THE UTILIZATION OF MULTIPOTENT MESENCHYMAL STROMAL CELL TRANSPLANTATION TO IMPROVE FASCIA REPAIR

by

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ABSTRACT

Incisional hernias, defined as the protrusion of abdominal contents through a weakness in abdominal muscle at a previous surgical incision site, are a recurrent complication of abdominal surgeries. Stem cell therapy is being investigated as an alternative for improving wound healing. Bone marrow-derived mesenchymal stem cells (MSC) possess the potential to develop into multiple cell lineages and have been found to modulate the wound healing process. In this study we have determined the ideal MSC transplant dose needed to improve fascia recovery following an abdominal incision by testing five MSC doses: 2.5x10^4, 5.0x10^4, 1.0x10^5, 2.5x10^5 and 5.0x10^5. Thirty-five age matched rats were divided into 5 equal groups (n=7), all receiving a 6cm abdominal incision along the linea alba. Surgically closed wounds, treated with CollaTape, platelet rich plasma (PRP) and a designated MSC dose, were histologically assessed after 4 weeks to determine which dose had a greater affect on improving wound healing based on three criteria: collagen organization, collagen abundance and myocyte regeneration. MSC treated wounds were also analyzed to determine the optimal method for determining the type of collagen produced at the wound site. Following recovery, harvested wound tissue was analyzed using trichrome staining to identify total collagen and type III collagen location was identified using both Immunohistochemistry and Gridley silver staining. Results indicated the ideal dose for MSC transplantation was 1x10^5 MSCs while the best staining procedure for identifying type III collagen in paraffin embedded tissues was the Gridley stain.
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DEDICATION

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1. INTRODUCTION

Despite the ever-increasing number of medical advancements, one area of concern, which still requires much improvement, is fascial healing. Fascia, a sheet or band of fibrous loose to dense connective tissue derived from the mesoderm, which is found in large quantities within the abdominal region, is known to envelop, separate, or bind together muscles, organs and other soft bodily structures (Ou et al., 2013). Following surgery, improper healing of the wound site can result in extreme patient discomfort as well as numerous medical complications. Such complications may include wound dehiscence, which can lead to exposure of abdominal contents, infection, or incisional hernia formation. Understanding proper fascial repair is vital to the medical profession due to the frequency of fascial dehiscence. Dehiscence rates are known to occur in 3% to 16% of cases following abdominal surgeries.

1.1 Hernia

A hernia is a condition in which a portion of an internalized tissue, normally contained within the abdominopelvic cavity, is irregularly displaced causing the tissue to protrude through the wall of the cavity. In total there are six types of abdominopelvic hernias: inguinal, femoral, umbilical, epigastric, hiatal, and incisional. The most common are inguinal hernias (Zheng et al., 2002).

An inguinal hernia occurs specifically when abdominal tissue pushes through a weakened region of the inguinal canal. The weakened region often arises near the deep inguinal ring, the area providing passage for both the testicular artery and vein and the ductus deferens. There are two types of inguinal hernias, direct and indirect. Direct
herniation occurs when abdominal contents herniate through a weak spot in the fascia of the posterior wall of the inguinal canal. Conversely, an indirect inguinal hernia, the more common of the two, occurs when abdominal contents protrude through the deep inguinal ring and down the inguinal canal, passing lateral to the inferior epigastric vessels. This type of herniation is most often experienced in males due to the larger opening into the inguinal canal, compared to that of females.

A femoral hernia passes into the proximal thigh, deep to the medial end of the inguinal ligament, due to a weakening of the femoral sheath. These hernias are relatively uncommon, accounting for approximately 3% of all hernias. Although this type of hernia is known to occur in both males and females, occurrence is most often observed in females due to wider bone structure in the pelvis. Five categories of femoral hernias are known to exist including reducible, irreducible, obstructed, strangulated and incarcerated.

An umbilical hernia occurs when a portion of the intestines protrudes through an opening of an abdominal muscle near the umbilicus. This specific type hernia is often most prevalent in those individuals with health problems that create abnormal pressure in the belly region, such as obesity, ascites, or pregnancy. Other health problems such chronic cough, constipation, or prostate enlargement can also lead to hernia development due to the acquired intra-abdominal strain, which results from these complications.

An epigastric hernia develops in the upper central part of the abdominal region located between the costal margins and the subcostal plane, otherwise known as the epigastrium. Upon weakening of abdominal muscle, underlying fat may push through a weak part of the belly wall, resulting in the occurrence of the hernia. Often times, these specific hernias are asymptomatic, but if fat or other tissue becomes trapped inside the
opening, serious pain and tissue damage can arise. Those most often affected include pregnant women or obese individuals.

A hiatal hernia is unique in its origin, as compared to other hernias, due to the involvement of the stomach rather than the intestines. This hernia occurs when the cardiac region of the stomach bulges superiorly through the esophageal hiatus of the diaphragm and into the thorax. Two variations of hiatal hernias exist which include sliding, the most common, and paraesophageal. During sliding hiatal herniation, the gastroesophageal junction along with additional regions of the stomach, are displaced above the diaphragm. Alternatively, during paraesophageal hiatal hernias, the fundic region of the stomach herniates through the esophageal hiatus without movement of the gastroesophageal junction and ultimately comes to rest adjacent to the esophagus.

An incisional hernia most often occurs following the incomplete healing of a surgical wound. Often during surgeries performed in the abdominal region, incisions (laparotomies) are oriented in a vertical fashion to prevent tearing of the surgical area during extension of the torso. However, when proper healing does not occur, any increased intra-abdominal pressure may cause rupture and successive displacement of underlying organs through the abdominal wall musculature or aponeurosis, the broad tendinous portion of the oblique and transverse abdominal muscles that attaches to the linea alba. These types of injuries are most prevalent when laparotomies are performed along the linea alba, the vertical fibrous band extending down the anterior midline of the abdominal wall. After injury, proper healing and scar formation are achieved during the maturation phase of wound healing. Previous research has largely focused on the
pathogenesis of incisional hernia formation, which is believed to occur as a result of collagen content abnormalities within the scar tissue (White et al., 2007).

The most common complication after laparotomy is incisional hernia formation, which requires approximately 200,000 reoperation repairs per year in the United States (DuBay et al., 2007; Franz et al., 2001). The occurrence of an incisional hernia results from laparotomy wound failure. Following surgery, the abdominal wall is continuously subjected to numerous internal forces and body movements that put strain on the muscle. If too much force is applied to the weakened region, the regenerating myofascial tissue may rupture, resulting in the protrusion of abdominal contents, thus leading to herniation (DuBay et al., 2007).

1.2 Chronic Wounds

Chronic wounds can typically be attributed to impaired vascular perfusion or venous hypertension (Hodde and Johnson, 2007). Vascular perfusion provides for the exchange of gas, nutrients and metabolites between blood and body tissues. Adequate perfusion is essential to both the integrity and function of tissue. If vascular perfusion is impaired, the healing process is severely retarded. During wound healing, many of the white blood cells responsible for secreting factors required for initiation of the healing process must travel to the site of injury. Once arriving at the site, these cells are faced with the task of successfully traversing from vasculature to the surrounding tissue. Without adequate vascular perfusion, the required cells do not reach the wound site and the critical steps responsible for wound healing are never initiated (Strodtbeck, 2001).
Conversely, venous hypertension can be attributed to a multitude of factors, some of which include increased blood volume, increased large vessel tone throughout the body, and persistent dilatation of the arterioles, which functions to decrease the peripheral resistance, ultimately allowing rapid flow of blood from the arteries into the veins (Guyton and Hall, 2011). In the case of chronic wounds, persistent dilation of the arterioles is the contributing factor. During the healing process, localized hyperemia allows for the implementation of the necessary immunomodulating agents, nutrients, and cells at the site of injury. The resulting rapid flow of blood from the arteries into the veins prevents ample time for the necessary blood constituents to successfully contribute to the healing process. Therefore, the wound healing process is never fully initiated due to the absence of these necessary blood constituents. Thus, either venous hypertension or impaired vascular perfusion can have a substantial impact on the healing process, often preventing complete healing even after prolonged standard treatment. In these cases, active intervention is required to promote successful wound closure.

1.3 Stem Cells

During healing following surgery, the dermis normally directs all phases. However, healing complications can act to prevent the dermal matrix from eliciting its regulatory role in the healing process. Therefore the addition of external factors, such as wound dressings, is often required to stimulate healing and eventual wound closure (van der Veen et al., 2010). Stem cell therapy is currently being investigated as a possible mechanism for enhancing the healing ability of difficult or chronic wounds (McFarlin et al., 2006). Adult bone marrow-derived mesenchymal stromal cells (MSCs) have been
found to be a viable additive to chronic wound sites due to their characteristic potency or differentiation potential (Goedecke et al., 2011).

A stem cell is an unspecialized cell that possesses the potential for self-renewal via mitosis while in an undifferentiated state (Cha and Falanga, 2007). During the self-renewal process, stem cells exhibit a special property known as asymmetric replication: a characteristic property which results in the production of two sister cells, each with varying destinies. Upon division, one of the sister cells retains its self-renewing capacity while the other cell enters a differentiation pathway and becomes part of a mature non-dividing subpopulation (Cha and Falanga, 2007). All stem cells, regardless of their source, possess three distinctive characteristics: obligatory asymmetric replication, the capability of continual division for prolonged periods, and the ability to give rise to distinct specialized cell types.

Stem cells have five levels of potency, defined as the differentiation potential of a specific stem cell (Cha and Falanga, 2007). These levels, from the greatest to the least potential, are as follows: totipotent, pluripotent, multipotent, oligopotent, and unipotent. Totipotency is the ability of a single cell to divide and produce all of the differentiated cells in an organism. Totipotent cells include zygotes and cells produced by the first few divisions of the zygote during embryonic development. Pluripotent stem cells are descendants of totipotent stem cells and are capable of differentiating into any cell derived from one of the three germ layers: endoderm, mesoderm or ectoderm. Therefore these cells, like totipotent cells, also possess the ability to differentiate into any cell type present within the mature adult body, but they are not able to produce a whole organism. Multipotent stem cells, on the other hand, are cells, which possess the potential to
differentiate into various, but a limited number of lineages. Examples of this type of stem cell include hematopoietic stem cells (HSC) and mesenchymal stem cells. Oligopotency is the ability of a progenitor cell to differentiate into limited types of cells, such as lymphoid or myeloid cell lineages. Lastly, unipotent stem cells, which are the least potent, retain the capacity to differentiate into only a single cell type (Cha and Falanga, 2007).

There are two types of bone marrow (BM) derived multipotent stem cells, both of which possess the ability to develop into a number of specific cell types. Adult BM derived HSC have the potential to give rise to all blood cell lineages. Thus, HSCs can differentiate into both erythroblast and megakaryocyte progenitors, which produce erythrocytes and thrombocytes, respectively. HSCs can also differentiate into myeloid progenitors that give rise to mast cells, eosinophils, basophils, neutrophils, monocytes, macrophages, and into lymphoid progenitors that become T-cells, B-cells, and natural killer cells. Additionally, some studies suggest that under the proper conditions, HSC are also capable of producing some non-blood cells, including hepatocytes, endothelial cells, smooth muscle cells, and cardiac myocytes (Wu et al., 2007). However, these findings regarding HSC plasticity remain controversial. The second main type of BM derived stem cells are the mesenchymal stem cells (MSC), which are capable of differentiating into any cell derived from mesenchyme, an embryonic connective tissue that arises from the mesoderm (Ogawa et al., 2010). Therefore these cells are capable of forming several cell types, including fibroblasts, osteoblasts, adipocytes, chondrocytes, and skeletal myocytes (Stoff et al., 2009; Rasmusson et al., 2007).

Mesenchymal stem cells are defined as plastic-adherent stem cells, which possess
both the capacity for multipotent differentiation *in vitro* and have a morphology similar to fibroblasts (Hocking and Gibran, 2010). Morphologically, MSCs possess a small, narrow, spindle-shaped cell body with a large nucleus containing numerous chromatin particles, and a predominant nucleolus. The cell body also exhibits thin, elongated cell processes that extend in all directions, similar to the morphology of fibroblasts (Vater et al., 2011). The definition of MSC arose as a direct result of the work of Friedenstein and colleagues in the 1960s and 1970s (Friedenstein, 1976). It was noted that upon plastic adherence of bone marrow derived cells, a rare cell population developed into colony forming units, which were fibroblastic in nature. After *in vitro* culture expansion and introduction into diffusion chambers, it was discovered that these cells were capable of producing bone, cartilage, and stromal elements (Jones and McGonagle, 2008). The term MSC was first established in the 1990s to better describe one of two specific populations of cells derived from bone marrow. These cells, which possess the ability to differentiate into any cell type derived from the mesenchyme, have several unique functions. These include the generation of osteoblasts, chondrocytes, myocytes, and fibroblasts and the production of bone, cartilage, muscle fibers, and collagen, respectively (Phiney, 2012).

