The Effect of rhBMP-7 on

Distraction Osteogenesis of the Rabbit Mandible

By

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ABSTRACT

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A rabbit model of Distraction Osteogenesis was used to study bone formation post-injection of rhBMP-7 at the distraction site. Twenty-four white New Zealand rabbits underwent osteotomy and distraction. The animals were divided into three groups. The control group was not injected. The comparison group was injected with lactate buffer, and the study group received an injection of 200 μg of rhBMP-7. All rabbits were sacrificed after 7 weeks. Results were obtained through imaging analysis. Bone densitometry enhanced after both: lactate buffer and rhBMP-7, when compared to control. This was statistically significant ($p < 0.05$). Micro-CT showed that rhBMP-7 injection resulted in a 6% increase in relative bone volume, compared to control group, and 4% increase, compared to the lactate buffer group. This trend did not reach statistical significance ($p=0.17$). Increased bone volume following rhBMP-7 injection is clinically important and may shorten the long consolidation period of device wearing for the patients.

RÉSUMÉ

L'Effet du rhBMP-7 sur L'Ostéogènèse lors de la Distraction du Maxillaire Inférieur chez le lapin

L'ostéogénèse par distraction est une technique chirurgicale qui produit de l'os de novo en utilisant les tissus adjacents au site d'ostéotomie et de distraction. Les forces mécaniques de distraction stimulent la nouvelle production osseuse. Les protéines morphogénétiques osseuses (BMPs) sont des médiateurs connus qui initient la formation d'os pendant le processus d'ostéogénèse par distraction. Les recherches qui ont évalué l'administration de BMPs recombinant en utilisant des méthodes de distraction, ont confirmé leurs effets positifs sur la formation d'os. L'objectif de ce projet est d'évaluer si l'ostéogénèse par distraction du maxillaire inférieur chez le lapin est augmentée suite à l'administration de la protéine morphogénétique osseuse humaine recombinante 7 (rhBMP-7). L'injection de rhBMP-7 est faite au site spécifique de distraction. Vingt-quatre lapins ayant subi une distraction du maxillaire inférieur ont reçu une injection de rhBMP-7 ou de solution placebo (tampon à base de lactate, soit le substrat du rhBMP-7). Un troisième groupe de contrôle n'a pas subi aucune injection après la distraction. Tous les lapins ont été sacrifiés 7 semaines après la chirurgie. Les mâchoires inférieures ont été soumises à des études radiologiques, de densitométrie osseuse ainsi qu'à une analyse de Micro-CT, la technologie la plus avancée qui existe pour ce genre d'analyse présentement. Nous avons noté une augmentation de formation osseuse suite à l'injection de la solution placebo comparée au groupe de contrôle ($p < 0.05$). L'injection locale de rhBMP-7 n'a pas augmenté la formation osseuse de façon statistiquement significative, une fois comparée par la densitométrie, à la solution placebo. Une fois analysée par Micro-CT, l'injection de rhBMP-7 a démontré une augmentation de 6% du volume relatif d'os, une fois comparé au groupe de contrôle, et une augmentation de 4% lorsque comparé aux groupes de solution placebo. Cependant, même si cette tendance est notée, la signification statistique n'est pas obtenue ($p=0.17$).

L'implication clinique de cette tendance obtenue à partir des résultats est importante. Par extrapolation, si une simple injection de rhBMP-7 augmente le volume d'os fabriqué par le processus de distraction, il est dès lors possible de réduire le période de consolidation et de morbidité associée.

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LIST OF ABBREVIATIONS

Ag	Antigen
β	Beta
bFGF	Basic fibroblast growth factor
BMA	Butyl methacrylate
BMC	Bone mineral content
BMD	Bone mineral density
BMP	Bone morphogenetic protein
cm	Centimetre
°C	Degree Celsius
DEXA	Dual Energy X-ray Absorptiometry
FB	Fibroblast
GDF	Growth factor and differentiation factor
gm	Gram
IGF	Insulin-like growth factor
IM	Intramuscular
Kg	Kilogram
Kv	Kilovolt
M	Molar
mAMP	Milliampere

mg	Milligram
mL	Millilitre
mm	Millimetre
MMA	Methyl methacrylate
OB	Osteoblast
OP-1	Osteogenic protein 1
PEG	Polyethylene glycol
®	Registered trademark
TGF	Transforming growth factor
VCT	Vascularized connective tissue
W/V	Weight per volume
μG	Microgram
μL	Microlitre
μM	Micrometer
%	Percent
1°	Primary
2°	Secondary
3°	Tertiary

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1.0 Introduction

1.1 *Distraction Osteogenesis*

1.1.1 Definition of Distraction Osteogenesis and Role of Bone Morphogenetic Proteins

Distraction osteogenesis (DO) is a method of producing large quantities of bone by means of stimulating local host tissues with mechanical distraction forces. It is a biologic process of new bone formation between the surfaces of bone segments that are gradually separated by incremental traction[1]. Specifically, this process is initiated when a traction force is applied to the bone segments and continues as long as the bone segments are distracted. Because of the law of tension-stress as outlined by Ilizarov, living tissues become metabolically activated by slow, steady traction, a phenomenon characterized by the stimulation of both proliferative and biosynthetic cellular functions called osteoinduction and osteoconduction[2,3]. Osteoconduction is a process that supports the ingrowth of sprouting capillaries, perivascular tissues, and osteoprogenitor cells into the separated bone segments[1]. Osteoinduction is a process that supports the proliferation of undifferentiated mesenchymal cells and the formation of osteoprogenitor cells with the capacity to form bone[1]. The traction force on the separated bone edges generates tension within the tissues that connect the bone segments, which stimulates new bone formation parallel to the vector of distraction. As the bone edges are distracted, a fibrin clot forms in the resultant gap. This is followed by deposition of collagen fibers parallel to the axis of distraction. As the distraction continues, bony trabeculae

form at the bone edges and over time, new bone is created over the entire length of the gap by intramembranous and endochondral ossification[4,5].

Distraction osteogenesis has revolutionized the treatment of craniofacial conditions. The technique has been used in the treatment of mandibular, midface and zygomatic hypoplasia, as well as for many other craniofacial anomalies such as Nager's syndrome, Pierre-Robin syndrome, and temporomandibular joint ankylosis[6]. Additionally distraction osteogenesis has applications in treatment following post-oncologic ablation, and post-traumatic growth retardation. The use of distraction osteogenesis obviates the need for endogenous tissue transfer that would otherwise be needed to treat these conditions, and prevents the considerable degree of morbidity associated with conventional bone graft surgery.

Despite the success of DO, one major limitation of this process is that it requires a considerable length of time in order to achieve the desired length of bone. The entire process of DO in the craniofacial bones takes from 2 to 4 months, during which the patient must wear a cumbersome distraction device[7,8]. This long treatment time results in a higher potential for complications such as a late return to normal social activities, and pin site infection[9]. Strategies to accelerate distraction osteogenesis would therefore significantly benefit the patient.

To facilitate maturation of the regenerate bone, several experimental approaches have been described. These have included mechanical stimulation[10,11], electrical and electromagnetic stimulation[12,13], low velocity ultrasound[14], transplantation of bone marrow cells or osteoblast-like cells[15,16,17], and stimulation with growth factors such as recombinant fibroblast growth factor (FGF)[18,19], and transforming growth factor beta (TGFB)[20]. Although these methods have shown some promise in the animal studies, they have not proven as beneficial in clinical trials.

One potential approach to accelerating bone regeneration involves the use of the bone morphogenetic proteins (BMPs, also called Osteogenic Protien1, OP-1).

BMPs form a unique group of proteins within the TGF B super-family of genes and have a pivotal role in the regulation of bone induction, maintenance and repair[21,22,23]. In adult mammals, BMP is synthesized by osteoblasts and osteocytes, and is primarily found in bone and dentin[23]. Bone morphogenetic proteins act at act at the earliest stage of bone induction, at the transcriptional level and higher, to promote osteogenesis by promoting the differentiation of pluripotent stem cells to differentiate into bone-forming cells (osteoblasts) cartilage-forming cells (chondrocytes)[22]. These osteoinductive actions are exerted on mesenchymal stem cells, osteoblasts, and osteoclasts, and result in modulation of gene expression and subsequent chemotaxis, cellular proliferation and differentiation[23]. Thus they play an important role in bone repair and bone regeneration.

Since the cloning of bone morphogenetic proteins, basic studies have been carried out using the recombinant human BMP produced by genetic bioengineering techniques. As a result, seventeen BMP's, (BMP1 to 17) have been identified[23]. The BMPs have been studied in DO models, and their use has been shown to accelerate bone deposition in the lower extremity DO, as well as in healing of critical-sized defects in a number of animal models. Of the seventeen identified BMP's, BMP 2, 4, and 7 appear to be the most potent inducers of new bone formation in numerous preclinical and clinical studies, including healing of critical sized defects of long bones, healing of diaphyseal nonunions, and enhancement of bone grafts and bone substitutes [24,25,26,27].

A recent study showed the efficacy of a single injection of BMP-7 in enhancing bone formation in distraction osteogenesis of long bones in a rat model[24]. To our knowledge, the present study is the first animal model study to examine the effect of BMP-7 on standard distraction osteogenesis of the membranous mandible bone.

We hypothesize that locally applied recombinant human BMP-7 will accelerate bone formation during mandibular distraction osteogenesis in a rabbit model. The improved understanding of the effects of exogenous rhBMP-7 administration

during mandibular distraction osteogenesis will have important clinical applications in the many craniofacial arenas, including the surgical management of pediatric congenital craniofacial anomalies, treatment of facial skeletal anomalies, reconstruction of oncologic defects, maxillofacial trauma and orthognathic aesthetic surgery.

1.1.2 Historical Background

Principles of mechanical manipulation of bone segments have been practiced in orthopedics since ancient times, when Hippocrates described the placement of traction forces on broken bones. He used an external apparatus consisting of two leather rings that were connected by four slightly bent rods made from the elastic cornel tree. The tension applied to the bone segments was controlled by the amount of bending of the rods[28]. See figure 1.

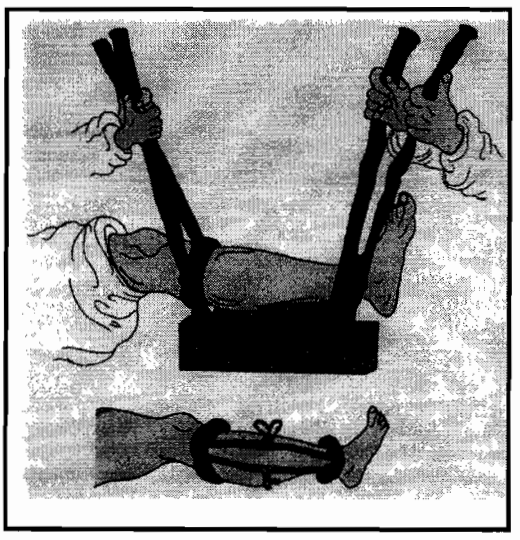

Figure 1. Hippocrates' fracture technique and external fixation device.[29]

Further evolution of distraction osteogenesis involved the development and integration of traction, bone fixation and osteotomy techniques. In the fourteenth century, de Chauliac, was the first to document the use of a pulley system, consisting of a weight attached to the leg by a cord, to place continuous traction on long bone fractures[30]. In 1826, Barton, was the first to perform an osteotomy, or surgical division of bone[31]. Later in the middle of the nineteenth century, Malgaigne developed the first external skeletal fixator device. He constructed an apparatus that was directly attached to bone by two double hooks connected by a screw, and inserted through the skin in to the bone, and thus allowed direct transmission of a mechanical force to the skeleton[32]. Considerable evolution of external fixation has occurred since then.

At the turn of the twentieth century, Allessandro Codivilla performed the first limb lengthening by creating an oblique osteotomy of the femur, and placing external skeletal traction on the osteotomized bone segments[33]. As shown in figure 2, his distraction device utilized a traditional plaster cast, placed on the leg, and cut in half at the level of the osteotomy. The distal part of the cast was connected to a pin inserted into the calcaneus, and the proximal part of the cast was fastened to a stationary external frame Skeletal traction was then applied onto the transcalcaneal pin, and thus, limb elongation was achieved. This traction was repeated as often as necessary to achieve the desired result[33]. Later, Codivilla's skeletal traction procedure was modernized by modifying the osteotomy technique, distraction protocol, or device for bone fixation.

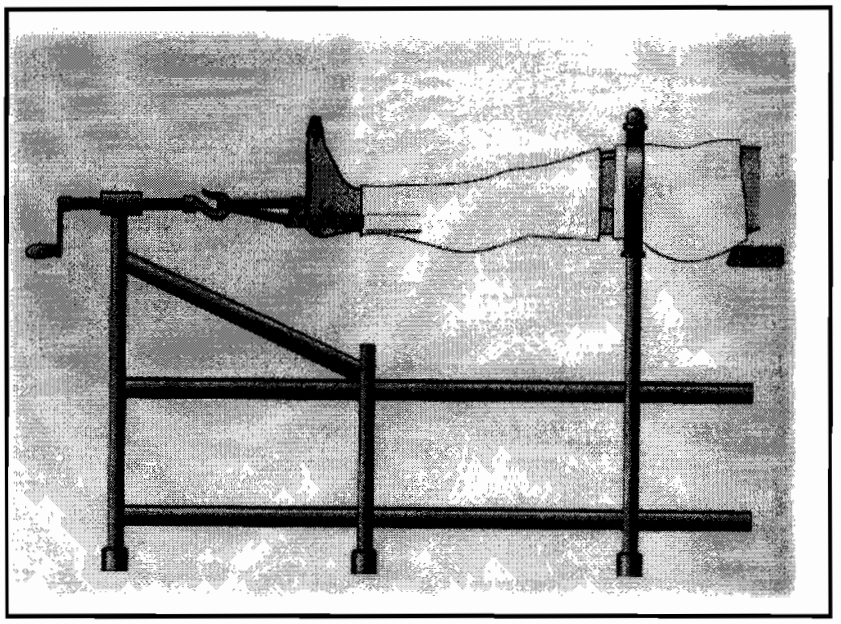

Figure 2. Illustration of Codivilla's device for limb lengthening [29]

The Russian surgeon Gavril Ilizarov contributed significantly to the development of distraction osteogenesis. In 1951, he designed a new apparatus for bone fixation, which consisted of two metal rings joined together with threaded rods[34]. As demonstrated in figure 3, two thin tension wires were inserted at right angles into each bone segment, and these wires were then secured to a ring around the bone segment. Ilizarov later developed a low-energy, subperiosteal osteotomy technique (corticotomy) and a unique protocol for limb lengthening using a 5 to 7 day latency period, followed by distraction at a rate of 1 mm per day performed in four increments of 0.25 mm[2,3].

