

Effects of repeated intra-articular administration of amikacin on serum amyloid A, total protein and nucleated cell count in synovial fluid from healthy horses

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No conflicts of interests

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Summary

Reasons for performing study: Serum amyloid A (SAA) in synovial fluid (SF) has recently been used as a marker for septic arthritis in horses but the effects of repeated intra-articular (IA) administration of amikacin on synovial SAA concentrations are unknown.

Objectives: To report the effect of repeated IA administration of amikacin on SAA, total protein (TP), nucleated cell count (NCC) and differential nucleated cell count (DNCC) in SF of equine healthy joints.

Methods: A controlled, two-period, cross-over study was performed on 5 clinically healthy horses. Each intercarpal joint received one of two treatments every 48 h for 5 consecutive times: arthrocentesis alone (CG) or arthrocentesis combined with IA administration of 500 mg of amikacin (TG). Clinical and lameness examinations were performed daily. Serum SAA and synovial SAA, TP, NCC and DNCC were measured and statistically compared. Significance was set at $p < 0.05$.

Results: Horses remained healthy and non-lame throughout the study. Baseline values for all variables were not statistically different between groups. Values for TP in TG were significantly higher than in CG after the first sample ($p < 0.05$). In both groups NCC increased significantly ($p < 0.05$) after the first sample. No significant changes were found in DNCC. In both groups, all synovial and most serum SAA concentrations remained below the lower limit of quantification

Conclusions: Repeated IA administration of amikacin caused increased values of TP and NCC in SF, with some TP values within the range reported for septic arthritis. In contrast, synovial SAA concentrations did not increase in either group.

Potential relevance: Synovial SAA could serve as a more reliable marker than TP and NCC when evaluating a joint previously sampled or treated with amikacin.

Keywords: Horse, SAA, amikacin, arthrocentesis, joint

Introduction

Septic arthritis is one of the most severe arthropathies affecting horses of all ages. Survival rates range from 62% in foals to 85% in adult horses [1] and rates of return to previous athletic activity range from 48.3% to 65.8% [2, 3]. An early, aggressive, multi-modal approach is recommended for treatment of septic synovitis. Antimicrobials are frequently administered intra-articularly since this is easy to perform and provides high synovial drug concentrations, increasing antimicrobial efficacy [4]. Amikacin is commonly the preferred antimicrobial because of its broadest activity against the most common pathogens isolated from equine synovial infections [5].

Diagnosis of sepsis in a synovial structure can be challenging. Bacteria are not always evidenced on cytology [6] or isolated from bacterial cultures [1, 6, 7]. Therefore, practitioners usually rely on cytological examination of samples of synovial fluid (SF) and measurement of inflammatory markers in SF for the diagnosis of septic synovitis [8-12]. Presence of septic synovitis is typically considered with synovial total protein (TP) >4 g/dl and nucleated cell count (NCC) >30 x10⁹ cell/l although lower values may be observed in some cases [13]. A percentage of neutrophils (PNEU) >80% (normal range <10%) is also suggestive of sepsis [8, 14, 15]. Degenerative changes in neutrophils are less frequently observed in synovial sepsis than in other body cavities due to the presence lower concentration of bacterial toxins in SF than in other body tissues [16]. Synovial TP and NCC determinations are usually repeated during the course of treatment and results of these tests serve as a guideline for therapy adjustments and prognosis [7]. Unfortunately, repeated arthrocenteses alone [17] as well as single IA administration of antimicrobials [18] can cause increased synovial TP concentrations and NCC values, which can confound the clinical interpretation of these parameters. Therefore, identification of more reliable markers for synovial sepsis is warranted.

