Evaluation of a biopolymer matrix for cell based bone repair

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ABSTRACT

DEEPALI JERE: Evaluation of a biopolymer matrix for cell based bone repair

(Under the direction of Dr. Lyndon Cooper)

Autogenous bone grafts are often associated with unpredictable success rates and donor site morbidity. There is clear medical need for cost-effective bone graft material that overcomes these short-comings.

Aim: To test a thermally stable, macro-porous collagen scaffold, DuraGen® for bone repair and study its ability to stimulate healing of critical size defects in rat calvaria engrafted with Mesenchymal Stem Cells(MSCs).

Methods: Craniotomy defects, 8.9mm diameter were created in 28 adult Sprague-Dawley rats, randomly divided into four groups, 6-Empty(E), 6-Collagen only(C), 8-Collagen+MSCs(C+M), 8-Collagen+rhBMP-2(C+B). Two transgenic rats served as stromal cell donors by means of femoral marrow lavage.

Results: Bone repair at 28 days was measured using radiographs and histology. Histology showed mean bone fill of 9.25%(10.82), 19.07%(17.38), 44.21%(3.93) and 66.06%(15.08) respectively. Statistical analysis demonstrated significant differences between: E & C, C & C+M, C+B; none between C+M & C+B.

Conclusion: This study clearly displays osteogenic properties of collagen scaffold implanted with MSCs.

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LIST OF ABBREVIATIONS AND SYMBOLS

%	Percentage
alpha-MEM	alpha- Minimum Essential Medium
BMPs	Bone Morphogenic Proteins
CO_2	Carbon-di-oxide
CSD	Critical Size Defect
DBM	Demineralized bone matrix
DLAM	Department of Laboratory Animal Medicine
Dlx5	Distal-less homeobox 5
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulphoxide
ECM	Extra Cellular matrix
EDTA	Ethylenediaminetetra acetic acid
FBS	Fetal Bovine Serum
FGF	Fibroblast Growth Factor
GBR	Guided Bone Regeneration
GFP	Green Fluorescent Protein
H & E	Hematoxylin and Eosin
IACUC	Institutional Animal Care and Use Committee
IGF	Insulin-like Growth Factor
MGV	Mean Gray Value
MSCs	Mesenchymal Stem Cells
Msx2	Msh-like homeobox 2

Osx2	Osterix 2
PDGF	Platelet-derived Growth Factor
PGA	Poly(glycolic acid)
PLA	Poly(lactic acid)
PLGA	Poly(DL-lacticlco-glycolic acid)
PPF	Poly(propylene fumarate)
rhBMP-2	recombinant human Bone Morphogenic Protein 2
R-Smad	Receptor regulated Smad
Runx2	Runt-related gene 2
TCP	Tricalcium phosphate
TGF	Transforming Growth Factor
VEGF	Vascular Endothelial Growth Factor

CHAPTER 1

INTRODUCTION

Multiple bacterial dental diseases, trauma, congenital defects and cancer therapy result in loss of alveolar (jaw) bone and teeth. As the population continues to age, the percentage of individuals with missing teeth is also increasing. Their desire for teeth is often limited by lack of supporting bone. This is especially true for replacement of teeth with dental implants. Optimal functional rehabilitation requires replacement of bone and teeth, the later often using endosseous implants. Bone regeneration procedures must be developed to be simple, rapid and devoid of second surgical donor sites which are frequently associated with increased morbidity.

Use of autografted bone from the iliac crest, the “gold standard” bone graft material for this procedure, results in non-union failure rates ranging from 5% to 35% (Boden SD, 1995; Betz RR, 2002) with morbidity and pain at the donor site reported in up to 25% of cases after two years. The most common alternative to autografts is allografts and they have associated problems with host rejection, excessive resorption and bone revascularization (Mankin HJ, 1983; Prolo DJ, 1985). Xenografts are less common alternatives due to their concerns with immunogenicity and disease transmission (Kenley RA, 1993).

There is clear medical need for a bone graft material that improves the success rates and eliminates donor site morbidity. This study is designed to produce a bone implant composite that eliminates use of autologous bone and results in successful bone grafting in a high percentage of procedures. In this study, DuraGen® was engineered for use in bone regeneration procedures. This research will provide the basis for a new therapeutic alternative for bone regeneration and specifically provide a cost effective approach for improving the outcome of bone regeneration procedures performed each year.

A. Specific aim

This research project will test thermally stable, macroporous cell regenerative scaffolds for bone repair; and evaluate the ability of the scaffolds engrafted with Mesenchymal Stem Cells (MSCs) to stimulate healing of 8.9 mm diameter critical size bone defects in rat calvaria.

B. Clinical Significance

Predictable bone regeneration can enhance the prognosis of dental and maxillofacial treatment when deficiency in bone mass can compromise or prohibit optimum treatment. It can be employed clinically for functional replacements of damaged or diseased bone tissues to improve the orofacial function and esthetics (Alsberg E, 2001). In the future, this approach of bone regeneration using collagen (DuraGen®) scaffolds and MSCs can potentially be applied in efficiently recreating missing osseous structures especially in case of vertical bone loss, repairing craniofacial

deformities and improving the long-term success and stability of implant therapy (Earthman JC, 2003).

C. Hypothesis

If a collagen / proteoglycan scaffold (DuraGen®) is a biomimetic agent providing features of embryonic extracellular matrix, then engraftment of mesenchymal stem cells using DuraGen® (type I collagen) will support tissue-specific differentiation.

CHAPTER 2

LITERATURE REVIEW

A. Tissue Engineering

Tissue engineering is an interdisciplinary field which aims to restore function or replace damaged or diseased tissues through the application of engineering and biological principles (Alsberg E, 2001). There is a significant need for functional replacement of missing tissues in the oral-maxillofacial complex due to dental disease, trauma, congenital defects and cancer therapy. Over 1 million operations in the United States annually involve bone repair (Langer R, 1993). While all of them may not need bone grafting, an estimated 426,000 bone grafting procedures were performed in the United States alone in 1996. Of these, 247,000 used autografts, 145,000 allografts, and 34,000 other materials (Boyce T, 1999). According to Boyce T in 1999, the number of these grafting procedures has enormously increased in the recent decades. The clinical consequence of this experiment is to evaluate the use of a collagen scaffold, DuraGen® with bone marrow stromal cells to more efficiently regenerate bone in surgical wounds and bone defects that otherwise will not heal or heal slowly.

A1. Approaches to tissue engineering

Three main approaches to tissue engineering have been discussed in literature, namely osteoinduction, osteoconduction and cell-transplantation (Alsberg E, 2001).

A1a. Osteoinduction

It is a process by which osteogenesis is induced by bioactive factors. It involves stimulation of undifferentiated or pluripotent cells to develop into osteogenic cells. A very common example of this process is fracture healing where immature, pluripotent cells are stimulated to differentiate into preosteoblasts (Albrektsson T, 2001). Many modern experiments in osteoinduction using bone morphogenic proteins have been performed ever since Urist discovered the inductive growth factors in 1965 (Urist MR, 1965).

A1b. Osteoconduction

As Wilson-Hench mentioned in 1987, osseoconduction is the process by which bone is directed to regenerate and adapt to a material's surface (Albrektsson T, 2001). This technique makes use of a barrier membrane placed over the bone defect in order to inhibit the nonosteogenic cells of the surrounding tissues from entering the defect site while permitting osteogenic cells from the bone marrow cavity to access the defect (Hermann JS, 1996). Guided bone regeneration which has been used for many years in Periodontics is a good example of osteoconduction.

A1c. Cell transplantation

In this technique, seeded cells with a potential to stimulate or differentiate into osteogenic cells are applied at the defect site. Some examples of such cells could be chondrocytes and marrow stromal cells which are often seeded on osteoconductive scaffolds (Alsberg E, 2001).

BMPs and associated osteoinductive agents have shown promising results and are currently in clinical trials (Kawai T, 2006). Osteoconductive approaches passively facilitate regeneration of tissues and may be employed in small bony defects (Alsberg E, 2001; Kaigler D, 2001). Cell transplantation provides a cost-effective approach for large bone regeneration procedures where transplanted cells and possibly entire bone structures can be grown in vitro (Kaigler D, 2001).

B. Types of grafts

B1. Autografts

The most commonly used and the gold standard for bone regeneration is autologous grafting. But, autografts are often challenging in cases where extensive grafting is needed since large volumes of autogenous bone may not be available (Alsberg E, 2001). In wide spread grafting situations, iliac crest bone graft is often used and it has been associated with significant morbidity (Arrington ED, 1996; Kenley RA, 1993; Aichelmann-Reidy ME, 1998). Apart from extensive donor site morbidity, a second surgical procedure may result in chronic pain which may be disabling in 25% of cases (Summers BN, 1989). Other associated problems could include poor contouring and the difficulty involved in harvesting bone (Alsberg E, 2001).

B2. Allografts

Historically, the most popular alternative to autograft has been human cadaver bone or allograft (Mankin HJ, 1983; Prolo DJ, 1985). For most part, it is used in situations where extensive grafting is necessary and autograft is not a viable alternative (Boyce T, 1999). However, allografts have higher failure rates than autografts and have additional disadvantages related to extensive resorption, unpredictable outcome, and potential host rejection (Mankin HJ, 1983; Prolo DJ, 1985). Although allografts these days may be safer with regard to disease transmission when satisfactorily packaged and stored, (Boyce T, 1999; Horowitz B, 1993; Friedlander GE, 1983) they still have problems related to bone revascularization (Mankin HJ, 1983; Prolo DJ, 1985).

B3. Xenografts

Xenogenic or animal grafts are widely available, but scarcely applied in extensive grafting procedures owing to concerns with immunorejection and disease transmission (Alsberg E, 2001; Kenley RA, 1993). Bovine-derived bone graft materials such as Bio-Oss® are popularly used in smaller defects.

B4. Alloplasts

This term is often applied to biocompatible, inorganic materials such as synthetic calcium phosphate ceramics, polymers and bioactive glasses which can be used in conjunction with autogenous or allografts. The main advantage of alloplasts is that they have no potential for disease transmission (no author, JADA 2002). With the

substantial increase in number of bone grafting procedures done every year (Boyle T, 1999), and their significant role in functional replacement and rehabilitation of damaged or diseased orofacial structures, there is a clear and pressing need for a cost-effective bone graft material that improves predictability of the procedures and eliminates donor site morbidity.

Reddi AH in 2000 suggested that bone tissue engineering primarily involves three important ingredients namely the osteoinductive signaling molecules, host cells / stem cells responding to the signal and suitable scaffolding for the stimulated cells.

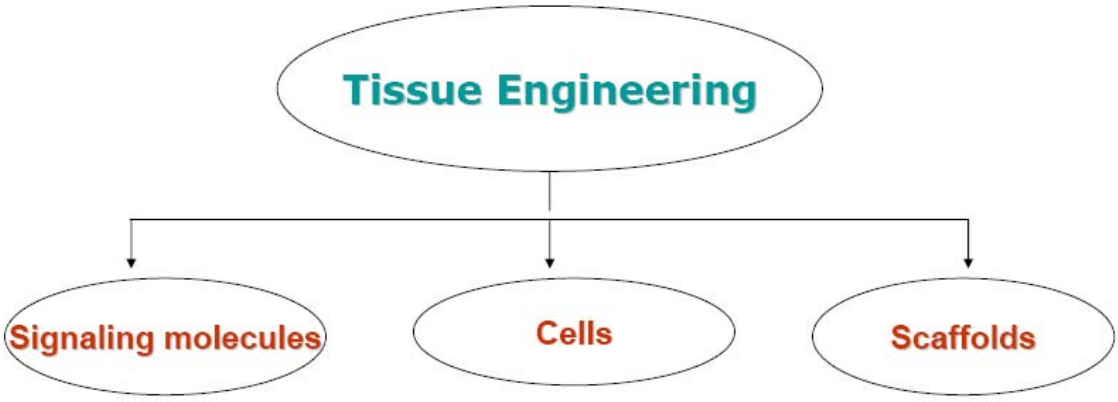

Figure 2.1. Diagram demonstrating the three important ingredients of tissue engineering

C. Signaling molecules

The first ingredient for tissue engineering as suggested by Reddi AH, 2000 are the signaling molecules. Several growth factors and hormones act as signaling molecules and they play an important role in osteogenesis by stimulation, differentiation and protein synthesis in osteoblastic cell cultures (Reddi AH, 2000). Different animal models have been used to identify the major bone growth factors

which include, bone morphogenic proteins (BMPs), transforming growth factor beta (TGF-b), insulin-like growth factors I and II (IGF-I and IGF-II), platelet derived growth factor (PDGF) and basic and acidic fibroblast growth factor (bFGF and aFGF). Usually, they are polypeptides or glycoproteins, present in specific tissues in extremely low concentrations where they modulate cell functions. Localization of various growth factors influences the spatial patterning and temporal sequence of bone healing (Solheim E, 1998).

C1. Bone Morphogenic Proteins (BMPs)

Marshall R Urist in 1965 discovered that demineralized bone extract could induce bone formation when implanted intramuscularly (Urist MR, 1965). His key discovery led to the identification of the osteoinductive molecule as bone morphogenic proteins and plenty of experiments have been done since then to isolate and as well as clone them (Croteau S, 1999; Solheim E, 1998; Wozney JM, 1988). BMPs have been identified as members of the TGF-b superfamily and are present in very low concentrations in the bone matrix (Croteau S, 1999). At least twenty types BMPs have been identified in humans so far (Gautschi OP, 2007).

