Comparison of the Osteoinductivity of Infuse® and OP-1® via *in vitro* and *in vivo* Assays

By

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ABSTRACT

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Masters of Science 2009

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Due to its significant morbidity, alternatives to autogenous bone grafting are required. Recent research has focused on application of growth factors, including bone morphogenetic proteins (BMPs). Two recombinant human BMP (rhBMP) containing bioimplants, Infuse® (rhBMP-2) and OP-1® (rhBMP-7), are approved for human application. Objective: to provide a direct comparison of the two approved rhBMP containing bioimplants in their clinically available forms. Methods: Activity of rhBMP-2 and -7 were tested using the C2C12 cell based assay comparing alkaline phosphatase (ALP) activity. Activity of Infuse® or OP-1® bioimplants containing 52.5µg of rhBMP-2 or rhBMP-7 were compared using a mouse muscle pouch assay and analyzed with micro CT (mCT) and histology. Results: in vitro: rhBMP-2 induced greater ALP production than rhBMP-7 at different time points. In vivo: OP-1® induced greater bone volume than Infuse® of equivalent quality based on mCT. Conclusions: In the clinically available form, OP-1® induced greater bone volume than Infuse®.
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LIST OF ABBREVIATIONS

ACS: absorbable collagen sponge
ADU: arbitrary density unit
ALP: alkaline phosphatase
BMP: bone morphogenetic protein
rhBMP: recombinant human bone morphogenetic protein
BSA: bovine serum albumin
CT: computed tomography
DBM: demineralized bone matrix
FBS: fetal bovine serum
FGF: fibroblast growth factor
HDE: humanitarian device exemption
αMEM: alpha-minimal essential medium
mCT: microCT
MWRST: Mann Whitney rank sum test
NaOH: sodium hydroxide
TBS: tris-buffered saline
PBS: phosphate-buffered saline
pNP: para-nitrophenol
pNPP: para-nitrophenol phosphate
ROI: region of interest
VEGF: vascular endothelial growth factor
CHAPTER ONE: INTRODUCTION

1.1 Study Objectives

When considering reconstructive options for critical sized osseous defects, surgeons are presented with several options. The defects are reconstructed most commonly using autogenous bone grafts. Should autogenous grafting prove an unavailable or undesirable choice, the surgeon may consider using alloplasts such as tricalcium phosphate or allogeneic grafts involving use of cadaveric bone. More recently a new option has been made available, the application of growth factors such as bone morphogenetic proteins.

Autogenous grafting is considered the gold standard for repair and reconstruction of bony skeletal defects and stimulation of bone formation. Bone is the second most commonly transplanted tissue after blood with over 500,000 bone grafting procedures performed annually in the USA and approximately 2.2 million procedures worldwide (Giannoudis, Dinopoulos et al. 2005). Autogenous grafts present no risk of disease transmission or graft rejection, and cancellous bone grafts include live osteocytes, osteoblasts and all the requirements to begin formation of new bone (Hubble 2002). Harvesting of autogenous bone is also associated with several disadvantages including increased operating room time and a significant increase in patient morbidity due to increased surgical time and the addition of a second surgical site.
Alternatives to autogenous grafting have been sought since the need for grafting was identified. Historically, the alternatives could be classed into three main categories: allogeneic, xenogeneic and alloplastic. Allogeneic implants involves use of human bone harvested from another individual. Their use has increased fifteen fold over the course of the last decade and now account for one-third of bone grafts performed in the United States (Giannoudis, Dinopoulos et al. 2005). Allogeneic implants undergo various treatments after harvest to limit the potential for disease transmission however, the risk cannot be eliminated entirely. During the course of these treatments, all osteogenic capabilities are eliminated. As such, allogeneic implants are not osteogenic and function primarily as a physical scaffold which assists growth of native bone tissue, a process known as osteoconduction.

When compared to autografts, alloplastic (synthetic substitutes) and xenogeneic options have the advantages of low cost and no significant increase in operating time or patient morbidity. However, both alloplasts and xenogeneic implants pose a risk for foreign body reaction with possible rejection and carry a risk of disease transmission. Historically, xenogeneic implants have been shown to stimulate such an intense foreign body reaction from the host, the graft may undergo encapsulation and sequestration with resultant obstruction of vascularization (Prolo and Rodrigo 1985). In order to minimize their antigenicity and immune response, xenogeneic and allogeneic implants must be subjected to a variety of treatments including freeze-drying and deproteinization which eliminates virtually all osteogenic potential within the implant. Xenogeneic and alloplastic implants function as aids to osteoconduction, providing a
mechanical framework across which vascular ingrowth and osteoid production can occur.

All of the currently available grafting options, including autogenous grafts, have significant disadvantages. The lack of an ideal grafting option has spurred extensive research into the potential of growth factors, including recombinant human bone morphogenetic proteins (rhBMPs). Members of this group of endogenous growth factors have been shown to have the ability to induce bone formation when implanted into a healthy tissue bed. The term osteoinduction has been utilized following the seminal paper by Urist: “Bone formation by autoinduction” (Urist 1965). Osteoinduction implies growth factor stimulated differentiation of mesenchymal stem cells (MSC) into osteogenic cells. With rhBMPs, there is no risk of disease transmission, no increased operating room time or patient morbidity, and incredible potential for bone production. Extensive laboratory and clinical research has led to the commercial availability and approval of two rhBMP based bioimplants for human application: Infuse®, containing rhBMP-2 and OP-1®, containing rhBMP-7.

To date, no direct comparison of the osteoinductive activity of these two rhBMP bioimplants has been carried out. The objective of this thesis was to pursue a direct comparison of the two commercially available rhBMP bioimplants (Infuse and OP-1) to elucidate the nature and magnitude of their osteoinductive activity.
1.2 Bone Graft Healing

1.2.1 Autografts

The healing of autogenous bone grafts was first proposed by Axhausen in 1956 as a two phase process. Phase one involves bone regeneration from transplanted cells and occurs during the first through fourth weeks post-transplant. Surviving osteocompetent cells produce new bone matrix which results in a slight increase in radiopacity in the grafted area. The new bone is immature and randomly oriented.

In Phase two, existing recipient site osteoblasts and newly differentiated recipient bed progenitor cells produce new bone while resorption and remodeling of the immature and irregular phase one bone occurs. The remodeling aspect of this phase continues indefinitely.

More recent reviews of the subject have described bone graft healing as incorporation (Burchardt 1983; Goldberg and Stevenson 1987; Marx 2007). Bone graft incorporation is defined as the process of envelopment of a complex of necrotic old bone with viable new bone. It is described as being a function of the recipient bed and is affected by several factors including contact with donor tissue, state of health of the recipient bed and the biomechanical properties of the graft (Burchardt 1983). Incorporation is described in five phases which suggests that the events within each phase are singular and that each phase is cleanly divided from the next. However, most authors agree that bone graft incorporation is a fluid process with multiple phases overlapping or occurring simultaneously. The recognized phases include: Inflammatory, vascularization, osteoinduction, osteoconduction and remodeling (Goldberg and Stevenson 1987). In a discussion of graft healing, osteoinduction refers to the formation
of new bone, regardless of source. For the purposes of this discussion and to avoid confusion with bone formation due to growth factor induced differentiation of mesenchymal stem cells, this phase of graft healing shall hereafter be referred to as the osteogenic phase.

The first phase of bone graft incorporation is the inflammatory phase. Inflammation begins almost immediately during surgery and continues for approximately the first one to two weeks post-operatively. Associated with the inflammation is hemorrhage due to surgical manipulation of the recipient area. Generally, hematoma formation is observed with its associated aggregation of platelets, erythrocytes and fibrin. The platelets degranulate, releasing platelet-derived growth factor (PDGF), transforming growth factor-beta 1 and 2 (TGF-β), vascular endothelial growth factor (VEGF) and epithelial growth factor. These growth factors have a powerful mitogenic and chemotactic effect on the surrounding tissues of the recipient bed. Surgical access and site preparation result in a recipient bed with compromised vascular support and a central hypoxic core (Knighton, Hunt et al. 1983). Placement of the graft into the recipient bed exceeds the capacity of the compromised vascular supply and results in a local tissue environment that is hypoxic (oxygen tensions approximately 3-10mm Hg), acidic (pH approximately 4-6) and rich in lactate (Marx and Garg 1998). This environment contrasts sharply with the normal adjacent tissues where oxygen tensions are 50-55mm Hg and pH is 7.35-7.45. Tissue and circulating monocytes are recruited to the wound and differentiate into macrophages. Attractants include components of the complement cascade stimulated by platelets and both general inflammatory cell and macrophage specific chemoattractants (DiPietro 1995). Macrophages are activated via
a number of avenues including bacterial and tissue factors (e.g. expression of fibronectin), inflammatory cytokines (e.g. TNF-α) and the local environmental factors such as hypoxia, low pH and high lactate levels (Knighton, Hunt et al. 1983; Sunderkotter, Steinbrink et al. 1994; DiPietro 1995; Dipietro, Reintjes et al. 2001). Thus, the combination of oxygen gradient, platelet degranulation and release of the macrophage-derived factors culminates in stimulation of early angiogenesis from host capillaries and mitogenesis of the transplanted osteocompetent cells (Marx, Ehler et al. 1996).

Vascularization of the incorporating graft occurs during and is a marker of the second stage in graft healing. This phase is strongly associated with the inflammatory phase and many of the events and timelines occur simultaneously. Initial attempts at revascularization begin shortly after graft insertion. Some authors have suggested that revascularization of cancellous grafts may be achieved within hours via end-to-end anastomoses between graft and recipient vessels and that the entire graft may be covered by vessels within two days (Burchardt 1983). However, most investigators agree that while revascularization may begin within hours, clinically significant results are not seen before approximately day 3 and completion is reached sometime between one to three weeks after graft placement (Prolo and Rodrigo 1985; Stevenson, Emery et al. 1996; Marx 2007). Inflammatory mediators supplied by platelets ceases near the end of the first week. However, macrophages, drawn to the area in the acute inflammatory phase take over and continue to produce similar growth factors until vascularization is complete. Vascular ingrowth is vital to graft viability and interference by infection, radiation, scar or movement may prevent proper healing and result in graft
failure. As vascularization nears completion, the arrival of growth factors, oxygen and nutrients stimulates the few remaining viable osteocompetent cells transplanted with the cancellous graft to proliferate and produce osteoid matrix.

The third phase of bone graft healing is the osteogenic phase which commonly demonstrates significant overlap with the vascularization phase. A key component of autogenous cancellous grafts is the viable cells transferred within the bony trabeculae. The majority of cells die during the transfer and the graft becomes necrotic except for the peripheral regions. Osteocompetent cells transferred within the graft include periosteal osteoblasts, endosteal osteoblasts, and cancellous marrow stem cells. Those cells within 0.3mm of defect margins may survive 3-5 days due to their superficial location which allows the opportunity to absorb oxygen and nutrients diffused from the recipient tissue bed (Burwell 1969). However, it has been noted that mesenchymal stem cells are resistant to ischemia and are able to survive in a hypoxic environment for a limited time (Bauer and Muschler 2000) and thus may survive deeper into the graft. MSCs are present in bone marrow at an approximate concentration of 1 in 50,000 to 1 in 1 million bone marrow cells (Perry 1999; Khan, Cammisa et al. 2005).

Although their nutritional supply is very limited, surviving osteocompetent cells are nevertheless stimulated to produce a limited quantity of osteoid in response to the platelet and macrophage derived growth factors discussed earlier. As vascularization progresses, a significant increase in the availability of oxygen and nutrients promotes a marked increase in the quantity of osteoid production. Additionally, host mesenchymal stem cells from neighbouring tissues are drawn to the graft site by the hypoxic and acidic graft environment as well as these same growth factors. As mentioned
previously, osteoid is first laid down by remnant osteocompetent cells such as endosteal osteoblasts as a rim surrounding a core of dead, necrotic bone. Further osteoid is produced in the intertrabecular spaces, presumably from stem cells transferred within the graft (Marx and Garg 1998). The stem cells drawn to the site are proposed to be a source of new osteoid production in the graft. The increased osteoid production is responsible for the initial increase in radiopacity of bone grafted areas described as part of Axhausen’s Phase I bone formation.

Vascularization provides improved availability of oxygen and nutrients to remaining viable osteocompetent cells and a supply MSC-like cells known as pericytes, which retain the ability to differentiate into multiple cell types, including osteoblasts (Doherty and Canfield 1999; Collett and Canfield 2005). Additionally, increased vascularity delivers osteoclasts from the systemic circulation which begin the process of resorption of the necrotic portions of the graft. Osteoclastic resorption of the necrotic cancellous trabeculae may result in release of entrenched BMP. Released BMPs could further stimulate mesenchymal stem cell migration, proliferation and differentiation into osteoblasts and osteoblast precursors. BMPs have been implicated in multiple healing processes including angiogenesis, chemotaxis, mitogenesis, and cell differentiation and proliferation (Phillips 2005). We now have a good example of how the phases of vascularization, osteogenesis and osteoconduction are closely related and generally occur in concert.

