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Effects of Hyperbaric Oxygen on Healing of Bone, Bone Grafts and Bone Graft Substitutes in Calvarial Defects

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Tampereen Yliopistopaino Oy – Juvenes Print

To My Wife Fatima & My Sons Saeed & Yousef,

For Your Love, Support & Understanding.

To My Dear Beloved Father Sir MohammedSaeed Jan.

Abstract

This study was undertaken in four phases to evaluate the effect of hyperbaric oxygen (HBO) on the repair of critical-sized defects in the presence and absence of a non-vascularized autogenous bone graft (ABG) and bone graft substitutes namely, demineralized bone matrix (DBM) combined with Pluronic F127 (F127) to form a gel or putty, or a commercially available biphasic calcium phosphate (BCP), mixed either with blood or F127 to form a putty. A total of 50 New Zealand white rabbits were utilized in this study. Phase I utilized 20 animals which were randomly divided into 2 groups of 10 animals each. Calvarial defects were created in the parietal bones of each animal bilaterally. Defects were critical-sized, 15 mm on one side and supracritical-sized, 18 mm on the contralateral side. Group 1 received 90-min HBO treatment session at 2.4 absolute atmospheric pressure (ATA) per day for 20 consecutive days. Group 2 served as normobaric (NBO) controls, breathing only room air. Five animals in each group were sacrificed at 6 and 12 weeks. In phase II 20 specimens that were harvested in phase I were analysed for the presence of Vascular Endothelial Growth Factor (VEGF) expression using immunohistochemical staining. Phase III utilized an additional 10 animals which were randomly divided into 2 groups of 5 animals each. Bilateral 15 mm calvarial defects were created in the parietal bones of each animal. ABG were allocated to the left or right defect of each animal. Group1 received HBO treatment while Group 2 served as NBO controls. All animals were sacrificed at 6 weeks. In phase IV an additional 20 animals were used which were randomly divided into 2 groups of 10 animals each. Bilateral 15-mm calvarial defects were created. Group I defects were grafted with either DBM putty or DBM gel. Group II defects were grafted with either BCP or BCP putty. Five animals from each group received HBO treatment and 5 animals served as NBO. All animals were sacrificed at 6 weeks.

Calvarial specimens were analysed by plain radiography, micro-computed tomography (mCT) and histomorphometry. Both radiographic analysis and histomorphometric analysis demonstrated more new bone within HBO-treated defects compared to NBO defects ($p<.001$). There was no significant difference between the percentage of new bone forming in the 15-mm and 18-mm HBO-treated defects. VEGF expression in 6 week HBO samples was elevated compared to NBO ($p=0.012$). Staining of the 12 week HBO samples was reduced compared to 6 week HBO ($p=0.008$) and was similar to 6 and 12 week NBO samples. HBO reduced fibrous tissue formation in BCP grafted defects and promoted a small but significant increase in bone formation in DBM grafted defects. mCT analysis indicated a higher bone mineral density (BMD) and bone mineral content

(BMC) in ABG than Non-Grafted defects (p<0.05). Higher BMC (p>.05), bone volume fraction (BVF; p>.001), and BMD (p>.001) of the defects grafted with BCP compared with DBM grafted defects.

HBO was effective in enhancing the bony healing of full thickness critical-sized as well as supracritical-sized defects in the rabbit calvarial model. However, there was a significant decline in the bone mineral content (BMC) of HBO treated grafted defects compared to NBO treated grafted defects (p<0.05). HBO enhanced bony healing in non-grafted rabbit calvarial critical-sized defects and may increase the rate of residual graft resorption in ABG and DBM grafted defects.

Keywords: bony defects, bone healing, bone regeneration, calvarial defects, hyperbaric oxygen.

