UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

EFFECT OF REPEATED INTRA-ARTICULAR ADMINISTRATION OF AMIKACIN ON SERUM AMYLOID A CONCENTRATION, TOTAL PROTEIN AND NUCLEATED CELL COUNT IN SYNOVIAL FLUID FROM HEALTHY HORSES

A dissertation submitted to the

Faculty of Veterinary Science

University of Pretoria

In partial fulfilment of

the requirements for the degree

Master in Science (MSc)

Promoter: Prof. L. M. Rubio Martínez

Co-Promoter: Dr. Macarena Sanz

Department of Companion Animal Clinical Studies

Faculty of Veterinary Science

University of Pretoria

Dr. Andres F. Sanchez Teran

DECLARATION

I hereby declare that this dissertation, submitted for the degree of MSc to the University of Pretoria, is my own work and has not been submitted to another university for a degree, and that the data included in this dissertation are the results of my investigations.

Andres F. Sanchez Teran

29 November 2011

TABLE OF CONTENTS

AKNOWLEDGEMENTS

I would like to thank Prof. Luis Rubio Martinez and Dr. Macarena Sanz as my promoters and friends, for all their patience, personal support and the effort they put into helping me finish this dissertation in time.

Thanks to Dr. Nicolas Villarino for the statistical analysis performed in this study and his help with his corrections.

My especial thanks to Prof. Amelia Goddard, Carien Muller and Cheryl Booth and all the team at the Clinical Pathology Laboratory for their hard work and careful analysis of my samples.

To the students who helped me every day with lameness examinations, trotting horses up and down, making every research day a good time for fun work. Thanks.

DEDICATIONS

To Big Father, my God, who I have to blame for all my achievements, who has guided me along all my steps and has brought me to places I never thought about! Thank you with all my heart.

Dedicated especially to my parents, to whom I owe my life and my education. Without their support and love I could not have reached my dreams.

ABBREVIATIONS

^0C	degree Celsius
ANOVA	Analysis of Variance
APPs	acute phase proteins
APR	acute phase response
AAEP	American Association of Equine Practitioners
AUC	area under the curve
CG	control group
DFTS	deep flexor tendon sheath
DMSO	dimethyl sulfoxide
DNCC	differential nucleated cell count
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
g	grams
g	relative centrifugal force
g/dl	grams per deciliter
h	hours
H_2O_2	hydrogen peroxide
HDL3	high density lipoprotein
IL-1	interleukin – 1
IL-6	interleukin – 6
LPS	lipopolysaccharides

LRS	lactated ringer solution
mg	milligrams
mg/kg	milligrams per kilogram
MIC	minimum inhibitory concentration
ml	milliliter
mm	millimeter
MMPs	matrix metallo-proteinases
NCC	nucleated cell count
NO	nitric oxide
O_2^-	oxygen radical
OH	hydroxide
OTAU	Onderstepoort Teaching Animal Unit
OVAH	Onderstepoort Veterinary Academic Hospital
SAA	serum amyloid A
SD	standard deviation
spp.	species
TNF-α	tumour necrosis factor – α
TG	treatment group
TP	total protein
vs.	versus

LIST OF FIGURES

LIST OF TABLES

LIST OF APPENDICES

SUMMARY

Introduction:

Serum amyloid A (SAA) in synovial fluid has recently been used as a marker for septic arthritis in horses. The objective of this study was to report the effect of intra-articular administration of amikacin on 1) SAA; total protein (TP), nucleated cell count (NCC) and differential nucleated cell count (DNCC) in the synovial fluid of equine healthy joints, and 2) SAA concentration in systemic blood.

Material and Methods:

A prospective, two-period, cross-over study was performed using 6 horses determined to be healthy based on clinical, lameness and haematological examinations. Each horse's intercarpal joint received one of two treatments: repeated arthrocentesis (CG) or repeated arthrocentesis and intra-articular administration of 500 mg of amikacin sulphate (TG). Arthrocenteses, and synovial fluid and systemic blood sample collections were performed every 48h (baseline, 48, 96, 144 and 192h). Systemic SAA and synovial SAA, TP, NCC and DNCC were measured in all samples. Clinical and lameness examinations were performed daily. A 20-day washout was allowed between treatments.

Treatments were compared using cross-over ANOVA, Student T and MannWhitney U tests. Significance level was set at $p<0.05$.

Results:

Horses did not show lameness throughout the study. One horse was excluded from the study due to unrelated respiratory disease. Serum amyloid A concentrations in serum and synovial fluid did not vary during the study in either group and remained below the lower limit of quantification. Baseline values for TP, NCC and DNCC were not statistically different between groups. Total protein values for TG were significantly higher than for CG after the first sample ($p<0.05$). In both groups NCC

increased significantly ($p<0.05$) after the first sample but values remained within normal ranges throughout the study. No significant changes were found in DNCC.

Conclusion:

Repeated arthrocentesis alone or in combination with intra-articular amikacin did not affect systemic or synovial SAA concentrations in healthy horses. In contrast, synovial TP and NCC values increased over time; some of the TP values observed were within the range reported for septic arthritis. Synovial SAA could serve as a more reliable marker than TP and NCC when evaluating the clinical progression of a joint that has being treated with repeated intra-articular administration of amikacin.

CHAPTER 1: INTRODUCTION

Joint disease can reduce the performance and future competitiveness of sport horses due to non-responsive osteoarthropathy with consequences such as chronic pain, joint stiffness and overload laminitis of the contra-lateral limb (1-3). Therefore, research in the field of diagnosis and treatment of equine articular disease has been quite extensive over the last decades (4).

Septic arthritis is one of the most feared and severe osteoarthropathies in horses of all ages. Survival rates vary according to the age group affected. In foals, discharge rates from hospital after intensive treatment are lower (62%) than in adult horses (85%) due to concurrent conditions such as osteomyelitis, multiple joint involvement, hypogammaglobulinaemia and multisystemic disease (5). Only 48% of Thoroughbred foals discharged after being treated for septic arthritis started in at least one race in their career (6). Rates of return to previous performance in adult horses after synovial sepsis have been reported. In one study, only 56% of the adult horses were able to return to racing (5). In another report, 11 of 12 horses were able to return to racing (7).

Early recognition and aggressive treatment of joint sepsis has been associated with better prognosis for survival and return to athletic function than delayed treatment in adult horses (1, 4). Horses treated within the first 24 hours after contamination of a synovial structure (open joint injury) were less likely to develop infectious arthritis and more likely to return to normal performance than horses treated after 24h (8). However, in some cases, aggressive treatment after several days from the initial joint

contamination can also lead to satisfactory outcomes with horses returning to previous athletic function (7, 9).

Intra-articular antimicrobial therapy remains a mainstay in the treatment of septic joints (10). It is easy to perform and achieves high concentrations of antimicrobials in the synovial fluid, thus increasing the drug efficacy (11). However, successful treatment of synovial sepsis requires multi-modal therapy. In a report where open drainage of the infected joint was evaluated, the surgical technique used was found to be as important as the use of intra-articular antibiotics for the resolution of the synovial sepsis (12).

To diagnose and monitor the progression of joint sepsis, serial analysis of different inflammatory markers in synovial fluid are frequently used (13-18). Synovial total protein (TP) concentration and total nucleated cell count (NCC) typically increase in the presence of articular infection, and their decline generally serves as a guideline to discontinue the local administration of antimicrobials (13, 19). However, repeated arthrocentesis alone (18) as well as single intra-articular administration of antibiotics (20), both commonly performed during the treatment of septic joints, can cause an increase of total protein concentration and total cell count in the synovial fluid. Therefore, there is a need to find new synovial inflammatory markers that could help the equine practitioner recognize the progression of the disease and decide on the discontinuation of local administration of antibiotics when succeeding with the treatment.

Serum Amyloid A (SAA) is an inflammatory protein that has been recently used as a marker for inflammation in various species (21-25). Serum amyloid A values in serum and synovial fluid are increased in the presence of septic arthritis and did not

increase in response to repeated arthrocentesis (18, 26-29). However, the effect of the intra-articular administration of amikacin on the concentration of synovial SAA, TP, NCC and differential nucleated cell count (DNCC) in horses has not been evaluated.

Amikacin is commonly used in clinical cases since it has the best antimicrobial activity against the bacteria most frequently isolated from septic joints in horses (30). This study was designed to evaluate the articular inflammatory response to repeated intra-articular administration of amikacin sulphate by measuring SAA concentration, total protein concentration and total nucleated cell count in the synovial fluid of healthy horses. In addition, the effect on SAA concentration in the systemic blood was evaluated. If no changes on synovial SAA concentration are observed from baseline values after repeated intra-articular administration of amikacin, SAA could serve as a more reliable marker than total synovial protein and nucleated cell count when monitoring the progression of equine joints treated with intra-articular amikacin.

CHAPTER 2: LITERATURE REVIEW

2.1 The inflammatory response of the synovial structure to bacterial infection

The articular inflammatory response to infection is mediated by the innate immune system directed towards the elimination of the microorganism and the repair of damaged tissue (31). Healthy synovial structures are able to control synovial contamination with a certain amount of bacteria and impede their proliferation (32). In a previous *in vitro* study, 10g of normal equine synovial membrane were capable of neutralizing 100 colony-forming units of *Staphylococcus aureus* (33). When infection and proliferation of bacteria occur, the joint usually produces the most exaggerated inflammatory response described within the synovial environment, and this response is determined by factors such as host immunity, number of inoculated bacteria and bacterial virulence (32). The degree of tissue damage caused by the inflammatory response varies according to the host age, host debilitation, duration of infection, bacterial virulence and pre-existent joint disease (32).