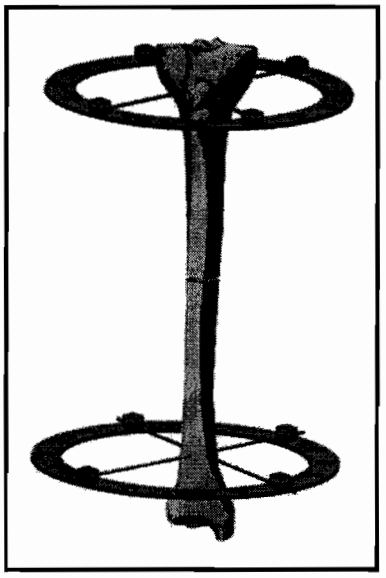

Figure 3. Illustration of Ilizarov's circular external fixator with tension wires.[29]

Mechanical tension, one of the key signals of osteogenesis during natural bone growth and development, was used by Ilizarov as the fundamental root of his distraction osteogenesis technique. Ilizarov discovered and described two biological principles of distraction osteogenesis, now known as the "Ilizarov Effects":

1. The Tension-Stress effect on the genesis and growth of tissues, and
2. The influence of blood supply and loading of the shape of bones and joints[2,3].

The first Ilizarov principle postulates that gradual traction creates stress that can stimulate and maintain regeneration and active growth of living tissues[2]. After distraction, clinically, the newly formed bone rapidly remodels to conform to the bone's natural structure[3]. The second Ilizarov principle theorized that the shape and mass of bones and joints are dependent on an interaction between mechanical

loading and blood supply[2,3]. If blood supply is adequate to support mechanical loading, then the bone will demonstrate compensatory hypertrophic changes. If the blood supply is inadequate, however, then the bone cannot respond favorably, and this would lead to atrophic or degenerative changes[34]. Since that time distraction osteogenesis has gained widespread recognition and become the preferred method for correction of limb length inequality, as well as congenital and acquired bone defects.

In 1973, Snyder and colleagues reported the first experimental distraction of the membranous bone of the canine mandible. They were successful in correcting an experimentally shortened hemimandible by gradually distracting it[35]. In 1976, Michaeli and Moitti reproduced Snyder's work, using an intraoral device[36]. Later, in 1984, Kutsevliak and Sukachev were able to lengthen a normal canine mandible by 1.2 cm, using the Ilizarov technique[37]. Following this, Karp and colleagues detailed the histological analysis of bone formation in the canine mandible following distraction osteogenesis. They reported that the bone was formed primarily by intramembranous ossification[38,39]. This experimental work paved the path for McCarthy and colleagues to perform the first human mandibular distraction osteogenesis in 1989. McCarthy and his team at New York University Medical group was the first to clinically apply extraoral distraction osteogenesis on four children with congenital craniofacial anomalies such as hemifacial microsomia and Nager's syndrome[40,41,4243].

About the same time, Guerroro began developing his midsymphyseal mandibular widening technique using an intraoral tooth borne δdevice. He first reported in 1990, demonstrating successful results of mandibular widening on eleven patients with transverse deficiencies[44].

Following the first reports of McCarthy and Guerroro, which demonstrated successful lengthening and widening of the human mandible by gradual distraction, the field of craniofacial distraction osteogenesis rapidly gained momentum. Many

authors have reported successful results with corrective osteotomies followed by gradual mandibular distraction for deformity correction, lengthening, widening, bone transport, and alveolar ridge augmentation[45, 46,47,48,49].

1.1.3 Biological Basis of New Bone Formation

Most historical investigations of the Ilizarov method of bone lengthening confirm that the mode of bone formation in distraction osteogenesis is primarily intramembranous ossification. This is in contrast with the different healing of a simple bone fracture, which is mainly endochondral bone formation[4,5,50, 51,52].

Distraction osteogenesis begins with the development of a hematoma between the edges of two bone segments divided by a low-energy osteotomy. After the hematoma has initially formed, a distraction force is applied to these bone segments and gradually pulls them apart. Gradual incremental separation of bone segments places the contents in the distraction gap under tension, and this aligns the intersegmentary gap tissues parallel to the direction of distraction. New bone is subsequently formed in the distracted zone[5,51,53]. After the desired amount of bone length is achieved, the distraction force is discontinued. The newly formed bone (distraction regenerate) then undergoes maturation and remodeling until it becomes indistinguishable from the residual host bone[54]. Clinically, distraction osteogenesis consists of five sequential periods:

1. Osteotomy,

2. Latency, the duration from bone division to the onset of traction,

3. Distraction, the time when gradual traction is applied and distraction regenerate is formed,

4. Consolidation, the period that allows maturation and corticalization of the regenerate after traction forces are discontinued, and,

5. Remodeling, which extends from the initial application of full functional loading to the completion of regenerate bone remodeling[1].

1.1.3.1 Osteotomy

An osteotomy divides a bone into two segments, resulting in a loss of continuity and mechanical integrity (Figure 4). Discontinuity of a skeletal segment triggers an evolutionary process of bone repair. This process involves recruitment of osteoprogenitor cells, followed by cellular modulation or osteoinduction, and establishment of an environmental template (osteoconduction)[55]. Osteoinduction is a process that supports the proliferation of undifferentiated mesenchymal stem cells and the formation of osteoprogenitor cells with the capacity to form bone. Osteoconduction is a process in which there is ingrowth of sprouting capillaries, perivascular tissues, and attraction of osteoprogenitor cells into the osteotomy site[55].

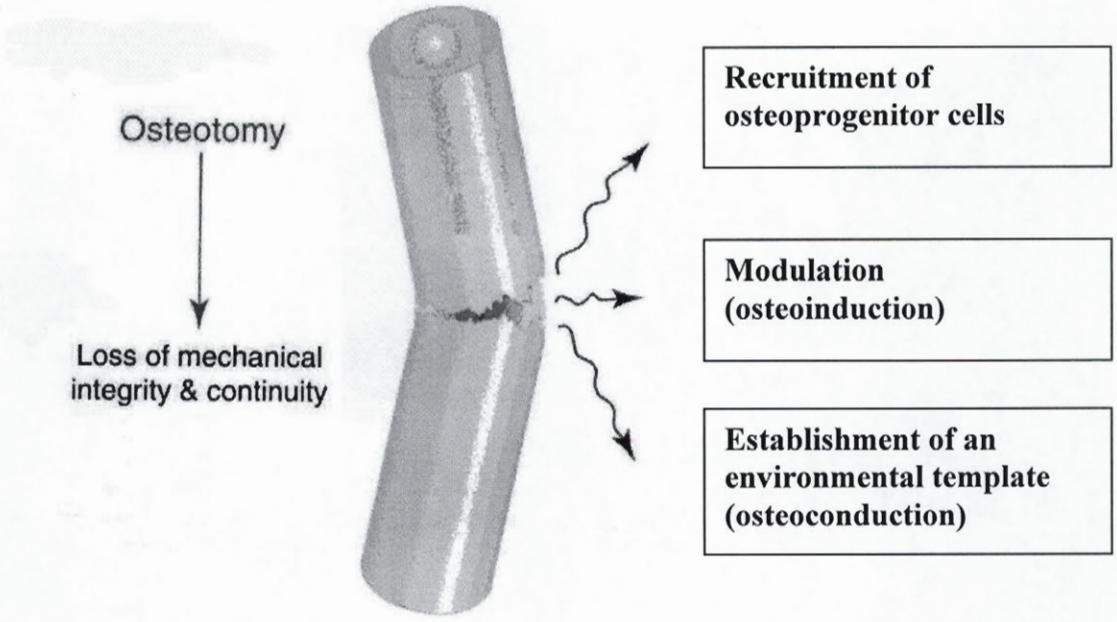

Figure 4. Schematic illustration of the osteotomy dividing the bone into two segments and triggering osteogenesis [56].

1.1.3.2 Latency Period

The latency period is the period from bone division to the onset of traction. This period represents the time allowed for hematoma formation, chemotaxis, mitosis and differentiation of stem cells in osteogenesis[1]. Following the surgical separation of bone into two segments, a cascade of events takes place.

Initially, a hematoma forms between and around the bone segments as a result of vascular disruption. See figure 5. The hematoma is converted to a clot, and bony necrosis occurs at the ends of the fracture segments. There is an ingrowth of vasoformative elements, and capillaries for the restoration of blood supply, and a tremendous amount of cellular proliferation[57]. This inflammatory stage of osteogenesis lasts from 1 to 3 days, at which time inflammatory cells, fibroblasts

and collagen, and invading capillaries are abundant within the clot[57].

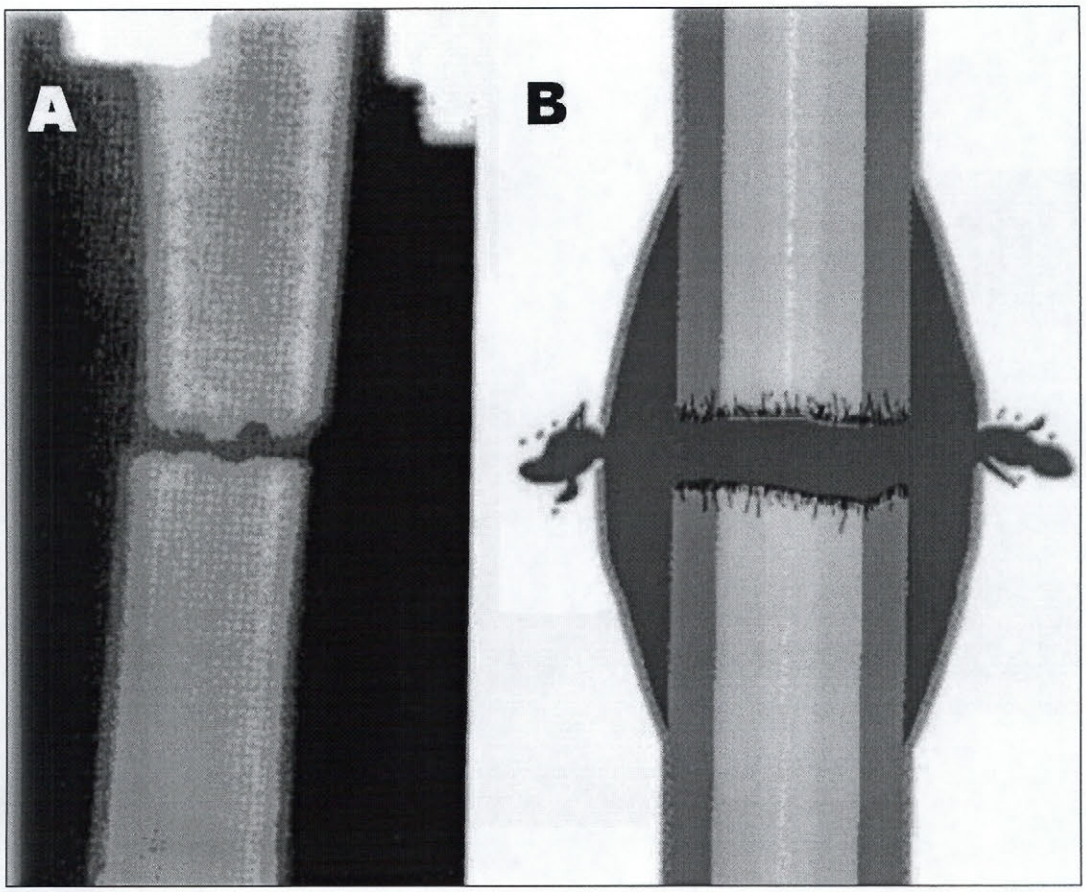

Figure 5. Radiograph, and schematic image of the cascade of events that occur during the inflammatory Latency stage. A, Osteotomy radiograph. B, Hematoma. Due to the ingrowth of capillaries and cellular proliferation, the hematoma between the bone segments triggers induction of osteogenesis. [56]

On around the fifth day following osteotomy, a minicellular network or growing capillary loops is formed in the medullar canal of both proximal and distal segments in the areas adjacent to the fracture line[57,58]. Less differentiated, free

circulating osteogenic cells are located inside the terminals of the newly formed capillaries[5,58]. During this stage, hematoma is converted to fibrous tissue by fibroblasts. Fibrous tissue formation is the response of determined osteoprogenitor cells, originating principally in the periosteum and endosteum, to a number of activating factors released from freshly injured bone tissue[5,58].

1.1.3.3 Distraction Period

The distraction period is characterized by the application of controlled traction forces to separate the osteotomized bone segments. Bone segments are gradually pulled apart, resulting in formation of new bony tissues: 'a generate', which is the term for the intersegmentary gap[1]. We are proposing the term 'osteogenerate' for more precision.

Through the application of tensional stress to the intersegmentary edges of the osteotomized bone, a dynamic microenvironment is created. The tension stress that develops in the gradually stretched tissues stimulates changes at the cellular and subcellular levels[2,3]. These changes can be characterized as a growth-stimulating effect and a shape-forming effect.

The growth-stimulating effect of tension activates the biological elements of the intersegmentary connective tissues. This includes:

1. Prolongation of angiogenesis with increased tissue oxygenation, and
2. Increased fibroblast proliferation with intensification of biosynthetic activity[2,3].

The shape-forming effect of tension causes an altered phenotypic expression of fibroblasts. This effect also orients these "distraction fibroblasts" and their secreted

collagen parallel to the vector of distraction. In addition, this environment encourages new tissue formation in a direction parallel to the vector of traction[5,58].

As distraction begins, the fibrous tissue in the hematoma becomes longitudinally oriented along the axis of distraction. The spindle-shaped fibroblast-like cells located between the collagen fibers form collagen fibrils that are grouped into fibers between distal and proximal ends of the intersegmentary tissues[5,57,58].

Between days 3 and 7 of distraction, capillaries grow into the fibrous tissues, thereby extending the vascular network not only toward the center of the gap, but also towards the medullar canal of both adjacent bone segments. The newly formed capillary loops are parallel to each other as well as to the axis of distraction. Capillary terminals actively invade the fibrous tissues, supplying them with stem cells that differentiate into fibroblasts, chondroblasts or osteoblasts [54, 57 ,58].

During the second week of distraction, primary trabeculae begin to form. The osteoblasts located among the collagen fibers, lay down osteoid tissue on these collagen fibers, and eventually become enveloped as bone spicules gradually enlarge by circumferential apposition of collagen and osteoid. Osteogenesis is initiated at the existing bone walls, and progresses toward the center of the distraction gap. By the end of the second week, the osteoid tissue begins to mineralize [5, 58].

At that time, the distraction osteogenerate has specific zonal structure, as shown in figure 6. A poorly mineralized, radiolucent fibrous interzone is located in the middle of the distraction gap, where the influence of tensional stress is maximal. This zone consists of longitudinally oriented parallel bundles of collagen with spindle-shaped fibroblast-like cells. The matrix is filled with undifferentiated mesenchymal stem cells [53, 56, 58]. The interzone functions as the center for fibroblast proliferation and fibrous tissue formation. The mixture of fibrous and cartilage tissues within the interzone suggests that during distraction, both

membranous and endochondral processes play an important role in the process of bone formation [53, 56, 58]. At the periphery of this fibrous interzone, there are two mineralization zones with longitudinally oriented cylindrical primary trabeculae, which are covered by a layer of osteoblasts that grow towards eachother[56].

