The effect of age and exercise on the size of collagen fibrils of the chicken gastrocnemius tendon was studied. Broiler female chickens were randomly assigned to control or runner groups. Runners were subjected to a moderate treadmill walking program for 3 weeks, 5 days a week at 50% of their maximum capacity. Chickens were killed at the age of 1-day, 1-week, 2.5 weeks (1 week of preconditioning exercise), 5 weeks (1.5 weeks of full exercise), and 6.5 weeks (3 weeks of full exercise). Immunohistochemistry method was performed to see the change in the amount of decorin in tendon. TEM and SEM were used to measure the average diameter and cross-sectional area of collagen fibrils. The exercise program relatively increased the amount of decorin in tendon. The average diameter and the cross-sectional area of collagen fibrils increased significantly with age and as an effect of exercise. These results suggest that moderate exercise cause greater decorin synthesis in growing chickens, as well as rapid growth and fusion of collagen fibrils.

INDEX WORDS: Collagen fibrils, Exercise, Age, Chicken gastrocnemius tendon
THE EFFECT OF AGE AND EXERCISE ON CHICKEN GASTROCNEMIUS TENDON

by

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B.S., The University of Georgia, 1998

A Thesis Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirement for the Degree

MASTER OF SCIENCE

ATHENS, GEORGIA

2001
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December 2001
ACKNOWLEDGMENTS

I would like to thank Dr. Jaroslava Halper, my major professor, for her encouragement, support and guidance from day one. I would also like to thank Dr. G.N. Rowland, Dr. W.L. Steffens and Dr. Timothy Foutz for serving on my reading committee. To my regret, Dr. Rowland could not continue serving on my committee. I am grateful to the entire faculty and staff of the Pathology Department, especially, Mary Ard, Randy Brooks, Gwin Kerce, Eric Lineman, Dr. Hongjie Pan, and Dr. Jung Hae Yoon for their excellent technical assistance and advice. I thank Dr. Dezso Benedek, the director of Japanese Language Program in the Comparative Literature Department, for providing me with Teaching Assistantships throughout my studies. Finally, I thank my family and friends for their love and support.
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CHAPTER 1
LITERATURE REVIEW

Tendon and Collagen:

Extracellular matrix (ECM) is a complex network of polysaccharides (such as glycosaminoglycans) and proteins (such as collagen) secreted by cells. It serves as a structural element in tissues and also influences their development and physiology (Alberts et al 1994). Extracellular matrix is a part of connective tissue. ECM is arranged in very different ways in different organs, depending on their functions, for example, matrix can be calcified to form hard structures of bone or teeth, or it can form the transparent matrix of the cornea (Alberts et al 1994). Cartilage and tendon are connective tissues as well. Skin and other organs contain connective tissue. Tendon is known to be a force transmitter. Researchers found that it also serves as a dynamic amplifier during rapid muscle contraction, an elastic energy store, and more commonly as a force attenuator during rapid and unexpected movement (Barnett et al 1961; Dawson and Taylor, 1973; Pins et al 1997).

Not all tendons have identical structure; for example, tail tendon has 2 dimensional planar wave. When the tendon is viewed under polarized light at low magnification, light and dark bands appear alternatively along the collagen fibers.
This is probably because of zigzag or crimps of collagen fibrils within the fibers. This light and black bands’ pattern undergoes changes until it disappears under stress. It suggests that extension of the tendon initially involve straightening of the crimp (Cribb and Scott 1995).

Collagen is a fibrous protein, comprising 25% of total protein in mammals, and is secreted by connective tissue cells and other types of cells. It is the primary structural element in extracellular matrices (Horton et al 1996). There are many types of collagen, 25 distinct collagen alpha chains, and at least 20 different types of collagen molecules found. They can be divided into three groups; fibrillar collagen, fibril-associated collagen, and network-forming collagen. Fibrillar collagens include collagen type I, II, III, V, and XI (Mayne and Burgeson 1987). In mammalian connective tissues, collagen type I is the most abundant one. Tendon is mainly composed of type I collagen fibers and submicroscopic fibrils (Alberts et al 1994). Type I collagen is fibrillar, which has long, hard, and triple-stranded helical structures, called collagen polypeptide chains (alpha chains).

Collagen is rich in glycine and proline (Piez and Reddi 1984). Glycine helps three helical alpha chains to pack tightly together to form the final collagen superhelix. Proline is believed to stabilize the helical conformation in each alpha chain by its ring structure (Alberts et al 1994). The collagen chains can resist tensile forces, while glycosaminoglycans (GAGs) resist compressive forces (Scott 1988).
Glycosaminoglycans are unbranched polysaccharide chains composed of repeating disaccharide unit, which is amino sugar and uronic acid. GAGs are highly negatively charged because of SO3- or SO4- group from amino sugar and COO- group from uronic acid. Different sugar residues, type of linkage between residues, number and location of sulfate groups give different GAGs. There are four different groups of GAGs; 1) Hyaluronan, 2) Chondroitin sulfate and Dermatan sulfate, 3) Heparan sulfate and Heparin, and 4) Keratan sulfate. GAG chains form porous hydrated gels to fill most of extracellular space (Hay 1991). They provide mechanical support to tissues and as such are mediators of rapid diffusion of water-soluble molecules and migration of cells (Alberts et al 1994). Deficiency in synthesis of dermatan sulfate disaccharide causes dwarves with prematurely aged appearance and generalized defects in skin, joints, muscles, and bones (Alberts et al 1994). In invertebrates and plants, other types of polysaccharides dominate extracellular matrix (Hay 1991).

Tendon contains surface waveform, which is defined as a three-dimensional twisting of collagen fibrils within the primary tendon bundles. In tendon, unidirectional arranged bundles of fibers are lying between tendon fibroblasts, called tenocytes or tenoblasts. Collagen molecules assemble together and become ordered polymers, which contribute collagen fibrils (Weiss and Jayson 1983). The covalent intramolecular and intermolecular cross-links are formed between modified lysine side chains within a collagen fibril.

In human tendons, hierarchical organization of the tendon can be observed. Collagen fibrils visualized by electron microscopy make up collagen
fiber. Collagen fibers get assembled together and make a primary fiber bundle (15-400um in diameter), that is also called subfascicle. Several primary fiber bundles make up secondary fiber bundle (150-1000um in diameter), that is called fascicle. Secondary fiber bundles make up tertiary fiber bundle (1-3mm in diameter). Finally, tertiary fiber bundles make up tendon. The primary, secondary, and tertiary fiber bundles are surrounded by endotenon, while whole tendon is surrounded by epitenon (Reynolds and Worrell 1991). A collagen fibril is several hundreds micrometer long and measures 10-300 nm in diameter. Several fibrils make up collagen fibers, which are cable-like bundles and measures 0.5-3 micrometers in diameter.

Collagen fibrils are visible in electron micrographs, while collagen fibers are visible in light microscopes (Alberts et al 1994). Collagen fibrillogenesis is discussed later. Collagen fibrils are organized differently in different tissues (Yurchenco et al 1994). Tendon had been thought to be metabolically inert for a long time. People often referred to it as dead tissue. It is actually slow to repair and degenerate. In bone, collagen molecules persist for about 10 years before they are degraded and replaced (Alberts et al 1994). However, some tissues, which are disrupted by scurvy like ones in fragile blood vessels, loose teeth, and bleeding gums should have relatively rapid degradation and replacement of collagen (Weiss and Jayson 1983). Turnover rate is tissue specific, even though when it was compared with that of muscle tissue, the metabolic turnover of tendon tissue is many times slower because of poorer vascularity and circulation.
(Barfred, 1971; Kannus et al., 1992a). Barfred (1971) also mentioned that tendon may adapt slower to the training experiment than muscle does.

Type III collagen is also a fibrillar collagen. Type III collagen has an important role in fibrillogenesis, which is an important part of the development of organs such as the cardiovascular system, intestine, and skin. Lack of type III collagen disturbs fibrillogenesis and results in defective development and functional failure of these organs (Liu et al 1997). Under physiological conditions, type III collagen is not only essential component of fibrils in tissues such as the media of aorta but also an important regulatory element in type I collagen fibrillogenesis. Type III collagen regulates the diameter of type I collagen fibrils, which serves as a mechanism to meet the physiological requirement of different tissues or of a tissue at different developmental stages (Liu et al 1997). Two other different types of collagen assemble in different way and have more flexible structure than fibrillar collagen. Fibril-associated collagen includes type IX and XII collagens. They do not aggregate with one another, but instead, they bind to fibrils formed by fibrillar collagens and mediate interactions of collagen fibrils with one another and with other matrix macromolecules (Kreis and Vale 1993). Network-forming collagen contains type IV and VII collagens. Type IV collagen form multilayered network with one another, which help form basal laminae, while type VII collagen form anchoring fibrils that connect basal laminae to underlying connective tissue (Ayad et al 1998).
Decorin:

Decorin is a major regulator of collagen fibrillogenesis and works as a spacer, which determines the distance between each molecule, during lateral assembly of collagen molecules, which are monomers, assemble into oligomers.

Decorin is a member of the small leucine-rich repeat proteoglycans since it is a small dermatan sulfate proteoglycan characterized by a core protein (45kDa) containing leucine-rich repeats (LRR) (Ayad et al 1998; Kreis and Vale 1993). LRR proteoglycan have 10-12 tandem repeats of 24 amino acid with hydrophobic residues in conserved positions (Kreis and Vale 1993). LRR is a molecular recognition motif found in proteins with roles in cell adhesion, signal transduction, DNA repair, and RNA processing (Alberts et al 1994). Other SLRPs are involved in maintenance and growth of diverse tissues, like cornea, bones, teeth, skin, and blood vessels (Hobby et al 2000). Biglycan, decorin, fibromodulin, lumican, keratocan, and mimecan are members of SLRPs.

Decorin has three attachment sites for N-linked oligosaccharides and central domain harboring 10 LRRs flanked by disulfide bonded terminal sequences. Sequence homologies can be observed among species. Most tissues express decorin (Hay 1991). Usually, amino terminus of decorin contains a single attachment site for either chondroitin or dermatan sulphate.

It also has high affinity binding site for type I, II, III, and VI collagens. Type VI collagen acts as linking moiety between collagen type I and decorin (Takahashi et al 1993). Type I collagen binds to LRRs 4-5, which is composed of some 40 amino acid residues (Svensson et al 1995). Decorin binds to a specific
binding site on the surface of type I collagen fibrils at the d bands (gap zone) in every D period. This location is very close to intermolecular cross-linking sites of collagen heterotrimers (Keene et al 2000). Fibromodulin also binds to type I collagen, but at a different attachment site on collagen. Binding of decorin and fibromodulin to type I collagen does not affect each other (Svensson et al 1998; Olderberg et al 1989).