Several groups have acknowledged the bone inductive capacity of recombinant forms, BMP-2 and BMP-7 in healing critical size bone defects in various animal models such as sheep, rats, dogs (Harakas NK, 1984; Gerhart TN, 1993; Stevenson S, 1994; Itoh T, 1998). During bone healing, activation of BMP receptors plays a major role in regulating osteoblastic differentiation and subsequent osteogenesis

(Reddi AH, 2000; Gautschi OP, 2007; Solheim E, 1998). These factors seem to be expressed in mature bone as well as during embryonic development (Urist, 1977).

Many in vitro experiments have tried to investigate the intracellular events which include alkaline phosphatase monitored BMP-2 activity and receptor activation with kinase activity (Heldin CH, 1997; Ryoo HM, 2005). This cascade of events may in turn lead to receptor regulated Smad (R-Smad) activation, and stimulation of osteogenic transcription factors, such as Runt-related gene 2 (Runx2), Osterix 2 (Osx), Distal-less homeobox 5 (Dlx5), and Msh-like homeobox 2 (Msx2). In BMP-2 induced osteogenesis, it was found that Dlx5 was the earliest to be stimulated by R-Smads and that it regulated other well-known master transcription factors such as Runx2 and Osx (Ryoo HM, 2005).

Despite numerous animal studies (Wang EA, 1988) on bone induction potential of BMPs, there is still a huge scarcity of well-designed clinical trials (Gautschi OP, 2007; Chen D, 2004). BMP-2 and BMP-7 have been clinically used in patients with non-union fractures. However their routine usage is not yet a possibility particularly due to the extremely high costs of manufacturing these glycoproteins (Gautschi OP, 2007).

C2. Transforming Growth Factor beta (TGF-β)

They are also members of TGF-β super family with five subtypes (Solheim E, 1998). They stimulate cells of mesenchymal origin while inhibiting cells of ectodermal origin

(Lind M, 1996). Bone contains the largest concentration of TGF-β since they are produced by osteoblasts and stored in bone matrix (Bonewald LF, 1990). Animal experiments with TGF-β have shown increased osteoblastic activity and enhanced bone in growth (Lind M, 1996). TGF-β along with BMPs may have a potential for clinical use, according to Lind.

C3. Insulin-like Growth Factors (IGFs)

IGF-1 has been proven to have the potency to accelerate bone repair in critical- size defects in rat calvaria (Thaller SR, 1993). IGF-1 is supposed to be two to seven times more potent than IGF-2 as suggested by Lind, 1996. The major effect of IGF in bone formation is that they stimulate preosteoblastic cell replication which in turn enhances bone collagen synthesis and matrix formation (Hock JM, 1988; McCarthy TL, 1989).

C4. Platelet derived growth factor (PDGF)

It is synthesized by blood platelets, monocytes, macrophages and endothelial cells and chiefly affects the mesodermal cells (Solheim E, 1998). They mainly influence by mitogenesis which is not specific for osteoblastic cell line (Abdennagy B, 1992). Upon local application of PDGF in rat muscle along with demineralized bone, it enhanced osteogenesis (Howes R, 1988). Two receptor subunits for PDGF, namely alpha and beta have been characterized (Solheim E, 1998).

C5. Fibroblast Growth Factor (FGF)

FGFs are made up of a family of polypeptides which influence the mitogenic activity of the mesodermal and neuroectodermal cells (Mabilleau G, 2008; Lind M, 1996). They are characterized in two forms, acidic and basic FGF. Acidic FGF is most commonly manifested in humans (Solheim E, 1998). In recent studies by a French group, FGF was able to stimulate bone regeneration in rabbits with the usage of an appropriate vehicle (Mabilleau G, 2008). FGFs play a major role in revascularization of bone during wound healing due to their angiogenic potential (Lind M, 1996).

D. Cell sources for bone repair

The second ingredient suggested by Reddi AH in 2000 for successful tissue engineering is cells.

D1. Embryonic Stem cells

Most often, embryonic stem cells are derived from inner cell mass of a blastocyst or from the primordial gonadal ridge of the fetus (Elisseeff J, 2005; Handschel J, 2006). They have great potential to differentiate into any tissue in the body. However, they have major limitations concerning purity of the isolated cell populations and immunogenicity.

D2. Mesenchymal stem cells (MSCs)

Experiments using multipotent bone marrow stromal cells can be dated back to 1968 through the work of Friedenstein and his team (Friedenstein AJ, 1968). These cells

have the capacity to divide and form two equal daughter cells and differentiate into tissue-specific cells on stimulation by signaling molecules (Elisseeff J, 2005). These multipotent cells can proliferate and differentiate into various cell types to repair tissues (Bianco P, 2001). MSCs can be cultured in the lab and combined with appropriate carrier to the site of defect. On transplantation, these progenitor cells can differentiate to form the entire bone organ which includes bone, cartilage, adipocytes and blood vessels (Bianco P, 2001). While the haemopoitic cells are provided by the host, the bone trabaculae, cortices, matrix and adipocytes are of donor origin (Friedenstein AJ, 1966). Several animal studies have demonstrated the effectiveness of mitotically expanded MSCs in supporting bone regeneration either alone or along with growth factors (Krebsbach PH, 1998; Petite H, 2000). The cultured stem cells require a suitable scaffold or vehicle before implantation for bone regeneration.

E. Delivery systems or Scaffolds

The third ingredient required for bone tissue engineering as suggested by Reddi AH, 2000 are scaffolds or delivery vehicles for the graft materials. Various materials have been investigated as delivery systems for bone tissue replacements. Some of them include ceramics (Gazdag AR, 1995), demineralized bone matrix, collagen composites, fibrin, calcium phosphate, polyacrylic acid, hydroxyapatite, dental plaster, titanium (Croteau S, 1999).

Ideally a scaffold should have a biomimetic matrix similar to the bone tissue environment and possess osteoinductive capacity. Biocompatibility is also desirable in that, the material should not be immunogenic or cause unexplained inflammation (Temenoff JS, 2000). Additionally, mechanical properties of the material should match that of the tissue to be regenerated (in this discussion, bone) and these are often tested under functional loads. The following most widely studied scaffolds will be discussed in this section.

E1. Ceramics

Ceramics have been applied in multiple bone regeneration studies. Several combinations of calcium phosphate have been evaluated, the most common among them being hydroxyapatite and biphasic calcium phosphate. The material has been used in powder and paste forms. Pastes seem to have better initial mechanical properties compared to powders. But powders can be sterilized by gamma radiation. Although they have good compressive strengths, the tensile strengths are still not close to that of bone. The injectable ceramics have several advantages, in that they can easily conform to the shape of the bone defect and produce efficient bone regeneration. Some studies have shown that they degrade very slowly and sometimes also cause inflammation due to the acid formation (Bermudez O, 1994). However a recent study confirmed the bone forming ability of calcium phosphate and they found minimal inflammatory cell infiltrate with complete resorption and replacement by new bone (Moon, 2005). Additionally, recombinant forms of TGF–β1 in combination with the cement form have shown to produce bone formation (Blom

EJ, 2001). Ectopic osteoinduction was observed when injectable biphasic calcium phosphate was packed with bone mesenchymal stem cells (Trojani C, 2006).

E2. Polymers

Many polymers have been evaluated for bone repair. In general they have distinct advantages: low cost, injectable, acceptable mechanical properties and degradation times. Poly(propylene fumarate) (PFF) has been successfully studied as a osteoconductive scaffold for osteoblasts to stimulate bone formation without producing long-term inflammatory effects in rats (Payne RG, 2002; Temenoff JS, 2000).

Poly (lactic-co-glycolic acid) (PLGA) has been tested as a delivery vehicle for BMP-2. It is a biocompatible synthetic polymer easily manufactured with predictable biodegradability (Woo BH, 2001). Other copolymers of PLGA such as Poly (alpha-hydroxy acids) (PHA) have been thought to be promising due to their biodegradability (Hollinger JO, 1995).

Recently a study by Mylonas D concluded that early bone healing was promoted when polyaxomer, thermoplastic carrier and a granular inorganic matrix was used in combination with allogenic mesenchymal stem cells in non-critical size defects in dogs (Mylonas D, 2007).

E3. Hydrogels

Elisseeff J, 2005 defined hydrogels as “crosslinked polymeric systems that are capable of absorbing large volumes of aqueous solution”. Hydrogels can be modified to be very similar to the bone matrix due to their permeability and mechanical integrity. Alginate, pluronics, chitosan, and fibrin glue are few examples of hydrogels (Elisseeff J, 2005). Alginate seems to act by enhancing cell attachment and mineralization (Nyugen H, 2003). Cross-linking induction chemistries have been used to alter mechanical properties. However, ionic cross-linking of alginates reduces its mechanical properties over time (Rowley JA, 1999). Bone marrow stromal cells applied locally in rats using temperature-dependent polymerizing polyethylene oxide hydrogel vehicle revealed new bone formation (Chen F, 2003). Elisseeff J and coworkers also developed multilayered hydrogel systems to engineer tissues such as bone and cartilage (2005).

E4. Collagen scaffolds

Previous studies have demonstrated the ability of type I collagen to act as a carrier matrix to bring about bone formation in critical sized bone defects in rats at 6 weeks (Saadeh, 2001). Studies by Lutolf and co-workers showed critical sized defects in rat calvaria treated with collagen matrix in conjunction with rhBMP-2, demonstrated high density of localized bone regeneration, especially close to bone-tissue interface (Lutolf MP, 2003). The collagen matrix used in our study, DuraGen® has been successfully used for dural repair and containment of CSF (Narotam, 2004).

The polymeric matrix (a collagen / proteoglycan co-polymer) to be evaluated for bone repair has been investigated as a collagen matrix for primary dural closure. It easily conforms to complex surfaces of any shape and size and is fully absorbed after complete tissue closure of the dural defect. Unlike some of the hydrogels which have been tested by our laboratory, the dural graft material is pliable and easy to handle. It is made from bovine tendon which is supposed to be one of the purest sources of Type I collagen. This kind of collagen is currently used in manufacture of artificial skin, absorbable sponges and wound dressings. The manufacturers claim that inactivation of the pathogens is done using sodium hydroxide. It is suggested to promote repair by supporting neovascularization, growth and differentiation of cells.

The collagen / proteoglycan co-polymer is designed to mimic the open polar structure of early embryonic extracellular matrix (ECM). Tissue morphogenesis during development and tissue homeostasis and remodeling throughout life depend on interactions between the ECM and neighboring cells (Adams JC, 1993; Damsky CH, 1997). Signals contained within the ECM and released during ECM remodeling bind to cells through receptors such as integrins and modulate gene expression, tissue differentiation and the survival of osteoblast and fibroblast (Damsky CH, 1999). If a collagen / proteoglycan material can offer suitable handling for bone repair, then it must also present morphological cues to the healing tissue. It is the aim of this project to determine if the collagen / proteoglycan copolymer maintains its biological activity when implanted with mesenchymal stem cells.

F. Critical Size Defects

Frequently, bone healing in large alveolar bony defects and extraction sites is less than ideal. An unfortunate consequence of orofacial cancer survival is that surgical resection of affected bone and soft tissue is limited by current technology and techniques. Current methods of regenerating bone have moderate morbidity and/or risks, providing only a limited framework for the bone to refill the defect. Results with these techniques can be unpredictable. Bone loss beyond healing capacity of the body to regenerate bone is called a "critical size defect". In this situation the body heals by forming scar tissue not restoring the functionality of the area.

Schmitz and Hollinger established the use of ‘critical size defect’ as an acceptable experimental model for bone research in the mid-1980s. They suggested a rationale for testing materials in a hierarchy of animal models with the initial testing to be done in the calvaria of rats and rabbits followed by mandibles of dogs and monkeys (Schmitz-Hollinger, 1986). Subsequently, several research groups have successfully adopted this rationale for testing of bone substitutes (Sikavistas VI, 2002). In general, adult rats are more reliable models to evaluate bone grafting materials due to the innate capacity of weanling rats to spontaneously heal extensive defects (Takagi K, 1982). The size of CSD for adult rat model varies in scientific literature from 5 to 8mm (Jager M, 2005).

The regeneration or repair of craniofacial osseous defects of dimensions exceeding those associated with spontaneous repair as well as larger defects exceeding autogenous sources of bone for grafting require bone-substitutes.

CHAPTER 3

MATERIALS AND METHODS

Based on a recent literature review, the rat model is a commonly employed model by the majority of researchers in this field. We utilized this species and followed standardized procedures in order to minimize the number and risk to the animal subjects, and to be able to compare our results with those obtained in the past by other institutions.

We selected the rat cranium as the site of our experiments based on literature review due to its poor healing response. The critical size defect in the rat calvarium is a particularly good model, since it provides an excellent challenge to the tested materials due to the natural lack of a primary nutrient artery in the rat, and also because of its relatively low marrow content. Our preliminary studies using the collagen scaffold demonstrated partial to complete bone repair of the rat calvarial defects and compared favorably with BMP-2 directed healing.

To determine efficacy of the DuraGen® scaffold, four different treatment groups were compared using the standardized calvarial defect. A total of 30 rats were used for this experiment. 2 rats served as bone marrow stromal cell donors by means of femoral bone marrow lavage. They were divided randomly into four groups of with 6

or 8 animals each. Animals were treated using implants with bone marrow stromal cells-containing matrix and compared to untreated defects as well as implants with bone morphogenic protein. The animals and treatments will be distributed as follows Table 3.1.

Group #	Number of animals	Treatment
1	6	Empty / Negative control
2	6	Matrix + Saline
3	8	Matrix + MSCs
4	8	Matrix + BMPs / Positive control

Table 3.1. Distribution of animals in the four treatment groups. Note that the matrix used in this experiment is DuraGen®

Prior to beginning the experiments, the protocol was submitted for approval by Institutional Animal Care and Use Committee (IACUC). UNC Department of Laboratory Animal Medicine (DLAM) requirements were fulfilled.