The initiator for the local inflammatory response is the release of molecules by the injured tissue. Reactive oxygen species (O_2^-, H_2O_2, OH and NO), arachidonic acid metabolites and modified host proteins are released and induce the migration and activation of cells such as neutrophils and macrophages, and the production of large protective molecules like antibodies and complement components (31). Synoviocytes and neutrophils respond to the stimulus by releasing cytokines such as interleukin-1 (IL-1), interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α), free oxygen radicals, and destructive enzymes such as lysozime, elastase, cathepsin G, gelatinase and collagenase (34). All these products contribute to the disruption of the

blood-synovial barrier, facilitating the articular access of many nonspecific mediators that amplify the inflammatory cascade leading to further activation of synoviocytes (32). Total protein content in the synovial fluid also increases because of the increased capillary permeability, which allows high molecular weight proteins (mainly globulins) to enter the joint (35). Activation of chondrocytes is mediated by IL-1, TNF-α and free radicals. Activated chondrocytes release matrix metallo-proteinases (MMPs) (stromelysin, collagenase, gelatinase) which decrease proteglycan synthesis (34). These MMPs are responsible for the cartilage breakdown leading to the development of osteoarthritis (3).

If the amount of inflammatory molecules released by the injured tissue is high, a systemic reaction may be induced, leading to an acute phase response (APR) (31). The APR is composed of a serial of local and systemic changes that involve many cell types and organs (36). Several proteins are released into the systemic circulation during an APR and are called acute phase proteins (APPs) (31).

The acute phase proteins are produced mainly by the hepatocytes after being stimulated by IL-1, TNF-α, and especially IL-6 (31). Their synthesis and release usually begin few hours after injury (31). The APPs include complement components, clotting molecules, protease inhibitors, and metal-binding proteins (31). The major APPs have very low or undetectable plasma levels in healthy animals, but their concentrations can rise more than 100-1000 times during an APR (21). The clinical use of serum amyloid A (SAA), an APP very sensitive to inflammation, has recently been investigated in equine medicine (21).

2.2 The acute phase protein equine serum amyloid A (SAA) and its use as a marker for inflammatory processes

The acute phase protein SAA is an immunomodulatory protein that regulates the immune response by attracting neutrophils, monocytes and T cells (31, 37). Serum amyloid A is an apolipoprotein and circulates in plasma bound to the fraction 3 of high density lipoprotein (HDL3) (38, 39). It is degraded in the liver (40) and has a short plasma half-life, which has been estimated to be from 75 to 80 minutes in mice, with a 95% clearance from plasma 6 hours after the synthesis has stopped (41). Therefore, plasma levels of SAA decrease soon after synthesis ceases (21).

Serum amyloid A is a good marker for inflammation. It has very low basal values which can increase up to 1000 times during APR (24). Its serum levels are not affected by sex (except for a moderate increase after parturition), and mild changes have been reported between different age groups (24). Concentrations of SAA can rise to different ranges depending on the amount of tisular damage. These concentrations decrease quickly after resolution of the tisular damage because of the SAA's short half-life (21, 24). In humans, its concentration in serum rises to higher levels in response to bacterial stimulus than to viral infection or non-infectious inflammation (42).

Equine SAA is a 9-11 kilodalton apolipoprotein (24, 39) mainly synthesized by the liver in response to inflammation and infection (22, 39). Three SAA isoforms have been recognized in the equine serum (22, 28). The basal values of SAA in serum of healthy horses are <30 mg/l (24, 43-45), with certain variability observed depending on the measurement assay used (21). In horses with experimentally-induced inflammation (by intramuscular administration of turpentine oil) SAA concentrations

rose within 6 hours and reached their serum peak concentration 2 days later with an up to 20-fold increase from pre-treatment values (24).

Extrahepatic synthesis has been reported in various species such as rabbits, minks, mice (46), horses (28) and humans (46, 47). The presence of SAA in human synovial fluid has an important role in the pathogenesis of inflammatory arthritis (rheumatoid arthritis, psoriatic arthritis, sarcoid arthritis, and undifferentiated arthritis) by inducing the production of MMPs, which are associated with cartilage degeneration (48-50). In horses, five isoforms were reported in synovial fluid in experimental arthritis induced by intra-articular injection of lipopolysaccharides (LPS) (28). Three of these isoforms were also found in serum and the remaining two only in synovial fluid. These findings indicate that isoforms produced in the liver can access the synovial cavity and that synthesis of equine SAA also occurs intra-articularly. In humans it has been demonstrated that synoviocytes, macrophages and endothelial cells are responsible for the extrahepatic synthesis of SAA (48).

Serum amyloid A concentration in synovial fluid in horses have recently been shown to serve as a good marker for infectious arthritis and tenovaginitis, reflecting changes in inflammatory activity (18). In healthy equine joints, synovial concentrations of SAA were lower than the lower limit of detection of the assay (0.48 mg/l) (28). When the effect of repeated arthrocentesis on inflammatory markers in the equine joint was evaluated (18), synovial TP concentration remained significantly increased from baseline from the second to the last arthrocentesis. In contrast, in the same study, concentrations of SAA in synovial fluid remained at baseline values (18). In another study, the intra-articular inoculation of LPS caused an increase in synovial fluid SAA concentration that reached a 100-fold increase peak 48 hours after inoculation (28). A systemic response also occurred and was characterized by increased production

of SAA in serum by the liver. The magnitude of the systemic and articular responses was dependent on the intra-articular LPS dose.

2.3 Determination of SAA concentration in equines

Different methods have been used for measurement of SAA in equines. These methods include electroimmunoassay (25), single radial immunodiffusion (24), slide reverse passive latex agglutination test (51), enzyme-linked immunosorbent assay (ELISA) (52) and latex agglutination immunoturbidometric assay (43). In addition, a commercially available immunoturbidometric assay (LZ test SAA)[1] that was first developed for the measurement of human SAA, has been validated in horses (44). This method has an acceptable intra- and inter-assay variability with better precision at intermediate and high SAA concentrations compared to low SAA concentrations. This imprecision at low SAA concentrations has limited clinical significance as horses develop high SAA concentrations during the inflammatory response (25, 27, 43, 44). This immunoturbidometric assay is automated and fast, and is being used in some diagnostic laboratories in Europe and the United States for routine SAA measurements on horse serum and synovial fluid (21).

2.4 Amikacin sulphate as a local antibiotic for septic arthritis

Amikacin sulphate belongs to the family of aminoglycoside antimicrobials, which also include streptomycin, neomycin, gentamicin, tobramycin and kanamycin (51). Aminoglycosides are composed of an hexose nucleus, which is bound to two or

[1] Eiken Chemical Co, Ltd., Tokyo, Japan.

more amino sugars by a glycoside linkage (51). Aminoglycosides act like mild polar basic structures and are hydrosoluble, which difficults their diffusion through cell membranes (51). Their mechanism of action is exerted intracellularly where they bind to 30S ribosomal subunit inhibiting the synthesis of proteins with a bactericidal effect (51). Their spectrum of activity includes mainly gram negative bacteria (53). As their penetration into the bacteria is oxygen-dependent, they are ineffective against anaerobic bacteria and their activity is also diminished in acidic environments such as abscesses (53). Aminoglycosides are concentration-dependent antibiotics and exert prolonged post-antibiotic effects. They have a half-life of 1-2 hours and are eliminated mainly by glomerular filtration (53). The use of most aminoglycosides is associated with toxic effects to the kidney, ear and vestibular apparatus (51).

Amikacin sulphate has become the preferred intra-articular antimicrobial for the prevention and treatment of equine joint sepsis (53). In a retrospective study, amikacin was the only antimicrobial highly effective against 95% of the bacteria isolated from horses with postoperative septic arthritis including *Staphylococcus* spp., *Enterobacteriaceae* spp. and *Pseudomonas* spp. (30). Intra-articular administration of 250-500 mg of amikacin sulphate every 24 to 48 hours is commonly used for the treatment of septic arthritis (54-56). This therapy achieves high peak antimicrobial concentrations in synovial fluid and these concentrations remain above the reported minimum inhibitory concentration (MIC) for most equine pathogens for prolonged periods of time (56, 57). A single administration of 500 mg of amikacin into the radiocarpal joint of horses produced synovial concentrations of amikacin that remained above the reported MIC for most equine pathogens for 72 and 48 hours in normal and inflamed joints, respectively (11). Repeated intra-articular administration

of amikacin is most commonly performed via arthrocentesis every 24 to 48 hours when treating septic arthritis in horses (55, 56).

A single intra-articular administration of 500 mg of amikacin did not produce any cytological or clinical evidence (i.e., lameness) of chemical synovitis in healthy horses (11). However, amikacin exerted toxic effects on equine chondrocytes in explant cultures (58). The effect of repeated intra-articular administration of amikacin on synovial SAA concentration, TP, NCC and DNCC has not been investigated in horses.

CHAPTER 3: OBJECTIVES AND HYPOTHESES

3.1 Study objectives

- To evaluate the articular inflammatory response to repeated arthrocentesis and repeated intra-articular administration of amikacin sulphate by measuring SAA concentrations, total protein and total nucleated cell count in synovial fluid of equine healthy joints.

- To evaluate the systemic inflammatory response to repeated arthrocentesis and repeated intra-articular administration of amikacin sulphate by measuring SAA concentrations in serum.

3.2 Hypotheses

- Serum amyloid A synovial concentrations in the equine intercarpal joint will not show increased values, after repeated (every 48 hours) arthrocentesis or after repeated (every 48 hours) intra-articular administration of amikacin in healthy horses.

- Total protein and total nucleated cell count in synovial fluid of the intercarpal joint will increase after repeated (every 48 hours) arthrocentesis or after repeated (every 48 hours) intra-articular administration of amikacin in healthy horses.

- Serum amyloid A concentrations in the systemic blood will not increase from baseline values, after repeated (every 48 hours) arthrocentesis or after repeated (every 48 hours) intra-articular administration of amikacin in healthy horses.

CHAPTER 4: MATERIALS AND METHODS

4.1 Study Design

The study was approved by the Animal Use and Care Committee of the Faculty of Veterinary Science, University of Pretoria. A prospective, two-period, cross-over study was conducted on a total of 6 horses. Each horse received a total of 2 treatments that were administered to the front limbs in a randomized sequence. Randomization was performed by the flip of a coin. A wash-out period of 20 days was allowed between treatments (Appendix A).