Decorin inhibits type I collagen fibrillogenesis. GAG chains of decorin are linked to collagen fibrils to stay in place and may determine the distance between collagen fibrils, determining the shape of the tissue (Scott et al 1998). However, GAG chains do not influence interactions between collagen fibrils. Gu et al (1997) found that the interactions between decorin and collagen depend on the core protein of decorin, but is not affected by removal of GAG chains, presenting core protein’s inhibitory effect on collagen fibrillogenesis without GAG. Neither the N-terminal half nor the central leucine-rich repeats of the decorin core, by itself, interacts completely with fibrillar collagen. When a glutamate in a central LRR of decorin was disturbed, it influenced the binding greatly (Kresse et al 1997). It seems that there are different independent binding sites of decorin for TGF-beta and type I collagen (Schönherr et al 1998).

Lack of decorin is known to cause skin fragility and abnormal collagen morphology, which were indicated by uncontrolled lateral fusion of fibrils, in humans (Weber et al 1996). Significantly decreased levels of mRNA for decorin were found in fibroblast cultures of lethally sick Marfan Syndrome patients and in infantile progeroid patients, who exhibit abnormal connective tissue formation.
Decorin knockout mice showed abnormal collagen fibril morphology and skin fragility with significantly reduced tensile strength (Danielson et al 1997). Masse et al (1997) observed that the amount of decorin synthesis was greatly decreased in articular cartilage in chicken osteoarthritis. Their data suggested that reduced decorin content lead to thicker collagen bundles in the tangential zone of pyridoxine deficient articular cartilage (Masse et al 1997).

Injured ligament heals with scar tissue, which exhibits worse mechanical properties than normal ligament. In a ligament scar, normal large thick collagen fibrils are replaced by small thin collagen fibrils. Downregulation of decorin by antisense decorin oligodeoxynucleotides improved the restoration of large collagen fibrils, which resulted in restoring mechanical properties of the scar (Nakamura et al 2000). When decorin is over-expressed, slowed tumor cell growth and inhibited TGF (transforming growth factor)-beta-induced proliferation are observed. Moscatello et al (1998) investigated the biochemical mechanism by which decorin suppresses the growth of several tumor cell lines by inhibiting cell cycle progression. Decorin caused rapid phosphorylation of the epidermal growth factor (EGF) receptor and at the same time activation of mitogen-activated protein kinase signal pathway. This induced active inhibition of cyclin-dependent kinases and blocked cell cycle completely (Moscatello et al 1998).

The function of decorin is to regulate extracellular matrix assembly and growth factor activity. It can bind TGF-beta and neutralize its biological activity. Biglycan and fibromodulin also bind to TGF-beta with slightly different affinities
Decorin also plays an important role in inhibition of tumor growth through its regulation of extracellular matrix assembly and growth factor activity. By blocking TGF-beta activity, decorin inhibits cell proliferation. By blocking tumor cells in the early stage of the cell cycle, de novo expression of decorin can suppresses malignant phenotype of colon carcinoma cells. This decorin-induced growth suppression is associated with an upregulation of the cyclin-kinase inhibitor p21 (Luca et al 1996). Patel et al (1998) reported effect of decorin on EGF receptor and cytosolic Ca2+ in A431 carcinoma cells. Decorin may also function as an inhibitor of tumor cells due to its interaction of the oncogenic protein ErbB2, a homology of the EGF receptor. Increased levels of decorin were found around invasive carcinoma cells (Santra et al 2000). Function of decorin strongly depends on the iduronation degree of the dermatan sulfate chain of decorin and on the glycosylation of collagen molecule (Sini et al 1997). Decorin binds to collagen through interaction, which is tissue specific, between collagen and decorin core protein. In tendon, decorin binds to type I collagen and inhibits collagen fibrillogenesis (Ayad et al 1994). Decorin reduces the rate of fibril growth, but increases diameters of fibrils formed (Kuc and Scott 1997).

Type I collagen fibril diameter is increased by fibrous connective tissue and larger fibrils can tolerate stronger tensile force. Pins et al (1997) investigated self-assembly of collagen fibers. They found better fibrillar orientation, packing, and ultimate tensile strength after they stretched fibers before cross-linking occurred. The presence of decorin increased final tensile strength of uncross-linked fibers. They concluded that decorin might promote fibrillar slippage
during collagen fiber deformation and therefore improve the tensile properties of collagen fibers (Pins et al 1997).

When effects of decorin on bovine skin, sclera, and tendon collagen fibrillogenesis were examined, decorin in those organs reduced the rate of fibril growth and increased the diameters of the fibrils formed with slightly different rate. Core protein of decorin by itself reduced the rate of fibril growth as effectively as intact decorin, but it did not affect the diameter of fibrils formed (Kuc and Scott 1997). However, decrease in fibril-associated decorin might be necessary for fibril growth as tissues mature (Birk et al 1995). Birk et al (1995) suggested that decorin might regulate the maturation of collagen fibrils into larger fibrils and fiber network.

Collagen fibrillogenesis is retarded by increased concentration of phosphate. Neither the N-terminal half nor the central leucine rich repeats of the decorin core protein can, by itself, interact fully with fibrillar collagen. (Pogány and Vogel 1992) The expression of decorin is seen during skeletal muscle differentiation and is up regulated in dystrophic muscle.

When the role of decorin in TGF-beta-dependent inhibition of myogenesis was investigated, it was found that reduction of decorin expression or of decorin availability resulted in a decreased responsiveness to TGF-beta. These date suggests that decorin might be involved in skeletal muscle differentiation by activating TGF-beta-dependent signaling pathways (Riquelme et al 2001).

The interactions between collagen and decorin have been studied under the electron microscope. Arch-shaped decorin molecules, which are 2.5nm in
diameter, can bind to one cylinder or rod shaped collagen fibril, which is 1.5nm in diameter and about 300nm in length. The overall thickness of these fused molecules is about 3nm (Weber et al 1996). Decorin is made of outer convex shaped alpha helices and inner concave shaped beta-sheets. LRR can force two parallel sets of alpha helices and beta strands into curved shape (Svensson et al 1995). Polar and charged ionic side chains are exposed on the concave surface of decorin and they bind to polar region of collagen. It was thought that binding of decorin to d band of collagen happens near the carboxyl end of collagen, but recent study showed that it happens near the amino-terminal end of collagen. Major binding site of decorin to collagen, is between collagen 158 and 198 residues and about 60% of total interaction occur at this site (Sini et al 1997). This region corresponds to d band of collagen quarter stagger. Many charged residues are involved with this association and also additional charge enhances binding. Different combinations of charged residues, which are acidic and basic, make up salt bridges interconnect three strands in triple helix (Weber et al 1996). A highly charged GAG chain is located orthogonally or parallel to major axis of collagen fibril. Three N-linked oligosaccharides all lie on the same surface of decorin and glycosylation increases thickness of decorin molecule. Secondary binding sites are found on collagen molecules (Weber et al 1996). Decorin binds to these less active sites in the carboxyl terminal of the core protein. These sites stabilize the primary binding. Decorin is able to block the aggregation of collagen molecule because only one helix fit inside decorin and the same binding site can not be used to make up continuous collagen fibrils. The concave surface of
decorin has a major collagen-binding site. Also decorin contacts a secondary site on an adjacent molecule with a possibly carboxyl end. In the presence of decorin, correct positioning of the collagen molecules within staggered conformation of fibrils happens. As a consequence of the binding of decorin to the surface of collagen fibrils, the lateral assembly of triple helical collagen molecules is delayed (Vogel et al 1984; Rosenberg et al 1993), and the final diameter of the collagen fibrils becomes thinner (Vogel 1987). Other SLRPs and cross-linked triple helices provide additional help for proper collagen fibrillogenesis.

When decorin and biglycan were immunolocalized in human intervertebral discs, researchers found that decorin and biglycan might play a major role in collagen network formation and biomechanical stress resistance, by having identical or adjacent binding sites on the fibril used by both proteoglycans. But further research is required to confirm the exact interaction of biglycan with type I collagen (Shönherr et al 1995). Also they found that the amount of both proteoglycans decreased in the annulus of 50 years or older individuals, reflecting an age-related change (Gotz et al 1997). Decorin also is known to influence binding of LDL (low-density lipoproteins), which is the major factor for atherosclerosis development, to collagen type I. In atherosclerotic lesions, LDL, decorin, and collagen type I contents were all increased. In the lesions, LDL and collagen interact with each other. LDL can bind to collagen type I with or without decorin, but when decorin was bound to collagen type I and then decorin-collagen complex was bound to LDL, the rate of binding was significantly higher than before (Pentikainen et al 1997).
Studies on the relationship between decorin and exercise are scarce. Visser and others studied decorin content of articular cartilage from the knee joint in female beagle dogs, which were put on long distance running exercise (40km/day for 15 weeks, five days a week). The running exercise protocol did not change the total GAG content of the cartilage, but increased the amount of decorin significantly. They concluded that decorin synthesis was stimulated by exercise, independent of the synthesis of other proteoglycan such as aggrecan (Visser et al 1998). In our study, chicken gastrocnemius tendon was used instead of cartilage, but tendon tissue should respond to exercise in similar way as cartilage did since both tissues are connective tissues. Tendons contain more decorin than cartilage or muscle does. Abundant decorin contents in tendon should make the observation of change in the amount of decorin by exercise easier.

Exercised Tendon:

Stone (1988) suggested that physical activity can increase connective tissue strength and mass. Even though activation of muscles is required to stimulate connective tissue adequately, the exercise training volume, intensity, and load bearing are important factors in causing connective tissue adaptations (Stone 1988). In animal studies, physical training seems to make tendons stronger, larger and more resistant to injury. Growing animals may adapt to the exercise routine better than adult and their responses are better. Vilarta and de Campos Vidal (1989) noticed biochemical and anisotropic changes in exercised
tendons. Their experiment was similar to the one Rollhauser (1954a, 1954b) performed, where tendon strength improved as adaptive response to exercise. Under the microscope, collagen fibrils were better packed and aligned with tendon stress lines than control. Many researchers observed positive changes (i.e., larger fibril diameters) in tendon structurally and functionally after controlled exercise program. (Archambault et al 1995; Curwin et al 1988; Heikkinen et al 1975; Kiiskinen and Heikkinen 1973; Maffulli and King 1992; Michna 1984; Michna and Hartmann 1989; Sommer 1987; Tipton et al 1975; Vilarta and de Compos Vidal 1989; Woo et al 1981; Woo et al 1980; Woo et al 1982; Zamora and Marini 1988).

