AN IN VIVO AND IN VITRO MORPHOLOGICAL STUDY OF EPITENON CELL POPULATIONS IN CHICKENS

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PREFACE

The following study was conducted to classify cell populations found in the epitenon of the deep digital flexor tendons of chickens. Tendons heal very slowly and in many cases the prognosis of mechanical recovery following repair is very poor.

Although various surgical and grafting procedures exist, satisfactory healing is dependent on achieving a balance between initial immobilization and passive controlled motion following repair. Well-defined post-operative exercise regimens are not readily available to deal with the diversity of tendon injuries and patient variability. What is needed is an additional treatment option to reduce post-operative healing time.

One approach would be the incorporation of tendon cells at the site of injury to decrease the time of immobilization. Cell populations present in the epitenon are actively involved in healing, however, the various epitenon populations have not been well characterized. The following study was designed to classify epitenon cell populations of the chicken deep digital flexor tendon which has been previously used as a model for the study of tendon healing.

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Chapter I

INTRODUCTION AND LITERATURE REVIEW

HISTORICAL PERSPECTIVE

In reviews of the historical background of tendons by Mason and Sheardon (1932) and Forster (1989), it is concluded that tendons were described as early as the second century A.D. by Galen and that his description overlapped with that of nerves. It was not until the eighteenth century that von Haller distinguished tendons from nerves by showing tendons to be relatively insensitive structures compared to nerves (Mason and Sheardon, 1932; Forster, 1989). According to Forster (1989), tendons were identified as a form of connective tissue by Bichat (1802) who observed tendons to be made of the same fibers (now known as collagen) that are also present in ligaments, joint capsules and fasciae.

GROSS MORPHOLOGY

Tendons are dense white fibrous structures that are distinguished from other connective tissue structures such as ligaments, interosseous membranes and fasciae by the fact that tendons join skeletal muscle fibers to their points of attachment, which are often on bone (Forster, 1989).

Tendon shape and size vary considerably. They may be thick bands with concave lateral borders such as the prepubic tendon, which is the common insertion for the abdominal muscles in the horse. They may pass through the length of the muscle belly itself from origin to insertion - such as the internal tendon of the biceps brachii in the horse. Tendons may be short such as the common tendon of insertion of the flexor carpi ulnaris muscle in the forelimb of dog which extends from the level of the distal third of the radius to the accessory carpal bone (Dyce et al., 1987). Tendons may be long such as the deep digital flexor tendons on the caudal and palmar surface of the forelimb in the dog, which extend from the level of the distal third of the radius and traverse the length of the metacarpals and phalanges before insertion onto the distal phalanges (Dyce et al., 1987).

Based on the presence or absence of a protective covering of synovial tissues, tendons are classified as either sheathed or non-sheathed tendons. The synovial sheath consists of outer and inner synovial membranes which are continuous on the deep surface of the tendon forming a double layered structure called the mesotendon (Dyce et al., 1987). Sheathed tendons are usually long tendons subject to significant gliding movement or change in direction over joints such as the tendon of the deep digital flexor muscles on the plantar surface of the pelvic limb in chickens. Tendons that do not possess a synovial

sheath such as the common calcanean tendon in dog is an example of paratenon covered or non-sheathed tendon.

The nature of the vascular supply in tendons depends on whether the tendon is sheathed or non-sheathed. Chaplin (1973) demonstrated with microradioangiography that paratenon covered tendons are substantially more vascular than sheathed tendons. In non-sheathed tendons, blood vessels that branch out to supply the tendon matrix in a diffuse manner are found throughout the paratenon along the entire length of the tendon. In sheathed tendons, blood supply is restricted to the mesotendon found along the deep surface of the tendon (St. Clair, 1975). In regions of extensive gliding, the mesotendon is modified to form intermittent elastic structures called vinculae (Peacock, 1959). Vinculae occur at mid-phalangeal regions in chickens and serve as conduits for the blood vessels, lymphatics and nerves that supply the tendon (Beckham and Greenlee, 1975).

HISTOLOGY

Histologically, tendons are a dense regular form of connective tissue consisting of vast quantities of extracellular matrix and relatively few cells (Wheater, 1993). Parry et al. (1978) demonstrated that fibrillar collagen content increases steadily from birth to adult age where it remains relatively constant and then slowly drops in aged horses.

Extracellular Matrix Components

The collagen content of adult flexor tendons is 75 % by dry weight in chickens (Banes et al., 1987). Collagen types include 85 - 95 % of type I collagen fibers, type II collagen in fibrocartilage areas, type III associated with blood vessels and epitenon, type V found in the basal lamina of endothelium, and type VI found diffusely throughout the myotendinous junction of the chicken tendon (Swasdison and Mayne, 1989). According to a review by Elliott (1965), collagen fibers are embedded in a small quantity of viscous mucopolysaccharide which constitutes less than 1% of the tendon. Variable amounts of structural matrix proteins such as elastin (Wortham, 1948), fibronectin and laminin (Swasdison and Mayne, 1989) are also present.

A transverse section from the phalangeal region of the deep digital flexor tendon from chicken is usually elliptical or round demonstrating the presence of collagen fibers arranged in bundles or fascicles. The assembly of tropocollagen into its structural hierarchy occurs extracellularly with its formation of the collagen fiber (Kastelic et al., 1977). Several fibers are organized together to form a primary bundle or fascicle which is surrounded by connective tissue called endotenon. Several primary bundles are grouped

together to constitute secondary bundles. Occasionally, two or three secondary bundles are organized into tertiary bundles. Connective tissue surrounding secondary and tertiary bundles is known as peritenon (Figure 1, pg. 48).

The outermost connective tissue investment is called the epitenon. It is usually two to three cell layers in depth (Gelberman et al.,1983) and demonstrates regional variation along the length of the tendon (Greenlee et al., 1975). The epitenon also contains type I and type III collagen fibers (Riederer-Henderson et al., 1983) and in areas of cartilage, type II collagen fibers are found.

In the case of sheathed tendons, the visceral (inner layer of the synovial sheath) synovial membrane has a layer of synovial cells which is the outermost cell layer of the epitenon. The parietal (outer layer of synovial sheath) synovial membrane blends with periosteum and loose connective tissues of the skin and fascia. Between the outer parietal and inner visceral synovial membranes, lies the synovial space in which synovial fluid circulates (Greenlee et al., 1975). In non-sheathed tendons, the epitenon blends into adjacent loose areolar connective tissue called paratenon.

In summary, a histological description of tendon fiber organization reveals that collagen bundles consisting primarily of dense regular type I collagen are held together by means of a dense irregular connective tissue frame-work. This connective tissue frame-work is named epitenon, peritenon and endotenon based on topographical location. These connective tissue investments are oriented perpendicular to the collagen fibers (Greenlee et al., 1975), and may allow independent movement of collagen fibers or fascicles (Woo and Buckwalter, 1987).

Cellular Components

Tendon fibroblasts or tendinocytes are the most common cells observed in histological sections of tendons. Tendinocytes produce and maintain type I collagen within the primary collagen bundles and have been referred to as wing cells or stellate cells because of their extensive cytoplasmic processes which surround the collagen fibers (Elliott, 1965). They are star shaped in transverse sections between collagen fibers in primary collagen bundles, and are characteristically spindle-shaped, oriented parallel to the collagen fibers in longitudinal sections. The cell boundaries are not readily distinguished from the surrounding eosinophilic type I collagen fibers under standard light microscopic techniques.

Cell types found in the connective tissue investments, namely, endotenon, peritenon and epitenon are mainly fibroblasts (Greenlee et al., 1975) though not necessarily oriented parallel to the longitudinal axis of the tendon (Woo and Buckwalter, 1987). Their characteristic spindle-shaped nuclei are often seen in transverse histological sections of the tendon.

The cell populations of the epitenon include synovial cells, epitenon fibroblasts and cartilage cells. Tendon synovial cells in the chick embryo constitute the outermost layer adjacent to the synovial space. Electron microscopy reveals fine filaments in these cells (Greenlee et al., 1975). Literature on chicken tendon synovial cells in vivo is limited to this study.