- Control Group (CG): Arthrocentesis of the intercarpal joint was performed 5 times with an interval of 48h between arthrocenteses. A sample of synovial fluid (~1.5 ml) was collected each time. Amikacin sulphate (500 mg) was injected into the intercarpal joint after the last synovial fluid collection (Appendix B).
- Treatment Group (TG): Arthrocentesis of the intercarpal joint was performed 5 times with an interval of 48h between arthrocenteses. A sample of synovial fluid (~1.5 ml) was collected each time. Amikacin sulphate (500 mg) was administered into the intercarpal joint after collection of the synovial fluid sample at each sampling time (Appendix C).

4.2 Study Setting

The study was performed at the Equine Clinic of the Onderstepoort Veterinary Academic Hospital (OVAH), Faculty of Veterinary Science, University of Pretoria, South Africa.

4.3 Study Population and Sampling

Six Nooitgedacht mares from the Onderstepoort Teaching Animal Unit (OTAU) breeding herd of the University of Pretoria were included in this study. The mares were determined to be clinically healthy based on physical examination (temperature, heart rate, respiratory rate, thoracic and abdominal auscultation and evaluation of mucous membranes). Horses were free of musculoskeletal disorders as determined by complete lameness examination including palpation of limbs and gait evaluation at the walk and trot. In addition, a complete blood cell count was performed in all horses before the beginning of each study period. Horses with abnormal results in the complete blood cell count were rejected. Horses did not receive any medical treatment for at least six weeks prior to entry the study.

All horses were sedated with romifidine (0.02 mg/kg – 0.03 mg/kg, intravenously) for collection of synovial fluid samples. The dorsal aspect of the selected intercarpal joint was clipped and aseptically prepared as previously described (59). Using sterile technique and with the carpus in semiflexion, approximately 1.5 ml of synovial fluid was collected in each horse. A 38 mm long, 22 gauge needle attached to a 3 ml sterile syringe was inserted into the intercarpal joint using a dorso-lateral approach (lateral to the extensor carpo-radialis tendon) (60). After collection of the sample the needle and syringe were removed from horses in the control group except for the last arthocentesis. After the last arthrocentesis, the syringe was detached from the

needle and the needle was used to inject 500 mg of amikacin sulphate (2 ml) (Amikacin-Fresenius[2]) into the joint. In horses in the treatment group, after collection of the each synovial fluid sample 500 mg of amikacin were injected into the joint in the same manner. The same procedure was repeated every 48 hours for a total of 5 arthrocenteses during 10 days. All arthrocenteses were performed by the same investigator (AST)

A sample of venous blood was collected from all horses at each time point by puncture of the right or left jugular vein using a 21 gauge, 38mm needle attached to a tube holder with a clot activator vacuum tube[3]. These blood samples were then used for the determination of systemic SAA concentrations.

Physical and lameness examinations were performed on the horses every 24 hours during the study and 24 hours after collection of the last sample. Lameness was graded in a scale from 0 to 5 according to the AAEP (American Association of Equine Practitioners) guidelines (60). The lameness examinations were videotaped for blind evaluation by one of the authors of the study (LRM).

4.4 Sample analysis

An aliquot of the synovial fluid sample was used to measure TP concentration (g/dl) by use of a standard refractometer[4]. The remaining synovial fluid was then

[2] Intramed, SA

[3] Becton, Dickinson and Company© BD Vacutainer®, USA

[4] A.S.T. Inc., Japan

transferred into an EDTA tube. An aliquot of this fluid was immediately analysed for NCC using an automated haematology analyzer[5].

The synovial sample was centrifuged for 5 minutes, on a 1341*g* centrifuge[6]. The DNCC was obtained by examination of a smear of the precipitate after staining with a commercially available haematoxylin-eosin (H&E) stain[7] following the manufacturer's instructions. The percentage of neutrophils was calculated. All the smears were evaluated by the same experienced clinical pathologist that was blinded to the treatment. Supernatant was stored under -80 ºC for further SAA determination.

The venous blood samples were centrifuged for 5 minutes on a 1341*g* centrifuge[8]. The serum was separated and stored at -80ºC for further SAA determination.

Serum and synovial SAA determinations from all samples were performed at the same time once the collection of samples was finalized. The measurement of SAA concentrations was performed using an automated chemistry analyser[9] according to manufacturer's instructions with a commercially available human SAA turbidometric immunoassay[10] previously validated for equine use (44).

[5] ABBOTT, Cell-Dyn® 3700 System, USA

[6] Hettich Zentrifugen, Rotofix® 32A, UK

[7] Rapidiff 1®, Clinical Sciences Diagnostics, SA

[8] Hettich Zentrifugen, Universal® 320, UK

[9] COBAS INTEGRA 400 plus, Roche Laboratories, Switzerland

[10] Eiken SAA TIA

All measurements were made at the Clinical Pathology Laboratory of the Department of Companion Animal Clinical Studies, Faculty of Veterinary Science, University of Pretoria.

4.5 Evaluation of performance of the assay

The in-house performance of the assay was evaluated using synovial fluid and serum samples obtained from clinical cases. For serum, a sample from a horse with increased SAA because of African Horse Sickness was used. For synovial fluid, a sample from a horse with increased SAA concentration in synovial fluid because of septic arthritis was used. Three independent, 5-serial dilutions for each sample type (synovial and serum) were prepared using the diluents provided with the SAA measurement kit. The SAA concentration in all these dilution samples was measured and analytical validation parameters calculated. All these dilutions and measurements were performed by the same trained laboratory technician.

Based on these measurements, for SAA in synovial fluid the lower limit of quantification was 0.05 mg/l, the upper limit of quantification was 129 mg/l, the coefficient of determination was >0.90, and the intra-assay variability range from 1 to 10%.

For SAA in serum, the lower limit of quantification was 0.21 mg/l, the upper limit of quantification was 186 mg/l, the coefficient of determination was >0.90 and the intra-assay variability range from 0.8 to 16%.

4.6 Data Analysis

Descriptive and comparative statistic analyses were applied to the results. All the statistical procedures were done by Statistical Analysis System[11]. Dependent variables were evaluated over a relatively long period of time. Therefore, a scheme for repeated samples based on the area under the curve (AUC) was applied (61). The area under protein concentration curve, area under the number of nucleated cells curve, and area under the percentage of neutrophils in synovial fluid were estimated following the trapezoidal rule (62). The areas under the curve for each dependent variable were calculated with the following equation (61).

$$AUC_{0\text{-}96}=0.5\ i=0n-1AUC_{0\text{-}192}=0.5\ i=0n-1(Y_i + Y_{i+1})^* (t_{i+1}\text{-}t_i)$$

Where “t” is sampling time and “y” the observed outcome.

Prior to the selection of the statistical test, the normality of the data was assessed by Shapiro-Wilk and Kolmogorov-Smirnov statistical tests (level of significance $P<0.05$). Cross-over analysis of variance (ANOVA) was implemented for the statistical comparison of areas under the curve of total protein, nucleated cell count and percentage of neutrophils between the two groups. Mann-Whitney U test was applied to compare statistically the dependent variables between both treatment groups at each sampling time and each sampling time *versus* (*vs.)* baseline within each group. Unpaired Student T test was applied to compare statistically the changes from baseline of protein concentrations. Significant differences for fixed effects for all the statistical comparisons were set at the $p<0.05$ level.

Mean values and standard deviation for total protein and total nucleated cell count at each sampling period were calculated.

[11] SAS Institute, version 9.2, Cary, NC, 2011.

Due to the small number of samples with values of SAA above the limit of quantification in serum and synovial fluid, SAA results were not compared statistically but analyzed descriptively.

CHAPTER 5: RESULTS

Horses used in this study ranged from 2 to 3.5 years of age (2.67 ± 0.76 years) and weighed between 302 and 450 kg (400 ± 58.4 kg). All horses were mares.

All 6 mares were healthy on clinical and haematological evaluations at the beginning of each study period. Daily clinical examinations performed during the study revealed no abnormalities in 5 of the six horses. One mare (Horse 4) was excluded from the study as on day 7 of CG because of developing a mild increase in rectal temperature (38.4 ºC), increased respiratory rate (24 breaths per minute), abnormal respiratory sounds on thoracic auscultation, increased abdominal respiratory effort and intermittent non-productive cough. Haematological analysis performed on the day when the abnormal clinical signs were noted (day 7) showed increased concentration of fibrinogen (9.00 g/l; normal reference values 2.00 to 4.00 g/l). Serum SAA concentration was also increased (86.1 mg/l; normal reference values <30.0 mg/l) (24) (Table 5). Repeated haematological analysis on day 9 revealed fibrinogen of 6.00 g/l. These findings were not considered to be a complication of the study, and the mare was excluded from the study. All mares remained free of lameness for the duration of the study.

Horse 5 required the administration of a higher dose of romifidine (0.04 mg/kg – 0.08 mg/kg) to allow safe arthrocentesis. All arthrocentesis where performed successfully. The needle was only replaced on Horse 2 on the 1st sampling time of TG, Horse 3 on the 4th sampling of TG, and on Horse 6 on the 4th sampling of CG (Tables 6, 9 and 12).

5.1 Protein concentrations in synovial fluid

Synovial protein concentrations (mean ± SD; g/dl) at each point in time for CG and TG are presented in Figure 1 and Table 1. Individual synovial protein concentrations are included in Table 6. Synovial TP baseline values (mean ± SD; g/dl) were not significantly different ($p>0.05$) between CG (1.06 ± 0.18) and TG (1.02 ± 0.13). Protein AUC_{0-192} (g*h/dl) for CG (262 ± 79.8) was significantly lower ($p<0.01$) than for TG (621 ± 91.2).

Synovial protein concentrations in CG ranged from 0.80 to 3.10 g/dl with a mean ± SD of 1.30 ± 0.54 g/dl during the study. When synovial protein values in CG were compared with baseline values a significant difference was only found at 96h ($p=0.03$) (Figure 1 and Table 7). In TG, synovial protein concentrations ranged from 0.90 to 6.00 g/dl with a mean of 3.12 ± 1.36 g/dl during the study and values increased significantly when compared with baseline after the first injection of amikacin and remained increased throughout the study ($p<0.01$) (Figure 1 and Table 7). When protein values at each sampling time were compared between treatments significant differences ($p \leq 0.03$) were found at sampling times 48h, 96h, 144h, and 192h (Table 1 and Table 8).