There are some limitations of those exercise programs. In most exercise experiments, young-trained animals are often compared to young untrained animals. Since caged animals have possibly reduced connective tissue size and tensile strength, training may just bring those values up to the baseline values before they were put on confinement. They also stated that those exercised animals were strained more than their normal physiological condition. Generalizing those results from exercise programs is difficult (Butler et al 1978). Stone (1991) mentioned that interpreting animal exercise data on connective tissue strength must be done cautiously. Since age can be a confounding factor in adaptation of exercise for tendons, mature animals’ studies are more applicable to human (Archambault et al 1995). Studies on exercise experiments with rats, mice, swine, and roosters, are described below.
Rats:

Langhoff and Munzenmainer (1973) studied changes in Achilles tendons of rats participating in different distance and intensity training program. Both long and short distance exercise programs increased adenosine triphosphatase, alkaline and acid phosphatases, and leucine amino-peptidase activities three times more in exercised tendons than in control tendons. When they compared the exercise programs, high-speed training had a greater effect. Also, exercise increased insulin-like growth factor I (IGF-1) expression in rats’ Achilles tendon. IGF-1 stimulates collagen synthesis and cell replication (Hanson et al 1988) and may be involved with tendon remodeling (Archambault et al 1995). Intensive running exercise increased cross-sectional area of Achilles tendon, as well as it increased its total collagen content, but it decreased its maximum tensile strength (Sommer 1987). When rats had restricted food availability, exercise had less impact on positive adaptations of tendons (Enwemeka et al 1992). Simultaneous treatment with anabolic steroids appears to intensify influence of exercise.

Extension of the tendon initially involves straightening of the crimp of fibrils (Cribb and Scott 1995). Wood et al (1988) found that exercise increased the angle of the crimp and decreased length of fibrils. The changes suggest that collagen fibrils elongate more under stress and elongation of fibrils causes tendon to be ruptured more easily (Wood et al. 1988).

When synergistic muscles were removed, positive morphology change was observed in rat plantaris tendon. More active tenoblasts were seen in cells. Increased protein synthesis was indicated the presence of more vacuoles in cell
cytoplasm (Zamora and Marini 1988). Ultrastructural change was also observed and it included disturbed collagen bundles and more empty spaces in between fibers. These changes suggest that tendon may collapse once before remodeling itself. Tendons in this transition phase tend to be much weaker than normal and much easier to break (Archambault et al 1995). Tittel and Otto (1970) had similar results as Rollhauser (1954a & 1954b) said was those growing animals’ tendon, and mature animals’ tendons undergo different changes during exercise.

Mice:

After three weeks of running exercise, Kiiskinen and Heikkinen (1973) found that tensile strength of the patellar tendon of growing mice increased. Also, the exercise increased the activity of tenocyte and accelerated collagen and proteoglycan matrix synthesis. Due to the acceleration of those syntheses, hydroxyproline content was also increased. Hydroxyproline content indicates concentration of collagen in the tissue. (Kiiskinen and Heikkinen 1973). The increase of activities of enzymes, which stimulate metabolism of tendon, was seen after 15 days of exercise program. Enzymatic activities of malate dehydrogenase, isocitrate dehydrogenase, succinate dehydrogenase, and NADH-diaphorase were increased (Heikkinen et al 1975; Heikkinen and Vuori 1972). With endurance training program, Takala et al (1983) found that enzyme activity, which stimulating the posttranslational modifications of collagen biosynthesis, was increased.
Mice response to a one-week short-term treadmill running program was significant. The running increased the collagen fibrils’ size and number as well as it increased digital flexor tendon’ cross-section area, when it was compared to immobilized control groups (Michna 1984, Michna and Hartman 1989). When they were exercised for 10 weeks, numbers of fibrils were increased, but the average diameter decreased. Since thickened fibrils have more intermolecular and intrafibrillar cross-links, they can withstand greater tensile forces (Maffulli and King 1992).

Swine:

After 12 month of training, swine’s extensor digitorum longus tendons were examined. They increased in weight, strength, collagen content, and stiffness. They became similar to flexor digitorum longus tendons, which are naturally strong.

Compared to the large change extensor digitorum longus tendon underwent flexor digitorum longus tendons experienced not very significant hypertrophy and no change in collagen content (Woo et al. 1980, 1981, 1982). Authors concluded that when muscle became stronger, it gained maximum tensile strength in connective and elastic energy storage capabilities. Since different tendons have different biochemical composition and mechanical properties, responses to exercise may vary among different types of tendons (Stone 1991; Woo et al 1982).
Rooster:

Young roosters were put on a progressive treadmill running program for 8 weeks, 5 days/wk at 70-80% maximal O$_2$ consumption (VO$_2$ max) and their immature Achilles tendons were examined (Curwin et al 1988). One of the significant biochemical changes was an increase in collagen and collagen content in the tendon. Dry weight and DNA and proteoglycan concentrations did not change. The amount of pyridinoline cross-links decreased 50%. They found that too strenuous exercise routine could make collagen-matrix turnover rate faster in growing roosters. Therefore, maturation of collagen that slows turnover rate in tendon will be retarded by exercise (Curwin et al. 1988).

Aging of tendons:

The components of tendons go through qualitative and quantitative changes as tendons get older. In human tendons, the amount of collagen increases from 35% of dry weight of whole tendon tissue to 80% from birth to adult. The content of proteoglycans and glycoproteins decreases from 5 and 6 % to 2 and 3 %, respectively (Ippolito, 1986). Collagen content does not change significantly after it matures, while the content of polysaccharides and glycoproteins further decreases. Water in tendon decreases from 80-85% at birth to 30-70% in older age (Hess et al 1989; Ippolito 1986). The decrease in water content may result in reduced elasticity of tendons. Aging of tendon decreases the solubility of collagen because aging increases cross-linking of tropocollagen molecules (Best and
Garrett 1994; Houck et al 1967; O’Brien 1992). Tendons have very slow turnover rate, but the rates seem to decline even more with age. The number of tenoblasts decreases as the animal grow and cells become longer and thinner. Also the average area and diameter of collagen fibrils, and the numbers of thick fibrils decrease as tendons get very old (Nakagawa et al 1994).

Collagen Fibrillogenesis:

Formation of collagen fibrils can occur inside the cell and outside the cell. First, pro-alpha chain is synthesized in the membrane-bound ribosome. Pro-alpha chain is a fairly large precursor with an amino-terminal signal peptide, which directs nascent collagen polypeptide to endoplasmic reticulum, and propeptides, which have both amino- and carboxyl terminals. Propeptides direct intracellular formation of triple-stranded collagen molecules and avoid intracellular formation of large collagen fibrils. Second, in endoplasmic reticulum, hydroxylation of prolines and lysines occurs. Hydroxyproline and hydroxylysine participate in formation of interchain hydrogen bonds, which helps stabilize the triple-stranded helix. Deficiency of vitamin C (ascorbic acid) or scurvy prevents proline hydroxylation. In scurvy, defective synthesized pro-alpha chains can not form a stable triple helix and they are immediately degraded within the cell. Blood vessels become extremely fragile and teeth become loose in their sockets. Individuals with scurvy also have bleeding of their gums. Preexisting normal collagen in the matrix in scurvy is lost gradually. Third, after hydroxylation, some hydroxylysines are glycosylated. The final size of collagen fibril is
correlated to the degree of glycosylation (Valli et al. 1986) and by decorin binding (see below). Fourth, three pro-alpha chains self-assemble and form procollagen triple-helix.

Procollagen is a hydrogen-bonded and triple-stranded helical molecule. Procollagen is secreted to extracellular space by secretory vesicles. In plasma membrane, propeptides are removed and those secreted forms of fibrillar procollagen become collagen molecules. Collagen molecules self-assemble together to form fibrils. This event happen close to cell surface, where deep infoldings of plasma membrane are formed by tandem fusion of secretory vesicles with cell surface. Since cortical cytoskeleton is lying underneath of that cell surface, cytoskeletons greatly influence sites, rates, and orientation of fibril assembly. Finally, collagen fibrils accumulate to form a collagen fiber.

Different arrangements of fibrillar collagen molecules can be found in different tissues. In skin collagen fibrils are arranged in wickerwork pattern, which can resist forces in multiple directions. Tendons have parallel bundles of fibrils aligned along the major axis of tension. Mature bone and cornea, and tadpole skins are plywood layers of collagen fibrils, which means that fibrils in each layer lie parallel to each other (Yurchenco et al. 1994). Collagen-secreting cells and plasma membrane can regulate disposition of collagen molecules after its secretion. Those cells also influence spatial organization and interactions of collagen fibrils with other molecules in the extracellular matrix. Fibroblasts influence the alignment of collagen fibers, while collagen fibers affect the distribution of fibroblasts (Hay 1991). Fibroblasts help create tough and dense
layers of connective tissues. Graham et al (2000) found that fibroblasts synthesize transient early fibril intermediates (1 µm in length), which fuse tip-to-tip to form longer fibrils in older tissues. There are two different early fibrils formed by fibroblasts. One is a unipolar fibril with carboxyl and amino ends. The other one is a bipolar fibril with two amino ends. Tip-to-tip fusion requires carboxyl ends of unipolar fibrils. The proteoglycan decorin is known to coat fibrils along with its axis, but not at the end of fibrils. Fibrils aggregate through side-to-side interactions (cross-linking) without decorin. Decorin promotes tip-to-tip fusion and inhibits side-to-side fusion (Graham et al 2000). Lumican and fibromodulin, which like decorin are both small leucine-rich repeat proteins (SLRP), also regulate collagen fibrillogenesis (Ezura et al 2000).

