MOLECULAR PATHOBIOLOGICAL AND SCANNING ELECTRON MICROSCOPIC CHANGES IN HORSE TENDON CELLS TREATED WITH ENROFLOXACIN

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ABSTRACT

Fluoroquinolone (FQNL) antibiotics have been used widely in horses because of their broad-spectrum bactericidal activity and relative safety, however, their use is not without risk. Tendonitis and spontaneous tendon rupture have been reported in people during or following therapy with FQNLs. To evaluate the potential damage of enrofloxacin (ENRO) on the equine superficial digital flexor tendon (SDFT), an equine cell culture system as an *in vitro* model of equine tendon injury and repair was developed. The effects of ENRO on tendon cell cultures established from equine SDFT were studied. The data thus collected demonstrated that ENRO inhibited cell proliferation, induced morphological changes and altered proteoglycan synthesis in equine tendon cell cultures. Interestingly, these effects were more pronounced in juvenile tendon cells as compared to adult horses. It is hypothesized that morphological changes and inhibition of cell proliferation is a result of impaired production of proteoglycans and other glycoproteins in the extra cellular matrix of ENRO-treated tendon cells.

Key words: Horse tendons, cell culture, scanning electron microscopy, Western blotting, Northern blotting

INTRODUCTION

Fluoroquinolone (FQNL) antibiotics have been used widely in horses because of their rapid, broad-spectrum bactericidal activity, infrequency of bacterial resistance and relative safety (Beluche *et al.*, 1999). One such FQNL, enrofloxacin (ENRO), is effective against most Gram-negative and some Gram-positive aerobic bacteria, with limited activity against *Streptococcus spp.* and anaerobes (Haines *et al.*, 2000). The benefits of FQNL antimicrobials, however, are not without risk. Tendonitis and spontaneous tendon rupture have been reported in people during or following FQNL therapy (Takada *et al.*, 1994; Lewis *et al.*,1999). Although the exact pathogenesis of the deleterious effects of FQNLs on tendon physiology is not known, several studies suggest that FQNL alter proteoglycan contents in soft tissues, including tendons, and thereby alter collagen fibrillogenesis (Svensson *et al.*, 1995; Gu and Wada, 1996).

Lesions, especially in the form of tendonitis, of the superficial digital flexor tendon (SDFT) have a bad reputation of healing and are a major (7-43 %) cause of wastage in the horse racing industry (Kobayashi *et al.*,1999; Kane *et al.* 2000; Cherdchutham *et al.*,2001). Current extra-label systemic treatment with FQNL antimicrobials in these performance horses may further predispose them to tendonitis or acute rupture of tendons and ligaments.

To evaluate the potential damage of ENRO on equine tendons, an equine cell culture system as an *in vitro* model of equine tendon injury and repair was developed in our laboratory. Using this system, we have studied the effects of ENRO on tendon cell cultures established from equine SDFTs. Previous studies from our and other laboratories have shown that FQNL-induced inhibition of cell proliferation is a cross-species phenomenon. It is hypothesized that FQNLs have similar effects on the proliferation of equine tendon cells. Our results demonstrate that ENRO not only inhibits the proliferation of equine tendon cell cultures, but it also induces morphological changes likely due to impaired proteoglycan synthesis in these cells. Interestingly, these effects appear to be much more pronounced in juvenile tendon cells as compared to adult horses.

MATERIALS AND METHODS

Cell cultures

Short-term cultures were established from SDFTs from a young 7-month old foal and three older horses

(2, 7 and 16 years of age). Aseptically removed tendons were washed with sterile phosphate-buffered saline (PBS) containing antibiotics, minced finely into 1-2 mm pieces with scissors and digested with 3 mg type I or type II collagenase (Sigma Chemical Co., St. Louis, MO, USA)/ml Dulbecco's modified Eagle medium (DMEM) at 37°C for 12 to 16 hours. The cell suspension was filtered through a 70 µm nylon mesh, washed in PBS and maintained in DMEM supplemented with 10% fetal bovine serum. Cells were cultured in 25 or 75 cm² tissue culture flasks until 60-70% confluent when enrofloxacin (Baytril® for s.c. injection, Bayer Corp., Pittsburgh, PA, USA) was added at concentrations of 25, 50, 100 or 300 µg/ml for 2-3 days. Cell viability was ascertained with trypan blue exclusion assay.