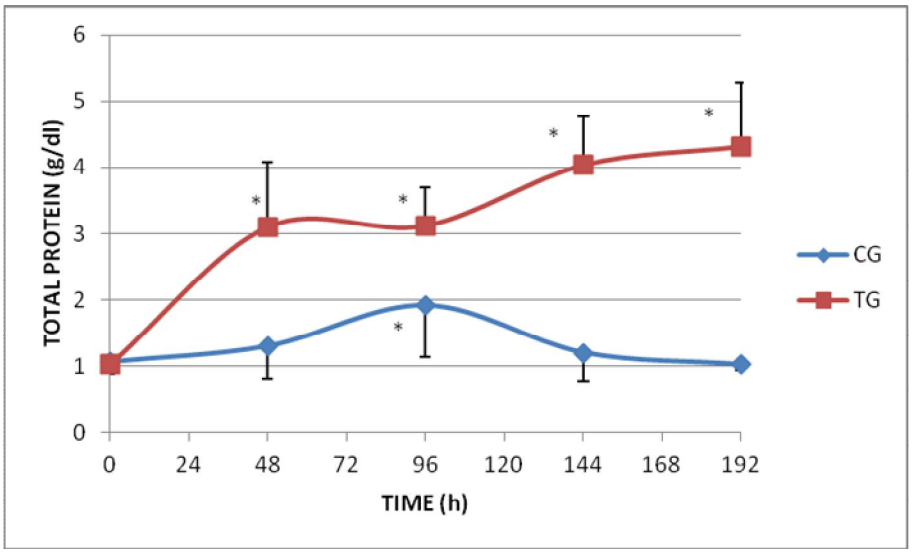


Figure 1 - Mean ± SD total synovial protein (TP) (g/dl) in 5 horses after repeated arthrocentesis (CG) or repeated arthrocentesis and administration of amikacin (TG) into the intercarpal joint. Synovial fluid samples were collected at time points 0, 48, 96, 144 and 192 hours. *Significant difference (p<0.05) with baseline value (time 0h).

Treatment	0h	48h*	96h*	144h*	192h*
CG	1.06 ± 0.18	1.33 ± 0.48	1.92 ± 0.78	1.2 ± 0.43	1.02 ± 0.08
TG	1.02 ± 0.13	3.1 ± 0.98	3.12 ± 0.58	4.04 ± 0.72	4.32 ± 0.95

Table 1 - Mean ± SD synovial total protein (TP) (g/dl) in 5 horses after repeated arthrocentesis (CG) or repeated arthrocentesis and administration of amikacin (TG) into the intercarpal joint at each sampling time (hours). Sampling was performed at 0, 48, 96, 144 and 192 hours (h). *Significant difference (P=<0.05) between CG and TG.

5.2 Nucleated cell count (NCC) in synovial fluid

Nucleated cell count values (mean ± SD) at each point in time for CG and TG are presented in Figure 2 and Table 2. Individual synovial NCC concentrations are included in Table 9. Baseline NCC values (mean ± SD; value x 10^9 cells/l) did not differ significantly between CG (0.06 ± 0.03) and TG (0.06 ± 0.04) (p= 1). Nucleated cell count $AUC_{0\text{-}192}$ (count x10^9*h/l) of CG (136 ± 95.9) and TG (281 ± 252) did not differ significantly (p=0.3).

Values of NCC in CG range from 0.04 to 3.36 x 10^9 cells/l with a mean ± SD of 0.61 ± 0.70 x 10^9 cells/l; and in TG, values range from 0.02 to 6.31 x 10^9 cells/l with a mean ± SD of 1.34 ± 1.67 x 10^9 cells/l. When compared with baseline, NCC values were significantly higher at all sampling times ($p<0.05$) in both CG and TG (Figure 2 and Table 10). When NCC values at each sampling time were compared between treatments a significant difference ($p<0.05$) was observed only at 192h (Table 2 and Table 11). Values of NCC peaked at 96h in both CG and TG (Figure 2).

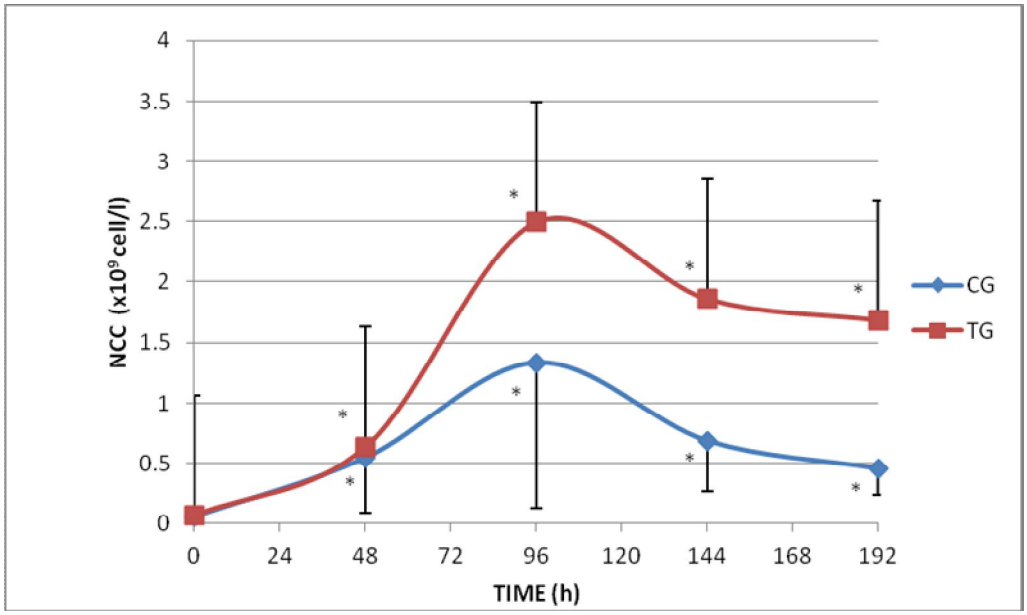


Figure 2 - Mean ± SD synovial nucleated cell count (NCC) (x10^9 cells/l) in 5 horses in response to repeated arthrocentesis (CG) or repeated arthrocentesis and administration of amikacin (TG) into the intercarpal joint. Sampling was performed at time points 0, 48, 96, 144 and 192 hours (h). *Significant difference (<0.05) with baseline value (time 0h).

Treatment	0h	48h	96h	144h	192h*
CG	0.06 ± 0.03	0.54 ± 0.46	1.33 ± 1.20	0.68 ± 0.42	0.45 ± 0.22
TG	0.06 ± 0.04	0.63 ± 0.37	2.49 ± 2.38	1.85 ± 2.21	1.68 ± 1.14

Table 2 - Mean ± SD synovial nucleated cell count (NCC) values (x10^9 cells/l) in 5 horses in response to repeated arthrocentesis (CG) or repeated arthrocentesis and administration of amikacin (TG) into the intercarpal joint at each sampling time (hours). Sampling was performed at 0, 48, 96, 144 and 192 hours (h). *Significant difference (P=<0.05) between CG and TG.

5.3 Percentage of neutrophils in synovial fluid

The percentage of neutrophils (mean ± SD) at each sampling time for CG and TG are presented in Table 3. Individual values of percentage of neutrophils for horses included in the study are presented in Table 12. The percentage of neutrophils at baseline did not differ significantly between treatments ($p>0.05$). The percentage of neutrophils AUC_{0-192} (count $x10^9$*h/l) revealed no significant difference between CG and TG ($p= 0.22$).

Values for percentage of neutrophils in CG range from 0.00 to 79.0% with a mean ± SD of 17.2 ± 17.4%. In TG, the percentage of neutrophils range from 0.00 to 24.0% with a mean ± SD of 9.16 ± 8.20%. No significant difference was found when comparing percentage of neutrophils in synovial fluid at baseline with each time point in both treatments (Table 13). When comparing percentage of neutrophils between CG and TG at each point in time, no significant differences were obtained (Table 14).

TREATMENT	0h	48h	96h	144h	192h
CG	11.8 ± 14.8	24.2 ± 10.2	32.0 ± 29.3	8.00 ± 6.32	9.80 ± 7.29
TG	5.60 ± 9.53	5.80 ± 4.60	8.80 ± 3.35	10.4 ± 9.07	15.2 ± 10.9

Table 3 - Mean ± SD percentage of neutrophils (%) in synovial fluid of 5 horses in response to repeated arthrocentesis (CG) or repeated arthrocentesis and administration of amikacin (TG) into the intercarpal joint at each sampling time (hours). Sampling was performed at 0, 48, 96, 144 and 192 hours (h).

5.4 Serum amyloid A in synovial fluid

Synovial SAA concentrations are detailed in Table 4. Only horse 4 in TG showed values higher than the lower limit of quantification (LOQ; 0.05 mg/l) (Table 4). Horse number 4 was eliminated from the statistical analysis in both treatments but SAA concentrations are included in Table 4.

TREATMENT	HORSE	SAA 0h	SAA 48h	SAA 96h	SAA 144h	SAA 192h
TG	1	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	2	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	3	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	4	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	5	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	6	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
CG	1	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	2	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	3	<LOQ	<LOQ	0.03	<LOQ	<LOQ
	4	<LOQ	<LOQ	0.23	<LOQ	<LOQ
	5	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	6	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ

Table 4 - Serum amyloid A (SAA) values (mg/l) obtained in synovial fluid of 6 horses subjected to repeated arthrocentesis (CG) or repeated arthrocentesis and administration of amikacin (TG) into the intercarpal joint. Time 0 corresponds to baseline values. Sampling was performed at time points 0, 48, 96, 144 and 192 hours. Lower limit of quantification (LOQ) was 0.05 mg/l.

5.5 Serum amyloid A in serum samples

Systemic SAA protein concentrations are detailed in Table 5. Horse number 4 showed increased SAA values at time 144h during CG and this horse was eliminated from the study due to clinical signs of systemic respiratory disease. The rest of the samples showed SAA values ≤ 18.42 mg/l and most of the samples remained below the lower limit of quantification (LOQ; 0.21 mg/l).

