].

While it has been shown that extending semen appears to mitigate the effects of urine contamination on sperm motility [4], the effects of urospermia on the motility or fertility of cooled stallion semen is not documented. A large amount of urine contamination can be easily detected in raw semen by evaluation of odor and color [3,7], but diagnosis of urine contamination in extended cooled-shipped semen can be challenging. In practice, urine contaminated ejaculates may be shipped due to lack of knowledge of the personnel collecting and processing the semen, or for fraudulent reasons without disclosure to the practitioner breeding managing the mare. Thus, objective means to detect urine contamination in cooled-shipped semen are warranted from both the practical standpoint and legal standpoint (e.g. grounds for another semen collection and shipping). Creatinine (>2mg/dl) and urea (>25-30mg/dl) concentrations have been suggested to be useful to diagnose urine contamination in raw stallion semen [3,12,13] and the use of strip-paper has been used as a screening tool for urine contamination in raw semen, where urea levels >39 mg/dl indicate urospermia [12]. However, it remains unknown whether urine contamination can be properly diagnosed in cooled-extended semen using common means to assess urine contamination. We hypothesized that urine contamination affects sperm motility in extended cooled-stored semen in an amount-dependent manner, and that measuring creatinine and urea can accurately diagnose urospermia in extended cooled-stored semen but not color, odor, or semen pH assessments. The objectives of this study were: (i) to assess the effects of different amounts of urine contamination on sperm motility of extended fresh and cooled-stored stallion semen, (ii) to evaluate the usefulness of semen color, odor, pH, creatinine and urea concentrations for diagnosis of urine contamination in fresh extended and extended-cooled stallion semen, and (iii) to evaluate the accuracy of a commercial blood urea nitrogen test strip in diagnosing urine contamination in extended-cooled stallion semen.

MATERIALS AND METHODS

Animals, urine and semen collection

Eleven reproductively healthy light breed stallions (4 Standardbreds, 4 Quarter Horses, 3 Thoroughbreds), averaging 9 yrs. old (range 5 to 15 yrs. old), with no known history of urospermia were enrolled in this study. All stallions were handled in accordance with the United States Department of Agriculture Guide for Care and Use of Agricultural Animals in Research, in accordance with the University of Illinois Institutional Animal Care and Use Committee protocol #1400.

All animals were housed at the same research facility in small turnout runs, and fed similar grass/alfalfa hay diets. Free catch urine samples were obtained from three of the enrolled stallions, pooled and frozen at -20^{o}C until further use. Each stallion was collected on a dummy phantom, with a teaser mare present in the breeding shed using a Missouri model artificial vagina (Nasco^{TM,} Fort Atkinson, Wisconsin). Stallions were collected at two to three day intervals over a two-week period in November of 2014 until a total of thirty-seven ejaculates were obtained. The artificial vagina was lubricated (Priority Care1, First Priority Inc., Elgin IL) and fitted with a clean inline sperm filter (Har-Vet^{TM} ,Spring Valley, WI) for each semen collection.

Raw semen evaluation & processing

Immediately after semen collection, each ejaculate was assessed for initial motility using Computer-Assisted Sperm Analysis (CASA) using settings recommended by the manufacturer (Spermvision, Minitube of America, Verona, Wisconsin). Semen was extended with a milk protein-based extender (INRA 96 IMV, Maple Grove, Minnesota) to 25 million sperm/mL. A small aliquot (10 µL) of extended semen was placed on a heated slide with a coverslip, and assessed using CASA. Parameters analyzed included total percent of sperm motility, progressive sperm motility, sperm velocity parameters (VCL: curvilinear velocity (µm/s), VAP: average pathway velocity (µm/s), VSL: straight-line velocity (µm/s)) and coefficients (LIN: linearity (%), STR: straightness (%), WOB: wobble (%)) were assessed for all samples using CASA.

Sperm concentration in raw semen was analyzed in standard fashion using a spectrophotometer according to the manufacturer's recommendations (Equine Densimeter, Animal Reproduction Systems, Chino, California). Measurement of semen pH was attained using a hand-held pH meter (LAQUA Twin, Horiba Instruments, Irvine, California). The sample well

was filled until the bottom of the chamber was covered, and a reading was recorded when a stable measure was achieved. A standard buffer solution (pH=7) was used to calibrate the pH meter immediately before each analysis. The loading chamber was thoroughly rinsed with bi-distilled water and gently wiped dry between each analysis.

Each ejaculate was divided into five 5 mL aliquots, and either 0, 0.25, 1, 1.5, or 5 mL of pooled stallion urine was added (Figure 1). Every semen sample was then assessed for urea nitrogen concentration using a semi-quantitative test strip designed to assess urea nitrogen in whole blood (Azostix®, Siemens Healthcare Diagnostics, Tarrytown, New York) using a method similar to that used by Althouse et al [12]. Briefly, each Azostix® strip has a reagent pad that contains both urease and bromothymol blue. When a sample is placed on the reagent pad, urea present in the sample is hydrolyzed by urease to ammonium hydroxide and carbon dioxide. Ammonium hydroxide is alkaline and increases the pH. Bromothymol blue is used to indicate the change in pH, and will cause a concentration dependent change in reagent pad color. Simply, the more urea in a sample, the darker green the strip will become. For this study, the reagent pad was fully immersed with the sample being tested. After 60 seconds, the pad was rinsed thoroughly with distilled water, and immediately directly compared to the color chart on the side of the Azostix® bottle. The corresponding category closest to the color of the reagent strip was recorded, and if there was any ambiguity, the higher concentration was chosen. No change in color was recorded as negative for the presence of urea. The four color categories corresponded, from lightest to darkest, to (5-15 mg/dl), (15-26mg/dl), (30-40 mg/dl) and (50-80 mg/dl). Azostix® strips were graded as positive for urine contamination if the strip read >5 mg/dl in the raw and 5mg/dl in the extended cooled semen samples.

Semen odor was assessed as positive or negative for urine by three observers. Photographs were acquired of each sample by placing the test tube against a white background (Figure1). All tubes were identified by a random number, and semen color was graded at a later time as positive or negative by three observers who assessed the color via photograph, blinded to treatment group.

The semen aliquots were reassessed for sperm concentration and semen pH, and 1 mL of each sample was removed and frozen for later analysis of urea and creatinine using a quantitative automated analyzer (Beckman Coulter, Pasadena, California). The remainder of each aliquot was then extended with a commercial milk-based extender (INRA96, IMV Technologies, Maple Grove, Minnesota 55369) to a final concentration of 25 million sperm/mL. Total and progressive

motility were re-assessed with CASA, and the samples were then packaged in Whirl-paks (Nasco, Fort Atkinson, Wisconsin) and stored in commercial semen containers (Equitainer I, Hamilton Research, Inc., Ipswich, Massachusetts) for 24 hours.

Semen cooling and evaluation

At 24 hours, the semen containers were opened, and the samples were allowed to warm in an incubator to 37°C for 10 minutes before analysis. Semen pH, motility, color and odor were assessed for all samples as described above. Assessors were blinded to degree of urine contamination for both color (Fig 2) and odor assessments. Each sample was then retested using the commercial urea test strip as published elsewhere [12] and described above.

Statistical analyses

Statistical analyses were carried out with a commercially available software (JMP 11, SAS Institute, Cary, NC). Sperm motility, concentrations of creatinine and urea, and semen pH were analyzed using mixed models and when significant differences found, post hoc comparisons were made with LSD's test. Sensitivity and specificity for odor, color, and Azostix® were calculated using the urea nitrogen and creatinine concentrations. Agreement's tests were performed between evaluators for color and odor. Mean concentrations were calculated for urea nitrogen and creatinine in the control samples. The threshold for a urine free sample was calculated as three standard deviations above the mean concentration for a negative sample. Significance was set at $p<0.05$. Data were expressed as means ± SEM. Arbitrarily, specificity and sensitivity were classified as low (<60%), moderate (60-80%) and high (>80%).

RESULTS

None of the ejaculates presented spontaneous urine contamination as demonstrated by the low concentrations of urea (26 ± 1 mg/dl) and creatinine (0.6 ± 0.3 mg/dl). There were no significant effects of stallion for motility parameters and urea and creatinine levels in raw semen samples. Total and progressive motility decreased in all samples pre and post cooling in a dose-dependent urine contamination ($p<0.05$) (Table B.1). There were significant effects of treatment ($p<0.001$) and time by group interaction for total and progressive motility ($p<0.0001$). As expected, total and progressive sperm motilities were significantly reduced in urine contaminated

samples, both in raw and extended cooled-stored semen (Table 1). Sperm velocity parameters (VCL: curvilinear velocity (μm/s), VAP: average pathway velocity (μm/s), VSL: straight-line velocity (μm/s)) and coefficients (LIN: linearity (%), STR: straightness (%), WOB: wobble (%)) were assessed for all samples using CASA. Results for VCL and VAP for samples contaminated with 50% urine were significantly different ($p<0.01$) than all other samples at both 0 and 24h (Table1). VSL was significantly greater for control and 9% contaminated samples than for 23% and 50% contaminated samples at both 0 and 24h. No difference was observed between any samples for sperm coefficient measurements (LIN, STR, and WOB) at either 0 or 24 h (Table B.1).

Initial raw semen pH was lower than urine contaminated semen ($p< 0.05$) (Table B.2). This difference was not observed after extension and cooling for 24h, as the pH of the control group was not significantly different than the urine contaminated groups. The group with high percent of urine contamination (50%) had a pH significantly higher than the control groups and remaining contaminated groups (9 to 23%) (Table 2).

As expected, creatinine and urea concentrations were greater as increasing amounts of urine was added to the raw semen (Table B.2). Specificity and sensitivity for creatinine and urea were very high (Table B.3). Overall odor and color assessment of raw and cooled extended semen presented a wide range of sensitivity from low to high depending on the amount of urine contamination (Table 4). However, color and odor assessment of raw and extended semen contaminated with varying amounts of urine presented high specificity (Table B.4).

All negative controls tested negative at 0 h (37/37), and one positive sample tested negative (1/148). Azostix® test strips were highly sensitive and specific for raw semen (Table B.5)**.** All but one negative control tested negative (36/37) after extension and cooling, and all but six positive samples tested positive after extension and cooling (142/148). All false negatives were in the small degree of urine contamination group (9%). Azostix® were highly sensitive and specific for detecting urine in cooled extended semen (Table 5).

DISCUSSION

We demonstrated that creatinine, urea and Azostix® are highly specific and sensitive methods to evaluate urine contamination in fresh and cooled extended stallion semen. Creatinine and urea determinations in raw semen have been used to confirm urospermia in practice [3,8]. Here we documented that these two analytes can be used to diagnose urospermia in extended

semen samples as well. Previously, it had been demonstrated that Azostix® is an effective tool to diagnose urine contamination in raw semen samples [12], however, its effectiveness for extended semen has been questioned [3,13]. Using an experimental approach we confirmed that Azostix® can be a useful screening tool to detect even small amounts of urine contamination. Recently, in our practice, we have had the opportunity to put the findings of the present study to test in a Quarter Horse stallion and an Arabian stallion presenting with recurrent urospermia. The ejaculates were contaminated with apparently moderate and small amounts of urine and smelled like urine. We determined urea and creatinine concentrations and used Azostix® in raw semen samples and cooled extended semen (25 million sperm/mL) in 3 ejaculates. Results of these clinical cases fully support the findings of the present study obtained under experimental conditions (IC and RE unpublished).