Scanning Electron Microscopy

Juvenile or adult tendon cells were placed into wells of 12-well plates containing Thermanox cover slips at density 20,000 cells/well. When monolayers reached 60-70% confluence, ENRO (50 or 100µg/ml) was added to one half of the wells. Two to three days later, the medium was discarded from all wells. Cells growing on cover slips were fixed with a standard EM fixative (2% paraformaldehyde, 2% glutaraldehyde, 0.2% picric acid in 0.1 M cacodylate-HCl buffer) for 1-2 days at 4°C. The fixed cover slips were dehydrated, dried and mounted on aluminum stubs before coated with gold. Cell morphology was evaluated with a JSM-5800 Scanning Electron Microscope (JEOL USA, Inc., Peabody, MA, USA).

Proteoglycan extraction

Control cells and cells treated for 3 days with 100 µg/ml ENRO were extracted twice in 4 M guanidine HCl, 0.5% (w/v) 3-[(3-cholami-dopropyl) dimethyl ammonio]-1-propanesulfonate CHAPS, 100 mM sodium acetate buffer, pH 5.8, with protease inhibitors (5 mM benzamidine-HCl, 10 mM Na₂EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 mM iodoacetamide and 0.4 mM pepstatin A) for 24 hrs (Hascall and Kimura, 1982). The extracts were centrifuged at 17,000 x g for 1 hr at 10°C. The supernatants from the two extractions were combined and protein content was determined using spectrometry at 220 nm absorbance.

Western Blotting

Cell extracts were dialyzed against three changes of water and clarified by centrifugation. Aliquots of 50 µg of protein from cells were digested with chondroitinase ABC (Chase ABC) (*Proteus vulgaris*, Sigma Chemical Co.) at 0.0025 units/1 µg of glycosaminoglycans (GAGs) in 50 mM of Tris, 0.02% NaN, pH 7.5 at 37°C overnight. Digested samples were denatured for 10 min at 95°C, suspended in Laemmli loading buffer, separated on standard 12.5 % SDS polyacrylamide gels and transferred onto a nitrocellulose membrane using standard methanol-glycine buffer. The core proteins of decorin and biglycan were identified using rabbit polyclonal antibody LF-30 or LF-136 to decorin and LF-106 to biglycan (generous gift from Dr. Larry Fisher, NIDCR, NIH). Biotinylated secondary anti-rabbit antibody, avidin-biotin complex solution (Vectastain® *Elite* ABC kit) and DAB substrate kit (all from Vector Laboratories) were used to visualize antibody-antigen complexes.

Northern Blotting

To determine whether ENRO regulates the content of decorin and biglycan at the transcriptional or post-transcriptional level we compared the level of decorin and biglycan mRNAs in horse tendon cells which were cultured in the presence or absence of ENRO. Because the cDNA sequences for the protein cores of both proteoglycans have been known and entered in the GenBank (Decorin accession No. AF038127; Biglycan accession No. AF035934), we have been able to construct DNA probes. The horse decorin probe is 1091 bp large and represents a segment of the open reading frame between position 183 and 1274 in the decorin gene. The biglycan probe, 1232 bp, is derived from the open reading frame for biglycan gene between positions 71 and 1303.

Confluent control cell cultures or cell monolayers exposed to ENRO (100 µg/ml for 8 (T_1) and 24 (T_2) hrs) were scraped off with a sterile plastic cell scraper and used to extract total RNA with TRIZOL reagent (Life Technologies, Inc., Grand Island, NY, USA). Total RNA was denatured at 60°C for 10 min, separated in 1.4% formaldehyde/agarose gel (3 µg RNA/lane), transferred to a nylon membrane and hybridized with a digoxigenin (DIG)-labeled DNA probes for horse decorin and biglycan. The DNA probes were labeled using the PCR DIG Probe Synthesis Kit from Roche. Both prehybridization and hybridization were executed at 60°C for 1 hr and overnight, respectively. After hybridization, membranes were washed with 2 x SSC, 0.1% SDS for 15 min at room temperature (RT), then with 0.1 x SSC, 0.1% SDS for 2 x 15 min at RT, and with 0.1 x SSC, 0.1% SDS for 2 x 15 min at 68°C. The membranes were then treated with blocking (maleic acid) solution for 30 min at RT, block solution with enzyme (Anti-DIG-AP Fab. Fragment, Roche) for 30 min at RT an maleic acid buffer for 2 x 15 min at RT. Finally, the membranes were placed in DIG washing and block buffer, DEPC water and NBT/BCIP (Roche) for 10 min to 2 hrs at RT in dark place for color development.

