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ASSESSMENT OF THE CLOSURE OF CRITICAL SIZED DEFECTS IN THE RABBIT CALVARIUM UTILIZING DEMINERALIZED BONE MATRIX PUTTY AS AN ALLOGENIC GRAFT MATERIAL

A THESIS SUBMITTED TO THE FACULTY OF GRADUATE STUDIES
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Abstract

Closure of bone defects that do not heal spontaneously require some form of bone inducing agent in order to ensure complete repair. Autogenous bone is the clinical gold standard for the management of these types of defects. Present research is aimed at finding acceptable alternatives to harvesting autogenous bone grafts in patients for obvious reasons. Recent literature supports that demineralized bone matrix (DBM) is osteoinductive, although this is not the case for all commercially available forms of DBM. It is now known that the methodology used in producing the DBM has a dramatic effect on the "osteoinductivity" of these materials. Additionally, research into the carriers of these osteopromotive substances is ongoing to determine which excipient facilitates the integration and osteoinductive nature of these materials.

An in vivo study was conducted which attempted to evaluate the healing of critical sized defects in New Zealand white rabbit calvarium using various grafting materials. By combining demineralized bone matrix and a poloxamer gel carrier, a putty-like material that is surgically convenient can be delivered to these defects and allowed to heal. The resultant healed defect can then be studied and compared to other models. Twenty rabbits were divided into two groups each having bilateral oval 15mm defects created in their parietal bones. This animal model was chosen because the rabbit critical size defect is a widely supported model in the literature to research such bone promotive substances. The first group of animals had untreated defects on one side and defects filled with Plaster of Paris in the second defect. The untreated defects were used as controls to confirm the critical size nature of the wound (<10% bone fill), and validated our choice to use this model. Plaster of Paris was chosen, as it is the gold standard of osteoconductive grafting

materials. The second group of animals had one defect grafted with the putty mixture of DBM and poloxamer gel and the other defect grafted with the excipient (poloxamer gel) alone. By looking at the excipient alone, it allowed for an observation of it's intrinsic ability to promote bone healing. Animals were sacrificed at 6 and 12 weeks post grafting and evaluated clinically, radiographically, and histomorphometrically using previously published methods.

Control defects healed with fibrous scar and some new bone growth at the periphery only. This confirmed and validated the use of this animal model in this study. The Plaster of Paris filled defects healed with a thick, primarily fibrous scar with variable amounts of bone within the tissue. The poloxamer gel only defects healed via a thin, fibrous scar and the occasional bony island within the scar. Clinically and radiographically, only the DBM putty group yielded a calcified, hard bony filled defect that showed large amounts of vital bone within the full volume of the defect. The 6-week group displayed new bone formation surrounding the allogenic grafting material, which was being resorbed as evidenced by the amount of osteoclastic activity. By 12 weeks, the graft material was almost completely replaced by new woven bone. Therefore, significant differences in bone fill were found ($p < 0.001$, ANOVA) at both time points between defects reconstructed with DBM putty as compared to all other groups that were evaluated.

The addition of demineralized bone matrix in a gel carrier appears to allow complete closure of critical sized calvarial defects in New Zealand white rabbits with viable new bone. These results provide evidence that DBM putty is osteoinductive and may be clinically useful for the management of critically sized cranial vault defects. Human studies are needed to assess the effectiveness in that scenario.

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Résumé

L'ajout d'une matrice osseuse déminéralisée et d'un mastic en gel de poloxamère dans une brèche de taille critique et son effet sur la cicatrisation ont été observés.

Une étude in vivo a tenté d'évaluer l'effet de l'ajout d'une matrice osseuse déminéralisée dans des brèches de taille critique pratiquées dans la calotte crânienne de lapins. Ces brèches ont été étudiées sous un angle histomorphométrique, radiographique et clinique. Des brèches de 15mm de diamètre ont été pratiquées bilatéralement dans l'os pariétal de lapins. Certaines brèches témoins ont été laissées se cicatriser seules. Dans d'autres brèches, on a par contre introduit des disques de plâtre de Paris de manière à pouvoir établir une comparaison avec les matériaux de remplacement des greffes osseuses ostéoconductrices, qui constituent l'étalon-or en la matière. Deux matériaux de remplissage expérimentaux ont été utilisés. Certaines brèches ont été remplies de gel de poloxamère seul et d'autres d'une matrice osseuse déminéralisée et d'un vecteur de gel de poloxamère. La cicatrisation a été évaluée après sacrifice des animaux 6 ou 12 semaines en post-opératoire. Les calottes crâniennes échantillons ont été examinées sur le plan clinique au titre des tissus durs et mous, dans les brèches. Ils ont été radiographiés pour examiner la calcification des tissus en voie de cicatrisation et tous les échantillons ont fait l'objet d'un examen histomorphométrique pour évaluer la nature exacte des tissus qui remplissaient la brèche et les dimensions des tissus représentatifs dans la brèche.

Toutes les brèches témoins étaient porteuses de cicatrices fibreuses et affichaient une nouvelle croissance osseuse en périphérie seulement. Cela a permis de confirmer la taille critique des brèches pratiquées pour les besoins de l'étude. Les brèches remplies de plâtre de Paris ont donné des cicatrices

fibreuses assez épaisses avec une quantité variable de tissu osseux. Les brèches remplies de gel de poloxamère ont donné une cicatrice fibreuse fine avec présence occasionnelle d'îlots osseux dans la cicatrice. Toutes les brèches remplies d'une matrice osseuse déminéralisée et de gel de poloxamère ont cicatrisé avec l'os sur toute l'étendue de la brèche. Ce phénomène est évident sur le plan clinique, examen qui révèle en outre l'abondance de tissus durs dans la brèche. Sur le plan radiographique, les brèches sont complètement refermées avec un tissu radiopaque dense, contrairement aux autres sites expérimentaux. Le groupe sacrifié après six semaines affiche une nouvelle formation osseuse autour du matériau de greffe allogénique qui s'est résorbé comme en témoigne l'intense activité ostéoclastique. Après 12 semaines, le matériau de greffe a entièrement cédé la place à un nouvel os.

L'ajout de matrice osseuse déminéralisée au gel semble favoriser la fermeture complète des brèches de taille pratiquées sur la calotte crânienne de lapins New Zealand blancs et la formation d'un nouvel os viable. De recherches sur l'être humain s'imposent pour évaluer l'efficacité de cette technique.

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Introduction

Defects in the bony skeleton occur from a wide range of clinical conditions including trauma, developmental anomalies, oncologic resections, infection and pathology. To restore lost tissue, or to prevent fracture nonunion, orthopedic, reconstructive, and maxillofacial surgeons perform many thousands of autogenous bone grafts yearly. The corticocancellous bone graft, such as that harvested from the iliac crest, continues to be the workhorse of present day surgeons. Despite being highly effective, autogenous bone graft harvesting subjects patients to a second surgical site, and with it, an associated increase in morbidity, complications, hospital stay, and recovery duration. There is also more risk of wound infection, higher blood loss, and a slower return to normal function from surgery to a site on the body distant from the original problem.

Allogenic (human cadaver) bone is the most common alternative to the autograft, but this material suffers from the potential risk of disease transmission, potential rejection, and resorption. Research has been very active in the field of bone graft replacement materials, specifically, osteoinductive materials. Original work by Marshall Urist in the 1960's revealed that implanting acid-demineralized bone into extraskeletal sites lead to the production of bone in several animal models (Urist, 1965). This principle is known as osteoinduction. It describes the properties of a material that allows it to stimulate local undifferentiated mesenchymal cells to become osteoprogenitor cells, which will eventually form new bone. Osteoconductive materials, on the other hand, are substances that assist bone formation by providing microstructural scaffolding allowing for bone formation to occur.

From this original work, Urist was able to identify proteins within the demineralized bone matrix (DBM), which he called bone morphogenic proteins. Since then, these proteins have been isolated and reproduced by recombinant means, and are known to be the osteoinductive factors within the DBM. Along with other growth factors, such as transforming growth factor beta, present research continues and aims to identify the ideal bone graft substitute that provides both osteoinductive factors and an osteoconductive framework. Ideally, the resultant material would ideally be easy to handle and surgically predictable to use.

The work presented here was designed to assess the healing of critical sized calvarial bone defects in skeletally mature New Zealand white rabbits. Demineralized bone matrix was combined with poloxamer gel yielding a putty-like material that was inserted into the experimental defects. Healing of defects treated with the DBM putty was compared to unfilled controls as well as defects filled with Plaster of Paris, and others filled with the gel carrier alone. The defects were evaluated for healing using clinical, radiographic and histologic methods at 6 or 12 weeks post implantation.

Review of the Literature

The Biology of Bone

Bone is a remarkable tissue. It is unique in that while most other tissues have a limited capacity for regeneration, and therefore heal by the formation of fibrous scar tissue, bone is constantly remodeling and can repair itself despite being disrupted by fracture or by being osteotomized during surgery. Bone also has an amazing ability to be transferred with or without a continuous blood supply to another area of the body, and continue to grow and become incorporated into its new site. With the exception of blood, bone is the most common tissue to be transplanted (van Heest et al., 1999).

Bone provides many important functions. It supports the human frame and provides for the origin and insertion of tendons and muscles to allow for locomotion and movement. It acts to protect the vital organs from blows to the body. It is the major reservoir of calcium in the body and continually releases and stores this important cation when induced to do so. Despite its relative light weight, bone is very strong and can withstand significant compressive forces. It has limited flexibility and will bend or twist and return to its original shape, but will fracture with excessive torsional, rotational, or compressive forces (Feinberg, et al., 1991).

Bone is a connective tissue that has two main structural types: tubular and flat. One serves as weight bearing and for locomotion, the other to protect vital structures. A soft tissue envelope known as periosteum covers bone. This tissue can be divided into three layers. The cambium layer next to the bone can provide for appositional bone formation. The middle layer provides for osteogenic reserve cells. The outer fibrous layer supports the periosteum with a vascular network and structural integrity (Manson, 1994). Bone generally has a dense, compact zone of bone called the cortex, which

surrounds a softer marrow cavity, composed of cancellous bone, myeloid and fatty tissues. In long bones, there is a central cavity, which is lined by a fibrous sheet called the endosteum. The functioning unit of mature bone is known as an osteon or haversian system. This is comprised of a central haversian canal surrounded by concentric layers of bone. The surrounding lamellae have lacunae, each containing an osteocyte with cytoplastic processes extending through canaliculi to communicate with the haversian system central blood vessel and other osteocytes. The diffusion distance to the nearest central vessel limits the size of the osteon, which is generally less than 100 microns (Feinberg, et al., 1991).

Immature bone, known as woven bone, is seen in areas of repair or growth. This bone is comprised of loosely organized collagen fibrils, which are relatively coarse and randomly arranged. The osteocytes within the lacunae are not positioned within an organized haversian system, and the canaliculi are less frequent and less organized. As the bone matures, osteoclasts break down the bone and destroy its architecture, paving the way for osteoblasts to lay down new bone in an organized, stress-influenced pattern. This results in the haversian system which gives the lamellar bone greater strength. This process of turning over bone continues for life and is a normal process of living bone (Manson, 1994).

Bone is composed of roughly 8 per cent water and 92 per cent solid material. The solid portion is 21 per cent organic in nature; the remaining 71 per cent is an inorganic phase (Feinberg, et al., 1991). The main organic constituent is type I collagen. Surrounding these collagen fibrils is an extracellular matrix composed of a variety of glycosaminoglycans and proteoglycans. The bulk of the matrix is chondroitin sulfate, which is interconnected with hyaluronic acid. Proteins, like the bone morphogenic

proteins, are produced by various cells within the bone and are contained within this matrix (Feinberg, et al., 1991).

The inorganic component of bone is a crystalline salt, which forms on the individual fibrils of collagen. Hydroxyapatite crystals are approximately 20 Angstroms thick and 200 to 400 Angstroms long and have the chemical formula $Ca_{10}(PO_4)_6(OH)_2$. This is the major storage form of calcium in the body. The calcium salts give bone its compressive strength, while the collagen gives its tensile strength. During the formation of the bone, the specific banding pattern of the collagen fibrils allows for a densely packed deposit of hydroxyapatite crystals yielding a very compact structure (Feinberg, et al., 1991).

The cellular component of bone is dominated by the osteocyte. These cells lie within the lacunae of formed bone. They maintain the homeostasis of the bone and communicate and transfer metabolic products via the canaliculi. These cells are presumably derived from the osteoblast, which is encased in the calcifying osteoid matrix during the laying down of bone. The osteoblast is the cell responsible for bone formation and the deposition of osteoid, the uncalcified protein matrix, which is the foundation for bone formation. These cells are derived from the differentiation of osteoprogenitor mesenchymal cells when stimulated by bioactive substances like bone morphogenic protein (Caplan, 1994). During the normal turnover of bone, resorption initially occurs as a result of the actions of the osteoclast. Thought to be derived from the circulating monocyte, these multinucleated cells contain and release various proteolytic enzymes that degrade the organic components of bone. The inorganic component is broken down when lysosomal acids are released from the microvillus surface of the advancing osteoclast. This results in a burrowing effect by the cell, allowing

for neovascularization and new bone formation by osteoblasts (Feinberg, et al., 1991).

Bone is formed from mesenchymal tissue through one of two processes. Long bones and certain other bones, such as portions of the mandible are formed via endochondral ossification. This process begins by the generation of a cartilagenous model that ossifies, allowing for the ingrowth of blood vessels and the differentiation and proliferation of osteoblasts from osteoprogenitor cells. These cells then lay down osteoid which calcifies to form initially woven bone, which is remodeled to form mature lamellar bone (Manson, 1994). Intramembranous bone formation does not develop through a cartilagenous model. Much of the craniofacial complex is formed when mesenchymal cells differentiate directly into osteoblasts and osteoid is laid down directly within the membranous tissue (Feinberg, et al., 1991).