The Azostix® manufacturer recommends waiting at least 60 seconds to read the test strip against the scale provided in the back of the container. We followed the manufacturer recommendations, but Althouse and collaborators [12] reported the occurrence of false positives if rinsing and reading was performed after 10 seconds. We did not find an increase in false positives when preforming the Azostix® analysis per manufacturer recommendations (waiting for 60 seconds) for blood urea nitrogen analysis. Even after waiting 60s, control samples remained negative for urine contamination. It is unknown if Azostix® has changed over the years but based on our present findings, we recommend following the manufacturer instructions. It is notable that the only false negative results were recorded for samples in the 9% urine contamination group. As demonstrated here, Azostix® is highly sensitive and specific when used to diagnose urine contamination in cooled semen, regardless of the percentage of urine contamination.

Practitioners in need of further diagnostics can submit semen samples for urea and creatinine concentrations. Samples with a urea concentration greater than 30 mg/dl or creatinine greater than 2.0 mg/dl are considered contaminated with at least a small amount of urine according to Hurtgen [14]. Our findings agree with Hurtgen's value [14] for creatinine for raw semen samples, but we found that his urea concentration was much lower than all 37 ejaculates evaluated in the present study, as the urea concentrations for all control semen samples were averaged 26 mg/dl ± 1 while all contaminated samples had a urea concentration greater than 95mg/dl.

Similarly, with the exception of one sample, all control samples had a creatinine of less than 1.7 (0.8 ± 0.3), and all contaminated samples had a creatinine greater than 3.5mg/dl. Our

findings suggest that potential thresholds for urea and creatinine concentrations in cooled extended samples should be >12 mg/dl and >1.3mg/dl respectively. Although these values could be highly variable in a clinical setting, these suggested values may offer some potentially useful reference values for practicing clinicians.

On the other hand, odor and color assessment are not sensitive methods to detect small amounts of urine in cooled extended semen samples, though both methods were highly specific regardless the amount of urine contamination. Color is not only influenced by urine contamination, but also by semen concentration, extender used, and the cleanliness of the ejaculate. Failure to properly wash the penis and remove smegma before collection can result in debris in the ejaculate and a change in color. Similarly, odor was a poor diagnostic tool for small amounts of urine contamination, but it was highly specific for moderate to high amounts of urine contamination in raw and cooled-extended semen. Odor may also be influenced by the stallion, extender used, or failure to assess odor after collection. If urine smell or color can be detected in cooled semen, the semen is likely contaminated with urine, which warrants further confirmation by objective methods such creatinine, urea and Azostix®.

While semen pH appears to be a highly useful tool to diagnose urine contamination in raw semen [4,15], it does not appear to be useful to assess urine contamination in extended-cooled samples. In our study, pH of control samples at 24h did not differ statistically to groups containing 9% to 23% of urine. However, pH of the group containing a high amount of urine contamination was significantly higher than the control group. While this is an interesting finding, its use in clinical practice may be difficult as there was a small numeric difference between the samples.

As expected, urine contamination affects initial sperm motility and also motility after cooling for 24 hours, even when a small amount of urine is present. Urine contamination was found to have a more pronounced effect on reduction of sperm motility in raw semen as compared to the cooled stored samples. Semen extension with a commercial milk protein-based semen extender may mitigate some of the urine's detrimental effects on stallion semen survival as suggested by Griggers et al. [4], perhaps by correcting the detrimental effects of a hypertonic solution and alkaline pH. However, as demonstrated here even a small amount of urine contamination (less than 10% of total semen volume) has deleterious effects on sperm motility, and may go unnoticed by personnel packaging and shipping semen. Thus, Azostix® strips represent a point-care means of testing for the presence of urine for practitioners presented with a semen shipment that has

unexplained poor motility or a suspicious color or odor. While it has been shown that urine contamination affects sperm motility [4], there have been no controlled studies showing effects of urine on fertility, but apparently reduction in fertility was associated with urospermia in a Thoroughbred stallion [1], but its effect on fertility of urine contaminated and then cryopreserved semen has not been demonstrated. In addition to decreased motility, it has been hypothesized that urine in the ejaculate may cause uterine irritation similar to that seen in mares with urovagina which can subsequently affect fertility. Naylor and collaborators reported no pregnancies from an HYPP H/H stallion with confirmed recurrent urospermia [10]. Other case reports have documented pregnancies from cooled shipped urine-contaminated samples where processing involved centrifugation [3]. Previous studies have reported stallion pregnancy rates per season have ranged from 35% to 80% for horses with urospermia [1,3,6], and variations may be in part due to the fertility of mares being bred. In our practice, we have had multiple ejaculates presented with perceived variable amounts of urine contamination resulting in pregnancies when young fertile mares were bred, but pregnancies were not confirmed when older subfertile mares were bred with urine contaminated semen.

In conclusion, semen pH, color and odor are not reliable tests for detection of small amounts of urine contamination in cooled-shipped samples, but a commercial blood urea nitrogen test strip can detect the presence of even small concentrations of urine in raw samples, and moderate degrees of urine contamination in cooled and extended samples. Practitioners in need of further diagnostics can submit semen samples for urea and creatinine concentrations. While this study provides practitioners information regarding potential threshold values for urea and creatinine concentrations when presented with ejaculates with suspected urospermia, work still needs to be done on the effects of urospermia on fertility, and improving semen quality of urospermic samples.

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TABLES

Table B.1.

Sperm motility parameters (Mean ± standard error of the mean) after urine contamination and extension to 25 million sperm/mL with INRA 96 at 0 h and after cooling for 24 h.

Group	TM (%)	PM (%)	VCL	VAP	VSL	LIN	STR	WOB
0 h								
0	80 ± 1.5^{a}	72 ± 2^{a}	178 ± 27.8^{a}	98.6 ± 15.8^{a}	76.1 ± 13.0^{a}	0.43 ± 0.06^{a}	0.77 ± 0.04	0.55 ± 0.06^{ab}
9%	71 ± 2.2^{b}	60 ± 3^{b}	175.6 ± 31.2^{a}	94.7 ± 15.0^{a}	73.3 ± 13.4^{ab}	0.42 ± 0.07^{a}	0.77 ± 0.06	0.54 ± 0.06^{ab}
17%	63 ± 2.7^{c}	50 ± 3.2^{c}	167.7 ± 39.8^{a}	86.6 ± 18.2^{a}	65.2 ± 14.6^{b}	0.39 ± 0.08^{a}	0.75 ± 0.07	0.52 ± 0.07^{ab}
23%	56 ± 2.6^{c}	42 ± 3.1^{d}	151.8 ± 40.1^{a}	73.5 ± 20.64^{a}	57.0 ± 13.9^{c}	0.38 ± 0.06^{a}	0.74 ± 0.06	0.51 ± 0.05^{a}
50%	30 ± 1.3^{d}	14 ± 1.4^{e}	86.5 ± 37.0^{b}	46.8 ± 13.2^{ab}	$35.1 \pm 10.8.^{d}$	0.45 ± 0.14^{a}	0.76 ± 0.06	0.57 ± 0.12^{b}
24 h								
0	67 ± 2^{ab}	53 ± 2.6^{a}	137.2 ± 30.7^{a}	71.6 ± 14.0^{a}	55.8 ± 10.3^{a}	0.41 ± 0.06	0.78 ± 0.06	0.52 ± 0.05
9%	61 ± 2.2^{b}	44 ± 3^{b}	133.6 ± 36.4^{a}	70.2 ± 17.2^{a}	54.6 ± 13.2^{a}	0.41 ± 0.07	0.78 ± 0.07	0.53 ± 0.05
17%	52 ± 2.2^{c}	35 ± 2.7^{c}	124.7 ± 52.6^{a}	65.6 ± 25.2^{a}	49.5 ± 17.9^{a}	0.42 ± 0.08	0.76 ± 0.07	0.54 ± 0.08
23%	46 ± 2.2^{c}	28 ± 2.7^{c}	121.3 ± 45.1^{a}	62.9 ± 19.8^{ab}	47.0 ± 15.2^{ab}	0.4 ± 0.06	0.74 ± 0.07	0.53 ± 0.07
50%	27 ± 1.3^{d}	8.8 ± 2.2^{d}	93.2 ± 37.9^{b}	49.7 ± 15.18^{ab}	36.5 ± 12.3^{ab}	0.41 ± 0.14	0.73 ± 0.09	0.55 ± 0.08

Different superscript letters within columns denotes statistical differences between groups ($P < 0.05$).
LIN, coefficient of linearity (%); PM, percent of progressive motile sperm; STR, coefficient of straightness (%); TM, percent of total motile sperm; VAP, average pathway velocity (μm/s); VCL, curvilinear sperm velocity (μm/s); VSL, straight-line velocity (μm/s); and WOB, coefficient wobble (%).

Table B.2.

Mean semen pH, urea (mg/dL), and creatinine (mg/dL) ± standard error of the mean at initial semen collection and after extension with INRA96 to 25 million/mL and cooled storage for 24 h for five different degrees of urine contamination (n = 185).

Groups	pH (0 h)	pH (24 h)	Urea (0 h)	Urea (24 h)	Creatinine (0 h)	Creatinine (24 h)
0%	7.54 ± 0.03^{a}	7.16 ± 0.02^{a}	29 ± 4^{a}	8 ± 0.3^{a}	0.6 ± 0.3^{a}	0.9 ± 0.03^{a}
9%	7.85 ± 0.02^{b}	7.22 ± 0.01^{a}	158 ± 5^{b}	33 ± 2^{b}	11.6 ± 0.3^{b}	2.9 ± 0.1^{b}
17%	7.94 ± 0.02^{b}	7.32 ± 0.01^{a}	277 ± 7^{c}	58 ± 3^{c}	21.2 ± 0.6^{c}	5.1 ± 0.2^{c}
23%	7.99 ± 0.03^{b}	7.37 ± 0.01^{ab}	356 ± 6^{d}	86 ± 6^{d}	29.3 ± 0.7^{d}	7.1 ± 0.4^{d}
50%	8.07 ± 0.03^{b}	7.56 ± 0.02^{b}	775 ± 17^{e}	228 ± 12^{e}	64.9 ± 0.9^{e}	18.8 ± 1.6^{e}

Different superscript letters within columns denotes statistical differences between groups ($P < 0.05$).

Table B.3.

Overall sensitivity and specificity for urea and creatinine concentrations.

Time	Methods				
	Groups	Creatinine		Urea nitrogen	
		Sensitivity	Specificity	Sensitivity	Specificity
0 h	9%	100%	97%	95%	100%
	17%	100%	97%	100%	100%
	23%	100%	97%	100%	100%
	50%	100%	97%	100%	100%
Overall means		100%	97%	99%	97%
24 h	9%	100%	100%	100%	100%
	17%	100%	100%	100%	100%
	23%	100%	100%	100%	100%
	50%	100%	100%	100%	100%
Overall means		100%	100%	100%	100%

Table B.4.

Sensitivity and specificity of different methods for analyzing urine contamination in raw stallion semen (0 h), and semen extended with INRA 96 to 25 million sperm/mL and cooled for 24 h (24 h; n = 185 samples).

