An Investigation of Collagen, Platelet-Rich Plasma and Bone Marrow Derived Mesenchymal Stem Cells on Achilles Tendon Repair in a Rat Model.

By

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Abstract

The goal of this study was to improve the healing rate and strength of the Achilles tendon injury (rupture) following treatment with collagen, platelet-rich plasma (PRP) and bone-marrow derived mesenchymal stem cells (MSC). Achilles tendon rupture is the most frequent tendon injury in the body and it takes longer to heal due to the poor blood supply to it. This research investigated the effort on healing rate and strength of the tendon, following the addition of collagen, platelet rich plasma and bone marrow derived mesenchymal stem cells to a repaired Achilles tendon in a rat model. Platelet rich plasma was prepared from blood obtained from the rats and Bone marrow mesenchymal stem cells were also obtained from the femur and tibia of the same rats. A differentiation assay was performed to determine, if the cells used for this study were indeed stem cells. The cells differentiated into osteocytes, chondrocytes and adipocytes when given growth factors needed for these cell lineages. Rat surgeries were performed on the right legs of rats to cut the Achilles tendon 3mm above the calcaneal bone, which was then repaired. Treatments were given based on the groups the rats belong to; collagen only group, collagen and platelet rich plasma group, collagen and MSC or the three biologics group. Rats were euthanized at week 1 and 2 and their Achilles were harvested for biomechanical testing. Rats that received the three biological materials recorded the highest stress (tensile strength) and strain (elasticity) after a week of recovered however, strains and stress of the collagen only group was almost as high as that of the three biologics group. The MSC+ collagen group recorded the same strain as the three biologics group. There was no significant difference between the stress and strain among the groups at week two. The results indicated that all the treatment groups healed at the same rate at week two. However, MSC and PRP may play a significant role in increasing the tensile strain and elasticity of the tendons.

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List of Abbreviations

BM-MSC: Bone Marrow-Derived Mesenchymal Stem Cell

DMSO: Dimethyl Sulfoxide

ECM: Extracellular Matrix

EDTA: Ethylenediaminetetraacetic acid

EGF: Epidermal Growth Factor

FACIT: Fibril Associated Collagens with Interrupted Triple helices

FBS: Fetal Bovine Serum

FGF: Fibroblast Growth Factor

GF: Growth Factor

IBMX: 3-isobutyl-1 methylxanthine

IFN: Interferon

IGF: Insulin-like Growth Factors

IL: Interleukin

ITS: Insulin-Transferrin-Selenium

MMP: Matrix Metalloproteinase

MSC: Mesenchymal Stem Cell

MTJ: Muscle-Tendon Junction

PBS: Phosphate Buffered Saline

PDGF: Platelet-Derived Growth Factor

PPP: Platelet-Poor Plasma

PRP: Platelet-Rich Plasma

RPMI: Roswell Park Memorial Institute

SDF: Superficial Digital Flexor

SDF-1: Stromal-Derived Factor-1

TGF: Transforming Growth Factor

VEGF: Vascular Endothelial Growth Factors

α-MEM: Alpha-Minimum Essential Media

INTRODUCTION

The current trend in regenerative medicine has led to several studies examining the effect of different biologics on wound repair. Many of these are seen to accelerate the rate of wound healing. Achilles tendon injury is a sport related injury which has which has been seen in increasing amounts over the years, constituting 35% of all tendon injuries. It occurs when the fibers of the tendon tear and start to separate (Gulati et al., 2015). Achilles tears are primarily found in males between the ages of 30 to 50. Treatment can either be operative or conservative, also known as the non-operative method (Maquirriai, 2011). Treatment options are chosen based upon the age and activeness of the patient. Both methods have advantages and disadvantages. The non-operative method is seen to have up to about a 30% rate of re-rupture, while the operative method may lead to infection and poor wound healing (Metzl, et al, 2008).

There have been several studies done on additions to the surgical site to accelerate the rate at which the the Achilles tendon heals, including platelet-rich plasma (PRP), collagen, growth factors, and mesenchymal stem cells (MSCs), among others. One study examined Achilles tendon healing in a rat model using the addition of PRP, which resulted in improvement in tendon repair (Virchenko and Aspenberg, 2006). A similar study was done on the Achilles tendons in a rat and rabbit models, using MSCs to repair the wounds. These studies demonstrated accelerated healing of the tendon (Young et al., 1998 and Chong et al., 2007). An investigation was also done to check the effect of PRP and MSC addition to repaired Achilles tendons in a rat model (Yuksel et al., 2016). These studies showed increased levels of anti-inflammatory cytokine, suggesting improved healing, but did not test the strength of the tendons.

To improve the time it takes to heal an Achilles tendon injury and also prevent scar formation, this study investigates the healing rate of rat Achilles tendon when combining collagen, platelet rich plasma and mesenchymal stem cells. The study includes three phases. Phase one involves harvesting and culturing mesenchymal stem cells (MSC's) from rat tibias and femurs. In phase two, MSC's are transplanted onto a rat tendon following transection of the Achilles tendon and repair. Phase three includes harvesting tendons, both control and treated, following 1 and 2 weeks of recovery, and measuring the effect upon tendon strength in the different study groups. Group one will have standard Achilles repair with a collagen carrier (CollaTape) added, group two will have collagen and platelet-rich plasma, group three will have collagen and mesenchymal stem cells and group four will have collagen, platelet-rich plasma and mesenchymal stem cells added to the repaired tendon.

As described, the three biologics added to the wound in this study will be collagen, PRP and MSCs. Fibroblasts produce collagen, which helps keratinocytes migrate to the wound site. (Gelsea et al., 2003). This leads to the formation of new blood vessels and the formation of new epithelial cells, which are able to move to the wound site and then form a barrier between the wound and its surrounding environment. Collagen serves as a substrate on which new tissues grow and helps to accelerate the rate of wound healing. Autologous platelet-rich plasma (PRP) may also have an important role to play in the healing of the wound, as it contains cytokines and multiple growth factors in higher concentrations than that found in natural blood plasma (Smith, 2016). These cytokines and growth factors, play crucial roles in the regulation of other cells. They are able to attract cells to the wound site and stimulate them to upregulate protein production. They also influence the migration and proliferation of cells in and around the wound (Pinedo et al. 1998 and Folkman et al. 2007). PRP also offer structural support by stimulating the

formation of healthy new tissues and blood vessels at the cellular level, which help with the formation of new blood vessels, regulate the production of the various inflammatory cytokines released during wound healing. PRP also controls the healing events of the extracellular matrix and helps to reestablish the proper function of the skin. In a study done by Heffner et al, (2012) PRP was combined with MSC to produce a faster healing rate in a rat abdominal surgery model. MSCs assist in the formation of new blood vessels, regulate the production of inflammatory cytokines, and help to accelerate the closure of wounds (Heffner et al., 2012). This study hopes to see a significant improvement in the Achilles tendon healing rate with the addition of the described biologicals as determined by testing the tensile strength.

Achilles tendon

The Achilles tendon, also known as the heel cord or the calcaneal tendon, is a tough, white band connecting the calf muscles to the heel bone, which is also known as the calcaneal bone. It is made of fibrous tissues and it is the largest and strongest tendon in the body. It is about 15 centimeters (6 inches) long in humans. The gastrocnemius and soleus muscles are at the proximal end of the Achilles tendon, forming the calf muscles (Benjamin et al., 2007). The Achilles tendon functions by connecting the calf muscles to the heel bone to allow for a flexible movement of the ankle joint. The position of the Achilles tendon enables it to support the weight and movement of the body (Maquirriai, 2011). Tendons function by transferring the energy produced by the muscles to the bone and therefore play a crucial role in the musculoskeletal system (Yuksel et al., 2016).

Structure of the Achilles Tendon

The formation of the Achilles tendon from the gastrocnemius and soleus muscles has been described in detail by Cummins and co-workers (Cummins et al., 1946). This tendon is surrounded by fascia and integument and prominently stands behind the calcaneal bone, forming a gap. This gap is filled with areolar tissue, a common form of loose connective tissue found at many locations in the body of an organism, and adipose tissue (Benjamin et al., 2007).

The Achilles tendon has small fluid filled sacs called bursae cushioning the tendon at the calcaneal bone (Ackermann et al., 2001). The bursae can also be found between the skin and tendon to promote easy skin movement around the tendon and also at the upper end of the tendon (Frey et al., 1992). Along the side and exterior to the muscle is the small saphenous vein which delivers blood from the ankle, lower leg and thigh to the femoral vein. There are minute branches of nerve from the neighboring cutaneous nerves, known as the sural nerves, connecting to the Achilles tendon (Benjamin et al., 2007). The tendon is able to tolerate high loads of stress, about 4 times the body weight when walking and 8 times during activities such as running, because it is made up of type one collagen, which accounts for its considerable tensile strength. Being the largest and strongest tendon in the body does not prevent it from getting injured. The Achilles is prone to injury like the other tendons in the body and this may be due to the limited supply of blood to it and the amount of tension it receives from the other body parts.

Achilles Tendon Injuries

The Achilles is the tendon that is most frequently injured in the body (Cummins et al., 1946; Gulati et al, 2015). Injury of the Achilles tendon has been rising over recent years, constituting 35% of all tendon injuries. It is a debilitating injury which lasts longer than most

injuries and can lead to losing the functionality of the tendon. Achilles tendon rupture is seen in 18 of every 100,000 patients (Chiodo et al., 2006). This injury is common among 30 to 50 year old male patients. The region 3 to 6 cm above the heel bone is poorly vascularized. This area is the commonest site of rupture (Gulati et al., 2015).

There has been attention brought to the treatment of the Achilles tendon injury. This attention is based upon the fact that the rupture is the most common tendon injury in the body and a serious one. It does not heal as quickly as seen with other tendons (Maquirriai, 2011). Some of the causes of the rupture include the use of oral and intratendinous steroids which leads to spontaneous Achilles rupture. Other risk factors include high levels of glucose in the blood, and different types of arthritis. It can also occur as a side effect of long-term dialysis and renal transplantation (Gulati et al., 2015). Commonly known symptoms of Achilles injury are swelling of the leg, inability of the leg to support the body's weight, pain, sharp sensation in the leg after an activity and less flexibility of the knee and ankle joints (Gulati et al., 2015).

Achilles Tendon Repair

Several new techniques have been developed relating to the treatment of Achilles tendon injury. Despite all these new treatments, this injury is still considered a problem in athletes and adults between the ages of 30-50, due to the high rate of re-rupture and the long recovery period after treatment (Chiodo and Wilson, 2006 and Benjamin et al., 2007). The lack of better treatment options has led to the difficulty in healing of the tendon, which can take from months to several years to heal. The human body is normally able to regenerate and heal most injuries to tissues. The question about why this does not happen in injuries relating to this tendon is what scientist and medical professionals are working hard to solve (Cummins et al., 1946).

There are several studies showing that the Achilles remains the same throughout an individual's adult life, unlike other tissues.. The cells do not regenerate and so cannot repair themselves quickly like other cells. This discovery has unveiled the mystery behind the long recovery period of the Achilles tendon injury and suggests possibilities for finding new methods of treatment to accelerate the healing of an Achilles tendon injury. Treatment of Achilles tendon rupture remains an issue worldwide. Many studies have looked at several therapeutic options for the tendon injury/rupture, including surgical repair and non-surgical repair. (Maquirriai, 2011).

The operative or surgical repair method includes the minimally invasive and the open method. Surgery has several advantages, including low incidence of re-rupture, being able to move around within a short period of healing which will help to prevent muscle atrophy. The non-surgical treatment option includes casting the leg to prevent movement. This treatment is aimed at maintaining the contact between the tendon ends, which can facilitate healing. (Gulati et al., 2015). The advantages of the non-operative treatment option include a better cosmetic view of the leg, low to no infection of wound site, no iatrogenic sural nerve lesion, that is lesions induced accidentally by a surgeon during a procedure. Unfortunately, prolonged periods of immobilization of a limb or joint after the non-operative repair method may be complicated by atrophy of muscles and articular cartilage, osteoarthritis, skin necrosis, tendocutaneous adhesions and thrombophlebitis (Orhan et al., 2004).

New methods are required to accelerate the healing of the tendon. According to Orhan et al, such healing involves the proliferation of fibroblasts in the synovial layer, paratendon (the areolar tissue filling the space between a tendon and its sheath) and at the injured ends (Orhan et al., 2004). Any method which stimulates tissue healing without causing associated damage due to immobilization may decrease the incidence of complications after tendon injuries. Aside from

the treatments mentioned above, therapies such as bone morphogenic protein administration (BMP), genetically modifying the tissue by adding certain growth factors, ultrasound therapy, local growth factors and platelet administration, local extracorporeal shock therapy (this involves the use of pressure waves to treat the tendon using a minimally invasive approach), administering anti-inflammatory drugs systemically and the use of bone marrow derived MSCs. These therapies have resulted in a significant improvement in healing of the Achilles tendon in several animal models (Reviewed in Aspenberg, 2007).

