Optimization of Adult Multipotent Stromal Cell-Bioscaffold Interactions for Tissue Regeneration with Bioreactors

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OPTIMIZATION OF ADULT MULTIPO TENT Stromal CELL-BIOSCAFFOLD INTERACTIONS FOR TISSUE REGENERATION WITH BIOREACTORS

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Interdepartmental Program in
Veterinary Medical Sciences
through the
Department of Veterinary Clinical Sciences

by
Lin Xie
B.S., Xi’an Jiaotong University, 2006
M.S., Louisiana State University, 2011
May 2012
DEDICATION

This dissertation is dedicated to my father, Youzhong Xie, for giving me unconditional support and courage to pursue PhD degree in US. He is always my role model and is the best father in the entire world that a daughter could ever ask for. To my mother, Jinping Zhu, the kindest person I ever know, for loving me every second of her life. To my parents in law, Jianhua Huang and Chunjin Zheng, for loving me unconditionally from the first time they met me. Particularly to my wonderful husband, Wei Huang, for always being here loving, supporting and inspiring me. His work ethic and strong sense of responsibility encourage me to continue when I am deeply frustrated. I am so grateful that he influences me in all good ways and our love is so deep and mature that we grow together all these years while I can still and will always be that happy little girl every single day of my life just like when we first fell in love. Just like our big laughs when we walked out of lab at midnight. To my dearest daughter, Claire Lily Huang, for all her beautiful smiles, sweet voices and cutest kisses. No matter how much stress I have, the second I see her, it dissolves completely. She is and will always be my angel.
ACKNOWLEDGEMENTS

I would like to sincerely address that all of my accomplishments would not be possible without the support from all my colleagues at Laboratory of Equine and Comparative Orthopedic Research and Equine Health Studies Program. I am particularly grateful to the mentorship of my major professor Dr Mandi J Lopez. She has provided me a significant amount of support and advice during the past five years. She has always been making great efforts to maintain a very good laboratory for me to learn and apply my knowledge. She never hesitates to provide more opportunities for me to grow academically and professionally. I would also to express my appreciation to my committee member, Dr Jeffrey M. Gimble, who has offered me valuable insights on stem cell research. I am very thankful to my committee member, Dr Rebecca S. McConnico, who has always been there giving me encouragement and valuable advice when I am in need. Last but not the least, I am much obliged to my committee member, Dr W. Todd Monroe, for his help and support. I would still be very lost in research without their patience and guidance.

I would like to acknowledge and extend my heartfelt gratitude to my co-workers in the laboratory: Nakia Spencer, Nan Huff, Yanru Zhang, Prakash Bommala, Masudul Haque, Nan Zhang, Vanessa Marigo, Laura Kelly and Patrick Daigle. I could not have completed my research without all their help and unconditional support. I am so blessed that I can work in this lab with so many wonderful people. Thank you so much for being there for me like my family. I will always love you guys and we will continue to be part of each other’s life. I am also grateful for all the inspirations and encouragements from Dr Martin M Vidal, who is a pioneer in equine stem cell research.
My great appreciations also go to Dr Mustajab H. Mirza, Dr Laura M. Riggs, Mike Keowen and all EHSP personnel who helped with providing facilities, taking care of horses, and collecting cells. I would also like to show my deepest appreciation to Jackie Bourgeois, Rama Ramachandran and Michelle B Reed for their kind help and to Marilyn Dietrich and Xiaochu Wu for their expertise. Moreover, I would like to express my gratitude to all those who gave me the opportunity to complete my degree, Dr Thomas R. Klei, Dr Frank M. Andrews and Dr Dale L. Paccamonti, who are always so nice, kind and supportive. I will always remember your encouragements and will follow you as my models to treat others with a warm and sincere heart.
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ABSTRACT

Adult multipotent stromal cells (MSCs) in combination with biocompatible scaffolds may augment well-established medical and surgical techniques. Pre-implantation cell loading and viability on scaffold carriers followed by *in vitro* differentiation and extracellular matrix (ECM) formation contribute to the efficacy of *in vivo* tissue formation. Given the specificity of the parameters for each cell-scaffold construct, it is vital to assess the relative potential of various combinations to select the best option for various clinical needs. High demand for equine and human bone regeneration especially non-union fracture repair is still largely unaddressed. Therefore osteogenesis potential of both equine and human MSCs engrafted on bioscaffolds was comprehensively investigated in this study. After loading onto scaffolds, cells attached to scaffolds were assessed immediately and following different time of culture. Cell number, viability, distribution and differentiation were evaluated with DNA levels, confocal laser, light, and scanning electron microscopy (SEM) and RT-PCR. Both perfusion bioreactor and spinner flasks loading proved to be reliable and efficient procedures for seeding cells within bioscaffolds as well as achieving uniform cell distribution. Tissue specific micro- and ultrastructural changes were evident in equine cell-scaffold constructs cultured in induction medium. The versatility and suitability of type I collagen (COLI) scaffolds for equine adult MSC tissue regeneration was confirmed by generation of distinct mesenchymal tissues by paired adipose- (ASCs) and bone marrow derived multipotent stromal cells (BMSCs). *In vivo* study of equine MSCs on COLI scaffold further supported bone formation by BMSC constructs after short-term induction. However, ASC constructs produced comparative ECM as that of BMSC constructs but under commitment into dermal-like tissue. Pre-induction times as well as osteoinductive biomaterial addition to Coll
scaffold were optimized to improve efficiency of human ASC's constructs to commit osteogenesis. The customized bioscaffold was proved \textit{in vitro} as a promising carrier of MSCs to meet diverse clinical needs for osteogenesis. Overall, this research established straightforward and reproducible procedures to support customized tissue engineering with bioreactor loading of MSCs onto proper scaffolds. This achievement extensively supports customized equine tissue engineering to meet various clinical needs of equine bone regeneration in the near future.
CHAPTER 1. STEM CELL RESEARCH FOR REGENERATIVE MEDICINE: PRINCIPLES AND METHODOLOGIES
1.1 The Past Overview, Present Perspective and Future Challenges

In 2001, President Bush imposed boundaries on embryonic stem cell research because of the debated ethical issues. This caused limited research funding attributed to stem cell research. Since President Barack Obama lifted restrictions on federal funding for stem cell research in 2009, the previous policy that had restrained the development of stem cell research has been reversed. With recent advancement in the clinical application of stem cell research and the rapidly increased demand of stem cell application in clinics, an effort has been made to combine stem cell research with tissue engineering methods to broaden its prospective in regenerative medicine.

1.1.1 Major Stem Cell Research Breakthrough in Last Two Decades

From perspective of clinical value, the history of stem cells dates back to 1896, when E. Goujon showed that autologous marrow induces bone at heterotopic sites. A Keith conducted a classic experiment in 1919 revealing osteogenic activity from marrow, which was further confirmed by RG Burwell in 1961. The fact that multipotent stromal cells (MSCs) can be isolated from adult whole marrow was first proposed and proved later by AJ Friedenstein [1]. The importance of MSCs was further established by their key role in supporting hematopoietic cell proliferation *in vitro* [2] and transplantation *in vivo* [3]. Stem cells isolated from mouse embryos have the ability to proliferate indefinitely while maintaining pluripotency [4-5]. With the finding of MSCs and investigation of their behavior, bone marrow was applied for treating non-union bone fractures [6]. Bone marrow application was also found to provide cells for chondrogenesis and promote articular cartilage healing process. The milestone of this field was successful isolation of stem cell from human embryos [7]. Pluripotent stem cells were successfully cultured a few days later
from germ cells [8]. The research on stem cells has moved forward at rapid speed subsequently. Human embryonic stem cells were found to be capable of dedifferentiating into myeloid precursors by cell-cell fusion [9], which provides a model for investigation of nuclear reprogramming. The factors that induced this reprogramming were unveiled by groundbreaking work from Shinya Yamanaka when he identified a few defined factors (Oct3/4, Sox2, c-Myc and Klf4) crucial to cell stemness. He confirmed this finding by introducing these factors into fibroblasts, which successfully produced induced pluripotent stem (iPS) cells [10]. iPS cells, which displayed the essential characteristics of embryonic stem cells, were successfully produced one year later by introducing four factors (Oct4, Sox2, NANOG and Lin28) into human somatic cells [11]. A total of 13 lines embryonic stem cells were approved by US regulators for use in scientific research in 2009. Food and Drug Administration approved the study using embryonic stem cell research to treat human spinal cord injuries in 2010. It did not take long to apply this research accomplishment in clinics. On October 2010, the first patient with a spinal cord injury was treated in a U.S.-government-approved study by receiving millions of human embryonic stem cells.

1.2 Multipotent Stromal Cells Characteristics

1.2.1 Definition of Adult Multipotent Stromal Cell

Adult stromal cells represent a heterogeneous population. For example, only an estimated of 1 in 10,000 or 15,000 cells in bone marrow are marrow derived MSCs (BMSCs) [12]. BMSCs along with other stromal cells dispersed in tissues throughout the mature animal are referred to adult stromal cells in general. Adult stromal cells have the common characteristics as all stromal cells: self-renewal and differentiation. Cells divide into identical copies of themselves and maintain their differentiation capacity for a long period of time. For
example, equine MSCs proliferated for up to 30 passages before senescence in vitro [13]. Their ability to differentiate is a two-step process. Normally adult stromal cells give rise to intermediate cell types called precursors or progenitor cells. Later, these partially differentiated precursors or progenitor cells divide and give rise to specific differentiated cells. Although these two common characteristics of adult stromal cells are acknowledged worldwide, the definition of adult stromal cells is expanded into many levels with updated discovery in this field. To characterize adult stromal cells, more strict criteria have been set besides spindle cell morphology and cell capacity of differentiation [14].

1.2.2 Adult Stromal Cell Plasticity

Adult stromal cells from one tissue type have the capacity to differentiate into different tissue specific cell types. This is frequently referred to as “plasticity”, “transdifferentiation” or “unorthodox differentiation”. Various studies have demonstrated that adult stromal cells are capable of producing cell types besides the cell type in the tissue of origin under certain microenvironmental conditions. BMSCs give rise not only to skeletal muscle cells [15-16] and cardiac muscle cells [17-18] but also to liver cells [19-20], which are from the different mesoderm derivative. Furthermore, BMSCs can differentiate into neural tissue, which arises from the ectoderm germ layer [21-22]. Interestingly, adult brain tissue derived neural stromal cells could differentiate into hematopoietic cells [23]. The most possible reason might be heterogeneous populations of cells and co-infusion of multiple different stromal cells. Therefore for specific stromal cell plasticity research, it is established that stromal cells should be purified to ensure only one cell type is used and it should be labeled as well to track the stromal cells behavior and evaluate their engraftment into their new tissue environment.
1.2.3 Adult Stromal Cell Niches

The stromal cell niche and its interaction with stromal cells is an essential research topic in multipotent stromal cells biology. To date, majority of studies on adult stromal cell are conducted in vitro. Normally stromal cell niches cannot be reconstructed in vitro. Therefore adult stromal cell self-renewal and multipotentiality cannot be maintained for a long period of culture outside of the original niches in most cases. The complex molecular interplay of stromal cells and their niches has been intensely investigated so that artificial niches can be reconstructed in vitro.

Stromal cell niches are defined as a 3-D complex and controlled microenvironment that physically hosts the stromal cells in an anatomically well-defined location within a tissue[24]. Stromal cells are surrounded by extracellular matrix (ECM), growth factors, cytokines and other cells that provide a complex mixture of extrinsic cues. A variety of interactions are included: 1) cell-cell interactions such as cadherins, cell adhesion molecules and notch ligands; 2) ECM adhesion such as fibronectin, laminin, collagens and proteoglycan; 3) metabolic cues such as ions, hormones; 4) biophysical aspects such as physical forces, substrate elasticity and spatial arrangement; 5) soluble signals such as growth factors, cytokines and chemokines.

There are several proposed mechanisms for stromal cell niches [25]. Development of effective method is still in progress to identify niche cells from other specific cell types. These questions need to be addressed to inspire the application of stromal cell niche in vitro and eventually promote tissue regeneration in clinics. Tissue engineering plays an important role on providing robust spatial and temporal control of biophysical and biochemical microenvironmental cues for adult stromal cell to maintain their multipotentiality.
Bioscaffolds, as temporary structural supports for directing cell growth, create powerful artificial microenvironments to regulate cell function to promote tissue regeneration and repairs [26]. Secreted ECM surrounding cells on bioscaffolds is capable of attracting cytokines, growth factors and nearby progenitor cells to accelerate tissue regeneration. Optimization of cell-bioscaffolds interactions promise to have a profound impact on adult stromal cell biology and provide insights that will advance adult stromal cell-based clinical approaches to tissue regeneration.

1.2.4 Multipotent Stromal Cell Homing
The capacity of MSCs in tissue regeneration depends largely on its migration to injury sites. The homing of leukocytes is directed by various chemokine receptors, ligands and adhesion molecules, which also stimulated trafficking of hematopoietic precursors into and through tissue [27]. Adhesion molecules, such as selectins, β1-integrins and their respective counterligands, play an important role in mediating contacts between microvascular endothelial cells and blood leukocytes [28]. Then integrin activation is triggered by chemokines presented on endothelial cells. The leukocytes that carry the corresponding receptors are arrested by corresponding chemokines as well. The adherent leukocytes move across the endothelial cell and then get access into the injured tissue.

Similar to leukocytes, MSCs is capable of migration into tissues from the circulation in response to upregulated signals under injury conditions. It is possible that the similar group of adhesion molecules, chemokines and their receptors are involved. Overall, there are a variety of chemokine receptors that play an important role in MSCs trafficking. For example, stromal cell-derived factor-1 (SDF-1) and its receptor have been reported to assist migration of certain progenitor cells and homing of hematopoietic stromal cells, respectively. Research
on SDF-1 has proved its therapeutic potential in tissue regeneration [29]. In response to injuries, cells with highly expressed SDF-1 caused increased SDF-1 levels at damaged sites. This leads to recruitment and retention of circulating progenitor cells. For tissue engineering applications, corporation of SDF-1 protein into scaffolds improved the regeneration of the damaged tissue or organ [29].

1.2.5 Multipotent Stromal Cell Immunophenotyping

The immunophenotyping of adult stromal cells has recently become very popular for the purpose of identifying the presence and proportions of the various cell populations of interest. Flow cytometry is preferred because of its single cell based accuracy and multiple efficient measurements to characterize the specific antigens expressed on cells. Research has been conducted to characterize specific stromal cell population and thus achieve the goal of accurate cell sorting.

The following antibodies have been previously validated as specific markers for their respective molecules in human and horses. CD29, also known as integrin beta 1, has diverse roles in several biological processes including cell migration during development and wound healing, cell differentiation, and apoptosis by mediating interactions between adhesion molecules on adjacent cells or the extracellular matrix. CD 44 is a cell-surface glycoprotein involved in cell-cell interactions, cell adhesion and migration as well. It is a receptor for hyaluronic acid (HA) and also interacts with other ligands, such as osteopontin, collagens, and matrix metalloproteinases (MMPs). CD73, known as ecto-5’-nucleotidase, is largely expressed on the vascular endothelium and is useful for lymphocyte adhesion and differentiation. CD90, known as Thy-1 membrane glycoprotein, is considered as a fundamental regulator in cell-cell and cell-matrix interactions, with important roles in nerve
regeneration, metastasis, inflammation, and fibrosis. CD105, known as an endoglin, participates in cell adhesion and embryonic angiogenesis expressed in vascular endothelial cells and some bone marrow cells and activated macrophages. Both CD34 and CD45 are considered as common hematopoietic cell surface markers.

It has been found that the phenotype of the culture-expanded MSCs was similar for all fetal tissues (bone marrow, liver, lung, and spleen), i.e. CD90, CD105, CD166, SH3, SH4 and HLA-ABC positive and CD34, CD45 and HLA-DR negative [30]. Rat MSCs have been tested to be CD11b-, CD45-, CD29+, CD49e+, CD73+, CD90+, CD105+ and Stro-1+ [31]. In human, MSC from bone marrow, umbilical cord and placenta were positive for multipotent cell marker (CD29, CD44, CD105, CD73 and CD90) [32]. Equine BMSCs had shown to be negative for CD45 and CD34, and positive for CD44, CD29 and CD172a [33]. It has been shown that cell immunophenotype varies not only based on the specific stromal cell type but also in response to passage changes. For example, MSCs are identified by a CD45- and CD106+ phenotype normally. However, mouse MSCs achieve this characteristic phenotype only at later passages. With increasing passages, CD45 expression shifted to negativity, whereas CD106 expression became increasingly positive [34]. Because cell population becomes more homogenous after passages but its multipotentiality may decrease over time. Therefore optimal cell passage should be chosen based on the profile of its cell surface marker.

1.2.6 Isolation and Culture of Multipotent Stromal Cells

MSCs isolation and expansion techniques are vital to substantial progress in MSCs application in clinical trials of tissue regeneration. The low isolation rate, slow MSCs proliferation and low differentiation rate is partly attributable to suboptimal isolation and
expansion conditions. There are several influential factors that will be discussed, including oxygen level, cell plating density, substrate used for cell isolation and culture as well as mechanical forces applied on cells. Among these, oxygen level and mechanical stimulation can be controlled by bioreactors to provide a suitable environment for cell growth to promote tissue regeneration.

1.2.6.1 Oxygen

The physiology of MSCs is affected by the oxygen level in many aspects, including cell attachment, growth, differentiation and tissue regeneration capability [35]. MSCs respond differently to hypoxia condition in vivo and in vitro. It is important to characterize their behavior in order to acquire optimal in vitro culture and successful in vivo implantation by adjusting oxygen level [36].