TREATMENT	HORSE	SAA 0h	SAA 48h	SAA 96h	SAA 144h	SAA 192h
TG	1	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	2	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	3	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	4	0.02	<LOQ	<LOQ	<LOQ	<LOQ
	5	<LOQ	<LOQ	<LOQ	0.01	<LOQ
	6	0.15	<LOQ	<LOQ	<LOQ	<LOQ
CG	1	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	2	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	3	<LOQ	18.42	0.43	<LOQ	<LOQ
	4	0.86	5.04	14.63	86.06	0.77
	5	<LOQ	<LOQ	<LOQ	8.64	0.73
	6	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ

Table 5 - Serum amyloid A (SAA) values (mg/l) obtained in blood samples from 6 horses subjected to repeated arthrocentesis (CG) or repeated arthrocentesis and administration of amikacin (TG) into the intercarpal joint. Time 0h corresponds to baseline values. Sampling was performed at time points 0, 48, 96, 144 and 192 hours (h). Lower limit of quantification (LOQ) was 0.21 mg/l.

CHAPTER 6: DISCUSSION

The study presented in this dissertation was designed to imitate the common practice performed when evaluating and treating an equine septic joint in the clinical setting. For this reason, collection of synovial samples and intra-articular administration of amikacin where performed every 48h. Amikacin was selected for this study because it has best antimicrobial activity against the bacteria most frequently isolated from septic joints in horses (30). The injection of a placebo substance such as lactate ringer solution (LRS) or saline solution, was not performed in the control group because it is it is not relevant clinically as equine practitioners do not inject a placebo solution when evaluating a suspected septic joint. The need for administration of amikacin after the last sample collection in the control group is questionable but was performed as protective measure. The study protocol simulated clinical practice and therefore offered an accurate representation of what happens within a joint after performing several sample collections and repeated intra-articular administration of amikacin. This brings clinical relevance to this study.

All arthrocentesis where successfully performed except in three different times where the needle was replaced and arthrocentesis was performed again (Tables 6, 9 and 12). Statistical analysis of the effect of these three incidents could not be performed due to the small number of animals.

The normal value for TP in synovial fluid is < 2.5 g/dl (3) and values can increase in mild synovitis (<3 g/dl), after arthrocentesis (1.5-2.5 g/dl), and arthrocentesis combined with balanced electrolyte solution injection (3-4 g/dl), local anesthetics (2.5-4 g/dl), gentamicin (4.5-6 g/dl) or 10% DMSO solution (2.5-4 g/dl) (3). In our

study, TP significantly increased from baseline only at 96h after repeated arthrocenteses; however, all TP values remained within the normal range with the exception of one sample (horse 5 at 96h). This is in agreement with a previous study where repeated arthrocentesis with injection of LRS in the digital flexor tendon sheath (DFTS) did not increase protein significantly at any sampling time (20). In contrast, repeated arthocenteses combined with a single intra-articular saline solution administration produced a significant increase of synovial TP concentration within 4h after the first arthrocentesis and TP remained increased thereafter for the remaining of the study (18). Mean TP values in that study seem to be slightly higher than those observed in this study, but most of the values remained ≤ 3.5g/dl (18). The use of a more frequent sampling protocol (18, 20), along with intra-articular saline solution injection (18), rather than LRS (20) after each arthrocentesis might have caused a more prominent inflammatory reaction accounting for the observed differences between those studies and the present, where no fluid was injected into the joint.

On the other hand, repeated intra-articular administration of amikacin produced TP values above normal published values (< 2.5 g/dl) (1, 3, 13, 63) in all horses from time 48h (4 horses) or 96h (1 horse) and these values remained elevated until the end of the study. In fact, many of the samples at 144h and 192h were ≥4.00 g/dl, values that are typically associated with septic synovitis (1, 3, 13, 63). When the effect of a single intra-thecal administration of amikacin (250 mg) was evaluated in the DFTS, TP values increased significantly after 12h (2.3 ± 0.8 g/dl) and decreased slowly afterwards (20), but those values remained below those typically associated with septic arthritis. Similarly, in a study performed by Taintor *et al* (11), a single intra-articular administration of 500 mg of amikacin did not produce an increase in

synovial TP values above clinically normal published ranges. Differences in the frequency of amikacin administration (single dose *vs.* 5 doses in this study), the response to centesis of the synovial structures being evaluated (joint *vs.* tendon sheath), antimicrobial pharmaceutical preparation as well as total dose administered per horse (large horses receiving a lower net dose than smaller horses) might explain different results.

Amikacin sulphate and mepivacaine hydrochloride have been shown to cause mild toxic effects on equine articular cartilage explants (58). Transient synovitis has also been observed after intra-articular administration of pharmaceuticals such as gentamicin (64, 65), mepivacaine hydrochloride and lidocaine (60) in healthy horses. Such synovitis was attributed to a direct chemical effect (gentamicin) (64, 65) or a reduction of pH in the synovial environment (lidocaine) (66). There is a possibility that amikacin causes chemical inflammation of synovial structures; however, the study presented here was not designed to evaluate such hypothesis and further research is needed in this area.

From the horses included in our study, synovial TP values were highest in horse 3, reaching values of 5.20 g/dl and 6.00 g/dl at 144h and 192h, respectively, and after repeated intra-articular administration of amikacin. This horse had the lowest body weight (302 kg) and therefore received the largest amikacin dose in relation to its body weight in comparison with the rest of the horses. This may indicate a relationship between the intra-synovial dose and the magnitude of the inflammatory response and should be further investigated.

Blood contamination during sampling could have increased synovial TP values. The effect of blood contamination on synovial TP levels was not evaluated; however,

arthrocentesis of the intercarpal joint using a dorsal approach has a low degree of difficulty (1 out of 3, being 3 the most difficult) (59) and all arthrocenteses were performed by the same operator (AST).

Normal reference values for NCC in synovial fluid are <0.5 x 10^9 cells/l (3). Increased NCC are typically observed in the presence of a septic process (>20 x 10^9 cells/l), mild synovitis (<0.25 x 10^9 cells/l) and after arthrocentesis (1-4 x 10^9 cells/l) (3). Intra-articular injection of different compounds also has an effect on NCC. Increased NCC in responses to intra-articular injection of balanced electrolyte solution (6-45 x 10^9 cells/l), local anaesthetics (2-10 x 10^9 cells/l), gentamicin (8-40 x 10^9 cells/l) and 10% DMSO solution (6-20 x 10^9 cells/l) have been reported (3). In our study, repeated arthrocentesis as well as repeated arthrocentesis combined with amikacin administration caused an increase in synovial NCC when compared with baseline values, and NCC values remained ≤6.31 x 10^9 cells/l. Differences between groups were only observed at 192h where the group receiving amikacin had higher NCC than the control group. The increased NCC values found after repeated arthrocentesis in our study are similar to those previously reported (3). The highest NCC after amikacin administration was observed in horse 3 and this is probably the result of the higher relative dose received by this horse.

The single intra-articular administration of amikacin produced an increase in synovial NCC reaching a peak (median value of 1.16 x 10^9 cells/l) 24h post-administration (11). In our study, the highest mean NCC value was reached after the second administration of amikacin (96h from baseline) with a mean ± SD NCC of 2.49 ± 2.38 x 10^9 cells/l.

The NCC values observed in our study after repeated intra-articular administration of amikacin are similar to those reported after administration of local anesthetics (3). Higher values, consistent with those observed on septic synovitis were observed after single administration of LRS or amikacin into the DFTS (20). Greater difficulty in accessing a tendon sheath (difficulty grade of 3 out of 3, being 3 the most difficult) (59) linked to a higher risk of blood contamination of the synovial fluid sample and a greater sensitivity of sheaths to centesis might explain differences between studies.

For the comparison of DNCC between groups, the percentage of neutrophils was used as this marker is commonly used clinically and normal and pathological values have been widely published (1, 3, 13, 63). The normal value for percentage of neutrophils in equine joints is <10% and a marked increase (>90%) typically occurs in septic arthritis (3). Toxic changes to the neutrophils are also relatively common in the presence of sepsis (3). Increased percentage of neutrophils is also observed in mild synovitis (<10%), after arthrocentesis (50%), and after intra-articular injection of balanced electrolyte solution (80%), local anesthetics (60%), gentamicin (50%) and 10% DMSO solution (>50%) (3). In our study, some horses showed values higher than those reported to be normal; however, values remained lower than the values observed in septic processes and toxic changes of the neutrophils were not observed. Centesis of the DFTS showed values higher than those reported here (20). As previously discussed greater difficulty in accessing a tendon sheath leading to more trauma as well as greater sensitivity of sheaths to centesis might account for the observed differences.

A high percentage of neutrophils was observed in one of the mares (79%) (CG, 96h); however, the total NCC was 1.29 x 10^9 cells/l and no toxic changes to the neutrophils were observed. Values of percentage of neutrophils should always be

correlated directly with NCC values as they are mathematically dependent and sepsis should be considered in the presence of other consistent parameters and clinical signs.

In humans, synovial SAA synthesis is associated with cartilage degeneration during rheumatoid processes and it accurately reflects changes in inflammatory joint disease being the best marker available (26, 48). In our study, repeated arthrocentesis every 48h did not show a detectable increase in SAA concentrations in serum or synovial fluid supporting previous reports (18). Repeated arthrocentesis with and without the intra-articular administration of amikacin might not exert a sufficient inflammatory response to stimulate serum and synovial SAA synthesis to reach values higher than the detection range of the test used. Another explanation for these results is that as described in humans, SAA concentrations in serum rises to higher levels in response to bacterial stimulus than to viral infection or other kinds of inflammation (42). The same phenomenon could be occurring in horses. Synovial and serum SAA in horses suffering from septic arthritis were reported to be >1000 mg/l and >1500 mg/l respectively (18). In our equine hospital, concentrations of SAA in synovial fluid collected from septic arthritis typically ranges from 60 mg/l to 850 mg/l (unpublished data). In addition, SAA has a very short half life in serum. In mice, the half-life of SAA in serum is 75-80 minutes with a 95% clearance from plasma 6h after the synthesis has ceased (41). This brings the possibility that SAA concentrations could have risen but then decreased before the next sampling was performed (48h after) and the increased levels were not detected in our measurements. However, to the authors' knowledge the half-life of SAA in synovial fluid has not been reported. The fact that SAA values were not detected after repeated arthrocentesis and repeated arthrocentesis with the administration of

amikacin every 48h, and that synovial SAA increased to high levels during septic arthritis (18, 28), suggest that measurement of synovial SAA might be valuable when monitoring clinical cases.

