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Clinical efficacy of intravenous administration of marbofloxacin in a *Staphylococcus aureus* infection in tissue cages in ponies

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Tissue cages (TC), implanted subcutaneously in the neck in eight ponies, were inoculated with *Staphylococcus aureus* (*S. aureus*) to determine the clinical efficacy of marbofloxacin in the treatment of this infection. From 21 h after inoculation, marbofloxacin (6 mg/kg) was administered intravenously (i.v.) once daily for 7 days. Samples of the tissue cage fluid (TCF) were taken to determine marbofloxacin concentrations (days 1, 3 and 7), using high-pressure liquid chromatography, and numbers of viable bacteria [colony forming units (CFU)] (days 1, 3, 7, 14 and 21). Statistical analysis was used to compare CFU before and after treatment. Clinical signs and CFU were used to evaluate the efficacy of treatment. Although, there was a slight decrease in CFU in all TC initially, the infection was not eliminated by marbofloxacin treatment in any of the ponies and abscesses formed. As the *MIC* (0.25 μg/mL) did not change during treatment and the concentration of marbofloxacin during treatment (mean concentration in TCF was 0.89 μg/mL on day 1, 0.80 μg/mL on day 3 and 2.77 μg/mL on day 7) was above *MIC*, we consider that the treatment failure might be attributable to the formation of a biofilm by *S. aureus*. Based on the present results, i.v. administration of marbofloxacin alone is not suitable for the elimination of *S. aureus* infections from secluded sites.

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INTRODUCTION

In cases of infectious arthritis, there is a localized infection in a body compartment. State-of-the-art treatment consists of local joint lavage and intra-articular administration of antimicrobial agents, often supported by concomitant systemic administration of antimicrobial agents. Although the prognosis for infectious arthritis has greatly improved in recent years, there are still many cases where the infection cannot be controlled, indicating that the quest for improved antimicrobial treatment, locally and/or systemically needs to continue.

A wide range of bacterial species can be found in infected synovial structures. One of the regularly encountered pathogens is *Staphylococcus aureus* (*S. aureus*), which belongs to the normal skin flora of both horses and humans (Meijer *et al.*, 2000).

In a previous TC study, we investigated whether local administration of ceftiofur was effective in the treatment of *S. aureus* infection. It was concluded that ceftiofur did not resolve infection in all cases and should therefore only be used with adjunctive therapy. One of the probable causes of this therapeutic failure was the ability of *S. aureus* to migrate into neutrophils, which was confirmed by microscopical evaluation of cell smears (Bosch *et al.*, 2006).

Marbofloxacin is a fluoroquinolone antimicrobial agent, developed for veterinary use, which has characteristics that make it potentially promising for use as adjunctive systemic treatment in equine infectious arthritis. Like all third generation fluoroquinolones, the mechanism of action of marbofloxacin leads to killing of bacteria by inhibition of DNA-gyrase and topoisomerase IV. Fluoroquinolones are broad spectrum antimicrobial agents and are bactericidal at concentrations eight times the minimum inhibitory concentration (*MIC*) of pathogens. At concentrations approximately equal to or less than the *MIC*, bacteriostatic effects predominate (Frazier *et al.*, 2000; Walker, 2000). Marbofloxacin usually has time-dependent activity in Gram-positive bacteria and a concentration-dependent activity in Gram-negative bacteria; anaerobic bacteria are not susceptible (Aliabadi & Lees, 2002). Fluoroquinolones in general have good tissue and cell penetrating capacity. Marbofloxacin differs from other fluoroquinolones on account of its oxadiazine ring, which

may give this molecule some pharmacokinetic advantages, such as longer half-life, a larger volume of distribution and a higher bioavailability. Classically, fluoroquinolones have not been recommended for the treatment of infections in horses owing to the concerns about the potential risk of arthropathy in young horses, although there are no published data on the fluoroquinolones inducing arthropathy in mature horses. There are reports of long-term oral administration of enrofloxacin to mature horses with successful outcomes and no apparent side effects (Carretero *et al.*, 2002).

