Dose Response Analysis of Bone Marrow-Derived Mesenchymal Stem Cells for Treatment in Fascial Wound Repair

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ABSTRACT

The use of biological products such as platelet-rich plasma (PRP) and mesenchymal stem cells (MSCs) has become an incredibly promising and advantageous product in modern regenerative therapeutics. The occurrence of incisional herniation following invasive abdominal surgeries remains a particularly complicating issue in post-surgery recovery with about 10% of patients experiencing herniation following laparotomy (Bucknall et al., 1982). This study aims to provide a cell-based therapy for the treatment of surgical wounds and enhance healing. Using a rat model, bone marrow-derived MSCs (BM-MSCs), PRP, and a collagen scaffold (CollaTape) are applied to the surgical site following an abdominal laparotomy. The rats were allowed to heal for 4 weeks before tissue samples of the wound site were harvested to analyze wound characteristics. Histological examination of wound sites treated with BM-MSCs indicates important information for differences in specific events involved in the wound healing process including collagen deposition, connective tissue organization, and muscle regeneration. It was found that applying PRP and CollaTape to the abdominal incisions was able to increase collagen abundance and stimulate new muscle growth. The application of MSCs to the injury was able to further enhance the deposition of organized collagen fibers in a dose-dependent manner. Additionally, characteristics of the BM-MSCs, including their ability to differentiate into chondrogenic, adipogenic, and osteogenic cell lineages was performed in order to exhibit their stem potential.

LIST OF ABBREVIATIONS

α-MEM:	Alpha-Minimum Essential Media
BM-MSC:	Bone Marrow-Derived Mesenchymal Stem Cell
DMSO:	Dimethyl Sulfoxide
ECM:	Extracellular Matrix
EDTA:	Ethylenediaminetetraacetic acid
EGF:	Epidermal Growth Factor
FBS:	Fetal Bovine Serum
HLA:	Human Leukocyte Antigen
IBMX:	3-Isobutyl-1-Methylxanthine
IFN:	Interferon
IL:	Interleukin
ISCT:	International Society for Cellular Therapy
ITS:	Insulin-Transferrin-Selenium
MCP:	Monocyte Chemoattractant Protein
MIP:	Macrophage Inflammatory Protein
MMP:	Matrix Metalloproteinase
MSC:	Mesenchymal Stem Cell
PBS:	Phosphate Buffered Saline
PDGF:	Platelet-Derived Growth Factor
PRP:	Platelet-Rich Plasma
RPMI:	Roswell Park Memorial Institute

SDF-1:	Stromal-Derived Factor-1
TGF:	Transforming Growth Factor
VEGF:	Vascular Endothelial Growth Factor

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INTRODUCTION

Regenerative Medicine

Regenerative medicine involves the ability to restore or replace cells, tissues, or organs to reinstate or establish normal tissue function. This course of clinical therapeutics represents an incredibly diverse and emergent field with expanding capabilities. Through regenerative medicine, effective treatments are being developed for countless medical pathologies.

The methods and strategies utilized in regenerative therapies hold a tremendous capacity for the amelioration of wound healing. Using cell-based techniques, an individual's own repair mechanisms can be stimulated to mend or fix damaged tissues. Furthermore, cells, tissues, or even organs can be grown *in vitro* and used to replace those which are damaged, diseased, or improperly functioning. This ability bypasses the necessity for organ donation and reduces the risk for rejection, since the tissues can essentially be individualized for each person or entity. The primary goal for regenerative medicine lies in the understanding of disease mechanisms, so that they may be exploited to bring about a desired result and aid in the repair of tissues.

At present, regenerative medicine relies heavily on the use of stem cells, other types of multipotent cells, and various biological products. This includes mesenchyme-derived cells such as mesenchymal stromal/stem cells (MSCs) and blood isolate products like platelet-rich plasma (PRP). The term "mesenchymal stromal cell" is often used interchangeably with "mesenchymal stem cells" since there is a bit of controversy over whether certain cell populations can in fact be called stem cells. However, for the remainder

of this article, when using the abbreviation "MSCs" it will refer to "mesenchymal stem cells" and not "mesenchymal stromal cells". MSCs and PRP are of particular interest in one area of regenerative medicine, wound repair, since they may be easily obtained and have a large influence on the process of healing. MSCs and PRP can induce cell migration to wound sites and stimulate the proliferation and differentiation of these cells through secreted trophic factors (Chen et al., 2008). MSCs also possess immunomodulatory properties which could prove to be very beneficial for the treatment of tissue rejection following transplantation, as well as therapy for some autoimmune reactions (Lee et al., 2015). The lack of immunogenicity of MSCs, even when the cells come from different individuals (allogeneic) or different species (xenogeneic) indicates a capacity for the cells to be used in widespread clinical applications (Gutierrez-Fernandez et al., 2015; Ryan et al., 2005).

What are Mesenchymal Stem Cells?

Mesenchymal stromal cells are a diverse population undifferentiated, plastic-adherent cells with a fibroblast-like growth morphology which contain MSCs (Dominici et al., 2006). Bone marrow would include populations of both mesenchymal stromal and mesenchymal stem cells. The nomenclature of these multipotent cells is rather convoluted, as it is quite difficult to utilize any one term to fully encompass their functional properties. Mesenchyme is an embryonic tissue that can differentiate into hematopoietic or connective tissues. However, MSCs cannot differentiate into hematopoietic cells except macrophages in some instances (Freisinger et al., 2010). Stromal cells, in general, are defined as the connective tissue cells that form the supporting framework which make up an organ. For example, fibroblasts are stromal cells which produce the extracellular matrix and collagen

that form the structure for various organs. While this may adequately describe one function of MSCs, it does not address their role in wound healing and ability for multipotent differentiation. A defining characteristic of stem cells includes their plastic-adherent capability and capacity for indefinite self-renewal *in vitro*, which means they can undergo numerous cycles of cell division while retaining an undifferentiated status (Dominici et al., 2006). The manipulation of culture conditions cells can then be utilized to induce the cells to differentiate into a certain cell type (Vater et al., 2011). Current evidence suggests that some of the cells in a population of MSCs may lack the ability for self-renewal and differentiation; therefore, they do not fit the complete description for "stem" cells (Horwitz et al., 2005). Thus the terms "stem" and "stromal" both fail to fully characterize these cell populations. The International Society for Cellular Therapy (ISCT) have recommended that "multipotent mesenchymal stromal cell" is the correct terminology for the plastic-adherent cells isolated from bone marrow and other tissues, rather than the term "stem" cell in order to reduce confusion and reserve the term "mesenchymal stem cell" for cells which meet the criteria for stem cell activity (Dominici et al., 2006). The differentiation of the cells into various lineages and detection of certain cell surface protein markers are the *de facto* methods for exhibiting the stem potential of cells growing in culture.

Currently there are no specific cell surface markers identified for only MSCs. The methodology for MSC isolation and expansion can vary, sometimes quite significantly between laboratories. As a consequence of using different methods and assorted animal strains, cell surface marker expression can be inconsistent and quite heterogeneous (Haggman et al., 2013). Because of this, accurate comparisons between experimental results can be difficult and standardization for identifying MSCs is necessary. The ISCT

has provided standards to define MSCs for greater consistency in the identification and designation of these cells (Dominici et al., 2006). There are three minimal criteria for the definition of MSCs: (1) the cells are plastic-adherent under standard culture conditions; (2) greater than 95% of the cells exhibit positive expression for the cell-surface protein markers CD105 (endothelial cells), CD73 (follicular dendritic cells, epithelial, and endothelial cells), and CD90 (hematopoietic stem cells); while less than 2% of the cells are positive for CD45 (hematopoietic cells), CD34 (hematopoietic stem/progenitor cells), CD14 (monocytes, macrophages, B cells, dendritic cells, hepatocytes) or CD11b (granulocytes, monocytes, NK cells, myeloid dendritic cells), CD79α (B cells) or CD19 (follicular dendritic cells), and HLA class II (triggers T lymphocyte activation); (3) The cells must be able to differentiate into osteocytes, adipocytes, and chondrocytes under standard conditions (Dominici et al., 2006).

MSCs hold particular promise for clinical applications, tissue engineering and regenerative medicine due to certain inherent properties. They can be easily obtained from bone marrow and various other sources including adipose tissue, peripheral blood, umbilical cord blood, and connective tissues have been identified as reservoirs for these cells (Haggman et al., 2013). MSCs retain a state of multipotency, which means they have the ability to undergo differentiation into more specialized and mature cells types (Sasaki et al., 2008; Chen et al., 2014). Furthermore, they have exhibited an ability to promote wound healing and tissue renewal while also supporting cell engraftment and immune modulation (Chen et al., 2012).

Platelet-Rich Plasma

Concentrated blood platelets are yet another autologous component which is being studied for its potential in tissue repair. When blood clotting occurs at a wound site, platelets degranulate to release bioactive growth factors. The growth factors secreted from platelets bind to receptors on MSCs, osteoblasts, fibroblasts, endothelial cells, and epidermal cells to induce cell processes such as proliferation and tissue synthesis (Marx 2004).

PRP Use in Cell Media

It has been suggested that PRP, which is blood plasma with a high concentration of autologous platelets, can be used as an alternative serum source for the expansion of MSCs to be utilized in clinical applications (Goedecke et al., 2011). This means that platelets obtained from the same individual can be used during cell expansion in place of serum from other species, such as a fetal bovine serum, to reduce immunogenicity during subsequent therapy. The use of human platelet lysate in place of fetal bovine serum during cell expansion has been shown to induce no modifications of MSC immunophenotype or differentiative capacity. However, differences in cytokine expression were apparent and MSCs expanded in media containing platelet lysate secreted a higher amount of IL-6 (inflammatory cytokine) and IL-8 (acts as a chemoattractant for granulocytes and stimulates the formation of new blood vessels) (Azouna et al., 2012).

The Trophic Effects of PRP

PRP is known to contain multiple growth factors which induce MSC proliferation and migration (Goedecke et al., 2011). There are seven principle growth factors that are

released by platelets which induce healing: three isomers of platelet-derived growth factor (PDGFαα, PDGFββ, and PDGFαβ), two types of transforming growth factors-β (TGFβ1 and TGFβ2), vascular endothelial growth factor (VEGF), and epidermal growth factor (EGF) (Marx 2004). PDGF is a protein that regulates cell growth and division and acts as a potent mitogen for undifferentiated mesenchymal cells. Both PDGF and VEGF play a significant role in promoting the formation of blood vessels through angiogenesis, an important process necessary to restore blood flow to damaged tissues (Wu et al., 2007b). EGF promotes cell growth, proliferation, and differentiation of various cells, including fibroblasts, mesenchymal cells, and endothelial cells (Berlanga-Acosta 2009). TGF-β also causes increased cell proliferation. Along with a high concentration of soluble factors, PRP also provides cell adhesion molecules such as fibrin, fibronectin, and vitronectin which aid in cell migration and the process of wound healing (Lubkowska et al., 2012).

Bone Marrow-Derived MSCs

Bone marrow-derived mesenchymal stromal cells (BM-MSCs) are clonal precursors for non-hematopoietic mesodermal cells. Given specific conditions, MSCs can differentiate both *in vitro* and *in vivo* (following transplantation) into multiple cell types. These include adipocytes, osteoblasts, chondrocytes, fibroblasts, skeletal and cardiac myocytes, endothelial cells, and neurons (Jiang et al., 2002). Normally MSCs can leave the marrow and enter the systemic circulation in response to tissue damage (Sasaki et al., 2008). They can subsequently migrate to the specific site of injury. Bone marrow is typically viewed as an easily obtainable source for MSCs since they may be readily isolated and expanded *in vitro* despite being found in only small quantities.

