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Clinical efficacy of local administration of ceftiofur in a *Staphylococcus aureus* infection in tissue cages in ponies

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Ceftiofur concentrations in an infected and uninfected environment were compared and the efficacy of locally administered ceftiofur was evaluated in an experimental infection with *Staphylococcus aureus* in tissue cages.
Eight ponies had tissue cages (TCs) implanted s.c. on each side of the neck. Into one of the cages 150 mg of ceftiofur was administered and fluid samples were taken to determine ceftiofur concentrations. After 1 week the other TC was infected with *S. aureus* and subsequently treated with 150 mg ceftiofur administered locally into the TC once daily for 21 days. Samples of fluid were taken to determine ceftiofur concentrations and for bacterial counts. Ceftiofur concentrations did not differ significantly in the infected and uninfected environments after single dose of 150 mg of ceftiofur. Concentrations were considerably in excess of the minimum inhibitory concentration (MIC) of the *S. aureus* strain used. A marked decrease of viable bacteria in tissue cage fluid (TCF) occurred. In five of seven ponies; however, the infection was not eliminated and abscess formation occurred. Therefore, local application of ceftiofur alone is not advisable for infections with *S. aureus* in secluded sites in horses, but should be used only with adjunctive therapy.

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INTRODUCTION

Treatment of a bacterial infection in a secluded site, like an abscess, may sometimes be straightforward. Usually drainage of the abscess, if required supported with systemic administration of antibiotics, generally quickly resolves the problem. However, despite the development of new treatments and more potent antimicrobial agents, local bacterial infections in some secluded sites, especially joints and tendon sheaths, continue to be a serious clinical problem in horses. After bacterial colonization of a joint or tendon sheath there is an influx of neutrophils and an accumulation of fibrin and inflammatory exudate. Proteolytic enzymes and other inflammatory mediators may cause damage to the articular cartilage and synovial membrane and may subsequently lead to degenerative joint disease and chronic lameness (Tulamo *et al.*, 1989; Schneider *et al.*, 1992a,b). A wide range of bacteria can be found in infected synovial structures. One of the regularly encountered pathogens is *S. aureus*, which belongs to the normal skin flora of both horses and humans (Meijer *et al.*, 2000).

For successful treatment rapid clearance of bacteria and removal of fibrin, inflammatory cells and mediators is necessary. The currently recommended approach includes joint lavage with or without arthroscopy, administration of systemic and local antibiotics with or without anti-inflammatory medications (Bertone *et al.*, 1987, 1992; Meijer *et al.*, 2000; Ter Braake, 2002). For antibiotics with a time-dependent killing action, like ceftiofur, success of antimicrobial treatment depends on maintaining a concentration of the antimicrobial drug above the minimum inhibitory concentration (MIC) of the pathogen for an extended period of time. Intra-articular administration is an appropriate route for drug administration in order to reach high intra-articular concentrations of antimicrobial drugs and to minimize the risk of systemic side-effects. Ideally, locally administered antibiotic should be sufficient on its own to terminate the infection. Furthermore, these antibiotics should not have adverse effects on articular cartilage or the synovial membrane (Adamson *et al.*, 1985; Lloyd *et al.*, 1990).

Ceftiofur sodium is a third-generation cephalosporin with broad-spectrum bactericidal activity. The third-generation cephalosporins, typically containing an aminothiazole group, are active against Gram-negative bacteria, retain good activity against Gram-positive bacteria and are resistant to most β-lactamase enzymes (Prescott, 2000). Upon injection, ceftiofur is rapidly metabolized to desfuroylceftiofur. Desfuroylceftiofur is

microbiologically equipotent to ceftiofur against most veterinary pathogens, but *S. aureus* is two- to eightfold less sensitive to desfuroylceftiofur than to ceftiofur (Brown *et al.*, 1991; Salmon *et al.*, 1996; Anonymous, 2003). Albeit registered for systemic use only, administration of a single dose of 150 mg ceftiofur into joints was shown not to have adverse effects on articular cartilage (Mills *et al.*, 2000). Ceftiofur therefore potentially is a good candidate to be tested for its clinical effectiveness after local application as a single agent.