REGULATION OF BONE

Bone turnover occurs continually throughout life after the growth of the bone is completed. Bone remodels itself according to the stresses placed upon it so that it can maintain its integrity in response to the forces that the muscles and tendons transmit to them. The piezoelectric effect is the phenomenon whereby bending forces on bone results in the creation of a negative electrical potential at the site of compression on the bone and a positive electrical potential being created at the site of tension. This minute electrical current stimulates the deposition of bone by osteoblasts in the area of compression, and an increased activity of osteoclasts and bone removal in the areas of tension (Basset, 1968).

Local factors within the bone substance can stimulate bone formation. Substances such as the bone morphogenic proteins have been shown to be osteoinductive. That is to say that they have the potential to stimulate mesenchymal stem cells to differentiate into osteoprogenitor cells and thus osteoblasts leading to the formation of bone tissue. Marshall Urist attributed bone induction to 'a complex entity of unknown character, which produces differentiation of osteoblasts,' (Urist et al., 1967). There are several such entities, most of which are included in the transforming growth factor beta superfamily including the bone morphogenic proteins (Hollinger et al., 1994). Once these factors are released, they can stimulate bone formation and are entrapped in the formed bone. If the bone is damaged, or is resorbed by osteoclasts, the proteins are thus released again, leading to the stimulation of bone formation.

Calcium ion concentration is regulated closely in the human body for many reasons due to its importance in many physiologic processes such as cell membrane potentials and molecular reactions. With bone being the major storage source of calcium in the body, storage and release of calcium to maintain a homeostatic calcium concentration within the tissues is carefully controlled by humoral hormones (Guyton, 1981). Parathyroid hormone can stimulate the resorption of bone and thus release calcium and phosphate from the stores within the bone in both a rapid and slow phase. The rapid phase involves increased osteoclast activity and the release of calcium into the extracellular space, which diffuses into the canaliculi and eventually the main circulation. The slow process leads to the stimulation of mononuclear cell differentiation into osteoclasts, which cause the release of calcium ions in the same way. This process takes longer to reach an effect, but this effect is longer lasting (Guyton, 1981). Parathyroid hormone also acts by slowing the activity of osteoblasts, therefore less calcium ion is taken up and stored in hydroxyapatite. The negative feedback of this is the release of

osteopromotive proteins when the bone is broken down leading to the stimulation of bone formation.

Calcitonin is a hormone with the opposite effects. The parafollicular clear cells of the thyroid gland produce it when calcium levels are too high. The effect of this hormone is to slow down the activity of osteoclasts and activate osteoblasts to deposit more osteoid, thereby 'locking in' more calcium ions. This hormone has also been thought to have the ability to stimulate the differentiation of osteoblasts from undifferentiated osteoprogenitor cells (Guyton, 1981).

HEALING OF BONE AND BONE GRAFTING

Bone has a unique ability to heal and repair its structure after being disrupted by fracture or an osteotomy. Once the bone structure has been disturbed, a hematoma forms locally around the ends of the bone due to disruption of the blood vessels within the bone itself and that of the periosteum and other soft tissue surrounding it. Depending on the alignment of the bone ends, healing will progress via primary healing or secondary (gap) healing. There are many other factors that will affect the healing process, including mobility of the fracture site, the presence of foreign bodies, infectious agents, osteosynthesis hardware, and host factors. Aside from these factors, the general mechanism of uncomplicated bone healing follows a rather specific pathway. Rarely, the ends of the bone meet end to end at the microscopic level. This can occur when the bones are anatomically reduced, rigidly fixed, and compressed with bone plates. Primary bone healing involves a release of osteogenesis factors locally (described later), activation of osteoclasts, resorption of the bone ends, and direct deposition of osteoid and bone

calcification by osteoblasts. There is no cartilagenous stage and the bone fracture site heals by a remodeling process to its original form.

In most areas of the fracture ends, bone heals via gap healing. After the hematoma is formed, there is early invasion of polymorphonuclear leukocytes followed by an induction of mesenchymal cells by local bone proteins to form chondroblasts. The cartilage stage involves a deposition of cartilage that ultimately calcifies, and along with the development of new vessels, osteoblasts differentiate from their precursors and begin to deposit osteoid on the calcified tissue in an appositional manner. This new bone is disorganized woven bone that is ultimately remodeled by osteoclasts and new lamellar bone is formed by osteoblasts and maintained by the entrapped osteocytes. This callus formation of bone healing develops from the original hematoma, leading to hyaline cartilage deposition and endochondral bone formation. The remodeling stage lasts many months (Manson, 1994). After a bone fracture, the osteoinductive proteins are exposed to blood and vascular-derived cells and proteins. It is this interaction of the osteoinductive proteins with the blood-borne osteoprogenitor cells and noncellular constituents that initiates reparative osteogenesis and fracture healing (Deatherage, et al., 1988). Fracture healing leads to fibrous nonunion when the basic requirements for gap healing fail. This includes persistent mobility at the fracture site, infection, excess gap between the fracture ends, and disruption of the blood supply, among other events.

Bone graft placed in a void within the bone heals in a similar pattern depending on the type of graft material being placed. Bone grafts aid new bone formation in several ways. Osteogenic grafts provide viable cells with the direct ability to form new bone. Osteoconductive grafts provide scaffolding for new bone to grow into from an adjacent osseous bed. Osteoinductive grafts provide factors that induce undifferentiated tissue to

differentiate into bone (van Heest, et al., 1999). Autogenous bone grafts provide for all of these and are thus considered the gold standard or ideal bone graft material. Despite this, autogenous bone graft harvesting has several disadvantages, the most significant of which is the morbidity at the donor site. Allogenic grafts (cadaveric bone from the same species) have been used successfully but pose the risk of disease transmission and have a higher failure rate for many reasons, since they do not possess viable cells.

The specifics of graft healing depends on the material being used, whether it is particulate or solid, made up of cancellous, cortical, or both types of bone, whether the bone graft is fixed rigidly, or placed passively. Soft tissue quality around the graft recipient site plays a vital role in the successful integration of the graft material, especially in irradiated tissue with or without hyperbaric oxygen therapy. The healing of autogenous grafts has been well described by Marx (1996) in the jaw reconstruction model and can be summarized as follows: The viable graft contains endosteal osteoblasts and the cancellous marrow stem cell population (osteogenesis) as well as the structural lattice (osteoconduction) and bioactive proteins (osteoinduction) to maximize graft healing. The recipient bed is normally hypoxic, acidotic, and rich in lactate. The soft tissue surrounding the graft will provide the initial blood supply for nutrient delivery and waste removal. Some of the viable cells within the graft will ultimately perish, but this induces chemotaxis and the delivery of macrophage and platelet derived growth and angiogenesis factors. Within the original clot and graft, angiogenesis commences bringing other factors and osteoprogenitor cells to the graft by day 14. This activation phase is followed by a resorptive phase whereby osteoclasts begin to break down the nonviable areas of bone while the new bone formation stage involves the direct induction of osteoblast deposition of osteoid onto the graft matrix (Ludwig, et al., 1999). This immature woven bone is

ultimately remodeled completely and lamellar bone is formed along the lines of stress according to Wolff's law.

OSTEOINDUCTIVE GROWTH FACTORS

In an attempt to obviate the need for autogenous bone grafting procedures, there has been much research in the area of osteoinductive bone growth factors. It would be ideal to be able to place a material in a bony defect that could induce local host cells to form bone. In the years since Marshall Urist's landmark study in the 1960's which showed that a bone ossicle could be induced to form in a rodent muscle pouch using decalcified bone matrix, intense research has identified several protein factors that can induce bone formation from undifferentiated mesenchymal cells (Urist, 1965, Perry, 1999, Ludwig, et al., 1999). In 1981, Sampath and Reddi identified an assay designed to quantify osteogenic activity of suspected compounds, which was the missing link in this area of research since Urist's initial work. A potential compound would be injected subcutaneously into immunosuppressed rats. The osteogenic activity of the compound was assessed histologically and by measuring the alkaline phosphatase and calcium content in the area. Several such compounds have since been identified and purified.

Growth factors are small proteins that function as signaling agents for cells and affect function of specific cellular events, such as proliferation or differentiation and extracellular matrix synthesis (Ludwig, et al., 1999). Urist initially showed that demineralized bone matrix (DBM) had osteoinductive effects, and the search for the active components within the matrix began (Urist, 1965). DBM is created by the acid extraction of bone, the specific process of which will greatly alter it's ultimate activity (Russell and Block, 1999). The remaining components in DBM include the noncollagenous

proteins; bone osteoinductive growth factors, the most important of which is bone morphogenic protein (BMP); and type I collagen.

Five growth factors have been associated with bone healing and maintenance: platelet derived growth factor (PDGF), transforming growth factor beta (TGF-B), insulin-like growth factors, fibroblastic growth factors, and epidermal growth factor. Bone morphogenic proteins have been shown to be the most important factors in bone formation (Hollinger and Seyfer, 1994). BMP's comprise a family of at least 15 structurally related growth factors (Ludwig, et al., 1999, Van Heest, et al., 1999, Hollinger and Seyfer, 1994). A property of the original members (BMP 2-8) was their ability to stimulate new bone formation. These BMPs are able to initiate a cascade of cellular events that mimic endochondral ossification in an ectopic location by stimulating mesenchymal cells to differentiate into chondroblasts and eventually osteoblasts (Hollinger and Seyfer, 1994).

With the cloning and sequencing of the genes for specific BMPs, recombinant protein is now available for these substances (Wozney, et al., 1988). This has allowed for the production of large quantities of these materials for study and clinical use since their relative available amounts in bone is low. Their use in comparison to demineralized bone has been shown to be superior in some studies (Marsden, et al., 1994). Recombinant human BMP-2 (rhBMP-2) and rhBMP-7 (osteogenic protein 1) are the two most commonly studied (Winn, et al., 1999, and Ludwig and Boden, 1999). BMP-3, or osteogenin, was previously studied extensively as well (Hollinger, et al., 1989 and Hollinger, 1993). This is likely the area where most study will continue as it offers an endless supply of osteoinductive material with specific and controlled mechanisms of action. The problem is finding the appropriate osteoconductive carrier for the recombinant proteins. So far, collagen, demineralized bone matrix, resorbable synthetic polymers,

hydroxyapatite granules, and dextran beads, among others, have been used as carriers. The ideal carrier and osteoinductive substance combination has not been found, but much research continues.

Current leading edge research is looking at the possibilities of gene therapy for the control of bone formation (Ludwig and Boden, 1999). Several strategies have emerged. A gene-activated matrix involves a plasmid DNA including the cDNA for an osteoinductive gene is suspended in a matrix, which is taken up by cells when the matrix is placed into a tissue bed. Ex vivo transduction of marrow cells followed by percutaneous injection into muscle tissue with an osteoinductive cDNA such as BMP-2 is also being looked at (Alden, et al., 1999). Another recent idea involves the use of adding responsive osteoblast precursor cells (OPC's) to an rh-BMP-2 preparation and carrying these to a critical sized defect with a resorbable carrier (Winn, et al., 1999). Their early results show no added advantage to the rhBMP-2/carrier graft alone. All efforts are being made to provide for the surgeon an ideal "off the shelf" bone repair material which reduces the requirement for harvesting bone from the host and subjecting them to further biological and economical costs.

Demineralized Bone Matrix

The study of demineralized bone matrix started over a century ago. Nicolas Senn reported in 1889 on a technique of demineralizing ox tibiae by using dilute hydrochloric acid and using this bone to repair bone defects secondary to tibial osteomyelitis and cranial defects in dogs (Senn, 1889). The first experimental model for induced ectopic bone formation was provided by Huggins in 1931, which established that urinary tract epithelium could induce bone formation. He showed that grafting proliferating epithelium

from the kidney, ureter, or gallbladder into guinea pig fascia induced intramembranous bone formation. The phenomenon of osteoinduction was recognized by Lacroix in 1945, when he demonstrated that epiphyseal bone extracts with acid alcohol produced heterotopic bone formation in rabbit muscle. He called this bone extract "osteogenin". Attempts were made to induce bone formation by implanting undemineralized bone without success. Urist's landmark study described the phenomenon of bone formation by autoinduction using demineralized bone matrix in several animal models (Urist, 1965). Reddi and Huggins demonstrated in 1972 that both acid-demineralized bone and dentin were osteoinductive.

The standard demineralization protocol was originally described by Urist (1965) and has since been modified by many researchers. Initially segments of long bones (endochondral), stripped of their soft tissues, are placed in 0.6 N HCL for 24 hours, using 1 gram of bone per 100mL acid solution. The acid is removed by washing the samples in sterile water and then the samples are lyophilized (freeze-dried) (Russel, and Block, 1999). Modifications have included pulverizing the bone sample first, adjusting the concentration, duration, and temperature of the acid demineralizing, altering the method of freezing the bone, washing the bone with ethanols, ethyl ether, chloroform as well as sterilizing the samples with ionizing radiation or gases. Some processes have been shown to reduce the osteoinductive effect of the samples, such as excessive pulverization, or certain sterilization and storage techniques (Russel and Block, 1999).

Following the findings by Marshall Urist in 1965, there was a scramble to characterize the nature of the demineralized bone matrix in all respects. Most importantly, what factor was contained within the matrix that allowed for the induction of bone formation, and why did the bone have to be subjected to a demineralizing process first. Urist had coined the term "bone

morphogenic protein" to describe the factor within bone that allowed for undifferentiated mesenchymal cells to induce endochondral bone formation. Many others have isolated osteoinductive proteins, one in particular, "osteogenin", turns out to be a member of the ever-enlarging bone morphogenic protein family (Hollinger, et al., 1989). This idea of inducing local tissues to form bone was significantly different than the traditional "creeping substitution" process seen with autogenous bone grafts where the implanted bone was slowly resorbed and replaced by new bone by the normal osteoclast-osteoblast activities (Khouri, et al., 1991).

