Age-dependent effects of systemic administration of oxytetracycline on the viscoelastic properties of rat tail tendons as a mechanistic basis for pharmacological treatment of flexural limb deformities in foals

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Objective—To describe the effect of systemically administered oxytetracycline on the viscoelastic properties of rat tail tendon fascicles (TTfs) to provide a mechanistic rationale for pharmacological treatment of flexural limb deformities in foals.

Sample—TTfs from ten 1-month-old and ten 6-month-old male Sprague-Dawley rats.

Procedures—5 rats in each age group were administered oxytetracycline (50 mg/kg, IP, q 24 h) for 4 days. The remaining 5 rats in each age group served as untreated controls. Five days after initiation of oxytetracycline treatment, TTfs were collected and their viscoelastic properties were evaluated via a stress-relaxation protocol. Maximum modulus and equilibrium modulus were compared via a 2-way ANOVA. Collagen fibril size, density, and orientation in TTfs were compared between treated and control rats.

Results—Viscoelastic properties were significantly decreased in TTfs from 1-month-old oxytetracycline-treated rats, compared with those in TTfs from 1-month-old control rats. Oxytetracycline had no effect on the viscoelastic properties of TTfs from 6-month-old rats. Collagen fibril size, density, and orientation in TTfs from 1-month-old rats did not differ between oxytetracycline-treated and control rats.

Conclusions and Clinical Relevance—Results confirmed that systemically administered oxytetracycline decreased the viscoelastic properties of TTfs from 1-month-old rats but not those of TTfs from 6-month-old rats. The decrease in viscoelastic properties associated with oxytetracycline treatment does not appear to be caused by altered collagen fibril diameter or organization. The age-dependent effect of oxytetracycline on the viscoelastic properties of tendons may be related to its effect on the maturation of the extracellular matrix of developing tendons. (*Am J Vet Res* 2012;73:1951–1956)

Contracted tendons are a common flexural limb deformity in neonatal foals.[1–3] This condition manifests as deviation of a limb in the sagittal plane and as persistent hyperflexion of a joint region.[1–3] Foals with this deformity are unable to extend their digits appropriately because of a shortening of their flexor tendons. Investigators of clinical studies[2–6] have shown that the short-term administration of large systemic doses (up to 3 g, IV) of oxytetracycline is successful in correcting tendon contracture in very young foals but not older foals. However, the mechanisms by which this tendon lengthening develops are unclear.[3,5–7] Because oxytetracycline chelates calcium, it has been hypothesized that systemically administered oxytetracycline causes inhibition of calcium-mediated muscle contraction, which results in the observed relaxation of joint angles.[3,5,8] However, in some circumstances of flexural limb deformity (eg, distal interphalangeal deformity), the key anatomic structure that tethers the deformity, the accessory ligament (distal check ligament),[1,3] is not directly associated with a muscle. Therefore, inhibition of muscle contraction cannot be the only mechanism by which oxytetracycline aids in the correction of contracted tendons in foals.[7]

Because neonatal foals respond to oxytetracycline more dramatically than do adult horses,[2–6] another possible mechanistic theory is that oxytetracycline inhib-

Abbreviations

MMP	Matrix metalloproteinase
TTf	Tail tendon fascicle

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its collagen remodeling during rapid growth by myofibroblasts, which comprise most of the cells within the distal check ligament of foals.[7,9] Results of in vitro studies[7,10] indicate that oxytetracycline inhibits myofibroblast expression of various MMPs (including interstitial collagenase), which are required for the growth and organization of collagen fibrils by fibroblasts in developing tendons. Therefore, it was hypothesized that oxytetracycline may inhibit collagen remodeling and collagen fiber organization by tendon fibroblasts in rapidly growing foals, thereby decreasing the mechanical properties of the tendons and allowing them to elongate under normal weight bearing conditions.[7] Additionally, the age-related decrease in cellularity and increase in extracellular matrix properties of tendons in both rats[11,12] and horses[13] may also be involved in the age-dependent effect of oxytetracycline on the correction of flexural limb deformities in foals.[2–6]