Time	Methods						
	Groups	Color		Odor		Azostix	
		Sensitivity	Specificity	Sensitivity	Specificity	Sensitivity	Specificity
0 h	9%	51%	82%	27%	96%	97%	100%
	17%	74%	82%	77%	96%	100%	100%
	23%	80%	82%	95%	96%	100%	100%
	50%	98%	82%	100%	96%	100%	100%
Overall means		76%	82%	75%	96%	99%	100%
24 h	9%	26%	79%	19%	100%	83%	97%
	17%	41%	79%	55%	100%	100%	97%
	23%	46%	79%	86%	100%	100%	97%
	50%	77%	79%	100%	100%	100%	97%
Overall means		47%	79%	65%	100%	95%	97%

REFERENCES

1. Lowe J. Diagnosis and management of urospermia in a commercial thoroughbred stallion. Equine Vet. Educ. 2001;13(1):4-7.
2. Turner RO, Love C, McDonnell S, Sweeney R, Twitchell E, Habecker P, et al. Use of imipramine hydrochloride for treatment of urospermia in a stallion with a dysfunctional bladder. J. Am. Vet. Med. Assoc. 1995; 207:1602-6.
3. Dascanio JJ, Witonsky SG. Theriogenology question of the month. urospermia. J. Am. Vet. Med. Assoc. 2005; 227:225-7.
4. Griggers S, Paccamonti DL, Thompson RA, Eilts BE. The effects of pH, osmolarity and urine contamination on equine spermatozoal motility. Theriogenology 2001;56:613-22.
5. McDonnell SM. Physiology and dysfunction. Vet. Clin. North Am.: Equine Pract. 1992; 8:57-70.
6. Nash JG,Jr, Voss JL, Squires EL. Urination during ejaculation in a stallion. J. Am. Vet. Med. Assoc. 1980; 176:224-7.
7. Voss J, McKinnon A. Hemospermia and urospermia. Equine Reproduction.Lea and Febiger, Philadelphia 1993:864-70.
8. Turner RMO. Urospermia and hemospermia. In: Samper J, Pycock J, McKinnon AO, editors. Current Therapy in Equine Reproduction. Saint Louis: W.B. Saunders; 2007, p. 258-65.
9. Varner DD, Schumacher J, Blanchard TL, Johnson L. Diseases affecting semen. In: Varner DD, Schumacher J, Blanchard TL, Johnson L, editors. Diseases and management of breeding stallions. 1st ed. Goleta CA: American Veterinary Publications; 1991, p. 337-40.
10. Naylor JM, Nickel DD, Trimino G, Card C, Lightfoot K, Adams G. Hyperkalaemic periodic paralysis in homozygous and heterozygous horses: A co-dominant genetic condition. Equine Vet. J. 1999;31:153-9.
11. Hoyos Sepulveda M, Quiroz Rocha G, Brumbaugh G, Montiel Q, Rodríguez M, Candanosa de Morales E. Lack of beneficial effects of bethanechol, imipramine or furosemide on seminal plasma of three stallions with urospermia. Reproduction in Domestic Animals 1999;34:489-93.
12. Althouse GC, Seager SWJ, Varner DD, Webb GW. Diagnostic aids for the detection of urine in the equine ejaculate. Theriogenology 1989;31 :1141-8.
13. Dascanio JJ. Diagnosis and management of urospermia. In: Dascanio JJ, McCue P, editors. Equine Reproductive Procedures. 1st ed. John Wiley & Sons; 2014, p. 404,-405.
14. Hurtgen J. Stallion genital abnormalities. In: Robinson NE, editor. Current Therapy in Equine Medicine. 2nd ed. Philadelphia: WB Saunders Co; 1987, p. 561.
15. Ball BA. Diagnostic methods for evaluation of stallion subfertility: A review. J Equine Vet Sci 2008; 28:650-65.

APPENDIX C: EFFECTS OF URINE CONTAMINATION ON STALLION SEMEN FREEZING ABILITY

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ABSTRACT

Urospermia is a common ejaculatory dysfunction of stallions. Current practice suggests that urine contaminated semen should not be used for cryopreservation. The aim of this study was to determine effects of urine contamination on semen cryopreservation. Sixty-five ejaculates from eight stallions were divided into no urine (CONT), low (20% urine, LOW), and high (50% urine, HIGH) samples. Semen was extended with a commercial cooling extender, cushion-centrifuged, resuspended to 200 million/mL in a commercial egg-yolk based extender, and cryopreserved. A subset of ejaculates (n=20) were split in two after cushion-centrifugation, and one half of the ejaculate was submitted to a single-layer gradient centrifugation before cryopreservation. Sperm

motility were assessed pre- and post-freezing with an automated sperm analyzer. Semen pH, creatinine, and urea concentrations were assessed in raw samples, after urine contamination and after centrifugation and extension. Statistical analyses were performed with ANOVA and Tukey's posthoc. There was a significant reduction in total and progressive sperm motilities (i.e., %TM and %PM, respectively) with increasing urine contamination pre-freezing (%TM 67±1.7, %PM 50 ± 2.2, CONT), (%TM 60.3 ±1.7, % PM 42.5 ± 2.1, LOW), and (%TM 41.3 ± 2, %PM 21.3 ± 1.5, HIGH). Post-thaw motilities for CONT (%TM 54 ± 2.3, %PM 40.8 ± 3.3) and LOW (%TM 51.7 ± 1.8, %PM 36.2 ± 2.1) were not different, but were higher than the HIGH (%TM 31.5 ± 1.2, %PM 17.1 ± 1.0) ($p<0.05$). Post-thaw sperm viability was significantly lower in the HIGH (54.7 ± 2.4) than in the CONT (63.8 ± 2.3) or LOW (64.6 ± 3.4) groups. Semen creatinine and urine levels were significantly higher with increasing urine contamination, and were significantly decreased after centrifugation and resuspension in freezing extender. Pre-treatment semen pH was significantly lower than semen contaminated with low or high amounts of urine, and pH decreased significantly after centrifugation and resuspension. Semen contaminated with a small amount of urine may be suitable for cryopreservation, whereas highly contaminated semen might not be usable. Although urine was mostly removed in this fashion, the initial exposure to high quantities was sufficient to decrease sperm motility pre- and post-freezing, whereas low urine contamination was not as detrimental.

Keywords: Urospermia, sperm parameters, semen cryopreservation, horses

INTRODUCTION

Urine contamination of semen is one of the most common ejaculatory dysfunctions in stallions[1]. Occurrence of urospermia is unpredictable and may present as a rare, isolated event, intermittently, or frequently [2-4]. Unfortunately, some stallions may present with urospermia throughout their entire breeding career, which makes the breeding management for optimal fertility a true challenge in clinical practice [3,5] .

Urospermia has been associated with a wide range of conditions, such as neoplasia or fractures that interfere with normal lumbosacral neurological pathways, osteomyelitis, equine herpesvirus 1, sorghum poisoning, cystitis, and hyperkalemic periodic paralysis [5,10,11]. However, idiopathic causes appear to be the most prevalent, which challenges the ability to properly treat or manage the condition [11,12]. Different management practices have been

implemented for stallions presenting with recurrent urospermia, including encouraging stallions to urinate before collection or mating, urinary bladder catheterization before collection, fractionated semen collection, and pharmacological intervention with drugs such as imipramine hydrochloride to improve bladder sphincter control [2-4,13-15]. Unfortunately, even implementing these techniques does not prevent urospermia in some stallions [2,5,11,14].

Detrimental effects of urine on semen appear to be mediated by alkaline pH, high osmolality and possibly the high concentration of crystals [2,5-7]. However, it is currently unknown how the different sperm cell compartments and functions are affected by urine contamination. Urine might also interfere with fertility by inducing endometrial inflammation in mares mated by affected stallions [5]. The adverse effect of urine on both sperm quality and endometrium may be mitigated by extension and centrifugation [8,9]. Immediate extension in semen extender, followed by cushioned centrifugation and resuspension seems to help preserve quality of urine contaminated semen [9]. However, to the best of our knowledge, this method does not account for damage to semen in collections where urospermia was not expected and sperm are exposed to urine for a prolonged period during processing.

Up until now, no studies have evaluated the effects of urine contamination on stallion semen cryopreservation. Unfortunately, genetically and economically valuable stallions may present ejaculates that are intermittently or constantly affected by urine contamination, which imposes a major barrier to effectively cryopreserve semen from these stallions. Currently, the most acceptable clinical practice is to discard ejaculates contaminated with urine and repeat semen collection in hopes of obtaining a urine-free sample. It is assumed that semen cannot be cryopreserved from stallions with recurrent urospermia. Our clinical experience suggests that it might be possible to freeze mildly urine-contaminated semen with apparent success. Therefore, we felt compelled to critically address this question in the present study and provide evidence-based recommendations for the use of urospermic semen during cryopreservation. The results would be particularly relevant for valuable studs that experience urospermia but have low sperm counts or are difficult to re-collect due to orthopedic, neurologic or behavioral impairment. Additionally, we were interested in determining if using gradient centrifugation, which selects for viable sperm, would improve standard semen freezing in urine-contaminated samples.

We hypothesized that semen experimentally contaminated with low, but not high, amounts of urine can be successfully cryopreserved with only marginal changes in sperm quality compared

to control samples. Therefore, the objectives of this study were to evaluate the effects of urine contamination on sperm parameters of cryopreserved stallion semen and to determine if single-layer gradient centrifugation improves semen quality of cryopreserved semen.

MATERIALS AND METHODS

The present study was performed from January to February 2017. The Institutional Animal Care Unit Committee under the protocol # 14200 approved all procedures carried out in the present study.

Animals and semen collections

Eight healthy mature light breed stallions (four Standardbreds, one Appaloosa, one Paint and two Quarter Horses) from 5-18 years of age (mean 9.5 ±1.4 years) were enrolled in the study. The animals were housed in stalls at the Illinois Veterinary Teaching Hospital, College of Veterinary Medicine, University of Illinois Urbana-Champaign and fed similar grass/alfalfa hay diets.

Free catch urine samples were collected one week before the experiment from three of the stallions enrolled in the study. Urine was pooled, split into 100 mL aliquots, and then froze at -20^{o}C until use. One week before the experimental collections, three clean out collections were performed per stallion. Stallions were then collected at 2 to 3 day intervals on a dummy mount, using a Missouri artificial vagina (Nasco Fort Atkinson, Wisconsin) until five (n=3 stallions) to ten (n=5 stallions) ejaculates were obtained. The artificial vagina was lubricated (Priority Care1 First Inc., Elgin Illinois) and fitted with a clean in-line sperm filter (Har-Vet, Spring Valley, Wisconsin) and a collection plastic bag (Whirl-Paks Nasco, Fort Atkinson, Wisconsin) for each semen collection.

The gel-free portion of each ejaculate was weighed and grossly assessed for the presence of contaminants. Semen pH was measured using a commercially available hand-held pH meter (LAQUA Twin, Horiba Inc., Irvine, CA, 92618) [8]. A small aliquot of raw semen was frozen in cryovials for measurement of creatinine and urea using an automated quantitative analyzer (Beckman Coulter, Pasadena, CA 92821). Urine free ejaculates were defined as those with no visible gross contamination with urine (e.g., high volume, pungent urine smell, and yellow-tinged), presenting semen pH ≤7.7, and urea and creatinine concentrations of ≤40 mg/dl and ≤ 2ng/ml,

respectively [2,6-8,15,16]. All sixty-five ejaculates collected attained the definition criteria of urine free ejaculates, therefore, further processed and included for the analyses.