Collagen

Collagen is one of the main structural proteins of the body, which contributes to the formation connective tissue by providing a substrate for cells to attach to. Examples of some of these connective tissues are tendons, bones, cartilage, blood vessels, skin, ligament and muscles. Collagen is the principal tensile element of these vertebrate tissues, occurring in the extracellular matrix as long fibrils (Kadler et al., 2007). Collagens are the main structural element of all connective tissues and are found in in the interstitial tissue of almost all parenchymal organs. Collagens contribute to the stability of tissues and organs and maintain their structural integrity (Kadler et al., 2007). It is the most common and abundant protein found in the body, comprising about 35% of the total body protein. Collagen has the ability to withstand a high amount of stress and therefore provides strength to the various tissues. In the skin for example, collagen provides strength, elasticity and the firmness to withstand harsh environmental conditions. It is essential for maintaining strength, firmness and elasticity of the tissues (Kadler et al., 2007).

Fibroblasts are cells that produce collagen. They are constantly broken down, while constantly being newly produced to meet the demands of the body. A breakdown in this system

may lead to the death of some tissues. An imbalance in the collagen producing system may occur due to stress or a lack of certain nutrients. This results in collagen loss in the body. Collagens are composed of two amino acids, glycine and proline. These proteins are made by the body and are also found in foods containing proteins. Enzymes producing collagen require vitamin C to function well.

Collagen is used as a general term for proteins with a triple helix of three polypeptide chains, a characteristic of the collagen family. All collagens have this unique structure, but differ in their function, size and tissue distribution (Myllyharju and Kivirikko, 2001). Collagens can be grouped into transmembrane collagens, anchoring fibrils, basement membrane collagens, network-forming collagens, fibril-forming collagens and fibril associated collagens based upon their molecular structure and organization. These different collagens are grouped based on their splice variants, structural diversity and complexity, their assembly and function, and as well as the presence of non-helical domains (Kadler et al., 2007). The fibril-forming collagens are the most abundant and widespread collagen family in the body. They make up about 90% of the total collagen in the body. A common feature among the collagen family is the presence of a right-handed triple helix with three chains, although individual groups have different structural diversities. Degradation and the disturbed metabolism of collagen are relevant in the cause of certain diseases such as osteoarthritis and osteoporosis. Knowledge of the different properties of the diverse groups of collagen is essential in treating certain diseases (Kadler et al., 2007). The binding abilities of collagen make them useful in almost every part of the body. They are used as drug delivery systems and reservoir for growth factors. They form scaffolds and help in tissue regeneration and repair due to their networking and anchoring abilities (Gelsea et al., 2003). Aside from these abilities, transmembrane collagens are found on a variety of cells and they

serve as precursors for some bioactive peptides. The paracrine function of these peptides has resulted in resurgence in the interest in collagens (Kadler et al., 2007).

Collagens play a significant role in tissue repair, organ development and wound healing by entrapping, storing and delivering certain growth factors and cytokines needed for these processes (Gelsea et al., 2003). Collagen type I is known to bind decorin, a small proteoglycan found in all connective tissues which opposes the actions of transforming growth factor. It is possible that collagen might block TGF action within the tissue indirectly through decorin (Ortega and Werb, 2002). Transforming growth factor (TGF) is one of the several proteins secreted by macrophages and T cells that can stimulate the growth of normal cells. TGF encourages the formation of new blood vessels in cells and tissues (Reviewed by Shiel et al., 2018). Collagens also bind several cytokines and some growth factors including insulin-like growth factor I and II (IGF I and II). IGF-1 is a hormone that stimulates the growth of cells and tissues, including muscles and bone. IGF-II is a protein hormone known to control the proliferation of cells, the growth and migration of these cells, as well as their differentiation and survival. Insulin-growth factor I and II are attached to collagen matrices of bones and bones serve as the main reservoirs of various growth factors in the body (Borza et al., 2001). During bone degradation, osteoclasts destroy bone networks, leading to the release of matrix bound IGFs which are then used to form new bones through the stimulation of the osteoblasts by nearby cells.

The induction of new cartilage formation may be due to the release of matrix bound IGFs which may be due to the activation of cartilage cells through the release of bound growth factors after the degradation of the cartilage matrix. Collagen also serves as a scaffold for cell migration and provides strength to the forming scar. These properties of collagen have been utilized for the

growth of neuronal and vascular tissues and the repair of bony defects (Reviewed in Xue and Jackson, 2015).

Role of Collagen in Wound Healing

Collagen plays a significant role in the wound healing process due to its chemotactic role. Studies show that when collagen is degraded during tissue remodeling, collagen-derived degradation products can serve as chemotactic stimuli for human peripheral blood monocytes (Postlewaite and Kang., 1976). Collagen also encourages the removal of dead cells and tissues, the formation of new blood vessel and re-epithelialization (Hochstein and Bhatia, 2014). In order to protect the collagen being produced in a healing wound, collagen dressings are often used (David Brett, 2008). Collagen dressings are wound dressings obtained from different animal sources, such as cattle, pigs and horses. They are produced in powder, gel, paste or freeze-dried sheet forms. Collagen stimulates tissue re-epithelization and deposits new collagen fibers and granular tissues in and around wound sites (Hochstein and Bhatia, 2014). The collagen dressings chemically bind to certain endopeptidases known as matrix metalloproteinases (MMPs) which are found in the extracellular matrix of wounds. The roles of these MMPs are to breakdown collagen in the wound. The presence of the collagen wound dressings provide alternative source of collagen for the MMPs to break down, leaving the normal collagens formed during the wound healing process to perform their functions (Hochstein and Bhatia, 2014). Aside from preserving the natural collagen in the wound, these dressings aid in maintaining the right temperature in and around the wound.

In this study, CollaTape will be used as a collagen dressing due to its soft and sponge-like nature, its ability to serve as the matrix in which the other biological materials will be injected.

CollaTape is also able to control bleeding and acts as an anti-microbial agent while helping to rapidly heal the wound (Mahesh et al., 2015).

Platelet Rich Plasma (PRP)

Blood is made primarily of plasma, which is the liquid component. The solid components include the red and white blood cells and platelets. The major role of platelets in the body is their role in blood clotting. They however contain several proteins known as growth factors needed in the acceleration of wound healing. (Hsu et al, 2013). Platelet rich plasma, commonly referred to as PRP, is plasma with higher platelet concentration compared to normal blood. They are suspended in a small volume of platelet poor plasma. Platelet-rich plasma has not only a higher platelet concentration, but also contains three proteins, fibrin, fibronectin and vitrinectin, that act as cell adhesion molecules for osteoconduction (when undefined cells of loose makeup undergo mitosis and form bone making cells). They also serve as matrix for the migration of bones, connective tissues and epithelial cells (Marx, 2004). The high platelet concentration, correlating with higher amount of growth factors, can be ten times greater than found in normal blood. Blood platelets release a cocktail of growth factors from their α-granules when activated. Some of these growth factors are known to initiate and stimulate tissue repair in the body (Smith, 2016). Examples of some of these growth factors are vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), and transforming growth factor beta (TGF-b), which are found in abundance when the wound healing process is in the proliferative phase. This encourages the formation of new tissues in and around the wound environment (Heffner et al, 2012).

Platelets also contain Insulin-like Growth Factors (IGFs) and platelet-derived angiogenic factor. This was determined by enzymatic analyses pf PRP and normal blood. The result showed 7, 30 and 10 times higher amounts of TGF-J31, PDGF and EGF, respectively, in PRP compared to normal blood (Babbush et al., 2003). A study was done by Jo and co-workers to find the effects different types of growth factors in PRP have on human tendon cells from rotator cuff tendons with degenerative tears. Tenocytes were cultured in platelet-poor plasma (PPP), PRP activated with calcium and PRP activated with calcium and thrombin at different platelet concentrations. Their results indicated that cell proliferation was increased in the PRP groups at days 7 and 14 as the doses increased. The PRP + thrombin group recorded the highest rate of proliferation. Their data suggested that injecting platelet-rich plasma into the wound helped the healing of the rotator cuff tendon (Jo et al, 2012).

PRP therapy is a non-surgical treatment used for conditions like ligament and tendon sprains or tears, and arthritis. This therapy involves preparing concentrated platelets which are then introduced into the damaged tendon, ligament or joints to help repair the damaged tissues and also to help increase the healing rate. The effectiveness of the PRP therapy relies on the technique used during the preparation, the dose and the concentration used. The recovery potential of a tissue is increased when PRP is injected into it after an injury, due to the presence of the large pool of growth factors in PRP (Freymiller, 2004). Due to the rich content of growth factors in PRP, an individual injected with PRP after an injury can get back to a normal life after about six weeks (Kadler et al., 2007). This came to light when professional athletes used it to treat season-ending symptoms such as pain, swelling, inflammation and stiffness. Some researchers thought platelets play a role in the formation of tumors and in trying to understand how it worked, they conducted several studies. These studies investigated the role of platelets in

wound healing, tissue repair, new blood cell formation and homeostasis (Folkman et al., 2007). The investigations in this field reported that platelets influence the function of endothelial cells and leucocytes and enable autoactivation (when a gland is stimulated by its own secretions) with autocrine (binds to receptors and affects the function of the same cell that produced it) and paracrine (affects the nearby cells and affects their function) effects, leading to the differentiation of cells, coagulation, regulation of inflammatory responses and chemotaxis (Pinedo et al. 1998 and Folkman et al. 2007).

Jo and co-workers worked with different concentrations of PRP and human tenocytes obtained from patients during an arthroscopic rotator cuff repair surgery. They concluded that the concentration of platelets used directly correlated with the rate of tenocyte proliferation, the synthesis of collagen and glycosaminoglycans and the rate of matrix gene expression (Jo et al., 2012). Another study was done to compare PRP treatment and exercise therapy in treating sub acromial impingement syndrome. The study showed that the difference recorded between the PRP treatment group and the exercise therapy group were not significant but the rotator cuff index scores and the abduction range of motion scores were higher in the exercise therapy group compared to the PRP treatment group (Nejati et al., 2017).

Mesenchymal Stem Cells

Mesenchymal stem cells (MSCs) are undifferentiated cells capable of differentiating into different progenitor cells (Gulotta et al., 2012). Bone marrow-derived mesenchymal stem cells (BM-MSCs) in adults are able to renew themselves and their precursor cells are capable of differentiating into several cell lineages, such as bone, cartilage, fat, and tendon cells (Caplan and Dennis, 2006). They participate in autocrine and paracrine activities due to the different

cytokine and growth factors they secrete. The cytokines and growth factors limit the function of the local immune cells, prevent the formation of scars and apoptosis (programmed cell death). They also encourage the stems cells to differentiate into the cells needed, based upon their location and help with the formation of new blood vessels. The roles played by the cytokines and growth factors are known as trophic effects.

Stem Cell Therapy

Looking at the feasibility, the cost, the effectiveness and ethical issues associated with the use of biological materials, MSCs have become the better option among all cell therapies (Liang et al., 2014). MSCs have great therapeutic effects in different human diseases like cancers, diabetes, cardiovascular diseases, immune diseases, spinal cord injuries, hematological disorders and graft versus host disease (Liang et al., 2014). The roles of MSCs in wound healing include controlling the secretion of pro-inflammatory, inflammatory and anti-inflammatory cytokines and regulating the functions of the immune cells in and around the wound site (Huang et al., 2015). Transplanting MSCs in some parts of the body can prevent the formation of fibrotic tissues and also restore the functions of the cells and tissues in that part of the body (Salibian et al., 2013).