Many investigations have demonstrated the therapeutic capacity of BM-MSCs in repairing or regenerating damaged tissues in almost all of the major organs of the body, including the brain, heart, lung, liver, eye, kidney, and skin (Hocking and Gibran, 2010). The diverse uses for MSC therapy indicates a vast potential for clinical applicability.

Heterogeneity in MSC Populations

MSC populations display considerable heterogeneity when it comes to morphological, differentiative, and proliferative properties (Azouna et al., 2012). Bone marrow itself consists of several different types of adherent cells, including osteogenic cells, phagocytic cells, and endothelial cells. Thus, these other adherent cells could potentially contaminate MSC cultures. Furthermore, cell surface protein expression has been shown to vary between different cell populations, likely due to differences in cell isolation and expansion procedures, making it even more difficult to properly identify MSCs (Hagmann et al., 2013). Additionally, differences have even been found in MSCs from the bone marrow of different strains of inbred mice, even when using identical isolation and expansion protocols (Peister et al., 2004). The heterogeneous quality of these stromal cells adds a level of complexity and uncertainty for their use in clinical settings, and it is something that must be addressed in order to introduce standardized procedures involving MSCs as treatments for certain pathologies and conditions.

Effects of Media on MSCs

MSCs are found in low quantities from harvested primary bone marrow so it is necessary to isolate and expand the cells *in vitro* for later use. Currently, the expansion of MSCs relies heavily on the utilization of animal-based serum. The addition of animal

serum adds an inherent risk of exposure to disease causing agents and to xenogeneic proteins. There is a motivation to eliminate the use animal-derived additives and instead utilize serum-free media supplemented with alternative additives such as platelet lysate solutions. By eliminating the use of serum while during preparation of MSCs, the risk for immunogenic reactions and rejection of xenogeneic materials can be significantly reduced. Additionally, differences between animal serum sources prevents methodological standardization that is necessary for the extensive use of MSCs in clinical applications.

Further contributing to cell heterogeneity, expansion media has been shown to alter the proteomic profile of human MSCs (Hocking and Gibran, 2010), as well as their growth characteristics and differentiation potential (Haggman et al., 2013). MSCs are influenced by signals resulting from culture conditions and factors such as cell seeding density, growth media, and added media supplements, which can compound the amount of heterogeneity the cell populations exhibit.

Cutaneous Wound Healing

MSC therapy is of particular interest for the promotion of cutaneous wound healing. A critical part of reforming viable tissue at wound sites is the proper reorganization of the underlying collagen fibers which increase dermal tissue strength and thus decrease the likelihood for rupture of a healed wound (Lokmic et al., 2006). Ideal healing of cutaneous injuries involves intricate and well-coordinated cellular and molecular mechanisms. MSCs can influence those complex processes at wound sites to bring about improved tissue regeneration (Jeon et al., 2010; Sasaki et al., 2008; McFarlin et al., 2006).

The Phases of Cutaneous Wound Healing

The progression of typical wound healing involves a complex succession of reactions. The molecular events occurring at wound sites can essentially be divided into four overlapping sequential phases with undefined beginnings and ends. The phases are: coagulation, inflammation, migration/proliferation, and remodeling. The first phase, coagulation, occurs almost immediately following injury and involves a cascade of molecular and cellular mechanisms which bring about hemostatic control via platelet degranulation and release of clotting factors. Blood platelets become embedded in clumps of fibrous proteins such as fibronectin, vitronectin, and fibrin that stops bleeding and forms an initial barrier to prevent infection. These protein aggregates also provide a matrix for cell adhesion and migration during the following healing phases.

Directly following hemostatic control, the inflammatory phase occurs. The platelets also release growth factors, including PDGF and TGF, which begin the recruitment of inflammatory and structural cells such as fibroblasts and endothelial cells to the wound site (Falanga 2005). Inflammatory cells recruited to the injury have multiple purposes including the neutralization and elimination of foreign pathogens. In addition, neutrophils and macrophages secrete mediators that stimulate the growth of connective tissue and also assist in debridement of the wound (Falanga 2005). Damage to blood vessels following cutaneous injury results in decreased oxygen availability to the wound microenvironment due to reduced blood flow. The local state of hypoxia at the wound site actually induces the migration of various cells, such as fibroblasts and keratinocytes (Falanga 2005). However, prolonged hypoxia causes excessive inflammation, leading to increased oxidative stress which may impair healing resulting in a chronic wound (Zou et

al., 2012). The disproportionate degree of inflammation resulting from persistent hypoxia can also cause disorganization in the synthesis of new connective that forms the granulation tissue which provide preliminary wound closure (Zou et al., 2012). Extended hypoxia can also result in the impairment of vessel perfusion during angiogenesis and delay reepithelialization necessary for contraction (the movement of wound edges toward a center point to accomplish closure of a cutaneous injury) (Zou et al., 2012).

As the inflammation stage diminishes, the recruited fibroblasts and endothelial cells begin to form granulation tissue which begins the process of wound contraction. Contraction occurs through the synthesis of granulation tissue, vasculature, and extracellular matrix (ECM). The ECM is the structural material made up largely of collagen, elastin, and proteoglycans that surrounds cells. It is also very useful for cell adhesion. New blood vessels are formed to restore oxygen and nutrient allocation to the cells. The formation of new vasculature from existing blood vessels is an important aspect of wound repair to replace those damaged at the wound site and to restore blood supply. This process, called neovascularization, is essential to provide damaged tissue with nutrients and oxygen, plus the circulating cells and factors necessary for healing. Neovascularization can occur either by angiogenesis, where endothelial cells located at the wound site proliferate and form new vessels, or through vasculogenesis, where systemically circulating progenitor cells are directed to the wound site and begin the formation of producing completely new vasculature (Sasaki et al., 2008).

The formation of ECM in the initial stages of healing serves to provide early stability for the wound and to act as a medium for cell migration. However, the initial extracellular matrix does not provide adequate structural integrity to the healing wound, so

it is subsequently degraded by proteolytic enzymes. Large deposits of collagen are then put in place of ECM; first type III collagen and then later type I collagen (Lokmic et al., 2008). Type III collagen can be quickly made, however it is mechanically weaker (Henriksen et al., 2010). The type I collagen that replaces it forms much stronger connections to enhance the integrity of the resulting scar. The collagen fibers can then be reorganized by dermal fibroblasts to further increase the tensile strength of the newly formed tissue during the tissue remodeling phase (Lokmic et al., 2008).

Chronic Wounds

If the process directing wound repair is interrupted, cutaneous injuries will fail to heal, triggering the formation of chronic non-healing wounds. Typically, non-healing wounds do not follow the natural progression of healing. Furthermore, portions of the wound may be unsynchronized with other parts, so several different phases of the healing process are occurring simultaneously. Dysregulation and inhibition of wound contraction frequently occur in wounds exhibiting pathophysiological abnormalities (Zou et al., 2012). This causes non-healing wounds to often be quite heterogeneous in nature. Dysfunction of the molecular mechanisms occurring in wound healing may alter the normal inflammatory events and prevent mesenchymal cell proliferation, differentiation, or migration to the wound (Zou et al., 2012). Angiogenesis, reepithelialization, and appropriate alignment of collagen fibers may also be hindered (Zou et al., 2012).

There are a multitude of factors that may contribute to the pathogenesis of impaired wounds, including a reduction in the proliferation and migration of fibroblasts and keratinocytes. Since there are so many aspects which may be contributing to the chronic pathology, it is difficult to identify and address one problem at a time. Even with the

utilization of current treatments such as physical debridement, hyperbaric oxygen therapy, and antibiotics, chronic wounds often remain unresponsive and will not undergo normal healing processes (Zou et al., 2012). At best, modern treatments can only induce the closure of chronic wounds with a success rate of about 50% (Chen et al, 2008). Cells in non-healing wounds may be fundamentally impaired and may exhibit higher rates of senescence and less sensitivity to growth factor stimulation (Chen et al., 2012). Since current remedies for chronic wounds are ineffective in many cases, novel treatments are needed.

Those suffering from chronic diseases, such as diabetes, are particularly susceptible to the development of non-healing wounds. There are more than 150 million people diagnosed with diabetes and, of these, 15% experience chronic wounds (Chen et al., 2008). With an increasing prevalence of type II diabetes mellitus, the creation of dynamic treatments for chronic wounds is becoming more and more important. The use of cell-based therapies represents an incredibly promising therapeutic approach which may significantly increase wound closure. For example, realization of the importance for growth factor stimulation in healing has led to topical applications of regulatory proteins such as PDGF (found in PRP) which were able to increase wound closure (Falanga 2005). Ultimately, understanding the mechanisms of wound repair will allow the development of new approaches in treating cutaneous injuries.

The Role of MSCs in Tissue Regeneration

MSCs can be naturally signaled to leave the bone marrow and enter the systemic circulation where they are subsequently directed via molecular mechanisms to wound sites. These multipotent cells have been found to be immensely responsive to growth factor

stimulation and their migration can be stimulated by several molecular signals (Akino et al., 2005). Once arriving at the site of injury MSCs exert powerful mediation for the progression of wound healing in various ways.

Engraftment and Integration into Regenerating Tissue

There are two principal theories for how MSCs actually contribute to the process of healing to promote increased tissue repair. Transdifferentiation, the ability of MSCs to home to wound sites and integrate as differentiated cells specific for the regenerating tissue, was originally thought to be a primary mechanism for their role in tissue repair. MSCs from bone marrow have been shown to express keratin following their application to cutaneous wounds in mice (Sasaki et al, 2008). These data suggested a contribution to wound healing through differentiation into the keratinocytes which form the epidermis. However, in studies examining the addition of labelled bone marrow-derived MSCs into mature skin following cutaneous injury, MSC incorporation was quite minimal (Badiavas et al., 2003). While MSC engraftment can provide some benefits to improve healing, its contribution may be somewhat marginal as a result of weak cell engraftment, low cell retention, and low survival of MSCs at application sites. The high rate of cell mortality and low retention is likely caused by an increased level of reactive oxygen species located at wound sites, particularly those exhibiting ischemia (Chen et al., 2012). The contribution from direct integration of differentiated MSCs into the wound bed may be less important as the healing process progresses, since the engraftment of MSCs will decline as the regenerated tissue becomes more mature (Chen et al., 2009b). Changes in the wound microenvironment likely cause a decrease in MSC engraftment as the healing process advances. Cytokines and ECM molecules that promote MSC survival and integration into

tissue decline during later healing phases (Chen et al., 2009b). Thus the involvement of MSC transdifferentiation into new cell types during healing may be initially important only as a way to stabilize the injury (Zou et al., 2012).

Paracrine Signaling

MSCs are also able to mediate the repair and regeneration of tissue by recruiting inflammatory cells, as well as additional stem and progenitor cells. Current evidence suggests that paracrine signaling from MSCs plays a much more important role than transdifferentiation in the beneficial effects of applying MSCs to injuries (Gnecchi et al., 2008). Benefits of MSC therapies have been readily witnessed even in the absence of noticeably sustained engraftment of cells within damaged tissues. Conditioned media which contains the paracrine secretions from MSCs can be applied to wounds to elicit similar results as seen with the use of MSCs themselves (Chen et al., 2008). The collective MSC secretome acts to trigger and stimulate the recipient's endogenous repair mechanisms at the site of injury. Thus, the ability of MSCs to differentiate may provide negligible benefits to promote wound healing. Conflicting evidence has been exhibited when analyzing the degree MSC engraftment to tissue post-injury. This may be due to cell heterogeneity present within MSC populations.