Because hypoxic conditioning modulates MSCs paracrine activity, activates signaling pathways that control cell cycle rates and upregulate various important genes that increase their migratory phenotypes [35, 37]. In cell culture, hypoxia results in increased cell proliferation rates and cell migration. Furthermore, hypoxia enhanced cell differentiation along different mesenchymal lineages. It contributed to adipogenesis of human MSCs without affecting PPAR-gamma2 maturation pathway [38]. A previous study on rat MSCs showed that hypoxic conditioning had beneficial effects on chondrogenesis [39]. In order to adapt to low oxygen tension in vivo, MSCs were pretreated with hypoxic environment, which avoided massive hypoxia-induced cell death after implantation [36]. Hypoxic pretreatment was also considered as an effective way to overcome hypoxia-induced inhibition on osteogenic differentiation in vivo [40].
Although the effects of hypoxia have not been well established, the current achievement in finding its role in cell survival, proliferation and differentiation provides us the guidance for its application in tissue engineering. Proliferation rates of MSCs can be increased and its multipotency towards different mesenchymal lineages can be enhanced under hypoxia condition, which could potentially decrease the time needed for cell expansion and induction \textit{in vitro}. Long-term cell survival \textit{in vivo} could be achieved by hypoxic preconditioning of MSCs. Perfusion bioreactors can provide cells with controlled oxygen level. It has been shown that perfusion bioreactors in the culture environment of 30\% O2 level improved cell adhesion and expansion in contrast to those in 5\% O2 where expansion rate was 3 times lower. Bioreactors provided controlled O2 conditions to promote efficient cell productions [41].

\textbf{1.2.6.2 Mechanical Stimulation}

Cells are surrounded by a highly complex biomechanical environment \textit{in vivo}. Unveiling the mechanical effects on cells \textit{in vivo} could inspire the design of bioreactors and thus provide suitable environments for cell growth \textit{in vitro}. In general, mechanical conditioning has the potential to stimulate cell growth and tissue formation. But there exist wide variations for the effects of mechanical conditioning among different cell types and different mechanical forces may exert different effects as well. It is vital to establish the effects of specific mechanical forces on the specific tissue formation. Thus optimized bioreactors can be tailored to meet the needs of specific cell type.

Osteocytes, the most mechanosensitive cell type in bone [42-44], are active in bone adaption to mechanical stimulation. Signaling molecules such as nitric oxide (NO) and prostaglandins are produced in response to shear stress stimulation exerted by fluid flow, which play
important roles in the change of functional and structural properties of bone adapted to mechanical load in vivo [45-46]. Rapid increases in prostaglandin production induce fully differentiated bone cells in vitro [43, 47-48]. Evaluation of osteopontin (OPN) and collagen type Iα1 (COL1A1) expression is a common tool to assess bone cell mechanoresponsiveness [49-51]. This established mechanism of bone cell response to mechanical forces provides a tool to investigate responses of BMSCs and ASCs to mechanical conditioning [52]. The mechanosensitivity of BMSCs and ASCs indicate their potential for musculoskeletal tissue engineering.

1.3 Bioscaffolds

Scaffolds play a significant role in regenerative medicine by creating a structural environment to support cellular interactions and new tissue formation. Scaffold selections take into consideration the biodegradability and biocompatibility properties, which are determined by the material composition. Researchers must further modify the porosity and pore size of scaffolds to improve nutrient transfer and cell adhesion capacity. This is key to successfully decreasing the possibility of necrotic center formation. The challenge of scaffold design, however, is to improve a scaffold’s porosity and pore size without compromising its mechanical properties.

1.3.1 Biomaterial

To present, scaffolds used in tissue regeneration can be categorized into three main types: ceramics, natural and synthetic polymers. To meet a variety of clinical needs, copolymers (made up with different polymers) and composite scaffolds (made up with both ceramics and polymers) are introduced to combine desired properties from different biomaterials.
1.3.1.1 Ceramics

The two main components of ceramics are β-tricalcium phosphate (β-TCP) and hydroxyapatite (HA). The chemical composition for β-TCP and HA is Ca$_3$(PO$_4$)$_2$ and Ca$_{10}$(PO$_4$)$_6$(OH)$_2$, respectively. Biocompatibility of β-TCP and HA makes it an excellent candidate for osteoinductive scaffolds because of their resemblance to bone inorganic component. These scaffolds provide osteoconductivity as well and may bind directly to bone to support bone ingrowth [53-55]. Previous studies have unveiled that calcium phosphates support the attachment, differentiation and proliferation of osteoprogenitor cells [56]. But the low mechanical strength limits their direct application in bone regeneration, especially at load-bearing positions.

Bioactive glass is currently regarded as the most biocompatible material in the bone regeneration field because of its excellent bioactivity, osteoconductivity and osteoinductivity [57]. This material, which shows direct bonding to living bone, is likely to open the door to the development of new nanostructured bone regeneration materials. Moreover, this scaffold can accelerate osteoconduction in bone defects with coated bone-like apatite on the surface [58].

1.3.1.2 Natural Polymers

The common natural polymers are collagen and silk. Collagen, a major component of ECM, has the advantage of biocompatibility, biodegradability and efficient cell capture for tissue repair. In previous studies, collagen/hyaluronate matrix demonstrated high biocompatibility and osteoconductivity in treating cranial defects in rats [59]. Tibia rat defects implanted with collagen scaffolds presented a low inflammatory response and bone formation within a short period of time [60]. Collagen is considered a promising choice for bone regeneration
because of the low cost of production associated to the biocompatibility and osteoconductivity. In addition to its osteoconductivity, collagen scaffold has also been used for mice dermal regeneration because it supports cell infiltration and vascularization [61]. However, collagen scaffolds also have low biomechanical properties and relatively high degradation rates, which excludes them from use in load-bearing sites [62]. To overcome these issues, chemical modification is introduced by cross-linking to decrease degradation rates and improve mechanical strength. Collagen scaffold, which was cross-linked by carboxyl-amine chemistry, demonstrated significantly decreased degradation rates compared with collagen scaffold [63].

Another alternative to collagen scaffolds are silk-based scaffolds. This idea was originally inspired by long-term use of silk as suture material because of its biocompatibility as well as excellent mechanical properties [64-67]. Porous silk scaffold can be produced by dissolving the silk fibroin produced after lyophilizing the aqueous silk solution [68]. Previous studies showed similar degradation rates between silk-based scaffolds and cross-linked collagen constructs [63]. Human BMSCs proliferate and differentiate on silk scaffolds [69] and form trabecular-like structures under static culture conditions [70]. Moreover, osteogenesis on silk scaffolds could be improved by increasing its pore size [71].

1.3.1.3 Synthetic Polymers

The customized scaffolds for different tissues are required with the rapid development of tissue engineering. Synthetic polymers provide diversity by altering fabrication parameters such as changing monomeric units, their connectivity and microstructure. The morphology and porosities as well as pore sizes can be modified to match tissue specific needs for mechanical properties and rates of degradation. Increase in porosities allows for adequate
diffusion but it should be adjusted without compromising its mechanical properties. Saturated poly-α-hydroxy esters, such as poly-l-(lactic acid) (PLLA), poly-(glycolic acid) (PGA) and poly-(lactic-co-glycolide) (PLGA), polyethylene glycol (PEG) are considered among the most biodegradable synthetic polymers for 3D scaffolds and have wide applications in tissue engineering.

Because of their porous tubular structures, PLGA and PLLA scaffolds have promising potential for tubular tissue regeneration like blood vessels, peripheral nerves, intestines and long bone [72]. Previous study have also shown that fabricated PLGA with an interconnected porosity was capable of promoting tissue in-growth in teeth [73]. For in vitro culture, scaffolds with the multi-pore sizes and the 2-layer provided more efficient nutrient and oxygen exchange environments for cell culture. With this advantage, synthetic polymers promoted more efficient human dermal fibroblast cells proliferation significantly compared with uniform-pore size scaffold [74].

In the other studies, synthetic polymers were coupled with peptide to improve human osteoprogenitor cells adhesion, migration, expansion and differentiation to promote new bone formation [75]. Furthermore, better bone ingrowth into the scaffold and bone formation was shown in skull defects of rabbits by PLGA/polyvinyl alcohol blended scaffold compared with PLGA only scaffold [76]. In addition to chemical modifications, extracellular biomaterials can also be introduced to scaffolds to promote cell attachment and proliferation. PLGA scaffolds mixed with porcine ECM recruited a significantly larger number of BMSCs that contributed to the subcutaneous implant site remodeling [77].
In addition to chemical modifications, physical modifications were introduced as well to change geometry of a scaffold and thus verify its regulations of cell activities. Early studies on a PGA scaffold made of 13 µm microfibers demonstrated highly effective chondrogenesis based on morphology and composition of its constructs seeded with chondrocytes [78]. PLLA scaffolds composed of nanofibers and seeded with chondrocytes promoted more efficient cartilage regeneration compared with that of microfibers [79]. Nanofibrous PLLA scaffolds were also loaded with human BMSCs to accelerate cartilage regeneration in clinics. Compared with autologous cartilage grafts, cell-scaffold constructs grew into better predefined dimensions and shape to repair cartilage defects [80].

1.3.2 Scaffolds for Bone Regeneration

In order to find a compatible scaffold for bone regeneration, the material composite of bone should be considered. Bone consists of two parts, the inorganic part and the organic part. The inorganic component is mainly calcium and other mineral salts. Calcium is present in the form of hydroxyapatite with composition \( \text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2 \). About 60% to 70% of the dry weight is made by these minerals. The organic part is comprised of type I collagen and ground substance. The ground substance, primarily formed by proteoglycans, is interconnected with mineralized collagen fiber, and thus develops into extracellular matrix[81]. This explains why majority highly investigated scaffolds have these two components.

1.3.2.1 Osteogenesis, Osteoinductivity and Osteoconductivity

These three properties of implants, osteoconductivity, osteoinductivity and osteogenesis, are essential to the success of bone healing. Osteogenesis means that there are viable cells from implants that contribute to the new bone formation. Cytokines, growth factors, hormones are
transferred by implants to modulate host cells, which is osteoinductivity. The matrix provided by implants supports new bone formation that is perpetuated by the native bone, which is called osteoconductivity.

1.4 Bioreactor Designed for Tissue Engineering

The uses of equine stromal cells to address equine tissue regeneration needs have become increasingly popular over the last decade. However, no current applications of equine stromal cells include cell seeding, culture and differentiation on a scaffold inside of a bioreactor[82]. In order to establish a protocol for efficient in vitro tissue regeneration by equine stromal cell, a custom tailored bioreactor should be designed. There are three established types of bioreactors. The three types of bioreactors will be described here are spinner flask, perfusion bioreactors and rotating wall vessel (RWV) bioreactors.

1.4.1 Bioreactor Components and Assembly

Spinner flasks are cost effective, easy to operate and fit scaffolds with different sizes. Additionally, using spinner flasks can increase the mixing of oxygen and nutrients throughout the medium during cell culture compared with static culture. One spinner flask can accommodate higher number of scaffolds compared with other two bioreactors. All the scaffolds within the same spinner flask are provided with the identical loading condition. However, spinner flasks certainly have limitations that other dynamic cell cultures do not have. A needle is applied through the neural axis of scaffold for fixation during loading. Therefore, this will affect the integrity of subsequent tissue formation given the center of scaffold including a hole penetrated by the needle. Moreover, the amount of time required for seeding is longer and the cell seeding efficiency is lower at low cell concentrations compared with other dynamic systems [83]. In addition, spinner flask culture causes non-
uniform distribution of cells [84] with a higher density of cells on the scaffold surface[83]. The turbulence generated by stirred medium causes tissue disintegration by releasing newly formed matrix in medium[85]. If it is gently stirred, the mechanical stress generated may not be sufficient to induce differentiation [86-87].

Perfusion bioreactor shows higher seeding efficiency, more uniform cell distribution [84] in a relative short loading time compared to the other two bioreactors. Its capability of both seeding and culture could minimize the chance of contamination [88-89]. This bioreactor can also enhance matrix deposition because of its adjustable flow rate to balance between the nutrition transfer and the waste disposal as well as to balance between the growth of matrix and the fluid shear stress [35]. Compared to spinner flasks, the perfusion bioreactor is more expensive, more time-consuming for assemble and requires adjustments periodically. Additionally, perfusion bioreactors cannot avoid cell aggregates forming within scaffolds because cell seeding and culture is still in 2-dimensional (2D) space without any diffusional flow at horizontal direction. There will be a necrotic center in the cell aggregate. For stromal cell differentiation, there is enhanced osteogenesis supported by BMSCs [90-92]. Compared with static loading on starch-based polymer scaffold, more homogeneous bone graft with better quality was achieved by perfusion bioreactor loading [93].

RWV bioreactor has higher mass transfer rate because of dynamic laminar flow to reduce diffusional limitations and exerts low shear stress [83]. Cells are naturally gathered, which is called cell apposition, without forming cell aggregates. Close cell apposition provides optimum distance among cells and allows normal cell-cell interactions, which promote cell proliferation and differentiation via specialized cell adhesion molecules and autocrine or paracrine cell signaling [86-87]. In addition, RWV bioreactors can develop a spatially
extended ECM because they provide 3-dimensional (3D) communications for both cells and scaffolds [94]. In conclusion, among these three bioreactors, RWV bioreactor provides controlled microenvironment more analogous to natural cells and tissues. It has been shown that RWV bioreactors promote osteogenesis of stromal cell. The condition of RWV bioreactors should be optimized based on different requirements for differentiation [95].

1.4.2 Requirement for Bioreactor Designs

The first and most important step of 3D stromal cell culture is cell seeding and it is crucial in determining the progression of subsequent tissue formation. For clinical application, there would be less amount of biopsy required for tissue collection and less time needed for cell expansion if high cell seeding efficiency can be achieved. Higher seeding enhances tissue formation by increasing the number of cells attached to scaffolds. The initial distribution of cells in the scaffold after seeding determines the distribution of the subsequent tissue formation. Uniform cell seeding could facilitate uniform tissue generation. Therefore, to compare the advantages and limitations of these three bioreactors, efficiency and uniformity of cell seeding are important factors to be considered. Stromal cell differentiation is mainly determined by mechanical stimulation controlled by bioreactors [95-96]. There are established studies on mechanical stimulation to induce ligament regeneration from MSCs [97], which displays the possibility of achieving multipotentiality or pluripotentiality of stromal cell in vitro by controlled bioreactors and lays the ground for tissue repair through regeneration medicine.
1.5 Applications

1.5.1 Adipogenesis
The fact that BMSCs and ASCs can both show adipogenesis potential is not only used to confirm the multipotentiality of BMSCs and ASCs but also have potential applications for clinical uses. In human medicine, there is a wide range of adipose tissue applications such as in treating burns, avulsions, lipodystrophy, post-surgical defects and so on. In equine medicine, the applications are limited due to fewer uses in these problems for horses and thus they are still largely unexplored. Because of the capacity of BMSCs and ASCs differentiation into adipocytes and the similar cell types, it is highly anticipated that BMSCs and ASCs transplantation would promote wound healing. It has been shown that BMSCs played a significant role in treating wound healing in rats [98-99]. In addition, adipocytes carry out important endocrine functions and can be produced by BMSCs and ASCs to meet different endocrine needs.

1.5.2 Chondrogenesis
Up to date, there is a continuous demand for seeking effective remedy for cartilage repair in equine medicine. Horses are at high risk of getting focal articular cartilage injuries especially for sporting horses. A lot of surgical techniques have been developed to promote cartilage regenerations. For example, cell transplantation using autogenous or allogenous chondrocytes is used frequently to support healing over the past decade [100-106]. However, because of the inner environment of cartilage, it is normally isolated from circulation and thus the repair is not effective especially for adults. The chondrogenic capacity of BMSCs and ASCs provides a more effective alternative to promote repair. Currently a lot of cartilage repair stimulated by adult MSCs implant have been successfully conducted in rats [107],
rabbit [108-109], dog[110] and goat[111]. These studies showed promising results. The study of using this method for cartilage repair conducted in equine showed that the BMSCs implantation accelerated healing in a short term, proving it as a straightforward, cost-effective and clinically effective approach for cartilage repair[33, 112-113].

1.5.3 Osteogenesis

Although the surgical and medical techniques for bone repair are well established in human medicine, there is still a largely unmet need for efficient, quick and satisfactory bone healings to accommodate the variety of patient conditions such as non-union fracture, congenital cleft palate, lesions caused by removing bone cancer, periodontal disorders and injuries associated with a severe car accident. Acute bone injuries are common for athletic horses. If they cannot be addressed efficiently in time, horses may develop degenerative disease and lameness. High demand for equine efficient bone regeneration is still largely unmet.

Both autograft and allograft are applied to provide fine alternatives for bone repairs. However, their limitations should be taken into considerations. Autograft is quantitatively limited and has risks in causing morbidity, pain and possible infection of the donor site. Allograft could cause immune rejection and disease transmissions[55, 107, 114]. Therefore, current methods for bone grafts should be optimized on osteogenesis of MSC alone or with them engrafted on the biocompatible scaffolds suitable for bone generations.

It has been shown that bone healing of rat with a femoral segmental defect was facilitated with MSCs derived from bone marrow[107]. MSCs with a high aldehyde dehydrogenase activity showed efficient fracture healing with a short period of time[114]. In equine patient, cells mediated with osteoinductive gene promoted the rib drill defects by increased bone
filling volume and mineral density[115]. However, long-term risks of viral vectors need to be taken into considerations such as immune and inflammatory responses, toxicity and potential risk for wrong DNA integration. Pre-induced adult equine MSCs seeded on bioscaffolds can provide a promising alternative for bone repairs.

The cell-scaffold constructs have been applied to produce inductive bone graft both in vitro and in vivo. BMSCs loaded onto biophasic calcium phosphate scaffolds by perfusion bioreactors system produced clinically relevant and viable amount of tissue-engineered bone after implantation in nude mice[116]. BMSCs and ASCs loaded on mineralized collagen sponges showed new bone formation at a critical size defect on the sheep tibia[117]. Therefore engineered bone constructs can provide a customized and potentially permanent cure of specific bone injuries in equine medicine.