Experimental design

Immediately after collection, semen concentration was determined using a spectrophotometer (Equine Densimeter, Animal Reproduction Systems, Chino, California) according to the manufacturer's instructions. Semen was then allocated into six 50 mL conical tubes, and for each ejaculate, a control, low, and highly contaminated group were created. The control group (CON) had 10 mL of raw semen added to the tube, whereas, the low (LOW) and high (HIGH) urine contamination groups had 8 mL and 5 mL of raw semen added to the tubes. Urine aliquots of 100 mL were thawed overnight in a refrigerator and warmed at 37^{o}C immediately before use. Two milliliters of urine was added to each LOW tube, and 5 mL of urine was added to each HIGH tube. Once the semen had been exposed to urine for two minutes at room temperature, each of the six tubes was extended to 40 mL with a milk-based semen extender (Equi-Pro® Cool Guard®, Timentin, Minitube of America, Vernon, Wisconsin). This length of exposure accounts for the estimated time that the semen would be directly exposed to urine before semen extender could be added during routine collection and processing. Final semen concentrations pre-centrifugation ranged from 20 million/ml to 79 million/ml.

Following extension, 1 mL of cushion solution (Maxifreeze®, IMV, Maple Grove, MN) was added to the bottom of each conical tube using a long spine needle (18G 3.5'') as previously described [17]. Semen was centrifuged at room temperature (1000 x *g*, 20 minutes). Following centrifugation, the supernatant was aspirated with a Sink Vacuum Aspirator Pump® (Animal Reproduction System, Chino, California) and discarded. The cushion solution was aspirated with a long spinal needle and discarded. The sperm pellet was resuspended and concentration was determined with a hemocytometer. For a subset of ejaculates (n=20), a portion of the resuspended semen was submitted to gradient centrifugation before freezing using a commercially available silane-coated silica solution (EquiPure™ Nidacon, Mölndal, Sweden). Three milliliters of Equi-Pure™ were placed on the bottom of 15 mL conical tube, thereafter, 1 billion of sperm cells resuspended on 6 mL of INRA 96 (IMV, Maple Grove, MN) were carefully loaded on top of the colloid solution. Then the semen was centrifuged at 400 g for 30 minutes. After centrifugation, the EquiPure™ and any semen within the gradient, or still resting on top of the gradient were aspirated

and discarded. The selected sperm pellet was aspirated from the bottom of the tube, the percent of yield was calculated, and the resulting sperm were resuspended in a freezing extender. Thereafter, the semen (with/out gradient) was extended to a final concentration of 200 million sperm/mL, with a commercially available freezing extender (Botucrio® Botupharma, Botucatu Sao Paulo, Brazil). The extended semen was manually loaded into 0.5 mL straws and cooled at 5°C for 20 minutes. Straws were then placed 4 cm above liquid nitrogen for 15 minutes before plunging into liquid nitrogen. Cryopreserved samples were stored in nitrogen tanks until further analyses.

Sperm motility analyses

Immediately before semen freezing (i.e., after centrifugation and extension), and after freezing and thawing, each ejaculate was assessed for sperm motility parameters by Computer-Assisted Sperm Analysis (CASA) (Spermvision II®, Minitube of America, Verona, Wisconsin) [238]. Thawing was performed by submerging straws into a 37°C water bath for at least 30 seconds. Semen was extended with a temperature matched milk protein-based extender (INRA 96® IMV, Maple Grove, Minnesota) to 20 million sperm/mL. A small aliquot (5 µL) of extended semen was placed on a heated slide and motion characteristics of a minimum of 1000 cells were analyzed in a least ten different fields. The preset values for the computer-assisted sperm analyzer system were as follows: static cell area 14 to 80 μm^2; straightness (STR) threshold for progressive motility, 90; average path velocity threshold for progressive motility, 40; average path velocity threshold for static cells < 9.5; cell intensity, 106; and light-emitting diode illumination intensity, 1800 to 2550. Parameters assessed included total percent of sperm motility, progressive sperm motility, sperm velocity parameters (curvilinear velocity [VCL, µm/s]; average path velocity [VAP, µm/s]; straight-line velocity [VSL µm/s]; and coefficients (linearity [LIN, %] and straightness [STR %]).

Statistical analysis

Data were analyzed using RStudio v 0.99.489 (RStudio Team, Boston, MA). Normality of data was confirmed within histograms and using a Shapiro-Wilk test of the residuals. Sperm motility were analyzed by mixed models, with treatment group considered a fixed effect and stallion and ejaculate number as random effects. When significant, posthoc comparisons were made with Tukey's HSD test. Simple correlations were determined between urine contamination

measures and motility variables. Data are expressed as mean ± SEM. Statistical significance was set at $p < 0.05$.

RESULTS

Semen creatinine and urine levels were significantly higher with increasing urine contamination, and were significantly decreased after centrifugation and resuspension in freezing extender (Table C.1). Pre-treatment semen pH was significantly lower than semen contaminated with low or high amounts of urine, and pH decreased significantly after centrifugation and resuspension. There was a significant reduction in total and progressive sperm motility with increase in urine contamination pre-freezing (Table C.2). Post-thaw motilities for control and low were not different from one another but were higher than the high group, and significantly lower than pre-freeze parameters (Table C.2). Similarly, VCL, VAP, and VSL were all significantly lower in highly contaminated semen than in the control or low groups on both pre-freeze and post-thaw examinations (Table C.2). Semen pH, creatinine, and urea concentrations were strongly correlated with total and progressive motility on pre-freeze (Table C.3), and post-thaw analysis (Table C.4).

Gradient centrifugation did not improve total motility parameters in the control groups, but did improve pre-freeze total and progressive motilities in the low and high groups, and improved significantly post-freezing total and progressive motility in the high contaminated group (Table C.5). After gradient centrifugation, there were no differences in post-thaw total motility between treatment groups. While progressive motility improved, post-thaw progressive motility in the high group was still significantly lower than the control group (Table C.5). No differences were seen pre-freeze in the other sperm motility parameters (VCL, VSL, and VAP) between the non-gradient samples, and the samples subjected to gradient centrifugation other than a slight increase in pre-freeze VAP in the high group after gradient centrifugation (Table C.6). This difference disappeared on post-thaw evaluation. Percent yield after gradient centrifugation did not differ between treatment groups (Control 49.7 ± 5.5 %; Low 45.6 ± 5.4%; High 55.8± 5.5%, p = 0.43), or between horses (p= 0.16).

DISCUSSION

Our findings in the present study suggest that urine contamination affects stallion semen in a dose-dependent manner. Additionally, our results appear to support our hypothesis and clinical experience that mildly contaminated ejaculates from stallions affected by conditions causing persistent urospermia can still be processed for cryopreservation. While the *in vitro* results in this study suggest that semen freezing ability is not affected by low urine contamination, further studies are needed to determine the effects clinical urospermia on the cryopreservation of semen, and to determine the effects on fertility after insemination with the frozen-thawed urospermia samples.

It is hypothesized that urine is detrimental to semen motility because urine increases semen pH and osmolarity. Griggers et al [6] demonstrated that both alkaline pH and high semen osmolarity have negative effects on semen motility. Studies have suggested that the deleterious effects of urine contamination can be mitigated by extension of the urine contaminated semen with milk-based extenders in both raw [6] and cooled-stored semen [8]. A subsequent study suggested that cushion centrifugation was also useful for mitigating the effects of urine contamination on cooled stallion semen [9]. Herein, we demonstrated that cushion centrifugation and standard extension for cryopreservation appeared to effectively remove urine from the experimentally contaminated ejaculates, as evidenced by the reduction in creatinine and urea after centrifugation. Although urine was mostly removed in this fashion, the initial exposure to high quantities was sufficient to decrease sperm motility pre- and post-freezing, whereas low urine contamination was not detrimental.

While pre-freeze motilities were different between groups, no differences in motility were seen between the control and low contamination groups on post-thaw examination, suggesting that the centrifugation and freezing process eliminated the effects of low amounts of urine on semen parameters. Highly contaminated samples (50% urine) had significantly lower progressive and total motilities both pre-freeze and on post-thaw examination. Parameters used to assess degree of urine contamination (pH, urea, and creatinine) were highly correlated with total and progressive motility on both pre -freeze and post-thaw evaluations, suggesting that these parameters might be useful for determining when an ejaculate is still suitable for cryopreservation.

When semen was subjected to additional gradient centrifugation before freezing, pre-freeze total motility improved in the both urine-contaminated groups, and pre-freeze progressive motility improved in all groups. Similarly, post-thaw total and progressive motilities were improved in

highly contaminated ejaculates subjected to gradient centrifugation before freezing, suggesting that additional processing may improve semen quality in contaminated ejaculates. This suggests that highly contaminated samples might be used if further processed, and if the breeding dose is adjusted for the lower post-thaw motilities. When faced with a stallion with persistent urospermia, the combination of semen processing, coupled with an increase in breeding dose, may allow these stallions to be successfully cryopreserved. Interestingly, post-thaw progressive motility decreased in the control group that was subjected to gradient centrifugation, suggesting that single layer gradient centrifugation may be detrimental to normal sperm.

Gradient centrifugation selects for viable sperm, and increased yield would be expected in higher quality ejaculates with little to no urine contamination. This is important to consider when the decision is made to retain and process highly contaminated ejaculates, as lower yields would be expected. Interestingly, no differences were detected between treatment group yields after gradient centrifugation in the current study. It is important to note that this may be due to small sample size, as yield data was not available for all ejaculates, and there was a large variation in yield between ejaculates. Nevertheless, the data herein suggests a reasonable use of gradient centrifugation to improve semen quality in highly contaminated ejaculates of valuable stallions with potentially comparable yields.

Given the effects of urine on both pre-freeze [6,8,9] and post-thaw semen parameters, the next logical steps would be to confirm that gradient centrifugation can improve semen quality in clinical urospermia cases and to evaluate effects of different semen processing methods on fertility. Urospermia is thought to affect female fertility by inducing endometrial inflammation after breeding [5]. In the current study, we have demonstrated that centrifugation removes the majority of urea and creatinine, which may prevent endometrial irritation when using processed urine-contaminated samples for artificial insemination. Therefore, it would be interesting to determine if the removal of urine from a highly contaminated sample combined with an increase in sperm dose to overcome the effects on sperm motility can produce comparable pregnancy rates. Developing a protocol such as this may prove useful for valuable stallions who are otherwise difficult to collect.

In conclusion, this study has demonstrated that cushioned centrifugation can remove the majority of urea and creatinine from urine contaminated ejaculates, and that ejaculates with 20 % or less urine contamination may still be cryopreserved with satisfactory results. Highly

contaminated ejaculates may be improved with processing before cryopreservation but will still have lower post thaw progressive motility parameters than uncontaminated ejaculates. Therefore, use of these samples will depend on individual stallion fertility and management of the mare being bred. We predict that stallions and horse owners will directly benefit from the results of the present study, as it provides suggestions on how to manage urospermic samples, and potential parameters for deciding when an ejaculate is freezable.

Authors' contribution

IFC, JLS, FSL, and CCL participated in the conceptualization of the study and grant application. IFC, REE, JH, and BRC cryopreserved the semen. REE and JLS performed the post-thaw sperm motility and flow cytometry assessments. REE and IFC analyzed the data and wrote the manuscript. All authors reviewed the manuscript.

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TABLES

Table C.1. Mean ± SEM – Semen pH, urea (mg/dL), and creatinine (mg/dL) concentrations pre- and post-centrifugation (n=40 ejaculates from eight stallions).

