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# BMP2 induced osteogenic differentiation of human umbilical cord stem cells in a peptide-based hydrogel scaffold

Shruthi Lakshmana  
*Nova Southeastern University*

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SCAFFOLD.

Shruthi M. Lakshmana, B.D.S, D.M.D.

A Thesis Presented to the Faculty of the College of Dental Medicine of  
Nova Southeastern University in Partial Fulfillment of the Requirements for the Degree of  
MASTER OF SCIENCE IN DENTISTRY

December 2014

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BMP2 INDUCED OSTEOGENIC DIFFERENTIATION OF HUMAN UMBILICAL CORD STEM  
CELLS IN A PEPTIDE-BASED HYDROGEL SCAFFOLD.

By

SHRUTHI M. LAKSHMANA, B.D.S, D.M.D.

A Thesis Submitted to the College of Dental Medicine of Nova Southeastern  
University in Partial Fulfillment of the Requirements for the Degree of  
MASTER OF SCIENCE IN DENTISTRY

Orthodontic Department  
College of Dental Medicine  
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December 2014

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**I certify that I am the sole author of this thesis, and that any assistance I received in its preparation has been fully acknowledged and disclosed in the thesis. I have cited any sources from which I used ideas, data, or words, and labeled as quotations any directly quoted phrases or passages, as well as providing proper documentation and citations. This thesis was prepared by me, specifically for the M.Sc.D. degree and for this assignment.**

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## **Dedication**

To my loving husband, adorable daughter and wonderful family for all of your love and support throughout my education. Thank you for standing by me in my endeavors.

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## **Abstract**

BMP2 INDUCED OSTEOGENIC DIFFERENTIATION OF HUMAN UMBILICAL CORD STEM CELLS IN A PEPTIDE-BASED HYDROGEL SCAFFOLD.

DEGREE DATE: DECEMBER 12, 2014

SHRUTHI M. LAKSHMANA, B.D.S, D.M.D.

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Craniofacial tissue loss due to traumatic injuries and congenital defects is a major clinical problem around the world. Cleft palate is the second most common congenital malformation in the United States occurring with an incidence of 1 in 700. Some of the problems associated with this defect are feeding difficulties, speech abnormalities and dentofacial anomalies. Current treatment protocol offers repeated surgeries with extended healing time. Our long-term goal is to regenerate bone in the palatal region using tissue-engineering approaches. Bone tissue engineering utilizes osteogenic cells, osteoconductive scaffolds and osteoinductive signals. Mesenchymal stem cells derived from human umbilical cord (HUMSCs) are highly proliferative with the ability to differentiate into osteogenic precursor cells. The primary objective of the study was to characterize HUMSCs and culture them in a 3D hydrogel scaffold and investigate their osteogenic potential. PuraMatrix™ is an injectable 3D nanofiber scaffold capable of self-assembly when exposed to physiologic conditions. Our second objective was to

investigate the effect of Bone Morphogenic Protein 2 (BMP2) in enhancing the osteogenic differentiation of HUMSCs encapsulated in PuraMatrix™. We isolated cells isolated from Wharton's Jelly region of the umbilical cord obtained from NDRI (New York, NY). Isolated cells satisfied the minimal criteria for mesenchymal stem cells (MSCs) as defined by International Society of Cell Therapy in terms of plastic adherence, fibroblastic phenotype, surface marker expression and osteogenic differentiation. Flow Cytometry analysis showed that cells were positive for CD73, CD90 and CD105 while negative for hematopoietic marker CD34. Alkaline phosphatase activity (ALP) of HUMSCs showed peak activity at 2 weeks ( $p < 0.05$ ).

Cells were encapsulated in 0.2% PuraMatrix™ at cell densities of  $10 \times 10^4$ ,  $20 \times 10^4$ ,  $40 \times 10^4$  and  $80 \times 10^4$ . Cell viability with WST and proliferation with Live-Dead cell assays showed viable cells at all cell concentrations ( $p < 0.05$ ). A two- fold upregulation of ALP gene was seen for cells encapsulated in PuraMatrix™ with osteogenic medium compared to cells in culture medium ( $p < 0.05$ ). HUMSCs encapsulated in PuraMatrix™ were treated with BMP2 at doses of 50ng/ml, 100ng/ml and 200ng/ml. A significant upregulation of ALP gene in BMP2 treated cells was seen compared to HUMSCs treated in osteogenic medium ( $p < 0.05$ ). Peak osteogenic activity was noted at BMP2 dose of 100ng/ml ( $p < 0.05$ ). We have developed a composite system of HUMSCs, PuraMatrix™ and BMP2 for repair of bone defects that is injectable precluding additional surgeries.

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## List of Abbreviations

ALP	Alkaline Phosphatase
BMP2	Bone morphogenic protein 2
CM	Culture medium
Col I	Collagen Type I
DMEM	Dulbecco's modified eagle medium
FGF	Fibroblast growth factor
HUMSCs	Human umbilical cord derived mesenchymal stem cells
MSCs	Mesenchymal stem cells
MSCM	Mesenchymal stem cell medium
NDRI	National Disease Research Interchange
OM	Osteogenic medium
OPG	Osteoprotegrin
OPN	Osteopontin
PBS	Phosphate buffer saline

## 1 CHAPTER 1 INTRODUCTION

### 1.1 Cleft Palate



**Figure 1-1 Newborn infant with cleft lip and palate.**

Cleft palate is the second most common congenital malformation in United States affecting 225,000 children every year<sup>1</sup>. Cleft palate is defined as a developmental defect of the palate characterized by a lack of fusion of the two lateral portions of the palate resulting in a communication between the oral cavity and the nasal cavity<sup>2</sup>. Two main regions are involved in palatal clefts, the primary palate- a triangular shaped piece of bone that will include the four incisor teeth and secondary palate which makes up 90% of the hard and soft palates posterior to the primary palate<sup>1</sup>. Cleft palate babies suffer from several problems such as feeding difficulties, speech abnormalities, dentofacial anomalies and psychosocial problems<sup>3</sup>.

## 1.2 Current protocol for Cleft Lip and Palate management

Numerous efforts have been made to date in order to repair cleft lip and palate defects using surgical procedures in combination with bone graft techniques<sup>4</sup>.

These surgeries begin as early as few weeks after birth followed by surgeries during mixed and permanent dentition as well<sup>5</sup>.

**Table 1-1 Current surgical protocol for repair of cleft lip and palate.**

Age	Surgical procedures	References
3-6 months	Cheiloplasty/Lip repair	Farronato et al, 2014 <sup>6</sup>
6 months	Soft palate closure	Precious et al, 2001 <sup>7</sup>
12 months	Gingivoperioplasty	Losquadro et al, 2007 <sup>8</sup>
Before 24 months	Primary bone grafting	Farronato et al, 2014 <sup>6</sup>
Late mixed dentition	Secondary bone grafting	Jeyaraj, 2014 <sup>9</sup>

## 1.3 Complications of Cleft Lip and Palate repairs

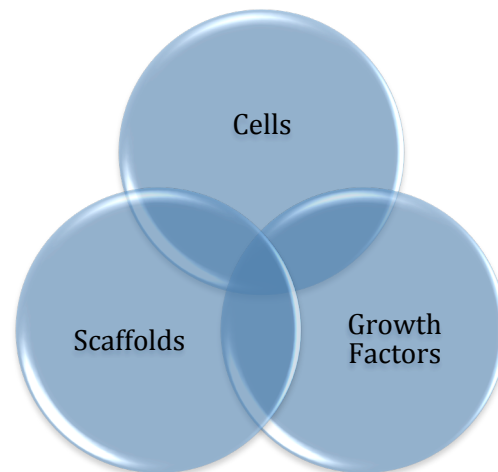
Although these surgeries (Table1-1) are aimed at reducing the adverse effects on maxillofacial growth and development while improving social and psychological development of the child, it does present with certain unfavorable effects. Some of these complications include wound dehiscence, residual lip and/or nose deformity, impaired healing, prolonged period of disability, hypertrophic or keloid scar formation, pain, postoperative hemorrhage and death<sup>4</sup>. These residual deformities often require more corrective surgical procedures. 25% of patients treated by standardized clinical protocol from infancy through adolescence required orthognathic surgery to correct anteroposterior discrepancy of the jaws<sup>10</sup>.

## 1.4 Application of bone grafts in Cleft Lip and Palate patients

Autologous bone graft remains a gold standard for the repair of this defect, which requires large amount of bone graft that may lead to donor site morbidity<sup>11</sup>. On the

other hand, allografts present potential risk of infections with additional threat of immune response of host tissue towards implant<sup>12</sup>. All these methods are aimed to ease the surgical procedures while improving the clinical outcomes of cleft palate treatments, reducing the incidence of scar tissue formation and residual facial asymmetry. People perceive cleft lip and palate patients differently, even after reparative surgery due to residual asymmetry<sup>13</sup>. Recent developments in stem cell based tissue engineering approaches offer an alternative solution.

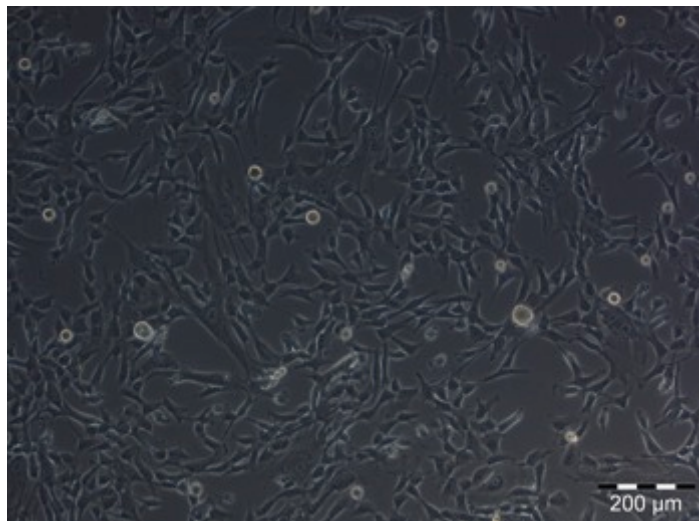
### 1.5 Tissue Engineering



Tissue engineering involves three basic elements- cells, scaffolds and growth factors. Bone tissue engineering involves the above elements with osteogenic stem cells, osteoconductive scaffolds and osteoinductive growth factors. Osteogenic tissue engineering involves regeneration of bone with stem cells from various sources (Table 1-2) under different culture conditions. Osteogenic tissue engineering plays a crucial role in the repair and regeneration of tissue in craniofacial defects.

## 1.6 Mesenchymal Stem Cells

Mesenchymal stem cells are adult stem cells capable of giving rise to tissues of mesodermal origin<sup>1</sup>. MSCs are originally derived from neural crest cells. During embryological development cells from dorsal part of neural tube (neural crest cells), migrate and form frontonasal processes, first, second, third and fourth pharyngeal arches. Neural crest cells contribute to neural, dermal and mesenchymal structures<sup>14</sup>. Mesenchymal cells derived from neural crest cells, after birth, are called “Mesenchymal stem cells” (MSCs)<sup>15</sup>.



**Figure 1-2 Mesenchymal stem cell isolated from Human Umbilical Cord.**

**Table 1-2 Source of mesenchymal stem cells in tissue engineering.**

<b>Author</b>	<b>Source</b>
Warotayanont et al, 2009	Embryonic stem cells- inner cell mass <sup>16</sup>
Peng et al, 2004	Muscle derived <sup>17</sup>
Friedenstein et al, 1968	Bone marrow <sup>18</sup>
Covas et al, 2003	Umbilical Cord <sup>19</sup>
Rodbell et al, 1964	Adipose tissue <sup>15</sup>
Gronthos et al, 2000	Dental Pulp stem cells <sup>20</sup>

Autologous mesenchymal stem cells are cornerstone cells most often used in many tissue-engineering applications. Additionally, they appear to be the best choice because of the minimal risks associated with immune-rejection in the host<sup>21-24</sup>. MSCs as a cell source for craniofacial tissue engineering, have widely been used in repair and regeneration of tissue in the past few years<sup>25</sup>. The MSCs are undifferentiated cells with high proliferation rate, capable of giving rise to diverse tissues including bone, cartilage and other tissues of mesenchymal in origin<sup>26</sup>.

The purpose of present study was to develop an injectable cell- scaffold system for regenerate missing bone in the palatal region.