There have been several trials done to show the healing capacity of MSCs from different sources in the body, such as the bone marrow, adipose, platelets and umbilical cord. The use of MSCs to improve tissue healing has been demonstrated in several studies using different models (Chong et al., 2007). Others have shown that BM-MSCs migrate to the wound bed during cutaneous wound healing. An example is research done by Badiavas and Falanga, which demonstrated that BM-MSC differentiate and become incorporated into normal skin tissue

during growth and dermal rebuilding, resulting in the closure of chronic wounds considered to be non-healing. Autologous bone marrow cells (patients' own stem cells) were applied to the wounds of more than one year duration, considered to be non-healing chronic wounds in patients. Patients in this study were previously given autologous grafts and bioengineered skins but were reported not responsive to those treatment. At the end of their study, their observation was that there were complete closures of the wounds after the MSC administration. This result was associated with the ability of MSCs to help with rebuilding of the dermal tissues in the patients (Badiavas and Falanga, 2003). Another study was carried out to evaluate the healing of the hallucis longus tendon (a tendon that helps to easily move the big toe) in a heel bone tunnel using mesenchymal stromal cells derived from rabbits. The stromal cells for this study were delivered in a fibrin glue carrier. It was observed that the mesenchymal stromal cells improved healing of the hallucis longus tendon by forming fibrocartilaginous attachments (a type of attachment found between soft tissue-to-bone which is made of cartilage that contains type I and II collagens). The tendons were allowed to heal two weeks post-surgery before the results were taken. No biomechanical test was done on the tendons (Ouyang et al., 2004). A study done by Gulotta et al, (2012) examined some ways to improve the wound site to encourage the regeneration of the indigenous insertion site following a rotator cuff injury and also to inhibit scar tissue formation. A β-galactosidase assay is an assay that uses adenovirus containing a reporter gene lacZ, coding for the protein β-galactosidase. This protein interacts with its substrate, X-Gal and emits a blue color. MSCs were transduced *in vitro* with Ad-lacZ 24 hours before surgery. A β-galactosidase assay was performed to determine the presence of the MSCs. There was evidence of their presence and metabolic activities when blue stains were observed after performing the assays on the surgical tissues. Unfortunately, improved healing was not

observed in the tissues. The authors suggested that the MSCs used for this study may not be sufficient to stimulate tissue regeneration (Gulotta et al., 2012).

Summary of Project Goals

There has been an increase in the number of Achilles tendon injuries recorded over the years drawing the attention of health professionals and scientists to the limitations of the available treatment options. Current treatments result in the inability to use the leg over a long period of time, due to the slow healing of the tendon and the high re-rupture rate of the Achilles. Researchers are now focusing on using regenerative medicine to increase the healing of this tendon and to prevent re-ruptures if possible. The use of biologics, such as collagen, PRP and MSCs to accelerate wound healing has been a current trend in regenerative medicine. Collagen, PRP and MSCs accelerate wound healing rates, help with the formation of new blood vessels, and regulate the production of various inflammatory cytokines released during wound healing. They also control the healing events of the extracellular matrix and reestablishe the proper structure and function of the skin during cutaneous wound healing.

This study will compare collagen, PRP and MSCs as additives to Achilles surgery for their ability to promote tendon repair in a rat model. One characteristic of MSCs is the ability to differentiate into chondrogenic, adipogenic and osteogenic cells. This will be examined for the MSCs used in this study in order to demonstrate their competence for multipotency. The effects of media and culture conditions on the morphological, phenotypical, and functional properties of MSCs derived from rat bone barrows will be examined. The MSCs used for this study will be expanded *in vitro*.

Four different study groups will be examined. Rats in the first group will have only surgical repair and collagen treatment and will serve as the control group. The second group will have surgical repair plus collagen and PRP, group three will have surgical repair plus collagen and MSCs, and group four will have surgical repair plus collagen, PRP and MSCs. Rats will be euthanized at weeks one and two from each of the four groups to compare the healing rate of each group. The healing rates will be determined by measuring the tensile strength of the Achilles tendons in each group.

Studies on improving the healing rate of the Achilles tendon using collagen only, PRP only and PRP and MSCs have been done previously (Huang et al., 2015, Aspenberg and Virchenko, 2014, Aktas et al., 2016). However, these studies did not examine this combination of therapeutics by measuring tensile strength and time of maximum healing of the repaired tendon. The current study hopes to aid in the search for a better way of repairing Achilles tendon injuries.

MATERIALS

Male Lewis Rats were purchased from Charles River Laboratories International Inc. (Wilmington, MA). 3-isobutyl-1 methylxanthine, Serum free alpha-minimum essential medium, Alcian Blue, Alizarin Red S, Oil Red O, Ascorbic acid 2-phosphate, β-Glycerophosphate, Dexamethasone, Fetal Calf Serum, GlutaMAX, Penicillin-Streptomycin, Trypsin/EDTA were purchased from Sigma-Aldrich (Saint Louis, MO). CollaTape was purchased from Zimmer Dental Inc. (Warsaw, IN). Vinyl sutures were purchased from Ethicon (Cornelia, GA). Cell titer 6 AQ Solution was purchased from Promega (Madison, WI). Heparin was purchased from STEMCELL Technologies Inc. (Cambridge, MA).

METHODS

Surgical Manipulations

The experimental protocol for this study was approved by the Institutional Animal Care and Use Committee at Youngstown State University. All surgical manipulations were performed under aseptic conditions. Surgeons wore masks, sterile gloves and hairnets. All instruments were sterilized by autoclave or with a dry sterilization unit. Between each procedure, instruments were wiped with a 70% ethanol solution, wiped dry and then re-sterilized using a Germinator 500 dry bead sterilizer. All procedures were performed under isoflurane (3-5%) inhalation anesthesia for induction and 1-3% for maintenance of anesthesia. Time under isoflurane anesthesia ranged from 20 to 40 minutes. Lewis rats (Charles River Laboratories International, Inc) (IACUC protocol #06-18) were maintained under standard housing conditions and fed ad libidum. Respiration, tissue color and toe pinch reflex were monitored during surgery. Rats receive buprenorphine (0.01-0.05 mg/kg, s.c.) as an analgesic before every surgery at 12 and 24 hours after surgery. For all nonsurgical and surgical procedures, rats were monitored once per day for the first week of recovery for signs of infection and autophagia (biting oneself). After each procedure, the rats were placed in clean bedding and monitored during the recovery period. Three different procedures were performed. The first was the collection of blood for platelet rich plasma and the harvesting of bone marrow for mesenchymal stem cells.

Platelet Rich Plasma Preparation

The protocol for collection of PRP was modified from the work performed by Maekawa et al., 2003. Blood was collected from male Lewis rats via a heart puncture using a 21-gauge needle and a 10mL syringe containing 1/10 volume of citrate dextrose. The blood was centrifuged at

room temperature in a conical tube for 10 minutes at 200 x *g*. The plasma layer was removed and centrifuged at 700 x *g* for 10 minutes. The upper (platelet-poor) layer was removed and stored at-20^{O}C leaving about 1 mL portion with the platelet pellet. The pellet was re- suspended following the addition of 5% DMSO. The PRP was frozen slowly by gradually decreasing temperature to -80^{O}C. Subsequently, the frozen pellets were stored in liquid nitrogen. In order to thaw PRP for later use, the frozen pellet was quickly melted by adding it to 1 mL warm plasma and mixed. The PRP was then centrifuged at 700 x g for 10 minutes at 4^{O}C. The plasma was then aspirated off and the remaining pellet was re-suspended in 1 ml plasma.

Isolation of MSCs

The acquisition and processing of BM-MSCs was performed as in Dai et al. (2005), Javazon et al. (2001), and Heffner et al. (2012). Three male Lewis rats were euthanized by CO_2 inhalation and the femurs and tibias were removed from the hind limbs. The epiphyses of the bones were cut and the bone marrow was flushed with 10 mL of RPMI 1640 media using a 21-gauge needle. The clumps were allowed to settle for 5 minutes in a conical tube and all but 0.5mL of the supernatant was removed. The cells remaining in the supernatant were centrifuged at 400 x *g* for 10 minutes at room temperature. The pellet was re-suspended in 10 mL of complete medium (αMEM containing 20% fetal bovine serum, 2mM glutaMAX, 100 U/mL penicillin, and 1 mg/mL streptomycin). A 10μL sample of the solution was taken and the nucleated cells were counted using a hemacytometer following treatment with 4% acetic acid. The cells were diluted in complete medium to about $1x10^6$ cells/mL and 10 ml was then added a T75 culture flask for expansion.

Expansion of MSCs

The cells (1 x 10^7cells in a T75 cell culture flask) were incubated for 4 days at 37^{o}C and 5% CO_2. The non-adherent cells were aspirated off and 10 mL of fresh media was added. The cells were fed every 3-4 days until the cells reached 80% confluency. The cells were washed with PBS and trypsinized using a treatment of a 2 mL solution of 1X trypsin and EDTA (0.25% trypsin and 1.0 mM EDTA) for 7 minutes at 37^{O}C. The cells were dislodged by tapping the flask and the reaction was ended by the addition of 20mL of complete medium. The cells were then split into two flasks. The expansion of MSCs was continued until the second passage. The cells were then counted using a hemacytometer, centrifuged for 5 minutes at 600 x g, and re-suspended in complete media containing 10% DMSO to achieve a concentration of 1x10^6 cells/ml. The MSCs were then placed in cryovials, frozen slowly for 24 hours at -80°C, and then stored in liquid nitrogen.

Osteogenic Differentiation

The protocols for all differentiation assays were adapted from procedures in Chen et al. 2009. In order to evaluate osteogenic potential, 2 x 10^5 MSCs in the third passage were seeded into a 6 well plate. The cultures were incubated at 37^{O}C in 5% atmospheric CO_2 and allowed to become confluent. Half of the wells were maintained with complete media while the remaining were maintained with osteogenic medium (200 μM ascorbic acid 2-phosphate, 100 nM dexamethasone, 10 mM β-glycerophosphate, 10% FBS, 2 mM glutaMAX, and 1% penicillin/streptomycin in DMEM high glucose) for three weeks (Chen et al., 2009). Media was aspirated off the cells and fresh media was added every 2-3 days. To identify the presence or absence of osteogenic differentiation, the cells were washed with PBS and then fixed with a 10%

formalin solution for one hour at room temperature. Formalin was removed and the cells were washed with distilled water. Alizarin Red S (2%) was dissolved in distilled water. The pH was adjusted to 4.2 using 0.1% NH4OH and the solution was filtered. The wash solution was aspirated from the cells; the bottom of the well was covered with 2% Alizarin Red S stain (pH 4.2) and allowed to incubate at room temperature in the dark for 45 minutes. The cells were washed four times with 1mL of distilled water and PBS was added to each well. The cells were visualized using an inverted phase contrast microscope.

Adipogenic Differentiation

To evaluate adipogenic potential, 2 x 10^5 MSCs in the second or third passage were added to each well of a 6 well plate. The cultures were incubated at 37^{O}C in 5% atmospheric CO_2 for 48 hours with complete media until they reached confluence. To induce differentiation, the cells in three of the wells were maintained in adipogenic media containing 1µM dexamethasone, 500 µg/mL insulin, 1 µM indomethacin, 500 µM 3-isobutyl-1 methylxanthine (IBMX), 10% FBS, 2mM glutaMAX, and 1% penicillin/streptomycin in DMEM high glucose for three weeks (Chen et al., 2009).The remaining 3 wells were maintained with complete media. The media was aspirated and replaced every 2-3days. Cells were taken from the incubator and washed with PBS. The solution was aspirated off and about 1mL of neutral buffered formalin (10%) was added and allowed to incubate at room temperature for 30 minutes. A 0.3% stock solution was made by dissolving 300mg Oil Red O in 100 mL isopropanol. The staining solution was prepared by diluting 3 parts of the stock solution with 2 parts distilled water and filtering with a syringe filter. The fixation solution was aspirated off the cells which were subsequently washed with distilled water. About 1mL of 60% isopropanol was added and allowed to incubate for 5 minutes before being aspirated off. Enough Oil Red O staining solution was added to cover the cells (about

1mL) and incubated for 15 minutes. The stain was removed and the cells were washed with water until wash became clear. PBS was added to cover the cells and images were taken using an inverted phase contrast microscope.

Chondrogenic Differentiation

In order to evaluate chondrogenic potential, 2 x 10^5 MSCs in the second or third passage were added to each well of a 6 well plate. The cultures were incubated at $37^{O}C$ in 5% atmospheric CO_2 for 48 hours with complete media until the cells reached confluence. Chondrogenic media (1% insulin-transferrin-selenium (ITS), 50 µg/mL ascorbic acid, 100 nM dexamethasone, 10 ng/mL TGF-β1, 2mM glutaMAX, and 1% penicillin/streptomycin in DMEM high glucose) was then used to maintain half of the wells while complete media was used for the remaining wells (Chen et al., 2009a). Media was aspirated off the cells and fresh media was added every 2-3 days for 3 weeks. The cells were washed with PBS and then fixed with a10% formalin solution for one hour at room temperature. The formalin was removed and the cells were washed twice with distilled water. The staining solution was prepared by dissolving about 10mg of Alcian Blue 8 GX dye in a 60:40 solution of ethanol and acetic acid. Enough stain was added to the wells in order to cover the cells and allowed to incubate overnight in the dark at room temperature. The stain was aspirate and 1 mL of a de-staining solution consisting of 120 mL ethanol and 80 mL acetic acid was added and incubated for 20 minutes. The de-staining solution was aspirated off and PBS was added to cover the cells which were then visualized and imaged with an inverted phase contrast microscope.