Osteoconduction is defined as an ordered, spatial three-dimensional ingrowth of capillaries, perivascular tissue and osteoprogenitor cells from the recipient bed (Burchardt 1983) and is closely associated with osteogenesis. The fibrin matrix in the
organized clot or hematoma as well as the cancellous trabeculae serve as a mechanical scaffold across which host tissues may invade. It has also been proposed that osteoblasts “creep” along the scaffold and lay down osteoid along the framework (Marx and Garg 1998). Osteoblasts produce osteoid around the necrotic trabeculae and in the intertrabecular spaces, which results in coalescence of the osteoid. This occurs approximately 4 weeks post graft placement. In general, the term “creeping substitution” is applied to the process of laying down new bone (osteogenesis and osteoconduction) and remodeling of the graft with more mature bone. Creeping substitution is a translation of the German phrase “schleichenden Ersatz” coined by Barth in 1895 (Burwell 1994) to describe the replacement of necrotic bone by new connective tissue and bone in his animal experiments. This process of gradual laying down of immature bone and early remodeling may last for several months in cancellous grafts and longer in cortical grafts (Stevenson 1999). The bone formed during the osteogenic and osteoconduction stages has a random organization and is hypercellular, similar to that seen in a fracture callus (Marx and Garg 1998). It is weak and incapable of withstanding the functional load normally borne by an intact skeleton. Thus, this immature bone must be replaced with stronger, mature bone to allow a return to function of the affected area of the skeleton.

Remodelling is the final stage of bone graft incorporation. Remodeling begins during the revascularization stage with the arrival of osteoclasts. The process involves the resorption of the immature Phase I bone and necrotic graft trabeculae and subsequent replacement with mature, lamellar bone. As is true with the entire process of graft incorporation, remodeling occurs simultaneously along with vascularization,
osteogenesis, and osteoconduction. During the course of remodeling, an endosteum and periosteum develop approximately 4 months after graft placement (Marx, Ehler et al. 1996). Graft integration into the recipient site via remodeling is considered functional by six months and complete by one year post surgery (Goldberg and Stevenson 1987). Long term bony remodeling occurs throughout life in response to functional loads placed on the area.

1.2.2 Allograft & Xenografts

Currently, allografts and xenografts are utilized with similar expectations. It is well known that the required tissue processing of these grafts leaves them with no osteogenic capability, due to the death and removal of the cellular components. Processing of allografts may leave entrenched BMP intact, however, the defatting and deproteinization of xenografts required to limit the immune response effectively destroys any osteoinductive proteins (Bauer and Muschler 2000). As such, these grafts are implemented with the aim of improving osteoconduction and often to provide structural support.

The healing of allografts and xenografts attempts to follow the same five stages as autografts. The initial inflammatory stage closely mirrors the events of autogenous healing. Revascularization has traditionally been described as being impaired by the intense inflammatory response to foreign tissue. The vessels become occluded due to the dense cellular inflammatory infiltrate surrounding them leading to ischemia and ultimately necrosis (Goldberg and Stevenson 1987). There is no osteogenic phase.
Ultimately, healing occurs via osteoconduction and a slow process of remodelling and turnover.

1.2.3 Alloplastic (Synthetic) grafts

The search for an alternative to autogenous grafts has also included research on alloplastic grafts. The available synthetic grafts vary considerably in physical properties and chemical characteristics. Synthetic grafts do not have any osteogenic or osteoinductive capabilities and attempt to provide an osteoconductive scaffold which has undergone osteointegration with the native bone. Many alloplasts are formed as rigid implants which are often used to provide initial mechanical support and stability to the graft area.

Healing of alloplastic grafts depends on their individual physicochemical makeup. In general terms, they heal in a similar manner to allografts, via resorption and remodelling. Functioning as an osteoconductive scaffold, new osteoid is laid down along the alloplast which is later resorbed during the normal remodelling process. Some alloplasts such as hydroxyapatite are more resistant to resorption and are present in the graft site for longer periods.

1.2.4 Growth factor based grafts

Bone graft substitutes utilizing growth factors are a relatively new addition to the surgeon’s armamentarium. They attempt to modify the complex interplay of cellular function and differentiation, growth factors, inflammatory cells and angiogenesis in
favour of osteogenesis. Several growth factors have been investigated for their abilities to improve bone healing including PDGF (platelet derived growth factor), TGF-β (transforming growth factor beta), VEGF (vascular endothelial growth factor), FGF (fibroblast growth factor) and BMPs. Each growth factor influences bone healing in different, but connected ways. In addition to stimulating and regulating angiogenesis, VEGF influences osteoblast differentiation and is involved in mineralization of fracture callus. PDGF has been shown to be chemotactic and mitogenic for osteoblasts and to increase production of osteopontin (Schilephake 2002). With application of growth factors to bone fractures or grafts, site physiology can be modulated to increase speed and efficiency of healing. For example, application of exogenous VEGF could increase angiogenesis at the graft or fracture site (Eckardt, Ding et al. 2005). Increased angiogenesis would provide greater oxygen and nutrient supply, perivascular precursor cells as well as increase differentiation of perivascular and mesenchymal stem cells into osteoblasts.

The ability of BMPs to stimulate chondro- and osteogenic differentiation of MSCs is well known (Reddi 2001). Differentiation of MSCs to osteoblasts increases the number of osteogenic cells at a graft site, resulting in greater bone formation. BMPs have also been shown to stimulate chemotaxis of endothelial progenitor cells which may then differentiate into endothelial cells participating in angiogenesis (Raida, Heymann et al. 2006).

Additionally, BMPs, and most other osteogenic growth factors, are capable of stimulating the expression of VEGF at a local site (Carano and Filvaroff 2003). VEGF has been shown to play crucial roles in the recruitment, differentiation and activity of
osteoblasts, osteoclasts and endothelial cells (Engsig, Chen et al. 2000; Mayr-Wohlfart, Waltenberger et al. 2002; Ferrara, Gerber et al. 2003). Thus, healing of growth factor based grafts is significantly different from autogenic or allogeneic grafts. Growth factor based graft healing involves manipulation of the normal cellular wound physiology in favour of promotion of osteogenesis.

1.3 BMP History and Discovery

Bone is a vital structure in the human skeleton and serves many key functions including structural support, attachment points for muscles, tendons and ligaments and repository for minerals such as calcium and phosphate. A unique feature of bone physiology is its ability to heal without scar formation, that is, to heal via regeneration. It was this feature which spurred interest in the pursuit of soluble factors which could stimulate or activate this healing process. Experiments demonstrating heterotopic osteogenesis in the early part of the 20th century (Neuhof 1917; Huggins 1931) led to the theory that “bone regeneration takes place as a result of some specific bone formation substance activating the nonspecific mesenchymal tissue” (Levander 1938). In accordance with this theory, Dr. M. Urist performed a series of simple experiments involving the implantation of decalcified allografts of cortical tibial bone into muscular pouches of rabbits, mice, rats and guinea pigs. The results demonstrated new bone formation through a process involving proliferation of pleuripotent host cells, which he called autoinduction. He further postulated that the inductive agent must interact with host cells and induces primitive connective tissue cells to become osteoprogenitor cells and osteoblasts (Urist 1965). Further studies led to the proposal that bone induction
could be due to a discrete chemical compound (referred to as bone morphogenetic protein) which Urist and Strates defined as “the osteogenic chemical components of the matrix of bone, dentine and other hard tissues that are deinsulated by demineralization and associated intimately with collagen fibrils” (Urist and Strates 1971). In 1979, Urist reported isolation of an osteoinductive, hydrophobic, low-molecular-weight protein from insoluble bone matrix gelatin (Urist, Mikulski et al. 1979).

Following the initial isolation of the active compound, several authors have successfully elucidated the amino acid sequence of most commonly occurring bone morphogenetic proteins (BMPs) (Wozney, Rosen et al. 1988; Celeste, Iannazzi et al. 1990; Wang, Rosen et al. 1990) as well as the chromosomal location (Tabas, Zasloff et al. 1991). These discoveries fueled our general understanding of the nature of BMPs and allowed a greater exploration of their biologic functions not only in bone and bone healing, but in embryogenesis and skeletal development.

1.4 BMP Classification

Bone morphogenetic proteins comprise the largest subfamily of the TGF-ß superfamily of growth and differentiation factors. The constituent proteins of this superfamily are subdivided by degree of sequence homology (see Figure 1). It is well known that the amino acid sequence of BMPs is highly conserved across multiple species and is thought to have existed for more than 600 million years (Kingsley 1994). The sequence conservation is primarily seen in the carboxy terminus.
The BMP subfamily is further broken down into subclasses of BMPs based on their sequence homologies. BMP-2 and BMP-4 exhibit 80-92% homology, BMP-5 through -8 share 78-89% homology and BMP-3 and GDF-10 (BMP-3b) also demonstrate high amino acid sequence homology. Additionally, GDF-5, GDF-6 (BMP-13) and GDF-7 (BMP-12) are strongly related and comprise a fourth subclass of the BMP subfamily. The classification of BMPs is discussed in several excellent reviews (Sakou 1998; Hoffmann, Weich et al. 2001; Rengachary 2002; Canalis, Economides et al. 2003). There are currently 15 proteins labelled as BMPs with many more placed in the same sub-family but named as “growth and differentiation factors” (GDF) rather than BMPs, with some members being given both a BMP and a GDF name (i.e. BMP-3a is also called GDF 10) (Li, Li et al. 2003; Dimitriou and Giannoudis 2005). BMP-1 does not retain the conserved amino acid sequence and is thus not a member of the TGF-ß superfamily. Evidence suggests it is a procollagen proteinase involved in processing procollagen to collagen (Hofbauer and Heufelder 1996; Kessler, Takahara et al. 1996).
Figure 1: Phylogenetic tree of the TGF-β superfamily (with permission, R&D Systems)
1.5 BMP Structure

Most constituents of the TGF-β superfamily are produced as large precursor proteins which undergo proteolytic cleavage to liberate the mature protein (Groeneveld and Burger 2000). BMP precursors contain amino terminal signal sequences and variable prodomains in addition to the mature peptide. During secretion from the cell, the precursor molecules undergo dimerization and are then cleaved at a consensus Arg-X-X-Arg cleavage site to release the mature carboxy terminal regions (Granjeiro, Oliveira et al. 2005; Rosen 2006). The mature regions are comprised of approximately 110-140 amino acids and become linked to a second mature region to form the physiologically active BMP dimer.

The mature regions of BMPs universally contain seven cysteine residues, six of which are involved in the formation of three disulfide bonds resulting in the classic cysteine-knot structural arrangement. The free seventh cysteine residue is utilized to form a dimer with a second monomer. It is after the formation of the dimer that the BMP protein becomes active (Xiao, Xiang et al. 2007). Mature BMPs are active as both homo- and heterodimers and it has been suggested that biologic activity may be enhanced via formation of heterodimers of two different BMPs (Granjeiro, Oliveira et al. 2005).
Figure 2: BMP structure and pre-secretion processing. BMPs are produced as precursor proteins containing a pro-domain and a mature region. The BMP precursors dimerize prior to undergoing cleavage to release the mature BMP regions.
1.6 BMP Receptors and Signalling

1.6.1 BMP receptors

Following secretion from cells, BMPs initiate signalling via interactions with surface membrane receptors. Receptors for BMP are one of two types of transmembrane serine-threonine kinase receptors which in turn activate cytoplasmic SMAD proteins. Type I and II BMP receptors are distinct entities, yet both receptors are involved in initiation of the intracellular signalling cascade of SMAD activation.

Type I receptors are smaller than Type II and contain a highly conserved cysteine rich region in the extracellular region of the receptor (Rosen, Cox et al. 1996). Currently, seven Type I receptors have been identified, three of which bind BMPs, including Alk3 (BMPRIA) and Alk6 (BMPRIB) and type IA activin receptor (Alk2 or ActRIA) (Miyazono, Maeda et al. 2005). The Alk3 receptor is found in most cells, including osteoblasts whereas Alk6 receptors are found only in chondrocytes and osteoclasts. It has been noted that BMP-2 and BMP-4 preferentially bind to Alk3 or Alk6 receptors (Abe 2006) (Table 1). The Type I receptor primarily mediates the specificity of the intracellular signals (Miyazono, Maeda et al. 2005).