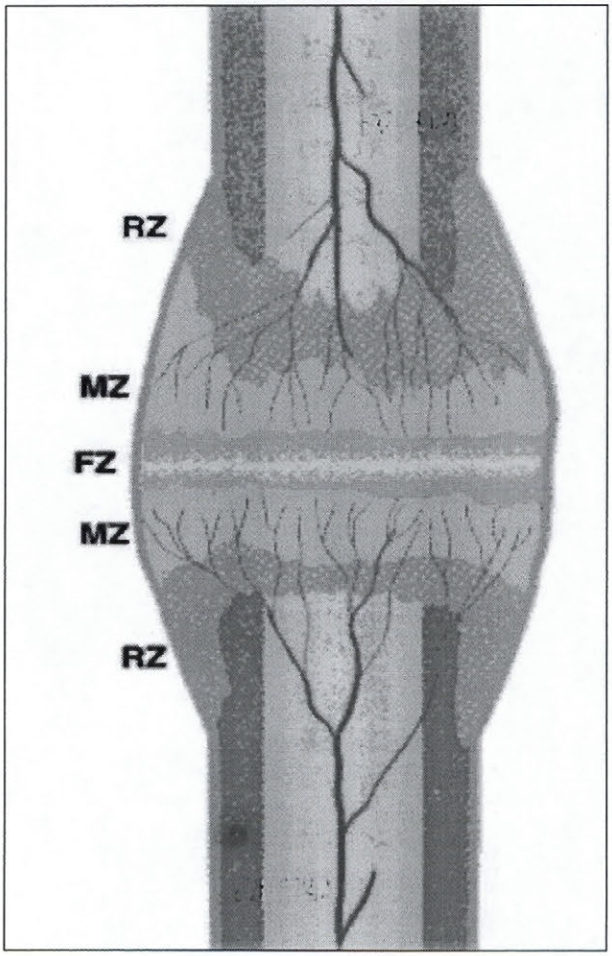

Figure 6. Schematic illustration showing the three-zone structure of distraction osteogenerate. FZ., Fibrous interzone. MZ, Mineralization zone. This zone is primarily trabecular bone formation. RZ, Remodelling zone [56].

Bone formation occurs along the vector of tension, and is maintained by the growing apices of the primary trabeculae during the distraction period. These areas therefore function as the "growth zone" of the distraction regenerate, providing active osteogenesis throughout the period of elongation [5, 56, 58]. This zonal distribution of newly formed tissues remains until the end of the distraction period. In addition, two new zones of primary trabeculae remodeling may become evident at the junction of the osteogenerate, which is also the host bone segments edges[5, 56].

1.1.3.3.1 Rate and Frequency of Distraction Osteogenesis:

The bony regeneration after distraction osteogenesis is affected by several factors, including the latency period, stability of distraction device, age of patient, vascularization of the distraction site, timing of distraction device removal, environmental effects such as muscle prolapse on bone formation in the distraction gap, and rate and frequency of distraction[59].

The rate and frequency of distraction are important to osteogenesis, on the basis of the influence of the tension-stress effect. A short latency period can result in a poor osteogenic response, with decreased vascularity, whereas a long latency period can result in premature ossification[59].

In a series of experiments using a canine tibia model, Ilizarov described that distraction at a rate of 0.5 mm/day often led to premature consolidation of the lengthening bone, whereas a distraction rate of 1.0 mm/day produced the best results[34]. As such, he observed that the greater the distraction frequency, the better the outcome. Ilizarov also described that a distraction rate of 2.0 mm/day yielded a large regenerate zone filled with dense fibrous connective tissue, with virtually no osteogenic activity 4 weeks after distraction. This rapid

rate also led to deleterious changes within the overlying tissues including a decreased biosynthetic activity within the cells of small vessels or microvasculature, fascia, and neuronal elements[34].

In other experimental studies, the rabbit model has been used to study the effects of different rates of distraction upon mandibular bone. In 1998, Stewart and colleagues studied bilateral mandibular distraction at rates of 0.5 mm twice a day and 1.5 mm twice daily. At both rates, the distraction zone was composed of a mixture of woven and maturing lamellar bone with a loose fibrovascular stroma. However, non-union was more common in the rapidly distracted group[60]. In 1999, Meyer and colleagues also reported that the gradual distraction of bone in physiologic magnitudes at higher frequencies (0.22 mm per day) seemed to be desirable for bony differentiation [61].

The effects of rates of distraction have also been studied in other animal models. In 1998, Rowe and colleagues reported their results in a rat model of mandibular distraction. This group applied distraction rates of 0.25 mm daily and 0.25 mm twice daily, but found no significant difference in the formation of bone between the two groups[62]. Carls and Sailer described their distraction osteogenesis in the mandible of sheep, and reported that a distraction rate of 1 mm/day produced stronger biochemical and histological properties than a rate of 2 or 3 mm/day. Elongation by 1 mm/day in four divided equal increments (0.25 mm every 6 hours) led to more favorable results than either a slower or faster rate in long bones. However, in the craniofacial bone, elongation by 1 mm/day in one step produced more favorable results because of good vascularization[63, 64]. In 2000, Farhadieh and colleagues also employed a sheep model and found that bone formed at a distraction rate of 1mm per day was superior to that formed at a rate of 4 mm per day[65]. In that same year, Troulis et al. also studied the effects of rate and latency on bone formation in a porcine model of mandibular distraction osteogenesis. They

found that bone formation and stability was greatest in the group distracted at a rate of 1 mm per day [66].

From a clinical perspective, faster distraction rates would be expected to decrease the overall treatment time however, it would challenge the quality of the new tissue formed and would not be as successful.

1.1.3.4 Consolidation Period

The consolidation period is the time between cessation of traction forces and removal of the distraction device. This period represents the time required for complete mineralization of the distraction regenerate. After distraction ends, the fibrous interzone gradually ossifies and one distinct zone of bone completely bridges the gap [5, 58]. Although the distraction regenerate forms predominantly by membranous ossification, isolated islands of cartilage may also be observed, suggesting endochondral bone formation. As the regenerate matures, the zone of primary trabeculae significantly decreases and later is completely resorbed[5,58].

The factors that affect this consolidation period include the amount of distraction and the age of the patient[59]. The reported consolidation period for mandibular distraction osteogenesis is 1-4 weeks for a child and 6-12 weeks for a youth or adult[7, 8]. Fischgrund et al. found that patients younger than 20 years of age healed faster than those between 20 and 29 years of age, who in turn healed faster than those over 30 years old[7]. The consolidation period for craniofacial bones ranges from 6 weeks to 6 months, and is even longer for long bones[8].

The consolidation period determines timing for the device removal. In summary, a shorter enhanced consolidation phase, which is the longest phase, is a major advantage for the patients. This translates a short period with external deformity and/or intra-oral discomfort as well as less medical visits and less risk of pin sites

infections. Accordingly, a procedure is needed to decrease the consolidation period through stimulating new bone formation in the distracted zone.

1.1.3.5 Remodeling Period

The remodeling period is the time from the application of full functional loading to the complete remodeling of the newly formed bone. During this period, the initially formed bony scaffold is reinforced by parallel fibered lamellar bone. Both the cortical bone and marrow cavity are restored[5,58]. Haversian remodeling, representing the last stage of cortical reconstruction, normalizes the bone structure[5]. It takes at least one year before the structure of newly formed bony tissue is comparable to that of the pre-existing bone[1].

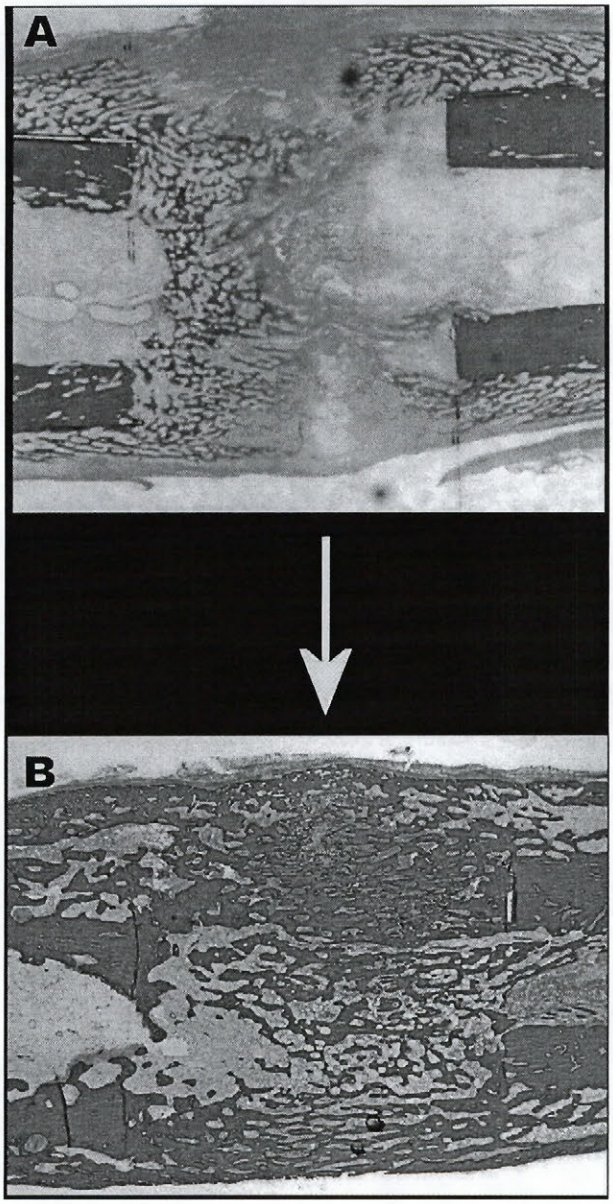

Figure 7. Histology sections showing A. Osteotomy and hematoma between the osteotomized bone edges, and B. New bone formation between the osteotomized bone edges. (Slides courtesy of Dr. R.C. Hamdy)

1.1.4 Membranous Bone Distraction Osteogenesis – Animal Model

Several studies have revealed that the dynamics of new bone formation during distraction osteogenesis of membranous bone is similar to that of long bones.

In 1992, McCarthy, Karp and colleagues at the New York University Medical Center examined the histological changes of membranous bone in a canine model in response to distraction osteogenesis[38]. They noted that the gap between the distracted bone edges was first occupied by fibrous tissue. As distraction continued, the fibrous tissue became longitudinally oriented in the direction of distraction. Early bone formation then advanced along the fibrous tissue, starting from the cut bone edges. Eventually, the area was converted to mature cortical bone, predominantly by intramembranous ossification[38]. In 1993, Costantino and colleagues described the long-term functional, morphological and biomechanical results of canine mandibular distraction osteogenesis one year after surgery[67]. Histological evaluation of the generate bone revealed a normal cortical and medullary architecture.

In 1994, Komuro et al detailed a histological analysis of distraction osteogenesis of the mandible in rabbits. Thirty rabbits underwent unilateral mandibular distraction at a rate of 0.18 mm every 12 hours for 24 days. At the completion of distraction, longitudinal new bone trabeculae were examined in the distraction zone. The new bone was remodeled resulting in cortical bone by 8 to 10 weeks after completion of distraction. The newly formed bone had features of intramembranous and endochondral ossification[58]. Using another rabbit model, Guerrissi and colleagues examined the radiographic and histological effects of unilateral and bilateral

mandibular distraction osteogenesis. At the end of the distraction period, ossification extended from both ends towards the central fibrous zone[68].

Despite the success of distraction osteogenesis, its major limitation is the considerable length of time required to achieve the desired bone formation. The long treatment time results in a higher potential for complications and strategies to accelerate distraction osteogenesis would therefore be significantly beneficial.

To facilitate maturation of the regenerate bone, several experimental approaches have been described, including stimulation of osteogenerate formation with growth factors.

1.1.5. Osteoinductive Growth Factors

A number of growth factors have been identified at the distraction site in distraction osteogenesis (Figure 8). These growth-promoting substances have been shown to enhance and even accelerate bone formation during distraction osteogenesis in a variety of animal models[69, 70, 71]. Among these compounds are transforming growth factor (TGF)-β, fibroblast growth factors (FGFs), insulin-like growth factors (IGFs), platelet-derived growth factors (PDGFs), and bone morphogenetic proteins (BMPs).

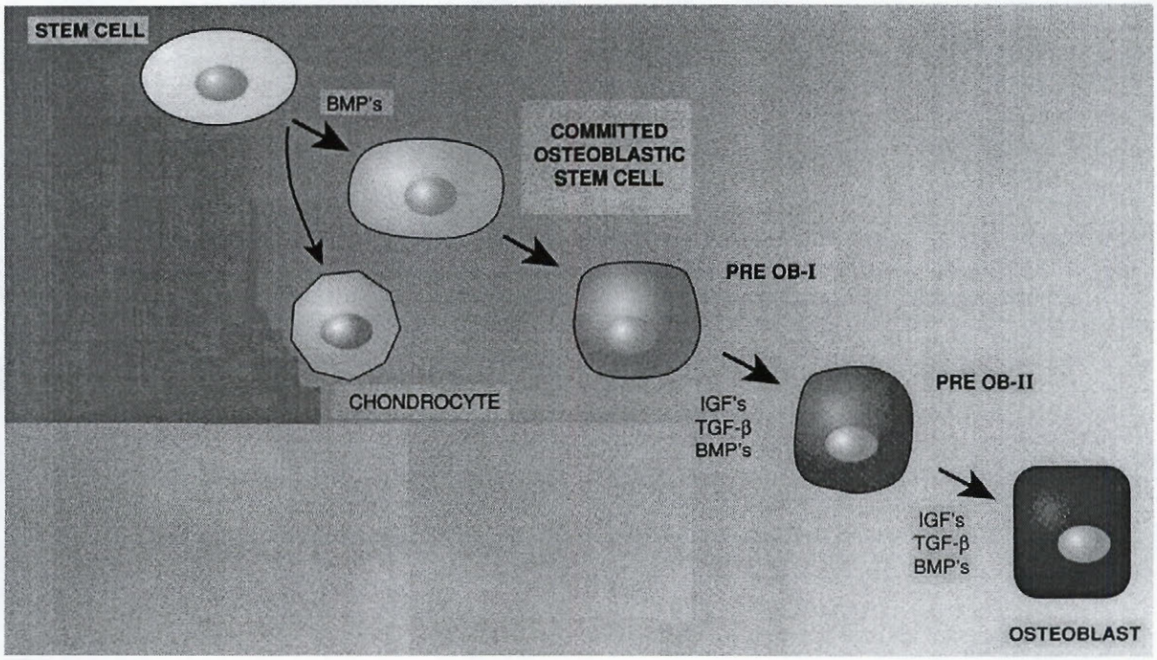

Figure 8. Schematic illustration of growth factors and the levels at which regulate cells involved in distraction osteogenesis. (Illustration courtesy of Dr. R.C. Hamdy)

1.1.5.1 Transforming Growth Factor – Beta

The transforming growth factor beta (TGF-β) family of growth factors has emerged as critical regulators of osseous repair and development. TGF-β is a multifunctional regulatory growth factor integrally involved with extracellular matrix deposition in both soft tissue and bone[72]. It potently stimulates the recruitment and proliferation of osteoblasts at the site of a defect. This results in the rapid deposition of bony matrix followed by a normal remodeling process thereafter. Data to support the concept that exogenous TGF-β stimulates bone formation are substantial, and may potentiate the osteoinductive activities of the bone morphogenetic proteins[73,74]. The overall osteoinductive capacity of TGF-β, however, is weak compared to BMPs[75,76].