A. Surgical procedures

Adult, male Sprague-Dawley rats weighing 250-300g and 5 months old were used for our project. They were gently restrained by a surgical assistant and anesthetized presurgically by intraperitoneal injection of ketamine/xialzine (40-80mg/kg; 2-10mg/kg). The animals were monitored for respiration rate, toe-pinch and eye reflex throughout and after the procedure as a way to ensure an appropriate level of anesthesia. Immediately after the surgery, each animal was given a subcutaneous injection of buprenorphine (0.15mg/kg; Henry Schein, Melville, NY) for post-

operative analgesia, and an intraperitoneal injection of 3 ml of normal saline (0.9% Sodium Chloride NaCl; Henry Schein). This is to compensate for sensible and insensible fluid losses during the post-op recovery periods. In order to prevent excessive drying of the conjunctiva, 'Perlube' lubricant in the form of ointment was applied in the rats' eyes. The incision site and wound area were shaved gently with an electric shaver. The surgical field was cleaned with Providine, 10% Povidone-Iodine antiseptic solution.

Adhering to aseptic technique guidelines, a midline skin incision from the mid-nasal bone area to the posterior nuchal line was made, and the underlying periosteum incised and dissected. A trephine bur with an internal diameter of 8.0mm and external diameter of 8.9mm was attached to a rotating slow-speed, straight dental hand-piece which was used to create a 0.89-cm circular defect in the rat calvaria. The dura and surrounding blood vessels were carefully guarded to avoid any perforation or damage. Constant irrigation with a mixture of normal saline was used on the drilling site to minimize bleeding. Once the bone defect was prepared, the circular piece of bone was saved for future evaluation. After ensuring that the animal was stable, implants were set in place. Collagen (DuraGen®, Integra, NJ) scaffolds were precut using 8.0mm tissue punch into 2mm thick implants. The scaffolds were saturated with 2-3 drops of sterile 0.9% saline and/or 5μg/ml of rhBMP-2 solution or 5 x 10^6 cells/ml of rat MSCs as required by the treatment groups.

The surgical wound was closed by approximating the overlying tissues and suturing in layers with resorbable 4 or 5-0 Vicryl sutures (Ethicon, Somerville, NJ). The animals were then housed individually at room temperature, and observed by trained personnel until they ambulated and did not show any visible signs of distress. Once they were ambulant, they were transferred in individual labeled cages with soft bedding and were given free access to water and soft diet. Approximately 8 hours after surgery, once the animals recovered from sedation, each animal was given another subcutaneous injection of buprenorphine (0.15mg/kg; Henry Schein, Melville, NY), an opioid analgesic for prolonged post-operative analgesia. Each animal was given a subcutaneous injection of ketoprofen (dose of 5mg/kg; Henry Schein) 24 hours after the surgery in order to provide long-term analgesia. Then the animals were monitored daily for signs of complications related to surgery or illness. They were housed at room temperature and had unrestricted access to food and water. Daily records of each animal were kept in writing. The animals were euthanized by Carbon-di-oxide (CO_2) inhalation as per the standard protocol, 28 days after surgery. Death was ensured using thoracotomy on the rats.

After the euthanasia was complete, 10% formalin was injected for perfusion fixation. Upon debriding the soft tissue, craniotomy site was separated along with surrounding bone using bone rongeurs. Care was taken during the procedure to maintain the calvarial periosteum intact. The recovered bone was then placed in 4% paraformaldehyde solution. It is immediately placed in the cold room at 4^0C with the vibrator operating at low speed. The paraformaldehyde was changed out after 24

hours. Another 24 hours later, the bone specimens were washed with PBS and micro CT scanning was done. Then, 45ml of 0.5M EDTA, pH 8.0 was added to initiate the process of decalcification. Every 3 days, fresh EDTA was replenished. After 14 to 30 days of being immersed in EDTA, the decalcified bone specimens were ready for paraffin embedding.

A1. Bone marrow isolation

6-week old Green Fluorescent Protein (GFP) transgenic rats of Sprague-Dawley type were used for bone marrow isolation. The rats were euthanized using the protocol for CO_2. Immediately after that, the rats' femur was isolated. The femur was excised at its head as well as above the knee at the diaphysis. Medium was premade by mixing alpha Minimum Essential Medium (alpha-MEM), 10% Fetal Bovine Serum (FBS) and 1% antimycotic agent. Using a 5ml syringe and 18 gauze needle, medium was passed through and through the shaft of bone into a 50ml tube. This was done repeatedly using additional 15-25ml of media until the bone becomes white. The entire mixture of medium and cells are pipetted several times to remove cell clumps. Each femur was plated into two 150ml dishes, labeled and allowed to culture in an incubator. The plates were rinsed extensively with PBS several times and the culture medium was replaced on second, fourth and sixth days. When the cells were approximately 90% confluent, they were split and plated.

Splitting was done by removing the medium and washing with PBS. About 5ml of Trypsin or EDTA was added to a 150mm dish with at least 90% confluent cells. The

dishes were lightly tapped on the bottom and incubated for approximately 5 minutes to allow the cells to detach. 10ml of medium was added and centrifuged for 4 minutes at 1000 rpm. The supernatant was removed and resuspended in growth medium. The solution was pipetted to remove all cell clumps and then plated in additional 150mm dishes.

A2. Freezing MSCs

The following protocol was used for freezing the MSCs.

- Label vials with date, cell type, initials.
- Trypsinize, centrifuge cells as explained previously to get a cell pellet.
- Re-suspend cell pellet in non-supplemented, cold culture media, using half the final volume. Keep on ice.
- Once the cells were resuspended, 10µL was used and placed in the haemocytometer for counting. Average cells per square were counted.
- Then cold Supplemented Media (40% FBS, 20% sterile DMSO - Dimethyl Sulphoxide) was slowly added drop-by-drop until the desired volume was obtained.
- Aliquot into freezing vials and cap by keeping on ice for 30 minutes.
- The vials were transferred into Mr. Frosty.
- Mr. Frosty was stored in -80^{o}C freezer.
- After freezing overnight, the vials were transferred to liquid Nitrogen for long term storage.
- Each plate was frozen in 2 vials to yield about 1.5 x 10^{6} cells per vial.

8 plates of confluent rat MSCs were resuspended in 200μL of media. It was then added to precut Duragen scaffolds in a 24-well dish and allowed to sit for 2 hours. Then 1ml of growth media was added.

B. Evaluation

B1. Radiomorphometry

After debriding the soft tissue, harvested tissues were placed in 4% para formaldehyde. Radiographs were obtained using table-top micro-CT scanner (Skyscan, Belgium) to demonstrate defect size and the amount of mineralized bone within the defects. The bone specimens were gently rinsed with water. The periosteoum was cut with a #15 blade or diamond disc to leave about 2mm of the surrounding bone intact. The specimens were positioned on a holder in the digital image receptor at right angles to the source of the beam and exposed to 66kVp, 8mA for 0.06 seconds. The images were reconstructed in 3 dimensions. Densitometric tracing of the radiographs was used to estimate bone fill. Mean grey values (MGV) was calculated for each radiograph using the Image J 1.37 for Windows software. The range of values noted was between 0 (Black) and 255 (White). Denser bone would look darker.

B2. Histomorphometry

All excised bone samples were decalcified using EDTA and bisected along a line parallel to the sagittal suture. Two sections were made along the same plane for all

specimens to enable accurate comparison of the groups. The specimens were processed for regular histology. All paraffin-embedded specimens were sent to NC State University College of Veterinarian Medicine where they were sectioned at 5μm and stained with hematoxylin and eosin (H&E) and Mason's Trichrome. The total defect and bone areas were measured using the Image J 1.37 software. Those two parameters excluding the area occupied by voids were measured in each of the two histological sections in $mm^{2.}$ The bone fill estimates were calculated as a percentage of the total defect area. The estimates for each of the two sections were averaged for an individual specimen. Group means and standard deviations were calculated for the percentage of defect area occupied by bone in the microradiographs and the histological sections.

Mason's Trichrome histochemical staining was also carried out on all the slides for identifying areas of osteoid production within the critical size calvarial defects.

Two evaluators were calibrated for measurement of bone fill using both micro CT and histology. However, the measurement of bone in histological sections was carried out by one evaluator only and consensus was obtained regarding certain questionable areas of new bone formation.

CHAPTER 4

RESULTS

A. Radiography

After the bone specimens were fixed with 4% paraformaldehyde solution, radiographs were taken using the Micro CT scanner (Skyscan, Belgium). The Mean Gray Values (MGV) obtained using the measurements from Image J 1.37 were noted for each specimen.

Descriptive statistics was done using SPSS and as represented in Table 4.1., the average MGV and standard deviation measurements were noted for the four treatment groups.

Groups	N	Average MGV	Standard deviation
Empty	6	217	13.45
Matrix + Saline	4	192.92	32.64
Matrix + MSCs	6	168.74	7.67
Matrix + BMP	8	149.46	20.99

Table 4.1. Average MGV and Standard deviation for the four treatment groups obtained by Image J measurements of Micro CT radiographs

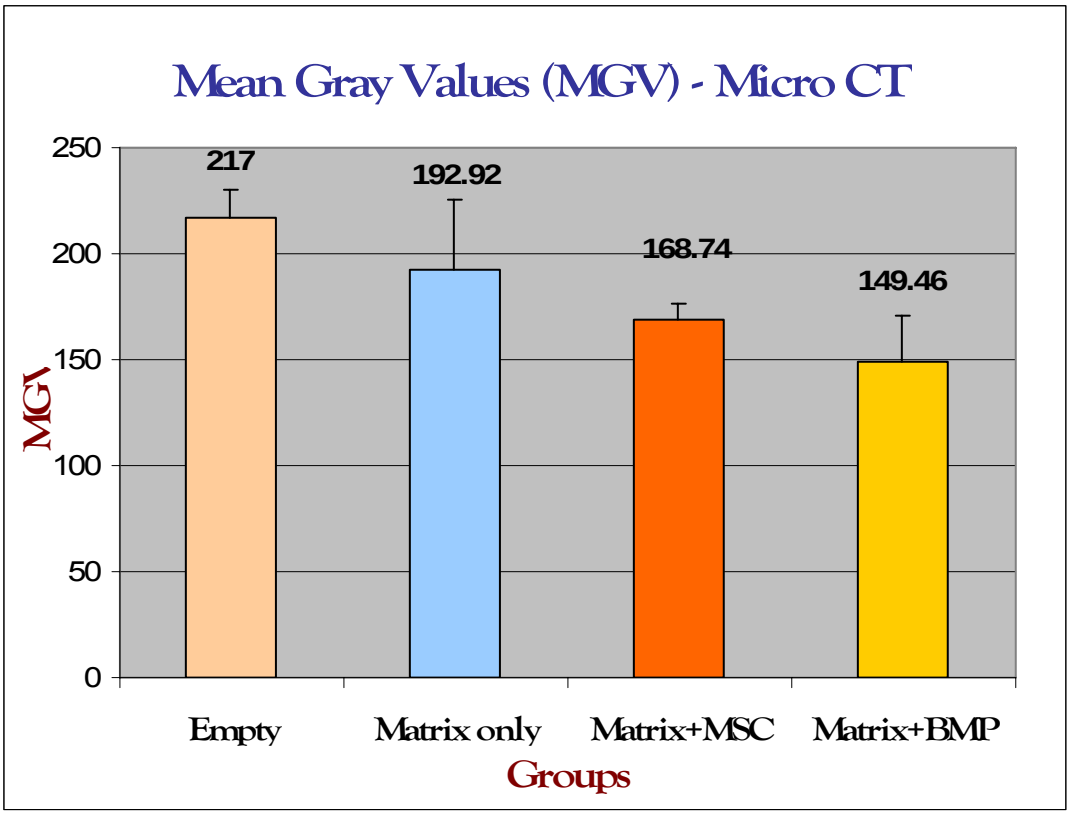

Figure 4.1. Bar graphs comparing average MGVs and standard deviation for the four treatment groups

The above bar graph (Figure 4.1.) displays the average of mean gray values measured using the micro CT radiographs for each of the four treatment groups. The higher mean gray value correlates to lesser density of bone. Accordingly, the empty and DuraGen® matrix groups showed significantly less density of bone formation when compared to the groups with MSCs and rhBMP-2 along with DuraGen® matrix.

Some representative micro CT images of each of the groups tested are included.