Mesenchymal stem cells are thought to have great potential for a variety of cell based therapies. Many of their clinical uses include utilization during tissue regeneration, support of engraftment, and immune modulation (Goedecke et al., 2011). MSCs have been isolated from multiple tissue sites, including BM, adipose tissue, human adult liver, peripheral blood, amniotic fluid, bronchial lung tissue, and articular synovium. Each MSC, regardless of source, is capable of differentiating into adipogenic, osteogenic, myogenic, chondrogenic, tendogenic, marrow stromal, or neurogenic cell types, although
the ability to differentiate into neurogenic lineages remains widely debated (Lorenz et al., 2008; Oreffo et al., 2005; Ozaki et al., 2007). The versatility of these cells to differentiate into such a large range of tissue types provides an extremely powerful tool for potential cell therapies.

In wound healing, the incorporation of MSCs at the site of injury has been shown to increase the tensile strength of the healed tissues, to produce increased levels of collagen, and to accelerate extracellular fiber formation, while also promoting histologic maturation of previously injured tissue (McFarlin et al., 2006). These healing benefits can be primarily attributed to the MSC’s ability to secrete multiple types of growth factors and cytokines, which are essential to the repair of injured tissue (McFarlin et al., 2006). To understand why MSC multipotent potential is significant to the wound healing process, one needs to better understand the molecular and cellular biology behind the wound healing process.

1.4 Wound Healing

The healing process of a wound occurs in three successive phases: inflammation, proliferation and maturation. These three phases usually last for 12-24 hours, 3-7 days and 1-2 weeks, respectively. Inflammation is a multistep process, which consists of vasodilation, increased vascular permeability, cellular infiltration and activation of leukocytes. The degree to which these phases occur is dependent upon and proportional to the severity of injury and extent of infection (Kondo and Ishida, 2010). Upon injury, damage to blood vessels and disruption of their tunica intima, the innermost layer of an artery or vein, leads to the release of internal cellular contents. This stimulates nearby
sensory neurons, which in turn produce vasodilation of local blood vessels and increased capillary permeability. The increased permeability will then allow for leukocyte extravasation. Once arriving at the site of injury, cytokines released by neutrophils and macrophages cause vasodilation of blood vessels in the surrounding area. The enlargement of blood vessels cause localized hyperemia, the increase of blood flow to the injured tissue. The onset of these three physiologic responses, vasodilation, increased vascular permeability and cellular infiltration, results in edema due to the accumulation of extracellular fluid at the injury site and inflammatory cell aggregation.

The increased vascular permeability that arises following injury allows for the movement of blood constituents into the surrounding tissues, resulting in thrombogenesis and eventual hemostasis (Wynn, 2008). Coagulation, a multistep process, can be divided into three essential steps: 1) induction of a complex cascade of chemical reactions in the blood involving numerous coagulation factors. These chemical reactions result in the formation of a complex of activated substances collectively deemed the prothrombin activator. 2) The newly synthesized prothrombin activator functions to catalyze the conversion of prothrombin into thrombin. 3) Activated thrombin, an enzyme, mediates conversion of fibrinogen into fibrin fibers that serve to entangle erythrocytes, platelets (thrombocytes), and plasma to form a clot (Guyton and Hall, 2011). Blood clot formation is an essential mechanism in the wound healing process, that allows for the cessation of blood loss from damaged blood vessels.

During clot formation, thrombocytes release multiple growth factors, including epidermal growth factor (EGF), platelet-derived growth factor (PDGF), transforming growth factor-β (TGF-β) and chemokines, which function to stimulate cell growth,
proliferation, division and differentiation (Tarnuzzer and Schultz, 1996). These factors also play an essential role in promoting the localization of inflammatory leukocytes to the site of injury. Additionally, keratinocytes release both interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α) in response to disruption of the epidermal barrier (Kondo and Ishida, 2010; Mooney et al., 1990). The release of these cytokines stimulates the recruitment of surrounding neutrophils and macrophages through specific cellular receptor binding and activation via intracellular signaling pathways. Upon stimulation, these cells will travel to the location of epidermal damage, where they will function to remove bacteria from the contaminated region. Macrophages also function to augment the inflammatory response by releasing the proinflammatory cytokines IL-1 and IL-6, as well as specific growth factors which include fibroblast growth factor (FGF), EGF, TGF-β and PDGF (Tarnuzzer and Schultz, 1996; Hubner et al., 1996). All of these signaling factors will serve to initiate the formation of granulation tissue by facilitating cellular infiltration (Kondo and Ishida, 2010).

Proliferation, the second phase of wound healing, can be divided into two stages: 1) the formation of granulation tissue along with neovascularization and 2) reepithelialization (Vidinsky et al., 2006). Within 3-5 days after injury, the initial fibrin clot is replaced by vascularized fibrous connective tissue known as granulation tissue. During this process the wound site becomes impregnated with macrophages, fibroblasts, and endothelial cells. The localization and secretion of vascular endothelial growth factor (VEGF) by macrophages is critical for promoting angiogenesis at the wound site (Marneros and Olsen, 2001). The development of new blood vessels supplies the tissue with the means of producing and developing a new extracellular matrix. The newly
formed vascular network allows non-native fibroblasts to migrate to the injury site where they will act to produce and secrete connective tissue fibers and ground substance. Likewise, the secretion of FGF by macrophages stimulates fibroblast proliferation. Prolonged exposure of fibroblasts to TGF-β and PDGF, which are also secreted by macrophages, ultimately results in the phenotypic differentiation of fibroblasts to myofibroblasts. These site-specific cells align along the border of the wound where they then generate smooth muscle actin and myosin, which then produce a constrictive force to aid in wound closure (Kondo and Ishida, 2010).

Following injury, the release of EGF and TGF-α by macrophages facilitates epithelial cell migration and growth, as well as development. The presence of FGF, which is also secreted by macrophages, stimulates proliferation of epithelial cells, ultimately commencing the reepithelialization process. Recruitment and stimulation of epithelial cells occurs in response to signal transduction, initiated through the binding of these factors to specific receptors located on the surface of epithelial cells. For epithelial cell migration to occur successfully, the cell-to-cell macula adherens (desmosomes) and cell-to-substrate hemidesmosomes contacts must first be dissolved. This process, which is initiated by EGF, then allows keratinocytes to freely migrate over the extracellular matrix (ECM). The ECM, consisting of connective tissue fibers and ground substance, serves as a substrate upon which recently migrated keratinocytes continue to proliferate and upon completion of wound closure, undergo keratinization to reform the epidermal barrier (Wu et al., 2007).

The maturation process, which is the third phase of wound healing, is characterized by sequential collagen and elastin fiber degradation, deposition and
remodeling (White et al., 2007). During this process, the previously formed connective tissue is first degraded by fibroblasts. Next, revascularization of the wounded area, achieved via angiogenesis, allows for the establishment of a novel collagen and elastin fiber network produced by newly localized fibroblasts. This network subsequently becomes heavily saturated with proteoglycans and glycoproteins upon maturation. Macrophages, which continue to release TGF-β, mediate the synthesis and deposition of new collagen by fibroblasts, which are stimulated by continued exposure to the growth factor. Simultaneously, macrophages, epidermal cells, endothelial cells and fibroblasts secrete matrix metalloproteinases (MMPs), which function to degrade the collagen network (van der Veen et al., 2010; Zheng et al., 2002). The continued cycling of collagen fiber synthesis, deposition and catabolism results in the formation of scar tissue as the final product.

Complications can arise during the maturation phase of wound healing when high levels of MMPs are released into the surrounding extracellular matrix (ECM) (Hodde and Johnson, 2007). The increased protease activity results in a greater rate of collagen fiber degradation compared to collagen deposition by fibroblasts. The proper healing of wounds is extremely dependent on this balance between collagen fiber degradation and deposition, and alterations in this balance can have an adverse effect on the healing process. Subsequently, the expression and distribution of growth factors and cytokines by macrophages is also altered during increased MMP activity. Recent studies have shown that the application of exogenous forms of type I collagen to a chronic wound site will function to bind excess MMPs, thus restoring the proper matrix degradation-deposition rate (Hodde and Johnson, 2007). The decreased rate ultimately allows for proper
fibroblast functioning, organization of the ECM and tissue remodeling. Thus, proper collagen fiber formation and deposition is essential for completion of the wound healing process.

1.5 Collagen Synthesis

The production of the elastin and collagen fiber network is essential to the maturation phase of the wound healing process and therefore crucial for directing wound closure. As the new framework of connective tissue is deposited, the injury site gains a new scaffolding network, which will be used to remodel the dermis and eventually produce scar tissue (Kondo and Ishida, 2010). The remodeling of the collagen network is directly dependent on continued synthesis and catabolism of collagen during the transition from granulation to scar tissue. For this reason, collagen fibril production is vital to facilitating wound closure.

Biosynthesis of fibril forming collagens can be summarized in two successive multistep processes: 1) intracellular synthesis and secretion of the precursor molecule, procollagen, and 2) extracellular cleavage of procollagen to tropocollagen and the formation of collagen fibrils. In order to produce the macromolecular procollagen, amino acids are first taken up by fibroblasts, the cells responsible for collagen synthesis. Two amino acids, which are essential for the production of this complex are proline and lysine. Following uptake of the necessary amino acids, transcription of multiple genes encoding the various α-chains unique to each procollagen molecule is performed within the nucleus of fibroblasts (Raghow and Thompson, 1989). Each of these alpha chains is then synthesized in the cytosol along with their respective propeptides. Upon entry of the α-
chains into the cisternae of the rough endoplasmic reticulum, post-transcriptional modification occurs, which consists of hydroxylation of varying quantities of the amino acids proline and lysine. While inside the cisternae, glycosylation of specific hydroxylysyl residues also occurs. Each of these recently modified α-chains is then combined in triplets to generate the procollagen triple helix. Finally, procollagen is subsequently transferred to the golgi complex where additional glycosylation and packaging of the product occurs prior to exocytosis.

Once the procollagen molecule is transferred to the extracellular space, procollagen peptidases cleave the propeptides from procollagen, thus transforming the triple helix into tropocollagen (Kadler et al., 2008). Completion of the synthesis process occurs upon the polymerization of these intermediate molecules. Tropocollagen molecules arrange in a staggered conformation with each row being 56nm in length with a gap between the C-terminal of one intermediate and the N-terminal of the successor (Gelse et al., 2003).

Variations in collagen can arise via three means. First, α-chains can vary by amino acid composition. Because α-chains are the basic components of collagen, variations in these primary tropocollagen molecules can produce significant differences in the final collagen fibril composition. Second, posttranscriptional modification can also change the molecular composition of the procollagen intermediates. While inside the cisternae of rough endoplasmic reticulum, glycosylation and hydroxylation occur at specific locations, which are unique to each α-chain. Last, the combination of various α-chains can also produce characteristic differences in the procollagen triple helix composition and ultimately the collagen fibril type (Gelse et al., 2003).
Currently, twenty-eight types of collagen have been identified and characterized. Although a large number of types exist, the most common include types I, II, III, V and XI. These five types are described as the fibril forming collagens, which are known for their ability to assemble into highly oriented aggregates with a defined suprastructure. The two types of collagen which are of specific interest to studies of wound healing are types I and III. Type I collagen is known to be the most abundant of all types of collagen and is the major collagen component of bone, tendons, dermis, fascia and ligaments (Raghow and Thompson, 1989). Reticular fibers, a type of fiber in connective tissue composed of type III collagen, are found in high concentrations within lymphatic tissues. Reticular fibers can also be found in many of the same tissues as type I collagen, with the exception of bone. During the maturation phase of wound healing, type III collagen, which is prevalent during proliferation and initial deposition, is gradually degraded and replaced by the stronger type I collagen. Because both of these fiber types play a critical role in the establishment of proper scar tissue, the relative amounts of both fibers observed around the injury site will be increased. The ratio of collagen type III to collagen type I is greater than one directly following injury and is subsequently diminished as remodeling of the scar tissue occurs (Rosch et al., 2003; Klinge et al., 2000). To test whether this hypothesis holds true, we have implemented both an immunohistochemical and silver staining technique, which can be used to differentiate between the two collagen types.