Results of in vivo studies[14,15] indicate that systemic administration of oxytetracycline results in a decrease in the material properties of bone and skin in young rats. Therefore, the purpose of the study reported here was to examine the effect of oxytetracycline on the mechanical properties of rat tail tendons from young and mature rats. We hypothesized that short-term (4-day) systemic administration of oxytetracycline in young rats would cause a decrease in the viscoelastic properties of tendons, compared with the viscoelastic properties of tendons in young untreated rats, and would have no significant effect on the viscoelastic properties of tendons in older rats. We also hypothesized that the decrease in viscoelastic properties of tendons following oxytetracycline treatment would be a result of altered collagen fiber organization.

Materials and Methods

Animals—Ten 1-month-old and ten 6-month-old male Sprague-Dawley rats were used in the study. All study procedures were approved by the Institutional Animal Care and Use Committee at Michigan State University.

Study design—Five rats in each age group were given oxytetracycline (50 mg/kg, IP) daily for 4 consecutive days. The remaining 5 rats in each age group served as untreated controls. The IP dose of oxytetracycline administered was chosen on the basis of the IV dose recommended for foals.[2–6] In rats, results of a study[16] indicate that IP administration of oxytetracycline produces a plasma concentration of oxytetracycline similar to that following IV administration of oxytetracycline. The rats were weighed daily starting 7 days prior to the initiation of the injections. Rats were euthanized via an overdose of pentobarbital sodium[a] 4 days after the initial IP injection. Tail tendon fascicles were harvested from each rat and either frozen at –80°C for mechanical evaluation or placed in neutral-buffered 10% formalin for electron microscopic analysis of collagen fiber size, distribution, packing density, and fiber orientation.

Mechanical evaluation—A stress-relaxation protocol was chosen to determine the viscoelastic properties of the rat TTfs. Just prior to testing, the rat TTfs were thawed in PBS solution at room temperature (approx 22°C). Five TTfs from each rat were tested. To determine the cross-sectional area of each fascicle, a 10-mm segment was removed and photomicrographs of the segment were taken with a calibrated microscope. The fascicle diameter was determined via calculation of the mean of 3 measurements perpendicular to the long axis of the fascicle with software.[b] A circular cross section was assumed for the computation of initial area.[17] A 60-mm-long segment of each rat TTf was then used for the stress-relaxation tests. The rat TTfs were gripped at each end in sawtooth clamps for a final gage length of approximately 43.5 mm. To eliminate failure at the clamps, the segments of tendon within the clamps were air-dried and placed between 2 pieces of emery board, and the test area of tendon between the grips was kept moistened with PBS solution. Each tendon was mounted onto a custom-made material testing system in a PBS solution bath at room temperature. The testing system was equipped with a 5-lb load cell,[c] a linear variable differential transformer[d] to measure grip-to-grip displacement, and a motion controller.[e] Tendons were first loaded to 1 g to determine the initial length, or 0% strain mark, and then displaced to 3% strain at a constant rate of 0.41 mm/s (approx 0.94% strain/s). The tendons were then maintained at this displacement for 10 minutes of stress-relaxation. Load and displacement values were recorded at 50 Hz with an analog-to-digital computer data acquisition system for the duration of the experiment. With the use of tendon diameter and load and displacement values, the viscoelastic material properties of maximum modulus (maximum stress divided by applied strain) and equilibrium modulus (equilibrium stress divided by applied strain) were computed (**Figure 1**).