After formation in extracellular space, collagen fibrils are greatly strengthened by the formation of covalent cross-links (four-dimensional stagger) between lysine residues of the constituent collagen molecules (Yurchenco et al 1994). First, the extracellular enzyme, lysyl oxidase, deaminates selected lysine and hydroxylysine residues to produce highly reactive aldehyde groups. Those aldehydes react spontaneously to form covalent bonds with each other or with other lysine or hydroxylysine residues. Most of the cross-links are formed between the short nonhelical segments at each end of the collagen molecule (Alberts et al 1994). Intramolecular cross-links are formed by condensation of 2 allysines, while condensation of allysine and lysine produces intermolecular cross-links, which are called Schiff base cross-links. These bridges between collagen fibrils play a role in transmitting and resisting tensile stresses in tendons,
contributes to the strength of the tissue (Weiss and Jayson 1983). Appropriate positioning of collagen fibrils in the matrix decides the shapes of the extracellular matrices (Balazs 1970). Collagen fibrils are linked together by GAG chains of decorin to stay in place. Anti-parallel form of GAGs are stabilized by hydrophobic and hydrogen bonding. Repeating series of collagen fibrils, decorin, and GAG aggregates are called “shape modules”. The length of GAG chains may be able to regulate the distance between each collagen fibrils, determining the shape of the tissue (Scott et al 1998). The distribution of decorin is consistent with matrix-centered functions, related to regulation of collagen fiber growth.

There are numerous human genetic diseases, which affect fibril formation (Myllyharju and Kivirikko 2001). Mutations of type I collagen cause osteogenesis imperfecta, which leads to weak bones, which are easily fractured (Chevrel and Meunier 2001; Weiss and Jayson 1983). Chondrodysplasias caused by mutation of type II collagen cause abnormal cartilage, and bone and joint deformities (Weiss and Jayson 1983). Type III collagen mutation cause Ehlers-Danlos syndrome. Patients with Ehlers-Danlos syndrome have fragile skin and blood vessels, and hypermobile joints (Iurassich et al 2001; Weiss and Jayson 1983). In chicken, reovirus affects the structure and mechanical function of tendons. Reovirus infected gastrocnemius tendon and sheath from male broilers showed cytoplasmic vacuolization, disruption of membranes, and loss of ribosomes from rough endoplasmic reticulum, mitochondrial degeneration, and cell disruption (Hill et al 1989). Rosenburger et al (1989) found that slow, intermediate, and high pathogenicity isolates when the virus was inoculated orally
or intratracheally into broiler chickens of various ages, different isolates caused different condition in those chickens. The low-pathogenicity isolate did not cause mortality, weight depression, or clinical disease. Intermediate-pathogenicity isolates caused low mortality, some weight depression, and microscopic lesions in the intestine and gastrocnemius tendon. High pathogenicity isolates produced mortality rates as high as 84%, and also microscopic lesions in liver, intestine, pancreases, and/or gastrocnemius tendon. Age-associated resistance to this virus was observed as well. Younger chickens were more susceptible to reovirus-induced-disease than older chickens. Embryos had equal mortality rate with low, intermediate, and high pathogenicity isolates (Rosenberger et al 1989).

References:


Barnett; Davis; McConaill: 1961


Reynolds; Worrell: 1991


CHAPTER 2

THE EFFECT OF AGE AND EXERCISE ON CHICKEN GASTROCNEMIUS TENDON

Introduction:

Tendon is mainly composed of collagen fibers. Collagen fibrillogenesis is highly influenced by the growth, age, and use of animal and by other biochemical components of the tendon. Three collagen alpha chains make a triple helical arrangement in a collagen molecule. Collagen molecules aggregate in parallel to form a fibril (Alberts et al 1994). The collagen molecules arrange themselves into a structure where the individual molecules overlap each other by three-quarters of their length. This overlap or aggregation of collagen molecules gives rise to a D periodicity or D-staggered array of fibrils in which each D period is divisible into a collagen overlap zone or a gap zone. It has been proposed that decorin binds to collagen in the gap zone between collagen molecules. The correct positioning of decorin will properly position the collagen molecules. The precise spatial arrangement of collagen molecules is critical in the formation of collagen cross-links (Sini et al 1997). If decorin binds to other potential binding sites on the collagen molecule, the inaccurate spacing of the collagen molecules will result in a lateral fusion or increased cross-linking between collagen molecules (Scott et al
The amount of decorin in tendon will be changed by stress regardless of its age-dependent amount change. The change in the amount of decorin will influence collagen fibrillogenesis and biomechanical function of tendon.

Physical activity can increase connective tissue strength and mass. In animal studies, physical training seems to make tendons stronger, larger and more resistant to injury (Stone 1988). Young growing animals may adapt to the exercise routine better than adult and their responses are faster and more distinct. Biochemical and anisotropic changes in exercised tendons, improvements in tendon strength were observed as adaptive response to exercise (Vilarta and de Campos Vidal 1989). Under the microscope, collagen fibrils were better packed and aligned with tendon stress lines than control. Many researchers observed positive changes in tendon structurally and functionally after controlled exercise program (Archambault et al 1995; Curwin et al 1988; Heikkinen et al 1975; Kiiskinen and Heikkinen 1973; Maffulli and King 1992; Michna 1984; Michna and Hartmann 1989; Sommer 1987; Tipton et al 1975; Vilarta and de Compos Vidal 1989; Woo et al 1981; Woo et al 1980;Woo et al 1982; Zamora and Marini 1988), even though there are some limitations of those exercise programs (Butler et al 1978). Positive changes mean larger, stronger, and more resistant to injury.

The components of tendons go through qualitative and quantitative changes as tendons get older. The contents of proteoglycans, glycoproteins, polysaccharides, glycoproteins, and water go through change, while collagen content does not change (Ippolito 1986; Hess et al 1989). Aging of tendon decreases the solubility of collagen because aging increases cross-linking of
tropocollagen molecules (Best and Garrett 1994; Houck et al 1967; O’Brien 1992). Tendons have very slow turnover rate, but the rates seem to decline even more with age. The number of tenoblasts decreases as the animal grow and cells become longer and thinner. Also the average area and diameter of collagen fibrils, and the numbers of thick fibrils decrease as tendons get very old (Nakagawa et al 1994).

The purpose of this study was to further define the effect of age and exercise on the size of collagen fibrils in tendon. We randomly assigned growing chicken to runner or control groups. Runners were subjected to a treadmill running program for 3 weeks. They were exercised three times a day for 10 minutes with 10-min. rest in between the exercise. Immunohistochemistry and immunocytochemistry methods were used to visualize decorin and collagen fibrils in tendon under light microscope and electron microscope.

Material & Methods:

Chicken Exercise Protocol:

Gastrocnemius tendons were removed from 1-day, 1, 2.5, 5, and 6.5 weeks old female broiler chickens, and frozen at –70 °C. Two hundreds 1-day-old broiler chickens were obtained. Males were separated from females by feather sexing at 1 day of age with 75% accuracy. At 2.5 weeks of age, 83 female chickens were randomly divided into a control (43 chickens) and exercise (40 chickens) group. The exercise group was preconditioned to treadmill walking by
pacing at 0.5 mph for 5 min a day, 5 days a week for one week, starting at 2.5 weeks of age. After preconditioning, the exercise group ran on the treadmill three times for 10 minutes each time with 10-min. between intervals, 5 days per week, for the next 3.5 weeks. The chickens were paced at approximately 1 mph which corresponds to 50% of their maximum capacity or the speed at which chickens will attempt to fly (Biewener et al., 1986). Between exercise periods, the chickens were kept in a standard pen where they were allowed free movement. Control chickens of the same strain and age was kept in standard pens. Both groups were kept under the same standard conditions, regarding space, food and water. Flooring consisted of pine shavings. The chickens had free access to food (standard feed was used) and water.

At the age of 1 day and 1 week, 5 female chicks were killed (10 chickens as total) by CO₂ overdose. At 2.5 weeks of age, 5-exercised (female) and 6 control (female) were killed. At 5 weeks old, 12 exercised (6 females and 6 males) and 5 controls were killed. At the end of exercise period, all of the chickens, which were 6.5 weeks old, were killed with CO₂ gas. This means that 16 exercised (10 females and 6 males) and 21 controls (all female) were killed (37 as total). Blood taken from these killed birds was tested for reovirus, which result was negative. Every time chickens were killed, 2 tendons were randomly selected from each group for immunohistochemistry and 2 different tendons for EM studies. Remaining tendon were used for other biochemical study in our laboratory.
Immunohistochemistry:

Decorin was detected by sensitive and specific immunohistochemical analysis with a biotin-avidin horseradish peroxidase method (Leppert et al 2000). By this method, decorin was analyzed at each stage of development for spatial distribution (Velleman et al 1999).

Chickens were selected as described above. Gastrocnemius tendons were removed from those chickens. All the tendons were measured by ruler and were divided into three sections: top, middle, and bottom. Those were fixed in formalin and paraffin-embedded. Thin sections from paraffin-embedded tissues were prepared for immunohistochemistry. Slides were dried overnight at 55°C and deparaffinized in xylene for 3 X 10 minutes. Ethanol was used for hydration after deparaffinization. Slides were incubated in 3% H₂O₂ for 5 minutes and in PBS + Brij (blocking solution from Zymed). Blocking solution from Zymed was used to block any background staining. Slides were washed with PBS + Brij after 10 minutes incubation with another blocking solution from Zymed, which was labeled as A solution from ABC kit from Zymed. CB-1, monoclonal antibody to chicken decorin, was used as primary antibody with 4 hours incubation at room temperature and 1:10 dilution. Slides were rinsed with deionized H₂O, and then washed with PBS for 3 X 5 minutes. Super Sensitive Multilink (biotinylated second antibody) from Zymed was applied for 20 minutes. Super Sensitive Label (HRP-Streptavidin; enzyme conjugate) was applied for 20 minutes after rinsing with dH₂O and PBS. Aminoethyl carbazole (AEC) chromogen was applied for
10-15 minutes to stain antigen-antibody complex and then rinsed with deionized H$_2$O. Slides were counter-stained with haematoxylin for 1 minute, rinsed in tap water and then dipped into Scott’s tap water for 30 seconds. After tap water rinsing, slides were dipped into 1- % acetic acid three times quickly and washed in running tap water for about 1 minute. Stained sections were covered with liquid coverslip called Crystal Mount (Zymed) and placed at 70°C overnight. Cover slip was put on tissues with mounting medium, Flo-Tex. After drying by air, sections were examined under light microscope to see the age- or exercise associated differences in distribution and localization of decorin in those tissues.

**Scanning electron microscopy:**

Scanning electron microscopy (SEM) is used to study the three-dimensional features of individual cells and even whole organisms (Bozzola and Russel 1992). Scanning electron microscopy method was employed in order to reveal the three-dimensional organization of the collagen fibrils in gastrocnemius tendon (Vizza et al 1995).