1.6 Immunohistochemistry

Immunohistochemical assays are a diagnostic tool that can be used to both
accurately identify specific extracellular structures as well as biologically characterize various tissue types (Torlakovic et al., 2010). This technique has been found to be increasingly valuable in defining the distribution of various collagens in connective tissues. Established methods for collagen immunohistochemistry (IHC) utilize frozen tissue sections preserved by liquid nitrogen or formalin preserved paraffin embedded tissue. However, complications are known to arise with the latter technique. Formalin, a commonly used fixative in histology, acts to cross-link primary amino groups in proteins, in turn possibly sacrificing structural detail and antigenic specificity in an attempt to assure proper retention and accessibility to antigens and their respective epitopes (Horton et al., 1983).

The real benefit of the IHC technique lies in its ability to identify specific cells or subcellular structures with more precision than the more standard approach of histochemical staining. This highly intricate technique, originally developed in the 1940s and then subsequently refined by the incorporation of enzymes in the 1960s, utilizes a method, which provides both the identification and labeling of specific sites. Throughout the development of IHC, many additions have been made in an attempt to improve the technique, such as the integration of biotin-streptavidin and other labeling enzymes. These chemical substituents aid in the visualization of specific antibody-labeled antigens within the two-dimensional histology of a tissue section (Tangrea et al., 2011).

Tissue preparation for histologic analysis can be summarized in seven successive steps. Initially, tissue collection must be performed. During this time it is vital that collection of the tissue from the specimen is done in a timely manner to prevent tissue degradation on a cellular level, otherwise epitope conformational changes could arise.
These epitopes are localized to regions on the surface of an antigen that are capable of eliciting an immune response and binding with a specific antibody. Therefore, changes to this site will act to diminish antibody specificity leading to decreased labeling. Next, the excised tissue is fixed with a preservative and then dehydrated to remove water and allow for the complete immersion of the tissue in wax. Once dehydrated, the tissue is embedded in paraffin, which provides the tissue with support and shape in preparation for cutting. Afterwards, the newly embedded tissue is sectioned. This step of the procedure is vital to assure that the correct thickness and proper orientation of the tissue is selected. Tissue thickness and orientation can play a pivotal role in allowing for successful interaction between the reagents used during staining of cellular components. Sectioning often produces sections approximately one cell thick to be placed on microscopic slides. Finally, the sectioned tissue is mounted onto glass slides and stained to accent the structures of choice.

For successful staining, the appropriate IHC method must be selected and refined to ensure staining specificity for the desired antigen. This step of the procedure is the most critical. Successful staining of selected tissues is multifactorial and is dependent on numerous factors such as correct antibody selection, incubation time and temperature, proper epitope retrieval, chromogen selection, counter staining, controls, preservation of tissue, and quality of reagents.

1.7 Silver Staining

Though immunostaining for collagen fiber types exists as an extremely powerful tool for pathologists, its usage is known to become somewhat diminished when utilized
on fixed, paraffin embedded tissues. Many fixative agents have been known to cause destruction of tissue antigens, in turn leading to false negative histopathology results. Much of the corresponding literature discusses such conventional fixatives, including formalin and Bouin’s fluid, which are commonly used in combination with paraffin and are known to produce inconsistent results (Gerhard et al., 1985). Commonly, when the aforementioned complications arise, many histologists turn to silver impregnation, more generally known as silver staining, to obtain successful identification of reticular fibers.

Ever since Maresch (1905) first utilized the Bielschowsky silver impregnation technique for the identification of connective tissue fibers in paraffin embedded tissue, the Bielschowsky-Maresch silver impregnation technique has been extensively employed (Gömöri, 1937). The continual popularity of this technique can be directly attributed to its unique ability to provide sharp delineation of the finest reticular fibrils. Like many other techniques, various modifications have been made over the years to the original technique in attempts to achieve the best contrast in coloration based upon tissue preparation methods. However the integrity of the technique remains unchanged.

For those requiring direct visualization of reticular fibers, the application of silver staining is ideal. The use of the silver staining technique is often utilized to differentiate between type III collagen and the other collagen types, by exploiting the characteristic high carbohydrate-rich matrix present in close proximity to type III collagen (Hwang et al., 1990). During staining, cleaved carbohydrates are first chemically altered to produce numerous aldehydes. Once subjected to an ammoniacal silver nitrate compound, these aldehydes facilitate the precipitation of metallic silver at the site of the reaction, in turn causing the tissue to convert from a previous brown color, to varying shades of black
throughout development (Fujimori, 1991). The unmistakable color of type III collagen can then be later studied for their location in various tissues. Other structures besides reticular fibers stain with silver impregnation, including the “I” bands of skeletal muscle, polysaccharides, egg white, and gliadin (Putchler and Waldrop, 1978). Although these other structures are known to react positively when subjected to silver impregnation, the method remains the top choice to those needing to differentiate between collagen types.

The goal of this study is twofold. First, we will seek to determine the ideal MSC transplant dosage needed to improve fascia recovery, by testing five MSC doses and analyzing the experimental tissue histologically to determine the effect of the stem cell addition on the wound healing process. Secondly, MSC treated wounds will also be analyzed to determine the optimal method for determining the type of collagen produced at the wound site. During this investigation, MSCs will be harvested from the bone marrow of rats and subsequently applied to a recently generated surgical incision on a rat specimen. Following designated times of recovery, each of the experimentally treated rats will be euthanized and their surgical tissue sites excised for examination. Analysis will include the use of trichrome stains to determine the total collagen deposition. Trichrome staining, however, non-specifically stains all collagens and thus, does not differentiate between collagen types. For this reason, both an IHC and silver staining method will be developed to directly analyze the amount of collagen type III present in the healed incisional area. The relative amount of collagen type III present will provide an indication of how quickly and efficiently the wound has recovered. I hypothesize that the MSC addition will function to accelerate scar tissue production, therefore, accelerating type III
collagen deposition, degradation and subsequent replacement by its stronger counterpart, type I collagen.

2. MATERIALS

Adult male Lewis white rats weighing 250–300g were obtained from Charles River Laboratories International, Inc. (Wilmington, Massachusetts). Buprenorphine, isoflurane and an EZ-AF9000 Auto Flow System anesthesia device were obtained from Anesthesia Plus, Inc. (Elk Grove, California). Wounds were sutured using 5-0 Vicryl sutures obtained from Chinook Medical Gear, Inc. (Durango, Colorado). CollaTape™ type I bovine collagen was obtained from Zimmer Dental (Mississauga, Ontario, Canada). Fascia tissue removal was performed using a customized cutting die fitted to a manual press (exclusively constructed). Dose response histologic wound evaluation materials included Microsoft PowerPoint and Microsoft Excel from Microsoft Corporation (Redmond, Washington), Adobe Acrobat Pro (San Jose, CA) and IBM SPSS, (Armonk, New York). Immunohistochemistry materials included a biotinylated α-Rat IgG (H+L) primary antibody obtained from Vector (Bulingame, California), Pierce collagen III rabbit polyclonal primary antibody obtained from Thermo Scientific (Rockford, Illinois), and α-skeletal myosin primary antibody developed in rabbit and obtained from Sigma-Aldrich (St. Louis, Missouri) (‘α-’ designates antibody against stated antigen). Secondary antibodies included two biotinylated goat α-rabbit IgG antibodies. One was obtained from Invitrogen (Eugene, Oregon), while the second was obtained from Millipore (Temecula, California). The label, Horseradish Peroxidase
Conjugated Streptavidin, was purchased from BioGenex (Fremont, California). 3,3'-Diaminobenzidine (DAB)-Plus Substrate Kit was obtained from Invitrogen (Eugene, Oregon). The protein-blocking agent (#407501) Immunon was obtained from Thermo Shandon (Pittsburgh, Pennsylvania). All other chemicals and solutions were obtained from Sigma-Aldrich (St. Louis, Missouri). Culture flasks (T25 and T75) and all other chemicals and solutions, including Trypsin, PBS, DMSO, EDTA, cobalt chloride (CoCl₂), Imidazole, sodium hydroxide (NaOH), silver nitrate, 28% ammonia, periodic acid, silver nitrate, concentrated formaldehyde, gold chloride (Au₂Cl₆) and sodium thiosulfate (Na₂S₂O₃), were obtained from Sigma-Aldrich (St. Louis, Missouri).

3. METHODS

3.1 Rat Surgeries

The Institutional Animal Care and Use Committee at Youngstown State University approved the experimental protocol for this study. Rats were housed and acclimated for 1 week prior to experimental use in the animal care facility at Youngstown State University. Standard rat chow and water was provided ad libitum. All surgical manipulations were performed under aseptic conditions. The instruments utilized were either sterilized via autoclave or with a dry sterilization unit. Between each procedure, all surgical instruments were wiped with a 70% ethanol solution, dried, and then re-sterilized using a hot bead sterilizer.

Prior to surgery, the rats received a dose of buprenorphine (0.025 mg/kg), given subcutaneously, for pain alleviation. Additional buprenorphine doses were given at 12
and 24 hours post-surgery. All procedures were performed under isoflurane (3-5%) inhalation anesthesia for induction and 1-3% isoflurane for maintenance of anesthesia. The total time each rat spent under isoflurane anesthesia ranged from 20 to 40 minutes. Following anesthetization, the abdominal area of the rat was shaved, washed with betadine and rinsed with 70% isopropanol. An approximate 6cm, in length, midline full-thickness fascial incision was made along the linea alba of the rat abdomen. Fascial incisions were closed using 4-0 running vicryl sutures while skin incisions were closed with individual 4-0 vicryl subcuticular sutures. Animal respiration, tissue color and toe pinch reflex were monitored throughout the surgery.

Following all surgical procedures, each rat was placed in clean bedding and monitored during the recovery period, consisting of either 4 or 8 weeks dependent on subgroup designation. Initially the rats were monitored once per day during the first week of recovery, paying particular attention for signs of infection and autophagia. If infection was noted, a combination antibiotic treatment, consisting of Procaine penicillin and Benzathine penicillin (40,000 units/Kg), was administered intramuscularly. A total of three treatments were administered every other day throughout the week. Following the completion of antibiotic treatments, the rats were monitored daily for adverse conditions for an additional week. Animals experiencing chronic (longer than two days) pain (indicated by cat-like stretching, horizontal stretching, twitching, or chewing at the surgical site), significant signs of pain (significant depression or aggressiveness), unresolved infection, wound dehiscence (rupture of a surgical wound) or autophagia were removed from the study and euthanized. Following the first week of recovery, the animals were monitored 2-3 times per week.
3.2 Rat Experimental Groups

The first experiment examined wound healing in response to different MSC doses. For this, 35 age-matched rats were separated into five equal groups, each consisting of 7 rats per group. All rats received a similar midline fascial incision along the linea alba and underwent suture repair of the wound, as described above in Rat Surgeries. In addition, these animals also received the addition of CollaTape™ (CoTa), PRP and MSCs directly at the site of injury. Groups were varied by altering the number of transplanted MSCs applied to each of the five groups of rats. The five doses included: 2.5x10⁴, 5.0x10⁴, 1.0x10⁵, 2.5x10⁵ and 5.0x10⁵ fresh MSCs. Following surgery, all rats were allowed to heal for a total of 4 weeks before being sacrificed and analyzed postoperatively.

In a separate experiment, a second group of rats were used to determine the type of collagen produced at the wound site. A total of four test groups, consisting of 14 rats per group, were employed during this experimentation: Group 1) primary repair receiving only sutures, or surgical correction of the abdominal wound without an intervening stage, Group 2) receiving primary repair plus the addition of CoTa and platelet-rich plasma (PRP), Group 3) receiving primary repair plus the addition of CoTa, PRP and frozen MSCs, and Group 4) receiving primary repair plus the addition of CoTa, PRP and fresh MSC. Rats receiving transplanted MSCs, groups 3 and 4, were treated with a dose of 1.0x10⁶ MSCs. During recovery, each group was then divided into two equal subgroups, A (n = 7) and B (n = 7). Subgroups received the same conditions during recovery, however, subgroup A animals were allowed to recover for a total of four weeks, while
subgroup B animals were allowed to recover for a total of eight weeks, prior to sacrifice and tissue harvesting.

