The Behavior of Rotator Cuff Tendon Cells in Three-Dimensional Culture

by

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ABSTRACT

The rotator cuff is composed of the supraspinatus, infraspinatus, subcapularis, and teres minor tendons. Rotator cuff injuries are common athletic and occupational injuries that surgery cannot fully repair. Therefore tendon tissue engineering can provide alternatives to surgical solutions. Tendons are composed of parallel lines of bundles of collagen fibers and fibroblasts called fascicles and a glycoprotein, superficial zone protein (SZP), which is expressed by the gene, proteoglycan 4 (PRG4) may play a role in joint and intrafascicular lubrication. Studies have shown that a smooth muscle actin isoform (SMA), which plays a role in the contraction of smooth muscle cells, is expressed in the rotator cuff tendon cells. Previous investigations have been conducted to study PRG4 expression and distribution in different regions of the infraspinatus (ISP) tendon. The aim of this study was to investigate the behavior of adult goat ISP tendon cells and bovine bone marrow-derived mesenchymal stem cells (BMSCs) cultured in three-dimensional pellets in chondrogenic (CM), expansion (EM), and tenogenic media(TM). The focus was on the effects of such growth factors as TGF-β1 and hormones such as dexamethasone and various culture methods, such as the use of 96-well plates and 15 ml tubes, on the ISP tendon cells' and BMSCs' cell proliferation, chondrogenesis, and expression of PRG4 and SMA. ISP tendon cells and BMSCs were obtained from five adult Spanish goats ranging. After 14 days, the pellet cultures were analyzed using Safranin-O staining and immunohistochemical staining for SZP and SMA. The biochemical contents of the cell pellet cultures were also evaluated using a DNA assay on days 0 and 14 and a GAG assay on day 14. It was found that CM, containing TGF-β1 and dexamethasone, induced the most cell proliferation and chondrogenesis. SZP was expressed in all of the ISP tendon cells pellet cultures that were cultured in tubes. In comparison to the larger CM-pellets, the ISP tendon and BMSC EM- and TM- pellets cultured in tubes had higher percentages of SMA present. However SMA was also expressed in the CM-pellets cultured in the 96-well plates. The results of our study showed that environmental differences can change SMA expression. Further investigations on tendon cells and the effects of growth factors, bone morphogenetic proteins (BMPs), and culture methods on the cell proliferation, chondrogenesis, and SZP and SMA expression need to be conducted.

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1.1 The Rotator Cuff and Ligaments and Tendons

The rotator cuff is a combination of tendons and ligaments that with the synovial capsule stabilizes the shoulder by holding the head of the humerus in the glenoid cavity of the scapula. Since the shoulder is a comparatively unstable joint due to the shallowness of the glenoid fossa and weak supporting ligaments, its stability is dependent mostly on the rotator cuff tendons and muscles. The main components of this support system, as shown in Fig. 1, are the supraspinatus, infraspinatus, subcapularis, and teres minor.[1]

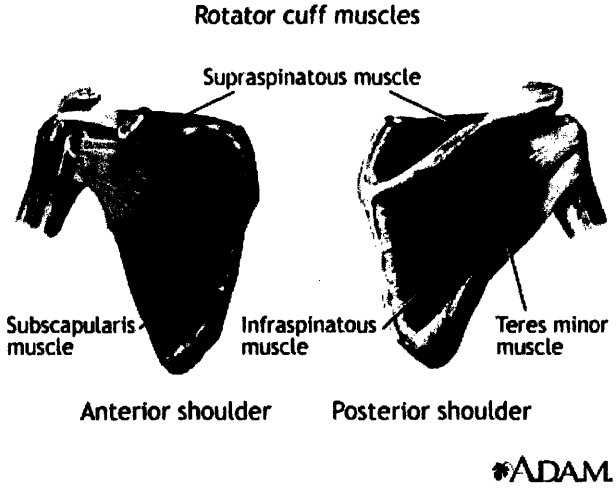


Figure 1: Diagram of rotator cuff tendons and muscles: supraspinatus, infraspinatus, subcapularis, and teres minor.[2]

Tendons and ligaments are fibrous connective tissues that attach muscles to bones and bones to bones, respectively. Their high tensile strength allows for the range of motion and stability of the joints. Tendons are complex composite materials that are mostly water, which is 55% of the net weight, proteoglycans, which are less than 1% and consist of glycosaminoglycan (GAG) chains, cells and type I collagen, which make up 85% of the dry weight, and smaller amounts of other collagens, such as collagens type III, V, XII, and XIV.[3] The production and maintenance of the collagen in the tendons is the main role of tenocytes.[4]

The primary structures of tendons are collagen polypeptides that consist of a glycine molecule at every third amino acid. The three polypeptides form triple-helical collagen molecules which then form larger collagen molecules by the cleavage of N- and C-terminal polypeptides. The collagen monomers further form the fibrils which make bundles of collagen fibers. The fibers combined with fibroblasts are bundled into

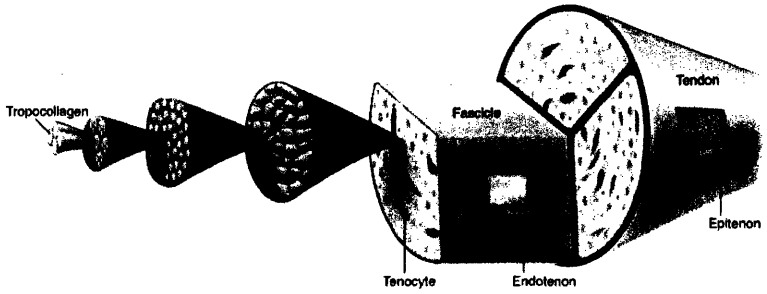


Figure 2: Structure and composition of a tendon.[5]

fascicles.[6] As seen in Fig 2, the fascicles are formed by fibers being surrounded by a layer of a fine loose connective tissue sheath of endotenon. The epitenon bundles parallel lines of fascicles to form the tendon.[7]

1.2 Tendon Injury and Repair and Restoration Challenges

Since all of the support in the shoulder depends on the tendons comprising the rotator cuff, the shoulder is actually quite unstable. Rotator cuff injuries are common athletic and occupational injuries, which can lead to chronic pain and disability.[8]

(a) 

(b) 

Figure 3: Anthroscopic views of (a) a normal rotator cuff and (b) a small-to-medium tear of a rotator cuff.[9]

When a tendon is torn or injured, surgery is unable to fully repair and restore its function.[10] According to Ahmed, *at al.*, under normal conditions, a fully developed

tendon is a tissue with a low density of cells and poor vascularization.[11] These are believed to be reasons for the large amount of time required for the healing of tendons and the production of an extracellular matrix of lesser quality than before injury.[12]

1.3 Significance of Tendon Tissue Engineering

While surgeries, such as the margin convergence procedure in Fig. 4, on a torn

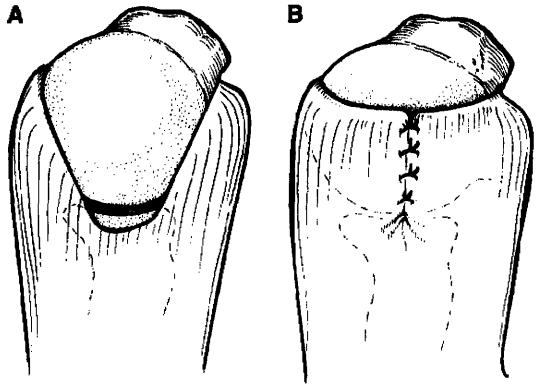


Figure 4: Diagram of the margin convergence procedure to repair the rotator cuff before (A) and after (B) the procedure.[13]

tendon are routine, it is clear that a more effective solution is necessary in providing improved solutions for the healing of tendons. It has been purposed that the development of tendon tissue engineering could provide alternatives to existing surgical solutions.[14] Surgical procedures such as autografts, allografts, and prosthetic devices are currently used to treat tendon and ligament injuries. There have been many disadvantages identified with the use of biological grafts and there are still questions about the lifetime and quality of prosthetic devices. A large gap caused by a tendon tear is usually difficult to repair. When a tendon has been completely removed, a graft or replacement device is used. However, the developmental process of tendon and ligament tissues has not yet been completely understood. Tissue-engineering solutions such as the use of growth factors, gene transfer, biodegradable biomaterials, and cell therapy have shown to be successful in improving the quality of the healing of tendons and ligaments. [15] With this progress in the research of tissue-engineering and its applications in tendon and ligament repair, it is essential to increase the understanding of tendon cell growth and repair to help develop alternatives to surgical repair procedures.

Chapter 2- Research on Rotator Cuff Tendons and Injuries

2.1 A Review of Research in Tendon Repair and Tendon Tissue Engineering

The investigations of therapeutic approaches for rotator cuff repair and regeneration, reported in this section, have been conducted in human trials, animal models, and in cell/tissue culture.