Reddi, Weintroub, and Muthukumaran bets described the cellular and chemical processes of bone induction by demineralization (1987). They demonstrated that the major phases of osteoinduction are chemotaxis, mitosis, and differentiation. The implantation of DBM promotes chemotaxis of cells (polymorphonuclear leukocytes and mesenchymal cells) and plasma fibronectin binds avidly to the implanted material. This occurs within the clot along with the degranulation of platelets (PDGF's). Proteolytic enzymes are released and an accumulation and adhesion of cells to the graft occurs within the first 24 hours. Fibroblasts move in over the first few days as the initial breakdown of the DBM releasing the BMP's occurs. By the third day, cell proliferation occurs in response to these factors, which continues until these cells differentiate into chondroblasts by day 5, and the early synthesis of type II collagen, a specific marker for cartilage formation. Type III collagen, specific for mesenchymal cell protein synthesis is seen just prior to this as these cells are proliferating. Hypertrophy of the chondrocytes follows cartilage matrix secretion on day 7 and the calcification of the matrix commences about day 9. Type IV collagen is seen at this point, which coincides with the endothelial invasion of the matrix. Once calcification starts, basophilic osteoblasts appear and true bone formation occurs on days 10 to 12 along with and increase in alkaline phosphatase activity and

calcium uptake and the appearance of type I bone specific collagen. Remodeling of the early woven bone is seen when osteoclasts start to dissolve the matrix and the bone marrow cells (hematopoietic and megakaryocytes) begin to differentiate and populate the area by three weeks post implantation.

The process by which DBM induces osteogenesis was thought to be well known, but what is it about the DBM that induces this process? Was it a single factor, multiple factors, or a combination of factors and the matrix itself? Sampath and Reddi showed in 1981 that the osteoinductive capacity of dissociative extracted DBM was lost. Neither the soluble components extracted nor the insoluble matrix alone could induce bone formation. When the insoluble matrix was reconstituted with the soluble factors, the mixture regained it's ability to induce bone to be formed. This was very important to understand, since it was not just one or the other, but the two combined, that would allow for osteogenesis to proceed. The osteoinductive factor also needed an appropriate osteoconductive vehicle as, for example, muscle tendon matrix was unsuccessful as a carrier.

The mechanism(s) by which DBM stimulates new bone formation (as a complex sequence of events) presumably involves residual calcium levels (perhaps as nucleation sites for the redeposition of calcium phosphates), degradation of the organic (collagen/proteoglycan) matrix (as elements which may diffuse from the implant stimulating cellular chemotaxis into the implant and/or as sites to which cells attach and receive appropriate regulatory signals), release of growth factors (which may not only stimulate cell infiltration, but induce differentiation of mesenchymal cells into matrix and bone-forming cells), and preparation of a matrix which facilitates the infiltration and attachment of the right type of cells in some proper, but as yet unknown, sequence (Zhang, Powers, and Wolfinbarger, 1997B).

Isolating and characterizing the osteoinductive factor or factors was then the main goal of research in the late 1970's and this has continued ever since Urist and coworkers described what they called "bone morphogenic protein" as the osteoinductive factor responsible for bone formation (Urist, et al., 1979). Since then, several factors in the bone morphogenic protein family have been identified. Research has also isolated cDNA clones encoding the human equivalents of bovine BMP (Kale, et al., 1995). This has allowed for the molecular cloning and production of 13 distinct BMPs (Van Heest et al., 1999). They have been described as being in the transforming growth factor beta family of growth hormones.

Many studies have since been performed to assess the effectiveness of using DBM in bony defects. Specific areas of possible clinical application include bone tumors and cysts, long bone defects, spinal fusion, craniofacial reconstruction, and dental applications (Russell and Block, 1999). Most reports indicate good results in animal models.

The ideal model for studying the effects on bony healing is that of the critical sized defect, since it will not heal in spontaneously unless a bone graft is placed into the defect (Schmitz and Hollinger, 1986). Human use has been extensive, however, recent papers have begun to question the validity of their clinical results (Becker, Urist, et al., 1995). Most papers are case reports or limited clinical series with short-term follow-up. The main controversy revolves around the actual 'osteoinductivity' of the DBM used in these experiments. Commercially prepared DBM varies considerably in its ability to induce bone formation within an identical in vivo model (Bowers, et al., 1991, Becker, Urist, et al., 1995, Niederwanger and Urist, 1996). Several processing factors will influence the 'quality' of the DBM including donor characteristics (age, delay in procuring bone, handling), particle size,

demineralization protocol, residual mineral content (esp. calcium), and sterilization techniques.

Randomized, prospective clinical trials are being conducted on several combinations of the use of demineralized bone matrix, bone morphogenic proteins, recombinant factors, and the appropriate carriers of these factors in many different clinical scenarios. In addition to the ones mentioned above, studies involving distraction osteogenesis using grafts and whether or not intramembranous-formed bone heals differently with endochondral bone derived factors are being evaluated (Rabie, et al., 1996). Eventually, the ideal bone graft "material" may be found that can replace the need to harvest autogenous bone. There appears to be a significant difference in the success of animal studies and that seen in the early human experiments. The clinician faces many different variables in the operating theatre than he does in the research institute. Further animal and human studies are needed in this promising field.

Poloxamer Gel

Poloxamer gel (Pluronic F-127) is a member of a large group of high molecular weight block copolymer surface-active agents derived from propylene oxide and ethylene oxide in propylene glycol (Schmolka, 1972). The synthesis of this copolymer involves the creation of a hydrophobe of desired molecular weight by the controlled addition of propylene oxide to the two hydroxyl groups of propylene glycol. Ethylene glycol is then added to make the molecule hydrophilic. The relative percentage by weight of the ethylene oxide gives the resultant gel unique handling and functional properties, which may be custom mixed depending on the intended use of the substance (BASF monograph).

In industry, the gels have been used as defoaming/antifoaming agents that remove or prevent the formation of foam when added to certain substances. They are also used as detergents and have excellent wetting capabilities (BASF monograph). The structure of Pluronic surfactants can be seen in figure 1 below.

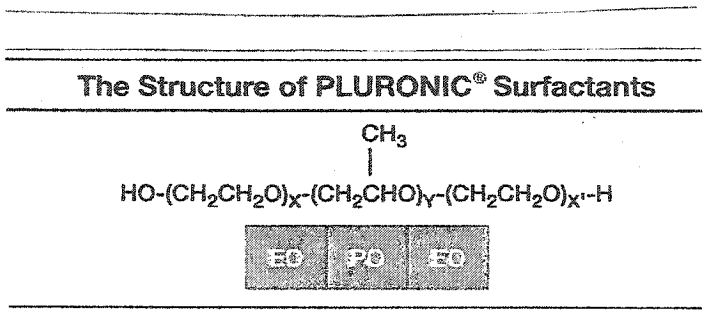

Figure 1 The structural design of Pluronic Surfactants (Poloxamer).

Poloxamer contains approximately 70% by weight of ethylene oxide and 30% of propylene oxide and has a molecular weight of 12600. The gels are thermally reversible, which means that it is a liquid both below 4 degrees and above 54 degrees centigrade. It is in gel phase between these temperatures (BASF monograph). Clinically, therefore, this substance can be stored as a liquid and then either applied to the epidermis or injected within the body from which it warms to body temperature becoming a gel and maintaining its position appropriately (Cao et al, 1998). It can also be washed away easily if necessary. It is important to note that this nonionic substance is biodegradable and biocompatible. The physical properties of the substance do not change with repeated heating and cooling. Any substances dissolved within the gel will also remain in the gel with these temperature changes as well (Schmolka, 1972).

The toxicity of the substance has been tested both in acute and chronic exposure situations and has been successfully included in the Inactive Ingredient Guide published by the Food and Drug Administration. For these

compounds, the toxicity is lowered by increasing the ethylene oxide concentration and by an increased molecular weight, of which the Poloxamer molecule is the highest in this group of copolymers for both characteristics (BASF monograph). There is also a minimal inflammatory response to these gels.

As Poloxamer gels were found to be biologically inert, and considering the favorable handling characteristics of the material, medical applications were considered. The early use of Pluronic F-127 gel was in thermal burn wound therapy to replace or add to the present use of occlusive dressings (Schmolka, 1972). The gel could be applied as a cool liquid, which would congeal to a stable gel over which other dressings could be applied. Just as importantly, bactericidal agents such as silver nitrate or silver lactate could be added to the gel, making this "artificial skin" an effective barrier in the treatment of burn wounds (Nalbandian et al., 1972).

Further applications have been studied with the use of Poloxamer gel as a delivery agent for several materials including drugs and tissues. Drug delivery to different body sites have included ophthalmologic (Miller and Donovan, 1982), rectal (Miyazaki et al., 1986), subcutaneous (Morikawa et al., 1987), nasal (Jain et al., 1991), and the topical application of drugs in cancer therapy (Miyazaki et al., 1992). Many investigators have used Poloxamer as a delivery vehicle for drugs such as ketoprofen (Chi and Jun, 1990), indomethacin (Miyazaki et al., 1986 and 1995), mitomycin C (Miyazaki, 1992), interleukin-2 (Morikawa et al., 1987), metoprolol (Jain et al., 1991), and theophyline (Xu and Lee, 1993).

Desai and Blanchard (1998) added methylcellulose or hydroxyproply methylcellulose to the Pluronic gel to form a sustained release ocular delivery system for pilocarpine as a retardation of the delivery of the dissolved drug

was necessary in certain applications. The delivery of tissues has also been looked at. Cao and colleagues (1998) have studied the subdermal injection of autogenous chondroctyes dissolved in Pluronic gel. The resultant material was used to create nipple-like structures that could be tattooed in such a way as to appear like the nipple-areolar complex for women post mastectomy.

Considering the biocompatibility and convenient handling characteristics, Poloxamer gel appears to be an ideal delivery agent for particular clinical scenarios. The gel has wetting properties that make it suitable for use with demineralized bone matrix yielding a putty-like substance that is easy to handle surgically. For these many reasons, poloxamer gel was chosen for this investigation to evaluate its ability to deliver DBM and facilitate bone induction in an applied clinical model.

Plaster of Paris (Calcium Sulphate)

Calcium sulphate has been used as a 'bone grafting' material since the late 1800's when it was described by clinicians at the Trendelenberg Clinic in Germany to fill defects in long bones (Snyders, et al., 1993). Medical grade calcium sulphate (MGCS), otherwise known as Plaster of Paris, and it's hemihydrate form, is made by heating gypsum. It comes in several forms including small granules, or pellets, such as those used in this study (OSTEOSET, Wright Medical Technology, Inc.) Finally, a powder anhydrous form is available to be reconstituted with water when the material is needed. When water is added to the dry powder, an exothermic reaction results in crystallization and hardening of the preparation after a few minutes.

This hard substance can withstand significant compressive forces, but is relatively inflexible and brittle. It is useful for many reasons. Firstly, it is

biocompatible with a long clinical use history. It resorbs in vivo, but the timing of this varies with its preparation and placement (Bell, 1960). The material is absorbed by osteoclasts, and osteoblasts will deposit osteoid onto the material and form bone (Sidqui et al., 1995). It has good handling characteristics, which allows for the variation of working consistency by altering the powder to liquid ratio. This will yield a material with a suitable working time to place the unset material into a defect, and then allow time for the adaptation of the material to the recipient site prior to it hardening. It can then only be adjusted by shaving the material with an instrument. It must be noted that varying the powder to liquid ratio will alter the performance and setting characteristics of the material.

Other advantages include the space filling nature of the material, which prevents soft tissue prolapse into a defect. This occurs both macroscopically and microscopically, the latter being considered by many to be a distinct disadvantage. There is also the potential benefit of the leaching of calcium ions into the immediate area and their potential effect on bone healing (Snyders et al., 1993).

In terms of bone graft materials, plaster of paris can be grouped along with the collagen preparations and the calcium phosphate materials (Snyders et al., 1993). These are all osteoconductive agents. In this capacity they will provide a matrix or scaffold, facilitating vascular invasion, resorption, and finally new bone formation (Ludwig and Boden, 1999). They do not induce bone formation like the osteoinductive bone morphogenic proteins or transforming growth factors, but rather help with the formation of new bone due to their inherent structural characteristics. MGCS has been used as a binder or filler material and mixed with several possibly osteoinductive materials such as hydroxyapatite (Rawlings et al., 1988), and negatively charged dextran beads (Snyders et al., 1993).

The disadvantages of its use include migration of particulate or pellet forms of the material post implantation. In addition, if the powder and liquid form is made and the material is placed into the body, the exothermic reaction may release enough heat to damage nearby tissues similar to that seen with polymethylmethacrylate. This is especially true when filling calvarial defects. A final disadvantage is seen with early resorption coupled with a possible lack of bony in growth leaving a deficit, which in a clinical scenario may become obvious, resulting in a cosmetic deformity.

On the microscopic level, a dense calcium sulphate material may actually impede bone healing by slowing the ingrowth of blood vessels and osteogenic precursors, thereby slowing bony transformation (Snyder et al., 1993). The powder preparation, when mixed with water, tends to yield a densely packed final product, which may interfere with this process more than the particulate or pellet preparations. Although these will not give initial structural integrity to the area, they will allow clot formation to occur and normal bone healing pathways to proceed. They will also be resorbed more quickly due to the earlier presence of osteoclasts invading the area. Development of a biologically suitable porous form of the calcium sulphate could maximize osteoconductive properties while providing variable structural characteristics (Snyder et al., 1993).

Plaster of Paris has been used in this study as it is considered to be the predicate device when comparing experimental bone grafting materials (FDA). No other material besides autogenous bone has such a lengthy clinical use record.

Critical Size Bone Defect

Substantial interest exists in the development of bone graft substitutes and human recombinant bone-inducing factors to enhance bone regeneration both in the craniofacial complex and in other parts of the skeleton (Bosch, et al., 1998). Demineralized bone matrix may provide a potential source for hope. When faced with a bone defect that may not heal spontaneously surgeons are continually searching for ways of obviating the need for harvesting autogenous bone grafts..

The search for an appropriate, accurate and reliable animal model to study these bone graft substitutes is ongoing. Materials that are osteoinductive and/or osteoconductive need to be studied in animal models prior to being used in human clinical cases. Researchers and clinicians require histologic evidence confirming the ability of a potential substance to enhance bone healing. This should be a precondition for the application of osteopromotive substances in the human clinical situation (Bosch et al., 1998). Hollinger (1993) proposes that potential bone inducing factors and their delivery systems must be assessed in characterized, reproducible animal wound models that can mimic normal wound progression. In his study to find the ideal animal model for testing bone substitute materials, Frame (1980) expanded on earlier studies by Kramer et al. (1968) by testing various sized full thickness calvarial defects and their inherent ability to heal spontaneously with only soft tissue closure. He found that for the adult rabbit calvarium, a 15mm defect would not heal in with bone spontaneously, but that a 10 mm defect would heal with bone.