Serum amyloid A (SAA) is an acute phase inflammatory protein, which has various clinical applications in horses [19-22]. It is mainly synthesized by the liver in response to inflammation and infection [19, 23] but can also be synthesised by synoviocytes in the presence of synovial inflammation and sepsis [24]. Serum amyloid A concentration in SF has been shown to serve as a good marker for septic arthritis and tenovaginitis, reflecting changes on inflammatory activity in the equine joint [17, 24]. When the effect of repeated arthrocentesis on inflammatory markers in the equine joint was evaluated [17], synovial TP concentration remained significantly increased from baseline from the second to the last arthrocentesis. In contrast, concentrations of SAA in SF remained at baseline values throughout the study [24]. Even though IA administration of antimicrobials is often repeated during treatment of synovial sepsis, the effect of repeated IA administration of amikacin on SF concentrations of SAA, TP, NCC and differential nucleated cell count (DNCC) has not been evaluated in horses.

The objective of this study was to evaluate the synovial inflammatory markers SAA, TP and NCC in healthy horses undergoing repeated IA administration of amikacin. We hypothesized that synovial SAA

would not increase in response to repeated arthrocentesis with or without IA administration of amikacin, whereas synovial TP and NCC would increase.

Materials and methods

Horses

This study was approved by the Institutional Animal Care and Use Committee. A controlled, two-period, cross-over study was performed on 6 "detail to be provided on acceptance" mares, with a mean \pm SD age of 3.8 ± 0.76 years (range 2-6 years) and mean \pm SD bodyweight of 400 ± 58.4 kg (range 302-450 kg). Horses had not received any medical treatment for at least 8 weeks prior to the study and were determined to be healthy and free of musculoskeletal disorders based on physical and lameness examinations as well as complete blood cell count, fibrinogen and systemic SAA measurement. Horses with abnormal results were rejected.

Procedure

Each horse underwent 2 study trials (control and treatment group) randomly distributed between the front limbs with a 20-day wash-out period in-between.

- Control Group (CG): Arthrocentesis of the intercarpal joint was performed every 48 hours for a total of 5 times. A SF sample (~1.5 ml) was collected each time. Amikacin sulphate (500 mg; 2 ml) [Amikacin-Fresenius]^a was injected into the intercarpal joint after the last SF collection.
- Treatment Group (TG): Arthrocentesis and SF collection were performed as in CG. Amikacin sulphate (500 mg; 2 ml) was administered into the intercarpal joint after each SF collection.

Horses were sedated with romifidine (0.02 – 0.03 mg/kg bwt i.v.) and the carpal area was clipped and aseptically prepared. Arthrocentesis was performed on the dorso-lateral synovial pouch with the carpus in semiflexion and using a 22-G needle [25]. Blood was collected from the jugular vein at each time point. The same investigator (AFST) performed all sample collections.

Physical and lameness examinations were performed in all horses every 24 hours during and for 2 days after each study period. Lameness was graded in a scale from 0 to 5 [26] by one investigator (LRM) who was unaware of group distribution.

Sample analysis

An aliquot of the SF sample was used to measure TP concentration with a standard refractometer (Protein Refractometer)^c. The remaining SF was transferred into an EDTA tube and used for NCC analysis using an automated haematology analyzer (Cell-Dyn® 3700 System)^d. The remaining sample was centrifuged for 5 minutes at 1341g (Rotofix® 32A)^e. Supernatant was stored at -80°C for SAA determination. A smear of the precipitate was stained with haematoxylin-eosin (Rapidiff 1®)^f and used for calculation of DNCC and PNEU by an experienced pathologist unaware of group distribution. Venous blood samples were centrifuged for 5 minutes at 1341g (Universal® 320)^g and serum was stored at -80°C for SAA determination.

SAA measurement

An automated chemistry analyser (COBAS INTEGRA 400 plus)^h with a human SAA turbidometric immunoassay (Eiken SAA TIA)ⁱ previously validated for equine use [27] was used for SAA concentration determination. In-house performance of the assay was assessed using SF and serum samples from clinical cases with elevated SAA concentrations. Three independent, 5-serial dilutions for each sample type (SF and serum) were prepared using the diluents provided with the SAA measurement kit.