In vitro studies provide the opportunity to evaluate the behavior of cells in well-controlled environments. One of the culture conditions which can affect the behavior of cells *in vitro* is the configuration in which the cells are grown: whether they are grown in monolayer on the surface of a conventional tissue culture dish or in a three-dimensional culture. The latter configuration may more closely simulate the environment of the cells *in vivo*. Such three-dimensional culture configurations can be achieved by employing culture methods that allow cells to aggregate into a "pellet" or by seeding cells into sponge-like scaffolds.

2.1.1 Effects of Growth Factors

Many studies have attempted to define the effects of growth factors on the healing process of tendons and ligaments. Growth factors such as those from the transforming growth factor (TGF), epidermal growth factor (EGF), platelet-derived growth factor (PDGF), and insulin-like growth factor (IGF) families have been able to improve matrix formation and tendon and ligament cell growth both *in vitro* and *in vivo*. However, since there are still many remaining questions about the regulatory signals that direct the proliferation of tendon and ligament cells, further studies about these growth factors need to be performed.[16]

2.1.2 Effectiveness of the Gene Transfer Method

Using the gene transfer technique, specific genes are transferred into cells *in vitro* or *in vivo* to change their functions. Due to the continuous expression of the exogene, a high concentration of growth factors can be maintained at the repair site. The exogene may improve tendon and ligament repair and prevent adhesion. For example, for flexor tendon injuries, gene therapy can help promote tissue regeneration and prevent adhesion. Several studies have also demonstrated successes in the transfer of marker genes to tendons.[17] Many groups have been successful in transferring genes encoding PDGF-BB, TGF-β, and pp125^{FAK} to tendons.[18 19 20]

2.1.3 Use of Prostheses and Augmentation Devices

Biological grafts were the first ligament and tendon reconstruction solutions.[21 22] However major problems occurred due to issues such as donor site morbidity, limited sources, transmission of pathogens, and difficulties with storage. Artificial ligaments were also designed. However there were more factors which prevented their complete success. For example, there were continuous inflammatory reactions in the new ligaments, small amounts of new collagen fibers which were oriented poorly were produced, there were negative responses to the wear particles of the synthetic materials, and the articular cartilage underwent reactive degeneration.[23 24 25] Therefore now biosorbable polymers are used as materials for scaffolds in the area of tendon and ligament tissue engineering.[26]

2.1.4 Role of Cell-Seeded Implants

One major factor in the tissue repair and regeneration process is the presence and availability of necessary cells. Cells need to be accessible due to their proliferation potential, cell-to-cell signaling processes, biomolecule production, and the production of extracellular matrix (ECM). Therefore the quantity of the initially seeded cells can strongly influence cell-mediated processes.[27] It has been established that there may be a required minimum number of cells at a repair site for normal neotissue formation.[28] Thus many groups have developed fibroblast-seeded collagen scaffolds for ligament regeneration on which fibroblast viability and proliferation was studied. Mesenchymal

stem cells (MSCs) have also been isolated from various types of animals and humans. MSCs can develop into progenitors of different structural and connective tissues such as bone, cartilage, fat, tendon, and muscle.[29] It has also been reported that autogenous MSCs can significantly improve the structure and biomechanics of injured tendons.[30 31]

2.1.5 Bioreactor Method

One approach to tissue engineering is the implantation of a cell-scaffold mechanism directly into the repair site so that the body acts as a "bioreactor." Another solution is the use of an *ex vivo* bioreactor in which a cell-scaffold composite can be cultured for a certain amount of time before transplantation into the body. With an *ex vivo* bioreactor, biochemical and physical regulatory signals that direct cell differentiation, proliferation, and tissue development can be introduced in a controlled manner. An *ex vivo* bioreactor allows for a better understanding of tissue development.[32]

2.2 Previous Studies on Superficial Zone Protein (SZP)/ Lubricin/ Proteoglycan 4 (PRG4) and Alpha-Smooth Muscle Actin (α-SMA)

2.2.1 Studies on Superficial Zone Protein (SZP)/Proteoglycan 4 (PRG4)/Lubricin

Articular cartilage found at joint surfaces has surface, middle, and deep layers that have different cell architecture, biochemical composition, and mechanical properties.[33 34] A glycoprotein called superficial zone protein (SZP) is produced and secreted by chondrocytes in the superficial layer of the articular cartilage into the synovial fluid. SZP is not retained in the ECM. [35 36] SZP has also been found in synovial fluid lining tendons.[37] After a glycoprotein was first identified and isolated to have a role in joint lubrication, it was named *lubricin*.[38 39 40] Further studies showed that it was related to SZP; lubricin and SZP are commonly referred to by the name given to the gene which has been found to encode them, proteoglycan 4 (PRG4).[41 42] Studies have shown that SZP is not only involved in joint lubrication, but also growth promotion and cytoprotection.[43 44 45] Since there is so much potential in the roles of SZP, further investigations are needed.

Khalafi, *et al.*, studied the influence of bone morphogenetic protein 7 (BMP-7) on SZP accumulation in cell culture models of bovine superficial articular cartilage. They also investigated the effects of BMP-7 in combination with other growth factors and

cytokines, such as TGF-β1, FGF-2, IGF-1, and PDGF, on bovine superficial articular chondrocytes. Chondrocytes treated with the growth factors produced significantly more SZP than those treated with other growth factors and cytokines. Also the addition of BMP-7 to the growth factors did not lead to a significant increase in the amount of SZP produced. In fact, TGF-β1 led to the most SZP accumulation.

2.2.2 Studies on Smooth Muscle Actin (SMA) Isoform

As discussed above, tendon fibroblasts in the rotator cuff are important for the production and maintenance of tendon tissue. During the repair process of an injured tendon, fibroblasts may display characteristics of a smooth muscle cell and express the gene for a smooth muscle actin isoform (SMA).[46 47] Alpha-smooth muscle actin (α-SMA) plays a role in contraction and is usually expressed in vascular smooth muscle cells.[48] Premdas, *et al.* investigated the effects of different growth factors (TGF-β1, PDGF-BB, and IFN-γ) on the regulation of SMA in rotator cuff cells. The group discovered that a significant portion of the nonvascular cells expressed SMA in all of the seven rotator cuffs. It was the first identification of the expression of SMA in rotator cuff cells and in any type of human tendon.[49]

In another study, α-SMA was expressed by human MSCs during chondrogenesis undergone by cells cultured in pellet cultures. The addition of TGF-β1 significantly increased differentiation of the human MSCs which led to an increase in GAG and type II collagen synthesis and α-SMA expression. The pellet cultures were grown in chondrogenic media (CM) and growth media (GM). The cells in the peripheral layers of the CM pellets that were positive for α-SMA mimicked the cells found within the superficial layer of the articular cartilage and are believed to play an important role in cartilage development and maintenance.[50]

2.3 Previous Studies on PRG4 Expression in Infraspinatus Tendon Tissue and in Infraspinatus Tendon Cell Pellet Cultures in Various Media

2.3.1 Previous Investigation of PRG4 expression in Infraspinatus Tendon Cells

In the study conducted immediately before this investigation, the goal was to understand the PRG4 expression and distribution in different regions of the infraspinatus

(ISP) tendon using tendons from eight different goat rotator cuffs.[51] PRG4 may act as a lubricant between fascicles and help separate the collagen bundles during normal shoulder movement.[52] Lubrication between the fascicles helps minimize the shear stress caused by the movement of the fascicles relative to each other. In this study, the crimped fascicles were defined as collagen bundles separated by loose connective tissue, as shown in Fig 5.[53]

Immunohistochemical staining for PRG4 showed positive staining in the tendon, in the synovial fluid of the synovium, and on the humeral head, as shown in Fig 6. There was no staining in the bone. Inside the tendon, the endotenon surrounding the fascicle expressed positive staining for PRG4. Cells inside the fascicles and the intrafascicular region between the fascicles were also positively stained.

The fascicle diameter and crimp length of the bursal side of the tendon were compared to those of the joint side. The crimp length of the joint side was significantly shorter than that of the bursal side which led to the conclusion that ISP tendons function under various mechanical conditions. It was also concluded that perhaps intrafascicular PRG4 expression also changed under various mechanical conditions.[54]

After verifying the expression of PRG4 in the ISP tendon tissue between the fascicles, the next steps were to explore PRG4/SZP expression *in vitro* in monolayer and pellet cultures.