Abbreviations

ABG	Autogenous Bone Graft
BCP	Biphasic Calcium Phosphate
BMC	Bone Mineral Content
BMD	Bone Mineral Density
BMP	Bone Morphogenic Protein
BV	Bone Volume
BVF	Bone Volume Fraction
CT	Connective Tissue
DBM	Demineralized Bone Matrix
HA	Hydroxylapatite
HBO	Hyperbaric oxygen
HBOT	Hyperbaric oxygen therapy
MB	Mature bone
MRI	Magnetic Resonance Imaging
mCT	Microcomputed tomography
NB	New Bone
NBO	Normobaric Room Air Oxygen
OD	Margin of Original Defect
ORN	Osteoradionecrosis
RA	Room air
ROI	Region of Interest
RT	Radiation therapy
SD	Standard Deviation
TCP	Tricalcium Phosphate
TGF-β	Transforming Growth Factor Beta
TMD	Tissue Mineral Density
TMC	Tissue Mineral Content
VEGF	Vascular Endothelial Growth Factor

Glossary of terms

Hyperbaric oxygen: Oxygen at a level higher than atmospheric pressure.

Hyperbaric oxygen therapy: Treatment during which a patient breathes 100% oxygen inside a closed chamber pressurized above 1 atmosphere absolute (ATA).

Critical-sized Defect: The smallest full thickness osseous wound that will not heal spontaneously during the life time of the subject during the experimental period.

Bone Grafting: A procedure done by reconstructive surgeons to augment volume, width or height of deficient or missing bone.

Bone Graft: A material used to augment volume, width or height of deficient or missing bone. It can be either autogenous, allogenic, xenogeneic, or alloplastic.

Autogenous Bone Grafting: Transfer of bone harvested from the same individual undergoing the bone grafting.

Allogeneic BoneGrafting: Transfer of bone harvested from an individual of one species into a different individual of the same species.

Xenogenic Bone Grafting: Transfer of bone between two different species.

Alloplastic Bone Grafting: Transfer of synthetic products.

Radiomorphometrics: The quantitative measurement of areas of radiopacities of bone in plain radiographs using a digital image analysis.

Histomorphometrics: The quantitative measurement of different histological elements of bone using a digital image analysis.

Microcomputed Tomography Bone Analysis: Analysis of a region of interest in a reconstructed three dimensional image for bone mineral density parameters.

Bone mineral content (mg/mm3): Microcomputed tomography based measurement of bone mass in the organic matrix.

Bone Mineral Density (BMD): Microcomputed tomography based percentage of bone mineral content in the total defect volume.

List of original papers

The thesis is based on the following original articles, which are referred to in the text by the Roman numerals I - IV:

I. **Jan A**, Sándor GKB, Iera D, Mhawi, A, Peel SA, Clokie CML (2006). *Hyperbaric oxygen results in an increase in rabbit calvarial critical sized defects*. Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology and Endodontology. 101(2): 144-149.

II. Fok, TCO, **Jan A,** Peel SA, Clokie CML, Sándor GKB (2008). *Hyperbaric oxygen results in an increase in vascular endothelial growth factor (VEGF) protein expression in rabbit calvarial critical sized defects*. Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology and Endodontology. 105(4): 417-422. Epub 2008 Jan 16.

III. **Jan A**, Sándor GKB, Brkovic BMB, Peel SA, Evans AW, Clokie CML (2009). *Effects of hyperbaric oxygen on grafted and non-grafted on calvarial critical-sized defects*. Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology and Endodontology. 107(2): 157-163. Epub 2008 September 19.

IV. **Jan A**, Sándor GKB, Brkovic BMB, Peel SA, Kim YD, Xiao WZ, Evans AW, Clokie CML (2010). *Effects of hyperbaric oxygen on demineralized bone matrix and biphasic calcium phosphate bone substitutes.* Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology and Endodontology. 109(1):59-66. Epub 2009 Oct 20.