Data Analysis

Descriptive and comparative statistic analyses were performed by Statistical Analysis System^j. Normality of data was assessed by Shapiro-Wilk and Kolmogorov-Smirnov statistical tests (level of significance $p < 0.05$). For dependent variables (TP, NCC, PNEU), a repeated samples scheme based on the area under the curve (AUC) was applied [28]. The AUC for each dependent variable was estimated following the trapezoidal rule [29] with the following equation [28].

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

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

Cross-over analysis of variance (ANOVA) was implemented for the statistical comparison of AUC of dependent variables between both groups. In addition, Mann-Whitney U and unpaired Student T tests were applied to compare dependent variables between both treatment groups at each sampling time and each sampling time vs. baseline within each group. Significance was set at $p < 0.05$.

Results

All horses remained free of lameness throughout the study. Five horses remained clinically healthy but one mare was excluded from the study because of developing respiratory disease unrelated to the study. This horse showed hyperfibrinogenemia (9.00 g/l) and increased SAA in serum (86.1 mg/l).

Protein concentrations in SF

Synovial TP (mean \pm SD; g/dl) for both groups are included in Figure 1 and Table 1. Synovial TP baseline values were not significantly different between groups. 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 TP in CG ranged from 0.80 to 3.10 g/dl during the study (1.30 ± 0.54 g/dl) and values were significantly higher than baseline only at 96h ($p = 0.03$) (Figure 1). In TG, synovial TP ranged from 0.90 to 6.00 g/dl during the study (3.12 ± 1.36 g/dl) and values increased significantly when compared with baseline after the first injection of amikacin and remained increased thereafter ($p < 0.01$) (Figure 1). Except for baseline, TP concentrations between both groups were significantly different ($p \leq 0.03$) at all collection time points (Figure 1 and Table 1).