Regardless of whether engraftment occurs, significant evidence has suggested the advantages of MSC application to injury may be overwhelmingly due to paracrine mechanisms. While dermal fibroblasts normally provide much of the growth factors in cutaneous wound healing, MSCs have been shown to express substantially more VEGF-A, erythropoietin, SDF-1α, and EGF (Chen et al., 2008). So instead of MSCs migrating to the site of inflammation and undergoing transdifferentiation into fibroblasts, endothelial,

inflammatory, or other structurally significant cells, MSCs may be providing the growth factors to promote the migration and proliferation of surrounding native cells (Chen et al., 2008). Signaling from MSCs results in various processes important in wound healing, including proliferation, migration, genetic expression and cellular survival. Vascularization and keratinization are also increased in epithelial wounds following the application of MSCs (Shokrgozar et al., 2012).

MSCs secrete VEGF-A, EGF, erythropoietin, and stromal cell-derived factor-1α (SDF-1α) (Chen et al., 2012). As mentioned previously, VEGF contributes to angiogenesis and helps restore blood perfusion to damaged tissue. EGF binds to its receptor on many types of cells to induce cell adhesion, proliferation, and migration. The primary role of erythropoietin is the stimulation of red blood cell development, but it can also contribute to angiogenesis. SDF-1 is very important for the recruitment of endothelial progenitor cells (Zheng et al., 2007) and acts as a potent chemoattractant for T lymphocytes (Bleul et al., 1996) and MSCs (Nakamura et al., 2013). In fact, MSCs transfected with a plasmid to increase secretion of SDF-1 were able to promote significantly higher rates of migration and accelerate wound healing when compared to normal MSCs (Nakamura et al., 2013). Numerous other factors may be secreted by MSCs, including substantial amounts of the cytokines MIP-1, MIP-2, and MCP-5, which act as chemoattractants for macrophages and monocytes. These factors also stimulate macrophage penetration into the tissues at wound sites to promote debridement and to clear infections (Chen et al, 2008).

MSCs have been identified neighboring newly formed blood vessels but not integrated as a part of the actual vessel wall (Wu et al., 2007b). Accordingly, this suggests the MSCs are exerting a paracrine effect to induce angiogenesis rather than differentiating

and incorporating themselves directly into the vasculature structure. MSCs secrete various soluble factors, including SDF-1, which contribute to the process of angiogenesis (Goedecke et al., 2011). SDF-1 is also needed to promote the survival of the endothelial cells which make up the inner lining of the vasculature, as well as for the recruitment of pericytes, specialized contractile cells that regulate blood flow through capillaries (Zou et al., 2012). Following cutaneous injury, damage to blood vessels causes a state of ischemia due to a lack of adequate oxygen supply to the tissues at the wound site. The resulting hypoxia activates the synthesis of VEGF, stimulating the formation of new vasculature within the newly synthesized extracellular matrix (Lokmic et al., 2006). Capillary reperfusion restoring oxygen supply to the wound tissue instigates the formation of oxidative free radicals. Excessive oxidative damage can lead to substantial inflammation and the formation of non-healing wounds. MSCs promote the production of antioxidative enzymes, such as superoxide dismutase, which catalyzes the destruction of superoxides, and glutathione peroxidase which catalyzes the reduction of organic hydroperoxides, prompting a reduction in the oxidative stress and toxicity that may contribute to the pathogenesis of chronic wounds (Jeon et al., 2010). Detoxification of reactive oxygen species results in upregulation of the expression and activity of VEGF in wounds, thus promoting increased angiogenesis (Bir et al., 2012).

Fibroblasts are the predominant cells found in cutaneous injuries and they contribute to healing and scar formation by inducing wound contraction, extracellular matrix deposition, and tissue remodeling (Smith et al., 2010). These cells are responsible for synthesis of new collagen and fibrous proteins to regenerate and strengthen tissues following injury. Immediately following cutaneous damage, fibroblasts in neighboring

tissues are stimulated to proliferate and to migrate into the wound bed. MSCs can stimulate the proliferative phase of cutaneous wound healing by inducing fibroblast migration and proliferation to form granulation tissues (Jeon et al., 2010). MSC conditioned media has also been shown to increase collagen and elastin synthesis by fibroblasts to form the extracellular matrix that makes up granulation tissue (Jeon et al., 2010). Furthermore, the conditioned media suppresses the secretion of matrix metalloproteinases (MMPs) by dermal fibroblasts (Jeon et al., 2010). These proteins degrade extracellular matrix molecules and hydrolyze the type I collagen found in the dermis and mature scars thus weakening the wound (Varani et al., 2001). MSCs can diminish the destructive action of MMPs, resulting in higher amounts of collagen deposition in the dermis and to improve the healing of cutaneous injuries.

To investigate the paracrine signaling effects MSCs have on the migration of dermal fibroblasts, scratch assays were performed utilizing co-cultures of MSCs and fibroblasts (Walter et al., 2010). The co-cultures were scored in certain areas to remove all cells. The scored regions were then monitored to observe the rate of migration back into those areas. In these experiments, fibroblast migration was accelerated by the presence of MSCs. A different cell migration assay using MSC-conditioned media (which contains the soluble secretions from MSCs) was able to confirm MSCs do in fact induce fibroblast chemotaxis by the production of soluble factors, since the conditioned media induced the same increased rate of fibroblast migration as the cells (Smith et al., 2010). However, there can be differences in the functional abilities of MSCs to signal fibroblast migration. For instance, MSCs taken from normal healthy individuals induced a higher rate of fibroblast migration *in vitro* when compared to MSCs taken from an individual experiencing chronic

wounds (Rodriguez-Menocal et al., 2012). Collectively, this information suggests that MSCs play an extensive role in stimulating the proliferation and migration of the cells involved in wound repair.

Immunomodulation

MSCs have also been observed to possess immunomodulatory capabilities. More precisely, they have exhibited immunosuppressive roles both *in vivo* and *in vitro*. The addition of MSCs to lymphocytes in a mixed lymphocyte reaction suppresses T cell proliferation, even in the presence of T cell mitogens (Le Blanc et al., 2002). MSCs can suppress CD4+ and CD8+ T cell proliferation by releasing soluble factors and by modifying dendritic cells, effector T-cells, and natural killer cells to adopt a more anti-inflammatory profile (Chen et al., 2012). Human leukocyte antigen-G5 (HLA-G5) is produced by MSCs and exerts several regulatory actions on immune response, including the induction of osmotic lysis of NK and cytotoxic T lymphocytes, increased interferon-γ (IFN-γ) secretion, and the promotion of regulatory T cells (Selmani et al., 2007). The IFN-γ contributes to both the innate and adaptive immunity, primarily through the activation of macrophages. Increased macrophage stimulation would likely assist in the debridement of cutaneous wounds. T regulatory cells normally suppress activation of the immune system to prevent autoimmune reactions or inappropriate recognition of self-antigens by the lymphocytes. Understandably, the primarily immunosuppressive actions prompted by MSCs can be beneficial in preventing the rejection of engrafted cells by suppressing possible immune responses.

MSCs have been used to reduce rejection and complications resulting from transplanted tissues and have been utilized to treat autoimmune disorders and conditions

such as graft-versus-host disease following allogeneic tissue transplantation (Ozaki et al., 2007). MSCs themselves have been shown to provoke only minimal immunogenicity and even those from allogeneic sources have been shown to generate little to no immunogenic response (Chen et al., 2012). The degree of MSC engraftment using allogeneic cells was similar to that of syngeneic cells, with neither resulting in graft rejection (Chen et al., 2009b). Therefore, the use of MSCs from sources other than the patient's bone marrow shows promise for therapeutic uses without causing immunogenic reactions in hosts. This is promising information for potential treatments, since injuries would often necessitate immediate MSC application and the employment of syngeneic MSCs would require significant time delays necessary for their isolation and *in vitro* expansion. Thus pre-prepared allogeneic cells could be readily utilized without fear of rejection or adverse immunogenic responses. However, there is little information on whether the immunosuppressive characteristics of MSCs can lead to increased sensitivity to tumor formation and opportunistic pathogens.

MSC Delivery

Yet another reason why MSCs are so actively studied for clinical uses is their ease of application. Various delivery methods are being researched to maximize the therapeutic potential of MSCs. There are three primary modalities for clinical applications of MSCs: intradermally, topically, and systemically. Extensive research has been performed to investigate usage of both synthetic and non-synthetic biomaterials including fibrin sprays, hydrogels, and composite grafts to provide vehicles and scaffolds for MSC application. One study used a gel developed to mimic the hygroscopic nature of extracellular matrix (Lutolf and Hubell 2005). MSCs were seeded and cultured in the gel and then applied to

wounds leading to accelerated wound closure. Furthermore, the number of MSCs within the wound were about three times greater after one week and ten times more numerous at day 10 compared to wounds treated with a local injection. This is promising since difficulties remain to allow cellular homing and preservation of transplanted cells in a localized area. Additionally, recent studies have utilized treatments to increase MSC homing. Exposing MSCs to certain short peptides which increase surface integrin expression can allow a greater amount of cell adhesion and a higher rate of systemically infused cells migrating to sites of inflammation (Levy et al., 2015). Alternatively, MSCs may be modified to express specific ligands through DNA or mRNA transfection to promote homing to injury (Sackstein et al., 2008; Levy et al., 2013). Further analysis is required to understand optimal dosage, timing, and manner of administration for MSCs to offer better outcomes in the clinical translation to treatment for individual cases.

Summary and Project Goals

Current evidence clearly suggests that cell based therapies, including those which utilize MSCs and PRP have an enormous potential in therapeutic applications and the improvement of wound healing. The capacity for MSCs and PRP to accomplish this largely results from their ability to secrete paracrine factors, which enhance normal responses to cutaneous injury. These factors can stimulate the movement of various cells that contribute to healing and recruit them to the wound site. Furthermore, the MSCs themselves have been found to be enormously responsive to stimulation by growth factors and cytokines. Their ease of attainability and their lack of immunogenic response in hosts indicates that they have potential for widespread use. The multiple characteristics of MSCs, including their immunomodulatory, trophic, and differentiative properties,

represent incredibly beneficial and promising traits which can be exploited in clinical service.

One of the defining characteristics of MSCs is their ability to differentiate into various cell lineages. The capacity for MSC differentiation into chondrogenic, adipogenic, and osteogenic cells will be examined in order to demonstrate their competence for multipotency. The effects of media and culture conditions on the morphological, phenotypical, and functional properties of MSCs isolated from rat bone marrow and expanded *in vitro* will also be examined. The current analysis will contribute to understanding MSCs and their use in regenerative therapy. The presented here seeks to evaluate the effectiveness of MSCs and PRP in wound healing, and to determine the optimal dose for MSC use. This work will examine the healing of surgical incisions in rats in response to various MSC dosages. Histological analysis will also be used to quantitate the degree of improved wound healing following treatment with MSCs, PRP, and CollaTape. Increases in characteristics such as collagen deposition and organization can contribute to increased tissue strength that would prevent wound herniation.

MATERIALS

3-isobutyl-1 methylxanthine (Sigma-Aldrich)

α-MEM (Sigma-Aldrich)

Alcian Blue (Sigma-Aldrich)

Alizarin Red S (Sigma-Aldrich)

Ascorbic acid 2-phosphate (Sigma-Aldrich)

β-Glycerophosphate (Sigma-Aldrich)

CollaTape (Zimmer Dental)

Dexamethasone (Sigma-Aldrich)

Fetal Bovine Serum (Sigma-Aldrich)

GlutaMAX (Sigma-Aldrich)

Indomethacin (Sigma-Aldrich)

Male Lewis Rats (Charles River Laboratories International Inc. in Wilmington, Mass.)