Schmitz and Hollinger (1986) then coined the term 'critical size defect' and defined this as a 'defect of a size that will not heal during the lifetime of the animal' when created surgically and no bone grafting material was added to

the area. They expanded by saying that a critical size defect (CSD) was the smallest size intraosseous wound in a particular bone and species of animal that will not heal spontaneously and that attempts at healing results in the formation of fibrous connective tissue rather than bone.

Wound models that are based on this premise are helpful in measuring the capacity of bone repair materials to initiate and promote the return of form and function to skeletally deficient wound beds (Hollinger, 1993). Hollinger and Kleinschmidt (1990) extended the definition of a CSD to include the smallest sized intraosseous wound that will heal with less than 10% of bone growth, unless augmented with a bone repair agent. Finally, the quality of the bony repair under optimum experimental conditions (noninfected, stabilized defects) is influenced significantly by the following variables: species, age of the animal, location of the defect, size of the defect, and the intactness of the periosteum upon closure of the wound (Schmitz and Hollinger, 1986).

With this in mind, the experimental model used in this study was that of a 15 millimeter circular, full thickness, parietal bone defect in a skeletally mature New Zealand white rabbit.

The Rabbit Model

The development and study of autogenous bone graft substitute materials and human recombinant bone inducing factors to enhance bone regeneration requires a strict model so that results can be accurately analyzed and the results deemed valid. Prior to a material being accepted for clinical use on human patients, adequate animal studies must be conducted. Many different animal models have been used to evaluate such materials, varying the species, anatomic location, and size of the defect created. As well,

histologic and radiologic evidence of the ability to enhance bone healing is a precondition for the application of osteopromotive substances in clinical situations (Bosch et al., 1998). It is also imperative that the animal model be carefully chosen to ensure that it is appropriate for the material and application being studied. This will prevent the inappropriate allocation of animal lives, funding, and resources. The materials must also be studied using a critical size defect, which is the standard testing defect when studying osteopromotive materials (Schmitz and Hollinger, 1986).

For this investigation, the adult rabbit calvarial bone model was chosen because it has been shown to satisfy the previously mentioned prerequisites and has been studied extensively (Frame, 1980). In an effort to find the best animal model, Frame looked closely at all the possible animal models and outlined various advantages and disadvantages with each to come up with features of a potential ideal animal model. He claims that in order to study substitutes that may have craniomaxillofacial applications, this animal model should have certain features including low expense, ease of handling and care for the animal, and uncomplicated anesthesia. The site should provide adequate bulk of bone and should include both cortical and cancellous bone. The site should also allow for the preparation of a critical size defect for that animal species and should be similar to the jawbone, both embryologically and physiologically. Finally, the animal model should allow for accurate follow-up and assessment of implant behavior, both radiographically and histologically. Adult rabbits satisfy all of these requirements.

The use of the critical size defect in adult rabbit calvaria is useful for many other reasons as well. The location is subject to low or nonexistent functional stresses as compared to jaw defects that are subjected to masticatory forces. It does not disable the animal where a limb defect would create a significant disability for the animal. Additionally, it is easily

accessible surgically and is relatively free from contamination in contrast to those defects created in the jaws via the oral cavity. Furthermore, calvarial bone is membranous in embryological origin, which is similar to the majority of the remaining bone of the craniomaxillofacial complex. It also allows for the creation of two critical size defects in the same surgical wound without disrupting the sagittal sinus within the cranium. This allows for a paired design so that each animal can act as its own control if different substances are placed in each defect (Bosch, et al., 1998). The design is also easily reproducible, so that other investigators can substantiate each other's findings.

The age of the animal is also important since immature animals have an immense capacity to spontaneously repair large bone defects (Takagi and Urist, 1982). Thus, it is important to use skeletally mature animals to control for this variable. It should also be noted that care must be taken prior to extrapolating animal model results to the human scenario since animals may have superior regenerative abilities to humans.

The use of the 15-millimeter defect is the accepted critical size defect in the adult rabbit calvaria as shown by Frame (1980). Schmitz and Hollinger (1986) agree that this is an appropriate model and they propose that initial material testing should first be performed in 8-mm rat calvaria to rule out obviously unsuccessful materials. They then recommend that the rabbit calvarial model be utilized to substantiate the findings of the rat studies and to determine if it is appropriate to study the bone graft substitute in nonhuman primates or mongrel dogs before advancing to the human clinical scenario. The final testing in the nonhuman primates allows the direct comparison of the material to the gold standard of autogenous cancellous bone grafting. Successful bone graft substitutes at this stage would then be appropriately looked at in the human model.

The animal model used in this study is supported by previous investigators and is appropriate for the intended purpose of the study. The 15-mm defect has been shown to be critical size and will allow for the accurate analysis of the demineralized bone matrix material used here.

Materials and Methods

SURGICAL PROTOCOL

Before initiating the surgical procedures for this investigation, all protocols regarding animal care and handling were approved by the animal research ethics review committee at the institutions involved in the project. Twenty adult male New Zealand White rabbits of skeletal maturity were used in this study. The animals were each weighed prior to the procedure (range: 3.2 to 3.8 kg). Each animal was premedicated according to their weight with an intramuscular injection of a mixture of ketamine 35 mg/kg, acepramazine 1 mg/kg and xylazine 2 mg/kg. Following an adequate level of sedation, the marginal ear vein was canulated with a 25-gauge angiocatheter and a 5% dextrose solution was infused at roughly 30 ml/hour to maintain venous patency. The animals' scalps were shaved with an electric shaver. The animals were then positioned on the operating table and general anesthesia was induced with an intravenous injection of sodium pentobarbital 20 mg/kg titrated slowly to effect. After induction, the animals were endotracheally intubated with a cuffed 3 mm tube. The position of the tube was confirmed via auscultation and was secured with tape. Maintenance of anesthesia was with a mixture of 1-1.5% isoflurane and oxygen delivered with mechanical ventilation. The animals were monitored with pulse oxymetry. The animals were then positioned prone and padding was placed under their mandible and neck to support the head. The surgical areas were prepped three times with a proviodine solution. After a standard surgical scrub, gowning, and gloving, the surgical area was draped and the operating theatre was set up for a sterile procedure. The temperature of the animal was monitored and maintained via an external heating source.

Using protocols previously published by Alberius et al. (1989), a solution of 2% lidocaine with 1:100 000 epinephrine was injected subcutaneously using a 25-gauge needle on a syringe. Approximately 3-5 mls of solution was injected along the anticipated incision site. After waiting for an adequate vasoconstriction effect

of the anesthetic solution, an incision was carried out along the midline of the scalp from a point midway between the base of the ears to approximately 5 centimeters anteriorly through full thickness skin and galea. A #20 blade was used for the incision. Blunt dissection above the pericranium was carried out and the pericranium was incised sagittally the same length as the skin incision. Sharp subperiosteal dissection reflected the pericranium off the parietal bones to the lateral aspect of the superior portion of the skull. The coronal suture was identified, as was the lamdoid suture (see figure 2).

Figure 2. The rabbit calvarium is exposed prior to creating bilateral parietal bone critical size defects

An electric drill and various burs were used along with copious saline irrigation to create full thickness calvarial defects. The defects were ovoid in shape measuring 15 mm by 13-15 mm. A surgical template was used to define the

defect margins. Care was taken to avoid the midline, as this is the location of the sagittal sinus. Care was also taken to protect the dura mater from contact with the burs by using a rounded molt #9 instrument in the extradural space. Two defects were created, one through each parietal bone on each side of the midline. The defects were cleaned of sharp bony spurs and irrigated thoroughly with saline. The dura was inspected for perforation and bleeding was controlled. Photographs were taken of the procedure and the defects (see figure 3).

Figure 3. The rabbit calvarium is exposed with newly created bilateral critical cized (15mm) defects. Notice the intact dura mater.

Each animal was preselected to an experimental group. The grafting protocol will be described shortly. After preparation of the sites and the grafting procedure completed, the closure of the wound was performed. The area was cleansed with saline for a final time ensuring that minimal debris or bone dust

remained within the wound. The pericranium was approximated as closely as possible with 4-0 vicryl interrupted sutures. The skin was closed in two layers, using deep sutures of 4-0 vicryl placed to approximate the skin margins and the skin was closed with 3-0 plain catgut sutures in an interrupted fashion. An antibacterial (polysporin) skin ointment was applied to the wound margins after the area was cleaned. The animals were then taken off the ventilated anesthetic gases, allowed to awaken and extubated once they were breathing spontaneously. The animals were transferred carefully to their cages and a heating lamp was provided for the animals. Buprenorphine, an analgesic , and Baytril, an antibiotic , were given intramuscularly in the immediate postoperative period. The animals were monitored closely in the postoperative period and were given adequate food and water supplies. Analgesic injections were carried out every 8 hours for two more doses and then only if the animal showed signs of discomfort (i.e. abnormal activities of grooming, eating, etc.). The antibiotics were given twice daily for two days.

Animals were grouped as follows. 10 animals had defects filled with Plaster of Paris pellets on one side and the remaining defect was left unfilled. A final ten animals had demineralized bone matrix putty placed into the defects on one side of the animals, whereas the opposing defects were filled with poloxamer gel only. Each group of 10 animals was randomly assigned to either a six-week subgroup, or a 12-week subgroup. These animals were allowed to recovery for exactly that amount of time before being sacrificed and examined clinically, radiographically, and histomorphometrically for comparison. Since three animals died shortly after their surgery, a previously unassigned animal was grafted and was used to replace the lost group member.

OPEN DEFECTS (CONTROL GROUP)

Establishment of the control group involved several considerations. Facility animal use protocols limit the number of animals that can be used by an

investigator. This goal is universal. Considering that the critical sized defect in the rabbit calvarium is very well established in the literature (Frame, 1980, Schmitz and Hollinger, 1986, Bosch, et al., 1998), and considering that the healing of a defect on one side of the calvarium should not in any way affect the healing of a defect on the opposite side of the calvarium, it was decided that the number of animals used could be reduced by 50% if two defects per animal could be studied, even if it involved different materials. Various review bodies have accepted this. Therefore, the animals in the control group had one of their defects left unfilled and were allowed to heal spontaneously without the use of any grafting material. The defect was prepared, cleansed thoroughly and the pericranium was sutured over the defect and the skin closed. This left only the bony margins laterally, the dura mater deep and the pericranium superficial to the defect, with only blood filing the defect.

Figure 4. Bilateral critical sized defects with Plaster of Paris filled defect and open (control) defect.

PLASTER OF PARIS GROUP

In the defect opposite the open group, Osteoset Plaster of Paris (calcium sulphate) pellets (Wright Medical Technology Inc., Arlington, USA) were placed into the defects. No attempt was made to fractionate the pellets in order to completely fill the void (see figure 4). Rather, The pellets were placed directly onto the dura and the pericranium was closed gently over the defects followed by skin closure over the cranium.

POLOXAMER GEL ONLY GROUP

After the defect was prepared and cleaned of excessive blood clot, Pluronic F127 gel (poloxamer 407) (BASF Canada Inc., Toronto, Canada) was placed into the defect until the entire defect was filled. The gel was leveled off with a spatula such that the bone that was removed was replaced in volume with the gel. The soft tissue was closed as described previously. Excess gel was suctioned away. See figure 5 below.

DEMINERALIZED BONE MATRIX AND POLOXAMER GEL PUTTY GROUP

The final defect was packed with a rabbit allograft material in a carrier base. Previously prepared rabbit demineralized bone matrix in a poloxamer gel carrier yielded a putty-like material that was placed into the defects to replace the volume of bone removed. Excess was removed with a spatula and the soft tissue closed as before (see figure 5). The demineralized bone matrix was tested by in vivo and in vitro means to confirm its osteoinductive activity prior to being accepted for use in this study. This is an important consideration, as many commercially available bone grafting materials that claim to have "osteoinductive" properties do not attempt to quantify just how "osteoinductive" their material really is. When the material used in this study is manufactured, a representative

sample of that "batch" is studied via both in vivo and in vitro means prior to distribution. This key step gives the manufacturer and the purchaser the assurance that the material that they are using is as effective as it claims. As will be explained later in this document, variations in the manufacturing technique, which includes harvesting of the bony specimens, tissue storage, physical and chemical specimen breakdown (i.e. demineralization and freeze-drying), and reconstitution with the excipient, all have a direct effect on it's potential clinical performance. Therefore, for the clinician, it is invaluable to know beforehand that the "batch" of bone grafting material that he or she is about to use has been proven by currently acceptable testing techniques (i.e. in vitro and in vivo studies) to be effective.

Figure 5. Bilateral critical sized defects with DBM putty and Poloxamer gel-only filled defects.

SACRIFICE

The animals were randomly placed into the six or twelve week groups. Prior to sacrifice, the animals were weighed and premedicated with an intramuscular injection of a mixture of ketamine 35 mg/kg, acepramazine 1 mg/kg and xylazine 2 mg/kg. Following an adequate level of sedation, the marginal ear vein was canulated with a 25-gauge angiocatheter. The animals were anesthetized with an intravenous injection of 30 mg/kg sodium pentobarbital and euthanized by an intravenous injection of 120 mEq of potassium chloride.

The soft tissue of the scalp was removed carefully anterior of the defects to posterior staying close to the galea and leaving the soft tissue covering and pericranium intact. The skullcap was removed with bone shears away from the defects so that there was native bone surrounding all of the defects. The specimens were labeled, placed in moist gauze and transferred to a separate laboratory for further investigations.

CLINICAL EXAMINATION OF THE SPECIMENS

Each specimen was examined visually after procurement and the observations recorded and photographed. The skull caps were then radiographed in a standardized fashion as follows. The specimen was carefully secured to a blank sheet of paper and placed in a cephalostat machine and radiographed for a standardized 1:1 film. Each specimen was radiographed with the same parameters, namely 60 KVP, 5 mA, and for 0.2 seconds. The films were processed in a standard dental radiograph processor. The films were labeled and the observations recorded. The specimens were then prepared for histologic preparation and examination.