Type II receptors also exhibit cysteine conservation albeit in a different pattern than type I receptors. Three different type II receptors have been identified to date: Type II BMP receptor (BMPRII or T-Alk), Activin type II (ActRII) and activin type IIB (ActRIIB) receptors. All are known to bind BMP ligands including BMPs 2, 4 and 7 (Massague, Attisano et al. 1994).
<table>
<thead>
<tr>
<th>Serine-Threonine Receptor Class</th>
<th>Receptor Type</th>
<th>Alternative Name</th>
<th>BMP Binding Ligands</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Type I</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>BMPR-IA</td>
<td>ALK-3</td>
<td>BMP-2,4,7,13</td>
</tr>
<tr>
<td></td>
<td>BMPR-IB</td>
<td>ALK-6</td>
<td>BMP-2,4,7,14,15</td>
</tr>
<tr>
<td></td>
<td>ActR-IA</td>
<td>ALK-2</td>
<td>BMP-2,7,14; Activin</td>
</tr>
<tr>
<td></td>
<td>ActR-IB</td>
<td>ALK-4</td>
<td>BMP-3,11</td>
</tr>
<tr>
<td><strong>Type II</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>BMPR-II</td>
<td>T-ALK</td>
<td>BMP-2,4,6,7,14</td>
</tr>
<tr>
<td></td>
<td>ActR-II</td>
<td></td>
<td>BMP-2,6,7,14; Activin</td>
</tr>
<tr>
<td></td>
<td>ActR-IIB</td>
<td></td>
<td>BMP-2,6,7,14</td>
</tr>
</tbody>
</table>

**Table 1:** Serine-Threonine receptors and the BMP-ligands bound.
2.6.2 Intracellular Signalling

Ligand binding leads to formation of a heterodimer complex of both the Type I and Type II receptors. Type II receptor kinases activate the type I receptor via phosphorylation, allowing recognition and subsequent phosphorylation of pathway-specific receptor-regulated Smads (R-Smads). Smads (Sma and Mad related proteins) are a group of proteins functioning as mediators of transcriptional activation for members of the TGF-β superfamily. Different members of the TGF-β family activate different Smad proteins intracellularly. For BMPs, R-Smads include Smads 1, 5 and 8. Following activation by the type I receptor, the R-Smads are released from the receptor and form a complex with Smad 4 (Figure 3). Smad 4 is referred to as a common mediator Smad, or co-Smad as it is utilized by all members of the TGF-β family in their respective signalling pathways. The R-Smad/Smad 4 complex then translocates into the nucleus where it exerts its effects on gene transcription via a core binding factor. Activated Smad 1 or 5, in association with Smad 4 induce expression of the transcription factor PEBP2αA/Cbfa1. Cbfa1 is essential for osteogenesis and regulates gene expression of key bone matrix proteins. PEBP2αA/Cbfa1 is expressed primarily in the fetal development period and damage or mutation of this transcription factor results in severe skeletal defects including complete lack of skeletal ossification in homozygous mice (Ebara and Nakayama 2002). Cbfa1 directly binds and activates the osteocalcin transcriptional promoter region and results in the production of osteocalcin. Osteocalcin is an accepted marker of osteoblast differentiation and its production is only induced by BMP (Schmitt, Hwang et al. 1999). Human Cbfa1 mutations, seen in cleidocranial
dysplasia patients, lead to a truncated Cbfa1 protein which ultimately manifests with the classic stigmata of that syndrome including hypoplastic or absent clavicles.

**Figure 3**: BMP receptor binding and intracellular signalling pathway (Adapted from Sakou, 1998). Ligand binding stimulates phosphorylation of BMPR-I by BMPR-II. Activated BMPR-I in turn phosphorylates one of the R-Smads (1,5 or 8). Activated R-Smad then couples with Smad-4 (co-Smad) and the complex translocates into the nucleus where it modulates gene expression with cooperation from transcription factors. (Modified from, Sakou 1998)
1.7  Physiologic Roles of BMPs

Bone morphogenetic proteins or their species specific analogues have been identified in a wide range of species, from *Drosophila* and its decapentaplegic protein (dpp) to *Xenopus* and its Vg1 to the BMPs in mammals. Indeed, dpp shares 75% homogenicity with BMP-2 and 4 (Kaplan, Tabas et al. 1990). Throughout development and into adult skeletal healing and remodeling, BMPs play roles in a wide array of key biologic functions, including osteoinduction and embryonic patterning (see Table 2).

1.7.1  Developmental functions

Bone morphogenetic proteins have diverse roles in development. At a very early stage, BMPs are essential in dorso-ventral patterning of the mesoderm layer, with BMP-4 being the most potent ventralizing factor. BMP 3 and 3-b induce head-tail orientation and development (Hino, Kangawa et al. 2004). Loss of BMP signalling results in severe dorsalization of the embryo with loss of epidermis, ventral and lateral mesoderm and expansion of dorsal-lateral mesoderm (Schier 2001). They have been associated with development of many organ systems including the heart, kidney, lung, nervous system, gonads and teeth (Hogan 1996; Mehler, Mabie et al. 1997; Schneider, Gaussin et al. 2003).

Knockout mice are commonly investigated to elucidate the roles of BMPs in development. Disruption of BMP-8 results in infertility and defects in spermatogenesis while mice deficient in BMP-2 have severe defects of the heart and amnion which are usually fatal in the perinatal period (Zhang and Bradley 1996; Zhao, Deng et al. 1996).
Mice with a knockout of the gene encoding BMP-7 demonstrate multiple defects including reduced nephron development leading to renal failure, lens and retinal malformation and multiple skeletal anomalies (Jena, Martin-Seisdedos et al. 1997). Kingsley (1994) examined the short ear mouse strain which demonstrated a defect in the gene coding for BMP-5. The animals generally showed multiple mesenchymal defects including stunted perichondral and periosteal growth leading to diminished growth potential of the affected structures (e.g., short ears, thin long bones, altered skull morphology).

1.8 Clinical Applications of BMPs

The intensive laboratory and clinical research devoted to members of the BMP family has driven speculation on the wide range of potential human applications. Turgeman found that systemic administration of rhBMP-2 increased MSC activity and reversed ovariectomy-induced and age related bone loss (Turgeman, Zilberman et al. 2002). This finding has led researchers to suggest systemic administration of rhBMP-2 for treatment of osteoporosis. Other authors have investigated the potential application of BMP to the field of endodontics. Several authors have demonstrated induction of secondary (reparative) dentin formation after exposure to BMP (Nakashima 1994; Rutherford, Spangberg et al. 1994), leading to a possible role in pulp capping and maintaining tooth vitality. Other possible dental applications investigated include aid to the osseointegration of endosseous dental implants, and periodontal regeneration (Ripamonti and Renton 2006).
1.8.1 Use of BMPs containing bioimplants for bone repair

Of all the members of the BMP family, two have received the most attention for their ability to induce bone formation: BMP-2 and BMP-7. They are both approved for specific indications and have also been used for off label uses such as reconstruction of skeletal bony defects (Moghadam, Urist et al. 2001; Clokie and Sandor 2008). It has been estimated that over one million patients worldwide have been treated with BMPs (Pecina and Vukicevic 2007).

There are two commercially available rhBMPs which have received FDA approval for human application. rhBMP-2, marketed as Infuse® by Medtronic, has received full FDA approval for multiple indications. In 2002, approval was granted for use as an alternative to autogenous grafting for spinal fusion surgery with the LT-Cage device. A second approval was granted in 2004 for use in the treatment of acute open fractures of the tibial shaft. Most recently, Medtronic received approval for application of Infuse® for maxillary sinus augmentation and augmentation of the oral alveolar ridges in preparation for dental implant therapy.

Stryker Biotech’s OP-1® was granted a humanitarian device exemption (HDE) in 2001 for the application of OP-1 to long bone fracture non-unions. In 2004, OP-1 received a second HDE for use in revision posterolateral lumbar fusion surgery when autogenous bone harvest and grafting is either not feasible or not expected to result in a stable outcome. It should be noted that HDE approval is significantly different than full FDA approval for human application. HDE status is bestowed on a product that is intended to treat a medical condition which affects fewer than 4000 patients annually.
An HDE application is not required to contain the results of scientifically valid clinical investigations demonstrating that the device is effective for its intended purpose, but does require adequate information to demonstrate that the product does not pose unreasonable health risks to the intended patients.

A review of the literature quickly reveals an overwhelming disparity in the supporting literature for the two products. Infuse® is supported by volumes of in vitro and in vivo studies including both animal and human trials. A PubMed search using the terms rhBMP-2 vs. rhBMP-7 results in a 12:1 ratio of sources identified in favour of rhBMP-2. The apparent bias towards rhBMP-2 would suggest its superiority or greater availability over rhBMP-7 when applied. However, few studies have compared rhBMP-2 and -7 directly to assess their respective osteoinductive potentials. Data from in vitro and in vivo studies currently available have consistently indicated that BMP-2 elicits a greater osteoinductive response than BMP-7 (see discussion).

In spite of these publications there are no available studies where the osteoinductive capacities of both rhBMP-2 and rhBMP-7 based bioimplants have been compared directly. As such, the aim of this thesis was to provide a direct comparison of the osteoinductive activity of Infuse® and OP-1.
<table>
<thead>
<tr>
<th>BMP Type</th>
<th>Alternative Name</th>
<th>Developmental &amp; Physiologic Functions</th>
</tr>
</thead>
</table>
| BMP-1    |                  | No longer considered a member of TGF-ß superfamily  
            |                  | A metalloprotease which acts on procollagen I, II, III |
| BMP-2    | BMP-2a           | Induces bone and cartilage formation in embryogenesis  
            |                  | Active role in osteogenesis via osteoblastic differentiation from mesenchymal progenitor cells  
            |                  | Role in apoptosis signalling  
            |                  | Expressed in lung, spleen and colon |
| BMP-3    | Osteogenin       | Role in cartilage and bone formation  
            |                  | Induces synthesis & secretion of TGF-ß1  
            |                  | Expressed in lung, ovary & small intestine |
| BMP-4    | BMP-2b           | Regulates formation of teeth, limbs & bone from mesoderm  
            |                  | Role in fracture repair  
            |                  | Induces formation of embryonic hematopoietic tissue  
            |                  | Expressed in lung & kidneys |
| BMP-5    |                  | Role in early developmental skeletal patterning  
            |                  | Functions in cartilage development  
            |                  | Expressed in lungs & liver |
| BMP-6    |                  | Role in cartilage & bone development  
            |                  | Key role in adult joint integrity  
            |                  | Role in osteoblast differentiation from Mesenchymal stem cells (MSCs) |
| BMP-7    | OP-1             | Role in cartilage & skeletal patterning, lens formation  
            |                  | Vital role in glomeruli formation  
            |                  | Role in bone and calcium homeostasis  
            |                  | Induces production of SMAD 1  
            |                  | Expressed in brain, kidney & bladder |
| BMP-8    | OP-2             | Role in bone & cartilage development  
            |                  | Role in maintenance of spermatogenesis |
| BMP-9    | GDF-2            | Role in osteogenesis via osteoblastic differentiation from (MSCs) and stimulation of mature osteoblasts  
            |                  | Role as hematopoietic hormone |
| BMP-10   |                  | Role in cardiac development |
| BMP-11   | GDF-11           | Role in development of embryonic mesodermal & neuronal tissue |
| BMP-12   | GDF-7            | Role in chondrogenesis  
            | CDMP-3            | Role in tendon/ligament formation & repair |
| BMP-13   | GDF-6            | Role in chondrogenesis  
            | CDMP-2            | Role in tendon/ligament formation & repair  
            |                  | Expressed in long bones during embryogenesis |
| BMP-14   | GDF-5            | Role in chondrogenesis  
            | CDMP-1            | Role in tendon healing  
            |                  | Expressed in long bones during embryogenesis |
| BMP-15   | GDF-9b           | Role in ovarian development & function |

**Table 2**: Summary of biologic roles of BMP family members (Adapted from Samarzis, 2005)
CHAPTER TWO: MATERIALS & METHODS

2.1 Study Aims & Research Design

The aim of this study was to compare the osteoinductive activity of the 2 commercial bioimplants that contain rhBMPs. Two separate approaches were taken to address this aim, an in vitro series of experiments which directly compared the activity of the 2 different rhBMPs used in the bioimplants, and an in vivo study which compared the bioimplants formulated with the BMPs.