**Table 1-3 Clinical applications of MSC for osteogenesis.**

Source of MSCs	Clinical application of MSC for osteogenesis	Author
Bone marrow	Treatment of bone defects with bone aspirate	Jager et al, 2009 <sup>27</sup>
Bone marrow	Treatment of long bone defects with culture-expanded osteoprogenitor cells and HA scaffolds	Marcacci et al, 2007 <sup>28</sup>
Bone marrow	Treatment of non-unions with culture expanded marrow cells with macroporous HA scaffolds	Quarto et al, 2001 <sup>29</sup>
Bone marrow	Treatment of tumor defect with culture expanded cells in HA scaffold	Morishita et al, 2006 <sup>30</sup>
Bone marrow	Jaw rehabilitation with BMP7 and MSCs in HA blocks	Warnke et al, 2004 <sup>31</sup>
Allogeneic bone marrow	Treatment of osteogenesis imperfecta with allogeneic MSCs	Horwitz et al, 2002 <sup>32</sup>
Bone marrow	Treatment of osteonecrosis with autologous bone marrow grafts	Hernigou et al, 2002 <sup>33</sup>
Adipose tissue	Treatment of osteonecrosis in femoral heads with adipose tissue MSCs, hyaluronic acid, PRP and calcium chloride	Pak et al, 2011 <sup>34</sup>
Bone marrow	Treatment of steroid induced osteonecrosis with cultured MSCs in $\beta$ -TCP ceramic	Kawate et al, 2006 <sup>35</sup>
Bone marrow	Bone marrow derived stem cells and PRP in areas of distraction osteogenesis	Kitoh et al, 2004 <sup>36</sup>
Bone marrow	Bone marrow stem cells in $\beta$ -TCP scaffold in spinal fusion treatment	Gan et al, 2008 <sup>37</sup>

HA: Hydroxyapatite, PRP: Platelet rich plasma,  $\beta$ -TCP- beta tricalcium phosphate

## 1.7 Human Umbilical Cord Derived mesenchymal Stem Cells

Human umbilical cord derived mesenchymal stem cells (HUMSCs) are highly advantageous potential source for cell-based therapies as umbilical cord can be obtained as postnatal tissue that is usually discarded after birth<sup>38</sup>. Collection of tissue is not an invasive procedure and these cells have a high proliferative capacity<sup>39</sup>. They possess self-renewal and have multilineage differentiation potential<sup>40,41</sup>. The UC contains two arteries and one vein, surrounded by a mucoid connective tissue known as Wharton's jelly<sup>42</sup> (Figure 1-3). HUMSCs are pluripotent, indicating their ability to differentiate into ectodermal, mesodermal or endodermal origin<sup>42</sup>. HUMSCs possess properties of embryonic stem cells and mesenchymal cells<sup>42,43</sup>. Retaining properties of embryonic stem cells, HUMSCs promise a unique ability of stemness (undifferentiated nature of stem cells) with a potential to evolve into MSCs that have the potential for self-renewal and ability to differentiate into multiple mesenchymal lineages such as adipocytes, chondrocytes and osteocytes<sup>42</sup>. HUMSCs have been used as an autologous source of cells for regenerating a wide variety of tissues of cardiac, osteogenic, chondrogenic and adipogenic origins<sup>44</sup>.



**Figure 1-3 Cross-Section of Human Umbilical Cord**

## 1.8 Scaffolds

In cell-based therapies, choosing an appropriate scaffold to deliver the cells is given high priority. Scaffolds that promote cell adhesion; proliferation and migration characterized by biocompatible and biomechanical parameters are essential<sup>45</sup>. Specifically, craniofacial bony defects like cleft palate require appropriate bone substitute scaffold to fill the 3D anatomical defect. The scaffold should have the capacity to carry, deliver and house the cells. Additionally, it should provide a temporary load bearing capacity until the bone is formed<sup>46</sup>. Hydrogel scaffolds are able to mimic natural extracellular matrix of many tissues and are able to form solid constructs that permit homogenous distribution of the cell<sup>47</sup>. Hydrogels offer the convenience of incorporating growth factors and cells prior to injection into the *in vivo* site to enable gel formation<sup>48</sup>. Injectable hydrogels present a novel approach of cell delivery in tissue engineering enabling surgeons to transplant cells in a minimally



invasive way<sup>49</sup>. They are naturally biocompatible, as they do not cause an immune response or inflammatory reaction<sup>50,51</sup>. These gels are degraded by hydrolysis, action of enzymes and/or dissolution<sup>51</sup>. Efficient palatal repair and reduced facial growth distortions in cleft palate patients using hydrogels has been achieved<sup>25</sup>. Self-assembling peptides are a new class of molecules with the ability to form stable hydrogels and have been used *in-vivo* animal studies for repairing bony defects<sup>25,52</sup>. Cleft palate defects are irregular and are 3 dimensional. Consequently, a 3D scaffold that mimics the defect while being rigid enough to support cells and flexible to blend into host tissue would be ideal. 3D scaffolds increase cell proliferation, migration and viability compared to preformed 2D scaffolds<sup>45</sup>. Our study intends to use a 3D peptide based hydrogel biomaterial, PuraMatrix, with over 99% water content that can self-assemble into 3D interweaving nanofibres<sup>53</sup>.

### 1.9 PuraMatrix™

PuraMatrix™ is a liquid self-assembling peptide scaffold that became commercially available in 2001<sup>54</sup>. They are also called self-assembling peptide nanofiber scaffolds (SAPNS) and RADA peptides because of its component Arginine, Aspartic acid and Alanine residues. Under physiologic salt conditions they are known to form nanostructured fibrillar hydrogels<sup>53</sup>. Nanostructured biomaterials are gaining popularity in regenerative medicine because they mimic natural extracellular matrix in a nano scale<sup>55</sup>. Physical and biological parameters of this scaffold can be modified due to its synthetic nature. Bioactive modifications can be made, which makes it versatile in terms of cell adhesion while increasing its stability<sup>56</sup>. PuraMatrix™ hydrogel is capable

of both ionic and hydrophobic interactions<sup>54</sup>. These interactions trigger spontaneous self-assembly enabling cell encapsulation and filling in both *in vitro* and *in vivo* applications. We intend to use this property to encapsulate HUMSCs within the PuraMatrix™ for the purpose of site-specific delivery of cells and growth factors.

### 1.10 Bone Morphogenic Proteins

Bone morphogenetic proteins are a family of osteoinductive proteins that promote differentiation of mesenchymal cells into osteoblasts and promote neovascularization<sup>57</sup>. Among the 15 identified BMPs, BMP-2 and BMP-7 (i.e., osteogenic protein-1) are now commercially available and have been investigated as an alternative to bone auto grafting in a variety of clinical situations, including spinal fusions, internal fixation of fractures, treatment of bone defects, and reconstruction of maxillofacial conditions<sup>58-62</sup>. While BMP2 is used widely in many applications, adverse effects such as enhanced bone formation in undesired site<sup>63</sup>, inflammation<sup>64</sup> and respiratory distress<sup>65</sup> have been reported. The conventional use of BMPs for therapeutic applications is to administer large quantities<sup>66</sup>. However, minimal and optimal dosage is essential when it is used for pediatric population. Keeping this in view, the project is intended to investigate an optimal dosage of use of BMP2 while developing a cell based composite scaffold system. *In- vitro* studies report-using doses of BMP2 ranging from 100- 400ng/ml for osteogenic differentiation with mesenchymal stem cells as cell source<sup>67</sup>. Doses as low as 0.1ng/ml and as high as 1000ng/ml have been used for osteogenic differentiation of Human Marrow Stromal Precursor Cells<sup>68</sup>. HUMSCs have been used for osteogenic differentiation using osteogenic medium, however, they have not been used in

combination with BMP2<sup>69</sup>. The aim of this study is to find an optimum dose of BMP2 that can be used for osteogenic differentiation of HUMSCs with Puramatrix<sup>TM</sup> as a scaffold system.

### 1.11 Focus of our research

The current protocol for repair of cleft lip/palate defects involves multiple surgeries to correct both hard and soft tissue defects<sup>4</sup>. Numerous efforts are being made to reduce the incidence of surgical procedures and develop procedures aimed at tissue self-renewal and regeneration<sup>51</sup>. Cell based therapies involving osteoinductive growth factors and biocompatible scaffolds with stem cells offer great promise<sup>70</sup>.

**Innovation:** This study intends to develop a novel procedure using PuraMatrix<sup>TM</sup>, a new biomaterial that helps serve as synthetic extracellular matrix to support growth and differentiation of cells. This hydrogel scaffold helps organize cells in a 3D architecture and enhances osteogenic differentiation. Our study uses a composite system of HUMSCs as source cells, PuraMatrix<sup>TM</sup> as an injectable scaffold in combination with BMP2 as a growth factor. This combination has never been investigated before. Published studies report the use of BMP2 ranging from 100ng/ml to 400ng/ml for osteogenic differentiation of MSCs<sup>67</sup>. Previous literature has reported adverse effects when BMP2 is used clinically. Our aim is to determine an optimum dose of BMP2 for the purpose of osteogenic differentiation in young patients with cleft lip/palate and this *in-vitro* study is the pioneering step in developing an *in-vivo* procedure.

## 1.12 Objectives

The long-term goal of this project is to develop a 3D injectable scaffold for the purpose of bone regeneration in patients with cleft lip and palate defects using HUMSCs and minimal dose of BMP2.

## 1.13 Specific aims and Hypothesis

### 1.13.1 Specific Aim #1: Encapsulating HUMSCs in PuraMatrix

The first aim of this study was to investigate the capability of PuraMatrix™, a 3D self-assembled peptide-based hydrogel, to support osteogenic differentiation of human umbilical cord derived mesenchymal stem cells (HUMSCs).

### 1.13.2 Specific Aim #2: Effect of BMP2 on Osteogenic differentiation of PuraMatrix™ encapsulated HUMSCs

The second aim of this study was to assess the effect of different doses of BMP2 on osteogenic differentiation of PuraMatrix™ encapsulated HUMSCs.

### 1.13.3 Hypothesis

**Null Hypothesis Specific Aim 1:** PuraMatrix™ does not support osteogenic differentiation of HUMSCs.

**Alternative Hypothesis Specific Aim 1:** PuraMatrix™ supports osteogenic differentiation of HUMSCs.

**Null Hypothesis Specific Aim 2:** Low doses of BMP2 do not enhance osteogenic differentiation of HUMSCs.

**Alternative Hypothesis Specific Aim 2:** Low doses of BMP2 enhance osteogenic differentiation of BMP2.

#### 1.14 **Location of Study**

This study was conducted in Craniofacial Research Center (Room #7391), College of Dental Medicine Nova Southeastern University, Fort Lauderdale, FL 33328.

## **2 CHAPTER 2 MATERIALS AND METHODS**

### **2.1 Materials**

Human umbilical cord was obtained from NDRI (National Disease Research Interchange, Philadelphia, PA) after Nova Southeastern University's Institutional Review Board approval. Commercially available PuraMatrix™ hydrogel (BD Biosciences, San Jose, CA) was used for the study. Mesenchymal stem cell medium was obtained from Sciencell (Carlsbad, CA). Commercially available rhBMP2 (R&D systems, Minneapolis, MN) was used. All other necessary chemicals and lab supplies were obtained from Sigma (St. Louis, MO) and VWR international (Atlanta, GA), respectively.

### **2.2 Overall study design**

Human umbilical cord tissue was obtained from National Disease Research Interchange (NDRI; Philadelphia, PA). HUMSCs were isolated from umbilical cord using explant method. The detailed procedure is discussed in section 2.3.2. Cells isolated from the tissue were cultured and expanded under standard culture conditions. Cells at 70-80% confluency were induced with osteogenic supplements and osteogenic differentiation of HUMSCs was determined by gene expression of various osteogenic differentiation marker genes. Mineral deposition of osteogenically induced cells was confirmed by Alizarin Red and Von kossa staining techniques. Cells were encapsulated in 3D PuraMatrix™ scaffold and then supplied with CM in one group and OM in other

group. Cells were encapsulated in PuraMatrix™ in different cell concentrations ( $1 \times 10^4$ ,  $2 \times 10^4$ ,  $4 \times 10^4$  and  $8 \times 10^4$  cells/ml). Cell viability was assessed by using live/dead cell assay. Cell proliferation was assessed by WST assay. Cells were encapsulated in PuraMatrix™ with different concentrations of BMP2 (50ng/ml, 100ng/ml, 200ng/ml) for 1 week. Cells grown in osteogenic medium served as a control. Osteogenesis was determined by gene expression studies, mineralization studies and ALP assay.