HISTOLOGIC EXAMINATION OF THE SPECIMENS

The specimens were fixed in a 10% neutral buffered formalin solution. For histologic examination, bone cores specimens were either decalcified in formic acid and embedded in paraffin or dehydrated in graded 2-propanol and directly imbedded in paraffin. A section 2-4 microns in width was obtained from each defect and stained with hematoxylin and eosin for light microscopic examination. The section was taken from the middle of the defect were it was 15mm wide (i.e. critical size). Observations were once again recorded with reference to healing pattern, graft material/scar tissue interaction, time scale differences, and tissue types in the defects.

HISTOMORPHOMETRIC ANALYSIS

Specimens were visualized with a light microscopic to include the entire defect and the bony margins left behind during the initial preparation of the defects. These images were then scanned onto a computer using the Quartz PCI image acquisition software version 4.20 (Quartz Imaging Corporation, New York). These images were then processed by SigmaScan Pro Image Analysis program version 4.01 (SPSS Inc., Chicago) which allowed for histomorphometric analysis to be completed. The following parameters were measured in relation to the total surface area of the defect. In most cases, the "tissue" (i.e. bone, soft tissue, bone graft material) did not completely fill the area left after the removal of the original calvarial bone. This meant that the grafted material (or non material as in the open defect as the control), was unable to maintain the space left once the original bone was removed, and therefore was unable to replace it properly in either surface area or by extension, volume (not measured). In order to determine and quantify the ability of each material to replace the void created by the critical sized defect as a percentage fill of the original defect size, the photomicrograph of each

defect cross sectioned at it's widest point was measured (width and length) to determine the original surface area of bone removed to create the defect. By then measuring the surface area of each new healed tissue within that defect, one could then calculate the percentage fill that that tissue occupied as compared to the original defect (surface area, and volume by extension). Therefore, the parameters measured included the surface area of: new bone within the defect, graft material, soft tissue scar, and marrow space. The same power of magnification was used in all cases. The Sigma Scan Image analysis software allows one to identify multiple surface areas on a screen and total the area of each material very easily. These results can then be compared easily between materials, groups and time periods. Five animal sites were available for each defect type and for both healing time periods. The measurements were taken from histologic 'cuts' taken at the middle of the defect that represented the full critical size width of the defect (i.e. 15 mm). The values were compared within and between groups for statistical significance using analysis of variance (ANOVA). A percentage of new bone fill versus soft tissue versus graft material was looked at and analyzed for statistical significance between the groups

Results

All animals survived past the immediate post-operative period. There were 3 animals that expired in the immediate perioperative period. One animal died post intubation and on post mortem examination appeared to have a cardiomyopathy. A second animal died late in the surgery and examination revealed pus in one entire lung from a preexisting condition. The third animal died immediately postoperatively and examination again revealed an infected lung. None of these animals were used for the experiment. Rather, the next animal to have surgery was used to replace the lost specimen in that experimental group. All specimens used were obtained from healthy animals that were sacrificed at the predetermined and specified time period (i.e. 6 or 12 weeks post operatively).

One animal developed a swelling consistent with a hematoma, which drained spontaneously but incompletely, and the animal survived the remainder of the study. The skin crusted and eventually sloughed several weeks after the surgery, revealing an intact skin surface underneath. The healing of the defects (open (control) and Plaster of Paris) appeared to be unaffected as there were no obvious changes histologically nor clinically after the specimen was retrieved.

One final animal developed seizure-like activity on post-operative day #1 which was initially observed but when it did not resolve, the animal was given an intramuscular injection of Diazepam that resulted in a resolution of the seizures and remarkably, the animal recovered normally thereafter.

All other animals recovered without incident. They typically resumed drinking within hours of their surgery and food intake started on postoperative day #1. They all gained weight. The mean preoperative weight was 3.48 kg and the presacrifice weight at 6 weeks was 3.95 kg and at 12 weeks was 3.92 kg (see Table 1). All wounds healed without problem. There were no wound infections and no wound dehisced.

Group	Pre-Op Weight	Post sacrifice Weight	Average Weight Gain
6 Weeks	3.38 kg	3.95 kg	570 gm (16.7%)
12 Weeks	3.57 kg	3.92 kg	350 gm (9.9%)

Table 1. Presurgical and Post sacrifice animal weights.

As the specimens were being retrieved, the skin of the scalp was incised anterior to the defects down to pericranium and reflected posteriorly over and behind the defects. There was no evidence of adhesions of the healing defects with the overlying aponeurotic layer. There were moderate amounts of soft tissue scar over the defects and these were included with the specimen. Once the skullcap was removed, the dura reflected away from the defect site easily. Specific observations of the defects will be described next.

CLINICAL OBSERVATIONS

Control Group (Open Defect)

The defects in this group were identical clinically at 6 and 12 weeks. There was an obvious thin, flexible, fibrous scar that filled the defect at the time of specimen retrieval (see figure 6). Radiographically, the defect was radiolucent throughout with no evidence of central radiopacities. The margins were smooth and the radiodensity tapered rapidly from bone to scar. Figure 7 shows an example of a radiograph of an open defect and Plaster of Paris at 12 weeks post surgery.

Figure 6. Clinical photograph of healed open defect.

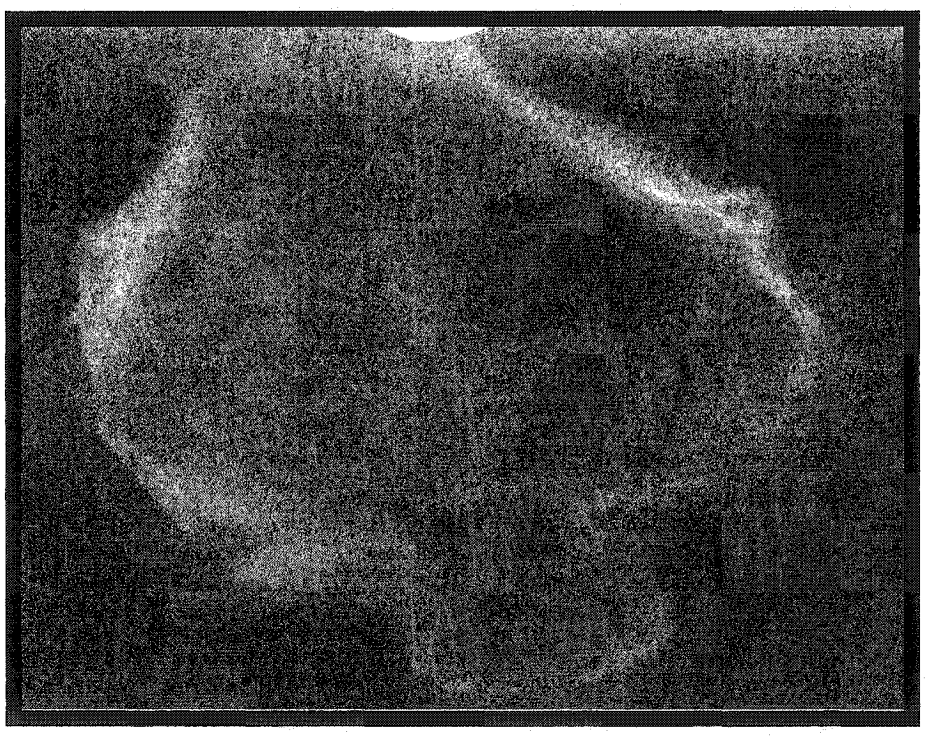

Figure 7. Radiograph of Open and Plaster of Paris defects at 12 weeks.

Histologically, as can be seen in Figure 8, the defect was filled with a thin, dense fibrous scar throughout the defect length. There was some evidence of bony in growth at the margins as evidenced by the length of the scar versus the original defect size. The bony margins showed a rapid tapering of osseous tissue to fibrous scar tissue, which was smooth in nature. Occasional bone islands could be seen within the fibrous scar of the defect, but this was uncommon. One rabbit site had a greater amount of bone in relation to all others in his group, but this was isolated and the defect was still predominately scar tissue.

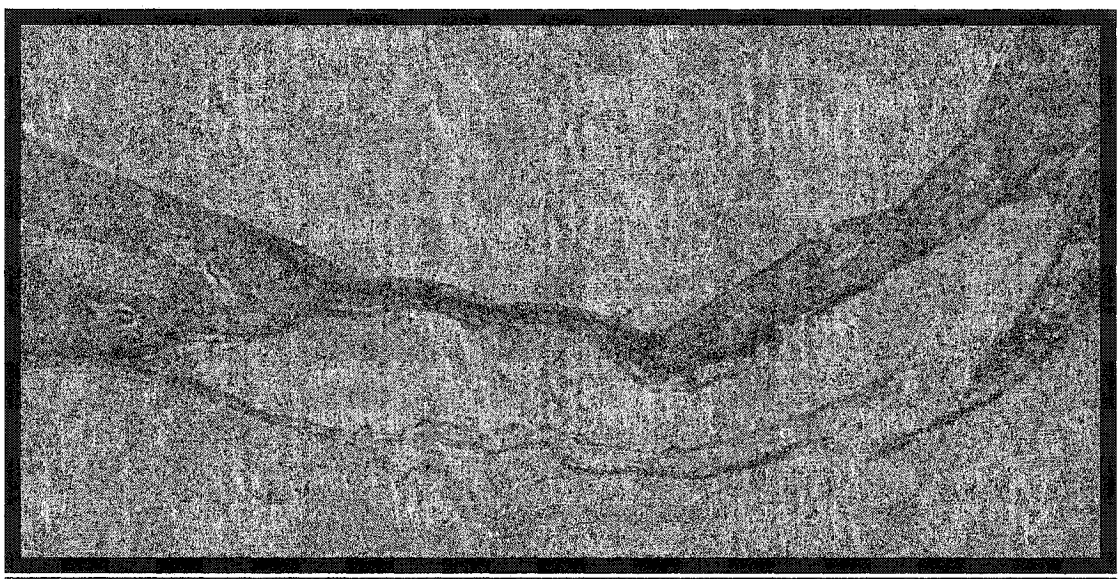

Figure 8. Histology of Open defect at 12 weeks.

Plaster of Paris Group

This group healed primarily with a fibrous scar with progressive dissolution of the Plaster of Paris disks and in growth of soft and bony tissues around and in the center portion of the disks. Clinically, the scar tissue rigidly held the disks to the defect but the defect was flexible between the fingers. There was no bone obvious to the eye. Radiographically, notice the relative radiolucent areas around the Plaster of Paris discs and within the Open defect demonstrating poor

or incomplete bony fill. Therefore, bony healing was not able to bridge the critical sized defect. Notice that although some bone surrounds the pellets in scanty amounts, the plaster material has not been replaced by bone. As well, even though there is significantly more bone in the 12-week group, it still does not completely bridge the defect. See figure 7 previously. Both 6 and 12-week groups were clinically the same.

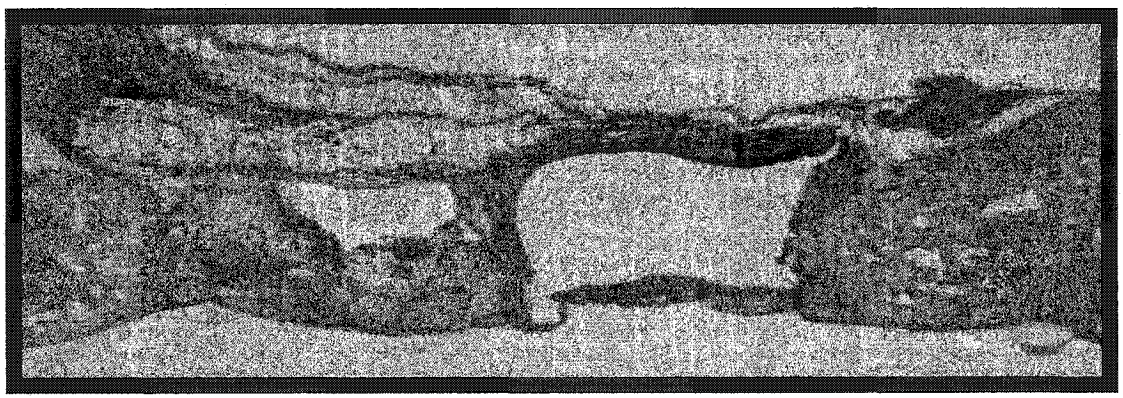

Figure 9. Histology of 6 week Plaster of Paris group. Some bone surrounds the pellets, but in scanty amounts and the plaster material has not been replaced by bone.

Histologically, there were obvious differences (see figure 9 and 10). There appeared to be some progression between the two time groups. In comparison to the 6-week group, the disk of Plaster of Paris at the 12-week time period exhibited greater breakdown, with definite loss of the original shape of the disk. A greater portion of the disk material was missing although this is difficult to comment on because of the processing that it was subject to. It was obvious that there was a greater amount of encapsulation of the material in adjacent soft and hard tissue areas. Both bone and fibrous scar healed on to the material. The edges of the disk seemed to resist breakdown longer and the central portion hollowed out and filled with mostly fibrous scar and some bone. Bone was more prevalent at the 12-week time period as is shown in Table II.