Gastrocnemius tendons were removed from described above chickens. All the tendons were measured by rules and divided into three sections; top, middle, and bottom. For SEM, tissues were fixed in yellow fixative (2% (para) formaldehyde, 2% glutaraldehyde, 0.2% picric acid in 0.2M cacodylate-HCl buffer, pH 7.25) for 24 hours first. Then, they were washed in 0.1M Cacodylate-HCl buffer, pH 7.25 3 X 15 minutes and dehydrated in ethanol. Autosamdrri-814 Critical Point Dryer from Tousimis Research Corporation in Rockville, MD was
used for the complete dehydration. Finally, tissues mounted on aluminum stubs and sputtered with gold in an SPI-Module Sputter Coater (SPI Supplies of Division of Structure Probe, Inc., West Chester, MA). Stubs were examined in a JSM-5800 Scanning Electron Microscope (JEOL USA, Inc.; Peabody, MA) to see the age- or exercise associated changes in thickness or coordination of collagen fibrils and fibers. Two to three images per each section were prepared and diameters of each fibril in each electromicrographs were measured manually and calculated to get averaged values.

Transmission electron microscopy:

Transmission electron microscopy is used to view ultrathin sections of biological specimens (Bozzola and Russel 1992). Diameters of collagen fibrils were examined under transmission electron microscope (Devessa et al 2001). Chickens were used as described above. Gastrocnemius tendons were removed from those chickens. All the tendons were measured by ruler and divided into three sections: top, middle, and bottom. For TEM view, tissues were fixed in yellow fixative (2% paraformaldehyde, 2% glutaraldehyde, 0.2% picric acid in 0.2M cacodylate-HCl buffer, pH 7.25) for 24 hours first. Then, they were washed with 0.1M Cacodylate-HCl buffer, pH 7.25 and post-fixed in osmium/1% osmium tetroxide in 0.1M Cacodylate-HCl buffer for 1 hour and half. Tissues were rinsed in deionized water 4 X 15 minutes. Then, they were en bloc stained with 0.5% aqueous uranyl acetate for 1 hour (Dawes 1971), washed in deionized H2O and dehydrated in ethanol. After rinsing in acetone for 30 minutes twice,
tissues were immersed in Propylene Oxide for 2 X 20 minutes, then in increasing concentration of Epon-Araldite (Dawes 1971). Polymerization was in fresh Epon-Araldite at 75ºC for 24 hours. The plastic embedded gastrocnemius tendon sections were cut with a Reichert ultramicrotome and mounted on formvar-carbon coated nickel grids. Post-staining was in methanolic uranyl acetate and lead citrate. Those grids were examined under the transmission electron microscope to see the age- and exercise-related changes in diameters of collagen fibrils at magnification of x20,000. Four images per section were prepared and measurements made using Image Pro Plus (Media Cybernetics). Average diameters and area of each collagen fibrils in each section were measured.

Image Pro Plus:

Image Pro Plus expresses all spatial measurements in terms of pixels by default. The spatial scale can be changed to microns with proper calibration. In digital images of electron micrographs, fibrils and the connective tissues around fibrils were selected through image thresholding. Most of the time fibrils were identified as bright objects and surrounding connective tissues were as dark objects. Outlines of fibrils were obtained through SCM-automated means, and diameters and cross-sectional area of each fibril were measured. When fibrils could not be selected through thresholding, outlines around fibrils were drawn manually. Four to eight images per each portion (i.e., top, middle, bottom) from each group and age were obtained for each data point and used for statistical analysis.
Results:

Immunohistochemistry:

Decorin was immunolocalized under the light microscope. Immunohistochemical staining method with CB-1 antibody (1:10 dilution) was used. Aminoethyl carbazole (AEC) chromogen was used as the chromogen. It reacts with the antigen-antibody complex in tissues, staining the complex red. For background staining, haematoxylin was used. Results are shown in Table I.

All staining was extracellular, confined to extracellular matrix surrounding tendon fibers. Younger tissues (1 day and 1 week) had more intensive staining through the top, middle, and bottom sections than older tendons. This indicated there is more decorin present in younger tendons. Younger tendons (1-day- and 1-week-old (Figure I (2)) also contained less collagen which may account for the appearance as containing more decorin than older (i.e., 6.5 weeks old) tendons. One-day-old gastrocnemius tendon sections had the strongest reaction. The top part of one-day-old tendons stained very strongly, while middle and bottom parts stained somewhat less intensively (Figure I (1)). In sections from older tendons (5 and 6.5 weeks old), immunostaining was more diffuse and less intense in the middle portion when compared with top and bottom (Figure I (3)). In 6.5 weeks old tendon sections, from the top and bottom parts of the tendon, though staining more for decorin than the middle part, had less staining than the equivalent sections of 1-day-old tendon (Figure I (5)). The connective tissues separating muscle cells in the top sections reacted also strongly. The amount of connective
tissue between muscle cells changed with the age of tendon. Tendon fibers reacted much stronger than muscle tissue or cartilage adjacent to tendon because they contain much more decorin bound to collagen than muscle or cartilage tissues do. Tendon sheath stained intensively for decorin. Exercise changed the amount of decorin present in tendon only slightly (Figure (3)&(4) and Figure (5)&(6)). Only a few tendon cells, separated by collagen fibers, reacted very slightly, in all examined tendons. Tendon cells and nuclei were getting more elongated with age.

Scanning Electron Microscopy:

Scanning electron microscopy was used to study the three-dimensional organization of the collagen fibrils in gastrocnemius tendon. Results are shown in Table II and Chart I.

At 1 day of age, fibrils were not tightly packed or aligned under the scanning electron microscope (Figure III (1)), compared to fibrils in older 6.5 weeks old tendons, which looked tightly arranged (Figure III (2)). It was very difficult to distinguish single fibril from a bundle of fibrils because of their packed arrangement. However, it is easily observed from figures that diameters of fibrils progressively increased as chicken aged from 1 day to 6.5 weeks. Large increase in diameter was observed at age of 1 week (top and middle-0.0174µm, bottom-0.0131µm) and 2.5 weeks (all part-0.0261µm) (Figure (3)). Fibrils in bottom parts increased 0.0043µm in diameter between 1 day (0.0131µm) and 1
week (0.0174µm). The average diameters of fibrils in bottom and middle parts increased 0.0056µm between 5 weeks (0.0261µm) and 6.5 weeks (0.0305µm).

Size of the diameters of fibrils from all parts were similar between males and females of the same age (i.e., 1-day-old male (Figure II (4)) and female). The fibrils in the top and middle parts of 6.5 weeks control male tendons had 0.0056µm and 0.0043µm larger diameters than fibrils from top and middle portions at 6.5 weeks control female (Figure III (5)). Exercised female and male tendons at 5 weeks old had the same size of fibril diameter, except for the top part, where the average diameter of fibrils was 0.0261µm in the female tendon, while the average diameter of fibrils of male tendon was 0.0244µm (Figure III (6) & (7)). This corresponds to the TEM results.

When diameters of fibrils were compared between control and exercise tendons, changes in the diameters between exercised and unexercised fibers were not very significant. The surface of the exercised fibrils looked smoother than control ones. When control and exercised fibrils of the same age were compared, control fibrils’ diameters increased from 0.0174µm to 0.0261µm in between 1 week and 2.5 weeks, while exercised fibrils increased in its diameters from 0.0261µm to 0.0305µm between 5 weeks and 6.5 weeks. At the end of exercise period, fibrils from exercised female tendons had larger average diameters in all sections (top-0.0305µm, middle-0.0348µm, and bottom-0.0348µm) than control ones (top-0.0261µm, middle-0.0305µm, and 0.0305µm). The fibrils in the top, middle, and bottom sections in control-female and exercised-female tendons had same diameters, except for the fibrils in bottom parts of 1 day (0.0131µm) and top
part of control and exercised 6.5 weeks (0.0261 and 0.0305µm) (Figure III (8)). Statistical analysis was not performed because of small sample size and unreliable measurements by hand.

Transmission Electron Microscopy:

Under the transmission electron microscope, the transverse sections revealed thin collagen fibrils in younger (1-day and 1-week-old) tendons. Fibrils could be divided into 2 groups or peaks, according to their diameters. Percentages show the number of collagen fibrils in each peak per each part (i.e., top, middle, and bottom). Image Pro Plus program was used to measure the cross-sectional area and diameters. Results are shown in Chart I.

At 1 day of age, female tendons contained 2400-3672 fibrils per image (x20, 000) in each section (Figure II (1)). Fibrils aggregated in 2 distinct peaks: The average diameter of fibrils in the first peak of 1 day old female was 0.0082µm (bottom portion), 0.0106µm (middle portion), and 0.0106µm (top portion), which contained 4.9%, 7.7% and 9.5% of all fibrils, respectively, while the average diameter of the second peak were 0.075µm (bottom), 0.075µm (middle) and 0.077µm (top), containing 2.8%, 5.8% and 3.4% of all fibrils, respectively (Chart I (1)-A). The remaining fibrils (not included in the 2 peaks) were situated between the 2 peaks or after the2 peaks. This was true for all the measurements in all groups and ages. Mean average diameters were 0.062 (top), 0.057 (middle), and 0.056µm (bottom). The first peak when evaluated by the cross-sectional area, contained 9.3% in top, 12.5% in middle and 14% in bottom
of all fibrils and the area was an average of 0.00041µm², 0.00042µm² and 0.00046µm², respectively. The cross-sectional area in second peak was 0.0042µm², 0.0039µm² and 0.0044µm², respectively, and the top contained 3.4% fibrils, the middle 3.7% and the bottom part 4.2% of all fibrils. Mean areas were 0.0041 (top), 0.0036 (middle), and 0.0036 µm²(bottom) (Chart I (1)-B).

Sections from one-day-old male tendons had 2900-3800 fibrils per image (x20, 000) in each section (Figure II (5)). The top part of 1-day-old male tendons contained most fibrils in 2 large peaks. The first peak contained 39.6% of all fibrils (0.074µm) and the second peak had 37.1% (0.098µm). The average diameter of fibrils in the first peak for middle portion was 0.010048µm (4.9%) and bottom was 0.0087µm (3.6%). Middle and bottom parts had second peaks with an average fibril diameter of 0.075µm and 0.070µm. The average cross-sectional area of the first peak was 0.000367µm², 0.00062µm² and 0.00049µm², and the top part contained 4.3%, the middle 10.3% and the bottom 6.9% of all fibrils, respectively. The 2nd peak contained only 7.3, 8% and 3.9% fibrils in the top, middle, and bottom portions. The cross-sectional area of the second peak was 0.0044µm², 0.0042µm² and 0.0044µm², respectively (Chart I (2)-A & B).