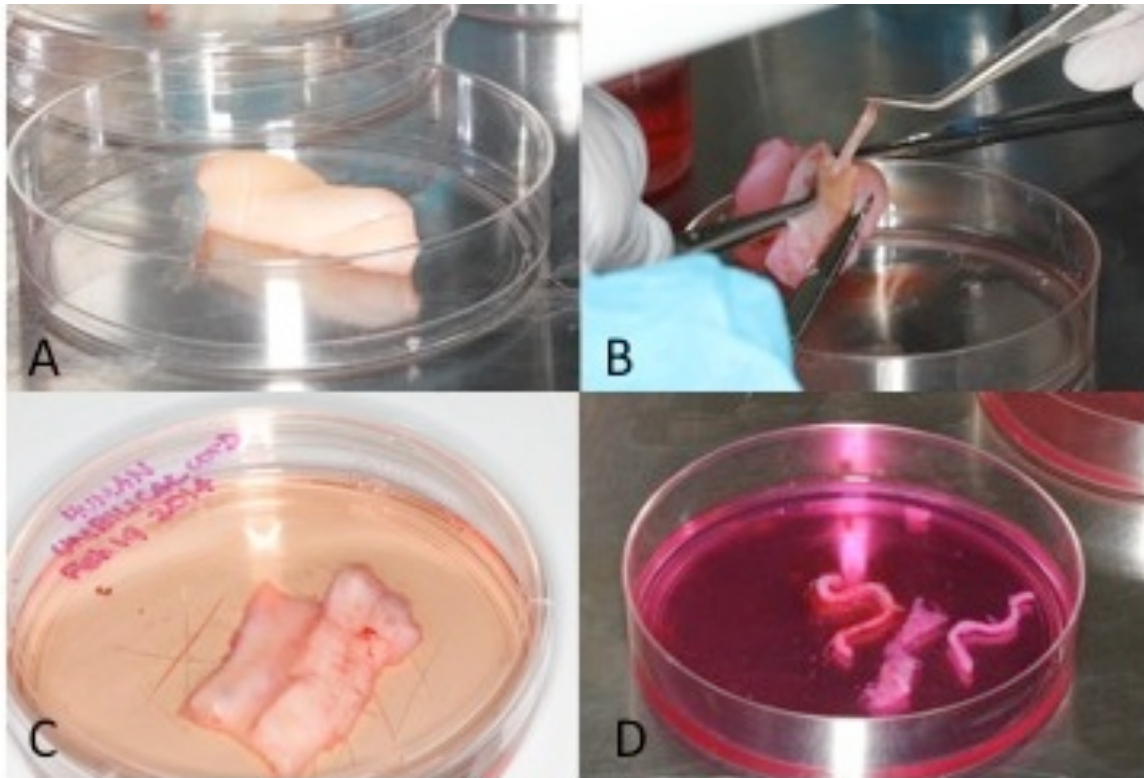
### **2.3 Isolation protocol**

In this study, we employed both Explant culture and Enzymatic digestion methods for isolating cells from human umbilical cord.

#### **2.3.1 Ethics Statement**

This study was conducted in accordance with Nova Southeastern University's institutional review board guidelines to obtain human umbilical cord from NDRI (Philadelphia, PA). Once umbilical cord was obtained, isolation procedure was carried out within 48 hours after collection. We have primarily used explant method to isolate cells from tissue.

### 2.3.2 Explant method



**Figure 2-1 Isolation protocol with Explant method. A- Section of Umbilical Cord before longitudinal incision. B- Removal of vessels from the umbilical cord. C- Explant tissue in culture medium, D- Vessels removed from umbilical cord tissue.**

Umbilical cord was cut into 5 cm sections. Umbilical cord was washed with sterile phosphate buffer saline (PBS) to remove blood<sup>71</sup>. A longitudinal section was made into the umbilical cord parallel to the umbilical vein to enable visualization of the vessels before removal. Umbilical vessels- 2 umbilical arteries and 1 umbilical vein were dissected and removed completely. 5 cm sections of umbilical cord were placed in a 10 cm<sup>2</sup> petridish with 5 ml of culture medium (CM-DMEM supplemented with 10% FBS and 1% antibiotics) and incubated at 37<sup>0</sup> C with 5% CO<sub>2</sub>. Culture medium was changed every three days. After 5 days, cells were found attached to the surface of the petridish.



Sections of the umbilical cord were discarded and fresh culture medium was added. The cells were then expanded until they reached 70-80% confluence with medium changed twice every week.

### 2.3.3 Enzymatic Digestion



**Figure 2-2 Isolation of HUMSCs using Enzymatic Digestion.**

After removal of umbilical vessels, umbilical cord was cut into pieces each measuring approximately 2 cms. Tissue was washed with PBS. The tissue was further minced and kept for digestion in 0.1mg per ml type I collagenase and 2mg/ml dispase at 37° C with continuous shaking for one hour. Cell suspension with enzyme solution was collected and enzyme digestion was continued for the remaining tissues. The cell suspensions were pooled and centrifuged at 900 rpm for 10 minutes. The supernatant was discarded and the pellet was suspended in culture medium (Dulbecco's Modified Eagles medium- DMEM, Fetal Bovine Serum- FBS, Penicillin 1%, Streptomycin 1%) and cultured for further expansion in a 37°C humidified environment with 5% CO<sub>2</sub><sup>72</sup>.

## 2.4 Cell Culture

HUMSCs were cultured in monolayers in T75 flasks with Mesenchymal Stem Cell Medium (MSCM). The cells were fed with fresh medium every 2-3 days. Cultures were propagated at 37 °C under humidified conditions using 5% CO<sub>2</sub>. Cells with 70-80% confluency were treated with trypsin to dislodge from the flask and plated again until 3<sup>rd</sup> passage. Cells from passage 3 or 4 were used in this study.



**Figure 2-3 Incubator, B and C- Cell culture in T75 flask.**

## 2.5 Characterization of HUMSCs

In order to verify the mesenchymal origin, HUMSCs must meet several criteria set forth by the International Society for Cellular Therapy<sup>73</sup>. The mesenchymal origin of HUMSC's was confirmed by their plastic adherence, observation of fibroblastic

phenotype under light microscope, differentiation capacity to osteogenic lineage *in vitro* and by surface marker expression.

### **2.5.1 Flow Cytometry surface marker analysis**

All flow cytometry experiment procedures were performed in University of Miami. Passage 3 or Passage 4 cells after attaining sub-confluence were used. Cells at the concentration of  $10^6$  cells were used to measure the surface markers using Miltenyi Kit according to manufacturer's instruction. The specific markers positive for mesenchymal stem CD73, CD90 and CD105 and negative for CD34 were identified at the facilities at University of Miami using a fluorescent activated cell sorter FACARIA IIIu (BD Biosciences, San Jose, CA) with adjusted fluorescence compensation setting. Negative samples were used to set up the thresholds of quadrant markers.

### **2.5.2 Osteogenic Differentiation**

The monolayer cultures were grown in culture medium (CM) consisting of DMEM, 10% FBS and 1% antibiotics. The cells at 70-80% confluency were replaced with growth medium supplemented with osteogenic reagents, which include 50mM ascorbic acid and 10mM of  $\beta$ -glycerophosphate and 100nM dexamethasone. The osteogenic differentiation was measured at 1 week, 2 weeks and 3 weeks intervals. Osteogenic differentiation potential of HUMSCs was determined by measurement of ALP activity, gene expression studies, western blotting and mineralization studies. The osteogenic potential of HUMSCS induced with osteogenic medium will be compared with the cells grown in CM.

### **2.5.2.1 Alkaline Phosphatase (ALP) Activity Assay**

Cells at 70-80% confluency were trypsinized and plated in 6 well plate at  $60 \times 10^3$  cells per well. Cells grown with or without osteogenic medium were collected from each well at different time points (1, 2 and 3 weeks) and lysed with m-per mammalian protein extraction reagent (Thermo Scientific, Rockford, IL). ALP activity was measured by a pNPP assay (Sciencell, Carlsbad, CA) according to manufacturer's instructions. Briefly, 15 $\mu$ L of cell lysate was placed in a 96 well plate and 35  $\mu$ L of alkaline reaction buffer was added to each well followed by 5 $\mu$ L of pNPP substrate and the cells were incubated at 37°C for 30 minutes and pNPP production was measured by a microplate reader at 405 nm. The ALP activity of cells grown in OM was compared with cells grown in CM.

### **2.5.2.2 RNA Isolation, reverse transcription and Polymerase Chain Reaction**

mRNA expression of ALP, Osteoprotegrin, Osteopontin and Collagen Type I was measured at 1, 2 and 3 weeks for cells grown with or without osteogenic supplements. Cells were cultured in t-25 flasks and total RNA was extracted using TRIzol method (Ambion, Carlsbad, CA) (Figure 2-6). RNA was quantified by using smart spec spectrophotometer (Bio-Rad, Hercules, CA). RNA was reverse transcribed and cDNA was synthesized using high capacity reverse transcriptase kit (Life Technology, Carlsbad, CA) according to manufacturer's instructions. Osteogenic marker genes ALP, Osteopontin and Osteoprotegrin were assessed at 7-day interval using Q semiquantitative PCR (Biorad, Hercules, CA) using specific primers (Table 2-1). The

PCR products were separated by 2% agarose gel and the relative density was measured using a densitometry analysis.

**Table 2-1 Specific Primers Table**

<b>Gene</b>	<b>Sequence</b>
Col I (sense)	5'-ctgaccttctgcgctgatgtcc-3'
Col I (antisense)	5'-gtctggggcaccaacgtccaagg-3'
ALP (sense)	5'-ccacgtctcacattggtg-3'
ALP (antisense)	5'-agactgcgctgtagttgt-3'
OPN (sense)	5'-tgaaacgagtcagctggatg-3'
OPN (antisense)	5'-tgaaattcatggctgtggaa-3'
beta-actin (sense)	5'-catgtacgttgctatccaggc-3'
beta-actin (antisense)	5'-ctcctaatgacgcacgat-3'

### **2.5.2.3 Mineralization Assays**

Alizarin red and Von Kossa staining was performed in order to analyze matrix mineralization.

### **2.5.2.4 Alizarin Red Stain**

Cells were plated in a 12 well plate. Osteogenesis was induced on day 3 using OM. A quantitative Alizarin red S method was used at the end of 1, 2 and 3 weeks. Briefly the cells were fixed with 10% formalin followed by staining with 2% Alizarin red S solution (Sigma- Aldrich) for 20 minutes. Cells were photographed under the microscope.

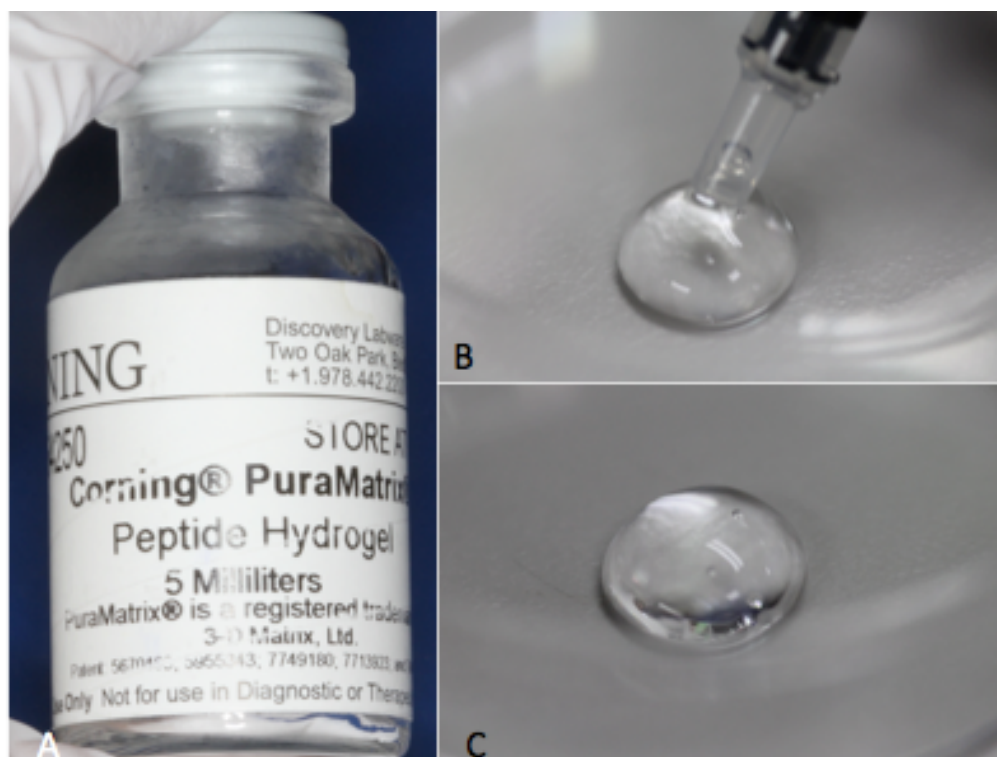
### **2.5.2.5 Von Kossa stain**

To determine the presence of phosphate based mineral, cells were stained with 2% silver nitrate (Sigma-Aldrich, St Louis, MO) solution for 1 hour under bright light. The reaction was stopped by adding the developing solution, viz. 1% sodium thiosulphate (Sigma-Aldrich, St Louis, MO) for 1 min. Sections were counterstained with 0.5%

nuclear fast red (Sigma, Aldrich, St. Louis, MO) and dehydrated in saline and mounted with DPX and observed under phase contrast microscope (Olympus, XI 50).