Achilles Tendon Laceration and Surgical Repair

For the second surgical procedure, forty rats were anesthetized with isoflourene and were randomized to one of the four treatment groups and then subjected to a 3 mm Achilles tendon segmental defect. All surgeries were performed using aseptic techniques. Rats were placed in prone position and the right ankle was secured in neutral under general anesthesia. A 1.5 cm longitudinal midline skin incision was made over the Achilles tendon, exposing the Achilles and superficial digital flexor (SDF) tendons. A 3 mm segmental defect was created in the mid-substance of the Achilles, half way between the calcaneal insertion and the musculotendinous junction. Approximately 3 mm tendon remained on each side, distal and proximal to the gap defect. The defect was reconstructed with one of the four treatments; (i) empty carrier (CollaTape = collagen) only, (ii) collagen with platelet rich plasma (iii) collagen with mesenchymal stem cells and (iv) collagen with platelet rich plasma and mesenchymal stem cells. Following Achilles tendon reconstruction, the skin incision was closed with 5–0 Vicryl sutures using an interrupted stitch.

Animals recovered in the animal facility. One, two, and three weeks later, the third surgical procedure was performed. Animals were deeply anesthetized, euthanized by exsanguination and pneumothorax and the Achilles tendon excised for analyses.

Biomechanical Testing

Mechanical testing was performed to measure the effect of combining PRP, CollaTape and MSCs on functional outcome of the healing Achilles tendons using standard tensiometric analysis. The methods for the biomechanical testing were modified from the works of Aktas et al., 2016 and Bolt et al., 2007. Achilles tendons were excised from the rats leaving the calcaneal

insertion and the muscle-tendon junction (MTJ) intact. Tendons were covered with guaze soaked in PBS and frozen at -20^{o}C until testing date. Tendon length was measured using a 0–150 mm(0.01 mm resolution) digital caliper. Tendon width and thickness were measured optically using Image J (measurements taken at three locations along the tendon length and averaged) and the cross-sectional area (assumed to be an ellipse) was calculated. Tendons remained hydrated using phosphate buffered saline (PBS) throughout testing. The calcaneus was trimmed and press-fit into a custom bone grip. The soft tissue end was fixed to strips of 100 grade sandpaper with acyanoacrylate adhesive, which were held in a soft-tissue grip. Tendons were tested in a custom-designed load frame along their longitudinal axis. Loading was constrained to prevent twisting of the grips or out-of-plane movements. The distance between grips at the onset of loading (7.13 0.19 mm) was the gauge length. Pull-to-failure testing was applied at a rate of 6.0 mm/sec. This rate (84% per second) was standardized across all treatment groups and was chosen to produce clinically relevant failure modes. Axial force was measured with a load transducer. The load and displacement data from a linear variable differential transformer were collected and analyzed. Failure load and failure stress were determined as the maximum load and stress reached during testing, respectively. Stress was calculated by dividing the load by the initial cross-sectional area. Stiffness was calculated by determining the slope of the most linear portion of the load displacement curve.

RESULTS

Mesenchymal stem cells (MSCs) were prepared from the femur and tibia of two Lewis rats. In this study, we needed passage 3 undifferentiated cells. Previous studies have shown that passage three mesenchymal stem cells have the least amount of contaminating cells while still retaining stem cell characteristics (Dai et al., 2005; Javason et al., 2001). After expansion of our BM-MSC to passage three, the cells were stored in liquid Nitrogen until the day preceding surgery. They were then thawed and plated and then trypsinized on the surgery day. A study was done to determine the number of days needed to thaw and plate cells before being used on the day of the surgery and the type of flask (either T75 or T25) needed. This experiment was performed twice. Passage three MSCs were counted and plated in T75 and T25 flasks. Half of them were trypsinized and counted on day one and the other half, on day two (Tables 1 and 2, Figures 1 and 2). The results indicate that the MSCs plated in T75 and counted on Day 2 in the 1^{st} trial had the highest number of cells recovered, representing 150% of the cells plated (Fig.1), followed by T25 (113% of the plated cells) on Day 1 in the 2^{nd} trial (Fig 2). The lowest number of cells recovered was found in T75 flasks on days 1 and 2 of the 2^{nd} trial (Fig 2). This is not likely to be a result of cell death resulting from the cell number being too low for the cells to provide growth factors needed for survival, as the number plated was higher than in the first trial. This low recovery is likely due to human error, such as improper cell counts or trypsinization. Plating cells in T25 flasks a day before the surgery could lead to differentiation of the cells, since MSCs begin to differentiate when there is direct cell-to-cell contact between them. Day 1 counts for both T25 and T75 on the 1^{st} trial were good (Fig 1). We needed 5×10^5 cells for each group of 5 rat surgeries. The figures indicate that plating the MSCs in either T25 or T75 for a day prior

Table 1: Passage 3 MSC counts: 1st Trial

MSC	1st Cell Count (cells/ml)	Flasks		[b]Trypsinization		2nd Cell Count (cells/ml in 10ml of media)	Percentage of Cells recovered (%)
		T25	T75	Day 1	Day 2		
[a]DF P3	$2.3x10^5$	√		√		$2.0x10^5$	87
DF P3	$2.2x10^5$		√	√		$1.9x10^5$	86
DF P3	$3.5x10^5$	√			√	$1.7x10^5$	49
DF P3	$2.0x10^5$		√		√	$3.0x10^5$	150

[a]DF Passage 3 BM-MSCs were plated at the indicated total number of cells in 5 ml complete media for T25 plates or 10 ml complete media for T75 plates.

[b]Cells were washed with PBS and dettached from the bottom of the plates using trypsin/EDTA, then counted using a hemactometer.

Table 2: Passage 3 MSC counts : 2nd Trial

MSC	1st Cell Count (cells/ml)	Flasks		[b]Trypsinization		2nd Cell Count (cells/ml in 10ml of media)	Percentage of Cells recovered (%)
		T25	T75	Day 1	Day 2		
[a]DF P3	$1.5x10^6$	√		√		$1.7x10^6$	113
DF P3	$1.5x10^6$		√	√		$1.5x10^55$	10
DF P3	$1.1x10^6$	√			√	7.5x105	68
DF P3	$1.6x10^6$		√		√	$1.3x10^5$	8

[a]DF Passage 3 BM-MSCs were plated at the indicated total number of cells in 5 ml complete media for T25 plates or 10 ml complete media for T75 plates.

[b]Cells were washed with PBS and dettached from the bottom of the plates using trypsin/EDTA, then counted using a hemactometer.

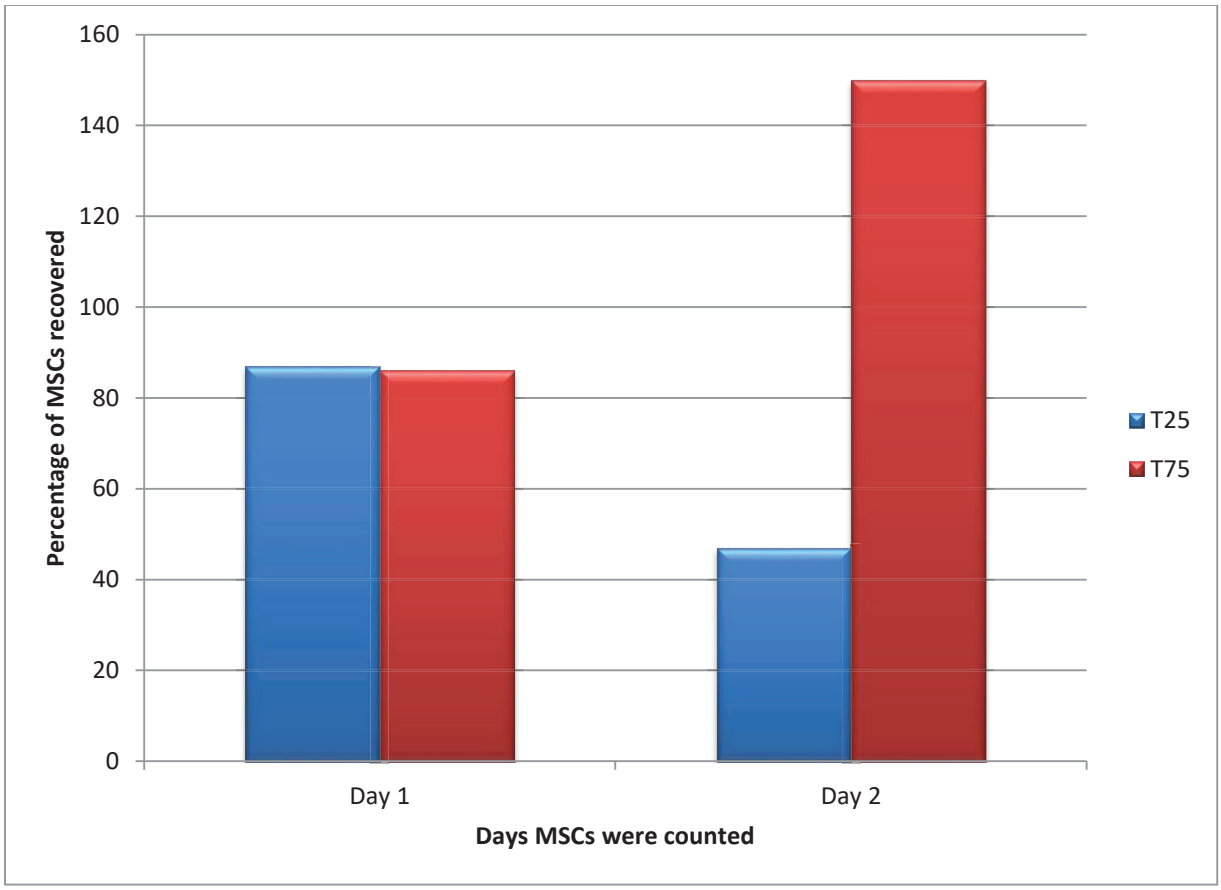

Figure 1: MSC counts for the 1st trial. Passage 3 MSCs were thawed, counted and plated in MSC media in two T25 and two T75 flasks. They were then incubated in complete media at 37^{O}C in 5% atmospheric CO_2. A day after the plating, cells in one of each of the flasks were trypsinized (detaching adherent cells from the bottom of the flask) and counted to determine the number of cells recovered a day after plating them. The cells in the other two flasks (one T25 and one T75) were trypsinized and counted on the second day after plating. The percentage recovery of cells was determined by dividing the number of cells recovered by the number of cells plated and multiplied by 100.

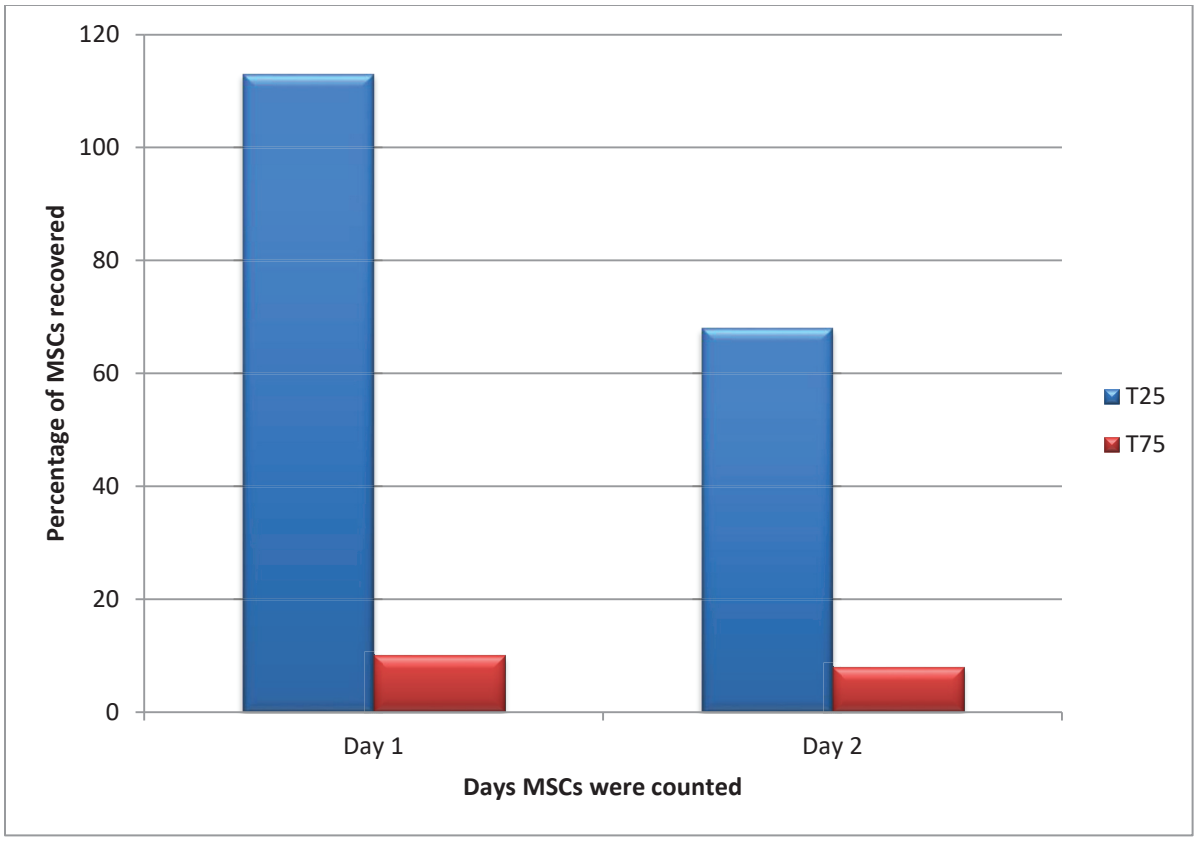

Figure 2: MSC counts for the 2nd Trial. Passage 3 MSCs were thawed, counted and plated in MSC media in two T25 and two T75 flasks. They were then incubated and harvested as described in Figure 1. The percentage recovery of cells was determined by dividing the number of cells recovered by the number of cells plated and multiplied by 100.

to the surgery would ensure the right amount of cells were recovered. T75 flasks were chosen for this study because they are big enough to provide enough nutrients to the cells a day before the surgery and cells plated in the larger flasks have the least chance of differentiation due to cell contact. The study therefore used BM-MSCs plated in T75 flasks a day before the surgery and trypsinized on the surgery day.