### 3.3 Bone Marrow Extraction

Two rats were sacrificed by CO$_2$ inhalation. Following death, the entire rat body was dampened with 70% ethanol in order to reduce the possibility of hair and dander becoming airborne. The rat was placed on paper towels also soaked with 70% ethanol. The scissors and mouse tooth forceps utilized during dissection were sterilized by dipping into a beaker of 95% ethanol and then quickly passed through the flame of an alcohol lamp. A vertical cut was made through the loose skin of the inguinal region of the rat. Excess skin was pinned back on both sides. The subepithelial layer was then flooded with 70% ethanol to remove any loose hair. While grasping the hind legs with the mouse tooth forceps, the muscle layer in close proximity to the hip joint was cut away with scissors. The hind limbs of the rat were then separated from the body at the hip joint. The feet were carefully removed, taking care not to damage the epiphyses of the long bones during separation of the joints. The newly separated limbs were immediately transferred to a culture dish suspended on ice containing stem cell media (MEM/Ham’s F-12 media) without fetal calf serum (FCS). Removal of additional muscle tissue from the femoral and crural regions was performed by grasping the femur and tibia with the mouse tooth forceps and trimming the majority of the muscle away with scissors. Residual muscle tissue was removed by scraping the bones with flat forceps and a scalpel.

The partially cleaned bones were transferred to a fresh culture dish suspended on ice, containing stem cell media without FCS. Following transfer, the tibia and femur were
separated with scissors. Once separated, the culture dish containing the bones was relocated to a cell culture hood where the epiphyses of each bone were removed using scissors. The bone ends were punctured with a 21-gauge needle and the bone marrow was flushed from the bone with stem cell media without FCS. The marrow was then drawn in and out of the needle and syringe to obtain a single-cell suspension, which was transferred to a 12mL conical tube and allowed to let settle for 4 minutes to remove clumps. The resulting supernatant was transferred to a fresh tube. A cell count was performed on the supernatant, after using 4% acetic acid to lyse the red blood cells. The sample was then centrifuged at 600 x g for 8 minutes at 4°C. The pelleted cells were resuspended in 10mL of stem cell culture media (Alpha-MEM media containing 10% FCS and Glutamax), then added to a T75 culture flask at an initial concentration of 1x10^5-1x10^6 cells/mL. The stem cells were maintained in cell culture until they reached a concentration of 1x10^6-1x10^7 cells/mL (DuBay et al., 2006).

3.4 Rat Bone Marrow Stem Cell Culture

Stem cell culture was performed in a NUAIRE™ IR AUTOFLOW CO₂ Water-Jacketed Incubator (Model NU-8500), with 5% CO₂ and a temperature of 37°C. After three to four days in culture, non-adherent cells were removed from the culture flask by removing the stem cell culture media. The remaining adherent cells were fed every three days until they became 85% confluent (Dai et al., 2005; Javazon et al., 2001). Expansion to 85% confluence took approximately one week to achieve. Next, these cultured stem cells were passaged by first washing the stem cells with phosphate buffered saline (PBS) (10x solution), followed by treatment with approximately 3mL of a 0.25% trypsin
solution in 1.0 mM EDTA for 7 min at 37°C. The trypsin was inactivated by adding 30mL of complete stem cell culture medium (Alpha-MEM media containing 20 % FCS, 2 mm of L-glutamine, 100 U/mL of penicillin, and 100µg/mL of streptomycin) to the cell culture flask. Afterwards, the cells were separated into two flasks by dividing the media in half and adding 15mL to each T75 culture flask. MSC expansion was then continued until passage 3. After passage 3, the cells were collected via trypsinization and centrifuged for 5 min at 600 x g. The pellet was resuspended and a cell count was taken. Cells to be used at a later time were frozen in complete media containing 10% DMSO at 10⁶ cells/ml by decreasing the temperature slowly overnight and then storing in liquid nitrogen.

When needed, recovery of the frozen stem cells was performed by first thawing the frozen mixture slightly and transferring the frozen pellet into 5mL of complete media at room temperature (RT) (21°C). The contents were mixed quickly to facilitate thawing, washed once to remove DMSO, and then plated in a T25 culture flask. The recently thawed cells were incubated overnight. The cells were harvested by trypsinization. Next, the cells were centrifuged for 10 min at 400 x g at RT, and resuspended in either 0.5mL platelet-rich plasma, or in 0.5mL of complete media (Dai et al., 2005; Javazon et al., 2001).

3.5 Preparation of Platelet Rich Plasma (PRP)

A total of 5-10mL of blood per rat was collected from each rat being sacrificed for bone marrow harvest. Blood was collected by heart puncture of anesthetized rats using a 21-gauge needle and a 10mL syringe containing 1/10 volume of the anticoagulant
citrate dextrose (ACD). The collected blood was then centrifuged for 10 minutes at 200 x g at RT. The plasma layer was aspirated off and re-centrifuged for 10 minutes at 700 x g. The resulting platelet poor upper layer was aspirated off, frozen and stored at -80°C. Next, 5μL of 5% DMSO (diluted with PBS) was added to approximately 1mL of the remaining platelet pellet and re-suspended. The final mixture was placed in a cryovial and frozen by slowly decreasing the temperature to -80°C. The vial was then stored in liquid nitrogen until needed.

Platelets were thawed by first bringing the previously collected frozen platelet poor plasma (5-10mL) to 37°C. Once the plasma was thawed, 1mL of plasma was transferred to a separate centrifuge tube. Next, the frozen platelet pellet was thawed until the pellet could be dislodged from the conical tube and rapidly thawed by mixing with the 1mL of previously warmed plasma. This mixture was centrifuged for 10 minutes at 700 x g at 4°C. Following centrifugation the plasma containing DMSO was aspirated off, discarded and the platelet pellet was re-suspended in the remaining originally thawed plasma (5-10mL). This final mixture was then applied to the surgical incision area of all rat specimens in the dose response study and to rats in groups 2-4 of the type III collagen study, at a volume of 0.5mL per injury site (Maekawa et al., 2003).

3.6 Fascia Recovery

The abdominal wall musculature and fascia of each rat was harvested at 4 weeks for all rats belonging to the dose response study, and at 4 weeks (subgroup A) or 8 weeks (subgroup B) for rats belonging to the type III collagen study. For recovery (collection) of fascia, rats were initially placed under deep inhalational anesthesia, as previously
described. Next, a midline sternotomy was performed and whole blood was obtained via cardiac puncture for PRP isolation. Afterward, bilateral pneumothoraces were created to euthanize the rat. Once successfully euthanized, the cutaneous surgical scar was incised, and bilateral skin flaps were carefully removed, avoiding inadvertent fascia disruption. Abdominal musculature was then excised along the subcostal and pelvic margins using a customized cutting die fitted to a manual press. Two I-shaped segments spanning the healed incision site were removed, immediately fixed in 10% formalin in PBS while being transferred to the Pathology Center at Saint Elizabeth Medical Center for histological preparation. All tissues obtained from both the dose response study and the type III collagen study, were transferred to Saint Elizabeth Medical Center for histological preparation.

3.7 Histology

The previously excised and fixed rat tissue was processed by the Pathology Center at Saint Elizabeth Medical Center into smaller pieces, dehydrated, paraffin embedded, sectioned into five-micron sections and placed on glass slides. One section from all tissues obtained from rats were either stained with Masson’s trichrome or with Hematoxylin and Eosin (H&E). Technicians at Saint Elizabeth Medical Center performed all trichrome and H&E staining. The remaining sections were left unstained for type III collagen content analysis, using IHC or silver staining. In total 6 sections were cut for each rat specimen belonging to the dose response and type III collagen studies.
3.8 Dose Response Histologic Wound Evaluation

After obtaining the trichrome stained slides from the Pathology Center at Saint Elizabeth Medical Center, high-resolution photographs were taken at 40x magnification for all representative specimens. In total, 35 images were taken, each corresponding to one experimental rat. Next, the tissue images were randomized, placed into an electronic slideshow and assessed by three professionals trained in histology. Three categories or criteria were used to evaluate wound recovery. The three categories included: collagen organization, collagen abundance, and myocyte regeneration. Each observer was blinded to individual study groups and a score of 0 (worst) to 3 (best) three was assigned to each category for each slide, using a semi-quantitative scale (Table 1) (Badylak et al., 2002; Konstantinovic et al., 2005). Statistical analysis was then performed for each of the five groups to analyze the effect of MSC dosage on each of the three analysis criteria.

3.9 Immunohistochemical Staining

For IHC staining, the tissue mounted glass slides were placed on a heating block for 15 minutes at 60° C to partially melt the paraffin. Next, paraffin was dissolved by passing the slides through three separate xylene baths (7 minutes each). The tissue sections were rehydrated and prepared for staining by first passing the slides through a series of alcohol baths: 100%, 100%, 95%, 70% and 50% (1 minute each) followed by washing the slides in distilled water (5 minutes) before washing the slides in a PBS bath (5 minutes).

To quench endogenous peroxidases, 100-150µL of 0.3% H₂O₂ was pipetted directly onto the tissue sections on each slide and incubated for 20 minutes at RT in a
Each image of a histologic tissue section was analyzed based on these three categories and appointed a score ranging between 0 (least) and 3 (best).

<table>
<thead>
<tr>
<th>Category</th>
<th>Score</th>
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<tbody>
<tr>
<td></td>
<td>0</td>
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<tr>
<td><strong>Collagen Organization</strong></td>
<td>Disorganized</td>
</tr>
<tr>
<td>Collagen Amount</td>
<td>None</td>
</tr>
<tr>
<td><strong>Myocyte Regeneration</strong></td>
<td>None</td>
</tr>
</tbody>
</table>

*Each image of a histologic tissue section was analyzed based on these three categories and appointed a score ranging between 0 (least) and 3 (best).*
humidified chamber. Following incubation, the slides were rinsed with water and placed in a PBS bath for 10 minutes with gentle stirring.

If antigen retrieval was required to optimize the epitope for the primary antibody (1° Ab), pretreatment of the tissue was then performed. This involved incubating the slides in a 0.01M sodium citrate buffer at 95-100°C. A hot water bath was used to maintain the buffer temperature, and the slides were placed in a staining dish containing buffer, with the lid loosely attached, for 20 minutes. Following incubation, the staining dish was allowed to cool for approximately 20 minutes or until buffer solution reached room temperature. The slides were then rinsed twice in a PBS bath, with each bath lasting for two minutes.

To prevent nonspecific binding of the 1° Ab, all tissue sections were pre-incubated with 100-150μL of a protein-blocking agent at room temperature for 20 minutes in a humidified chamber. Following incubation, the slides were drained and blotted dry to remove residual blocking agent. The slides were then incubated for one hour in a humidified chamber with 100-150μL of the desired 1° Ab. Each 1° Ab solution was diluted in PBS to the distributor’s recommended concentration. Each experiment utilized a negative control that consisted of a slide incubated with PBS, but without a 1° Ab. Following incubation, the slides were rinsed with PBS and then placed in a stirred PBS bath for ten minutes. The slides were subsequently removed from the PBS bath and placed back into the humidified chamber where 100-150μL of secondary antibody (2° Ab) was added to each tissue sample for 20 minutes. The slides were removed from the incubation chamber, rinsed with PBS and then washed in a stirred PBS bath for ten minutes. After removal, the slides were placed flat in a humidified chamber where 100-
150µL of streptavidin horseradish peroxidase (S-HRP) was added to each tissue sample for 20 minutes. After incubation, the slides were rinsed with PBS and placed in a stirred PBS bath for ten minutes. The slides were again placed flat in a humidified chamber where 100-150µL of substrate (chromogen) solution was added to each tissue section. The chromagen or substrate solution was made fresh by combining one drop (approximately 50 µL) of DAB chromogen concentrate (20X), one drop of substrate buffer concentrate (20X), and one drop of concentrated hydrogen peroxide (20X) in a microcentrifuge tube where the three reagents were mixed (Invitrogen, DAB-Plus Reagent Set, #002020). The solution was then brought up to a final volume of 1mL by adding 850µL of deionized water.

If DAB enhancement was to be performed, one of three other solutions was added to the above chromagen solution. These additional solutions included 1M imidazole (10µL) or 0.3% cobalt chloride (CoCl) (60 or 10µL). These enhancers were added prior to bringing the final volume up to 1mL with the addition of deionized water. The amount of water added was dependent on the amount of enhancer added to the above DAB solution volume. Stock solutions for the two enhancement solutions were prepared in advance. The 1M Imidazole and 0.3% CoCl aqueous solutions were prepared, aliquoted and stored at -20°C to prevent degradation of the solutions.

The slides were incubated with DAB between 2-30 minutes, depending on the speed of tissue coloration or staining, with their respective DAB solutions. To stop the reaction, the solution was discarded and the slide was submerged in a distilled water bath for five minutes. Tissue dehydration was performed through five successive alcohol baths
(50%, 70%, 95%, 100% and 100%), followed by a single xylene bath at 1 minute each. Lastly, coverslips were applied using toluene Acrytol Mounting Media (IHC World).