A review of literature on joint capsule synovial cells in mammals reveals the presence of two cell types (Barland et al., 1962; Williamson et al., 1966; Southwick and

Bensch, 1971). The first cell type is phagocytic having vacuoles as the type A cell (Barland et al., 1962), which is described as "V" cell containing large vacuoles by Southwick and Bensch (1971), or as type I cell reported by Williamson et al. (1966) containing membrane bound organelles or lysosomes. This phagocytic cell type contains a large round or oval nucleus (Castor, 1960: Williamson et al., 1966).

The second cell type is involved in protein synthesis, having large amounts of ergastoplasm as the type B cell described by Barland et al. (1962) which is the ER cell with dilated cisternae described by Southwick and Bensch (1971), or as the type II cell which accounts for the basophilia described by Williamson et al. (1966). This synthetic cell type has a dark staining nucleus (Southwick and Bensch, 1971; Williamson et al., 1966).

An ultrastructural study of joint synovial membrane in chickens (Luckenbill and Cohen, 1967) suggested that there is no morphologic division of synoviocytes into distinct phagocytic or synthetic types. Information distinguishing synovial cells and epitenon fibroblasts in vivo and in vitro has not been found.

Electron microscopy revealed epitenon fibroblasts deep to the synovial cells with extensive rough endoplasmic reticulum and secretory vesicles (Mass and Tuel, 1990). Whether or not the epitenon fibroblasts are the type B synovial cells is unknown. Cartilage cells are found on the surface in regions along the length of the tendon closer to bone. They are seen as cuboid cells in lacunae with foamy cytoplasm (Greenlee et al., 1975).

Epitenon cell populations have been demonstrated to contribute to successful tendon healing (Gelberman 1983; 1985; Mass and Tuel 1990). In a study of flexor tendon healing in dogs treated with total immobilization and early controlled passive mobilization, it was shown that with total immobilization, the site of healing consisted of ingrowth of reparative tissue from the digital sheath leading to adhesion formation and loss of gliding function. Comparatively, early passive mobilization resulted in fibroblast proliferation from the epitenon itself, presumably reducing adhesion formation and resulting in the maintenance of gliding function of tendons.

In summary, three epitenon cell populations are identified, namely, synovial cells, epitenon fibroblasts and cartilage cells. However, no definite markers or morphological criteria currently exist to distinguish epitenon fibroblasts from synovial cells and cartilage cells in vitro. It is important to know more about epitenon fibroblasts so that they can be cultured in vitro and incorporated directly into the site of injury. The following study was directed at providing distinguishing criteria for synovial cells, epitenon fibroblasts and cartilage cells in vitro.

TENDON INJURY, HEALING AND REPAIR

Tendon injuries can be divided into two broad categories, namely, mechanical damage and degenerative damage (Fackleman, 1973). Rupture or mechanical damage occurs through abrupt loss of continuity between the tendon fibers, whereas degenerative changes produce gradual loss of elasticity leading to disruption.

Tendon injuries range from relatively small tears to complete rupture of the tendon collagen bundles. Following injury, the healing process proceeds slowly and is associated with vascularity (Peacock, 1959). Healing of vascular or paratenon covered tendons is well described (Flynn and Graham, 1965), and, although adhesion formation occurs, it does not usually affect function. Such tendons generally regain satisfactory mechanical strength post-healing. Healing of sheathed tendons however, is relatively complicated because of limited vascularization and the presence of a tendon sheath. Sheathed tendons take a longer time to heal and adhesion formation interferes with satisfactory gliding function post-healing.

Hence, satisfactory tendon healing depends on a delicate balance between immobilization to allow strong repair and early passive motion to prevent adhesion formation (Mason and Sheardon, 1932).

Tendons heal by extrinsic and /or intrinsic healing mechanisms (Gelberman et al. (1985). Early studies (Potenza, 1962; Lindsay and Birch, 1964; Peacock, 1964) demonstrated the importance of extrinsic sources of cell populations for healing. These populations include fibroblasts from the outer layer of the synovial sheath, periosteum, subcutaneous tissue, the dermis and elements of the immune system in blood. More recent

studies however, demonstrate the importance of intrinsic cell populations involved in the healing process (Lundborg and Rank, 1980; Gelberman et al., 1982). The latter populations are derived from within the epitenon and endotenon connective tissues of the tendon. It is clear that tendons heal by both extrinsic and intrinsic activity and the relative contributions of these two cell populations depends on the type and site of the tendon injury as well as post-operative management (Gelberman, 1983). However, post-operative healing regimens aimed at optimization of the duration and the level of exercise have been essentially empirical, since it is dependent on variables such as the diversity of type of tendon injuries, patient variability and temperament. Although intrinsic healing mechanisms have been demonstrated, showing proliferation and production of collagen by cell populations of epitenon and endotenon, the specific cell types in the epitenon and endotenon have not been studied.

Surgical approaches available for repair include numerous suture techniques that attempt to take advantage of the mechanical strength of the tendon (Pijanowski et al., 1989). Suturing is used to conjoin both ends of a ruptured tendon. Tensile strength of repair during the first two weeks is primarily dependent on the strength and pattern of the suture (Mason and Allen, 1941). Orientation of the collagen fibers in tendon does not support suture patterns which are applied parallel to the longitudinal axis. On the other hand, patterns arranged perpendicular to the longitudinal axis of the tendon provide mechanical strength but at the expense of blood flow that compromises repair. Mechanical strength of a suture pattern alone is an insufficient measure of the likelihood of successful tendon healing. Also, the presence of sutures as foreign body material

magnifies the inflammatory response which contributes to an extrinsic population of cells responsible for adhesion formation.

Another approach used is grafting wherein a gap between the ruptured tendon ends precludes conjoining with sutures. In a comparative study (Abrahamsson and Gelberman, 1994) between healing of extrasynovial and intrasynovial tendon grafts within synovial sheaths, it was observed that the surface cells of the intrasynovial tendons show greatly reduced adhesion formation with an epitenon primarily composed of 2 - 3 layers of spindle-shaped cells and a few rounded cells. Cell proliferation and neovascularization is not extensive, leaving the endotenon preserved. It is apparent from this study that the surface cells of intrasynovial tendons are well suited for cellular survival and tendon gliding. However, survival of grafts depends on host compatibility and immune response mechanisms. Since grafting necessiates suturing, the associated problems of suture patterns, excessive inflammatory response, adhesion formation, diminished circulation and gap formation occur.

Additional treatment approaches to tendon healing which may augment intrinsic healing mechanisms are needed. One such possibility may be the incorporation of in vitro reared epitenon fibroblasts at the tendon injury site. These cells may decrease the time of immobilization, thereby initiating early post-operative passive motion sooner. It is important to incorporate cultured cells that are known to be involved in healing such as the epitenon or endotenon cell populations.

TENDON CELL ISOLATION TECHNIQUES

Dehm and Prockop (1971) digested "leg" tendons from the phalangeal region of 17 day old White Leghorn chick embryos with 2.5 % trypsin and 0.1 % collagenase in approximately 40 minutes at 37 °C in 5 % carbon dioxide atmosphere with continuous agitation. The hydrolysate was centrifuged at 600 X *g* for 3 minutes at room temperature and the cells obtained were 88 % viable. No quantitative data was reported to indicate total numbers of cells isolated. Their incomplete morphological description of isolated cells indicate that at least two cell populations were present. One population consisted of rounded cells with sharp borders. This constituted the majority of the cells and were presumably tendinocytes. The other population described consisted of a few cells with large vacuoles with a low nuclear to cytoplasmic ratio. It is possible that these cells are similar to the phagocytic synovial cell type described by Barland et al. (1962), or the Type I cells described by Williamson et al. (1966), or to the V cells described by Southwick and Bensch (1971).

Another study of 17 day old chick embryo tendon cell cultures was done by Riederer-Henderson et al. (1983). Here, two cell populations, namely, synovial cells and tendon fibroblasts are reported. The cell population presumed to be synovial cells was reported to take a longer time to contact the substrate than the tendon fibroblast population. Synovial cells spread out more rapidly and were quite adherent to the substrate. It is postulated (Riederer-Henderson et al., 1983) that the synovial cells probably need to secrete attachment proteins of a different type or in different amounts before attachment to the substrate compared to the tendon fibroblast population.