Electron microscopic evaluation—To determine whether systemic oxytetracycline treatment had an effect on collagen fibril size distribution, packing density, or orientation, TTfs from each 1-month-old control and oxytetracycline-treated rat were processed for transmission electron microscopy. Fixed tissue was rinsed in 0.1M phosphate buffer solution, placed in 1% osmium tetroxide in 0.1M phosphate buffer solution for 3 hours, dehydrated in graded ethanol solutions (30%, 50%, 65%, 75%, 95%, and 100%), and transferred to propyl-

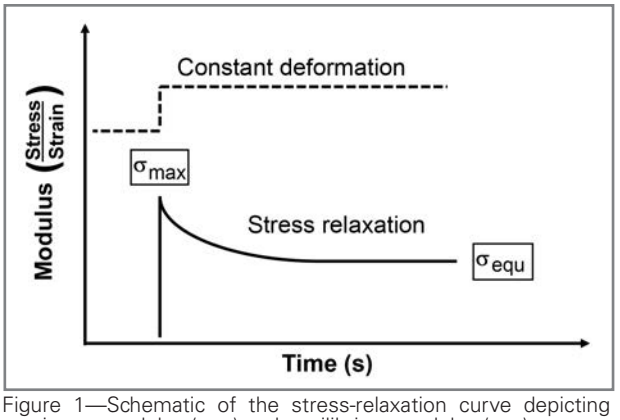

Figure 1—Schematic of the stress-relaxation curve depicting maximum modulus (σ_{max}) and equilibrium modulus (σ_{equ}).

Table 1—Mean ± SD viscoelastic properties of TTfs obtained from 1-month-old (n = 10) and 6-month-old (10) male Sprague-Dawley rats, of which 5 rats from each age group were treated with oxytetracycline (50 mg/kg, IP) daily for 4 days and the other 5 rats from each age group served as untreated controls.

	1-month-old rats			6-month-old rats		
Variable	Oxytetracycline-treated	Control	*P* value	Oxytetracycline-treated	Control	*P* value
Maximum modulus (MPa)	133.9 ± 21.6	164.6 ± 15.9	0.030	254.7 ± 20.1	284.1 ± 44.9	0.218
Equilibrium modulus (MPa)	35.6 ± 3.6	46.4 ± 5.2	0.005	180.7 ± 15.9	195.5 ± 32.9	0.392

Table 2—Mean ± SD for collagen fibril number, diameter, and packing density in the TTfs from ten 1-month-old male Sprague-Dawley rats, of which 5 were treated with oxytetracycline (50 mg/kg, IP) daily for 4 days and the other 5 served as untreated controls.

Variable	Oxytetracycline-treated rats	Control rats	*P* value
Fibril number	293 ± 64	293 ± 61	0.993
Fibril diameter (nm)	123.00 ± 14.01	124.72 ± 14.91	0.856
Fibril density (nm^2/nm^2)	0.658 ± 0.024	0.675 ± 0.082	0.661

Table 3—Mean ± SD number collagen fibrils with various diameters in the TTfs of the ten 1-month-old rats in Table 2.

Fibril diameter	Oxytetracycline-treated rats	Control rats	*P* value
0–50 nm	12.97 ± 4.87	12.08 ± 5.93	0.657
51–100 nm	31.32 ± 4.87	31.82 ± 4.21	0.736
101–150 nm	21.94 ± 2.60	20.31 ± 4.36	0.225
151–200 nm	18.65 ± 3.47	19.54 ± 4.17	0.533
201–250 nm	11.75 ± 5.69	14.68 ± 8.22	0.267
251–300 nm	3.36 ± 5.19	1.57 ± 2.17	0.228

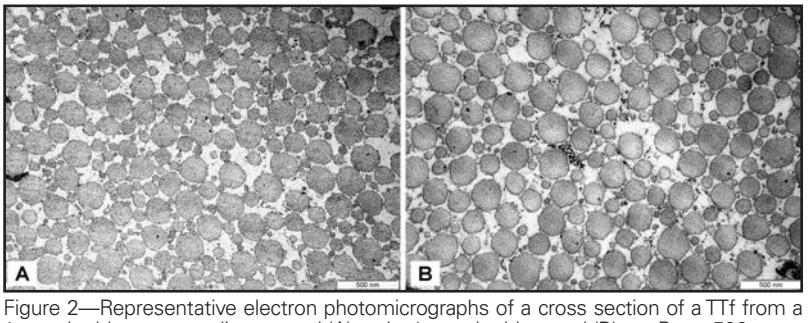

Figure 2—Representative electron photomicrographs of a cross section of a TTf from a 1-month-old oxytetracycline-treated (A) and a 1-month-old control (B) rat. Bar = 500 nm.

ene oxide. Specimens were then infiltrated with an epoxy resin (embedding media,[f] adhesive,[g] and dodenyl succinic anhydride [5:4:12]). The resin was infiltrated in 3 steps (50%, 75%, and 100% resin to propylene oxide). These 3 infiltration processes were performed for 12 hours each. The specimens were then hardened at 60°C for 48 hours.