Differentiation Assays

Mesenchymal stem cells (MSCs) are cells that have spindle shapes and capable of adhering to plastic plates and flasks. They are able to differentiate into different cell lineages when the growth factors needed for differentiation *in vitro* are provided (Horwitz et al., 2006). There have been several studies done to show the differentiation capacities of BM-MSCs (Ju et al., 2007), since stem cells are known to be capable of differentiating into different cell lineages under the right conditions. In vitro differentiation assays were done to confirm the multipotent nature of stem cells used in this study. Cells were incubated in osteogenic, chondrogenic and adipogenic media to promote their differentiation towards osteocytes (bone cells), chondrocytes (cartilage cells) and adipocytes (fat cells), respectively. BM-MSCs were seeded in 6-well plates and incubated in differentiation media for 3 weeks while the control group received complete media for same amount of time. These assays were done twice in triplicate.

Cells differentiating towards the osteocyte lineage (Fig 3C) and their controls (Fig 3D) were stained with Alizarin Red. Reddish brown areas of the culture were indicative of cells that differentiated into osteoblasts (Fig 3A). Alizarin red S stain is used to assess the deposition of calcium by bone cells in cultures (Gregory et al., 2004) used to evaluate Calcium rich deposits by cells in culture (Gregory et al., 2004). Calcium matrix formation indicates the development of

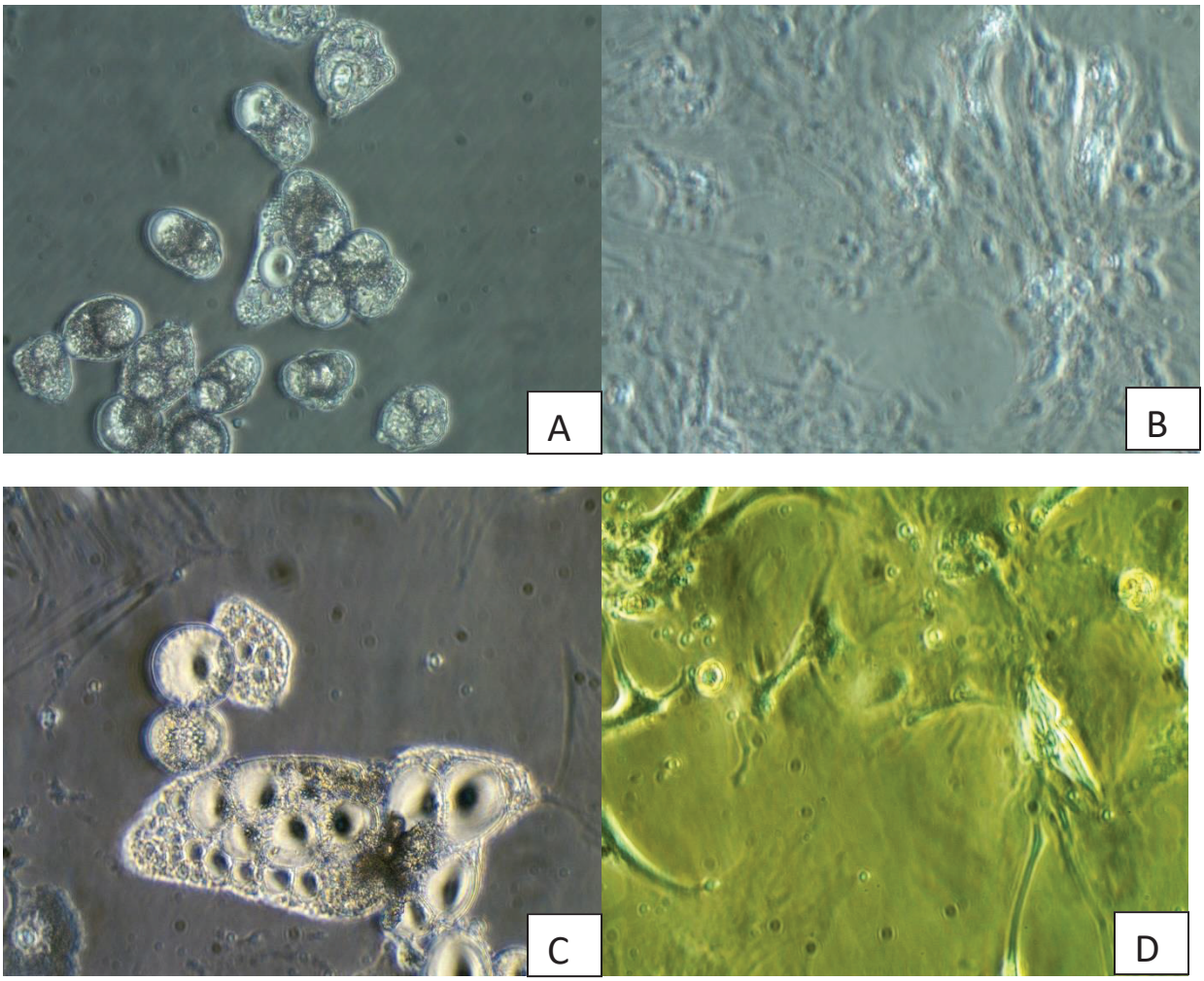

Figure 3: Osteogenic Differentiation. BM-MSC cells cultured for 3 weeks in the absence (B and D) or presence (A and C) of osteogenic medium. Both cultures were stained with 2% Alizarin Red S to indicate the presence of calcium deposition. Positive staining, seen as the pale reddish-brown areas in (A), suggests the formation of calcified extracellular matrix.

osteocytes which are bone cells which later develop into bone tissues. These negative controls, which were maintained in complete media, were negative for Alizarin red S staining. (Fig 3B)

Oil-red O stain is a fat soluble dye used for staining of neutral triglycerides and lipids (Vater et al., 2010). BM-MSCs maintained in adipogenic media for 3 weeks were positive when stained with Oil-red O. The cells were stained red, which indicates the accumulation of intracellular lipid vesicles (Fig 4A). This suggests that the MSCs may have differentiated into adipocytes. The controls maintained in complete media were negative for Oil-red O staining (Fig 4B).

Alcian Blue is a dye, specific for glycosaminoglycan and all types of collagen (Vater et al., 2010). Cells maintained in chondrogenic media were stained blue which is indicative of deposits of collagen and extracellular matrix in the cell culture (Fig 5A). This suggests that the MSCs may have differentiated into chondrocytes. The control cells maintained in complete media were negative for Alcian Blue staining (Fig 5B).

Mesenchymal stem cells are known to improve the healing of tendons (Ju et al, 2008; Aktas et al, 2016; and Yuksel et al., 2016). This research was also performed to check the healing capacities of combining MSCs, PRP and collagen on an Achilles tendon injury. The Achilles tendon on the right legs of the rats were cut 3mm above the calcaneal bone and repaired with absorbable sutures. Dr. Stuart Drew from the Department of Osteopathic Surgery at Mercy Health Youngstown performed the surgeries. The animals were then treated with only collagen (CollaTape), collagen and PRP, collagen and MSCs or collagen, PRP and MSCs, as shown in Table 3. Rats were euthanized at weeks one and two, weighed and tendons from both right and

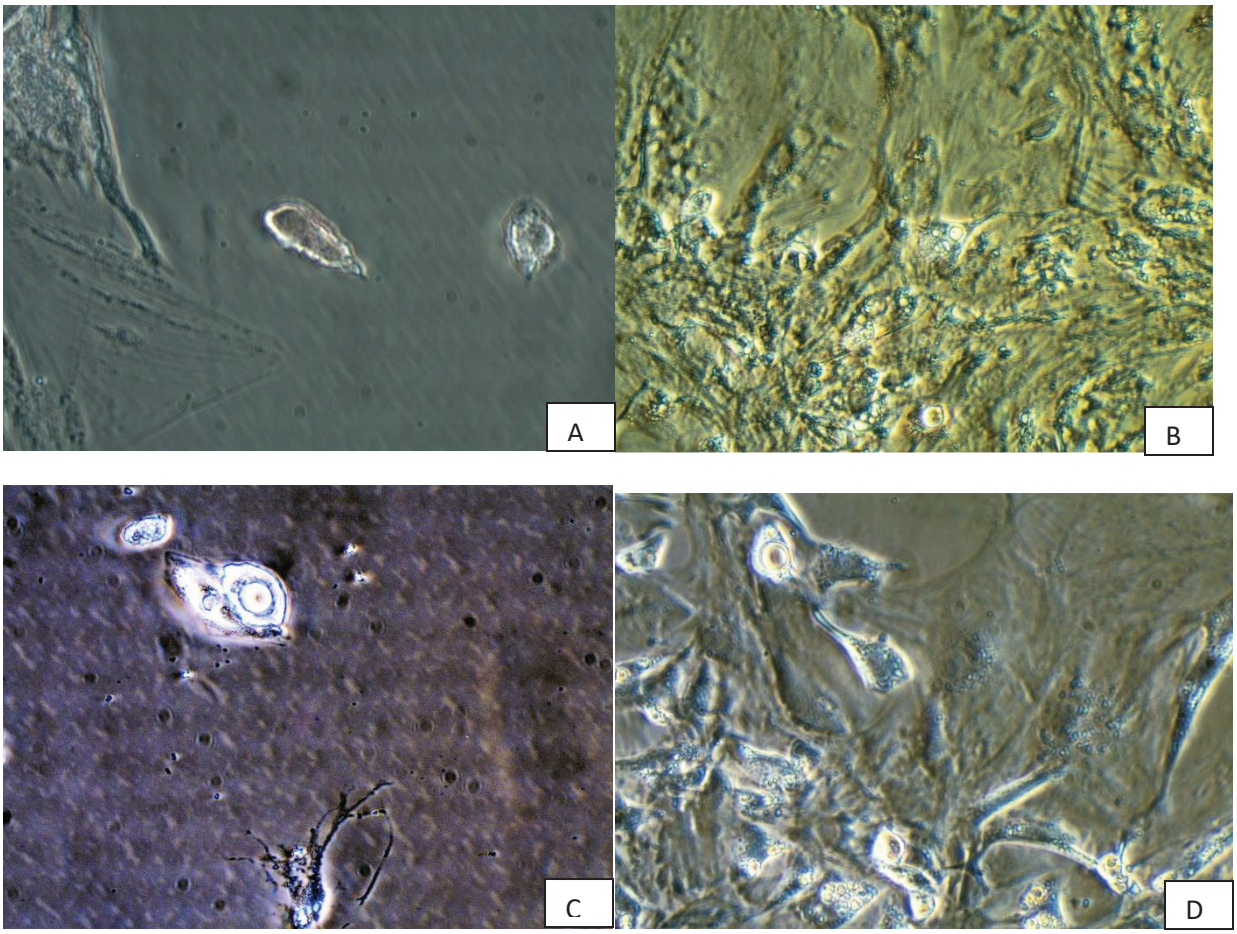

Figure 4: Adipogenic Differentiation. BM-MSC cells cultured for 3 weeks in the absence (B and D) or presence (A and C) of adipogenic medium. Both cultures were treated with 0.2% Oil Red O to stain for intracellularlipid droplets. The red staining of cells in (A) indicates the occurrence of lipogenesis.