1.6 Applications in Equine Veterinary Medicine

Injection of MSCs isolated from bone marrow aspirates, adipose tissue or umbilical blood into lesions is most commonly used in equine clinics. This technique is referred to as equine stem cell therapy. Stem cell therapy is considered as a simple, one-step, autogenously, and arthroscopically applicable treatment. Equine adult MSCs has potential to restore normal tissue structure and function. This is shown not only by in vitro laboratory work but also by a large number of clinical cases treated successfully by current stem cell therapy with fast recovery rate and low rate of reinjures compared to other therapy. Full thickness cartilage defects have been successfully treated with BMSCs injection [33]. Majority of MSC therapy applied to treat orthopedic lesions are frequently used to accelerate tendon and ligament repair.
However, current stem cell therapy certainly has some limitations to impede its further development. It still takes time for the healing to take effect. For some conditions especially acute injuries, if it cannot be addressed efficiently, horse may develop degenerative disease and lameness. High demand for equine bone regeneration is still largely unmet. How stem cell therapy works is not completely understood and the treatment was applied mainly based on empirical data. For example, there is uncertainty of exact dose and frequency of the treatment to repair different injuries. More research is required to learn how and why stem cell therapy work in a certain way and to investigate how to employ MSCs to optimize its ability to heal a particularly tissue in order to reach their full potential. The wide availability of multiple cell sources and bioscaffolds provides more alternatives for clinician to consider, and may augment well-established medical and surgical techniques. But optimization of their interactions is still largely undone in equine medicine. Solid evidence-based research is necessary to evaluate their interactions in vitro as the first critical step, to promote its application for the best possible cell growth, differentiation and subsequent in vivo function.
CHAPTER 2. *IN VITRO* TISSUE GENERATION BY ADULT EQUINE MULTIPOTENT STROMAL CELLS ON COLLAGEN SCAFFOLDS
2.1 Introduction

Adult equine multipotent stromal cell (MSC) research supports their potential to restore normal tissue structure and function. The use of adult equine MSCs to address equine tissue regeneration needs is a promising research focus [118-130]. Use of adult equine MSCs in combination with biocompatible scaffolds may augment established medical and surgical techniques. To derive the most benefit from clinical equine MSC applications and propel the technology into clinical trials, it is necessary to optimize interactions between the cells and biocompatible scaffold carriers in vitro for the best possible cell growth, differentiation and subsequent in vivo function.

An appropriate scaffold, favorable environment and multipotent cell source are three major components of tissue engineering [82, 131-133]. Based on published information, cell growth and differentiation efficiency in vitro are different between adult equine BMSCs and ASCs [13, 134]. Biocompatible scaffolds are designed to support cell growth and extracellular matrix formation for efficient tissue repair. Given distinct differences between cells isolated from different equine stromal cell sources, scaffolds tailored to each type are critical to their application for tissue regeneration. Some considerations in this regard include scaffold composition, stromal cell loading and pre-implantation culture.

Cell seeding is one of the first processes of three-dimensional stromal cell-scaffold construct generation. The use of a bioreactor for cell loading provides a well-defined, controlled environment, and bioreactors are key to successful stromal cell tissue regeneration [82, 122, 133]. To date, bioreactors have not been used extensively with equine stromal cells [82]. High cell seeding efficiency reduces the required initial cell number and thus decreases time consumed by cell expansion, which may interfere with timely implantation. Cell distribution
in the scaffold affects subsequent tissue formation, and uniform cell seeding facilitates homogenous tissue generation [85]. Use of a perfusion bioreactor to load adult equine MSCs onto biocompatible scaffolds may have significant, positive effects on equine MSC tissue regeneration [84, 90-92, 135-138].

Collagen scaffolds have advantages for tissue engineering like biocompatibility, biodegradability and high cell adhesion that have been demonstrated in a number of species [59-61, 139-142]. They also support efficient cell capture and growth, both critical to tissue formation [143]. Collagen based scaffolds are used widely and have been applied for generation of bladder [144], bone [145], brain [146-147], cartilage [148-149], heart [143] and skin [150] tissue among others. Therefore it is anticipated that collagen scaffold is suitable for multiple tissue regeneration. However, a systematic and comprehensive in vitro evaluation of interactions between collagen scaffolds and equine MSCs is necessary to evaluate their potential to support diverse mesenchymal tissue production.

The overall goal of this study was to investigate adult equine MSC interactions with collagen type I (COLI) scaffolds for purposes of advancing clinical application of equine tissue regeneration technology. Several hypotheses were tested to support this aim. The first hypothesis was that equine BMSCs and ASCs have indistinguishable cell seeding, attachment, viability, and distribution on COLI scaffolds. The second hypothesis was that BMSC – COLI and ASC – COLI scaffold constructs have comparable capability for three major specific tissue formation: adipogenesis, osteogenesis and chondrogenesis. To test these hypotheses, adult equine ASC and BMSC seeding efficiency, viability and distribution on collagen scaffolds was quantified immediately after perfusion bioreactor loading. Additionally, adipogenesis, osteogenesis, and chondrogenesis of ASC- and BMSC-COLI
constructs were compared to each other and uninduced controls with micro- and ultrastructure analysis as well as mRNA levels of tissue specific target genes after loading and following 7, 14, and 21 days of culture.

2.2 Materials and Method

2.2.1 Experimental Design

Cell passage 0 or 1 paired equine ASC and BMSCs were thawed from cryopreservation and expanded to P3. Cells (10^6 cells/scaffold) were loaded onto 10 x 7 x 3 mm bovine corium collagen type I scaffolds (Davol Inc, Cranston, RI) by perfusion bioreactor. A total of 37 scaffolds were seeded with each cell type per animal (296 scaffolds/total). From each animal, one scaffold was used to evaluate cell viability and distribution directly after loading. Two construct for each medium, culture period, and animal was used to evaluate cell viability and distribution by confocal laser microscopy and microstructure by light microscopy (one construct each), and two were used to isolate DNA and RNA to determine cell proliferation and mRNA level, respectively. Additional MSC-constructs were cultured for 21 days in the three induction medium for scanning electron microscopy (SEM) ultra-structural analysis.

2.2.2 Animals

Tissue harvest procedures were reviewed and approved by LSU Institutional Animal Care and Use Committee prior to the initiation of the study. Cells were harvested from 4 Thoroughbred geldings (7 to 10 years). Bone marrow (BM) was collected from sternebrae and subcutaneous adipose tissue was collected superficial to the gluteal muscle fascia as previously described [13, 134]. Stromal cell isolation was performed immediately after tissue harvest.
2.2.3 Stromal Cell Isolation

The BM aspirate was diluted 1:1 with stromal medium consisting of DMEM-Ham’s F12 (Hyclone, Logan, UT) with 1% antibiotic/antimycotic solution (MP Biomedical, Irvine, CA) and 10% characterized fetal bovine serum (FBS, [Hyclone, Logan, UT]), layered over Ficoll-Paque PLUS (Stem Cell Technologies, Vancouver, BC Canada) and centrifuged (350g, 30 min). Total nucleated cell number per unit volume BM aspirate was calculated for each sample [134].

Adipose tissue was minced in a sterile petri dish, washed, briefly agitated with an equal volume of phosphate buffered saline (PBS), and then allowed to separate into two phases. The infranatant was digested in an equal volume of PBS containing 1% bovine serum albumin type V (BSA, [Sigma, St Louis, MO]) and 0.1% collagenase type I (Worthington, Lakewood, NJ). The mixture was continuously rotated at 37°C for 30 minutes with brief agitation every 15 minutes. The stromal-vascular fraction (SVF) pellet was then isolated by centrifugation (350g, 5 min) and suspended in 2 ml of stromal medium. A small aliquot of the cell suspension was centrifuged and resuspended in an equal volume of red cell lysis buffer for 5 minutes. The total nucleated cell number per unit volume of adipose tissue was calculated [134].

2.2.4 Cryopreservation

Passage 0 or 1 cells from each horse were cryopreserved in aliquots of 1 x 10^6 cells/ml in cryopreservation medium (80% FBS, 10% DMEM, and 10% dimethylsulfoxide).
2.2.5 Flow Cytometry

Freshly revitalized cells were cultured for 4-5 days to approximately 70% confluence. Cell aliquots (2 x 10^5/ul PBS) were incubated individually with each of the following PE or FITC conjugated antibodies CD 34, CD29, CD73, CD44, CD45, CD90 and CD105 (Table 1) for 30 minutes in room temperature. Autofluorescence was measured with cell aliquots without antibody. Samples were rinsed with PBS followed by centrifugation (350g, 5 minutes). Cell pellets were fixed in 30 ul of cold 1% formalin prior to FACS analysis.

2.2.6 Cell Seeding

Following revitalization, cells were expanded to P3 (70-80% confluence prior to passage) in stromal medium. Cells in stromal medium were loaded onto 7 x 10 x 3 mm bovine corium collagen I scaffolds in a perfusion bioreactor. The perfusion bioreactor has been previously described [151] (Figure. 1). Briefly, scaffolds were placed into custom-designed perfusion cartridges and held in place by rubber gaskets. Cells suspended in culture medium passed only through the area occupied by scaffolds. Two perfusion cartridges were connected at their dependent ends by a 13 cm length of 0.48 cm diameter tygon tubing attached with luer connectors. There was a side tubing with 28 cm length and 0.31 cm diameter attached vertically on the top of each cartridge by three way stopcocks (Smiths Medical Asd Medex). The system was closed by two filters (0.22 µm, Millipore, Billerica, MA) connected to side tubings to maintain sterility during the seeding process. Each system of two cartridges was loaded with 5 ml of stromal medium. A cell suspension (1 x 10^6 cell/scaffold) ASCs or BMSCs in 2 ml stromal medium) was added to the system through injection ports on one of the three way stopcocks. The perfusion system was connected to a peristaltic pump via pump tubings (Ismatec, Glattbrugg, Switzerland). The perfusion direction and flow rate were
controlled by a customized program (Labview, National Instrument, USA). Loading was performed for two hours in a humidified incubator (37º C, 5% CO₂) at a flow rate of 1.05 ml/min. The direction of fluid flow was reversed after the lowest level of the medium reached the top of a cartridge to ensure scaffolds immersed in the medium during loading.

**Table 1 Information of antibodies for flow cytometry**

<table>
<thead>
<tr>
<th>AB</th>
<th>Label</th>
<th>Manufacturer</th>
<th>Marker Specification</th>
<th>Antibody properties</th>
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<td>CD29 (1st)</td>
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<td>BD Biosciences</td>
<td>Integrin Beta 1, MSC Marker</td>
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<td>Cell-surface Glycoprotein, MSC Maker</td>
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<td>PE</td>
<td>BD Biosciences</td>
<td>Leukocyte, Hematopoietic cell marker</td>
<td>Mouse anti-canine</td>
</tr>
</tbody>
</table>

**Figure 1 Perfusion bioreactor system for scaffold seeding.**

COLI scaffolds were placed inside a rubber gasket. Scaffolds in bioreactors were fixed by rubber gasket. Two bioreactors were assembled in one closed perfusion system. Cell suspension was injected into system through injection sites.
2.2.7 Induction Medium

For adipogenesis, cell-scaffold constructs were cultured in adipogenic induction medium (DMEM-Ham’s F12, 3%FBS, 1% antibiotic/antimycotic solution, biotin [33 µmol/L], pantothenate [17 µmol/L], insulin [1 µmol/L], isobutylmethylxanthanine [0.5 mmol/L], dexamethasone [1 mM], and rosiglitazone [5 µmol/L]) for 6 days and then in adipogenic maintenance medium (induction medium without isobutylmethylxanthanine and rosiglitazone) until collection. For chondrogenesis, cell-scaffold constructs were cultured in chondrogenesis medium (high glucose DMEM-Ham’s F12, 1%FBS, 1% antibiotic/antimycotic solution, transforming growth factor-β3 [10 ng/ml], dexamethasone [100 nm], ascorbate-2-phosphate [50 µg/ml], proline [40 µg/ml], sodium pyruvate [100 µg/ml], and 1% ITS + Premix [BD Biosciences, Bedford, Mass; 6.25 µg/ml insulin, 6.25 µg/ml transferrin, 6.25 ng/ml selenious acid, 1.25 mg/ml bovine serum albumin, and 5.35 mg/ml linoleic acid]). For osteogenesis, cell-scaffold constructs were cultured in osteogenic medium (DMEM-Ham’s F12, 20% FBS, 1% antibiotic/antimycotic solution, β-glycerophosphate [10 µmol/L], dexamethasone [20 nmol/L], and sodium 2-phosphate ascorbate [50 µg/mL]).

2.2.8 Loading Efficiency

After removal of the scaffolds, the remaining medium was removed from the system, centrifuged (350g, 5 minutes), and the total number of cells was determined using a hemocytometer and light microscopy. Subsequently, 4 ml of trypsinase (Hyclone, Logan, UT) was added to the system and manually distributed through the system for 5 minutes. The number of cells within the extracted trypsinase was determined as for the stromal medium. Loading efficiency was calculated as follows:
Percent loading efficiency = 100% x [1 – (cell #medium + cell #trypsinase/Initial cell number)]

Where cell #medium = total # of cell in the medium, cell #trypsinase = total # of cells in trypsinase.

2.2.9 Cell Viability

Cell viability was determined following incubation of cell−scaffold constructs with a live/dead stain containing calcein and ethidium bromide (EB) for 30 minutes according to the manufacturer’s instructions (Sigma, St Louis, MO). Live cells stained green by metabolizing calcein AM and dead cells stained red by intercalation of EB into DNA double helix (Figure 2). Scaffolds were viewed at 10x using a confocal laser microscope equipped with argon and rhodamine filters (Leica TCS SP2, Buffalo Grove, IL). A total of 20 sequential digital photomicrographs were generated every 1µm from one surface to the other in the perfusion plane of each scaffold. Digital images were exported as JPEG images and evaluated using graphics software (Image-Pro, Media Cybernetics, Bethesda, MD). Z plane images were assigned to regions; the 10 sections closest to each surface were defined as surface regions, and the 10 sections between the surface regions were defined as the center region. The spatial uniformity ratio was calculated as follows: Spatial uniformity ratio = % viable cells_surface/% viable cells_center.

2.2.10 Cell Numbers

Cell-scaffold constructs were stored in TRI Reagent (Molecular Research Center, Cincinnati, OH) at -80˚C after each culture period. DNA was isolated from the interphase, washed and solubilized according to the manufacturer’s protocol. Total DNA content was quantified using nanodrop reader (Nanodrop, Wilmington, DE). The cell number in each scaffold was
then determined from the total DNA content following generation of a standard curve from known numbers of MSCs.

![Live/dead cell stain on one sequential section of scaffold generated by confocal laser microscopy](image)

**Figure 2 Live/dead cell stain on one sequential section of scaffold generated by confocal laser microscopy**

Green color introduced by calcein stain indicating live cells (A), and red color introduced by ethidium homodimer indicating dead cells (B).

### 2.2.10 Light Microscopy

Constructs were fixed in 4% neutral buffered formalin, embedded in paraffin and stained with Masson’s Trichrome (5 µm sections) for light microscopy (Leica DM 4500B, Allendale, NJ). Constructs cultured in osteogenic medium were also stained with Von Kossa’s stain and those cultured in chondrogenic medium with alcian blue. Scaffolds cultured in stromal medium and scaffolds without cells were stained similarly for comparison.

### 2.2.12 Scanning Electron Microscopy

Cell-scaffold constructs cultured in stromal, adipogenic, osteogenic, or chondrogenic medium were evaluated with scanning electron microscope after 21 days of culture. Constructs were fixed in 2% paraformaldehyde and 1.25% glutaraldehyde in 0.1 M sodium cacodylate (CAC) buffer, pH 7.4. Samples were rinsed in 0.1M CAC buffer with 5% sucrose and incubated with 1% osmium tetroxide in 0.1 M CAC buffer. They were dehydrated in a
series of ethanol-distilled water solutions, critical point dried in a Polaron CO₂ bomb, and then sputter coated with gold and palladium. Samples were imaged with a scanning electron microscope (Quanta 200, FEI Company, Hillsboro, OR) at 15 kVP.

2.2.13 RT-PCR
Constructs were stored in TRI Reagent at -80°C following culture. Constructs were homogenized in tissue lyzer (Qiagen, Valencia, CA) and then RNA was isolated according to the manufacturer’s protocol. After DNase treatment, RNA was reversely transcribed to cDNA using oligo dT and M-MLV reverse transcriptase (Sigma, St Louis, MO). Real time polymerase chain reaction was performed using SYBR Green supermix (Bio-Rad, Hercules, CA) with ABI Prism 7900HT sequence detection system (Applied Biosystem, Foster City, CA). Equine-specific primers for adipogenic (peroxisome proliferator-activated receptor gamma [PPARγ], lipoprotein lipase [LPL] and leptin), osteogenic (collagen type I [COLI], osteocalcin [OC], and alkaline phosphatase [ALP]) and chondrogenic lineages (collagen type II [COLII] and sex determining region Y-box 9 [Sox9]) were used for all analyses (Table 2). The housekeeping gene, Glyceraldehyde 3-phosphate dehydrogenase gene (GAPDH), was used to determine the ΔCT value for each gene of interest.

2.2.14 Statistical Analysis
All outcomes are reported as mean ± standard deviation (SD). Student’s t test was performed to compare effects of cell type on loading efficiency. Three-way ANOVA was used to determine effects of cell type, culture time and induction medium on cell viability. Two-way ANOVA was used to compare the effects of cell type and culture time on mRNA expression levels. Different combination effects were evaluated with Tukey’s post-hoc tests. Significance was considered at P<0.05.
Table 2 Equine-specific primers designed for RT-PCR

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<th>Reverse Primers</th>
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2.3 Results

2.3.1 Loading Efficiency

The loading efficiencies, 74.6% ± 14.4% and 85.8% ± 9.2% for BMSCs and ASCs, respectively, were not significantly different between cell types.

2.3.2 Flow Cytometry

Stromal cell surface marker expression was higher in BMSCs for four out of five of those investigated in this study. Specifically, 99.86% of BMSCs and 87.81% of ASCs were CD29+; 77.96% of BMSCs and 31.06% of ASCs were CD44+; 43.71% of BMSCs and 1.35% of ASCs were CD73+; 40.57% of BMSCs and 1.34% of ASCs were CD90+; and 79.27% of
BMSCs and 97.01% of ASCs were CD105+. ASCs were negative for CD34 and CD45, but 16% of BMSCs were positive for CD34 and 8% were positive for CD45 (Figure 3).