3.10 Gridley (Silver) Staining

Silver staining was performed using the Gridley Method (Humason, 1962). Similar to the IHC protocol, all solutions were prepared prior to staining. The ammoniacal silver hydroxide solution was prepared by adding 20 drops of 10% sodium hydroxide (10g NaOH in 100mL) to 20mL of 5% silver nitrate (1g/20mL distilled water) solution. Fresh 28% (reagent) ammonia was then added drop-by-drop to the sodium hydroxide/silver nitrate mixture, until the silver hydroxide precipitate was almost redissolved. The final solution was brought up to a total of 60mL with the addition of distilled water.

The slides were prepared for staining as described in the IHC section of the methods. Briefly, the slides were immersed in three separate xylene baths, for 7 minutes each, followed by a series of alcohol washes each lasting for 3 minutes (100%, 100%, 95%, 70% and 50%). Next, the slides were treated with 0.5% periodic acid (0.5g/100mL water) for a period of 15 minutes and then rinsed in distilled water. Afterwards, the slides were incubated with 2% silver nitrate solution (2g/100mL water) for 30 minutes at room temperature and washed twice in a distilled water bath. Following the washes, the slides were impregnated with the ammoniacal silver solution for 15 minutes at room temperature and then rinsed in distilled water. Following silver impregnation, the tissue sections were incubated for three minutes in 30% formalin (30mL/70mL water) with continual and gentle agitation. They were then rinsed in four successive distilled water
baths. The slides were incubated with gold chloride (10mL 1% stock solution/40mL water) until the yellow brown tissue color gave way to a lavender-gray coloration. Again the slides were rinsed in a distilled water bath. The tissue staining was fixed by incubating the slides for three minutes in 5% sodium thiosulfate (5g/100mL water). The slides were washed in running water for 5 minutes, dehydrated through five successive alcohol baths (50%, 70%, 95%, 100% and 100%), 1 minute each, and cleared using a single 1 minute xylene bath. Lastly, the slides were coverslipped for light microscopy.

4. RESULTS

The goal of this study is twofold. First, we will seek to determine the ideal MSC transplant dosage needed to improve fascia recovery and second, to determine the optimal staining method for determining the type of collagen produced in MSC treated wounds. During this investigation, MSCs were harvested from the bone marrow of rats and subsequently transplanted on recently generated surgical incisions in two independent groups of rat specimen. Experimental groups for the dose response study differed only in the concentration of transplanted MSCs, whereas experimental groups belonging to the type III collagen study varied by transplantation material. By comparing and analyzing the healed tissue obtained from the each of the experimental groups within each individual study, one can determine the ideal MSC concentration needed to facilitate wound healing and whether the inclusion of MSCs act to accelerate the wound healing process, respectfully.
4.1 MSC Concentration Histologic Wound Evaluation

The purpose of varying the MSC dose followed by histologic wound evaluation was to determine what number or concentration of MSCs added were most efficient at improving wound healing in injured rat tissue. Rats, surgically injured as described above, received MSC additions at various doses. In total, five cell concentrations, \(2.5 \times 10^4\), \(5.0 \times 10^4\), \(1.0 \times 10^5\), \(2.5 \times 10^5\) and \(5.0 \times 10^5\) were tested and the amount of wound healing was analyzed using three criteria: collagen organization, collagen abundance and myocyte regeneration. Three blinded observers each scored a total of 35 randomized trichrome stained slides. Each slide corresponded to a different tissue, obtained from a single rat specimen utilized during experimentation. All slides received a score between 0 (worst) and 3 (best) for each criteria (See Table 1) based on how the tissue samples appeared (Badylak et al., 2002).

Collagen organization was scored by observing fascia connective tissue orientation located near the injury site. Erratic orientation of the collagen fibers yielded lower scores while comparatively, smooth, parallel collagen fiber orientation within the fascia received higher scores. Collagen abundance was scored based on the relative amount of blue coloration resulting from trichrome staining of collagen, present throughout the wound tissue. Tissue samples containing significant amounts of blue coloration and therefore large quantities of collagen, received higher scores than those with limited blue coloration. Trichrome staining also stains muscle tan to light pink. Areas with this coloration were analyzed for myocyte regeneration. Uninjured skeletal muscle fibers possess a uniform diameter, despite shape, when viewed in cross section. Areas exhibiting myocyte regeneration contain muscle fibers of a smaller diameter and
appear shrunken. Tissue samples were analyzed for smaller skeletal muscle fiber character along the edge of the injury site. Those samples possessing numerous small muscle fibers near the injury site received higher regenerative score compared to those samples possessing uniform diameter muscle fibers near the injury site.

Score analysis, performed by plotting average scores (+/- SEM) for each MSC concentration (Figure 1), revealed that both collagen organization and abundance within the recovered tissue steadily rose between MSC doses of $2.5 \times 10^4$ to $1 \times 10^5$. Following the initial increase, both categories then showed a reduction in scores as the doses continued to increase from $1 \times 10^5$ to $5 \times 10^5$ MSCs added. Myocyte regeneration initially exhibited a slight, but not significant decrease in scores between the doses of $2.5 \times 10^4$ to $5 \times 10^4$ MSCs added. Following the initial decline, myocyte regeneration scores rose slightly with dosages between $5 \times 10^4$ to $5 \times 10^5$ MSCs added.

Statistical ANOVA analysis revealed significant differences among dosages, both in collagen organization and collagen abundance. Significance was found in collagen organization between the dosages of $2.5 \times 10^4$ and $1 \times 10^5$ ($p < 0.002, n = 7$). Similarly, statistical significance was also found in collagen abundance between the dosages of $2.5 \times 10^4$ and $5 \times 10^4$ ($p = 0.013, n = 7$); $2.5 \times 10^4$ and $1 \times 10^5$ ($p < 0.001, n = 7$); $2.5 \times 10^4$ and $2.5 \times 10^5$ ($p = 0.013, n = 7$); and $1 \times 10^5$ and $5 \times 10^5$ ($p < 0.001, n = 7$). No significance was found between dosages in the degree of myocyte regeneration. These results indicate that optimal collagen organization and abundance within the recovered tissue was at a dosage of $1 \times 10^5$ MSCs. MSC dose does not appear to have a significant effect on myocyte regeneration, however, a slight increase was seen at the dose of $5 \times 10^5$ MSCs. Comparisons between slides receiving the best and worst scores for collagen organization
Figure 1. Mesenchymal stem cell dosage histologic scoring comparison of collagen organization and abundance and myocyte regeneration. Different MSC dosages were tested to ascertain which produced the best recovery of injured rat tissue. Wound healing was evaluated utilizing three histologic criteria: collagen organization, collagen abundance and myocyte regeneration. Graphed values are score averages for each dosage. Error bars represent standard error of the mean. Statistical significance was found in collagen organization between the dosages of 2.5x10^4 and 1x10^5 (*: $p < 0.002$, n = 7). Similarly, statistical significance was also found within the collagen abundance group between the dosages of 2.5x10^4 and 5x10^4 (**: $p = 0.013$, n = 7); 2.5x10^5 and 1x10^5 (***: $p < 0.001$, n = 7); 2.5x10^5 and 2.5x10^5 (****: $p = 0.013$, n = 7); and 1x10^5 and 5x10^5 (*****: $p < 0.001$, n = 7). No significance was found between any dosages in the myocyte regeneration.
(Figure 2), collagen abundance (Figure 3) and myocyte regeneration (Figure 4) are shown.

4.2 Immunohistochemical Staining

The purpose of immunohistochemistry is to determine whether the antigen or protein of interest is present within the experimental tissue. By exploiting the specificity and character of antibodies, precise distinctions in collagen types can be made within tissue. During experimentation, a primary antibody, specific for the antigen of interest, is first incubated with the tissue containing the antigen. Next, a secondary antibody, specific for the primary antibody and conjugated to an enzyme that facilitates precipitation of a substrate, is then incubated with the tissue. Upon the addition of the substrate, a precipitate is formed at the location of the enzyme, which corresponds to the location of the antigen of interest. The precipitate is then observed using light microscopy.

As represented in Table 2, a total of three distinct primary antibodies (1° Ab) were utilized to evaluate the surgically repaired abdominal rat tissue. Positive controls for IHC staining were conducted using α-skeletal myosin (‘α-’ read “anti” designates antibody against the stated antigen) to determine the effectiveness of the IHC protocol. Four variants of the α-skeletal myosin 1° Ab mixture were employed with each diluted using either PBS, or potassium PBS (KPBS). Additionally, additives including rabbit plasma and sample buffer (SB), were incorporated with the 1° Ab mixture. Each diluent and additive was employed in attempts to diminish background staining that often occurred as a result of non-specific binding of either the primary or secondary antibody with various other reactive sites present within the tissue. Furthermore, the primary antibody, α-Col
Figure 2. MSC Concentration Collagen Organization Comparison  Histologic view of rat abdominal tissue that has been (Masson’s) trichrome stained to identify regions of collagen and muscle, blue and tan regions respectively. Images depict tissue sections scored by three blinded observers. These specific images received the lowest ‘A’ and highest ‘B’ scores based on collagen organization. (A) Healed incisional area depicting minimally organized collagen (arrows) in a location normally possessing dense irregular connective tissue (2.5x10⁴ MSC dosage) (40x). (B) Conversely, this healed incisional area shows a highly organized network of collagen (arrows) within the recovered tissue (1.0x10⁵ MSC dosage) (40x). Suture site was visible in this image and was excluded from analysis (encircled area).
Figure 3. MSC Concentration Collagen Abundance Comparison Histologic view of rat abdominal tissue that has been (Masson’s) trichrome stained to identify regions of collagen and muscle, blue and tan regions respectively. Images depict tissue sections scored by three blinded observers. These specific images received the lowest ‘A’ and highest ‘B’ scores based on collagen abundance. (A) Low amounts of collagen can be seen within the injury site (arrows) (2.5x10^4 MSC dosage) (40x). (B) Incisional area displays significant collagen abundance (arrows) at the wound site (1.0x10^5 MSC dosage) (40x). Suture sites were visible in these images and were excluded from analysis (encircled areas).
Figure 4. MSC Concentration Myocyte Regeneration Comparison Histologic view of rat abdominal tissue that has been (Masson’s) trichrome stained to identify regions of collagen and muscle, blue and tan regions respectively. Images depict tissue sections scored by three blinded observers. These specific images received the lowest ‘A’ and highest ‘B’ scores based on myocyte regeneration. (A) Image displays injury site (double-ended arrow) and recovered muscle tissue (arrows) showing low amounts of regeneration ($5.0 \times 10^4$ MSC dosage). (B) Incisional area (double-ended arrow) has been incorporated with collagen and shows significant muscle regeneration (arrows) in the underlying muscle ($5.0 \times 10^5$ MSC dosage).
During immunohistochemical analysis each primary antibody was diluted to the specified concentration and incubated with the rat tissue following blocking of endogenous proteins. In total three different primary antibodies were used, however, nine different combinations were employed.

<table>
<thead>
<tr>
<th>Primary Antibody (1° Ab)</th>
<th>Dilution</th>
<th>Tissue Staining Results and Observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>No 1° (PBS)</td>
<td>----</td>
<td>No staining specificity resulting primarily in background coloration</td>
</tr>
<tr>
<td>α-IgG</td>
<td>1:1000</td>
<td>No significant staining, all tissue coloration appears to simply arise from background staining</td>
</tr>
<tr>
<td>α-Col III</td>
<td>1:500</td>
<td>Darker staining of collagen, but decreased specificity</td>
</tr>
<tr>
<td>α-Col III</td>
<td>1:1000</td>
<td>Dark staining, which appeared to be unspecific, throughout entirety of tissue despite type</td>
</tr>
<tr>
<td>α-Skeletal Myosin</td>
<td>1:40</td>
<td>No specificity of staining; all areas of the tissue appeared to stain everything nonspecifically</td>
</tr>
<tr>
<td>α-Col III + R.P.</td>
<td>1:500</td>
<td>Nonspecific coloration or background decreased with the addition of R.P.</td>
</tr>
<tr>
<td>α-Col III + R.P.</td>
<td>1:1000</td>
<td>Nonspecific coloration or background decreased with the addition of R.P.; increased dilution failed to produce any distinguishable difference</td>
</tr>
<tr>
<td>α-Skeletal Myosin + R.P.</td>
<td>1:40</td>
<td>Dark staining with no specificity throughout entirety of slide; all tissue appeared universally colored the same shade</td>
</tr>
<tr>
<td>α-Skeletal Myosin + KPBS</td>
<td>1:40</td>
<td>Uniform darkened coloration throughout entire tissue</td>
</tr>
<tr>
<td>α-Skeletal Myosin + S.B.</td>
<td>1:40</td>
<td>Interesting darkened coloration of peripheral (cross sectional) myofibrils along with some interior skeletal muscle coloration. Again collagen and other tissue types are colored (unspecific)</td>
</tr>
</tbody>
</table>

During immunohistochemical analysis each primary antibody was diluted to the specified concentration and incubated with the rat tissue following blocking of endogenous proteins. In total three different primary antibodies were used, however, nine different combinations were employed.