Contents

1 Introduction

Congenital bony defects in the craniofacial and maxillofacial skeleton arise as a result of areas of failed development such as in cleft lip and palate patients. Ablative surgery may produce bony defects when segments of bones are resected to treat tumors. Trauma may result in large boney defects when tissue may have been traumatically avulsed. Such osseous defects can be reconstructed using bone grafts, or hopefully, in the future with bone graft substitutes. The healing of such wounds relies on the vascularity of the affected tissues.

Hyperbaric oxygen (HBO) has been used to aid in the healing of hypoxic or compromised wounds (Brown et al., 1998; David et al., 2001; Feldmeier, 2003) such as hypoperfused grafts, radiation induced side effects (Bui et al., 2004) and necrotizing anaerobic bacterial infections (Larson et al., 2002). Muhonen *et al* have demonstrated that HBO treated rabbits have more osteoblastic activity and osteogenic potential in their irradiated distracted mandibles when compared to a non-HBO treated group (Muhonen et al., 2002a; Muhonen et al., 2002b). HBO is thought to act by increasing the oxygen partial pressure gradient between vessels and interstitial fluids. This results in increased wound healing by increasing the amount of oxygen dissolved in the blood (oxygen tension) which in turn can increase the amount of oxygen delivered to the hypoxic wound site (Shirely and Ross, 2001). HBO can promote angiogenesis (Sheikh et al., 2005) and results in an increase in the vessel density in irradiated tissue (Marx et al., 1990). Studies have demonstrated that HBO increases bone formation in bone harvest chambers in rabbits (Nilsson et al., 1988) and elevates alkaline phosphatase activity, a marker of bone formation, in rats following mandibular osteotomy (Nilsson, 1989), and increased osteoblastic activity and angiogenesis in irradiated mandibles undergoing distraction (Muhonen et al., 2004; Muhonen et al., 2002c). Marx et al. have shown an eight to nine fold increase in vascular density with HBO in irradiated tissues (Marx et al., 1985).

HBO's mode of action in the treatment of decompression sickness and carbon monoxide poisoning is well understood based on its effects on reducing gas emboli and hastening carboxyhaemoglobin dissociation (Feldmeier, 2003). However, it has also demonstrated effectiveness in the treatment of, necrotizing soft tissue infections, soft tissue radiation necrosis, diabetic wound healing, and now osseous defect repair where other mechanisms are believed to involved (Al-Waili and Butler, 2006; Coulson, 1985). It has been well established that the formation of new blood vessels (angiogenesis) is essential in the process of soft tissue and bone repair (Bauer et al., 2005; Glowacki, 1998). Vascular disruption, caused by traumatic injury has been shown to lead to the formation of a hypoxic zone. Wound hypoxia is necessary to stimulate angiogenesis and revascularization. HBO increases the amount of oxygen dissolved in the blood (oxygen tension) which can in turn increase the amount of oxygen delivered to these hypoxic

tissues reducing the effects of the hypoxia (Shirely and Ross, 2001). While this is helpful in cases of chronic hypoxia, which blunts the repair process, it is not very clear as to how this would stimulate the normal repair process. HBO was shown to be an effective adjunct to enhance membranous bone healing and bony union of bony defects (Al-Waili and Butler, 2006). HBO treatment also results in increased vascular endothelial growth factor (VEGF) protein expression (Bauer et al., 2005). *Ex-vivo* studies have shown elevated titers of alkaline phosphatase activities in HBO treated rabbits (Glowacki, 1998). *In-vivo* studies have also demonstrated increased bone formation in bone harvest chambers (Byrne et al., 2005; Klagsbrun and D'Amore, 1991). The need for additional grafting may thus be minimized (Marx et al., 1985).

Vascular Endothelial Growth Factor (VEGF) has been identified as one of the primary growth factors responsible for neo-vascularization during wound healing and embryonic development (Klagsbrun and D'Amore, 1991). Oxygen tension is a key regulator of VEGF expression in vitro and in vivo (Byrne et al., 2005; Nanka et al., 2006; Shweiki et al., 1992).

