Intra-articular administration of doxycycline in calves

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Objective—To evaluate local tissue compatibility of doxycycline hyclate (DOX) in antebrachiocarpal joints of calves.

Animals—10 healthy calves between 80 and 110 kg.

Procedures—Calves were assigned to 2 treatment groups. Calves in groups DOX and NO were administered 5 and 10 mg of DOX, respectively, locally in 1 antebrachiocarpal joint. The contralateral joint served as a control joint and was injected with 0.9% NaCl solution. General and local clinical findings were scored. Several variables were assessed in blood and synovial fluid for 9 days. Calves were euthanatized and pathologic changes and drug residues evaluated.

Results—Throughout the study, none of the calves had clinical changes or abnormal hematologic values. Signiﬁcant differences between treatment and control joints were evident only for matrix metalloproteinases at 0.5 hours after injection, with less activity for the DOX-treated joints in both treatment groups. Values for all synovial fluid variables, except nitric oxide, increased signiﬁcantly during the ﬁrst 12 to 72 hours after arthrocentesis in control and DOX-treated joints. Histologic examination revealed minimal inﬁltration of inﬂammatory cells independent of the treatment. No drug residues were detected 9 days after arthrocentesis in any tissues obtained from the liver, kidneys, fat, and skeletal muscles.


Septic arthritis is a major problem in horses and cattle, and the prognosis is guarded, with rates of return to soundness ranging from 27% to 81%. Infection results from traumatic perforation, arthrocentesis, or local or hematogenous spread of infectious agents, with the latter most often the cause in young animals. Diagnosis relies on examination of synovial fluid and may be confirmed by isolation of microbial organisms. However, it is often difficult to culture organisms from septic joints.

Immediate treatment of animals with septic arthritis is crucial to control infection and prevent degenerative joint disease. Treatment includes surgical debridement, joint lavage, joint drainage, and aggressive antimicrobial administration over a prolonged period. To enhance the effective antibacterial concentration and minimize adverse effects and drug residues, local administration of drugs and substances, such as gentamicin, amikacin, cephalosporins, and antiseptic preparations, have been used successfully. Additionally, some of these drugs have been tested in controlled-release formulations to decrease the number of arthrocentesis procedures needed for treatment. However, some of these drugs can induce tissue irritation and their antimicrobial spectrum does not always match the microorganisms isolated from cattle.

Doxycycline is a semisynthetic antimicrobial of the tetracycline group. It was introduced into human
medicine in 1967 and provides decreased antimicrobial resistance and fewer toxic events when compared with other agents of the tetracycline group.\textsuperscript{17,18} The antimicrobial spectrum includes \textit{Staphylococcus aureus} and other \textit{Staphylococcus} spp, \textit{Streptococcus} spp, \textit{Escherichia coli}, \textit{Klebsiella} spp, \textit{Salmonella} spp, \textit{Pseudomonas} spp, and anaerobic bacteria such as \textit{Bacteroides} spp, \textit{Fusobacterium} spp, \textit{Clostridium} spp, \textit{Actinobacillus} spp, and \textit{Arcanobacterium pyogenes}.\textsuperscript{17,19–22} All these organisms are isolated commonly from septic arthritis or osteomyelitis lesions of cattle and horses.\textsuperscript{1,4,6,23} Compared with other tetracyclines, DOX causes less interference with calcium binding in bony tissue.\textsuperscript{17,18} In 1 study,\textsuperscript{24} DOX inhibited the onset of clinical signs of osteoporosis in ovariectomized rats.

Several in vitro and in vivo studies\textsuperscript{25–34} have revealed anti-inflammatory or chondroprotective activities of DOX, a fact confirmed in clinical studies\textsuperscript{10–17} in humans with osteoarthritis or rheumatoid arthritis. The mechanism of action for DOX involves modulation of inflammatory mediators (such as PGE\textsubscript{2} and NO) and reduction of MMPs.

Local application of drugs of the tetracycline group causes tissue irritation. Currently, oxytetracycline is used to induce acute endometrial inflammation in cows with pyometra or related chronic uterine inflammation. In human dentistry, local application of DOX into the periodontal pocket can be a successful means of treatment.\textsuperscript{38,39} We are not aware of any data on local tolerance of DOX in musculoskeletal tissues.

Because it combines antimicrobial and chondroprotective activities, DOX may be the ideal drug for local treatment in animals with septic arthritis. However, before it can be recommended for intra-articular use, in vivo testing of tissue compatibility is required. Thus, the objective of the study reported here was to determine the local reaction of joint tissues in calves after intra-articular injection of 2 concentrations of DOX.