To determine directly whether ceftiofur as a local therapeutic is effective against pathogens in infectious arthritis *in vivo* would require a study in patients or in experimental animals. However, failure of the therapy might easily lead to painful and fatal consequences. Tissue cage (TC) models have been proven to be very useful to study standardized experimental infections in secluded sites (Clarke *et al.*, 1989; Ensink *et al.*, 1996a, 2003, 2005). In this study, we therefore used a TC model to compare the elimination of ceftiofur in a normal and an infected environment after local application and to study the efficacy of locally administered ceftiofur as single antimicrobial agent in an experimental infection with *S. aureus*.

MATERIALS AND METHODS

Animals

Eight Shetland pony geldings, 3–14 years of age, were used. Their body weights ranged between 135 and 210 kg. The animals were housed indoors in individual box stalls and were fed a maintenance ration of hay twice daily. Water was freely available. All ponies had been vaccinated against influenza and tetanus and treated with an anthelmintic (Equimectin® 600, AST Farma BV, Oudewater, the Netherlands) before the experiment started. In each pony a TC had been implanted s.c. on both sides of the neck 17 months before this experiment as described by Beadle *et al.* (1989). Internal volume of the TCs is about 10 mL after ingrowth of tissue buds (Ensink *et al.*, 1996b). The TCs had been used previously for endotoxaemia experiments that had ended more than 3 months prior to the start of the present study but not for infection experiments. Sterility of the TCs was assessed by culture of aspirates from the cages at the start of the experiment. All punctures of the TCs were performed using an 18-gauge needle and a 1 or 2 mL syringe after surgical disinfection of the skin. Rectal temperature was monitored twice daily for 10 days after inoculation of the TCs and once daily for the rest of the experiment. Clinical evaluation included a daily check for swelling around the TC, and recording of the animal behaviour and appetite. The experiments were approved by the Animals Ethics Committee of Utrecht University in compliance with the Dutch Act on Animal Experiments.

Experimental design

First, one TC in each pony was injected with a single dose of 150 mg ceftiofur and tissue cage fluid (TCF) samples were taken after 1 h and at 2, 4, 10 and 24 h for ceftiofur concentration measurements and cell counts.

One week later, the other TC in each pony was inoculated with *S. aureus* (day 0). Starting at 21 h postinfection, 150 mg of ceftiofur was administered once daily for 21 days into the *S. aureus*-infected TC. TCF samples were taken at 1 h and at 2, 4, 10, 24, 48 and 52 h and on days 4 and 8 after the first administration of ceftiofur for drug concentration measurements. Additional samples were taken on days 1, 3, 7, 14, 21 and 28 for bacterial counts and white blood cell (WBC) counts.

Analysis of samples for bacteriology and cell counts was performed immediately after sampling and samples for drug concentration measurements were stored in aliquots at −80 °C until analysis.

Antimicrobial agent

Ceftiofur was administered in the form of commercially available ceftiofur sodium (Excenel® Sterile Powder, Pfizer Animal Health BV, Capelle a/d IJssel, the Netherlands), which was reconstituted with sterile pyrogen-free water (Braun Melsungen AG, Melsungen, Germany). In order to reduce the volume of injected fluid in the TCs, the concentration of the solution was 100 mg/mL, which is twice the concentration recommended by the manufacturer. Of this solution 1.5 mL was injected into the TC daily.

Ceftiofur determination

Concentrations of ceftiofur and its metabolites were determined by high-performance liquid chromatographic (HPLC) analysis as described by Beconi-Barker *et al.* (1995) and Okker *et al.* (2002). In this method metabolites, including desfuroylceftiofur–protein conjugates, are converted into desfuroylceftiofuracetamide (DCA), which is then quantified by HPLC.

In brief, 500 μL TCF was mixed with 5 mL 50 mM potassium tetraborate pH 9.0 (Merck, Darmstadt, Germany), containing 0.5 M sodium chloride (Merck) and 130 mM dithioerythritol (Sigma-Aldrich, Zwijndrecht, the Netherlands), and incubated at 50 °C for 15 min with intermittent mixing at 5-min intervals.