One way to assess the healing of a bony wound is to use a critical-sized defect model. A critical-sized defect is by definition the smallest full thickness osseous wound that will not heal spontaneously during the lifetime of an animal (Schmitz and Hollinger, 1986). Such a defect requires an adjunctive technique to permit its complete bony healing. The rabbit calvarial critical-sized defect model has been used to study the efficacy of a variety of bone substitute materials in promoting defect healing. This model was also effective to study cranio-maxillofacial bone regeneration (Clokie et al., 2002; Haddad et al., 2006; Moghadam et al., 2004). In the rabbit calvarium, a critical-sized defect is defined as a defect 15 mm in diameter (Schmitz and Hollinger, 1986).

Clinically, critical-sized osseous defects may lead to numerous complications including fracture, non-union and pseudo-arthrosis (Schmitz and Hollinger, 1986). Surgical treatments are utilized to prevent further complications, which may involve the use of a fixation device and autogenous bone graft material to bridge the gap in the defect. All such reconstructive procedures that require a second surgical site for the harvesting of tissue are associated with potential morbidity (Clokie et al., 2002; Haddad et al., 2006; Moghadam et al., 2004). Synthetic biomaterials have been used in place of autogenous bone grafts (Moghadam et al., 2004).

Reconstruction of maxillofacial defects aims at restoring form and function. Following maxillofacial trauma there is a vascular disruption which leads to the formation of a hypoxic zone. While hypoxia is necessary to stimulate angiogenesis and revascularization, extended hypoxia will blunt the healing process. Hypoxia inhibits fibroblast proliferation, collagen synthesis and granulation tissue formation (Tandara and Mustoe, 2004). Hyperbaric oxygen therapy (HBO) is the exposure of the patient to 100% oxygen at elevated pressures. HBO has been used to improve the healing of a variety of compromised or hypoxic wounds including diabetic ulcers, radiation induced tissue damage, gangrene and necrotizing anaerobic bacterial infections (Broussard, 2004; Fenton et al., 2004; Hunt et al., 2004).

Assessment of bone mineral density (BMD) to determine the quality of bone regenerate remains undefined. Quantitative computer tomography has been shown to be the most accurate method to measure bone mineral density (Grampp et al., 1997; Weigert, 1997). It would therefore be useful to evaluate the quality of bone regenerated within critical-sized calvarial defects with and without HBO and to compare the quality of bone produced in similar defects with a non-vascularized autogenous bone graft by assessing BMD using micro CT techniques.

The current standard of practice for the treatment of critical-sized defects is the use of autogenous bone grafts. Large defects require the harvesting of bone extra-orally which requires a second surgical site and results in increased risk of complications (Kainulainen et al., 2002b). Smaller defects can be treated with bone grafts obtained intra-orally (Kainulainen et al., 2005). Animal studies proved that defects are treatable by bone substitutes including demineralised bone matrix, calcium phosphate cements with or without bone morphogenic proteins (Clokie et al., 2002; Haddad et al., 2006; Moghadam et al., 2004) and fiber-reinforced composites (Tuusa et al., 2007; Tuusa et al., 2008).

This thesis hypothesizes that HBO treatment would promote the healing of a rabbit critical-sized calvarial defect, possibly even allowing a supra-critical-sized defect to heal. It also examines the healing of bony defects under hyperbaric and normobaric oxygen conditions when the defects are treated with autogenous bone grafts and a variety of bone substitutes.

2 Review of the literature

2.1 Biology of Bone Graft Healing

Three types of bone healing are described: primary, secondary and gap healing. The difference between the three is dependent on the size of the osseous defect and the rigidity of fixation. The three bone healing models gives different information that relates to bone regeneration within non-grafted as well as grafted defects.