## **2.6 Encapsulating HUMSCs in 3D peptide hydrogel scaffold PuraMatrix™**

PuraMatrix™ from BD Biosciences was assembled using CM following manufacturer protocol in a 24 well plate. The cells were suspended in 20% sterile sucrose solution that was previously autoclaved. The cells suspended in sucrose solution were encapsulated in 0.2% Puramatrix™. Commercial PuraMatrix™ is available at a concentration of 1%. It was further diluted with sucrose solution to prepare 0.2% gel. Cell seeding densities were  $1 \times 10^4$  HUMSCs in monolayer 2D culture. And increasing cell concentrations of  $100 \times 10^3$ ,  $200 \times 10^3$ ,  $400 \times 10^3$  and  $800 \times 10^3$  were used per well for encapsulation in PuraMatrix™. For Cell viability assays 50 $\mu$ L of PuraMatrix™ was used. Cells mixed with 50 $\mu$ L of PuraMatrix™ were slowly dropped into 150 $\mu$ L of culture medium. And the cell- gel constructs were incubated in 37<sup>0</sup>C at 5% CO<sub>2</sub>.



**Figure 2-4 A- Commercially available PuraMatrix™, B and C- Injectable hydrogel PuraMatrix™.**

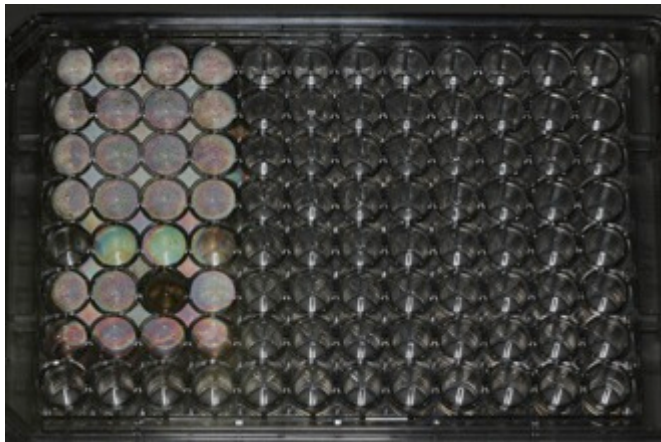
## 2.7 Cell Viability and Proliferation of HUMSCs in 3D PuraMatrix™ Culture

In order to assess cell viability and proliferation, WST and Live-Dead assay were performed respectively.

### 2.7.1 WST Assay

Cells were encapsulated at  $100 \times 10^3$ ,  $200 \times 10^3$ ,  $400 \times 10^3$ ,  $800 \times 10^3$  per well in a 96 well plate.  $50 \mu\text{L}$  of 0.2% PuraMatrix™ was used per each well. In order to prepare cell-gel constructs,  $200 \mu\text{L}$  of growth medium was placed in each well of the plate. Cells suspended in 0.2% gel were slowly released into the growth medium. After 30 minutes of incubation at  $37^\circ\text{C}$ , growth medium was replaced and cells were fed with new growth medium and gelation was examined under microscope. Cell proliferation was assessed

by addition of WST-1 (2,4-dioxophenyl)-3,4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) reagent to a 1:10 final concentration. WST-1 cell proliferation assay (Roche, Indianapolis, IN, USA) is a mitochondrial activity assay. WST reagent is a soluble tetrazolium salt that can react with metabolically active cells and gives a deep red color. The cell-gel constructs were finally incubated at 37°C with 5% CO<sub>2</sub>. The assay was conducted after 72 hours of incubation and the absorbance was measured using micro-plate reader (Figure 2-5). The cell viability was measured and compared.



**Figure 2-5 Cells encapsulated in PuraMatrix™ for WST assay.**

### **2.7.2 Live Dead Cell Assay**

Cell viability of the HUMSCs in PuraMatrix™ was also confirmed by a Live/Dead cell assay (Molecular Probes, Carlsbad, CA). Cells at  $100 \times 10^3$ ,  $200 \times 10^3$ ,  $400 \times 10^3$ ,  $800 \times 10^3$  were encapsulated in 0.2% Puramatrix™. Cell proliferation was examined after 72 hours of incubation using a Live-Dead cell assay kit (Life Technology, Carlsbad, CA) according to manufacturer's protocol. Briefly, the cells encapsulated in PuraMatrix™ gel



was washed twice with PBS and Live/Dead cell stain was added. Live/Dead cell assay kit was provided with two molecular probes, calcein AM and ethidium homodimer-1 (Eth-D). These probes were used for simultaneous visualization of the live cells and dead cells. Live cells emit Green Fluorescence, when calcein AM enters the cells and is hydrolyzed to calcein by intracellular esterase. Eth-D 1 enters into nucleic acids to produce bright red Fluorescence that indicates dead cells. The 2µm ethidium homodimer-1 and 2µm calcein AM was reconstituted in PBS. Cells were incubated at 37°C for 30 minutes. Micrographs were then taken using a fluorescent microscope (Olympus IX 51) equipped with a digital camera (Olympus XC 30).

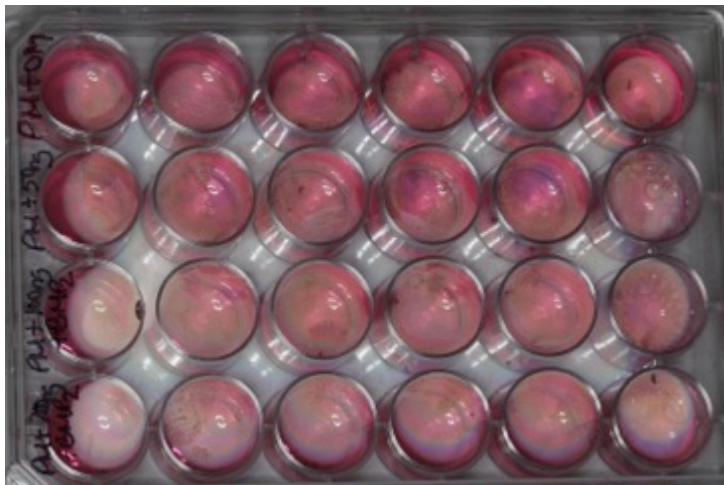
### **2.7.3 Cell-Gel Constructs - Osteogenic differentiation**

PuraMatrix™ in combination with stem cells can induce osteogenic differentiation in the presence of osteogenic supplements<sup>74</sup>. Medium was replenished every 3-4 days. For differentiation assays 24- well plates were used. Cells suspended in 20% of sterile sucrose (120 µL) with 80 µL of 1% PuraMatrix™ and dropped slowly into growth medium in which the scaffold can self-assemble to acquire gel morphology. The gelation was observed under microscope. Gene expression of osteogenic markers was investigated using RT-PCR and matrix mineralization was monitored after one week.

### **2.8 BMP2 treatment and cell seeding on to scaffolds**

Cells were suspended and were loaded onto the scaffolds as previously described in the encapsulation section. Briefly, a total of  $2 \times 10^6$  cells were seeded in a drop wise manner in the PuraMatrix™ solution and the cell-scaffold insert was placed in each well

of 24-well culture plates and allowed to solidify. The cells in the scaffold were provided with 1ml of culture medium and incubated at 37°C and 5% CO<sub>2</sub>. Osteogenic medium containing increasing concentrations of BMP2 50ng/ml, 100ng/ml and 200ng/ml was introduced on the third day. The cell-scaffold inserts were replenished with fresh medium every 48 hours. The cell-scaffold insert supplemented with osteogenic medium only was considered as control group. Experiments were performed for osteogenic induction and mineralization. All experiments were repeated at least three times.



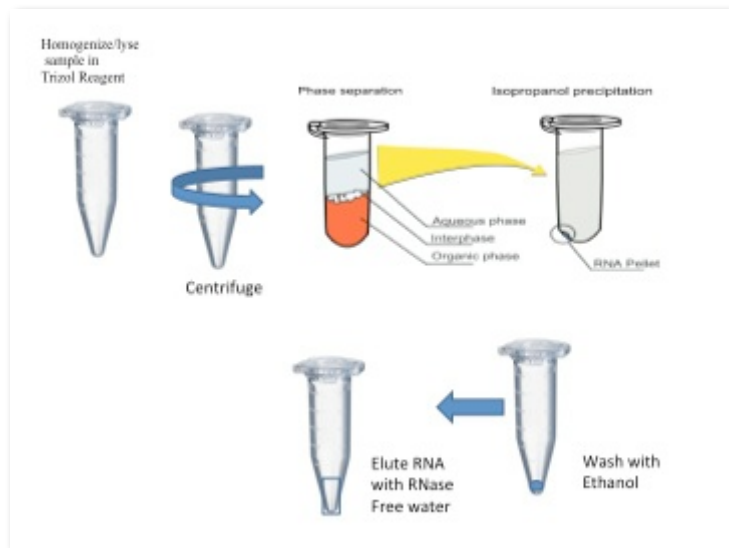
**Figure 2-6 BMP2 treatment of cells encapsulated in PuraMatrix™.**

## 2.9 Osteogenic Differentiation

### 2.9.1 Gene Expression

Cells were plated in T-25 flasks in culture medium (CM). Osteogenic medium was introduced on day 3. Medium was changed twice each week for 2 weeks. Quantitative real-time reverse transcription polymerase chain reaction was used to analyze the expression of osteogenic genes. Total RNA was isolated from cells at the end of 1 and

2 week time-points using RNeasy Mini kit following manufacturer's instruction. The concentration of RNA was determined by spectrophotometer. RNA was reverse transcribed and cDNA was synthesized. Osteogenic marker genes ALP, Osteopontin and Osteoprotegrin were assessed at 7-day interval using Quantitative PCR (Step-One plus Applied Biosystems, Foster City, CA) using specific primers (Table 2-1). Expression levels were determined by using  $2^{-\Delta\Delta C_t}$  methods.



**Figure 2-7 Steps involved in RNA isolation**

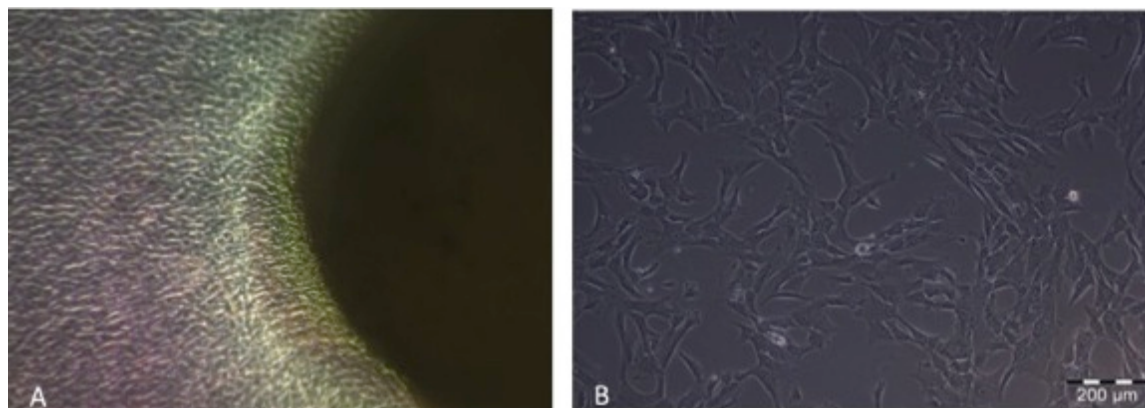
### **2.9.2 Statistical Analysis**

Data was expressed as mean  $\pm$  standard deviation (SD) of at least three independent samples. Statistical comparisons between groups were performed with a two tailed student's t-test,  $P < 0.05$  was considered as significant.