Oil Red O (Kodak)

Penicillin-Streptomycin (Sigma-Aldrich)

Recombinant human insulin (Sigma-Aldrich)

RPMI (Sigma-Aldrich)

Transforming growth factor-β1(PeproTech)

Trypsin/EDTA (Sigma-Aldrich)

METHODS

ISOLATION OF MSCS

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

EXPANSION OF MSCS

The cells ($1x10^7$ cells in a T75 cell culture flask) were incubated for 4 days at 37°C and 5% CO_2. The non-adherent cells were aspirated off and 10mL of fresh media was added. The cells were fed every 3-4 days until the cells reached 80% confluency. The cells were washed with PBS and trypsinized using a treatment of a 3mL solution of 1X trypsin and EDTA (0.25% trypsin and 1.0mM EDTA) for 7 minutes at 37°C. The cells were dislodged by tapping the flask and the reaction was ended by the addition of 30mL of complete medium. The cells were then split into two flasks. The expansion of MSCs

was continued until the third passage. The cells were then counted using a hemacytometer, centrifuged for 5 minutes at 600 x g, and resuspended in complete media containing 10% DMSO to achieve a concentration of $1x10^6$ cells/mL. The MSCs were then placed in cryovials, frozen slowly for 24 hours at -80°C, and then stored in liquid nitrogen.

Osteogenic Differentiation

The protocol for all differentiation assays were adapted from procedure in Chen et al. (2009a). In order to evaluate osteogenic potential, $2x10^5$ MSCs in the third passage were seeded into a 6 well plate. The cultures were incubated at 37°C in 5% atmospheric CO_2 and allowed to become confluent. Half of the wells were maintained with complete media while the remaining were maintained with osteogenic medium (200µM ascorbic acid 2-phosphate, 100nM dexamethasone, 10mM β-glycerophosphate, 10% FBS, 2mM glutaMAX, and 1% penicillin/streptomycin in DMEM high glucose) for three weeks (Chen et al., 2009a). Media was aspirated off the cells and fresh media was added every 2-3 days.

To identify the presence or absence of osteogenic differentiation, the cells were washed with PBS and then fixed with a 10% formalin solution for one hour at room temperature. Formalin was removed and the cells were washed with distilled water. Alizarin Red S (2%) was dissolved in distilled water. The pH was adjusted to 4.2 using 0.1% NH_4OH and the solution was filtered. The wash solution was aspirated from the cells, the bottom of the well was covered with 2% Alizarin Red S stain (pH 4.2) and allowed to incubate at room temperature in the dark for 45 minutes. The cells were washed four times with 1mL of distilled water and PBS was added to each well. The cells were visualized using an inverted phase contrast microscope.

Adipogenic Differentiation

To evaluate adipogenic potential, $2x10^5$ MSCs in the third passage were added to each well of a 6 well plate. The cultures were incubated at 37°C in 5% atmospheric CO_2 for 48 hours with complete media until they reached confluence. To induce differentiation, the cells in three of the wells were maintained in adipogenic media containing 1μM dexamethasone, 500μg/mL insulin, 1μM indomethacin, 500μM 3-isobutyl-1 methylxanthine (IBMX), 10% FBS, 2mM glutaMAX, and 1% penicillin/streptomycin in DMEM high glucose for three weeks (Chen et al., 2009a). The remaining 3 wells were maintained with complete media. The media was aspirated and replaced every 2-3 days.

The cells were stained with Oil Red O to detect lipogenesis. Cells were taken from the incubator and washed with PBS. The solution was aspirated off and about 1mL of neutral buffered formalin (10%) was added and allowed to incubate at room temperature for 30 minutes. A 0.3% stock solution was made by dissolving 300mg Oil Red O in 100 mL isopropanol. The staining solution was prepared by diluting 3 parts of the stock solution with 2 parts distilled water and filtering with a syringe filter. The fixation solution was aspirated off the cells which were subsequently washed with distilled water. About 1mL of 60% isopropanol was added and allowed to incubate for 5 minutes before being aspirated off. Enough Oil Red O staining solution was added to cover the cells (about 1mL) and incubated for 15 minutes. The stain was removed and the cells were washed with water until wash became clear. PBS was added to cover the cells and images were taken using an inverted phase contrast microscope.

Chondrogenic Differentiation

In order to evaluate chondrogenic potential, $2x10^5$ MSCs in the third passage were added to each well of a 6 well plate. The cultures were incubated at 37°C in 5% atmospheric CO_2 for 48 hours with complete media until the cells reached confluence. Chondrogenic media (1% insulin-transferrin-selenium (ITS), 50μg/mL ascorbic acid, 100nM dexamethasone, 10ng/mL TGF-β1, 2mM glutaMAX, and 1% penicillin/streptomycin in DMEM high glucose) was then used to maintain half of the wells while complete media was used for the remaining wells (Chen et al., 2009a). Media was aspirated off the cells and fresh media was added every 2-3 days for 3 weeks.

The wells were then stained with 0.01% Alcian Blue to visualize the formation of cartilage and collagen secretion indicative of chondrocyte formation. The cells were washed with PBS and then fixed with a 10% formalin solution for one hour at room temperature. The formalin was removed and the cells were washed twice with distilled water. The staining solution was prepared by dissolving about 10mg of Alcian Blue 8 GX dye in a 60:40 solution of ethanol and acetic acid. Enough stain was added to the wells in order to cover the cells and allowed to incubate overnight in the dark at room temperature. The stain was aspirate and 1mL of a destaining solution consisting of 120mL ethanol and 80mL acetic acid was added and incubated for 20 minutes. The destaining solution was aspirated off and PBS was added to cover the cells which were then visualized and imaged with an inverted phase contrast microscope.

Platelet-rich Plasma Preparation

The protocol for collection of PRP was modified from the work performed by Maekawa et al. (2003). Blood was collected from male Lewis rats via a heart puncture

using a 21-gauge needle and a 10mL syringe containing 1/10 volume of citrate dextrose. The blood was centrifuged at room temperature in a conical tube for 10 minutes at 200 x g. The plasma layer was removed and centrifuged at 700 x g for 10 minutes. The upper (platelet-poor) layer was removed and stored at -20^{O}C leaving about 1 mL portion with the platelet pellet. The pellet was resuspended following the addition of 5% DMSO. The PRP was frozen slowly by gradually decreasing temperature to -80^{O}C. Subsequently, the frozen pellets were stored in liquid nitrogen. In order to thaw PRP for later use, the frozen pellet was quickly melted by adding it to 1 mL warm plasma and mixed. The PRP was then centrifuged at 700 x g for 10 minutes at 4^{O}C. The plasma is aspirated off and the remaining pellet is resuspended in plasma.

SURGERIES

A wound healing analysis was performed using male Lewis rats. Isoflourane (3-5%) anesthesia was induced and the abdomen of was shaved and sterilized by washing with 70% isopropanol. An abdominal skin incision was made from the xiphoid to pubis region. The skin flaps were pulled back and a full-thickness midline incision of 60mm in length starting at the xiphoid process and extending inferiorly was made in the abdominal wall. The incision was closed using about 15 running Vicryl sutures. Each corner of a 6x1 cm strip of CollaTape was fixed over the wound using sutures to the rectus sheath and a suspension of varying amounts of MSCs ($2.5x10^4$, $5x10^4$, $1x10^5$, $2.5x10^5$, $5x10^5$, and $1x10^6$ cells) in 0.5 mL platelet-rich plasma were applied to the CollaTape. The skin was repaired using 5-0 Vicryl subcuticular sutures. A control group received no treatment and was repaired with only sutures. One group also received a sham treatment of CollaTape and 0.5mL PRP, but no MSCs. The rats were monitored and allowed to recover for 4 weeks in

normal bedding/conditions and then sacrificed via heart puncture under isoflourane anesthesia. For sample collection, incisions were made horizontally across the abdominal wall above and below the area of tissue to be collected. A metal plate was inserted under the musculature and a metal stamp was used to cut out precise shapes from the tissue for various analyses as shown in Figure 1. One of the wound samples were taken for trichrome staining and histological analysis.

Histological Scoring for Wound Analysis

Midline sections consisting of the healed incision site were paraffin embedded and cross-sections were fixed on slides. The samples were then subjected to Masson's trichrome staining staining as performed in Hefner et al. (2012). Images of the wound site were taken using 40x magnification. The images of the trichrome stained slides were assessed similar to the procedure by Stanwix et al. (2011), based on the grading scale in Figure 2 in order to determine how treatment with MSCs and PRP affected the wound healing process. The images were randomized and the degree of collagen abundance, collagen organization, and myocyte regeneration was determined and the resulting data was averaged. Criteria explaining the how scores for specific tissue characteristics were given is explained in Figure 2.

Data Analysis

Difference of histological scores between groups receiving varying treatments was analyzed by performing a one-way ANOVA using Microsoft Office Excel 2016. Mann-Whitney testing was performed using IBM SPSS Statistics 20 to provide pairwise analysis between groups. Statistical significance was accepted with a p value of less than 0.05.

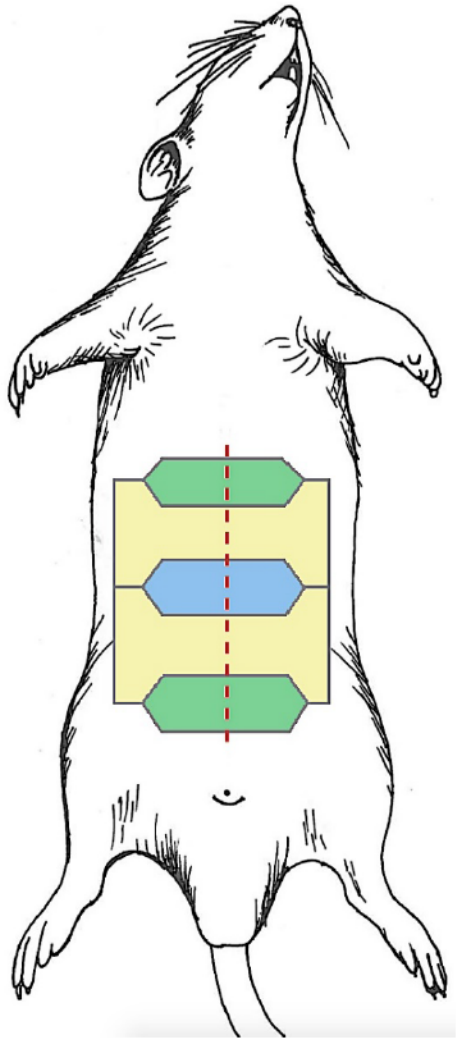

Figure 1: Sample collection diagram. This illustration depicts the wound location and sample collection sites from the ventral portion of the male Lewis rats utilized in this study. All wounded tissues received a 60mm full-thickness incision through the abdominal musculature extending from the xiphoid process to the pubis (dashed red line). The samples colored yellow and green were sent to other laboratories. The yellow strips were used to test wound breaking strength using a tensiometer. The green samples were used to determine composition of type I and type III collagen at the wound site. The blue strip in the middle was used for Masson's Trichrome staining to determine histological characteristics.