2.1.1 Primary Bone Healing

Primary bone healing occurs without callus formation when the bone ends are in direct contact and rigidly fixated, or anatomically reduced and are compressed together by bone plates (Danis, 1949). This process is usually called contact healing and it was initially described after observing radiographs of long bone fractures that were treated by plating, it was noticed that they failed to show callus formation. Osteoclasts began to cut away cores on either side in the area of compression, progressing towards the fracture. The osteoclastic cutting cone proceeded at a rate of 50 to 80 μm per day. Cores were 200μm which provides space for vessel ingrowth, osteoblastic proliferation and new bone formation (Simmons, 1980). Intermediary cartilage is not seen with primary bone healing.

Gap healing is another type of primary bone healing. It occurs when a small gap exists after rigid fixation. A critical distance of 0.3 mm was thought to be required for surviving cells to acquire nutrients from canaliculi at the bone surface and form lamellar bone. If the gap was larger than 0.3 mm but up to 1 mm, then woven bone formed first and further transformed or remodeled into lamellar bone (Schenk and Willenegger, 1977).

2.1.2 Secondary Bone Healing

Fractures treated without rigid fixation heals with secondary bone healing. A good example of non-rigid fixation is maxillomandibular fixation by wiring. The initial injury elicits an inflammatory response and activation of the complement cascade. Damage to blood vessels initiates cellular extravasation and cell signaling. Chemotactic factors (C5a, leukotriene B4) attract monocytes and macrophages. Activated macrophages release FGF, stimulating endothelial cells to release plasminogen activator and procollagenase. Growth factors (PDGF, TGF-β1 and TGF-B2) released from the alpha granules of degranulating platelets, are stimulants for PMN, lymphocytes, monocytes and macrophages. The blood clot acts as a hemostatic plug, contains the growth factors in the injured site and provides an environment for cell signaling. The injured tissue is normally hypoxic with Oxygen partial pressure of 5-10 mmHg as well as acidotic (pH, 4-6). Acidosis and hypoxia are required for PMN's and macrophages stimulation (Marx et al., 1998). A proliferation phase starts the healing by day 3 and can lasts up to 40 days after fracture occurrence. A reparative phase follows with new blood vessels, collagen, and cells. Osteoprogenitor cells are then stimulated to proliferate and differentiate into active chondroblasts and osteoblasts, laying down large amounts of extracellular matrices forming a bridging callus. Chondroblasts lay down extracellular matrix then become chondrocytes, which eventually hypertrophy and die, leaving empty lacunae in a calcified matrix. These empty spaces (lacunae) allow for vascular ingrowth resulting in a higher oxygen supply and normalized pH, which in turn favour differentiation of osteoblasts. The cartilage is then removed by osteoclasts while osteoblasts lay down immature woven bone. Mobility at the regenerate site will disrupt blood supply and result in cartilage and fibrous tissue predominance.

2.1.3 Bone Graft Healing

Three processes are involved in the healing of bone grafts. Osteogenesis, osteoinduction and osteoconduction (Burchardt, 1983).

1. Osteogenesis is defined as the formation of new bone from osteocompetent cells contained within the bone graft.
2. Osteoinduction is defined as bone formation from primitive mesenchymal cells in the recipient bed, which have been stimulated to differentiate into bone forming cells by inductive proteins within the graft.
3. Osteoconduction is defined as ingrowth of capillaries and osteoprogenitor cells from the recipient bed into and around the grafted material.

Various elements of bone tissue contribute toward the healing of bone grafts through the processes of osteogenesis, osteoinduction and osteoconduction, (Gray et al., 1982). Endosteum, periosteum osteocytes and marrow spaces contributes via different percentages. Gray, Phil and Elves estimated this contribution as:

1. Endosteum 60%
2. Periosteum 30%
3. Osteocytes 10%
4. Marrow 0%

Osteoblasts are immature bone cells responsible for synthesis and secretion of osteoid which is rapidly mineralized to form bone. Osteocytes are entrapped osteoblasts responsible for the maintenance of the extracellular matrix of bone. Osteoclasts on the other hand are large multi-nucleated cells derived from macrophage-monocyte cell lines and responsible for the resorptive processes and remodelling of bone. Organic Extracellular Matrix is made of inorganic salts namely hydroxylapatite crystals and ground substance namely glycoproteins. The extracellular matrix also has a fibrous component, the most prominent of which is type I collagen.