2.2 In Vitro Experiments

These experiments were based on the observation that osteoinductive BMPs stimulate mouse muscle derived C2C12 cells to change their differentiation pathway from a myogenic to an osteogenic one (Katagiri, Yamaguchi et al. 1994; Peel, Hu et al. 2003). When C2C12 cells are cultured they will form myotubes and express very low levels of alkaline phosphatase activity. However, when C2C12 are exposed to BMPs they undergo osteoblastic differentiation, develop a cuboidal morphology and express high levels of alkaline phosphatase (ALP) activity and secrete osteocalcin. The ALP activity of the cells increases proportionately to the amount of BMP present in the medium. The level of ALP can thus be used as an index of the osteogenic potential of the test agents.
2.2.1  Cell culture of C2C12 cell line

2.2.1.1  Stock & Cultures

Frozen aliquots of C2C12 cells, stored in liquid nitrogen, were thawed in a 37°C water bath. The thawed 1 ml of cell suspension containing $10^6$ cells/mL was added to 14mL of pre-warmed culture medium ($\alpha$-minimal essential medium ($\alpha$MEM) + 15% heat-inactivated fetal bovine serum (FBS)). After suspending the cells in the medium, they were then plated into a T-75 flask and incubated at 37°C with 5% carbon dioxide.

The culture was monitored and upon reaching 70% confluence (approximately 72 hours), was subcultured to ensure that the cells did not undergo myogenic differentiation, but remained in an undifferentiated state. To subculture the C2C12 cells, the culture medium was removed, cells were rinsed with warmed sterile phosphate buffered saline (PBS) and 5ml of 0.05% trypsin-EDTA solution was added. The progress of cell detachment is monitored via phase contrast microscopy. When cell detachment was complete, 5mL of culture medium was added to neutralize the trypsin. Following transfer of the trypsinized cell suspension to a test tube, a 1 mL aliquot was placed into a ViCell cell counter (Beckman) to determine cell number and that the viable proportion of cells is at least 95%.

The remainder of the mixture was centrifuged at 300g for 5 minutes. The supernatant was discarded and the cells resuspended in culture medium at a concentration of $0.5\times10^5$ cells/mL. If the cells were to be continued as a stock culture, 10mls of the cell suspension were seeded into a new T75 flask. If the cells were to be used for an assay then they were seeded at 1ml per well into wells of a 24-well plate.
2.2.2 Testing the Bioactivity of rhBMP-2 and rhBMP-7 in vitro

A 1mL aliquot (0.5 x 10^{-5} cells/mL) of the resuspended cells was added to each of the test wells in a premarked 24 well cell culture plate. The plate was marked to denote the groups: negative control (assay medium only), rhBMP-2, rhBMP-7, and positive control (40ng/mL rhBMP-2) with four wells per group. The plate was then incubated overnight to allow cell attachment to the wells. The following day, the medium was changed from the culture medium (αMEM + 15% heat inactivated FBS) to the assay mediums containing the experimental substance. In addition to the assay medium, the rhBMP experimental groups contained 50ng/mL of rhBMP-2 and -7 respectively. Three duplicate plates were prepared to allow testing at 1,3 and 6 days post-exposure.

At the appropriate time points, the cultures were terminated and prepared for the alkaline phosphatase assay. After removing the culture medium, the cells were rinsed with tris buffered saline (TBS – 20mM tris, 137mM NaCl, pH 7.4) three times, then 1 mL of mammalian cell lysis buffer was added to each culture well. The culture plate was then sealed with Parafilm and frozen at -30°C. After allowing adequate time to ensure cell lysis, the samples were then thawed and scraped into 1.5mL Epindorf microfuge tubes using 1 mL pipette tips. The samples were then placed in a centrifuge at 12,000g for 10 minutes at 4°C. The supernatant from each sample well was then transferred to clean Epindorf tubes and stored at -20°C until they were assayed.
2.2.3 Alkaline Phosphatase and Protein Assays of Cell lysates

2.2.3.1 Alkaline Phosphatase (ALP) Assay

The ALP assay is based on a chemical reaction involving the conversion of p-nitrophenol phosphate (pNPP) to p-nitrophenol (pNP) in the presence of ALP. At a pH of 10.5 and a temperature of 37°C, pNPP is hydrolysed by ALP to pNP and free phosphate. Prior to the addition of ALP, the pNPP solution is nearly colourless. The pNP produced is yellow. Greater production of pNP due to a higher concentration of ALP results in a more densely coloured pNP solution. The darker solution will then display greater absorbance at 405nm compared to pNP standards.

\[
\text{p-Nitrophenyl Phosphate (pNPP) } \xrightarrow{\text{Alkaline Phosphatase (ALP)}} \text{p-Nitrophenol (pNP)} + \text{Inorganic phosphate}
\]

The assay requires preparation of reagents prior to running the assay. The phosphatase substrate (Sigma 104 – 100mg capsules pNPP) is first allowed to warm to room temperature prior to separating and emptying the contents of the capsules into a dark tinted bottle. The solution is then diluted with 25mL of laboratory grade water per capsule. The dissolved pNPP substrate is then mixed with 221 alkaline buffer solution (2-amino-2-methyl-1-propanol, 1.5mol/L, pH 10.3) in equal portions (1:1) and stored in a darkened bottle at 4°C.

The assay can then be performed after preparation of pNP standards. Standards for this assay were prepared as follows: A stock solution of 50μL p-nitrophenol (pNP, 10μmol/mL) is diluted in 10mL of 0.02M sodium hydroxide (NaOH). Serial 1:1 dilutions are performed to generate a series of solution standards: 200, 100, 50, 25, 12.5, 6.25,
Two hundred and forty microliters (240µl) of each standard were then pipetted into a standard 96 well assay plate, two wells per standard.

Cell lysates were thawed, vortexed and held on ice. Twenty microliters (20µl) aliquots were pipetted into the 96 well plate wells, 4 wells per sample. Additionally a blank sample of cell lysis buffer alone was included to correct for the low level absorbance caused by the reagent buffer. Two hundred microliters of the pNPP substrate buffer was added to each of the sample and blank wells (but not the standards). The plate was incubated at 37°C for 30 minutes, and the reaction terminated with the addition of 20µL of 1M NaOH. The absorbance was then read at 405nm using a plate reader (Versamax, Molecular Devices). The amount of alkaline phosphatase activity was calculated by comparison with the absorbance from the pNP standards and the results for each sample were taken to be the mean of the 4 aliquots measured for each sample. The results were reported in units of activity (U) with each unit being equivalent to 100nmol pNP produced per 20µl sample per 30 minutes.

2.2.3.2 Protein Assay

To normalize the results of the alkaline phosphatase activity to the number of cells in each well a protein assay was performed as follows:

Protein standards were prepared from stock solutions of 2mg/ml bovine serum albumin (BSA) provided with the protein assay kit (Coomassie Plus Kit, Fisher Scientific, Mississauga ON), by diluting the BSA with cell lysis buffer. Twenty microliters aliquots of each sample or standard were pipetted into the 96 well plate (4 wells per sample/standard). To each well was added 200µl of the Coomassie Plus reagent which
reacts with the protein in the samples turning from a brown to a blue colour. The samples were left for 5 minutes before the absorbance at 595nm was determined by the microplate reader. The amount of protein in each sample was determined by comparison of the absorbances of the samples with that of the known concentrations of the standards.

To normalize the ALP activity of the samples the value obtained from the ALP assay was divided by the mean protein concentration obtained for the same sample and the normalized results were reported U/µg protein.

2.3 IN VIVO

2.3.1 Experimental Design

The overall design of the in vivo portion was based on previous studies involving insertion of a bioimplant containing 50µg of BMP into a mouse muscle pouch (Urist, DeLange et al. 1983; Volek-Smith and Urist 1996; Clokie and Urist 2000) as well as a pilot study performed in our lab. The pilot study was also based on the mouse muscle pouch assay but differed from the current study in two important aspects. First, it included more experimental groups: Mouse DBM, Human DBM, Bovine DBM, Infuse and OP-1. Second, and most importantly, a majority of the animals had two different experimental groups in the left and right muscle pouches. As such, a cohort of 10 animals had OP-1 and Infuse on opposite sides of the same animal. During sacrifice, it became clear that a continuous bony bridge had been formed, effectively linking the two surgical sites. As such, it was impossible to determine to which experimental agent
bone growth should be attributed, making comparison of their osteoinductivity impossible. In order to focus on the direct comparison of bone induction by OP-1 and Infuse, we eliminated the other experimental groups and ensured that each animal had only one experimental group and a contralateral control. It was hoped that these changes would allow better assessment of the volume from each agent and thus allow a direct comparison.

For the current experiment, the protocol utilized twenty male CD-1 mice aged 37-42 days which were divided into 2 groups of 10 animals each. Both the OP-1® and Infuse® animals had an experimental side and a control side. Within each group, half the animals received the bioimplant on the left and the other half on the right. The control side in both groups consisted of an empty #5 gelatin capsule containing collagen sponge alone, which had been sterilized over chloroform vapour.

2.3.2 Fabrication of bioimplants

2.3.2.1 OP-1®

OP-1® is available as a single use vial containing 3.5mg of rhBMP-7 lyophilized onto 1g of purified bovine Type I bone collagen (Stryker Biotech). Based on published data, the implant would thus require 15mg of the lyophilized OP-1® powder to provide 52.5μg of rhBMP-7. Using an analytical balance (Sartorius, Germany), 15mg of OP-1® powder was measured and placed into a #5 gelatin capsule (Torpac Inc., USA). The capsules were placed in sterilization pouches, sealed and then sterilized by exposure to
chloroform gas for 4 hours. The pouches were then placed in a running biological safety hood overnight to remove all the chloroform vapours.

2.3.2.2 INFUSE®

Infuse® is designed to be used as a reconstituted powder added to an absorbable collagen sponge (ACS). Absorbable collagen sponges have a long history of medical application. Their fabrication involves lyophilization of bovine Type 1 collagen obtained from the Achilles tendon. It then undergoes chemically based cross-linking and sterilization (Geiger, Li et al. 2003). The kit supplied by Medtronic contains 1 vial of 4.2mg of lyophilized rhBMP-2; 2 -1" x 2” ACS; and 5mL vial of sterile water. The ACS was cut into 4x8mm sections in order to fit into the #5 Gelatin capsules and sterilized with chloroform vapors as described above. On the morning of the surgery, the 4.2mg of rhBMP-2 powder was reconstituted according to the package instructions with sterile water provided in the Infuse package, yielding a total graft of 2.8mL, with 1.5mg of rhBMP-2/mL (Medtronic Infuse® package insert). In order to achieve the 52.5μg necessary for the implant, a 200 μL micropipette was used to draw a 35 μL sample from the reconstituted graft. At the time of surgery, the 35μL was then added to an open gelatin capsule with the inserted ACS for each experimental site.
2.3.3 Surgical Phase

2.3.3.1 Implant placement

The surgery was performed in the animal surgical suite at the Faculty of Dentistry. The animals were labelled using ear tags and then anesthetized using 2% isoflurane inhalational anesthetic via a nasal cone. Electric clippers were used to remove fur over the dorsal aspect of the pelvis and hind quarters. The skin was prepared using Providone iodine, and the animal draped using disposable sterile paper drapes.

A #10 scalpel blade was used to make an incision along the dorsal midline through skin and subcutaneous tissue for approximately 3cm. Blunt dissection was utilized to gain access to and subsequently beneath the gluteus superficialis muscle. The muscle pouch was developed and the capsule inserted (Figures 4a-d). In the case of the OP-1® and control capsules, insertion into the pouch was aided by lubrication with a small drop of sterile saline. The unsealed Infuse® capsules were inserted dry and then had the aqueous BMP-2 added to the section of ACS within the open capsule via a micropipette. The gelatin capsule was not sealed with its cap due to diminished capsule integrity once the liquid was added. This finding was confirmed during previous use of this model.

The skin wound was then reapproximated and closed using 2-3 surgical clips. Each subject then received buprenorphine injected subcutaneously for post-operative pain control and was then allowed to recover in a private cage prior to being returned to
its original cage population. The cages were then returned to the small mammal
dormitory of the animal care facility.

The subjects were checked on post-op day one to ensure no wound dehiscences
or complications occurred. All subjects appeared well and their progress was monitored
regularly throughout the 28 day period. Wound clips were removed on post-op day 4.
None of the subjects demonstrated any significant post-op complications and all
regained excellent mobility of the hind legs.

2.3.3.2 Sample Harvest

The subjects were euthanized on post-op day #28 using a sealed carbon dioxide
chamber followed by cervical dislocation. Surgical scissors were used to dissect the
skin off the hind quarters. A bone cutting forceps was then utilized to sever then spinal
column cephalad to the pelvis as well as to remove the feet at the level of the ankles.
Subjective assessment of the ectopic bone formation suggested that OP-1® had
resulted in a greater volume of bone formation. The samples were then placed into
labelled 50mL sealable polypropylene test tubes (BD Falcon, Canada) containing 10%
buffered formalin for tissue fixation.
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**Table 3**: Cohort Breakdown for *in vivo* experiment
2.3.4 Radiographic Evaluation

2.3.4.1 CT protocol, calibration and reconstruction

The specimens were imaged using a General Electric Healthcare eXplore Locus SP microCT scanner. The scanner is equipped with two specimen tubes for scanning, a larger diameter tube and a smaller diameter tube. Due to the size of the mouse hindquarter specimens, the larger diameter tube was required. Specimens were placed individually into the large standardized scanning tube oriented with the pelvis inferiorly. In addition to the sample, the tube was packed with moistened cheesecloth to stabilize the specimen and ensure no shifting occurred during the scan. With each sample we also included the standard provided by the manufacturer, to act as an internal control.