Figure 3 A). Histogram of flow cytometry results. B). Cell morphology of both cell types.

A). Histogram of CD29, CD44, CD73, CD90, and CD105 antibody stained cell (black) with auto fluorescent control cell (green) for both BMSCs and ASCs. B). BMSCs and ASCs morphology of P1 before flow cytometry taken by microscope at 10x.

2.3.3 Cell Viability

Immediately after loading, percentages of viable cells were similar between center and surface regions in all scaffolds. There were no effects of cell type, culture time or medium
Figure 4 A). Mean±SD of spatial uniformity to evaluate BMSC viability on COLI scaffolds. B)-E). BMSCs morphology on scaffolds demonstrated by live/dead stain over time (1 – 0, 2 – 7, 3 – 14, 4 – 21 days) in adipo- (B), chondro- (C), osteogenic (D) and stromal medium (E).

A). Spatial uniformity (mean±SD) of viable cells in BMSC constructs after 0, 7, 14, or 21 days of culture in adipogenic, chondrogenic, osteogenic and stromal medium. B)-E). BMSCs changed from round shape at 0 day immediately after loading to spindle shape after 14 days of culture when they started to form cell aggregates. BMSCs appeared to be embedded in amorphous collagogenous matrix after 21 days of induction.
Figure 5 A). Mean±SD of spatial uniformity to evaluate ASC viability on COLI scaffolds. B)-E). ASCs morphology on scaffolds demonstrated by live/dead stain over time (1 – 0, 2 – 7, 3 – 14, 4 – 21 days) in adipogenic (B), chondrogenic (C), osteogenic (D) and stromal medium (E).

A). Spatial uniformity (mean±SD) of viable cells in ASC constructs after 0, 7, 14, or 21 days of culture in adipogenic, chondrogenic, osteogenic and stromal medium. B)-E). ASCs changed from round shape at 0 day immediately after loading to spindle shape after 14 days of culture when they started to form cell aggregates. ASCs appeared to be embedded in amorphous collagenous matrix after 21 days of induction.
on spatial uniformity ratios. Notably, cells were round and distinct from each other immediately after loading. After 14 days of culture, cells assumed a spindle shape and formed cell aggregates. Cells appeared to be embedded in amorphous collagenous matrix after 21 days of induction (Figs. 4 and 5).

2.3.4 Cell Number

Overall, there were no significant differences between ASCs and BMSCs based on DNA content. Cell number on the scaffolds for BMSCs was affected significantly both by culture time and medium type and for ASCs was significantly affected by medium type. There were significantly more cells in BMSC constructs cultured in chondrogenic medium than in adipogenic or stromal medium after 21 days of culture (Fig. 6). The ASC constructs in stromal medium had significantly higher cell numbers than those in the three induction medium after 21 days. ASC constructs had significantly more cells after 14 and 21 days of culture in stromal medium than after 7 days (Fig. 6).

2.3.5 Light Microscopy

Both ASC and BMSC constructs cultured for 21 days in induction medium tended to colonize the intrafibillar spaces of the scaffold and produce their own extracellular matrix (ECM) within formed cell aggregates compared with constructs in stromal medium (Fig. 7A-H). The BMSC constructs cultured in chondrogenic or osteogenic medium showed similar cellular morphology with clusters of heterogenous cell populations composed of round chondrocyte-like, polygonal osteoblast-like and undifferentiated longitudinal stromal cell-like structures within ECM (Fig. 7B&C). In constrast, ASC constructs cultured in chondrogenic or osteogenic media were showed distinct differences between chondro-induced and osteo-induced types. There were cell aggregates emmeshed in fibrous ECM in
Figure 6 Mean ± SD of relative cell number of both BMSCs and ASCs on collagen scaffold over time.

Mean±SD of relative cell numbers of both BMSCs and ASCs on collagen scaffold over culture time (7, 14 and 21 days) in adipogenic, chondrogenic, osteogenic and stromal medium. * indicates significant differences at 14 and 21 days compared with 7 days. |---| indicates significant differences between groups with different medium at the same time points.
constructs cultured in chondrogenic medium, while those cultured in osteogenic medium tended to have all cell aggregates of polygonal osteoblast-like cells with less spindle and round shaped cells (Fig. 7 F&G). ASC constructs cultured in adipogenic medium contained ellipsoid lipoblast-like cells containing lipid droplets while BMSC constructs contained sparse round cells that were smaller in size.

Both tissue density and proteoglycan staining with alcian blue increased with culture time in chondrogenic medium in all constructs (Fig. 8). There was greater alcian blue staining in BMSC constructs (Fig. 8A-C) compared to ASC constructs (Fig. 8D-F) at all time points. Scaffold alone did not stain with alcian blue (Fig. 8G). Von Kossa staining of calcium deposition also increased in constructs with longer culture time (Fig. 9) and was consistently most intense in BMSC constructs (Fig. 9A-C). Scaffold alone did not stain (Fig. 9G).

2.3.6 Scanning Electron Microscopy

Extra cellular matrix was apparent on constructs cultured in osteogenic (Fig. 10 and 11 A) and chondrogenic medium (Fig. 10 and 11 B) for 21 days, compared to scaffold alone (Fig. 10 and 11 C). There were polygonal, osteoblasts like cells on scaffold cultured in osteogenic medium (Fig. 10 and 11 A). Mineral deposits were apparent surrounding spindle-shaped (Fig. 10F) and polygonal cells (Fig. 11F). Cells appeared to adhere to scaffold and assume a flattened appearance. The cell morphology was distinct from constructs cultured in chondrogenic medium which has spherical cells embedded in dense, organized ECM (Fig. 10 and 11 B). After 21 days culture in adipogenic medium, ellipsoid lipoblast-like cells containing lipid droplets were apparent in BMSC (Fig. 10D) and ASC (Fig. 11D) constructs. The newly formed extra cellular matrix was distinguishable by its distinct ultra structure for both constructs cultured for 21 days in chondrogenic medium (Fig. 10 and 11 E).
Figure 7 Masson’s Trichrome staining for both BMSCs and ASCs – scaffold constructs after 21 days culture

BMSCs - scaffold constructs after 21 days in adipogenic (A), chondrogenic (B), osteogenic (C), stromal (D) medium by Masson Trichrome staining (100x). ASCs - scaffold constructs after 21 days in adipogenic (E), chondrogenic (F), osteogenic (G), stromal (H) medium (100x, Masson’s Trichrome, scale bar – 10 µm).
Figure 8 Alcian blue staining for both BMSCs and ASCs – scaffold constructs over culture time in chondrogenic medium

BMSCs – scaffold (A-C) and ASCs – scaffold constructs (D-F) cultured in chondrogenic medium for (A&D) 7 days, (B&E) 14 days and (C&F) 21 days (63x for upper right image, scale bar – 40 µm; 20x for major image, scale bar – 100 µm; Alican blue stain with nuclear fast red counterstain).
Figure 9 Von Kossa staining for both BMSCs and ASCs – scaffold constructs over culture time in osteogenic medium

BMSCs – scaffold (A-C) and ASCs – scaffold constructs (D-F) cultured in chondrogenic medium for (A&D) 7 days, (B&E) 14 days and (C&F) 21 days (63x for upper right image, scale bar – 40 µm; 20x for major image, scale bar – 100 µm; Von Kossa’s stain with nuclear fast red counterstain).
Figure 10 Scanning electron photomicrographs of BMSCs constructs after 21 days of culture in induction media at low (A,B) or high (D,E,F) magnification. COLI scaffold without cells at low magnification used for comparison (C).

Scanning electron photomicrographs of BMSCs constructs after 21 days of culture in osteogenic (A) or chondrogenic (B) medium at low magnification. Collagen scaffold without cells or culture is provided for comparison (C). Higher magnification images of construct after 21 days of culture in adipogenic (D), chondrogenic (E), and osteogenic (F) medium. Red arrows point to a cell with adipocyte-like morphology (D), neo extracellular matrix (E), and a cell with osteoblast-like morphology (F).
Figure 11 Scanning electron photomicrographs of ASCs constructs after 21 days of culture in induction media at low (A,B) or high (D,E,F) magnification. COLI scaffold without cells at low magnification used for comparison (C).

Scanning electron photomicrographs of ASCs constructs after 21 days of culture in osteogenic (A) or chondrogenic (B) medium at low magnification. COLI scaffold without cells or culture is provided for comparison (C). Higher magnification images of construct after 21 days of culture in adipogenic (D), chondrogenic (E), and osteogenic (F) medium. Red arrows point to fat droplets (D), neo extracellular matrix (E), and crystals (F).
2.3.7 RT-PCR

The COLI mRNA levels increased with culture time in ASC and BMSC constructs cultured in osteogenic medium, and levels were significantly higher in BMSC compared to ASC constructs after 7, 14 and 21 days of culture. The ALP mRNA levels peaked significantly in BMSC constructs after 7 days of culture while mRNA levels were highest significantly after 14 days of culture for ASC constructs. The OCN mRNA levels were significantly higher in BMSC constructs after 21 days of culture compared to other time points. The mRNA levels of COLII and SOX9 were significantly higher in BMSC constructs compared to ASC constructs cultured in chondrogenic medium at all time points but day 21 (Fig. 11A). There were significantly higher mRNA levels of all three adipogenic specific genes in ASC versus BMSC constructs after 14 days of culture while LPL was higher after 7 and 21 days of culture and Leptin was higher after 7 days of culture. There were no differences among time points within cells type for progenitor genes NANOG and SOX2. OCT4 mRNA levels were significantly lower in BMSC constructs compared to ASC constructs at all time points (Fig. 11B).

2.4 Discussion

Adult stromal cells have played a vital role in human tissue regeneration over the last decade. In equine medicine, clinical applications of adult stromal cells have historically been geared toward soft tissue and cartilage regeneration[33, 126]. For translational tissue engineering applications, characterization of three major components, cell, scaffold and bioreactor, is required. This study investigated adult equine BMSC and ASC loading and behavior on COLI scaffolds in vitro following perfusion bioreactor loading. Overall, perfusion bioreactor loading was efficient and resulted in effective cell distribution for both cell types. COLI
Figure 12 Tissue-specific gene expressions of both ASCs and BMSCs after 7, 14 and 21 days in induction media.

A. ΔCT value change in osteogenic genes (ALP, Osteocalcin and Collagen I) and chondrogenic genes (Sox9 and Collagen II) of both ASCs and BMSCs on collagen scaffolds in osteogenic medium and chondrogenic medium respectively for 7, 14 and 21 days. B. ΔCT value change in adipogenic genes (PPARγ, Leptin and LPL) and stemness genes (Oct4, Nanog and Sox2) of both ASCs and BMSCs on collagen scaffolds in adipogenic medium and stromal medium respectively for 7, 14 and 21 days. * indicates significant differences between BMSCs and ASCs at the same time points; # indicates significant differences over time compared with 7 days for ASCs; & indicates significant differences over time compared with 7 days for BMSCs.
scaffolds supported equine MSCs adhesion, proliferation and differentiation into three distinct tissue lineages. Both BMSC and ASC constructs promoted osteogenic, chondrogenic and adipogenic tissue formation after induction in vitro. This study established straightforward and reproducible procedures to support customized tissue engineering with perfusion bioreactors loading of two easy to be isolated equine MSCs onto COLI scaffold to grow aimed tissue type. This achievement significantly support customized tissue engineering by adult MSCs for therapeutic applications in the horse.

This study evaluated paired ASCs and BMSCs from four equine subjects and thus included the variations introduced by individual animals, which provided meaningful statistical replicates. The relative small error bar in result section indicated low animal differences and proved the consistency of success specific tissue formation using the animal selection criteria, isolation techniques and induction conditions in this study. This revealed great potential of using equine MSCs as reliable cell sources for tissue regeneration in equine clinics.

Based on the results of this study, perfusion bioreactor loading is a reliable, consistent, and reproducible procedure for adult equine MSC loading. Cell loading onto scaffolds is a critical step in tissue engineering, and both ASCs and BMSCs had high loading efficiency on collagen scaffolds at the perfusion rate used in this study. The findings are supported by the studies with the adhesion of ASCs and BMSCs on channeled elastomeric scaffolds in earlier report [44]. In this study, the perfusion rate was determined for efficient mass transfer and low shear force stimulation. Fluid flow is critical to the balance between nutrient exchange and shear force stimulation and affects loading efficiency. There is an inverse correlation between flow rates for seeding proficiency and seeding efficiency. Seeding efficiency decreases as the flow rate increase at the same seeding density [152]. Applied flow rates
vary for different perfusion systems [116, 153] but still remain with a range of 0.1085 ml/min ~ 1.0850 ml/min (calculated from 0.1 mm/s ~ 1.0 mm/s) which includes that of this study. For other perfusion seeding, the maximum average seeding efficiency achieved was 51.9% [153]. Average seeding efficiency with 87% has been achieved with the same perfusion seeding technique under equal flow rate for 2 hours loading for both myocytes and endothelial cells [151], which is consistent with high seeding efficiency reached for both equine BMSCs and ASCs. Therefore this perfusion seeding through the scaffolds accomplished a critical and success step in acquiring high cell density, which is necessary for subsequent tissue generation.

Immunophenotyping was applied to confirm the multipotentiality of isolated equine MSCs through established MSC markers in human [154] and equine [33]. The high percentage of BMSCs positive for CD29 and CD44 are comparable with the previous findings of equine mesenchymal progenitor cells after 7 days culture, with much higher expression of CD 90 [155]. The consistency among all these three markers indicated more accurate cell sorting and high percentage of multipotential cells derived by the protocol established in this study. Similar pattern of surface antigen expression between BMSCs and ASCs was supported by previous studies [156]. However, there are relatively lower percentage of MSC surface marker of both BMSCs and ASCs expressed in equine compared with human [154]. The homogeneity of cells increases with higher passage cells. The heterogeneity of both equine MSCs may be due to their lower passage number used in this study compared to that of human cells. Taking advantage of cell immunophenotyping characterization before loading can be useful and beneficial for enhanced isolation of MSCs and screening cells guaranteed capable to commit further multiple differentiations.
Cell viability on scaffolds was evaluated to assess cell distribution on scaffold. It is an important parameter to estimate the effects of loading and subsequent cell growth situation on scaffolds. After 2 hours of perfusion loading, cell viability maintained high on scaffold, indicating the flow rate and loading time were not damaging. The limitations of tissue generation on scaffolds needed to be considered here is that cells reside in the center of scaffold died, forming necrotic center [157]. This is largely caused by the diffusional restrictions of oxygen penetration (less than 10 µm) [158]. However, all cell/scaffold constructs in center sections showed relative similar cell viability compared with surface sections as shown in results. This may lead to the conclusion that with efficient loading, static culture could provide appropriate environment for cell growth on this highly porous COLI foam scaffold structure with 2 mm thickness. Further studies should be conducted to evaluate the cell viability with dynamic culture as well as cell viability on thicker scaffold. It is expected that the high cell seeding efficiency on scaffolds can be achieved to mimic the physiologic cell density in engineering tissues.

Collagen, as an important component of bone, has the advantage of biocompatibility and biodegradability. In previous studies, collagen/hyaluronate matrix demonstrated high biocompatibility and osteoconductive in treating created cranial bone defects in rats[59]. Created bone defects in rat tibia implanted with collagen scaffolds presented a low inflammatory response and bone formation within a short period of time [60]. Collagen matrix is considered a promising choice for bone defects treatment because of its low cost of production associated to the biocompatibility and osteoconductivity. It was also demonstrated in a recent study that collagen has the potential to induce and maintain chondrogenesis of equine ASCs, which promoted application of ASCs for cartilage repair in
equine tissue regeneration [159]. In this in vitro study, both equine BMSCs and ASCs demonstrated early commitment to osteogenic and chondrogenic lineages, which is confirmed respectively by fibro-cartilaginous and hyaline like extra cellular matrix integrated properly with collagen scaffold indicated by Masson’s Trichrome staining. It is also mentioned that the osteoblast needs to adhere and spread on the surface of the implant in order to proliferate and subsequently produce ECM components, which was also shown by results of scanning electron microcopy [160]. Besides, collagen scaffold has also been used for dermal regeneration in a nude mice full skin defect model because it can serve as a backbone for cell infiltration and vascularization [61], which was presented in this study as well by the cell high affinity to collagen scaffold and contents. These morphological results further confirmed multipotentiality of adult equine stromal cells and supported the ability of cell - scaffold construct as an efficient biocompatible implant for tissue generation.

Overall, tissue specific micro- and ultrastructure changes supported suitability of both BMSC and ASC construct into adipogenic, osteogenic and chondrogenic lineages. Temporal analysis of tissue specific marker genes expressions were especially useful to confirm differentiations and showed consist results. Some common bone-specific genes and proteins are ALP, COLI and OCN [161-162]. There were significant upregulations of ALP in both cell types on scaffolds after 14 days induction compared with day 0, indicating higher percentage of osteoblasts phenotype cells. The upregulations of COLI over time confirmed the osteogenic progression of both cells on scaffolds. The level of COLI in BMSCs was significantly higher than that in ASCs at all time points, which showed the advantage of BMSCs in osteogenic differentiation on scaffold compared with ASCs. The OCN level of BMSCs was significantly higher than ASCs at day 21, supporting better osteogenic
maturation of BMSCs on scaffolds. Increases expression levels of COLII and SOX9 are normally corresponding to enhanced and stronger matrix production, which provides cues for chondrogenic differentiation [163]. The significant increase of COLII in both cells demonstrated their chondrogenic maturation over time. The quicker progression of BMSCs into chondrogenesis on scaffolds compared with ASCs can be proved by the fact that both COLII at day 7 and 14 and SOX9 at all time points were expressed significantly higher in BMSCs than ASCs. The adipogenic induced cells with upregulated genes such as PPARγ, Leptin and LPL were positive for lipid accumulation and were committed to adipogenesis by Oil Red O stain [164-165]. Significantly increase in Leptin expression of BMSCs confirmed its progression into adipogenic lineage. Both leptin at day 7 and 14 and LPL at all time points was significantly upregulated in ASCs compared with BMSCs. This indicated ASCs higher tendency to commit to adipogenesis differentiation on scaffolds. COLI scaffold proved able to support adipogenic, chondrogenic and osteogenic differentiation of both cells and provides a promising platform for specific tissue formation in vitro, demonstrated by their micro- and ultrastructure as well as tissue specific gene expressions. With gene level quantification, it was further revealed that BMSCs are more commitment to chondrogenic and osteogenic differentiation while ASCs are easier into adipogenic differentiation.