Category	0	1	2	3
Collagen Organization	Disorganized	Mildly Organized	Moderately Organized	Well Organized
Collagen Abundance	None	Mild	Moderate	Abundant
Myocyte Regeneration	None	Mild	Moderate	Abundant

Figure 2: Histological scoring criteria. These standards were utilized to determine and quantify tissue characteristics and morphology. Samples which exhibited tightly packed and unidirectional collagen fibrils with limited cellular infiltration were given a score of 3 while those which did not exhibit these traits received a score of 0. Samples which displayed some of the characteristics were given a 1 or 2. Collagen abundance was semi-quantitatively determined through subjective analysis and given a score from 0 to 3 based on perceived collagen amount. Myocyte regeneration was based on the presence of small muscle packets forming at the edges of the incision site. The degree was of muscle regeneration was visually determined and given a score from 0 to 3.

RESULTS

Previous studies have shown that bone marrow MSC cells can differentiate into a variety of mesenchymal-derived cell types (Azouna et al., 2012; Wu et al., 2007b; Goedecke et al., 2011). Thus, the rat BM-MSC cells grown in culture for the current study were investigated for their *in vitro* osteogenic, adipogenic, and chondrogenic differentiation to determine their inherent multipotentency. Cells seeded into a 6-well plate were allowed to reach confluence while grown with complete media. Differentiation media was then added to the test the cells (3 weeks in osteogenic and chondrogenic medium; 2 weeks in adipogenic medium), while control cells were maintained with complete media for the same amount of time. Cells undergoing induced differentiation and their respective controls were performed in triplicate (N=3).

The occurrence of calcified matrix mineralization is indicative for the development of osteocytes which act to form bone tissue (Vater et al., 2010). Mineralization can be identified early in culture by the accumulation of calcium phosphate crystals which can be detected by staining with Alizarin Red S (Birmingham et al., 2012). Positive staining was indicated by areas of reddish-brown staining in cultures treated with osteogenic media, suggesting the presence of extracellular calcium deposits (Figure 3B). This indicates that treated cells may have differentiated into osteoblasts. Cells maintained in normal growth media served as a control and were negative for Alizarin Red S staining (Figure 3A).

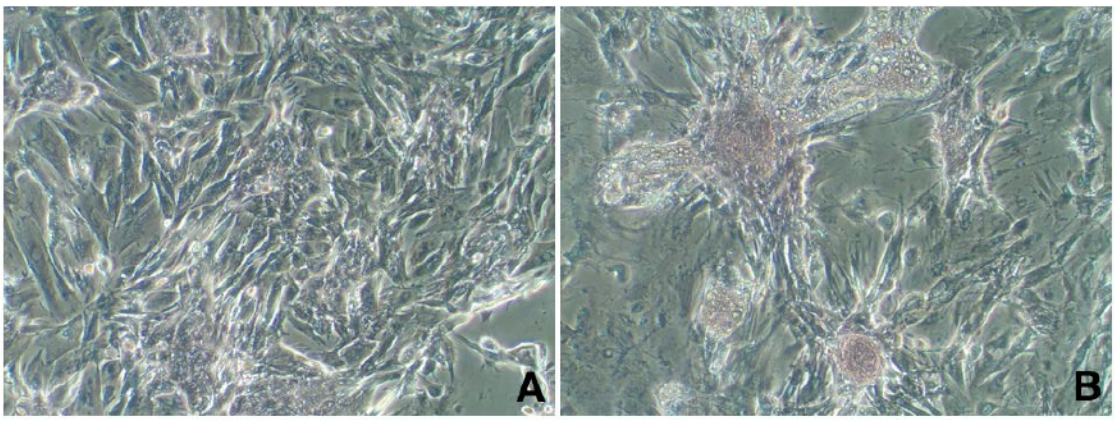

Figure 3: Osteogenic Differentiation. BM-MSC cells cultured for 3 weeks in the absence (A) or presence (B) of osteogenic medium. Both cultures were stained with 2% Alizarin Red S to indicate the presence of calcium deposition. Positive staining, seen as the pale reddish-brown areas in (B), suggests the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

Oil Red O is a stain specific for neutral triglycerides and lipids (Vater et al., 2010). Cultures treated with adipogenic media for 2 weeks showed positive staining with Oil Red O as indicated by accumulations of intracellular lipid vacuoles staining red (Figure 4B). This suggests MSCs may have differentiated into an adipocyte lineage. Cells maintained in normal growth media served as a control and were negative for Oil Red O staining (Figure 4A).

Chondrogenic media induces the cells to undergo differentiation and produce extracellular matrix composed of cartilage-specific molecules including type II collagen and aggrecan (Solchaga et al., 2011). Alcian Blue, which is specific for all types of collagen and glycosaminoglycans (Vater et al., 2010) stained the deposits of collagen and extracellular matrix in the cell culture to suggest the generation of chondroblasts (Figure 5B). Cells maintained in normal growth media served as a control and were negative for Alcian Blue staining (Figure 5A).

Since the application of MSCs to cutaneous wounds has shown to provide therapeutic benefit and accelerate healing (Chen et al., 2012; Nakamura et al., 2013; Jeon et al., 2010; Liu et al., 2013), an investigation was also performed to examine the effectiveness of topical applications of BM-MSC cells and to determine the optimal concentration of allogeneic MSCs in the treatment of abdominal wall incisional wounds. A histological analysis was performed using a rat model to determine changes in wound healing characteristics following varying treatments to promote tissue regeneration after invasive laparotomy surgeries. MSCs were suspended in PRP at six different concentrations ($2.5x10^4$, $5x10^4$, $1x10^5$, $2.5x10^5$, $5x10^5$ and $1x10^6$ cells per 0.5mL of PRP)

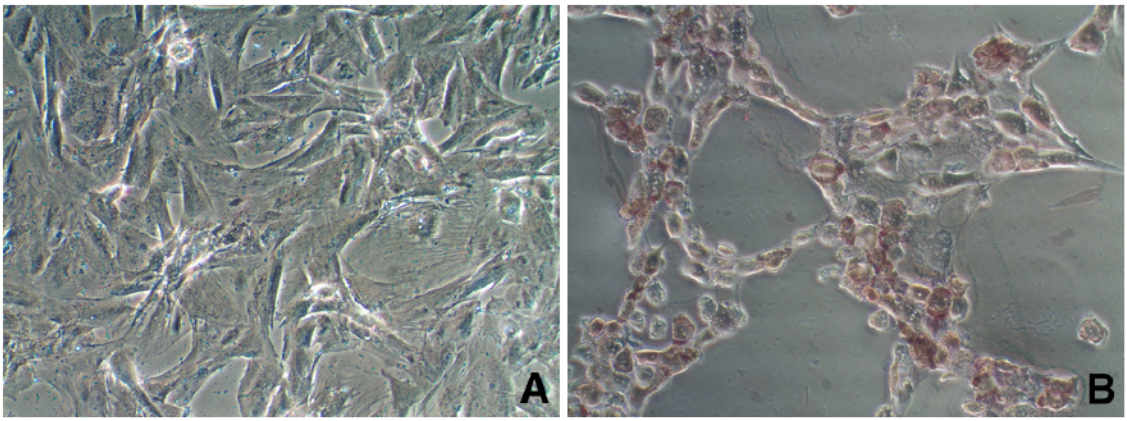

Figure 4: Adipogenic Differentiation. BM-MSC cells grown for 2 weeks in the absence (A) or presence (B) of adipogenic medium. Both cultures were treated with 0.2% Oil Red O to stain for intracellular lipid droplets. The red staining of cells in (B) indicates the occurrence of lipogenesis.

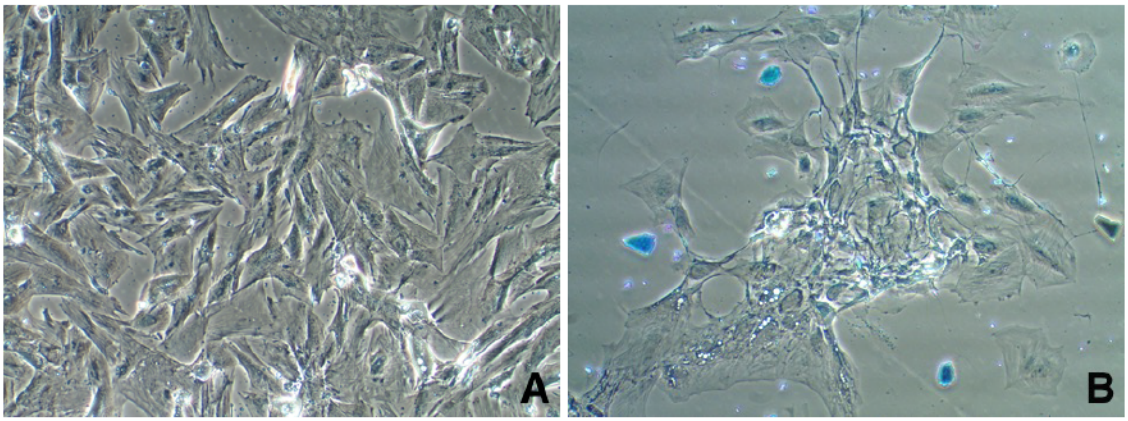

Figure 5: Chondrogenic Differentiation. BM-MSC cells cultured for 3 weeks in the absence (A) or presence (B) of chondrogenic medium. Both cultures were stained with 0.01% Alcian Blue to determine the presence of collagen deposition.

and the suspensions were applied to CollaTape fixed over a full-thickness abdominal incision which was closed with suture repair. A control group received only suture repair and a sham group had CollaTape and PRP applied over the sutures. The abdominal wall tissue samples from the wound site were harvested at 4 weeks of healing, paraffin embedded, sectioned, and trichrome stained (performed by the St. Elizabeth Hospital Pathology Department) to demonstrate collagen deposition and tissue morphology. The trichrome stained tissue sections were also used for semi-quantitative scoring of collagen fiber abundance after 4 weeks of healing. Scores from 0 to 3 were subjectively given to each of the samples for three criteria: collagen abundance, collagen organization, and myocyte regeneration. Criteria for deciding scores are detailed in Figure 2.

Representative images of the abdominal wall tissue samples for the control group, sham group, and the treated groups receiving the lowest ($2.5x10^4$) and highest ($1x10^6$) number of cells are shown in Figure 6. The presence of collagen fibers was indicated by bright blue staining and muscle fibers are stained red. The stained tissue samples showed a noticeable increase in both collagen amount and organization for the groups receiving MSC treatment when compared to wounds with no added MSCs. Animals treated with the highest number of MSCs ($1x10^6$) exhibited numerous collagen fibers which were largely arranged unidirectionally and densely packed against one-another, and displayed limited cellularity among the collagen fibers. Control individuals (Figure 6B) had little collagen deposited at the wound site while the samples from the sham group (Figure 6C) had slightly more collagen abundance. The control tissues also had little to no myocyte regeneration (Figure 6B). Myocyte regeneration was indicated by the formation of small collections of new muscle tissue at the edges of the incision. Groups receiving MSC therapy (Figures

