ENROFLOXACIN INDUCED CHANGES IN DECORIN PRODUCTION BY EQUINE TENDON EXPLANTS IN CULTURE

by

JOHN A. BRYAN, II

(Under the Direction of Jaroslava Halper)

ABSTRACT

Fluoroquinolone antimicrobials are a fluorinated, synthetic group of broad-based antibiotics that share a common mechanism of inhibiting bacterial deoxyribonucleic acid (DNA) gyrase, resulting in the replicative failure of bacterial DNA. However, their use has also been associated with tendonitis and tendon rupture due to altered collagen assembly and the sequela of decreased tensile strength. Specifically, fluoroquinolated antibiotics exert an influence on the actions of proteoglycans, which are a class of proteins instrumental in the proper assembly of collagen. This study examined the effects of the fluoroquinolated antibiotic enrofloxacin on the small, leucine-rich repeat proteoglycan decorin. Experimentation via immunoblotting, enzyme-linked immunosorbent assays, dot blotting, and immunohistochemistry revealed decreased amounts of decorin in enrofloxacin treated tendons, and decreased binding of enrofloxacin treated decorin to antibody and to collagen. These changes are most likely due to enrofloxacin induced alterations in the glycosaminoglycan attachments of decorin.

INDEX WORDS: Enrofloxacin, Proteoglycans, Decorin, Collagen, and Glycosaminoglycans

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DEDICATION

This work is dedicated to my son: Alexander Hugh Bryan

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I would like to express my deepest gratitude to Dr. Jaroslava Halper for her patience, understanding, and guidance throughout graduate school. I would also like to thank my committee members Dr. Corrie Brown and DR. Eric Mueller for their insight and support during my pursuit of this degree, and to the late Dr. Barry Harmon for his enthusiastic encouragement during his tenure on my committee. He will be missed. Profound thanks are also imparted to Jian Zhang, whose tireless assistance made almost anything possible.

Most importantly, I would like to extend my most profound appreciation and love to my wife Karen for her undying perseverance through this time.

Lastly, I give thanks to the horses who sacrificed all for this study. May our efforts be equal to their contribution.

TABLE OF CONTENTS

Page

INTRODUCTION

The Structure and Function of Tendons

Tendons facilitate body motion. They function in mechanical concert with the forces of gravity to propel limbs through space. Through their structural properties, tendons endow their bearers with the freedom to pursue food, the speed to escape predators, and the resilience to migrate long distances.

As muscle fibers contract, they exert powerful forces upon connective tissues, specifically tendons. Tendons in turn transmit these contractile forces to bone, initiating the act of movement. Body extremities rely on the tensile strengths of tendons to carry out motion. The main structural component of tendon connective tissue is collagen type I, with III and IV in lower concentrations (Tsuzaki et al, 1993; Fan et al, 1997). As the primary structural component of tendons, collagen must maintain the durable character of its molecular matrix while concurrently bearing multiple stresses. Collagen achieves this harmony through the integrity of its construction.

The basic structure of a collagen molecule is characterized by a helix of three interwoven alpha polypeptide chains. Collagen fibrils result when adjacent triple helices are woven together. Collagen fibrils aggregate in turn to form collagen fibers. This arrangement is precise, and its construction must be tightly regulated in order that the resulting tendon possess an appropriate tensile strength sufficient to its function.

Collagen Assembly and Decorin

Collagen is the primary structural component of connective tissues (Pins et al, 1997). The proteoglycan decorin is a regulatory protein involved in collagen fibrillogenesis and structural assembly (Pins et al, 1997; Sini et al, 1997). Thus the normal functions of connective tissues, specifically tendons, rely upon the successful interplay of decorin and collagen fibril assembly.

The structure of a collagen molecule is comprised of a helix of three interwoven alpha polypeptide chains. The structures of these alpha chains are primarily made of a triad repeat of amino acids; specifically in the order of glycine – X – Y. Amino acids X and Y in this series are usually represented by proline and hydroxyproline, respectively. The ring-like conformation of proline provides the alpha helix with stability, while the small size of glycine facilitates close packing of the chains (Alberts et al, 2002).

Collagen polypeptide chains are synthesized on ribosomes and then transferred to the endoplasmic reticulum (ER) as pro-alpha chains. Once in the ER, specific proline and lysine amino acids on the alpha chains become hydroxylated, resulting in the formation of hydroxyproline and hydroxylysine, respectively. Each of the newly formed pro-alpha chains then combines with two others via hydrogen bonding to make the triple-stranded helix of procollagen. Propeptides attached to each of the procollagen molecules prohibit premature assemblage of collagen molecules within the cell. Procollagen molecules containing propeptides are then excreted out of the cell via secretory vesicles (Alberts et al, 2002).

Propeptides are removed from procollagen molecules outside of the cell and procollagen then becomes collagen. The next step of extracellular self-assembly of collagen molecules into collagen fibrils is facilitated by the low solubility of collagen in the extracellular matrix (ECM).

Aggregation and covalent cross-linking between fibrils lead to the formation of collagen fibers which function to form the final tendon product (Alberts et al, 2002); however, the successful aggregation of collagen fibrils into fibers is dependent upon many regulatory proteins. Among the most important of these proteins is the small, leucine rich repeat proteoglycan decorin. This proteoglycan is characterized by a small core protein of approximately forty kilo-Daltons (kDa) with a series of ten leucine amino acid repeats (Patthy et al, 1987; Reed and Iozzo, 2003; and Yoon et al, 2005). Glycosaminoglycan chains are also associated with decorin, and may vary in size depending upon the action or localization of decorin (Hauser et al, 1998). These constituent parts of decorin function in concert to facilitate the normal assembly of collagen fibrils (Danielson et al, 1997). The leucine-rich core protein of decorin mediates noncovalent interactions between decorin and collagen type I fibrils, specifically the inhibition of fibrillogenesis (Vogel et al, 1984 and Pogany et al, 1992). Decorin has been shown to prevent uncontrolled lateral binding between collagen fibrils via the inhibition of fibril diameter growth, thus promoting greater tensile strength (Scott and Parry, 1992). Laterally extended glycosaminoglycan chains on the surfaces of decorin molecules function in the regulation of interfibrillar spatial arrangements (Scott et al, 1988). Biochemical associations between decorin and collagen fibrils form a scaffold upon which the tensile strength of tendons depends. Collagen fibrils must align in a particular orthogonal fashion in connective tissue, and the assemblage of this specific arrangement is facilitated by decorin (Scott et al, 1998). Due to its role as a structural regulator, decorin has been described as a shape molecule. Through the attachment of its core protein at roughly sixty-five nanometer intervals along collagen fibril surfaces, decorin is able to exercise structural control by setting appropriate distances between fibrils (Scott et al, 1998). The result of this regulation is an assembly of collagen fibers that

provides maximum tensile strength and support. Decorin also mediates the stages of collagen fibrillogenesis with respect to fibril development (Sini et al, 1997). Through the regulation of fibrillogenesis and fibril assembly, decorin plays a seminal role in the development of normal tendon function.

Fluoroquinolated Antibiotics and Collagen Assembly

Preliminary data from our laboratory indicate that decorin-mediated processes in cell proliferation, morphology, and carbohydrate content can be influenced by fluoroquinolated antibiotics. Derived from the initial double-ringed construct of nalidixic acid, fluoroquinolated antimicrobials are a fluorinated, synthetic group of broad-based antibiotics that share a common mechanism of action. This action is primarily characterized by the inhibition of bacterial DNA gyrase (Harrel, 1999). As a topoisomerase involved in uncoiling DNA to facilitate prokaryotic replication and transcription, DNA gyrase plays a central role in bacterial survival. The fluoroquinolated antibiotic enrofloxacin is believed to specifically target and inhibit the function of DNA gyrase. Through blocking the action of DNA gyrase, enrofloxacin causes a breakdown in replication, resulting in bacterial death. Employed in this fashion, enrofloxacin has historically provided highly effective treatment against gram-negative spectrum bacteria, and moderately effective treatment against gram-positive spectrum bacteria.

Fluoroquinolated antibiotics have also been associated with tendonitis and tendon rupture (Van Der Linden et al, 2001). Moreover, it has been demonstrated that fluoroquinolones effect biochemical alterations in the processes of proteoglycan synthesis, resulting in damage to collagen (Simonin et al, 2000). Fluoroquinolone influence on proteoglycan synthesis culminates in a general decrease in levels of production, providing fewer shape molecules in the assemblage of collagen fibrils (Simonin et al, 2000; Harrell, 1999).

Expectations

The focus of this research was to demonstrate an altered form of the proteoglycan decorin following exposure to the fluoroquinolone enrofloxacin. Employing the techniques of Western blotting, enzyme-linked immunosorbent assay (ELISA), dot blotting, and immunohistochemistry; two groups were compared: 1.) decorin from control, untreated equine superficial digital flexor tendon explants in culture, and 2.) decorin from enrofloxacin treated equine superficial digital flexor tendon explants in culture.

These data herein suggest that fluoroquinolones decrease levels of decorin synthesis in tendons, likely resulting in abnormal extracellular matrix formation and tendon failure. Previous data from our group indicated via northern blotting of enrofloxacin treated and untreated equine cells that decorin messenger ribonucleic acid (mRNA) was unaffected by fluoroquinolone exposure, suggesting that enrofloxacin has an effect on the posttranslational regulation of decorin (Yoon et al, 2004).

Western blotting, ELISA, dot blot, and immunohistochemistry experimentation suggest that enrofloxacin targets the glycosaminoglycan (GAG) chains on decorin, resulting in an altered glycosylation of the proteoglycan. A better understanding of the deleterious relationship between enrofloxacin and decorin as a regulator of collagen assembly may facilitate greater judicious use of fluoroquinolated antibiotics, and lessen the physiological and economic burdens of tendon failure secondary to antibiotic treatment.

Experimental Hypothesis and Objectives

We hypothesized that enrofloxacin treated tendon tissue explants in culture would exhibit a decrease in decorin synthesis. Moreover, we also hypothesized that enrofloxacin treated decorin would demonstrate a decrease in binding with regard to collagen and anti-decorin antibody. It was our intent to demonstrate these changes by juxtaposing and comparing enrofloxacin treated tendon explants and decorin against control, untreated tendon explants and decorin.