Thin cross sections of each rat TTf were stained with aqueous uranyl acetate and lead citrate and then examined with an electron microscope.[h] Three fields per section were photographed at a magnification of 40,000X, avoiding areas containing artifacts. Quantitative assessment of each image was performed with an acquisition and analysis software package.[i] Major and minor collagen fibril diameters and collagen fibril density in the image field of view were calculated for each 1-month-old control and oxytetracycline-treated rat. The minor collagen fibril diameter was the value interpreted as the actual fibril diameter in all measurements. Collagen fibril density was determined as the ratio of the total cross-sectional area of fibrils in a standardized area to that standardized area.

Statistical analysis—All data were reported as mean ± SD. For each age group, Student *t* tests were used for all comparisons between treated and control rats. Comparisons were made for each of the following mean variables: daily weight, maximum modulus, equilibrium modulus, collagen fibril density, and collagen fibril size distribution within the TTfs. Values of $P \leq 0.05$ were considered significant for all comparisons.

Results

Animals—For the oxytetracycline-treated rats, the IP injections had no detectable adverse effects (ie, signs of pain, decrease in activity, or change in appetite) on the health of the rats. Throughout the experimental period, the mean daily weight did not differ significantly between the control and treated rats in either the 1-month-old (P = 0.28) or 6-month-old (P = 0.17) group. During necropsy, the oxytetracycline-treated rats had no gross signs of peritonitis or other pathological changes in the abdominal cavity as a result of the IP injections.

Mechanical evaluation—The TTfs of the 1-month-old oxytetracycline-treated rats had a lower mean maximum modulus (P = 0.030) and equilibrium modulus (P = 0.005), compared with those of the TTfs of the 1-month-old control rats (**Table 1**). In the 6-month-old group, the mean maximum modulus (P = 0.218) and equilibrium modulus (P = 0.392) for the TTfs did not differ significantly between the control and oxytetracycline-treated rats.

Electron microscopic evaluation—The mean number of collagen fibrils, fibril diameter, and fibril density in the TTfs of the 1-month-old rats did not differ significantly between the control and oxytetracycline-treated rats (**Table 2**). Similarly, comparisons of the number of fibrils with various diameters within the TTfs of the 1-month-old rats revealed no significant difference between the control and oxytetracycline-treated rats (**Table 3**). The fibrils in the TTfs from control and oxytetracycline-treated rats were uniformly oriented along the long axis of the TTfs and had no evidence of disorganization or random orientation (**Figure 2**).

Discussion

The results of the present study indicate that systemic administration of oxytetracycline decreased the viscoelastic properties of TTfs from young (1-month-old) but not old (6-month-old) rats. The viscoelastic, or time-dependent, properties of ligaments and tendons are directly related to the structure and makeup of their extracellular matrices.[18] The combination of highly oriented, cross-linked collagen fibrils embedded in an organized, hydrated matrix allows viscoelastic tissues such as ligaments and tendons to respond to loads in a rate-dependent manner.[18] The 2 classically described manifestations of viscoelastic behavior are creep (the slow progressive elongation of a tendon or ligament over time in response to a constantly applied load [stress]) and its inverse, load (stress) relaxation (a microstructural reorganization of the extracellular matrix of tendons or ligaments held under a fixed elongation to minimize internal stresses).[18] Therefore, the less organized that the extracellular matrix of tendons or ligaments is, the more extensive is the creep and the more rapid is the stress -relaxation of those tendons or ligaments. Both creep and stress-relaxation tests can be used to evaluate viscoelastic properties, but stress-relaxation tests are more sensitive than creep tests in the identification of differences in viscoelastic properties[18–20]; therefore, a stress-relaxation test was used in the present study.