Limitations of this study include the limited sample size (5 horses after one was excluded). In addition, all horses received the same amount of amikacin (500 mg) intra-articularly regardless of their body weight, and a specific dose per kilogram was not calculated. Although this is a common clinical practice, potential local side-effects of a 500 mg dose of intra-articular amikacin might be more pronounced in smaller animals as suspected in horse 3. Joint distention was not recorded and graded even though it was subjectively noticed in some horses and information of sampling difficulty or percentage of blood contamination was not systematically recorded. Although this information might have been useful for evaluation of synovial TP and NCC, this is consider of marginal importance due to the low degree of difficulty of the arthrocentesis technique and the fact that all the centesis were performed by the same person (AST). Based on the short half life of SAA in serum, more frequent arthrocenteses might have helped elucidate if a short-lived increase in synovial SAA occurred. However, the authors elected not to sample more frequently as such practice does not reflect the clinical approach and such detailed evaluation of SAA response was beyond the scope of this study.

An interesting and incidental finding not related to the purpose of this study is the SAA levels found in Horse 4. The increase in SAA measurement in serum and its correlation with clinical signs of systemic illness shows the quick SAA response to inflammation and its potential use as a real time monitoring APP. Various studies have demonstrated that SAA in serum is a reliable marker of most forms of tissue

injury, infection and inflammation that is rapid and reflects the extent and activity of disease (24, 25, 43, 67).

The methods used to measure TP, NCC and DNCC are widely accepted and used in clinical equine practice (13). All these measurements were performed by the same experienced professional in the Clinical Pathology laboratory of our institution. The method used in this study to measure SAA in synovial fluid and serum (human SAA turbidometric immunoassay) has been validated previously for use in horses (44) and has been used by other equine investigators (18, 28). In addition, this method is currently being used in some diagnostic laboratories in Europe and the United States for routine SAA measurements on horse serum and synovial fluid (21). An in-house evaluation of performance of the assay was also performed. The results from this evaluation show a lower limit of quantification and intra-assay variability for serum and synovial fluid, similar to previous studies (44). Lower limits of quantification are not considered necessarily important as in clinical cases, SAA in serum and synovial fluid increase to high levels (18, 28). A clinical case of African Horse Sickness was used for the evaluation of the assay for serum samples, as this pathology produces a high inflammatory response in the body with the concurrent increase in SAA on serum.

The results of this study suggest that repeated intra-articular administration of amikacin produces a mild to moderate synovitis characterized by increased synovial total protein concentration and nucleated cell count. These values, especially TP, could rise to ranges consistent with septic arthritis and therefore, special attention should be paid when evaluating these synovial markers in joints being treated with repeated intra-articular administration of amikacin. Contrary to these findings and as hypothesized initially, concentrations of SAA in synovial fluid and serum did not

exhibit any change when evaluations were performed every 48h. Values were lower than the test's detection range in most of the samples. Based on the findings in our study, and considering the highly increased synovial SAA concentrations in the presence of joint sepsis as observed in previous studies (18, 28) and in our hospital population, synovial SAA could be a more specific marker for septic processes than TP and NCC since it was not influenced by repeated arthrocentesis and repeated administration of amikacin in the commonly used schedule (every 48h). These findings contribute to the knowledge of SAA behavior in normal horses and its use when evaluating a joint that has been subjected to intra-articular amikacin administration. To the best of the authors' knowledge no previous studies have evaluated the effect of repeated doses of amikacin on synovial SAA, TP, NCC and DNCC.

CHAPTER 7: CONCLUSIONS

Based on the results of the present study we can conclude that:

- Repeated anthrocentesis and intra-articular administration of amikacin every 48h can cause an increase in the synovial total protein concentration to values consistent with septic arthritis.

- Repeated anthrocentesis alone or in combination with intra-articular administration of amikacin can cause a mild increase in synovial nucleated cell count, although these values were typically lower than those commonly observed in the presence of joint sepsis.

- Serum amyloid A in synovial fluid was not affected by either the repeated arthocenteses or repeated intra-articular administration of amikacin. Therefore, SAA could serve as a better marker for synovial sepsis than synovial total protein or nucleated cell count when evaluating a joint that has been previously sampled or treated with intra-articular amikacin.

- The test used (Eiken SAA TIA) for the measurement of SAA seems to perform reliably in different laboratories.

TABLES 6 to 14

TREATMENT	HORSE	TP 0h	TP 48h	TP 96h	TP 144h	TP 192h
TG	1	1	3.9	3.9	3.9	4
	2	0.9*	1.5	2.3	4	4
	3	1.1	3	3.3	5.2*	6
	5	1.2	3.2	2.9	3.2	4
	6	0.9	3.9	3.2	3.9	3.6
CG	1	0.8	0.8	1.3	0.8	1
	2	1.1	1.1	1.1	1	1.1
	3	1.3	1.3	2.1	1.3	1.1
	5	1	2.1	3.1	1.9	0.9
	6	1.1	1.2	2	1*	1

Table 6 - Total protein (TP) (g/dl) in synovial fluid of horses included in the study (n=5), in response to repeated arthrocentesis (CG) or repeated arthrocentesis and administration of amikacin (TG) into the intercarpal joint. Time 0h corresponds to baseline values. Sampling was performed at time points 0, 48, 96, 144 and 192 hours (h). *Samplings where the needle was replaced.

Treatment	0h *vs* 48h	0h *vs* 96h	0h *vs* 144h	0h *vs* 192h
TG	<0.01	<0.01	<0.01	<0.01
CG	>0.05	0.03	>0.05	>0.05

Table 7 - P values when comparing total protein (TP) (g/dl) in synovial fluid at 48, 96, 144 and 192 hours (h) with baseline values (0h) of horses included in the study (n=5), in response to repeated arthrocentesis (CG) or repeated arthrocentesis and administration of amikacin (TG) into the intercarpal joint.

Treatment	0h	48h	96h	144h	192h
CG *vs.* TG	>0.05	<0.01	<0.03	<0.01	<0.01

Table 8 - P values when comparing total protein (TP) (g/dl) in synovial fluid at 0, 48, 96, 144 and 192 hours (h) between repeated arthrocentesis (CG) and repeated arthrocentesis with administration of amikacin (TG) into the intercarpal joint.

TREATMENT	HORSE	NCC 0h	NCC 48h	NCC 96h	NCC 144h	NCC 192h
TG	1	0.04	0.74	0.96	0.38	2.53
	2	0.05*	0.79	3.26	1.78	0.84
	3	0.15	1.12	6.31	5.69*	3.24
	5	0.02	0.21	0.46	0.92	1.14
	6	0.06	0.30	1.48	0.51	0.65
CG	1	0.04	0.96	1.05	0.69	0.36
	2	0.05	0.05	0.82	0.43	0.29
	3	0.12	0.52	1.29	1.22	0.63
	5	0.04	1.07	3.36	0.96	0.76
	6	0.05	0.14	0.16	0.14*	0.25

Table 9 - Nucleated cell count (NCC) (count x10^9 cells/l) in synovial fluid of 5 horses included in the study, in response to repeated arthrocentesis (CG) or repeated arthrocentesis and administration of amikacin (TG) into the intercarpal joint. Time 0h corresponds to baseline values. Sampling was performed at time points 0, 48, 96, 144 and 192 hours (h). * Samplings where the needle was replaced.

Treatment	0h *vs* 48h	0h *vs* 96h	0h *vs* 144h	0h *vs* 192h
TG	0.01	0.01	0.01	0.01
CG	0.04	0.04	0.01	0.004

Table 10 - P values when comparing nucleated cell count (NCC) (count x10^9 cells/l) in synovial fluid at 48, 96, 144 and 192 hours with baseline values (0h) of 5 horses included in the study, in response to repeated arthrocentesis (CG) or repeated arthrocentesis and administration of amikacin (TG) into the intercarpal joint.

Treatment	0h	48h	96h	144h	192h
CG *vs.* TG	1	>0.05	>0.05	>0.05	<0.05

Table 11 - P values when comparing nucleated cell count (NCC) (count x10^9 cells/l) in synovial fluid at 0, 48, 96, 144 and 192 hours (h) between repeated arthrocentesis (CG) and repeated arthrocentesis with administration of amikacin (TG) into the intercarpal joint.

TREATMENT	HORSE	%N 0h	%N 48h	%N 96h	%N 144h	%N 192h
TG	1	0	5	10	15	19
	2	0*	1	11	4	10
	3	22	7	9	24*	32
	5	0	13	11	7	12
	6	6	3	3	2	3
CG	1	4	20	11	16	16
	2	3	14	25	4	18
	3	6	41	79	12	10
	5	8	21	39	8	3
	6	38	25	6	0*	2

Table 12 - Percentage of neutrophils for each horse included in the study (n=5), in response to repeated arthrocentesis (CG) and repeated administration of amikacin (TG) into the intercarpal joint. Sampling was performed at time points 0, 48, 96, 144 and 192 hours (h). Time 0h corresponds to baseline values. *Samplings where the needle was replaced.

Treatment	0h *vs* 48h	0h *vs* 92h	0h *vs* 144h	0h *vs* 192h
TG	>0.05	>0.05	>0.05	>0.05
CG	>0.05	>0.05	>0.05	>0.05

Table 13 - P values when comparing percentage of neutrophils (%) in synovial fluid at 48, 96, 144 and 192 hours with baseline values (0h) of 5 horses included in the study, in response to repeated arthrocentesis (CG) or repeated arthrocentesis and administration of amikacin (TG) into the intercarpal joint.

Treatment	0h	48h	96h	144h	192h
CG *vs.* TG	>0.05	>0.05	>0.05	>0.05	>0.05

Table 14 - P values when comparing percentage of neutrophils (%) in synovial fluid at 0, 48, 96, 144 and 192 hours (h) between repeated arthrocentesis (CG) and repeated arthrocentesis with administration of amikacin (TG) into the intercarpal joint.

