In vitro effects of oxytetracycline on matrix metalloproteinase-1 mRNA expression and on collagen gel contraction by cultured myofibroblasts obtained from the accessory ligament of foals

Steven P. Arnoczky, DVM; Michael Lavagnino, MS; Keri L. Gardner; Tao Tian, PhD; Zachary M. Vaupel, BS; John A. Stick, DVM

Objective—To determine the effects of oxytetracycline on matrix metalloproteinase-1 (MMP-1) mRNA expression and collagen gel contraction by equine myofibroblasts in an effort to explain the mechanistic basis for the pharmacologic treatment of flexural deformities in foals.

Sample Population—Cultured myofibroblasts from the accessory ligament (distal check ligament) of 6 foals.

Procedure—Collagen gel scaffolds seeded with equine myofibroblasts were cultured in individual culture dishes containing complete media (Dulbecco’s modified Eagle medium with 10% fetal bovine serum) and oxytetracycline (0, 12.5, 25, or 75 µg/mL) for 48 hours. After 24 hours, the gels were released from the bottom of the culture plate and allowed to contract. Photographs were taken at 0, 1, 2, 4, 6, 8, and 24 hours after release to assess the degree of collagen gel contraction. Additional gels were harvested at 2 hours after release for RNA isolation and reverse transcriptase-polymerase chain reaction assessment of the degree of MMP-1 mRNA expression.

Results—Oxytetracycline induced a dose-dependent inhibition of collagen gel contraction by equine myofibroblasts. Oxytetracycline also induced a dose-dependent decrease in MMP-1 mRNA expression by equine myofibroblasts.

Conclusions and Clinical Relevance—Results of this study indicate that oxytetracycline inhibits tractional structuring of collagen fibrils by equine myofibroblasts through an MMP-1 mediated mechanism. In young foals, oxytetracycline administration may make the developing ligaments and tendons more susceptible to elongation during normal weight-bearing. Inhibition of normal collagen organization may provide the mechanistic explanation for the results seen following the pharmacologic treatment of flexural deformities in foals by oxytetracycline administration. (Am J Vet Res 2004;65:491–496)

Flexural deformities (contracted tendons) are a common, crippling problem in foals.1 A flexural defor-
months (1) old. Myofibroblasts were expanded to passage 3 and used in experiments.

**Collagen gel contraction**—To determine the effect of oxytetracycline on the tractionsal structuring of collagen, 1-mL collagen gels (type-I bovine collagen [2.4 mg/mL]) were seeded with equine myofibroblasts (200,000 cells/mL), placed in individual 60-mm-diameter culture dishes, and incubated with oxytetracycline (0, 12.5, 25, or 75 µg/mL) in complete medium (Dulbecco's modified Eagle medium with 10% fetal bovine serum, ascorbate, and penicillin-streptomycin-amphotericin B). Concentrations of oxytetracycline used in this study were chosen on the basis of a previously published study, which used a therapeutic dose of 44 mg/kg to produce alterations in joint angles of clinically normal foals and foals with contracted tendons. To calculate the maximum concentration of oxytetracycline that would be available to myofibroblasts following IV administration, the following formula was used:

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C (\mu g/mL) = \text{dose (mg/kg)}/\text{volume of distribution (L/kg)}
\]

where \( C \) equals the maximum theoretical concentration. The volume of distribution for oxytetracycline is 2 L/kg, which was determined in a previous study that examined the pharmacokinetics of oxytetracycline in foals. Thus, the in vitro concentration of 25 µg/mL would approximate the previously published effective clinical dose of oxytetracycline (44 mg/kg). The low (12.5 µg/mL) and high (75 µg/mL) concentrations were added to determine whether this effect was a dose-dependent or threshold effect.

After 24 hours, the collagen gels were photographed and released from the bottom of the culture dishes. Additional photographs were taken at 1, 2, 4, 6, 8, and 24 hours after release. The digital images were used to measure the area of each collagen gel over time. Each image was scaled, and areas were measured by use of a computer program. For cultured myofibroblasts from each foal, 5 collagen gels/dose were examined. To determine whether foal age affected the response of myofibroblasts to oxytetracycline, areas of the contracted collagen gels (determined on the basis of the percentage of original area) were compared among cultured myofibroblasts from each of the 6 foals.