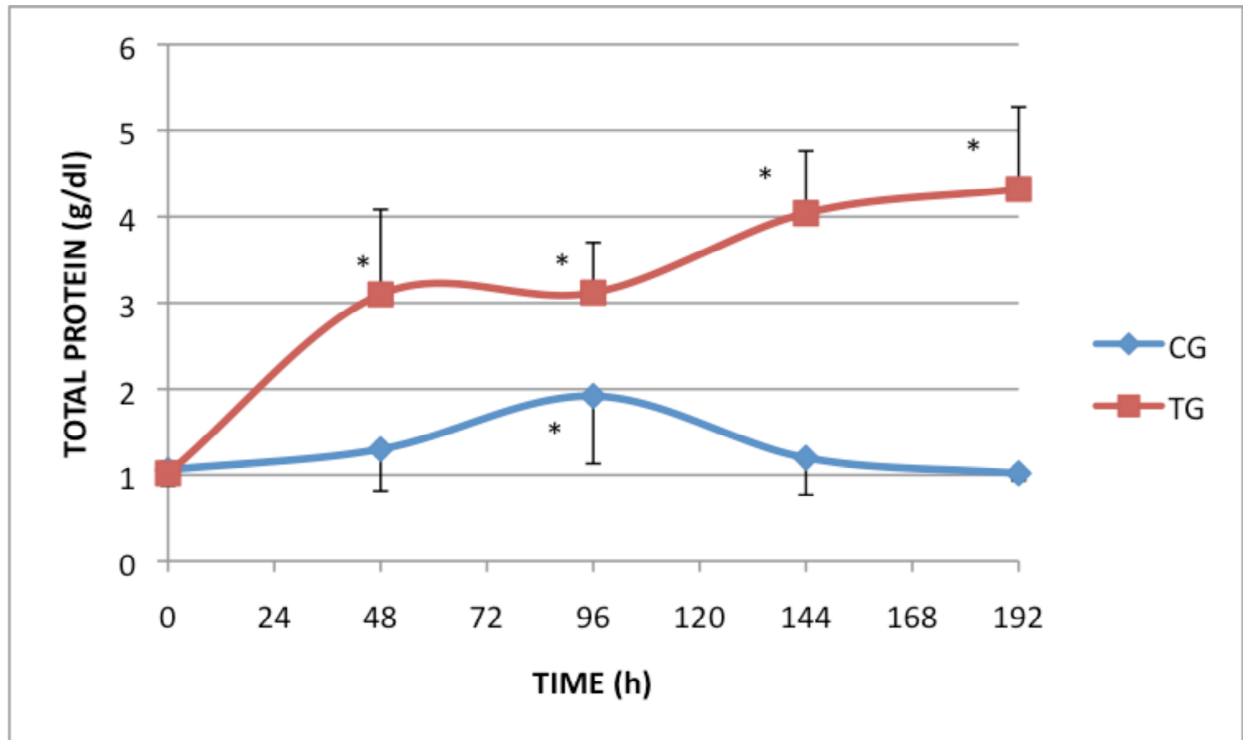


Figure 1 - Mean \pm SD total synovial protein (g/dl) in 5 horses after repeated arthrocentesis (CG) or repeated arthrocentesis and administration of amikacin (TG) into the intercarpal joint. *Significant difference ($p < 0.05$) with baseline (time 0h).

Table 1 - Mean \pm SD synovial total protein (TP; g/dl), nucleated cell count values (NCC; $\times 10^9$ cells/l), percentage of neutrophils (PNEU; %), and serum amyloid A (SAA; mg/l) in SF and serum samples from 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)

| | TIME (h) | TP (g/dl) | NCC ($\times 10^9$ cells/l) | PNEU (%) | Synovial SAA (mg/l) | SAA in serum (mg/l) |
|----|----------|--------------------------------|--------------------------------|----------------------------|---------------------|---------------------|
| TG | 0 | 1.02 \pm 0.13 (0.9-1.2) | 0.06 \pm 0.04 (0.02-0.15) | 5.60 \pm 9.53 (0-22) | <LOQ | <LOQ |
| | 48 | 3.10 \pm 0.98 (1.50-3.90) | 0.63 \pm 0.37 (0.21-1.12) | 5.80 \pm 4.60 (1-13) | <LOQ | <LOQ |
| | 96 | 3.12 \pm 0.58 (2.30-3.90) | 2.49 \pm 2.38 (0.46-6.31) | 8.80 \pm 3.35 (14-41) | <LOQ | <LOQ |
| | 144 | 4.04 \pm 0.72 (3.20-5.20) | 1.85 \pm 2.21 (0.38-5.69) | 10.4 \pm 9.07 (2-24) | <LOQ | <LOQ |
| | 192 | 4.32 \pm 0.95 (3.60-6.00) | 1.68 \pm 1.14 (0.65-3.24) | 15.2 \pm 10.9 (3-32) | <LOQ | <LOQ |
| CG | 0 | 1.06 \pm 0.18 (0.80-1.30) | 0.06 \pm 0.03 (0.04-0.12) | 11.8 \pm 14.8 (3-38) | <LOQ | <LOQ |
| | 48 | 1.33 \pm 0.48 (0.80-2.10) | 0.54 \pm 0.46 (0.05-1.07) | 24.2 \pm 10.2 (14-41) | <LOQ | (0-18.4) |
| | 96 | 1.92 \pm 0.78 (1.10-3.10) | 1.33 \pm 1.20 (0.16-3.36) | 32.0 \pm 29.3 (6-79) | <LOQ | (0-0.43) |
| | 144 | 1.2 \pm 0.43 (0.80-1.90) | 0.68 \pm 0.42 (0.43-1.22) | 8.00 \pm 6.32 (0-16) | <LOQ | (0-8.64) |
| | 192 | 1.02 \pm 0.08 (0.90-1.10) | 0.45 \pm 0.22 (0.25-0.76) | 9.80 \pm 7.29 (2-18) | <LOQ | (0-0.73) |

Nucleated cell count in SF

Values of NCC (mean \pm SD) for both groups are included in Table 1. Baseline NCC values did not differ significantly between groups. Nucleated cell count AUC₀₋₁₉₂ in CG ($136 \pm 95.9 \times 10^9 \text{h/l}$) and TG ($281 \pm 252 \times 10^9 \text{h/l}$) did not differ significantly.