Periosteum contains condensed fibrous tissue located on the outer surface of bone. The inner layer of the periosteum contains osteoprogenitor cells that have the capability to differentiate into osteoblasts. With the exception of the articular surfaces of bone, periosteum is bound to bone by Sharpey's fibers which exist at the sites of insertion of tendons and ligaments into bone. Periosteum plays an important role in healing of critical-sized defects (Ozerdem et al., 2003). Bone marrow contains dividing pluripotent stem cells which are located in the intertrabecular spaces of cancellous bone. Bone marrow can be active (Red marrow) or fibrofatty (Yellow marrow), which may be reactivated if the need for haemopoiesis arises.

Woven Bone is immature bone with randomly organized collagen fibers. It is much coarser than lamellar bone. Woven bone is the first version of bone to form during development and also during gap healing of bony defects if the gap is more than 0.3 mm. Woven bone continuously remodels over approximately 6 months to form lamellar bone. The time needed for transforming woven bone into lamellar bone is known as "Sigma", which is a species specific value. The Sigma for humans is 18 weeks, which explains why surgeons wait at least 4 months before applying functional loads to autogenous bone grafts. The sigma for rabbits on the other hand, is only 6 weeks (Parfitt, 1976).

Lamellar Bone forms most of the mature skeleton. It comprises a solid mass "compact bone" and spongy mass "cancellous bone". Compact bone has a unique physical structure. Bony columns in compact bone are parallel to the axis of stress representing concentric bony layers or lamellae. Central channels contain lymphatic vessels, blood vessels, nerves and are known as Haversian canals. Volkmann's canals are also neurovascular bundles which interconnect haversian canals at right angles. As osteoblasts lay down bone peripherally, they get trapped as osteocytes in lacunae with connecting canaliculi. Cytoplasmic extensions within canaliculi connect osteocytes together and with osteoblasts as well.

The outermost layers of a bone consist of concentric lamellae of dense cortical bone while the inner layer is the medullary aspect. The medullary bone has irregular lamellae and trabeculae of spongy bone. Cancellous bone is synonymous with spongy bone. The network of irregular bony trabeculae separates the bone marrow spaces from each other. Trabeculae are lined by endosteum that is structurally similar to periosteum with osteoprogenitor cells, osteoblasts and osteoclasts. Bony lacunae contain osteocytes

with canaliculi. Cancellous trabeculae have no haversian systems. Metabolite exchange occurs via canaliculi and blood sinusoids in the bone marrow.

Axhausen described autogenous bone grafts as inert collections of transplanted bone which eventually loose their vitality and become replaced by new bone through neoangiogenesis and cell differentiation (Axhausen, 1907). This process was termed creeping substitution. Others have demonstrated *in vivo* new bone formation with grafted autogenous bone in the muscle pouches of dogs (Ham and Gordon, 1952). The theory that Ham and Gorlin adopted was that some cells survived within the graft and continued to grow new bone.

Many investigators described two phases of bone formation in autogenous bone graft healing (Axhausen, 1956; Gray and Elves, 1979; Marx, 1993)

I) Phase I: transplanted cells within the graft formed new bone during the first week of healing and continued for approximately four weeks. Nutrition was acquired through diffusion from the recipient bed. The amount of new bone formed correlated with the number of surviving transplanted cells.
II) Phase II: Bone formation began in the second week of grafting and peaked around the fourth or fifth week. Phase II continued for life as bone remodeling. The graft integrated during this period of remodeling.