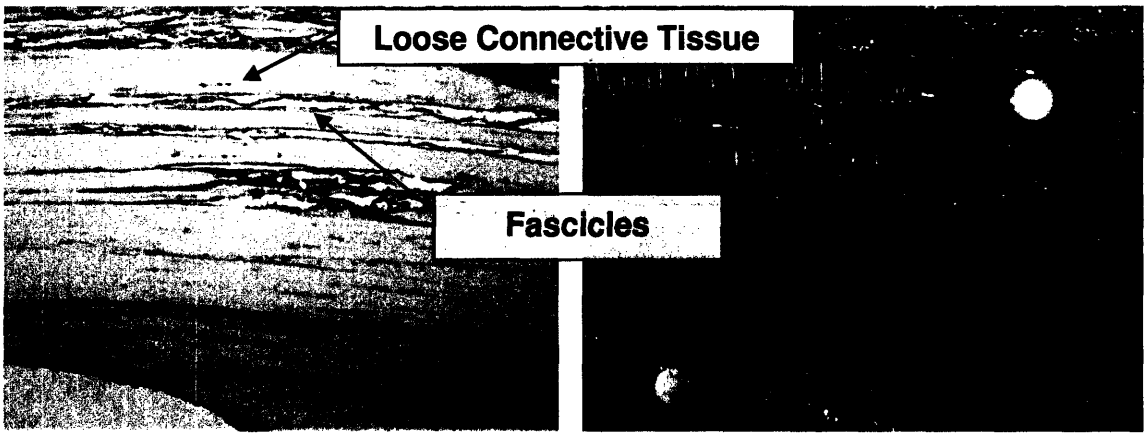


Figure 5: Photographs of ISP tendon tissue showing the crimped fascicles separated by loose connective tissue.[55]

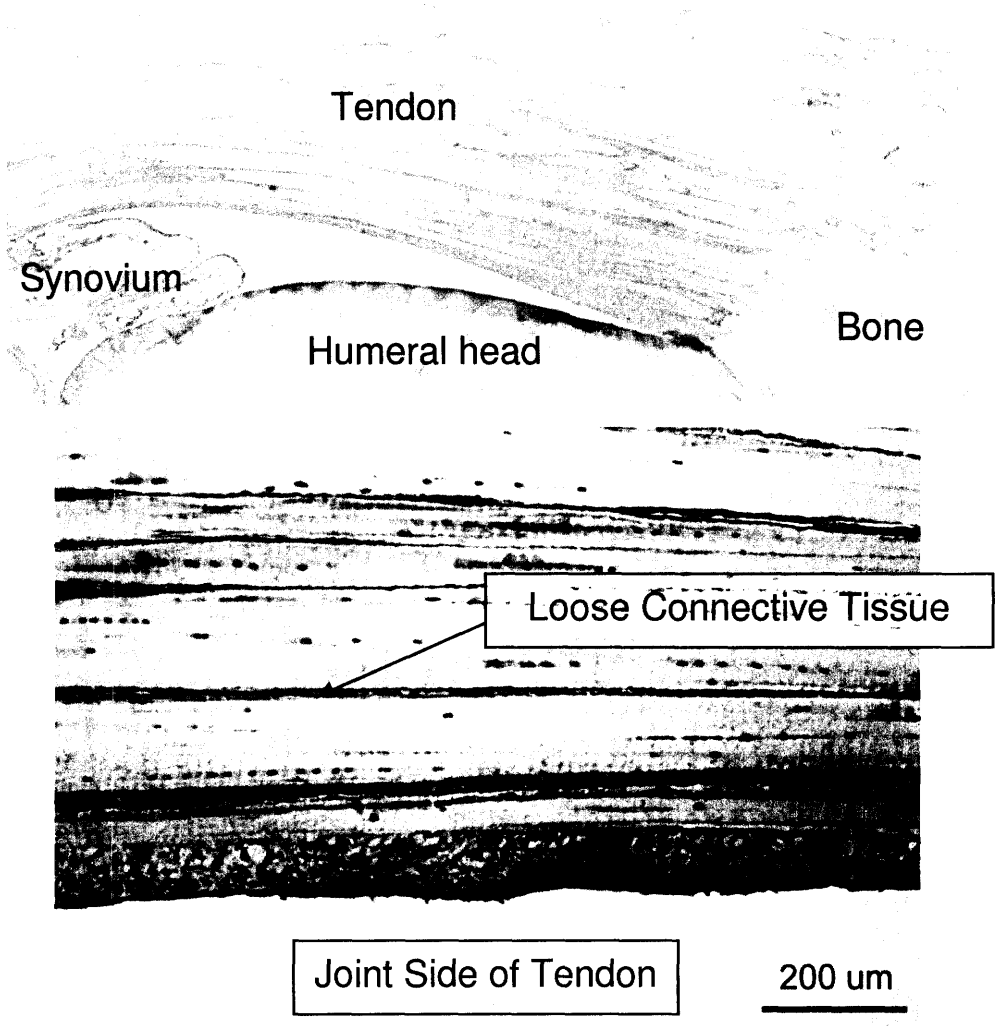


Figure 6: (a) Photograph of ISP tendon section after immunohistochemical staining for PRG 4. Note positive staining for PRG4 in the tendon, synovium, and on the humeral head and negative staining for PRG4 in the bone. (b) Positive immunohistochemical staining for PRG4 in ISP tendon.[56]

2.3.2 Investigation of ISP Tendon cell Cultures in Chondrogenic, Expansion, and Tenogenic Media

The aim of the study following the previous investigation was to understand PRG4/SZP expression in the rotator cuff and determine the best media for tendogenesis using monolayer and pellet cultures of ISP tendon cells cultured in chondrogenic media (CM), expansion media (EM), and tenogenic media (TM). The samples were obtained from five Spanish goats: #60(+), #60(-), #217(+), #140, #171 and four types of cells were investigated. Table 1 indicates the cell types.[57]

Table 1: Goat and Cell Type of the samples and their origins

Goat and Cell Type	*Origin*
# 60 (+)	Exclusively from articular side of ISP tendon
#60 (-)	From remainder of ISP tendon
#217 (+)	Exclusively from articular side of ISP tendon
#140	Whole tenocyte from ISP tendon
#171	Whole tenocyte from patellar tendon of the kneecap

After 14 days, the pellet sizes were measured. Chondrogenic media stimulated the largest pellet sizes, followed by tenogenic media, with expansion media having the smallest pellet sizes, as seen Fig. 7.

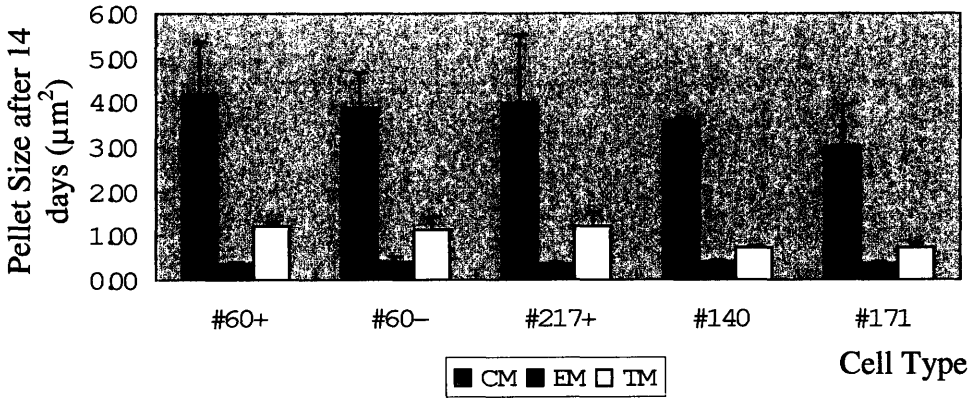


Figure 7: Histogram comparing the five cell pellet culture sizes after 14 days of cells being cultured in CM, EM, TM.[58]

The pellet cultures were immunohistochemical stained for SZP and stained with Safranin-O. According to the results, seen in Fig. 8, expansion media seemed to stimulate SZP expression for all of the cell types. The #140 cell type pellet culture cultured in tenogenic media was completely positively stained for SZP. The #217 cell type pellet culture cultured in tenogenic media was partially positively stained for SZP. The remaining TM pellet cultures were not stained for SZP. Therefore it was unclear if tenogenic media stimulates SZP expression. Even though the EM pellet cultures expressed positive staining for SZP, the effects of expansion media in comparison to other types of media on SZP expression needed to be investigated further.

As seen in the Safranin-O staining results, shown in Fig. 9, CM stimulated ECM production, chondrogenesis, while the TM pellet cultures did not produce any ECM. Questions also remained about EM's ability to stimulate chondrogenesis and TM's ability to stimulate tenogenesis.[59]