Collagen fibrils from 1-week-old female tendon had 1190-1700 fibrils per image (x20, 000) in each section. The sections showed dramatically decreased numbers of fibrils per image when compared to 1-day-old tendons, but fibril diameter increased (Figure II (3)). The average diameter of fibrils in the first peak was 0.015µm (7.6%), 0.016µm (9.8%), and 0.026µm (14%) of the section’s fibrils in the top, middle, and bottom sections, respectively, while the average
diameter of fibrils in the second peak was 0.091µm (27.7%), 0.11µm (19.3%), and 0.10µm (37.6%) in the top, middle, and bottom portions, respectively. The second peak was wider, i.e.; it contained more fibrils than the first peak. Mean average diameters were 0.078, 0.080, and 0.075µm for top, middle, and bottom parts. The average cross-sectional area of fibrils in the top, middle, and bottom sections of the first peak was 0.0011µm², 0.0014µm², and 0.00097µm², which contained 9.3%, 15%, and 15.3% of all fibrils, respectively. The average areas in the second peak was 0.0072µm², 0.0055µm², and 0.0065µm², 11.3%, 11.5%, and 9% of all fibrils in the middle and bottom portions, respectively. Mean areas were 0.0059, 0.0066, and 0.0056µm² for top, middle, and bottom portions (Chart I (3)-A & B).

At 2.5 weeks of age, the area and average diameters of control female fibrils were examined (Figure II (4)). Each image contained 640-1000 fibrils (x20, 000). The average diameter of the first peaks for top portion was 0.047µm (30.4%), for middle portion was 0.012µm (12.7%), and for bottom portion was 0.012µm (13%). Second peaks were not distinct and showed a lot of trailing, with a range of 0.071µm-0.19µm for middle part and 0.058µm-0.18µm for bottom part. No second peak was recognized for the top part. Mean average diameters were 0.078, 0.089, and 0.099 µm for top, middle, and bottom portions. For all fibrils, the average cross-sectional area was at 0.0030(top-33%), 0.0015 (19.7%-middle), 0.0019 µm² (21.2%-bottom) in the first peak. The second peaks showed a lot of trailing with the most frequent cross-sectional area of 0.0087µm² (20.3%) for top part, 0.0027µm² (8.32%) for middle, and 0.017µm² (6.8%) for bottom,
respectively. Mean areas for all fibrils were 0.0069 (top), 0.0090 (middle), and 0.011µm (bottom) (Chart I (4)-A & (5)-A).

In 2.5 weeks exercised female fibrils, 580-790 fibrils were present per image (x20,000) in each section, which represents a marked decrease when compared to the numbers of fibrils in 2.5 weeks old control tendon (Figure II (5)). The average diameter of fibrils in the first peak was 0.010µm (10.6%) for top, 0.011µm (11.6%) for middle and 0.015µm (10.4%). The average diameters in the second peaks of average diameter were 0.10 (top), 0.15 (middle), and 0.18µm (bottom), which contained 2.9, 4.3, and 3.5% of all fibrils. These peaks had wide distributions with trailing in the range of 0.020µm-0.24µm for the top, 0.028µm-0.20µm for the middle, and 0.029µm-0.21µm for the bottom part. Mean average diameters were 0.11, 0.10, and 0.10µm for top, middle, and bottom parts. The average cross-sectional area in the first peak was 0.0018µm² (18.6%) for top, 0.0015µm² (19.8%) for middle and 0.0015µm² (17.8%), respectively. The cross-sectional area of fibrils in the second peaks of areas was 0.015 (top), 0.013 (middle), and 0.012µm² (bottom), which contained 3.3, 4.7, and 4% of all fibrils in each section, respectively. These peaks had wide distributions ranging from 0.0050-0.016µm² for top, 0.0044-0.021µm² for middle, and 0.0043-0.012µm² for bottom portion. Mean areas were 0.014 (top), 0.011 (middle), and 0.012µm² (bottom) (Chart I (4)-B & (5)-B).

At 5 weeks of age, control female tendons contained 700-750 fibrils per image (x20,000) in each section (Figure II (6)). The average diameter in the first peak was 0.0090µm (12.1%) for top, 0.013µm (14.5%) for middle and 0.024µm
(13.6%) for bottom. No clear second peaks were observed, but the most frequent average diameter of the larger fibrils was 0.081µm (top), 0.079µm (middle) and 0.094µm (bottom). The second peak contained 4.4%, 6.6%, and 10.5% of all fibrils, respectively. Mean average diameters were 0.12, 0.11, and 0.13µm for top, middle, and bottom portions. The average cross-sectional area in the first peak was 0.0047µm² (36.3%) for the top, 0.0031µm² (27%) for the middle and 0.0044µm² (28.5%) for the bottom portion. Also the frequent average cross-sectional area of the large fibrils was 0.0093µm² (14.1% in the top part), 0.0059µm² (11.8% in the middle part) and 0.0085µm² (12.5% in the bottom), respectively. The average cross-sectional areas were 0.018 (top), 0.015 (middle), and 0.022µm² (bottom) (Chart I (6)-A & (7)-A).

Exercised 5 weeks old female tendons had 495-760 fibrils per image (x20, 000) in each section (Figure II (7)). The average diameter in the first peak was 0.010µm (11.1%) for top, 0.0075µm (13.8%) for middle, 0.013µm (9.3%) for bottom. Fibrils in second peaks had average diameters of 0.060, 0.068, and 0.10µm, which contained 6.1, 3.2, and 3.2% of fibrils in each part. Mean average diameters were 0.12 (top), 0.12 (middle), and 0.12µm (bottom). The average cross-sectional area in the first peak was 0.0039µm² (35.5%) for top, 0.0042µm² (33.4%) for middle and 0.0025µm² (24.6%) for bottom. The average cross-sectional area in the second peak was 0.0079 (top), 0.0082 (middle), and 0.0072µm² (bottom), which contained 14.9, 10.3, and 7.8% of all fibrils. Mean areas were 0.019µm in top part, 0.020µm in middle part, and 0.017µm in bottom part (Chart I (6)-B & (7)-B).
Exercised 5 weeks old male tendons had 680-920 fibrils per image (x20, 000) in each section (Figure II (8)). The average diameter of fibrils in the first peak was 0.012µm (8.8%) for top, 0.0080µm (13%) for middle and 0.0097µm (11.4%) for bottom. The most frequent average diameters of fibrils in the less distinct second peak was 0.072µm (3.6%-top), 0.14µm (3.6%-middle) and 0.11µm (3.2%-bottom), respectively. The average cross-sectional area in the first peak was 0.0020µm² (20.5%) for top, 0.0027µm² (29.2%) for middle and 0.0017µm² (17.4%) for bottom. Second peaks had an average cross-sectional area of 0.0037µm² (10%-top), 0.0053µm² (9.5%-middle) and 0.0032µm² (6.6%-bottom) (Chart I (10)).

At 6.5 weeks of age, control female tendons had 540-770 fibrils per image (x20, 000) in each section (Figure II (9)). The average diameter in the first peak was 0.022µm (9%) for top, 0.011µm (7.9%) for middle and 0.013µm (8.6%) for bottom. Fibrils in the second peaks had an average diameter of 0.0074µm (15.9%-top), 0.0082µm (middle-13.8%) and 0.011µm (14.2%-bottom), respectively. Mean average diameters were 0.13µm in the top, 0.13µm in the middle, and 0.14µm in the bottom part. The average cross-sectional area in the first peak was 0.0037µm² (26.6%) for top, 0.0042µm² (24.7%) for middle and 0.0054µm² (30.9%) for bottom. The second peak for top section (0.090µm²) contained more (13.3%) fibrils than the first peak. Middle and bottom parts showed second peaks with an average cross-sectional area of 0.089µm² (5.7%) and 0.076µm² (6.2%). Mean areas were 0.020µm² (top), 0.020µm² (middle), and 0.024µm² (bottom) (Chart I (8)-A & (9)-A).
Exercised 6.5 weeks old female tendon contained 660-700 fibrils per image (x20, 000) in each section (Figure II (10)). The average diameter of fibrils in the first peak was 0.0083 µm (6.3%) for the top, 0.0069 µm (7.5%) for the middle and 0.0078 µm (6.2%) for bottom. Second peaks contained fibrils with average diameter of 0.083 µm (4.7%) for top, 0.083 µm (3.9%) and 0.12 µm (3.8%) for bottom. Mean average diameters were 0.14 (top), 0.13 (middle), and 0.14 µm (bottom). The average cross-sectional area of the fibrils in the first peak was 0.0031 µm² (22.6%-top), 0.0026 µm² (19.6%-middle) and 0.0029 µm² (17.4%-bottom), respectively. Fibrils in the second peaks had an average cross-sectional area of 0.0061 µm² (13.2%-top), 0.0050 µm² (10.6%-middle) and 0.0055 µm² (8.9%-bottom). Mean areas were 0.025 µm² for top part, 0.022 µm² for the middle, and 0.024 µm² for the bottom (Chart I (8)-B & (9)-B).

Control 6.5 weeks old male tendon had 640-750 fibrils per image (x20, 000) in each section (Figure II (11)). The average diameter of fibrils in the first peaks was 0.012 µm (13.3%) for top, 0.019 µm (10.3%) for middle and 0.021 µm (5.2%) for bottom. Distinct second peaks were observed as well. The average diameter of fibrils was 0.079 µm (2.9%) for top, 0.094 µm (10.7%) for middle and 0.10 µm (11.3%) for bottom. The average cross-sectional area of fibrils in the first peak was 0.0044 µm² (36.7%) for top, 0.0039 µm² (25.1%) for middle and 0.0074 µm² (34.4%) for bottom (Chart I (11)).
Statistical Analysis:

As statistical analysis tool, t-test was used because of our small sample size. Areas and average diameters from different age groups were evaluated under the t-test to see if their means were significantly different. Analysis showed that the differences between 1-day-old and 1-week-old, 1-week-old and 2.5 weeks old, 2.5 weeks old and 5 weeks old, and 5 weeks and 6.5 weeks old, were statistically significant with p<0.05. There were some exceptions: top parts in 5 week, and middle and bottom parts in 6.5 week old tendons did not show a significant effect.