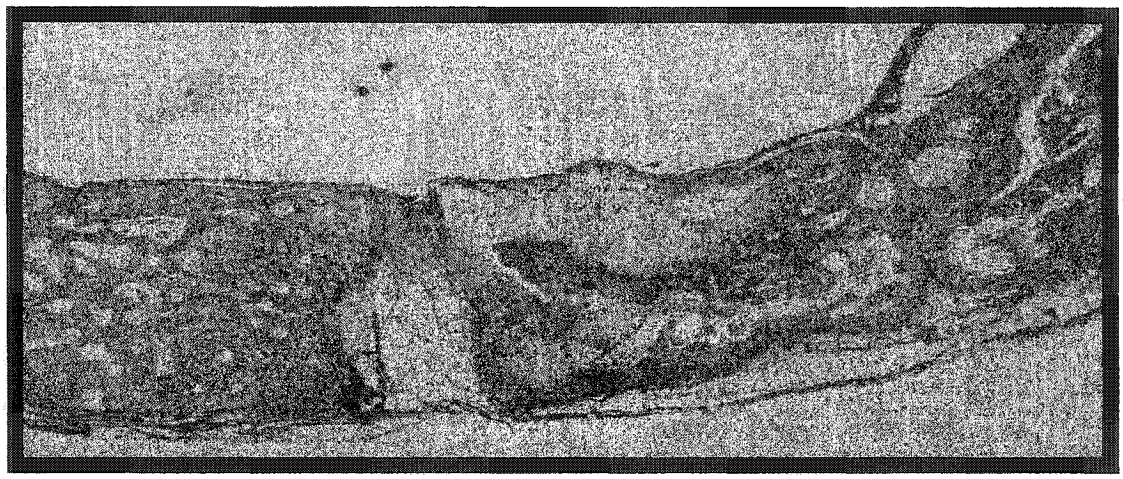

Figure 10. Histology of the 12 week Plaster of Paris group. Degradation of the original pellet is evident, as is more new bone within the defect.

Poloxamer Gel Only Group

These defects were identical to the open defects clinically and radiographically. Histologically, they were also very similar with a primary fibrous scar and occasional bony islands within the length of the defect scar. The variability of the scar composition was greater in this group. There were some animals that had more bone than others that looked identical to the open defect group (see figure 11). Table III shows the histomorphometric analysis of the Poloxamer gel only group.

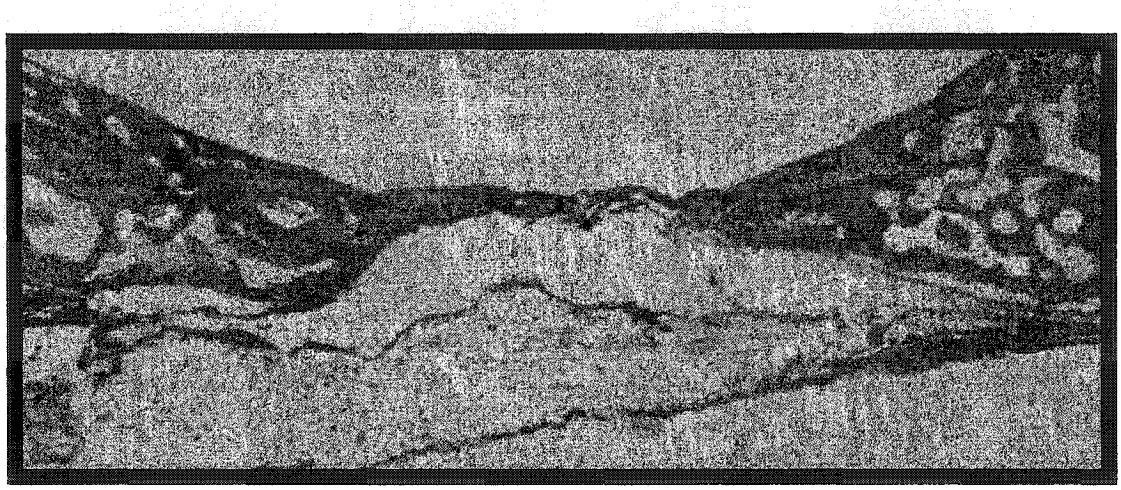

Figure 11. Histology of the Poloxamer gel only group at 12 weeks.

DBM Putty Group

Clinical examination of the specimens in this group was remarkable for the hard tissue-like feel to the defect areas. The tissue was firmly attached to the poorly defined defect margins. There was no flexibility or movement to the tissue such as would be expected if one were to palpate a fibrous union between two hard tissues. Radiographs revealed the same abundance of radiopaque tissue within the defect that was more radiodense than the native cranial bone. See figures 12 and 13 showing a clinical photograph and a radiograph of a skull with the DBM-Poloxamer gel putty filled defects at 12 weeks. The tissue surface was irregular but smooth under the soft tissue covering.

Figure 12. Clinical photograph of the DBM putty filled defect at 12 weeks.

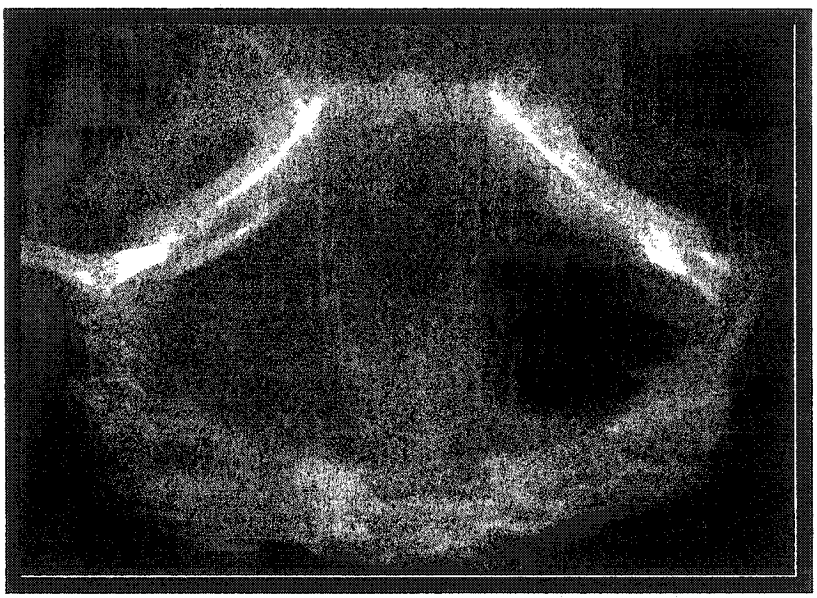

Figure 13. Radiograph of DBM putty and Gel only defects at 12 weeks.

Histologic examination revealed a completely closed defect with new bone and graft material. The new bone had viable marrow spaces surrounded by immature woven bone. The bone was growing onto and around the demineralized bone matrix particles with obvious osteoclastic and osteoblastic activity occurring. The DBM particles appeared to be pushed to the deep and superficial margins of the defect by the in growth of new bone coming from the native bony margins. There was an obvious union at the original surgical margins. There were differences between the 6 and 12-week groups. See the histology in figures 14 and 15. The 12-week group had a greater proportion of marrow space and there was turnover of immature woven bone to mature trabecular bone. The amount of residual DBM particles in the 12-week group was less and in some cases, was hard to find within the defect. Tables IV and V show the differences between the 6 and 12 week groups for tissue makeup. There was definite appearance of greater amounts of new bone growth from the periosteum covering the skull superficially and this caused a generalized 'pushing' of the remaining DBM particles towards the deeper aspect of the healing defect.

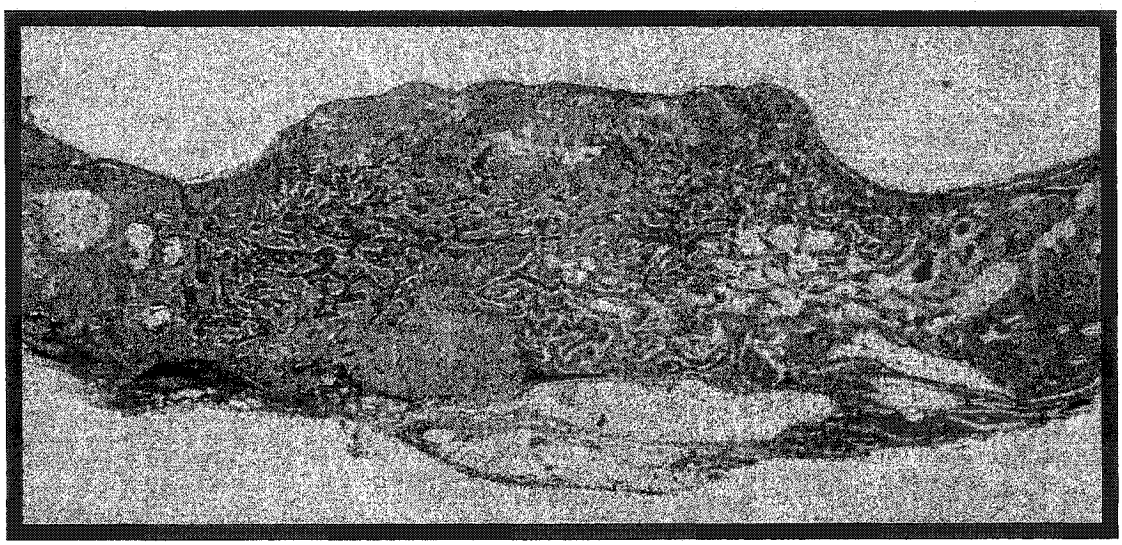

Figure 14. Histology of the DBM putty group at 6 weeks. The original graft material (DBM) is still evident in significant amounts.

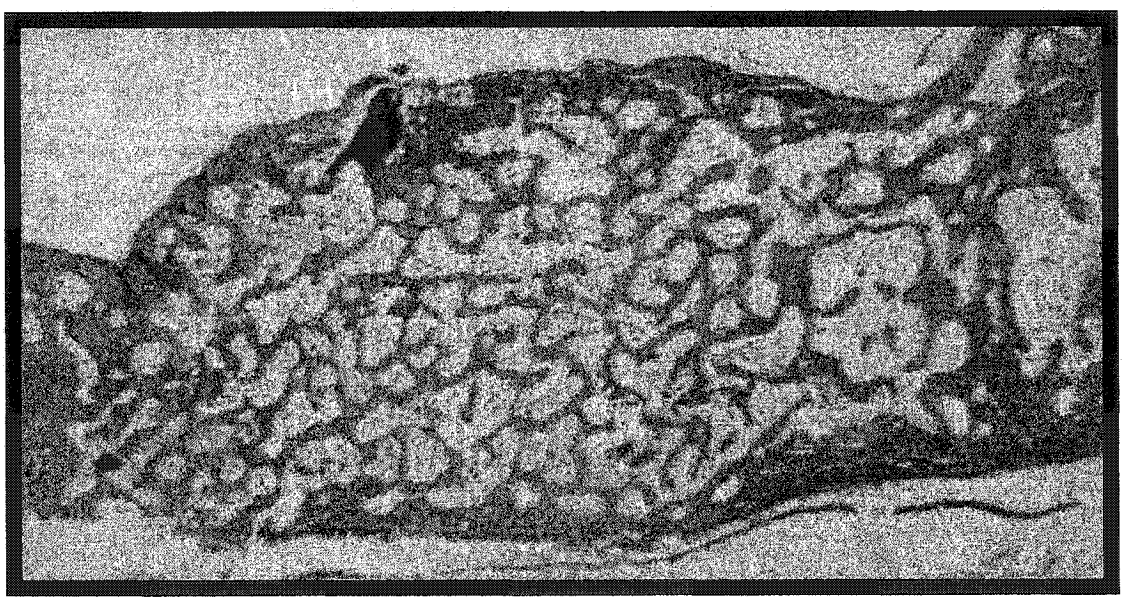

Figure 15. Histology of the DBM putty at 12 weeks. Almost completely remodeled new bone with very little original graft material remaining.

HISTOMORPHOMETRIC ANALYSIS

All specimens were examined histomorphometrically.
This meant that the entire histologic slide of each specimen was examined thoroughly and to allow for adequate surface area measurements, specific anatomic landmarks were identified. From these, various surface area measurements could be calculated and then compared with others from the same specimen and finally with other specimens. These results could then be looked at to see the relative amounts of bone and soft tissues within the defects at the different time periods for each grafting material. These materials could then be characterized for their ability to facilitate bony bridging of these critical sized defects. Of course, the Open (control) group was looked at first to confirm that these were indeed "critical sized defects". This would then validate the model used and give credence to the values obtained with the other grafting materials.

The following measurements were taken and surface areas calculated. Total defect surface area was determined by first identifying the inner and outer table defect margins. These were easily identified by the observing the mature osseous anatomy adjacent to some form of newer woven bone intermixed with grafting material. The circumference of the inner and outer tables of the calvarium was measured utilizing the Sigma Pro software and these two lengths were averaged and multiplied by the average thickness of each defect margin to quantify the total defect surface area. This, therefore, represented 100% of the surface area to be replaced by any potential grafting material. By definition, the control group or open defect must not have a bony surface area greater than 10% of this value, or else the validity of this model could not be substantiated and the remaining graft material results unusable.

The following tissue surface areas were calculated using the Sigma Pro software that allowed the examiner to identify under higher power the type of tissue and "paint" this material separate from the other tissues. The software could then calculate the total surface areas of all the different tissues measured. The tissues looked at were connective tissue or no tissue, new bone, graft material, and marrow space. The resultant values could then be compared to the total surface area to determine how effective the grafting material was in forming new bone and described as a percentage of the original defect area (or volume by extension). For example Control = 8% new bone, DBM putty = 80% new bone, etc. An Analysis of Variance (ANOVA) would then determine statistical significance and bone grafting effectiveness implied. The results of the following variables were evaluated statistically for significance within and between groups. The goal was to establish the amount of bone growth within each defect at the 6 and 12 week time periods of healing. Each experimental group will be described in detail.

Open Defect (Control Group)

Table I shows the values obtained in the analysis for the control defects only at 6 and 12 weeks.

Group	Animal	Defect Area (sqr. mm)	%	Connective Tissue (sqr. mm)	%	Total New Bone (sqr. mm)	%
	A	41.18	100	39.49	95.9	1.69	4.1
	B	43.59	100	41.5	98.2	0.79	1.8
6 week	C	43.23	100	42.19	97.6	1.04	2.4
Group	D	43.21	100	41.09	95.1	2.12	4.9
	E	48.05	100	44.25	92.1	3.41	7.9
	Average	**43.85**	**100**	**41.70**	**95.8**	**1.81**	**4.22**
	F	40.41	100	38.36	94.9	2.21	5.01
	G	45.47	100	35.9	78.9	9.57	21.04
12 week	H	45.58	100	44.04	96.6	1.53	3.36
Group	I	45.26	100	36.03	79.6	9.23	20.39
	J	43.79	100	42.43	96.9	1.36	3.1
	Average	**44.1**	**100**	**39.35**	**89.4**	**4.78**	**10.6**

Table I. Histomorphometric evaluation results from the Open group at 6 and 12 weeks of healing. The average values for the 5 animals from each time group are provided.