		Pre-centrifugation (raw)			Post Centrifugation		
Groups	n	pH	Urea	Creatinine	pH	Urea	Creatinine
Control	40	7.5 ± 0.04^{a}	28 ± 1.5^{a}	0.8 ± 0.2^{a}	6.8 ± 0.03^{d}	2.4 ± 0.2^{d}	1.8 ± 0.1^{d}
Low	40	8.3 ± 0.03^{b}	322 ± 10^{b}	40 ± 1.3^{b}	7 ± 0.03^{e}	15 ± 2.0^{e}	3.1 ± 0.1^{d}
High	40	8.6 ± 0.02^{c}	721 ± 17^{c}	87.2 ± 1.7^{c}	7.2 ± 0.02^{f}	48 ± 5.1^{a}	7.1 ± 0.6^{e}

Control group represented samples not contaminated with urine; Low group was contaminated with 20% of urine contamination; High group was contaminated with 50% urine. Mean extender pH was 7.05. Different superscripts within columns and rows for the same parameter denote differences ($p<0.05$).

Table C.2. Mean ± SEM – Stallion sperm motility parameters pre-freezing (after cushion centrifugation and extension) and post-thawing (n=8 stallions/64 ejaculates).

		Pre-freezing				
	n	TM	PM	VCL	VAP	VSL
Control	64	67 ± 1.7^{ax}	50 ± 2.2^{ax}	152 ± 2.5^{ax}	76 ± 1.1^{ax}	60 ± 3^{ax}
Low	64	60 ± 1.7^{bx}	42 ± 2.1^{bx}	145 ± 2.8^{bx}	73 ± 1.2^{ax}	57.5 ± 2^{ax}
High	63	41 ± 2^{cx}	21 ± 1.5^{cx}	118 ± 6.7^{cx}	58 ± 1.5^{bx}	45 ± 2.3^{bx}
		Post-thaw				
	n	TM	PM	VCL	VAP	VSL
Control	64	54 ± 2.3^{ay}	41 ± 3.3^{ay}	102 ± 3^{ay}	56 ± 1.4^{ay}	43 ± 1.2^{ay}
Low	64	52 ± 1.8^{ay}	36 ± 2.1^{ay}	102 ± 2.7^{ay}	57 ± 1.3^{ay}	44 ± 1.1^{ay}
High	63	31 ± 1.2^{by}	17 ± 1^{by}	86 ± 2.3^{by}	48 ± 1.1^{ay}	37 ± 1^{by}

C: control group; L: low urine contamination group; H: high contamination group. TM: percent of total sperm motility; PM: percent of progressive sperm motility; VCL curvilinear sperm velocity (μm/s); VAP average path velocity (μm/s); VSL straight-line velocity (μm/s); LIN coefficient of linearity (%). Control group represented samples not contaminated with urine; Low group was contaminated with 20% of urine contamination; High group was contaminated with 50% urine. Different superscripts within columns (^{abc}) and rows (^{xy}) for the same parameter denote differences ($p<0.05$).

Table C.3. Coefficients of correlation between urine contamination parameters and semen parameters, before cryopreservation (pre-freeze) (n=8 stallions/64 ejaculates).

Parameter	pH	Creatinine	Urea
Total Motility	-0.52**	-0.69**	-0.67**
Progressive Motility	-0.53**	-0.70**	-0.73**
VAP	-0.50**	-0.67**	-0.77**
VCL	-0.49**	-.54**	-0.63**
VSL	-0.38**	-0.60**	-0.73**
pH		0.86**	-0.86**

**p<0.0001, * p<0.005 x p<0.05

Table C.4. Coefficients of correlation between urine contamination parameters and semen parameters after cryopreservation (post-thaw). Pre – and Post- specify parameters before extension and centrifugation, or after centrifugation and re-suspension in freezing extender (n=8 stallions/64 ejaculates).

Parameter	pH	Pre Creatinine	Post Creatinine	Pre Urea	Post Urea
Total Motility	-0.43**	-0.77**	-0.63**	-0.84**	-0.69**
Progressive Motility	-0.39**	-0.70**	-0.60**	-0.77**	-0.70**
VAP	-0.30*	-0.49**	-0.52**	-0.58**	-0.64**
VCL	-0.30*	-0.43*	-0.47**	-0.52**	-0.60**
VSL	-0.29*	-0.43*	-0.47**	-0.51 **	-0.61**
Pre Creatinine			.80**		
Pre Urea		0.99**			0.84**

**p<0.0001, * p<0.005 x p<0.0

Table C.5. Mean ± SEM –Stallion sperm motility parameters after cushion centrifugation followed by single layer centrifugation (GR) or not (NGR) pre-freezing and post-thawing (n=5 stallions/20 ejaculates).

Groups	Pre-freezing				Post-thawing			
	Total motility (%)		Progressive motility (%)		Total motility (%)		Progressive motility (%)	
	Non-gradient	Gradient	Non-Gradient	Gradient	Non-gradient	Gradient	Non-gradient	Gradient
Control	79 ± 2.1^{ax}	80 ± 2.4^{ax}	63 ± 3.1^{ax}	67 ± 2.7^{ay}	61 ± 2.8^{ay}	60 ± 3.1^{ay}	54 ± 2.3^{az}	47 ± 3.7^{axy}
Low	75 ± 2.1^{abx}	79 ± 2.0^{ay}	60 ± 3.1^{abx}	67 ± 2.1^{ay}	61 ± 2.9^{az}	59 ± 3.8^{az}	52 ± 1.8^{az}	45 ± 3.4^{abz}
High	69 ± 2.1^{bx}	73 ± 2.6^{ay}	46 ± 3.1^{bx}	54 ± 3.5^{ay}	48 ± 3.1^{bz}	52 ± 3.2^{ax}	32 ± 1.2^{bz}	35 ± 3^{bx}

TM: percent of total sperm motility; PM: percent of progressive sperm motility; VCL curvilinear sperm velocity (µm/s); VAP average path velocity (µm/s); VSL straight-line velocity (µm/s); LIN coefficient of linearity (%). Control group (n=20) represented samples not contaminated with urine; Low group (n=20) was contaminated with 20% of urine contamination; High group (n=20) was contaminated with 50% urine. Different superscripts within columns (^{abc}) and rows (^{xyz}) for the same parameter denote differences ($p<0.05$).

Table C.6. Mean ± SEM –Stallion sperm motility parameters after cushion centrifugation followed by single layer centrifugation (GR) or not (NGR) pre-freezing and post-thawing (n=5 stallions/20 ejaculates).

Groups	Pre-freezing						Post-thawing					
	VCL		**VSL**		VAP		**VCL**		**VSL**		VAP	
	NGR	GR	NGR	GR	NGR	GR	NGR	GR	NGR	GR	NGR	GR
Control	160 ± 8^{ax}	158 ± 3^{ax}	61 ± 2^{ax}	61 ± 3^{ax}	80 ± 1.4^{ax}	82 ± 1.3^{ax}	105 ± 5.2^{ay}	99 ± 4^{ay}	44 ± 2.4^{ay}	42 ± 2^{ay}	57 ± 2.4^{ay}	54 ± 2.2^{ay}
Low	157 ± 5.5^{ax}	156 ± 4^{ax}	60 ± 1.2^{ax}	63 ± 1.7^{ay}	79 ± 1.3^{ax}	81 ± 3.8^{ay}	104 ± 4^{ay}	96 ± 3.1^{ay}	43 ± 2.0^{az}	40 ± 1.6^{az}	56 ± 2.1^{az}	53 ± 2.1^{az}
High	139 ± 12^{bx}	135 ± 5^{by}	53 ± 2.2^{bx}	53 ± 1.7^{bx}	67 ± 3^{bx}	70 ± 4.5^{by}	101 ± 4.4^{az}	90 ± 4.2^{az}	41 ± 2.2^{axy}	37 ± 1.8^{ay}	54 ± 2.4^{az}	50 ± 2.1^{az}

TM: percent of total sperm motility; PM: percent of progressive sperm motility; VCL curvilinear sperm velocity (µm/s); VAP average path velocity (µm/s); VSL straight-line velocity (µm/s); LIN coefficient of linearity (%). Control group represented samples not contaminated with urine (n=20); Low group was contaminated with 20% of urine contamination (n=20); High group was contaminated with 50% urine (n=20). Different superscripts within columns (^{abc}) and rows (^{xyz}) for the same parameter denote differences ($p<0.05$)

REFERENCES

1. McDonnell SM. Ejaculation Physiology and Dysfunction. Vet Clin North Am - Equine Pract 1992;8:57–70.
2. Varner DD, Schumacher J, Blanchard T. Diseases affecting semen. In: Varner DD, Schumacher J, Blanchard TL, Johnson L, editors. Dis. Manag. Breed. stallions. 1st ed., Goleta, CA: American Veterinary Publications; 1991, p. 337–40.
3. Turner R, Love CL, McDonnell SM, Sweeney R, Twitchell E, Habecker P, et al. Use of imipramine hydrochloride for treatment of urospermia in a stallion with a dysfunctional bladder. J Am Vet Med Assoc 1995;207:1602–6.
4. Lowe J. Diagnosis and management of urospermia in a commercial thoroughbred stallion. Equine Vet Educ 2001;13:4–7.
5. Dascanio J. Diagnosis and management of urospermia. In: Dascanio J, McCue P, editors. Equine Reprod. Proced. First, John Wiley & Sons; 2014, p. 403–5.
6. Griggers S, Paccamonti DL, Thompson RA, Eilts BE. The effects of pH, osmolarity and urine contamination on equine spermatozoal motility. Theriogenology 2001;56:613–22.
7. Althouse GC, Seager SWJ, Varner DD, Webb GW. Diagnostic aids for the detection of urine in the equine ejaculate. Theriogenology 1989;31:1141–8.
8. Ellerbrock R, Canisso I, Feijo L, Lima F, Shipley C, Kline K. Diagnosis and effects of urine contamination in cooled-extended stallion semen. Theriogenology 2016;85:1219–24.
9. Voge J, Varner DD, Blanchard TL, Meschini M, Turner C, Teague SR, et al. The effects of urine concentration, and cushion centrifugation to remove urine, on the quality of cool-stored stallion sperm. Theriogenology 2016;86:1294–8.
10. Naylor J, Nickel D, Triminio G. Hyperkalemic periodic paralysis in homozygous and heterozygous horses: A co-dominant genetic condition. Equine Vet J 1999;31:153–9.
11. Turner R. Urospermia and Hemospermia. In: McKinnon A, editor. Curr. Ther. Equine Reprod., St Louis: 2007, p. 258–65.
12. Voss JL, McKinnon A. Hemospermia and Urospermia. Philadelphia: Lea and Febinger; 1993.
13. Nash J, Voss J, Squires, EL. Urination during ejaculation in a stallion. J Am Vet Med Assoc 1980:224–7.
14. Hoyos S, Quiroz R, Brumbaugh G. Lack of beneficial effects of bethanechol, imipramine or furosemide on seminal plasma of three stallions with urospermia. Reprod Domest Anim 1999;34:489–93.
15. Dascanio J, Witonsky S. Theriogenology question of the month. Urospermia. J Am Vet Med Assoc 2005;227:225–7.
16. Ball BA. Diagnostic Methods for Evaluation of Stallion Subfertility: A Review. J Equine Vet Sci 2008;28:650–65.
17. Schnobrich M, Roach J, Ellerbrock R, Bradecamp E, Alvarenga M, Kline K, et al. Comparison of cushioned centrifugation and Sperm-Filter filtration on sperm longevity and morphology of cooled-stored equine semen. J Equine Vet Sci 2016;43:S67–8.