Following this reduction, 5 mL 0.1 M ammonium acetate (J.T. Baker, Deventer, the Netherlands) containing 0.2 M iodoacetamide (Sigma-Aldrich) was added, mixed and incubation was continued at ambient temperature for 30 min in the dark under gentle agitation at 500 rpm. After centrifugation at 4000 ***g*** for 30 min at 5 °C, supernatants were transferred onto preconditioned C_{18} solid-phase extraction (SPE) cartridges (1 g; Bond Elut, Varian, Bergen op Zoom, the Netherlands). Cartridges were washed with 5 mL 0.1 M ammonium acetate and 5 mL 2% (v/v) acetic acid (Sigma-Aldrich), and then eluted with 5 mL of a mixture of acetonitrile (Sigma-Aldrich) and 2% (v/v) acetic acid at 2:8 (v/v). Analyte-containing eluates were passed through activated and dried SCX-SPE cartridges (100 mg; Bond Elut, Varian). The SCX-retained DCA was eluted with 1.0 mL of a mixture of 1.0 M ammonium acetate and acetonitrile at 85:15 (v/v).

The HPLC analysis of 20 μL samples was carried out on a combination of 3 μm C_{18} (50 × 4.6 mm; Phenomenex, Torrance, CA, USA) and 3 μm phenyl-hexyl (50 × 4.6 mm; Phenomenex) columns connected in line. Elution of analytes was performed using a binary linear gradient using 10 mM ammonium acetate at pH 6.8 (eluent A) and acetonitrile (eluent B) at a flow rate of 1.0 mL/min as follows: 99% A (v/v) for 1.9 min, to 92% A (v/v) in 0.1 min, to 82% A (v/v) in 12 min and to 0% A for 7 min. Sample series were analysed with a repetitive analysis time of 30 min per sample. The HPLC eluate was monitored at a wavelength of 266 nm.

The limit of quantification of the method was 0.1 μg/mL of ceftiofur in TCF. The accuracy is partly dependent on the conversion of the original residue to a detectable compound, and inherently to this method the recovery of the residues of ceftiofur could not be determined. As an alternative, control samples were prepared in the same matrix and would therefore undergo comparable complete or incomplete chemical conversion reactions. In fact, these quality control samples prepared including at least five different concentration points between 25 and 750 μg/mL, were used to determine a standard curve as well. The precision of the method was assessed through the day-to-day variability of these standard curves, i.e. the relative standard deviation of the slope of the curves measured at 10 different days was 4%. Furthermore, the correlation coefficients of the curves were better than 0.9965 with an average of 0.9989 ± 0.0011.

Bacterial strain

A *Staphylococcus aureus* strain which had originally been 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 MIC for this strain was determined to be 1.0 μg/mL for ceftiofur, using an agar dilution method with microtitre trays. 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 thawed and cultured in BHI broth at 37 °C for a total of 24 h. The overnight culture was centrifuged and the bacterial pellet was washed twice with 0.9% saline. The pellet was then resuspended in pyrogen-free phosphate-buffered saline (PBS) at pH 7.2. A serial dilution was prepared in PBS to provide a concentration of approximately 1.0×10^5 colony-forming units (CFU)/mL. The actual number of CFU was 8.0×10^4 CFU/mL. About 2 mL of this suspension was used as inoculum.

Bacterial counts and cell counts

The aspirates 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 the plates growing 30–300 colonies. The detection limit of this plate count method is 1.0×10^2 CFU/mL of TCF. For calculation purposes, samples with a count of $<1.0 \times 10^2$ CFU/mL were arbitrarily set at 10^1 CFU/mL.

Total WBC counts in TCF were performed using a Coulter Counter (type industrial D, Coulter Counter, Luton, UK). Gram-stained smears were prepared of some samples, which were examined microscopically for the presence of bacteria.