Intravenous administration of oxytetracycline has been recommended as a pharmacological treatment for the correction of congenital contracted tendons in foals.[2–6,21] However, the precise mechanism by which oxytetracycline affects the supporting structures of clinically normal and affected limbs of foals is unclear.[3,5–7] On the basis of results of an in vitro study,[7] our laboratory group postulated that oxytetracycline may impair the structural remodeling of developing tendons by its inhibition of MMPs. Matrix metalloproteinases are a family of zinc-dependent proteinases that are capable of digesting the various structural components of the extracellular matrix,[22] which, in turn, permits the remodeling of collagen fibrils in developing ligaments and tendons through a mechanism called tractional structuring.[23,24] During tractional structuring, cells within a developing ligament or tendon exert a tractional force on collagen fibrils, aligning them in a compacted and oriented fashion.[23,24] Horses tendon cells exposed to therapeutic concentrations of oxytetracycline in vitro were unable to exert these tractional forces on collagen gels.[7] Thus, it was theorized that oxytetracycline-induced inhibition of MMP activity would alter the structural remodeling of collagen fibers in growing ligaments and tendons, resulting in a more disorganized extracellular matrix.[7] This biomechanically compromised tissue would then be more easily subjected to elongation when exposed to weight-bearing loads. However, results of the present study failed to reveal any significant differences in collagen fibril diameter, packing density, or orientation in the TTfs of 1-month-old control and oxytetracycline-treated rats. The distribution of collagen fibrils of various diameters in the TTfs from both 1-month-old control and oxytetracycline-treated rats was a mixture of large- and small-diameter fibrils similar to the distribution of collagen fibrils in the tendons of horses.[25] Additionally, the mean diameter of the fibrils in the rat TTfs was also similar to that reported in the tendons of horses.[25]

Investigators of a study[16] suggest that the decrease in the material properties of the skin of rats that received IP administration of oxytetracycline for 2 weeks may be caused by an increase in soluble collagen concentrations in the skin. Collagen molecules are generally linked together by specific covalent bonds known as cross-links.[26] If alterations occur in the cross-linking process, the hierarchic structure and thus the tensile strength of the collagenous component of the extracellular matrix is adversely affected.[26,27] In addition to causing a reduction in the material properties of the collagen structure, interference in the cross-linking process also increases the solubility of collagen.[28] It has been postulated that oxytetracycline may affect the cross-linking of collagen by inactivation of the enzyme lysyl oxidase.[16] This is similar to the mechanism of action of lathyrogens, which reduce the mechanical strength and increase the solubility of collagen by inhibiting the formation of collagen cross-links.[29–31] Lysyl oxidase catalyzes the first step in the collagen cross-linking process and requires copper for this reaction.[31,32] Given that tetracyclines are potent chelators,[33] oxytetracycline may inhibit lysyl oxidase by binding copper.[16] It has also been theorized that oxytetracycline may affect the conversion of procollagen to collagen by its chelation of calcium and the subsequent inhibition of the calcium-dependent enzyme aminoterminal procollagen protease.[16] The persistent presence of the aminoterminal extension on collagen can result in deficient formation of the intermolecular cross-links and altered material properties of collagen.[16] Additional studies are needed to identify the precise mechanisms by which oxytetracycline may exert its effect on collagen cross-linking.

It has been suggested that the tensile properties of collagen in tendons are more dependent on fibril length than fibril diameter.[26] The increase in collagen fibril length (as a result of the end-to-end cross-linking of collagen fibrils), which is thought to be specific to tendons, has also been associated with increased elastic storage capabilities.[26] Conversely, shorter (less cross-linked) collagen fibrils may be more prone to viscous slippage.[26] Such fibril slippage could explain the more rapid stress relaxation (ie, decreased equilibrium modulus) detected in the TTfs from the 1-month-old oxytetracycline-treated rats, compared with that in the TTfs from the 1-month-old control rats. Future studies should focus on evaluation of the effect of oxytetracycline on collagen solubility and fibril length in vivo.