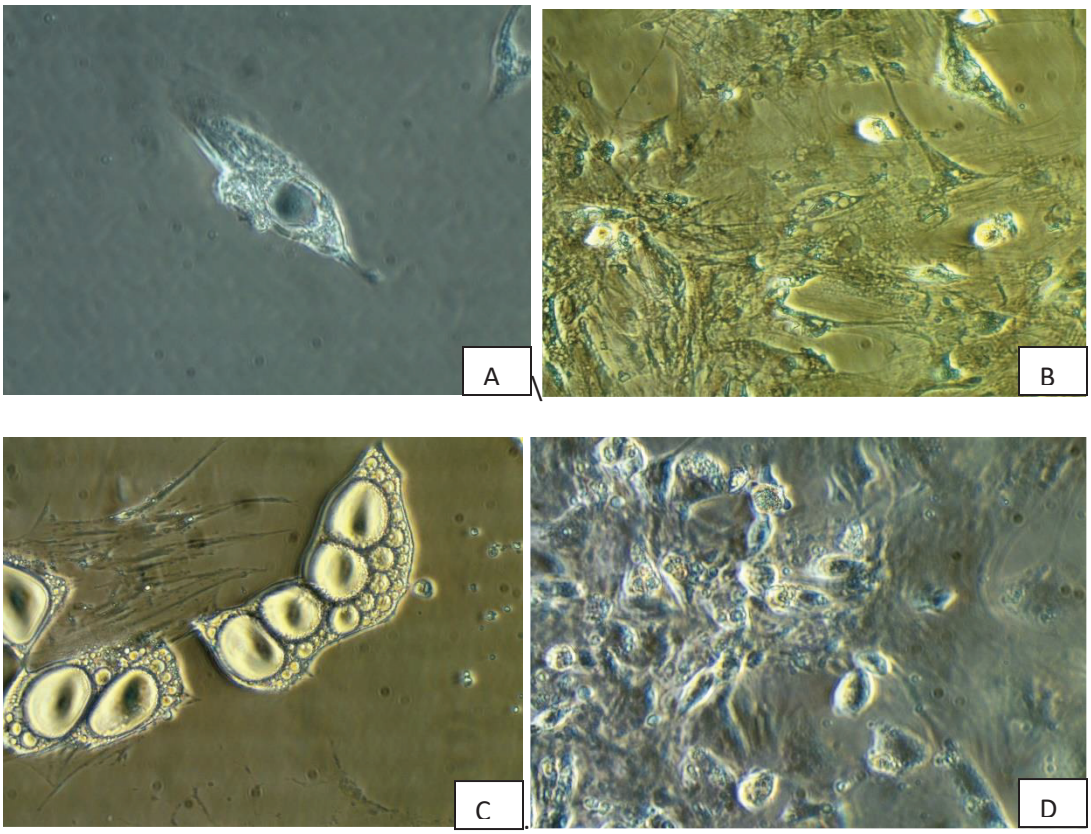

Figure 5: Chondrogenic Differentiation. BM-MSC cells cultured for 3 weeks in the absence (B and D) or presence (A and C) of chondrogenic medium. Both cultures were stained with 0.01% Alcian Blue to determine the presence of collagen deposition A and B.

Table 3: Study Design

Experimental Groups	Treatments	Time of sacrifice (Weeks)
1A	*CollaTape[a] only*	1
1B	CollaTape only	2
2A	CoT + PRP[b]	1
2B	CoT + PRP	2
3A	CoT + MSC[c]	1
3B	CoT + MSC	2
4A	*CoT + PRP + MSC*	1
4B	CoT + PRP + MSC	2

[a]CollaTape (CoT) = collagen wound dressing. Collatape (8 x 13 mm) was wrapped around achilles tendon after repair and before closing skin.

[b]PRP = 100 µl of Platelet-Rich Plasma, recovered from 6 ml total plasma, was injected adjacent to the repaired incision.

[c]MSC = 1 X 10^5 rat mesenchymal stem cells in 100 µl PBS or PRP was injected adjacent to the repaired incision.

left legs were harvested (Table 4 and Figure 6). The left Achilles served as a normal control for each rat. The tendons were then given random numbers to blind the analysis of the tensile strength of the tissues. The tendons were kept frozen in PBS until the date of biomechanical testing. The biomechanical test was done to determine their tensile strength and elasticity after one and two weeks of recovery (Figure 7).

All treatment groups had an apparent increase in the amount of strain they could withstand prior to tissue failure (Figure 8). Unfortunately, statistical analysis using a 2-way ANOVA did not demonstrate a significant difference between the groups. Interestingly, collagen +PRP group recorded the lowest stress at one week recovered, which is different from the results from some of the previous studies done using PRP on the Achilles tendon (Aspenberg et al., 2014).

At week 2, there was no observed or statistically significant difference between the stress and strain of the various treatment groups (Fig. 9). Comparing the 1 and 2 weeks of recovery, all the groups healed almost the same at week two and their stress and strained doubled at week 2, compared to week one.

Table 4: Rat Achilles experimental groups

random #	treatment group	recovery time	surgery date	harvest date
74	col	1 wk a	2/6/2019	2/13/2019
45	col	1 wk a	2/6/2019	2/13/2019
36	col	1 wk a	2/6/2019	2/13/2019
76	col	1 wk a	2/6/2019	2/13/2019
21	col	1 wk a	2/6/2019	2/13/2019
47	col	1 wk c	3/1/2019	3/8/2019
26	col	1 wk c	3/1/2019	3/8/2019
61	col	1 wk c	3/1/2019	3/8/2019
25	col	1 wk c	4/9/2019	4/16/2019
65	col	1 wk c	4/9/2019	4/16/2019
29	col+PRP	1 wk a	2/6/2019	2/13/2019
13	col+PRP	1 wk a	2/6/2019	2/13/2019
72	col+PRP	1 wk a	2/6/2019	2/13/2019
31	col+PRP	1 wk a	2/6/2019	2/13/2019
75	col+PRP	1 wk a	2/6/2019	2/13/2019
67	col+PRP	1 wk c	3/1/2019	3/8/2019
63	col+PRP	1 wk c	3/1/2019	3/8/2019
79	col+PRP	1 wk c	3/1/2019	3/8/2019
8	col+PRP	1 wk c	3/1/2019	3/8/2019
37	col+PRP	1 wk c	4/9/2019	4/16/2019
20	col+MSC	1 wk b	2/27/2019	3/6/2019
70	col+MSC	1 wk b	2/27/2019	3/6/2019
4	col+MSC	1 wk b	2/27/2019	3/6/2019
49	col+MSC	1 wk b	2/27/2019	3/6/2019
68	col+MSC	1 wk b	4/9/2019	4/16/2019
81	col+MSC	1 wk b	4/9/2019	4/16/2019
53	col+MSC	1 wk d	4/4/2019	4/11/2019
77	col+MSC	1 wk d	4/4/2019	4/11/2019
78	col+MSC	1 wk d	4/4/2019	4/11/2019
56	col+MSC	1 wk d	4/4/2019	4/11/2019
7	col+MSC	1 wk d	4/4/2019	4/11/2019
66	col+both	1 wk b	2/27/2019	3/6/2019
80	col+both	1 wk b	2/27/2019	3/6/2019
39	col+both	1 wk b	2/27/2019	3/6/2019
11	col+both	1 wk b	2/27/2019	3/6/2019
15	col+both	1 wk b	2/27/2019	3/6/2019
34	col+both	1 wk d	4/4/2019	4/11/2019
60	col+both	1 wk d	4/4/2019	4/11/2019

69	col+both	1 wk d	4/4/2019	4/11/2019
64	col+both	1 wk d	4/4/2019	4/11/2019
2	col+both	1 wk d	4/4/2019	4/11/2019
30	col	2 wk a	2/13/2019	2/27/2019
50	col	2 wk a	2/13/2019	2/27/2019
6	col	2 wk a	2/13/2019	2/27/2019
71	col	2 wk a	2/13/2019	2/27/2019
24	col	2 wk a	2/13/2019	2/27/2019
48	col	2 wk c	3/22/2019	4/5/2019
23	col	2 wk c	4/5/2019	4/5/2019
35	col	2 wk c	3/22/2019	4/5/2019
12	col	2 wk c	3/22/2019	4/5/2019
40	col	2 wk c	3/22/2019	4/5/2019
51	col+PRP	2 wk a	2/13/2019	2/27/2019
55	col+PRP	2 wk a	2/13/2019	2/27/2019
1	col+PRP	2 wk a	2/13/2019	2/27/2019
5	col+PRP	2 wk a	2/13/2019	2/27/2019
52	col+PRP	2 wk a	2/13/2019	2/27/2019
73	col+PRP	2 wk c	3/22/2019	4/5/2019
46	col+PRP	2 wk c	3/22/2019	4/5/2019
10	col+PRP	2 wk c	3/22/2019	4/5/2019
33	col+PRP	2 wk c	3/22/2019	4/5/2019
32	col+PRP	2 wk c	3/22/2019	4/5/2019
42	col+MSC	2 wk b	3/8/2019	3/22/2019
59	col+MSC	2 wk b	3/8/2019	3/22/2019
28	col+MSC	2 wk b	3/8/2019	3/22/2019
41	col+MSC	2 wk b	3/8/2019	3/22/2019
57	col+MSC	2 wk b	3/8/2019	3/22/2019
18	col+MSC	2 wk d	3/29/2019	4/12/2019
27	col+MSC	2 wk d	3/29/2019	4/12/2019
54	col+MSC	2 wk d	3/29/2019	4/12/2019
38	col+MSC	2 wk d	3/29/2019	4/12/2019
9	col+MSC	2 wk d	3/29/2019	4/12/2019
3	col+both	2 wk b	3/8/2019	3/22/2019
43	col+both	2 wk b	3/8/2019	3/22/2019
16	col+both	2 wk b	3/8/2019	3/22/2019
17	col+both	2 wk b	3/8/2019	3/22/2019
62	col+both	2 wk b	3/8/2019	3/22/2019
22	col+both	2 wk d	3/29/2019	4/12/2019
14	col+both	2 wk d	3/29/2019	4/12/2019
44	col+both	2 wk d	3/29/2019	4/12/2019
58	col+both	2 wk d	3/29/2019	4/12/2019
19	col+both	2 wk d	3/29/2019	4/12/2019

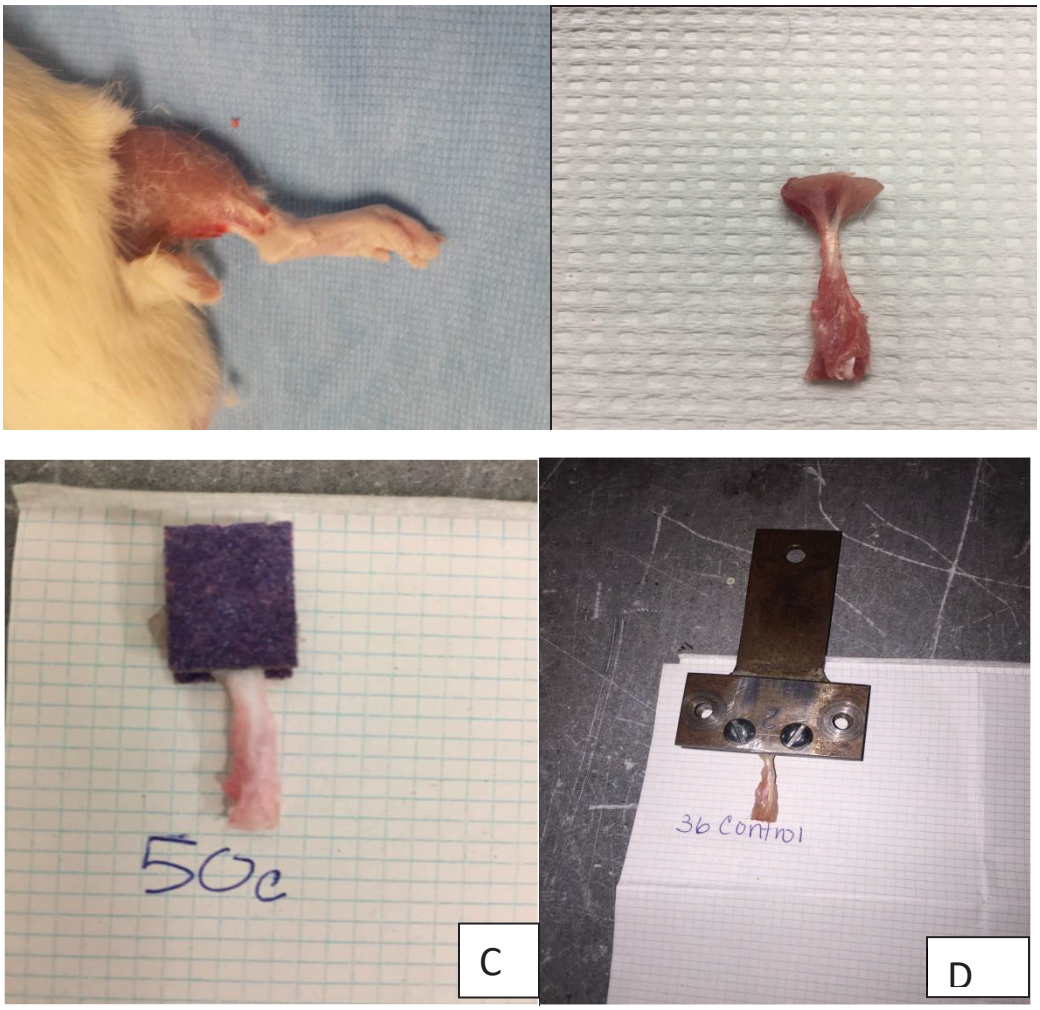

Figure 6: Achilles tendon excision and preparation for biomechanical testing. *A*- Leg skinned in preparation for Achilles excision. *B*- Achilles excised, given a random number and trimmed for Biomechanical testing. *C*- Tendon glued between grade 100 sand paper to prevent muscle from tearing during testing. *D*- Glued tendon fix between metal to begin the biomechanical testing.