Treatment of abscessation

When antibiotic treatment in the TC model is not successful abscessation of the TC occurs: the infection ruptures through the skin over the TC. In these cases the TC was removed in the standing animal using sedation with detomidine 10 μg/kg (Domosedan, Orion Pharma Corporation, Espoo, Finland), nalbufine 0.1 mg/kg (Nubain, Schering-Plough, Amstelveen, the Netherlands) and local analgesia (Lidocaine HCl 2%, Eurovet, Bladel, the Netherlands). In case of abscessation a final sample was taken for confirmation of the presence of pathogens and for susceptibility testing by an agar diffusion method.

Statistical analysis

Data were analysed statistically using the SPSS® 10.0 (SPSS Inc., Chicago, IL, USA) statistical software package for Windows. Differences between the two matrices (infected TCF and noninfected TCF) were evaluated using ANOVA followed by a multiple comparison using Tukey's *post hoc* test. Significance was set at $P < 0.05$.

RESULTS

The initial culture of TCF at the start of the experiment showed that one of the TCs had a latent infection; this pony was therefore excluded from the experiment.

At 24 h after single dose of 150 mg of ceftiofur, average concentrations of ceftiofur and its metabolites in TCF were 0.86 mg/mL and 0.40 mg/mL in uninfected and infected TCs respectively. Concentrations remained slightly lower in the infected environment at all sampling times, but differences were not significant ($P < 0.05$; Fig. 1). The interindividual differences

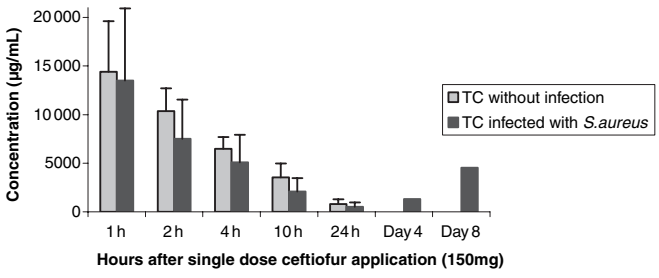

Fig. 1. Tissue cage fluid concentrations of ceftiofur during 24 h after a single dose of 150 mg ceftiofur in infected and uninfected tissue cages and at D4 and D8 in the infected tissue cage after daily administration. Data are the mean ± SD of seven ponies, at D8 data are of five ponies.

in ceftiofur-like concentrations were rather large, but at all times concentrations remained well above the MIC (1.0 μg/mL) of the *S. aureus* strain used in this experiment. Following repeated daily administration of ceftiofur, the average concentration rose to 4.5 mg/mL on D8 (Fig. 1), 24 h after the previous and just before the following ceftiofur administration.

Infection of the TCs did not result in any signs of inflammation on the day of inoculation. On the following day all ponies showed signs of inflammation; they had a rectal temperature over 39.5 °C and a painful swelling around the TC. In addition, they appeared depressed and had a poor appetite. After the start of treatment appetite returned within 1 day and the depression cleared over a period of a few days. Body temperatures; however, decreased very slowly, reaching normal values only 1 week after inoculation.

In two ponies abscessation of the infected TC occurred on D7, during the treatment with ceftiofur. One of these ponies had by far the highest bacterial count at D7, whereas the bacterial count in the other pony was of the same order as the bacterial counts of the five other ponies at D7. In three ponies abscessation of the TC occurred long after ending the ceftiofur administration, on D35, D45 and D48 respectively. In all ponies the TCs were removed upon abscessation and all abscesses healed within 3 weeks thereafter. In the two remaining ponies the TC remained in place without any signs of infection for over 12 months.

From D0 to D1 the number of bacteria in the inoculated TCs rose from approximately 1×10^4 to 1×10^7 CFU/mL. Ceftiofur caused a marked decrease in the numbers of viable bacteria in the TCs from the first administration of ceftiofur to D14. While ceftiofur administration was continued daily until D21, the number of bacteria on D21 and D28 was similar to that found on D14 (Fig. 2) Three ponies yielded TCF with no viable bacteria on D21 and D28. Two of these ponies did not show abscessation; the third was the last pony to form an abscess on D48. On the day of abscessation it was possible to determine a final TCF sample in three ponies. In all three samples only *S. aureus* was present, which was confirmed to be susceptible to ceftiofur by agar diffusion testing; no MIC determination was performed at this time.