In the Riederer-Hendersons study, the cells were isolated following a 20 minutes digestion of deep digital flexor tendons with 0.25 % trypsin and 0.2 % collagenase. Upon isolation, epitheloid cells were occasionally observed but the morphology of the majority of the cells isolated was not described. Upon plating, the synovial and tendinocyte populations were both reported to assume fibroblast-like characteristics.

In a study of tendon cell populations from eight week old chickens by Banes et al. (1988), a population of presumed synovial cells was obtained using elaborate enzymatic and physical treatment of flexor tendons obtained from the region over the tarsometatarsus bone of the pelvic limbs.

Two procedures were utilized for the isolation of the synovial cell population. One procedure involved isolation of cells from the supernatant of a hydrosylate (900 X *g*) from tendons that were digested for 4 hours with 0.5 % collagenase. The second procedure provided cells in a pellet formed following digestion with 0.25 % trypsin in Hank's Salt Solution for 15 minutes at 37 °C following centrifugation at 1,200 X *g* . Viability and total cell counts were not reported.

Compared to cells isolated from the tendon core, the synovial cells were larger, more flattened cells with granules. The size of the granules was not reported. On contact with the substratum, spherical cells from the presumed synovial cell population measured 28 ± 8 μm in diameter. On staining with Sudan black, it was noted that although some vesicle-containing cells demonstrated the presence of lipid, such cells were found adjacent to similar cells that did not stain at all.

In an abstract by Brigman et al. (1994), flattened cells filled with large multiple vacuoles were described as synovial cells. It was postulated that these vacuoles contained phospholipids secreted by the cells for boundary lubrication of the tendon (Brigham et al., 1994).

In the study by Banes et al. (1988), three morphologically recognizable forms of presumed synovial cells has been reported two hours post-plating. The first form measured 71 ± 8.5 μm X 64 ± 2.1 μm and consisted of round to oval cells with clear cytoplasm and large refractile bodies, with relatively little ruffling around their edges. The size of the large refractile bodies was not measured. The second form measured 111 ± 9.2 μm X 98 ± 19 μm , and were round to oval cells with clear cytoplasm, short pseudopods and an active ruffling border. The third form measured 49.5 ± 9.2 μm X 57 ± 16 μm and were irregular cells with 3 - 5 blunt pseudopods having dense granules at their ends. The cytoplasm also possessed dense granules and refractile bodies. The refractile bodies may have been lipid containing vacuoles or cell organelles. The size and nature of the dense granules was not reported.

In summary, the cell populations isolated from 17 day old embryos and 8 week old chickens obtained from the surface of tendons by brief enzyme digestion were presumed to be synovial cells. However, it was also demonstrated that the cell isolate consisted of a non-homogenous population of cells whose morphological descriptions remain presently unclear. The following study is a quantitative approach designed to provide in vitro and in vivo information on epitenon cell populations.

Chapter II

EXPERIMENTS

Experiment I: In vitro isolation of epitenon cell populations from deep digital flexor tendons from four and five week old chickens

Objective

The following experiment was conducted in order to isolate the epitenon cell populations from the deep digital flexors of four and five week old chickens.

Materials and Methods

Two male Cobb - X chickens (Tyson Farms, AR, USA) at the ages of four weeks and five weeks were killed by carbon dioxide asphyxiation. The deep digital flexor tendons over digits II, III and IV were isolated in a sterile manner. A transverse incision on the plantar surface was made at the level of the tarsometatarso-phalangeal joints. Subsequently, transverse incisions were made over the head of the ungual phalanx of each digit. The deep digital flexor tendons were individually extracted using a pair of artery forceps following a gentle tug and release action. Sterile isolated tendons were placed in a petri-dish containing sterile transport medium (100 ml Hank's Balanced Salt Solution (Cat. # 14060-016, Gibco BRL, N.Y., USA), 0.05 ml of 10mg/ml Gentamicin (G 1272, Sigma Chemical, St.Louis, MO, USA). Segments measuring 2-5 mm in length were

removed from isolated tendons of each digit, fixed in 10 % neutral buffered formalin (NBF - 10 ml of 37-40 % formaldehyde in 90 ml of water, 0.034 M $NaH_2PO_4 \cdot H_2O$ and 0.031 M $Na_2 HPO_4$) and used as controls to verify epitenon digestion. The isolated tendons were pooled within an age group. Their total weight was determined and they were placed in a 125 ml sterile flask with a magnetic stirrer for digestion in 10 ml of collagenase solution (50 mg type V-S, C-2014, Sigma Chemical, St.Louis, MO, USA). Collagenase solution (0.5 %) was freshly prepared using 100 ml of Dulbecco's Minimal Essential Medium (D-8913, Sigma Chemical, St.Louis, MO, USA), 50 mg of type V-S collagenase, 2 ml of Seru-Max (S-8894, Sigma Chemical, St.Louis, MO, USA) and 0.05 ml of 10mg/ml Gentamicin. Trypsin (as used by Banes et al., 1988) was not preferred being a less specific proteolytic enzyme and more harsh on the cell membranes than collagenase. Digestion was conducted under constant agitation in a water bath maintained between 35-37 °C for 20 minutes. The digested hydrolysate was collected and centrifuged at 1,000 X *g* for 5 minutes at 4° C. The cell pellet was re-suspended in growth medium

(100 ml Dulbecco's MEM with 10 ml Seru-Max and 0.05 ml of 10mg/ml Gentamicin). Viability and total cells were determined using 0.4 %, w/v trypan blue stain (T-8154, Sigma Chemical, St.Louis, MO, USA) and a standard hemocytometer. Cell measurements (length and width) were taken for at least 100 viable cells using a 100 division eyepiece micrometer scale. Cells were classified based on shape and size. Based on shape, cells were classified as spherical if the

length was equal to the width. They were classified as oval if the ratio of length to width was ≥ 2 and < 3, and as elongate if the length to width ratio was ≥ 3. Based on length, cells were tabulated in the ranges as ≤ 9.99 μm, 10-14.99 μm, 15-19.99 μm, 20-24.99 μ m and ≥ 25 μm.

The remaining undigested tendon segments were fixed in 10 % neutral buffered formalin. Segments were embedded in paraffin and 5 μm transverse histological sections were routinely prepared and stained with hematoxylin and eosin (Coolidge and Howard, 1979). Sections were examined under bright-field microscopy and the extent of epitenon removed was noted.

Results

Epitenon digestion: Comparative examination of tendon histological sections taken prior to (Figure 1, pg. 48) and following enzymatic digestion (Figure 2, pg. 48) for 20 minutes with collagenase revealed incomplete epitenon digestion in all sections for both age groups.

As seen in figure 3 (pg. 49), 83 ± 9 % of isolated surface cells in both age groups were found to be spherical cells 10 - 19.99 μm in diameter. Spherical cells with a diameter ≤ 9.99 μm constituted 4.5 ± 0.5 % of the cells. The percentage of cells ≥ 20 μm in diameter was 10 ± 7 %.

Viability and Total Cells: The total number of cells obtained per mg of tendon tissue in four week old birds was $1.13 \times 10^6 \pm 0.07 \times 10^6$ with a viability of 41.1 %. The total number of cells obtained per mg of tendon tissue in five week old birds was $4.26 \times 10^6 \pm 0.13 \times 10^6$ with a viability of 60.4 % (Table 1, pg. 43).

Discussion

On comparative examination of histological sections prior to and following enzymatic treatment tendons with collagenase from both four and five week old birds demonstrated incomplete epitenon digestion. Possible reasons for incomplete epitenon digestion as compared to the study by Banes et al. (1988) include age, functional and regional variation. In this study, four and five week old birds were used compared to eight week old birds used by Banes et al. (1988). Functionally, tendons over the tarsometatarsal region are relatively less flexed during movement compared to the tendons over the phalangeal region. Anatomically, tendons over the phalangeal region of the chicken are similar to the human deep digital flexors of the fingers (Lindsay and Thomson, 1960). Also, tendons in the tarsometatarsal region have more extensive connective tissue between them and from the report by Banes et al. (1988), it is unclear if the tendons digested were separated from these outer layers of parietal tissue. The phalangeal region used in this study was preferred over the tarsometatarsal region used by Banes et al. (1988) since tendon injuries in the phalangeal region are common and have been studied extensively.