In order to test our hypotheses, we elected to pursue several experiments. Through electrophoresis and Western blotting we expected to demonstrate differences in decorin concentrations between enrofloxacin treated decorin and control decorin. These discrepancies in decorin concentrations would be revealed in band thickness inequalities between the two groups at appropriate molecular weight markers. By employing ELISA and dot blotting experiments, we tested the binding characteristics of enrofloxacin treated decorin with regard to both collagen and anti-decorin antibody. In both experiments (ELISA and dot blotting) we expected to find decreases in decorin-collagen and decorin-antibody binding compared to control as interpreted through spectrophotometry absorbance (ELISA) and densitometric quantification (dot blotting). Immunohistochemical assays were designed to microscopically demonstrate differences in decorin concentrations between enrofloxacin treated tendon tissue explants and control. In this capacity, enrofloxacin treated tissue explants were expected to show less specificity of stain uptake versus control.

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LITERATURE REVIEW

The Effects of Fluoroquinolones on Connective Tissues

Broad based antibiotics of the fluoroquinolone family have been associated with musculoskeletal problems involving tendons (Simonin et al, 2000; Casparian et al, 2000; and Kashida et al, 1997). In-vitro studies have demonstrated that high doses of fluoroquinolone antibiotics induce tendon failure through disruption of normal collagen assembly (Simonin et al, 2000; Beluche et al, 1999; and Movin et al, 1997). It has also been suggested that tendon collagen failure from exposure to fluoroquinolones is associated with a disruption in the synthesis of proteoglycans, specifically decorin (Bernard-Beaubois et al, 1998 and Simonin et al, 1999).

As the precursor of the fluoroquinolated antibiotic class, the quinolone nalidixic acid was first used with much success in the treatment of gram-negative urinary tract infections in domestic animals, and in 1989 the fluoroquinolated antibiotic enrofloxacin was approved for veterinary use in the United States (Greene and Budsberg, 1993). Unfortunately fluoroquinolated antibiotics brought about negative orthopedic side effects in patients, especially juveniles. In studies using immature beagle dogs receiving the fluoroquinolated antibiotic difloxacin, histological evidence of abnormal collagen fibrils and proteoglycan loss was identified in the ECMs of dogs receiving daily doses of the drug (Burkhardt et al, 1990). Similar issues concerning the connective tissues of young horses were also identified with the use of enrofloxacin (Berg 1988). Concerning effects in people, human kidney transplant patients receiving the fluoroquinolated antibiotic pefloxacin as a peri-operative antimicrobial control

measure suffered higher rates of tendonitis and/or spontaneous rupture of the Achilles tendon (12 %) than patients who never received fluoroquinolones (4 %) (Donck et al, 1994). Similar events related to fluoroquinolated antibiotic usage and tendonitis or rupture of Achilles tendons in human patients were also reported by Zabraniecki et al, 1995. Mounting evidence supporting a harmful association between the use of fluoroquinolated antibiotics and tendonopathies was documented in patients in Europe, especially in France where multiple retrospective studies were undertaken (Pierfitte and Royer, 1996). The documentation of approximately four-hundred twenty-one human cases involving either fluoroquinolone-associated tendonitis or tendon rupture was presented by French physicians, and in approximately ninety-eight percent of cases involving fluoroquinolated-associated tendonopathies, the tendon primarily affected was the Achilles (Pierfitte and Royer, 1996).

Moving from retrospective research to the laboratory, in-vitro studies involving rats receiving fixed doses of either pefloxacin and/or ofloxacin revealed evidence of edema with inflammatory cell infiltrates of the Achilles tendon after one day (Kato et al, 1995). Investigations of rat Achilles tendon microstructures revealed that collagen bundles were irregular, and often detached following fluoroquinolone treatments (Kato et al, 1995). Early microscopic evidence of tendonopathies occurring secondary to fluoroquinolone administration showed irregular arrangements of collagen fibers, hypercellularity, and increased interfibrillar glycosaminoglycans (Movin et al, 1997). Prior to investigations centered around the deleterious effects of fluoroquinolones on connective tissues, such aberrant presentations of tendon architecture were almost exclusively associated with athletic overuse (Movin et al, 1997). By the late 1990's, the emergence of appreciable tendonopathies as consequences of fluoroquinolated antibiotic use became more accepted.

By the year 1997, approximately one thousand cases of tendonopathies associated with fluoroquinolone use were reported by the French drug surveillance agency; however, an accurate description outlining the precise mechanism by which fluoroquinolated antibiotics effect tendonitis was still under investigation (Stahlmann and Lode, 1999). Fluoroquinolone antibiotics were known for readily forming complexes with metals possessing either divalent or trivalent cations, examples of which are iron and magnesium (Stahlmann and Lode, 1999). It is this relationship that possibly facilitates the binding of fluoroquinolated antibiotics with DNA gyrase via magnesium ions (Stahlmann and Lode, 1999). Moreover, a possible mechanism to explain the negative effects of fluoroquinolone antibiotics on connective tissues emerged from the metal chelating properties of the drug and the importance of magnesium in developing chondrocytes. Studies from the nineteen fifties suggested a relationship between magnesium-deficiency and joint abnormalities in young dogs (Syllm-Rapoport I et al, 1958). Extrapolating upon the theme of magnesium chelation as necessary for connective tissue development, Stahlmann et al in 1995 reported that rats fed a magnesium-deficient diet for nine days produced joint cartilage lesions that were indistinguishable from those induced by fluoroquinolone usage. However, the role of magnesium chelation inhibition through fluoroquinolone sequestration alone does not provide a complete explanation of the mechanism behind fluoroquinolone-induced tendonopathies. The role of proteoglycans and their relationship with fluoroquinolones is essential in order to gain a full understanding of the events surrounding antibiotic-induced tendonopathies.

Decorin is a member of the small leucine-rich repeat group of proteoglycans (the SLRP family or group). The SLRP family of proteoglycans has been subdivided into approximately five classes based on one or more of the following categorizations: gene organization, amino acid sequence similarities, cysteine spacing, or numbers of leucine repeats (P.G. Scott et al,

2004; Schaefer and Iozzo, 2008; and Zhang et al, 2009). The leucine rich repeats of decorin are usually flanked on either side by clusters of cysteine residues, (P.G. Scott et al, 2004). The 40 kDa decorin core protein possesses a single glycosaminoglycan (GAG) attachment to a serine residue in close proximity to the N-terminus of decorin (P.G. Scott et al, 2004). The decorin GAG attachment is characterized by a link trisaccharide consisting of (distally from the core protein) xylose, galactose, and second galactose, (Alberts et al, 2002). The GAG chain attaches to the end of the link trisaccharide and usually consists of uronic acid and an N-acetylated sugar (Alberts et al, 2002).

The Effects of Decorin Deficiency on Connective Tissues

The proteoglycan decorin contributes in many ways to the formation of collagen fibrils. Decorin acts as a shape molecule and in this fashion regulates the interfibrillar arrangements of collagen fibrils (Scott et al, 1998). This seminal role in fibrillogenesis places decorin at the forefront of research concerning abnormal tendon assembly. Moreover, the importance of decorin as a regulator and shape molecule make it an excellent target for investigation of collagen failure mechanisms.

Despite research on the effects abnormal decorin variants have upon collagen synthesis, much remains unknown about the specific mechanisms that define this relationship. However, efforts toward a characterization of the relationship between abnormal decorin and collagen synthesis have involved multiple approaches.

Among the varied and elegant research efforts made to distinguish the abnormal decorin-collagen synthesis mechanism involve the targeted disruption of decorin (Danielson et al, 1997), comparisons of decorin concentrations between normal and post-burn tissue fibroblasts (Scott et al, 1998), the effects on ECM development of cells unable to produce decorin (Scott et al, 1998), and the role of abnormal decorin production in Ehlers-Danlos syndrome (Seidler et al, 2006).

Decorin null mice and fibrillogenesis. To better understand the functional role of decorin as it pertains to the assembly and arrangement of collagen, decorin deficient mice were investigated (Danielson et al, 1997). Decorin null mice were generated via a targeted disruption of the mouse decorin locus employing targeting vectors, germ-line transmission, husbandry, and finally genotype determination (Danielson et al, 1997). Assessments of connective tissue development in different mouse populations revealed quantifiable differences in collagen assembly and arrangement between homozygous (wild type) decorin expressing mice and homozygous (nullizygous) decorin null mice (Danielson, 1997). Wild type mice exhibited normal collagen fibril development characterized by consistent fibril diameters and appropriate spacing between fibrils (Danielson et al, 1997). Moreover, this group of mice developed no skin lesions or appreciable loss of connective tissue tensile strength (Danielson, 1997). On the contrary, nullizygous mice were viable, but displayed inconsistent fibril diameters, uncontrolled lateral fusion between fibrils, and fragile skin that easily ruptured upon handling (Danielson et al, 1997).

By creating a population of decorin null mice, Danielson's group demonstrated a direct, quantifiable relationship between the inability to express decorin and the loss of tensile strength through abnormal collagen development and misalignment via the uncontrolled lateral fusion of fibrils. This research provided the first genetic appreciation of a link between decorin and collagen fibril synthesis and arrangement. It cemented the importance of decorin gene expression in the normal development of connective tissues by showing that a loss of decorin directly resulted in connective tissue failure.

Scar tissue fibroblasts synthesize less decorin than unaffected fibroblasts. Through the development of comparative cell cultures expressing different levels of decorin synthesis, Scott et al demonstrated similar findings to those of Danielson (1988). Scott's group established two lines of cell culture from multiple human burn patients. This work demonstrated, chiefly through Western blot analyses, that control cell cultures of normal human dermal fibroblasts exhibited higher levels of decorin synthesis than experiment cell cultures of abnormal human hypertrophic scar tissue fibroblasts (Scott et al, 1998). Moreover, in all cell strains tested, levels of decorin synthesized in hypertrophic scar tissue were consistently lower than those in normal dermal fibroblasts (Scott et al, 1998). Scott's group proposed that the observed decrease in decorin content of hypertrophic scar fibroblasts directly influenced the reduced elasticity and overall strength of scar tissue when compared to normal dermis (Scott et al, 1998).

Cells unable to express decorin and the development of disorganized extracellular matrices. Connective tissue extracellular matrices are comprised largely of proteoglycans and collagen arranged in a precise architecture, the purpose of which is to allay the gravitational and contractile stress forces of movement. This relatively fixed and consistent design of connective tissue extracellular matrices is dependent upon the activity of decorin as a shape molecule in

addition to that of other proteoglycans and molecules. Distinct orthogonal associations between collagen fibrils must be achieved in order to meet and withstand the stress forces placed upon connective tissues (J.E. Scott, 1975). Attached to collagen fibrils via their protein moieties at specific binding sites at sixty-five nanometer intervals, decorin molecules ensure the correct arrangement of fibrils within the matrix (J.E. Scott, 1995).