### 3 CHAPTER 3 RESULTS

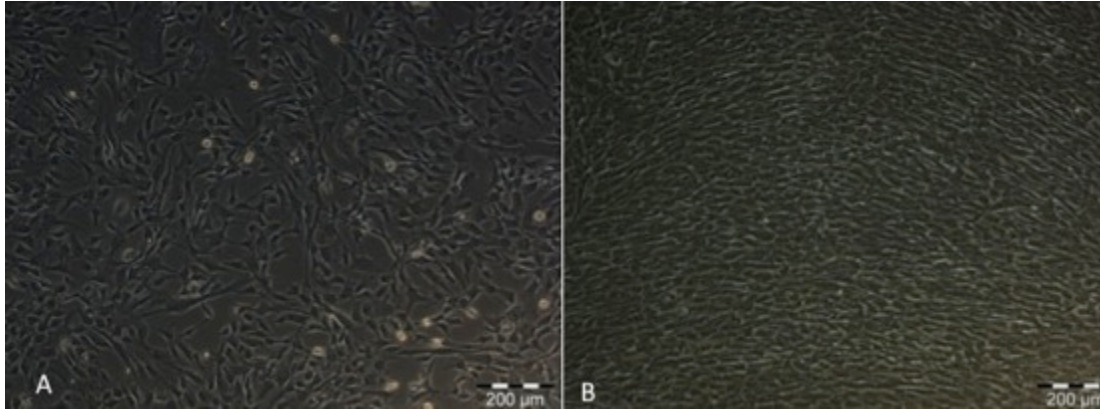
#### 3.1 Characterization of HUMSCs

Cells were extracted from the Wharton's jelly region of the umbilical cord using both Explant and Enzymatic digestion methods. We preferred the explant method due to ease of processing. We obtained more cell yield from the Explant method. Isolated cells demonstrated a fibroblast-like phenotype when observed under light microscope <sup>75</sup>.



**Figure 3-1 A- Cells after plating B- Fibroblastic phenotype of HUMSCs**

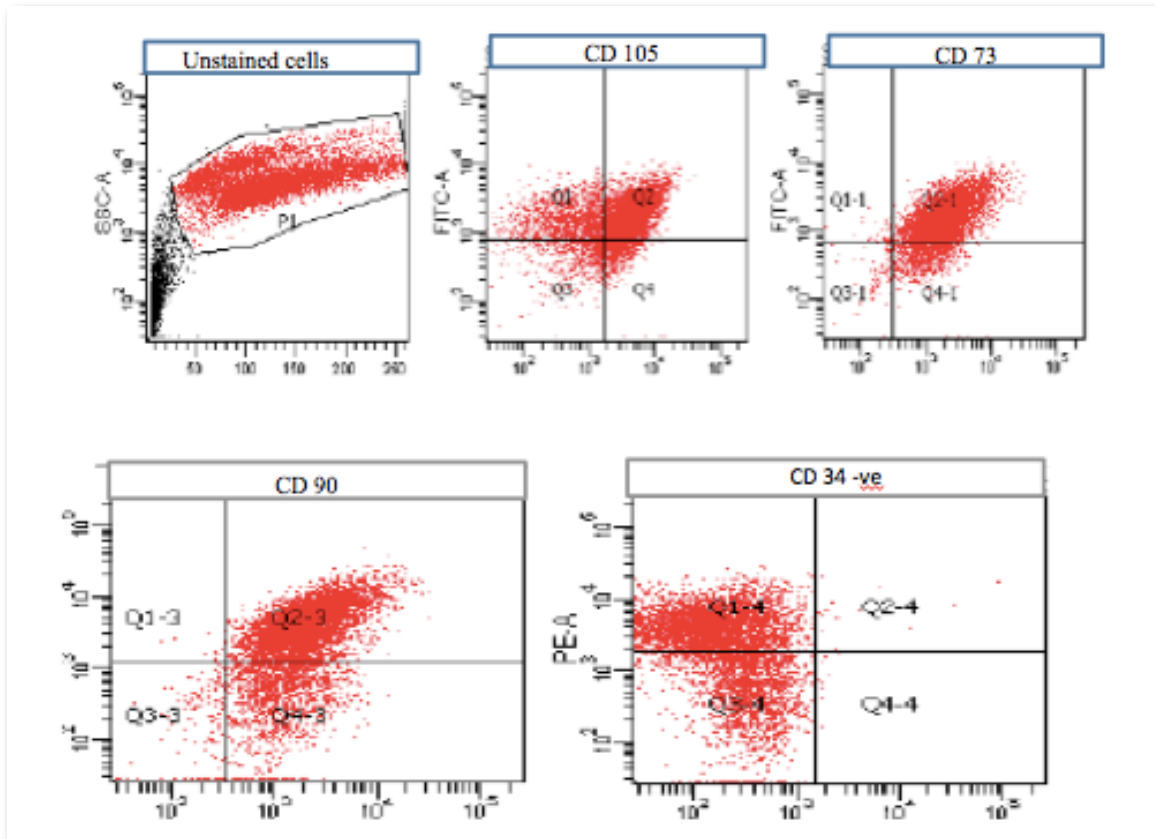
The cells obtained from explant culture or enzyme digestion of umbilical cord fragments were seeded on to a T75 flask at a density of  $0.5 \times 10^6$ . Fig 3-1 shows cells after plating. After 24 hours the cell morphology was observed under phase contrast microscopy. Adherent cells demonstrated typical fibroblast morphology (Fig 3-2). This represented the 100% of harvest efficiency. The cells reached confluency 4 days after plating.



**Figure 3-2 A- Cells 2 days after plating, B- Cells at confluence after 4 days**

### **3.1.1 Immunophenotype of HUMSCs**

For all experiments cells from passage 3 to passage 5 were used. HUMSCs must meet certain criteria as defined by the International Society for Cellular Therapy<sup>73</sup>. Mesenchymal origin of HUMSCs in this study was confirmed by their plastic adherence, fibroblastic phenotype, surface marker expression and their ability to differentiate into osteogenic lineages *in-vitro*. Flow cytometric analysis of HUMSCs after passage 3 demonstrated that cells were 90% positive for surface markers CD73, CD90, CD105 and negative for hematopoietic marker CD34. Results verify the mesenchymal origin of HUMSCs and the lack of hematopoietic markers (Figure 3-3).



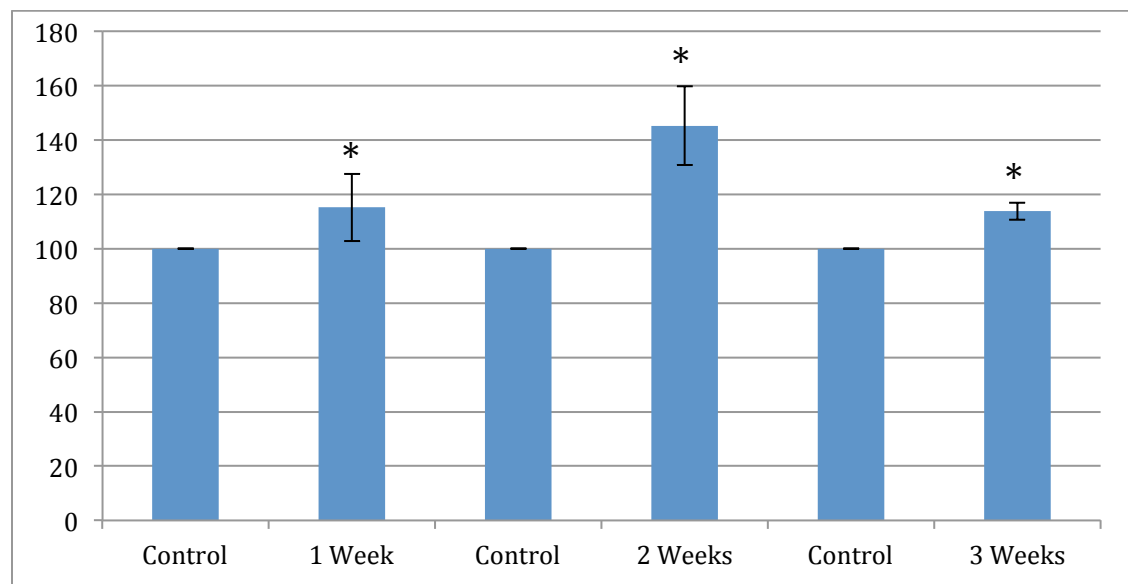
**Figure 3-3 Flow Cytometry Data showing surface marker of MSCs**

### 3.1.2 Osteogenic Differentiation of HUMSCs

Osteogenic differentiation potential of monolayer HUMSCs was assessed by using passage 3 to passage 5 cells. Cells in culture medium (CM) were considered as control group and cells in osteogenic medium as experimental group. The osteogenic differentiation was monitored at 1, 2 and 3 weeks. Osteogenic differentiation was determined by ALP activity assay, gene expression and mineralization studies using Alizarin Red and Von Kossa stain.

### 3.1.2.1 ALP activity

Alkaline phosphatase is an early marker for osteogenic differentiation. Our results showed significant increase of ALP activity (15%) at the end of 1 week in OM group compared to CM group. There was a significant increase (>45%) in OM group compared to control group at 2 weeks. The enhancement of ALP activity of OM group was at its peak at day 14 ( $P=0.027$ ) than at day 7. Although there is significant increase in ALP activity when compared to cells grown in culture medium, the cells showed more than 30% decrease in ALP activity at the end of 3 weeks.

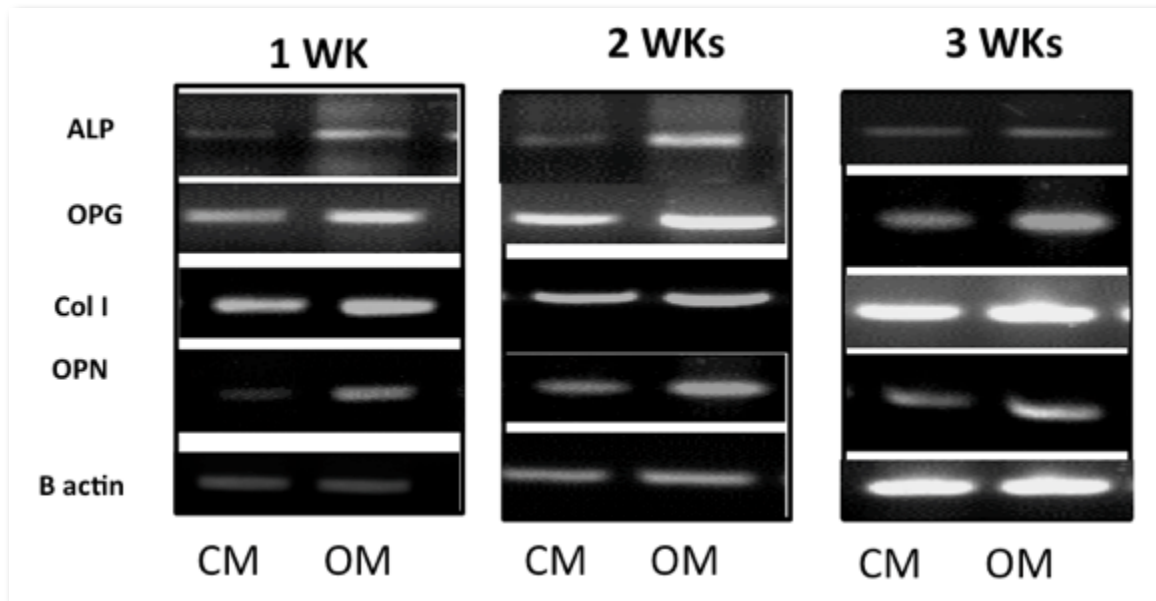


**Figure 3-4 ALP activity of HUMSCs in CM compared to OM samples at 1,2 and 3 weeks.**

### 3.1.2.2 Gene Expression

Gene expression of osteogenic lineage was assessed by semi quantitative PCR. Osteoblast specific genes Alkaline phosphatase (ALP), Osteoprotegrin (OPG), Collagen type I (Col1), Osteopontin (OPN) were assessed at 1, 2 and 3 weeks

intervals. B actin served as endogenous control. The results indicated that ALP gene expression upregulated at 1 and 2 weeks. The peak up regulation was observed at 14<sup>th</sup> day. On the other hand, Collagen type I started up regulating from week 1 and continued until 3 weeks.