To determine whether marbofloxacin is effective against *S. aureus in vivo* would require a study in patients. As it is not be expected that systemic administration of antimicrobial agents alone would be sufficient to suppress such an infection entirely and because of the possibly fatal consequences of longer term insufficient treatment of infectious arthritis, the use of an *in vivo* arthritis model was considered unethical. Instead, tissue cages (TC) were used as a model, as has been performed successfully in earlier trials (Beadle *et al.*, 1989, Ensink *et al.*, 1996a,b). Previous studies with marbofloxacin were performed using a dose of 2 mg/kg, which would be effective against bacteria with low *MIC*, such as *Enterobacteriaceae*, but probably not for bacteria with *MIC* > 0.25 μg/mL, like *S. aureus* (Bousquet-Melou *et al.*, 2002).

Before using marbofloxacin in the TC infection model, we performed a pharmacokinetic study in eight healthy Shetland ponies with TC implanted. The results of PK/PD modelling, using an *MIC* of 0.25 μg/mL, showed that a dose of 6 mg/kg intravenously (i.v.) once daily should be clinically effective in treatment of *S. aureus* infections (Fink-Gremmels unpublished data).

The objective of the present study was to determine the efficacy of marbofloxacin (6 mg/kg, i.v.) in the treatment of a TC infection with *S. aureus*.

MATERIALS AND METHODS

Ponies

Eight healthy Shetland pony geldings were used. The ponies were 5–15 years old and weighed 138–216 kg. The animals were housed indoors in individual box stalls and were fed a maintenance ration of hay twice a day. Water was provided *ad libitum*. All ponies had been vaccinated against influenza and tetanus and treated with an anthelmintic before the experiment started. All ponies had one s.c. TC implanted surgically on the left or right side of the neck. In five ponies the TC was implanted surgically 37 months before the present experiment, as described by Ensink *et al.* (1996a). The ponies had been used previously for an endotoxaemia study, an infection study for determination of the clinical efficacy of local administration of ceftiofur and in a pharmacokinetic study with marbofloxacin. None of the TC had been infected before. New TC was implanted in three ponies, 3–5 months before the present experiment started. So although all of the ponies in the present experiment had been infected with *S. aureus* in a TC, but none of the TC used in the present experiment had been infected before. Sterility of the TC was assessed by bacterial culture of aspirates from the TC at the start of the present experiment. Rectal temperature was monitored twice daily for 10 days after inoculation of the TC with *S. aureus* and once daily for the rest of the present experiment. The ponies were checked daily for swelling around the TC, and behaviour and appetite were recorded. The experiments were approved by the Animals Ethics Committee of Utrecht University in compliance with the Dutch Act on Animal Experiments.

Experimental design

In each pony the tissue cage was inoculated with *S. aureus* (day 0). Starting at 21 h postinfection, marbofloxacin was administered i.v. once daily for 7 days. TCF samples were collected before inoculation of the TC (day 0) and again on days 1, 3, 7, 14, 21, 28 after inoculation for viable bacterial counts and white blood cell (WBC) counts. Additional samples were taken on days 1, 0.5–1.5 h after treatment, on day 3 just before treatment (trough concentration) and on day 7, 4 h after treatment (corresponding to the expected peak concentration) for the measurement of marbofloxacin concentrations. Analysis of samples for bacteriology and cell counts was performed immediately after sampling and samples for the measurement of drug concentrations were stored at −80 °C until analysis.

Inoculation

A strain of *S. aureus*, originally isolated from a clinical case of infectious arthritis of the tibiotarsal joint in a horse, was used as the inoculum.