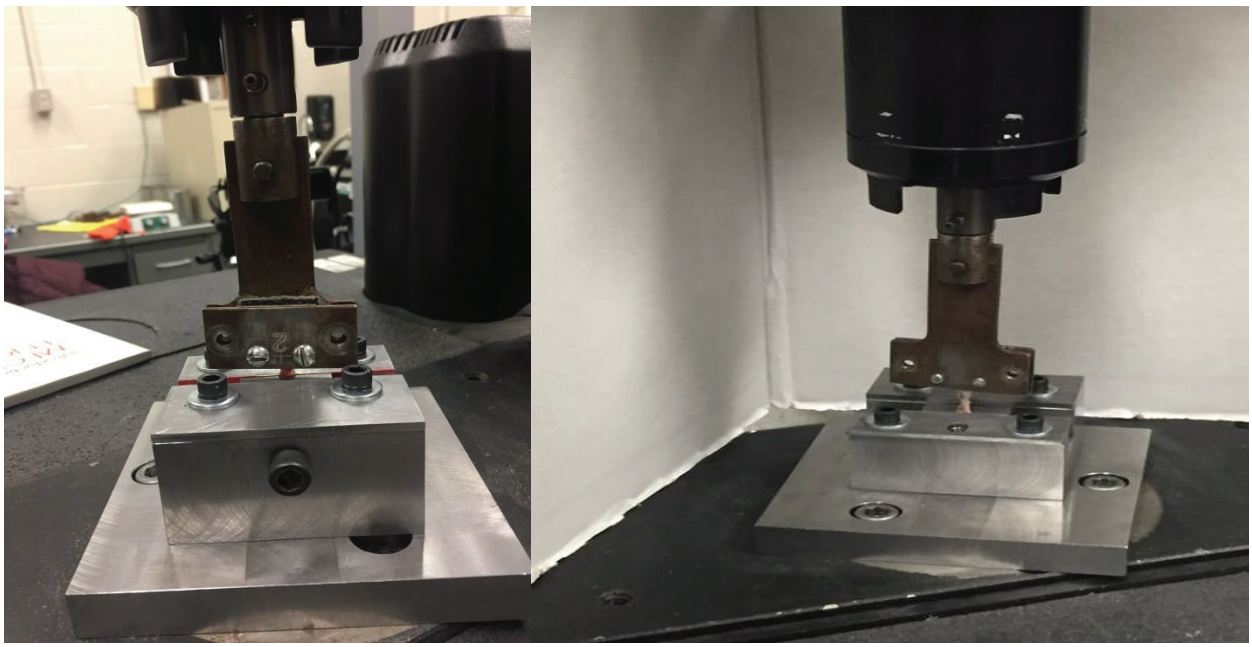

Figure 7: Biomechanical testing in tensiometer.

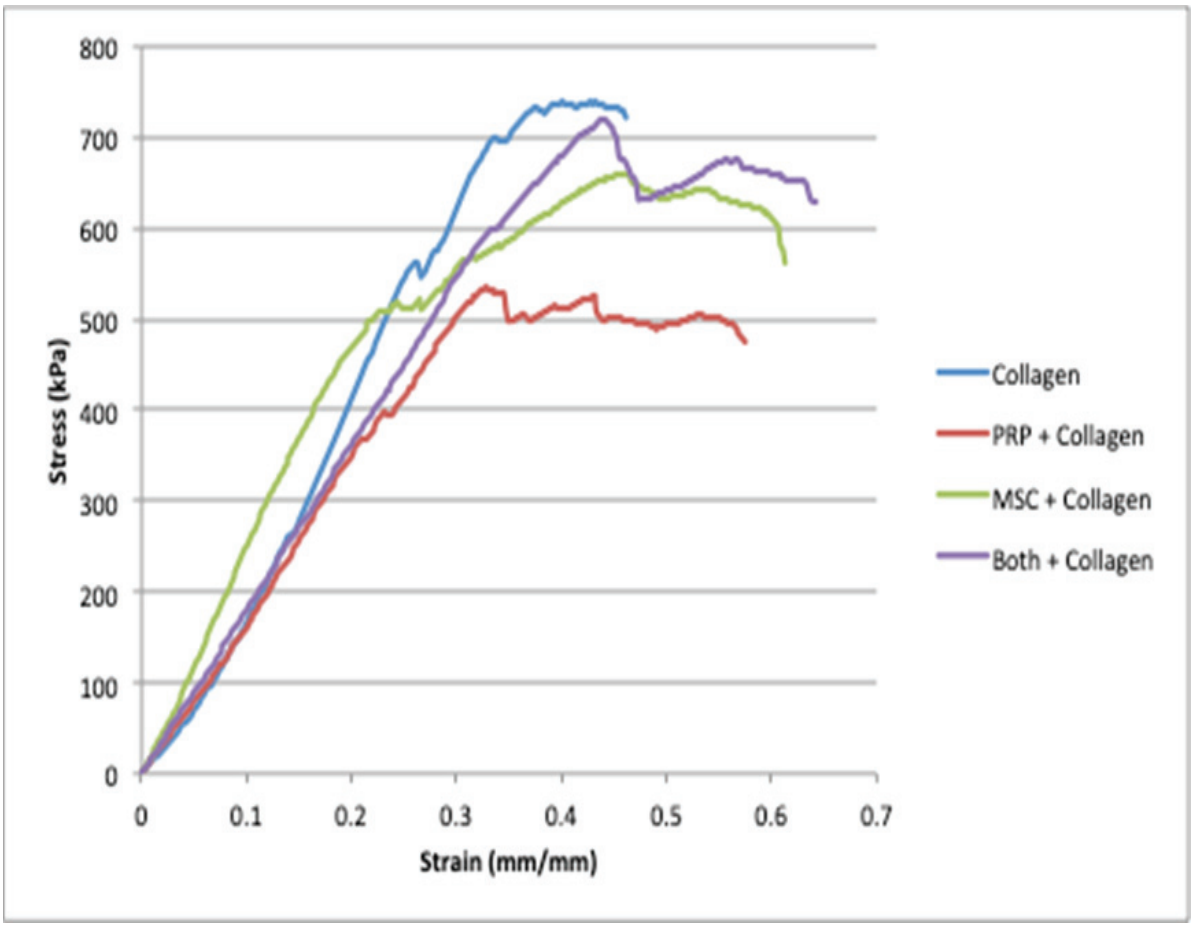

Figure 8: 1 Week Recovery: Stress-Strain to Failure, Original Treated Cross-Section.

Biomechanical tests were done as described in Figures 5 and 6. The peak of the curve represents the ultimate stress (tensile strength) point. The area below the curve illustrates the amount of energy absorbed by the tendon (elasticity) before ultimate failure. Collagen only represents group one treatment group and collagen + both, group four (collagen+PRP+MSC).

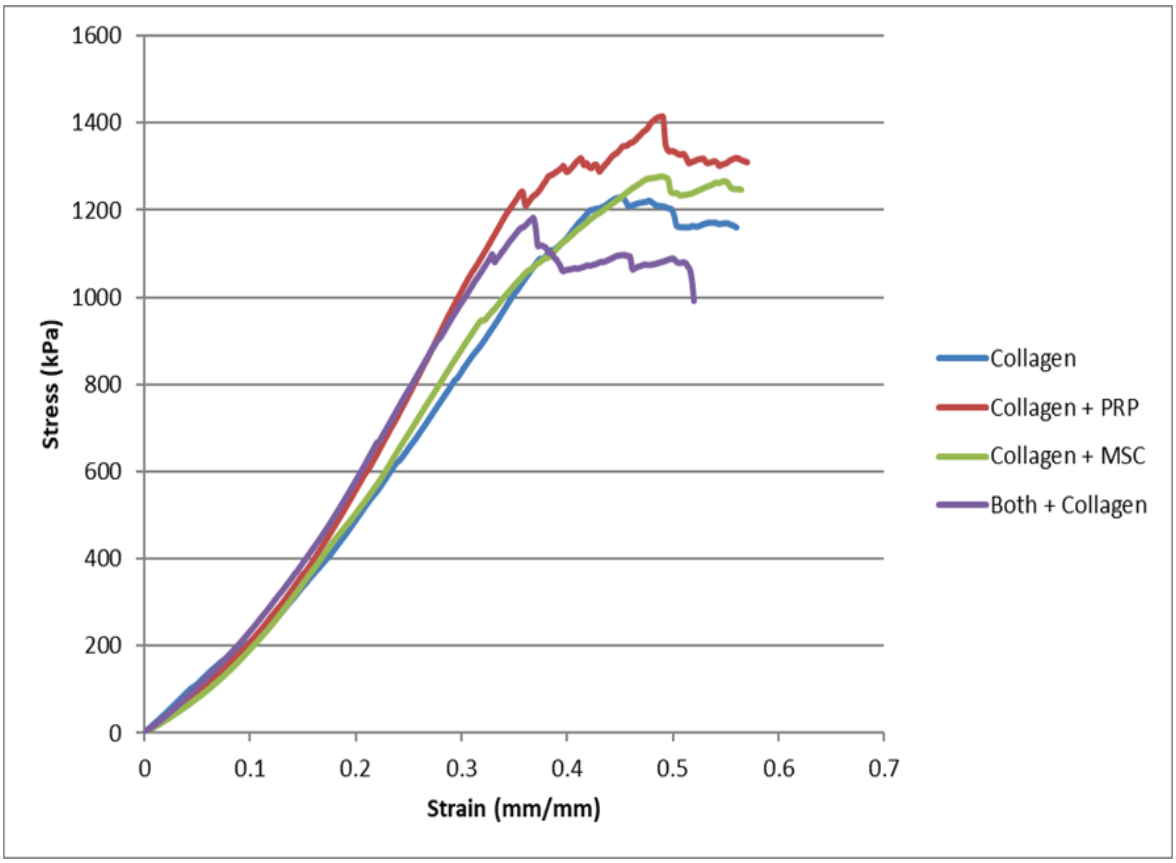

Figure 9: 2 Week Recovery: Stress-Strain to Failure, Original Treated Cross-Section.

Biomechanical tests were done as described in Figures 5 and 6. The peak of the curve represents the ultimate stress (tensile strength) point. The area below the curve illustrates the amount of energy absorbed by the tendon (elasticity) before ultimate failure. Collagen only represents group one treatment group and collagen + both, group four (collagen+PRP+MSC).

DISCUSSION

Achilles tendon injury has been on the rise over recent years, constituting 35% of all tendon injuries. There are several options to consider for treating this injury. The non-surgical method has about a 30% rate of re-rupture and takes longer to heal, while the surgical methods (open and minimally invasive) may lead to infection, sural nerve damage and poor wound healing (Metzl, et al, 2008). The disadvantages associated with these treatments led scientists to investigate using biological materials such as platelet-rich plasma, collagen and mesenchymal stem cells as additives to help accelerate the healing rate of the Achilles tendon. No study has combined collagen, platelet rich plasma and mesenchymal cells as additives to the surgical repair of the Achilles.