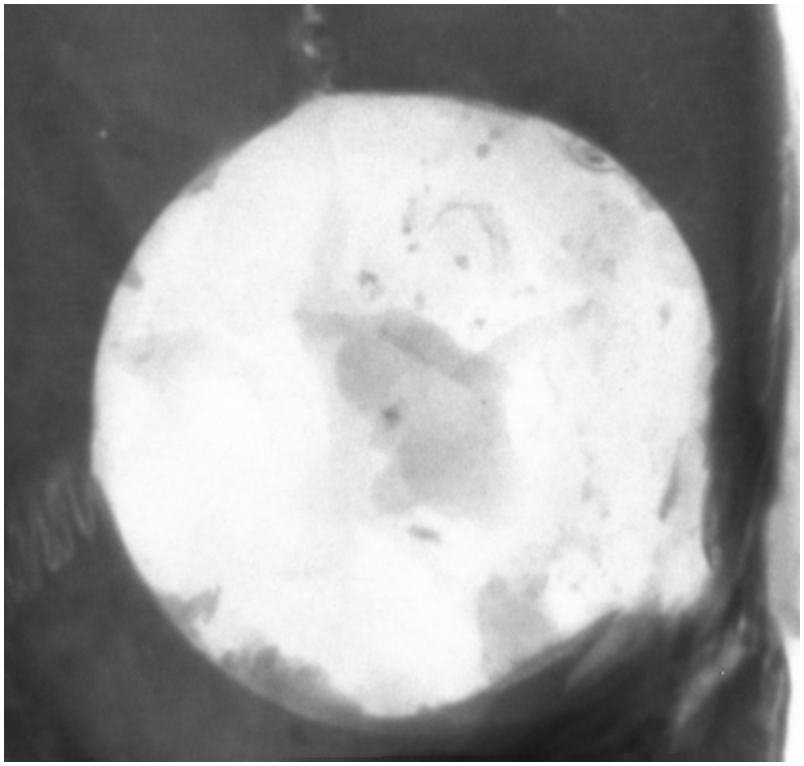

Figure 4.2. Micro CT image of Empty / negative control at 4 weeks

As seen in the radiographs (Figure 4.2.) in the empty group, there is almost no evidence of bone formation at 4 weeks. The average MGV and standard deviation for this group was calculated to be 217 and 13.45 respectively (Table 4.1.).

Radiographs of DuraGen® + saline group (Figure 4.3.) show some evidence of bone formation, especially around the periphery of the circular defect. The average MGV was shown to be 192.92 and the standard deviation was 32.64 (Table 4.1.).

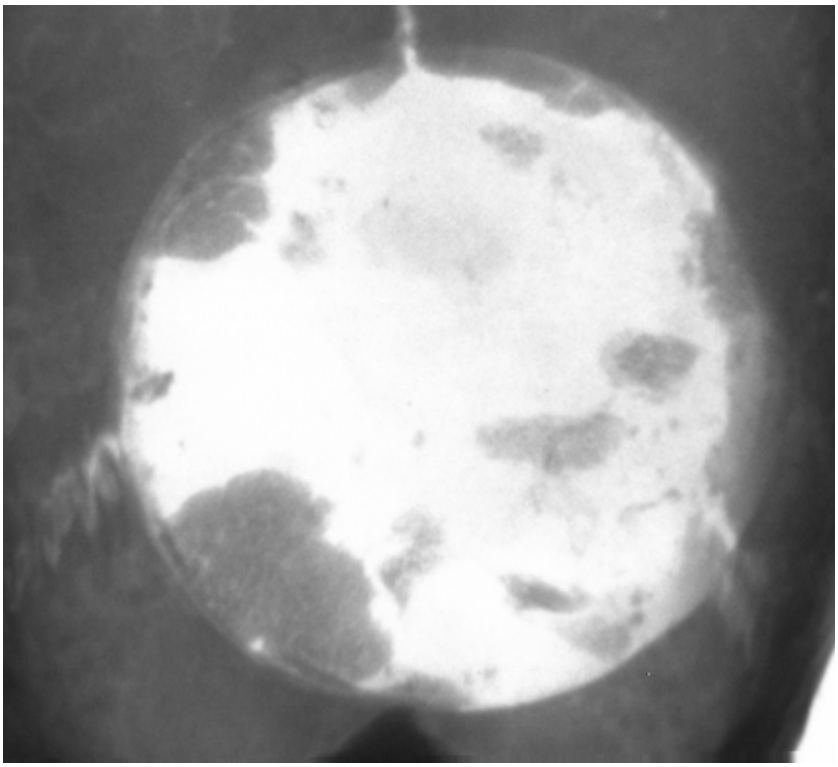

Figure 4.3. Micro CT image of DuraGen® + saline at 4 weeks

However, in the group implanted with DuraGen® and MSCs (Figure 4.4.), there is definite evidence of bone formation both peripherally and centrally in the critical size cranial defect. The amount and density of bone formation seems very similar to that seen in rhBMP-2 group, if not better. The average MGV and standard deviation values for this group were 168.74 and 7.67 respectively (Table 4.1.).

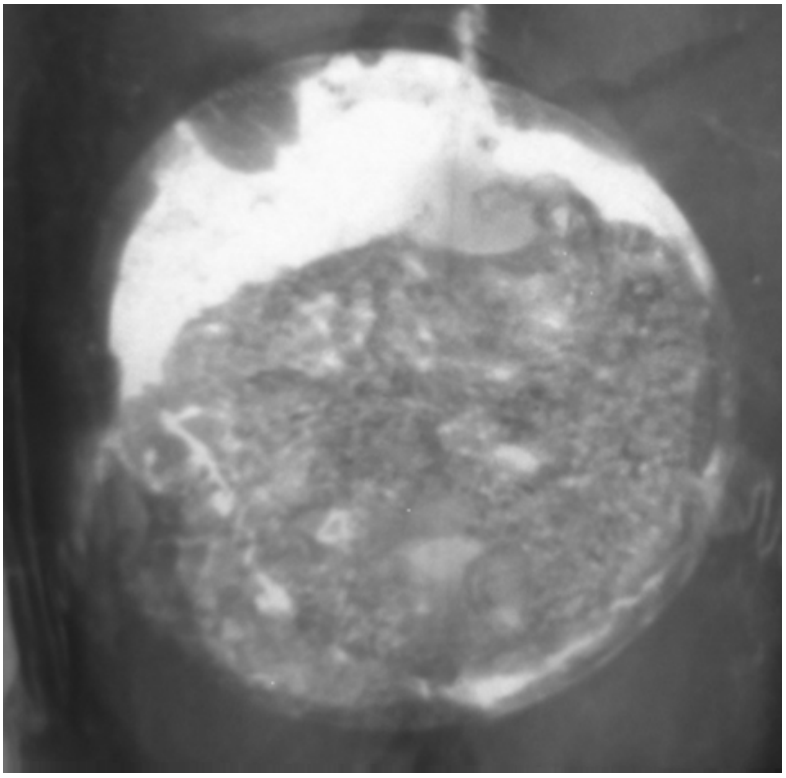

Figure 4.4. Micro CT image of DuraGen® + MSCs at 4 weeks

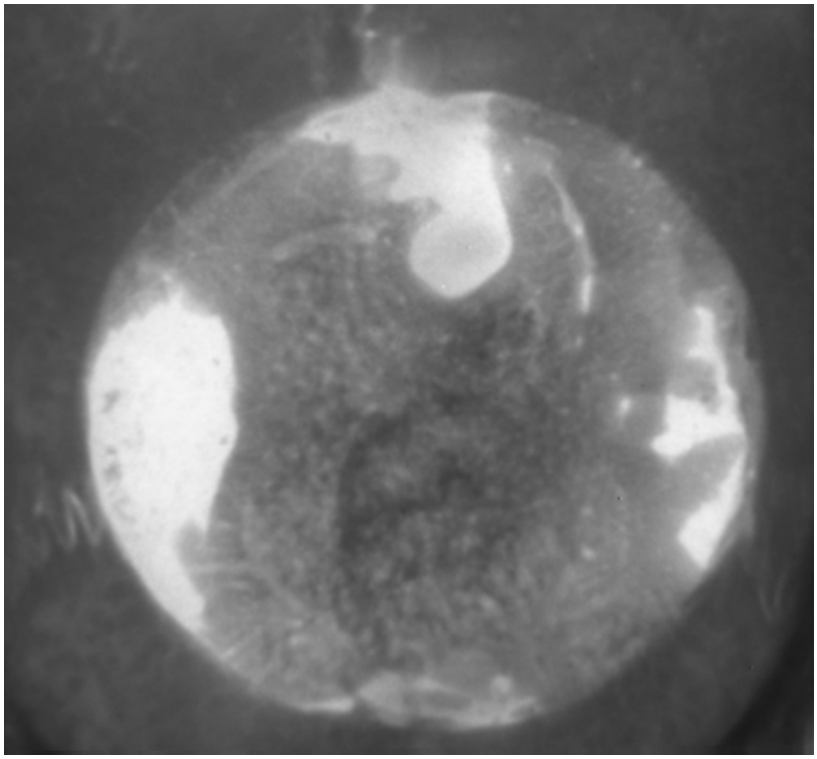

Figure 4.5. Micro CT image of DuraGen® + BMP / positive control at 4 weeks

As expected, the DuraGen® + BMP group, which was our positive control had the highest density of new bone. The average MGV for this group was 149.46 and the standard deviation was 20.99 (Table 4.1.).

B. Histology

Table 4.2. and Figure 4.6. summarize the descriptive statistics for percentage of bone fill measured using the H&E histological sections for each specimen of the four treatment groups.

Groups	N	Mean bone fill (%)	Standard deviation
Empty	6	9.25	10.82
Matrix + Saline	4	19.07	17.38
Matrix + MSCs	6	44.21	3.93
Matrix + BMP	8	60.06	15.08

Table 4.2. Mean bone-fill (in percent) and standard deviation for the four treatment groups obtained by Image J measurements of the Histological images

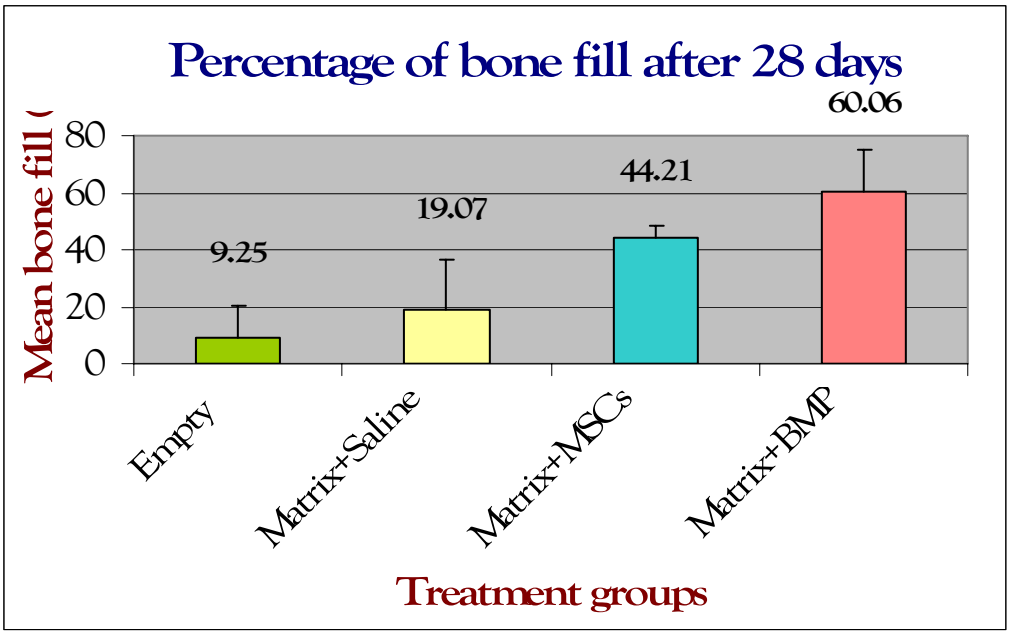

Figure 4.6. Bar graphs comparing the mean bone-fill (in percent) and standard deviation for the four treatment groups

The empty and DuraGen® + saline groups had only 6 animals of which one rat died after intraperitoneal injection of ketamine/xialzine (40-80mg/kg; 2-10mg/kg) just before the surgery was completed. 8 animals were surgically operated for the rhBMP-2 group. Although all 8 animals in the MSC group survived, two of them had severe inflammation at the surgical site which was evident during the histological evaluation. Hence, values obtained from them were discarded for statistical analysis.

Two representative photographs of the histological sections for each group are included to demonstrate bone regeneration at four weeks.

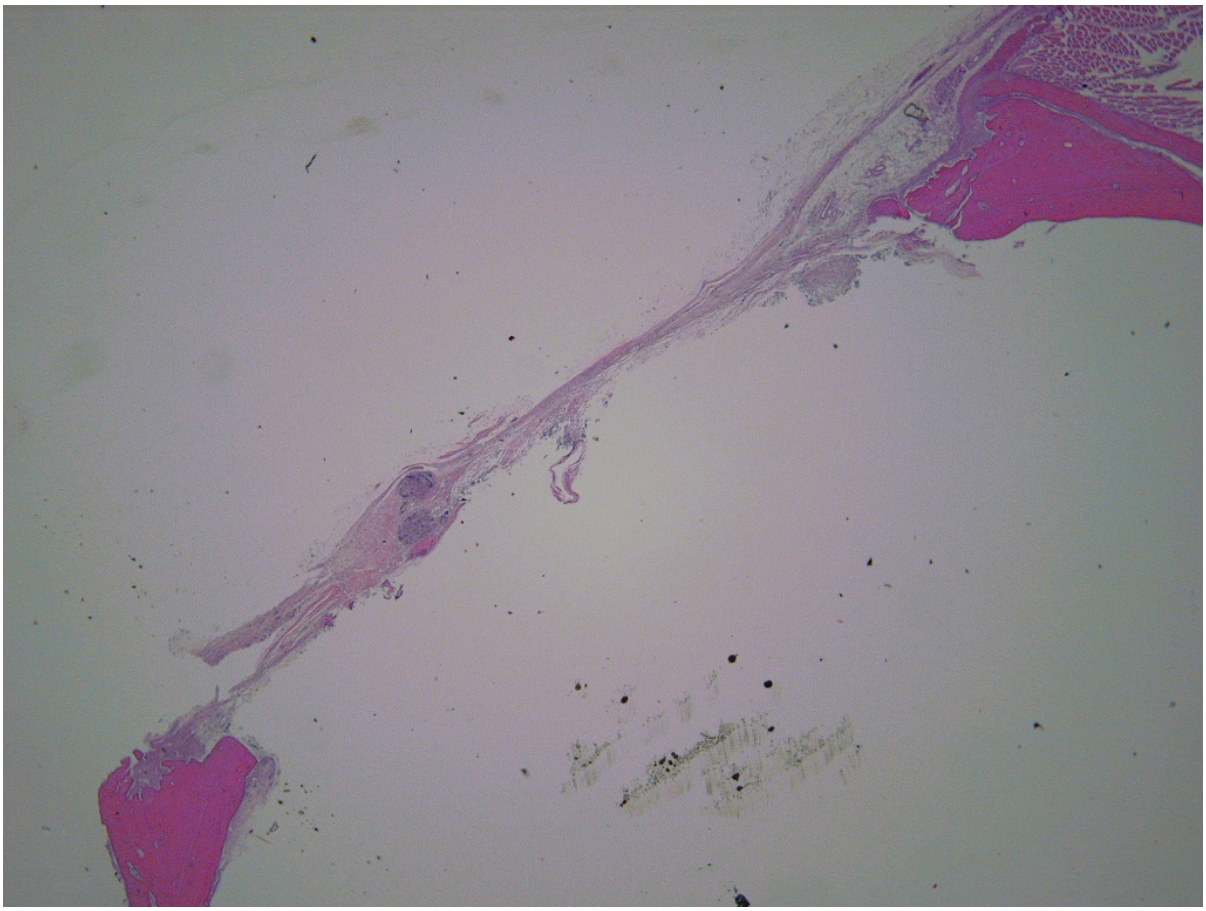

Figure 4.7. H and E image of Empty / negative control at 4 weeks

The specimens in empty group which was used as the negative control in our study showed very little evidence of osteoid formation. The mean bone fill was calculated to be 9.25% (<10%) and the standard deviation was 10.82 (Table 4.2. and Figure 4.6.).