This study evaluated the in vitro behavior of two types of adult equine MSCs on COLI scaffolds. Both cell types had high viability and DNA contents, confirming the suitability of collagen scaffolds for cell growth. Furthermore, collagen scaffolds supported cell differentiation into distinct mesenchymal tissues committed to adipogenic, chondrogenic and osteogenic lineages. This study, as one of the first, offers a promising platform for equine tissue regeneration. More attention should be paid to the cell/scaffold construct interaction
with native tissue in vivo and especially to how the initial induction of cells in vitro influences cell behavior on scaffold and cell integration with specific tissue type in vivo. The developed cell adhesion, growth and induction behavior on collagen scaffold will be further applied in vivo in nude mice to investigate their great potential role in equine veterinary tissue regeneration.
CHAPTER 3. *IN VIVO* OSTEOGENESIS OF EQUINE MULTIPOTENT STROMAL CELLS ON COLLAGEN SCAFFOLDS
3.1 Introduction

Our previous studies revealed that collagen (COLI) scaffolds supported MSC adhesion, proliferation and differentiation into three distinct tissue lineages. The established, straightforward and reproducible procedure supports customized tissue engineering with bioreactor loading of equine multipotent stromal cells (MSCs) onto COLI scaffolds to generate specific tissue type.

Infection, delayed repair and inappropriate fixation devices can cause failures of equine bone repair. The investigation of equine bone regeneration is considered to be a promising method for accelerating bone healing as well as providing an alternative besides the conventional approaches. Research of equine tissue regeneration became increasingly popular over the last decade [118, 162, 166-169]. But few previous studies have been aimed to focus on investigation of bone regeneration. Majority of MSC therapy are applied to promote tendon and ligament repair. However, this application is mainly based on empirical data and there is still a great potential to improve healing efficiency using proper engraftment of cells into the lesion site. MSCs engrafted on bioscaffolds showed success to treat cartilage degeneration supported by solid evidence-based studies. But high demand for equine bone regeneration is still largely unmet.

The previous study accomplished the first critical step to establish suitability of MSCs engrafted by Coll scaffold for osteogenesis in vitro. Further in vivo studies are needed to investigate their osteogenesis in athymic mice before its application in equine bone regeneration. This study was designed to test the hypothesis that osteogenesis of MSCs supported by COLI scaffolds in vivo can be achieved as well as its in vitro and in vivo osteogenesis is comparable between BMSC- and ASC constructs. The objective of this study...
was to compare osteogenesis at mRNA level and ECM formation on COLI scaffolds of both cell types, monitor bone formation process by radiograph and reconstruct and quantify tissue generated by each constructs at both 2D and 3D view by microcomputed tomography.

3.2 Materials and Methods

3.2.1 Experimental Design

Two cohorts of nude mice (N = 4 nude mice/cohort) were subcutaneously implanted with scaffold loaded with BMSCs or with ASCs, which had been pre-induced in osteogenic medium during 2 hours loading. One control nude mice was implanted with scaffold only. All nude mice were harvested 9 weeks later (Fig. 13) and assessed shortly post-mortem using microcomputer tomography to evaluate perimeter, porosity, volume and surface area of the formed callus. The osteogenesis of cell scaffold constructs was confirmed with compositional and mRNA analysis. This procedure was approved by the Institutional Animal Care and Use Committee.

Figure 13 Subcutaneous implantation of cell constructs.

The black circle depicts a pocket containing one cell construct. A total of 6 replicates were implanted in each nude mice.
3.2.2 Radiography

Radiographs were performed at 0, 3, 6 and 9 weeks after implantation on dorsal aspect of nude mice. Since the Coll scaffold was radiolucent, it was impossible to tell the position of scaffold if there was no bone formation, which provided a reliable measurement of newly generated bone formation.

3.2.3 Microcomputed Tomography

Cell scaffold constructs were evaluated by microcomputed tomography (Micro-CT) shortly after postmortem according to previous published protocol [170]. Briefly, 2D micro-CT imaging was taken by slides with 0.04 mm thickness (SkyScan 1074, Skyscan n.v., Belgium). 2D and 3D reconstructions were generated subsequently (Mimics v 10.1, Materialise, Ann Arbor, MI). Total area (µm2), bone perimeter (µm), and percent porosity (%) of generated tissues were measured on 2D images and total volume (mm3) and surface area (mm2) of generated tissues were measured on 3D images by Mimics or CTan software packages, respectively.

3.2.4 Compositional Analysis

All compositional analysis was performed according to published manufacture’s protocols.

3.2.4.1 DNA Content

Cell quantity on scaffolds was evaluated by DNA content using the quantative picogreen assay kit (Invitrogen, Carlsbad, CA) according to manufacturer’s protocol. Briefly, 50 µl of a papain digested sample was mixed with 50 µl picogreen reagent in a clear 96 well plate and incubated at room temperature for 5 min in the dark. The plate was read immediately using fluorescence microplate reader (Synergy HT, Winooski, VT) at the wavelength for
fluorescein (excitation 485 nm and emission 538 nm). Amount of DNA content was calculated based on the standard curve.

### 3.2.4.2 Protein Assay

Modified lowry method was used to measure the quantity of total proteins [171]. 150 µl of biuret reagent (Sigma, St Louis, MO) was added to 50 µl of sample and standard (bovine serum albumin) in a clear 96 well plate and incubated for 15 min at room temperature. 15 µl of 1N Folin and Ciocalteau’s reagent (Sigma, St Louis, MO) was added to each well and mixed. Absorbance at 650nm was measured after 30 min incubation at room temperature. Samples were diluted to fit into the standard curve range if needed.

#### 3.2.4.3 BMMB Assay

Quantification of sulfated glycosaminoglycan (sGAG) was performed according to a published protocol [172]. DMMB solution was prepared by dissolving 21 mg of 1,9-dimethylmethylene blue (Sigma, St Louis, MO) in 5ml ethanol and 2.0 g of sodium formate in water separately. pH of the combined solution was adjusted with concentrated formic acid to 3.0 in a final volume of 1 liter. In a clear 96 well plate, 200µl DMMB solution was added to 40 µl of sample and standard. Absorbance was measured at 520 nm immediately after mixing using a microplate reader (Synergy HT, Winooski, VT). Chondroitin sulfate (Sigma, St Louis, MO) was used as standard and concentration of GAG in samples was determined from the standard curve.

#### 3.2.4.4 DMBA Assay

Determination of hydroxy proline (total collagen) was measured according to the published protocol [173]. Briefly, 50 µl of concentrated HCl was added to 50 µl of papain digestion
buffer and incubated for 18 hrs at 110°C. Samples were dried by heating to about 50°C in a fume hood after hydrolysis and resuspended in acetate citrate buffer. 50 µl of 1.27% chloramines (Sigma, St Louis, MO) prepared in water was added to 50 µl of hydrolyzed sample and incubated at room temperature for 25 min. 15% of p-dimethylaminobenzaldehyde (DMBA) (Sigma, St Louis, MO) was dissolved in n-propanol/perchloric acid (2:1) after addition of 50 µl of Ehrlich’s reagent. The mixture of DMBA and samples were incubated at 60 °C for 20 min and cooled to room temperature. The plate was read at 550 nm. The standard curve was generated with hydroxyproline (Sigma, St Louis, MO).

3.2.5 RT-PCR

Constructs were stored in TRI Reagent at -80°C following culture. Constructs were homogenized in tissue lyzer (Qiagen, Valencia, CA) and then RNA was isolated according to the manufacturer’s protocol. After DNase treatment, RNA was reversely transcribed to cDNA using oligo dT and M-MLV reverse transcriptase (Sigma, St Louis, MO). Real time polymerase chain reaction was performed using SYBR Green supermix (Bio-Rad, Hercules, CA) with ABI Prism 7900HT sequence detection system (Applied Biosystem, Foster City, CA). Equine-specific primers for osteogenic (osteocalcin [OCN], alkaline phosphatase [ALP], cannabinoid1 [CB1] and bone sialo protein [BSP]) were used for analyses (Table 3). The housekeeping gene, Glyceraldehyde 3-phosphate dehydrogenase gene (GAPDH), was used to determine the ΔCt value for each gene of interest.

3.3 Results

The size of COLI scaffolds in control group decreased dramatically, which indicated the degradation of scaffold. Both ASC- and BMSC constructs remained the same position with
Table 3 Primer sequence of bone specific genes

<table>
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<th>Reverse Primers</th>
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<td>GCCGGAGTCTGTTCACTACC</td>
</tr>
<tr>
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<td>CATGCTGGCTGTGTTATTTGG</td>
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<tr>
<td>BSP</td>
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<td>CTTGTGGCTCTGATGTTCCA</td>
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Figure 14 Pictures showing the representative anatomical view of constructs progression after 9 weeks postmortem. A). COLI scaffolds only as control, B). ASC constructs, C). BMSC constructs.

A). Very small portion of COLI scaffolds in control group remained at the implantation sites. B). ASC constructs progressed into a uniformly structured tissue integrated with the skin tissue of nude mice. C). BMSC constructs progressed into denser tissue embedded within the constructs.

This finding at harvest was further confirmed by radiograph taken 9 weeks after implantation. Because COLI scaffolds were radiolucent, the degraded COLI scaffolds were invisible on radiograph (Fig. 15A). Based on the fact that the newly generated tissue by ASC constructs
were also invisible on radiograph, it was confirmative that ASC constructs did not generate bone tissue but more like part of dermal tissue (Fig. 15B). After comparison, BMSC constructs generated bone tissue according to this radiograph (Fig. 15C). The newly generated bone was present as early as 3 weeks post-implantation (Fig. 16B). The size of newly generated bone did not increase dramatically over time, which indicated the bone formation was guided and controlled by original scaffold size (Fig. 16C&D).

**Figure 15** Radiograph evaluation of bone formation 9 weeks after implantation (A. COLI scaffolds only as control, B. ASC-COLI constructs, C. BMSC-COLI constructs).

A). COLI scaffolds only were radiolucent. B). Tissue generated by ASC constructs were also radiolucent. C) BMSC construct generated bone tissue captured by radiograph indicated by red circle

There were significantly higher 2-D surface area and 3-D total volume and lower porosity for ASC constructs, indicating more uniform distributed ECM formation (Fig. 17). Tissue generated on both constructs showed similar shape directed by ColI scaffold, indicated by their comparable 2-D perimeter and 3-D surface area (Fig. 17). These findings were further
demonstrated by reconstructed 3D structure of newly formed tissue with higher porosity of
BMSCs constructs and more uniform structure of ASC constructs (Fig. 18). In summary, the
fact that the newly generated tissue was guided by original shape of scaffold suggested that
the COLI scaffold, serving as a temporary structure support, its degradation rate is balanced
with new tissue formation rate.

There were no significant differences between ASCs and BMSCs for HPR, GAG and protein
expressed on scaffolds normalized to DNA contents, which indicated indistinguishable
ability between ASCs and BMSCs to synthesize ECM in the form of collagen and GAG (Fig.
19).

There were significant upregulations of ALP, OCN and BSP, indicating enhanced
osteogenesis of BMSC constructs. Because BSP is an important component of bone ECM,
significant increased expression of BSP suggested that ECM formed by BMSC constructs is
likely to contribute to bone formation. After 9 weeks of implantation, bone formation in
BMSC constructs suggested activities of mature osteoblast. No differences of CB1
expression between ASC- and BMSC constructs might suggest CB1 receptor did not play a
role in mature osteoblasts (Fig. 20).

3.4 Discussion

Adult equine MSCs for multiple tissue regeneration have gained great attention as the
treatment choice for equine orthopedic lesions over the last decade [14]. Current equine
regenerative medicine is largely geared to MSCs administration as a single product or in
combination with the heterogeneous non-expanded progenitor cell population. Besides,
clinical applications of adult stromal cells therapy have been mainly focused on soft tissue
Figure 16 Radiograph evaluation of bone formation by BMSC-COLI constructs (A. 0 week, B. 3 weeks, C. 6 weeks and D. 9 weeks post-implantation).

A). BMSC constructs were radiolucent immediately after implantation. B). Bone tissue generated by one of BMSC constructs was captured by radiograph as early as week 3. C). Two of BMSC constructs generated bone tissue and the size of bone tissue generated at week 3 increased after 6 weeks of implantation. D). Generated bone tissue remained similar size and shape after 9 weeks of implantation.
Figure 17 Micro-CT measurements of tissue formation in 2D and 3D view for both ASCs and BMSCs.

Significant higher 2-D surface area and 3-D total volume and significant lower porosity for ASC constructs indicated more uniform distributed ECM formation on ASC constructs. Tissue generated on both constructs showed similar shape indicated by their indistinguishable 2-D perimeter and 3-D surface area.
Figure 18 Representative reconstructed 3D images of generated tissue by BMSC constructs (A) by selecting bone density and ASC constructs (B) by selecting soft tissue density.

Bone tissue produced by BMSCs constructs (A) showed higher porosity than soft tissue generated by ASCs constructs (B). ASC constructs demonstrated more uniform structure.
Figure 19 ECM formation on BMSC- and ASC constructs

Quantification of HPR, GAG and protein on BMSC- and ASC constructs which has been normalized by DNA content.
**Figure 20** Bone specific gene expressions by BMSCs and ASCs on COLI scaffolds implanted *in vivo.*

ΔCT value change in osteogenic genes (ALP, OCN, BSP and CB1) of both ASCs and BMSCs on COLI scaffolds after 9 weeks of implantation. * indicates significant differences between BMSCs and ASCs.

and cartilage regeneration. Although recent development of MSCs engrafted on bioscaffolds showed success for tissue regeneration, they were mainly aimed to treat cartilage degeneration. There are still largely unmet needs for equine bone regeneration that can be addressed by studies of optimization of MSC-bioscaffold interaction and pre-induction condition to achieve bone regeneration in clinics in the near future. This study investigated adult equine BMSC and ASC osteogenesis on COLI scaffolds *in vivo* based on established procedures in a previous study. Overall, COLI scaffolds supported equine MSCs adhesion, ECM production and differentiation with BMSC constructs more committed towards
osteogenesis and ASC constructs more committed towards dermal-like tissue. This achievement significantly supported engineered tissue by adult MSCs for therapeutic applications in the horse especially for bone regeneration.

Based on results from radiograph, there was radiopaque bone tissue formation of BMSC construct as early as 3 weeks post-implantation, which proved BMSC-COLI constructs as a promising and efficient therapy for equine bone repair especially for acute injuries. Although ASC constructs did not show any bone formation based on radiograph, they produced similar ECM as BMSC to form dermal-like tissue. Based on micro-CT, both ASC- and BMSC construct generated tissue had similar size, indicating that the degradation of COLI scaffold was balanced with newly formed tissue. COLI scaffolds served successfully as a temporary structure for new tissue to form.

It was proved by this study that short-term induction in vitro is sufficient to trigger osteogenesis of BMSC subcutaneously in vivo, which significantly improves efficiency of bone repair in equine clinics in near future. However, lack of consistent distribution and uniform shape for bone formed by BMSC constructs may be due to insufficient vascular supplies subcutaneously. ASC constructs produced comparative ECM as that of BMSC constructs but under commitment into dermal-like tissue, indicating ASCs more responsive to signal cues encountered subcutaneously due to their original niches. This finding was consistent with significant upregulation of osteogenic genes in BMSC constructs, confirming bone regeneration by BMSC construct.

Further study should be conducted to optimize the pre-induction time as well as to introduce osteoinductive component to COLI scaffold to improve efficiency of ASCs constructs to
commit osteogensis differentiation. Considering the key advantage of ASCs with high harvest rate, easy isolation from autologous fat tissue, minimal injury to donor site and still with equal multipotentiality as BMSCs, it is necessary to investigate and establish the role of ASCs in equine bone regeneration to provide more alternative for clinicians as well as improve the efficiency of bone repair in equine clinics.
CHAPTER 4. OPTIMIZED LOADING AND PRE-CULTURE CONDITIONS FOR ADULT HUMAN ADIPOSE-DERIVED MULTIPOTENT STROMAL CELL OSTEOGENESIS ON BIOPATIBLE SCAFFOLDS
4.1 Introduction

There are more than 500,000 bone graft procedures in the United States and approximately 2.2 million are performed worldwide each year [174]. The associated annual cost of these procedures is estimated to 2.5 billion US dollars. Either autografts or allografts are the primary choice of grafts and are used in 90% of procedures. Harvesting the autografts requires an additional surgery at the donor site and quantities of bone tissue available for harvest are also limited. Due to these limitations of autologous bone graft, various commercially available synthetic bone graft substitutes have been developed. However, bone graft substitutes usually do not have the same osteogenic capacity as autologous bone [175]. Functional tissue can be regenerated using differentiated cells or multipotent stromal cells (MSCs) engrafted on biocompatible scaffolds. Huge selections of commercially available scaffolds have been developed to promote bone formation but information about their interactions with MSCs is limited. There are distinct differences among scaffolds such as components, porosity, surface area and osteoinductive properties [176]. Since all these properties of the scaffolds play an important role in osteogenic tissue formation, it is critical to optimize scaffold and cell interactions to promote the development of functional bone tissue [177-178].

Research on bone regeneration by MSCs has largely focused on bone marrow derived MSCs (BMSCs) because of their superiority in osteogensis. Similar to bone marrow, adipose tissue also derived from the embryonic mesendoderm. It has been reported that adipose derived MSCs (ASCs) have high proliferative capability and multipotentiality, including osteogenic, chondrogenic, and adipogenic differentiation [179-181]. Recent findings proved ASCs as a promising cell source in terms of their osteogenic potential [117, 169, 182-183].
Accessibility of ASCs in abundance from autologous fat tissue with minimal injury to the donor site is a key advantage over BMSCs. The objective of this study was to evaluate ASCs viability, ECM formation, osteo-specific mRNA expression and ultrastructure on three biocompatible scaffolds, which was aimed to test hypothesis that ASCs on collagen I (COLI) scaffold is more superior in osteogenesis by combining osteoinductive components than COLI only scaffold.