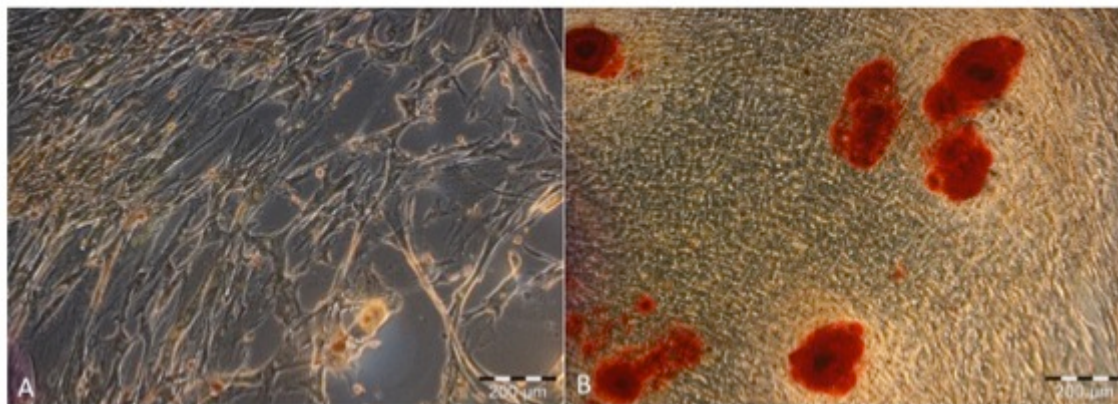


**Figure 3-5 Gene expression showing upregulation of osteogenic genes ALP, OPG, Col I and OPN. B-Actin was used as an endogenous control.**

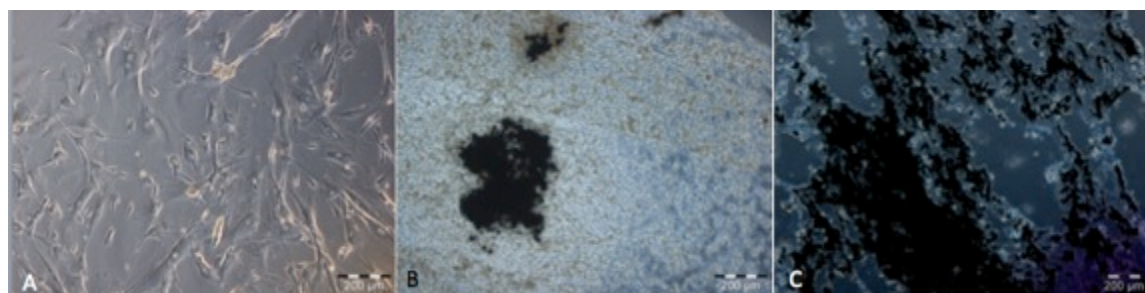
### **3.1.2.3 Mineralization studies with Alizarin Red and Von Kossa stains**

Matrix mineralization with calcium and phosphate is a late indicator of osteogenesis and maturation of osteoblasts. Calcium deposits were discerned using Alizarin Red stain. There was a significant increase in calcium deposits at the end of 3 weeks for cells in OM compared to cells in control group with CM. Matrix mineralization for phosphate deposits was determined by von Kossa staining technique.





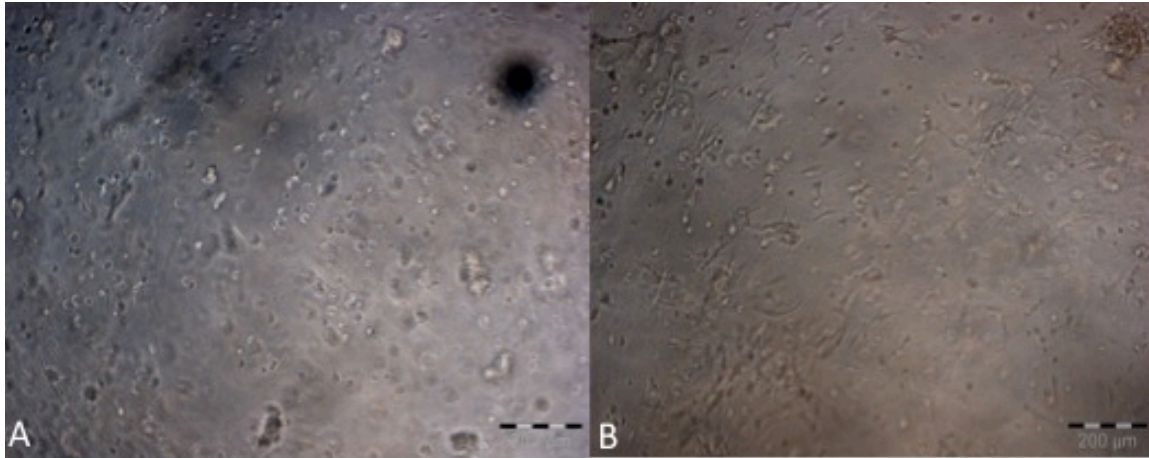
**Figure 3-6 A- Alizarin red staining in CM after 4 weeks. B- Alizarin red staining in OM after 4 weeks.**



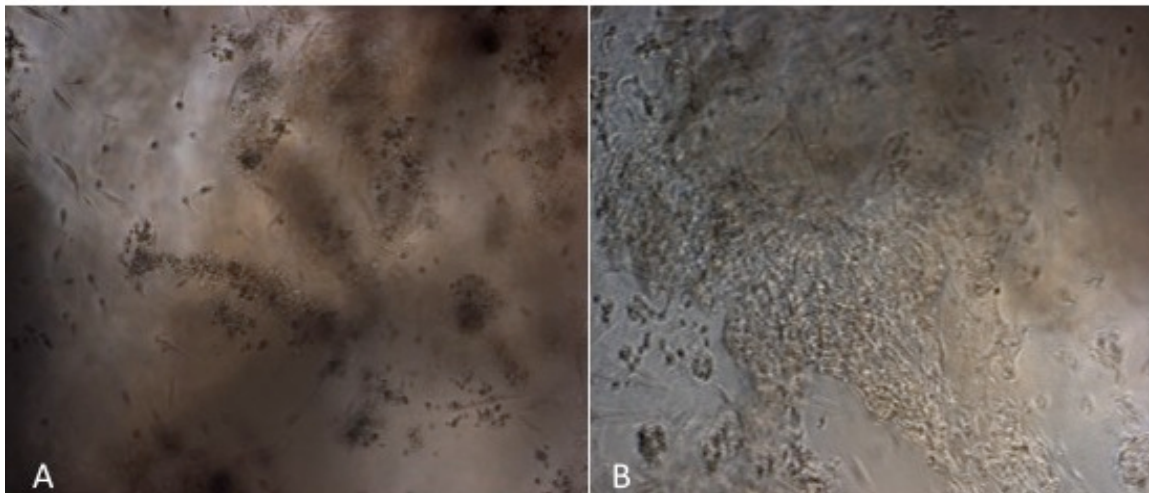
**Figure 3-7 Von Kossa Staining of cells, A- Cells in control group CM at 4 weeks, B- Cells in OM at 4 weeks, B- Cells in OM at 8 weeks.**

### 3.2 Cell morphology of cells encapsulated in PuraMatrix™

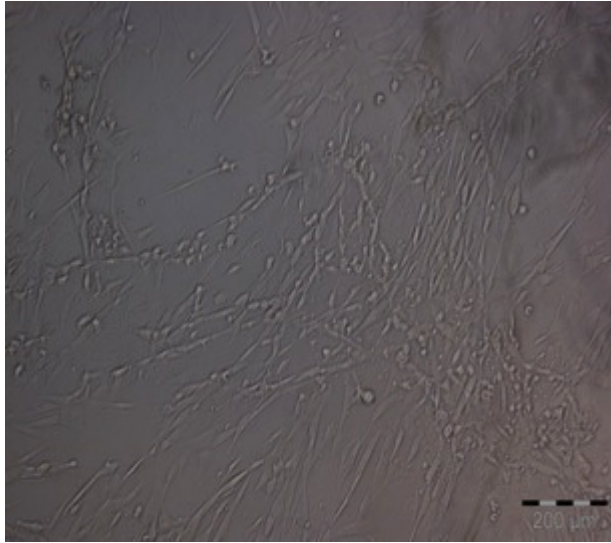
Hydrogels containing HUMSCs were observed under phase contrast view of Olympus IX 51 (Center Valley, PA). Figure 3-8 A shows cells encapsulated in PuraMatrix™. Cells were spherical immediately after encapsulation and cell growth was observed within 24 hours. The cells attained spindle shaped after 24 hours (Fig 3-8 B). On day 3, cells started forming a network (Fig 3-9 A). At higher concentrations, cell aggregate formation was observed in PuraMatrix™ gels (Fig 3-10). Cell survival and proliferation was examined by WST assay and Live Dead Cell assay.



**Figure 3-8 A- 100K cells in PuraMatrix™ on Day 0, B- 100K cells in PuraMatrix™ on Day 1.**



**Figure 3-9 A- 100K cells in PuraMatrix™ on day 3, B- 100K cells in PuraMatrix™ on day 4 showing cellular interconnections.**



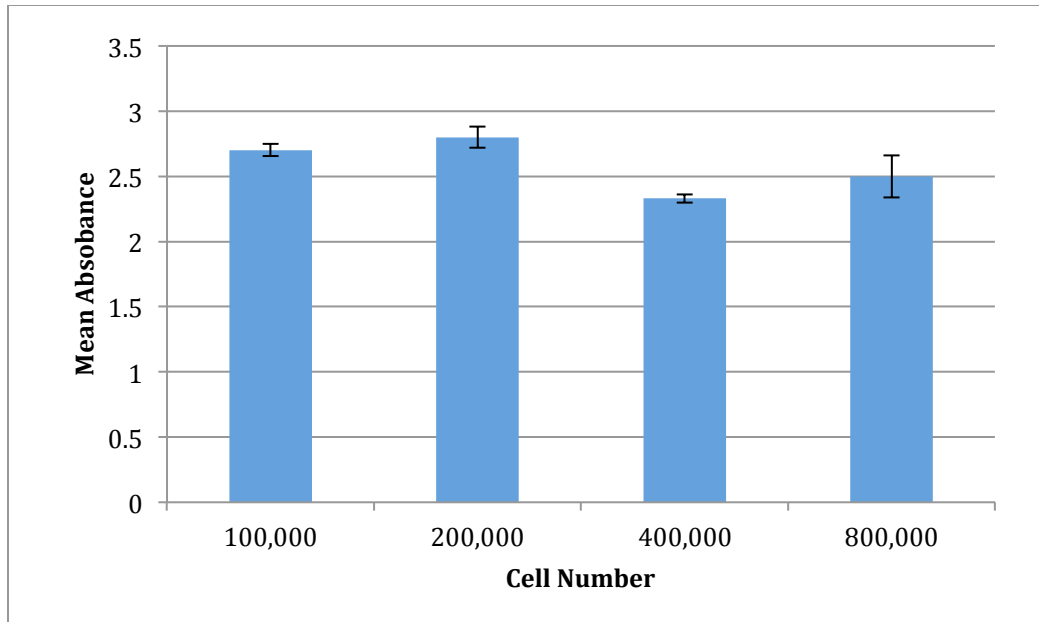
**Figure 3-10 800K cells in PuraMatrix™ on day 10.**

### **3.3 Cell proliferation and viability in PuraMatrix™**

WST and Live- Dead Assay as described below assessed cell proliferation and viability.

#### **3.3.1 WST assay**

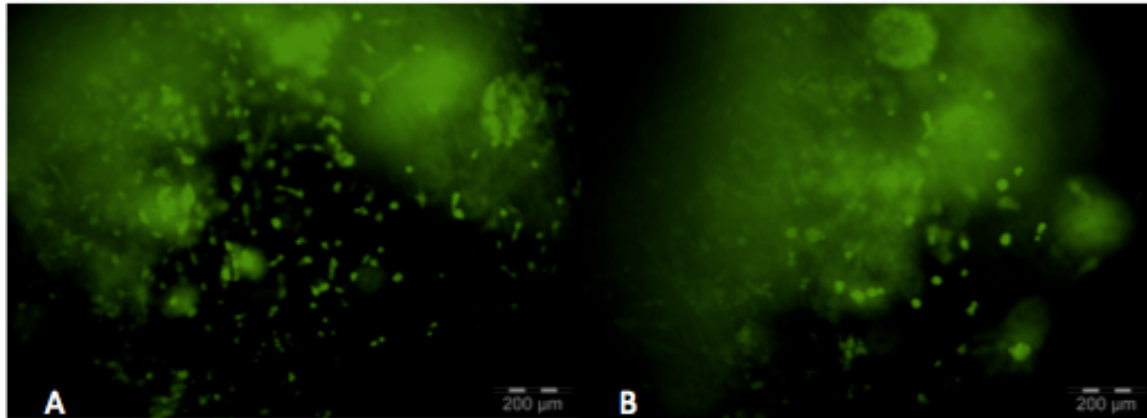
Cell proliferation was observed by seeding cells in different concentrations. Cells were encapsulated at a density from  $100 \times 10^3$  to  $800 \times 10^3$  cells per well in a 96 well plate. Cell proliferation was assessed after 72-hour time point. Cells survived at all concentrations, however, survival rate was higher at  $100 \times 10^3$  and  $200 \times 10^3$ . There was slight decrease in cell viability of cells with increase in cell number (Fig 3-11).