Materials and Methods

Animals—Ten calves (8 males and 2 females; 8 Brown Swiss and 2 Holstein-Friesian) that weighed between 80 and 110 kg were used in the study. They were housed in groups (3 or 4 calves/group) in a free-stall barn and fed milk twice daily. Hay and water were available ad libitum. The study was approved by the National Animal Protection Authorities (No. 6/2004; Kantonales Veterinäramt Graubünden).

Experimental protocol—Milk and hay were withheld for 12 hours before surgery. Each calf was examined clinically by the principal investigator (CHL). Subsequently, a pretreatment blood sample was collected from a jugular vein, and xylazine hydrochloride (DOX 1 mL of sterile saline (0.9% NaCl) solution) was then injected. Calves were positioned in left lateral recumbency. Hair on the right carpus was clipped and the skin disinfected, followed by centesis of the antebrachio carpal joint with a 22-gauge sterile needle. One milliliter of synovial fluid was withdrawn and 1 mL of sterile saline (0.9% NaCl) solution (DOX\textsubscript{low} control or DOX\textsubscript{high} control treatments). Each calf was then repositioned in right lateral recumbency, and the same procedures were performed on the left carpus, except that the solution injected contained 5 (group DOX\textsubscript{low}) or 10 (group DOX\textsubscript{high}) mg of DOX,\textsuperscript{2} respectively, dissolved in 1 mL of saline solution (treated joints). Time of the DOX injection in each animal was designated as time 0.

Additional samples of blood and synovial fluid were collected after clinical examination conducted at 0.5, 12, and 24 hours and 3, 5, and 7 days after injection. For the samples collected after arthrocentesis, additional sedation was not provided, and the calves were positioned in sternal recumbency with the head and neck positioned to 1 side, which allowed the investigators access to the carpus on the ipsilateral side. The head and neck were then repositioned to the other side, which provided the investigators access to the other carpus. Blood and synovial fluid samples were collected into tubes containing EDTA for analysis of RBC and WBC counts and into blood tubes that did not contain an anticoagulant. Samples were allowed to clot, and the serum was then harvested.

Nine days after injection, calves were examined clinically, blood samples were collected, and the calves were euthanatized by use of a captive bolt followed by exsanguination. Samples of synovial fluid were collected within 10 minutes after calves were euthanatized. Gross inspection of both antebrachio carpal joints was performed within 0.5 hours after calves were euthanatized. Samples of synovial membrane and cartilage were collected and placed in 4% formalin. For the evaluation of drug residues, samples of the liver, kidneys, and renal fat and muscle tissues from the carpal and digital flexors located proximal to the treated joints were collected and stored at −20°C until analysis.

Clinical assessment—General clinical condition was evaluated by examination of general behavior, appetite, excretions, discharges, and rectal temperature. Joints were assessed for evidence of pain, localized heat, and swelling.

Laboratory analyses—Blood and synovial fluid films were prepared immediately after sample collection. All samples were stored at 4°C and analyzed (RBC and WBC counts) within 24 hours after collection by personnel at a quality-certified veterinary laboratory\textsuperscript{4} or frozen at −20°C until analyzed for PGE\textsubscript{2}, NO, and DOX concentrations and MMP activity. Concentrations of PGE\textsubscript{2} and NO were measured by use of a PGE\textsubscript{2} immunoassay\textsuperscript{7} and total NO assay, respectively. Total MMP activity was determined by use of a fluorochrome-labeled substrate test.\textsuperscript{1} The DOX content of serum, synovial fluid, and tissue was measured by use of high-pressure liquid chromatography.\textsuperscript{8}

Articular tissue samples were processed for routine histologic examination and stained with H&E. The inflammatory reaction of the synovial membrane was evaluated to determine the type and degree of inflammatory cell infiltrate, fibrin adherent to the synovial intimal surface, and proliferation of synovial intimal cells and vessels; these evaluations were performed independently by 2 investigators (CHL and MMS) who were not aware of the treatment group. Investigators assigned grades in accordance with a scoring system (Appendix). In addition to samples obtained from the
control and treatment joints, synovial membrane samples obtained from 5 healthy calves that had not been subjected to arthrocentesis were included as negative control samples, and samples obtained from 5 clinical patients (calves with septic arthritis) were included as positive control samples.