Each experiment was repeated at least twice. Using Microsoft excel program, one-way analysis of variance was applied to analyze the data thus obtained.

RESULTS

Effect on cell proliferation

Enrofloxacin (ENRO) led dose-dependent decrease in number of cells in culture. Another effect was partial detachment of cells started from the plastic surface. Cultures established from young horses were more susceptible to the effect of ENRO than cultures established from tendons from older horses (Table I).

Table I: Effect of enrofloxacin concentration on juvenile and adult equine tenocyte proliferation

Dosage level (µg/ml)	Juvenile cells	Adult cells
0 (Control)	750 ± 25a	525 ± 35a
25	600 ± 39b	560 ± 42a
50	500 ± 33c	490 ± 39a
100	350 ± 27d	470 ± 48a
300	50 ± 16e	110 ± 19b

Figures bearing different letters in a column differ significantly ($P<0.05$).

Morphological changes

Scanning electron microscopy of control equine tenocytes demonstrated a smooth intact cell surface (Fig. 1A). Treatment with ENRO, however, induced pores and perforations in the cell membranes of equine tenocytes (Fig. 1B). Morphological changes were more pronounced in juvenile cell cultures as compared to adult cells.

Western Blotting

Adult tendon cells exposed to 0, 50 and 100 µg ENRO/ml for 2 days were extracted with 4 M guanidine HCl. Undigested aliquots and aliquots treated with chondroitinase ABC were separated on 12.5% SDS polyacrylamide gels and transferred onto a nitrocellulose membrane. The core proteins of decorin and biglycan were identified using rabbit polyclonal anti-decorin LF-30 or LF-136, and anti-biglycan LF-106 antibodies. As Fig. 2 demonstrates, ENRO had opposite effects on decorin and biglycan synthesis. ENRO decreased the amount of biglycan (Fig. 2A), and increased the amount of decorin in horse tendon cells

(Fig. 2B). It is of interest that biglycan was identifiable even without chondroitinase ABC treatment (Fig. 2A), whereas decorin could be detected only after treatment with chondroitinase ABC (Fig. 2B).
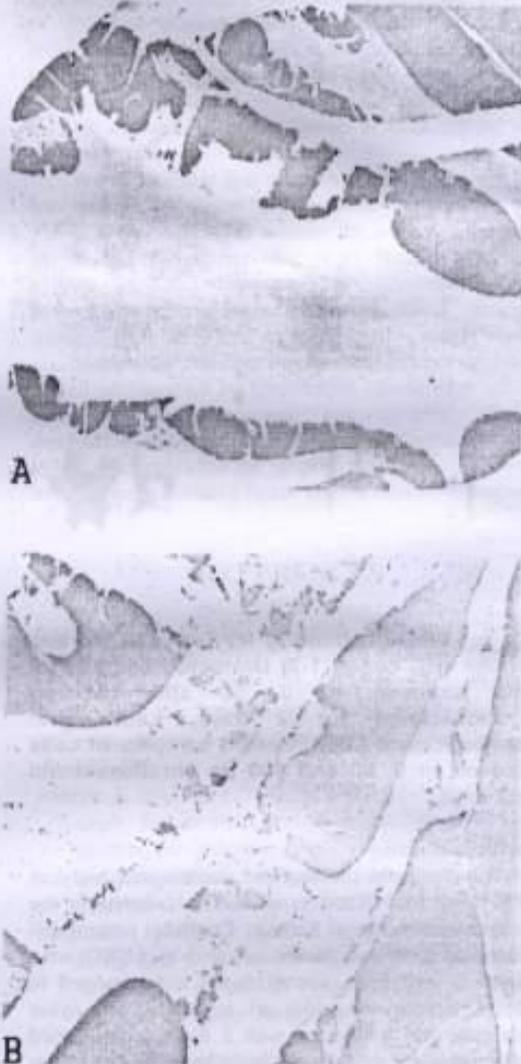