1.1.5.2 Fibroblast Growth Factor

Fibroblast growth factors (FGFs) are present in normal fracture healing and have both mitogenic and angiogenic activities. These factors have been linked to osteoblast and chondrocyte proliferation and synthetic activity[77]. Whether or not exogenously applied FGF enhances distraction osteogenesis bone formation remains unclear.

Several authors investigated the stimulation of bone formation by adding recombinant FGF during distraction osteogenesis. Okazaki et al investigated the effects of a single local injection of recombinant human FGF in rabbits. Injection of rhFGF into the center of the distracted callus on the final day of distraction increased bone formation, as demonstrated by an increase in bone mineral content in the distracted site[78].

1.1.5.3 Growth Hormone and Insulin-Like Growth Factor-I

Growth hormone and IGF-I clearly play a critical role in skeletal growth and development, but whether they play a role in distraction osteogenesis is less certain. A number of studies have demonstrated that growth hormone has a stimulatory effect on bone formation, whereas others have shown no such response. The differences have been explained by the variety of experimental designs used, the dosage of the growth hormone, and the species of the animals that were studied.

The application of recombinant homologous growth hormone (rhGH) has been proven to show stimulation effect on bone healing during distraction osteogenesis. Raschke and colleagues administered 100 μg of rhGH per kg bodyweight per day in the distraction zone of porcine tibia. The final regenerate bone was stronger and stiffer in the treatment group when compared to the control group. They concluded that rhGH, when added during distraction phase, lead to a stimulating effect the generate[79].

Similarly, Stewart et al reported that recombinant IGF-I infusion significantly enhanced osteoblast activity at distraction sites in the rabbit mandible, and resulted in bony union[80]. They concluded that exogenous IGF-1 has a positive influence on osteoblast activity during distraction osteogenesis.

1.2 Bone Morphogenetic Proteins

1.2.1 Definition

In 1965, Urist was the first to describe ectopic bone formation after the implantation of a demineralized bone matrix in intramuscular sites in rats, and the factor responsible for this was later named bone morphogenetic protein (BMP), also known as osteogenic protein 1 (OP-1)[81].

Bone morphogenetic proteins are a subdivision of the transforming growth factor-β (TGF-β) super-family that plays a crucial role in this process of cell growth and differentiation. These growth factors act at the earliest stage of osteoinduction, by influencing gene transcription of pluripotent stem cells, to promote their differentiation into osteoprogenitor cells[22,23](see Figure 10).

Seventeen BMPs, designated as BMP-1 through BMP-17, have been identified and cloned to date. Out of these 13, there are eight classes of BMPs that have been identified as osteogenic regulatory molecules, BMP-2 through BMP-9. BMP-1 is not part of the TGF-β family; it is a proteinase and possesses different properties[82]. These have been further subdivided into three subsets based upon similarities in their amino acid sequences. BMP-3 is the sole member of its subset; BMP-5, BMP-6, BMP-7 and BMP-8 form a second set, with 82% homology; and finally BMP-2

and BMP-4 are categorized together and are the two most closely related BMPs, with 92% homology[82,83]. Of the seventeen identified BMP's, BMP 2, 4, and 7 appear to be the most potent inducers of new bone formation in animal studies.

1.2.2 Molecular Characterization

Many cell types synthesize BMPs as large precursor molecules. They are composed of an amino acid terminal sequence of 15 to 25 amino acids, a poorly conserved pro-domain that varies from 20 to 375 amino acids, and a carboxyl terminal domain of 110 to 140 amino acids[84]. Proteolytic cleavage releases the mature carboxyl terminal domain from the pro-domain, and following folding and dimerization, it becomes biologically active[82]. See figure 9.

BMPs become homodimers or heterodimers, but the heterodimers have shown more potent induction of cartilage and bone formation.

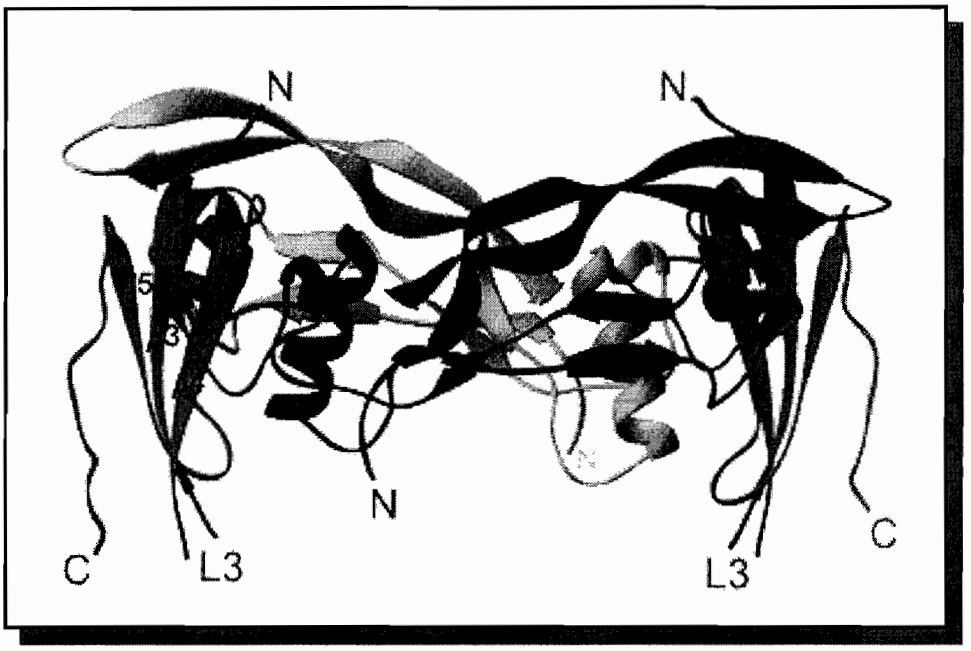

Figure 9. Schematic drawing of the molecular structure of bone morphogenetic proteins (Illustration courtesy of Dr. R.C. Hamdy)

BMPs bind and interact with specific receptor proteins in order to express their biological effects. Two types of receptors have been characterized and cloned, type I and type II. In mammalian species, type I receptors have been further subdivided into 7 subtypes. Similarly, type II receptors have 5 subtypes[85]. Receptor subtypes IA and IB demonstrate the greatest affinity for BMPs. Binding of a BMP dimmer to its type II receptor recruits type I receptors, so that a heterotetramer is formed with two receptors of each type. The proximity of the receptors allows the type II receptor to phosphorylate the type I receptor. Two downstream pathways have

been identified. One of these pathways, called the Smad cascade, is initiated by phosphorylation of certain Smad proteins by type I receptors[85]. The other pathway involves two mitogen-activated protein cascades. In either case, regulation of gene transcription is the result. The gene transcription mediated by BMPs serves to promote osteogenesis by promoting the differentiation of pluripotent stem cells to differentiate into bone forming cells (osteoblasts), and cartilage-forming cells (chondrocytes)[85].

1.2.3 Role in Embryo genesis

Since the time of their discovery by Urist, molecular biology techniques have been used to elucidate the spatial and temporal localization of BMPs and their specific receptors during chondro-osteogenesis in various mammalian tissues, including limb buds during embryonic development. They have been shown to play a vital role in cell growth and differentiation of pluripotent mesenchymal progenitor cells and in apoptosis of various cell types, including osteoblasts, chondroblasts, neural cells, and epithelial cells.

BMPs are also expressed in many other tissues, including the CNS, heart, prostate, kidney, oocytes, and tooth buds, which give rise to the speculation that they are involved in the morphogenesis and development of many organ systems[86].

1.2.4 Role in Bone formation

Evidence suggests that BMPs play an essential role in the formation and maturation of skeletal tissues. BMPs and their receptors also have been shown to be essential in osseous regeneration in distraction osteogenesis.

Bone formation by BMPs is an orderly sequence that involves the recruitment of undifferentiated mesenchymal progenitor cells, and driving osteogenic precursors down osteoblast, osteoclast, and chondrocyte differentiation pathways, stimulating bone formation in vivo[81]. It has been speculated that the many BMPs act in concert, such that the induction of one BMP lead to modulation of gene induction and expression of other BMPs and subsequent chemotaxis, cellular proliferation and differentiation. Moreover, BMPs may stimulate the production of other growth factors such as insulin like growth factor (IGFs), Fibroblast growth factor (FGFs), transforming growth factor B (TGF B) involved in new bone formation[87].

Two different BMP monomers may bind to form a heterodimer with greater osteogenic potential. Heterodimers of BMP-2 with BMP-7 or BMP-2 and BMP-6 have demonstrated more potent induction of cartilage and bone formation when compared to BMP-2 alone[23]. Biologic functions of the individual BMPs have been reported differently in different cell lines, culture conditions, and anatomic sites depending on the concentration and the target tissues.

1.2.5 Role in Distraction Osteogenesis

Lessard and colleagues characterized the temporal and spatial expression of BMP in membranous bone of the rabbit mandible[86]. Interestingly, a very intense signal for BMP-7 was detected in the vascularized connective tissue, and was also expressed in osteoblasts, chondrocytes and fibroblasts, during the first 2 weeks of distraction. These signals decreased significantly or were absent during the consolidation period[86]. These findings suggested that an enhancement of bone formation during distraction osteogenesis might be possible if exogenous recombinant BMP is administered during the consolidation period during which the endogenous BMP protein abruptly tapers. Applied as such, BMP-7 may provide an

alternative to the use of autogenous graft and allograft bone in the reconstruction of bone defects caused by trauma, neoplasia, or infection.

Through genetically modified cell lines, recombinant BMP has been produced as singular molecular species in unlimited quantities. Recombinant human BMP (rhBMP) does not cause a host-versus-graft immune response and is free of infectious agents and contaminants[87]. This is what we have used in this study.

In vitro studies have shown that recombinant human BMP-7 is a potent inducer of differentiation of the pluripotent mesenchymal cells into osteoblast cells. Yeh et al examined the effects of BMP-7 differentiation of a pluripotent mesenchymal cell line. BMP-7 enhanced the expression of several mRNA involved in cell differentiation into osteoblast cells, and significantly elevated the mRNA expressions of BMP-1, BMP-4, and BMP-5. They concluded that BMP-7 is a potent inducer of differentiation into osteoblast cells[88].

In addition, recombinant human BMP-7 can stimulate in vitro human osteoblasts when applied on various biomaterials for bone replacement[89].

1.2.5.1 ENDOCHONDRAL (LONG BONE) DISTRACTION OSTEOGENESIS MODEL

Previous in vivo studies on recombinant BMPs have centered on their administration into segmental mandibular bone defects. These studies have provided good evidence that recombinant BMP administration improved bone healing and formation in a static model. To date, only a few studies have investigated the concept of administering exogenous recombinant BMP-7 to promote osteogenesis.

A recent study by Hamdy et al examined the effect of a single injection of BMP-7, given locally at the end of the distraction phase, on the osteogenerate of the rabbit femur[90]. The study examined six groups of rabbits, which included a control

group, a lactate buffer group, and four groups of rabbits, which received varying doses of BMP-7. It was noted after histology, bone densitometry measurements, and biomechanical testing, that while the group of rabbits that received the highest dose of BMP-7 had modestly more successful osteogenerate results compared to the other groups, differences between the groups did not reach statistical significance. This was explained by immunohistochemistry examination of the osteogenerate, which revealed an abundance of BMP-7 receptors at the beginning of distraction phase, and a lack of receptors in the target tissue at the end of the distraction phase. It was then postulated that the paucity of BMP-7 receptors at the end of the distraction phase might have impaired the effect of the BMP-7 that was given at that time[90].

Another study by Mizumot et al lent some support to the above results[24]. This group used a rat model and injected one group with BMP-7 at a single dose of 20 micrograms, and injected another group, which served as a control, with the same volume of a carrier substance (hydrochloric acid). The substances were injected directly into the femur osteotomy site at the time of osteotomy, before distraction began. It was concluded that a single injection of BMP-7 at the time of osteotomy surgery stimulated the rate of regenerate ossification and increased bone mineral density during DO and increased biomechanical properties of the newly formed bone, as evidenced by radiographs showing larger amount of new bone compared to control, enhanced bone mineral density shown by DEXA densitometry scan, and increased normalized values of stiffness in comparison with the control group in biomechanical testing[24].

1.2.5.2 MEMBRANOUS (MANDIBULAR) DISTRACTION OSTEOGENESIS MODEL

Recently Kim et al showed that the use of BMP-4 has been shown to enhance bone formation in the distracted zone when compared to TGF-beta and a natural biopolymer[27]. The authors compared BMP4 (100 micrograms) Betaig-h3 (a TGF B inducible cell adhesion molecule) and chitosan (a polysaccharide that is a major constituent of the exoskeleton of crustaceous water animals, which may enhance bone formation) injected into the distraction zone at the end of distraction in mandibular distraction osteogenesis. The BMP group showed by histology, the firmest new bone formation, most radiodense opacity in radiographs, and highest radiodensity. Their findings suggest that BMP 4 seems to be very effective in early bony consolidation in distraction osteogenesis[27].

Ashinoff et al demonstrated the use of gene therapy and BMP-2 application in mandibular distraction osteogenesis. They used a recombinant adenovirus as a vector to deliver DNA encoding BMP-2 at the end of the distraction phase of mandibular distraction osteogenesis in a rat model. This was found to increased bone deposition when compared to a control group and a group that comparison group[91].

Very recently, Terheyden et al. examined the effect of locally injected BMP-7 in a rat model of mandibular distraction osteogenesis[92]. Positive results were obtained with the locally applied 50 micrograms of BMP-7 at the end of the distraction phase. In that study, however, only five rats were included, and there was no separate control group. Additionally, the authors increased the distraction rate to 0.7 mm twice daily, instead of the standard 5 mm daily rat mandible critical threshold, and intentionally created more demanding conditions, and a critical sized defect. These differences thus render the study incomparable to our study.

The present study, for the first time, uses a rabbit model to compare the administration of rhBMP-7 to the distracted site at the end of the distraction phase of distraction osteogenesis of the mandible, with separate control and comparison groups. The objective of the study is to examine the effects of rhBMP-7 on enhancing the quantity of bone generation in distraction osteogenesis of the rabbit mandible.