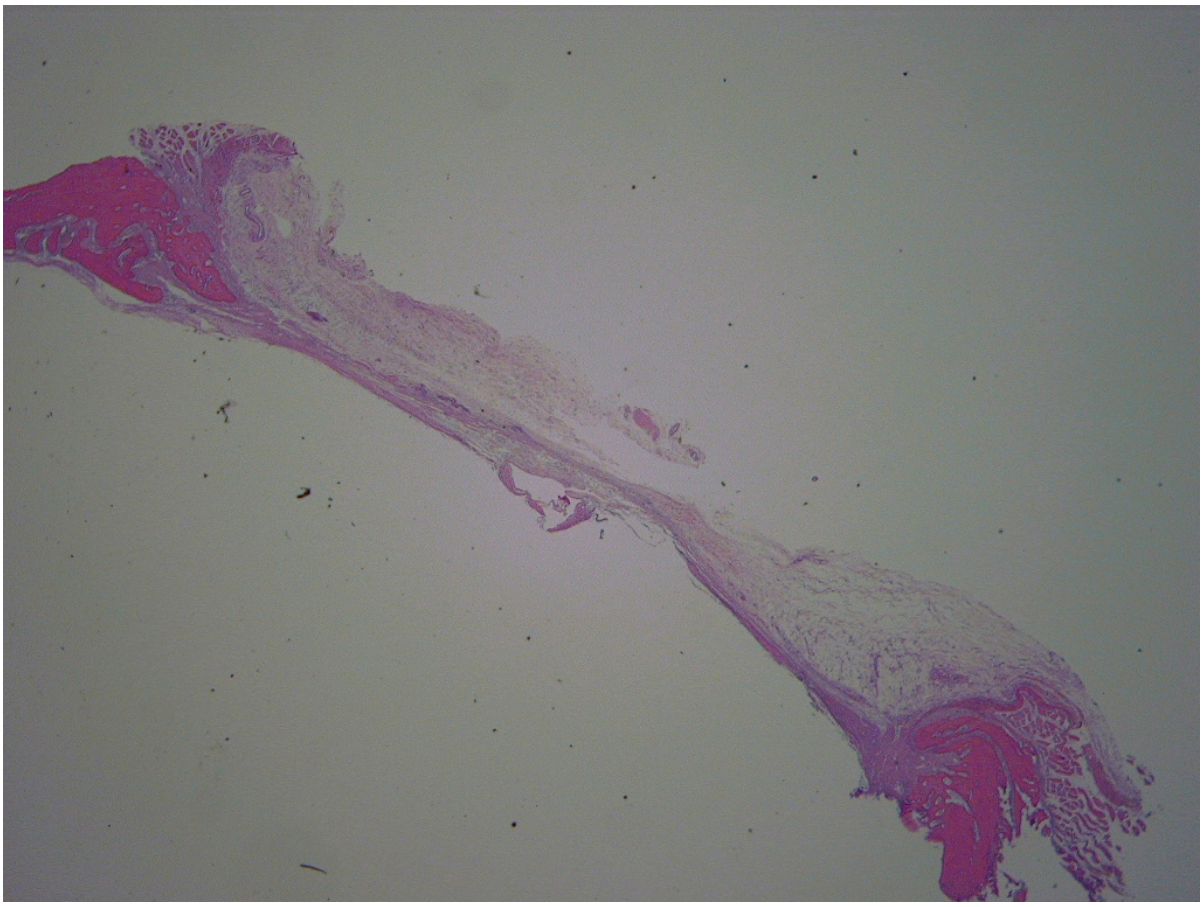

Figure 4.8. H and E image of Empty / negative control at 4 weeks

Figures 4.7. and 4.8. are low-magnification pictures of H & E sections showing minimal bone formation within the defect. Few suture fragments can be observed in the middle of the graft.

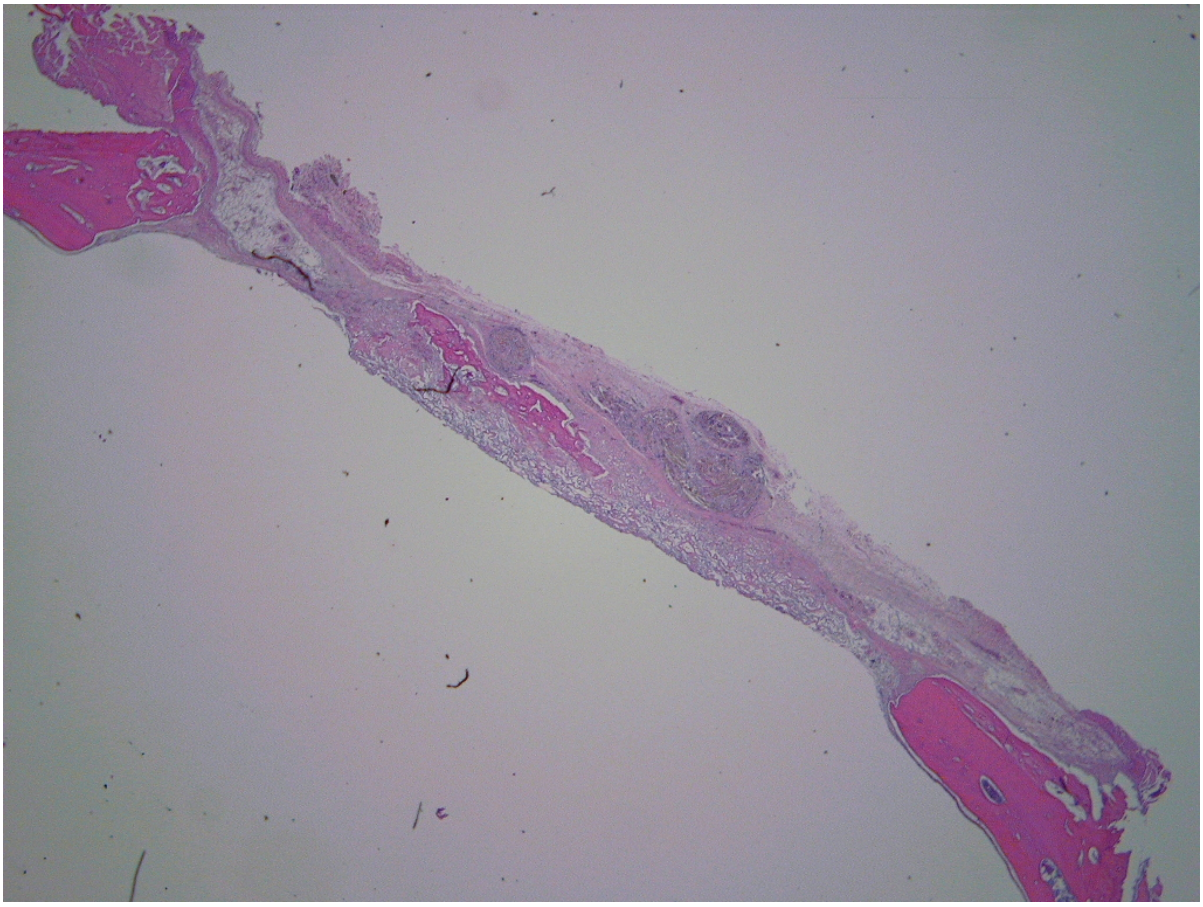

Figure 4.9. H and E image of DuraGen® + saline at 4 weeks

The DuraGen® + saline group, which is represented by Figures 4.9. and 4.10. in low-magnification photographs of H & E stained sections, there is some evidence of bone formation. The mean bone fill for this group was calculated to be 19.07% & while the standard deviation was 17.38 (Table 4.2.).

Note the graft material is harboring osteoid and bone formation in the center of the graft. Suture material is also seen at the superficial aspect of the wound. No

significant inflammation is seen. However, osteoid formation did not cover the entire length of the defect.

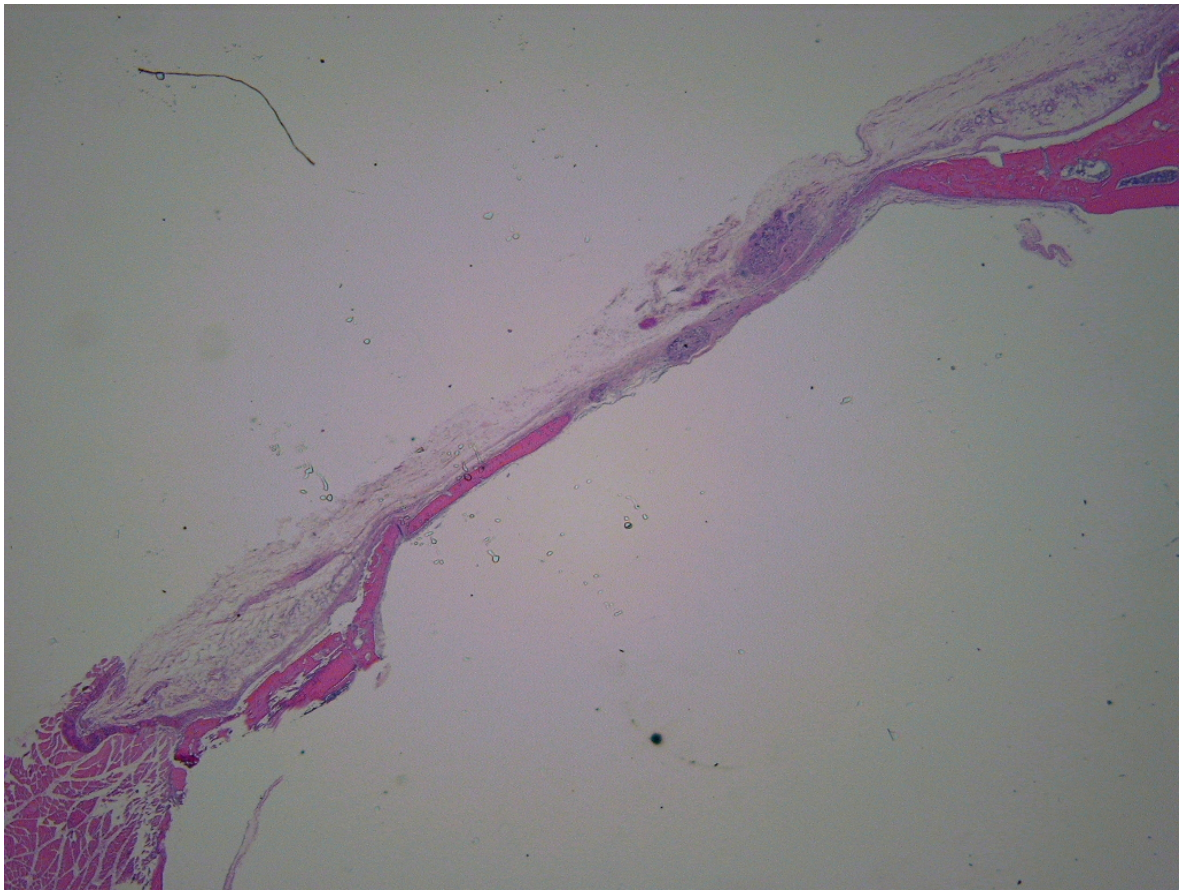

Figure 4.10. Hand E image of DuraGen® + saline at 4 weeks

In the DuraGen® with MSC group, bone formation was seen along the entire length of the cranial defect at 4 weeks. It seems to be consistent in all the specimens. The mean bone fill for this group was measured to be 44.21% and the standard deviation was 3.93 (Table 4.2.).