Note that here is a trend for more bone from the 6 to 12 week period but that it only approached the 10% threshold, which confirmed this defect as a "critical size defect" and thus validates this model's use for this study. Statistical analysis of these values reveals at 6 weeks, the percentage of new bone fill within the defects to be 4.2% (sd, 2.4) and at 12 weeks to be 10.6% (sd, 9.3). These values are compared with the other experimental groups in Table VI.

Plaster of Paris Group

The variables and tissues evaluated included: **total defect area**, which included all types of tissues within the healing defect lying between the defect margins as evidenced by an obvious native bone margin; **connective tissue area**, which included fibrous scar and Plaster of Paris remnants or that area of graft material obviously dissolved through the processing of the specimen; and **new bone area**. Table II shows the values obtained in the analysis for the Plaster of Paris filled defects at 6 and 12 weeks.

Group	Animal	Defect Area (sqr. mm)	%	Connective Tissue (sqr. mm)	%	Total New Bone (sqr. mm)	%
	A	40.12	100	39.23	97.8	0.89	2.22
	B	46.32	100	44.83	96.8	1.49	3.22
6 week	C	43.37	100	41.23	95.1	2.14	4.93
Group	D	43.82	100	38.18	87.1	5.64	12.87
	E	44.16	100	41.85	94.8	2.31	5.23
	Average	**43.56**	**100**	**41.06**	**94.3**	**2.49**	**5.69**
	F	40.87	100	27.94	68.4	12.93	31.63
	G	41.24	100	29.5	71.5	11.74	28.47
12 week	H	47.9	100	38.98	81.4	8.92	18.62
Group	I	46.25	100	30.62	66.2	15.63	33.79
	J	46.45	100	42.97	92.5	3.48	7.49
	Average	**44.54**	**100**	**34.0**	**76.0**	**10.54**	**24.0**

Table II. Histomorphometric evaluation results from the Plaster of Paris filled defects at 6 and 12 weeks of healing. The average values for the 5 animals from each time group are provided.

Both groups compared similarly except for a trend for more bone at the 12-week group. The 6-week group had 5.7% (sd, 4.2) bone fill versus the 12-week group that had 24.0% (sd, 10.9). These values are compared with the other experimental groups in Table VI.

Poloxamer Gel Only Group

Group	Animal	Defect Area (sqr. mm)	%	Connective Tissue (sqr. mm)	%	Total New Bone (sqr. mm)	%
6 week Group	K	39.32	100	35.99	91.5	3.34	8.5
	L	41.83	100	36.43	87.1	5.4	12.9
	M	44.82	100	42.17	94.1	2.65	5.9
	N	45.27	100	41.78	92.3	3.49	7.7
	O	47.24	100	42.36	89.7	4.88	10.3
	Average	**43.7**	**100**	**39.75**	**90.9**	**3.95**	**9.1**
12 week Group	P	40.55	100	39.33	97.0	1.22	3.0
	Q	42.55	100	39.37	92.5	3.18	7.5
	R	44.82	100	41.4	92.1	3.52	7.9
	S	47.57	100	41.38	87.0	6.19	13.0
	T	41.78	100	39.72	95.1	2.06	4.9
	Average	**43.45**	**100**	**40.22**	**92.7**	**3.23**	**7.3**

Table III. Histomorphometric evaluation results from the Poloxamer gel filled group at 6 and 12 weeks of healing. The average values for the 5 animals from each time group are provided.

Table III shows the values obtained in the analysis for the Poloxamer gel filled defects at 6 and 12 weeks. Evaluation of these results indicates very little

difference between the two time periods of healing. The 6-week group had 9.1% (sd, 2.7) and the 12-week group 7.3% (sd, 3.8) of their defects filled with new bone. These values are compared with the other experimental groups in Table VI. There is little difference between the open defects and the Poloxamer gel filled defects across these two time periods.

Demineralized Bone Matrix Group

Group	Animal	Defect Area (sqr. mm)	%	DBM Grafting Material (sqr. mm)	%	Marrow Space (sqr. mm)	%	New Bone (sqr. mm)	%	Total New Bone (sqr. mm)	%
6 week Group	K	42.05	100	5.09	12.1	13.8	32.8	41.8	99.4	55.6	132.2
	L	44.4	100	10.79	24.3	19.28	43.4	27.3	61.5	46.57	104.9
	M	46.99	100	5.22	11.1	20.1	42.8	32.1	68.3	52.2	111.1
	N	43.23	100	5.14	11.9	19.91	46.1	24.2	56.1	44.13	102.1
	O	42.08	100	1.69	8.4	28.31	67.3	24.6	58.4	52.87	125.6
	Average	**43.75**	**100**	**5.59**	**13.6**	**20.28**	**46.5**	**30.0**	**68.7**	**50.27**	**115.2**
12 week Group	P	46.39	100	3.34	7.2	31.12	67.1	19.29	41.6	50.41	108.7
	Q	44.91	100	3.78	8.4	29.01	64.6	22.01	49.0	51.02	113.6
	R	41.27	100	2.02	4.9	38.93	94.3	14.67	35.6	53.6	129.9
	S	43.01	100	0.56	1.3	34.01	79.1	18.12	42.1	52.13	121.2
	T	44.88	100	1.21	2.7	31.86	71.0	19.98	44.5	51.84	115.5
	Average	**44.09**	**100**	**2.18**	**4.9**	**32.99**	**75.2**	**18.81**	**42.6**	**51.8**	**117.8**

Table IV. Histomorphometric evaluation results from the 6 and 12 week Demineralized Bone Matrix group. The average values from each of the 5 animals are provided.

Measurement of the soft tissue aspect of these specimens was impossible considering that the entire defect was filled with bone between the native bone

margins. Therefore, the connective tissue covering the superficial and deep aspects of the bone (i.e. periosteum) was not measured because there would be no obvious superficial limit (except for the galea aponeurosis) and the deep limit was the potential epidural space. The variables and tissues evaluated included the **bone marrow tissue** area, which included loose connective tissue supporting blood vessels; **new bone** area; total of **new bone** and it's **marrow space**; remaining **Demineralized bone matrix** material; and finally the **total defect area**was determined to enable a calculation of the percentages of each component. Table IV shows the values obtained in the analysis for the Demineralized Bone Matrix filled defects at 6 weeks and 12 weeks respectively.

Evaluation of the results indicates some differences between the two time periods of healing. The 6-week group had 115.2% (sd, 8.1) and the 12- week group 117.8% (sd, 13.2) of their defects filled with new bone material. The amount of residual DBM material diminished from 13.6% of the total area to 4.9% in the 12-week group. This presumably represents continued remodeling of the graft material and replacement by new bone material. The amount of marrow space increased (46% to 75%) and the new bone proper decreased (68.7% to 42.6%) to indicate a turnover of bone material with remodeling leaving greater marrow space contribution to the more mature bony tissue. These values are compared with the other experimental groups in Table V. Figure 16 shows graphically the differences in percentage bone fill between the groups. Pair-wise multiple comparison demonstrated that the Demineralized bone matrix putty was significantly better than all other groups at both 6 weeks ($p<0.0001$, ANOVA) and 12 weeks ($p<0.0001$, ANOVA).

Group/Material	*6 Weeks*	*12 Weeks*
Open	4.2 (sd, 2.4)	10.6 (sd, 9.3)
Plaster of Paris	5.7 (sd, 4.2)	24.0 (sd, 10.9)
Poloxamer gel	9.1 (sd, 2.7)	7.3 (sd, 3.8)
Demineralized Bone Matrix Putty	115.2 (sd, 8.1)	117.8 (sd, 13.2)

Table V: Comparison of new bone fill within the critical sized defect as a percentage of the original defect area in cross section.

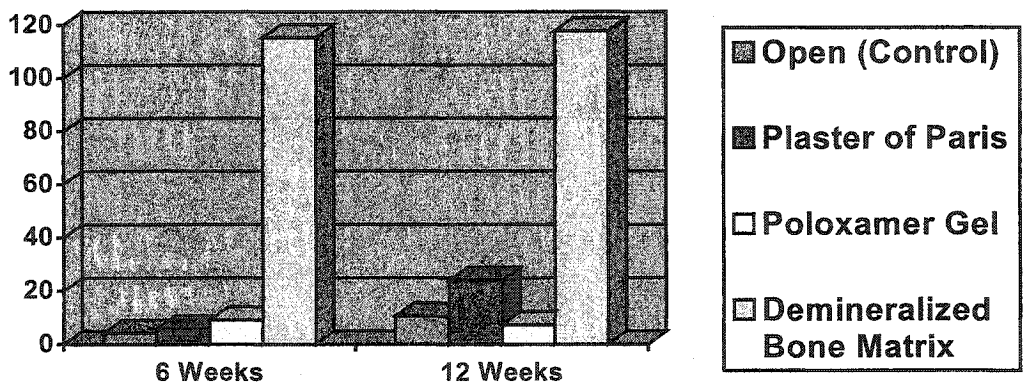

Figure 16. Bar graph comparison of new bone fill within the critical sized defect as a percentage of the original defect area in cross section.

Discussion

Demineralized bone matrix (DBM) has been in experimental and clinical use since the late 1880's when Senn sterilized a bovine xenograft with hydrochloric acid. Marshall Urist's early studies in the 1960's were the first to demonstrate that DBM was osteoinductive as it was able to stimulate the formation of bone at heterotopic sites. Since then, the scientific community has been active searching for ways to prepare DBM in order to maximize its osteoinductive and osteoconductive properties. More recent efforts have been directed towards identifying the ideal delivery vehicle for DBM so that the bioactive molecules within the DBM could be maximally effective at its designated implant site. The investigation outlined in this work evaluated a novel delivery agent for DBM, namely poloxamer 407, which is a thermally reversible block copolymer. The two major advantages of this material are its ability to allow for a controlled, sustained-release of dissolved osteoinductive proteins from the matrix and its natural resorption pattern. Within the first week of being placed, which coincides with the neovascularization stage of wound healing, it is replaced with newly regenerated tissue. The potential of this DBM putty as an allogenic bone graft material needs to be studied and validated in experimental models if it, or any other such bone graft replacement material, is to be used successfully in the surgical management of osseous defects.

Demineralized bone matrix has been studied extensively in several bone grafting scenarios, namely that of the orthopedic, craniomaxillofacial, periodontal, and spine models. Recent studies have discussed the actual osteoinductive potential of DBM in various animal models. Bowers, et al. (1991) showed that demineralized freeze dried bone allograft (DFDBA), which is essentially the same as demineralized bone matrix (DBM), could allow for a significant regeneration of periodontal bone attachment in both a submerged and non-submerged environment. Becker, Urist, et al. (1995) then refuted this message

by showing that commercially available DFDBA without added bone morphogenic protein (BMP) produced clinically insignificant amounts of bone in muscle pouches of athymic mice. This fact was reinforced by Niederwanger and Urist (1996) when they showed that only by adding recombinant human BMP to DFDBA could bone be induced to form in the same model. The importance of this study was that it suggested that not all DFDBA was the same in terms of its ability to induce bone formation. Many reasons were put forward to account for this discrepancy. Processing factors such as particle size, mineral content, calcium content, length of demineralization, sterilization protocols, harvesting techniques, and donor characteristics (especially age) all had a dramatic effect on the overall osteoinductivity of the graft. In other words, the actual osteoinductivity of commercially available DBM varied between manufacturers, and the search for a reliable method of determining this activity was initiated.

In an attempt to establish *in vitro* and *in vivo* models for a quantitative assessment of the osteoinductivity of DBM before clinical utilization, Zhang, Powers, and Wolfinbarger (1997) comparedstandard heterotopic DBM implantation within a muscular pouch with an *in vitro* model. The protocol included measuring alkaline phosphatase activity of human periosteal cells in culture as a measure of bone activity. They found a positive (0.74) correlation between the two and implied that the *in vitro* model was adequate for demonstrating osteoinductive potential in DBM before clinical use. This same group identified several important processing factors for optimizing DBM. The bone graft should be demineralized to roughly 2% residual calcium and the particle sized should be in the 500-710 micron range. The donors of the bone should be in their 30's for females, and in their 40's for male donors. The *in vitro* models continue to come under scrutiny as recent studies using immature osteoprogenitor cell culture assays failed to provide a strong correlation with *in vivo* models (Carnes, et al., 1999). A reliance on the histologic and histomorphometric analysis of animal models is still necessary to prove

osteoinductivity with present day standards. The search for an accurate *in vitro* model continues.

The allogeneic graft material that was studied for this investigation was demineralized bone matrix in a poloxamer gel delivery system. The resulting 'putty' has a doughy texture that is easily molded to adapt to any osseous cavity, and it does not flow once the shape has been established. These are admirable characteristics for a material to have when it is being used surgically to fill voids in bony structures. The demineralized bone was prepared in the standard fashion as has been previously described (Urist, 1965, Reddi and Huggins, 1972). The DBM is first particulated, which increases the surface area of exposed osteogenic factors and is then dissolved in poloxamer gel, which prevents migration of the particles away from the intended graft site. The osteoinductive activity of the implants was confirmed by both *in vitro* and *in vivo* models prior to their usage. The importance of which has been already discussed. The poloxamer gel was initially chosen for its biocompatibility, biodegradability, wetting characteristics, and the resultant putty characteristics that this combination yields. Poloxamer gel now appears to be the superior vehicle for the delivery of osteogenic proteins due to its ability to control the release of these substances while retaining the molecules within the desired surgical site (Coulsen, Clokie, and Peel, 1999).