APPENDIX D: COMPARISON OF CENTRIFUGATION AND NONCENTRIFUGATION METHODS TO CRYOPRESERVE STALLION EPIDIDYMAL SEMEN

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ABSTRACT

Most epididymal flushes are performed after a catastrophic injury, acute, or chronic illness and are the last chance to save genetic material from a valuable stallion; this not only prevents test freezing for the particular animal but also means that the starting semen quality is likely poor. This necessitates that the protocol used for epididymal flushes be optimized to achieve the best possible recovery of the least damaged sperm. The objective of this study was to compare the effects of direct flushing of the epididymis with freezing extender to flushing with cooling extender and centrifugation. Epididymides were dissected away from the testes, and each vas deferens was catheterized. For the centrifugation method, the epididymis was flushed using 5 mL of a skim milk–based extender, followed by 20 mL of air, and then, semen was diluted to 50 million/mL before centrifugation and processing. Epididymides used for the noncentrifugation method were flushed directly with a commercial semen freezing extender. Semen was loaded in 0.5-mL straws at 200 million/mL, cooled and frozen. Post-thaw samples were evaluated for total and progressive sperm motility using a computer-automated sperm analyzer. Additionally, post-thaw samples were stained with fluorescent probes and subjected to flow cytometry for assessment of sperm viability, mitochondrial potential, acrosome integrity, and chromatin stability. No significant differences were observed between the two groups for any of the sperm

parameters evaluated. In conclusion, although centrifugation does not appear to be detrimental to epididymal sperm, it also does not seem necessary for semen processing.

INTRODUCTION

Epididymal sperm is routinely harvested in clinical practice as a method to cryopreserve a stallion's genetics following unexpected death, injury, or routine castration [1]. Viable pregnancies from epididymal sperm have been achieved since 1957; however, pregnancy rates have historically been lower with epididymal sperm than with traditional frozen semen [2–4]. Although the basic principle of the technique is simple, much variation exists in protocols to harvest and process epididymal sperm. Recent efforts to improve the procedure have focused on the potential benefits of seminal plasma addition, optimal breeding technique, and identification of the best freezing extender [1,2,5–10]. Advances in technique and freezing extenders have resulted in improving pregnancy rates in recent years [2–5,11]. Despite these improvements, cryopreservation of epididymal semen has not been standardized.

Epididymal sperm can be harvested by direct aspiration, slicing and flotation, or retrograde flushing of the cauda epididymis [1,12,13]. Retrograde flushing appears to be the most practical and consistent technique, results in better sperm yield, and is most commonly used in clinical practice [1,3,6,14]. Still, there is a debate about how to process semen after collection via retrograde flushing with many practitioners considering semen centrifugation a necessary intermediate step. Centrifugation is required for cryopreservation of ejaculated semen to concentrate semen by removing seminal plasma and to improve sperm cell interaction with cryoprotectants [15]. Though traditional centrifugation (600g/10 minutes) yields a poor recovery of 55%–60%, cushioned centrifugation (1,000g/20minutes with 1- to 3.5-mL cushion solution) can result in higher sperm yields (89%–91%) [16]. Still, the necessity of centrifugation in processing epididymal semen is questionable because retrograde flushing yields very high sperm concentration. Additionally, it is possible to flush the epididymis with freezing extender, which eliminates the need for centrifugation and subsequent loss of sperm [5].

Despite the sperm loss associated with centrifugation, increased processing time, and cost of additional extenders and cushion solution, some practitioners still advocate the use of centrifugation [3,17]. It is suggested that epididymal proteins can block sperm capacitation and need to be removed by centrifugation to achieve appropriate fertilization potential. To date, there

have been no studies comparing sperm functions (e.g., membrane integrity, acrosome integrity, mitochondrial potential, sperm motility parameters) and DNA damage of epididymal semen cryopreserved with and without centrifugation. Fluorescent probes have been widely used to assess sperm quality of various species as other sperm functions than motility can be assessed.

The triple stain method has been used to simultaneously assess sperm viability and mitochondrial membrane potential in many species, including the stallion [18–20]. Metachromatic staining can assess chromatin stability with acridine orange (AO) based on the susceptibility of the sperm DNA to acid-induced denaturation in situ [21]. Acrosome integrity can be assessed using a combination of FITC-PNC (peanut agglutinin conjugated with fluorescein isothiocyanate) and propidium iodide (PI) [22]. Collectively, these stains can be used to provide a more in-depth assessment of semen quality when comparing semen processing protocols.

The objective of this study was to compare the quality of post-thaw sperm obtained either from direct flushing of the epididymis with freezing extender without centrifugation or from flushing the epididymis with cooling extender and processing by centrifugation. We hypothesized that there would be no difference in sperm motility parameters, sperm viability, acrosome integrity, or chromatin stability between the two methods.

MATERIALS AND METHODS

Animals and Semen Processing

Stallions presented for elective castration during the Junior Surgery course at the Veterinary Teaching Hospital. of the University of Illinois Urbana-Champaign were enrolled in this study. These horses were admitted under a teaching protocol (#14204) that has been reviewed and approved by the University of Illinois Institutional Animal Care and Use. One testicle from each stallion was used for each method evaluated here as described in the following section. Testes were collected at castration (n= 8 animals) and held at room temperature until processing within an hour of surgical removal. The testicles were rinsed using sterile saline, and the epididymides were dissected from the testicles to expose the cauda epididymis and vas deferens. The vas deferens was then catheterized using a 12-G catheter.

For the centrifugation group, the epididymis was flushed using 5 mL of a skim milk–based commercially available extender (Botu-Semen, Botupharma, Botucatu, Brazil) at room

temperature, followed by 20 mL of air. Semen was then processed by routine laboratory procedure [23]. Briefly, the concentration was assessed by hemocytometer, semen was diluted to a concentration of 50 million/mL with Botu-Semen, and then centrifuged with 1-mL cushion solution (MaxiFreeze, IMV Technologies, L'Aigle, France) (1,000 G 20 minutes) [17,23]. The supernatant and cushion were removed, and the remaining semen pellet was resuspended with a commercial egg yolk based semen freezing extender containing dimethylformamide and glycerol (Botu-Crio, Botupharma, Botucatu, Brazil) to a concentration of 200 million/mL. Semen was loaded in 0.5-mL straws and cooled at 5C for 20 minutes, frozen in liquid nitrogen vapor for 15 minutes, and then plunged in liquid nitrogen before storage [24]. The non-centrifuged sample was collected by flushing with 5mL of semen freezing extender at room temperature, followed by 20 mL of air [5]. The resulting sample was diluted to a concentration of 200 million/mL and processed for freezing in the same manner as the centrifugation group [24].

Semen samples were thawed in a 37C water bath for 30 seconds. Sperm motility parameters were evaluated with a computer-assisted sperm analyzer using default equine settings (Sperm-vision II, Minitube of America,Verona, WI) and a procedure previously described [25]. Parameters analyzed included percent total sperm motility, percent progressive motility, sperm velocity parameters (curvilinear velocity [VCL] [mm/s]; average pathway velocity [mm/s]; and straight-line velocity [VSL] [mm/s]), and coefficients (linearity [LIN] [%], straightness [STR] [%], and wobble [%]).

Fluorescent Probes for Sperm Evaluation

Additionally, post-thaw samples were stained with fluorescent probes and analyzed by flow cytometry for assessment of viability and mitochondrial potential using triple stain (SYBR-14/PI/JC-1), acrosome integrity (FITCPNA/ PI), and chromatin integrity (AO). The LIVE/DEAD Sperm Viability Kit (SYBR-14 and PI dyes; #L-7011) and JC-1 dye (#L-7381) were purchased from Molecular Probes, Inc. (Eugene, Oregon). Fluorescein isothiocyanate–conjugated pea (Pisum sativum) agglutinin (FITCPNA; #L-7381) was purchased from Sigma Chemical Co (St. Louis, MO). Acridine orange C.I. (#04539-500) was purchased from Polysciences, Inc (Warrington, PA). Dimethyl sulfoxide, anhydrous (#D12345), triton X-100 surfactant detergent solution (#85111), and phosphate- buffered saline (pH 7.4) (#10010-023)

were purchased from Thermo Fischer Scientific (Waltham, MA). All other chemicals used were reagent grade and purchased from Sigma-Aldrich.

Assessment of Sperm Viability and Mitochondrial Membrane Potential (SYBR-14/PI/JC-1)

The triple stain assay has been used to simultaneously assess sperm viability and mitochondrial membrane potential in many species, including the stallion [18–20]. Staining solution was prepared on the day of analysis by combining 5 mL SYBR-14 (0.02 mM), 5 mL PI (2.4 mM), and 1 mL JC-1 (3 mM) per milliliter of HEPES buffer (10 mM Hepes, 150 mM NaCl, pH 7.4), creating working concentrations of 100 nM(SYBR-14), 12 mM(PI), and 3 mM(JC-1); 5 mL of thawed semen was added to 0.5 mL of staining solution to create a final concentration of 2 million spermatozoa/ mL. Samples were mixed by reverse pipetting 10 times and subsequently incubated in the dark at 37C for 30 minutes before flow cytometric analysis.

Samples were analyzed using a BD LSR II Flow Cytometry Analyzer (BD Biosciences, San Jose, CA) at the Roy Carver Biotechnology Center, University of Illinois Urbana-Champaign. The three dyes were excited using a 488-nm argon excitation laser. A total of 10,000 gated events were recorded. Two-dimensional plots of green versus red fluorescence events were drawn, and debris was gated based on those events that emitted minimal fluorescence. As previously described [18], four sperm populations in the SYBR-14/PI/JC-1 stained sperm preparations were observed (Fig. D.1). Presumptively viable cells made up both populations 1 and 2, with the high green fluorescent signal from SYBR-14 uptake in the head. Population 1 included the viable sperm cells with midpieces that stained orange, exhibiting higher red fluorescence, due to J-aggregates formation from high mitochondrial membrane potential (LIVE, POLARIZED). Population 2 included the viable sperm cells with midpieces that only weakly stained orange, exhibiting lower red fluorescence, due to depolarization and monomer formation (LIVE, DEPOLARIZED). Population 3 represented the nonviable sperm cells, exhibiting high red fluorescence due to PI uptake in the head, which also fluoresced a faint green in the midpiece due to monomer formation (DEAD). Population 4 included sperm cells not closely associated with either population 1, 2, or 3 (DEBRIS). The percentage of cells in each of these populations was calculated.

Assessment of Acrosome Integrity

Acrosome integrity was assessed using a combination of FITC-PNC (peanut agglutinin conjugated with fluorescein isothiocyanate) and PI as described elsewhere [16,21]. Staining solution was prepared by combining 0.625 mL PI (2.4 mM) and 1 mL FITC-PNA (1 mg/mL) per milliliter of phosphate-buffered saline; 5 mL of thawed semen was added to 0.5-mL staining solution to create a final concentration of 2 million spermatozoa per milliliter. Flow cytometric analyses were carried out as described above with the exception that FITC-PNA fluorescence was detected at 515–545 nm fluorescence detector 1. Nonsperm events were gated out of analyses as judged by scattering properties. Four sperm populations in the FITC-PNA/PIstained sperm preparations were present (Fig. D.2). Population 1 included sperm cells that were viable (PI negative) with an intact acrosome (FITC-PNA negative). Population 2 encompassed sperm cells that were viable (PI negative) with a damaged acrosome (FITC-PNA positive). Populations 3 and 4 contained dead sperm (PI positive) with intact (FITC-PNA negative) or damaged (FITC-PNA positive) acrosomes, respectively. These populations were divided into quadrants, and the frequency of each population was quantified.