An initial scout scan was performed, giving an overall image from which an area of focus was then selected for scanning at full resolution (30μm). The full resolution scan utilized the manufacturer's protocol "large tube, 120 minutes".

Image calibration is then completed utilizing the GE issued calibration block. The block contains areas consistent with air, water, and cortical bone. The bone standard is actually a substance referred to as SP3 or Gammex. This standard is designed to give a calibration value of approximately 3500 Hounsfield units which roughly translates to a bone mineral density of 1050mg of hydroxyapatite per mL. The calibration block was run with each sample in order to serve as an internal control to assess the inter-scan variance. The variance in the value of the calibration block was analyzed to ensure results not due to variance in scan. The mean values for air, water and bone were
calculated and from that a standard deviation and coefficient of variance were then determined (Table 4). The coefficient of variance was found to be 6%, which suggests that the scans were well calibrated and the results should be repeatable.

Final image reconstruction was performed via the reconstruction utility within the GE Healthcare eXplore MicroView v.2.0 software. Reconstruction was performed using the “half resolution” option to limit file size and reconstruction time. The resultant image had resolution of 59μm.

2.3.4.2 Image formatting and selection of Region of Interest

Following image reconstruction, the image required further manipulation to ensure the analysis applications were administered on the appropriate area (the newly formed bone only, excluding the femur and pelvic girdle). As such, a region of interest (ROI) needed to be determined. This area was set to encompass all areas containing newly formed bone and would be the only data to be analyzed for bony content. The technique to accurately outline the ROI is referred to as splining.

Splining involves outlining the bony mass within the area of focus on individual CT slices in a peripheral halo technique. A series of points are selected manually shadowing the edge of the bony mass. The points are then connected by the software to outline external contour of the bony mass (Figure 5). The CT of each specimen comprised of approximately 700 slices. Manual splining was done approximately every
20-30 slices with auto-splining interpolating the contours of the remainder. The splining was completed by one individual who also reviewed every auto-splined slice to ensure the computer generated contour included all areas of new bone formation and no native bone. Thus a 3-dimensional ROI is developed for the bony mass (Figure 6).

2.3.4.3 Threshold Value

Images obtained via computed tomography (CT) are based on the interaction of the x-ray photons and the tissues they encounter and the degree to which the tissues attenuate or block the photons. Different tissues have different abilities to attenuate x-ray photons and are thus assigned different attenuation coefficients. The attenuation coefficient for a particular tissue type is a reflection of the tissue density. Tissues with greater density (e.g. bone) have a higher attenuation coefficient than lower density tissues (e.g. adipose).

Images are first obtained as single two-dimensional (2-D) images comprised of pixel values. Each pixel value is assigned an arbitrary density unit (ADU). When the 2-D images are reconstructed to 3-D volumes, the pixels become represented by a volume rather than a point and are referred to as voxels. Additionally, during the conversion from 2-D to 3-D, the ADUs are converted to CT units. These newly obtained CT units are then calibrated to Hounsfield units (HU), a standardized unit of CT contrast. This calibration is done via comparison with known standards of air, water and bone, hence the inclusion of the calibration block.
In order to analyze the quantity and quality of bone within the ROI, the CT images must be segmented into bone and non-bone phases. Following the detailed scan and subsequent reconstruction, the ROI will include not only bone but other tissues and constituents included in the scan. Segmentation is completed by comparing individual voxel greyscale values against a set threshold value (Fajardo, Ryan et al. 2002). The bone-non-bone boundary is formed more by a gradient of greyscale values than a discrete interface (Coleman and Colbert 2007). Those voxels with values equal to or greater than the threshold value are counted in the bone phase. Those voxels with values less than the threshold value are counted in the non-bone phase.

Our threshold value was chosen by first evaluating the calibration values of the standardized calibration block as discussed previously. We then began a process of utilizing several trial threshold values to analyze samples and compared the results of the software analysis. These trials pointed to an approximate value which maximized the bony volume without including other tissues. We then set this value as our threshold.

It was noted that the selected threshold value was approximately 30% of the calibration value. Coincidentally, a published abstract by Rosello (2005) suggested that the “optimal threshold for determining volume fraction of regenerated bone is 950”. By looking at our mean value for the “bone calibration values”, we see that 950 is approximately 30% of our mean value. This provided further support for our selected value and as such, a 30% multiplier was applied to the individual calibration values to set the overall threshold value at approximately 1/3 of 3392.
2.3.4.4 Image Analysis

To the newly created ROI, bone analysis functions associated with the post-CT scan software (MicroView v2.0.029) were performed including total volume (TV), bone volume (BV), bone mineral density (BMD), bone mineral content (BMC), tissue mineral density (TMD), tissue mineral content (TMC) and bone volume fraction (BVF). The descriptions of each of these parameters is listed in table 5.

Review of the reconstructed images demonstrated that the mass of induced bone was not necessarily contiguous. Over half (12/20) of the specimens demonstrated more than one distinct ossicle. In order to obtain the most accurate volumetric analysis, we opted to categorize the ossicles into two groups: primary and secondary. Primary ossicles were defined as the largest contiguous induced ossicle. Secondary ossicles were then all other induced bony ossicles in the ROI. Determining the primary versus secondary ossicles was not difficult as there was an obvious main ossicle with often several satellite ossicles adjacent to it (Figure 7).

The bone analysis parameters were applied individually to the primary and secondary ossicles as well as to the total bone volume which included primary and secondary ossicles.
### Table 4: Variance in Calibration Values of mCT

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<th>Definition</th>
<th>Threshold Dependant</th>
<th>Quantity vs. Quality</th>
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<td>Includes total volume selected for analysis. Includes all volume assumed by bone, soft tissues and fluids</td>
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<td>Quantity</td>
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<td>Quantity</td>
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<td>Bone Mineral Content</td>
<td>Determines mineral (calcium) content within the region of interest</td>
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<td>Quantity</td>
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<tr>
<td>Bone Mineral Density</td>
<td>Determines value of mineral density within the region of interest (Mineral mass/Volume of ROI)</td>
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<td>Quality</td>
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<td>Tissue Mineral Content</td>
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<td>Tissue Mineral Density</td>
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<td>Quality</td>
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<tr>
<td>Bone Volume Fraction</td>
<td>Compares the fraction of bone greater than the threshold value to the total volume within the ROI</td>
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<td>Quality</td>
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### Table 5: Overview of bone microCT bone analysis parameters
2.4 Statistics

2.4.1 Sample Populations and comparison of means

All categories of CT tissue analysis were analyzed using Sigma Stat v3.5 statistical software (Systat, Richmond CA). All data were evaluated for normality and equal variance. Results that were distributed normally were compared using Student’s t-test. If the distribution was found to be non-parametric and/or the variance was found to be abnormal, the t-test was aborted and the Mann-Whitney Rank Sum Test was used. The tests were carried out to assess for statistically significant differences between the OP-1 and Infuse groups with significance established using a ‘p’ value of < 0.05 for each CT analysis category as mentioned previously.

2.5 Histological Evaluation

Following completion of the microCT analysis, the specimens were prepared for histological assessment. The fixed specimens were decalcified in a solution of 45% formic acid in 20% sodium citrate for a period of 6 weeks. The decalcified specimens were then embedded in paraffin wax in preparation for serial sections through the induced bony spicule. Sections were performed perpendicular to the long axis of the native femur and subsequently stained with hematoxylin and eosin. Sections from each
specimen were then examined via light microscopy (Leitz, Wetzlar, Germany) to confirm bony makeup of the induced masses. Digital images of a representative section from each specimen were then obtained for purposes of reporting.
Figure 4: a – skin incision and muscle exposure; b - Development of hind quarter muscle pouch

Figure 4: c – insertion of bioimplant capsule, d – wound closure with clips
Figure 5: Screen shot of reconstructed image demonstrating splined outline of the primary bone ossicle

Figure 6: 3-D reconstructed image from image analysis software
Figure 7: Reconstructed 3-D image of mouse pelvis, spinal column and legs. Primary ossicle is outlined in green. Secondary ossicle is shaded yellow.
CHAPTER THREE: RESULTS

3.1 In vitro

3.1.1 In vitro comparison of osteoinductivity of rhBMP-2 and rhBMP-7

Exposure of the C2C12 cells to both experimental agents resulted in an increase in alkaline phosphatase (ALP) production. Production of ALP was markedly higher in the rhBMP-2 group at all time points. The mean ALP concentrations were approximately 6 times greater than the rhBMP-7 group at 3 days and 3 times greater at 6 days (Figure 8).

3.2 In Vivo

3.2.1 Necroscopy

During the process of harvesting the samples several recurring observations were noted. The induced bony ossicle was not limited to the side of the implant. In many specimens, the ossicle spanned the vertebral column. Subjective assessment of the ectopic bone formation suggested that OP-1® had resulted in a greater volume of bone formation.
3.2.2 Micro CT analysis

Results of the mCT bone analysis are represented graphically with use of box plots. Box plots are often used to display differences between populations regardless of the constituent data distribution. The box itself represents 50% of the data with the upper and lower borders at the 25th and 75th percentiles respectively. The size of the box can convey the dispersion of the data. The line within the box represents the median or 50th percentile. The whiskers represent all data 40% above and below the median. Although the meaning of the whisker ends can differ, in this study the whisker ends represent the 10th and 90th percentiles. Thus, the box plus whiskers represent 80% of the data. Closed dots near the whisker ends represent the highest and lowest values.

3.2.2.1 Entire Induced Bone
(see also Table 6 - Statistical Summary for Total Induced Bone)

**Total Volume** (mm$^3$): This group includes all bone and soft tissue within the region of interest. (Figure 9) Population passed the normality test (P=0.422). The mean total volume for the OP-1 group (529.528mm$^3$) was 2x greater than the Infuse group (225.811 mm$^3$) and was found to be significantly greater using a T-test (p<0.001).

**Bone Volume** (mm$^3$): The data did not demonstrate a normal distribution. Using a Mann-Whitney Rank Sum Test, a significant difference was found between the bone
volumes within the region of interest, with OP-1 (median = 106.290mm\(^3\)) significantly greater than Infuse (median = 50.652mm\(^3\)) with a p value of 0.031. (Figure 10)

**Bone Mineral Content** (mg): Data passed the normality test. T-test analysis showed significantly higher bone mineral content in the OP-1 group (p=0.023) with the mean OP-1 (66.187mg) almost double the mean of the Infuse group (34.044mg). (Figure 11)

**Bone Mineral Density** (mg/mm\(^3\)): Using a T-test, mean bone mineral density difference was not statistically significant (p=0.6). (Figure 12)

**Tissue Mineral Content** (mg): The data failed the normality test, thus analysis was carried out using the Mann-Whitney Rank Sum Test. Comparison demonstrated a significantly greater tissue mineral content in the OP-1 group (p=0.045). (Figure 13)

**Tissue Mineral Density** (mg/mm\(^3\)): The data failed the normality test and a Mann-Whitney Rank Sum Test was utilized. The Infuse group showed a higher mean tissue mineral density, however the difference was not statistically significant (p=0.186). (Figure 14)
**Bone Volume Fraction**: Using a T-test, the mean values for the Infuse and OP-1 groups compared but no significant difference existed between them (p=0.55). (Figure 15)

3.2.2.2 Primary Ossicle  
(see also Table 7: Statistical Summary for Primary Ossicles)

**Total Volume** (mm$^3$): The total volume was found to be significantly greater in the OP-1 group than the Infuse group (p=0.002). (Figure 16)

**Bone Volume** (mm$^3$): Comparison demonstrated a significant difference in the bone volumes within the region of interest with OP-1 significantly greater than Infuse (p=0.038). (Figure 17)

**Bone Mineral Content** (mg): Analysis showed significantly higher bone mineral content in the OP-1 group (p=0.026). (Figure 18)

**Bone Mineral Density** (mg/mm$^3$): The mean bone mineral density was greater in the Infuse group but the difference was not statistically significant (p=0.628). (Figure 19)
**Tissue Mineral Content** (mg): Sample population failed the normality test and Mann-Whitney Rank Sum Test performed. A significant difference was detected between the means (p = 0.045). (Figure 20)

**Tissue Mineral Density** (mg/mm$^3$): Sample population failed the normality test and Mann-Whitney Rank Sum Test performed. No significant difference was detected between the means (p = 0.212). (Figure 21)

**Bone Volume Fraction**: The mean values for the Infuse and OP-1 groups were very close and no significant difference existed between them (p=0.65). (Figure 22)

3.2.2.3 Secondary Ossicle
(see also Table 8: Statistical Summary for Secondary Ossicles)

**Total Volume** (mm$^3$): The total volume was found to be significantly greater in the OP-1 group than the Infuse group (p=0.292). This group includes all bone and soft tissue within the region of interest. (Figure 23)

**Bone Volume** (mm$^3$): Comparison demonstrated a significant difference in the bone volumes within the region of interest with OP-1 significantly greater than Infuse (p=0.558). (Figure 24)
**Bone Mineral Content** (mg): Analysis showed significantly higher bone mineral content in the OP-1 group (p=0.329). (Figure 25)

**Bone Mineral Density** (mg/mm$^3$): The mean bone mineral density was greater in the Infuse group but the difference was not statistically significant (p=0.959). (Figure 26)

**Tissue Mineral Content** (mg): Sample population failed the normality test and the Mann-Whitney Rank Sum Test was performed. No significant difference was found between the means (p=0.558). (Figure 27)

**Tissue Mineral Density** (mg/mm$^3$): Sample population failed the normality test and the Mann-Whitney Rank Sum Test was performed. No significant difference was found between the means (p=0.458). (Figure 28)

**Bone Volume Fraction**: The mean values for the Infuse and OP-1 groups were very close and no significant difference existed between them (p=0.484). (Figure 29)
3.2.3 **Histological Analysis**

Inspection of the histologic specimens revealed bone formation in both the Infuse and OP-1 groups. No bone formation was noted in the control samples.