- α-IgG = Biotinylated α-Rat IgG (H+L) (Vector, Burlingame, CA)
- α-Col III = Pierce Collagen III Rabbit Polyclonal Antibody (Thermo Scientific)
- α-Skeletal Myosin = α-Skeletal Myosin Antibody Developed in Rabbit (Sigma Aldrich)
- α-Col III + R.P. = Pierce Collagen III Rabbit Polyclonal Antibody + Rabbit Plasma (Mixture contained 10% Rabbit Plasma)
- α-Skeletal Myosin + R.P. = α-Skeletal Myosin Antibody Developed in Rabbit + Rabbit Plasma (Mixture contained 10% Rabbit Plasma)
- α-Skeletal Myosin + KPBS = α-Skeletal Myosin Antibody Developed in Rabbit + Primary Ab diluted in potassium PBS
- α-Skeletal Myosin + S.B. = α-Skeletal Myosin Antibody Developed in Rabbit + S.B. = Sample Buffer
III, was diluted with PBS, combined with rabbit plasma, and diluted to two different concentrations in an attempt to diminish observed background staining.

Observations of stained tissue using any of the four variants of the α-skeletal myosin 1° Ab were mostly similar and consisted of positive staining for skeletal myosin along with background or non-specific staining of other tissue areas not containing myosin. For example, dense irregular connective tissue was often mistakenly stained despite being comprised of collagen. In most instances tissue coloration, despite type, appeared uniform with limited exclusivity to muscle (Figure 5). Cross sectional areas of muscle fibers stained intensely, despite uniform background coloration. Negative controls were also conducted to ensure no staining resulted when tissue was treated with α-IgG. This 1° Ab, specific for white blood cells, produced no significant or specific staining within the treatment tissue.

To examine for both the presence of type III collagen in wounded abdominal rat tissue and the effect of exogenous wound additions, α-collagen III 1° Ab was employed. In this analysis, rats were first subjected to abdominal incisions and then treated with biologic additions specific for each experimental group. Tissue samples were subsequently harvested at four or eight weeks. Four experimental groups, each consisting of 14 rats and two subgroups, were employed. Groups varied by the addition of transplantation material, while subgroups varied by healing time, 4 or 8 weeks, prior to sacrifice and tissue harvesting. IHC examination was then performed utilizing multiple combinations of 1° Abs, secondary antibodies (2° Ab), labels and enhancers (Tables 3, 4 and 5, respectively). During this examination, four different α-collagen III variants were tested, with two variants differing based on dilutions. Dilutions were increased in an
Figure 5: Positive α-Skeletal Myosin Immunohistochemistry Staining
Immunohistochemistry results depicting staining achieved utilizing α-skeletal myosin, (a)-biotinylated goat α-rabbit IgG, (b)SA and DAB only. (A) Overview of the tissue and staining results, which shows various areas of concentrated staining of muscle fibers (darkened areas within circle). However, much of the tissue portrays background staining, including areas high in collagen content (arrow). These areas appear stained the same color as the muscle fibers (40x). (B) Magnified (encircled) area of multiple muscle fibers that have been positively stained and appear darker compared to the uniform background coloration (100x).
### Table 3. Immunohistochemistry secondary antibodies and their respective staining results

<table>
<thead>
<tr>
<th>Secondary Antibody (2° Ab)</th>
<th>Dilution</th>
<th>Tissue Staining Results and Observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>No 2° Ab (PBS)</td>
<td>----</td>
<td>Residual tissue discoloration with minimal staining</td>
</tr>
<tr>
<td>(a) B.G. α-R. IgG&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1:500</td>
<td>No significant staining; all tissue coloration appears to simply arise from background staining</td>
</tr>
<tr>
<td>(b) B.G. α-R. IgG&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Undiluted</td>
<td>Some specific myosin staining, however, on one edge of the tissue there appears to be an area of non-specific staining of collagen with some background. (Most specific, but lighter staining)</td>
</tr>
<tr>
<td>(b) B.G. α-R. IgG</td>
<td>1:5</td>
<td>Dark staining, which appeared to be unspecific, throughout entirety of tissue despite type</td>
</tr>
<tr>
<td>B.G. α-R. IgG (b) + R.P.&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Undiluted</td>
<td>Nonspecific coloration or background decreased with the addition of R.P.</td>
</tr>
</tbody>
</table>

<sup>a</sup>These secondary antibodies were used in conjunction with the nine primary antibody combinations. Secondary antibody incubation was performed following primary antibody incubation during the IHC protocol.

<sup>b</sup>(a) B. G. α-R. IgG = Biotinylated Goat α-Rabbit IgG Antibody (Invitrogen, Eugene OR)

<sup>c</sup>(b) B. G. α-R. IgG = Biotinylated Goat α-Rabbit IgG Antibody (Millipore, Temecula CA)

<sup>d</sup>(b) B. G. α-R. IgG = Biotinylated Goat α-Rabbit IgG Antibody + R.P. = Rabbit Plasma (Mixture contained 10% Rabbit Plasma)
Table 4. Immunohistochemistry streptavidin conjugated horseradish peroxidase labels and their respective results

<table>
<thead>
<tr>
<th>Label</th>
<th>Dilution</th>
<th>Tissue Staining Results and Observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) SA&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1:6000</td>
<td>No ideal staining and looks much like a negative</td>
</tr>
<tr>
<td>(a) SA</td>
<td>1:6100</td>
<td>Very faint coloration yielding virtually no staining; staining virtually indifferent compared to 1:6000 dilution</td>
</tr>
<tr>
<td>(b) SA&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1:6000</td>
<td>Dark staining, which appeared to be nonspecific throughout entirety of tissue</td>
</tr>
<tr>
<td>H.P.C.S.&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Undiluted</td>
<td>All parts of the tissue are stained very dark</td>
</tr>
</tbody>
</table>

<sup>a</sup> Labels were incubated with tissue following primary and secondary antibody addition.

<sup>b</sup>(a) SA = Streptavidin (Elisa Grade Streptavidin Horseradish Peroxidase Conjugates)

<sup>c</sup>(b) SA = Streptavidin (HRP-Conjugated Streptavidin)

<sup>d</sup>H.P.C.S. = Horseradish Peroxidase Conjugated Streptavidin (BioGenex)
Table 5. Immunohistochemistry chromogen enhancers and their respective results$^a$

<table>
<thead>
<tr>
<th>Enhancer</th>
<th>Quantity</th>
<th>Tissue Staining Results and Observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cobalt Chloride$^b$</td>
<td>10µL</td>
<td>Light coloration, however, still no staining specificity; all background coloration</td>
</tr>
<tr>
<td>Cobalt Chloride$^c$</td>
<td>60µL</td>
<td>Very dark coloration in comparison to the 10µL, however, no staining specificity resulting primarily in background coloration</td>
</tr>
<tr>
<td>Imidazole$^d$</td>
<td>10µL</td>
<td>Very faint coloration yielding virtually no staining</td>
</tr>
<tr>
<td>DAB$^e$</td>
<td>----</td>
<td>No significant staining; all tissue coloration appears to simply arise from background staining</td>
</tr>
</tbody>
</table>

$^a$ These chromogens were used in conjunction with each primary antibody, secondary antibody and label combinations. All slides received primary antibody, secondary antibody and label additions before being subjected to chromogens during the final step of Immunohistochemistry.

$^b$ Cobalt Chloride (10µL) + DAB

$^c$ Cobalt Chloride (60µL) + DAB

$^d$ Imidazole + DAB

$^e$ DAB only (Diaminobenzidine-Plus Substrate Kit; Invitrogen)
attempt to minimize dark, nonspecific background staining observed in both lower dilution variants. No specific staining was seen with any of the $1^\circ$ Ab dilutions used.

Similarly, four $2^\circ$ Ab variants were employed during IHC in combination with the $1^\circ$ Abs. All four $2^\circ$ Ab variants were biotinylated, produced in rabbit and specific for the constant region of the $1^\circ$ Ab. Despite the alternative combinations of $1^\circ$ and $2^\circ$ Abs, background staining was not alleviated. Positive controls engaging $\alpha$-skeletal myosin $1^\circ$ Ab combined with the four variant $2^\circ$ Abs resulted in strong positive myosin staining along with additional nonspecific staining of other tissue components, including collagen (Figure 6) Though skeletal muscle stained strongly, the coloration of adjacent dense irregular connective tissue demonstrates nonspecific binding.

To exclude both the label and enhancer components from possible causes of the observed nonspecific staining, four label and four enhancer variants were substituted into the IHC protocol. Despite alternative measures, all label variants consisting of horseradish peroxidase conjugated streptavidin produced non-ideal staining with minimal efficacy. Enhancer variants, including cobalt chloride, imidazole and diaminobenzidine (DAB), produced similar results, yielding light or very dark nonspecific tissue staining, all of which resulted in uniform background coloration of the entire tissue section.

4.3 Gridley (Silver) Staining

The purpose of the Gridley silver staining analysis, similar to that of IHC, was to identify reticular fibers or type III collagen present within the wound tissue (Hwang et al., 1990). Reticular fibers are unique in composition compared to other collagen types due to
Figure 6. Nonspecific α-Skeletal Myosin Immunohistochemistry Staining Imaging of experimental rat tissue analyzed utilizing α-skeletal myosin, (b)-biotinylated goat α-rabbit IgG, H.P.C.S and DAB only during immunohistochemical analysis. (A) Low magnification view of stained tissue displaying the site of injury (black arrow) and darkened tissue, which has stained positively during experimentation (40x). (B) Enhanced view of tissue injury site (black arrow) depicting both muscle tissue (red arrows) and connective tissue (encircled area) staining positively despite the absence of myosin in the connective tissue location (100x).
the fact that these fibers are embedded within a carbohydrate rich matrix. During staining, chemical conversion of these sugars allows for the collection of black colloidal silver deposits at the site of fiber formation when the chemically altered sugars are subjected to ammoniacal silver nitrate solutions (Samuel, 1953).

Rat tissue samples stained utilizing the Gridley method originated from Group 1:B rats. These rats received an abdominal incision along the linea alba, underwent primary repair only and were allowed to heal for a total of eight weeks before being sacrificed and harvested for tissue samples. Silver staining analysis of Group 1:B rat number 5 revealed positively stained areas for type III collagen in close proximity to the injury site and within scar tissue (Figure 7). Many of these positively stained areas were primarily found within the underlying rectus abdominis muscle. Specific locations (Figure 7, A and D) where dark staining is observed represents type III collagen. Similar positive staining was seen in tissue samples obtained from Group 1:B rat number 6 (Figure 8). This sample also depicts significant endomysium staining, located along the outer margins of the muscle fibers (Figure 8, A-C). Additional areas of interest within the underlying muscle also show positive staining (Figure 8, D).

To validate sites of positive type III collagen staining, adjacent serial sections to those stained using the Gridley method were Mason’s trichrome stained (trichrome staining performed at St. Elisabeth Hospital Histopathologic Center). Trichrome stained samples were then compared to silver stained samples to determine whether areas positive for type III collagen staining paralleled collagen staining in trichrome samples. Side-by-side examination of tissue obtained from Group 1:B rat
Figure 7. Gridley Silver Staining of Rat 5 Group 1:B Histologic view of group I rat (no exogenous additions) tissue, which has been stained using the Gridley silver staining method to identify type III collagen. Red arrows represent areas that have been positively stained for type III collagen and black arrows indicate the site of injury. This rat was sacrificed 8 weeks following surgical procedures. (A) Image of wound area that has formed scar tissue during recovery and one area of interest (red arrow) that appears to have reacted positively for staining (40x). (B) Enhanced view of the rectus abdominus, directly adjacent to the wound site shown in ‘A’. Intensely stained darkened regions (red arrows) correspond with the endomysium, which surrounds all skeletal muscle fibers (100x). (C) Additional area of interest (red arrows), which has reacted positively to the silver impregnation. (40x). (D) Area within a muscle fascicle exhibiting positive staining of the endomysium (area within oval) (40x).
Figure 8. Gridley Silver Staining of Rat 6 Group I:B Histologic view of group I rat tissue (no exogenous additions), which has been stained using the Gridley silver staining method to identify type III collagen. Red arrows represent areas that have been positively stained for type III collagen and black arrows indicate the site of injury. This rat was sacrificed 8 weeks following surgical procedures. (A) Strip of positively stained tissue is seen directly below the injury site (red arrow) (40x). (B) Positive staining seen within the rectus abdominis muscle (red arrow), located at regions corresponding to the endomysium (40x). (C) Additional muscle area depicting positive staining along the outside margins of muscle fibers (red arrow) (40x). (D) Area of tissue where noteworthy positive staining can be seen along the outer margins of the muscle fascicles (red arrows) (40x).
number 5 revealed positive staining for type III collagen directly beneath the injury site and on the fringe of underlying muscle (Figure 9). Locations stained positive for type III collagen in Group 1:B rat number 6 tissues, also paralleled areas staining positive for collagen in the corresponding trichrome stained sections. Here type III collagen staining can be observed along the outer margin of the rectus abdominis, directly beneath the injury site, and along the outer border of an underlying muscle fascicle (Figure 10).