Immunohistochemical Staining for SZP

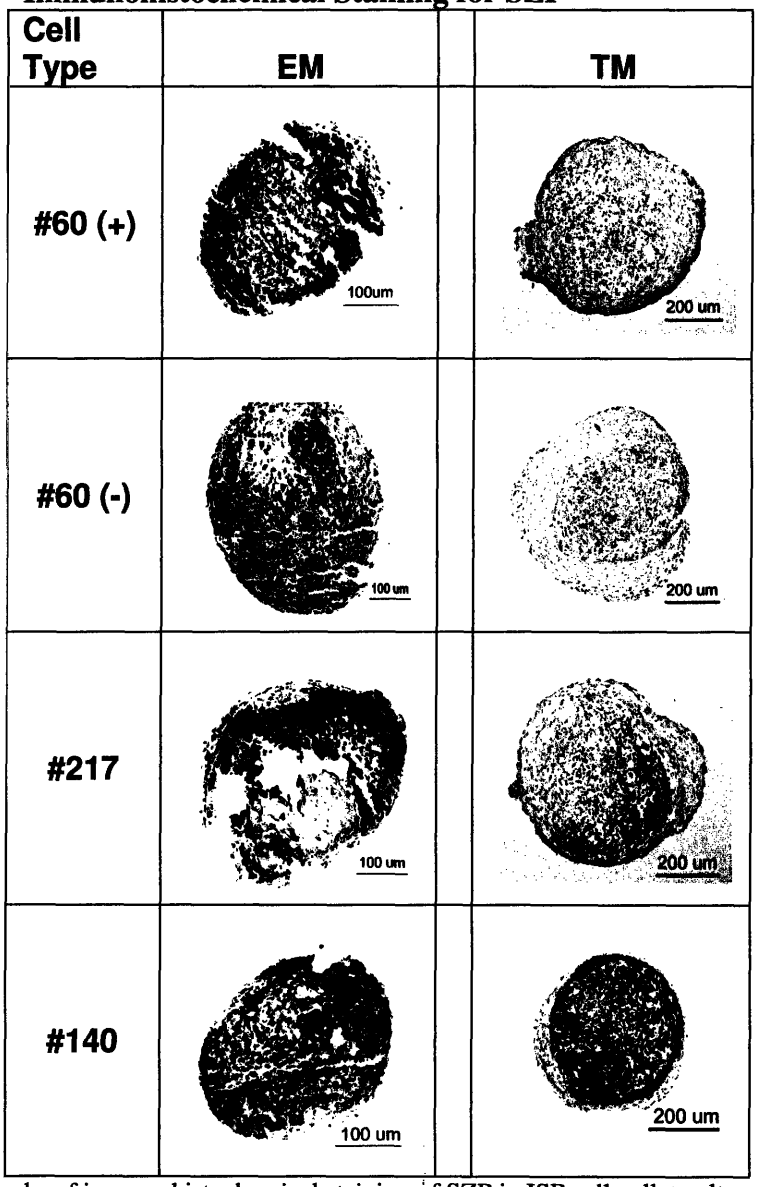


Figure 8: Micrographs of immunohistochemical staining of SZP in ISP cell pellet cultures (#60(+), #60(-), #217, and #140) cultured in EM and TM. All EM pellet cultures were positively stained for SZP.[60]

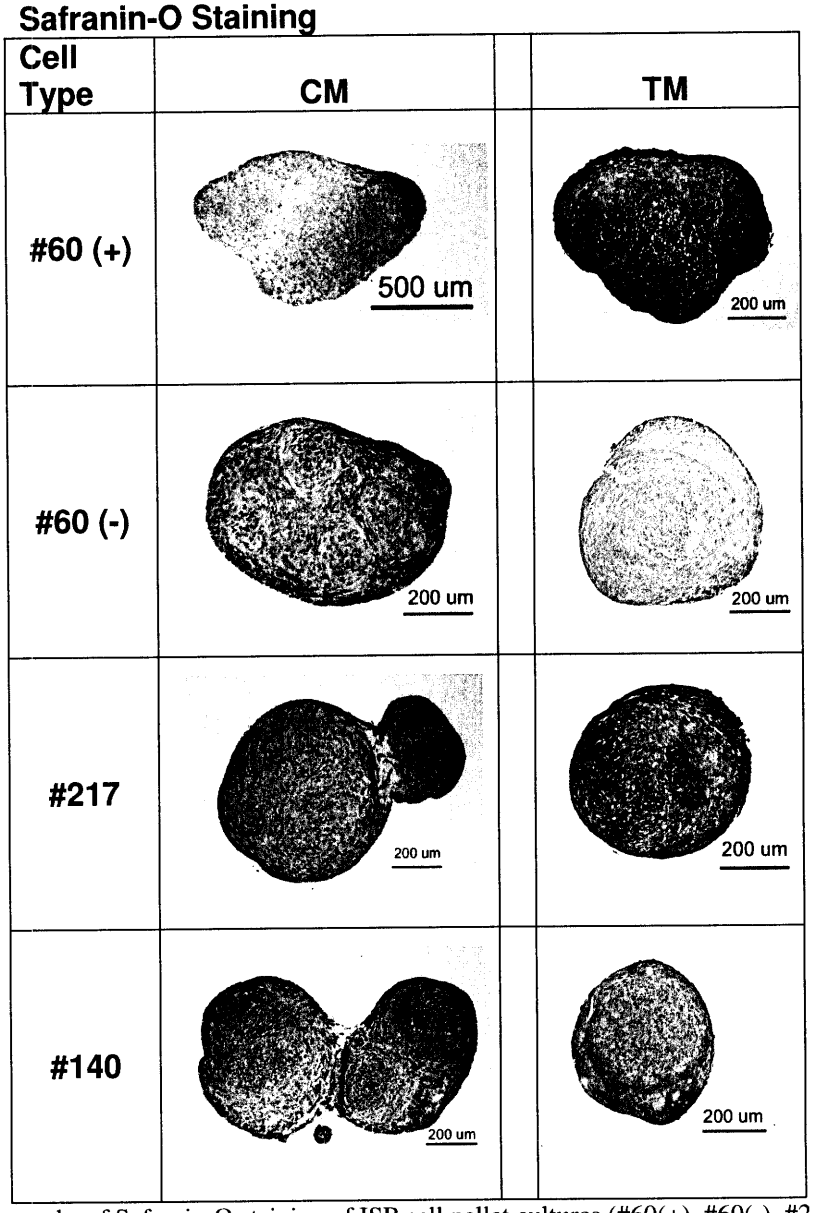


Figure 9: Micrographs of Safranin-O staining of ISP cell pellet cultures (#60(+), #60(-), #217, and #140) cultured in CM and TM. All CM pellet cultures were positively stained indicating GAG production.[61]

This study helped in gaining a basic, introductory understanding about ISP cell pellet cultures, effects of various media, SZP expression, and chondrogenesis. The next step was a thorough investigation comparing cell types cultured in high density pellet cultures in various media and their effects on PRG4/SZP and α-SMA expression and chondrogenesis.

2.4 Purpose of Current Study

The aim of this thesis was to investigate the behavior of adult goat infraspinatus tendon (ISP) cells and caprine bone marrow-derived MSCs (BMSCs) cultured in three-dimensional pellet cultures in chondrogenic, expansion, and tendon media. The reason that BMSCs were included in this thesis is that they could be of value in future therapeutic modalities for the treatment of rotator cuff injuries, and therefore it is important to compare their behavior with cells taken directly from the ISP tendon. The focus was on the effects of various culture media and culture methods on the ISP cells' and BMSCs' expression of PRG4/SZP and α-SMA and the stimulation of chondrogenesis.

2.4.1 Use of Cell Pellet Cultures to Investigate Cell Behavior

Other groups have successfully used pellet cell cultures in their studies. Tanaka et al found that collage type II was most expressed in pellet mass cultures. Sections of the pellet masses showed round cells which resembled hyaline chondrocytes and were forming cartilaginous lacunae.[62] It has also been found that a high-density microenvironment stimulates chondrogenic differentiation of embryonic stem (ES) cells.[63] Three-dimensional cultures and pellet cultures of chondrocyte have been used for *in vitro* production of large populations of chondrocytes which have the ability to maintain their phenotype.[64] A monolayer chondrocyte culture is unable to maintain the chondrogenic phenotype.[65]

A study by Zhang, *et al.* has shown that chondrocytes cultured using pellet cultures have similar characteristics of cellular distribution, matrix composition and density, and tissue ultrastructure as native cartilage.[66] Studies have shown that cells proliferated in pellet culture or high cell density micromass culture form three-dimensional masses that allow cell-cell interactions that are similar to those in precartilage growths during embryonic development.[67 68 69 70] In another study, cell-cell contacts such as gap junctions were identified in tendon high-density cultures using electron microscopy.[71]

Schulze-Tanzil, *et al.* concluded that the use of three-dimensional high-density cultures could be a significant new method to stimulate differentiation of tenocytes to be used for autologous tenocyte transplantation in tendon and ligament repair and to study the effects of various factors affecting the tendon *in vitro*.[72]

2.4.2 Characteristics of ISP Tendon cells and BMSCs

It has been suggested that tenocytes can be considered to act like myofibroblasts and tendons can be considered to act like a contractile organ.[73] Therefore it is appropriate to use ISP tendon cells to explore their capabilities in relation to tendon repair. Another challenge in tissue engineering is that when grown *in vitro*, primary chondrocytes lose their phenotype which does not allow them to be used for the repair process. However it has been found that BMSCs are pluripotential.[74 75] Therefore it has been suggested that BMSCs can act as seed cells to differentiate into chondrocytes and for use in tendon tissue engineering.[76]

2.4.3 Goal to Observe Chondrogenesis

Currently there is much discussion and debate about the identity and location of cells that stimulate collagen synthesis and chondrogenesis during the tendon healing process. It is believed that both tenocytes and external cells such as cells fron tendon sheath have roles in tendon repair.[77] It is still uncertain if the necessary number of tenocytes or connective tissue progenitor cells that are needed for repair of an injury are readily available within the body. There is a need for the use of exogenous cells for tendon tissue healing.[78] Therefore it would be a significant contribution to tendon tissue engineering if methods could be developed for the stimulation of chondrogenesis *in vitro* using cell cultures.