Other possible reasons for incomplete epitenon digestion may be either the absence of enzyme trypsin during tendon digestion or the ratio of collagenase to tendon collagen. Since collagenase is more substrate specific than trypsin, some extracellular matrix components may not have been digested sufficiently to release all cells. In previous studies by Banes et al. (1988) and Riederer-Henderson et al. (1983), trypsin was used and the epitenon was reported to be completely digested,

however, total cells obtained and cell viability were not reported. Trypsin is known to be a relatively harsh enzyme capable of damaging cell membranes compared to collagenase. Since recovery of viable cells for measurements was required in our study, the use of collagenase alone was preferred. In this experiment it was seen that an increase in the ratio of collagenase to tendon collagen from 0.05 ml per mg of tendon tissue in four week old birds to 0.21 ml per mg of tendon tissue in five week old birds still did not completely digest the epiternon. However, an increase in total cells and cell viability was observed (Table 1, pg. 43). The determination of a time period using only collagenase digestion to isolate epitenon cells became critical to future work.

Of the cells obtained, 83 ± 9% of the spherical cells were found in the 10 - 19.99 μm size-range which based on size are presumably surface synovial cells. This was in contrast to the majority of cells obtained by Banes et al. (1988) which measured 28 ± 8 μm in diameter. In our study, only 10 ± 7 % were found to measure ≥ 20 μm in diameter. It is possible that the cells in the size range reported by Banes et al. (1988) were not recovered because the epitenon was not completely digested in eight week old chickens or that such cells were not present in this study in four and five week old birds. Efforts were then concentrated on isolating all epitenon cell populations.

Experiment II: Determination of time period for complete epitenon digestion using deep digital flexor tendons of five week old chickens.

Objective

The following experiment was designed to determine what time period would be required to completely digest the epitenon from chicken deep digital flexor tendons using 0.5 % collagenase.

Materials and Methods

Two male five week old Cobb - X chickens were killed by carbon dioxide asphyxiation and the deep digital flexor tendons were removed using the technique outlined in experiment I. Tendons were placed in sterile transport medium. Segments measuring 2-5 mm in length were removed from isolated tendons of each digit, fixed in 10 % neutral buffered formalin and used as controls to verify epitenon digestion. The tendon pieces were then pooled and divided into three aliquots and digested separately with 10 ml of collagenase solution (0.5 %) for 45, 75 or 105 minutes respectively. Digestion was conducted under constant agitation in a water bath maintained between 35-37 °C. Following enzymatic digestion, hydrolysates were collected and centrifuged at 1,000 X *g* for 5 minutes at 4° C. Cell pellets were re-suspended in growth medium. Viability and total cells were determined for each sample using 0.4 % trypan blue stain. The remaining undigested tendons were fixed in 10 % neutral buffered formalin,

embedded in paraffin, sectioned and stained for examination to verify epitenon digestion.

Results

Epitenon digestion: Examination of histological sections taken following 45 minutes of collagenase digestion revealed the epitenon to be loosened but not removed. Also, all the epitenon cells were rounded up following enzymatic treatment. Digestion times of 75 and 105 minutes resulted in complete epitenon removal, similar to figure 6, pg. 51.

Viability and Total Cells: From Table 2 (pg. 43), it is seen that increasing the time and the ratio of collagenase solution to tendon collagen resulted in an increase in total number of cells isolated. Cell viability was not affected by length of digestion or by ratio of collagenase solution to tendon collagen used.

Discussion

This study was done to define the digestion time period for future experiments that would assure complete removal of the epitenon from flexor tendons. Examination of histological sections indicated that 75 or 105 minutes of collagenase digestion may be appropriate depending on the volume of collagenase used. The 105 minutes digestion time period was chosen for future studies to be conducted in 7-9 week old birds since no detrimental effects on cell viability was seen and since cross-linking between collagen fibers increases with age, a longer digestion time period would be required.

A standard digestion formulation of 0.02 ml / mg of tendon tissue was selected in order to have sufficient volume of enzyme solution to allow thorough mixing of tendon pieces. This volume was also sufficient to provide epitenon cells in concentrations siutable for future plating experiments.

Experiment III: In vivo and in vitro classification of epitenon cell populations in deep digital flexor tendons of seven, eight and nine week old chickens

Background

This study was conducted to describe cell populations of the epitenon in vivo and compare them to isolated epitenon cells in vitro. In addition, since spherical cells in the 28 ± 8 μm diameter range were not recovered in experiment I, the effect of centrifugation speed on recovery of cells larger than 20 μm was examined. Banes et al. (1988) reported that presumed synovial cells obtained from 8 week old birds measured 28 ± 8 μm in diameter upon isolation. In addition, these cells attached within two hours after plating to form spherical cells in three size ranges. It was also suggested (Banes et al., 1988; Brigham et al., 1994) that the population of cells obtained by brief enzyme digestion from the surface of the tendon contains phospholipid which aids in the smooth gliding of the tendon. Hence, the effect of varying centrifugation speed was tested under the hypothesis that larger cells would be found in the pellet at 1,500 X *g*.

Objectives

1) To measure synovial cells, epitenon fibroblasts and cartilage cells in vivo.

2) To isolate all the cell populations of the epitenon in seven, eight and nine week old birds.

3) To classify epitenon cells using shape and size measurements obtained following two hours post-plating.

4) To relate cells observed in vivo to cells classified in vitro.

5) To observe the effects of varying centrifugation speeds (500 X *g*, 1,000 X *g* and 1,500 X *g*), on cell viability, total cells recovered and cells greater than 20 μm in diameter.

Materials and Methods

Two male Cobb - X chickens at seven, eight and nine weeks of age were killed by carbon dioxide asphyxiation. The deep digital flexor tendons were isolated using the procedure reported in experiment I. Random samples measuring 2 - 5 mm in length were removed from tendons for histological evaluation and fixed in freshly prepared 10 % neutral buffered formalin. The remaining tendon pieces were then weighed and pooled for digestion in 2 ml of collagenase solution (0.5%) per 100 mg of tendon tissue. Enzymatic digestion proceeded with constant agitation in a water bath maintained between 35 - 37 °C for 105 minutes. Following collagenase digestion, the hydrolysate was equally divided into three conical tubes and centrifuged at 500 X *g*, 1,000 X *g* and 1,500 X *g* respectively for

5 minutes at 4° C. Cell pellets were re-suspended in growth medium. Aliquots (0.4 ml) of each suspension was used to determine total cells and viability using 0.4 % trypan blue stain. The remaining cell suspensions were plated at equal cell concentrations (0.5 X 10^6 cells per well in 24 well plates) and incubated for two hours at 37 ° C. Undigested tendon pieces were fixed in 10 % neutral buffered formalin. Cell measurements were recorded from at least 100 attached cells at each centrifugation speed using a 100 division eye-piece micrometer scale under phase-contrast microscopy. Cells were classified based on shape and size using the same criteria outlined in experiment I.

Formalin fixed pre- and post-enzymatic treated tendon segments were embedded in paraffin, sectioned at 5 μm and stained with hematoxylin and eosin. Sections from post-enzyme treated tendon pieces were used to evaluate epitenon digestion using bright-field microscopy. Sections from pre-enzymatic treated tendon segments were used to obtain cell measurements. Length versus width of at least 20 synovial and cartilage cells on transverse histological sections was recorded and used to calculate their size (mean ± SD). The following morphological and staining criteria were used to define epitenon cell populations in vivo.

Synovial cells: Since no histological description of tendon synovial cells was available, histological sections from interphalangeal joints of sheathed tendons from dog and opossum were studied. The synovial membrane with synovial cells were observed and assuming that synovial cells in the chicken epitenon are similar,

synovial cells were identified as plump cells in the outermost layer of the epitenon, with a round to oval nucleus and a deeply basophilic cytoplasm.