**Live-to-dead assay**—To determine whether the concentrations of oxytetracycline used in this study had any effect on cell viability, myofibroblasts were cultured in monolayer in a 24-well culture plate (20,000 cells/well). Myofibroblasts were exposed to oxytetracycline (0, 12.5, 25, and 75 µg/mL; n = 6 wells/concentration) for 48 hours and cell viability assayed by use of ethidium homodimer-1 and calcein AM. The effect of foal age on the response of cells to oxytetracycline was compared among cultured myofibroblasts from each of the 6 foals.

**MMP-1 mRNA expression**—To determine the effect of oxytetracycline on the expression of MMP-1, myofibroblasts (400,000 cells/mL) from each of 4 foals (1 neonatal foal and 3 foals that were 2 days [1 foal], 10 weeks [1], and 6 months [1] old) were seeded into 1 mL collagen gels and exposed to various concentrations of oxytetracycline as already described. An insufficient number of cells were available from the remaining 2 foals (8 days [1 foal] and 10 weeks [1] old) to conduct this aspect of the study. Two hours after release, the gels were harvested and digested with collagenase. Total RNA was extracted and purified by use of an RNA reverse transcriptase-polymerase chain reaction (RT-PCR) kit. The RNA (0.5 µg) was treated with deoxyribonuclease I ribonuclease-free for 15 minutes at 37°C. The RNA was then subjected to an RT-PCR assay by use of a single tube RT-PCR system and the equine oligonucleotide primers (5'-AACTTGTGCG-CAATTTCAG-3' and 5'-AAGGGATGTCTTAGTGAATG-3'). Five microliters of the PCR product was electrophoresed and band intensity evaluated densitometrically. This value was normalized to the constitutively expressed gene glyceraldehyde-3-phosphate dehydrogenase. This provides a semiquantitative assessment of MMP-1 mRNA expression.

**Statistical analysis**—The effect of oxytetracycline dose on the collagen gel contraction (area) was evaluated by use of an ANOVA. The effect of foal age on the response of cells to oxytetracycline was compared among cultured myofibroblasts from each foal by use of an ANOVA and a Tukey posthoc test. Percent cell viability with the various concentrations of oxytetracycline was compared by use of an ANOVA. The effect of foal age on the response of cells to oxytetracycline was compared by use of an ANOVA. The effect of oxytetracycline concentration on the expression of MMP-1 mRNA in the cultured myofibroblasts was examined by use of a polynomial regression analysis, \( f = 1/(a + bx) \). For all analyses, significance was set at a value of \( P < 0.05 \).

**Results**

Equine myofibroblasts induced a distinct contraction of collagen gels over a 24-hour period (Fig 1). The pattern of collagen gel contraction was similar in
response to cultured myofibroblasts from each of the 6 foals.

Oxytetracycline was found to inhibit collagen gel contraction by equine myofibroblasts in an apparent dose-dependent fashion (Fig 3). However, the extent of this inhibition varied among cultured myofibroblasts; in response to clinically relevant concentrations of oxytetracycline (12.5 and 25 µg/mL), a significant inhibition of collagen gel contraction was not found by all myofibroblasts obtained from the various foals. In contrast, oxytetracycline at a concentration of 75 µg/mL significantly inhibited collagen gel contraction by myofibroblasts obtained from all foals. Inhibition of collagen gel contraction at this concentration of oxytetracycline was greatest at 8 hours (after release) by myofibroblasts obtained from all foals, except by myofibroblasts obtained from the 6-month-old foal. However, by 24 hours, a significant decrease was found in the degree of collagen gel contraction inhibition (compared with controls during the same period) by myofibroblasts obtained from all foals. At an
oxytetracycline concentration of 75 µg/mL at 8 hours, the degree of inhibition of collagen gel contraction by myofibroblasts obtained from the 6-month-old foal was significantly less then by myofibroblasts obtained from the other (younger) foals. No significant effect of oxytetracycline concentration was found on the viability of equine myofibroblasts over a 24-hour period. A strong ($r^2 = 0.78$) and significant ($P < 0.001$) inverse correlation was found between the concentration of oxytetracycline and MMP-1 mRNA expression among examined cultured myofibroblasts from all foals (Fig 4 and 5).