Pre-implantation culture conditions are also crucial in ASCs osteogenesis. Studies have shown that cell-scaffold constructs developed bone more efficiently in vivo when constructs were first cultured in vitro with osteogenic medium [184-185]. However, long pre-implantation culture is time-consuming and not desirable from aspects of clinical applications. It has been shown that pre-culture of MSCs may on the other hand limit their proliferation potential and bone formation efficiency in vivo [186]. Therefore we hypothesize that short-term exposure to induction for ASCs have comparable effects on osteogenesis as long-term exposure after loaded on biocompatible scaffolds. The objective was to determine whether the short-term pre-induction is sufficient for osteogenesis of chosen cell-scaffold constructs based on ECM formation and mRNA expression level.

Scaffolds of different compositions and structures should have unique osteoinductive mechanisms and the knowledge of changes at molecular level will help determining the suitable scaffolds. Therefore we were interested in exploring the molecular mechanism of osteogenesis of cell-scaffold constructs. Bone tissue is subject to constant remodeling by osteoblasts and osteoclasts to balance between bone-forming and bone-resorbing activities, respectively. It has been characterized that osteoprotegerin (OPG) binds to receptor activator of nuclear factor kappa beta ligand (RANKL), a protein expressed on osteoblast membrane,
to limit the access of RANKL to RANK on osteoclast and thus inhibit activation and differentiation of osteoclasts [187]. Therefore OPG/RANKL system plays a major role in bone formation and absorption. High OPG/RANKL ratio indicates bone formation activities while low value is associated with bone resorption activities [188]. We investigated OPG/RANKL mRNA expression on ASC-scaffold constructs to confirm the involvement of this pathway in osteogenesis. It was further used as an indicator of osteogenesis progress to uncover the mechanism of other regulatory events that may be responsible for MSC osteogenesis.

Recent findings demonstrated the role of the cannabinoid system in bone formation and regulation of bone mass [189-191]. The endocannabinod system constitutes of two cannabinoid receptors (CB₁ & CB₂). Cannabinoid receptors belong to the G protein couple receptor family and mediators of cannabinoid action. Endocannabinoids are well known for their roles in the nervous system and the presence of cannabinoid receptors in bone and peripheral tissue have been reported as well [189-193]. Expression of CB₁ and CB₂ osteoblast progenitors are normally very low [189] and CB₂ mRNA expression increases when the cells are grown in osteogenic medium [194]. Studies reported that MSCs from CB₁ deficiency mice had reduced osteogenic capacity [192]. It has been shown that CB₁ level of mature bone tissue is as low as non bone-forming tissue [192]. The significantly increased level of CB₁ after osteogenic induction of osteoprogenitors may indicate their participation in early stage of osteogenesis [195]. In order to investigate the role of cannabinoid system in osteogenesis and further confirm their participations in ASCs osteogenesis, we investigated the mRNA expression level of CB₁ & CB₂ in ASC constructs and correlation between
CB1&CB2 and OPG, RANKL&OPG/RANKL to explore the mechanism of cannabinoid pathway in adult MSC osteogenesis.

In summary, human ASCs adhesion, viability, proliferation, ECM formation, ultrastructure and mRNA expression level on three commercially available scaffolds with common component of COLI have been investigated in both short-term and long-term osteogenic induction media with stromal media as control for 7, 14, 18 days to test the following hypotheses: 1) COLI scaffold is more superior in osteogenesis by combining osteoinductive components than COLI only scaffold for ASCs; 2) long- and short-term exposure to induction have comparable effects on osteogenesis of chosen constructs; 3) cannabinoid pathways are involved in ASCs osteogenesis.

4.2 Materials and Methods

4.2.1 Isolation of ASCs and Culture

All procedures were approved by the University Institutional Review Board. Subcutaneous adipose tissue was obtained from healthy donors after informed consent. ASCs were isolated from liposuction aspirates of three healthy adults [196]. Briefly, the tissue was finely minced and incubated in 0.01% collagenase type I (Worthington Biochemical, Lakewood, NJ) containing 0.1% bovine serum albumin (Sigma, St Louis, MO) in DMEM/F12 (Hyclone, Logan, UT) for 1 hour at 37 ºC with continuously shaking and brief agitation every 15 minutes. At the end of the incubation, the suspension was centrifuged at 1200 rpm for 10min. The resulting pellet containing ASCs was suspended in stromal media composed of DMEM/F12, 10% characterized fetal bovine serum (Hyclone, Logan, UT), and antibiotics (MP biochemical, Solon, OH). Primary isolated cells were incubated at 37 ºC in 5% CO₂
until reaching 70% confluence. Cells were then replated at a density of 5,000/cm² and expanded up to passage 2 (70-80% confluence prior to passage) in stromal medium. The culture medium was changed every 2-3 days until the end of the experiment. Cells were never allowed to reach 100% confluence.

4.2.2 Cells Loading on Scaffolds

Cells were trypsinized and loaded onto three types of scaffolds with size of 27 mm³: 1) Ultrafoam scaffold with bovine corium collagen type I (UF) (Avitene, Providence, RI); 2) Mastergraft scaffold with type I collagen and biphasic calcium phosphate ceramic composed of 15% hydroxyapatite and 85% β-tricalcium phosphate (MG) (Medtronic, Minneapolis, MN); and 3) Vitoss scaffold with 20% type I bovine collagen and 80% β-tricalcium phosphate (VI) (Orthovia, Malvern, PA) within a spinner flask bioreactor at a cell density of 55,000 cells/mm³ for 2 hrs at 60-80 rpm. Four scaffolds of the different composition were seeded in a spinner flask each time to ensure identical loading of different scaffold for the purpose of comparisons. After 48 hours of culture in stromal medium, scaffold replicates of each composition were assigned to one of three culture medium: 1) stromal medium, 2) 48 hours in osteogenic medium composed of stromal medium, 10mM beta-glycerophosphate (Affymatrix, Santa Clara, CA), 20nM dexamethasone (Sigma, St Louis, MO) and 0.05mM ascorbic acid (Sigma A, St Louis, MO) followed by stromal medium (osteostromal medium), or 3) osteogenic medium. Scaffolds were harvested for evaluation immediately after preculture period (0 day), and after 7, 14 or 28 days in culture.

4.2.3 Cell Viability

Cell viability and distribution in the scaffolds were determined using confocal laser microscopy following live/dead staining (Invitrogen, Carlsbad, CA). Scaffolds were washed
in PBS before incubation in staining solution containing calcein AM and ehtidiumhomodimer-1 (EthD-1) for 30 min in darkness. Live cells were stained green by calceinAM and dead cells were stained red by EthD-1. Inside the cells, calcein-AM is hydrolyzed by endogenous esterase into the highly negatively charged green fluorescent calcein, which is retained inside the cytoplasm. Scaffolds were viewed at 10x using a confocal laser microscope equipped with argon and rhodamine filters (Leica TCS SP2, Buffalo Grove, IL). A total of 50 sequential digital photomicrographs were generated from one surface to the other in the Z plane of each scaffold. Digital images were exported as JPEG images and total cell number was evaluated using graphics software (Image-Pro, Media Cybernetics, Bethesda, MD). The area of each section is 1.44 mm$^2$ and cells in 50 sections were counted. So a total cell area of 72 mm$^2$ was counted.

**4.2.4 RT-PCR**

Quantitative PCR was performed to determine the relative expression of the osteogenic genes as well as regulatory genes of the osteogenesis pathway. Tissue samples were placed in RNA later solution (Qiagen, Valencia, CA), stored at 4°C overnight, and then stored at -20°C until needed. Total RNA was isolated from the samples using tri reagent (Sigma, St Louis, MO) after lysis in a tissue lyser (Qiagen, Valencia, CA). Concentration and quality of isolated RNA was determined using spectrophotometer (Nanodrop, Wilmington, DE). After DNAase treatment, 2μg RNA was reversly transcribed to cDNA using oligo dT and M-MLV reverse transcriptase (Sigma, St Louis, MO). Real time polymerase chain reaction was performed using SYBR green supermix (Bio-Rad) with ABI Prism 7900HT sequence detection system (Applied Biosystem, Foster City, CA) to analyze relative gene expression. Primers (Sigma, St Louis, MO) were designed using Primer 3 software (MIT). Primer sequences are shown
PCR for all genes was run at the same standard cyclic condition, with an initial activation of taq polymerase at 95 for 3 min, followed by 45 cycles of 95 °C/ 15 sec and 60 °C/ 1 min. A dissociation step (95 °C/ 15; 60 °C/ 15 sec; 95 °C/ 15 sec) was added at the end to check PCR product by a dissociation curve. Each sample was run in duplicate with non-template samples of both water and reverse transcriptase negative RNA. Levels of mRNA corresponding genes were quantified using the 2^−ΔΔCt method. Ct values of target genes were normalized by the Ct of housekeeping gene, GAPDH, to obtain the ΔCt values.

**Table 4 Primer sequence for genes quantified in this study**

<table>
<thead>
<tr>
<th>Markers</th>
<th>Genes</th>
<th>Forward Primers</th>
<th>Reverse Primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Osteogenic</td>
<td>ALP</td>
<td>GCTGAACAGGAACACGTGA</td>
<td>CCTCCACCCAGCAAGAAGAA</td>
</tr>
<tr>
<td></td>
<td>Osteocalcin</td>
<td>GACTGTGACGAGTGGCTGA</td>
<td>AAAGAAGGCTGGAGAGGAG</td>
</tr>
<tr>
<td>Regulatory</td>
<td>OPG</td>
<td>AACCCCAAGAGGAAATAC</td>
<td>AAGAATGCCTCCTCACAC</td>
</tr>
<tr>
<td></td>
<td>RANKL</td>
<td>ACGGTCTGGAGGAAATCA</td>
<td>TCCAAAACGTGGGCTCA</td>
</tr>
<tr>
<td>Receptor</td>
<td>CB1</td>
<td>CGATACACTTGGCATTGACG</td>
<td>AACCACGAGCAAAGGAGAGA</td>
</tr>
<tr>
<td></td>
<td>CB2</td>
<td>CAATGAGGGACTTGGGAGA</td>
<td>GGCCTTGACAATTAGGCAGA</td>
</tr>
<tr>
<td>Housekeeping</td>
<td>GAPDH</td>
<td>ATGTTCTGCTAGGGTGAGA</td>
<td>GTCTTCTGGGATGGCAGTGA</td>
</tr>
</tbody>
</table>

**4.2.5 Sample Preparation for Compositional Analysis**

Lyophilized samples were digested with immobilized papain in digestion buffer (2 mg papain, 100mM sodium acetate and 10 mM EDTA) overnight at 60 °C on a shaker (Labnet, Woodbridge, NJ) at 60 rpm. Scaffolds without cells were used as controls. Supernatants were collected after centrifugation for 10min at 4000 rpm and stored at -80 °C until ready to be analyzed.
4.2.6 DNA Content

ASCs growth on scaffolds was evaluated by DNA content using the quantative picogreen assay kit (Invitrogen, Carlsbad, CA) according to manufacturer’s protocol. Briefly, 50 µl of a papain digested sample was mixed with 50 µl picogreen reagent in a clear 96 well plate and incubated at room temperature for 5 min in the dark. The plate was read immediately using fluorescence microplate reader (Synergy HT, Winooski, VT) at the wavelength for fluorescein (excitation 485 nm and emission 538 nm). Amount of DNA content was calculated based on the standard curve.

4.2.7 Protein Assay

Modified lowry method was used to measure the quantity of total proteins [171]. 150 µl of biuret reagent (Sigma, St Louis, MO) was added to 50 µl of sample and standard (bovine serum albumin) in a clear 96 well plate and incubated for 15 min at room temperature. 15 µl of 1N Folin and Ciocalteau’s reagent (Sigma, St Louis, MO) was added to each well and mixed. Absorbance at 650nm was measured after 30 min incubation at room temperature. Samples were diluted to fit into the standard curve range if needed.

4.2.8 BMBB Assay

Quantification of sulfated glycosaminoglycan (sGAG) was performed according to a published protocol[172]. DMBB solution was prepared by dissolving 21 mg of 1,9-dimethylmethylene blue (Sigma, St Louis, MO) in 5ml ethanol and 2.0 g of sodium formate in water separately. pH of the combined solution was adjusted with concentrated formic acid to 3.0 in a final volume of 1 liter. In a clear 96 well plate, 200µl DMBB solution was added to 40 µl of sample and standard. Absorbance was measured at 520 nm immediately after mixing using a microplate reader (Synergy HT, Winooski, VT). Chondroitin sulfate (Sigma,
St Louis, MO) was used as standard and concentration of GAG in samples was determined from the standard curve.

4.2.9 DMBA Assay

Determination of hydroxy proline (total collagen) was measured according to the published protocol [173]. Briefly, 50 µl of concentrated HCl was added to 50 µl of papain digestion buffer and incubated for 18 hrs at 110°C. Samples were dried by heating to about 50°C in a fume hood after hydrolysis and resuspended in acetate citrate buffer. 50 µl of 1.27% chloramines (Sigma, St Louis, MO) prepared in water was added to 50 µl of hydrolyzed sample and incubated at room temperature for 25 min. 15% of p-dimethylaminobenzaldehyde (DMBA) (Sigma, St Louis, MO) was dissolved in n-propanol/perchloric acid (2:1) after addition of 50 µl of Ehrlich’s reagent. The mixture of DMBA and samples were incubated at 60 °C for 20 min and cooled to room temperature. The plate was read at 550 nm. The standard curve was generated with hydroxyproline (Sigma, St Louis, MO).

4.2.10 Scanning Electron Microscopy

Surface morphology, ECM formation of scaffolds and cell distribution on scaffolds were evaluated using a scanning electron microscope (SEM) (Quanta 200, FEI Company, Hillsboro, OR). The cell constructs were rinsed with PBS and fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for an hour, post fixed for 48 h in 0.1% osmium tetroxide (OsO4), and then dehydrated through the ethanol series (50% to 100%). After critical point dry, the samples were then sputter coated with gold and imaged with SEM at 15 kVP.
4.2.11 Statistical Analysis

All results were expressed as mean ± standard deviation (SD). Data was analyzed with one-way analysis of variance (ANOVA), followed by Tukey’s post hoc test. For all comparisons, significance was set at p<0.05.

4.3 Results

4.3.1 Osteogenic Potential of ASCs

The osteogenesis of ASCs potential was verified by alizarin red staining of calcium deposition after 21 days of osteogenic induction (Fig. 21A). Calcium staining was negative for hASCs cultured for 21 days in stromal cultures (Fig. 21B).

![Figure 21 Alizarin red stain to show osteogenic potential of ASCs after 21 days induction in osteogenic medium (A) and stromal medium (B).](image)

Calcium deposition was positive indicated by Alizarin red stain to show osteogenesis of ASCs after 21 days induction in osteogenic medium (A) and no calcium deposition confirmed no osteogenesis of ASCs in stromal medium (B).
4.3.2 Cell Distribution and Viability

Cell viability was greater than 70% at all time points, indicating that cell scaffold constructs remained relatively viable at all time points. Number of viable cells was highest in the UF scaffold with lower viable cell numbers but comparable cell number in MG and VI scaffolds (Fig. 22).

![Graph showing cell counts on all three scaffolds with different culture time and culture medium.](image)

**Figure 22** Mean±SD of cell counts on all three scaffolds with different culture time and culture medium.

Mean±SD of cell counts on all three scaffolds (top-UI, middle-MG, bottom-VI) in stromal, osteo-stromal, osteogenic medium for 0, 7, 14 and 28 days.

4.3.3 Expression of Osteogenic Marker

mRNA levels of osteogenic markers ALP and OCN were upregulated at all time points in all scaffolds. There is a significant increase in mRNA levels of ALP after 14 days of induction for UF and 7 days for MG but no significant increase observed in VI (Fig. 23A). Short-term
exposure to osteogenic media showed early (7 day) induction only for MG but this is a transient induction which disappears gradually after withdrawal of induction media. There were significant differences in expression of ALP between UF and MG and also between VI and MG but not between UF and VI. After 14 days in induction medium, the mean fold change in ALP mRNA for UF, MG and VI was 18.13, 18.37 and 5.0 respectively. OCN were late responsive markers and showed increased expression after 28 days of induction (Fig. 23B) but only MG showed significant upregulation. MG induced the greatest expression of these osteogenic markers and VI had the least upregulation.

Figure 23 Mean±SD fold changes of both ALP (A) and OCN (B) expression of hASCs on all three different scaffolds in all three media for all time points. Mean±SD fold changes of both ALP (A) and OCN (B) expression of hASCs on all three different scaffolds (top-UI, middle-MG, bottom-VI) in stromal, osteo-stromal, osteogenic medium for 7, 14 and 28 days respectively. Significant differences are indicated by *, ** and *** within the same time points among different medium and by a, b and c within the same medium type among different time points.
4.3.4 Expression of Regulatory Genes

mRNA levels of RANKL were significantly decreased and OPG mRNA expression was upregulated on all scaffolds cultured in osteogenic medium, resulting in increases in the OPG/RANKL ratio. After 28 days in osteogenic medium, the mean OPG/RANKL ratio in UF scaffold was 67.8, MG 90.4 and VI 6.22 fold higher than that in pre-culture scaffolds (Fig. 24).

![Figure 24](image)

**Figure 24** Mean±SD fold changes of OPG/RANKL expression of ASCs on all three different scaffolds in all three media for all time points.

Mean±SD fold changes of OPG/RANKL expression of ASCs on all three different scaffolds (top-UI, middle-MG, bottom-VI) in stromal, osteo-stromal, osteogenic medium for 7, 14 and 21 days respectively. Significant differences are indicated by *, ** and *** within the same time points among different medium and by a, b and c within the same medium type among different time points.