Fig.1: Scanning electronmicrograph demonstrating A: normal equine control tenocytes .and B: pores and perforations in the cell membranes of enrofloxacin-treated equine tenocytes
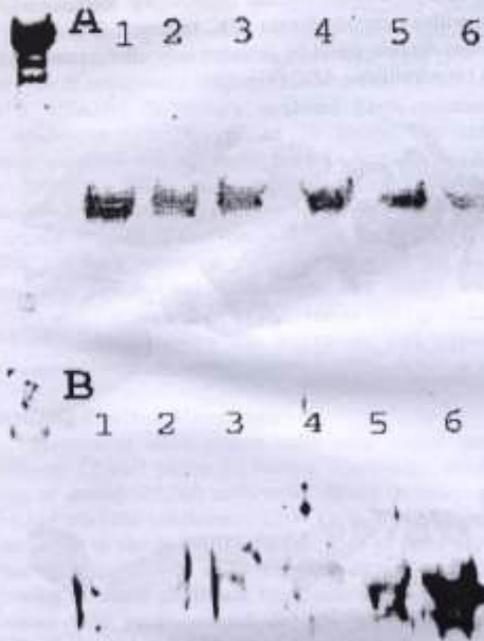
Fig. 2: Western blotting for biglycan (A) and decorin (B). Lanes 1-3: Untreated samples of cells exposed to 0, 50 and 100 µg enrofloxacin/ml for 2 days, Lanes 4-6: Chondroitinase ABC- treated samples of cells exposed to 0, 50 and 100 µg enrofloxacin/ml for 2 days.

Northern Blotting

We compared the level of decorin and biglycan mRNAs in adult horse tendon cells cultured in the presence or absence of ENRO. Confluent control cell cultures or cell monolayers exposed to ENRO were collected, total RNA was extracted and separated in 1.4% formaldehyde/agarose gel, transferred to a nylon membrane and hybridized with a digoxigenin-labeled DNA probes for decorin and biglycan. The expression of either biglycan or decorin mRNA did not change with ENRO treatment (Fig. 3).

DISCUSSION

The results of this study demonstrate that ENRO has multiple effects on cell cultures established from equine SDFTs. ENRO inhibits cell proliferation and induces morphological changes. These effects are more prominent in juvenile than in adult cells. ENRO also had an effect on glycosylation and post-translational regulation of decorin and biglycan, but not on their mRNA expression.
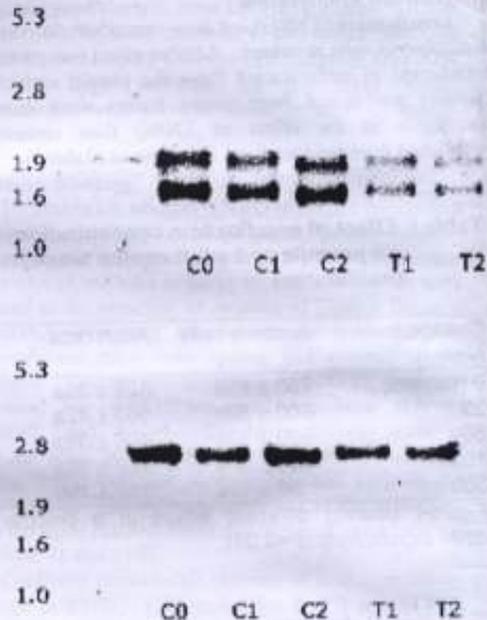

Fig. 3: Northern blot of total RNA from horse tendon cell cultures exposed to 100 µg/ml {T_1 (8 hrs) and T_2 (24 hrs)}, of enrofloxacin. Three µg of total RNA/lane was analyzed using a horse decorin (top) or biglycan (bottom) digoxigenin-labeled DNA probe.

The small leucine-rich proteoglycans decorin and, to lesser extent, biglycan have been shown to occur throughout the SDFT and play a major role in regulation of collagen fibrillogenesis, and the spatial organization of tendon fibers, thereby having a direct effect on tendon strength and function (Svensson et al., 1995; Gu and Wada, 1996). Decorin is a small leucine-rich proteoglycan, the primary role of which in the tendon is to bind to fibrillar collagen, one of the main components of tendon, and to regulate collagen fibril formation in vivo (Danielson et al., 1997). The majority of this fibrillar collagen is type I, which is primarily responsible for tensile strength (Jozsa and Kannus, 1997). Decorin is considered a key regulator

of collagen fibrillogenesis; in its absence collagen fibrils are coarse, irregular and haphazardly arranged (Danielson *et al.*, 1997). These changes are accompanied by decrease in collagen-bound proteoglycans in collagen in the skin and tendon. Decorin-deficient mice have fragile skin that is not able to withstand sudden tensile strain (Danielson *et al.*, 1997). Biglycans's role in collagen fibrillogenesis has been studied less. However, recent reports have linked biglycan deficiency to impaired gait, ectopic tendon ossification, and osteoarthritis (Ameye *et al.*, 2002). Biglycan deficiency in mice mimics Ehlers-Danlos syndrome, a human hereditary disorder characterized by joint and skin laxity (Ameye and Young, 2002; Corsi *et al.*, 2002).