This organism was identified using the API 32ID Staph system (Biomerieux SA, Marcy l'Etoile, France). The strain used had a *MIC* of 0.25 μg/mL for marbofloxacin. The organism had been stored at −70 °C in brain heart infusion (BHI; Oxoid, Basingstoke, UK) and glycerine (1:1). To prepare the inoculum, the organism was allowed to thaw and cultured on BHI at 37 °C in two stages for a total of 24 h. The overnight culture was centrifuged, the bacterial pellet washed twice with 0.9% saline, and the organism resuspended in pyrogen-free phosphate buffered saline (PBS, pH 7.2). A serial dilution was prepared in PBS to reach a concentration of approximately 1.0×10^5 viable colony forming units (CFU)/mL. The concentration was confirmed by counting CFU after serial dilution. The actual number of CFU inoculated per TC was 3.2×10^4 CFU/mL; 2 mL of this preparation was inoculated into each TC, so that the infection dose was 6.4×10^4 CFU.

Antimicrobial agent

A 10% marbofloxacin solution (Marbocyl® 10% solution injectable; Vetoquinol NV, Aartselaar, Belgium) was administered at a dose rate of 6.0 mg/kg i.v., via a catheter in the jugular vein.

TCF samples

Prior to sampling, the skin over each TC was clipped. After disinfection with alcohol, 2 mL TCF was aspirated from each TC using a 21-G needle. Where aspiration of TCF was not possible using a 21-G needle, because of increased viscosity of the fluid, an 18-G needle was used.

Marbofloxacin determination

TCF samples for determination of marbofloxacin concentration were taken on days 1, 3 and 7. Marbofloxacin concentrations were measured by high-pressure liquid chromatography (HPLC) according to the method described by Garcia *et al.* (1999), with minor modifications. Briefly, 100 μL of TCF was diluted with 400 μL of 0.1 M phosphate buffer (pH 7.4) and vortexed for 30 sec. After adding 3 mL of dichloromethane, the samples were vortexed for 1 min and centrifuged for 6 min (1000 ***g***, 4 °C). After removing the aqueous layer, the organic phase was evaporated in a vacuum evaporator at 40 °C. The residue was dissolved in 200 μL of demineralized Milli-Q-water and an aliquot of 50 μL was injected into an HPLC system. All samples were analysed in duplicate.

The HPLC system consisted of a High Pressure Pump Model 300, an autoinjector (Marathon Auto Sampler; Spark, The Netherlands) and a fluorescence detector (Detector Jasco, Model 821 FP; H.J. Ambacht, The Netherlands), set to an excitation and emission wavelength of 295 and 500 nm respectively. Separation was achieved with a Spherisorb ODS-2 [250 × 4.6 mm 5 (M)] column (Waters Corporation, Milford, MA, USA). Peak area integration was carried out by a Chromelion Computer Program (Seperations, H.I. Ambacht, The Netherlands). The mobile phase consisted of a mixture of acetonitrile and an aqueous solution (20:80, v/v) of potassium dihydrogenphosphate (0.02 M) and tetrabutylammonium hydrogenphosphate (0.02 M) in bidistillated water (20:80, v/v). The pH was adjusted to 3.0 by addition of phosphoric acid 85%. The flow rate was 0.8 mL/min.

For the calibration curve standard solutions ranging between 0.05 and 7.5 μg/mL marbofloxacin were prepared in TCF collected from the ponies before drug administration and subjected to HPLC analysis. The regressions coefficient (r) for the standard curve was 0.998. The limit of quantification in both plasma and TCF was 0.05 μg/mL.

Bacterial counts and cell counts

The samples of 0.5 mL TCF were immediately added to 4.5 mL of BHI and kept at 4 °C for a maximum of 4 h. Colony counts were determined by a plate count method using 10-fold dilutions in 0.9% saline of the TCF in BHI. After incubating the plates for 48 h, CFU were counted from plates where 30–300 colonies had grown. The limit of detection of this plate count method is 1.0×10^2 CFU/mL of TCF. For calculation purposes, samples with $<1.0 \times 10^2$ CFU/mL were arbitrarily set at 10^1 CFU/mL. The last sample taken from each pony was also used for reconfirmation of the pathogen as the *S. aureus* used for infection and for susceptibility testing using Mueller Hinton Broth and the microdilution method.