The goals of this study were many. Foremost, the ability of the DBM putty to allow healing of critical size defects was measured. The critical size defect is an accepted model for the evaluation of bone regeneration materials (Schmitz and Hollinger, 1986, Bosch et al., 1998). A non-grafted critical size defect was used as a negative control, in order to confirm the critical size nature of the defects and validate the model. Additional comparisons were made comparing defect healing using the poloxamer gel alone and calcium sulphate discs. Poloxamer gel filled defects were used to assess any potential contribution that the gel may

have on healing, such as any independent guided tissue regenerative capacity. Since it did not provide for any noticeable bony healing, this material satisfies the definition of an excipient, in that it is an inert or osteogenically inactive substance that is used to carry, then deliver, a bioactive agent (Clokie and Urist, 2000). The Plaster of Paris group was utilized as this material has been used to fill bone voids for over a century, and is well established for its osteoconductive properties. It is considered by some (FDA) to be the predicate bone regeneration material (Snyders, et al., 1993). Finally, the findings of this study will be used to substantiate previous investigations, and to provide evidence that supports the use of DBM in such defects. As Hollinger outlined in his paper on strategies for regenerating bone in the craniofacial complex (1993), materials must be studied in sequential experimental models starting in the rodent, progressing through rabbits, dogs, and finally nonhuman primates prior to being studied in human primate models. Only then can we safely and efficiently assess new materials for human surgical use.

The animal model itself has a proven history in terms of being appropriate for studying potential bone graft substitute materials (Schmitz and Hollinger, 1986). The advantage of having a paired defect model is that it allows for the comparison of two defects within the same animal, while avoiding the sagittal sinus. This also allows for surgery on fewer animals by doubling the experimental sites. The surgical area also has many advantages. The animal suffers minimal morbidity and normal activities were resumed shortly after the surgery. Other areas of the skeleton such as the mandible or a limb involve an interruption in masticatory or gait function for a definite period of recovery time after the surgery. One also has to consider that the cranial defect does not have the same stresses on it as the other sites do, therefore, extrapolating results from the cranial model to mandibular reconstruction, should be done with caution. It does extrapolate well to the craniofacial model well in that the bone has a similar membranous origin, and therefore bone grafting to areas of low

stress within the craniofacial area are well represented here. Can we extrapolate these results with confidence to the human model? Not at this point. Further research should be carried out in more highly evolved animals and eventually human clinical trials before any result can be considered representative. We also know that the healing capacity of various animals is different than humans. This is one of the reasons why we study healing in animals first. It is important to control for the variable of age by using skeletally mature animals when looking at healing, which was accomplished in this investigation.

The clinical observations of this study were generally as expected. The negative control group confirmed the critical size nature of the defects. The 15 mm circular defect in the rabbit calvarium healed with a flexible, but tough fibrous scar that was translucent to light when compared to the adjacent bone of the cranium. There was a smooth transition between the bone margin and the fibrous scar with some narrowing of the defect diameter. Histologically, this scar consisted of a dense fibrous band with the occasional bony island within the scar band. An obvious radiolucent area marked the defect with a smooth, but definite bony margin on radiographs.

Histomorphometric analysis of the control defects confirmed the high proportion of connective tissue scar. At six weeks, 95.8% of the defect area consisted of soft tissue scar. By 12 weeks10.6% of the defect was filled with bone tissue. At nearly 10% bony fill, our results confirm that the defects studied in this project were indeed critical sized defects and therefore the results from the other grafting materials can be validated within our model. It should be noted that more bone was seen in all animals at 12 weeks, indicating that some bone does tend to grow inside this connective tissue scar. The bone that was present at all time points consisted of bony islands, with obvious fibrous scar between them, so that there was not a continuous bony bridge in the animals with the higher bone content

The results provided by the Plaster of Paris group confirmed findings of previous studies. Discs of calcium sulphate healed amongst fibrous scar tissue with variable amounts of appositional bone growth around and throughout the dissolving graft material. Clinically, the scar tissue was less flexible due to the bulk effect of the discs within the tough fibrous tissue. Bone in growth was not obvious to the eye. Radiographic analysis demonstrated the relatively intact round discs within the defect, but the intervening area was radiolucent, suggestive of low amounts of calcified tissue. There were no significant differences between the 6 and 12-week group specimens at this stage.

Histomorphometric examination of the calcium sulphate group showed obvious differences. Between 6 and 12 weeks of healing, the overlying connective tissue scar was the same, but the changes in the appearance of the discs and the bony in growth was remarkable. Minimal breakdown of the discs and occasional bone island formation was seen at six weeks. The histomorphometric figures confirm the finding that the later group demonstrated more dissolution of the discs, especially in the middle of the disc structure. The overall shape of the disc showed 'softening' with a change from a manufactured block-shaped to a rounding of the superstructure of each disc. With further breakdown of the pellet, bone in growth was obvious in and around the new island of Plaster of Paris. New woven bone was identified growing directly onto the plaster and the soft tissue within the area had smaller granules of the material intermixed within the fibrous stroma. Indeed, the defect areas between the two time groups was similar, but the area of bone tissue increased from2.49 to 10.54 square millimeters; representing an increase from 5.7% to 24% bone tissue within the healed defect. This suggests that Plaster of Paris has some osteoconductive properties and validates its well-documented history as a bone graft substitute material.

The poloxamer gel was placed into defects and studied on its own merits to exclude any effect that it may have on the healing of the putty group. In other words, did this material fall within the definition of an excipient? It was also of interest to see if this biocompatible and biodegradable material had any guided tissue regenerative capacity. The safety of this material is well documented (Schmolka, 1972, Desai and Blanchard, 1998). All findings were similar to that of the open (control) defect group. The defects healed with a tough fibrous scar with occasional bony islands within the connective tissue band. There were no statistically significant differences between the two healing time periods. The amount of bone within the defect was 9.1% at 6 weeks, and 7.3% at 12 weeks. It appears that the presence of poloxamer gel does not interfere with the normal healing of a critical sized defect to a connective tissue scar band. There was no evidence of an inflammatory response present at either time periods. Earlier healing times were not looked at, but poloxamer gel is removed from the body soon after implantation (Schmolka, 1972, Clokie and Urist, 2000).

The primary goal of the study was to evaluate the osteoinductive potential of the DBM putty material utilizing poloxamer gel as a vehicle for demineralized bone matrix. The findings were extremely encouraging. Clinical examination of the surgical site revealed a densely packed, hard tissue filled defect. There was no mobility of the mass against the original surgical margins that might suggest a fibrous union between the graft and the native bone margins. The tissue was irregular but smoothly covered by a thin soft tissue sheet. There were no dural adhesions seen in any of the animals.

Radiographically, a densely radiopaque tissue, greater than the adjacent cranial bones filled the experimental defect. It was irregular in nature and the margins were similarly radiopaque and smooth. The images and clinical findings were similar across both time periods. Histologic examination confirmed the clinical findings. The entire defect was filled with bone tissue. Viable new bone that

was obviously remodeling surrounded the graft material. The DBM particles were visible at higher power and were being broken down by osteoclastic activity. The amount of DBM particles remaining at the 12-week time was approximately half that in area as was seen in the 6-week group. The particles appeared to be pushed to the deep and superficial aspects of the healing bony defect with abundant new bone being formed in the middle aspect of the 'sandwich' as well as around all sides of the particles. Although Takagi and Urist (1982) suggested that bone morphogenic proteins will induce perivascular dural connective tissue cells to become osteoblasts and form bone from the dura superficially, it seems that with the grafting of DBM, the main thrust of the new bone in growth is from the defect bony margins instead. As well, there was a significant subperiosteal apposition of new bone on top of the DBM particles appearing to push the particles deep in the direction of the dura. Presumably, the osteoinductive effect of the DBM on the periosteum has lead to appositional bone formation superficially as well as new bone invasion from the surgical bony margins. The surgical margin was difficult to identify exactly, but the architecture of the old, undisturbed bone was obvious. This suggests that the transition between the bony margin and the grafted defect site was a bony one, and that a rigid bone union had taken place.

Histomorphometric analysis revealed some interesting findings. Most importantly, the amount of bone fill was significantly greater ($p<0.0001$, ANOVA) at both time periods between defects reconstructed with the DBM putty and all other groups being evaluated. At 6 weeks, 115.2% of the defect was composed of new bone, with 13.6% of the original volume taken up by residual, non-remodeled DBM graft material. This, of course, was nonvital. At 12 weeks, 117.8% of the original defect volume (or surface area) had been replaced by vital, actively remodeling new bone, in the form of bone marrow tissue and new bone proper. The residual amount of DBM dropped by more than half to 4.9% of the remaining area. The standard deviations were small (8.1% and 13.2%)

indicating that all defects healed in a similar manner. This strongly supports the osteoinductive effect of the DBM putty graft material within this animal model, as well as the benefits of the poloxamer gel excipient.

Histologically, osteoclasts were observed to be responsible for the DBM that was being resorbed. By 6 weeks, the defect that was initially completely filled with the DBM putty (actually overfilled most likely) had only 13.6% of the total area attributable to the original DBM particles. By 12 weeks, continual remodeling reduced to less than half of the original value at 4.9%. On the other hand, the new bone was actively remodeling. The amount of new bone was seen to be decreasing (68.7% to 42.6%) as the area consisting of a more mature bone marrow tissue was expanding (46.5% to 75.2%. It was noted that a few of the specimens in the 12-week group had almost no remaining graft material. This is not surprising considering the findings of Marden, Hollinger and colleagues (1994) who showed significant new bone formation and resorption of a DBM incorporated graft material after only 14 days in rats. Rabie et al. (1996) showed similar findings at 14 days in New Zealand white rabbits although they used smaller than critical sized defects (10 x 5 mm).

Although several other aspects of the healing of DBM grafted CSD's could have been considered, the process of DBM induced bone healing has been well documented (Reddi 1999). The purpose of this study was to establish that DBM in a poloxamer gel vehicle could effectively induce the healing of CSD's in the rabbit calvarial model. This study has confirmed this hypothesis. The use of DBM and this particular vehicle has many advantages. Demineralized bone matrix has inherent osteoinductive and osteoconductive properties. Provided that the appropriate in vivo and in vitro models have confirmed the osteoinductive capacity of the DBM, this material appears to be a successful grafting agent. The osteoconductive nature of the matrix provides for the nidus of bone formation stimulated by the BMPs and other possible osteogenically active proteins within it.

Equally important is the benefit to the graft material that poloxamer gel provides. This thermally reversible gel yields a clinician friendly putty when combined with the DBM. It retains the graft in the surgical site and provides for a controlled, sustained release of the biologically active molecules into the local area so that the necessary processes of osteogenesis can occur. It also prevents the formation of a clot around and within the DBM graft material itself. This is important since the natural resorption of the gel away from the graft bed occurs within days at a pace that approximates that of the in growth of the budding granulation tissue (i.e. neovascularization). This appears to be one of the greatest benefits of using poloxamer gel. Osteogenesis requires a very precise, step-wise stage of events to occur (Bowers et al., 1991, Boyne, 1973). Poloxamer gel may be the best material studied to date to allow for this type of healing. It certainly seems obvious that this material would be more admirable than polyglycolic/poly-L-lactic acid resorbable carriers, since these materials leave voids within the healing graft that ultimately fill in with fibrous tissue (Dunn, 1999).

Tissue engineering of bone involves many aspects. In order to achieve the regeneration of hard tissues, a triad of factors (a scaffold, osteogenic cells and signaling molecules) is critical to ensure optimal results (Reddi, 1999). A scaffold must be present in order to provide a framework from which tissue regeneration may occur. The osteoconductive aspect of DBM as suspended by the poloxamer addresses this issue. Osteogenic cells, or their responsive stem cell precursors must be present to allow for the living tissue to form. In the system presented in this study the DBM's ability to stimulate osteoblasts and undifferentiated mesenchymal cells satisfy this requirement. Finally, signaling molecules such as the BMPs appear to be necessary to initiate the process. Time is, of course, the essence in which bone regeneration occurs and will follow a precise, step-wise course of events that lead to new bone, which is eventually remodeled by the host into mature, lamellar bone.

The ideal graft material will eventually use recombinant technology so that endless supplies of commercially produced substances will be available. The ideal carrier for this recombinant human bone morphogenic protein(s) has yet to be found, but poloxamer gel appears to be of great promise. This delivery vehicle when combined with DBM appears to be the state of the art for bone regeneration at this point in time. It delivers proven osteoinductive factors via an osteoconducive framework to a bone defect to provide for predictable bone regeneration. The material chosen as the delivery vehicle allows easy surgical manipulation of the graft, simplifying the graft procedure and making the bone graft more predictable.

This study has been able to show that demineralized bone matrix in a poloxamer gel vehicle was able to completely regenerate the osseous tissue in a critical sized rabbit calvarial defect model. It suggests and warrants study in higher species that may show that this material may be useful for the management of critically sized cranial vault defects and other bone grafting sites as an autogenous bone graft replacement or supplement.

Conclusions

Bone grafts are commonplace in the surgical fields of orthopedics, craniofacial and reconstructive surgery. Thousands of autogenous bone graft harvests are performed yearly to patients who require treatment of bony defects, nonunion, augmentation, and reconstructive procedures. These patients would undoubtedly benefit from the development of materials that would reduce or eliminate the need for harvesting these bone grafts, and thus leading to decreased surgical morbidity, complications, hospital stay duration, and ultimately health care costs.

By utilizing the body's own regenerative capacity to heal bone, the harvesting and placement of autogenous bone grafts may be unnecessary in certain circumstances. The understanding of the process of bone healing continues to improve. There now exists a great potential to place materials that have the ability to turn on the regenerative pathways for bony healing in a controlled way. Demineralized bone matrix is one of those materials. The problem now exists in finding the appropriate vehicle to carry this matrix to the desired location and allow it's action to proceed.

This thesis has presented a study that investigates the use of poloxamer gel as a vehicle for the demineralized bone matrix. The resultant putty was placed into critical sized defects created in New Zealand white rabbit calvarium and allowed to heal for 6 and 12 weeks. The results indicate that these defects are successfully closed with an abundance of new bone that eventually replaces the original graft material and remodels to lamellar bone with normal bone marrow. This suggests that this material may be used to supplement or even replace autogenous bone grafts when treating large defects in the calvarium. Further studies in higher species are needed before moving to human clinical situations.

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