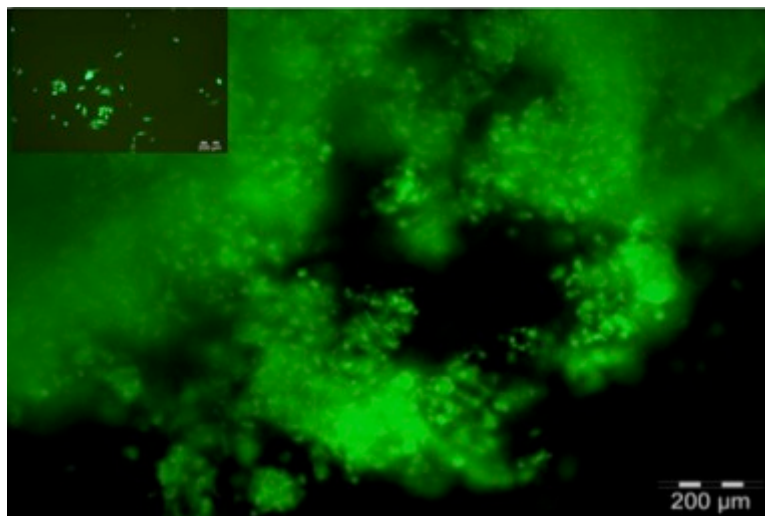
**Figure 3-11 Cell proliferation with WST Assay.**

### **3.3.2 Live dead cell assay**

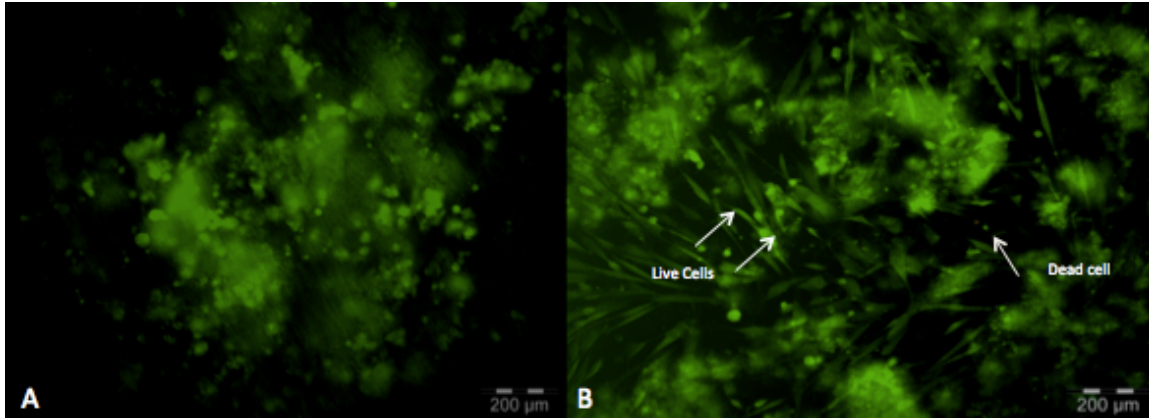
The results of live dead cell assay demonstrated that cells were viable at all concentrations at 72 hours. The cells encapsulated at higher densities showed clustered structures. Figure 3-12 to 3-14 show cells at various densities. Cells at all concentrations showed a typical spindle shaped structure 3 days post seeding. Overall results demonstrated that cells are viable at all concentrations.



**Figure 3-12 A- Live cells in PuraMatrix™ at 100K concentration, B- Live cells in PuraMatrix™ at 100K concentration.**



**Figure 3-13 Live cells in PuraMatrix™ at 400K concentration, inset picture shows cells growth in PuraMatrix™ gel.**



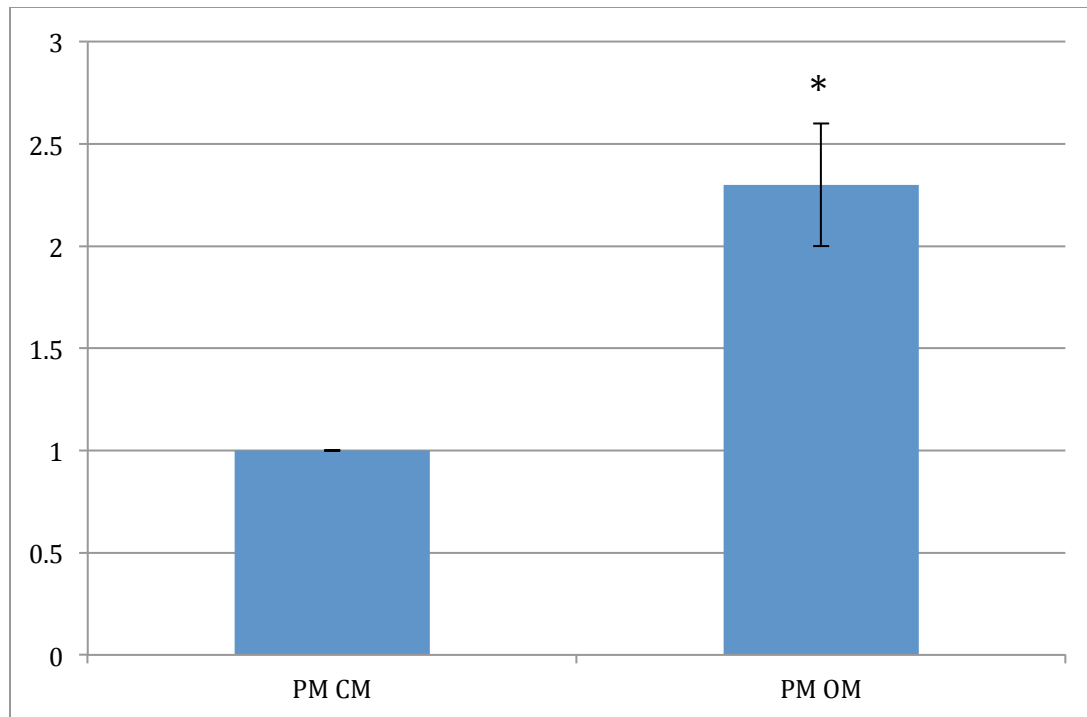
**Figure 3-14 A- Live cells in PuraMatrix™ at 800K concentration, B- Cells in PuraMatrix™ at 800K concentration displaying 1 dead cell.**

### 3.4 Osteogenic differentiation of cells in PuraMatrix™

Osteogenic differentiation of cells was determined by ALP gene expression as described below.

#### 3.4.1 Gene expression

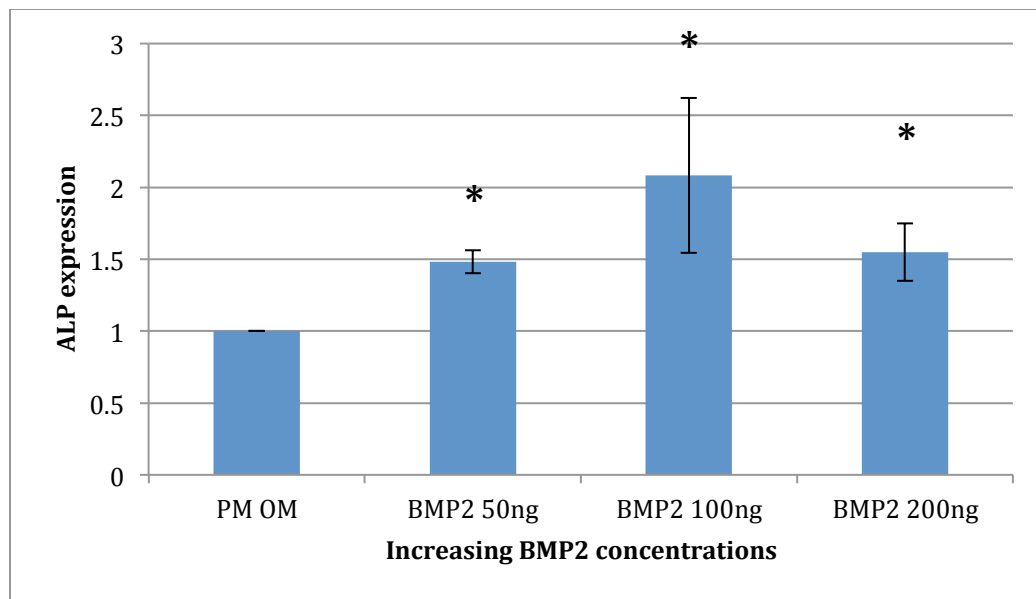
Gene Expression was monitored during the crucial early period of osteogenic differentiation. A cell seeding density of  $8 \times 10^5$  in 200 $\mu$ L gel in a 24 well plate was used. ALP gene expression of cell-gel constructs grown in osteogenic medium was compared with cell-gel constructs in culture medium on day 7 using Quantitative PCR method. ALP expression showed a 2-fold upregulation in OM cell-gel constructs compared to cell-gel constructs in culture medium used as control.



**Figure 3-15 2-fold upregulation of ALP gene in cell-gel constructs in OM.**

### 3.5 Effect of BMP2 on HUMSCS encapsulated in PuraMatrix™

Cell-gel constructs induced with increasing concentration of BMP2 were compared with cell-gel constructs in osteogenic medium. Cell-gel constructs in osteogenic medium were considered as control. The mRNA expression of ALP displayed gradual upregulation with increasing concentration. A 2-fold enhancement in ALP mRNA expression was evident at 100ng/ml of BMP2. However, there was a gradual decrease at 200ng/ml. A significant upregulation was noted at all concentrations of BMP2 compared to cell-gel constructs induced with osteogenic medium alone.



**Figure 3-16 Significant expression of ALP gene in BMP2 induced cell-gel constructs compared to cell-gel constructs in OM.**



## **4 CHAPTER 4 DISCUSSION AND CONCLUSIONS**

### **4.1 Discussion**

Current procedures for the repair of critical size bone defects such as cleft palate rely on various bone grafting methods. Emergence of stem cell based tissue engineering strategies is recognized as a promising source to regenerate biological tissue substitutes for critical size bony defects<sup>76-78</sup>. The objective of the proposed study was to develop an injectable cell-growth factor-scaffold system for repair of bony defects. In this study, HUMSCs were used as cell source, commercially available hydrogel, Puramatrix<sup>TM</sup>, was used as a scaffold and growth factor BMP2 was utilized as osteogenic inducer. First part of this study focused on the isolation and characterization of HUMSCs. Furthermore, HUMSCs were cultured in a 3-dimensional peptide scaffold and investigated for their osteogenic differentiation.

Human umbilical cord is a potential source of mesenchymal stem cells (MSCs) that are developmentally primitive, highly proliferative with potential to differentiate along mesenchymal lineages<sup>22,79</sup>. Recent evidence demonstrated their potential to form bone<sup>80</sup>. Isolation of MSCs from distinct parts of the umbilical cord and use of different methods for isolation has been reported<sup>38,44,81-83</sup>. In this study, we have isolated MSCs from Wharton's Jelly region using two different methods - explant culture and enzymatic digestion. In explant cultures, the cells arised from the tissue after one week and were confluent within 10 days. Enzymatic digestion resulted in successful isolation; nevertheless, the cell yield was low when compared with explant culture in our study. In

contrast, many authors have reported a high yield of cells after enzymatic digestion<sup>84</sup>. The low yield may be due to inappropriate digestion. Isolated cells exhibited fibroblastic morphology. Furthermore, isolated HUMSCs satisfied the criteria set forth by the International Society for Cellular Therapy in terms of plastic adherence, fibroblastic phenotype and differentiation into osteoblastic lineage. Immunophenotyping involves the detection of surface antigen on cells. The flow cytometry results revealed that they were positive for CD73, CD105, CD90 and negative for CD34. CD73, CD105 and CD90 are specific for mesenchymal cells<sup>73</sup>. In agreement with other studies, we found that 90% cells exhibited mesenchymal stem cell property.

In this study, HUMSCs obtained from passage 3 to 5 were used for all experiments. Researchers have reported that early passage cultures are needed to conduct the preclinical study as the MSCs gradually lose their proliferation capacity after several passages<sup>22</sup>. The advantages of using HUMSCs include their rapid proliferation rate and ability to expand over 7 passages without losing differentiation potential<sup>69</sup>.