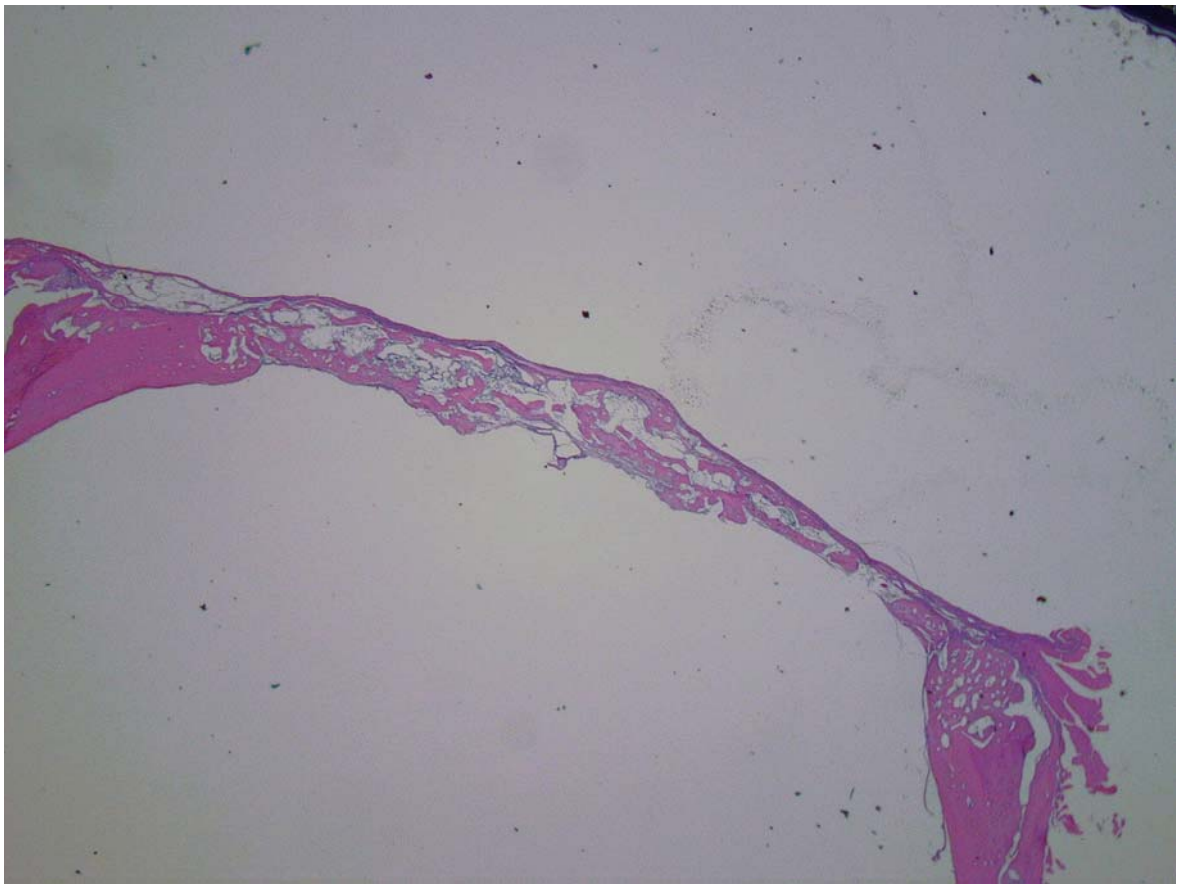

Figure 4.11. H and E image of DuraGen® + MSCs at 4 weeks

Low-magnification photographs of the histological sections of DuraGen® + MSCs group (Figures 4.11. and 4.12.) show there is complete osteoconduction of the defect by the graft. The density and thickness of the bone are slightly less than the

native bone at the defect's edge. Some adipose tissue was also present within the graft. Significant bone formation within the critical size defect was present.

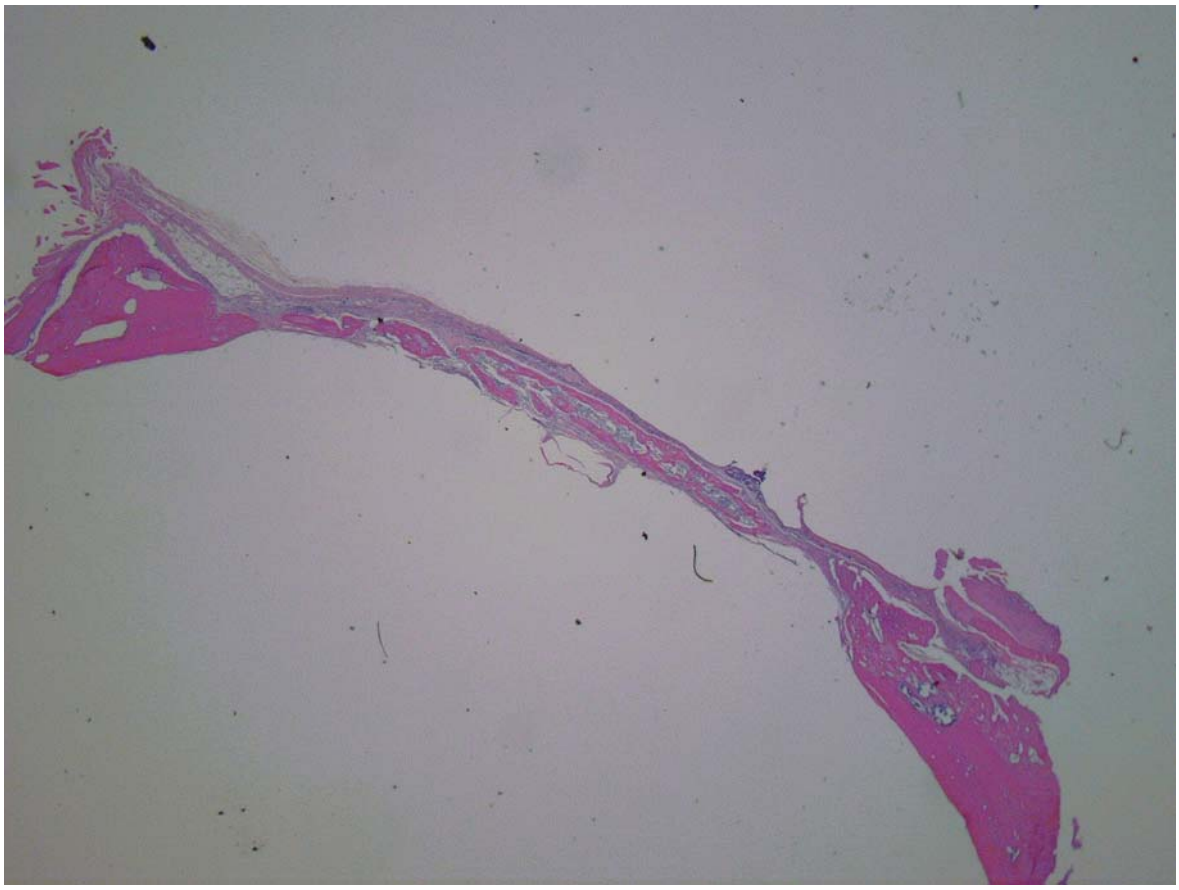

Figure 4.12. H and E image of DuraGen® + MSCs at 4 weeks

Similarly, in the Duragen with rhBMP-2 group, osteoid formation was quite dense and extended all along the length of the defect. Mean bone fill and standard deviation values for this group were 60.06% and 15.08 respectively (Table 4.2.).

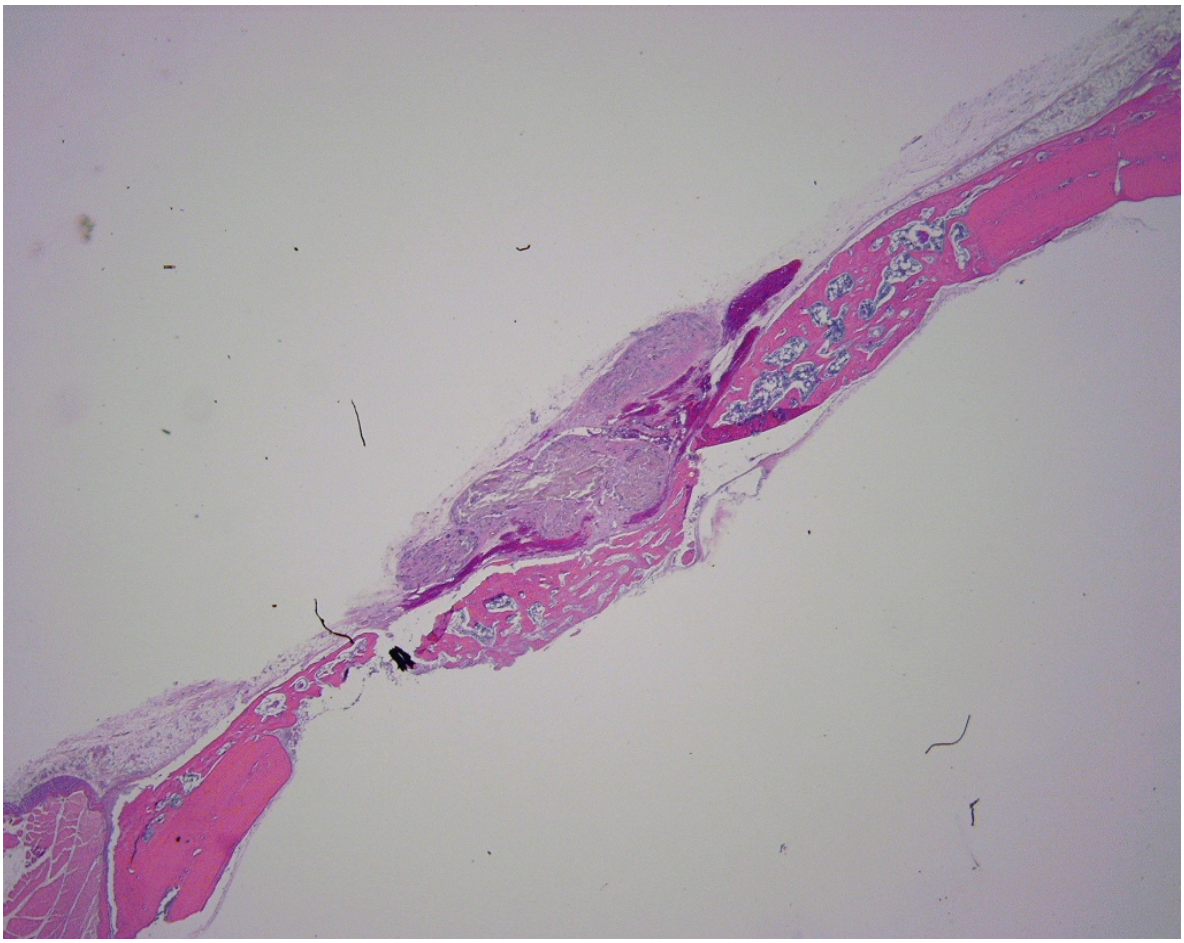

Figure 4.13. H and E image of DuraGen® + BMP-2 at 4 weeks

Low-magnification images of H&E section (Figures 4.13. and 4.14.) on the matrix and rhBMP-2 group showed complete osteoconduction and healing of the critical size defect. Note the focal lamellar architecture of the new bone. The thickness and density of the bone resembles that of the adjacent native bone. Suture material is also present at the superficial aspect of the wound. No significant inflammation was observed.

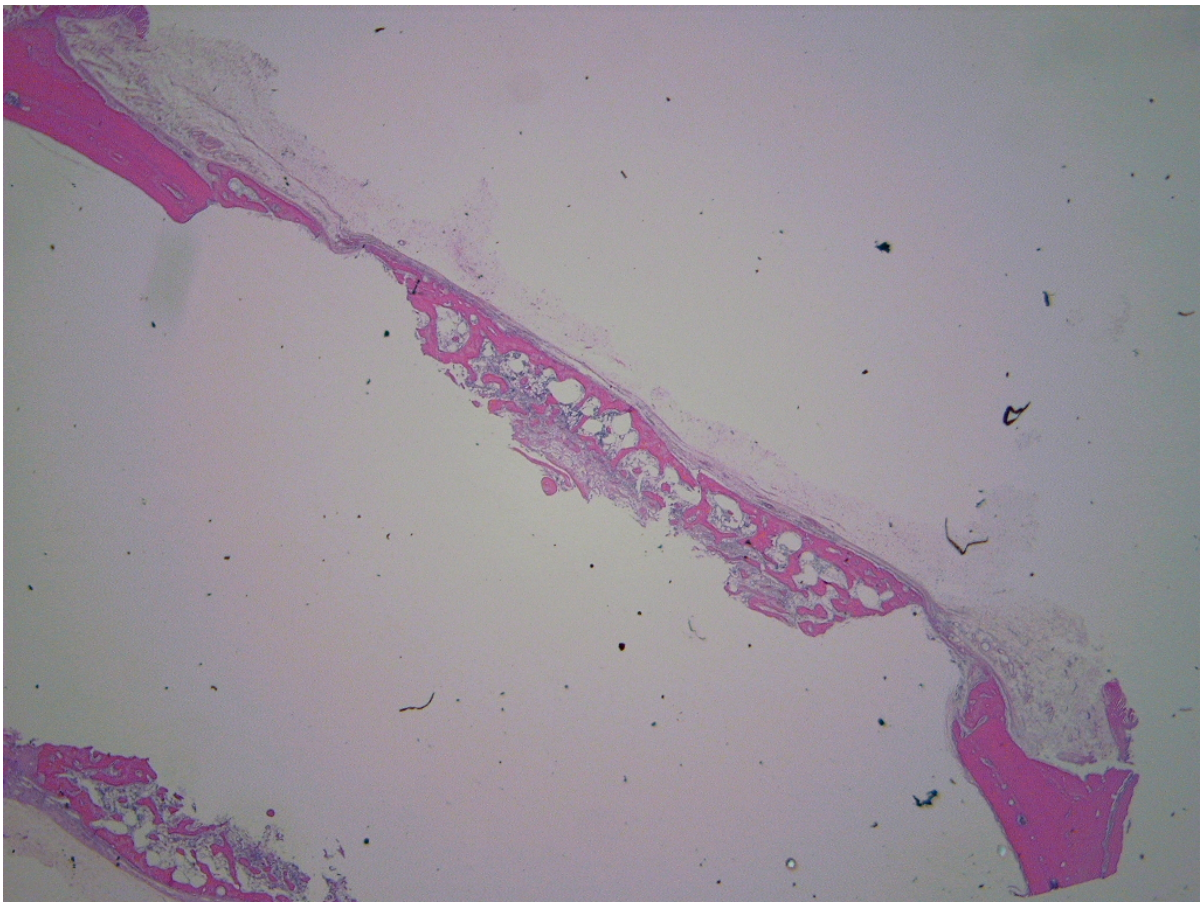

Figure 4.14. Histological image of DuraGen® + BMP-2 at 4 weeks

C. Statistical Analysis

Using SPSS 14 version, one-way ANOVA was used to evaluate the differences in bone fill among the treatment groups. When the F ratio was found to be significant, Tukey HSD test for multiple comparisons was done to determine the statistically significant differences between the groups. The Type 1 probability error (α) was set at 0.05.