Assessment of Sperm Chromatin Stability

Metachromatic staining assessed chromatin stability with AO based on the susceptibility of the sperm DNA to acid-induced denaturation in situ [21,26]; 10 mL of thawed semen was diluted in 990 mL phosphate-buffered saline to a final concentration of 2 million spermatozoa/ml. Acid-induced denaturation of DNA in situ was attained by adding 0.4 mL of an acid detergent solution (0.17% Triton X-100, 0.15 M NaCl, and 0.08 N HCl; pH 1.4) to 0.2-mL diluted semen. After 30 seconds, the cells were stained by adding 1.2 mL of a solution (0.1 M citric acid, 0.2 M Na2HPO4, 1 mM EDTA, 0.15 M NaCl; pH 6.0) containing 6 mg/mL AO. The stained samples were subsequently incubated in the dark at 37C for 3 minutes before flow cytometric analysis.

Flow cytometry analysis was carried out as above using a 488-nm argon excitation laser. Fluorescent data of all events were collected until at least 5,000 gated events were recorded. Cells were plotted based on red and green fluorescence intensity (Fig. D.3). Two populations were discerned by drawing a diagonal line between the groups of the cell with stable chromatin

(INTACT DNA) to the left of the line and cells with loose chromatin (DAMAGED DNA) to the right of the line. The frequency of each of these populations was quantified.

Data analyses were carried with R version 3.2.2 (https://www.r-project.org/). Normality was checked with Shapiro–Wilk, and data analyses were performed with one-way analysis of variance. Significance was set at $P < .05$. Data are presented as means standard error of the mean.

RESULTS

There were no significant differences between the centrifugation and non-centrifugation groups for any of the sperm motility parameters evaluated (Table D.1). Representative images of flow cytometry are presented in Figs. 1–3. Both groups demonstrated sperm motility parameters compatible with normal fertility in stallions (Table D.1). Similarly, there were no significant differences in DNA fragmentation index, acrosome intactness, or sperm mitochondrial potential (Table D.2). Representative dot plots for the triple stain can be seen in Fig. 1, which shows the similarity in profile between the two treatments, and the percent of live sperm with polarized membranes is shown in Table D.2. Fig. D.2 depicts dot plots representative of the FITC/ PI analysis showing similar acrosomal integrity between the two treatments. The sperm chromatin structure assay is depicted in Fig. D.3, with similar profiles for the two treatment groups.

DISCUSSION

This study demonstrates that centrifugation of epididymal stallion semen is not superior to the non-centrifugation method for cryopreservation. Post-thaw motility was high in this study and was consistent with the high motility seen in other studies that used Botu-Crio extender [5,17]. Epididymal sperm processed by either method showed similar post-thaw total and progressive motility to other recent studies that centrifuged semen, with other studies reporting total motilities of 44%–61% and progressive motilities of 19%–26% [5,27]. Motility was higher in this study than that observed by Alvarez et al [28], which is likely due to the percent glycerol used in that study and not differences in technique. Other semen motility parameters (VCL, VSL, LIN, and STR) were also similar to recent studies [5,27]. Overall, post-thaw epididymal sperm total motility has improved in the past few years from original reports of less than 30% [2,4,29–31] to greater than 50% in more recent work [3,5,27].

DNA fragmentation index was similar between groups and in line with another study evaluating the relationship between fertility and DNA damage in stallions [28]. Similarly, percent live, acrosome-intact sperm was close to other reported data. Although we did not see a decrease in membrane integrity or acrosome integrity after centrifugation, we also did not see an improvement in motility, sperm integrity, or acrosome integrity.

A recent study evaluating epididymal semen from stallions that died of colic found that initial semen parameters (total motility, progressive motility, and mitochondrial potential) were significantly poorer than parameters from epididymal flushes of testicles collected by routine castration [27]. Interestingly, the authors found that after successive centrifugation and cooling to 5C in freezing extender, all parameters improved in the colic group. They found no significant differences in motility, mitochondrial potential, or sperm membrane integrity between the healthy and colic groups on post-thaw examination. That study did not, however, have a group that was processed via direct flushing of the epididymis without washing, and it is unclear if the parameters would have improved post-thaw without additional processing [27]. Though we found no benefits in using centrifugation to process semen from healthy stallions, further studies would be needed to compare these two methods in stallions that died from certain diseases.

Previous studies have also addressed the benefits of adding seminal plasma to the flushing technique, to simulate normal semen exposure to seminal plasma [1,6]. The rationale behind this technique is the need for seminal plasma proteins to initiate motility in live but quiescent sperm. However, reports are contradictory, with some authors finding no difference in post-thaw parameters despite the addition of seminal plasma, whereas others found that seminal plasma improved post-thaw motility, at least in some stallions [6,7,10,27,32]. Our post-thaw motility results were comparable to the improving post-thaw motilities seen in recent years, without the addition of seminal plasma [27,33].

Although semen centrifugation is a necessary step for the cryopreservation of equine semen collected by an artificial vagina, the current results suggest that it is not necessary to cryopreserve semen collected from epididymal flushes from healthy stallions. Semen collected by flushing contains no seminal plasma and is already concentrated at a sufficient concentration to allow immediate extension and freezing. Centrifugation may still be of benefit to practitioners wishing to minimize straws per breeding dose by freezing at concentrations as high as 800

million sperm/mL. However, the advantage of centrifugation is questionable given the decreased total recovery of sperm. The extra steps involved in the centrifugation method can be time-consuming and do not result in improved motility, membrane integrity, or DNA integrity.

In summary, no differences were seen between epididymal sperm processing protocols for any of the post-thaw parameters evaluated. This suggests that there is no need to centrifuge semen collected via epididymal flushes before freezing and allows the practitioner to save time, money, and potentially increase total sperm yield.

Acknowledgments

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FIGURES AND TABLES

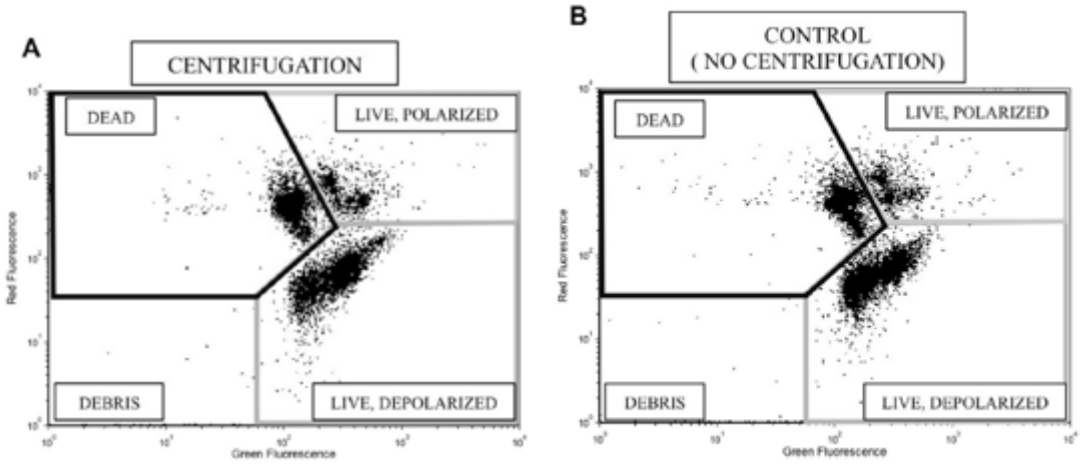

Figure D.1. Representative flow cytometry dot plot for the triple stain assay (SYBR-14/PI/JC-1) for the centrifugation group (A) and non-centrifugation group (B). Units indicate fluorescence intensity, with high SYBR-14 producing green fluorescence (x-axis) and high PI producing a red fluorescence (y-axis). The top left quadrant corresponds to dead sperm (high PI-positive events and low SYB-14). The bottom right quadrant corresponds to viable sperm (high PI-positive events and low PI). The top right quadrant represents moribund sperm, with both high PI and high SYBR-14 fluorescence. The bottom right quadrant corresponds to debris or low fluorescing particles. PI, propidium iodide.

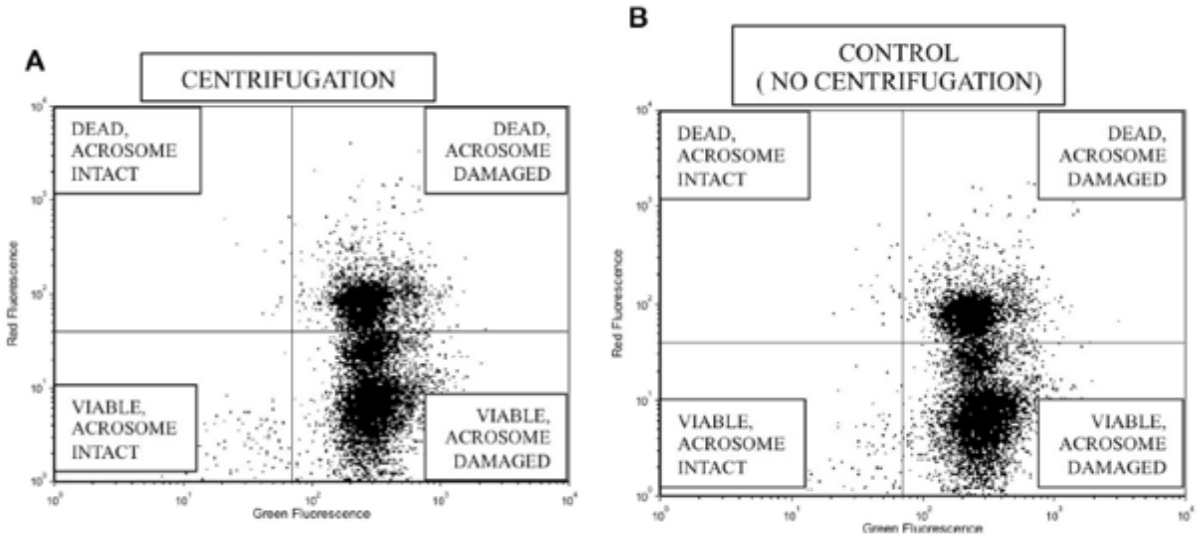

Figure D.2. Representative flow cytometry dot plots for acrosomal integrity assay, FITC-PNA, and propidium iodide for the centrifugation group (A) and noncentrifugation group (B). Units indicate fluorescence intensity, with high FITC-PNA producing green fluorescence (x-axis) and high PI producing a red fluorescence (y-axis). The first unstained population of viable sperm is in the lower left quadrant, with low PI and intact acrosomes (low FITC-PNA). The upper quadrants represent the populations of dead sperm (high PI), with damaged acrosomes (high FITC-PNA) on the right and intact acrosomes (low FITC-PNA) on the left. The fourth population in the lower right quadrant (low PI and high FITC-PNA) corresponds to viable acrosome damaged sperm. PI, propidium iodide.