Specimens from the Infuse group demonstrated woven bone formation as well as a significant cartilaginous component. The immature woven bone contained normal appearing osteocytes within lacunae and osteoblastic rimming. (Figure 30a & b). Several specimens showed patterns consistent with endochondral bone formation (Figure 31). Hypertrophic chondrocytes were also commonly seen in many specimens.

Histology of the OP-1 specimens demonstrated woven bone with embedded osteocytes in lacunae (Figure 32). Very little cartilage was noted with the exception being calcified cartilage matrix within bone trabeculae in some specimens (Figure 33). Bone formation was often found in the form of a thin peripheral shell (Figure 34). OP-1 specimens overall demonstrated greater organization of the bony trabeculae and marrow space with examples of both red and yellow marrow (Figure 35a & b), suggesting greater maturity of the specimens compared to the Infuse group.
Figure 8: Alkaline Phosphatase production over time following exposure to BMPs
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**Table 6**: Statistical Summary for Total Induced Bone

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**Table 7**: Statistical Summary for Primary Ossicle
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**Table 8**: Statistical Summary for secondary ossicles
Figure 9: Difference in the total volume of all bone induced in OP-1® or Infuse® treated mice (n=10). The difference in the mean values of the two groups is statistically significant (P=<0.001).

Figure 10: Difference in the bone volume of all bone induced in OP-1® or Infuse® treated mice (n=10). Using the Mann-Whitney Rank Sum Test, the difference in the mean values of the two groups is statistically significant (P=0.031).
**Figure 11:** Difference in the bone mineral content of all bone induced in OP-1® or Infuse® treated mice (n=10). The difference in the mean values of the two groups is statistically significant (P=0.023).

**Figure 12:** Difference in the bone mineral density of all bone induced in OP-1® or Infuse® treated mice (n=10). The difference in the mean values of the two groups is not statistically significant (P=0.600).
Figure 13: Difference in the tissue mineral content of all bone induced by OP-1® or Infuse® treated mice (n=10). Using the Mann-Whitney Rank sum Test, the difference in the mean values of the two groups was found to be statistically significant (P=0.045).

Figure 14: Difference in the tissue mineral density all bone induced by OP-1® or Infuse® treated mice (n=10). Using the Mann-Whitney Rank sum Test, the difference in
the mean values of the two groups was not found to be statistically significant (P=0.186).

**Figure 15:** Difference in the bone volume fraction of all bone induced by OP-1® or Infuse® treated mice (n=10). The difference in the mean values of the two groups is not statistically significant (P=0.550).
**Figure 16:** Difference in the total volume of the primary ossicle induced by OP-1® or Infuse® in mice (n=10). The difference in the mean values of the two groups is statistically significant (P=0.002).

**Figure 17:** Difference in the bone volume of the primary ossicle induced by OP-1® or Infuse® in mice (n=10). Using the Mann-Whitney Rank Sum Test, the difference in the mean values of the two groups is statistically significant (P=0.038).
**Figure 18**: Difference in the bone mineral content of the primary ossicle induced by OP-1® or Infuse® in mice (n=10). The difference in the mean values of the two groups is statistically significant (P=0.026).

**Figure 19**: Difference in the bone mineral density of the primary ossicle induced by OP-1® or Infuse® in mice (n=10). The difference in the mean values of the two groups is not statistically significant (P=0.628).
Figure 20: Difference in the tissue mineral content of the primary ossicle induced by OP-1® or Infuse® in mice (n=10). Using the Mann-Whitney Rank sum Test, the difference in the mean values of the two groups was found to be statistically significant (P=0.045).

Figure 21: Difference in the tissue mineral density of the primary ossicle induced by OP-1® or Infuse® in mice (n=10). Using the Mann-Whitney Rank sum Test, the
difference in the mean values of the two groups was not found to be statistically significant (P=0.212).

**Figure 22**: Difference in the bone volume fraction of the primary ossicle induced by OP-1® or Infuse® in mice (n=10). The difference in the mean values of the two groups is not statistically significant (P=0.650).
**Figure 23:** Difference in the total volume of secondary ossicles induced by OP-1® or Infuse® in mice (n=10). Using the Mann-Whitney Rank Sum test, the difference in the mean values of the two groups was not found to be statistically significant (P=0.292).

**Figure 24:** Difference in the bone volume of secondary ossicles induced by OP-1® or Infuse® in mice (n=10). Using the Mann-Whitney Rank Sum Test, the difference in the mean values of the two groups was not found to be statistically significant (P=0.558).
Figure 25: Difference in the bone mineral content of secondary ossicles induced by OP-1® or Infuse® in mice (n=10). Using the Mann-Whitney Rank Sum test, the difference in the mean values of the two groups was not found to be statistically significant (P=0.329).

Figure 26: Difference in the bone mineral density of secondary ossicles induced by OP-1® or Infuse® in mice (n=10). Using the Mann-Whitney Rank Sum Test, the difference
in the mean values of the two groups was not found to be statistically significant (P=0.959).

Figure 27: Difference in the tissue mineral content of secondary ossicles induced by OP-1® or Infuse® in mice (n=10). Using the Mann-Whitney Rank sum Test, the difference in the mean values of the two groups was not found to be statistically significant (P=0.558).
Figure 28: Difference in the tissue mineral density of secondary ossicles induced by OP-1® or Infuse® in mice (n=10). Using the Mann-Whitney Rank Sum Test, the difference in the mean values of the two groups was not found to be statistically significant (P=0.458).
Figure 29: Difference in the bone volume fraction of secondary ossicles induced by OP-1® or Infuse® in mice (n=10). The difference in the mean values of the two groups was not found to be statistically significant (P=484).
Figure 30a: Photomicrograph of Hematoxylin & Eosin stained induced ossicle from an Infuse subject (Mouse 129 Infuse R 10x). Specimen shows osteocytes within lacunae and osteoblastic rimming along immature bony trabeculae.
Figure 30b: Higher power (25x) photomicrograph of immature bony trabecula with osteocytes within lacunae (Mouse 124 Infuse L).
**Figure 31**: Photomicrograph of H&E stained induced ossicle in an Infuse subject (Mouse 126 Infuse L) demonstrating histologic pattern consistent with endochondral bone formation at higher power (10x).
**Figure 32:** Photomicrograph of a H&E stained section of an induced ossicle from an OP-1 subject (Mouse 119 OP-1 R 25x) demonstrating osteocytes in lacunae.
**Figure 33:** Photomicrograph of H&E stained section of induced bony ossicle from an OP-1 subject (Mouse 119 OP-1R 10x). Basophilic staining areas represent tissue of cartilaginous origin surrounded by woven bone.
Figure 34: Photomicrograph of H&E stained section of induced ossicle from an OP-1 subject (Mouse 112 OP-1L 10x). The form of the induced bone was often that of a cortical shell with immature marrow within. This specimen demonstrates a thin bony shell interface with the adjacent muscle.
Figure 35a: Photomicrograph of induced ossicle of subject in OP-1 group (Mouse 112 OP-1L 4x) demonstrating yellow marrow with bone trabeculae.
Figure 35b: Photomicrograph of H&E stained section of induced ossicle from OP-1 subject (Mouse 119 OP-1R 4x) demonstrating woven bone with red marrow.
Surgeons around the world are presented with a daily dilemma of finding the best methodology of reconstructing bony defects, solving fracture non-unions and augmenting existing bone volumes. Autogenous bone grafts have long been considered the gold standard and to that end, millions of autogenous bone grafts are performed each year to aid in surgical reconstruction of bony defects and fractures, spinal fusions and bony atrophy. Harvesting of autogenous bone necessitates a second surgical site with its own set of associated morbidities and complications including pain, neurovascular injury and infection and is reported to carry up to a 30% rate of unsatisfactory results (Dinopoulos and Giannoudis 2007). Alternatives to autogenous grafting have been extensively explored and utilized with mixed results. To date, no alternative has been able to replicate the success and relative predictability of autogenous grafting. One of the more recent alternatives suggested is the application of growth factors, most commonly bone morphogenetic proteins (BMPs). Initial studies have demonstrated incredible potential for induction of and replacement of large quantities of bone. Application of BMPs has not been without issue and concerns have been raised over the potential for a foreign body reaction, cost, predictability and safety.

Over the last decade, intensive research has been undertaken to prove the reliability and safety of rhBMP use in humans. It has been found that for rhBMPs to be effective in clinical application, they must be combined with a carrier or delivery system to form a bioimplant. Currently there are two rhBMP based bioimplants approved for specific human applications by both the United States Food & Drug Administration and
Health Canada: Infuse®, based on rhBMP-2, and OP-1®, utilizing rhBMP-7. A major disadvantage associated with the use of these rhBMPs is cost. A 1mg sample may cost thousands of dollars and may prove prohibitive for many patients and practitioners. Due to the high costs associated with their use, clinicians seek the highest return for their investment, namely the greatest quantity of induced bone.

Studies directly comparing the osteoinductive potential of BMPs are not common in the literature and there are no reports comparing the osteoinductive activity of the 2 commercially available BMP containing bioimplants. Therefore, the aim of this investigation was to compare the activity of the component BMPs alone and as formatted as the bioimplant as used clinically.

4.1 In vitro assay

This thesis set out to determine which commercially available rhBMP bioimplant had the greatest osteoinductive potential using \textit{in vitro} and \textit{in vivo} assays. The \textit{in vitro} assay utilized rhBMP-2 and -7 (R&D Systems, Minneapolis, MN) which should not be confused with the rhBMP-2 and -7 incorporated into the OP-1 and Infuse bioimplants. While the proteins involved should be identical, there are significant differences in their presentation and availability. The rhBMPs applied in the \textit{in vitro} experiments are purely mature bone morphogenetic protein. The rhBMPs in the bioimplants are combined with collagen carriers. These carriers serve to retain the BMP at the surgical site and control their availability to the local environment.
Both rhBMP-2 and rhBMP-7 were able to stimulate up-regulation of alkaline phosphatase production in mouse myoblast C2C12 cells with rhBMP-2 generating a significantly greater response than rhBMP-7. Studies from different investigators that compared the osteogenic activity of BMPs directly have shown repeatedly that BMP-2 consistently stimulates a greater osteogenic response than BMP-7 (Hazama, Aono et al. 1995; Cheng, Jiang et al. 2003; Li, Li et al. 2003; Kang, Sun et al. 2004; Luu, Song et al. 2007). These studies utilized baculoviral or adenoviral vectors to transfect cell lines and then measured alkaline phosphatase as a measure of osteoinductivity. Cheng et al. (2003) performed the largest direct comparison of BMP activity to date. In their study, the osteogenic activity of BMPs 2-15 were directly compared using adenoviral infection of multiple pleuripotent (C3H10 – mouse embryonic fibroblast derived), committed progenitors (C2C12 – mouse myoblast derived) and osteoblastic (TE-85 – committed human osteoblasts) cell lines. Alkaline phosphatase and osteocalcin production and matrix mineralization were then evaluated. The results demonstrated a clear hierarchy of osteogenic potential amongst the tested members of the BMP family. In the pleuripotent cell line, BMPs 6 and 9 showed strong activity with BMP-2 weaker but present and BMP-7 showing minimal activity. The C2C12 cell line the same line utilized in our study, showed especially strong activity of BMP-2, with BMP-6 and -9 close behind. However, BMP-7 again demonstrated weak activity which increased over the time course of the experiment, reaching a maximal value at the experiment terminus of 9 days. It is interesting to note that our results closely parallel those of Cheng et al., with rhBMP-7 reaching its greatest activity at the experimental terminus of 6 days, while rhBMP-2 peaked much earlier and attained considerably greater levels than rhBMP-7.
Another study provided an indirect comparison of the activity of BMP-2 and -7 while evaluating simultaneous gene transfer which also demonstrated greater ALP production from BMP-2 versus -7 in an *in vitro* model (Kawai, Bessho et al. 2006).