Also, the effect of exercise was tested with generalized linear model (GLM), as well as with t-test. By this method, the effect of age on fibrils’ average diameter and cross-sectional area should be eliminated. Analysis showed significant effect of age, exercise, and both on fibrils in top portion with p<0.0001. When middle portion of fibrils were tested to see if the effect of exercise was significant, it showed exercise, age, and interaction of both affected this portion significantly, but interaction of age and exercise was less significant (p=0.0012). Statistically significant effects by age, exercise, and both were noted in bottom portion of tendons. Exercise by itself affected this portion with less significant level (p=0.0161).

Discussion:

The effect of age and moderate exercise on collagen fibrils of gastrocnemius tendons of young broiler chickens was studied. Chickens were put
on treadmill running for 3x10 minutes a day, 5 days a week, and killed at 1-day, 1-week, 2.5 weeks, 5 weeks, and 6.5 weeks of age. Gastrocnemius tendons were removed and examined under light microscope with immunostaining for decorin and the size of collagen fibrils under transmission and scanning electron microscopes. The cross-sectional area and average diameters of fibrils in electronmicrographs were measured and t-test and GLM analyzed the results of measurements.

Age had a statistically significant effect on the fibril diameter and cross-sectional area on all 3 (i.e., top, middle, and bottom) parts of the tendon at all ages examined. We observed gradual growth of fibrils as chickens got older and also rapid increase in diameters of fibrils in tendons between 1 day and 6.5 weeks. Exercise had a significant effect on size of fibrils in parts of tendon at 2.5 weeks of age (after 5 days of light (conditioning) exercise). This effect was more pronounced in the top of the tendon. Most samples, regardless of age or exercise, had 2 populations or peaks as measured by the average diameter and the cross-sectional area of fibrils.

Younger tendons (i.e., 1-day-old) contained more decorin as detected by immunostaining and their collagen fibrils were much smaller in area and average diameter. All the sections from all 3 parts of younger tendon contained much more decorin than older tendons. As tendon gets older, the amount of decorin decreases, especially in the middle portion. Exercised tendons and control had similar amount of decorin in each tendon portion. This difference in the content of decorin between younger and older tendons likely is relative because older
tendons contain more collagen and the large collagen fibrils and fibers separate decorin. This difference in decorin content may also be related to how collagen fibrils grow under exercise as well as with growth and development. Decorin regulates collagen fibrillogenesis. Decorin increases diameters of fibrils already formed, but decrease the rate of fibril growth (Kuc and Scott 1997). In our study, exercise did not change the amount of immunostained decorin in top, middle, and bottom parts of tendons, however, it is known that it is difficult to use immunostaining for antigen quantification. We did not examine the tendons for the content of proteoglycans other than decorin. It is known that exercise increases collagen turnover in tendons of growing animals (Nakagawa et al 1994). To see the more specific interaction between collagen fibrils and decorin, further research using immunogold method under the TEM will be required.

In our study, the diameter and cross-sectional area increased with age, but only inconsistently with exercise. The cross-sectional area and average diameters were very similar in all three (top, middle, and bottom) parts of the 1-day-old tendons. Mean fibrils’ area and average diameters increased significantly as the chickens grew. In all three tendon parts, fibrils’ area and average diameter increased the most between 2.5 weeks and 5 weeks, especially, in the middle parts. Our data suggest that the middle part is also involved in the growth and strength of the tendon in 6.5 weeks old chickens. This correlate well with the time when broiler chickens gain a lot of weigh right before they go to the market.

Evaluation with t-test showed that comparison with exercised and control fibrils showed that the means of area and average diameter were significantly
different for some parts in certain age groups (all parts in 2.5 week, middle and bottom parts in 5 week, and top part in 6.5 week old tendon), suggesting that exercise did influence fibrils’ area and average diameters at least in some parts of the tendon. The means of cross-sectional areas and average diameters of fibrils from exercised tendons were always larger than control fibrils in every section. This suggests that there is a trend showing exercise might have an impact on the average diameter and the cross-sectional area of fibrils. Bottom parts from 2.5 weeks old tendon in exercised and control group showed less statistically significant difference in their means of average diameter. When control and exercised 5 weeks old tendon fibrils were compared, means of area and average diameter in the top portion did not show a significant difference, while middle and bottom parts did. Average diameter of the middle part and the cross-sectional areas and average diameters of bottom parts were not significantly different when compared to the same parts in control group. These findings indicate that exercise has impact on the middle and bottom parts of the gastrocnemius tendons. It is this portion of the tendon, which undergoes the most biochemical changes during exercise (Vilarta and de Campos Vidal 1989).

GLM analysis revealed the significant effect of age, exercise, and both in all three parts. Interaction of age and exercise had less significant effect on the middle part. Also age had less significant effect on fibrils in bottom portion. However, statistically significant effect by age, exercise, and both was seen in all parts of the tendon (p<0.05), suggesting age and exercise influenced the diameter and cross-sectional area of collagen fibrils.
When TEM and SEM results were compared, distinct differences between measurements of diameters were noted. This is most likely due to inherent problems of obtaining accurate measurements in SEM without using an internal calibration standard. However, trends of changes of increasing diameter of fibrils with age and exercise were observed using SEM.

Two peaks or populations of fibrils were recognized in most cases. A large percentage of fibrils with small diameter and cross-sectional area were in the first peak. Fewer fibrils with larger diameter and cross-sectional area were mostly present in the second peak with some exceptions. A large increase in cross-sectional area or average diameter of fibrils in both peaks was noted between the age of 1-day-old and 6.5 weeks old. The increased number of thicker fibrils in the 2nd peak was due to rapid growth, it was likely due to increased amount of newly synthesized collagen and also fusion of the fibrils as tendons age. Since thickened fibrils have more intermolecular and intrafibrillar cross-links, they can withstand greater tensile forces (Maffulli and King 1992). In human tendon, collagen content does not change much after completion of body growth. Very little fusion occurs in tendon at birth. Growth in thickness occurs very rapidly in younger tendons. It slows down gradually (Ippolito, 1986).

In our study, exercise induced significant changes in the average diameter and the cross-sectional area of fibrils in the middle and bottom parts of gastrocnemius tendons at 6.5 weeks of age. Many researchers observed that physical training made tendons stronger, larger and more resistant to injury. However, the presence of larger fibrils is also responsible for increased rate of
tendon rupture because of disturbed formation of collagen bundles and the presence of empty spaces between fibrils (Archambault et al 1995). Also the number of tenoblasts decreases as the animals grow and cells become elongated (Nakagawa et al 1994). When mice were put on one-week treadmill running program, the running increased the collagen fibrils’ size and number, compared to immobilized control groups (Michna 1984, Michna and Hartman 1989). After 10 weeks of exercise, numbers of fibrils were increased, but the average diameter decreased. Cross-section area did not changed significantly, compared to control tendons in this mouse study. In our protocol, exercise period was 3 weeks long. Numbers of fibrils decreased, while average diameter or area increased with age between 1 day and 6.5 weeks. There were clearly 2 different kinds of fibril size in 5 and 6.5 weeks old tendon under the transmission electron microscope. Our results showed that exercise did increase average diameter or cross-sectional area of fibrils at 2.5, 5, and 6.5 weeks of age. Though the increase was not statistically significant for all parts of the tendon, it still indicates the effect of exercise on collagen fibrils of a weigh-bearing tendon such as the gastrocnemius. In future studies, tensile strength of tendon should be measured to find out whether these chicken tendons undergo remodeling phase as a part of adjustment to exercise. Also collagen or hydroxyproline content in each part of tendon should be measured with Northern blotting or other assays (i.e., fibrillogenesis assay, colorimetric assay incorporation of measuring hydroxyproline into collagen) and compared to detect exact rate of synthesis or growth of collagen fibrils in exercise and control groups.
References:


APPENDIX

Chart I: Scanning Electron Microscopy Results
Chart II: Transmission Electron Microscopy Results

Figure I: Immunoreactivity for decorin, x100
Figure II: Scanning Electron Micrographs, x20,000
Figure III: Transmission Electron Micrographs, x12,000

Table I: Immunoreactivity for Decorin
Table II: Scanning Electron Microscopy Results
Chart I (1)-A: The change in the diameter of collagen fibrils from female chicken tendon by the effect of age and exercise at magnification of 12,000

Chart I (1)-B: The change in the diameter of collagen fibrils from male chicken tendon by the effect of age and exercise at magnification of 12,000
Chart I (1)-A: 1-day-old female chicken gastrocnemius tendon: collagen fibrils average diameter

Chart I (1)-B: 1-day-old female chicken gastrocnemius tendon: collagen fibrils cross-sectional area
Chart I (2)-A: 1-day-old male chicken gastrocnemius tendon: collagen fibrils average diameter

Chart I (2)-B: 1-day-old male chicken gastrocnemius tendon: collagen fibrils cross-sectional area
Chart I (3)-A: 1-week-old female chicken gastrocnemius tendon: collagen fibrils average diameter

Chart I (3)-B: 1-week-old female chicken gastrocnemius tendon: collagen fibrils cross-sectional area
Chart I (4)-A: 2.5 weeks old control female chicken gastrocnemius tendon: collagen fibrils average diameter

Chart I (4)-B: 2.5 weeks old exercised female chicken gastrocnemius tendon: collagen fibrils average diameter
Chart I (5)-A: 2.5 weeks old control female chicken gastrocnemius tendon: collagen fibrils cross-sectional area

Chart I (5)-B: 2.5 weeks old exercised female chicken gastrocnemius tendon: collagen fibrils cross-sectional area
Chart I (6)-A: 5 weeks old control female chicken gastrocnemius tendon: collagen fibrils average diameter

Chart I (6)-B: 5 weeks old exercised female chicken gastrocnemius tendon: collagen fibrils average diameter
Chart I (7)-A: 5 weeks old control female chicken gastrocnemius tendon: collagen fibrils cross-sectional area

Chart I (7)-B: 5 weeks old exercised female chicken gastrocnemius tendon: collagen fibrils cross-sectional area
Chart I (8)-A: 6.5 weeks old control female chicken gastrocnemius tendon: collagen fibrils average diameter

Chart I (8)-B: 6.5 weeks old exercised female chicken gastrocnemius tendon: collagen fibrils average diameter
Chart I (9)-A: 6.5 weeks old control female chicken gastrocnemius tendon: collagen fibrils cross-sectional area

Chart I (9)-B: 6.5 weeks old exercised female chicken gastrocnemius tendon: collagen fibrils cross-sectional area
Chart I (10)-A: 5 weeks old exercised male chicken gastrocnemius tendon: collagen fibrils average diameter