4.3.5 Expression of Cannabinoid Receptor Genes

There was a significant induction in the mRNA expression of CB1 in the induction group for all scaffolds, however, the UF showed the greatest induction. After 14 days in osteogenic medium, the fold changes in the UF scaffold was 46,000, MG 22,955 and VI 5,647 fold
higher relative to pre-culture scaffolds (Fig. 25A). On the other hand, CB₂ showed a moderate and mixed trend in mRNA expression. Spontaneous induction was observed in all scaffolds. When cultured in osteoinductive media, there was induction in MG but reduction both for UF and VI scaffold (Fig. 25B).

**Figure 25** Mean±SD fold changes of CB1 (A) and CB2 (B) expression of ASCs on all three different scaffolds in all three media for all time points.

Mean±SD fold changes of CB1(A) and CB2 (B) expression of ASCs on all three different scaffolds (top-UI, middle-MG, bottom-VI) in stromal, osteo-stromal, osteogenic medium for 7, 14 and 21 days respectively. Significant differences are indicated by *, ** and *** within the same time points among different medium and by a, b and c within the same medium type among different time points.

4.3.6 Correlations Between Regulatory Genes and Cannabinoid Receptor Genes

There were significant positive correlations between OPG/RANKL ratio and CB1 expressed on all three scaffolds. The positive correlation between OPG/RANKL and CB2 was significant only on MG scaffolds. Additionally, the correlations between RANKL and both
CB1 and CB2 were negative and significant on MG scaffolds. For OPG expression, it only had significant correlation with CB2 expressed on VI scaffolds (Table 5).

Table 5 Pearson correlations between OPG, RANKL, OPG/RANKL and CB1, CB2 expressed on all three different scaffolds.

<table>
<thead>
<tr>
<th></th>
<th>Variable</th>
<th>CB1</th>
<th>CB2</th>
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<tbody>
<tr>
<td><strong>UF</strong></td>
<td>OPG</td>
<td>0.18384 (p=0.6359)</td>
<td>-0.04509 (p=0.9083)</td>
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<td>RANKL</td>
<td>-0.50054 (p=0.1699)</td>
<td>0.50682 (p=0.1638)</td>
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<td>OPG/RANKL</td>
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<td><strong>MG</strong></td>
<td>OPG</td>
<td>-0.00221(p=0.9955)</td>
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<td><strong>VI</strong></td>
<td>OPG</td>
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<td>OPG/RANKL</td>
<td><strong>0.83097 (p=0.0055)</strong></td>
<td>-0.02481 (p=0.9495)</td>
</tr>
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</table>

4.3.7 Biochemical Analysis

There were increases in DNA content on all scaffolds with maximum DNA levels after 7 days in culture and the greatest increase on the MG scaffold (Fig. 26A). Total GAG was significantly increased on the UF scaffold after 28 days, while on the MG scaffold there was a significant increase after 7 days of culture and remained elevated until the end of the study (Fig. 26B). There were no significant changes of collagen contents on the VI. Only the UF scaffold showed significant induction of collagen after 14 days in induction media (Fig. 26C). No significant changes in protein content were observed (Fig. 26D).

4.3.8 Cell Adhesion and Morphology

Cell morphology and formation of ECM on the surface of cell-scaffold constructs was evaluated by SEM images. By comparing among three different scaffolds, UF had the most porous structure while VI had very few pores, indicating that porous structure was largely determined by ColI components (Fig. 27A, C and E). ASC adhered on the surface formed sheets covering the pores of scaffolds after 7 days of culture. There was apparent ECM
formation on all constructs under induction with most enhanced formation on UF. Cells and ECM appeared to cover pores of UF scaffold and assumed a uniform appearance (Fig. 27B). ECM mineralization was most apparent on VI scaffolds (Fig. 27F).

4.4 Discussion

Ideal biomaterials for osteogenesis should support cell attachment, migration, proliferation, and stimulate new bone formation. A wide selection of commercial scaffolds is available for clinical practice but physiochemical properties of these scaffolds are different. The multipotentiality of ASCs has been well established [16-18]. Adipose tissue is available in large quantities and is easily accessible, making ASCs a promising cell source for regenerative medicine. Therefore in the present study, we used an in vitro culture of ASCs to

![Figure 26](image)

Figure 26 Mean±SD fold changes of DNA (A), GAG (B), collagen (C) and total protein (D) quantified on all three different scaffolds.

Mean±SD fold changes of DNA (A), GAG (B), collagen (C) and total protein (D) quantified on all three different scaffolds (top-UI, middle-MG, bottom-VI) in stromal, osteo-stromal, osteogenic medium for 7, 14 and 21 days respectively Significant differences are indicated by *, ** and *** within the same time points among different medium and by a, b and c within the same medium type among different time points.
Figure 27 SEM images of ASCs on all three different scaffolds.

SEM images of ASCs on all three different scaffold (A. UF only; B. UF with cell; C. MG only; D. MG with cell; E. VI only; F. VI with cell) after 7 days culture in osteogenic medium, compared with scaffolds only without cells.
evaluate their seeding efficiency and osteogenesis potential on three different commercially available scaffolds. Overall, UF and MG were superior to VI in ASCs attachment, growth, differentiation and ECM secretion, indicating possible critical role of porous COLI components in efficient osteogenesis. Continuous exposure to osteogenic medium was proved to be the best for in vitro osteogenesis especially for ASCs on MG scaffolds. Short-term osteogenesis induction was able to trigger osteogenesis process similar to long-term osteogenesis after 28 days of culture. Substantial upregulation of CB1 in osteogenic medium demonstrated that cannabinoid pathway may play an important role in differentiation of osteoprogenitor cells. This study established procedures to optimize in vitro customized bone regeneration with spinner flasks loading of easy to be isolated human ASCs onto suitable commercially available scaffolds. This achievement significantly investigated the great potential of adult ASCs to meet the needs of human bone regeneration.

Seeding efficiency, viability, DNA contents and ultrastructure of ASCs noticeably differed among the scaffolds and showed consistent results with UF as the most suitable scaffolds for ASCs adhesion and proliferation. Spinner flasks provided identical environment for ASCs loading on all three different scaffolds. The differences existed among the scaffolds are due to their diversity in morphology and composition. It is possible that decreased capacity for cell adhesion and proliferation was caused by increased proportion of ceramics components and subsequent decreased porosity from UF to VI. Both UF and MG have porous structure which might favor the cell attachment, migration, proliferation and differentiation of the ASCs. Compact and crystal structure of VI may not be in favor of the cell adhesion process. This structural difference may be the key factor to the observed difference in scaffolds capacity to support cell growth.
Although MG had decreased cell adhesion compared with UF, MG is more appropriate for osteogenesis with addition of ceramics components without compromising its porous structure, indicated by its most enhanced osteogenic gene upregulation and ECM formation. ALP expression increased significantly as early as 7 days while significant OCN upregulation did not present until 28 days of induction on MG. This is in agreement with established concept that ALP is early and OCN is a late osteogenic marker. In comparison, ALP and OCN expression did not show any significant change over time or in different medium for VI, indicating the lack of support by VI for ASCs osteogenesis. The significant upregulation of ALP for ASCs on UF after 14 days of induction suggested the role of UF in osteoinductivity. ECM formation was consistent with changes of osteogenic gene expression. Newly formed ECM was most enhanced on UF in long-term osteoinduction medium. Long-term osteoinduction affects significantly for GAG formation on MG. VI still showed no difference by both medium and time for all outcomes. In summary, UF is considered the optimal scaffolds for ASCs adhesion and growth while MG is most suitable for osteogenesis with minimal compromise to support cell proliferation, indicating MG as the best candidate to support ASCs into functional bone specific tissue.

OPG/RANKL pathway plays an important role in the regulation of osteoclast maturation [25-27]. Many paracrine factors including cytokines regulate differentiation of osteoclasts by regulating RANKL and OPG expression. The osteogenic effects of BMP2 was confirmed by increased OPG/RANKL ratio in human MSCs on calcium phosphate substrate when treated with BMP2 at day 12 [197]. OPG/RANKL ratio expressed by ASCs increased significantly after 14 days of induction on MG and this increase peaked at day 28 for all scaffolds, which indicated that active osteogenesis. Substantial upregulations of CB1 receptor with ten
thousands fold increase on all scaffolds after 14 days induction suggested the important role CB1 plays in osteogenesis. Furthermore, there was consistent significant positive correlation between CB1 receptor and OPG/RANKL ratio on all three scaffolds, emphasizing the role of CB1 as osteogenesis regulator. CB2 receptor, though less influential as CB1, showed similar increasing trend as CB1 and may share the osteogenesis regulation function. CB1−/− mice had defects in osteoclast formation, which was caused by a decreased sensitivity of osteoclast precursors to RANKL as well as no sufficient RANKL expression to support osteoclast formation [21]. Bone marrow cells of CB2−/− mice also produce fewer osteoclasts than wild-type controls in response to RANKL [190]. Therefore with increased expression of CB1 and CB2, less RANKL is required to maintain balance between osteoblast and osteoclast formation. This is consistent with negative correlations between RANKL and both CB1 and CB2, which is significant on MG scaffolds. Cannabinoid pathway may play a role in adult MSC osteogenesis, especially having impacts on osteoprogenitor cells to switch them from self-renewal process to differentiation process. There were still a large proportion of progenitor cells existing as early as day 7. With increased expression for CB1 and CB2 caused by osteoinduction, their self-renewal process was suppressed and osteoprogenitor cells started to differentiate. This further triggered the OPG/RANKL pathway to regulate the differentiated osteoblast and osteoclast for bone formation. The correlation between OPG/RANKL and CB1/CB2 might suggest that they can provide positive feedback to each other. Further research should be conducted to confirm this preliminary finding. A further study using knockdown technique may be able to establish the exact role of cannabinoid receptor in human ASCs osteogenesis.
In this study we selected three scaffolds of different composition and structure and investigated their suitability for bone regeneration with ASCs. We also intended to test how osteogenic potential of ASCs on biocompatible scaffolds is affected by increased proportion of osteoinductive ceramic components. This study clearly showed the difference in the osteoinductive properties of these scaffolds when loaded with ASCs and established MG as the optimal scaffolds for osteogenesis of human ASCs. Osteogenesis was significantly enhanced by continuous exposure to osteogenic medium. Short exposure to osteogenic medium did not have any significant effect on osteogenesis although it had a transient effect on gene expression, suggesting that short-term exposure to osteogenic media might have an osteoinductive effect in vivo. Further studies are necessary to determine if similar results occur in vivo. One limitation of this study is the difficulty in discerning which properties of scaffolds (i.e., composition, surface chemistry, pore structure or porosity) are responsible for the observed differences. Because the scaffolds used in this study had different surface topography, porosity, physical characteristics and chemical composition. Further investigation using scaffolds with the same chemical composition but with different surface and pore structures may be able to acquire more information on interaction mechanism between cell and scaffold. The present study investigated systematically to solve the current issues associated with difficulties in selection of suitable bioscaffolds for bone regeneration and offers a basis for future studies involving stem cell-based therapy in order to improve bone regeneration process.
CHAPTER 5. THREE-DIMENSIONAL BIOSCAFFOLDS FOR ADULT EQUINE MULTIPOTENT STROMAL CELLS OSTEOGENESIS IN VITRO: A PRELIMINARY REPORT
5.1 Introduction

Synthetic polymers provide diversity by altering their fabrication parameters. The morphology and porosity as well as the pore size can be modified to match tissue specific needs for mechanical properties and rates of degradation. Increase in porosity allows for adequate diffusion and it should be adjusted without compromising its mechanical properties. Polylactic acids (PLLA) are considered as the most frequently used polymers in bone tissue engineering [198-199]. Polyethylene glycol (PEG) is often used to construct copolymer with PLLA to adjust the degradation rate [198, 200]. However, the surfaces of polymers are not very suitable for cell attachment. There are other bioscaffolds resemblance to bone inorganic components considered as promising candidates for producing bone construct, such as hydroxyapatite (HA) and β-tricalcium phosphate (β-TCP). But the low mechanical strength and relatively slow degradation may limit their application in bone regenerations. Therefore, it is critical to assess their effects alone as well as their combinations to choose the best bioscaffolds for bone regeneration.

The two most common and easy to access sites for isolating adult equine multipotent stromal cells (MSCs) are bone marrow from sternal bone and adipose tissue from superficial gluteal muscle [13, 134]. Therefore equine bone marrow- (BMSCs) and adipose derived multipotent stromal cells (ASCs) are chosen here to explore their potential application in equine tissue regeneration seeded onto bioscaffolds suitable for bone regeneration. Before applying them in equine patient, it is critical to evaluate their osteogenesis efficiency in vitro and thus can help to determine the optimal scaffold for later bone regeneration in vivo.

Another critical factor need to be investigated here is the sufficient time needed for induction before implantation. Cell-scaffold constructs should be pre-induced in osteogenesis
microenvironment before implantation to ensure their commitment into osteogenesis lineages. The shortest time required for osteogenesis should be determined to ensure the efficiency of this procedure. In this study, BMSCs and ASCs were seeded onto three different bioscaffolds composed with polymer alone (PLLA/PEG), polymer with bone-like component - ceramics (PLLA/PEG/HA/ β –TCP) and ceramics alone (HA/ β –TCP). Cell-scaffold constructs were then induced for different time points to assess and compare their osteogenesis capacity and efficiency in vitro.

5.2 Materials and Methods

5.2.1 Experimental Design

Cryopreserved passage 0 or 1 equine ASC and BMSCs were retrieved and expanded up to passage 3 to acquire adequate cell number for all the outcome measurements. Cells were then loaded onto round 60%/40% PLLA/PEG, round 36%/24%/24%/16% PLLA/PEG/ HA/β –TCP (generous gifts from Dr Daniel Hayes) and 60% HA/40% β – TCP (Scaffoldex, Tampere, Finland) by spinner flasks at a loading concentration of 10⁶ cells/per scaffold. Cell-scaffold constructs were evaluated immediately after loading and after 7, 14 and 21 days of culture in osteogenic induction media as well as stromal media when scaffolds were assessed for cell viability, cell proliferation, cell differentiation, extra cellular matrix synthesis and ultrastructures of cell as well as cell-scaffold constructs.

5.2.2 Animals

Cells used in this study were isolated from 3 Thoroughbred geldings (7 to 10 years old). Bone marrow (BM) was collected from sternebrae and adipose tissue was collected from superficial gluteal fascia[13, 134]. The collection procedure was approved by the
Institutional Animal Care and Use Committee. Stromal cell isolations were performed immediately after tissue harvest.

5.2.3 Stromal Cell Isolation and Cryopreservation

The BM aspirate was diluted 1:1 with DMEM-Ham’s F12 (Hyclone, Logan, UT) with 1% antibiotic/antimycotic solution (MP Biomedical, Irvine, CA) and 10% characterized fetal bovine serum (FBS, Hyclone, Logan, UT) added. It was then layered over Ficoll-Paque PLUS (Stem Cell Technologies) and centrifuged (350 g, 30 min). The total nucleated cell numbers per unit volume of BM aspirate were then calculated from BMSC - enriched layer.

Adipose tissue was minced in a sterile petri dish, washed and briefly agitated with an equal volume of phosphate buffered saline (PBS) to promote separation into two phases. The upper phase containing hematopoietic cells was removed. The tissue was then digested in an equal volume of a filtered (0.2 µm) PBS solution containing 1% bovine serum albumin type V (BSA) (Sigma, St Louis, MO) and 0.1% collagenase type I (Worthington, Lakewood, NJ). The tissue and solution were continuously rotated at 37°C for 30 minutes with brief agitation every 15 minutes. Subsequently, the sample was centrifuged (350g, 5 min). To complete stromal cell separation from primary adipocytes, the sample was briefly and vigorously agitated and then centrifuged (350g, 5 min) resulting in a stromal-vascular fraction (SVF) pellet. The SVF pellet was resuspended in 2 ml of stromal media. A small aliquot of the cell suspension was centrifuged and resuspended in an equal volume of red cell lysis buffer for 5 minutes. The total nucleated cell numbers per unit volume of adipose tissue were then calculated.
The fresh isolated cells were cultured for 4-5 days. Cells were passaged once if there were no sufficient cell number from passage 0. Then aliquots of cells (1 x 10^6 cells/ml) of passage 0 or 1 were frozen in cryopreservation medium, which was made up with 80% FBS, 10% DMEM, and 10% dimethylsulfoxide.

5.2.4 Flow Cytometry
Revitalized BMSCs and ASCs were cultured for 4-5 days until 70% confluence. Cells from 3 equine donors were pooled equally and cells (2 x 10^5) were suspended in 150 ul PBS and then mixed with 1.5 mL PE conjugated CD 34, CD29, CD73 and FIC conjugated CD 44, CD45, CD90 and CD105 (Table 1) in 5 ml round bottom glass tubes and incubated for 30 minutes in room temperature. Cell aliquots served as the autofluorescence controls. Samples were rinsed with 2 ml PBS followed by centrifugation (350g, 5 minute). The cell pellet was fixed in 30 ul of cold 1% formalin and stored at 4°C until FACS Analysis.

5.2.5 Cell Seeding
Revitalized passage 0 or passage 1 ASCs and BMSCs were expanded up to passage 3 in stromal medium (DMEM-Ham’s F12 (Hyclone, Logan, UT), 1% antibiotic/antimycotic solution (MP Biomedical, Irvine, CA), and 10% characterized fetal bovine serum (FBS, Hyclone, Logan, UT)). Seeding density was 5,000 cells/cm^2 for all passages and cells were passaged at 70-80% confluence. Passage 3 cells from 3 equine donors in stromal media were pooled and loaded onto 3 different scaffolds in spinner flasks. Loading was performed for two hours in a humidified incubator (37°C, 5% CO2).
5.2.6 Cell Proliferation

Cell proliferation was analyzed using [3-(4,5-dimethylthiazol-2-yl)-1, 5-diphenyl tetrazulium bromide] (MTT, Sigma, USA) mitochondrial reaction. This assay is based on the ability of live cells to reduce a tetrazulium-based compound, MTT, to a purplish formazan product. Briefly, cells/scaffold constructs either from day 0, 7 or 21 days of 3D culture were first washed with PBS, transferred into new 24-well plates containing a 5:1 ratio of media and MTT solution (5 mg/mL in PBS) respectively and incubated for 2h at 37ºC. After removing the culture media, 0.5mL of extraction solution (DMSO) was added. The constructs were washed extensively by pipetting up and down repeatedly to allow total color release. The absorbance of the supernatant was read with a microplate reader (BioTek, USA) at 540 nm. The cell number on scaffolds was determined via a standard curve that was established using a known number of cells counted by hemocytometer using light microscope.