REFERENCES

1. Bertone AL. Update on infectious arthritis in horses. Equine Veterinary Education. 1999;11(3):143-52.

2. Stokes AM, Eades SC, Moore RM. Pathophysiology and treatment of acute laminitis. In: Reed SM, Bayly WM, Sellon DC, editors. Equine Internal Medicine. Second Edition ed. United States: Elsiever; 2004. p. 522-531.

3. Frisbie DD. Synovial joint biology and pathobiology. In: Equine Surgery. Third Edition ed. St. Louis Missouri: Elsevier Inc.; 2006. p. 1036-1055.

4. Morton AJ. Diagnosis and treatment of septic arthritis. Veterinary Clinics of North America: Equine Practice. 2005 12;21(3):627-49.

5. Schneider RK, Bramlage LR, Moore RM, Mecklenburg LM, Kohn CW, Gabel AA. A retrospective study of 192 horses affected with septic arthritis/tenosynovitis. Equine Vet J. 1992;24(6):436-42.

6. Smith LJ, Marr CM, Payne RJ, Stoneham SJ, Reid SWJ. What is the likelihood that thoroughbred foals treated for septic arthritis will race? Equine Vet J. 2004;36(5):452-6.

7. Lapointe JM, Laverty S, Lavoie JP. Septic arthritis in 15 standardbred racehorses after intra-articular injection. Equine Vet J. 1992 Nov;24(6):430-4.

8. Gibson KT, McIlwraith CW, Turner AS, Stashak TS, Aanes WA, Trotter GW. Open joint injuries in horses: 58 cases (1980-1986). J Am Vet Med Assoc. 1989 Feb 1;194(3):398-404.

9. Ross MW, Orsini JA, Richardson DW, Martin BB. Closed suction drainage in the treatment of infectious arthritis of the equine tarsocrural joint. Veterinary Surgery. 1991;20(1):21-9.

10. Bertone AL, Cohen JM. Infectous arthritis and fungal infectious arthritis. In: Ross MW, Dyson SJ, editors. Diagnosis and Management of Lameness in the Horse. Second Edition. United States: Elsevier Inc.; 2011. p. 677-687.

11. Taintor J, Schumacher J, Degraves F. Comparison of amikacin concentrations in normal and inflamed joints of horses following intra-articular administration. Equine Vet J. 2006;38(2):189-91.

12. Schneider RK, Bramlage LR, Mecklenburg LM, Moore RM, Gabel AA. Open drainage, intra-articular and systemic antibiotics in the treatment of septic arthritis/tenosynovitis in horses. Equine Vet J. 1992;24(6):443-9.

13. Van Pelt RW. Interpretation of synovial fluid findings in the horse. J Am Vet Med Assoc. 1974 Jul 1;165(1):91-5.

14. Bertone AL, Palmer JL, Jones J. Synovial fluid cytokines and eicosanoids as markers of joint disease in horses. Veterinary Surgery. 2001;30(6):528-38.

15. Gibson KT, Hodge H, Whittem T. Inflammatory mediators in equine synovial fluid. Aust Vet J. 1996;73(4):148-51.

16. Korenek NL, Andrews FM, Maddux JM, Sanders WL, Faulk DL. Determination of total protein concentration and viscosity of synovial fluid from the tibiotarsal joints of horses. Am J Vet Res. 1992 May;53(5):781-4.

17. McIlwraith CW. Use of synovial fluid and serum biomarkers in equine bone and joint disease: A review. Equine Vet J. 2005 Sep;37(5):473-82.

18. Jacobsen S, Thomsen MH, Nanni S. Concentrations of serum amyloid A in serum and synovial fluid from healthy horses and horses with joint disease. Am J Vet Res. 2006 10/01; 2011/08;67(10):1738-42.

19. Kraus BM. Lacerations involving synovial structures: Initial treatment and novel therapy for infectious Arthritis/Tenosynovitis. Clinical Techniques in Equine Practice. 2006 6;5(2):82-92.

20. Dykgraaf S, Dechant JE, Johns JL, Christopher MM, Bolt DM, Snyder JR. Effect of intrathecal amikacin administration and repeated centesis on digital flexor tendon sheath synovial fluid in horses. Veterinary Surgery. 2007;36(1):57-63.

21. Jacobsen S, Andersen PH. The acute phase protein serum amyloid A (SAA) as a marker of inflammation in horses. Equine Veterinary Education. 2007;19(1):38-46.

22. Hultén C, Sletten K, Foyn Bruun C, Marhaug G. The acute phase serum amyloid A protein (SAA) in the horse: Isolation and characterization of three isoforms. Vet Immunol Immunopathol. 1997 7;57(3-4):215-27.

23. Jacobsen S, Nielsen JV, Kjelgaard-Hansen M, Toelboell T, Fjeldborg J, Halling-Thomsen M, et al. Acute phase response to surgery of varying intensity in horses: A preliminary study. Veterinary Surgery. 2009;38(6):762-9.

24. Nunokawa Y, Fujinaga T, Taira T, Okumura M, Yamashita K, Tsunoda N, et al. Evaluation of serum amyloid A protein as an acute-phase reactive protein in horses. J Vet Med Sci. 1993 Dec;55(6):1011-6.

25. Pepys MB, Baltz ML, Tennent GA, Kent J, Ousey J, Rossdale PD. Serum amyloid A protein (SAA) in horses: Objective measurement of the acute phase response. Equine Vet J. 1989;21(2):106-9.

26. Cunnane G, Grehan S, Geoghegan S, McCormack C, Shields D, Whitehead AS, et al. Serum amyloid A in the assessment of early inflammatory arthritis. J Rheumatol. 2000 Jan;27(1):58-63.

27. Hultén C, Grönlund U, Hirvonen J, Tulamo R-, Suominen MM, Marhaug G, et al. Dynamics in serum of the inflammatory markers serum amyloid A (SAA), haptoglobin, fibrinogen and 2-globulins during induced noninfectious arthritis in the horse. Equine Vet J. 2002;34(7):699-704.

28. Jacobsen S, Niewold TA, Halling-Thomsen M, Nanni S, Olsen E, Lindegaard C, et al. Serum amyloid A isoforms in serum and synovial fluid in horses with lipopolysaccharide-induced arthritis. Vet Immunol Immunopathol. 2006 4/15;110(3-4):325-30.

29. Sukenik S, Henkin J, Zimlichman S, Skibin A, Neuman L, Pras M, et al. Serum and synovial fluid levels of serum amyloid A protein and C-reactive protein in inflammatory and noninflammatory arthritis. J Rheumatol. 1988 Jun;15(6):942-5.

30. Schneider RK. In: Common bacteria encountered in septic arthritis. Proceedings of the annual convention of the AAEP; ; 1998. p. 152-158.

31. Tizard RI. How inflammation is triggered. In: Veterinary Inmunology: An Introduction. Seventh Edition ed. Philadelphia: Saunders, Elsevier; 2004. p. 10-23.

32. Bertone AL. Infectious arthritis. In: McIlwraith CW, Trotter GW, Saunders WB, editors. Joint Disease in the Horse. Philadelphia: Saunders, Elsevier; 1996. p. 397-409.

33. Hardy J, Bertone AL, Malemud CJ. Effect of synovial membrane infection in vitro on equine synoviocytes and chondrocytes. Am J Vet Res. 1998 Mar;59(3):293-9.

34. Palmer JL, Bertone AL. Joint structure, biochemistry and biochemical disequilibrium in synovitis and equine joint disease. Equine Vet J. 1994;26(4):263-77.

35. Curtiss PH,Jr. Changes produced in the synovial membrane and synovial fluid by disease. J Bone Joint Surg Am. 1964 Jun;46:873-88.

36. Gabay C, Kushner I. Acute-phase proteins and other systemic responses to inflammation. N Engl J Med. 1999 02/11;340(6):448-54.

37. Badolato R, Wang JM, Murphy WJ, Lloyd AR, Michiel DF, Bausserman LL, et al. Serum amyloid A is a chemoattractant: Induction of migration, adhesion, and tissue infiltration of monocytes and polymorphonuclear leukocytes. J Exp Med. 1994 Jul 1;180(1):203-9.

38. Benditt EP, Eriksen N. Amyloid protein SAA is associated with high density lipoprotein from human serum. Proc Natl Acad Sci U S A. 1977 Sep;74(9):4025-8.

39. Husebekk A, Husby G, Sletten K, Marhaug G, Nordstoga K. Characterization of amyloid protein AA and its serum precursor SAA in the horse. Scand J Immunol. 1986;23(6):703-9.

40. Bausserman LL, Saritelli AL, Van Zuiden P, Gollaher CJ, Herbert PN. Degradation of serum amyloid A by isolated perfused rat liver. J Biol Chem. 1987 Feb 5;262(4):1583-9.

41. Hoffman JS, Benditt EP. Plasma clearance kinetics of the amyloid-related high density lipoprotein apoprotein, serum amyloid protein (apoSAA), in the mouse. evidence for rapid apoSAA clearance. J Clin Invest. 1983 Apr;71(4):926-34.

42. Malle E, De Beer FC. Human serum amyloid A (SAA) protein: A prominent acute-phase reactant for clinical practice. Eur J Clin Invest. 1996;26(6):427-35.

43. Stoneham SJ, Palmer L, Cash R, Rossdale PD. Measurement of serum amyloid A in the neonatal foal using a latex agglutination immunoturbidimetric assay: Determination of the normal range, variation with age and response to disease. Equine Vet J. 2001;33(6):599-603.

44. Jacobsen S, Kjelgaard-Hansen M, Hagbard Petersen H, Jensen AL. Evaluation of a commercially available human serum amyloid A (SAA) turbidometric immunoassay for determination of equine SAA concentrations. The Veterinary Journal. 2006 9;172(2):315-9.

45. Hultén C, Tulamo R-, Suominen MM, Burvall K, Marhaug G, Forsberg M. A non-competitive chemiluminescence enzyme immunoassay for the equine acute phase protein serum amyloid A (SAA) – a clinically useful inflammatory marker in the horse. Vet Immunol Immunopathol. 1999 5;68(2-4):267-81.