Data analysis—Median, first quartile, and third quartile values of variables evaluated in synovial fluid were plotted over time by use of a spreadsheet program. Statistical evaluation was performed by use of commercially available software. For all time points, all maximum values, and the AUCs, the effect of DOX was calculated as the difference between treated and control joints and tested to detect significant differences against the null hypothesis (ie, not different from 0) by use of 1-sample t tests and to detect significant differences between groups DOXlow and DOXhigh by use of 2-sample t tests. Effects of arthrocentesis were tested by comparison (by use of the Wilcoxon signed rank test) of median values at time points from 0.5 to 72 hours with the baseline median value obtained before arthrocentesis. Scored variables were analyzed by use of rank sum tests. Significance was defined as values of P ≤ 0.05 (2 sided for t tests and 1 sided for the Wilcoxon signed rank test [approximated without continuity correction]).

Results

Clinical findings and hematologic variables—All calves were clinically normal throughout the observation period without any change in general clinical condition or local evidence of pain, swelling, or inflammation. All hematologic variables evaluated were within reference range values.

Synovial fluid analysis—Regarding TP ratio of PMNs to MNs, and WBC counts, no significant effects of DOX, calculated as differences between values for the control and DOX-treated joints, were detected during the entire study period at the time points measured and for the AUC values (Figures 1–3; Table 1). The only exception was the WBC count at 0.5 hours after injection, which had low absolute numbers but was significantly higher for the DOX-treated joints than for the control joints. For NO (data not shown) and PGE1 concentrations, DOX did not have significant effects, except at a single time point (12 hours after injection) for PGE1 (Figure 4). Finally, MMP activity was significantly lower at 0.5 hours after injection in DOX-treated joints, compared with values for control joints (Figure 5).

No significant difference was detected between the 2 DOX dosage groups (ie, DOXlow and DOXhigh) for any variable during the entire study period (Table 2). Significant effects were attributable to arthrocentesis (Table 3). Effects were evident as differences in values before and after arthrocentesis and were detected for most variables at several time points between 0.5 and 72 hours after injection. However, PGE1 and NO concentrations were not significantly affected by arthrocentesis (data for NO not shown).

Pathologic examination—Gross evaluation of the joints did not reveal pathologic changes. Histologic scores revealed slightly higher (but not significantly different [P = 0.07]) scores for DOX-treated joints than for control joints. In general, there was a minimal inflammatory response evident in all joint tissues after arthrocentesis independent of the DOX concentration, compared with responses for noninjected healthy control joints.

Figure 1—Median and interquartile range (25th to 75th percentiles) of TP content in synovial fluid obtained from the antebrachiocarpal joint of calves before (time 0) and at various times after intra-articular injection of DOX (5 [group DOXlow; black circles] mg of DOX or saline [0.9% NaCl] solution [control groups for the DOX] or DOXhigh [gray squares] groups). Saline solution or DOX (n = 5 calves/group) was injected during arthrocentesis.

Figure 2—Median and interquartile range (25th to 75th percentiles) of the ratio of PMNs to MNs in synovial fluid obtained from the antebrachiocarpal joint of calves before and at various times after intra-articular injection of DOX or saline solution. See Figure 1 for remainder of key.

Figure 3—Median and interquartile range (25th to 75th percentiles) of WBC counts in synovial fluid obtained from the antebrachiocarpal joint of calves before and at various times after intra-articular injection of DOX or saline solution. See Figure 1 for remainder of key.
Table 1—Mean values for the effects of intra-articular administration of DOX in antebrachiocarpal joints of calves.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Treatment group</th>
<th>Time after injection (h)</th>
<th>0.5</th>
<th>12</th>
<th>24</th>
<th>72</th>
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<tr>
<td>TP (g/L)</td>
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<td>−1.9</td>
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<td></td>
<td>DOX&lt;sub&gt;high&lt;/sub&gt;</td>
<td>1.2</td>
<td>3.9</td>
<td>7.4</td>
<td>4.3</td>
<td></td>
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<tr>
<td></td>
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<td>4,200</td>
<td>1,240</td>
<td>−420</td>
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<tr>
<td>PGE&lt;sub&gt;2&lt;/sub&gt; (pg/mL)</td>
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<td></td>
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<td>730&lt;sup&gt;*&lt;/sup&gt;</td>
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<td>MMP (RFU/s)</td>
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<td>0.180&lt;sup&gt;*&lt;/sup&gt;</td>
<td>−0.030</td>
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</table>

Values reported represent the mean difference between DOX-treated joints (n = 10) and control joints (10) injected with saline (0.9% NaCl) solution for 4 times after injection (time of injection was designated as time 0). Negative numbers indicate higher values for the control joints. *Values differ significantly (P < 0.05) from 0.

DOX<sub>low</sub> = Group injected with 10 mg of DOX. DOX<sub>high</sub> = Joints injected with 5 mg of DOX. RFU/s = Increase in fluorescence/s.