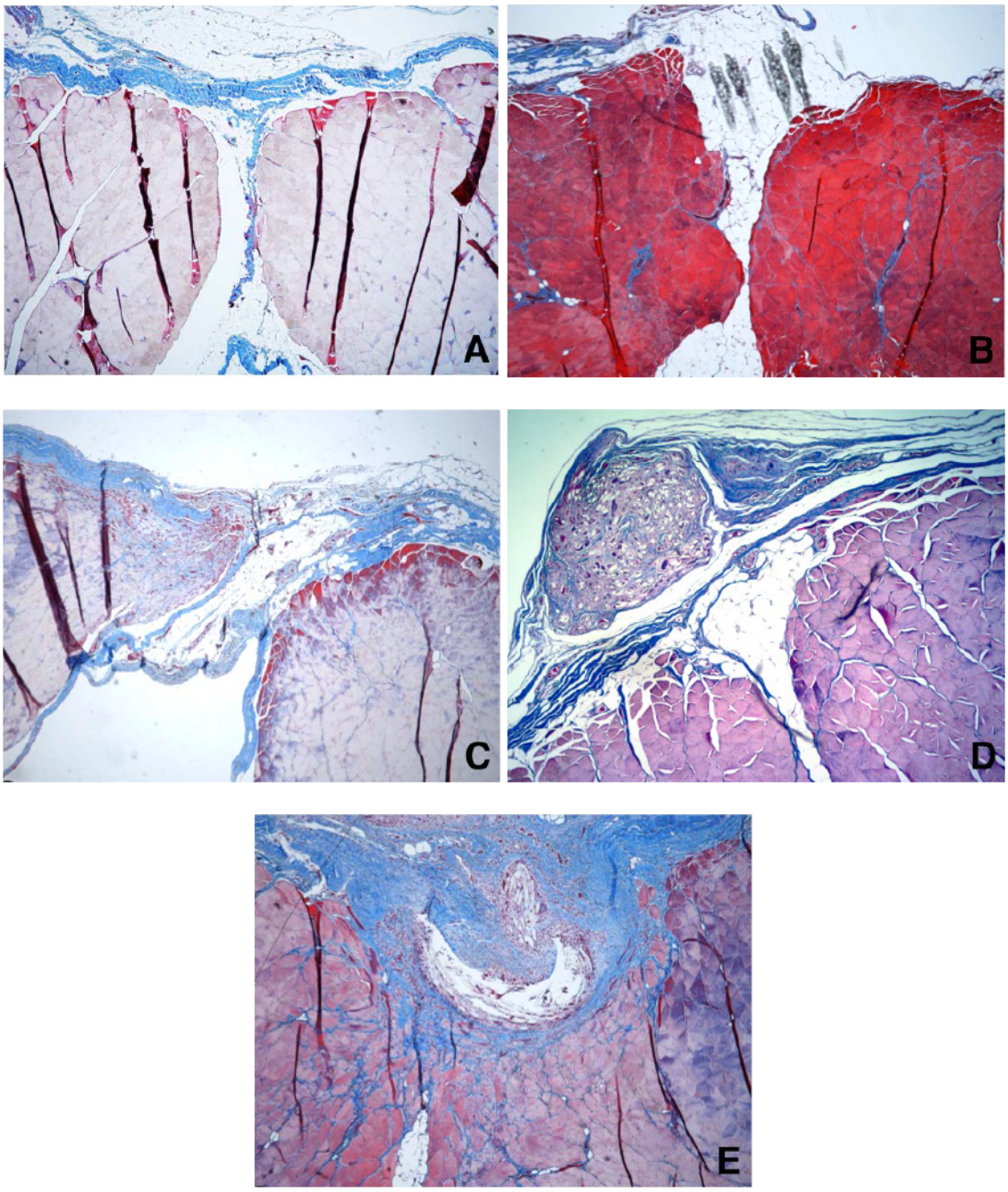

Figure 6: Wound Analysis of the Abdominal Fascia. Representative Masson's Trichome stained images of rat abdominal wall musculature following 4 weeks of varying treatments for fascial wound healing. Collagen appears as a vivid blue and muscle cells are stained pink/red. These images were used to determine wound characteristics including collagen abundance, collagen organization, and myocyte regeneration. All images were

taken at 40x magnification. (A) Unwounded abdominal wall tissue, with the image centered at the linea alba; (B) Wounded tissue (Control) which received only suture repair and no additional treatment; (C) Wounded tissue receiving 0.5mL PRP (Sham) applied over a band of 6x1 cm CollaTape; (D and E) Wounded abdominal tissue which received treatment with varying amounts of MSCs in a PRP suspension over CollaTape; (D) Tissue treated with $2.5x10^4$ MSCs; (E) Tissue treated with $1x10^6$ MSCs.

6D and 6E) exhibited increased muscle regeneration and were rich in collagen that was highly organized when compared to the control (Figure 6B).

Analysis of collagen abundance data showed that there was statistically significant difference between all groups receiving MSC therapy and the control ($p<0.01$) (Figure 7). There was a noticeable increase in collagen when treating the wound with only CollaTape and PRP (Sham group) when compared to the control group which received only suture repair. Furthermore, groups which also received MSC treatment generally had an increase in collagen deposition with increasing dosages of MSCs. However, only the groups receiving $1x10^5$ and $1x10^6$ MSCs were significantly higher than the sham group ($p<0.05$). There was a dip in the collagen abundance with groups receiving $2.5x10^5$ and $5x10^5$ cells. Furthermore, the group receiving the most MSCs ($1x10^6$) had more collagen deposition than the group receiving the least amount of cells ($2.5x10^4$ MSCs).

A similar trend was seen in collagen organization. The groups receiving MSC therapy generally had a higher degree of organization when compared to the control. There was also a mild decrease in organization for the groups receiving $2.5x10^5$ and $5x10^5$ cells. There was a statistically significant increase in collagen organization between the control and the groups receiving $1x10^5$ and $1x10^6$ MSCs ($p<0.05$). Only the group treated with $1x10^6$ cells was significantly higher than the sham group ($p<0.05$).

The sham group and the groups receiving MSC therapy exhibited much higher new muscle growth when compared to the control (Figure 9) and there was on average about an 8-fold increase in the sham and MSC-treated groups ($p<0.05$). However, there was no noticeable trend among the groups which received MSC therapy and were found to not be statistically different from one another or the sham group ($p>0.05$).

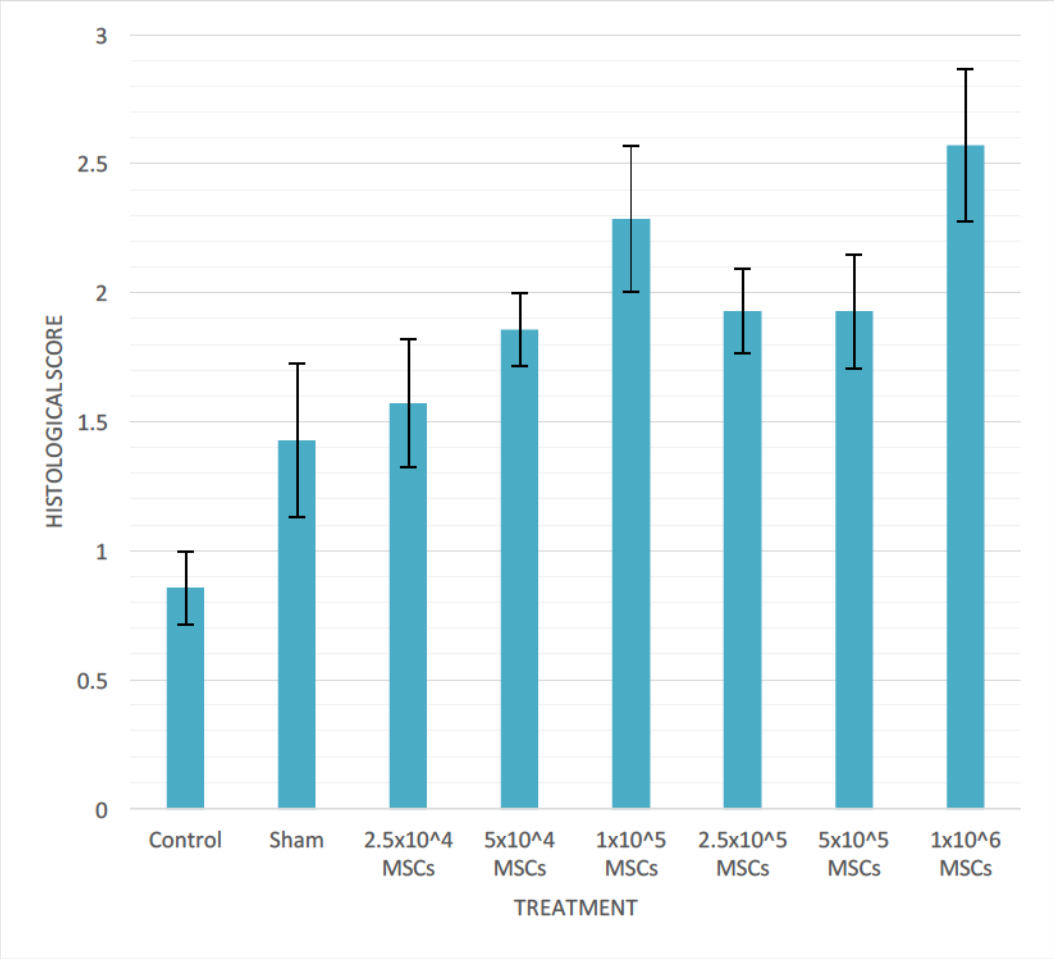

Figure 7: Collagen Abundance at MSC Treated Wounds. Histological determination of collagen abundance in wounds receiving varying MSC dosages. Abdominal wall tissue sections were stained with Masson's Trichrome to visualize collagen deposition at the wound site. Scores ranging from 0 to 3 were given to each sample by an observer to semi-quantitatively determine the amount of collagen fibers. Data represent the mean ± standard error of the scores for each group. Control groups received only suture repair and the sham group also had PRP and CollaTape but no MSCs applied to the wound. MSC treatment was performed using different concentrations of MSCs in a PRP suspension with CollaTape. The groups receiving MSC and sham treatment were statistically different from the control when analyzed by one-way ANOVA ($p=0.0019$). Groups receiving $1x10^5$ and

$1x10^6$ MSCs had elevated collagen deposition when compared to the sham group ($p<0.05$). N=14 for groups receiving $2.5x10^4$, $2.5x10^5$, and $5x10^5$ cells and N=7 for all other groups.