Epitenon fibroblasts: Found embedded in layers of dense irregular eosinophilic collagen fibers. These cells had eosinophilic cytoplasm with indistinguishable cell boundaries and a characteristic deeply basophilic spindle-shaped nucleus (Mass and Tuel, 1990).

Cartilage cells: Cuboid cells found in lacunae, frequently two to three cells together with eosinophilic foamy cytoplasm and round nuclei. Found in areas of fibrocartilage in the tendon (Greenlee et al., 1975).

Results

Examination of epitenon cell populations in transverse histological sections of pre-enzymatic tendon pieces taken from seven, eight and nine week old birds revealed the presence of three non-vascular cell populations in the epitenon. These cells were, synovial cells, epitenon fibroblasts, and cartilage cells. Cartilage cells in some areas were found in the peritenon.

The synovial cell population was found on the outermost layer of the epitenon. Synovial cells were present in a discontinuous layer. Two types of synovial cells were not morphologically evident. Synovial cells were plump cells with a deeply basophilic cytoplasm and a centrally located nucleus. The nucleus contained vesiculated heterochromatin. On an average, the cells measured 7.82 ± 1.44 μm X 5.59 ± 1.59 μm (Figure 1, pg. 48).

Epitenon fibroblasts were seen deep to the synovial cells. In areas where the synovial cells were absent, epitenon fibroblasts were present at the surface similar to figure 1 (pg. 48). Epitenon fibroblasts constituted the majority of the cells in the epitenon. Epitenon fibroblasts were recognized by their characteristic spindle-shaped nucleus. It was not possible to measure their size since cell boundaries could not be distinguished.

Cartilage cells were observed in two areas. Firstly, in areas of fibrocartilage (Figure 4, pg. 50), as in the plantar surface of the tendon. Here, the matrix was observed to be have eosinophilic collagen fibers. In all age groups, the

cartilage cells were observed in fibrocartilage areas where the epitenon merged into the peritenon between the deeper layers of the epitenon and the tendon fascicles. Secondly, close to areas of tendon insertion, the cartilage appeared to be a hyaline form (Figure 5, pg. 50). This form of cartilage was distinctly furrowed and the collagen fibers could not be seen. Cartilage cells in both forms contained uniform sized vacuoles measuring 2 μm within the eosinophilic cytoplasm. The nuclei were round and centrally located. The cells measured 14.81 ± 0.76 μm X 12.77 ± 0.57 μm.

Epitenon digestion: Examination of histological sections taken following 105 minutes enzymatic digestion with collagenase revealed complete epitenon removal in the seven (Figure 6, pg. 51) and nine week old birds, but incomplete digestion occurred in the eight week old birds.

A description of isolated cells from the epitenon plated for two hours revealed that the majority of cells from all age groups and at all centrifugation speeds were spherical in shape (Table 3, pgs. 44 - 46). Sizes of at least 100 cells post-plating from each centrifugation speed were recorded and classified based on shape and size ranges as in experiment I (Table 3, pgs. 44 - 46). It is seen from Table 3, that the majority of cells from all age groups and all centrifugation speeds are spherical in shape and in the ≤ 9.99 μm, 10 - 14.99 μm and 15 - 19.99 μm diameter size ranges. Relating cell measurements taken in vivo on transverse histological sections, to cells classified in vitro, it is postulated that cells in the

≤ 9.99 μm range are synovial cells and that a small proportion of cells in the 10 - 19.99 μm range are cartilage cells. Epitenon fibroblasts, which consituted the major epitenon population in vivo presumably constitute the majority of the cells found in vitro which lie in the 10 - 19.99 μm diameter size range.

An increase in the centrifugation speed did not effect the recovery of cells larger than 20 μm in diameter. In seven week old birds, the percentage of spherical cells ≥ 20 μm was 9 % at 500 and 1,000 X *g*, and 12 % at 1,500 X *g*. In nine week old birds, the percentage of spherical cells greater than 20 μm was 11 % at 500 X *g* and 14% at 1,000 and 1,500 X *g*. In eight week old birds, the percentage of spherical cells greater than 20 μm was 15% at 500 X *g*, 7% at 1,000 X *g* and 11% at 1,500 X *g* (Figure 7, pg. 52). Also, at all ages and centrifugation speeds, spherical cells ≥ 20 μm never accounted for more than 15 % of the total cells isolated. The majority of the cells were in the < 9.99 - 19.99 μm size range. An increase in centrifugation speed did not appear to have an effect on cells in the other size ranges.

Table 4 (pg. 47) demonstrates cell recovery in terms of the total cells isolated and their viability. The apparent decrease in viability with increasing centrifugation speed in all age groups was not significant ($p = 0.2$).

Discussion

The above experiment is an in vitro and in vivo study of the cell populations from the epitenon. Examination of histological sections following digestion confirmed complete digestion in seven and nine week old chickens and incomplete digestion in the eight week old chickens. Incomplete digestion may have occured due to individual variability. Previous experiments in eight week old birds (not reported here) revealed complete epitenon digestion using the same time period (105 minutes) digestion and same collagenase concentration (0.5% with standard formulation).

On examination of histological sections, it was observed that epitenon cell populations varied from region to region within the same tendon of a bird. For example, in regions at the tarsometatarsophalangeal joint and in regions of pulleys (close adherance of the synovial sheath to the tendon at interphalangeal joints), areas of fibrocartilage were seen. In mid-phalangeal regions, remnants of elastic vinculae were seen with blood vessels. The major part of the tendon however, constituted the epitenon with epitenon fibroblasts and synovial cells. Cell measurements of the synovial, epitenon fibroblasts and cartilage cell populations taken from transverse histological sections represent epitenon cell populations in vivo. Synovial cells and cartilage cells are spherical in vivo and in vitro. From partially digested epitenon histological sections, all epitenon cells (synovial cells and epitenon fibroblasts) were rounded up to form spherical cells in vitro.

On relating the cell populations found in vitro to the in vivo measurements, the in vivo cell measurements of synovial cells closely relate to the cells in the ≤ 9.99 μm diameter size range in vitro. The measurement of cartilage cells in vivo are in the middle of ranges 10 - 14.99 μm and 15 - 19.99 μm. The epitenon fibroblast population is presumably present in the range between 10 - 19.99 μm, since it constitutes the major population both in vivo and in vitro, and also since all epitenon cell populations round up to form spherical cells in vitro. Since the measurements of epitenon fibroblasts could not be taken in vivo, it is not clear whether they are larger, equal or smaller in size compared to cartilage cells. Also, it is not clear if the type, concentration and volume of collagenase used would have digested the cartilage matrix. One method to resolve the presence of cartilage cells would be to use a specific cell marker for cartilage cells such as antibodies to type II collagen.

For all age groups and at all centrifugation speeds, the population of spherical cells ≥ 20 μm was never more than 15%. The only population of cells close to this size range seen in vivo were adipocyte-like cells which measured 23.81 ± 5.63 μm X 15.50 ± 2.04 μm. However, these adipocyte like cells were found deep to the epitenon and not investigated in this study. It is therefore postulated that the cells representing the synovial population by Banes et al. (1988) and Brigham et al. (1994) may actually be the adipocyte-like cell population observed in histological sections.

On centrifugation at 500, 1,000 and 1,500 X g to locate spherical cells ≥ 20 μm , it was observed that increasing the centrifugation did not enhance the total number of cells recovered. It was observed that increasing centrifugation speed appeared to decrease viability of the cells isolated. ($p= 0.2$).

Chapter III

SUMMARY AND DISCUSSION

The objective of experiment I was to isolate epitenon cell populations in vitro. Cell measurements revealed that the a 83 ± 9 % of the cells were found in the 10 - 19.99 μm diameter size range. Spherical cells ≥ 20 μm in diameter constituted less than 10 % of the total cells contrary to the report by Banes et al. (1988) where the majority of cells isolated measured 28 ± 8 μm on contact with the substratum. Possible reasons for non recovery of cells in the size range reported by Banes et al. (1988) included age differences, functional variation, regional variation, differences in enzyme treatment and incomplete digestion of the epitenon. Efforts were then concentrated on isolating all epitenon cells for a future study (Experiment III) of in vivo cell populations (synovial, epitenon fibroblast and cartilage cells) using criteria that may be applicable in vitro to distinguish each cell type as a distinct population.