5. Discussion

The complexity of the wound healing process is decidedly influenced by the numerous components of the ECM. As previously stated, the ECM consists of two parts: 1) connective tissue fibers, namely collagen and elastin fibers, and 2) ground substance, which contains a myriad of components and functions as support for cells present within connective tissue. Following wounding or tissue defects, healing occurs, a process characterized by the formation of granulation tissue, reepithelization, and the replacement of granulation tissue with scar tissue (Ehrlich and Krummel, 1996). Scar tissue, the final product of the tissue remodeling sequence, is composed of collagen, newly synthesized by TGF-β stimulated fibroblasts (Kondo and Ishida, 2010). Though complete maturation of the scar may take up to one year to achieve, sutures within the wound site are absorbed within a few weeks post-primary repair. Research has shown that more than 85% of healthcare cases receiving surgical closure of the abdominal wall achieve sufficient stability, however, up to 15% of abdominal repairs have been shown to develop incisional hernias. Incredibly, additional attempts to repair the hernia may result in
Figure 9. Rat 5 Group I:B Comparison Between Gridley Silver Stain and Mason’s Trichrome Stain

Histologic view of group I rat tissue (no exogenous additions), which has been stained using either the Gridley silver staining method to identify type III collagen (left column: A and C) or Masson’s trichrome staining used to identify regions of collagen and muscle (right column: B and D). Darkened tissue regions in the left column represent areas that have been positively stained for type III collagen, while blue tissue regions in the right column indicate areas consisting collagen of all types. Black arrows indicate the site of surgical injury and this rat was sacrificed 8 weeks following surgical procedures. (A) Image depicts dark or positive silver staining on the edge of muscle tissue (red arrows) (40x). (B) Trichrome comparison of ‘A’ portraying significant blue coloration of tissue in the same region, which was positive for silver staining, thus confirming a positive match for type III collagen at this location (red arrow) (40x). (C) Additional underlying muscle area depicting distinct boundaries of sites positively silver stained (red arrows) (40x). (D) Trichrome comparison of ‘C’ illustrating regions (red arrows) positive for collagen at corresponding locations (40x).
Figure 10. Rat 6 Group I: Comparison Between Gridley Silver Stain and Mason’s Trichrome Stain

Histologic view of group I rat tissue (no exogenous additions), which has been stained using either the Gridley silver staining method to identify type III collagen (left column: A and C) or Masson’s trichrome staining used to identify regions of collagen and muscle (right column: B and D). Darkened tissue regions in the left column represent areas that have been positively stained for type III collagen, while blue tissue regions in the right column indicate areas possessing collagen of all types. Black single headed arrows indicate the site of surgical injury and this rat was sacrificed 8 weeks following surgical procedures. (A) Image depicts positive silver staining along the outside margin of the rectus abdominis and directly beneath the injury site (red arrows) (40x). (B) Trichrome stained tissue comparison of ‘A’ shows positive collagen staining (blue coloration) at corresponding locales (red arrows) (40x). (C) Additional underlying muscle region depicting a positive site for silver staining (red arrows) (40x). (D) Trichrome stained tissue comparison of ‘C’ illustrating positive collagen staining (blue coloration) in the same location depicting positive silver staining (red arrows) (40x).
greater than 50% hernia recurrence rates (Klinge et al., 2001).

With the use of trichrome staining, we assessed the effect of MSC concentration on three parameters of healing: collagen organization, collagen abundance and myocyte regeneration. Analysis of the tissue revealed that both collagen organization and abundance increased as MSC concentrations increased up to $1 \times 10^5$ MSCs applied. Following this improvement, any additional increase in dosage resulted in an overall decrease in scoring for both collagen organization and abundance. It is hypothesized that once MSCs are implanted at the injury site, these cells either differentiate into various cell types, such as fibroblasts or epidermal cells, or function to secrete cytokines which influence wound healing (Wu et al., 2007).

By differentiating into cell types present near the site of injury, MSCs may then play a direct role in the wound healing process. Differentiation of MSCs into fibroblasts would function to increase the amount of collagen synthesized and secreted at the injury site. Increasing the collagen content, would then serve to accelerate scar tissue formation, ultimately leading to improved wound healing. Conversely, the differentiation of MSCs into epidermal cells would provide additional cells that could function to facilitate the reepithelialization stage of wound healing.

However, the differentiation of MSCs into fibroblasts or epidermal cells could also serve to indirectly increase the MMP activity within the recovering tissue. As mentioned previously, MMPs are produced and secreted by macrophages, epidermal cells, endothelial cells and fibroblasts (van der Veen et al., 2010). An increase in MMP production and consequently, an increase in MMP activity could either improve the remodeling stage, or if increased adversely, initiate the excessive breakdown of collagen.
at the wound site. This theory may account for the observed decrease in collagen organization and abundance seen at higher MSC concentrations within the results.

On the other hand, MSCs have been shown to exhibit a paracrine effect by releasing cytokines that may play a role in wound healing. Cultured MSCs have been found to produce and secrete vascular endothelial growth factor (VEGF), placental growth factor (PlGF), basic fibroblast growth factor (bFGF), IL-6, and monocyte chemoattractant protein-1. Both VEGF and PlGF initiate the development of new blood vessels during the process of angiogenesis. Similarly, bFGF aids in the stimulation of fibroblast differentiation and proliferation. Lastly, monocyte chemoattractant protein-1 acts to recruit monocytes, white blood cells that aid in immune response initiation, to the site of injury. All four of these cytokines could aid in facilitating the wound healing process. Despite a minimal increase in wound tissue regeneration, research has shown that in some instances MSC-conditioned medium was found to promote in vitro proliferation and migration of endothelial and smooth muscle cells in a dose-dependent manner (Wu et al., 2007).

Collagen remodeling occurs during the transition from granulation tissue to scar. This remodeled scar tissue is responsible for maintaining both physiological and mechanical strength and the ability to resist tissue strain (Kondo and Ishida, 2010). Degradation of collagen, during the healing process, is achieved through the synergistic action of several matrix metalloproteinases (MMPs) (van der Veen et al., 2010; Zheng et al., 2002). Therefore, optimal wound healing is dependent on balanced synthesis, maturation, and degradation rates of collagen in the scar.
Collagen types I and III are the two primary fibrillar collagen forms involved in wound healing. During the early stages of scar formation, type III collagen is the most prevalent and the primary collagen type synthesized by fibroblasts. Type I collagen synthesis increases throughout the progression of scar formation and becomes the dominant collagen type within mature, remodeled scars (Klinge et al., 1999). Degradation of type I and type III collagen is almost entirely performed by MMP-1 and MMP-13 (Zheng et al., 2002). Thus, the enzymatic activity of these two matrix enzymes directly governs the amount of type I and type III collagen removed from connective tissue and ultimately the scar composition. Knowledge of the pathophysiologic activity of MMP-1 and -13, including active collagen turnover rate, is limited. A better understanding of regulatory mechanisms of these enzymes may provide insight into the development of herniation and conclusively the process of wound healing (Klinge et al., 1999).

Studies aiming to describe the collagen content in tissue samples often quantify the tissue using a type I/type III ratio. Tensile strength of connective tissue and scar tissue is directly proportional to the collagen type I/III ratio. As the ratio decreases, reduction in tensile strength is observed due to the smaller diameter and weaker nature of type III collagen (Klinge et al., 2001). Wound areas characterized by increased levels of type III collagen, and therefore a decreased type I/type III ratio, have been observed in the rectus sheath of patients suffering from abdominal wall hernia (Henriksen et al., 2011).

Dense bundles of connective tissue fibers consisting of type I collagen are present in anatomical structures bearing high amounts of tension including tendons, ligaments, aponeuroses, and dermis. These type I bundles are composed of large-diameter collagen fibrils that provide each of these structures with their mechanical strength.
Comparatively, type III collagen fibers are thinner in diameter to that of type I fibers and are found within structures possessing high amounts of flexibility and plasticity. Examples of these tissue types include secondary lymph organs such as the spleen and tonsils, in addition to the embryonic skin, blood vessels, bone marrow, liver and smooth muscle. Despite their limited strength, type III collagen fibers often form a highly ordered supporting network for various tissue types (Gelse et al., 2003). For this reason, early scar tissue is significantly plastic and mechanically unstable as a result of reduced cross-linkage between type III collagen fibrils. In contrast, highly stable intermolecular cross linkages are found in the type I fibers of mature scar tissue. Thus, the increased linkage and larger diameter of type I collagen plays a critical role in producing the observed increased tensile strength of wound tissue following remodeling (Zheng et al., 2002).

In this study we additionally investigated the effect of bone marrow MSC transplantation on the wound healing process in incisional abdominal fascia wounds. Previous studies have shown that MSCs improve the rate of healing in incisional wounds. In 2009, Stoff et al. found that following injury, the transplantation of human MSCs onto full-thickness incisional wounds in New Zealand rabbits decreased the time it took for treated tissue to reduce the epithelial gap and accelerated granulation tissue formation, compared to non-treated tissue. Similarly, Badillo et al. 2007 found that the treatment of diabetic wounds with MSCs corrected many of the wound healing impairments of diabetes and functioned to decrease the epithelial gap, while expediting granulation tissue formation. While both of these studies successfully demonstrated that the application of MSCs did in fact accelerate the wound healing process, neither study examined whether
the application of MSCs directly accelerated the transition from type III to type I collagen
during wound healing. After observing the beneficial effect of MSCs on wound healing,
identification of the tissue component being modified by the presence of MSCs was
assessed. To analyze our treated tissue for type III collagen content, we employed
immunohistochemistry (IHC) and silver staining analysis.

The benefit of immunohistochemistry lies in the ability of the technique to
distinguish between various molecules found in tissues. IHC harnesses the unique nature
of antibodies, immunologic agents used in the body to facilitate the identification and
neutralization of foreign objects. These biological proteins possess incredible specificity
for antigens (molecules that antibodies bind to), ultimately making them great analytical
tools. By exploiting this unique characteristic, antibodies can be used to identify specific
cellular components for histopathologic analysis. However, despite the incredible
specificity of each antibody to one antigen, proper binding to the epitope, or antigenic
determinant (the small part of the molecule that fits into the antigen binding site of the
antibody), is required for successful identification of an antigen. Thus, masking,
substitution, or complete epitope elimination can render this powerful technique
ineffective.

Despite proper staining technique and reagent variation, complications arose
during our histologic assessment of injured tissue utilizing immunohistochemical
analysis. Though slight staining was achieved in limited instances, background staining
and non-specific coloration primarily occurred as a result. Some reasons for the observed
poor staining could include non-specific binding of the primary or secondary antibody,
antigen masking, or inefficient antigen retrieval.
Although antibodies show preferential avidity to specific epitopes, nonspecific binding can occur due to structural similarities of antibody-binding sites present on both the target antigen and on non-specific proteins. Background staining is directly correlated and proportional to the amount of nonspecific binding occurring within treated tissue (Miller, 2001). The occurrence of background staining will function to conceal proper staining and therefore reduce the detection of the target antigen. In attempts to counteract background staining, tissue samples may be incubated with either a buffering agent or foreign animal plasma, prior to primary antibody incubation. The purpose of this technique is to minimize the non-specific binding of either the 1\textdegree or 2\textdegree Ab, during staining, to structurally similar reactive sites resembling the target antigen epitope.

During our experimentation, tissue samples were blocked to prevent non-specific binding by using a protein-blocking agent which functions to block the reactive sites to which the primary or secondary antibodies may otherwise bind. In an attempt to decrease resulting background staining, primary antibodies specific for skeletal myosin and type III collagen were mixed with rabbit plasma. Plasma contains numerous antibodies specific to various antigens. By incubating the tissue with these various antibodies, it is believed that additional reactive sites will be blocked after binding to other antibodies, ultimately decreasing the probability of nonspecific binding of the selected 1\textdegree or 2\textdegree Ab.