**Discussion**

The use of oxytetracycline has been advocated as a pharmacologic treatment for the correction of congenital flexural deformities in foals.1,2,4,6 However, the precise mechanism by which oxytetracycline affects the supporting structures of clinically normal foals and foals with contracted tendons was unclear. Results of our study suggest oxytetracycline inhibits the normal structural remodeling of collagen by equine myofibroblasts through a down regulation of interstitial collagenase (MMP-1) mRNA expression. This lack of organization of the extracellular matrix in developing ligaments and tendons may compromise the biomechanical characteristics of these connective tissues, making them more susceptible to creep during normal weight-bearing.

Movement of cells through the extracellular matrix and the resultant contraction of collagen containing tissues are fundamental to the biological process of tissue morphogenesis, embryonic development, and wound healing.8,11 Results of several studies 8,10 have shown that the movement of cells through the extracellular matrix produces a tractional force on the extracellular matrix resulting in the rearrangement of collagen into a compacted and orientated fashion. This has been elegantly demonstrated by the ability of fibroblasts to align and contract collagen fibers within isotopic gels in vitro.8,10,17,22 This process has been termed tractional structuring and is thought to be the basis for the formation and remodeling of developing ligaments and tendons.8,10

The structural remodeling of developing ligaments and tendons is mediated by proteolysis, which is facilitated through the production of MMPs by the tissue fibroblasts.19 Matrix metalloproteinases are a family of zinc-dependent proteinases that are capable of digesting the various structural components of the extracellular matrix.9,12 Interstitial collagenase (MMP-1) is known to target type-I collagen, the major component of the extracellular matrix of ligaments and tendons, and thus plays a key role in the tractional structuring response of these tissues.8,10 Therefore, it would appear that inhibition of MMP-1 could, in turn, inhibit the tractional structuring of developing ligaments and tendons. Inhibition of the normal structural remodeling of the collagen fibers in developing ligaments and tendons would result in a more disorganized, and thus biomechanically compromised, tissue.

Results of our study indicate that myofibroblasts harvested from the distal check ligament of foals are capable of contracting type-I collagen gels in vitro through the process of tractional structuring. A similar response has been reported for fibroblasts from other tissue sources.9,17,22 Results of our study also indicate that collagen gel contraction could be inhibited in a dose-dependent fashion by the addition of oxytetracycline to the culture media. These results are similar to a previously published study17 that revealed an inhibitory effect of tetracyclines on collagen gel contraction. The inhibition of collagen gel contraction has been linked to the inhibition of MMPs by tetracyclines.17

Tetracyclines have long been recognized as potent inhibitors of MMP activity because they can act as a chelator of the zinc ion.13-16 However, results of an in vitro study indicate that the micromolar concentrations of tetracyclines required for the direct inhibition of MMP enzymes would be difficult to achieve in vivo.19 Results of recent studies12,15,27 have suggested that the inhibition of MMPs by tetracyclines occurs primarily through the down-regulation of gene expression. This is thought to occur through a transcriptional inhibition that is mediated by pathways upstream of the transcription factor AP-1 binding site.27 In our study, the inhibition of MMP-1 mRNA expression in the myofibroblasts of foals was strongly correlated to the concentrations of oxytetracycline used. Because tetracyclines have been shown to inhibit MMPs in general,15,16 it is possible that other MMPs are also down regulated by oxytetracycline in a similar dose-dependent manner. However, because MMP-1 is most intimately related to the remodeling and reorganization of developing tissue, it was the focus of our study.8,10,12 In our study, neither MMP-1 protein concentrations nor MMP-1 activity were determined. However, because MMP-1 mRNA expression has been shown to correlate with MMP-1 protein synthesis in tendon cells,28 our findings suggest that MMP-1 synthesis is inhibited by oxytetracycline.