1.3 Clinical Applications: Craniofacial Surgery and Rational for the Study

Distraction osteogenesis has widespread clinical applications in the treatment of craniofacial deformities resulting from congenital anomalies, tumor resection, and secondary to trauma or bone grafts.

Since 1992, when McCarthy et al reported the results of the first clinical application of distraction osteogenesis to lengthen mandibles in children with congenital craniofacial anomalies, craniofacial distraction osteogenesis applications have increased dramatically. In the past 10 years, the most commonly applied craniofacial osteodistraction technique has been mandibular lengthening for the retrognathic or deformed mandibles. Distraction osteogenesis has been used primarily for treatment of pediatric patients and performed in patients with hemifacial microsomia, Treacher Collins syndrome, Goldenhar's syndrome, Nager's syndrome and other anomalies associated with micrognathia.

Conventional skeletal expansion techniques have included autologous bone grafts, allografts, xenografts and bone graft substitutes. The act of harvesting autologous bone grafts from the patient is associated with post-operative pain at the donor site, potential nerve injury, vascular injury, and potential for infection and post-operative mobility disturbances.

The advantage of distraction osteogenesis over conventional skeletal expansion techniques is that it has no bone donor site morbidity, and is associated with a lower incidence of relapse. In addition, distraction osteogenesis allows for an earlier age for reconstruction, and for shorter, less invasive surgical interventions.

The clinical stages of distraction osteogenesis can be divided into latency, distraction, and consolidation phases. In particular, the period of consolidation depends on the distraction site (such as the facial bone or long bone), the status of vascularization, and the age of the patient. The latency period ranges from 0 to 7 days in the craniofacial skeleton, and the bony consolidation period after distraction osteogenesis usually takes 3 to 5 weeks in children, and 6 to 12 weeks in adults[16]. Therefore, the entire process of distraction osteogenesis in the craniofacial skeleton takes from 2 to 6 months, including the distraction and long consolidation period. This long duration has remained a great limitation for the implementation of this technique. Because the patient suffers discomfort during this entire period caused by the distraction device, shortening the bony consolidation period by increasing the quality of bone formation would be of great clinical and economical benefit to the patient. In addition to improving patient comfort and compliance, a shorter treatment protocol would reduce the duration of convalescence and minimize the social burden imposed on children who require this treatment.

Previous attempts at shortening the distraction phase involved increasing the rate of distraction. These attempts have been unsuccessful, and were associated with unacceptably high incidences of bony non-union. By manipulating the molecular signals that mediate bone formation and consolidation, a more practical and effective solution to this clinical problem may be achieved.

Critically evaluating the existing literature concerning mandibular distraction osteogenesis and bone morphogenetic protein, several gaps in knowledge were identified. For example:

1. There is limited information available with respect to the use of exogenous recombinant BMPs in a dynamic model of osteodistraction.

2. Specifically, the use of rhBMP-7 to enhance bone formation during mandibular distraction osteogenesis has only been previously investigated one time in a study that is not comparable to the present research.

Indeed, the administration of rhBMP-7 during the consolidation period, may lead to an enhanced quality, and eventually rate, of bone formation.

Enhancement of bone formation during distraction osteogenesis may be possible if exogenous recombinant BMP is administered during the consolidation period during which the endogenous BMP protein abruptly tapers.

2. Hypothesis

We hypothesize that locally applied recombinant human BMP-7 (rhBMP-7) will accelerate the bone formation during distraction osteogenesis.

The present study, the first time according to our literature review, uses an animal model to administer rhBMP-7 to the distracted site at the onset of the consolidation phase in distraction osteogenesis of the mandible.

2.1 Specific Aims of the Study

Evaluate the effects of locally appled rhBMP-7 using the following techniques:

1. Radiography to qualitatively evaluate the opacity of the osteogenerate within the distraction zone.

2. DEXA scan to evaluate bone mineral content and bone mineral density of the osteogenerate.

3. Microcomputed tomography (micro-CT) scan to evaluate the total bone volume of mineralized tissue formed during distraction osteogenesis.

2.2 Future Directions

The general goal of the proposed series of experiments is to gain a better understanding of the role of exogenous recombinant BMP-7 administration to accelerate and enhance bone formation during distraction osteogenesis. This knowledge is important and may be clinically applied to the following uses:

1. The surgical management of pediatric congenital craniofacial anomalies.

2. Treatment of severe obstructive sleep apnea caused by facial skeletal anomalies.

3. The reconstruction of oncologic defects in irradiated mandibles, precluding the need for free bone grafts and microsurgery in some cases.

4. Maxillofacial trauma surgery

5. Orthognathic aesthetic surgery.

3. Materials and Methods

3.1 Animals

Twenty-four skeletally mature (9-month-old), male, white New Zealand rabbits weighing 3.5 to 4.5 kg were used in this study. The McGill University Animal Care and Ethics Committee approved the housing, care, and experimental protocol.

The rabbits were housed in the animal care facility of the Montreal Children's Hospital. They were housed individually and exposed to a 12-hour light and 12 hour dark cycle. The animals were provided with water, regular rabbit chow, and a modified soft diet as needed. McGill University staff veterinarians at the animal care facility were available for veterinary care, although their services were not needed during the research.

3.2 Medications

- Buprenex ® (Bupernorphine HCL, 0.3 mg/ml), Rechitt & Colman Pharmaceuticals Inc. Richmond, VA.
- Euthanly ® (Pentobarbital Sodium, 340 mg/ml), Schering Canada Inc., Pointe-Claire, QC.
- Isoflurane, MTC Pharmaceuticals, Cambridge, ON.

- Ketalean ® (Ketamine HCl, 100mg/ml) MTC Pharmaceuticals, Cambridge, ON.
- Lidocaine HCl 2% (with epinephrine 1: 100,000), Abbot Laboratories Ltd., Saint-Laurent, QC.
- OpSite ® Spray Dressing, Smith & Nephew Inc., Lachine, QC.
- Rompun ® (Xylazine, 20 mg/ml), Bayer Inc., Toronto, ON.
- Stanhexidine ® (Chlorhexidine Gluconate 2% W/V), Novopharm Ltd., Toronto, ON.
- Trimethoprim Sulfadiazine, Glaxo-Wellcome, Inc., Mississauga, ON.
- Xylocaine ® Endotracheal Spray (Lidocaine HCl, 12 mg/metered dose), Astra Pharma Inc., Mississauga, ON.

3.3 Surgical Instruments

Accu-Temp ® high temperature cautery (ref. 84-42000), Solan; Scalpel blade #15, W.R. Swan & Co. Ltd, Sheffield, England; Suture (4-0 Vicryl), Ethicon Inc., Somerville, NJ.; Suture (4-0 Silk), Cyanamid Canada Inc., Montreal, QC.; Hall Micro Oscillator saw blades (ref. 5053-38), Linvatec Canada ULC, Mississauga, ON.; Pilot Drill Shaft 1.1 x 35 mm (ref. 513.03), Linvatec Canada ULC, Mississauga, ON.; Self-retaining retractor; Freer elevator; Ragnell retractor; Gilles forceps; Needle driver; Mosquito forceps; Distraction device, Modified Orthofix uniplanar M-100 fixator, Orthofix Inc., Verona, Italy, Custom-made in Montreal; Adjustable screwdriver; Titanium pins, 2mm diameter.

3.2 Operative Technique

After weighing the rabbit, anesthesia was induced by intramuscular injection of ketamine (30 mg/Kg) and xylazine (5 mg/Kg). Following this, the rabbit was premedicated with a subcutaneous administration of trimethoprim sulfadiazine (30 mg/Kg) 30 minutes prior to surgery. The left mandibular area was then shaved and the animal was brought into the operating room to begin the surgery.

Using a #1 pediatric laryngoscope, the vocal cords were visualized and sprayed with 2 metered doses of lidocaine. The animal was then intubated with a #2 cuffless endotracheal tube (Mallinckrodt Inc., St. Louis, MO). The rabbit was then placed on its right side, and the endotracheal tube was secured in place. Anesthesia was then maintained with a gaseous mixture of isoflurane, nitrous oxide and oxygen by spontaneous ventilation. The eyelids were taped to protect the corneas.

The left mandibular area was prepped with stanhexidine and draped in a sterile fashion. A transverse skin incision was then made along the inferior border of the left mandible. The subcutaneous tissues were divided using a hand-held cautery. The platysma was transected and reflected in a supraperiosteal plane. The inferior alveolar nerve was identified as it emerged from the mental foramen and preserved. The first premolar tooth was exposed and identified. A Hall drill with 1.1 mm drill bit was used to drill through the lateral and medial cortices of the mandible in two standard locations. The first hole was drilled just inferior to the mental foramen. The second hole was drilled 1.4 cm posterior to the first hole. Two self-tapping 2 mm titanium pins were then inserted into each of these holes.

A minimal amount of periosteum was incised in a transverse direction along the inferior border of the mandible, and carefully elevated. The titanium pins were

then removed in order to facilitate performance of an osteotomy in the coronal plane with an oscillating saw, in the midline between the two pinholes. The titanium pins were then inserted in the holes and fixed to the modified Orthofix uniplanar M-100 fixator (Orthofix Inc., Verona, Italy), and the separate segments of bone were reapproximated. The wound was then closed in two layers using 4-0 vicryl for the platysma and subcutaneous tissues, and 4-0 silk for the skin. See the schematic and pictoral illustration in Figures 10 and 11.

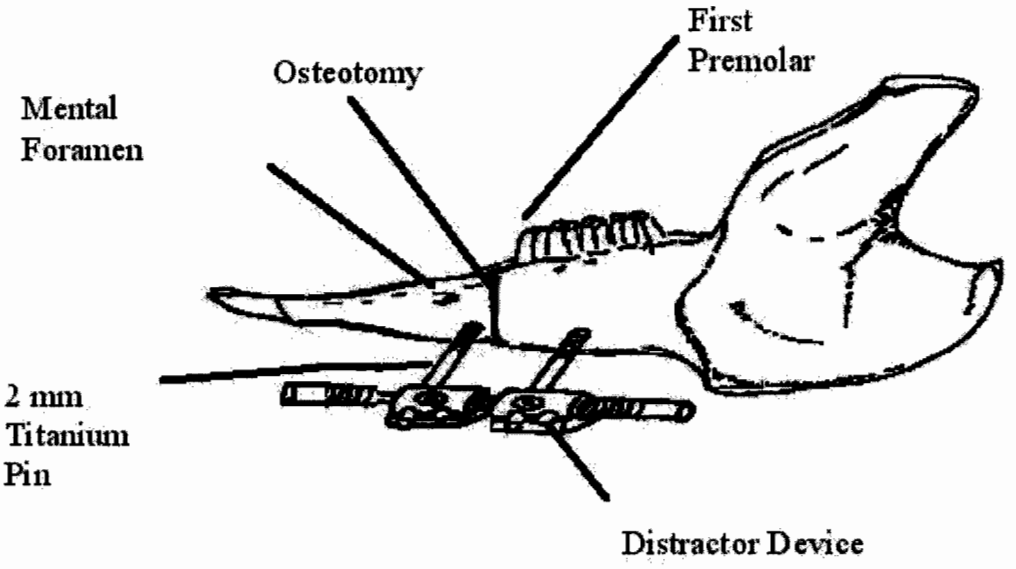

Figure 10. Schematic drawing of the left hemimandible with surgical landmarks, the osteotomy, and modified Orthofix M-100 uniplanar distraction device.

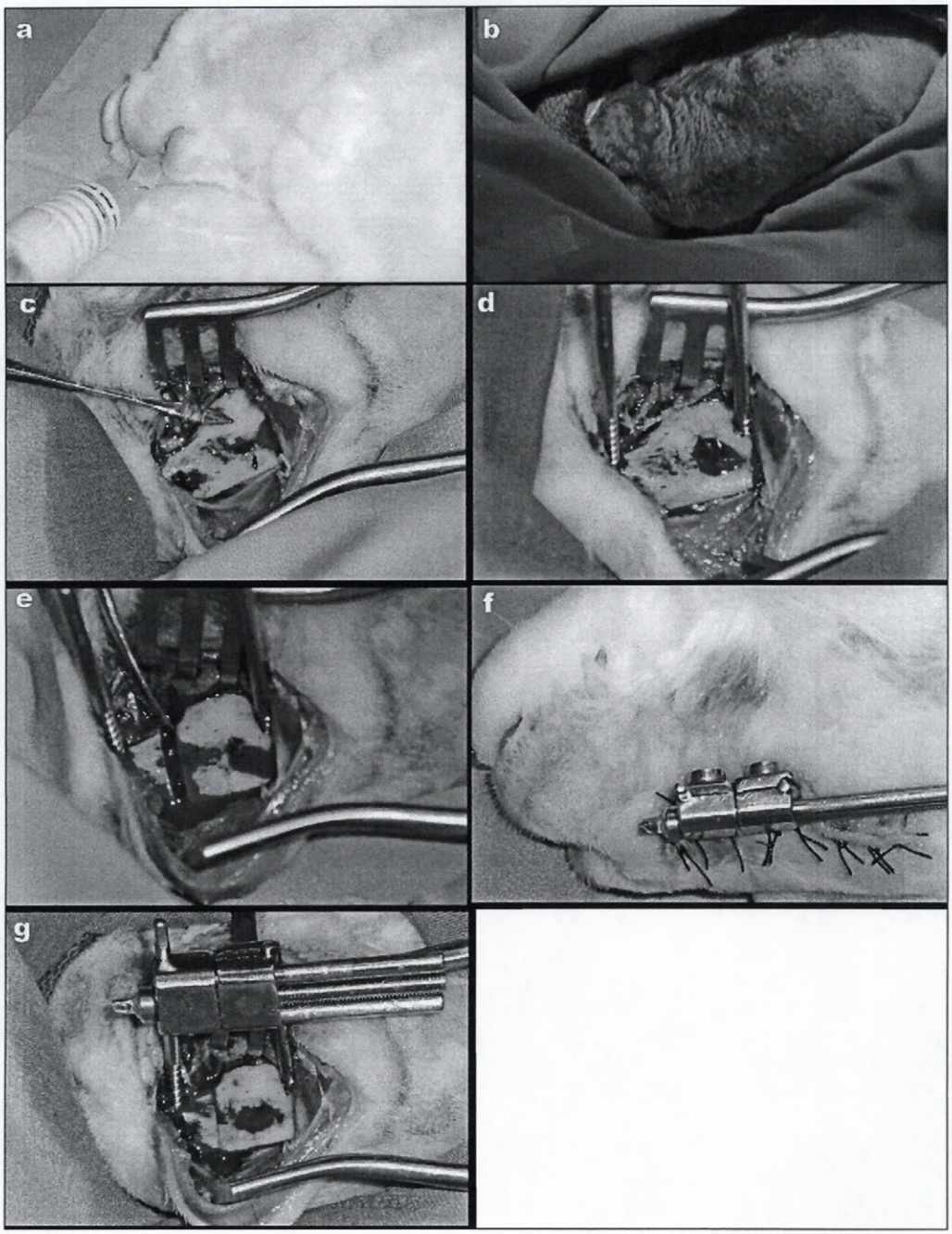

Figure 11. Photograph demonstrating the operative procedure. a, Intubation. b, Sterile draping. c, Isolation of inferior alveolar nerve in mental foramen. d, Placement of 2 mm titanium pins. e, Position of mandibular osteotomy. f, Application of distraction device . g,Wound closure.