Mayer et al. (Mayer, Scutt et al. 1996) monitored DNA synthesis activity in periosteal cells, epiphyseal and sternal chondrocytes in embryonic chicks after exposure to BMP-2, 4, 5, 6, and 7. They noted the greatest response from BMP-2 and 4 and that the lowest specific activity was found with the BMP-7 cultures.

The current study provided C2C12 cells direct exposure to the BMPs and demonstrated results consistent with the published literature, that BMP-2 elicits a stronger osteoinductive response than BMP-7. However, certain study limitations must be recognized prior to applying the *in vitro* results towards analysis of rhBMP activity. Firstly, the current study utilizes only one cell line in the *in vitro* assay. The mouse myoblast C2C12 cell line is a validated osteoblast precursor and commonly used in this particular assay but it may not be representative of cellular response as a whole. Our laboratory has performed the ALP assay with 2 other cell lines: a mouse stromal cell line (W17-20) and a rat myoblast cell line (L6). Both showed similar results to the C2C12 cells (Peel unpublished). Additionally, studies mentioned previously (Hazama, Aono et al. 1995; Cheng, Jiang et al. 2003) performed the ALP assay with other cell lines including MC3T3-E1 (mouse osteoblastic) and C3H10T1/2 (pluripotential mesenchymal) with similar results. Secondly, when attempting to relate the *in vitro* to *in vivo* results, it must be recalled that the sources of the rhBMPs differ. After recombinant proteins are produced, their biologic activity must be assessed and verified. If the commercial source and lot are not consistent for each rhBMP, it cannot be assured that
the biologic activity of each rhBMP is equivalent. Thus caution is required when attempting to directly link the in vitro and in vivo performance.

4.2 In vivo assay

The in vivo portion of this study was the primary focus and the portion with which we hoped to create a foundation upon which further study could be based. It is this portion of the experiment which has never been reported in the literature, thus, we have attempted to provide a direct comparison of the two commercially available rhBMPs approved for human applications in their clinically available and intended forms.

4.2.1 State of the Literature

Both rhBMP-2 and -7 have previously been shown capable of inducing ectopic bone in animal models (Wang, Rosen et al. 1990; Sampath, Maliakal et al. 1992; Cook, Baffes et al. 1994; Cook, Baffes et al. 1994; Aspenberg and Turek 1996; Okubo, Bessho et al. 2000). As with the in vitro comparison, very few studies have directly compared the osteoinductive activity of BMPs in an in vivo assay. In a follow up study to that performed by Cheng et al. (2003), Kang et al. (2004) evaluated the osteogenic ability of 14 BMPs via both in vitro and in vivo assays. The in vitro C2C12 assay confirmed the previous findings that BMPs 2, 6 and 9 elicited the greatest ALP production while a lesser response was found with BMPs 4 and 7. The in vivo portion
included two assays involving injection directly into the quadriceps muscle of athymic nude mice. In the first assay, C2C12 cells transfected with the adenoviral vector for each of the 14 BMPs were injected. The second assay involved the injection of the adenoviral vectors themselves into the quadriceps muscle. In both assays, the area was then radiographed at 3 and 5 weeks post injection and the bony ossicles evaluated for radiodensity and size with subsequent histologic analysis. The results for both assays revealed BMP-6 and -9 demonstrating the greatest osteogenic activity with the largest and most radiodense ossicles and most mature histologic patterns. BMP-2 and -7 did induce detectable ossicle formation at both the 3 and 5 week time points. Histologic examination of the BMP-2 samples revealed well calcified foci at 3 weeks which developed into mature bone by 5 weeks. BMP-7 exhibited small foci of woven bone and was found to “induce much weaker ossification”. Both BMP-2 and -7 were found to contain more cartilage-like histologic elements than BMP-6 or -9 in the direct adenoviral injection assay. The authors later concluded that “while BMP-7 exhibited apparent osteogenic activity, its ability to induce ossification was significantly less robust than that of BMP-2, BMP-6 and BMP-9”. It is also interesting to note that BMP-4 failed to produce any discernable ossicle in either the C2C12 or adenoviral vector injection assays, despite inducing notable ALP production in the \textit{in vitro} assay. The authors were unable to explain this finding or put forth any possible theories to account for it. Other studies have demonstrated the ability of BMP-4 to induce ectopic bone formation (Chen, Cheung et al. 2002; Jane, Dunford et al. 2002; Kishimoto, Watanabe et al. 2002). This discrepancy points out the inconsistencies in reported results as well as the limitations on extrapolation of results to other test models or species. In summary, the
Kang study leads to the conclusion that the *in vivo* activity of the tested BMPs, including BMP-2 and -7, parallels the *in vitro* activity. Furthermore, it supports the accepted finding that there is a hierarchy of osteogenic activity amongst the osteogenic BMP family members with BMP-6 and -9 having the greatest activity, BMP-2 with moderate activity and BMP-7 with the least osteogenic activity.

Further support for the hierarchy of osteogenic activity is found in a recent study by Li et al. (2003). In their study, Li et al. compared the osteogenic potential of rhBMP-2, -4, -6, -7, and -9 via *in vitro* (C2C12 ALP assay) and *in vivo* assays. The ALP assay utilized C2C12 cells transfected with adenoviral vectors. The results differed from previous studies (Hazama, Aono et al. 1995; Cheng, Jiang et al. 2003; Kang, Sun et al. 2004) in the hierarchical order of BMP activities. Li et al. found the greatest activity in BMP-9, with BMP-4 and -6 considerably less while BMP-2 and -7 demonstrated the least activity. The *in vivo* assay involved direct injection of adenovirus expressing one of the five BMPs (2, 4, 6, 7, or 9) into the thigh musculature of athymic nude mice and, in a second assay, into the thigh musculature of immunocompetent Sprague-Dawley rats. In both assays, the animals were radiographed with a CT scanner to assess and compare induced bone volumes. The mice were euthanized after CT scan and samples collected for histologic analysis. Data from the CT scan revealed the greatest induced bone volume from BMP-6, followed by BMP-4, -9, -2, and -7 in descending order. The activity hierarchy was duplicated at two different adenoviral injection dosages. The data was analyzed using single-factor ANOVA which demonstrated significant differences between all groups, suggesting that BMP-2 is induced significantly greater bone production than BMP-7. Interestingly, results from the immunocompetent Sprague-
Dawley rats was markedly different from the mice. Only BMP-6 and -9 induced detectable bony ossicles, while no bone was induced at the BMP-2, -4, and -7 injection sites. The authors go on to report that the osteogenic potential of injected BMP-9 adenovirus was inconsistent in a series of unpublished trials across 5 different rat strains. These results suggest that differences amongst animal species or strains impact the dosing required to achieve comparable results. The apparent hierarchy of osteoinductive activity amongst the various BMPs may due in part to receptor expression and binding affinity. BMP receptor expression is dependant on cell type, location and local physiology. Type I receptor expression is up-regulated in the periosteum and cells in the fracture callus shortly after stimulation of the healing cascade (Ishidou, Kitajima et al. 1995). BMP-2 and -4 bind preferentially to BMPR-1a (ALK and BMPR-1b (ALK-6) while BMP-7 binds preferentially to BMPR-1b (ALK-6) (Bessa, Casal et al. 2008).

Thus the literature available for comparison confirms that BMP-2 elicits a greater osteogenic response than BMP-7 in \textit{in vivo} experiments but that the magnitude may vary according to dose and experimental design. It must be noted however, that the available literature is comparing BMPs, not the approved bioimplants formulated with the BMPs. Thus caution must be used in extrapolating the previous \textit{in vivo} results to studies examining bioimplants.

The results of our study are in direct opposition to the consensus in the literature. We observed consistent superiority of OP-1 over Infuse in both the total induced volume and primary ossicles across all micro CT analysis variables related to bone volume. The variables “bone mineral density”, “tissue mineral density” and “bone volume
fraction” demonstrated no significant differences between the experimental groups. The volume is based on the bony perimeter but without any internal structure or trabeculae, the induced bone would be weak and incapable of providing structural support. Tissue mineral density provides a comparison of the amount of calcium per unit of bone. Bone maturity may be inferred by the degree of mineralization, with higher mineralization meaning greater maturity. Bone volume fraction is strongly related to density. It is an indicator of the proportion of bone within the ROI. A higher bone volume fraction indicates that a greater proportion of the ROI is occupied by bone and thus suggests a more developed bony lattice within the cortical shell. These parameters suggest that the quality of bone, i.e. its mineral content and density, does not differ significantly.

Taking into account all micro CT analysis variables, we see that OP-1 generates a greater volume of bone with equal quality. Again, we must recognize weaknesses within the current study that limit the extrapolation of our results. We have attempted to compare the osteoinductive activity of the two rhBMP based bioimplants approved for human application. The comparison was performed via a rodent model. Thus it is possible that the behaviour of the bioimplants in the animal model may differ from their behaviour in humans. Perhaps mouse pluripotential cells do not react to the human BMP in the same way they would react to mouse BMP. While this possibility must be considered, it is reported that the mature coding region of mouse and human BMP-2 are identical (Feng, Harris et al. 1994) and that mouse and human BMP-7 share 98% homology (R&D systems). As such, it would seem that mouse cellular response should closely mirror that seen in human cells.
Other authors have noted that results from animal models are often difficult to reproduce in humans and that there is a clear species-specific dose response (Luginbuehl, Meinel et al. 2004). There may be a difference in the recruitment of bone precursor cells in small animals versus humans (Bessa, Casal et al. 2008). It has also been postulated that there is a larger quantity of pluripotential cells in the bone and soft tissues of small animals and rodent models cannot predict the does of osteogenic factors needed to induce bone in humans (Seeherman, Wozney et al. 2002).

We must then endeavour to explain the marked disparity between our results and the consensus in the literature. There are several possible avenues to explore, primarily involving experimental design, but also the characteristics of the experimental agents themselves.

4.2.2 Explanation of Results – Experimental Design

Results from the studies previously discussed have reasonably consistent results likely due to similar experimental design. These studies involved implantation of either cells transfected with adenovirus expressing BMPs or direct injection of the adenovirus itself. We have attempted to compare the two commercially available bioimplants by applying them in their approved clinical form. By evaluating the performance of the bioimplants, we have taken the next step from comparison of the raw rhBMPs towards clinical comparison.

We must recall that the bioimplants are vastly different in their makeup and handling characteristics. According to the Stryker OP-1 website, the OP-1 implant contains exactly 3.5mg of rhBMP-7 on 1g of bovine bone type 1 collagen. The implant
is designed to be reconstituted with 2-3mL of sterile saline to form a paste with the consistency of wet sand. No other ingredients or additives are mentioned. It must be realized that the OP-1 bioimplant is not a single contiguous structure, rather it is a conglomeration of rhBMP-7 containing collagen granules. Thus the implant requires careful handling to avoid dispersion and seeding of adjacent tissues with the osteogenic growth factor. The Infuse bioimplant however, is markedly different, being aqueous in nature. Following reconstitution of the rhBMP-2 powder with sterile water, a solution is formed which contains: 1.5mg rhBMP-2, 5.0mg sucrose, 25mg glycine, 3.7mg glutamic acid, 0.1mg sodium chloride, 0.1mg polysorbate 80 and 1mL of sterile water. Due to the aqueous nature, certain additives are required to aid in maintaining the rhBMP-2 powder in solution and stability of the solution. The ACS is formed by lyophilization of bovine type 1 collagen obtained from the Achilles tendon. The fact that OP-1 utilizes bovine type 1 collagen obtained from bony sources and Infuse bovine type 1 collagen is from the Achilles tendon is interesting. However, type 1 collagen comprises more than 90% of extracellular proteins in bone and tendon and is biochemically identical from either source (Friess 1998). Thus the differences in collagen source between the two carrier systems would not be expected to affect their osteoinductive abilities. After addition to the ACS, the Infuse bioimplant is one contiguous structure and manipulation and implantation are relatively straightforward.