Considering this delicate relationship between decorin and the proper construction of connective tissue extracellular matrices, and employing cultures of tissue fibroblasts lacking the ability to synthesize decorin, Scott's group assessed the character of extracellular matrix produced by these cells. Extracellular matrices created by these decorin-deficient fibroblast cell lines were characterized by complete disorganization (J.E. Scott, 1998). Moreover, no discernible interfibrillar orthogonal arrangements were identified in the matrices manufactured by these cells, and proteoglycan bridges between fibrils were "totally absent" (J.E. Scott, 1998).

The results presented by Scott's group demonstrate a broader scope of the effects of decorin deficiency in connective tissue assembly. Previous investigations were able to demonstrate a link between decorin deficiencies (abnormalities) and collagen disruption (Danielson et al, 1997). Scott's work extrapolated the effects of abnormal decorin concentrations to include not only collagen fibrils, but the extracellular matrices with which they function in intimate association. A loss of architectural integrity in either construct has the potential to cause connective tissue failure.

Decorin and Ehlers-Danlos syndrome. Ehlers-Danlos syndrome (EDS) is characterized by a group of disorders affecting human connective tissues. Approximately 50% of traditional cases of EDS involve mutations in genes that encode type V collagen, specifically COL5A1 and/or COL5A2 (De Paepe, 2007). However, the progeroid (old age) form of EDS is rarer, and

involves an enzyme β4-galactosyltransferase (β4GALT-7) associated with GAG linkages of proteoglycans (Götte et al, 2007). Targeted regions in EDS patients often involve the skin and joints (Seidler et al, 2006). Moreover, particular molecular species affected by EDS include collagen type I and extracellular matrix proteins (Seidler et al, 2006).

D.G. Seidler and colleagues investigated an individual with EDS who demonstrated a particular genetic anomaly involving decorin expression. Specifically, compound heterozygous mutations of the enzyme β4GALT7 gene resulted in aberrant glycosylation of decorin (Seidler et al, 2006). In their investigations, Seidler et al discovered that almost 50% of decorin synthesized in their β4GALT7 -mutant EDS study patient was of the core protein-only variant instead of the wild-type decorin with a dermatan sulfate GAG attached (Seidler et al, 2006). This β4GALT7 point mutation in EDS patients which led to a decrease in decorin function was observed resultant to defective glycosylation (Seidler et al, 2006). Extrapolating further, Seidler postulated that this point mutation could provide an explanation for the molecular pathology observed in the connective tissues of EDS patients (Seidler et al, 2006).

Evidence of a Relationship between Fluoroquinolones and Decorin Deficiency

The proteoglycan decorin has been shown to play a regulatory role in the fibrillar formation of type I collagen and is suspected of effecting similar functions in the fibrillogenesis of collagen types II, III, and IV (Sini et al, 1997; Pins et al, 1997; Weber et al, 1996; and Danielson et al, 1997). Decorin has a dermatan sulfate chain attached to its protein core and is a member of the small-sized proteoglycans that share a similar structure. Decorin possesses a central core protein of approximately 40 kDa with ten leucine repeats, and a single GAG chain (Patthy et al, 1987; and Hausser et al, 1998).

With increased attention and focus toward the role of proteoglycans in collagen synthesis and the deleterious effects acted upon them by fluoroquinolated antibiotics, research in the late 1990's and the next decade provided increased evidence of a pathologic connection. Early studies investigating the effects of the fluoroquinolated antibiotic pefloxacin and its action upon proteoglycans revealed evidence of a relationship based on metabolic inhibition (Simonin et al, 1999). Simonin and his group quantified radiolabeled sulfate concentrations on mouse proteoglycan GAG chains at several time intervals following pefloxacin administrations via gavage or intraperitoneal injection. The results of this group demonstrated a clear drop in the concentration of sulfate concentrations associated with proteoglycan GAG chains within twenty-four hours of pefloxacin administration. Moreover, a measured decrease in the overall availability of sulfate in tissues following pefloxacin administration showed a direct effect on tissue metabolism, resulting in cellular damage (Simonin et al, 1999). Simonin's group proposed that this mechanism of pefloxacin-induced cellular damage (as demonstrated by decreased sulfate incorporation) secondarily inhibited proteoglycan anabolism resulting in abnormal collagen synthesis. Later studies conducted under similar conditions with similar parameters concluded that large single doses of the fluoroquinolated antibiotic pefloxacin inhibited proteoglycan anabolism via oxidative damage to connective tissue cells (Simonin et al, 2000). In this later research, Simonin's group demonstrated that reactive oxygen species generated from pefloxacin action played a central role in the shut-down of proteoglycan synthesis. Building on Simonin's experiments elucidating a cause and effect relationship between fluoroquinolated antibiotics and altered proteoglycans, investigations of other fluoroquinolones followed.

Research from our group at the University of Georgia College Veterinary Medicine (UGA) investigated the effects of the fluoroquinolated antibiotic enrofloxacin upon proteoglycans in equine tendon cells. Employing a model of cultured equine superficial digital flexor tendon cells, the group examined the effects of enrofloxacin on multiple cellular processes. Data from their work demonstrated that enrofloxacin inhibits the proliferation of cells, induces changes in morphology, and decreases the overall content of monosaccharides (Yoon et al, 2004). Moreover, an additional aspect of importance in these findings was the discovery that each of the aforementioned fluoroquinolone-induced effects was more pronounced in juvenile tendon cells than in mature cells (Yoon et al, 2004).

The inhibition of cell proliferation: In our UGA research, equine superficial digital flexor tendon cells in culture were exposed to enrofloxacin at different concentrations in order to evaluate potential dose-dependent effects. The procedure employed both adult and juvenile equine tendon cells. Results showed that cultured equine tendon cells exposed to enrofloxacin exhibited an overall decrease in number in contrast to unexposed cells (Yoon et al, 2004). Minimum effective concentrations of enrofloxacin were different for juveniles than for adults; specifically 50.0 μg of enrofloxacin/mL versus 100.0 μg of enrofloxacin/mL for juveniles and adults, respectively (Yoon et al, 2004).

Morphological changes: Working with electron microscopy, the UGA group demonstrated rough, porous cell membranes in enrofloxacin treated equine tenocytes (Yoon et al, 2004a). In comparison, control equine tenocytes displayed no changes in membrane integrity; instead showing smooth, intact cell membranes via scanning electron microscopy (Yoon et al, 2004a). Fluoroquinolone antibiotics effectively altered the synthesis of decorin, resulting in abnormal collagen assembly in extracellular matrices. Similar results were obtained in previous

work involving enrofloxacin treated (CE +) and untreated (CE -) cultures of embryonic chicken gastrocnemius tendon cells (Yoon et al, 2004b). These results revealed a marked decrease in collagen fibrils in treated (E +) cells (Yoon et al, 2004b). Electron microscopy studies of these chicken tendon cells showed less ground substance (proteoglycan concentration) as well in treated (E+) cells, (Figure 1).

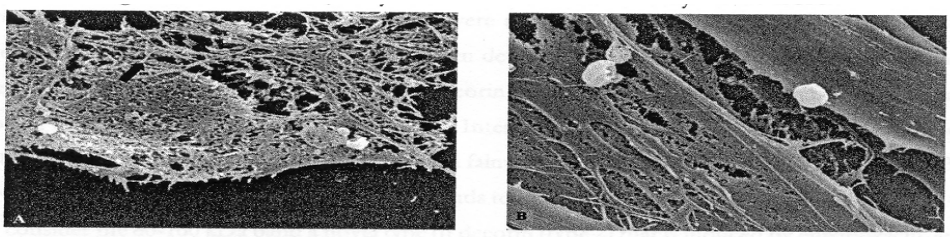

Figure 1 A: A scanning electron micrograph of E+ (enrofloxacin treated) chicken cells. Note the small amount of extracellular matrix present. **Figure 1 B:** A scanning electron micrograph of E- (untreated cells). Note the smooth surfaces of cells bodies and extensive extracellular matrix.

It has been previously shown that the inhibitory effect exhibited by fluoroquinolones upon tendon cell propagation is a cross-species phenomenon, (Kato et al, 1995; Bernard-Beaubois et al, 1998).

Decreases in overall monosaccharides: Through the use of mass-spectrometry Yoon's group assessed overall populations of monosaccharides (carbohydrates) in control and enrofloxacin-treated cells. Untreated control group cells contained an overall carbohydrate concentration of 2.8%, whereas enrofloxacin-treated group cells contained 1.5% carbohydrates

(Yoon et al, 2004b). Specific values reflecting individual concentrations of identified monosaccharides also highlighted further differences between the two groups of cells. Untreated control cells were comprised of 82% glucose, 12.8% galactose, 5% xylose, and trace concentrations of glucuronic acid (Yoon et al, 2004b). Enrofloxacin-treated cells were found to contain 85.3% glucose, 14.6% galactose, and trace concentrations of xylose and glucuronic acid (Yoon et al, 2004b). Of particular significance in these results were the differences in monosaccharide content between the two cell groups. The sugars xylose and galactose form the link trisaccharide structure of decorin, while glucuronic acid and N-acetylated sugars form the actual GAG chain. Differences between enrofloxacin treated and control groups in the concentrations of these constituent components suggest that enrofloxacin has a localized effect upon the GAG structure of decorin.

Enrofloxacin induced changes in decorin glycosylation. In Yoon's previous study concerning avian tendon cell cultures from the gastrocnemius tendons of eighteen day-old chicken embryos, it was demonstrated that enrofloxacin induced multiple changes in cell cultures (Yoon et al, 2004b). Among these findings was a 35% decrease in overall (total) monosaccharides in cells that received enrofloxacin (Yoon et al, 2004b). Moreover, Yoon's group found that the ratios of individual monosaccharides in this cell group were also altered (Yoon et al, 2004b).

Through Western blot analysis with chondroitinase ABC and N-glycanase treatment of enrofloxacin treated cells, Yoon's group discovered multiple lower molecular weight bands representing a glycosylation-altered form of decorin (Yoon et al, 2004b). Of significance in Yoon's research is the presentation of enrofloxacin induced glycosylation changes in decorin as a mechanism to explain the observed changes in cell proliferation and the creation of ECM in enrofloxacin treated cell cultures.