C1. Radiomorphometry

Group	N	Mean	Standard Deviation	Groups compared	p-value
Empty	6	217	13.45	Matrix + Saline Matrix + MSC Matrix + BMP	0.248 0.002* 0.000*
Matrix + Saline	4	192.92	32.64	Matrix + MSC Matrix + BMP	0.245 0.008*
Matrix + MSCs	6	168.74	7.67	Matrix + BMP	0.282
Matrix + BMP	8	60.06	20.99	-	-

* Significant $p<0.05$

Table 4.3. Summary results of Tukey HSD multiple comparison test using radiology data

SPSS 14 was used to analyze the radiology and histology data. Densitometric tracings using Image J 1.37 quantified the mean gray values (MGV) for each specimen. Table 4.3. summarizes the results of one-way ANOVA and Tukey's multiple comparison test. One way ANOVA revealed significant difference between the groups (Appendix A). When the F statistic was found to be significant, Tukey's test for multiple comparisons was done to compare the individual groups. Radiological data analysis showed that the density of bone regeneration was significantly different in the empty group when compared with MSC and BMP groups. However, no significant difference was found between the empty and DuraGen® groups. Although bone fill in DuraGen® group was significantly different from BMP group, it was not different when compared to the MSC group. The BMP and MSC groups were not significantly different in this analysis.

C2. Histomorphometry

Group	N	Mean (%)	Standard Deviation	Groups compared	p-value
Empty	6	9.25	10.82	Matrix + Saline Matrix + MSC Matrix + BMP	0.628 0.001* 0.000*
Matrix + Saline	4	19.07	17.38	Matrix + MSC Matrix + BMP	0.027* 0.000*
Matrix + MSC	6	44.21	3.93	Matrix + BMP	0.123
Matrix + BMP	8	60.06	15.08	-	-

* Significant $p<0.05$

Table 4.4. Summary results of Tukey HSD multiple comparison test using histology data

Using Image J 1.37, the bone forming areas were delineated and measured by an Oral Pathologist. Mean bone fill was calculated as a percentage of the total area of the defect. Group means and standard deviations are also included in Table 4.4. The first two specimens in the MSC group showed severe inflammation and it was clear that the grafts had failed. Hence those two specimens were excluded from the analysis. One of the specimens in the DuraGen® + saline group had to be excluded at the time of histological evaluation due to labeling error. ANOVA showed statistically significant differences between the groups. Then Tukey HSD multiple comparison test was performed which demonstrated mostly similar results as that for micro CT except that in histology, significant difference was found between saline and MSC groups. However, histological analysis confirmed that the bone

regeneration in MSC group was not significantly different from BMP group which was the positive control in this study.

CHAPTER 5

DISCUSSION

A. Validity of the experimental design

The rat model which was employed in our project is a convenient, inexpensive model that is very commonly used in several in vivo studies. The cranium was used as the site of our experiments due to its natural lack of nutrient artery. The rat cranium is a more suitable site than the long bone defects due to the relative lack of motion and elimination of the need for fixation (Wang J, 1999). A critical- size defect in such a site would mean unfavorable healing response. In contrast to non-unions which could result in inadequate bony or fibrous healing, a CSD would not heal by bone regeneration during the life time of the animal (Hollinger JO, 1990). Hence this makes a sound experimental design for in vivo evaluation of bone regeneration materials.

Rats have different bone remodeling patterns compared to humans with a higher bone turnover rate. But if the differences are accounted for while interpreting the data, the rat model seems excellent for initial testing of bone grafting materials (Whitfield JF, 1998).

B. DuraGen®

DuraGen® was used as matrix or carrier for MSCs and rhBMP-2 in our study in the form of pre-cut discs. DuraGen® was introduced in the market as a fully resorbable collagen matrix for primary dural closure. The collagen matrix has shown to be successful in cerebrospinal fluid containment in a vast majority of cases studied by Narotam and colleagues in 2004. It has been demonstrated to support survival of cerebral cortical neurons in vitro (Rabinowitz L, 2005). Danish and others compared the performance and complications of DuraGen® with AlloDerm as alternatives for duraplasty. The study concluded that both materials could be safe alternatives with DuraGen® requiring significantly shorter operative time since sutures are not necessary (Danish SF, 2006).

With the rat cranium being the site of critical size defect, DuraGen® seems like an appropriate choice to not only promote dural wound healing, but also to act as a vehicle for delivering growth factors and cells to the defect area. It has adequate tensile strength and porosity required to carry cells and/or growth factors to the surgical site. According to the manufacturer, DuraGen® is made from bovine achilles tendon which is known to be one of the purest sources of type I collagen. Several studies have observed the binding capacity of demineralized bone matrix (DBM) to osteoinductive growth factors of the TGF family such as BMPs (Zhao Y, 2008). DBM typically consists of 99% type I collagen. Sweeney and colleagues found in a similar study that animals treated with type I collagen gels alone showed bone repair of 92.5% at 20 weeks (Sweeney TM, 1995). Saadeh, 2001 also

demonstrated the ability of type I collagen to heal critical sized bony defects in rat mandible. He suggested that type I collagen would make a suitable carrier matrix for improved approaches to bone tissue engineering.

Bone matrix comprises predominantly of collagen type I which is thought to be a natural polymer. This kind of collagen can be extracted from various animal or human sources. Decalcification, purification, sterilization and modification may be some of the steps involved in extraction of commercial collagen (Wahl DA, 2006). Collagen and calcium phosphate as composites and individually have been found to increase osteogenesis. The rationale for using the composites to form bone substitute is that the collagen matrix will act as a medium in which the rigid hydroxyapatite crystals deposit and crosslink the fibers to form calcium ion bridges (Hellmich CH, 2002). However, the mechanical properties of these composites have been found to be lower than that of natural bone (Wahl DA, 2006).

B1. BMPs

The rhBMP-2 implanted on DuraGen® served as the 'positive control' or the 'gold standard' for bone regeneration in our experiments. It has been well-documented that rhBMP-2 has the potency to induce dose-dependent bone formation in animals and humans. However in therapeutic concentrations, the BMPs have a very short half life and are rapidly diffused by the body fluids. Chen 2007 developed a collagen-based BMP-2 targeting bone repair system and tested its efficacy in a rabbit mandibular CSD model. They concluded that it induced 'better and

homologous bone formation' possibly due to enhanced retention of BMP-2 by collagen fibrils (Chen B, 2007). The results of our study also validate this finding since the bone formed in the collagen (DuraGen®) and rhBMP-2 group was better in quantity and density when compared with the empty and collagen only groups. Although the mean bone formation for the rhBMP-2 group was 60.06%, the high variance as indicated by the standard deviation of 15.08 could lead us to speculate on the predictability of bone formation using rhBMP-2.

B2. MSCs

The multipotent mesenchymal stem cells, derived from rat bone marrow have exhibited their ability to proliferate extensively and differentiate into tissue-specific cell lineages. In an in vitro study, a compatible commercial collagen scaffold was found to possess the ability to support rat MSC osteogenic differentiation. Expression of osteopontin and osteocalcin, both proteins essential for Calcium binding and alkaline phosphatase actiivity were considered as specific markers for osteogenic differentiation (Donzelli E, 2006). The relative ease of obtaining these stem cells and the potential to expand them in vitro make them an attractive alternative for bone regeneration. In our study, we tried to combine the osseoconductive properties of the collagen matrix with the osseoinductive properties of the mesenchymal stem cells and evaluated them for bone regenerative capability in vivo.

C. Results

C1. Radiomorphometry

Radiographical assessment of bone formation was done using the Micro CT data by calculating the Mean Gray Values (MGV). This analysis served dual purposes. Firstly, to compare the accuracy of the radiomorphometry results with histology which is currently the 'gold standard' for evaluating bone formation. Secondly, a 3D reconstruction would allow us to visualize the cranial defect from axial view which is not possible in histological sections.

A significant advantage of radiomorphometry is that it can be done as early as 24 hours after surgical removal of the osteotomy defects. This analysis can provide preliminary results quite efficiently whereas the preparation of slides for histology can take up to a few months (Hollinger JO, 1990).

Radiography using a table-top system can inherently induce a discrepancy in the observed mean gray values and local bone density which has been related to the effect of beam hardening. However, this error is assumed to be similar for all specimens since they were comparably of the same size and composition. (Verna C, 2002)

In our study, the results of radiolographical assessment correspond fairly well with those of histology. The only variation using radiography was lack of significant

difference between DuraGen® + saline and DuraGen® + MSCs groups which was found in histological findings.

Many authors have ratified the use of micro CT data as a valid tool for 3D assessment of bone structure for preclinical trials, yet they recommend the use of histology for more conclusive results (Verna C, 2002; Engelke K, 1999).

C2. Histomorphometry

On histological examination, two rats in the DuraGen® + MSCs group which were the first to be operated on showed severe inflammation. Contamination could have occurred at any of the several steps involved in the study such as culture of MSCs, during or after surgery. The bone fill estimates for these two specimens were considered as outliers and they were not included in the statistical analysis.

During bone removal, the surrounding periosteum was maintained intact to delineate the defect site from newly formed bone. The area of the CSD between the margins of the periosteum was outlined and the area of newly formed bone was calculated as a percentage of the total area of CSD.

Histological measurements confirmed the findings from radiographical assessments. The results showed that bone fill in DuraGen® + MSC group was not significantly different from that in DuraGen® + BMP group. However, the density of bone in rhBMP-2 group seemed higher. The most significant finding from our study was the

consistency with which the DuraGen® + MSCs group made new bone. This was demonstrated by the narrow range of measurements (standard deviation: 3.93) for the MSC group.

Additional type of staining called the Mason's Trichrome was done to detect the areas of osteoid formation.

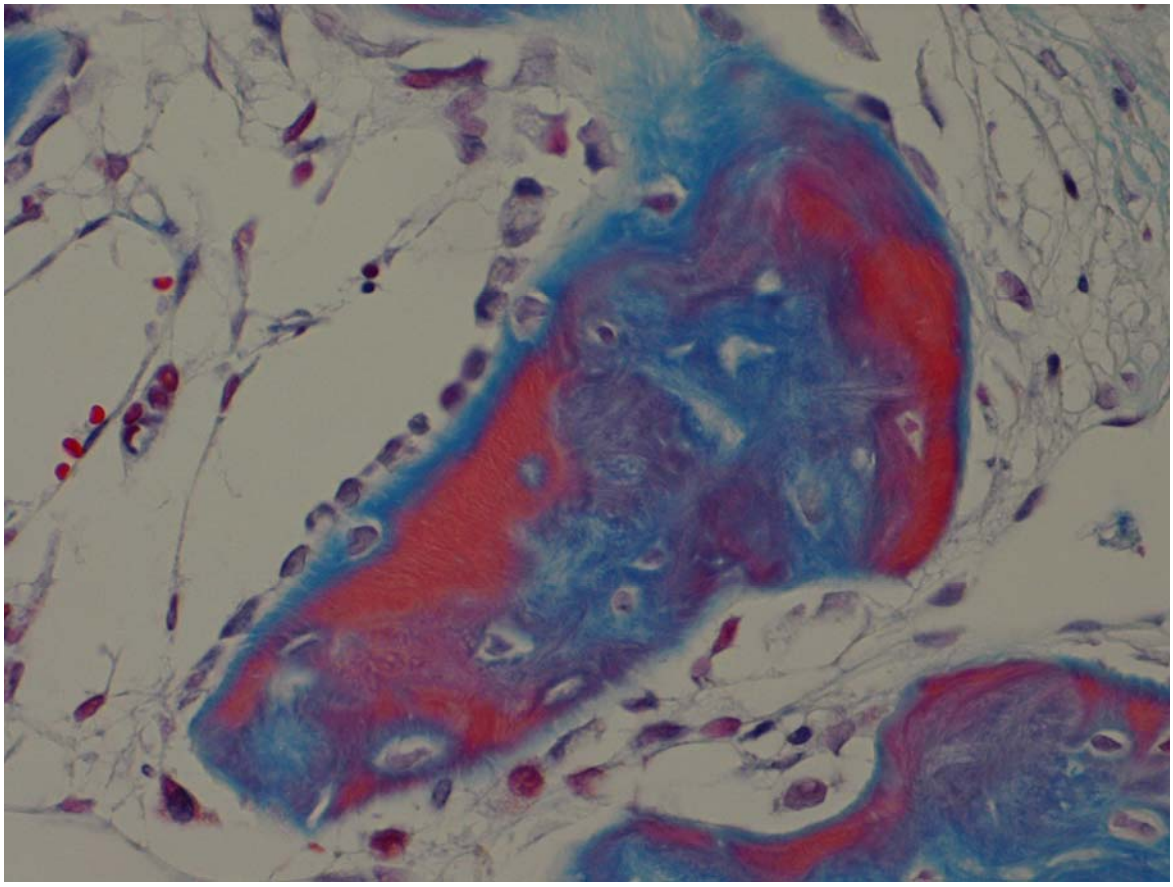

Figure 5.1. Mason's Trichrome histochemical staining photograph showing osteoblasts and osteoid formation