Because OP-1 is supplied as a dry powder, it could easily be pre-measured, placed into the capsules and sterilized preoperatively. Implant preparation did vary from the described preparation by the manufacturer (Stryker Biotech OP-1® package insert).
The described preparation involves the addition of 2-3mL of 0.9% sterile sodium chloride solution to the powder based BMP until the desired consistency is achieved. It then undergoes expansion over the course of the next 2 minutes to a maximum volume of approximately 4cc. It should then be used promptly.

Given the nature of the Infuse bioimplant makeup, certain adjustments needed to be made to the manufacturer’s directions prior to implantation. The supplied instructions dictate that following reconstitution of the rhBMP component, it is to be added to the ACS and allowed to soak for 15 minutes prior to insertion into the patient (Infuse package insert). This process is not conducive to placement of placement of multiple fragments of the soaked ACS into multiple patients. As such, we elected to pre-cut the ACS into sections that could be loaded into capsules destined for both the Infuse group and the control group. These capsules could then be pre-sterilized to ensure appropriate infection control and surgical efficiency. Additionally, sectioning of the ACS and insertion into the gelatin capsules pre-operatively allowed insertion of a ‘dry’ implant, similar to the OP-1 group. This becomes important during surgery as the gelatin capsules lose all structural integrity after coming into contact with fluids. The impaired physical properties and handling characteristics of a ‘wet’ implant or capsule would likely preclude its successful implantation. Additionally, because the Infuse capsules contained a section of ACS, the capsule lids were not applied to allow addition of the reconstituted aqueous rhBMP-2 via micropipette. During surgery, it was noted that the Infuse liquid was not well contained by the open capsule and thus communicated with the muscle pouch and surgical access point. It seems reasonable
that given the limitations on placement of the Infuse implant, its ability to induce bone formation may have been impaired by poor retention of the BMP at the site.

A study by Seeherman et al. (Seeherman, Wozney et al. 2002) evaluated retention of rhBMP-2 at a rabbit ulnar osteotomy site amongst delivery systems. They reported that use of an aqueous buffer delivery system resulted in only 5% of the initial BMP dose remaining at 7 days, whereas use of delivery vehicles such as gelatin foam, collagen sponge or calcium phosphate paste showed retention of 25-55% of initial BMP dose at 7 days. Ruhe and Boerman (Ruhe, Boerman et al. 2006) assessed radiolabelled rhBMP-2 release from Calcium-Phosphate discs and utilized an aqueous 5μg rhBMP-2 subcutaneous injection for control. Analysis revealed only 8±12% of rhBMP-2 remained at the surgical site after 24 hours. Einhorn et al. (Einhorn, Majeska et al. 2003) later clarified the issue further by suggesting that “BMP in an aqueous form may not result in a sufficiently long residence time for the osteoinductive protein to provide optimal bone induction…” The Infuse bioimplant does indeed have a delivery vehicle in the form of the ACS. However, the methodology of this study may not allow the ACS to reach its full potential. Following reconstitution with sterile water and addition to the ACS, the Infuse package insert recommends letting the wetted sponge stand for at least 15 minutes. This step in surgical implantation is made very clear and thus suggests its importance. It has been shown that in the Infuse system, rhBMP-2 binds to the ACS and that longer soak time is associated with an increase in rhBMP-2 incorporation within the sponge (Friess, Uludag et al. 1999; Friess, Uludag et al. 1999). Soak times ranging from 5-30 minutes were examined, and the authors commented that only a small increase in rhBMP-2 binding was found in soak times longer than 5
minutes. They also demonstrated that if allowed to soak and incorporate, rhBMP-2 loss was minimized during handling, with 44-90% of the rhBMP-2 dose retained with in the ACS depending on soak time and ACS mass. A minimum incorporation of 20% was noted due to the inability to express that portion of the applied liquid. The ACS in this study was not given any soak time. The incorporation and retention of the rhBMP-2 to the ACS at the surgical site may have been affected and thus may have been a contributing factor in the decreased bone induction associated with this implant. Perhaps a future study utilizing Infuse could more closely follow the manufacturer’s instructions by applying the reconstituted rhBMP-2 to the ACS for the prescribed 15 minute soak time, cleaving off an appropriate section of the impregnated ACS and placing the section into an implanted capsule.

Application of a growth factor such as a BMP, requires a carrier or delivery system to make it clinically feasible. These delivery systems serve a multitude of functions and are vital for successful osteoinduction. The have both physical and pharmacokinetic requirements. Qualities of the ideal delivery system have been reviewed elsewhere (Winn, Uludag et al. 1999; Dipietro, Reintjes et al. 2001; Seeherman, Wozney et al. 2002; Geiger, Li et al. 2003; Seeherman and Wozney 2005) and include:

- Biocompatible
- Biodegradable
- Structural porosity to allow cellular invasion and vascularization
- Adequate compressive and tensile strength
- Enhances cellular attachment
• Affinity to BMPs and host bone
• Sterilizable
• Enhancement of BMP activity
• Controlled release of BMP to adjacent tissues
• Adequate surgical handling characteristics and wound adaptability
• Maintenance of adequate space for osteogenesis

Delivery systems for rhBMP-2 have been extensively investigated (Uludag, D'Augusta et al. 1999; Uludag, Friess et al. 1999; Kirker-Head 2000; Uludag, Gao et al. 2001; Geiger, Li et al. 2003; Seeherman and Wozney 2005; Ruhe, Boerman et al. 2006) but literature assessing the currently utilized delivery system for rhBMP-7 (OP-1) is rare. It has been noted that increased retention of BMP at the implant site results in greater bone induction (Uludag, D'Augusta et al. 2000). Factors that have been shown to impact BMP retention within the ACS carrier include the protein isoelectric point (Uludag, D'Augusta et al. 2000; Uludag, Gao et al. 2001) and pre-implantation soak time (Friess, Uludag et al. 1999). There are no available studies evaluating BMP release from the OP-1 bioimplant. Studies utilizing the radiolabelled BMP-2 have assessed the pharmacokinetics of BMP release from multiple carriers including an absorbable collagen sponge. Utilizing radiolabelled rhBMP-2, Uludag, D'Agusta, et al (1999) found that following a 10 minute soak time with an ACS, 70% of the BMP does was retained at 3 hours post-implantation and that less than 5% remained by 14 days. They noted that the ACS degrades in approximately the same 2 week time frame. Interestingly, they also noted on histologic examination at the 3 hour mark, that no evidence of the gelatin
capsule used for other carriers in the study remained. This suggests that the gelatin capsule is completely dissolved within three hours of implantation. This finding has important implications for the results of the current study as well. The manner in which the rhBMP-2 was added to the ACS in the Infuse group did not allow any pre-implantation soak time and the physical vessel which may have aided in retaining the BMP at the surgical site was perhaps dissolved within 3 hours. Given the lack of literature outlining BMP release from OP-1 bioimplant and the various factors affecting the release and retention of BMP from the ACS, we cannot eliminate the possibility that OP-1 demonstrates greater retention of BMP at the surgical site, leading to greater bone induction.

Also relating to properties of the delivery system, Kim et al (Kim, Kim et al. 2005) found that when evaluating rhBMP-2 implantation into subcutaneous pockets with either ACS or beta-tricalcium phosphate as the carrier, induced bone was present in the ACS group at 2 weeks, but not 8 weeks post-implantation. The authors attributed this to the inability of the ACS to maintain space for bone induction. The current study may have been similarly affected. As mentioned previously, the bioimplant relies on the carrier portion to provide retention and stability at the surgical site. The current model utilized a muscle pouch which would also require the carrier to provide physical retention. The muscle pouch provides greater vascularity and sources of precursor cells than a bony site and thus hold the potential to foster greater bone induction.

4.2.3 Explanation of Results – Temporospatial Expression of BMPs in Bone Healing
Another avenue to explore in attempting to account for our results is to examine the temporospatial pattern of BMP expression in bone formation and healing. This study looked at the impact of two BMPs on bone formation at a specific time point, namely 28 days post-implantation. It is conceivable that differences in the expression of BMPs during bone healing and formation could manifest as an inequality in their ability to induce de novo bone formation.

It is well known that BMPs are intimately involved in bone formation. What is less well understood is the temporal expression of BMPs during bone formation. Most available studies have focused on fracture healing as a model of bone healing and formation. Several studies have demonstrated expression of BMP-2 early and throughout the fracture healing cascade (Ishidou, Kitajima et al. 1995; Onishi, Ishidou et al. 1998; Cho, Gerstenfeld et al. 2002; Phillips 2005; Ngo, Scherer et al. 2006). It has been suggested by multiple authors that BMP-2 may be responsible for initiation of the bone healing cascade (Cho, Gerstenfeld et al. 2002; Ngo, Scherer et al. 2006) and that BMP-2 plays important roles in the early stages of osteoinduction (Okubo, Bessho et al. 1999; Okubo, Bessho et al. 2002). These findings would lead to the belief that the Infuse implant should then have an advantage in total bone formed if it is truly better able to induce bone formation. However, other studies have found BMP-7 expressed early within the fracture healing cascade (Spector, Luchs et al. 2001) and that BMP-2 is not expressed until later in bone repair (Kitazawa, Kitazawa et al. 1998). Thus, no clear consensus exists to determine a definitive order of expression in the fracture healing cascade from which anticipated results could be extrapolated. It has been found that BMPs have site specific patterns of expression during bone formation (Zoricic, Maric et
al. 2003). As such, the surgical site chosen may impact the ability of a particular BMP to induce bone. Additionally, Rauch et al. (Rauch, Lauzier et al. 2000) found that during distraction osteogenesis, BMP-7 was not expressed in rats but was in rabbits leading to the conclusion that the animal chosen for study can impact results and impair the ability to extrapolate the results to another organism.

4.2.4 Explanation of Results – Influence of BMPs on angiogenesis in bone

Increasingly, evidence is suggesting that osteogenesis and bone healing is highly linked to angiogenesis. It has been found that vascularization occurs just prior to osteogenesis (Winet, Bao et al. 1990; Erlebacher, Filvaroff et al. 1995) and occurs with the transition of preosteoblasts to mature osteoblasts. Several members of the TGF-β family have been shown to possess angiogenic properties including BMP-2 and BMP-7 (Yamashita, Shimizu et al. 1997; Yeh and Lee 1999; Ramoshebi and Ripamonti 2000; Deckers, van Bezooijen et al. 2002). The mechanism of BMP induced angiogenesis is not entirely clear but seems to be related to the interplay between BMP, osteoblasts and vascular endothelial growth factor (VEGF). It is well known that BMP play a vital role in the regulation of differentiation and function of mesenchymal stem cells (MSCs). It has been shown that MSCs secrete VEGF resulting in blood vessel formation (Mayer, Bertram et al. 2005) and that BMP-2 in particular, can lead to an increase in angiogenic growth factors by MSCs (Raida, Heymann et al. 2006). Expression of VEGF may be induced by most growth factors involved with bone healing (Carano and Filvaroff 2003) but differences exist the ability of the BMPs to directly induce angiogenesis. Utilizing the
same chick chorioallantoic membrane assay, Ramoshebi and Ripamonti (2000) demonstrated that Osteogenic Protein-1 (BMP-7) directly stimulates angiogenesis, while Yamashita and Shimizu (1997) had showed that BMP-2 does not. In looking at BMP-2 induced angiogenesis in developing tumors, Langenfeld and Langenfeld (Langenfeld and Langenfeld 2004) found that BMP-2 does not directly stimulate endothelial cell migration and thus concluded that BMP-2 could not be the sole initiator of angiogenesis. The studies mentioned above suggest that BMP-7 possesses a greater ability to directly stimulate angiogenesis than BMP-2. It has been demonstrated that an increase in angiogenesis is linked to greater quantity, density and maturity of bone in healing fracture sites (Kleinheinz, Stratmann et al. 2005; Li, Stewart et al. 2008).

Earlier and more extensive angiogenesis in the favour of BMP-7 could then impact the number of circulating MSCs delivered to the surgical site in the current study and thus impact osteogenesis.
CHAPTER FIVE: CONCLUSIONS

This study suggests that the available literature does not accurately predict the *in vivo* behaviour of the two available rhBMP containing bioimplants. The osteogenic capabilities of the bioimplants is likely related to more than the osteoinductive activity alone. Delivery systems, release profiles and impact of the respective rhBMPs on post-surgical healing and recipient site physiology also play an important role. The results exhibited here suggest that further examination of the activity of bioimplants is required. Despite the prediction of the available literature, the rhBMP-7 containing implant OP-1 demonstrated greater induced bone of equal bone quality.
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