Recent Discoveries concerning Fluoroquinolones

Contemporary investigations characterizing the deleterious effects of fluoroquinolated antibiotics have elucidated ever more complex aspects of the relationship between this class of antibiotic and connective tissue cells. The effects of fluoroquinolated antibiotics have been unveiled on not only an intracellular level (Pouzaud et al, 2006), but also on an intranuclear level (Tsai et al, 2008 and Lim et al, 2008).

Moreover, age-related differences in the effects of fluoroquinolones have also been quantified (Pouzaud et al, 2006). Varying degrees of fluoroquinolone induced changes in tenocytes have also been shown to correspond with specific types of fluoroquinolones, demonstrating that some antibiotics of this class may cause fewer connective tissue problems in patients (Bae et al, 2006).

Age-related effects of fluoroquinolones on intracellular processes. Through the use of measured fluorescence signals via cold-light cytofluorometry, Pouzaud investigated the associations between fluoroquinolones and multiple intracellular processes within the context of age (Pouzaud et al, 2006). Pouzaud compared alterations in oxidation-reduction status (redox status), glutathione, and reactive oxygen species (ROS) in both juvenile and young adult rabbit tenocytes under the influences of nalidixic acid, pefloxacin, and ofloxacin, each at variable concentrations (Pouzaud et al, 2006).

Pouzaud employed the effects of three antibiotics: the quinolone nalidixic acid and the two fluoroquinolones ofloxacin and pefloxacin. At a maximum concentration of 1.0 mM, Pouzaud's group revealed that nalidixic acid, ofloxacin, and pefloxacin each caused significant reductions in the redox statuses and glutathione concentrations of both immature and young adult tenocytes while simultaneously increasing ROS values. However, the negative effects of these antibiotics were significantly greater in immature tenocytes than in young adult tenocytes (Pouzaud et al, 2006). In demonstrating the relationship between fluoroquinolone induced effects on cell processes as it related to age, Pouzaud concurrently revealed a deeper influence of action by fluoroquinolones and how they disproportionately affect younger, immature patients.

The effects of fluoroquinolones on intracellular/intranuclear processes: cell cycles. Wen-Chung Tsai investigated the effects of the fluoroquinolone ciprofloxacin on fibroblast cell cycling, particularly with regard to the arrest of fibroblast cell division at the G_2/M phase (Tsai et al, 2008). The ability of fibroblast cells to proliferate and synthesize materials necessary for connective tissue development is a crucial aspect of not only wound healing, but normal tendon synthesis. Tsai and his group established a link between the down regulation of key factors in cell cycling and ciprofloxacin that resulted in the arrest of cell cycling (Tsai et al, 2008).

Tsai exposed rat Achilles tendon cells to ciprofloxacin at variable concentrations up to 50 μg/mL followed by MTT assays, flow cytometry analysis, and fluorescence confocal microscopy. The MTT assay is a qualitative colorimetric assay for cell proliferation that measures yellow tetrazolium salt as it is metabolized to formazan in the mitochondria of proliferating cells (Buttke et al, 1993). Tsai's group discovered that ciprofloxacin treated cells became arrested in the G_2/M phase of the cell cycle, resulting the failure of tendon cell functions (Tsai et al, 2008). Through multiple experiments, Tsai's group showed that ciprofloxacin induced the down regulation of several key factors involved in tendon cell proliferation via cell cycling. Chiefly affected by ciprofloxacin were cyclin B and cyclin dependent kinase 1 (CDK-1); both of which regulate the passage of cells through the G_2/M phase of the cell cycle (Tsai et al, 2008). Moreover, Tsai's research also associated ciprofloxacin exposure with the simultaneous down regulation of checkpoint kinase 1 (CHK-1) and up regulation of polo-like kinase (PLK); which promote and restrict cell cycling, respectively (Tsai et al, 2008).

Through the quantification of ciprofloxacin induced reductions (and increases) in the concentrations of cell cycling kinases, Tsai proposes an intranuclear mechanism by which fluoroquinolones may manipulate cell processes resulting in tendon cell failure.

The effects of fluoroquinolones on intracellular/intranuclear processes: apoptosis. By investigating canine Achilles tendon cells in vitro, Lim and colleagues were able to demonstrate an association between enrofloxacin and the induction of apoptosis in this cell population (Lim et al, 2008). Following four days of exposure to enrofloxacin (maximum concentration 200 μg/mL), Lim's group quantified decreases in tendon cell growth and proliferation via colorimetric XTT assays (Lim et al, 2008). Furthermore, through Hoechst 33258 staining, Lim was able to demonstrate enrofloxacin induced apoptosis in these cells as evidenced by typical nuclear apoptotic condensed nuclei and DNA fragmentation.

Comparisons of different fluoroquinolone effects on tenocytes. Using Achilles tendons from Sprague-Dawley rats, Bae and colleagues compared the effects of three distinct fluoroquinolones: ciprofloxacin, gemifloxacin, and ofloxacin (Bae et al, 2006). Experiment group rats were challenged with oral doses of one of the three fluoroquinolones (maximum dosage 600 mg/kg of body weight) once daily for five days beginning four days postnatal (Base et al, 2006). Rat tendons were then compared by electron microscopy, and the effects of each fluoroquinolone group were assessed (Bae et al, 2006). Bae described multiple fluoroquinolone induced changes in tenocytes, specifically, indented cytoplasmic borders, dense nuclei with clumped chromatin, vacuoles, vesicles, organelle swelling, and decreased distances between collagen fibrils (Bae et al, 2006). Tenocytes with irregular borders suggesting membrane disruption were also noted, and characterized as having detached from the ECM (Bae et al, 2006). While, Bae's work found that all the fluoroquinolone rat groups demonstrated ultrastructural changes, these effects were least pronounced in the gemifloxacin group and most pronounced in the ofloxacin group (Bae et a;, 2006).

The findings of Bae's group suggest the effects of fluoroquinolones upon connective tissues are inconsistent from drug to drug. Thus, fluoroquinolones exhibiting lesser degrees of connective tissue disruption may be given priority in appropriate instances of treatment, especially concerning juvenile patients. The ability to select the most appropriate type of fluoroquinolone to maximize antibacterial action while minimizing connective tissue disruption would be optimal in almost any case.

The Effects of Enrofloxacin on another Proteoglycan similar to Decorin

In addition to decorin another small leucine-rich repeat proteoglycan known as biglycan also plays a significant role in the structure and function of connective tissue (Götz et al, 1997). The structure and composition of biglycan are similar to those of other proteoglycans, especially decorin, with which it shares a 55% structural identity (Neame et al, 1989). However, biglycan possesses a distinctive 38 kDa core protein with two GAG chains located on N-terminal serine-glycine sites (Neame et al, 1989).

Biglycan has been associated with extracellular matrix proteins, specifically fibrillar collagen types I, V, and VI, exhibiting an adjacent distribution to that of decorin along fibrils (Götz et al, 1997; Hocking et al, 1996; Krumdiek et al, 1992; and Schonherr et al, 1995). Moreover, Schonherr and associates demonstrated that decorin and biglycan compete for collagen binding sites suggesting the existence of identical or adjacent binding sites on fibrils used by both proteoglycans, and that biglycan may play a role in the organization of extracellular matrix assembly (Schonherr et al, 1995).

Abnormal associations between biglycan and collagen have also been investigated (Ameyé et all, 2002). Ameyé and associates demonstrated that biglycan deficient mice have progressive gait impairment, ectopic tendon ossification, and severe premature tendon

osteoarthritis (Ameyé et all, 2002). This group also asserted that biglycan is either directly or indirectly involved in the assembly of normal tendons, and that a deficit in biglycan results in abnormal, weakened tendon assembly leading to decreased tendon stiffness and loss of joint integrity (Ameyé et all, 2002). Additional research with biglycan and collagen revealed that biglycan alone possesses the ability to facilitate the organization of type VI collagen into hexagonal networks within a time frame of less than seven minutes (Wiberg et al, 2002). More importantly however, Wiberg's team revealed that biglycan lacking its GAG attachments fails to assemble collagen type VI, leading to the assertion that the ability of biglycan to function in the assembly of collagen type VI fibrils is dependent upon the presence of its GAG chains (Wiberg et al, 2002). Other proteoglycans such as decorin (with a single GAG attachment) and chondroadherin (with no GAG attachments) assemble collagen type VI at a very slow rate, or not at all, respectively (Wiberg et al, 2002).

Investigative research from our group at the University of Georgia demonstrates that enrofloxacin also negatively effects the synthesis of biglycan in horse tendon cells (Yoon et al, 2004). These results suggest that proteoglycans other than decorin that are active in the extracellular matrix are also targets of fluoroquinolone action, resulting in weakened collagen matrices (Bernard-Beaubois et al, 1998).

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ENROFLOXACIN INDUCED CHANGES IN DECORIN PRODUCTION BY EQUINE TENDON EXPLANTS IN CULTURE

Fluoroquinolated antibiotics have traditionally provided effective treatment against bacterial infections, especially gram-negative bacteria, with few intolerable side-effects. However, their associations with losses of tendon tensile strength and Achilles tendon rupture have also been reported (Pierfitte and Royer, 1996). Previous research from our laboratory highlighted a relationship between the fluoroquinolated antibiotic enrofloxacin and the small leucine-rich repeat proteoglycan decorin (Yoon et al, 2004a). Our laboratory has shown that enrofloxacin inhibits cell proliferation, induces morphological changes, and decreases overall monosaccharides in equine superficial digital flexor tendons cells (Yoon et al, 2004a). In a previous study, our research also demonstrated that enrofloxacin also altered decorin glycosylation in chicken tendon cells (Yoon et al, 2004b). Using equine superficial digital flexor tendon explants in culture, decorin synthesis was compared between enrofloxacin treated and untreated explants. The two types of decorin were also tested for their collagen binding abilities, and anti-decorin antibody binding abilities. Western blotting assays were carried out both with and without chondroitinase ABC (Chase ABC) to highlight differences between enrofloxacin treated decorin and untreated, control decorin. Enzyme-linked immunosorbent assays (ELISAs) and dot blotting assays were done to compare the binding abilities of enrofloxacin treated decorin and control decorin concerning collagen and anti-decorin antibody. Lastly, immunohistochemistry assays were carried out on tendon tissue explants in order to demonstrate differences in the binding specificities of enrofloxacin treated tissue versus control.