The rabbit was then reversed from anesthesia, extubated, and monitered closely for two hours post-operatively. During the following week, the animals received daily intramuscular injections of buprenorphine (0.01 mg/Kg) and subcutaneous injections of trimethoprim sulfadiazine (30 mg/Kg). For the duration of the experimental protocol, the animals were monitered twice daily for signs of infection, bleeding, discomfort, and dehydration.

3.3 Experimental Protocol

Following surgery, a latency period of one week was allowed. After this delay of seven days, distraction was initiated at a rate of 0.25 mm every 12 hours for 3 weeks (distraction period).

At the end of the distraction period, and immediately prior to the consolidation period (Day 28), each rabbit in the experimental group (8 rabbits) and the comparison group (8 rabbits) received an injection at the distraction site. The experimental group was injected with 200 micrograms of human recombinant BMP-7 (rhBMP-7) (kindly donated by Stryker Biotech. Inc, Hopkinton, MA, USA) dissolved in a lactate buffer. The comparison group was injected with 200 micrograms of only the lactate buffer (Stryker Biotech. Inc, Hopkinton, MA, USA). The distracted area was identified easily by palpation between the proximal and distal mandibular segments. A 30-gauge syringe was inserted into the mid-portion of the distracted site, and then 0.2cc of either the lactate buffer or the rh–BMP-7 was injected. No material was injected into the control group of 8 rabbits. This was followed by a 3-week period during which the

external fixator was held in place without distraction (consolidation period). The timetable of the study is represented schematically in Figure 12.

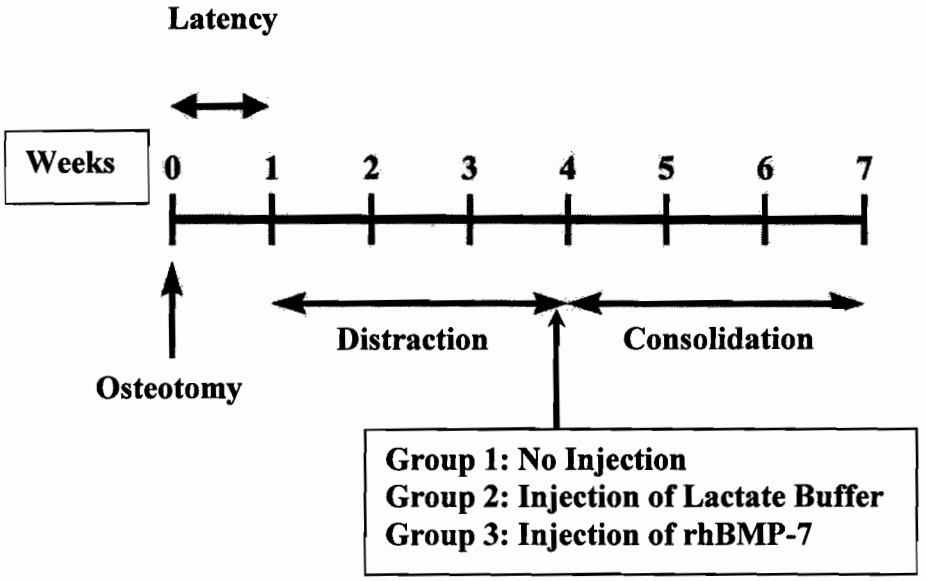

Figure 12. Schematic representation of the study protocol. The time points represent weeks after day of surgery.

After the consolidation period, the rabbits were euthanized with an intravenous injection of pentobarbital (100 mg/kg). A plain, superior-view radiograph of the mandible was obtained. The mandible was then resected en bloc, the surrounding soft tissues were then carefully removed, and the generate bone was harvested for radio-densitometry assessment using Dual Energy X-ray Absorptiometry (DEXA) analyses.

3.4 Mandibular X-rays

In the immediate post-operative period, and immediately after euthanization, plain x-ray films of the mandible were obtained using a General Electric portable X-ray machine. A superior view was obtained with the specimen 1 meter away from the

beam source. The X-ray machine was set at 100 mAmp, 58 Kv, and the exposure time was 1/40 seconds.

The radiographic appearance of the osteotomized site, and the distraction zone was subjectively assessed. Particular attention was paid to the radiodensity of the generate bone within the three different groups.

3.5 DEXA-Densitometry

Using the Hologic QDR 4500 X-ray Bone Densitometer (Hologic Inc., Bedford, MA), Dual Energy X-ray Absorptiometry (DEXA) scan of the mandible was performed in the axial plane. The specimen, including the mandible and the overlying soft tissues, was positioned at the center of the table with the long axis of the bone parallel to the long axis of the table. A laser marker was used to center the C-arm over the specimen. The global scanning region was then adjusted in order to ensure that equal amounts of soft tissue occupied both sides of the scanning region. The same radiology technician performed all the DEXA studies.

Beginning at the most anterior point of the specimen, the entire length of the mandible was scanned. The "Small Animal" menu was selected from the "Scan Selections" menu. Following the manufacturer's instructions, the "Regional High Resolution" scan mode was selected. The width, line spacing and point resolution were automatically set by the computer. The computer then calculated a bone mineral content (BMC) in grams, an area in square centimeter, and bone mineral density (BMD) in grams per square centimeters in the lengthened zone of the left mandible.

The BMD and BMC were systematically sampled within the confines of a computer generated box placed over the generate bone. The size of the sample box was tailored to the dimensions of the generate bone within the distraction site.

The values for each of the rabbits within the three groups were later compared.

3.6 Microcomputed Tomography (Micro-CT) Scan

To quantify regenerate bone volume, the distraction gaps of the harvested mandibles were imaged by three-dimensional micro-CT at an X-ray voltage of 100 kV, 98 μAmp, and resolution of 22.9 μm/voxel (volume elements). In total 206 Projections were captured over 360^0, resulting in 422 megabytes of data. Reconstructed micro-CT images were used to construct histograms of voxel intensity within the gap. Mineralized bone volumes were then calculated through voxel counts above a specified threshold value. In addition, a set of custom stereologic algorithms was implemented to analyze micro-CT images for determination of bone volume and relative bone volumes. The relative bone volume represents in percentage, the volume of osteogenerate relative to the entire volume of bone measured by the micro-CT analysis. In addition, measurements of bone volume and relative osteogenerate bone volume were determined, excluding the outer denser cortical layer of bone.

3.7 Statistical Analysis

Data analysis was performed using the SPSS Program Data Editor, Version 11. This program was used to perform One-way ANOVA testing and related Post Hoc tests, as well as T-test to compare means. Statistical significance was considered as a P value $p<0.05$.

4. Results

4.1 Animal Outcome and Complications of the Surgical Procedure

All but one of the 24 animals tolerated the surgery and distraction protocol. One rabbit from the rhBMP-7 group was omitted from the study, as his surgery was complicated by a comminuted fracture of the mandible, which resulted during the creation of the osteotomy. No postoperative complications such as aspiration pneumonia or wound infection were encountered. The animals were fed regular chow, and all gained weight. By the second week of distraction, the rabbits developed a significant overgrowth of their incisors and cross-bite all the animals required trimming of the incisors under sedation with bupernorphine for comfort and facilitation of feeding. This is a well-known problem in the relevant literature, and the solutions proposed by previous groups were applied in this study, and were successful.

4.2 Radiographic Appearance

Post-operatively, radiographs revealed good position of the osteotomy and proper alignment of the proximal and distal mandibular segments (Figure 4). The fracture line was also apparent, albeit subtly. These findings confirmed that the distraction device was properly applied. After euthanasia, the radiographs revealed bone formation within the distraction zone, confirming that the distraction device provided adequate rigid external fixation throughout the distraction and latency period, without abnormal torque (Figure 13).

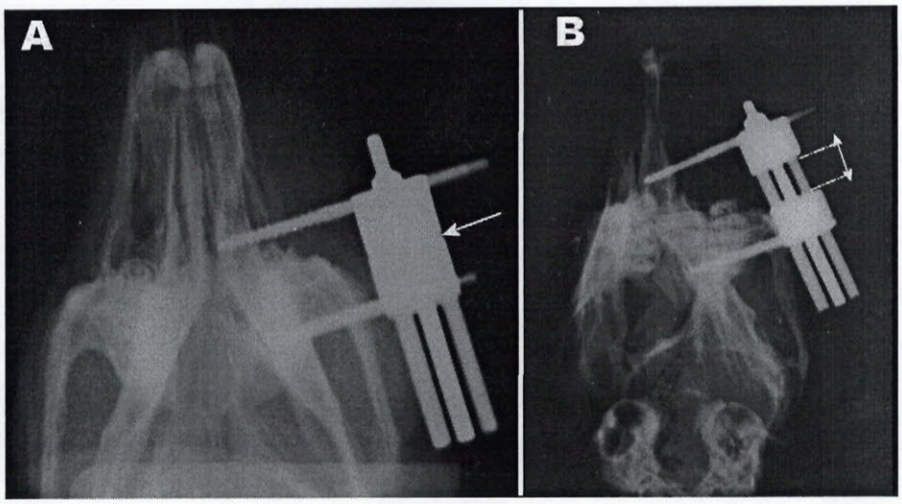

Figure 13. Sample plain radiographs of the mandible following surgery (A) and following completion of distraction and consolidation phases (B). All radiographs revealed good reduction of osteotomy and proper alignment of the proximal and distal mandibular segments. The single arrow reveals a closed distractor. Following euthanasia, all radiographs revealed bone formation within the distraction zone. The double-headed arrow reveals the ditractor opened by 10 mm at the completion of distraction.

4.3 Bone Mineral Density

After the consolidation phase, bone mineral density was lowest in the control group that was not injected with either lactate buffer or rh–BMP-7 (mean density 0.618 +/- 0.0506 g/cm^2). It was highest in the groups that had been injected with either the lactate buffer (comparison group, mean density 0.749 +/- 0.0588 g/cm^2) or rhBMP-7 (mean density 0.732 +/- 0.141 g/cm^2) (Figures 14 and 15).

A statistically significant difference existed between the control group and the comparison group, which had been injected with only the lactate buffer ($p= 0.03$). There was no statistical significance in the difference in mean bone mineral density between the rhBMP-7 group and the lactate buffer group ($p >0.05$). The difference

in mean bone mineral density between the BMP-7 group and the control group approached statistical significance (p=0.07).

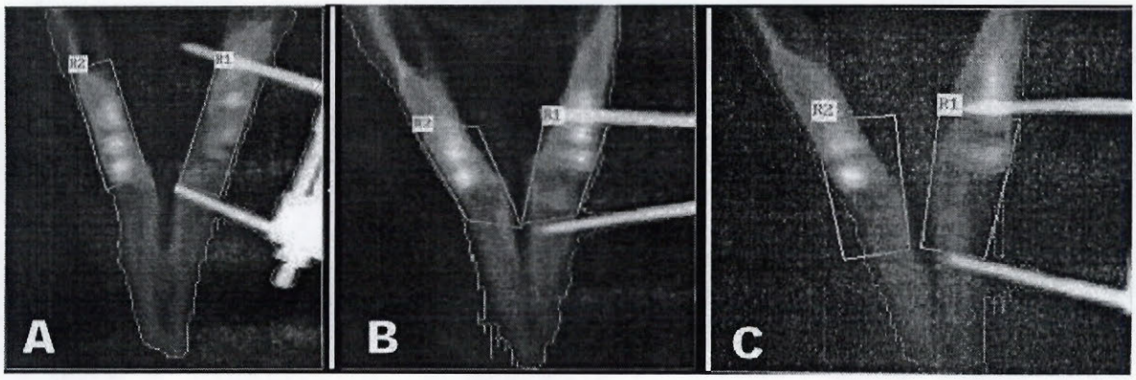

Figure 14. Sample densitometry of the distracted hemimandible (R1) and corresponding unaltered right mandible (R2) in the control (A), lactate buffer (B) and rh-BMP-7 (C) groups.

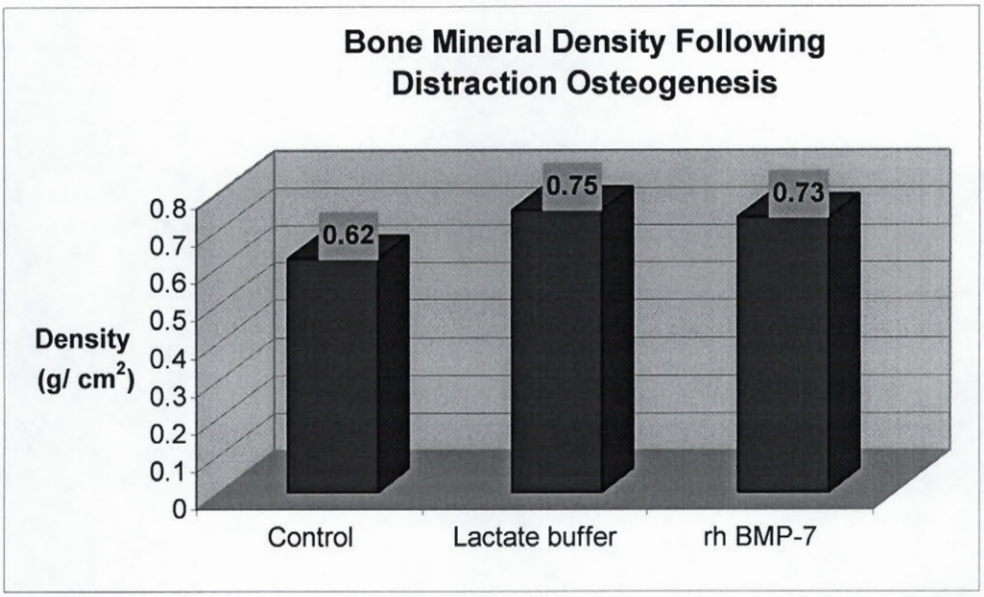

Figure 15. Diagrammatic representation of Radiodensity of the groups. Seven weeks after bone distraction, the radiodensity was highest in the Lactate buffer group, followed by the rhBMP- 7 group, and the control group. (Statistically significant by One-way ANOVA analysis, $p < 0.05$)

4.4 Bone Volume

Micro-CT images confirmed new bone formation in the distraction gap . The average relative new bone volume within the distraction gap was highest in the rhBMP-7 group (mean relative volume 36.44% +/- 7.02 %) when compared to the relative new bone volumes of the lactate buffer group (32.46% +/- 5.59%) and the control group (29.98% +/- 7.24%) (Figure 16).