Mesenchymal stem cells are able to differentiate into osteoblasts under appropriate stimuli. Several genes and proteins are upregulated during osteogenic differentiation. Of the several osteogenic markers, most remarkable ones are ALP, Osteopontin, Osteocalcin and Collagen type I. Several assays such as ALP activity, gene expression and mineralization studies were conducted to detect osteogenic differentiation in the current study. ALP activity assay is a commonly accepted biochemical marker assay for osteogenic differentiation. ALP is a metalloenzyme that is tissue specific encoded by separate genes<sup>85</sup>. ALP is important for hard tissue formation because it increases the

local concentration of inorganic phosphates, a phenomenon postulated as the 'booster hypothesis.'<sup>86</sup> ALP expression marks the success of osteogenesis because it inevitably leads to the formation of mineralization<sup>87,88</sup>. ALP activity in cells is primarily low but as osteogenic differentiation progresses; the progenitor cells undergo differentiation towards matrix maturation. ALP activity continues to increase during this process and finally decreases when osteoblasts turn into osteocytes<sup>89-91</sup>. Our results demonstrate similar pattern, the levels of ALP increased gradually with maximum increase on day 14 and a significant decrease on day 21. Gene expressions of ALP, Collagen type 1 and Osteopontin (OPN) were significantly increased at all-time points (1, 2 and 3 weeks) as well. Furthermore, HUMSCs have demonstrated ability to differentiate along osteogenic lineages which was confirmed by Von Kossa and Alizarin red staining

In this study, commercially available hydrogel scaffold PuraMatrix<sup>TM</sup> was used to encapsulate HUMSCs. PuraMatrix<sup>TM</sup> is a peptide hydrogel that has been used for multiple applications ranging from bone, cartilage<sup>92</sup>, vascular<sup>93</sup>, neural<sup>94</sup> to dental pulp tissue engineering<sup>74</sup>. It's unique features of self-assembly, injectability, nanofibre structure enables the cell attachment, migration and permeation of nutrients<sup>95,96,97</sup>. These properties of the scaffold can minimize surgical procedures and reduce scar formation<sup>98</sup>.

Data from WST and Live-Dead cell assay showed that HUMSCs encapsulated in PuraMatrix<sup>TM</sup> survived at all cell density ranges. However, cells at  $200 \times 10^3$  was the most suitable concentration. Although, there was no statistically significant decrease, there was a decrease in the cell number as observed at  $400 - 800 \times 10^3$  cells. Our

results are in agreement with the results of Cavalcanti et al<sup>74</sup>. Growth of dental pulp derived stem cells stopped at  $800 \times 10^3$  in this study<sup>74</sup>, on the other hand, our results show approximately 1% cell death 10 days after seeding. However, cells were able to migrate within the gel while maintaining spindle shaped structure with network formation. Our results indicate that 0.2% PuraMatrix<sup>TM</sup> is ideal concentration for cell encapsulation and growth. Previously it has been reported that 1% PuraMatrix<sup>TM</sup> forms a mechanically stable gel, nevertheless it was not suitable for cell survival<sup>74</sup>. Ability of osteogenic differentiation of cells encapsulated in PuraMatrix<sup>TM</sup> was investigated in this study. Cells encapsulated in PuraMatrix<sup>TM</sup> grown in culture medium (CM) were compared with the cells encapsulated in PuraMatrix<sup>TM</sup> induced with osteogenic differentiation medium (OM). Within one week of induction, a significant increase of over 2-fold was elicited for cells in OM compared to cells in CM. Chen J et al reported a periodic increase in the ALP gene expression<sup>99</sup>. The limitation of our study is a lack of data on comparison of osteogenic potential of 2D cultures compared to cells in PuraMatrix<sup>TM</sup> in complete medium.

The second part of this study focused on optimizing growth factor concentration. In bone tissue engineering strategies, growth factor remains one of the important components.

**Table 4-1 BMP2 application in cell based therapy**

Author	Cell source	Growth factor/Scaffold used	Significance
Chung et al <sup>100</sup> , 2007	Critical size defect in rats.	rhBMP-2- 200ng with Heparin functionalized nanoparticles in PLGA scaffold.	Heparin nanoparticles with VEGF for bone formation.
Luu et al <sup>101</sup> , 2006	Pre-osteoblast progenitors cells.	Adenoviral vectors express BMPs.	BMP2, 6 and 9 are most osteogenic.
Rickard et al <sup>102</sup> , 1993	Rat marrow cells.	10 <sup>-8</sup> dexamethasone (dex), Vitamin D and BMP2.	rhBMP2 and dex yield high ALP activity and increased osteoblastic mRNAs. Undifferentiated cells retain capacity to differentiate on further exposure to inducers.
Fan et al <sup>103</sup> , 2013	Adipose derived stem cells of mice.	Chitosan, chondroitin sulfate and apatite layer with controlled release of BMP2.	Osteogenic induction with noggin suppression and addition of exogenous BMP2 .
Alsberg <sup>104</sup> et al, 2002	Cotransplantation of rat calvarial osteoblasts, bovine articular chondrocytes in SCID mice.	MVG alginate hydrogel.	Cotransplantation of cells with cell adhesion ligands to engineer bone tissue that grew by endochondral ossification similar to long- bone growth.
Young et al <sup>105</sup> , 2009	VEGF and BMP2.	VEGF and BMP2 in Porous poly (propylene fumarate) scaffolds with gelatin microparticles.	BMP2 has more sustained release profile compared to VEGF. Decreasing amounts of BMP2 while increasing levels of VEGF does not increase percentage bone formation significantly.

VEGF- Vascular endothelial growth factor, SCID- Severe Combined Immunodeficiency, PLGA- Poly (lactic-co-glycolic acid).

BMP2 is the leading osteoinductive growth factor used for bone regeneration currently. Table 4.1 lists several clinical applications of BMP2. The Food and Drug Administration has approved the use of INFUSE bone graft material for anterior and lumbar interbody fusion and open tibial fractures<sup>106,107</sup>. However, BMP2 use in supraphysiological doses for these clinical applications has resulted in adverse effects ranging from ectopic bone formation<sup>108</sup>, spinal cord impingement<sup>109</sup>, bone resorption<sup>110</sup> and life threatening

cervical swelling<sup>65,111</sup>. Our aim was to find an optimum dose of BMP2, specifically for use in pediatric population.

Our study showed that BMP2 significantly enhanced osteogenic differentiation of HUMSCs compared to osteogenic medium. At a concentration of 100ng/ml, a peak expression of ALP mRNA was noted. There was more than two fold increase in ALP mRNA expression with 100ng/ml but this increase showed a decline at 200ng/ml. However, there was no significant difference between the doses of 50ng/ml, 100ng/ml and 200ng/ml in terms of ALP mRNA expression. This suggests that lower doses of BMP2 are equally effective in enhancing osteogenic differentiation of HUMSCs. PuraMatrix<sup>TM</sup> as a scaffold promotes osteogenic regeneration<sup>112</sup>. Results from Hanada et al showed that at 50ng/ml concentration BMP2 treatment alone showed a slight increase in ALP activity in bone marrow stem cells, whereas the effect was significant when it was used in combination with Fibroblast growth factor (FGF)<sup>113</sup>. The high doses of BMP2 (20mg/ml) induced osteogenic differentiation in bone marrow cells of Beagle<sup>114</sup>. Recently, Zachos et al investigated the feasibility of using BMP2 in a 3 dimensional model<sup>115</sup>. The results of the study indicated that BMP2 could be delivered using alginate as carrier system. However, the results of previous studies demonstrated either high doses or use of BMP2 synergistically with other growth factors. In contrast, our results indicate that 1) BMP2 can induce osteogenic differentiation in presence of a 3D culture model 2) BMP2 enhanced osteogenic differentiation with concentrations as low as 50ng/ml. In summary, our results indicate

that minimal doses of BMP2 are sufficient when used in an appropriate 3D scaffold system.

#### 4.2 **Conclusion**

PuraMatrix™ with HUMSCs and BMP2 is a viable composite system for osteogenic regeneration of bony defects. This composite system requires lower doses of BMP2 thus minimizing potential adverse affects reported from using higher doses of BMP2. As this system is injectable into the *in vivo* site it will reduce the number of surgeries and the incidence of scar formation. Future research involves incorporating BMP2 within the scaffold for site-specific delivery and to minimize its absorption systemically.

## 5 APPENDIX

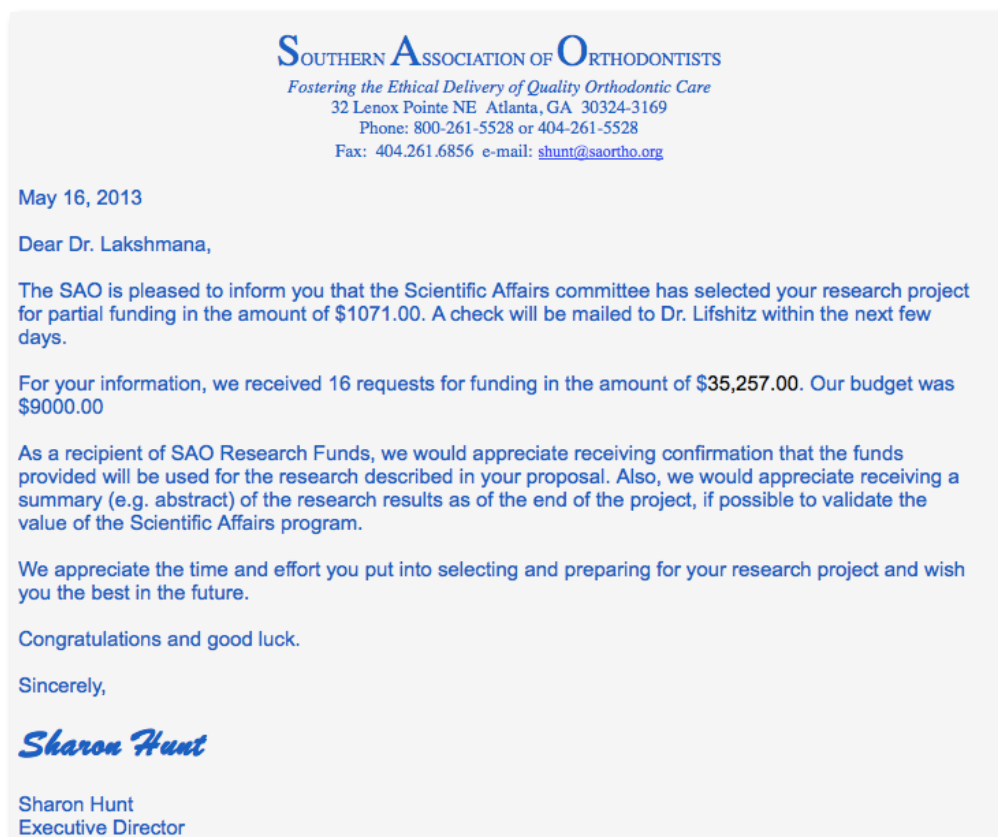


Figure 6-1 SAO Grant Award in support of this project.



## 6 RAW DATA

	A	B	C	D	E	F	G	H	I	J
1										
2				Control	1 Week	Control	2 Weeks	Control	3 Weeks	
3				100	115.2	100	145.2	100	113.72	
4			SD	0	12.4	0	14.5	0	3.08	
5			P value		P=0.0065		P=0.0083		P=0.0164	
6										
7										
8										
9					Control	100				
10					1 Week	115.2				
11					Control	100				
12					2 Weeks	145.2				
13					Control	100				
14					3 Weeks	113.72				
15										
16					1 week		2 weeks		3 weeks	
17		P Value		0.0065		P Value	0.0083		P Value	0.0164
18										
19						P value between 2 and 3 weeks			0.015	
20										
21			P value	1 week and 2 weeks		0.0027				
22			Between							
23										
24				P value between 1 week and 3 weeks 0.855						

Figure 6-1 ALP activity of HUMSCs in control CM and experimental group OM

	A	B	C	D	E	F	G	H	I	J
3										
4										
5										
6						Mean Absorbance	SD			
7				100,000		2.701	0.05			
8				200,000		2.8	0.08			
9				400,000		2.33	0.03			
10				800,000		2.5	0.16			
11										
12										

Figure 6-2 Mean absorbance values of WST assay

	A	B	C	D	E	F	G
1							
2							
3		Mean	1.483	2.083	1.55		
4		SD	0.08	0.54	0.20		
5							
6		P value	0.0085	P= 0.0043	0.0124		
7							
8							
9							
10							
11			PM OM	1	0		
12			BMP2 50ng	1.483	0.08		
13			BMP2 100ng	2.083	0.54		
14			BMP2 200ng	1.55	0.2		
15							
16							
17							

Figure 6-3 ALP activity of HUMSCs in PuraMatrix™ in BMP2

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