Depending on the antigen detection method and tissue type, quenching and or blocking of endogenous peroxidases may also need to be performed prior to IHC staining. The purpose of this technique is to physically block any endogenous enzymes, such as endogenous peroxidases, from chemically reacting with the substrate (chromogen) when incubated with the tissue in the last step. Improper blocking or
inactivation of these endogenous peroxidases will result in the precipitation of the chromogen in areas not corresponding to antibody binding. Therefore, improper staining of regions or structures can arise, ultimately leading to false analysis. During our IHC experiments, the tissue samples were initially quenched, using a 0.3% hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) incubation. For many peroxidase enzymes, H\textsubscript{2}O\textsubscript{2} is the optimal substrate. Therefore, the incubation with H\textsubscript{2}O\textsubscript{2} functioned to quench endogenous peroxidases. Despite taking extra precautions, each of these staining preparation techniques were deemed ineffective due to the continually observed non-specific results.

During histologic preparation, the experimental excised rat tissue was subjected to formalin fixation and paraffin embedding. Formaldehyde, an organic compound with the formula CH\textsubscript{2}O, is often used as a common tissue fixative or embalming agent. This chemical mediator acts to fix tissue or cells by inducing a mixture of both reversible and irreversible cross linkages of primary amino groups within proteins in close proximity to nitrogen atoms. These nitrogen atoms, present in either DNA strands or other proteins, act as a chemical intermediary of methyl (CH\textsubscript{2}-) linkages. The addition of methyl groups to specific sites within proteins can function to adversely affect, or in extreme cases, destroy epitope availability. Because antibodies are specific to distinct amino acid combinations, the introduction or substitutions of additional amino groups will decrease the antibody’s specificity for its antigen (Miller, 2001).

Depending on the fixation method employed, antigen masking or even epitope elimination can occur. During these instances, additional measures are required to improve epitope availability for antibody binding. Such measures include a deparaffinization step, often used with paraffin embedded tissue, or antigen retrieval. The
purpose of the deparaffinization step is to remove the embedding material and allow the
tissue, namely the antigen, to react with reagents during staining. Various antigen
retrieval methods also exist, and they all share the common purpose of breaking the
protein cross-linkages formed by formalin fixation and thereby function to uncover
hidden antigenic sites. The improved availability of the antigens allows for better
antibody-antigen interaction during IHC staining. Often, antigen retrieval techniques
involve the application of heat for varying lengths of time to formalin-fixed, paraffin-
embedded tissue sections, while inside an aqueous bath. This version of antigen retrieval
is termed heat induced epitope retrieval (HIER). Other antigen retrieval methods use
enzymatic digestions which function to cleavage of peptides that may be masking the
epitope (Shi et al., 2011). Both deparaffinization and antigen retrieval methods act to
reestablish epitope viability in formalin-fixed, paraffin-embedded tissue sections.

In 2007, White et al. performed deparaffinization and rehydration prior to
staining. In this study, antigen retrieval using a citrate buffer was also performed before
immunostaining. Both of these steps played a pivotal role in the preparation of the tissue
for analysis. During our IHC protocol, deparaffinization was performed by first heating
the slides on a heating block and subjecting the tissue to a series of xylene and alcohol
baths. As instructed by the primary antibody provider (Thermo Scientific), antigen
retrieval was not performed. Unsuccessful deparaffinization of our tissue could explain
our observed non-specific staining results. Even though antigen retrieval was not required
for our antibody, future IHC attempts may implement this technique in an attempt to
improve staining results.
Despite the threat of epitope masking, IHC is often performed on tissue previously formalin fixed and paraffin embedded. In a similar study conducted by Tyrone et al. (2000) IHC was performed in an attempt to identify proliferating cell nuclear antigen (PCNA) within surgically injured fascia tissue previously treated with transforming growth factor β3 (TGF-β3). Though antigen type and animal model varied between studies, IHC procedural differences were minimal. In both studies, tissue samples were formalin fixed and paraffin embedded during histologic preparation. IHC protocol comparison revealed similar technique in which both studies utilized a 1° Ab (specific to the antigen of choice) a biotinylated 2° Ab, streptavidin conjugated horseradish peroxidase (SA-HRP) and a chromogen label. One important similarity between both IHC methods was the exploitation of biotin-streptavidin amplification. By conjugating numerous biotin molecules to the secondary antibody and conjugating SA to the HRP enzyme, high affinity bonds are formed between the biotin-antibody complex and the SA-HRP enzyme complex. Four SA molecules will bind to each biotin, allowing for the localization of multiple HRP enzymes. High numbers of enzymes at the designated site will function to amplify tissue coloration following the addition of the chromogen. For our purposes, three different chromogen intensifiers were tested. However, each had little effect on the success of staining despite the utilization of this technique.

A large discrepancy between procedures was seen among 1° Ab incubation times. During our study, the 1° Ab (α-Col III) was incubated with the experimental tissue for a period of one hour, compared to the overnight incubation time applied by Tyrone et al. (2000). A longer incubation time may have provided the crucial time needed for
antibody-antigen interaction to occur within the tissue, ultimately improving the success of their IHC staining.

Though incubation length for 2° Ab and SA-HRP was identical between both studies, an additional variation was seen among incubation temperatures. During our procedure, incubation was performed at RT (20°C), whereas, incubation temperatures used by Tyrone et al. (2000) were 37°C. The observed incubation temperature difference is significant between experiments and functions to introduce kinetic variation between studies. By increasing the temperature, the chemical dynamics of the reagents are changed, possibly for the better. Higher temperatures can provide the reagents with enough kinetic energy to improve binding or reagents. However, if temperatures are raised too high, the additional kinetic energy can sever bonds or cause the reagents to bind to non-specific proteins, negatively affecting the analysis.

Although much research describes the application of formalin fixation, cryofixation is another technique commonly employed for IHC due to its less molecularly harsh character. During cryofixation, rapid freezing of specimens prevents the formation of ice crystals, which leads to the destruction of cells and the distortion of tissue. Flash freezing using liquid nitrogen is the most common practice because this method freezes the tissue in its entirety quickly and before degradation can occur. Following flash freezing, tissue is sectioned using a cryostat freezing microtome. Once mounted on glass slides, the tissue is ready for staining. The advantages of cryofixation are that tissue sections do not require a deparaffinization or protease digestion step prior to staining (Miller, 2001).
In 1999, Klinge et al., performed a study seeking to determine whether abnormal type I to type III collagen distribution was a significant factor for observed incisional hernias in patients. During experimentation, cryofixed sections were quenched, blocked, incubated with two different primary monoclonal antibodies for 1 hour at RT, incubated for 1 hour with horseradish-peroxidase-conjugated rabbit anti-mouse secondary antibody, and incubated with H$_2$O$_2$ for 5 minutes. Lastly, the slides were counterstained with hematoxylin. In their results, Klinge et al. found the ECM intensively labeled with the antibodies against type I and type III collagen.

When comparing these studies to our methods, Klinge et al. first fixed their tissue sections in acetone before incubating the sections in methanol containing H$_2$O$_2$. Because our tissue was formalin fixed, acetone fixation was not required. Despite the variation in preservation, both tissue samples were quenched using H$_2$O$_2$ and underwent reactive site blocking. Primary antibody utilization differed due to the variation of experimental tissue. However, the secondary antibody used by Klinge et al., horseradish-peroxidase-conjugated rabbit anti-mouse secondary antibody, was already bound to horseradish peroxidase enzyme. The use of this antibody simplified the procedure, eliminating the second incubation of a biotinylated secondary antibody with SA-HRP. After comparing our own IHC procedure to this and other studies, it appears that our staining problems most likely resulted from unsuccessful antigen retrieval prior to staining, rather than from mistakes made during IHC methodology. Because type III collagen analysis using IHC was unsuccessful, we sought to utilize another histologic analysis specific for type III collagen, silver staining.

Silver staining is a unique procedure that can be used to identify reticulin, a
scleroprotein present in connective tissue framework composed of reticular tissue.

Reticular fibers are comprised of one or more types of very thin and delicately woven strands of type III collagen embedded in a carbohydrate-rich matrix (Hwang et al., 1990). It is this carbohydrate-rich matrix, present in close proximity to the type III collagen fibers that allows type III collagen to react with silver stains and with the periodic acid-Schiff reagent. Ordinary histological stains, such as hematoxylin and trichrome, do not successfully demonstrate reticular fibers and therefore are not useful for the identification of type III collagen.

In 1953, Tomlin demonstrated that both reticular and regular collagenous materials contain the same four sugars: galactose, glucose, mannose, and fructose. The difference lies in the fact that reticular fibers possess a much greater concentration of these sugars compared to that of other collagenous material. In fact, reticular fibers do not show affinity for silver, but rather have to be chemically altered to allow successful combination with silver compounds. Reticular fibers are not the actual structures stained during silver staining (Irving and Tomlin, 1954). Instead, the closely associated carbohydrates are first oxidized causing the chemical conversion of glycol groups into aldehydes. When subjected to ammoniacal silver nitrate solutions, the aldehyde is oxidized to a salt, ultimately producing black colloidal silver deposits that can then be viewed (Samuel, 1953).

By comparing our silver stained tissue with trichrome stained tissue, we were able to validate that our silver staining method was successful in identifying type III collagen. However, the staining achieved during experimentation was faint in coloration and failed to produce the intense blackened pigmentation that is truly characteristic of silver
impregnation. Comparison of our staining techniques with that employed by Puchtler and Waldrop, (1978) revealed that increasing the ammoniacal silver solution incubation and gold chloride toning steps during experimentation may help to both intensify precipitation and improve coloration.

In our results, type III collagen was identified consistently within muscle tissue and in various other regions in close proximity to the injury site. Some areas showing strong staining included the endomysium, a sheath of delicate reticular fibrils surrounding each muscle fiber (Gelse et al., 2003). Because this structure is composed of reticulum, we know that it is a valid location for type III collagen. Hence, this observation confirms that our silver staining technique was successful. In other instances, small amounts of type III collagen were found outside of the muscle tissue, but near the injury site. Because the stained tissue was obtained from rats in group 1:B, the tissue was not treated with any additives (CoTa, PRP or MSCs), but rather served as a control receiving only primary repair and allowed to heal for eight weeks. Collagen remodeling occurs one to two weeks following injury (Kondo and Ishida, 2010). Therefore, type III collagen would be present in low quantities after eight weeks of healing. Though wound remodeling begins at different times at different regions within the body, type III collagen content is said to decrease from originally 30-10% post remodeling or scar formation (Strodtbeck, 2001). This could explain why limited amounts of staining were observed in our tissue samples. Despite unsuccessful IHC staining, silver staining provided another means of identifying type III collagen in our healed tissue. Though slight modifications can be made to the technique to improve staining results, the method we utilized shows great promise and validity.
In conclusion, the concentration response revealed that the optimal dose for MSC transplantation was $1 \times 10^5$ MSCs. This concentration successfully increased observed collagen organization and abundance within the observed tissue, but all MSC concentrations were unable to improve myocyte regeneration. Even though IHC did not provide reliable results, silver staining was successful at identifying type III collagen within the wound site. Therefore, the Gridley silver staining method was found to be the best staining method for identifying type III collagen. Although identification of collagen was obtained, quantification of the amount of type III collagen present within the wound site was not possible. Future experimentation will focus on additional modifications to the IHC protocol in the hopes of decreasing background staining. If background staining or lack of specific staining persists, possible cryopreservation and sectioning techniques may be implemented to avoid the use of paraffin-embedded tissues. Lastly, the silver staining protocol can be modified to improve type III coloration by increasing the incubation time of silver reagents.
References


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Friday, January 29, 2010

Dr. Diana Fagan
Biology Department
UNIVERSITY

Re: IACUC Protocol # 02-09
Title: Autologous Mesenchymal Stem Cell Transplantation in Rats to Improve Fascial Repair

Dear Dr. Fagan:

The Institutional Animal Care and Use Committee of Youngstown State University has reviewed
the aforementioned protocol you submitted for consideration titled “Autologous Mesenchymal
Stem Cell Transplantation in Rats to Improve Fascial Repair” and determined it should be
unconditionally approved for the period of July 2, 2009 through its expiration date of July 2, 2012.

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
July 2, 2010 and July 2, 2011. 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.

Sincerely,

Dr. Peter J. Kasinsky
Associate Provost for Research
Dean School of Graduate Studies and Research

PJK:oka

C: Dr. Walter Home, Consulting Veterinarian, NEOUCOM
   Dr. Robert Leipheimer, Chair IACUC, Chair Biological Sciences
   Dawn Amolsch, Animal Tech., Biological Sciences