Oxytetracycline did not have any adverse effect on the viability of equine myofibroblasts at the concentrations used in our study. Results of a previous study have also revealed no adverse effect of similar concentrations of doxycycline on cell viability in vitro.20

Experimental and clinical observations have revealed several caveats regarding the efficacy of oxytetracycline in the treatment of flexural deformities in foals that appear to be supported by the results of our study. It has been suggested that oxytetracycline produces a more dramatic effect in neonates (in terms of correction of flexural deformities).3 Although alterations in joint angles have been reported in foals up to 3 months of age, the effect is negligible in adult horses.5 These observations support the theory that the mechanism of action of oxytetracycline is the inhibition of normal collagen remodeling and orientation. Study findings indicate that the longitudinal growth of bone in the limbs of foals is most rapid during the first 10 weeks of life.29,30 Inhibition of the active collagen remodeling of ligaments and tendons, which must occur during this time to accommodate this rapid increase in limb length, could make these tissues more susceptible to creep when exposed to the forces of nor-
nal weight-bearing. Results of our study revealed a significant decrease in the inhibitory effect of oxytetracycline (75 µg/mL) on collagen gel contraction at 8 hours in the 6-month-old foal, compared with that of the remaining younger foals. Although the limited number of cultured myofibroblast specimens in our study precludes any definitive conclusions regarding the effect of donor age on the response of ligament myofibroblasts to tetracycline, these in vitro observations appear to parallel clinical experience.3

It has also been observed in clinical studies that the effect of oxytetracycline is transient.14 Clinically normal foals treated with oxytetracycline had a return to pretreatment metacarpal phalangeal angles 4 days after a single IV administration of the drug.2 This could be the result of the natural clearance of the oxytetracycline from the extracellular fluid compartment of treated foals23 allowing a return to normal collagen remodeling and alignment in the tendons and ligaments of the growing limb. Results of our study revealed a significant decrease in the inhibition of collagen gel contraction between 8 and 24 hours. Because oxytetracycline is not actually “cleared” from this in vitro system, a more plausible explanation for the observed transient effect would be a chemical degradation of the oxytetracycline in tissue culture over time. A decrease in the response of the cells to the drug over time is also a possibility.

Finally, it has been stated that the use of oxytetracycline IV at doses of <3 grams (approx 60 mg/kg for a neonatal foal) has been unrewarding in the treatment of flexural deformities in foals.4 Results of our study suggest that the extent of the inhibitory response of oxytetracycline on the tractional collagen structuring response of equine myofibroblasts is dose dependent. Although the highest concentration of oxytetracycline used in our study (75 µg/mL, which equates to approx 150 mg/kg or about 7.5 g for a 50-kg foal) produced the most profound results, such doses are not feasible in clinical situations. Indeed, nephrotoxicity has been reported in a foal following the IV administration of oxytetracycline at a dose of 70 mg/kg.21

The results of our study provide a potential mechanistic rationale for the clinical observations of joint angle relaxation in clinically normal foals and foals with contracted tendons following administration of large doses of oxytetracycline. The ability of oxytetracycline to inhibit tractional collagen structuring appears to be related to the inhibition of MMP-1 mRNA by equine myofibroblasts. The inhibition of MMP-1 mRNA expression during this time may impact the remodeling process and make the developing tissue more susceptible to creep. This increased susceptibility of the ligament and tendons to creep may explain the relaxation of this structure in clinically normal foals and foals with contracted tendons following oxytetracycline administration. Although the inherent limitations of the in vitro test system used in our study preclude any definitive conclusions regarding the clinical validity of this mechanistic theory, results of our study do provide a plausible explanation for the reported efficacy of large doses of oxytetracycline in the treatment of flexural deformities of foals.

References
19. Sadowski T, Steinmeyer J. Effects of tetracyclines on the production of matrix metalloproteinases and plasminogen activators as well as their natural inhibitors, tissue inhibitor of metalloproteinases-1 and plasminogen activator inhibitor-1. Inflamm Res 2001;50:175–182.
23. Daniels JT, Cambrey AD, Ockleston NL, et al. Matrix metalloproteinase inhibition modulates fibroblast-mediated matrix contrac-

Vitrogen, Cohesion Technologies, Palo Alto, Calif.
Oxybiotic, Butler, Dublin, Ohio.
GIBCO, Rockville, Md.
Scion Image, Scion Frederick, Md.
Molecular Probe Corp, L-3224, Molecular Probes, Eugene, Ore.
Stratagene, La Jolla, Calif.
KODAK ID Image Analysis Software, Rochester, NY.