The mechanism of FQNL's effect on the musculoskeletal system is unclear, but muscles, cartilage and tendon seem to be the most likely target (Kashida and Kato, 1997). In people, the Achilles tendon is the most commonly affected tendon, likely due to its weight bearing function (Kannus and Jozsa, 1991; Bernard-Beaubois *et al.*, 1998). Williams *et al.* (2000) observed a decrease in proteoglycan and collagen synthesis, and an increase in matrix-degrading proteolytic activity when canine tenocytes were treated with ciprofloxacin. Studies by Bernard-Beaubois *et al.* (1998) have indicated that in rabbit tenocytes FQNLs decrease the level of decorin mRNA, while the level of mRNA for type I collagen remains intact. It appears from these studies that FQNLs have cross-species effect on the musculoskeletal system. Simonin *et al.* (1999) reported a decrease in the content of cartilage proteoglycans but not collagen synthesis in mice after a single dose of pefloxacin, a FQNL widely used in Europe. They also observed damage to Type II collagen in the cartilage after repeated doses of pefloxacin. Williams *et al.* (2000) described that ciprofloxacin led to decrease in synthesis of not only proteoglycans but also of collagen synthesis which was attributed to increased matrix-degrading protease activity. We did not observe any increase in matrix-degrading protease activity secreted by ENRO-treated cells.

The results presented in this paper are consistent with other data from our laboratory. Using chicken embryonic cells cultures derived from gastrocnemius tendons, we have determined that cell proliferation was progressively inhibited with increasing concentrations of ENRO. This was accompanied by changes in morphology, extra cellular matrix content and collagen fibril formation as detected by electron microscopy (Yoon *et al.* 2003). We also observed ENRO-induced changes in glycosylation of decorin, the most abundant tendon proteoglycan, resulting in the emergence of another form of decorin. Because this form has a lower molecular weight than the common form of decorin only after chondroitinase ABC and *N*-glycanase treatment of extracts from ENRO-treated cells, we hypothesize that ENRO induces changes in the number of *N*-linked oligosaccharides attached to the core protein of decorin or changes in the decorin degradation process. In conclusion, our data suggest that ENRO affects cell proliferation and extra cellular matrix through changes in glycosylation in both equine and chicken cells.

We hypothesize that changes in tendon decorin and biglycan glycosylation and synthesis induced by the FQNL antibiotic ENRO weaken the integrity of equine SDFT, thereby predisposing it to failure. We also hypothesize that changes in glycosylation of proteoglycans and other proteins affect collagen assembly. In addition, we have identified that equine juvenile tendon cells are more susceptible to inhibition of cell proliferation by ENRO than equine adult tendon cells. A more complete understanding of the mechanisms involved in FQNL-induced tendonitis may help further elucidate the specific mechanisms involved in performance-related flexor tendon injuries in horses.

REFERENCES

Ameye, L., D. Aria, K. Jepsen, A. Oldberg, T. Xu and M. F. Young, 2002. Abnormal collagen fibrils in tendons of biglycan/fibromodulin-deficient mice lead to gait impairment, ectopic ossification, and osteoarthritis. J. Fed. American Soc. Exp. Biol., 16: 673-680.

Ameye, L. and M. F. Young, 2002. Mice deficient in small leucine-rich proteoglycans: novel *in vivo* models for osteoporosis, osteoarthrities, Ehlers-Danlos syndrome, muscular dystrophy, and corneal disease. Glycobiology, 12: 107R.

Beluche, L.A., A.L. Bertone, D.E. Anderson, C.W. Kohn and S. E. Weisbrode, 1999. *In vitro* dose-dependent effects of enrofloxacin on equine articular cartilage. American J. Vet. Res., 60:577-582.

Bernard-Beaubois, K., C. Hecquet, G. Hayem, P. Rat and M. Adolphe, 1998. *In vitro* study of cytotoxicity of quinolones on rabbit tenocytes. Cell Biol. Toxicol., 14: 283-292.