Values of NCC in CG ranged from 0.04 to 3.36×10^9 cells/l ($0.61 \pm 0.70 \times 10^9$ cells/l). In TG, NCC values ranged from 0.02 to 6.31×10^9 cells/l ($1.34 \pm 1.67 \times 10^9$ cells/l). When compared with baseline, NCC was significantly higher at all sampling times ($p < 0.05$) in both groups (Figure 2). When both groups were compared a significant difference in NCC was observed only at 192h ($p < 0.05$). Values of NCC peaked at 96h in both groups (Figure 2).

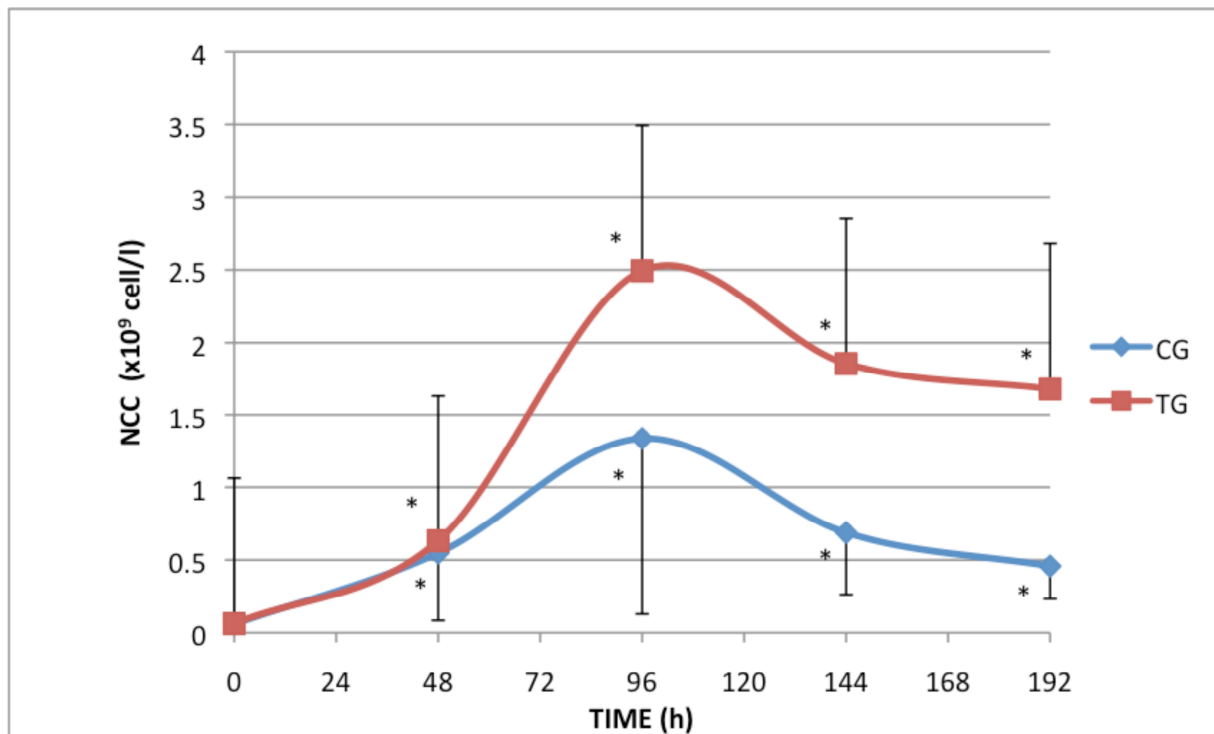


Figure 2 - Mean \pm SD synovial nucleated cell count (NCC; count $\times 10^9$ cells/l) in 5 horses in response to repeated arthrocentesis (CG) or repeated arthrocentesis and administration of amikacin (TG) into the intercarpal joint. *Significant difference (< 0.05) with baseline (time 0h).