5.2.7 Alkaline Phosphotase Staining for Osteogenesis

Osteogenesis was demonstrated by comparing the amount of staining on the composite scaffolds using an alkaline phosphotase (ALP) staining (Chemicon, USA). Constructs were washed in PBS and fixed in 10% formaldehyde for 1 hour at room temperature. ASMX and fast red stock solution was made by dissolving them respectively in distilled water with 50 mg/ml concentration. Then ASMX and fast red stock solutions were mixed and diluted in PBS to 1/200 and 1/400 respectively to make ALP staining solution. After washing three times with PBS, the constructs were incubated in ALP staining solutions for few hours. Scaffolds without cells were used as a control.
5.2.8 Scanning Electron Microscopy

Cell-scaffold constructs cultured in osteogenic medium were evaluated with SEM after 7 and 21 days of culture. Constructs were fixed in 2% paraformaldehyde and 1.25% glutaraldehyde in 0.1 M sodium cacodylate (CAC) buffer, pH 7.4. Samples were rinsed in 0.1M CAC buffer with 5% sucrose and incubated with 1% osmium tetroxide in 0.1 M CAC buffer. They were dehydrated in a series of ethanol-distilled water solutions, critical point dried in a Polaron CO2 bomb, and then sputter coated with gold and palladium. Samples were imaged with a scanning electron microscope (Quanta 200, FEI Company, Hillsboro, OR) at 15 kVP.

5.2.9 Transmission Electron Microscopy

Transmission electron microscopy was performed as described previously (Shakibaei et al, 1997). Cells cultured with scaffolds in osteogenic medium were trypsinized, centrifuged and washed in PBS. After the second centrifugation, cell pellets were fixed in 2% paraformaldehyde and 1.25% glutaraldehyde in 0.1 M sodium cacodylate (CAC) buffer, pH 7.4. Samples were rinsed in 0.1M CAC buffer with 5% sucrose and incubated with 1% osmium tetroxide in 0.1 M CAC buffer. The cell pellets were dehydrated in a series of ethanol-distilled water solutions and embedded in Epon (Plano, Marburg, FRG). Ultrathin sections of the constructs were prepared and evaluated with a transmission electron microscope (TEM).
5.3 Results

5.3.1 Flow Cytometry

Stromal cell surface marker expression was higher in BMSCs for four out of five of those investigated in this study. Specifically, 90.30% of BMSCs and 98.98% of ASCs were CD29+; 1.47% of BMSCs and 10.97% of ASCs were CD44+; None of BMSCs and ASCs were CD73+; 28.19% of BMSCs and 46.24% of ASCs were CD90+; and 58.83% of BMSCs and 87.23% of ASCs were CD105+. Both BMSCs and ASCs were negative for CD34 (Fig. 28).

![Flow Cytometry Results](image)

**Figure 28 Histogram of flow cytometry results.**

Histogram of CD 29, CD44, CD73, CD90, and CD105 antibody stained cell (black) with auto fluorescent control cell (green) for both BMSCs and ASCs.

5.3.2 Cell Proliferation

Overall, there were no significant differences between ASCs and BMSCs based on cell metabolic activity. Metabolic activity was affected significantly by time on all three scaffolds for BMSCs and on HA/β-TCP scaffold for ASCs. For BMSCs on all three scaffolds and ASCs on HA/β-TCP, their metabolic activities were significantly higher at day 21 compared with day 7 (Fig. 29).
Figure 29 MTT assay to quantify cell metabolic activities on all three scaffolds.

Mean±SD of fold change of cell metabolic activity of both BMSCs and ASCs on PLLA/PEG, PLLA/PEG/HA/TCP and HA/TCP scaffolds after 0, 7 and 21 days of culture in osteogenic medium. Different letters indicated significant differences between different culture time within the same cell type.
5.3.3 ALP Staining for Osteogenesis

For both ASC and BMSC on all three scaffolds, there were distinct differences of ALP stain between non-induced and osteogenic induced group at their outer surfaces (Fig. 30 and 31). Between constructs induced for 7 and 21 days, however, there were no apparent differences of the stain at both outer surfaces and inner surfaces (Fig. 30-33). Compared between outer surfaces and inner surfaces, it was obvious that much less stain existed on the inner surfaces than outer surfaces for all the stained groups especially for cell-HA/β-TCP constructs (Fig. 30-33).

Figure 30 ALP stained the outer surfaces of all three ASCs–scaffolds constructs under osteogenic induction for 7 and 21 days as well as under no induction at time 0 as control.

ALP stained the outer surfaces of ASCs-PLLA/PEG constructs (A-C), ASCs-PLLA/PEG/HA/β-TCP constructs (D-F), and ASCs-HA/β-TCP constructs (G-I) under osteogenic induction for 7 days (B, E, H) and 21 days (C, F, I) as well under no induction at time 0 as control (A, D, G).
Figure 31 ALP stained the outer surfaces of all three BMSCs–scaffolds constructs under osteogenic induction for 7 and 21 days as well as under no induction at time 0 as control.

ALP stained the outer surfaces of BMSCs-PLLA/PEG constructs (A-C), BMSCs-PLLA/PEG/HA/β-TCP constructs (D-F), and BMSCs-HA/β-TCP constructs (G-I) under osteogenic induction for 7 days (B, E, H) and 21 days (C, F, I) as well under no induction at time 0 as control (A, D, G).
ALP stained the inner surfaces of all three ASCs–scaffolds constructs under osteogenic induction for 7 and 21 days as well as under no induction at time 0 as control.

ALP stained the inner surface of ASCs-PLLA/PEG constructs (A-C), ASCs-PLLA/PEG/HA/β-TCP constructs (D-F), and ASCs-HA/β-TCP constructs (G-I) under osteogenic induction for 7 days (B, E, H) and 21 days (C, F, I) as well under no induction at time 0 as control (A, D, G).
Figure 33 ALP stained the inner surfaces of all three BMSCs–scaffolds constructs under osteogenic induction for 7 and 21 days as well as under no induction at time 0 as control.

ALP stained the inner surface of BMSCs-PLLA/PEG constructs (A-C), BMSCs-PLLA/PEG/HA/β-TCP constructs (D-F), and BMSCs-HA/β-TCP constructs (G-I) under osteogenic induction for 7 days (B, E, H) and 21 days (C, F, I) as well under no induction at time 0 as control (A, D, G).
5.3.4 Scanning Electron Microscopy

Three scaffolds demonstrated distinct ultra-structures visualized by SEM (Fig. 34). Compared with PLLA/PEG scaffolds, PLLA/PEG/HA/β-TCP scaffolds had much more organized pore structures. HA/β-TCP scaffolds were composed with much lower porosity and the macro-pores were much larger than the other scaffolds (Fig. 34A-C). However, after examined with higher magnification, the micro-pores of HA/β-TCP scaffolds were much smaller than the other two scaffolds. HA/β-TCP scaffolds were mainly composed by dense mineral granules while the other two scaffolds demonstrated fibrous and membranous structure. It was shown that the white and bright spots accumulated randomly were the mineral components of PLLA/PEG/HA/β-TCP scaffolds (Fig. 34E).

![SEM images of PLLA/PEG scaffold (A), PLLA/PEG/HA/β-TCP (B) and HA/β-TCP (C) under the same magnification (400x) as well PLLA/PEG scaffold (D), PLLA/PEG/HA/β-TCP (E) and HA/β-TCP (F) under the same magnification (3000x).](image-url)

Figure 34 SEM image demonstrated the ultrastructure of all three scaffolds at 400x and 3000x magnification.

SEM images of PLLA/PEG scaffold (A), PLLA/PEG/HA/β-TCP (B) and HA/β-TCP (C) under the same magnification (400x) as well PLLA/PEG scaffold (D), PLLA/PEG/HA/β-TCP (E) and HA/β-TCP (F) under the same magnification (3000x)
Because PLLA/PEG and PLLA/PEG/HA/β-TCP scaffolds were fibrous and membranous especially did not have organizable pattern at higher magnification, it was very difficult to differentiate cell shape and distributions on these two scaffolds (Figure not shown). For ultrastructure of cell-HA/β-TCP constructs, polygonal shaped cells were evident on both constructs after 7 days of osteogenic induction while ASC-HA/β-TCP constructs generated significantly more extra-cellular matrix (Fig. 35 A&D). The extra cellular matrix were matured and expanded to combine with scaffold, which were distinguishable by its distinct ultrastructure for ASC-HA/β-TCP constructs cultured for 21 days (Fig. 35E).

![Figure 35 SEM image of both ASC- and BMSC- HA/β-TCP scaffolds constructs at 3000x magnification.](image)

SEM image of ASCs - (A, B) and BMSCs - HA/β-TCP constructs (D,E) after 7 days (A,D) and 21 days (B,E). HA/TCP scaffold without cells is provided for comparison (C, F). Red arrows point to polygonal cells (A, D, E) attached to the scaffold and neo extracellular matrix (B).
5.3.5 Transmission Electron Microscopy

Preliminary results of TEM were reported here. ASCs at day 0 without any induction was a round cell containing well-developed organelles, mainly composed of large quantities of mitochondria, endoplasmic reticulum, lipid droplets, and few ribosomes in cytoplasm. ASC was observed with two set of euchromatin in its notched nucleus, which was filled with prominent nucleoli. Cell processes were present at the ASCs surface. All these characteristics indicated a very active cell metabolism (Fig. 36A). Compared with ASC without any induction, induced ASC for 14 days revealed a distinct difference with larger lipid droplets and less structured cell organelles. Cell did not have processes at the surface. Although the size of cell was bigger than ASC at day 0, the euchromatin was much smaller (Fig. 36B).

![Figure 36 TEM demonstration of ASCs after day 0 (A) and day 14 (B) of osteogenic induction.](image)

TEM demonstration of ASC without any induction (A) and ASCs under 14 days of osteogenic induction (1- nucleus, 2-mitochondria, 3- endoplasmic reticulum, 4-a lipid droplet, 5-a ribosome, 6-euchromatin)
ASCs on three scaffolds after 14 days of induction did not reveal any substantial differences between each other as well as compared with ASCs control. ASC on PLLA/PEG/HA/β-TCP, however, contained much larger quantities of lipid droplets. On the contrary, ASC on HA/β-TCP contained the least amount of lipid droplets. ASCs of all groups contained a number of mitochondria, a sign for active cell metabolism (Fig. 37).

Figure 37 TEM demonstration of ASC constructs of all three scaffolds as well as ASCs only as control after 14 days of osteogenic induction.

TEM demonstration of ASC constructs of all three scaffolds (A. PLLA/PEG, B. PLLA/PEG/HA/β-TCP, C. HA/β-TCP) as well as ASCs only as control after 14 days of osteogenic induction.
5.4 Discussion

This pilot study demonstrated the osteogenic lineage differentiation potential of both equine BMSCs and ASCs *in vitro* on three bioscaffolds suitable for bone regeneration. It was preliminarily confirmed that all three scaffolds are suitable for cell adhesion, proliferation and differentiation into functional osteo-specific tissue during *in vitro* development.

The pooled BMSCs and ASCs showed similar immunophenotype and had positive expressions for three mesenchymal stromal cell markers. ALP staining solved the difficulties that researchers encountered when referred to histology to confirm cell differentiation. Because of the fragile structure of polymers and hardness of ceramics, it was very difficult to obtain intact pieces of constructs on slides to investigate their microstructure. ALP staining of the whole constructs not only avoided unsuccessful sectioning but also provided three-dimensional view to demonstrate cell distribution on the scaffolds. It was confirmed by ALP staining that both cells can committee osteogenic differentiation on all three bioscaffolds under appropriate induction as early as day 7, which did not reveal dissimilarities from differentiation at day 21. Moreover, much less stain on the inner surface of HA/β-TCP scaffold for both cell types indicated the insufficient cell migration into the scaffolds that may be caused by its lack of micro-pores. The fact that cell metabolic activities was significantly increased at day 21 compared with day 7 for all BMSC constructs and for ASC-HA/β-TCP constructs may suggest that parts of cells at day 7 still maintain its ability of self-renewal.

The ultrastructure of all three scaffolds revealed the advantages of polymer components of scaffolds, which showed interconnected pore structures suitable for cell attachment. However, the dense structure composed by HA may indicate better mechanical properties.
The polygonal shaped cells existed for both cell-HA/\(\beta\)-TCP constructs with more developed extra cellular structure on ASC constructs. This indicated that compromised pore structures of HA/\(\beta\)-TCP did not cause insufficient cell adhesion onto the surface of this scaffolds. Moreover, this scaffolds provided excellent support for extra cellular matrix formation. However, there were no recognizable SEM images to demonstrate cell adhesion and extra cellular matrix formation on the other two scaffolds due to their structures.

In order to examine and compare cell progression into osteogenic lineages among all three scaffolds, TEM was introduced to compensate the incomplete results from SEM. Due to few technique issues, this preliminary study only included ASCs on all three scaffolds after 14 days of induction. Overall, it was obvious that ASCs under induction on all three scaffolds showed distinct cellular structure compared with ASCs without any induction. Besides, the differences existed among different constructs, which need further investigation to reveal the underlying mechanism.

In conclusion, these preliminary data has demonstrated, for the first time, that equine ASCs and BMSCs posses osteogenic potential when loaded onto bioscaffolds under induction. The displayed ultrastructure of cell on bioscaffolds further supported this finding. A comprehensive study with a deeper insight into the osteogenic differentiaton mechanism may help to uncover the interactions between cell and scaffolds and therefore promote the future use of equine MSCs in equine regeneration medicine, which may contribute to customized biomaterials to match different clinical needs.
CHAPTER 6. CONCLUSIONS AND FUTURE RESEARCH
The major contribution of these studies is that they established straightforward and reproducible procedures to support customized tissue engineering with proper bioreactors loading of easy to be isolated adult MSCs onto suitable bioscaffolds to grow aimed tissue type. The results showed that the suitability of bioscaffolds for MSC growth can be confirmed by high MSC viability and increased DNA contents over time. Furthermore, cell differentiation capacity into distinct mesenchymal tissues on bioscaffolds was successfully quantified by related mRNA upregulation and ECM formation. Therefore the optimization of cell-scaffold constructs can be achieved to ensure their high efficiency to commit to specific tissue lineages. These achievements expanded the application of equine regenerative medicine. Using proper engraftments of cells on bioscaffolds can expand application of equine stem cell therapy, which was previously limited to MSCs administration as a single product. MSCs integration into lesion sites guided by bioscaffolds as a tissue graft will significantly increase the efficiency of healing in clinics in near future.

It was shown by the first in vitro study that both equine BMSCs and ASCs demonstrated good cell adhesion and growth on COLI scaffolds. According to the requirement of current stem cell therapy, at least 10 million cells are needed for one injection to repair lesions. To obtain this amount of cells, it normally takes 3-4 weeks’ culture. One reason of doing this time-consuming cell culture is because of the fewer cells stayed at lesion sites due to cell migrations. Scaffolds play an important role in maintaining sufficient cells on the lesion sites for tissue repair. The potential of COLI scaffolds in targeting majority of cells to treat injuries in vivo was established by their strong capacity to support cell adhesion and growth in vitro. Therefore cell migration can be suppressed and much fewer cells are needed, which will shorten the culture time and improve the efficiency of stem cell therapy. Time is
important when considering the clinical applications especially for acute injury cases. Furthermore, multipotentiality of equine MSCs was supported on COLI scaffolds, which accomplished the first critical step to provide comprehensive results to evaluate cell-scaffold interactions and establish their potential in multiple tissue repairs. In summary, this study presented important information on how to design engineered tissue and stepped forward towards the long-term goal of meeting diverse clinical needs in equine tissue regeneration.

The developed cell adhesion, growth and induction behavior on COLI scaffolds was further applied in vivo using nude mice to investigate their potential in equine tissue regeneration. BMSC-COLI constructs under short-term induction produced bone formation 3 weeks after subcutaneous implantation while ASCs-COLI constructs required additional induction to committee osteogenesis in vivo. This will accelerate applications of engineered bone-forming constructs using BMSCs to expedite equine bone repair in near future. ASC constructs produced comparative ECM as that of BMSC constructs but under commitment into dermal-like tissue, indicating ASCs more responsive to signal cues encountered subcutaneously due to their original niches. Considering the key advantages of ASCs with high harvest rate, easy to isolate and minimal injury to donor site, pre-induction time and scaffolds components were further optimized in vitro to improve efficiency of ASCs constructs to commit osteogenesis.

Scaffolds component and porous structure were further confirmed as the two major factors that influence the ability of human ASCs to commit osteogenesis. Compared with COLI scaffolds, MG scaffolds added osteoinductive ceramics components while still maintaining similar porous structure. This study established MG as the optimal scaffolds for supporting osteogenesis of human ASCs without compromising its ability to maintain good cell
adhesion and high cell growth rate. Furthermore, osteogenesis was significantly enhanced by continuous exposure to osteogenic medium. By exploring the molecular mechanism of osteogenesis of cell-scaffold constructs, this study preliminarily demonstrated that cannabinoid pathway may play an important role in differentiation of osteoprogenitor cells. This accomplishment established the important role of human ASCs in osteogenesis under optimized conditions. Further studies are necessary to determine if similar results occur in vivo.

Our research solved the current dilemma of lack of solid-evidence based studies to support clinical use of MSCs in equine regenerative medicine. These studies investigated the mechanism of how MSCs function to restore tissue architecture and function and will improve the efficiency of stem cell therapy by introducing proper engraftment of MSCs through efficient bioreactor loading onto suitable bioscaffolds. The improved understanding of MSCs behaviors on bioscaffolds to commit to specific tissue regeneration especially bone regeneration should guide development of future regenerative therapies to address specific equine patients need in clinics.
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