2.4.4 Role of Transforming Growth Factor, TGF-β1 and hormone, Dexamethasone

Many studies show that members of the transforming growth factor (TGF) family stimulate chondrocyte development.[79] For example, the growth factor, TGF-β1, can stimulate mitotic activity, proteoglycan synthesis, and chondrogenic differentiation.[80] In

fact, Johnstone, *et al.* observed 100% chondrogenic differentiation in MSCs treated with TGF-β1 while 25% of marrow cell controls underwent chondrogenic differentiation.[81]

The hormone, dexamethasone, has been shown to induce multiple end-phenotypes.[82] [83] In several studies, dexamethasone has stimulated chondrogenic differentiation of undifferentiated mesenchymal cells.[84] In a study conducted by Zimmerann and Cristea, dexamethasone induced chondrogenesis of murine embryonic cells that were in organoid cultures.[85] In another study, it induced chondrogenesis in mesodermal progenitor cells.[86] However in the investigation conducted by Tanaka, *et al.*, dexamethasone did not seem to have had a significant effect on the stimulation of chondrogenic differentiation of the embryoid bodies (EBs) which were formed by ES cells after five days in culture and were encapsulated in alginate. It was suggested that further investigations were necessary to evaluate the effect of dexamethasone in such cultures as pellet or micromass cultures.[87]

Chapter 3- Investigation on the Behavior of Infraspinatus Tendon (ISP) Cell and Bone Marrow Mesenchymal Stem Cell (BMSC) Pellet Cultures

3.1 Purpose

In the study differences in behaviors were compared between ISP tendon cells and BMSCs cultured in pellet cultures in chondrogenic, expansion, and tenogenic media. We were also studying the effects of differences in growth methods by using 96 well plates and 15 ml tubes. The investigation's focus was on the effects of contents of the various media, such as growth factor, TGF-β1 and hormone, dexamethasone, and culture methods on the ISP cells' and BMSCs' expression of PRG4/SZP and α-SMA and the stimulation of chondrogenesis.

After 14 days, the pellet cultures were analyzed using Safranin-O staining and immunohistochemical staining for SZP and α-SMA. The biochemical contents of the pellet cultures were also analyzed using a DNA assay on day 0 and 14 and a GAG assay on day 14.

3.2 Materials and Methods

3.2.1 ISP Tendon Cells and BMSCs Isolation

The infraspinatus tendons were obtained from the rotator cuffs of five different Spanish goats ranging in ages two to five. The BMSCs were also taken from the same five goats (#208, 211, 253, 254, 256). After being minced, the ISP tendons were digested under shaking for three hours using 0.25% collagenase (M6C8665, Worthington Biochemical Corporation, Lakewood, NJ). The isolated tendon cells were then treated with protease, followed by being treated with 0.05% Trypsin/EDTA (GIBCO 25300, Grand Island, NY) and washed three times using Dulbecco's modified Eagle's medium with 1 g/l glucose (DMEM-LG; GIBCO 11885, Grand Island, NY) and 10% Feral bovine

serum (FBS). The cells used in the study were at passage 2. The cells were then spun in 20 ml of expansion media (HG-FBS) and 10 ml of media was added.

From the same five Spanish goats, bone marrow was aspirated from the iliac bone and the ISP tendon cells and MSCs were isolated as discussed above. The bone marrow sample was then washed with phosphate buffered saline (PBS) and Ficoll-Paque PLUS. After spinning in the centrifuge at 3000 rpm for 30 minutes, the whitish band at the interface was removed and washed with PBS. The BMSCs and ISP tendon cells were plated in a T75 Flask.

For cell suspension of 1 x 10^6 cells/ml, three types of media were used: CM, EM, and TM.

3.2.2 Preparation of ISP cells and Bone Marrow Mesenchymal Stem Cells pellet cultures.

The pellet cultures were cultured in 96 well plates and 15 ml tubes. 200 µl of aliquots were used to sterilize a 96 well, V-bottom, 300 µl polypropylene microplate (Phenix, Hayward, CA, USA). Each pellet culture consisted of 0.2 x 10^6 cells/well. A total of six pellet cultures were prepared for each of the five goats so that there would be three pellet cultures for histological analysis, one for DNA assay on day 0, one for DNA and GAG assays on day 14, and one for stock.

Six pellets per goat were cultured in sterile 15 ml falcon tubes. 0.5 ml of cells suspension was placed in each tube and was spun at 1500 rpm for 10 minutes. The cap was then loosened to allow ventilation and placed in an incubator. Five of the six pellets were for culture and one was for DNA analysis on day 0. The media of the pellet reserved for DNA analysis was removed and the pellet was frozen in -20°C. Three of the pellets were cultured for histology, one for DNA and GAG assay on day 14, and one for stock. Both the plate and tube were centrifuged for 10 minutes at 1500x g.

3.2.3 Chondrogenic, Expansion, and Tendon Media Preparation

The chondrogenic media (CM) was prepared using Dulbecco's modified Eagle's medium (DMEM) high glucose with 1% of Hepes (GIBCO, 15630 056), 1% of MEM non-essential amino acid (NEAA; GIBCO, 11140 050), 1% of Penicillin/Steptomycin/

Glutamate (PSG; GIBCO 10378 016), and 1% of insulin-transferrin-selenium (ITS+1; SIGMA, 12521). Also bovine serum albumin (BSA) was added so that the concentration was 17 µl/ ml of media. Immediately before experimentation, using 10 µl of stock aliquots per one ml of media, 0.1 mM of L-ascorbic acid 2-phosphate (A2P), 100 nm of dexamethasone (SIGMA, 2915), and 10 nm/ml media of TGF-β1 (240-B-002, R&D, Minneapolis, MN) was added. The final concentration of TGF-β1 was 10 ng/ml media.

The expansion media (EM) was prepared using 500 ml of DMEM low glucose. 50 ml of DMEM was then removed and kept separately. 45 ml of fetal bovine serum (FBS) and 5 ml of pen/strep (PS) was added. L-ascorbic acid 2-phosphate was added for a concentration of 10 µl/ ml.

The tendon media (TM) was also prepared using 500 ml of DMEM high glucose with 1% each of Hepes, NEAA, Pen/Step/Glutamate (PSG), and ITS+1 (100x). 9.37 ml of bovine serum albumin (BSA) was added so that the concentration was 17 µl/ ml of media. Then 45 ml of ham was removed and 45 ml of 10% FBS was added. L-ascorbic acid 2-phosphate was added for a concentration of 10 µl/ml.

The media in the 96-well plates and the 15 ml tubes were changed every other day for 14 days.

3.2.4 Histological Analysis using Safranin-O staining and Immunohistochemical Staining of SZP and α-SMA

To determine the effective diameters of the pellets, Image J software (NIH, Bethesda, MD) was used to find the area of each pellet. To prepare sections for immunohistochemical staining, the pellets were rinsed with PBS, fixed in 4% paraformaldehyde for three hours, embedded in paraffin, and cut into 5 µm thick cross-sections.

One of the immunohistochemical staining process was the safranin-O staining to stain sulfated glycosaminoglycans (GAG). The sections were also stained for SZP and α-SMA.

The following immunohistochemical staining processes were performed by the DakoAutostainor (DakoCytomation, Caprinteria, CA) using the program for PRG4. After deparaffinization with xylene, the sections were hydrated in ethanol and were

treated with a final wash of tris-buffered saline (TBS, S3001; DakoCytomation, Carpinteria, CA). They were then treated with 0.1% protease XIV (P5174; Sigma, St. Louis, MO) for 45 minutes to aid with the penetration of the antibody into the tendon tissue. Before incubation with the primary anti-body, the sections were treated with peroxidase-blocking regent (S2001; DakoCytomation) for ten minutes and 5% goat serum (Sigma) for 30 minutes. The primary antibody used for 30 minutes was a purified monoclonal antibody to PRG4 (#S6.79; from T.M. Schmid, Rush University Medical Center, Chicago, IL) at 1:1000 dilution (1 μg/ml protein concentration). The anti-body was produced in a mouse against human PRG4 and it reacts to different mammalian PRG4/lubricin molecules (Su, 2001 #67). Instead of being treated with the PRG4 antibody, the negative immunohistochemical control sections were treated with non-specific mouse myeloma immunoglobulin IgG_{2a} (cat. #02-6200; Zymed Laboratories, South San Francisco, CA). The stains could be seen by using biotinylated link as a secondary reagent, streptavidin-HRP as a tertiary reagent (K0675; DakoCytomation), and AEC substrate chromogen (K3464; DakoCytomation). After the staining procedures, the slides were counterstained with hematoxylin.

A MicroFire Model S99809 camera (Meyer Instruments, Houston, TX) mounted on an Olympus BX51 microscope (Olympus, Tokyo, Japan) was used to capture pictures of the stained sections.

3.2.5 Biochemical Analysis of Pellets

In preparation for the biochemical analysis of the cell pellets, the pellets were digested with protease K (Sigma, P6556). The amount of DNA was measured on days 0 and 14 using Quant-iT PicoGreen dsDNA Assay Kit (P7589, Invitrogen). The amount of GAG was spectrophotometically measured by using dimethylmethylene blue (Farndale, 1986 #396), with chondrotin sulfate as a standard and by being normalized to the amount of DNA.