Percentage of neutrophils in SF

Values of PNEU for both groups are included in Table 1. Baseline PNEU and AUC_{0-192} did not differ significantly between groups.

Values for PNEU in CG ranged from 0.00 to 79.0% ($17.2 \pm 17.4\%$). In TG, PNEU ranged from 0.00 to 24.0% ($9.16 \pm 8.20\%$). No significant differences were found when PNEU values were compared with baseline or between groups at any time point.

In-house validation of SAA measurement technique

For SAA in SF, the lower limit of quantification (LOQ) was 0.05 mg/l, the coefficient of determination was >0.90 , and the intra-assay variability ranged from 1 to 10%. For SAA in serum, the LOQ was 0.21 mg/l, the coefficient of determination was >0.90 and the intra-assay variability ranged from 0.8 to 16%.

Serum amyloid A in SF and serum samples

Synovial SAA values remained below the LOQ in both groups (Table 1). Systemic SAA concentrations remained ≤ 18.42 mg/l and most of the samples remained below the LOQ (Table 1). Due to the small number of samples with values of SAA above the LOQ, SAA results were not compared statistically.

Discussion

Synovial TP increases in the presence of synovitis or after IA injection of different substances [15]. In the present study, synovial TP after repeated arthrocentesis remained within normal range (< 2.5 g/dl) [15] with the exception of one sample (96h). These findings are similar to those previously reported in the digital flexor tendon sheath (DFTS), where repeated sheath centesis was combined with intra-theal administration of Lactated Ringer's solution [18]. In contrast, Jacobsen et al (2006) reported a significant increase in synovial TP concentration after repeated arthrocentesis combined with IA administration of saline solution [17]. The use of different sampling protocols, along with injection of different solutions [17, 18] might have caused varied grades of inflammatory reactions accounting for the different findings among studies.

In our study, repeated IA administration of amikacin produced TP values above normal range in all horses from the second (4 horses) or third (1 horse) arthrocentesis and these values remained elevated thereafter. Some of these samples had TP ≥ 4.00 g/dl, values typically associated with septic synovitis [8, 15]. In previous studies, single intra-synovial administration of amikacin (250 mg into the DFTS or 500 mg IA) produced a mild increase in synovial TP values, which remained below those associated with septic arthritis [4, 18]. Differences in the frequency of amikacin administration (single dose in previous studies vs. 5 doses in our study), the response to synoviocentesis (joint vs. tendon sheath), antimicrobial pharmaceutical preparations, as well as total antimicrobial dose per horse (larger horses receiving lower net doses than smaller horses) might explain different results among studies.

Nucleated cell count can increase in the presence inflammation, secondary to arthrocentesis or after injection of different substances [15]. In our study, increased NCC was observed in both groups when compared to baseline and the only difference between groups was at 192h, with NCC in TG being higher than in CG. In both groups, NCC remained $\leq 6.31 \times 10^9$ cells/l, similarly to previous studies after arthrocentesis or IA injection of local anaesthetics [15]. In contrast, Dykgraaf et al (2007) reported synovial NCC values consistent with septic synovitis ($>30 \times 10^9$ cells/l) [15] after single administration of LRS or amikacin into the DFTS [18]. Differences between studies might be attributed to a greater difficulty in accessing a tendon sheath linked to a higher risk of blood contamination of the sample and greater sensitivity of sheaths to centesis as compared with joints [30].

Blood contamination during sampling could have caused increased synovial TP or NCC values in our study. However, arthrocentesis of the intercarpal joint via a dorsal approach has low degree of difficulty [30], all arthrocenteses were performed by the same operator (AFST) and needle reposition was necessary only in 3 occasions. Due to the reduced number of times the needle was repositioned, it was not possible to evaluate statistically any potential association between needle reposition and SF markers. However, in one of the 3 horses that required needle repositioning, the variables (TP and NCC) remained within the normal reference range. In the second horse both outcome variables were already elevated above the reference range before the time of needle repositioning. And in the third horse TP and NCC rose above the reference range 144h after the time of needle repositioning.