This showed an approximately 4% increase in relative bone volume in the rhBMP-7 group, compared to the lactate buffer group and a 6.5% increase compared to the control group. This trend approached, but did not reach statistical significance ($p=0.17$).

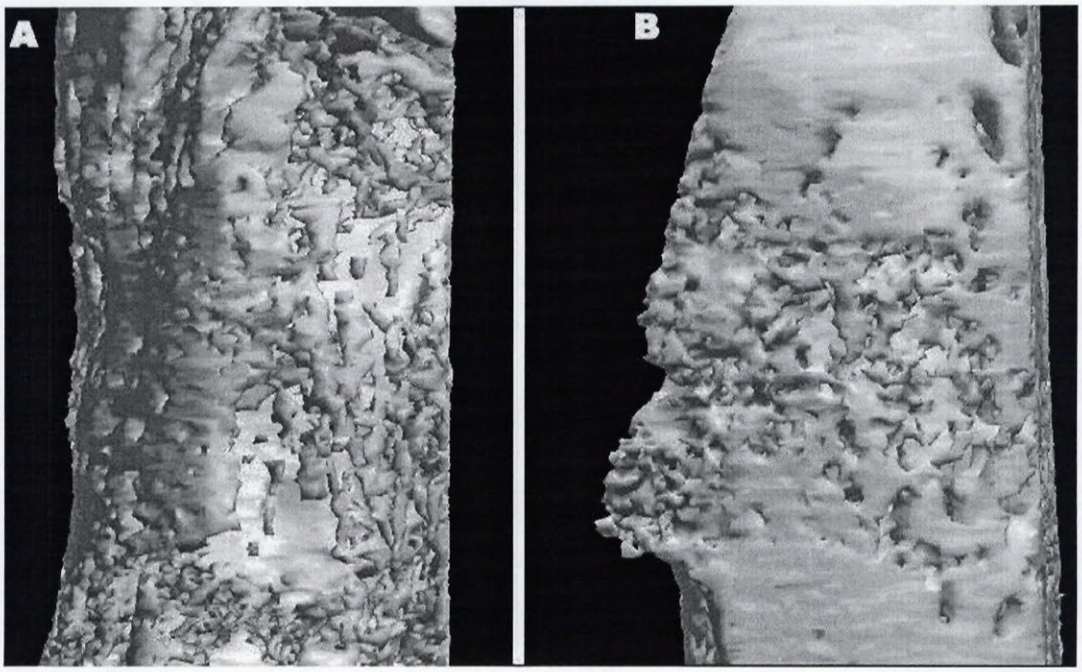

Figure 16. Sample very high-resolution 3-D micro-CT images of the distracted hemimandible showing more regenerate bone within the distraction gap in the rh-BMP-7 group (B) compared to the buffer group (A).

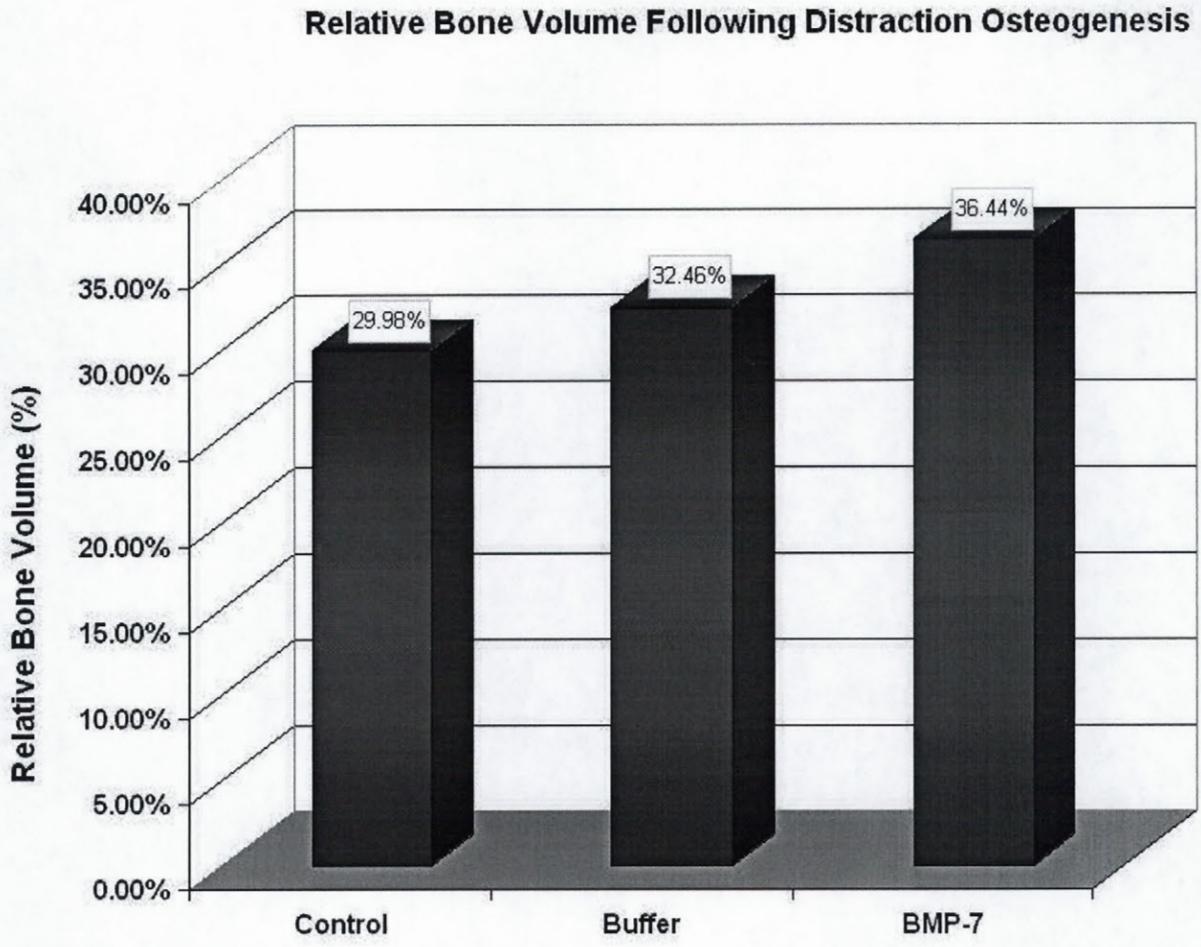

Figure 17. Diagrammatic representation of Relative bone volume of the groups. Seven weeks after bone distraction, the relative new bone volume was highest in the rhBMP- 7 group, followed by the buffer, then control groups. (p=0.17)

5. Discussion

Bone induction during regenerate ossification is a sequential cascade that includes chemotaxis, mitosis and differentiation of both bone and cartilage. Bone morphogenetic proteins (BMP) purified from demineralized bone matrix of a

variety of mammalian species govern these three key steps in new bone formation. They do so by inducing osteogenesis, both during embryological bone formation, and in distraction osteogenesis[23].

Recombinant BMP-7 (also called OP1) has been shown to accelerate the formation of new bone in numerous preclinical and clinical studies. In vitro studies of rhBMP-7 have demonstrated that it acts on the proliferation and differentiation of osteoprogenitor cells in bone-forming cells.

The therapeutic potential of rhBMP7 has been examined in animal models of critical sized bone defects, and in osteogenesis of long bones. To date, very few studies were conducted to examine potential of BMP in mandibular osteogenesis[27,91]. Additionally, the capacity of rhBMP-7 to promote regenerate ossification during distraction osteogenesis of the mandible or craniofacial bones has been previously investigated in one study in which the authors deliberately created a critical-sized defect, and thus rendered the study incomparable to standard distraction osteogenesis models[92]. In the present investigation, we studied the influence of a single injection of rhBMP-7 administered to the distraction site at the end of distraction, on the rate of new bone formation during mandibular distraction osteogenesis in a rabbit model. We hypothesized that the end of the distraction phase would be the ideal time to administer the recombinant BMP-7 since previous research demonstrated that at this phase, the endogenous BMP-7 abruptly tapered.

The present study demonstrated that a single injection of 200 micrograms of rhBMP-7 administered to the distraction gap at the end of distraction resulted in an increased bone mineral density value in the rhBMP-7 group and the comparison (lactate buffer) group compared to the control group. According to the densitometry evaluation, there was however, no significant increase in the bone mineral density between the group that was injected with the rhBMP-7 and the group that was injected with only the lactate buffer.

In contrast to this finding, micro-CT evaluation revealed that a larger volume of newly formed bone was remodeled in the rhBMP-7 group compared with both the control and the buffer groups. Although the statistical analysis revealed a trend towards greater bone formation in the BMP-7 group compared to the buffer group, this result did not reach statistical significance.

The DEXA scan bone densitometry finding suggests that there may be a dose-dependant or a temporal response to rhBMP-7, which was not elucidated in this study since the injection was given at in a single dose, and the mandibles were harvested at a single 7-week interval following surgery. This may be further studied in future investigations. It should be noted, that although a single dose of rhBMP-7 was used, the decision to apply a dose of 200 μg was based on an extensive literature research to investigate the range of BMP doses that were used in previous publications. It should also be noted that this dose was in the higher range of BMP doses that have been previously applied in previous DO studies.

5.1 *Temporal Response to rhBMP-7*

Lessard and colleagues have previously shown that the expression of BMP2, 4, and 7 was maximal during the distraction phase of DO, and that it tapered off during the consolidation phase[86]. We therefore hypothesized that the best time for local application of exogenous BMP's would be at the start of the consolidation phase.

A recent study by Hamdy and colleagues, finalized following the methodology development and start of the present study, included immunohistochemical analyses of BMP receptor expression during distraction osteogenesis of the rabbit femur, and showed a strong expression of BMP receptors during the early distraction phase, which then gradually decreased[90]. This pattern was similar to the

expression profile of BMP-7 during DO. It appeared that the expression of both BMP-7 and its receptors was related to the mechanical forces of distraction in DO, with strong expression as long as the distraction was maintained and rapid down-regulation as soon as the distraction stopped[90]. Thus, although our goal was to increase osteogenerate formation by applying exogenous BMP-7 to augment the diminishing expression of endogenous BMP-7 at the end of the distraction phase, BMP-7 given at the end of distraction period may not have a marked effect since only a small amount of receptor protein is present in the target tissue. The receptor expression findings by Hamdy and colleagues, suggest that BMP7 may be more effective when given at the start of distraction.

Indeed, there is evidence from a recent rat model that local injection of 20 micrograms of BMP-7 at the time of long bone osteotomy enhanced bone formation in the distracted zone significantly, even when given at a dose that was ten times lower than the dose used in this study[24]. This study was published after establishment of the present study's methodology, and following the experiment phase of this research. Since the above-mentioned findings were not available prior to, or during the conduction of the present research, it was not possible to incorporate this information into the development and execution of the present research's methodology. Future studies conducted by our group will compare the osteogenerate production after injection of rhBMP-7 given at the time of osteotomy in one group, and the osteogenerate production in a separate group injected with rhBMP-7 at the end of the distraction phase.

5.2 *Mechanical Stimulation as an Initiator of Distraction Osteogenesis*

An interesting finding in the present study, was that the buffer group demonstrated an increase in osteogenerate bone mineral density and relative bone volumes when

compared to the control group. BMPs are not alone in guiding skeletal modeling and repair. It has been suspected for many years that mechanical stimuli influence tissue differentiation and bone shape. This may be an alternative explanation for the apparent increase in bone mineral density caused by introducing a needle to inject the BMP-7, or the lactate buffer alone, when compared to the control group that did not receive the same mechanical stimulus.

The findings of this study may indicate that a contributing factor to the positive outcome could also be attributed to the mechanical stimulation of the needle microtrauma in the distracted zone, applied during the early consolidation period, accelerated the maturation of the bony callus. It has been reported that mechanical stimuli guide skeletal modeling and repair by influence tissue differentiation. Lee et al have suggested that micro-damage is a stimulus for bone remodeling[93]. In their study of sheep ulna, it was found that the location and timing of micro cracks, resorption cavities and secondary osteons were consistent with the activation-resorption-formation remodeling cycle, suggesting that microdamage is a stimulus for bone remodeling.

In a recent study, Mofid and colleagues demonstrated an increase in callus mineral deposition and volume through a simple regimen of daily alternating compression and distraction during the early consolidation period of the distracted mandible[94]. They showed that this daily alternating compression and distraction stimulated osteoblastic activity, as well as increased remodeling, and maturation of bone.

Another mechanism by which local wound trauma at the distraction zone may enhance bone formation implicates angiogenesis, and its role in distraction osteogenesis. Some authors have suggested that either vascular endothelial cells (ECs) or pericytes differentiate into osteoblasts or precursor cells, which means that vessels could directly participate in bone formation[95]. Wound trauma causes

mobilization of hematopoeitic cells, including pluripotent stem or progenitor cells in spleen, bone marrow and peripheral blood. Circulating and/or bone marrow-derived endothelial progenitor cells may migrate to sites of active angiogenesis, and differentiate there into EC's during distraction osteogenesis. These vascular EC's then differentiate into osteoblasts in the distraction site[95]. In 2002, Choi and colleagues implicated a close temporal and spatial relationship between periosteal and medullary vascular proliferation, and bone mineralization in the distraction gap in the rabbit tibia[95]. Lessard and colleagues also implicated the close temporal and spatial relationship between vascular proliferation and bone mineralization in the distraction gap[86].

5.3 *Lactate Buffer as an Initiator of Distraction Osteogenesis*

Another explanation for this outcome is that the lactate buffer alone may be effective in increasing the bone mineral density of the regenerate bone. When used for delivering osteo-inducing factors to enhance bone healing during distraction osteogenesis, the ideal biodegradable material should be biocompatible and completely absorbable.

Several studies have evaluated the ideal biomaterial for delivery of osteo-inducing agents, and although it is generally accepted that lactic acid is biocompatible, some authors have reported on the osteoinductive properties of lactate alone[96,97]. In 1996, Shimono and colleagues inserted poly-L-lactic acid films onto the periosteum of rabbit tibiae, and found that this alone promoted ossification[98].

At the same time, Otto and colleagues studied the effect of poly-Lactic acid on the proliferation and differentiation of primary bone cells in vitro. Their results

indicate that the proliferation and differentiation of bone cells in vitro can be modulated by lactic acid[96].

In contrast to the DEXA scan densitometry finding, the micro-CT analysis demonstrated that there was in fact a trend towards an increase in relative new bone volume within the distraction zone of the rhBMP-7 group when compared to both the buffer and the control groups.

High resolution imaging techniques such as microcomputed tomography (micro-CT) can provide more sensitive, complementary quantitative information on three-dimensional (3-D) skeletal morphology and bone volume following distraction osteogenesis.