Chart I (10)-B: 5 weeks old exercised male chicken gastrocnemius tendon: collagen fibrils cross-sectional area
Chart I (11)-A: 6.5 weeks old control male chicken gastrocnemius tendon: collagen fibrils average diameter

Chart I (11)-B: 6.5 weeks old control male chicken gastrocnemius tendon: collagen fibrils cross-sectional area
Figure I (1)-A: 1-day-old Control Female chicken Gastrocnemius Tendon
Longitudinal-section Bottom part: IHC shows moderate staining for decorin, light microscope x 100

Figure I (1)-B: 1-day-old Control Female chicken Gastrocnemius Tendon
Longitudinal-section Top part: IHC shows intensive staining for decorin, light microscope x 100
Figure I (2)-A: 1-week-old Control Female chicken Gastrocnemius Tendon
Longitudinal-section Bottom part: IHC shows intensive staining for decorin, light microscope x 100

Figure I (2)-B: 1-week-old Control Female chicken Gastrocnemius Tendon
Longitudinal-section Top part: IHC shows intensive staining for decorin, light microscope x 100
Figure I (3)-A: 5 weeks old Control Female chicken Gastrocnemius Tendon Longitudinal-section Bottom part: IHC shows moderate staining for decorin, light microscope x 100

Figure I (3)-B: 5 weeks old Control Female chicken Gastrocnemius Tendon Longitudinal-section Middle part: IHC shows weak staining for decorin, light microscope x 100

Figure I (3)-C: 5 weeks old Control Female chicken Gastrocnemius Tendon Longitudinal-section Top part: IHC shows moderate staining for decorin, light microscope x 100
Figure I (4)-A: 5 weeks old Exercised Female chicken Gastrocnemius Tendon Longitudinal-section Bottom part: IHC shows intensive staining for decorin, light microscope x 100

Figure I (4)-B: 5 weeks old Exercised Female chicken Gastrocnemius Tendon Longitudinal-section Middle part: IHC shows weak staining for decorin, light microscope x 100

Figure I (4)-C: 5 weeks old Exercised Female chicken Gastrocnemius Tendon Longitudinal-section Top part: IHC shows moderate staining for decorin, light microscope x 100
Figure I (5)-A: 6.5 weeks old Control Female chicken Gastrocnemius Tendon Longitudinal-section Bottom part: IHC shows moderate staining for decorin, light microscope x 100

Figure I (5)-B: 6.5 weeks old Control Female chicken Gastrocnemius Tendon Longitudinal-section Top part: IHC shows moderate staining for decorin, light microscope x 100
Figure I (6)-A: 6.5 weeks old Exercised Female chicken Gastrocnemius Tendon Longitudinal-section Bottom part: IHC shows intensive staining for decorin, light microscope x 100

Figure I (6)-B: 6.5 weeks old Exercised Female chicken Gastrocnemius Tendon Longitudinal-section Middle2 part: IHC shows weak staining for decorin, light microscope x 100

Figure I (6)-C: 6.5 weeks old Exercised Female chicken Gastrocnemius Tendon Longitudinal-section Middle1 part: IHC shows intensive staining for decorin, light microscope x 100
Figure I (6)-D: 6.5 weeks old Exercised Female chicken Gastrocnemius Tendon Longitudinal-section Top part: IHC shows weak staining for decorin, light microscope x 100
Figure II (1)-A: 1-day-old Female chicken Gastrocnemius Tendon Cross-section Bottom part: TEM x 20,000

Figure II (1)-B: 1-day-old Female chicken Gastrocnemius Tendon Cross-section Middle part: TEM x 20,000

Figure II (1)-C: 1-day-old Female chicken Gastrocnemius Tendon Cross-section Bottom part: TEM x 20,000
Figure II (2)-A: 6.5 weeks old Control Female chicken Gastrocnemius Tendon Cross-section Bottom part: TEM x 20,000

Figure II (2)-B: 6.5 weeks old Control Female chicken Gastrocnemius Tendon Cross-section Middle part: TEM x 20,000

Figure II (2)-C: 6.5 weeks old Control Female chicken Gastrocnemius Tendon Cross-section Top part: TEM x 20,000
Figure II (3)-A: 2.5 weeks old Exercised Female chicken Gastrocnemius Tendon Cross-section Bottom part: TEM x 20,000

Figure II (3)-B: 2.5 weeks old Exercised Female chicken Gastrocnemius Tendon Cross-section Middle part: TEM x 20,000

Figure II (3)-C: 2.5 weeks old Exercised Female chicken Gastrocnemius Tendon Cross-section Top part: TEM x 20,000
Figure II (4)-A: 6.5 weeks old Exercised Female chicken Gastrocnemius Tendon Cross-section Bottom part: TEM x 20,000

Figure II (4)-B: 6.5 weeks old Exercised Female chicken Gastrocnemius Tendon Cross-section Middle part: TEM x 20,000

Figure II (4)-C: 6.5 weeks old Exercised Female chicken Gastrocnemius Tendon Cross-section Top part: TEM x 20,000
Figure II (5)-A: 1-day-old Male chicken Gastrocnemius Tendon Cross-section Bottom part: TEM x 20,000

Figure II (5)-B: 1-day-old Male chicken Gastrocnemius Tendon Cross-section Middle part: TEM x 20,000

Figure II (5)-C: 1-day-old Male chicken Gastrocnemius Tendon Cross-section Top part: TEM x 20,000
Figure II (6)-A: 6.5 weeks old Control Male chicken Gastrocnemius Tendon Cross-section Bottom part: TEM x 20,000

Figure II (6)-B: 6.5 weeks old Control Male chicken Gastrocnemius Tendon Cross-section Middle part: TEM x 20,000

Figure II (6)-C: 6.5 weeks old Control Male chicken Gastrocnemius Tendon Cross-section Top part: TEM x 20,000
Figure II (7)-A: 5 weeks old Exercised Female chicken Gastrocnemius Tendon Cross-section Bottom part: TEM x 20,000

Figure II (7)-B: 5 weeks old Exercised Female chicken Gastrocnemius Tendon Cross-section Middle part: TEM x 20,000

Figure II (7)-C: 5 weeks old Exercised Female chicken Gastrocnemius Tendon Cross-section Top part: TEM x 20,000
Figure II (8)-A: 5 weeks old Exercised Male chicken Gastrocnemius Tendon Cross-section Bottom part: TEM x 20,000

Figure II (8)-B: 5 weeks old Exercised Male chicken Gastrocnemius Tendon Cross-section Middle part: TEM x 20,000

Figure II (8)-C: 5 weeks old Exercised Male chicken Gastrocnemius Tendon Cross-section Top part: TEM x 20,000
Figure III (1)-A: 1-day-old Female chicken Gastrocnemius Tendon Longitudinal section Bottom part: SEM x 12,000

Figure III (1)-B: 1-day-old Female chicken Gastrocnemius Tendon Longitudinal section Middle part: SEM x 12,000

Figure III (1)-C: 1-day-old Female chicken Gastrocnemius Tendon Longitudinal section Bottom part: SEM x 12,000
Figure III (2)-A: 6.5 weeks old Control Female chicken Gastrocnemius Tendon Longitudinal section Bottom part: SEM x 12,000

Figure III (2)-B: 6.5 weeks old Control Female chicken Gastrocnemius Tendon Longitudinal section Middle part: SEM x 12,000

Figure III (2)-C: 6.5 weeks old Control Female chicken Gastrocnemius Tendon Longitudinal section Top part: SEM x 12,000
Figure III (3)-A: 2.5 weeks old Exercised Female chicken Gastrocnemius Tendon Longitudinal section Bottom part: SEM x 12,000

Figure III (3)-B: 2.5 weeks old Exercised Female chicken Gastrocnemius Tendon Longitudinal section Middle part: SEM x 12,000

Figure III (3)-C: 2.5 weeks old Exercised Female chicken Gastrocnemius Tendon Longitudinal section Top part: SEM x 12,000
Figure III (4)-A: 6.5 weeks old Exercised Female chicken Gastrocnemius Tendon Longitudinal section Bottom part: SEM x 12,000

Figure III (4)-B: 6.5 weeks old Exercised Female chicken Gastrocnemius Tendon Longitudinal section Middle part: SEM x 12,000

Figure III (4)-C: 6.5 weeks old Exercised Female chicken Gastrocnemius Tendon Longitudinal section Top part: SEM x 12,000
Figure III (5)-A: 1-day-old Male chicken Gastrocnemius Tendon Longitudinal section Bottom part: SEM x 12,000

Figure III (5)-B: 1-day-old Male chicken Gastrocnemius Tendon Longitudinal section Middle part: SEM x 12,000

Figure III (5)-C: 1-day-old Male chicken Gastrocnemius Tendon Longitudinal section Top part: SEM x 12,000
Figure III (6)-A: 6.5 weeks old Control Male chicken Gastrocnemius Tendon Longitudinal section Bottom part: SEM x 12,000

Figure III (6)-B: 6.5 weeks old Control Male chicken Gastrocnemius Tendon Longitudinal section Middle part: SEM x 12,000

Figure III (6)-C: 6.5 weeks old Control Male chicken Gastrocnemius Tendon Longitudinal section Top part: SEM x 12,000
Figure III (7)-A: 5 weeks old Exercised Female chicken Gastrocnemius Tendon Longitudinal section Bottom part: SEM x 12,000

Figure III (7)-B: 5 weeks old Exercised Female chicken Gastrocnemius Tendon Longitudinal section Middle part: SEM x 12,000

Figure III (7)-C: 5 weeks old Exercised Female chicken Gastrocnemius Tendon Longitudinal section Top part: SEM x 12,000
Figure III (8)-A: 5 weeks old Exercised Male chicken Gastrocnemius Tendon Longitudinal section Bottom part: SEM x 12,000

Figure III (8)-B: 5 weeks old Exercised Male chicken Gastrocnemius Tendon Longitudinal section Middle part: SEM x 12,000

Figure III (8)-C: 5 weeks old Exercised Male chicken Gastrocnemius Tendon Longitudinal section Top part: SEM x 12,000
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0 = no reaction  
1 = weak reaction  
2 = reaction  
3 = intensive reaction

**Table I: Immunoreactivity for Decorin**

Decorin was detected by sensitive and specific immunohistochemical analysis with a biotin-avidin horseradish peroxidase method
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Table II: SEM results (um)

Diameters of each fibril in each SEM electronmicrograph were measured manually and calculated to get averaged values.