Total WBC counts in TCF were performed using a Coulter counter (type industrial D, Coulter Counter, Luton, UK).

Treatment of abscessation

When antimicrobial agent treatment of infected TC is not successful, abscessation of the TC occurs: the infection ruptures through the skin over the tissue cage. In these cases, the TC was removed from the standing animal using sedation with detomidine hydrochloride 10 μg/kg, (Domosedan®; Pfizer, Capelle aan de Ijssel, the Netherlands), methadone hydrochloride 0.1 mg/kg (Methadone HCl 10 mg/mL; Eurovet, Bladel, the Netherlands) and local analgesia (Lidocaine HCl 2%, Eurovet, Bladel, the Netherlands).

Data analysis

A paired t-test with Bonferroni correction was used to determine whether the increase and decrease in the CFU before and after starting treatment was significant.

RESULTS

Infection of the TC did not result in any signs of inflammation on the day of inoculation. Fever and a painful swelling were first seen 20–32 h after inoculation. The ponies appeared depressed, were reluctant to move and one pony was anorexic. After the start of the treatment, body temperatures dropped in a few days and were within normal range 6 days after inoculation. The signs of inflammation around the TC lessened after a few days and the ponies became brighter again.

During treatment with marbofloxacin no abscessation of the TC occurred. The first abscessation occurred 1 day after the last treatment.

From day 0 to day 1 the number of bacteria in the inoculated TC rose significantly from approximately 1×10^4 to 1×10^7 CFU/mL ($P = 0.000$). Marbofloxacin caused only a slightly decrease in the numbers of viable bacteria in the TC. CFU decreased significantly between day 1 and day 7 ($P = 0.037$) and between day 3 and day 7 ($P = 0.008$) (Fig. 1).

The mean WBC count increased from 0.9 (SD 0.85) × 10^9/L on day 0 to 13.5 (SD 21.13) × 10^9/L on day 1 and 141.4 (SD 53.49) × 10^9/L on day 7. On day 3 the samples were too viscous to be counted. The interindividual variation, reflected in SD of mean WBC counts, was relatively high. The high WBC counts indicate a purulent infection, in agreement with the macroscopic examination of the TCF.

Table 1 shows the concentration marbofloxacin determined on days 1, 3 and 7.

The *MIC* of *S. aureus* cultured from the inoculated TC after finishing treatment with marbofloxacin did not differ from the *MIC* (0.25 μg/mL) of *S. aureus* before inoculation.

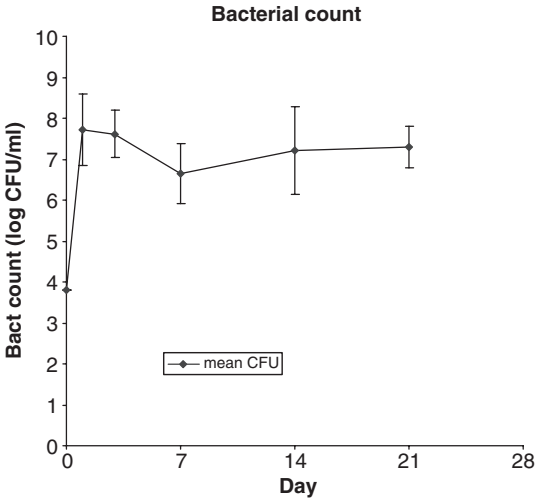

Fig. 1. Bacterial count in tissue cage fluid (log CFU/mL) (mean ± SD), $n = 8$. Day 0, inoculation of TC with *Staphylococcus aureus*. Day 1–7, treatment with marbofloxacin 6 mg/kg i.v. once daily. Day 0–7, $n = 8$; day 14, $n = 3$; day 21, $n = 2$.