Modest increases in PNEU have been observed in the presence of mild synovitis, after arthrocentesis, and after IA injection of different substances [15]. An overall mild increase of PNEU without toxic changes to neutrophils and non-consistent with sepsis was observed in our study.

Repeated arthrocentesis every 48h did not cause a detectable increase in SAA concentrations in serum or SF supporting previous reports [17]. Repeated arthrocentesis with or without the IA administration of amikacin might not exert a sufficient inflammatory stimulus for synthesis of serum and synovial SAA to values within the detection range. In humans, serum SAA concentrations rose to higher levels in response to bacterial stimulus than to viral infection or other kinds of inflammation [31], which could also be occurring in horses. Synovial and serum SAA in horses suffering from septic arthritis were reported to be >1000 mg/l [17]. In our equine hospital, concentrations of SAA in SF collected from septic arthritis typically are > 800 mg/l (unpublished data). In addition, SAA has a very short half-life in serum; in mice, the half-life is 75-80 minutes and 95% is eliminated from plasma 6h after the synthesis has ceased [32]. Therefore, SAA concentrations could have increased and decreased before the next sampling was performed (48h after). More frequent arthrocenteses might have detected increased synovial SAA concentrations; however, such sampling protocol would not reflect the clinical scenario. To the authors' knowledge the half-life of SAA in equine SF has not been reported but repeated arthrocentesis (4 h apart) combined with injection of saline solution did not produce increased synovial SAA in horses in a previous report [17]. The fact that SAA values were not increased after repeated arthrocentesis with or without IA administration of amikacin every 48h, and that synovial SAA increases to high levels during septic arthritis [17, 24], suggest that measurement of synovial SAA might be valuable when monitoring clinical cases with septic synovitis.

The study was designed to resemble a clinical scenario when evaluating and/or treating septic arthritis in horses. Amikacin was selected for its efficacy against common isolates [5] and administration of 500 mg of amikacin was repeated every 48h based on previous studies [4]. Intra-articular injection of amikacin produced significant changes on the synovial inflammatory markers, mainly TP and NCC. Whether these changes were due to a chemical effect of amikacin or to the act of introducing foreign fluid to the synovial structure cannot be concluded from this study. In previous studies single IA injection of amikacin did not

produce cytological or clinical evidence of chemical synovitis [4], but amikacin exerted mild toxic effects on equine chondrocytes *in vitro* [33]. An additional control group undergoing repeated arthrocentesis combined with injection of a placebo substance (lactated Ringer's or saline solution) could have been included to investigate if the changes observed in the treatment group can be related to the act of injecting a substance rather than just related to amikacin. However, in this study the sham group was not included due to economic limitations, and the inclusion of a control group without sham injection was considered preferable since this is routinely performed by equine practitioners to collect samples of SF and evaluate response to treatment.

The human SAA turbidometric immunoassay used in this study has been previously validated for equine use [27] and has been used by other investigators [17, 24] and diagnostic laboratories [34] on equine serum and SF. The in-house evaluation of assay performance revealed LOQ values and intra-assay variability similar to those in previous studies [27].

In conclusion, repeated arthrocentesis and IA administration of amikacin every 48h increases synovial TP and NCC values in some cases consistent with septic arthritis. However, synovial SAA concentrations were not affected and therefore could serve as a better marker for synovial sepsis when evaluating a joint previously sampled or treated with IA amikacin.

Manufacturers' details

^a Details to be provided on acceptance

^b Becton, Dickinson and Company©, USA

^c A.S.T. Inc., Japan

^d ABBOTT, USA

^e Hettich Zentrifugen, UK

^f Details to be provided on acceptance

^g Hettich Zentrifugen, UK

^h Roche Laboratories, Switzerland

ⁱ LZ test SAA, Eiken Chemical Co.

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