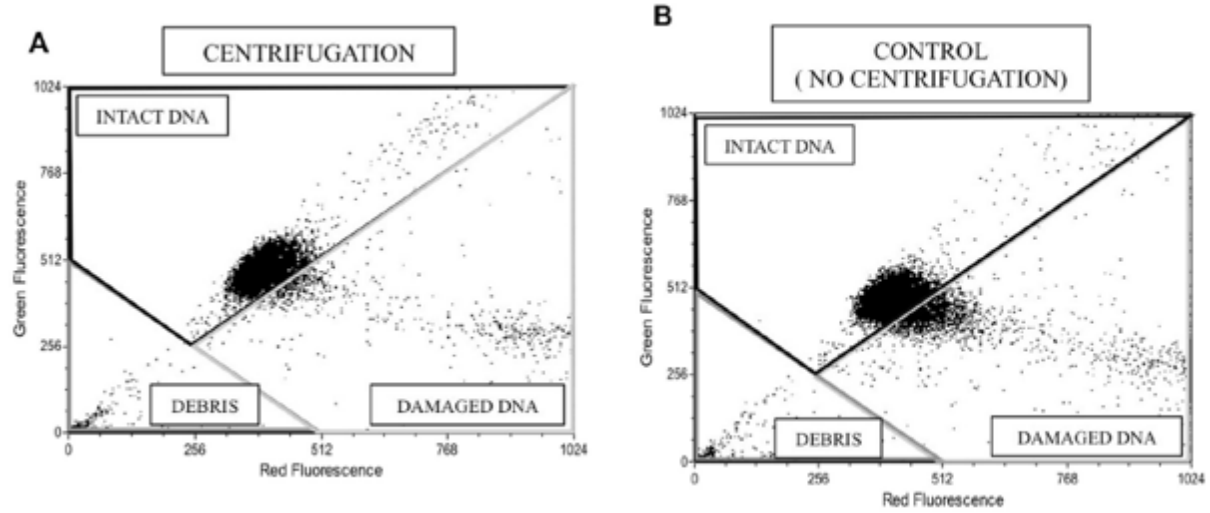

Figure D.3. Representative plots for the chromatin stability assay (acridine orange) for the centrifugation group (A) and the non-centrifugation group (B). Units indicate fluorescence intensity, with binding to double-stranded (intact) DNA producing green fluorescence (y-axis) and binding to single-stranded (damaged) DNA or RNA producing a red fluorescence (x-axis). Lower left quadrant represent unstained (discarded) debris. Upper left quadrants represent sperm with good chromatin integrity (intact DNA) that fluoresce green. Lower right quadrants represent sperm that have loose chromatin (damaged DNA) emitting red fluorescence.

Table D.1

Post-thaw sperm motility parameters of cryopreserved epididymal sperm processed either by centrifugation or without centrifugation.

Parameter	Centrifugation	Control (No Centrifugation)	*P* Value
Total sperm motility	60.1 ± 6.4	57.5 ± 4.6	.74
Progressive sperm motility	29.67 ± 6.9	33.2 ± 6.3	.70
VCL	109 ± 8.9	111.9 ± 15.1	.85
VAP	60 ± 4.85	55.4 ± 5.2	.52
VSL	48.4 ± 5	43 ± 4.2	.40
LIN	44 ± 0.05	41 ± 0.04	.28
STR	80 ± 0.07	76 ± 0.04	.30
WOB	0.55 ± 0.04	0.54 ± 0.06	.73

Abbreviations: LIN, linearity; SEM, standard error of the mean; STR, straightness; VAP, average pathway velocity; VCL, curvilinear velocity; VSL, straight-line velocity; WOB, wobble.
Data expressed as mean ± SEM.

Table D.2

Mean ± SEM–Sperm assessment using fluorescent probes of cryopreserved epididymal sperm centrifuged and noncentrifuged.

Parameter	Centrifugation (%)	No Centrifugation (%)	*P* Value
DNA fragmentation index	8.76 ± 1.66	11.08 ± 1.02	.26
Dead, intact acrosome	1.34 ± 0.9	2.53 ± 0.8	.48
Dead, damaged acrosome	37.9 ± 5.3	36.25 ± 4.6	.77
Live, intact acrosome	58.33 ± 6.5	52.4 ± 4.7	.49
Live, damaged acrosome	4.1 ± 0.6	7.1 ± 1.4	.12
Live, polarized mitochondria	6.5 ± 0.8	6.8 ± 1.1	.92
Live, depolarized mitochondria	54.25 ± 5.9	47.19 ± 3.7	.36
Dead (triple strain)	45.8 ± 3.9	39.5 ± 5.8	.40

Abbreviation: SEM, standard error of the mean.
DNA fragmentation index was obtained from acridine orange analysis. Acrosome integrity was assessed using FITC/PI. Mitochondrial polarization was assessed using the triple stain (SYB-14/PI/JC-1).

REFERENCES

1. Bruemmer JE. Collection and freezing of epididymal stallion sperm. Vet Clin North Am Equine Pract 2006;22:677–82.
2. Morris L, Tiplady C, Allen WR. The in vivo fertility of cauda epididymal spermatozoa in the horse. Theriogenology 2002;58:643–6.
3. Stawicki RJ, McDonnell SM, Giguère S, Turner RM. Pregnancy outcomes using stallion epididymal sperm stored at 5C for 24 or 48 hours before harvest. Theriogenology 2016;85:698–702.
4. Barker CAV, Gandier JCC. Pregnancy in a mare resulting from frozen epididymal spermatozoa. Can J Comp Med Vet Sci 1957;21:47–51.
5. Papa FO, Melo CM, Fioratti EG, Dell'Aqua JA, Zahn FS, Alvarenga MA. Freezing of stallion epididymal sperm. Anim Reprod Sci 2008;107:293–301.
6. Heise A, Thompson PN, Gerber D. Influence of seminal plasma on fresh and post-thaw parameters of stallion epididymal spermatozoa. Anim Reprod Sci 2011;123:192–201.
7. Neuhauser S, Rheinfeld S, Handler J, Rheinfeld S, Handler J. Evaluation of frozen-thawed stallion epididymal sperm with and without addition of seminal plasma. J Equine Vet Sci 2012;32:503.
8. Neuhauser S, Rheinfeld S, Handler J. Comparison of the effects of four freezing methods on motility characteristics, morphology, and viability of postthaw stallion epididymal sperm. J Equine Vet Sci 2014;34:882–8.
9. Sostaric E, Aalberts M, Gadella BM, Stout TAE. The roles of the epididymis and prostasomes in the attainment of fertilizing capacity by stallion sperm. Anim Reprod Sci 2008;107:237–48.
10. Neuhauser S, Dörfel S, Handler J. Dose-dependent effects of homologous seminal plasma on motility and kinematic characteristics of post-thaw stallion epididymal spermatozoa. Andrology 2015;3:536–43.
11. Monteiro GA, Guasti PN, Hartwig FP, Dellaqua JA, Alvarenga MA, Papa FO. Cryopreservation and fertility of ejaculated and epididymal stallion sperm. Anim Reprod Sci 2011;127:197–201.
12. Sharma RK, Padron OF, Thomas AJ, Agarwal A. Factors associated with the quality before freezing and after thawing of sperm obtained by microsurgical epididymal aspiration. Fertil Steril 1997;68:626–31.
13. Hewitt DA, Leahy R, Sheldon IM, England GCW. Cryopreservation of epididymal dog sperm. Anim Reprod Sci 2001;67:101–11.
14. Roels K, Leemans B, Ververs C, Govaere J, Hoogewijs M, Van Soom A. Collection and freezing of equine epididymal spermatozoa. Vlaams Diergeneeskd Tijdschr 2014;83:321–5.
15. Amann RP, Pickett BW. Principles of cryopreservation and a review of cryopreservation of stallion spermatozoa. J Equine Vet Sci 1987;7:145–73.
16. Bliss SB, Voge JL, Hayden SS, Teague SR, Brinsko SP, Love CC, et al. The impact of cushioned centrifugation protocols on semen quality of stallions. Theriogenology 2012;77:1232–9.

17. Stawicki RJ. How to process stallion epididymal sperm in your practice. Am Assoc Equine Pract 2015;61:485–9.
18. Love CC, Thompson JA, Brinsko SP, Rigby SL, Blanchard TL, Lowry VK, et al. Relationship between stallion sperm motility and viability as detected by two fluorescence staining techniques using flow cytometry. Theriogenology 2003;60:1127–38.
19. Volpe S, Leoci R, Aiudi G, Lacalandra GM. Relationship between motility and mitochondrial functional status in canine spermatozoa. Reprod Domest Anim 2009;44(Suppl 2):275–8.
20. Gaczarzewicz D, Udała J, Piasecka M, Błaszczyk B, Stankiewicz T. Storage temperature of boar semen and its relationship to changes in sperm plasma membrane integrity, mitochondrial membrane potential, and oxidoreductive capability. Turk J Biol 2015;39:582–94.
21. Martinez-Pastor F, Johannisson A, Gil J, Kaabi M, Anel L, Paz P, et al. Use of chromatin stability assay, mitochondrial stain JC-1, and fluorometric assessment of plasma membrane to evaluate frozen thawed ram semen. Anim Reprod Sci 2004;84:121–33.
22. Robles V, Martinez-Pastor F. Spermatogenesis methods and protocols. Spermatogenes. Hertfordshire: Methods Protoc.; 2013. p.175–86.
23. Schnobrich M, Roach J, Ellerbrock R, Bradecamp E, Alvarenga M, Kline K, et al. Comparison of cushioned centrifugation and Sperm-Filter filtration on sperm longevity and morphology of cooled stored equine semen. J Equine Vet Sci 2016;43:S67–8.
24. Canisso IF, Carvalho GR, Morel MD, Ker PG, Rodrigues AL, Silva EC, et al. Seminal parameters and field fertility of cryopreserved donkey jack semen after insemination of horse mares. Equine Vet J 2011;43:179–83.
25. Ellerbrock R, Canisso I, Feijo L, Lima F, Shipley C, Kline K. Diagnosis and effects of urine contamination in cooled-extended stallion semen. Theriogenology 2016;85:1219–24.
26. Stewart JL, Shipley CF, Katich AS, Po E, Ellerbrock RE, Lima FS, et al. Cryopreservation of white-tailed deer (Odocoileus virginianus) semen using soybean-, liposome-, and egg yolk-based extenders. Anim Reprod Sci 2016;171:7–16.
27. Gloria A, Carluccio A, Petrizzi L, Noto F, Contri A. Characteristics of frozen epididymal spermatozoa from stallions that died 12 to 36 hours after colic surgery. Theriogenology 2015;85:345–50.
28. Álvarez C, Gil L, González N, Olaciregui M, Luño V. Equine sperm post-thaw evaluation after the addition of different cryoprotectants added to INRA 96——————————— extender. Cryobiology 2014; 69:144–8.
29. Bruemmer JE, Reger H, Zibinski G, Squires EL. Effect of storage at 5Con the motility and cryopreservation of stallion epididymal spermatozoa. Theriogenology 2002;58:405–7.
30. Tiplady CA, Morris LHA, Allen WR. Stallion epididymal spermatozoa:pre-freeze and post-thaw motility and viability after three treatments. Theriogenology 2002;58:225–8.
31. Braun J, Sakai M, Hochi S, Oguri N. Preservation of ejaculated and epididymal stallion spermatazoa. Theriogenology 1994;41:809–18.

32. Aurich JE, Kühne A, Hoppe H, Aurich C. Seminal plasma affects membrane integrity and motility of equine spermatozoa after cryopreservation. Theriogenology 1996;46:791–7.
33. Monteiro GA, Guasti PN, Rocha AS, Martin I, Sancler-Silva YFR, Freitas Dell'Aqua CP, et al. Effect of storage time and temperature of equine epididymis on the viability, motion parameters, and freezability of epididymal sperm. J Equine Vet Sci 2013;33: 169–73