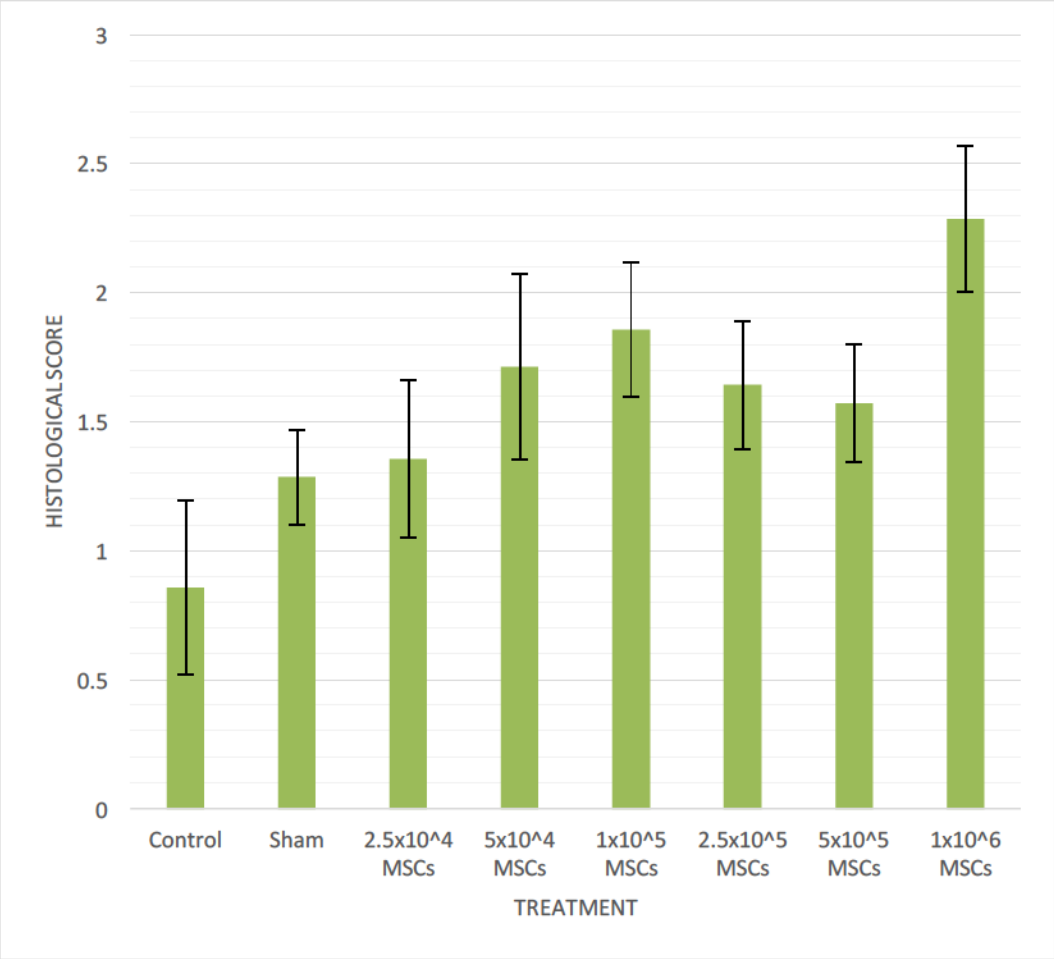

Figure 8: Collagen Organization at MSC Treated Wounds. Histological determination of collagen fiber organization in wounds receiving varying MSC dosages. Abdominal wall tissue sections were stained with Masson's Trichrome to visualize collagen fiber deposition within the wounded tissue. Scores ranging from 0 to 3 were given to each sample by an observer to quantitate the degree of collagen organization at the wound site, based on the degree to which collagen fibers were tightly packed and organized into parallel fiber bundles, with limited cellular infiltration. Data represent the mean ± standard error of the scores for each group. Control groups received only suture repair and sham groups had PRP and CollaTape but no MSCs applied to the wound. MSC treatment was performed using different concentrations of MSCs in a PRP suspension with CollaTape. The group

receiving $1x10^5$ MSCs had increased organization when compared to the control ($p<0.05$) and the group receiving $1x10^6$ MSCs had higher organization than both the control and sham groups ($p<0.05$). N=14 for groups receiving $2.5x10^4$, $2.5x10^5$, and $5x10^5$ cells and N=7 for all other groups.

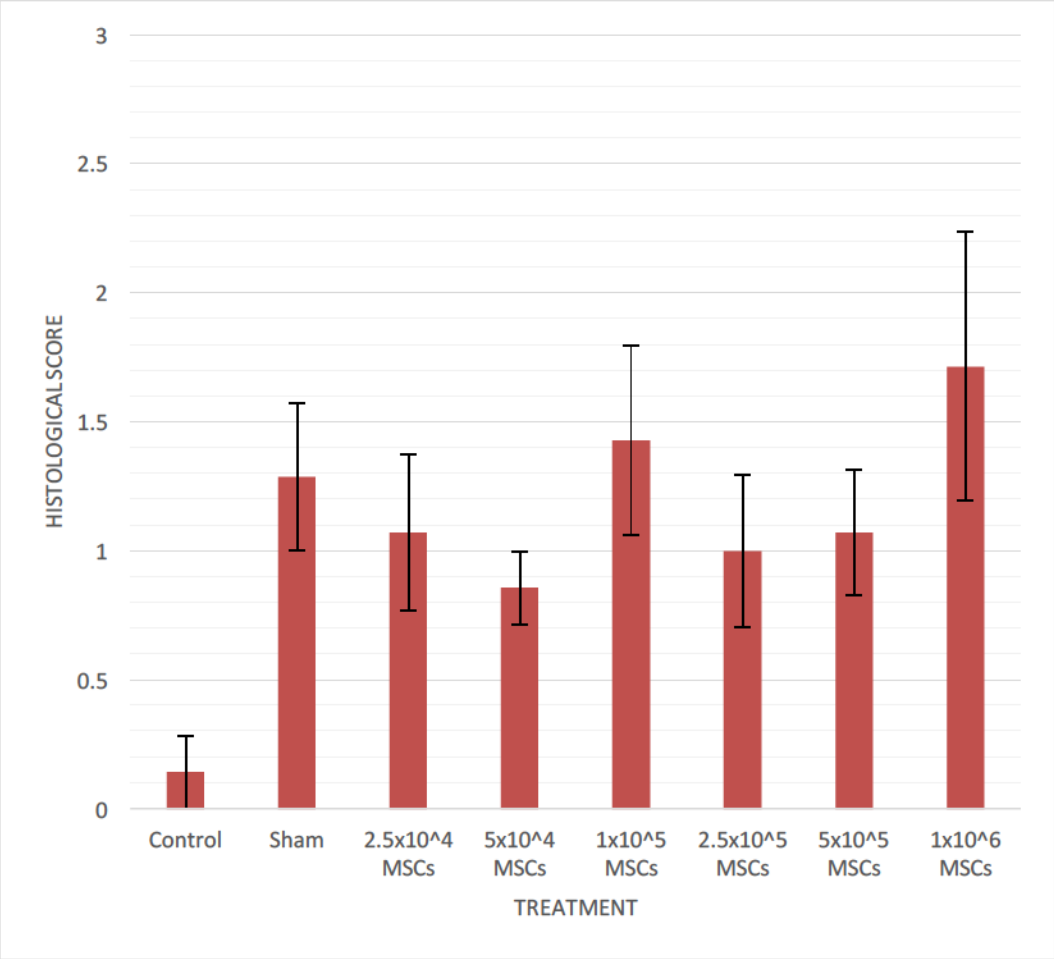

Figure 9: Myocyte Regeneration at MSC Treated Wounds. Histological determinations of myocyte regeneration in wounds receiving varying MSC dosages. Abdominal wall tissue sections were stained with Masson's Trichrome to visualize muscle regeneration at the wound site. Myocyte regeneration is evidenced by the presence of small packets of red-staining cells at the margins of the incision site.Scores ranging from 0 to 3 were given to each sample by an observer as a measure of new muscle growth at the wound edges. Data represent the mean ± standard error of the scores for each group. Control groups received only suture repair and the sham group also had PRP and CollaTape but no MSCs applied to the wound. MSC treatment was performed using different concentrations of MSCs in a PRP suspension with CollaTape. The groups receiving MSC and sham

treatment had increased muscle regeneration when compared to the control ($p< 0.05$). There were no differences among the groups receiving MSC treatment and the sham group. N=14 for groups receiving $2.5x10^4$ and $2.5x10^5$ and $5x10^5$ cells and N=7 for all other groups.

DISCUSSION

Experiments using rat and mouse models have been valuable in assessing the therapeutic potential of MSCs to promote tissue regeneration following injury (McFarlin et al., 2006; Wu et al., 2007b). The efficacy of these bone marrow-derived cells has been further demonstrated in the current study by determining their effect on abdominal fascia healing following a surgical incision. The purpose of this study was to determine effective concentrations of bone marrow-derived MSCs for improved wound healing. Specifically, the regeneration of tissue in the abdominal wall following surgical incisions was of interest. Herniation of incisional wounds remains a significant problem during surgery recovery with occurrence in about 10% of individuals receiving laparotomy procedures (Bucknall et al., 1982). With improved healing and strength of the fascia and abdominal tissue, the occurrence of incisional hernia post-surgery would decrease. The effectiveness of MSC application is dependent upon the number of cells applied to the incision (Falanga et al., 2007). Previous work from this laboratory has established the beneficial effects of using a dosage of $1x10^6$ cells in addition to PRP and CollaTape to treat incisional wounds (Hefner et al., 2012). The combination of supplemental biological and cellular additives such as PRP and CollaTape may synergistically magnify the therapeutic advantage of MSC therapy. We hypothesized that lower dosages of MSCs, in combination with PRP and CollaTape treatment, may provide similar benefits for enhancing fascial healing as seen in previous studies using higher doses of MSCs (Hefner et al., 2012). This study examined this hypothesis on abdominal wound healing in rats by looking for increases in collagen

organization, deposition, and myocyte regeneration following treatments with various dosages of MSCs.

Previous studies have demonstrated that MSCs can differentiate into a variety of mesenchymal-derived cell types (Azouna et al., 2012; Wu et al., 2007b; Goedecke et al., 2011). In the present study, it was found that the cells isolated from bone marrow and expanded in culture likely held a differentiative property consistent with mesenchymal stem cells by indicating an ability to develop into osteocyte, adipocyte, and chondrocyte cell lineages. Applying those BM-MSCs to incision sites resulted in augmented collagen deposition and organization after 4 weeks of healing. Wounds receiving $1x10^6$ cells had the highest degree of collagen abundance and organization while the individuals receiving only suture repair had the lowest. A significant difference in myocyte regeneration was not observed with an increasing dosage of MSCs; however, each group, including that which received only PRP and CollaTape, had increased new muscle growth when compared to rats which received only suture repair. Increasing the number of data points by performing surgeries on more subjects or by including histological scores assigned to the samples by several individuals could reveal statistical significance between the various groups for characteristics such as collagen organization and myocyte regeneration since trends in the current data suggest certain differences despite the lack of statistical significance.

Whether from bone marrow or adipose tissue MSCs obtained from different sources maintain similar phenotypic properties (Li et al., 2013; Lee et al., 2004). However, slight differences at the RNA and protein level have been observed, allowing the ability to trace the cells back to their tissue of origin (Lee et al., 2004). Therefore, a specific stem cell

source may be more desirable for treating a particular disease or a certain target tissue. No discernable differences resulting from MSC treatment of cutaneous wounds utilizing cells from varying sources have been identified, and therefore collection of MSCs from a readily available source, such as bone marrow, can be useful for studying their effects on healing (Liu et al., 2013).

Successful induction of cell differentiation into various cell types (adipocytes, osteocytes, and chondrocytes) demonstrates the stem nature of the cells isolated from bone marrow aspirates and subsequently expanded in culture (Dominici et al., 2006). The ability to differentiate into various tissues can also indicate the type of stem cell, since most are limited in which terminal lineages they may develop into (Seita and Weissman 2010). Thus demonstrating the differentiation of the cells isolated from the femoral and tibial bone marrow of the rats into adipogenic, osteogenic, and chondrogenic lineages, indicates that the cells utilized in the treatment of incisional wounds contain MSCs. Because the cells in culture appeared to differentiate, it disproves the notion that other plastic-adherent stromal cells or hematopoietic stem cells from the marrow are prevalent in the *ex vivo* culture as these other cell types cannot differentiate into mature, mesenchymal-derived cells (Seita and Weissman 2010). However, since we did not culture the cells from a pure population of stem cells and the cell cultures may still contain other stromal cells having similar morphology, the cells should be identified as mesenchymal stromal cells which include a stem population, as demonstrated by cellular differentiation.

The ability of MSCs to differentiate into discrete cell lineages provides a tremendous opportunity for treating numerous types of diseases and injuries. MSCs have proven to be a valuable for the treatment of damaged or malformed bone and cartilage

(Porada and Almeida-Porada, 2010). In addition, MSCs have been shown to produce fibroblasts, keratinocytes, functional skeletal muscle cells, neurons, cardiomyocytes, endothelium, and hepatocytes among other cell types (Parada and Almeida-Porada, 2010). With such an extensive differentiative repertoire, MSCs may be capable of integrating into a number of different tissues and organs and producing new cells.