Since our initial digestion time frame did not completely digest the epitenon and provide surface synovial cells as reported by Banes et al. (1988), experiment II was designed to determine how long 0.5 % collagenase digestion needed to be continued to remove epitenon completely thereby isolating all epitenon cells. From the results of experiment II, a standard formulation of 2 ml of 0.5% collagenase per 100 mg of tendon tissue was set with a 105 minutes of digestion time period. This time period and concentration of collagenase was found to be sufficient for complete epitenon digestion in seven, eight and nine week old chickens.

Experiment III was designed to study epitenon cell populations in vivo and to apply information gathered to cells seen in vitro. Also, since the cells reported by Banes et al. (1988) and Brigman et al. (1994) were not found in experiment I, it was thought that increasing centrifugation speed may concentrate the cells into a pellet. Hence, the experiment was conducted over a range (500, 1,000 and 1,500 X *g*) of centrifugation speeds. In vitro cell measurements 2 hours postplating revealed that the majority of the cells measured were again in the size range between ≤ 9.99 - 19.99 μm. No cells in any of the three size ranges reported by Banes et al. (1988) reported 2 hours postplating were observed. In fact, less than 15% of the cells isolated from tendons of any birds between four and nine weeks of age were greater than 20 μm in diameter.

In vivo observations of histological sections from experiment III revealed synovial cells to measure approximately 7.82 ± 1.44 μm X 5.59 ± 1.59 μm and cartilage cells to measure 14.81 ± 0.76 μm X 12.77 ± 0.5 μm. In vivo measurements of cells were taken on transverse histological sections only on cells with a visible nucleus measuring 5 μm to ensure that measurements were obtained at mid-sections of synovial and cartilage cells. Two measurements, namely, longest diameter (length) and shortest diameter (width) were recorded. In vitro measurements were taken on whole cells settled on the substratum following two hours postplating. Here also, two measurements, namely, longest diameter (length) and shortest diameter (width) were recorded. Longitudinal sections examined showed synovial and cartilage cells as spherical cells with centrally located nuclei, and epitenon fibroblasts as elongated cells in vivo. However, in sections revealing incomplete digestion of the epitenon with collagenase solution, all cells of the epitenon (synovial cells,

epitenon fibroblasts) were rounded up. In vitro, it was seen that 95 ± 2 % of the cells obtained in vitro were spherical. The size of epitenon fibroblasts could not be measured in vivo because the cell boundaries were not distinct. About 5 % of the cells were elongated or oval and these were probably epitenon fibroblasts which were not completely freed from the undigested collagen fibers. Of the three populations observed in vivo, the measurements of two (synovial cells, cartilage cells) are known. No histological sections showing cartilage digestion or release of cartilage cells from its matrix was seen. It is therefore not known if the cartilage cells were found in vitro. Of the spherical cells found in vitro following digestion and centrifugation, 17 ± 8% of the cells measured ≤ 9.99μm and 67 ± 9.5 % of the cells were in the 10 - 19.99 μm size range.

Correlating measurements taken in vivo to measurements of cells seen in vitro under the assumption that neither synovial cells, epitenon fibroblasts or cartilage cells were selectively altered during isolation procedures, synovial cells probably constituted the cells observed in the ≤ 9.99 μm size range. The spindle-shaped epitenon fibroblasts which is the major population in vivo is likely to be the major population in vitro constituting cells in the 10 - 19.99 μm size range in vitro, assuming that the epitenon fibroblasts were not selectively destroyed during digestion and centrifugation. Whether or not the epitenon fibroblasts deep to the synovial cells are, in fact, the synthetic type of synovial cells remains open to question. Although the cells in histological-sections have been identified by their characteristic spindle-shape, literature review indicates morphological characteristics of the synthetic synovial cells to have extensive rough endoplasmic reticulum and a dark staining nucleus to be involved in protein synthesis. Cartilage cells

are likely to lie in the size range of 10 - 19.99 μm on isolation in vitro, assuming that type V-S collagenase is capable of digesting cartilage. The only population ≥ 20 μm in vivo constitute an adipocyte-like cell population which measured 23.81 ± 5.63 μm X 15.50 ± 2.0 μm seen deep to the epitenon. It is postulated that these may be the cells observed by Banes et al. (1988) as synovial cells found in the supernatant. It is also postulated that the synovial cell population observed by Brigman et al. (1994) constituting cells with large vacuoles are adipocyte-like cells seen deep to the epitenon.

Correlation of in vivo measurements to in vitro measurements involves comparison of cells in section versus whole cells. The probability that synovial cells constitute cells in the ≤ 9.99 μm size range in vitro and that epitenon fibroblasts and possibly cartilage cells constitute the 10 - 19.99 μm size range of cells in vitro was based on the assumption that the difference in diameter measurements in vivo was not significantly altered following digestion and centrifugation to influence the relative proportions of each cell type measured in vitro.

Future studies include;

a) Testing whether or not cartilage cells are released by collagenase type V-S digestion.

b) Confirmation of a population of spherical cells in the 10 - 19.99 μm size range as cartilage cells with antibodies to type II collagen

c) An in vivo study of epitenon fibroblast rich regions in the tendon

d) Isolation of the epitenon fibroblasts

e) Epitenon fibroblast culture in vitro followed by incorporation at the site of tendon injury to study their effects on healing.

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Table 1

Classification of cells isolated in vitro based on shape and size in four and five week old chickens

Age (week)	Total Tendon Weight (mg)	Collagenase (ml/mg)	Cells (X 10^6) per mg tissue	Viability (%)
Four	184	0.05	1.13 ± 0.07	41.1 ± 0.9
Five	23	0.21	4.26 ± 0.13	60.4 ± 2.2

Table 2

Viability and recovery of isolated cells following different enzymatic digestion time periods in five week old chickens.

Time digestion	Cells (X 10^6) per mg tissue	Viability (%)	Number of ml of collagenase per mg of tissue
45 min	1.60 ± 0.15	68.6 ± 0.4	0.13
75 min	2.52 ± 0.48	66.9 ± 1.8	0.19
105 min	8.18 ± 0.21	70.8 ± 0.3	0.29

Table 3 A

Effect of centrifugation speed on cell sizes two hours post-plating in seven week old chickens

X *g*		≤9.99μm	10-14.99 μm	15-19.99 μm	20-24.99 μm	> 25μm
500	spherical	30	25	29	4	5
	oval			1		
	elongate				1	5
1000	spherical	21	32	33	6	3
	oval				1	1
	elongate				1	2
1500	spherical	26	41	17	9	3
	oval				1	1
	elongate				1	1

Table 3 B

Effect of centrifugation speed on cell sizes two hours post-plating in eight week old chickens

X *g*		< 9.99μm	10-14.99 μm	15-19.99 μm	20-24.99 μm	>25μm
500	spherical	9	32	38	10	5
	oval			2		
	elongate				1	3
1000	spherical	22	36	27	1	6
	oval			2		3
	elongate				1	2
1500	spherical	21	33	28	10	1
	oval			2		1
	elongate				1	3

Table 3 C

Effect of centrifugation speed on cell sizes two hours post-plating in eight week old chickens

X *g*		<9.99μm	10-14.99 μm	15-20 μm	20-25 μm	> 25μm
500	spherical	13	49	20	9	2
	oval				2	1
	elongate				3	1
1000	spherical	16	50	15	12	2
	oval					3
	elongate				1	1
1500	spherical	18	33	33	11	3
	oval				1	
	elongate					1

Table 4

Effect of centrifugation speed on viability and total cells isolated

Age (week)	Centrifugation speed	Viability (%)	Cells (X 10^6) per mg tissue
Seven	500 X *g*	60.22 ± 1.0	2.22 ± 0.07
	1000 X *g*	55.61 ± 0.6	1.71 ± 0.09
	1500 X *g*	47.16 ± 1.3	1.39 ± 0.02
Eight	500 X *g*	71.07± 1.1	0.64 ± 0.01
	1000 X *g*	64.61± 1.1	0.91 ± 0.01
	1500 X *g*	56.90± 1.3	0.81 ± 0.06
Nine	500 X *g*	73.86± 2.3	0.73 ± 0.03
	1000 X *g*	69.09 ± 1.8	0.77 ± 0.04
	1500 X *g*	64.19 ± 3.8	0.93 ± 0.01