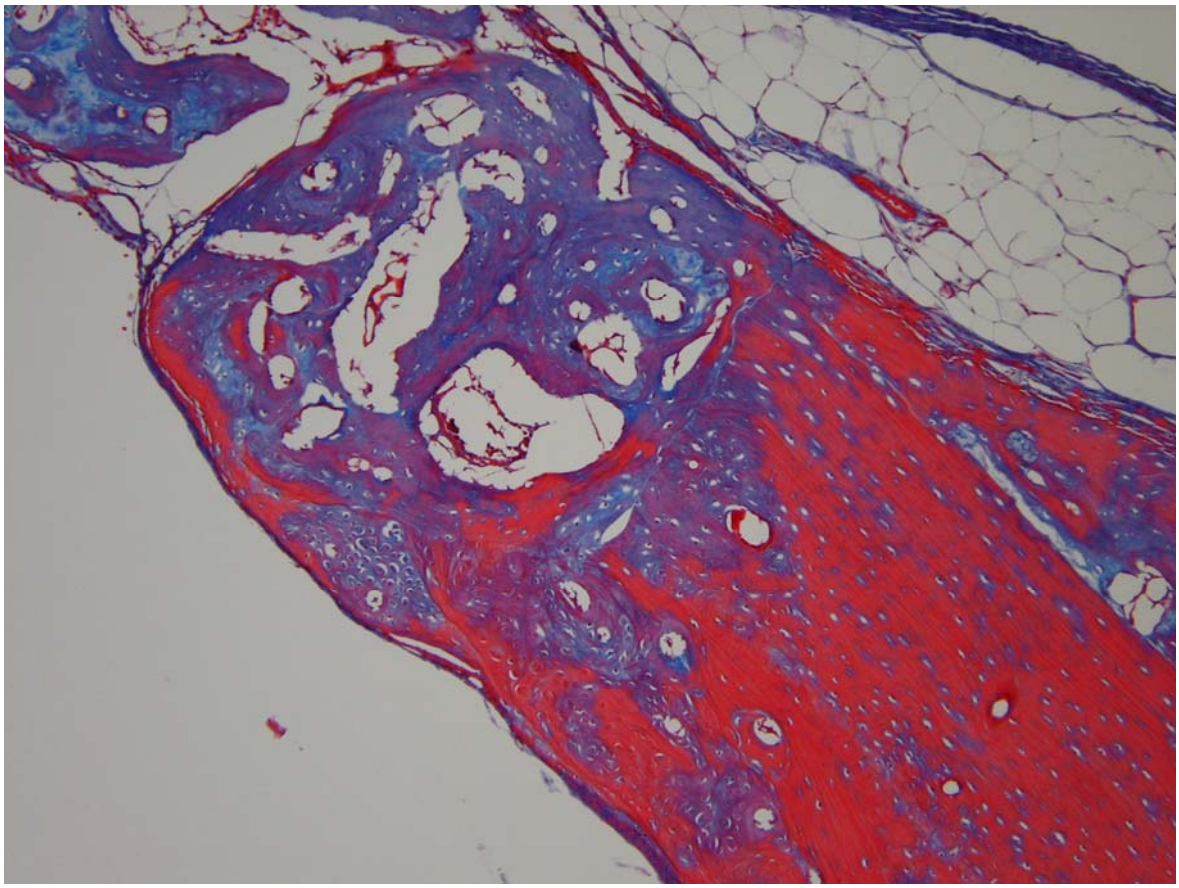

Figure 5.2. Mason's Trichrome histochemical staining photograph showing the border between intact periosteum and new bone formed at the defect site

Figure 5.1. demonstrates a photograph of the Mason's Trichrome stained slide. The lining of cells seen in the photograph are the osteoblasts and the blue layer adjacent to it denotes the osteoid tissue. The light blue areas represent unmineralized bone matrix while the pink areas indicate more mineralized bone. Figure 5.2. shows the border between the intact periosteum represented by pink and newly formed bone at the defect site which is seen in blue.

D. Advantages of the technique

Duragen has been shown to be a biocompatible, safe material which aids in accelerated wound healing. Clinical evidence suggests this material is immunologically well tolerated (Nartoram PK, 1995). Duragen is easy to handle, non-friable and resorbable onlay graft material. It conforms to surfaces like normal soft tissue. Its porosity helps deliver the implant material effectively to the site of defect.

The main advantage of using this scaffold seeded with MSCs could be its cost effectiveness especially in comparison with the gold standard in our study, rhBMP-2.

E. Limitations of the study

Resorption of the collagen scaffold prior to completion of bone formation may lead to incomplete bone fill. Though this may not be a problem with rat MSCs because of their ability to regenerate bone much faster, this could be a potential problem to consider while doing clinical studies using adult human MSCs.

The small sample size could be another limitation of this study. Similar results from a bigger sample size would be more valuable.

Delineation between the intact periosteum and new bone formation was not clear in the radiographs and sometimes also in histological specimens. Although standardized measurement criteria were applied for all specimens, there could be

some under or over estimation of bone fill. This error could be more pronounced in radiographical assessments.

Another variable which could affect the bone healing response in the rat calvaria is the surgical technique. Despite using a standard protocol, differences in the experience level of the surgeons could to some extent affect the outcomes.

Further validation of the results from this study needs to be done to evaluate the ability of DuraGen® matrix to support osteodifferentiation of human MSCs.

F. Potential mechanism of action

Osteoinduction involves recruitment and stimulation of multipotent stem cells to differentiate into osteogenic cell lineage. The intact periosteum (Agata H, 2007) and dura have been shown to positively influence bone healing by many studies. (Hobar PC, 1993; Wang J, 1999). The periosteum plays a role by providing osteogenic and angiogenic cells to the healing site. The preexisting osteoblasts at the defect site produce growth factors that induce osteogenic cells to differentiate into bone. But, it is well known that less than 10% of a critical sized defect can be repaired by these natural processes of bone healing (Albrektsson T, 2001; Hollinger JO, 1990).

The principle mode of action here seems to be osteoconduction. DuraGen®, the scaffold material used in our study is an excellent osteoconductive surface which permits bone growth on or through its porous surface. The collagen matrix provides

a suitable environment for the native bone cells and implanted MSCs to differentiate into osteoprogenitor cells. It enhances bone formation by providing mechanical support for osteogenic marrow stromal cells while acting as a barrier for ingrowth of nonosteogenic cells.

G. Clinical significance

The clinical consequence of this experiment is to develop a graft material to more efficiently regenerate bone in surgical wounds and bone defects that otherwise will not heal or heal slowly.

In the future, bone regeneration can be applied in cases of craniofacial tissue loss due to congenital diseases, severe injury or cancer therapy. In general, it can be applied clinically where lack of adequate bone mass can compromise or inhibit optimum treatment. In such situations, the prognosis of final treatment can be drastically improved by functional replacements of damaged or missing tissues.

Functional rehabilitation of the craniofacial structures involves recreating missing osseous structures for correction of orofacial defects as well as replacement of teeth which most often requires usage of endosseous implants. Bone regeneration in situations with extensive vertical bone loss is challenging, and if predictably done can enhance functionality and long-term stability of implant therapy.

H. Future directions

More studies need to be done using larger sample sizes in higher primates. According to Schmitz's protocol, the next step would be to use larger CSDs in a non-human primate.

Safety and immunogenicity of Duragen has already been tested in humans. But clinical application would require evaluating its biocompatibility to support osteogenesis using human MSCs.

CHAPTER 6

CONCLUSIONS

Within the limitations of this in vivo study, we can conclude that:

1. DuraGen® (type I collagen matrix) when implanted with bone marrow stromal cells clearly displays osteogenic properties comparable to that of BMP in rat critical size defects.
2. The predominant mechanism of action implicated is osseoconduction of MSCs by the collagen matrix while osseoinduction by the cells adjacent to the periosteum may be a contributing factor.
3. Further investigations are needed in larger number of CSDs in other non-human primates before clinical trials of these scaffolds using human MSCs are conducted.

APPENDICIES

A. SPSS 14 Statistical analysis of Radiography data

MicroCT

Descriptives

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Empty	6	216.9983	13.45139	5.49151	202.8820	231.1147	199.65	234.90
Dur + Saline	4	192.9200	32.63941	16.31971	140.9834	244.8566	153.96	223.66
Dur + MSCs	6	168.7383	7.67145	3.13186	160.6876	176.7890	156.21	176.65
Dur + BMP	8	149.4563	20.99194	7.42177	131.9065	167.0060	122.02	183.88
Total	24	178.4063	32.65358	6.66538	164.6179	192.1946	122.02	234.90

Test of Homogeneity of Variances

MicroCT

Levene Statistic	df1	df2	Sig.
5.583	3	20	.006

ANOVA

MicroCT

	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	17044.321	3	5681.440	15.192	.000
Within Groups	7479.579	20	373.979		
Total	24523.900	23			

Post Hoc Tests

Multiple Comparisons

Dependent Variable: MicroCT
Tukey HSD

(I) Group	(J) Group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Empty	Dur + Saline	24.07833	12.48297	.248	-10.8607	59.0174
	Dur + MSCs	48.26000(*)	11.16511	.002	17.0096	79.5104
	Dur + BMP	67.54208(*)	10.44400	.000	38.3100	96.7742
Dur + Saline	Empty	-24.07833	12.48297	.248	-59.0174	10.8607
	Dur + MSCs	24.18167	12.48297	.245	-10.7574	59.1207
	Dur + BMP	43.46375(*)	11.84239	.008	10.3177	76.6098
Dur + MSCs	Empty	-48.26000(*)	11.16511	.002	-79.5104	-17.0096
	Dur + Saline	-24.18167	12.48297	.245	-59.1207	10.7574
	Dur + BMP	19.28208	10.44400	.282	-9.9500	48.5142
Dur + BMP	Empty	-67.54208(*)	10.44400	.000	-96.7742	-38.3100
	Dur + Saline	-43.46375(*)	11.84239	.008	-76.6098	-10.3177
	Dur + MSCs	-19.28208	10.44400	.282	-48.5142	9.9500

* The mean difference is significant at the .05 level.

Homogeneous Subsets

MicroCT

Tukey HSD

Group	N	Subset for alpha = .05		
		1	2	3
Dur + BMP	8	149.4563		
Dur + MSCs	6	168.7383	168.7383	
Dur + Saline	4		192.9200	192.9200
Empty	6			216.9983
Sig.		.362	.187	.190

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 5.647.
b The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

B. SPSS 14 Statistical analysis of Histology data

Histology Bone fill

Descriptives

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
Empty	6	9.2517	10.81750	4.41623	-2.1006	20.6039	.37	23.27
Dur + Saline	4	19.0725	17.38454	8.69227	-8.5902	46.7352	3.41	43.38
Dur + MSCs	6	44.2117	3.93093	1.60480	40.0864	48.3369	39.09	48.41
Dur + BMP	8	60.0625	15.07618	5.33023	47.4585	72.6665	39.09	84.54
Total	24	36.5654	24.34177	4.96874	26.2868	46.8440	.37	84.54

Test of Homogeneity of Variances

Bone density

Levene Statistic	df1	df2	Sig.
1.565	3	20	.229

ANOVA

Bone fill

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	10467.948	3	3489.316	22.084	.000
Within Groups	3160.058	20	158.003		
Total	13628.006	23			

Post Hoc Tests

Multiple Comparisons

Dependent Variable: Bone fill
Tukey HSD

(I) Group	(J) Group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Empty	Dur + Saline	-9.82083	8.11385	.628	-32.5310	12.8893
	Dur + MSCs	-34.96000(*)	7.25725	.001	-55.2726	-14.6474
	Dur + BMP	-50.81083(*)	6.78853	.000	-69.8115	-31.8102
Dur + Saline	Empty	9.82083	8.11385	.628	-12.8893	32.5310
	Dur + MSCs	-25.13917(*)	8.11385	.027	-47.8493	-2.4290
	Dur + BMP	-40.99000(*)	7.69747	.000	-62.5347	-19.4453
Dur + MSCs	Empty	34.96000(*)	7.25725	.001	14.6474	55.2726
	Dur + Saline	25.13917(*)	8.11385	.027	2.4290	47.8493
	Dur + BMP	-15.85083	6.78853	.123	-34.8515	3.1498
Dur + BMP	Empty	50.81083(*)	6.78853	.000	31.8102	69.8115
	Dur + Saline	40.99000(*)	7.69747	.000	19.4453	62.5347
	Dur + MSCs	15.85083	6.78853	.123	-3.1498	34.8515

* The mean difference is significant at the .05 level.

Homogeneous Subsets

Bone fill

Tukey HSD

Group	N	Subset for alpha = .05	
		1	2
Empty	6	9.2517	
Dur + Saline	4	19.0725	
Dur + MSCs	6		44.2117
Dur + BMP	8		60.0625
Sig.		.566	.181

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 5.647.
b The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

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