Cherdchutham, W., L.S. Meershoek, P. R. van Weeren and A. Barneveld, 2001. Effects of exercise on biomechanical properties of the superficial digital flexor tendon in foals. American J. Vet. Res., 62: 1859-1864.

Corsi, A, T. Xu, X.D. Chen. A. Boyde, J. Liang, M. Mankani, B. Sommer, R.V. Iozzo, I. Eichstetter, P.G. Robey, P. Bianco and M. F. Young. 2002. Phenotypic effects of biglycan deficiency are linked to collagen fibrillar abnormalities, are synergized by decorin deficiency, and mimic Ehlers-Danlos-like changes in bone and other connective tissues. J. Bone Miner. Res., 17. 1180-1189.

Danielson, K.G., H. Baribault, D.F. Holmes, H. Graham, K. E. Kadler and R. V. Iozzo. 1997. Targeted disruption of decorin leads to abnrmal collegen fibril morphology and skin fragility. J. Cell. Biol., 136. 729-743.

Gu, J. and Y. Wada, 1996. Effect of exogenous decorin on cell morphology and attachment of decorin-deficient fibroblasts. J. Biochem., 119: 743-748.

Haines, G.R., M.P. Brown, R.R. Gronwall and. K. A. Merrit, 2000. Serum concentrations and pharmacokinetics of enrofloxacin after intravenous and intragastric administration to mares. Canadian J. Vet. Res., 64: 171-177.

Hascall, V.C. and J. H. Kimura, 1982. Proteoglycans: Isolation and characterization. Meth. Enzymol., 82: 769-799.

Jozsa, L.G. and P. Kannus, 1997. Structure and metabolism of normal tendons. In: Human Tendons: Anatomy, Physiology and Pathology, eds.: L.G. Jozsa and P. Kannus. Chapter 2, Human Kinetics, Champaign, IL, pp: 46-95.

Kane, A.J., J. Traub-Dargatz, W. C. Losinger and L.P. Garber, 2000. The occurence and causes of lameness and laminitis in the U.S. horse population. In: Proc. Amer. Assoc. Equine Pr., San Antonio, pp: 277-280.

Kannus, P. and L. Jozsa, 1991. Histopathologic changes preceeding spontaneous rupture of a tendon. A controlled study of 891 patients. J. Bone Joint Surg., 73; 1507-1525.

Kashida, Y. and M. Kato, 1997. Characterization of fluoroquinolone-induced Achilles tendon toxicity in rats: Comparison of toxicities of 10 fluoroquinolones and the effects of antiinflammatory compounds. Antimicrob. Agents Chemother., 41: 2389-2393.

Kobayashi, A., M. Sugisaka, K. Takehana, M. Yamaguchi, E. K. Iwasa, and M. Abe, 1999. Morphological and histochemical analysis of a case of superficial digital flexor tendon injury in the horse. J. Comp. Path., 120: 103-114.

Lewis, J.R., J. G. Gums and D.L. Dickensheets, 1999. Levofloxacin-induced bilateral Achilles tendonitis. American Pharmacother., 33: 792-795

Simonin, M.A., P. Gegout-Pottie, A. Minn, P. Gillet, P. Netter and B. Terlain, 1999. Proteoglycan and collagen biochemical variations during fluoroquinolone-induced chondrotoxicity in mice. Antimicrob. Agents Chemother., 43: 2915-2921.

Svensson, L., D. Heinegard and A. Oldberg, 1995. Decorin-binding sites for collagen type I are mainly located in leucine-rich repeats 4-5. J. Biol. Chem., 270: 20712-20716.

Takada, S. M. Kato and S. Takayama, 1994. Comparison of lesions induced by intra-articular injections of quinolones and compounds damaging cartilage components in the rat femoral condyles. J. Toxicol. Environ. Hlth., 42: 73-88.

Williams, R.J., E. Attia, T.L. Wickiewicz and J. A.Hannafin, 2000. The effect of ciprofloxacin on tendon, paratenon and capsular fibroblast metabolism. American J. Sports Med., 28: 364-369.

Yoon, J.H., R. Brooks Jr, Y. H. Kim, T. Masako and J. Halper, 2003. Proteoglycans in chicken gastrocnemius tendons change with age and exercise. American J. Vet. Res. (In Press).