MATERIALS AND METHODS

Tendon Explant Cultures

Short term explant cultures were established from equine superficial digital flexor tendons. Equine superficial digital flexor tendons (SDFTs) were excised aseptically from the limbs of a donated, healthy, adult, horse during necropsy. Proximal and distal tendon sections were then aseptically cut into 1 g portions and cultured at 37 ^{o}C in Dulbecco's Modified Eagle Medium with 10% fetal bovine serum for seventy two hours. Both enrofloxacin treated tendon sections and control tendon sections were derived from the left hind limb of the same animal, with the following arbitrarily chosen organization: control 1 (C1) from the distal medial aspect, enrofloxacin treated 1 (E1) from the distal lateral aspect, control 2 (C2) from the proximal lateral aspect, and enrofloxacin treated 2 (E2) form the proximal medial aspect. Experiment tendon explant cultures received 100 μg/mL of enrofloxacin (100 mg/mL Baytril®: Bayer Healthcare LLC; Kansas, 66201) and were exposed for seventy two hours. Both control and experiment group tendon culture sections of 1 g each were then finely chopped and minced by hand under a negative pressure vent hood prior to proteoglycan extraction.

Proteoglycan Extraction

Proteoglycans from sections C1, E1, C2, and E2 were extracted twice in 4 M guanidine hydrochloride, 0.5% (w/v) 3-[(3-chloaminodopropyl) dimethylammonio]-1-propansulfonate CHAPS, 50 mM trihydrous sodium acetate (NaOAc 3-H_2O), 50 mM di-sodium EDTA, (pH 6.0) and protease inhibitors [500 mM benzamidine hydrochloride, 500 mM iodoacetamide, 10 mg/mL pepstatin A, and 100 mM phenylmethylsulfonylfluoride (PMSF)]. Samples were

extracted for forty-eight hours on a mechanical rotator at 4 ^{o}C. Following extraction, samples were centrifuged at 14,000 rpm at 4 ^{o}C for twenty minutes. Supernatants from each of the extractions were collected. Four samples (C1, E1, C2, and E2) were run simultaneously. Proteoglycan extraction was followed by dialysis in de-ionized distilled water (ddWater) with six water changes over twenty four hours. Dialyzed samples were then frozen, lyophilized, and returned to storage at – 80 ^{o}C.

Column Fractionation

Column fractionation via the CL-2B gel column served for the separation of proteoglycans extracted from tendons. Based on the principle of size-exclusion, the CL-2B gel bead matrix separates different proteins (particularly proteoglycans) as they progress via gravity through the gel medium. Larger, asymmetrical, complex proteins negotiate the gel matrix at slower rates than those of smaller, symmetrical, less complex proteins. Comparisons in size between species of proteins can be evaluated through collection rates.

Each of the four lyophilized proteoglycan extraction samples (C1, E1, C2, and E2) was re-dissolved in 2 mL of 4 M guanidine buffer. Samples were then centrifuged at 3,500 rpm, at 25 ^{o}C for five minutes. Sample volumes of 2.1 mL from each sample were then separated on a 1.25 cm by 110 cm Sepharose CL-2B (2% agarose with 2, 3 di-bromopropanol: Amersham Biosciences; Uppsala, Sweden) resin column with 4 M guanidine buffer. The flow rate was adjusted to 0.1 mL per minute. The total collection time for each sample was approximately twenty-six hours. Each sample elution was collected in a total of one hundred-sixty 2 mL tubes

Protein Concentration Assay

A volume of 50 µL was removed from each sample tube and mixed with 2.5 mL of protein assay dye reagent concentrate solution (Bio-Rad Laboratories, Inc.). Each sample was read at 595 nm to determine its protein concentration. Tubes representing each of the four samples (C1, E1, C2, and E2) were assayed and compared via concentration curves.

Electrophoresis and Western Blotting

The process of separating proteins by size and charge via an electrical gradient in a sodium dodecyl sulfate polyacrylamide gel (electrophoresis, SDS PAGE) provides a means by which molecular species can be compared and/or identified. Further, more precise specification of proteins separated by gel electrophoresis can be attained through the transfer of proteins from the gel to a nitrocellulose membrane. This transfer process is then followed by the sequential application of primary and secondary antibodies with staining markers to identify molecular species and their sizes as compared to molecular weight standards. A picture of the identity and character of resulting proteins can then be made. Moreover, by removal of the GAG chain associated with decorin through the use of chondroitinase ABC (chase ABC), the size of the core proteins of control and enrofloxacin treated decorin can be compared.

Extracts were again dialyzed against ddWater and lyophilized. This was followed by precipitation with ethanol. Aliquots of 50 µg of protein from each of the four samples (C1, E1, C2, and E2) were suspended in 30 µL of standard loading buffer (20 µL of Tris buffer + 10 µL of dye buffer) and boiled in ddWater for five minutes.

Electrophoresis: Samples were centrifuged briefly for thirty seconds prior to being loaded on a 12% SDS-polyacrylamide gel. Samples were electrophoresed at an initial rate of 50 V for forty minutes (5% SDS-polyacrylamide stacking gel) followed by a final rate of 100 V for one hour and fifty minutes (12% SDS-polyacrylamide running gel).

Western Blotting: Following SDS-polyacrylamide gel electrophoresis, protein samples were electrophoretically transferred to a nitrocellulose membrane in standard transfer buffer (3 g Tris, 14.5 g glycine, 1 g SDS, 200 mL methanol, and 1 L ddWater) at a rate of 100 V for one hour. The nitrocellulose membrane was then briefly washed in Ponceau S stain to confirm the presence of transferred proteins. The nitrocellulose membrane was then washed once in standard wash buffer (100 mL 10x TBS, 900 mL ddWater, 1 mL Tween 20, and 1 g powdered milk) for five minutes followed by blocking in blocking buffer (100 ml standard wash buffer and 5 g powdered milk) for one hour. At a dilution of 1:500, primary antibody incubation of the nitrocellulose membrane was then carried out with LF-122 rabbit polyclonal anti-decorin antibody (a generous gift from Dr. Larry Fisher, NIDCR, NIH) and set overnight at 4 ^{o}C.

Following four consecutive five minute washes with standard wash buffer, secondary antibody incubation of the nitrocellulose membrane took place for one hour, at a dilution of 1:1000 with biotinylated anti-rabbit IgG (H + L) (Vector Laboratories; Burlingame, California). Four additional consecutive five minute washes with standard wash buffer were followed by the application of an avidin-biotin complex solution (Vector Laboratories; Burlingame, California) to the membrane for thirty minutes.

Four more five minute standard washes were then followed by the application of 3, 3' – diaminobenzidine solution (DAB SK-4100 kit: Vector Laboratories; Burlingame, California) to visualize membrane antigen-antibody complexes. Western blotting was completed with two consecutive five minute washes of the membrane in ddWater followed by drying overnight in dark, dry storage.

Western Blotting with Chondroitinase ABC: Western Blot experiments using chondroitinase ABC (Chase ABC) were achieved in a similar manner to that of previously described Western Blotting; however, aliquots of 50 μg of protein from each of the samples received 3 μL of Chase ABC (*Proteus vulgaris*, Sigma Chemical Co.) following ethanol precipitation and suspension in 100 mM, pH 8 Tris buffer. Samples were then incubated overnight at 37 ^{o}C. Following the incubation period, 30 μL of standard loading buffer were added to each sample, and thus proceeded as described previously for Western Blotting.

Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA standard curve for decorin: An ELISA was employed to quantify and contrast the binding relationships between control decorin with collagen type I and enrofloxacin treated decorin with collagen type I. Through the use of well plates containing calculated amounts of collagen with coated decorin, primary and secondary antibodies, and an absorbance reading via spectrophotometer at 405 nm differences in absorbance corresponding to binding affinity could be measured pertaining to control and experiment groups.

In order to establish a decorin binding curve, wells of a standard 96 well ELISA plate (CovaLink^{TM} NH Modules, Nunc Laboratories) were coated with 100 μL of coating buffer (0.1 M Na HCO_3, pH 9.6) containing bovine decorin (Sigma Laboratories, D8428) representing the following concentrations: 0; 1 pg; 10 pg; 100 pg; 1 ng; 5 ng; 10 ng; 20 ng; 50 ng; 100 ng; and

200 ng per well. The plate was then incubated with bovine decorin for twenty four hours at 4°C. Following incubation, non specific membrane binding sites were blocked using 100 μL of blocking buffer [1x PBST with 5% bovine serum albumin (BSA)] per well for two hours at room temperature. At the conclusion of the blocking incubation period, a primary polyclonal decorin antibody (LF-122 rabbit polyclonal anti-decorin antibody: a generous gift from Dr. Larry Fisher, NIDCR, NIH) in dilutions of 1: 500, 1: 1000, and 0 (PBST only) were added at 100 μL per well for two hours at room temperature (RT). Following primary antibody binding, a secondary biotinylated antibody of anti-rabbit IgG (H + L) (Vector Laboratories; Burlingame, California) was applied in dilutions of 1: 500 and 1: 1000 at 100 μL per well. The wells were then allowed to incubate for one hour at RT.

The secondary antibody incubation period was followed by thirty minutes of alkaline phosphatase incubation at RT. Antibody-antigen complexes were then incubated for another thirty minute interval at room temperature with via p-nitrophenyl phosphate solution (Vector Laboratories). Resulting color development was read at 405 nm (Ultraspec 3000: Pharmacia Laboratories; Sweden).

ELISA standard curve for collagen: In order to establish a collagen binding curve, each well of a standard 96 well ELISA plate (CovaLink^{TM} NH Modules, Nunc Laboratories) was coated with 100 μL of coating buffer (0.1 M Na HCO_3, pH 9.6) containing equine collagen in the following concentrations: 0; 1 pg; 10 pg; 100 pg; 1 ng; 5 ng; 10 ng; 20 ng; 50 ng; 100 ng; 200 ng; or 500 ng per well.

After coating for twenty four hours at 4°C, non specific membrane binding sites were blocked via 100 μL of blocking buffer (1x PBST with 5% BSA) for two hours at room temperature. Bovine decorin was then added at 200 ng per well for incubation in collagen coated wells overnight at 4°C.

Polyclonal decorin antibody (1:1000) was then added at 100 μL per well and incubated for two hours at room temperature. Following the primary antibody binding, biotinylated secondary antibody in a ratio of 1:500 at 100 μL pre well (Vector Laboratories; Burlingame, California) was added and incubated for one hour at room temperature. After alkaline phosphatase incubation for thirty minutes at room temperature, antibody-antigen complexes were detected with p-nitrophenyl phosphate solution (Vector Laboratories; Burlingame, California). After p-nitrophenyl phosphate incubation for thirty minutes at 37 ^{o}C, color development was read at 405 nm by spectrophotometer (Ultraspec 3000; Pharmacia Biotech).