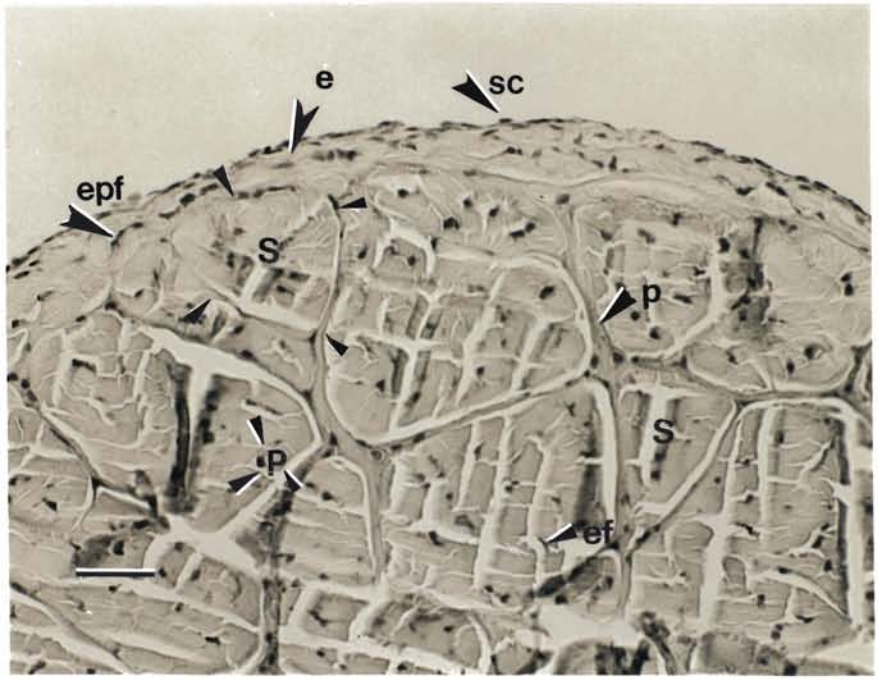


Figure 1: Transverse section of deep digital flexor tendon from a five week old chicken demonstrating primary (P) and secondary collagen bundles (S), endotenon fibroblast (ef), peritenon (p) and epitenon (e). Cell types seen are synovial cells (sc) and epitenon fibroblasts (epf). Magnification X 440. Hematoxylin and eosin.
Bar measures 20 μm

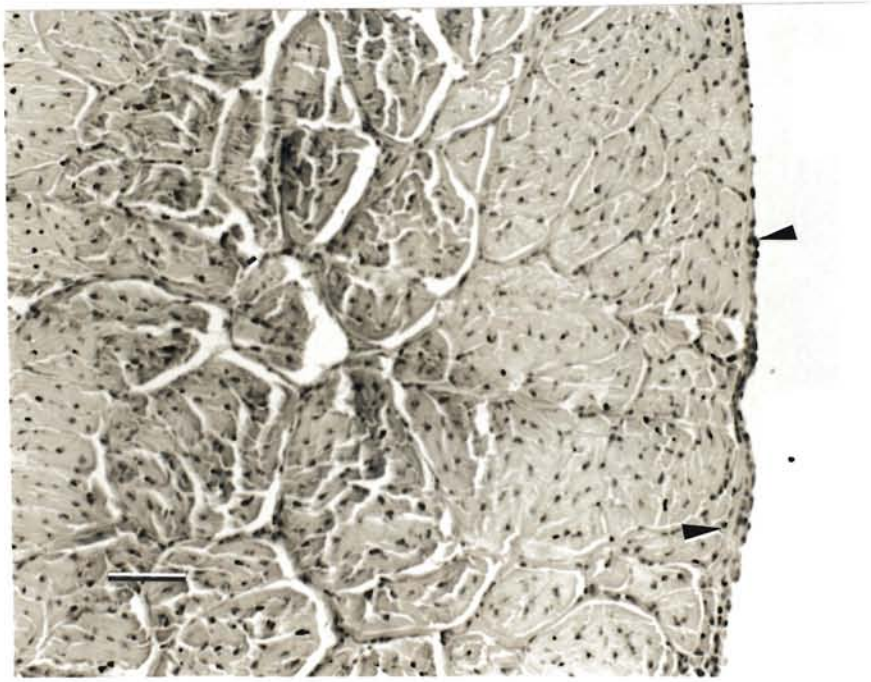
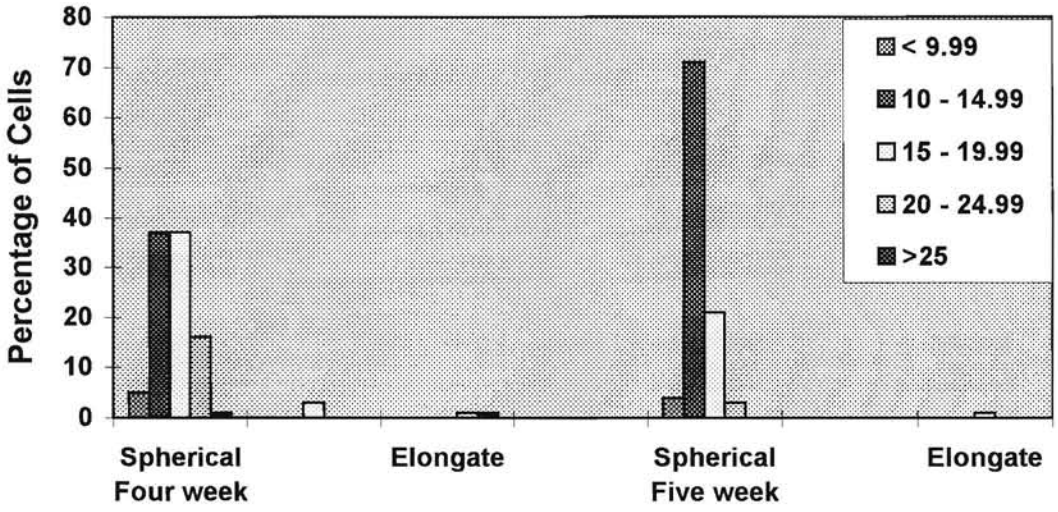


Figure 2: Transverse section of deep digital flexor tendon from five week old chicken following digestion with 0.5 % collagenase for 20 minutes showing incomplete digestion of epitenon. Arrows point to rounded epitenon cells. Magnification X 220. Hematoxylin and eosin. Bar measures 65 μm

Figure 3

Classification of cells isolated in vitro based on shape (spherical, oval, elongate) and diameter size ranges (μm) in four and five week old chickens



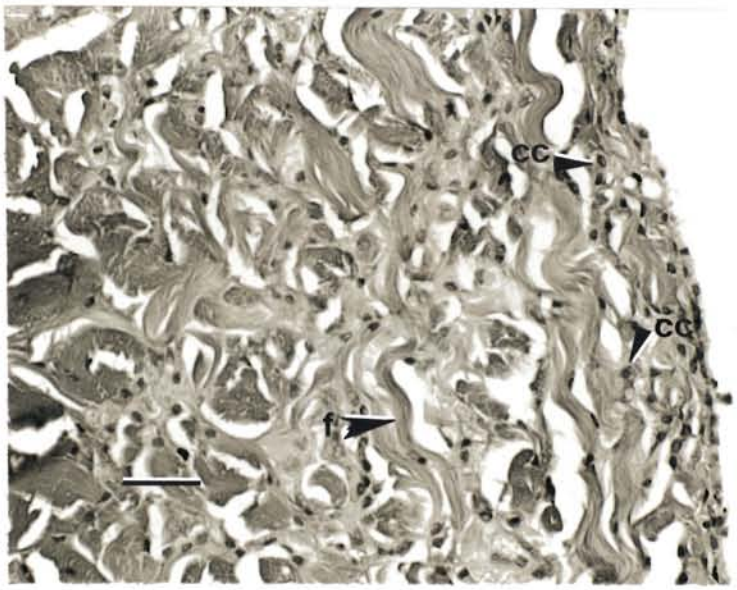


Figure 4: Transverse section of deep digital flexor tendon from nine week old chicken demonstrating fibrocartilage. Arrows point to cartilage cells (cc) and to eosinophilic collagen fibers (f).
Magnification X 220. Hematoxylin and eosin. Bar measures 65 μm