3.2.6 Statistical Analysis

An analysis of variance (ANOVA) was used to evaluate the effects of the three different media and two cell types on the results. To determine the DNA and GAG

content significance, Fisher's post hoc test was used. The data was collected and the mean ± SD was calculated. The significance level for the data was set at $p < 0.05$.

Chapter 4- Results and Discussion on the Behavior of ISP Tendon Cells and BMSCs Pellet Cultures

4.1 Results

4.1.1 Cell Pellet Culture Macroscopic Observations

All of the pellet cultures had smooth surfaces. However the CM pellets were the smoothest and most transparent, as seen in Fig. 11.

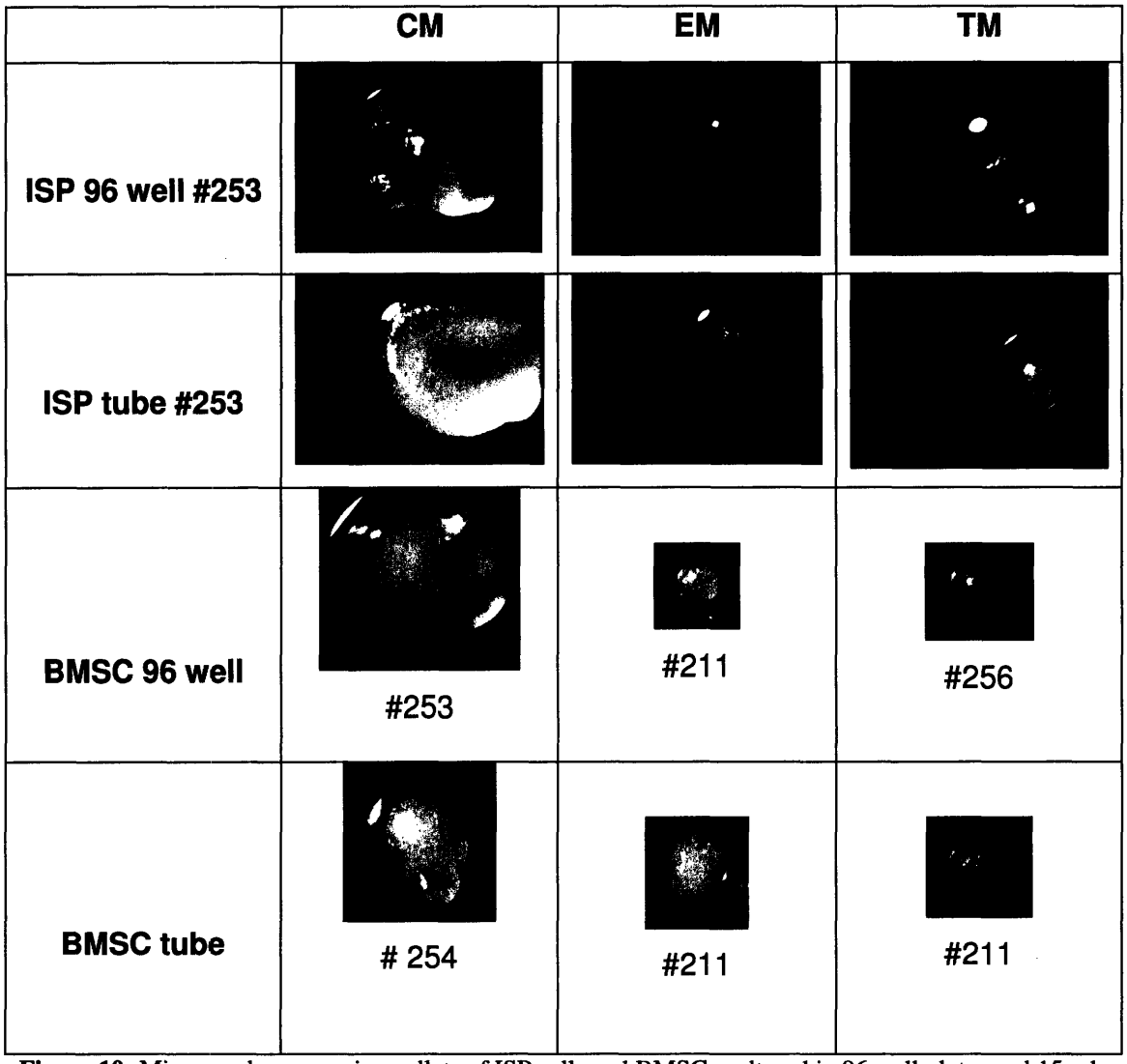


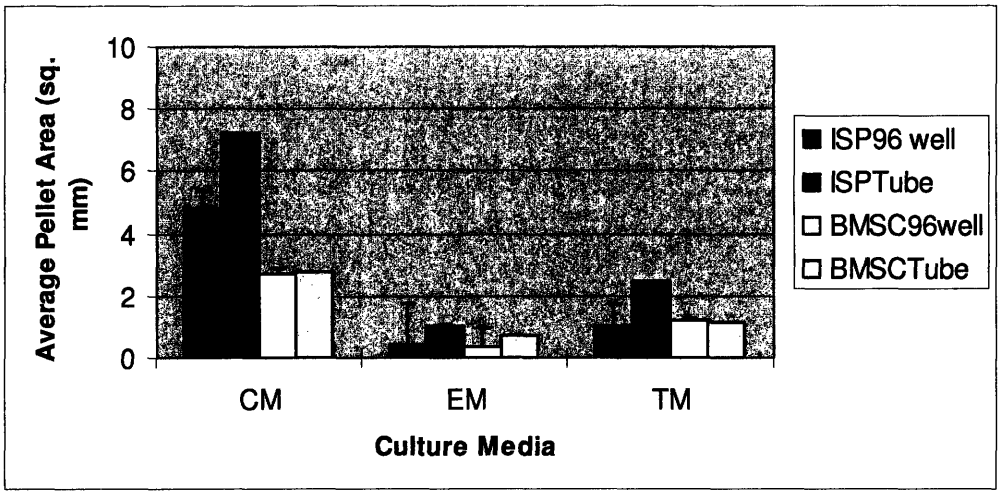
Figure 10: Micrographs comparing pellets of ISP cells and BMSCs cultured in 96 well plates and 15 ml tubes in CM, EM, and TM.

The EM- and TM-pellets were globular and white. The CM-pellets were much more irregular in shape that the EM- and TM-pellets because they consisted of small aggregates that combined together.

The largest pellet sizes were of those cultured in the chondrogenic media, as seen in Figs.10 and 11. Pellets cultured in tendon media were the second largest and those cultured in expansion media were the smallest.

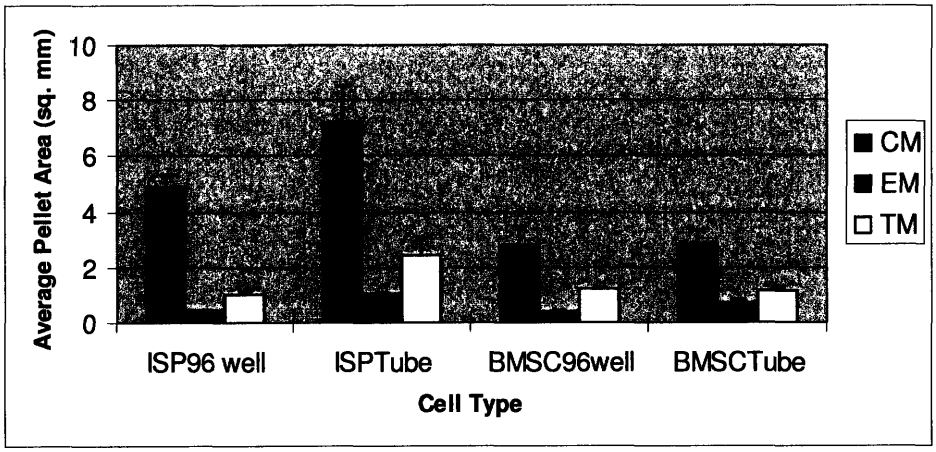
While the ISP tendon cells pellets cultured in CM were significantly larger than the EM and TM groups, there was a smaller difference between the BMSCs CM-, EM-, and TM-pellets, as seen in Fig. 11 (b).

The large sizes of the CM-pellets facilitated the experimentation and analysis process. Usually due to their small size, the EM-pellets were often lost during various procedures, such as changing of media and paraffin sectioning. The EM-pellets were difficult to distinguish and pick up inside the 96-well plates and 15 ml tubes.