ELISA for collagen-decorin binding: Wells of a standard 96 well ELISA plate (CovaLink^{TM} NH Modules, Nunc Laboratories) were coated with 500 ng/100 μL coating buffer (0.1 M Na HCO_3, pH 9.6). After coating for twenty four hours at 4°C, non specific membrane binding sites were blocked with 100 μL of blocking buffer (1x PBST with 5% BSA) for two hours at room temperature. Following the blocking period, 200 ng per well of experiment decorin was incubated in collagen coated wells at 4°C overnight, followed by the application of polyclonal decorin antibody (1:1000) at a volume of 100 μL per well, incubated for two hours at room temperature.

After primary antibody binding, biotinylated secondary antibody (1:500) at 100 µL per well was incubated for one hour at room temperature followed by an alkaline phosphatase incubation for thirty minutes at room temperature. Antibody-antigen complexes were detected with p-nitrophenyl phosphate solution (Vector Laboratories). After p-nitrophenyl phosphate incubation for thirty minutes at 37 ^{o}C, color development was read at 405.

Dot Blotting

Similar to ELISA, samples from control and enrofloxacin treated decorin were comparatively assayed in order to illustrate, via densitometric values, different collagen binding characteristics between the two groups. By dot blotting aliquots of collagen type I on polyvinylidene fluoride (PVDF) filters followed by incubation with either control or enrofloxacin treated decorin and the application of decorin antibody, binding between decorin and antibody and between decorin and collagen were assessed via radiographic development and densitometric quantification. Collagen-decorin binding experiments via dot blot were carried-out in complement to collagen-decorin binding assays via ELISA.

Dot blot for standard curve for collagen binding. In order to set an efficient binding concentration for collagen and decorin, a binding standard curve was established using bovine decorin prior to the applications of either control or enrofloxacin treated decorin samples. Incremental amounts of equine collagen type I [(0, 10, 20, 50, 100, 200, and 500) µg/dot] were applied (dotted) upon polyvinylidene (PVDF) paper. Collagen dots were then blocked with blocking buffer [PBST and 3% bovine serum albumin (BSE)] for one hour followed by probing with 2 µg/mL bovine decorin (Sigma Laboratories D8428) in buffer (PBST and 0.1% BSA) for two hours. Three buffer washes of the PVDF followed probing.

Collagen-decorin complexes were then incubated with a primary antibody of LF-122 rabbit polyclonal anti-decorin antibody (a generous gift from Dr. Larry Fisher, NIDCR, NIH) (dilution 1:1000) overnight at 4 ^{o}C. Following the primary antibody incubation period, dots received a secondary antibody (anti-rabbit IgG) conjugated with horseradish peroxidase (HRP) substrate (dilution 1:25,000) for one hour.

After incubation of the secondary antibody, dots were developed with a chemiluminescent (ECL) kit (GE Healthcare, Piscataway, NJ) followed by exposure to X-ray film. A standard collagen-decorin binding curve was established from dot density values using an Alphaimager^{TM} 2200 (Alpha Innotech; San Leandro, California).

Dot blot for collagen-decorin binding. Collagen aliquots at concentrations of 500 μg/dot were dotted onto PVDF filter paper. Four dots were made per group, specifically control groups C1 and C2 and enrofloxacin treated groups E1 and E2. Two additional dots representing bovine decorin and PBS only were also created for comparative binding standards and as a negative control, respectively.

Following nonspecific site blocking with buffer (PBST and 3% BSA) for one hour, the PVDF filter was probed with 2 μg/mL from each sample (C1, C2, E1, E2, bovine decorin, and PBS as a negative control). Binding took place in buffer (PBST and 0.1% BSA) for two hours. Three washes with buffer (PBST and 0.1% BSA) followed probing.

Collagen-decorin dots were then incubated with a primary antibody of LF-122 rabbit polyclonal anti-decorin antibody (dilution 1:1000) overnight at 4 ^{o}C. Following the primary antibody incubation period, dots received a secondary antibody (anti-rabbit IgG) conjugated with horseradish peroxidase (HRP) substrate (dilution 1:25,000) for one hour. After incubation of the secondary antibody, dots were developed with a chemiluminescent (ECL) kit (GE Healthcare, Piscataway, NJ) followed by exposure to X-ray film. A standard collagen-decorin binding curve was established from dot density values using an Alphaimager^{TM} 2200.

Immunohistochemistry

By fixing superficial digital flexor tendon tissues in 10% formalin followed by a paraffin embedding process and slide production, control and enrofloxacin treated tissues can be evaluated histologically for differences in overall architecture, cell structure and organization, and the character of their respective extracellular matrices. Immunohistochemistry provides a direct, microscopic evaluation of tissues as revealed through antibody binding and subsequent staining.

Phase I - Tissue Preparation: Two 1 g control equine superficial digital flexor tendon (SDFT) tissue samples and two 1 g enrofloxacin treated SDFT samples were removed from – 80 ^{o}C storage, and placed in 10% formalin for forty eight hours. Following fixation in 10% formalin, each of the samples was trimmed, embedded in paraffin, and processed into multiple Probe-On histopathology slides.

Phase II – Deparaffinization: Each of the slides was placed upon a standard heating block at 70 ^{o}C for fifteen minutes. Following the initial heating block period, each slide was set in Hemo-De I solution for ten minutes with successive removals and washes in Hemo-De II and Hemo-De III for five minutes each. Each of the slides was then set under a vented hood to dry for approximately five hours.

Phase III – The Primary Antibody: Dried tissue sections were encircled, and bordered with an ImmEdge Pen (Vector Laboratories; Burlingame, California) in order to prevent spillage and provide adequate exposure of tissues to reagents. Each slide tissue then received 400 µL of 3% H_2O_2, was placed on a covered humidity tray, and set at room temperature for ten minutes. Following H_2O_2 treatment, each slide was washed twice in ddWater at three minutes per wash with a third wash in phosphate buffered saline (PBS) containing Tween-20 (PBST) for five minutes. Antigen site exposure for each of the tissues was achieved by submersing each of the slides in 1x antigen-retrieval unmasking solution (Antigen Unmasking Solution H-3300: Vector Laboratories; Burlingame, California 94010) followed by five one-minute intervals of low-power microwave exposure and intermittent cooling. Antigen site exposure was completed in two consecutive washes with PBST at five minutes each. Tissue blocking was carried out using 400 µL of BioGenex 10x Universal Blocking Agent (Power Block ^{TM}) per slide. Each slide was then set at room temperature for seven minutes followed by two consecutive washed in PBST for five minutes each. Application of the primary antibody was achieved by dilution of LF-122 rabbit polyclonal anti-decorin antibody with PBST at 1:250. Each tissue received 250 µL of primary antibody solution, and was set overnight at 4 ^{o}C.

Phase IV – Staining: Tissues were removed from 4 ^{o}C and washed four times in PBST at five minutes per wash. Application of the secondary antibody was achieved by dilution of biotinylated anti-rabbit IgG (H + L) (Vector Laboratories; Burlingame, California) with PBST at a ratio of 1:250. Each tissue received 250 µL of secondary antibody solution followed by placement in a covered humidity tray at room temperature for one hour. Incubation with the secondary antibody culminated with two consecutive slide washes in PBST at five minutes each. An avidin-biotin complex (ABC) solution was created using 2.5 mL of ddWater with prescribed aliquots from the Elite PK-6100 Standard solution. Each slide received 500 µL of ABC solution, was placed on a covered humidity tray, and set at room temperature for one hour. The ABC treatment was followed by two consecutive washes in PBST at five minutes each.

Staining and visualization of antigen-antibody complexes was achieved via the application of 3, 3' – diaminobenzidine solution (DAB SK-4100 kit: Vector Laboratories; Burlingame, California). The DAB solution was created using 2.5 mL of ddWater with prescribed aliquots from the DAB SK-4100 kit. Each slide received 500 µL of DAB for approximate exposure time of fifteen seconds. All remaining DAB stain was then removed, and slides were allowed to dry for twenty four hours prior to the application of cover slips, and microscopic evaluation.

RESULTS

CL-2B Gel Fractionation and Protein Concentration Assay

Decorin samples C1, E1, C2, and E2 were eluted through a CL-2B gel column, collected in one hundred sixty 2 mL tubes per sample, and then assayed at 595 nm to locate and assess the peak protein concentrations of each. Protein peaks for each sample were collected, and protein concentrations for each sample (C1, E1, C2, and E2) were calculated using a line slope formula from previously established albumin standards. Measured absorbance values for each sample were used to calculate their respective protein concentrations.

Electrophoresis and Western Blotting

Aliquots of control and enrofloxacin treated decorin underwent comparative assays by gel electrophoresis and immunoblotting (Western Blotting). Aliquots 50 μg of protein from C1 and E1 samples were separated by SDS PAGE, transferred to a nitrocellulose membrane, and decorin was identified with LF-122 rabbit polyclonal anti-decorin antibody (Figure 8).

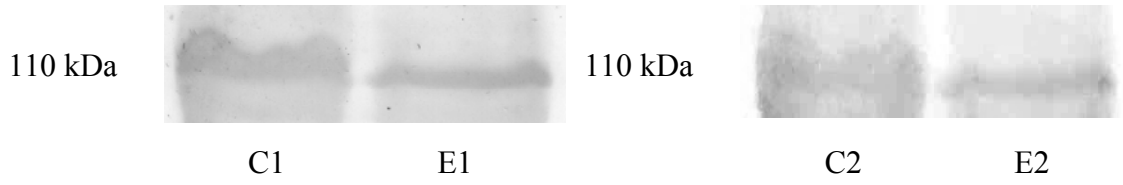


Figure 1. Western blots: C1, E1, C2 and E2

Samples C1 and E1 were run on a separate gel from C2 and E2. In Figure 1, protein bands corresponding to the 110 kDa position reveal differences in band intensities between control decorin and enrofloxacin treated decorin.

No differences between control and enrofloxacin treated decorin were shown with the use of Chase ABC. Following an overnight incubation period with Chase ABC, 50 µg protein aliquots of control and enrofloxacin treated samples were electrophoresed and processed in an identical fashion to that previously described for Western Blotting. Through removal of the glycosaminoglycan chain (GAG chain) of decorin via Chase ABC, only the protein cores of control decorin and enrofloxacin treated decorin samples were compared (Figure 2).