5.4 *Role of Micro-CT in the study of Distraction Osteogenesis*

X-ray computed tomography (CT) has been used extensively for clinical diagnostic imaging since its development in the early 1970's. Custom-built micro-CT systems were first constructed in the 1980's using microfocal spot X-ray sources and high-resolution detectors[99]. While clinical CT scanners typically produce reconstructed images composed of about 1.0 mm^3 volume elements (Voxels), microcomputed tomography (micro-CT) imaging systems have much better spatial resolution, producing voxels that measure 5 – 50 μm, or approximately one million times smaller in volume than CT voxels[99]. The resolution of the micro-CT scan is at least 500-700 times higher than the dimensions of the sample.

Micro-CT imaging offers several advantages over traditional 2-D imaging methods for the analysis of bone mineralization following distraction osteogenesis. Images collected from multiple viewing angles are reconstructed to produce unparalleled

evaluation of the entire three dimensional (3-D) spatial distribution maps of material density within attenuating materials or tissues, such as bone. By comparison, conventional radiography is limited to providing two-dimensional (2-D) images that represent the summation of material attenuation along the X-ray path, and is semi-quantitative, at best.

Micro-CT imaging also offers several advantages over traditional histological methods. The collection, reconstruction and morphometric analysis of micro-CT images can typically be completed in a few hours or less, depending on the size of the sample. In comparison, histological evaluation of under-mineralized bone samples, which includes fixation, embedding, sectioning and staining, can take a week or more. Additionally, in micro-CT reconstructed images, structures can be followed continuously from the level of the osteon, through to gross bone morphology, while evaluations of growth or bone regeneration based on 2-D histological sections are not necessarily representative of the entire 3-D structure. Micro-CT imaging and histological methods, however, provide complementary information since histological methods demonstrate cellular details and spatial distributions of protein or mRNA expression, which are not provided by micro-CT.

Early on, micro-CT imaging was applied primarily as a research tool to quantify structure-function relationships in trabecular bone[100]. It was coupled with stereological methods to estimate bone volume fraction and 3-D parameters of trabecular architecture, including trabecular number, thickness, orientation and connectivity[100]. Since then, several investigators have used micro-CT to assess 3-D matrix mineralization associated with fracture repair, and a few have used it to assess mineralization during distraction osteogenesis[101].

Two parameters that can be calculated from the 3-D images are total bone volume and bone mineral density, as measured by the average linear attenuation coefficient[99]. Linear attenuation, however, is more sensitive to voxel size than the

calculation of bone volume. This is due to a greater fraction of voxels at the coarser resolution being affected by artifacts at the boundaries of skeletal structures, causing underestimation of mineral density[99]. Since there is typically little effect of varying voxel size on calculation of bone volume, measurement of bone volume is a more reliable parameter[100].

Micro-CT has proven much useful information in recent years about bone and tissue regeneration and structure. This paper used micro-CT to demonstrate the usefulness of this technique of this technique in visualizing and calculating the increase in volume of osteogenerate following administration of rh-BMP-7 during the consolidation phase of distraction osteogenesis.

5.5 *Limitations of this Study*

Although much previous research was involved in strategically developing and supporting the methodology of this study, there are a few limitations in this investigation.

As mentioned previously, there may be a dose-dependant or a temporal response to rhBMP-7, during mandibular distraction osteogenesis, which was not elucidated in this study since the injection was given in one single dose, and the mandibles were harvested at a single 7-week interval following surgery. An approach to better elucidate this information would have included additional groups of rabbits that would have been injected with a dose-response curve for BMP-7 at the different phases of osteotomy, distraction and consolidation, and harvesting the mandibles at each of these phases to examine the osteogenerate.

Inherent to any animal study, is a certain amount of biological variability. Perhaps the differences between the groups may have reached statistical significance if

there were an increased number of rabbits in each group in order to override the biological variability.

Another possible criticism of this research is that immunohistochemistry and histology studies were not performed. The goal of this investigation was to evaluate the amount of bone formation after application of BMP-7, and not the cellular details, spatial distributions of protein or mRNA expression in the osteogenerate. Thus, 3-D bone volume measurement, quantified by micro-CT imaging, was thought to be the most sensitive and accurate measure of our parameter of interest, as opposed to the 2-D information provided by histology or immunohistochemistry.

The high resolution of the 3-D images provided by the micro-CT scan allowed a more detailed analysis and measurement of the volume of the osteogenerate. In addition to the standard bone volume measurement performed by the microcomputed tomography, the volume of only the soft cancellous osteogenerate bone was measured without the outer denser cortical bone. This was carried out by changing the parameters of the micro-CT machine and re-analyzing the specimens. Using the different measurement parameters, it was found that a statistically significant increase in relative cancellous bone volume was present in the rhBMP-7 group (27%) when compared to the buffer group (20 %), ($p<0.05$). It was difficult to scientifically interpret this result, since the osteogenerate bone mineral density previously calculated by the DEXA scan had been measured with the outer dense cortical bone. The DEXA scan provides a lower resolution 2-D image compared to the higher resolution and 3-D spatial distribution map of the bone provided by micro-CT technology. Since the DEXA scan images and calculation are not as accurate as those provided by the micro-CT, it would not have been possible to isolate and calculate only the soft cancellous bone mineral density with the DEXA scan. The statistically significant increase in soft cancellous relative bone volume in the rhBMP-7 group, compared to the buffer group, detected by the micro-CT

scan was an interesting finding. A new study by our group will be planned in the near future in order to further investigate the impact of this finding, by conducting biomechanical testing of the specimens to compare the quality of the increased osteogenerate in the BMP-7 group compared to the buffer group.

5.6 *Future Direction*

Over the past twenty years, investigators have applied the principles of distraction osteogenesis to the treatment of musculoskeletal conditions in a variety of animal studies. Despite the use of an appropriate distraction rate, formation of new bone is not always optimal. While BMP have shown promise for improving bone formation in distraction osteogenesis, their use has been hampered by their short biological half-life, which limits their in vivo effectiveness during the process of bone regeneration. Certain BMPs have shown a does-dependant and time dependant response. Future research should focus on ascertaining the ideal quantity and timing of BMP administration to achieve maximal acceleration of regenerate ossification.

The robust bone formation and healing in animal studies that have used BMP to accelerate bone formation in distraction osteogenesis, have been very promising. Although clinical trials have also produced promising results, they are not as impressive as those seen in the animal studies. The reasons for this are unclear, but may be related to the need for improved methods of BMP delivery.

The administration of recombinant BMPs requires the use of inactive carrier systems. An ideal bioconductive system should allow a substrate that would accommodate the proper dose of BMP, controlled release of the protein, have adequate exposure to inducible cells, be immunologically inert and biodegradable without producing toxic waste products that would inhibit the osteogenesis process[87]. Other important factors to consider are a delivery system that would allow enough void space to enable cell proliferation and angiogenesis to occur. In

addition, the kinetics of the release of BMP from its delivery system may need to be controlled in order to match the responsiveness of the host environment, and the delivery system would need to be able to distribute the limited quantity of BMP that is generally produced by current recombinant DNA technology. These carrier systems also must be sterilized and stored easily and, in certain circumstances, serve as a load-bearing device.

Numerous substances, including demineralized bone matrix, fibrin, ceramics, collagen, titanium, and polylactic acid have been used in ectopic bone formation assays and orthopedic experimental models[87]. As a result, the development of synthetic carriers has received much attention..

The development of a suitable carrier for BMPs still remains an active area of investigation for specific clinical scenarios in which induction of osseous regeneration is required[102,103].

An approach concept that may allow BMPs to produce therapeutic effects substantially greater than those achieved with recombinant proteins, is gene therapy. The transfer of genetic information to a cell that is normally found in the host would provide a setting in which endogenous BMP protein would be produced. Gene therapy has the potential to offer several advantages, including control of amount, timing, and duration of BMP production.

A recent report by Peng et al. illustrated how gene therapy may offer advantages that do not currently exist wit BMP technology[104]. There was an observed enhancement of stem cell recruitment and cell survival that primed the host environment by increasing the number of cells available to induce a strong bone formation response. This study had important implications for the genetic engineering of bone since it demonstrated that the use of gene therapy allows the titration of optimum expression of BMP. It supports the idea that gene therapies may provide the kinds of responses needed to regenerate large segments of bone,

such as increasing the host responsiveness to BMPs by providing a stimulus to increase the number of BMP receptors at the site of treatment. Gene therapy represents an attractive strategy for sustained production of osteoinductive proteins at sites of bone generation, such as in DO. This would have an important impact on improving clinical outcomes.

It is anticipated that as the field of gene therapy evolves, opportunities to apply these strategies to treatment of craniofacial conditions will be possible.
With improved osteogenerate formation in distraction osteogenesis, comes the need for a more precise and non-invasive method to quantitatively analyze and follow the bone formation. Micro-CT analysis has proven useful in a wide variety of applications by providing high-resolution images of opaque objects such as bone, and calculating quantitative parameters of skeletal morphology, without destroying the sample. As micro-CT instruments increase in resolution and computing resources become available, more high detailed information regarding osteogenerate volume will become available. The 3-D nature of this information means that much improved visualization of relationships is possible. This paper used micro-CT to demonstrate the usefulness of this technique of this technique in visualizing and calculating the increased relative bone volume of osteogenerate following distraction osteogenesis after administering rh-BMP-7 compared to the control and lactate buffer groups.

Recently developed micro-CT systems offer the potential for in vivo imaging, and therefore longitudinal studies of changes in bone microstructure[105]. Although not widely used for clinical diagnostic imaging, micro-CT is particularly well suited for imaging small animal models that are being established to study bone regeneration in distraction osteogenesis. The opportunity of perform multiple scans, at appropriate radiation doses, over time will reduce the number of animals needed, and have a tremendous impact on studies of bone regeneration during distraction osteogenesis. The ability to track changes over time in vivo will further establish

micro-CT as a standard evaluation technique for future studies of distraction osteogenesis.

6 Conclusions

Distraction osteogenesis has been described as in vivo tissue engineering. The ability to stimulate this process for the repair of bony defects or lengthening of congenital anomalies of the facial structures has already significantly impacted the field of craniofacial surgery in the last 10 years. Local application of osteoinductive agents such as BMP-7 by means of an injection is an appealing, minimally invasive technique to accelerate new bone formation in distraction osteogenesis. This has important clinical benefits in that it may lead to a decrease in the duration of distraction osteogenesis, and consequently, may decrease the morbidity associated with the entire surgical treatment. This decreased duration will also lead to improved results in children for whom a decrease physical activity secondary to a metallic device is a major disadvantage. Additionally, this will allow timely reconstruction for the oncological population and the associated radiotherapy treatment, which needs to be taken into consideration.

The only other reports to date on the use of BMP in distraction osteogenesis of the mandible, that were able to find a positive result were obtained with locally applied BMP2 in a rabbit model[91], and adenovirally mediated BMP-4 administration, also in a rabbit model[27], and locally applied BMP-7 in a rat mandibular critical-sized defect[92]. The present investigation is the first study to describe the effect of recombinant human BMP-7 injection in the distraction zone during distraction osteogenesis of the mandible in a rabbit model.

This investigation showed that the lactate buffer group had the highest bone mineral density compared to both the control and the BMP-7 group, when evaluated by DEXA densitometry scan. This may suggest that the mechanical trauma induced by the needle used for injection had some effect on stimulation of

new bone in the distracted zone. Alternatively, it may imply that the lactate itself may have osteoinductive properties

Our results also demonstrated a trend towards increased bone formation following the local application of 200 micrograms of rhBMP-7, as evidenced by the more sensitive micro-CT analysis. Thus, it is possible that rhBMP-7 has a beneficial effect in distraction osteogenesis, although the putative beneficial effect of rhBMP-7 applied at the end of the distraction phase, was not large enough, however to create a statistically significant effect in this study.

Future investigations that focus on the ideal dose and timing of the BMP administration, possibly early in the distraction phase, may lead to an effective method to enhance bone formation during this technique. Additionally, further studies are needed to determine an ideal delivery system for osteoinductive proteins, and developing gene therapy that may produce greater therapeutic effects than those achieved with recombinant proteins. Finally, the potential for in vivo imaging, such as with micro-CT scan, should be further developed in order to facilitate longitudinal studies of changes in bone microstructure during distraction osteogenesis. This will lead to a further understanding of the complex process of distraction osteogenesis, and improve its clinical application.

7. References

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Protocol #: 1602
Investigator #: 639
Approval End Date: Dec. 31, 2002
Facility Committee: ACC

McGill University
Animal Use Protocol – Research

Title: Effects of Recombinant BMP - 7 In Distraction Osteogenesis of the Rabbit mandible
(must match the title of the funding source application)

☒ New Application ☐ Renewal of Protocol # ____ ☐ Pilot **Category** *(see section 11)*: D

1. Investigator Data:

Principal Investigator: L. Lessard MD. FRCSC **Phone #:** 514 406-7854
Department: Surgery (Plastic Surgery) and ENT **Fax#:** 514 934-8207
Address: 1650 Cedar Avenue, H3G-1A4, Room L9-313 **Email:** Newningo@hotmail.com

2. Emergency Contacts: Two people must be designated to handle emergencies

Name: K. Zakhary MD **Work #:** 406-7854 **Emergency #:** Same (beeper)
Name: L. Lessard MD FRCSC **Work #:** 934-1934 ext 4-4341 **Emergency #:** 406-1603 (beeper)

3. Funding Source:

External ☒
Source (s):
1. PSEF Grant (American Society of Plastic Surgeons)

2. Striker US (BMP-7)
(Dr.Hamdy's BMP protocol, peer reviewed and accepted by the animal ethics committee)

Peer Reviewed: ☒ YES ☐ NO**
Status : ☒ Awarded ☐ Pending
Funding period: 1 year

Internal ☐
Source (s):
Peer Reviewed: ☐ YES ☐ NO**
Status: ☐ Awarded ☐ Pending
Funding period:

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ACTION		DATE
DCs	✓	June 20/02
DE		
APPROVE		

** All projects that have not been peer reviewed for scientific merit by the funding source require 2 Peer Review Forms to be completed e.g. Projects funded from industrial sources. Peer Review Forms are available at www.mcgill.ca/rgo/animal

Proposed Start Date of Animal Use (d/m/y): July 1, 2002 **or ongoing** ☐
Expected Date of Completion of Animal Use (d/m/y): October 1, 2003 **or ongoing** ☐

Investigator's Statement: The information in this application is correct. I agree that the care and use of animals in this proposal will be in accordance with the guidelines and policies of the Canadian Council on Animal Care and of McGill University. I shall request the Animal Care Committee's approval prior to any deviation from this protocol. I understand that this approval is valid for one year and must be approved on an annual basis.

Principal Investigator's signature: [signature] **Date:** June 18, 2002

Approved by:

Chair, Facility Animal Care Committee: [signature] **Date:** 15 July 2002
University Veterinarian: [signature] **Date:** 7/18/02

June 2002 ETHICS CHAIR: [signature] Aug 14, 2002

ADVANCED ANIMAL USE PERIOD: [illegible]