(a)

(b)



(c)

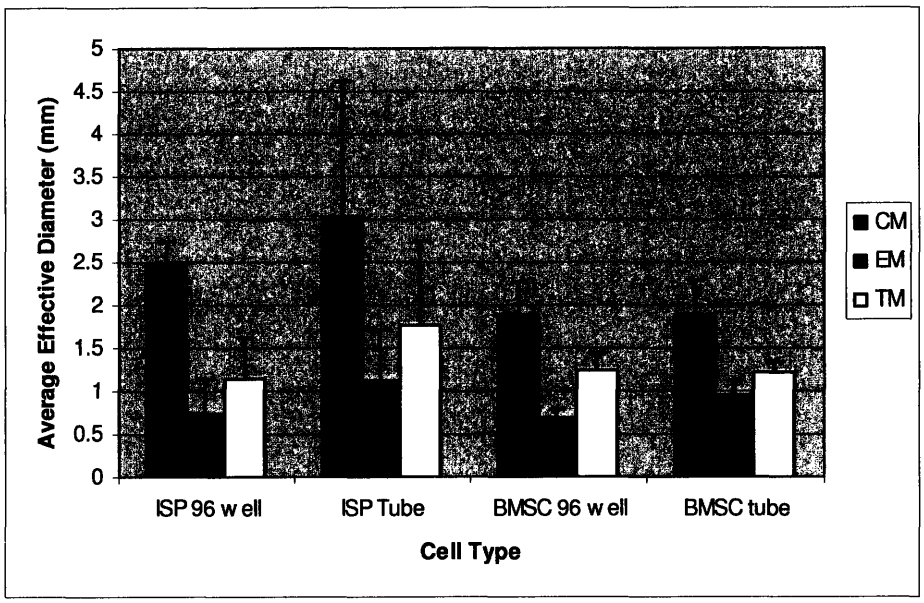


Figure 11: (a)-(b) Histograms comparing the average area of the ISP cell and BMSC pellets cultured in CM, EM, and TM. (c) Histogram comparing the average effective diameter of the ISP cell and BMSC pellets cultured in CM, EM, and TM. Mean ± SD.

As observed in Fig. 11 (c), the effective diameters of the pellets ranged from approximately 0.75 mm to approximately 3 mm, a difference of four-times. The ISP tendon cell pellet sizes were greatly affected by the type of medium as demonstrated by the two-fold difference between the sizes of the CM- and EM- pellets groups.

Three-factor analysis of variance (ANOVA) demonstrated that there were significant effects of cell type ($p < 0.0001$; power = 0.99), medium type ($p < 0.0001$; power =1), and culture condition (i.e., well or tube; $p = 0.001$; power =0.95) on the diameter of the pellets.

4.1.2 Safranin-O, SZP, and α-SMA staining

Safranin-O staining was used to evaluate the stimulation of chondrogenesis in the pellet cultures. As seen in Tables 2 and 3 and Fig. 14, all of the 96-well plate and tube ISP tendon cells and BMSCs pellet cultures cultured in the chondrogenic media, which contained TGF-β1 and dexamethasone, had positive staining for safranin-O staining, indicating chondrogenic differentiation. However none of the pellets cultured in either expansion or tenogenic media. Also neither culture media contained any growth factors or hormones, were stained by safranin-O. During the study, many EM-pellet cultures, indicated by a N/A in Tables 2 and 3, were lost during changing of media, cutting of paraffin sections, or other processes due to their small size. Therefore they could not be studied.

As shown in Tables 2 and 3, none of the pellets cultured in the 96-well plates stained positively for SZP. However a little less than half of the ISP tendon cells CM-pellets and all of the ISP tendon cells EM-pellets cultured in the tubes expressed SZP. None of the BMSC pellets indicated SZP expression.

All of the ISP tendon cells and BMSCs CM-pellets that were cultured in the 96-well plate stained positively for α-SMA. However a smaller portion of the CM-pellets cultured in the 15 ml tubes indicated the existence of α-SMA. While none of the 96 well plate EM-pellets had positive staining for α-SMA, all of the EM-pellets cultured in the tubes were positive. For the TM-pellets, the results varied depending on the cell type and culture methods. As shown in Table 2, TM did not affect the ISP tendon cells pellets in the 96-well plates. However most of the ISP tendon cells pellets in the tubes and all of the BMSCs pellets stained positively for α-SMA.

Table 2: Summary of staining of ISP tendon cells pellet cultures.

ISP	Saf-O		SZP		SMA	
	96well	Tube	96well	Tube	96well	Tube
CM	4 (4)	5 (5)	0 (5)	2 (5)	4 (4)	2 (5)
EM	N/A	0 (3)	N/A	3 (3)	N/A	2 (2)
TM	0 (4)	0 (5)	0 (4)	0 (5)	0 (4)	4 (5)

Note: Data represented in following form: Number of positively stained pellets (total number of pellets)

Table 3: Summary of staining of BMSCs pellet cultures.

BMSC	Saf-O		SZP		SMA	
	96well	Tube	96well	Tube	96well	Tube
CM	3 (3)	4 (4)	0 (3)	0 (4)	2 (2)	1 (4)
EM	0 (3)	0 (4)	0 (3)	0 (4)	0 (1)	3 (3)
TM	0 (4)	0 (4)	0 (4)	0 (4)	4 (4)	4 (4)

Note: Data represented in following form: Number of positively stained pellets (total number of pellets)

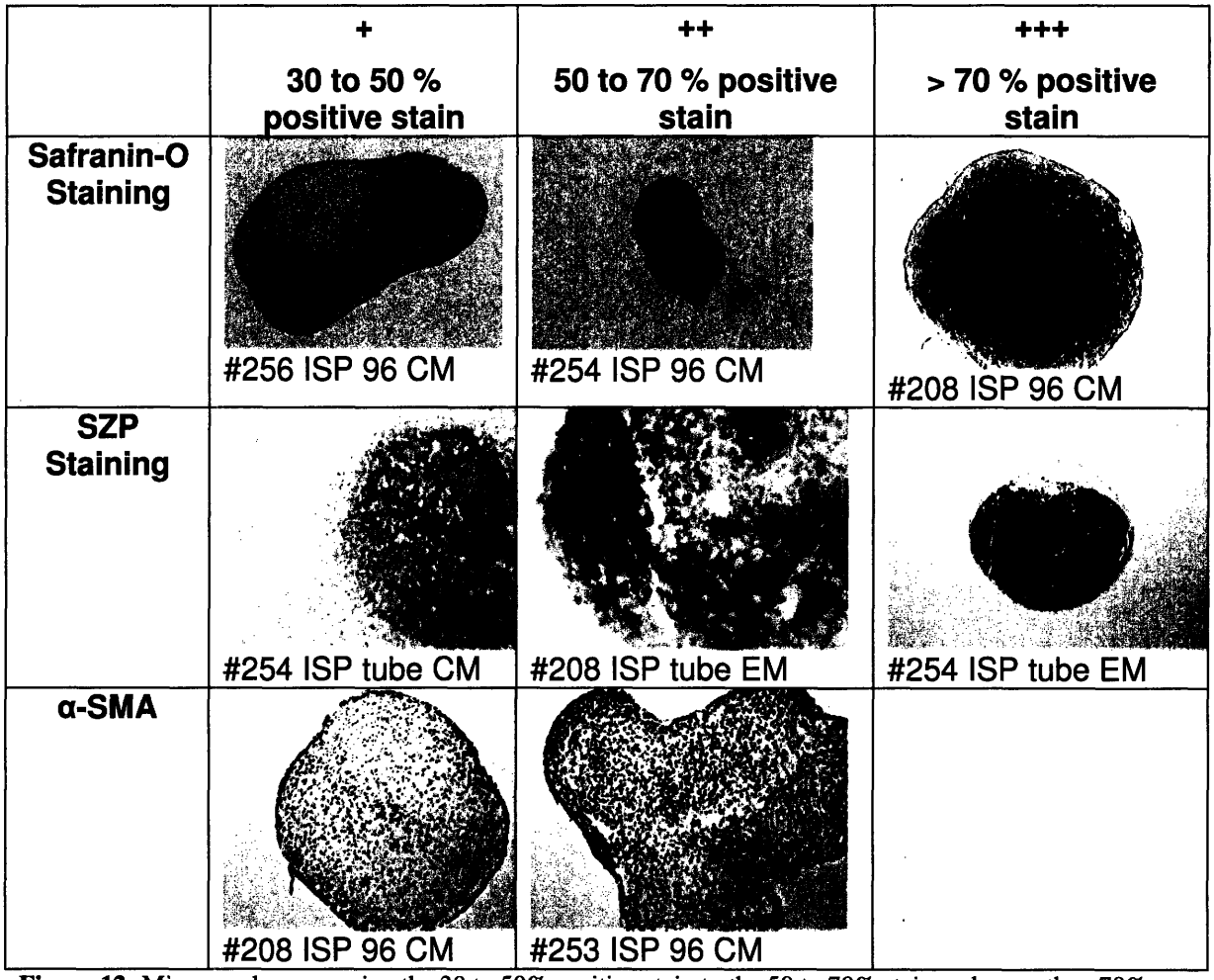


Figure 12: Micrographs comparing the 30 to 50% positive stain to the 50 to 70% stain and more than 70% stain of the immunohistochemical staining using safranin-O, α-SMA, and SZP staining.