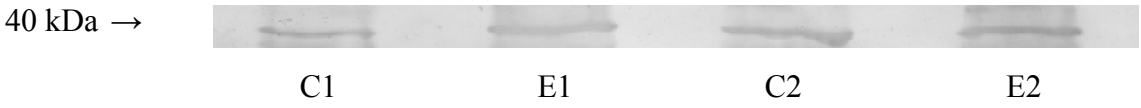


Figure 2. Chase ABC Western Blot: C1, E1, C2, and E2

As Figure 2 demonstrates, there was little to no difference between the control decorin bands and the enrofloxacin treated bands. In Figure 9 all four bands exhibit parity in thickness, and present sharp silhouettes as compared to those in Figure 1. The chief reason for this different presentation stems from the cleavage of GAG chains form the decorin core proteins by Chase ABC. The remaining core proteins lack the blurring characteristics that GAGs can produce in Western blotting. As Chase ABC removes GAG chains, leaving only decorin core proteins for comparison, the alterations induced by enrofloxacin most likely involve GAG attachments, particularly the monosaccharide constructs.

Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA standard curve for decorin. ELISA plate wells coated with incremental concentrations of bovine decorin received aliquots of primary polyclonal decorin antibody in ratios of 1:500 and 1:1000 followed by secondary biotinylated antibody, and were allowed to incubate. Subsequent incubations and detections were then carried out with alkaline phosphatase and p-nitrophenyl phosphate, respectively. Decorin concentrations were measured at 405 nm (Figure 3).

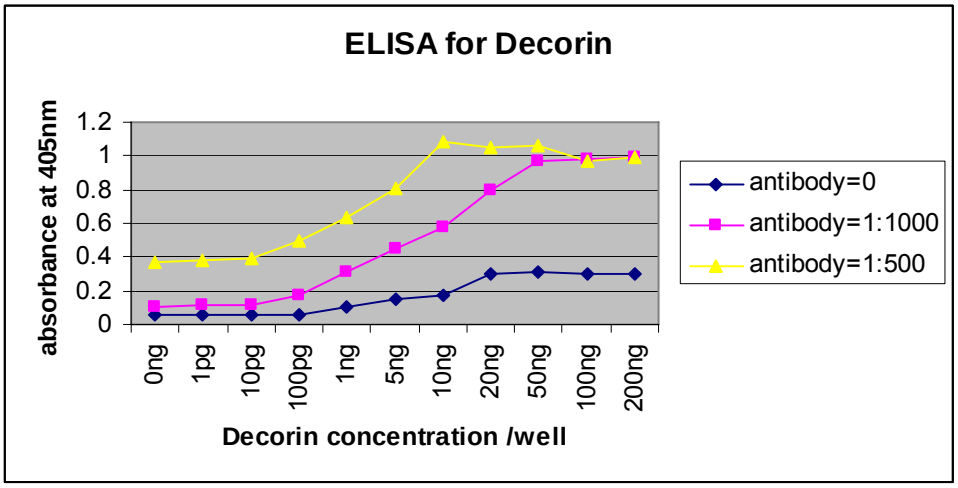


Figure 3. Decorin antibody binding curve comparing two ratios (1:500 and 1:100) at varying bovine decorin concentrations per well.

Both ratios of decorin antibody (1:500 and 1:1000) eventually reached an absorbance plateau at an approximate concentration of 50 ng per well. Absorbance values for both antibody ratios held relatively constant to a concentration of 200 ng per well. However, final evaluations revealed a 1:500 decorin antibody ratio that exhibited a great deal more background than the 1:1000 ratio, as was evidenced by the differences in absorbance values between the two dilutions from 0 ng through 50 ng (Figure 3). The 1:500 dilution began with an absorbance value of 0.4 at 0 ng of decorin antibody. It was thus decided that the 1:1000 ratio of decorin antibody would be used in the final collagen-decorin ELISA binding assay.

ELISA standard for collagen. ELISA plate wells coated with incremental concentrations of equine collagen each received identical aliquots of bovine decorin (200 ng) and were allowed to incubate overnight. Each of the wells then received primary polyclonal decorin antibody (ratio 1:1000) followed by secondary biotinylated antibody for a short incubation time.

Subsequent incubations and detections were then carried out with alkaline phosphatase and p-nitrophenyl phosphate, respectively. Evaluations of the best collagen-decorin binding concentrations were measured at 405 nm (Figure 6).

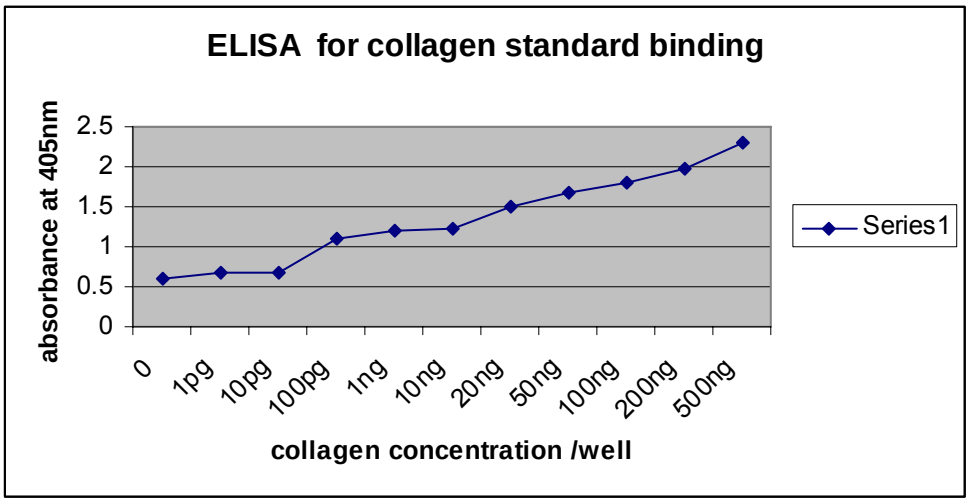


Figure 4. Collagen-decorin binding curve for varied equine collagen concentrations per well.

Absorbance values representing the most favorable binding concentrations for collagen and decorin are represented in the collagen binding curve of Figure 4. From these values collagen concentrations of 500 ng per well incubated with decorin concentrations of 200 ng per well were established as the optimal concentrations to employ for binding assays comparing control and enrofloxacin treated decorin with collagen.

ELISA for collagen-decorin binding: ELISA plate wells coated with 500 ng per well concentrations of equine collagen received 200 ng per well concentrations of control (C1 and C2) and enrofloxacin treated (E1 and E2) decorin. Each of the wells then received primary polyclonal decorin antibody (ratio 1:1000) followed by secondary biotinylated antibody for a short incubation time. Subsequent incubations and detections were then carried out with alkaline phosphatase and p-nitrophenyl phosphate, respectively. Comparative evaluations of control versus experiment collagen-decorin binding concentrations were measured at 405 nm (Figure 5).

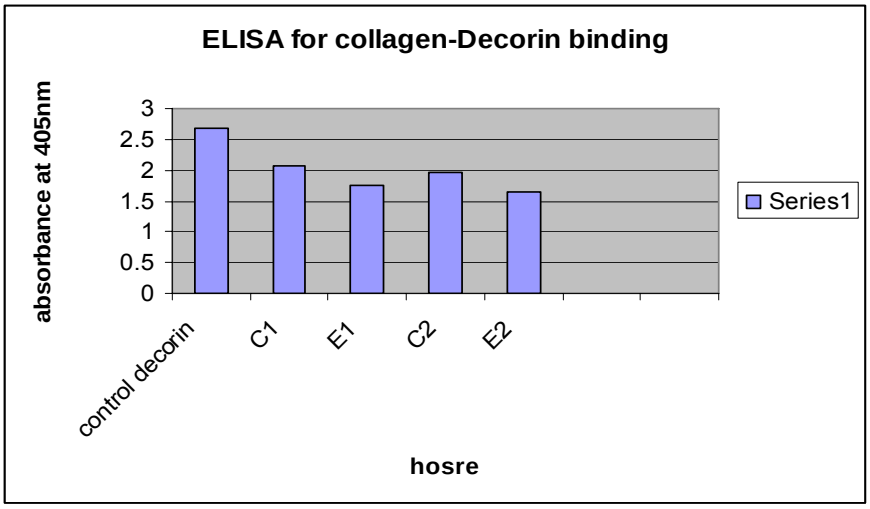


Figure 5. Collagen-decorin binding: control versus experiment decorin.

Comparative absorbance values are presented in Figure 5. Absorbance values represent binding concentrations between collagen and decorin, and correlate to the amount of binding between the two species. Despite the fact that the differences are not statistically significant, control decorin samples C1 and C2 have higher absorbance values (2.1 and 2.0, respectively) than those of enrofloxacin treated decorin samples E1 and E2 (1.7 and 1.6, respectively).

Dot Blotting

In order to establish appropriate collagen-decorin concentrations, equine type I collagen was dotted on PVDF filter paper at concentrations of 500 ng/dot and then incubated with bovine decorin at a concentration of 2 μg/mL. Incubation with a primary antibody of LF-122 rabbit polyclonal anti-decorin antibody was then carried out followed by incubation with a secondary biotinylated anti-rabbit antibody. Dot development took place with an ECL kit (GE Healthcare, Piscataway, NJ), and exposure to X-ray film.

Figure 6 demonstrates the set of collagen dots at incremental concentrations of 0, 10, 20, 50, 100, 200, and 500 ng/dot with 2 μg/mL bovine decorin per dot. Dot densities (darkness) correspond to quantities of collagen-decorin binding. Collagen concentrations of 500 ng (far right column) have the greatest density values with bovine decorin at 2 μg/mL. As a result, these concentrations were chosen for the control and enrofloxacin treated decorin samples.

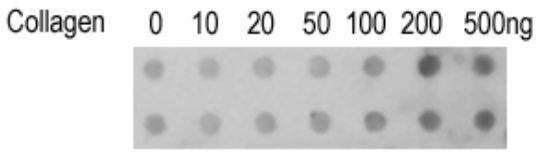


Figure 6. Standard binding curve for collagen-decorin binding. Incremental concentrations of collagen with bovine decorin show higher concentrations of binding at 500 ng collagen with 2 μg/mL bovine decorin.