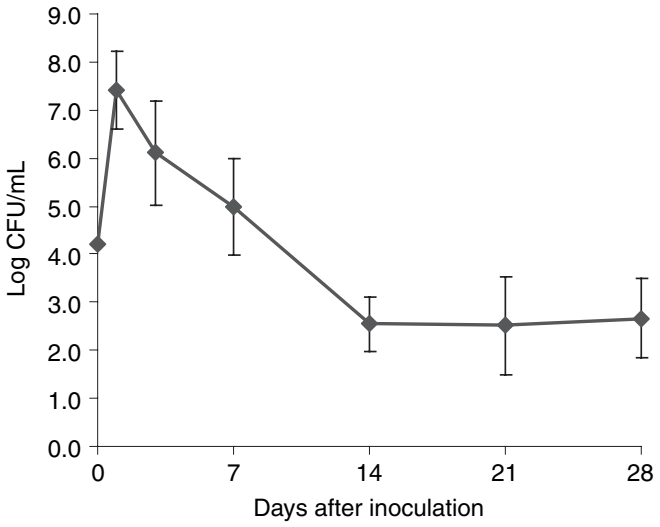

Fig. 2. Bacterial count in tissue cage fluid (TCF) in log CFU/mL in ponies treated with ceftiofur locally for 21 days. Data are the mean ± SD of seven ponies, except for days 14, 21 and 28 when only five ponies still had a TC. D0 is the known inoculation concentration of *Staphylococcus aureus* (2 mL of 8.0×10^4 CFU/mL administered in a TC with an average volume of 10 mL), which therefore has no SD.

Table 1. White blood cell count in tissue cage fluid (TCF; 10^9 cells/L) from TCs infected with 1.6×10^5 CFU of *Staphylococcus aureus*

	Mean ± SD (10^9 cells/L)	*n*
T = 0 h*	1.6 ± 1.7	7
T = 24 h*	2.4 ± 2.4	7
D0	5.6 ± 8.2	7
D1	7.1 ± 7.9	7
D2	282 ± 156	7
D3	215 ± 127	7
D7	216 ± 78	4†

*Samples of tissue cages, which were not infected.
†Five TCs still present, cell count performed on only four samples

Table 1 shows the WBC count in TCF. Administration of ceftiofur in an uninfected TC did not cause a rise in WBC count over a 24-h period. On the first day after infection there was no marked rise in the WBC count either, consistent with only mild signs of inflammation at that time. From D1 to D2 there was a dramatic rise in WBC count to levels above 200×10^9 cells/L, while there was a decrease in numbers of bacteria. On D7 one sample and on D14, D21 and D28 all five samples were too viscous to be counted by Coulter Counter. There was a large variation between individual animals.

In the cell smears intracellular *S. aureus* were encountered frequently.

DISCUSSION

In the horse ceftiofur is approved for systemic use only. Mills *et al.* (2000) showed, however, that there were no adverse side-effects after local administration of ceftiofur into a joint and that adequate levels of the antibiotic were reached for at least 24 h after single dose of 150 mg ceftiofur (Mills *et al.*, 2000). Therefore, local administration of ceftiofur could possibly play a role in infections in secluded sites, such as bacterial arthritis.

From the study of Clarke *et al.* (1996) it is known that in an infected environment the uptake and the antibiotic activity of ceftiofur are higher after systemic administration of the drug. Two possible explanations were given to explain this phenomenon. The first hypothesis was that a loss of endothelial integrity allowed a passive influx of protein and drug into the TC, resulting in a higher tissue distribution. The second explanation was a preferential localization of the drug at the infected site caused by specific protein-binding capacities of the drug (Clarke *et al.*, 1989). In this study, concentrations in an infected environment were lower at all stages when compared with the uninfected environment, but differences were not statistically

significant. This could well be explained by loss of endothelial integrity causing a more rapid efflux of antibiotic. If the specific protein binding was the cause for higher concentrations after systemic administration, one would expect higher concentrations in the infected TCs after local administration as well. The concentrations of ceftiofur that were reached in this experiment exceeded the MIC of most bacterial pathogens considered susceptible to ceftiofur (0.06–2.0 μg/mL; Brown *et al.*, 1991). The ceftiofur concentrations determined in the TCs after local administration were in excess of those determined in TCs and tissues after systemic administration (Clarke *et al.*, 1989; Meyer *et al.*, 1992; Okker *et al.*, 2002).