The goal of this study was to improve the healing rate and strength of a repaired Achilles tendon injury (rupture) following treatment with collagen, platelet-rich plasma (PRP) and bone-marrow derived mesenchymal stem cells (MSC). There have been several studies done on how to improve tendon healing in different animal models and mesenchymal stem cell addition has been examined in several studies. A study done on rats using mesenchymal stem cells from a hypoxic culture (deficiency in the amount of oxygen reaching the cells) recorded a significantly greater ultimate tensile strength of the tendon, when compared to untreated groups (Huang et al, 2013). Stem cell-bearing sutures also improved tendon healing in rats with ultimate failure load higher than the control group (Adams et al., 2014). Another study compared MSCs and PRP additions on the early healing properties of rats' Achilles tendon (Yuksel et al., 2016). They observed that the MSCs promoted a rapid recovery of the tendon when compared to those treated with platelet rich plasma and there was a significant increase in structural strength of the tendons treated with MSCs.

While all of these studies were done to check the ability of MSCs to accelerate tendon healing, no study has compared the healing capabilities of combining collagen, PRP and MSCs as additives to Achilles tendon surgical repair. In addition, no study has done a biomechanical test to check the ultimate tensile strength of the tendons, following the combination of materials added to the surgery site. This study combined CollaTape (a type of collagen dressing), PRP and MSCs as additives to Achilles tendon repair and examined wound healing using biomechanical tests to determine the tensile strength of the tendons.

A previous study in our lab combined these same three biological materials to treat abdominal fascial wound repair and used biomechanical tests to determine the strength of the tissues (Heffner et al., 2012). The data showed significantly accelerated healing and a higher tensile strength of the tissues in the treatment group that received the three combined biologics when compared to the other controls. Collagens are known to contribute to the wound healing process by trapping growth factors and cytokines, storing these factors and delivering them to the wound site when needed (Gelsea et al., 2003). CollaTape was the collagen dressing used for this study. CollaTape, a type of collagen wound dressing, is a soft, white sponge-like material. Application of CollaTape to wounds is easily controlled because of these properties (Mahesh et al., 2015). The flexible nature of CollaTape was important for this study, as it allowed the tape to be wrapped around the Achilles tendon following surgical repair. CollaTape was shown in a previous study to have the ability to absorb PRP (Marie et al., 2010). CollaTape was used in this study to serve as a scaffold in which the injected PRP and MSCs were trapped at the site of the wound. In addition, the collagen in CollaTape could be degraded by local enzymes and the degraded molecules used to form new collagen in the healed wound. All rats received CollaTape, CollaTape + PRP, CollaTape + MSC or CollaTape with both PRP and MSC. No surgical

procedure was performed on their left Achilles and these tendons served as the controls for the treated legs.

Our research used platelet-rich plasma because of its ability to enhance the wound healing environment. Platelet-rich plasma releases a cocktail of growth factors from their α-granules when they are triggered. These growth factors are known to play major roles in tissue repair (Smith, 2016). Most of these growth factors (VEGF, EGF, TGF-β etc.) are found in abundance during the proliferative phase of wound healing and help to promote tissue regeneration (Heffner et al., 2012). PRP has been seen to increase tenocyte proliferation significantly at days 7 and 14. Proliferation of tenocytes exposed to platelet-rich plasma was higher than those exposed to normal blood plasma, when these materials were used as additives fduring wound healing (Jo et al, 2012). PRP has been used in several studies to accelerate the rate of wound healing. In studies by Aspenburg and coworkers, it was injected percutaneously into repaired rat Achilles and observed to improve Achilles tendon repair in rats as indicated by increase in tensile strength (Aspenberg et al., 2014).

Mesenchymal stems cells have been shown in several studies to improve wound healing through their paracrine actions (Badiavas and Falanga, 2003, Lee et al., 2017). Many of these studies have been done to show the wound healing capacities of MSCs. McFarlin et al., (2006) did a study on using only MSCs to treat abdominal fascial healing in rats (McFarlin et al., 2006). An incision was made in the abdominal fascia and MSC was intradermally injected into the wound after closure. The wounds with the MSC treatments healed about two to three times better and had higher tensile strength than those without the MSC treatment. The improvement of wound tensile strength was attributed to various properties of the MSC, which include MSCs

being able to induce the formation of new collagen synthesis, matrix deposition, and the ability to promote the migration of cells to the site of the wound.

Mesenchymal stem cellss can be enriched by expanding and passaging in complete medium and ultimately eliminating contaminating cells (Javason et al., 2004). Currently, MSC expansion protocols are done based on the normal cell culture techniques where they are cultured into plastic tissue culture flasks. This method demands careful handling of cells (Madeira et al., 2012). In order to achieve useful cell numbers in an acceptable period of time, the cells need to be passaged at least 2 to 3 times (Ringden et al., 2006). Passaging MSCs for too long may lead to cell senescence, leading to the gradual loss of the differentiation and proliferation abilities of the expanded cells (Bonab et al., 2006). A study by Kim and coworkers was done to investigate the potential for growth, and the ability of MSCs to differentiate into bone and fat cells. This study checked the telomerase activity and length, the surface markers and gene expression relating to tumor formation in long-term cultured human MSCs (Kim et al., 2019). Their results indicated that long-term passaged human MSCs became senescent. Long term cell culture was reported to shorten telomere and arrested cells growth. They also reported a decrease in the adipogenic differentiation potential of the MSCs with increased passaging. The differentiation of the cells into osteocytes on the other hand was increased with increased passaging (Wagner et al., 2008). Madeira et al, (2012) investigated the underlying mechanism associated with cellular senescence observed when BM-MSCs were cultured for long term *ex vivo.* With regard to the proliferation of cells, they recorded maximum growth rate of the BM-MSCs at passages 2 and 3. The number of chaperone and stress response proteins decreased when passage 7 stem cells were compared to passage 3 stem cells. Their findings showed that BM-MSCs lose their proliferative and differentiation capacities when passaged for too long.

Madeira et al, (2012) and other investigators passaged their MSCs to a maximum of passage 4. Passage 3 stem cells were used in this study. Cells were passaged in complete medium to passage 3 and frozen in liquid nitrogen until a day before the surgery. At that time, they were thawed and plated. On the day of the surgery, MSCs were trypsinized, counted and resuspended in PBS or PRP. Each rat received $1x10^5$ MSCs. The optimal number of MSCs was determined by previous results on fascia healing (unpublished results).

Mesenchymal stem cells (MSC) are cells able to proliferate to renew themselves and are able to differentiate into different cell lineages under the right conditions (Ju et al., 2007). Differentiation assays were done to determine whether the cells used for this study retained their stem cell characteristics. Mesenchymal stem cells should be able to differentiate into different cell lineages *in vitro* (Javazon et al., 2004). The capacity for induced in vitro differentiation of MSCs to bone, fat, and cartilage is a critical requirement to identify putative MSC populations (Javazon et al., 2004). For this reason, the MSCs from this study were incubated *in vitro* in chondrogenic, adipogenic and osteogenic media to allow differentiation into cartilage, fat and bone cells respectively. The results from the differentiation assay showed that the cells used for this study were multipotent, indicating that they were indeed mesenchymal stem cells.

Biomechanical testing was done to determine the ultimate failure strength of the Achilles tendon following surgery and the addition of the different treatments (CollaTape only, CollaTape + PRP, CollaTape + MSC and CollaTape +PRP+MSC) used in this study. The biomechanical tests from the previous study of abdominal wound healing in our lab recorded 301% tensile strength and a 100% improvement in rat groups that received CollaTape, PRP and MSC treatment, compared to those that received only CollaTape and PRP (Heffner et al., 2012). Their group 2 (CollaTape + PRP) had a significant increase in tensile strength and energy absorption at

both 4 and 8 weeks after fascial repair when compared to group 1 (only CollaTape). Heffner et al., (2012) calculated a modulus of elasticity for the various groups to check the stiffness of the repaired tissues. Their results indicated that the tissues from the group three rats (CollaTape +PRP +MSC) recorded the highest elasticity and group 1 (no treatment), recorded the lowest. Based on these results, we hypothesized that our group four rats (CollaTape +PRP +MSC at week 2) would have the highest tensile strength and elasticity compared to the other treatment groups and controls.

In a similar study of rat Achilles tendons, biomechanical tests were performed on tendons treated with MSCs from a hypoxic culture (Huang et al., 2013). From their results, the tendons that received the MSC treatments recorded the highest ultimate fail load when compared to their controls and the other treatment groups at weeks 2 and 4. The ultimate failure load for tendons injected with MSCs in another study (Adams et al., 2014), were higher than those without the MSC injections. Results from Lee et al., (2017) indicated that rat Achilles tendon treated with human adipose-derived MSCs had a significantly higher resistance to tensile load compared to the other groups in their study.

Huang et al., (2013) injected MSC and PRP after repairing the tendons and observed them for four weeks before doing a biomechanical and histopathological test. The difference between their study and ours is they tested MSC only, PRP only and control. We examined the combination of the PRP and MSC on a collagen matrix. They observed their rats for four weeks and our study observed the rats for one and two weeks. We anticipated accelerated healing, which would require observing earlier time points. While their biomechanical tests showed a higher stress of the tendon treated with MSCs or PRPs, the tendons with MSCs recorded a significantly higher strain (ductility) than the PRP treated tendons. This results means MSCs may

have an important role to play in the elongation (strain) of the tendons (Huang et al., 2013). In this study 1 x 10^5 MSCs in PBS were used, while Huang et al., (2013) used 1 x 10^6 MSCs in PBS. This dose of MSC used in our study was chosen based upon a dose response study in the abdominal surgery model (unpublished data). In this study, MSCs were injected in rats receiving CollaTape at the wound site. CollaTape is adsorbent and should retain the other additives, possibly resulting in the need for fewer cells.

CollaTape may help in the early formation of collagen at the wound site, as well as preventing degradation of newly formed collagen by BMPs. This may play a role in increasing the tensile strength of the tendons. However, our collagen only treatment group at one week recorded a lower level of strain prior to failure than the other three treatment groups. The other additives appear to have more effect on the strength of the repaired wound. This was expected, because previous studies showed that collagen only recorded the lowest stress and strain compared to other treatment groups (Heffner et al., 2012). The other three treatment groups had higher strain, but no significant difference was seen between the groups.

It is anticipated that early collagen formation is influenced by the addition of CollaTape. Rapid proliferation of the MSCs should be stimulated by the growth factors in the PRP and paracrine actions of the MSCs. It is also likely that the combination of BM-MSCs, PRP, and CollaTape can further optimized tendon repair throughout the entire inflammatory and proliferative phase of wound healing. The data in this study does suggest that higher strength and elasticity was seen in the groups receiving MSCs, PRP and MSCs + PRP. The lack of statistical significance suggests that more studies are needed to increase the number of replicas and to include an untreated surgical control. Future studies should also be done to analyze the types of

collagen formed and collagen crosslinking. This may help in the understanding of the biomechanical test results.

Achilles tendon injuries are a burden to patient due to the inability to use the leg over long periods of time. Our study demonstrated that combining collagen, platelet rich plasma and mesenchymal stem cells as additives to an Achilles tendon repair accelerated the healing rate and capacities of the tendons after one week of recovery. The biomechanical tests of the excised tendons showed that the tensile strength (stress) of the group treated with the biologics was higher at one week when compared to the collagen only group. The biologics groups also showed a higher elasticity, when compared to the collagen only group. It is likely that combining collagen, PRP and MSC will provide a solution for the problems associated with healing of the Achilles tendon.

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YOUNGSTOWN STATE UNIVERSITY

One University Plaza, Youngstown, Ohio 44555
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November 7, 2018

Dr. Diana Fagan
Department of Biological Sciences
UNIVERSITY

Re: IACUC Protocol, 06-18
Title: An investigation of collagen, platlet-rich plasma, and bone marrow derived mesenchymal stem calls on the Achilles tendon in a rat model

Dear Dr. Fagan:

The Institutional Animal Care and Use Committee of Youngstown State University has reviewed the aforementioned protocol you submitted for consideration and determined it should be unconditionally approved for the period of August 14, 2018 through its expiration date of August 14, 2021.

This protocol is approved for a period of three years; however, it must be updated yearly via the submission of an Annual Review-Request to Use Animals form. These Annual Review forms must be submitted to the IACUC at least thirty days ***prior*** to the protocol's yearly anniversary dates of August 14, 2019 and August 14, 2020. If you do not submit the forms as requested, this protocol will be immediately suspended. You must adhere to the procedures described in your approved request; any modification of your project must first be authorized by the Institutional Animal Care and Use Committee.

Good luck with your research!

Sincerely,

[redacted]

Dr. Gregory Dillon
Interim Associate Vice President for Research
Authorized Institutional Official

GD:dke

C: Dr. Stanley Dannemiller, Consulting Veterinarian, NEOMED
Dawn Amolsch, Animal Tech., Biological Sciences