Histological analysis suggested the MSCs that were expanded in culture were able to differentiate into more mature cell types, and thus may be capable of becoming bone, fat, or cartilage cells. The media used for culture was supplemented with additives to promote specific differentiation of the MSCs into a mature cell type. Previous studies have shown that the addition of glucocorticoids, such as dexamethasone, to the culture media can stimulate the *in vitro* differentiation of multiple cell lineages, including bone, fat, and cartilage cells due to its ability to induce certain transcriptional activities (Derfoul et al., 2006; Vater et al., 2011). Thus dexamethasone was added to each of the differentiation media used in this study. Osteogenic and chondrogenic differentiation media also included ascorbic acid since it has a mitogenic effect on the cells and increases collagen hydroxylation (Choi et al., 2008). In addition, β-glycerophosphate was added to osteogenic media to provide a source of inorganic phosphate, which is needed to produce the mineralized extracellular matrix of bone tissue (Vater et al., 2011). The chondrogenic media contains transforming growth factor β to promote glycosaminoglycan and type II collagen accumulation (Vater et al., 2011). Adipogenic media includes insulin, which increases triglyceride accumulation, as well as the additives 3-isobutyl-1-methylxanthine and indomethacin to prevent osteogenesis and to promote cellular commitment to the adipogenic lineage via transcriptional alterations (Vater et al., 2011).

Ultimately, the apparent differentiation of BM-MSCs was observed following treatment using media with these additives. Cultures of bone marrow-derived cells showed positive staining with Alizarin Red S, Oil Red O, or Alcian Blue after treatment with osteogenic, adipogenic, or chondrogenic differentiation media. These findings suggest that immature cells were capable of differentiation into more mature cell types. Thus the cells taken from rat bone marrow can now be tentatively identified as MSCs. However, since the cells were not sorted based on their phenotypic characteristics, it is likely that there are some additional types of cells, including mesenchymal stromal cells, which may be found in our cultures. A large proportion of the cells demonstrated the ability to differentiate, indicating that our bone marrow-derived cell population has a high prevalence of MSCs and the cells are appropriate for the purposes of this studying the ability of MSCs to promote wound healing.

Efforts are underway to optimize the delivery methods necessary to preserve and enhance the therapeutic benefits of MSC usage in wound therapy. Generally speaking, most therapeutic applications of MSCs dictate that exogenous cells are expanded *in vitro* prior to administration either systemically via the circulation or through direct/topical application (Chen et al., 2012). Understandably, topical application of MSCs was chosen for this study since it provides the most direct and practical modality for delivery and represents a simple methodology that may be applied to a clinical procedure.

The benefits of MSC application have been evident within the first few days following injury (Wu et al., 2007b; Chen et al., 2008), suggesting the cells largely affect the early stages of healing, which is defined by a high degree of cell recruitment and angiogenesis. In the study by Wu et al. (2007b), BM-MSCs were applied via intradermal

injections surrounding excisional dermal wounds in the dorsal skin of Balb/C mice. The number of MSCs remaining at the wound was drastically reduced within the first two weeks after application. About 10% were engrafted into the wound tissue after one week and only about 3% remained after two weeks. With progression of the healing process, cytokines and extracellular matrix molecules which are advantageous for MSC survival are likely to decrease and thereby causing MSC engraftment to decline (Chen et al., 2009b; Von Bahr et al., 2012). Accordingly, early administration of MSCs may be beneficial to attaining maximum regenerative capabilities. To adhere to this logic, the application of MSCs to incisions in our study was performed directly after injury.

Diverse types of synthetic and nonsynthetic biomaterials are commonly used in attempts to increase the functional benefits of the MSC therapy, based upon the type of injury needing treatment (Falanga et al., 2007; Lutolf and Hubell 2005). As performed in the current investigation, applying a scaffold such as a collagen band (CollaTape) on top of the incision may provide benefits for increasing engraftment and preserving the cells at the wound following application of the cell suspension at the abdominal surgical site (Marie et al., 2010). The CollaTape may act as an external niche for the MSCs to remain at the wound site, so that they may provide a more sustained paracrine influence on the local environment. Additionally, PRP increases MSC proliferation through platelet secretion of specific growth factors such as PDGF and TGF-β (Goedecke et al., 2011). PRP may also provide an array of other factors which also act synergistically to those secreted by MSCs to direct cell processes involved in tissue regeneration (Goedecke et al., 2011). Increases in collagen abundance, organization, and myocyte regeneration seen when using PRP and CollaTape alone indicate the potential to provide beneficial therapy. Since the

MSCs were applied along with PRP to the abdominal fascia, the therapeutic benefit of the cell suspension may be amplified.

Previous work in this laboratory have shown that using MSC, PRP, and CollaTape treatment results in a tremendous increase in the tensile strength of the abdominal wall (Hefner et al., 2012). A study by McFarlin et al. (2006) similarly examined the use of BM-MSC treatment to promote fascial repair in rats. The investigators made a full thickness incision in the abdominal fascia of Sprague-Dawley rats and applied $6x10^6$ BM-MSCs through local intradermal injections immediately after closing the wound. The wound breaking strength of the abdominal tissue following MSC treatment increased two- to threefold when compared to untreated individuals at one and two weeks following surgery. Additionally, the abdominal fascia had increased collagen deposition following MSC treatment at both the 7 and 14-day time points (wound tissue was homogenized and total soluble collagen (types I-IV) was measured colorimetrically using a collagen assay). An increase in the organization of collagen fibers was also noted in histological analysis. Based on the histomorphological characteristics of the wound tissue, it was concluded that there was a swift completion of the early phase of tissue repair involving inflammation and granulation tissue production. These outcomes are consistent with the results of the current investigation. The improvement of wound tensile strength can be attributed to various factors including new collagen synthesis, matrix deposition, and cellular migration. Thus an increase in the regeneration of fascial tissue and increased strength can be best ascribed to an increased amount of collagen in the wound. In the present study, the enhanced generation of collagen with certain MSC dosages, including $1x10^6$, may directly translate

to improved tissue strength during and after healing. This improvement could possibly reduce the incidence of incisional herniation in patients receiving invasive surgeries.

While increased collagen was noted in most of the animals receiving high MSC concentrations, there was a decrease in collagen organization and abundance in the individuals receiving $2.5x10^5$ and $5x10^5$ cells. This is curious, since a relatively linear increase would be expected with increasing MSC concentration up to a maximally effective dosage. At that point, there would likely be a plateau in these collagen characteristics and higher doses would result in marginal increases in the promotion of healing mechanisms and characteristics. Thus the therapeutic benefit would no longer be augmented by administering increasing amounts of MSCs. However, using high concentrations of these cells may even have an inhibitory effect on healing because of their potent anti-inflammatory and immunosuppressive properties, thereby reducing the recruitment of cells such as fibroblasts, keratinocytes, and endothelial cells, which are necessary for tissue regeneration and remodeling (Aggarwal et al., 2015; Selmani et al., 2008). In this instance, the early stages of healing may be highly truncated, with a significantly shorter inflammatory phase which is normally required to induce migration of native cells to the wound microenvironment. Dosage has been shown to be important in other studies. For instance, one investigation using a fibrin spray with MSCs to treat chronic wounds showed that dosages of at least $1x10^6$ per cm^2 of wound were necessary to induce therapeutic benefits in db/db mice. Doses larger than $2x10^6$ resulted in similar outcomes, signifying a diminishing return from the addition of increasing cell concentrations (Falanga et al., 2007). Another investigation using a rat brain injury model established that there is a responsive plateau, where the addition of increased numbers of MSCs could not further

enhance a beneficial effect (Wu et al., 2007a). The data from our study indicates that doses of as little as $2.5x10^4$ MSCs can provide favorable influence on healing.

In the early stages of healing, inflammatory cells provide growth factors which induce the migration and proliferation of fibroblasts at the wound where they subsequently begin to synthesize matrix components including type I and type III collagen (Jeon et al., 2010). The paracrine activity of MSCs complements endogenously produced factors and cytokines produced during wound healing that are involved in cellular processes such as proliferation, signaling, extracellular matrix synthesis, and angiogenesis (Chen et al., 2014). For instance, factors secreted by MSCs have been shown to induce collagen synthesis from dermal fibroblasts *in vitro* (Kim et al., 2007). Typically type III collagen is produced quickly after injury (Lokmic et al., 2006). Increased prevalence of immature type III collagen rather than the stronger type I collagen in the abdominal wall results in higher rates of herniation (Henriksen et al., 2010). Tissues with more abundant type III collagen have thinner fibers with decreased biomechanical strength (Henriksen et al., 2010). As healing progresses, type III collagen is degraded by MMPs and replaced by the more robust type I collagen to provide a stronger and more cohesive tissue (Lokmic et al., 2006). In the studies by Lokmic et al. (2006) there is large spike in apoptotic cells 3-4 weeks after cutaneous injury in normally healing wounds which can be attributed to increased ECM remodeling. In the current investigation, sample collection was performed in later stages of the wound healing process (4 weeks), so there would likely be a great deal of collagen modification occurring. Collagen fiber synthesis, tissue maturation, and collagen degradation by MMPs all play a significant role in dictating collagen composition

(Henriksen et al., 2010). Unfortunately, the current study does not distinguish between the different types of collagen which are deposited at the wound site.

When examining the tissue samples, there was a noticeable increase in the amount of myocyte regeneration in the sham and MSC treated wound tissues when compared to the control. Accordingly, MSCs and PRP likely have an advantageous effect on muscle growth. Factors secreted from MSCs and found in PRP including insulin-like growth factor (IGF) and fibroblast growth factor (FGF) result in increased muscle proliferation (Lacci and Dardik, 2010). This suggests that application of PRP may have an influence on healing by promoting regeneration of muscle tissue following injury. The application of MSCs did not improve muscle regeneration and PRP alone was sufficient to produce increased myocyte regeneration

While the present study shows an overall improvement and acceleration of healing processes, such as the deposition of new collagen fibers, it does not address the mechanisms by which topically applied MSCs contribute to increased fascial regeneration and strength. Wounds treated with MSCs mature at an accelerated rate compared to those which are allowed to heal naturally (Hefner et al., 2013). It remains unclear whether MSCs contribute to tissue regeneration directly through cellular differentiation or indirectly via paracrine factors. Attention must be paid to delivery systems which can serve to augment the regenerative properties of therapeutic MSC application. Ultimately, the use of this cell therapy is a powerful technique which holds the capacity to enrich healing of clinically problematic wounds and promote increased strength in repaired fascial tissue to prevent the occurrence of incisional herniation following invasive surgery.

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APPENDIX

Youngstown
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Monday, June 17, 2013

Dr. Diana Fagan
Department of Biological Sciences
UNIVERSITY

Re: **IACUC Protocol # 04-13**
Title: Determining dose of mesenchymal stromal cells needed to improve wound healing in a rat model.

Dear Dr. Fagan:

The Institutional Animal Care and Use Committee of Youngstown State University has reviewed the aforementioned protocol you submitted for consideration and determined it should be unconditionally approved for the period of June 17, 2013 through its expiration date of June 17, 2016.

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 June 17, 2014 and June 17, 2015. If you do not submit the forms as requested, this protocol will be immediately suspended. You must adhere to the procedures described in your approved request; any modification of your project must first be authorized by the Institutional Animal Care and Use Committee.

Good luck with your research!

Sincerely,

Dr. Scott Martin
Interim Associate Dean for Research
Authorized Institutional Official

sm:dka

C: Dr. Walter Horne, Consulting Veterinarian, NEOUCOM
Dr. Robert Leipheimer, Chair IACUC, Chair Biological Sciences
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