Following the establishment of an appropriate collagen-decorin binding concentration, dot blots were repeated with the addition of control decorin, enrofloxacin treated decorin, and bovine decorin. A negative control consisting of PBS only was also added to the PVDF filter. Samples were processed similarly to that of the collagen-decorin binding dot blot. Following development, densitometric values were determined with the Alphaimager^{TM} 2200 (Figure 7) illustrates the resultant dot blotting values for control and enrofloxacin treated decorin samples.

Each sample is represented by four dots with the following assortment: 1 = PBS negative control; 2 = 2 μg/mL bovine decorin; 3 = 2 μg/mL control decorin sample C1; 4 = 2 μg/mL enrofloxacin treated decorin sample E1; 5 = 2 μg/mL control decorin sample C2; and 6 = 2 μg/mL enrofloxacin treated decorin sample E2.

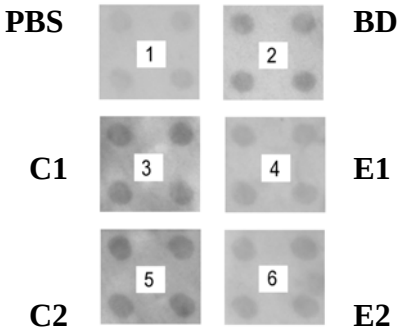
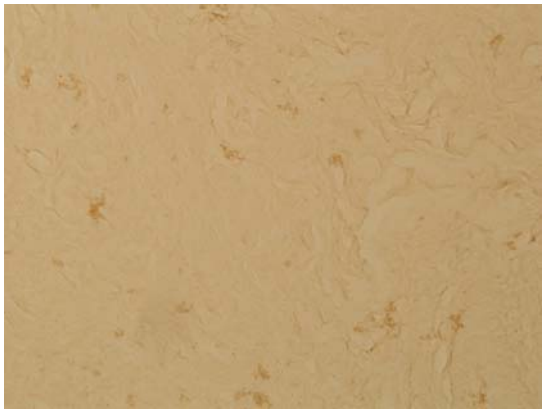


Figure 7. Squares: 1 (PBS negative control), 2 (bovine decorin), 3 (C1), 4 (E1), 5 (C2), and 6 (E2).

Figure 7 demonstrates differences in dot densities. Control decorin dots C1 and C2 (Figure 7; 3 and 5, respectively) are appreciably darker than enrofloxacin treated decorin dots E1 and E2 (Figure 7: 4 and 6, respectively). Control decorin dot densities exceeded (were darker than) those of enrofloxacin treated decorin.

Immunohistochemistry

Aseptically excised control and enrofloxacin treated sections of equine superficial digital flexor tendons were fixed in 10% formalin, embedded in paraffin, and mounted unstained upon histology slides. Slide mounted tissues of control and enrofloxacin treated tendons were probed with LF-122 rabbit polyclonal anti-decorin antibody followed by secondary antibody biotinylated anti-rabbit IgG (H + L), and finally stained with 3, 3' – diaminobenzidine solution, Figure 8.



A **B.**

Figure 8 A. Control tendon. Staining with decorin antibody reveals specific staining points with significantly less background staining. **Figure 8 B.** Enrofloxacin treated tendon. Staining with decorin antibody is non-specific with abundant background staining.

As Figure 10 illustrates, the application of decorin antibody to enrofloxacin treated tendon (Figure 10 A) produced non specific staining. In contrast to enrofloxacin treated tendon, control tendon (Figure 10 B) presents multiple specific points of stain uptake.

DISCUSSION

The seminal objectives of this research concerned the demonstration of differences between control, untreated decorin, and enrofloxacin-treated decorin. Through the use of equine superficial digital flexor tendon tissue explants and extracted decorin, this research attempted to shed further light upon the relationship between fluoroquinolated antibiotics, specifically enrofloxacin, and the small leucine rich repeat proteoglycan decorin. Decorin is a regulatory shape molecule involved in the synthesis and arrangement of collagen fibrils (Pins et al, 1997; Sini et al, 1997) and in this capacity is a vital component in the production of connective tissues, especially tendon (Pins et al, 1997; Sini et al, 1997). Failures in decorin synthesis have been shown to result in an abnormal assembly of collagen fibrils, which in turn results in the loss of connective tissue tensile strength and tendon failure (Scott et al, 1998 and Danielson et al, 1997). Our research focused on the effects of the fluoroquinolated antibiotic enrofloxacin on decorin, and by extension the repercussions of this relationship on connective tissues.

CL-2B Fractionation and Protein Concentration Assay

Extracted control and enrofloxacin treated decorin samples [(C1, C2) and (E1, E2), respectively] were passed through a CL-2B sepharose gel column, and their protein concentrations were compared at 595 nm. Resulting data revealed that both enrofloxacin treated samples of decorin (E1 and E2) eluted from the column sooner (faster) than control decorin samples.

Whether or not this demonstrates a difference in size between the control and enrofloxacin groups cannot be determined due to the samples not having been assessed for protein prior to loading on the column; however, the action of the CL-2B column is designed to gravitationally separate species of molecules based on size, with smaller species passing at faster rates than larger ones (Alberts et al, 2002). Peak protein concentrations as determined at 595 nm differed between the two groups as well.

Western Blotting

Disparities between enrofloxacin treated decorin samples and control decorin samples were evident on Western blot analysis. Bands representing enrofloxacin treated decorin (E1 and E2) and control decorin (C1 and C2) displayed unequal densities at 110 kDa. These findings indicate disproportionate concentrations of decorin with respect to control and enrofloxacin treated samples. Specifically, enrofloxacin treated decorin bands were smaller and less dense than control decorin bands, suggesting less decorin was available for antibody recognition and binding in samples E1 ad E2. Fluoroquinolones have been shown to decrease decorin synthesis (Simonin et al, 1999), further suggesting that band density differences between enrofloxacin treated samples and control samples are most likely due decreases in decorin synthesis in E1 and E2.

Western blots conducted with Chase ABC (to cleave GAG attachments from decorin core proteins) revealed little to no differences in band densities between control decorin and enrofloxacin treated decorin samples at 40kDa. These results differ from those of the Western blots carried out in the absence of Chase ABC.

Due to the fact that Chase ABC cleaves GAG attachments form the core proteins, Western blots with Chase ABC effectively compared only the core proteins from enrofloxacin treated and control samples, suggesting that enrofloxacin exerts a localized effect upon one or more of the decorin moieties; presumably the serine bound link trisaccharide GAG chain.

ELISA

Collagen binding assays of enrofloxacin treated decorin and control decorin revealed differences in the two groups. Through enzyme linked immunosorbent assays (ELISAs) followed by absorbance readings at 405 nm, the collagen binding capabilities of enrofloxacin treated decorin and control decorin were compared. Enrofloxacin treated decorin-collagen samples had lower absorbance values [E1 (1.7) and E2 (1.6)] than those of control decorin-collagen [C1 (2.1) and C2 (2.0)]. These data indicate fewer enrofloxacin treated decorin-collagen complexes as compared to control decorin-collagen, and may in addition suggest a lesser ability on behalf of enrofloxacin treated decorin to bind collagen. Moreover, the differences in decorin-collagen binding between the two groups may be further understood in the context of decreased decorin synthesis in enrofloxacin treated samples: specifically that samples E1 and E2 have lower concentrations of decorin, and thus generate fewer decorin-collagen complexes. Either interpretation presents an overall lower rate of association between enrofloxacin treated decorin and collagen as compared to control decorin.

Dot Blotting

Dot blot experiments designed to complement those of ELISA provided an additional set of data to support differences in collagen-decorin binding between control decorin and enrofloxacin treated decorin. Dotted areas of PVDF filter paper containing collagen were probed with samples of control decorin, enrofloxacin treated decorin, bovine decorin, and PBS as a

negative control. Densitometric values were determined and compared among the groups with quantifiable differences between control and enrofloxacin treated decorin. Similar to ELISA, comparisons in decorin-collagen binding were evaluated. Dot blot data revealed that control decorin dots had higher densitometric values corresponding to decorin-collagen binding than did enrofloxacin treated decorin dots. These data further indicate and support the compromised ability of enrofloxacin treated decorin to form complexes (bind) with collagen.

Immunohistochemistry

Control and enrofloxacin treated superficial digital flexor tendon tissues were formalin-fixed, paraffin embedded, and mounted on slides for immunostaining with polyclonal anti-decorin antibody. For the purpose of directly comparing the presence of decorin in the two groups of tissue, amounts of stain uptake and staining specificity were evaluated. Control decorin tissues revealed higher stain uptake, greater specificity of staining as compared to enrofloxacin treated tendon tissue. These results indicate that fewer antibody-decorin complexes were formed in enrofloxacin treated tissues as compared to control decorin tissues, suggesting an inability of enrofloxacin treated decorin to form associations with anti-decorin antibodies. Greater non specific staining (background) in enrofloxacin treated tissue also suggests a greater concentration of unassociated anti-decorin antibody in these tissues; i.e. antibody not bound to decorin.

While it has been shown that an initial lag time in decorin expression takes place in cultures lacking supplemental ascorbic acid (Hering et al, 1994 and Takeuchi et al, 2009), and that this could account for lower overall decorin binding and staining in both control and enrofloxacin treated samples, the discrepancies between the two groups remain consistent with enrofloxacin treated tissues demonstrating overall lower levels of decorin synthesis and binding. Specifically, if the absence of ascorbic acid in our explant cultures effectively lowered levels of decorin in both groups, then the comparative results obtained would still demonstrate differences between enrofloxacin treated tissue explants and untreated control tissue explants.

These results indicate that enrofloxacin treated tendons contain lesser concentrations of decorin, suggesting that available decorin has undergone alteration to the point of being incompatible either with anti-decorin antibody and/or collagen.

We propose that the fluoroquinolated antibiotic enrofloxacin induces fundamentally detrimental changes in the small leucine rich repeat proteoglycan decorin. Specifically, we believe these enrofloxacin induced changes result in a decrease in decorin synthesis, and an altered form of decorin with less capacity to bind collagen and anti-decorin antibody. We further suggest that this enrofloxacin altered form of decorin is also incapable of carrying out pivotal functions in collagen fibril synthesis and assembly, providing a potential mechanism for losses of connective tissue tensile strength and subsequent tendon failure secondary to fluoroquinolone therapy. It is hoped this research will help to further the cause of a more complete understanding of the overall deleterious effects of fluoroquinolated antibiotics on connective tissues, and thus a more judicious usage of these drugs to the benefit of all patients and their care-givers.

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