Table 1. Day 0 inoculation of tissue cages with *S. aureus*, day 1–7 treatment with marbofloxacin 6 mg/kg i.v. once daily

	Day 1	Day 3	Day 7
Pony 1	0.87	0.57	4.59
Pony 2	0.96	0.82	4.23
Pony 3	0.07	0.93	1.81
Pony 4	1.69	0.82	3.36
Pony 5	0.50	0.49	1.60
Pony 6	0.71	0.39	1.70
Pony 7	1.55	1.17	3.19
Pony 8	0.79	1.18	1.69
Mean	0.89	0.80	2.77
SD	0.53	0.30	1.23

Concentration (μg/mL) of marbofloxacin in tissue cage fluid. Day 1, samples taken 30–90 min after the first treatment. Day 3, samples taken just before treatment (trough concentration). Day 7, samples taken 4 h after treatment (approximately peak concentration).

DISCUSSION

Following systemic administration of marbofloxacin (6 mg/kg) there was only a slight reduction in the numbers of viable bacteria in the TC. Unfortunately no negative control values (viable bacterial counts in the inoculated TC in the absence of treatment) are available, although these probably would have been much higher. Failure to eliminate the pathogen despite initial improvement during antimicrobial treatment has also been encountered in other studies using infections models in ponies (Ensink *et al.*, 1996b). There are a number of possible explanations for the survival of staphylococci: (i) the concentration of marbofloxacin in infected TC was too low; (ii) sequestration in inflammatory exudates or intra-cellular migration; (iii) a higher *MIC* of staphylococci in TCF than *in vitro* medium; (iv) enrichment of resistant mutants: an increase in *MIC* during antimicrobial agent treatment or (v) the formation of a biofilm by the pathogen.

- Fluoroquinolones exert concentration-dependent bactericidal activity against Gram-negative bacteria and some Gram-positive bacteria, but time-dependent bactericidal activity is assumed for Gram-positive bacteria as well (Walker, 2000; Aliabadi & Lees, 2002; Bousquet-Melou *et al.*, 2002). Other studies (Bousquet-Melou *et al.*, 2002; Carretero *et al.*, 2002 and Peyrou *et al.*, 2004) have shown that a dose of 2 mg/kg given every 24 h could be effective against Gram-negative bacteria with low *MIC*, but probably not for treating infections with bacteria, such as *S. aureus*, with a *MIC* > 0.2 μg/mL. Before starting this experiment, we performed a pharmacokinetic study using the TC model and determined a suitable dosage of marbofloxacin for treatment of an infection with *S. aureus* (*MIC* 0.25 μg/mL). Although, we measured the concentrations of marbofloxacin in TCF on day 1 only 30–90 min after the first treatment, the concentrations were already above *MIC* in all but one of the ponies. On day 3, we measured the trough levels of marbofloxacin in TCF and, as Table 1 shows, these were all above *MIC*. This indicates that this dose of marbofloxacin should be effective when the action of marbofloxacin against *S. aureus* is time-dependent. On day 7, peak concentrations were measured. The peak concentration was 6.4–18.4 times the *MIC*. In four (pony 1, 2, 4 and 7) of the eight ponies the concentration reached eight times the *MIC*. Therefore 6 mg/kg marbofloxacin might be effective when the action of marbofloxacin against *S. aureus* is concentration-dependent. It can be concluded that the concentration of marbofloxacin is not likely to be the cause of failure to eliminate the infection.
- Marbofloxacin treatment was started when signs of inflammation were present. The staphylococci might be sequestered in inflammatory exudates (e.g. fibrin) and marbofloxacin might have poor access to these intracaveal tissues as the tissue penetration into inflamed tissue could be different from penetration into normal tissue. However, Aliabadi and Lees (2002) determined the tissue penetration and distribution of marbofloxacin in inflamed (exudate) and unstimulated (transudate) TCF in calves and found similar rates and extents of passage into exudate and transudate. This illustrates the ready penetration into TC, regardless of whether inflammation is present, reflecting the low binding to plasma proteins and the high lipid-solubility of marbofloxacin.
- The *MIC* values of the *S. aureus* used in this study were determined *in vitro* using a microdilution method with Mueller Hinton broth. In *in vitro* susceptibility tests, *MIC* may vary with the medium used. Biological fluids, such as plasma, urine, milk, etc., may be more relevant to prediction of efficacy *in vivo* than conditions in culture media, such as Mueller Hinton Broth, which may differ in respect of pH, cation concentrations and protein concentrations (Barger *et al.*, 2003). Therefore, it is possible the *MIC* would be different if TCF were used as an *in vitro* medium. However, Aliabadi and Lees (2002) also showed that the *MIC* values of marbofloxacin