46. Marhaug G, Hackett B, Dowton SB. Serum amyloid A gene expression in rabbit, mink and mouse. Clin Exp Immunol. 1997 Feb;107(2):425-34.

47. Urieli-Shoval S, Cohen P, Eisenberg S, Matzner Y. Widespread expression of serum amyloid A in histologically normal human tissues. predominant localization to the epithelium. J Histochem Cytochem. 1998 Dec;46(12):1377-84.

48. O'Hara R, Murphy EP, Whitehead AS, FitzGerald O, Bresnihan B. Local expression of the serum amyloid A and formyl peptide receptor-like 1 genes in synovial tissue is associated with matrix metalloproteinase production in patients with inflammatory arthritis. Arthritis & Rheumatism. 2004;50(6):1788-99.

49. Kumon Y, Suehiro T, Hashimoto K, Nakatani K, Sipe JD. Local expression of acute phase serum amyloid A mRNA in rheumatoid arthritis synovial tissue and cells. J Rheumatol. 1999 Apr;26(4):785-90.

50. Vallon R, Freuler F, Desta-Tsedu N, Robeva A, Dawson J, Wenner P, et al. Serum amyloid A (apoSAA) expression is up-regulated in rheumatoid arthritis and induces transcription of matrix metalloproteinases. J Immunol. 2001 Feb 15;166(4):2801-7.

51. Lemos ML. Antimicrobianos que inhiben la sintesis de proteinas. In: Botana LM, Landoni MF, Martin-Jimenez T, editors. Farmacologia y Terapeutica Veterinaria. Spain: McGraw-Hill, Interamericana; 2002. p. 468-483.

52. Satoh M, Fujinaga T, Okumura M, Hagio M. Sandwich enzyme-linked immunosorbent assay for quantitative measurement of serum amyloid A protein in horses. Am J Vet Res. 1995 Oct;56(10):1286-91.

53. Dowling PM. Antimicrobial therapy. In: Bertone JJ, Horspool LJ, editors. Equine Clinical Pharmacology. China: Saunders, Elsiever; 2005. p. 13-46.

54. Schneider RK. In: Treatment of posttraumatic septic arthritis. Proceedings of the annual convention of the AAEP; ; 1998. p. 167-171.

55. McIlwraith CW. Treatment of infectious arthritis. Vet Clin North Am Large Anim Pract. 1983 Jul;5(2):363-79.

56. Yarbrought T. Intraarticular medication. In: Bertone JJ, Horspool LJ, editors. Equine Clinical Pharmacology. Philadelphia: Saunders Co.; 2004. p. 121-134.

57. Sedrich SA, Moore RM, Barker SA. In: Pharmacokinetics of single dose intra-articular administration of amikacin in the radiocarpal joints of normal horses. Proceedings of the american college of veterinary surgeons meeting; Illinois: ; 1994. p. 437.

58. Bolt DM, Ishihara A, Weisbrode SE, Bertone AL. Effects of triamcinolone acetonide, sodium hyaluronate, amikacin sulfate, and mepivacaine hydrochloride, alone and in combination, on morphology and matrix composition of lipopolysaccharide-challenged and unchallenged equine articular cartilage explants. Am J Vet Res. 2008 07/01; 2011/08;69(7):861-7.

59. Moyer W, Schumacher J, Schumacher J. A guide to equine joint injection and regional anesthesia. USA: MediMedia USA; 2007.

60. Bassage II LH, Ross MW. Diagnostic analgesia. In: Ross MW, Dyson SJ, editors. Diagnosis and Management of Lameness in the Horse. Second Edition ed. USA: Elsevier Inc.; 2011. p. 100-135.

61. Matthews JN, Altman DG, Campbell MJ, Royston P. Analysis of serial measurements in medical research. British Medical Journal. 1990 January 27;300(6719):230-5.

62. Itoh K, Sasaki Y, Fujii H, Minami H, Ohtsu T, Wakita H, et al. Study of dose escalation and sequence switching of administration of the combination of docetaxel and doxorubicin in advanced breast cancer. Clinical Cancer Research. 2000 October 01;6(10):4082-90.

63. Wereszka MM, White NA,2nd, Furr MO. Factors associated with outcome following treatment of horses with septic tenosynovitis: 51 cases (1986-2003). J Am Vet Med Assoc. 2007 Apr 15;230(8):1195-200.

64. Stover SM, Pool RR. Effect of intra-articular gentamicin sulfate on normal equine synovial membrane. Am J Vet Res. 1985 Dec;46(12):2485-91.

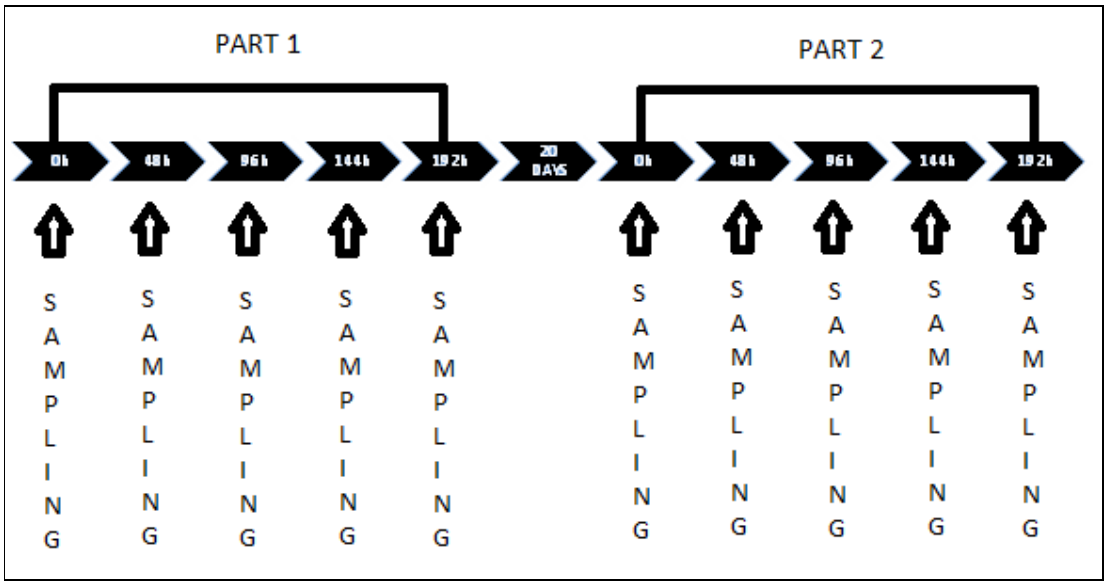
65. Kent Lloyd KC, Stover SM, Pascoe JR, Adams P. Synovial fluid pH, cytologic characteristics, and gentamicin concentration after intra-articular administration of the drug in an experimental model of infectious arthritis in horses. Am J Vet Res. 1990;51(9):1363-9.

66. Karpie JC, Chu CR. Lidocaine exhibits dose- and time-dependent cytotoxic effects on bovine articular chondrocytes in vitro. Am J Sports Med. 2007 Oct;35(10):1621-7.

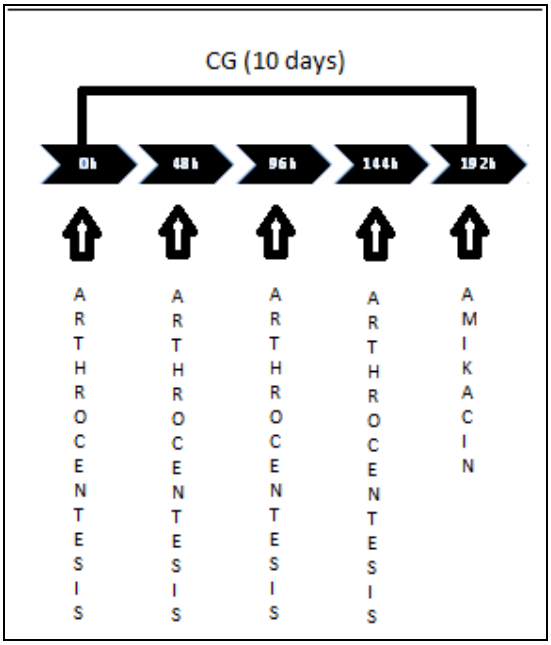
67. Busk P, Jacobsen S, Martinussen T. Administration of perioperative penicillin reduces postoperative serum amyloid A response in horses being castrated standing. Veterinary Surgery. 2010;39(5):638-43.

APPENDICES

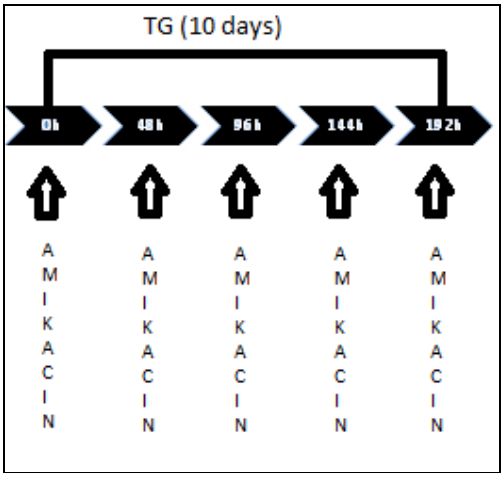
Appendix A – Sampling time line scheme. Each part was assigned to either CG or TG by the flip of a coin.



Appendix B – Time line scheme for horses subjected to repeated arthrocentesis (CG).



Appendix C - Time line scheme for horses subjected to repeated arthrocentesis with intra-articular administration of amikacin (TG).



Appendix D - Standardized data recording sheet for synovial total protein.

PART __	Clinician: _____				

		PROTEIN				
HORSE	**TREATMENT**	DATE	DATE	DATE	DATE	DATE
1						
2						
3						
4						
5						
6						

COMMENTS:

Appendix E - Standardized individual data recording sheet for clinical and lameness examination.

HORSE: _____	Clinician:_____

ORDERS	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 6	DAY 7	DAY 8	DAY 9	DAY 10												
TEMP																						
HR																						
RR																						
MM																						
CRT																						
DP																						
LAMENESS																						