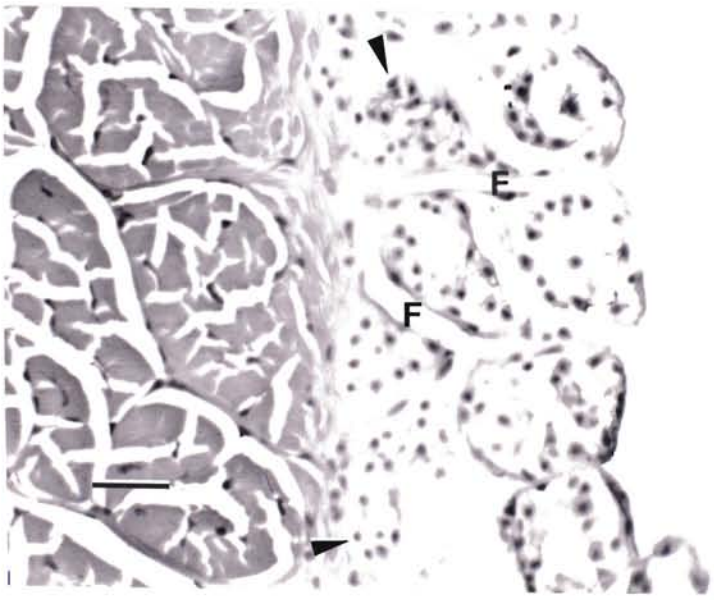


Figure 5: Transverse section of deep digital flexor tendon from nine week old chicken showing hyaline cartilage. Arrows point to chondrocytes in lacunae. Note furrows (F).Magnification X 220. Hematoxylin and eosin. Bar measures 65 μm

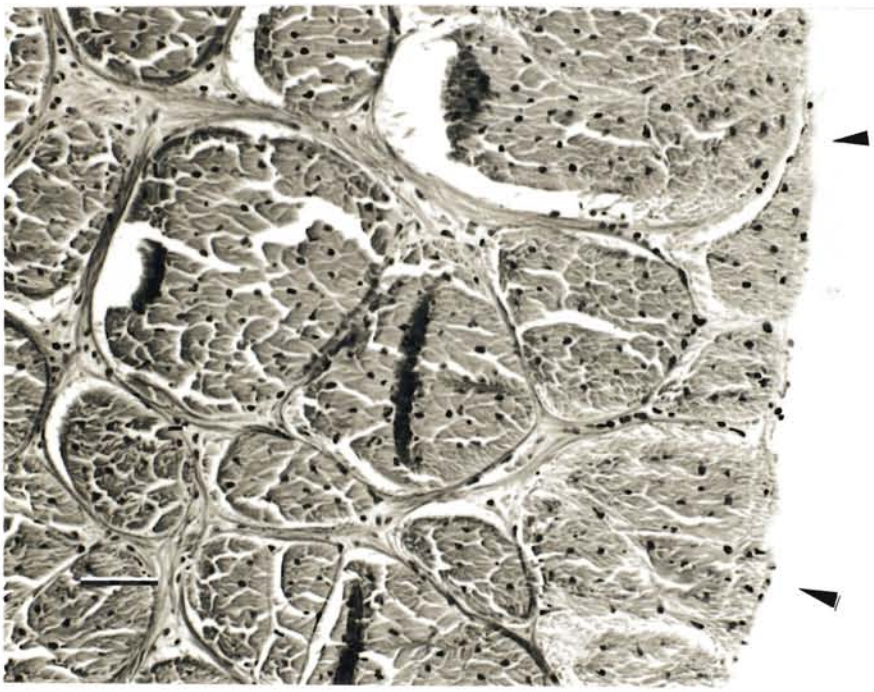
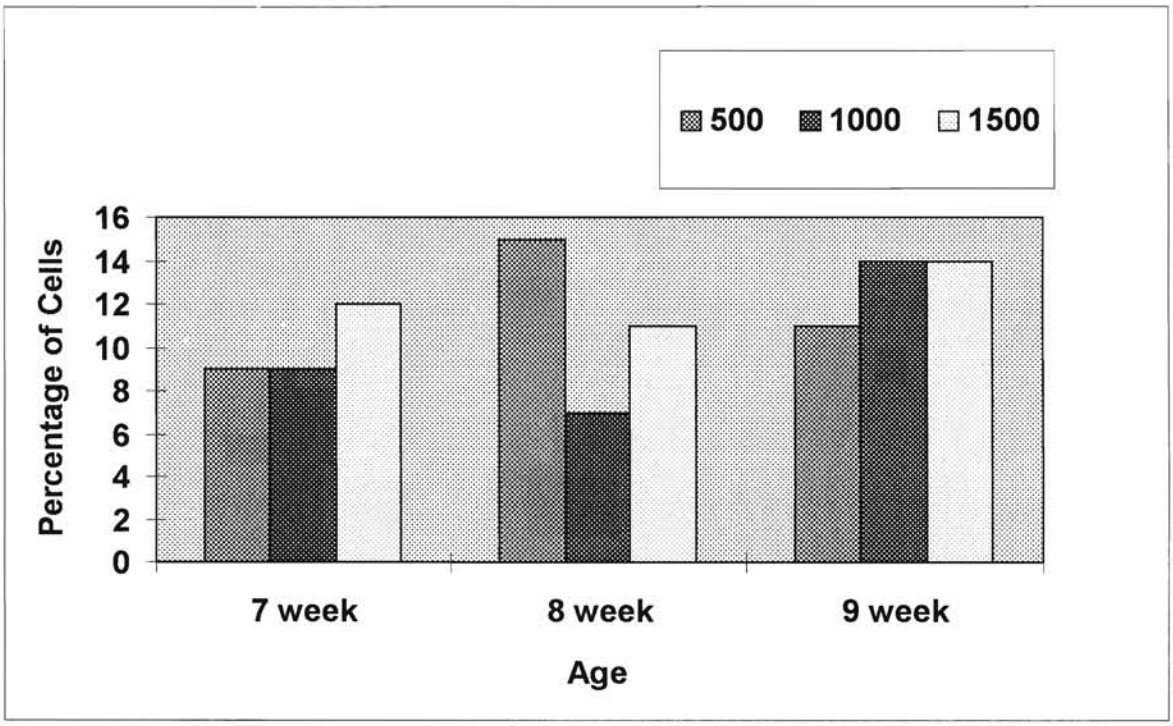


Figure 6: Transverse section of deep digital flexor tendon from seven week old chicken showing complete digestion of the epitenon following 0.5 % collagenase digestion for 105 minutes. Magnification X 220. Hematoxylin and eosin. Bar measures 65 μm

Figure 7:

Effect of centrifugation speed on isolated spherical cells greater than 20 μm in diameter



VITA

Deepa Bandi Rao

Candidate for the Degree of

Master of Science

Thesis: AN IN VITRO AND IN VIVO MORPHOLOGICAL STUDY OF EPITENON CELL POPULATIONS IN CHICKENS

Major Field: Physiological Sciences

Biographical:

Personal Data: Born in Bangalore, India, on November 19, 1970, the daughter of Prema and Bhasker Rao.

Education: Graduated from The Frank Anthony Public School, Bangalore in 1985; received the Bachelor of Veterinary Science degree in 1993. Completed requirements for the Master of Science degree in Physiological Sciences, May 1996.

Experience: Worked as a teaching assistant in Veterinary Gross Anatomy, Department of Physiological Sciences, College of Veterinary Medicine, Oklahoma State University, January 1993 to December 1995.