Figure 4—Median and interquartile range (25th to 75th percentiles) of PGE<sub>2</sub> concentrations in synovial fluid obtained from the antebrachiocarpal joint of calves before and at various times after intra-articular injection of DOX or saline solution. See Figure 1 for remainder of key.

joints. Saline-injected joints for the DOX<sub>low</sub> and DOX<sub>high</sub> groups (DOX<sub>low</sub> control and DOX<sub>high</sub> control joints, respectively) were not combined because of a potential general effect of DOX. Median, minimum, and maximum sums of scores were 6, 3, and 6 for the saline-injected control joints for DOX<sub>low</sub> and 6, 3, and 7 for the DOX-injected joints of group DOX<sub>low</sub>, whereas scores were 3, 2, and 8 for the saline-injected control joints for DOX<sub>high</sub> and 4, 1, and 10 for the DOX-injected joints of group DOX<sub>high</sub>. Mean, minimum, and maximum sums of scores for the negative control (no arthrocentesis) samples were 1, 0, and 2, whereas scores for the positive control samples (synovial membrane from septic joints) were 28, 20, and 32.

DOX concentrations—Concentrations higher than the limit of detection (i.e., > 0.025 µg/mL) were detected only in the DOX-treated joints. Blood, synovial fluid of the control joints, and organ samples obtained after the calves were euthanatized had negative results when tested for DOX. Median, maximum, and minimum DOX concentrations at 0.5 hours after injection were 116, 50, and 588 µg/mL for the DOX<sub>low</sub> group and 365, 15, and 733 µg/mL for the DOX<sub>high</sub> group. Concentrations higher than the breakpoint for susceptibility (4 µg/mL) were detected until 12 hours after injection in the DOX<sub>low</sub> group and until 24 hours after injection in the DOX<sub>high</sub> group. Elimination was complete at 72 and 120 hours after injection for the DOX<sub>low</sub> and DOX<sub>high</sub> groups, respectively.

Discussion

Local administration of antimicrobials in healthy joints can cause inflammation as has been reported for gentamicin, or antimicrobials (such as those in the

Table 2—Mean differences of the effects of intra-articular administration of 5 or 10 mg of DOX between groups DOX<sub>low</sub> and DOX<sub>high</sub> in antebrachiocarpal joints of calves.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Time after injection (h)</th>
<th>0.5</th>
<th>12</th>
<th>24</th>
<th>72</th>
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<td>TP (g/L)</td>
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<td>MMP (RFU/s)</td>
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<td>−0.058</td>
<td>−0.178</td>
<td>0.086</td>
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</table>

Values reported represent the mean difference of DOX effects between DOX<sub>low</sub> (n = 5) and DOX<sub>high</sub> (5) for 4 times after injection (time of injection was designated as time 0). Negative numbers indicate higher values for the DOX<sub>low</sub> group. *Values differ significantly (P ≤ 0.05) between DOX<sub>low</sub> and DOX<sub>high</sub> group.

See Table 1 for remainder of key.

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An increase in WBCs and TP concentration as well as a high ratio of PMNs to MNs in the synovial fluid have been described as variables that are useful for identification of joint inflammation, with corresponding values of WBCs of all joints were low. Thus, joint compatibility of DOX, as determined on the basis of TP concentration, ratio of PMNs to MNs, and WBC counts, was excellent in our study. Similarly, good tissue compatibility has also been described for ceftiofur. However, β-lactamase antimicrobials are subjected to high rates of hydrolysis when used in controlled-release settings. In contrast to DOX and cephalosporins, gentamicin induces a considerable increase in WBCs and refractive index (an indicator for protein content) in horses during a period of 3 to 5 days.

In the literature, it has been hypothesized that the chondroprotective activity of tetracyclines is based on modulation of NO, PGE\textsubscript{2}, and MMPs. The significantly lower total activity of MMP found in DOX-treated joints 0.5 hours after injection in the study reported here is indicative of a chondroprotective action of DOX in joints of cattle. Tetracyclines reportedly inhibit MMP activity via various mechanisms. Direct inhibition of stromelysin\textsuperscript{1,2} and gelatinase A\textsuperscript{3} has been described. Down-regulation of mRNA of MMP-1 and -13 and modulation of the autocrine production of several proinflammatory cytokines, such as interleukin-1 and cytokine receptors, are other mechanisms.

Dose-dependent effects may be the reason for significant differences found in our study in which the DOX\textsuperscript{high} treatment led to a significantly higher MMP activity at 24 hours after injection, compared with activity for the DOX\textsuperscript{low} treatment. This can be interpreted as...
loss of protection after 24 hours for the lower dose of DOX.