against *M. haemolytica* were similar (0.040 μg/mL) in inflammatory exudates, serum, transudate and Mueller Hinton broth. This is probably because the degree of binding of marbofloxacin to plasma protein is relatively low (Aliabadi & Lees, 2002).

- The fourth explanation for treatment failure could be that *S. aureus* developed resistance during marbofloxacin therapy. Resistance is reflected by an increase in *MIC* of the staphylococci during treatment (Walker, 2000). The *MIC* of the bacteria was tested before inoculation and after finishing treatment with marbofloxacin. There was no rise in *MIC* during treatment and therefore a rise in *MIC* cannot be the explanation of survival of *S. aureus* in the TC in the present study.
- Biofilms can be described as 'a structured community of bacterial cells enclosed in a self-produced polymeric matrix and adherent to an inert or living surface' (Costerton, 1999). Infections that involve a biofilm mode of growth are generally chronic and are often difficult to cure. Several publications are available which show that *S. aureus* isolates from human and veterinary resources are able to grow *in vitro* in biofilms. Subsequently these experiments also show that biofilm growing *S. aureus* are 10–1000 times more resistant to antimicrobial agents than the same bacteria growing as free-floating bacteria. However, some fluoroquinolones have been shown to be effective against bacteria growing in biofilms (Olsen *et al.*, 2002).

Using PK/PD modelling and *MIC* values for marbofloxacin according to CLSI (previously NCCLS) guidelines a therapy was determined for this TC model. However, several *S. aureus* strains are able to grow in biofilms on the surface of artificial devices. From this point of view it would be more appropriate to measure the MBEC concentration (Minimal Biofilm Eradication Concentration) for eradication of *S. aureus* from the TC. Further studies have to be performed to show whether biofilms play a role in infectious arthritis and if biofilms can be studied in a TC model. In addition, its histological/microscopical evidence of biofilm formation in the TC model and determination of whether the bacteria sampled are planctonic or biofilm bacteria would be an interesting additional aspect to future work.

In conclusion, formation of a biofilm is the most likely reason for the survival of *S. aureus* as the other possibilities appear to have been are ruled out in the present study. The results of the present study show that i.v. administration of 6 mg/kg marbofloxacin only slightly reduced the number of viable bacteria in infected TC and failed to eliminate the infection completely.

The study of Bosch *et al.*, 2006, is comparable with this study because it used the same ponies and the same strain of *S. aureus*. The conclusion of that study was that local administration of ceftiofur did not resolve infection in all cases. However, ceftiofur did eliminate the infection in two out of the seven animals and the number of CFU/mL decreased more rapidly and to a greater extent than in the present study using marbofloxacin. It is clear that neither antimicrobial agent alone is satisfactory as a stand-alone therapy for infectious arthritis caused by *S. aureus* and better treatment is needed. The search for antimicrobial agents that are more effective in the treatment of *S. aureus*-induced infectious arthritis should concentrate on agents that may be effective against biofilms.

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