Another interesting finding in the present study was the apparent age-dependent effect of oxytetracycline on the viscoelastic properties of the rat TTfs. Although oxytetracycline administration significantly reduced the viscoelastic properties of the TTfs from 1-month-old rats, it had no significant effect on the viscoelastic properties of the TTfs from 6-month-old rats. This age-dependent effect in rats is similar to that in foals, in which systemic administration of oxytetracycline has greater tissue lengthening effects in younger animals,

compared with that in older animals.[2–6] The decrease in tissue lengthening following oxytetracycline administration in older animals may be the result of increased collagen cross-linking in maturing tendons.[25] Increases in collagen cross-linking and tendon stiffness as a function of age have been reported in both rats[31,34,35] and horses.[13,36,37] Collagen cross-links in tendons become more stable as animals mature and are less likely to be affected by chemical or pharmacological agents.[34] The structural and functional maturation of tendons with age may explain the ineffectiveness of oxytetracycline on altering the viscoelastic properties of the TTfs from 6-month-old rats in the present study.

A potential limitation of the present study was the use of rat TTfs as a surrogate for the ligaments and flexor tendons of foals. Despite the obvious difference in size, rat TTfs have a collagen fibril structure similar to that of tendons in horses.[25] Additionally, rat TTfs have been used in studies[16,26,31,34,35] for evaluation of the form and functional relationship of the hierarchic structure of tendons. The methods used in the present study allowed us to test multiple TTfs from each rat, and we felt that calculation of mean data from multiple TTfs provided a more accurate representation for each rat than would the use of data from only 1 TTF from each rat.

Another potential limitation of the present study was the administration of oxytetracycline via an IP route rather than an IV route. However, results of another study[16] indicate that IP administration of oxytetracycline to rats achieved a plasma concentration of oxytetracycline similar to that achieved after IV administration. In the present study, oxytetracycline was administered for 4 consecutive days to rats at the dose (50 mg/kg)[2–6] recommended for the treatment of flexural limb deformity in foals. Although the duration (4 days) of oxytetracycline treatment used for the rats of the present study was longer than that (2 days)[2,5,6,21] recommended for the treatment of flexural limb deformity in foals, it was felt that the oxytetracycline dosage (50 mg/kg, q 24 h for 4 d) used in the present study was clinically relevant given that the half-life of oxytetracycline is considerably shorter in rats than it is in horses.[38,39]

The results of the present study provided a potential mechanistic explanation for the beneficial effect of systemic oxytetracycline administration on the correction of contracted tendons in young foals. Systemically administered oxytetracycline decreased the viscoelastic properties of TTfs in young (1-month-old) but not old (6-month-old) rats, and this decrease in the viscoelastic properties of the TTfs did not appear to be caused by an alteration in collagen fibril organization or density as theorized.[7] Therefore, it is likely that the age-dependent effects of oxytetracycline on the viscoelastic properties of tendons are associated with other aspects of tendon extracellular matrix maturation, such as collagen cross-linking.

a. Fatal-Plus, Vortech Pharmaceuticals, Dearborn, Mich.
b. Scion Image Software, Scion Corp, Frederick, Md.
c. 5-lb Load Cell, Sensotec, Columbus, Ohio.
d. Linear Variable Differential Transformer, Lucas Shaevitz, Pennsauken, NJ.
e. Motion Controller, Newport, Fountain Valley, Calif.
f. Poly/Bed 812, Polysciences Inc, Warrington, Pa.
g. Araldite, Huntsman Advanced Materials, Salt Lake City, Utah.
h. Philips 301 TEM electron microscope, Philips Electronic Instrument Co, Roselle, Ill.
i. ImageJ, version 1.42q, National Institutes of Health, Bethesda, Md. Available at: rsbweb.nih.gov/ij/index.html. Accessed Jun 8, 2009.

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