A similar dose-dependent effect (ie, loss of chondroprotectivity) may explain the significantly higher PGE$_2$ value at 12 hours after injection in the DOX$_{10}$ group. However, all PGE$_2$ values were highly variable in our study, including baseline measurements obtained before arthrocentesis and injection of DOX or saline solution. This is in contrast to results for healthy equine joints in which PGE$_2$ concentrations are low and within a narrow range.$^{44}$ It can be speculated that immature joints may physiologically produce variable amounts of mediators involved in matrix homeostasis of cartilage, depending on the stage of body growth, and PGE$_2$ plays a role in matrix homeostasis of cartilage.$^1$ In any case, the number of calves and joints in the study reported here was too small and the variances too large to yield conclusive results.

Production of NO is enhanced in synovial fluid of humans with rheumatoid arthritis and osteoarthritis.$^{45}$ After DOX treatment, NO production is reduced in vitro in cultured chondrocytes$^{39}$ and in vivo in cartilage.$^{28}$ Surprisingly, in the study reported here, neither arthrocentesis nor DOX treatment affected the NO content. Equine and canine synovial membrane explants can produce only extremely low amounts of NO.$^{26,47}$ However, rabbit and bovine synoviocytes are able to produce NO when stimulated in vitro.$^{1,48}$ Thus, it is not possible on the basis of our data to determine whether NO synthase was insufficiently stimulated in vivo or whether there was an insufficient amount of inducible-type NO synthase available in vivo in the synovial membranes of our calves.

Pathologic evaluation did not reveal significant differences in inflammation between DOX-treated and control joints; however, there was evidence for a minimal irritation effect, as indicated by the slightly higher values in the DOX-treated joints than in the control joints. Considering results for the clinical variables, this evidence would not preclude the use of DOX for intra-articular administration. A more pronounced but still clinically irrelevant effect was attributed to arthrocentesis because a residual inflammatory response was detected in nearly all joints, compared with results for negative control joints without arthrocentesis. In contrast to changes induced by DOX, pathologic changes induced by administration of gentamicin in another study$^{14}$ resolved by 6 days after injection. Authors of that study$^{14}$ described severe lesions with areas of necrosis, capillary thromboses, microscopic hemorrhages, serum exudate, clumps of fibrin at day 1 after injection, and edematous stroma of villi at days 2 and 3 after injection. In our study, in which histologic evaluation was conducted only at the end of the study, a minimal inflammatory cell infiltrate; slightly highercellularity of the synovial membrane tissue; and rare small, organized fibrin clots were detected on day 9 after injection. However, because these findings were minor and were detected in DOX-treated and control joints as well as in some negative control samples obtained from healthy calves, we do not believe that they were related to antimicrobial treatment. This is in contrast to the data reported in the aforementioned study.$^{14}$ Therefore, it is difficult to make comparisons between the studies, and it is assumed that the descriptive evaluation used in that other study$^{14}$ does not account for the minimal differences detected in our study.

Residues of DOX were detected only in DOX-treated joints. Blood, synovial fluid of control joints, and specimens of organs had negative results. This is in contrast to studies$^{11,13}$ performed with gentamicin in horses. However, in each of those studies, gentamicin application was of longer duration because of the use of collagen sponges and continuous infusion, respectively. Pharmacokinetics may vary substantially with continuous infusion. Kinetics described for gentamicin release from the sponge are in agreement with results for our study because no long-term effect attributable to the sponge has been reported. Therefore, with respect to drug residues, DOX may be a safer drug than gentamicin for intra-articular use in food animals.

In the study reported here, intra-articular administration of DOX in joints of calves did not evoke clinically relevant inflammatory reactions that differed substantially from those of saline-treated control joints. Furthermore, DOX exerted a short-term chondroprotective effect. No drug residues were detected throughout the entire observation period, except in the DOX-treated joints. Therefore, further evaluation of DOX for intra-articular application in cattle with septic arthritis is warranted.

References


Appendix appears on the next page
### Appendix

Scoring system for histologic assessment of joint tissue samples obtained from the antebrachiocarpal joints of calves.

<table>
<thead>
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<th>Variable</th>
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<td>PMN</td>
<td>None</td>
</tr>
<tr>
<td>Fibrillation of lining cells</td>
<td>None</td>
</tr>
<tr>
<td>Fibrin</td>
<td>None</td>
</tr>
<tr>
<td>Fibrillation of cartilage surface</td>
<td>None</td>
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NA = Not applicable.