In five ponies staphylococci survived despite achieving an appropriate concentration of an antibacterial drug to which the staphylococci were susceptible. Failure to eliminate the pathogen despite initial improvement during antimicrobial treatment has also been encountered in other studies with infection models in horses (Bertone *et al.*, 1987; Ensink *et al.*, 2003, 2005).

There are a number of possible explanations for the survival of bacteria: too low concentrations of antimicrobial agent in the infected site, increase in MIC during antibiotic treatment, sequestration of bacteria in inflammatory exudate, formation of a biofilm by *S. aureus*, or a higher MIC of staphylococci in infected TCF than in the *in vitro* medium.

In serum, hence likely in TCF as well, ceftiofur is rapidly hydrolysed into desfuroylceftiofur. The method for drug concentration measurements used in this study cannot differentiate between desfuroylceftiofur and ceftiofur, therefore only total ceftiofur-like concentrations were measured. *Staphylococcus aureus* is two- to eightfold less sensitive to desfuroylceftiofur than to ceftiofur (Brown *et al.*, 1991; Anonymous, 2003). Total concentrations were at least 80 times the *in vitro* MIC for ceftiofur of the *S. aureus* strain used, but it is unknown how much of the, for *S. aureus*, less active desfuroylceftiofur was present. Nevertheless, even with a two- to eightfold less susceptibility for desfuroylceftiofur of *S. aureus*, total concentrations of ceftiofur and its derivatives were more than adequate throughout the entire experiment.

The MIC of the bacteria was not tested throughout and at the end of the experiment. However, in the three cases where it was possible to determine a sample after abscessation the bacteria were found to be susceptible to ceftiofur by an agar diffusion method.

A probable cause of failure of the treatment is the ability of *S. aureus* to migrate intracellularly, which was confirmed in this experiment by microscopical evaluation of cell smears. Intracellular penetration of ceftiofur is very poor, because the drug does not easily penetrate cellular membranes because of its high-water and low-lipid solubility (Prescott, 2000). Another possible cause for the failure of treatment would be the capacity of *S. aureus* to form a biofilm over implanted material. For the eradication of bacteria in biofilms concentrations of ceftiofur that are over 250 times the MIC for *S. aureus* may be needed (Olson *et al.*, 2002). In both cases viable bacteria can survive and start multiplying again when ceftiofur concentrations in TCF drop. This could explain why 2–3 weeks after completion of the ceftiofur treatment abscessation still occurred in three ponies.

Both possibilities do not explain why in two ponies abscessation occurred actually during ceftiofur treatment, when the antibiotic concentration was clearly markedly in excess of the MIC. It was observed that the inflammation caused by *S. aureus* was more severe than the inflammatory reaction that was determined in previous experiments using *Streptococcus equi* ssp. *zooepidemicus* (Ensink *et al.*, 2003, 2005). It is therefore possible that in the 21 h before the first ceftiofur administration too much cell destruction and toxin formation had already occurred, leading to an inexorable process of abscessation.

It can be concluded that local application of ceftiofur did dramatically reduce the number of viable bacteria during the first 2 weeks of treatment, although it did not eliminate the infection in five of seven ponies. Because of the good capacity to reduce the number of bacteria, ceftiofur can possibly play a role in the therapy of bacterial arthritis by intra-articular application, but it is clear that this should be adjunctive to other therapies such as through-and-through lavage, arthroscopy and systemic antibiotic treatment. Local administration of ceftiofur is not an option as a stand-alone therapy for an infection with *S. aureus* in secluded sites, such as infected joints.

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