4.1.3 Cell Proliferation and DNA Assay Results

To study cell proliferation of the pellet cultures, the DNA content of the pellets was measured using DNA assay on 0 and 14 days after culture. As shown in Fig. 13, after two weeks, the DNA content per pellet decreased in all of the pellet cultures. In the ISP tendon cells CM-pellet cultures the DNA content was significantly higher than that in

the EM- and TM-pellet cultures. There was also a significant difference between the DNA content of the pellets cultured in the 96-well plates and 15 ml tubes. For both ISP tendon cells and BMSCs groups, the 96-well plates had significantly less DNA content than the tubes.

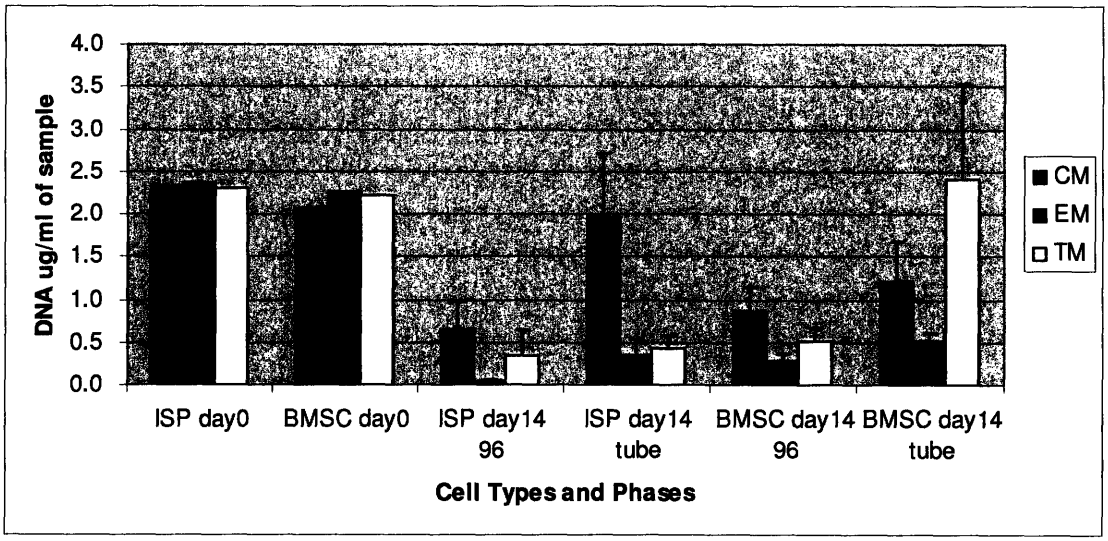


Figure 13: Histogram comparing the DNA assay results for ISP tendon cells and BMSCs cultured in CM, EM, and TM in 96-well plates and 15 ml tubes. Mean±SEM.

4.1.4 GAG content and GAG Assay Results

The results from the GAG assay matched the immunohistochemical staining results. GAG content was significantly higher when ISP tendon cells were cultured in chondrogenic media. The ISP tendon cells CM-pellets grown in tubes had the highest GAG content in comparison to all of the other pellet cultures. However the GAG content in the ISP tendon cells pellets cultured using 96-well plates was significantly lower than that in the ISP tendon cells pellets cultured in tubes.

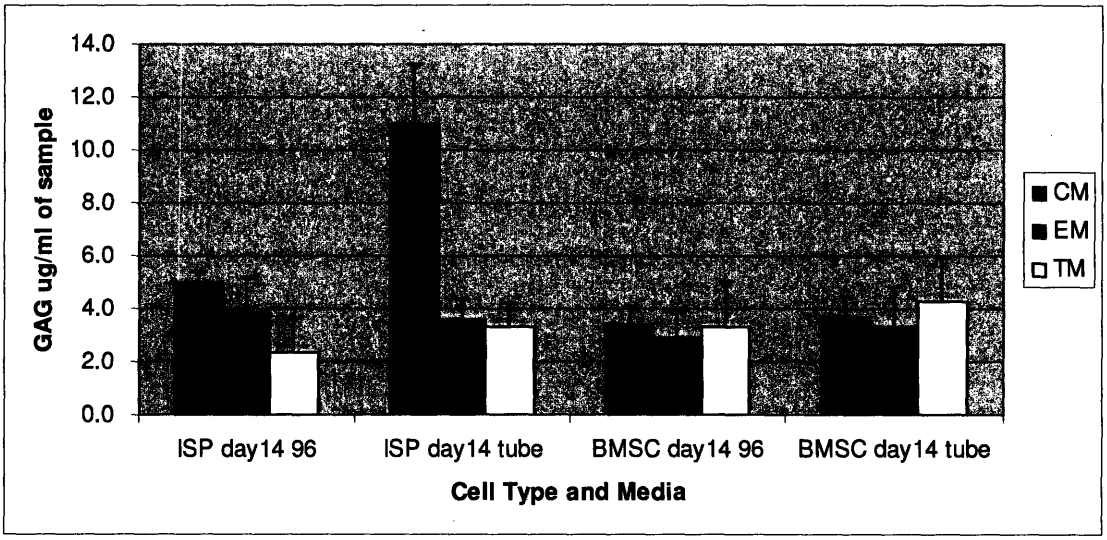


Figure 14: Histogram comparing the GAG assay results for ISP tendon cells and BMSCs cultured in CM, EM, and TM in 96-well plates and 15 ml tubes. Mean±SEM.

4.2 Discussion and Future Studies

From the study we learned that adult goat infraspinatus tendon (ISP) cells can survive in pellet cultures for at least two weeks. Previous work has demonstrated this for BMSCs. Similar to previous studies, it appeared that TGF-β1 and dexamethasone, which were two of the contents of the chondrogenic media, encouraged the most cell proliferation. Chondrogenic media also stimulated the most chondrogensis due to the combination of TGFβ-1 and dexamethasone. Of importance, the results of our study showed that ISP tendon cells as well as BMSCs can undergo chondrogenesis *in vitro* under appropriate conditions. This finding is consistent with the presence of cartilaginous regions within tendons, particularly at sites under compressive loading. Moreover, the 15 ml tubes would be recommended over the 96-well plates to produce higher DNA and GAG content.

Another notable finding of this thesis is that ISP cells were found to express the gene for SZP. Interestingly, this expression was dependent on the medium type, with no such expression seen in ISP cells in TM. This observation is consistent with the finding of SZP within tendons at certain locations, likely serving to lubricate regions of the tissue.

Khalafi, *et al.* reported that in their study on the effects of growth factors, BMPs, and cytokines on SZP accumulation, TGF-β1 induced the largest response.[88] Their

results corresponded with those of other studies which indicated that TGF-β1 is a strong stimulator of SZP expression.[89 90 91] However the results from our study did not indicate that TGF-β1 had a large effect on SZP synthesis. In fact a significantly smaller percentage of ISP tendon cells CM-pellets cultured in tubes were stained positively in comparison to the 100% of the ISP tendon cells EM-pellets which expressed SZP. Therefore no definite conclusion could be made about the contributions of the contents of the media culture on SZP expression. Further studies would be able to clarify the findings of this study.

For the ISP tendon cells and BMSCs pellets cultured in tubes, a higher portion of those in expansion media and tenogenic media were positively stained for α-SMA than those cultured in chondrogenic media. The smaller EM and TM-pellets had higher percentages since α-SMA acts to contract the smooth muscles, which matches with results of previous studies. Smooth muscle actin expression leads to the generation of higher contractile forces by the musculoskeletal tissues to help produce tissue specific architecture.[92] On the other hand, the bigger CM-pellets cultured in the 96 well plate were also positively stained for α-SMA. It has been reported that TGF-β1 can stimulate SMA expression.[93 94] "The SMA-positive cells in the peripheral layers of the chondrogenic pellets mimic those within the superficial layer of articular cartilage and are speculated to play a major role in cartilage development and maintenance."[95] The results of this study show that different biomechanical environments can affect SMA synthesis.

This study helps us understand some of the factors which contribute to cell proliferation and chondrogenesis of tendon cells. We also verified that it is reasonable to use pellet cultures to study the behavior of tendon cells. One of the considerations for future investigations is the use of bone morphogenetic proteins (BMPs). They have been shown to promote chondrogenesis from commitment to terminal differentiation.[96] It has been proposed that BMSCs can be induced to differentiate into tenocytes using BMP12, a BMP in the TGF-β family. Wang, *et al.* reported they were successful in introducing an exogenous BMP12 gene into BMSCs from rhesus monkeys using a gene transfection technique. Using morphological and molecular biological techniques, they confirmed the irreversible differentiation of BMSCs into tenocytes.[97]

It is also believed that TGF-β3 plays a role in chondrogenic maturation. Mackay reported that human MSCs differentiated into chondrocytes when cultured in cell pellet cultures and treated with TGF-β3.[98]

It has been suggested that high-density cultures are promising methods for long-term growth of human tenocytes in vitro. They could be applied to study the effects of drugs and for autologous tenocyte cultivation.[99] Further studies of factors, such as those suggested above and those from the current and previous studies, affecting the chondrogenesis of ISP tendon cells and BMSCs and the differentiation of BMSCs into tenocytes are necessary to near the goal of producing effective methods for tendon tissue engineering and alternatives to surgical solutions.

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