2.2 Bone Graft Substitutes

2.2.1 Allogeneic Demineralized Bone Matrix

Although allogeneic bone is one of the most commonly used alternative to the autogenous harvested bone, it offers the potential risk of disease transmission, rejection, and resorption. In the 1960s, Marshall Urist and his coworkers revealed that the implantation of acid-demineralized bone into extraskeletal sites led to the development of bone ossicles. This phenomenon has become known as osteoinduction (Urist, 1965) a process by which a bioimplant stimulates local undifferentiated mesenchymal cells to become osteoprogenitor cells, which will eventually form new bone. It is now generally accepted that the osteoinductive potential of demineralized bone matrix (DBM) is due to endogenous bone morphogenetic proteins (BMPs) that function to signal for embryonic bone induction.

Urist hypothesized that BMPs are released from a “supramolecular aggregate of noncollagenous proteins” during bone turnover or in injury (Urist, 1989). Mineralized bone matrix has no osteoinductivity, and BMPs are hidden by minerals in bone, once demineralized, BMPs will be exposed to the appropriate cells. It has been shown that if demineralization of the DBM is increased such that the residual calcium content of the

DBM falls below 2% by weight, the osteoinductivity of the DBM is lost (Zhang et al., 1997).

It is hypothesized that remineralisation of bone requires an initial nucleation event; by leaving some residual calcified component. Foci of hydroxyapatite (HA) facilitate calcium deposition onto its surface and start the mineralization process. Therefore, demineralization is a compromise between removing enough of the mineral content to expose the BMPs while at the same time leaving a sufficient amount of calcified component to facilitate remineralization. Providing calcium ions in the microenvironment during mineralization has been shown to be beneficial. The use of calcium hydroxide (CaOH) has showed increased mineralization, bone metabolism, total protein synthesis, and collagen synthesis (Murakami et al., 1997).

Others have postulated that early remineralization may have an effect on stimulating osteogenesis and may improve the osteoinductive potential of DBM (Garraway et al., 1998). The effect of CaOH on remineralization of DBM is largely unknown. It is clear that CaOH directly placed into a bony defect will not have stimulatory effects (Mitchell and Shankwalker, 1958). Particulate DBM has been shown to allow for bony healing of critical sized defects. Rabbit DBM grafted into cranial vault defects showed 50% to 75% new bone fill at 12 weeks; however, these were not critical sized defects (Lindholm et al., 1993). Dogs' calvarial defects grafted with fresh autogenous bone showed 99% bone fill, whereas those filled with DBM showed 77% bone fill (Oklund et al., 1986). A number of other studies have supported these findings as well and therefore suggest that DBM may be a reasonable alternative to autogenous bone (Moghadam et al., 2001; Salyer et al., 1992).

2.2.2 Poloxamer 407

Particulate DBM can be very technically challenging to use. It is dehydrated fat free material. Delivery of DBM in an appropriate vehicle may allow the surgeon to more effectively control its placement. Studies have shown that certain carriers may also allow for a more effective release of BMPs from the DBM (Clokie and Urist, 2000). One such medium is poloxamer 407.

Poloxamer 407 is a reverse-phase block copolymer that, once warmed by body fluids, will form a viscous gel and allow for improved handling characteristics (Clokie and Urist, 2000). Poloxamers are hydrophobic copolymers with solubility in aromatic solvents (Clokie and Urist, 2000; Schmolka, 1972). Poloxamers are commercially used as additives, defoamers, anti-static agents, demulsifiers, detergents, gelling agents, dispersants, and dye levellers (Clokie and Urist, 2000).

When poloxamer 407 is mixed with DBM or even mineralized bone graft substitutes, the resultant bioimplant will harden at the recipient site, making it easy to adapt, facilitating surgical application. Due to its molecular structure, poloxamer 407

allows for the slow release of an active ingredient (BMP) (Moghadam et al., 2004). When poloxamer 407 was evaluated against other delivery agents such as HA, collagen, and other noncollagenous proteins, it was found to be superior in its ability to deliver and release BMP.

In study IV, a commercially available poloxamer 407 (Pleuronic F-127 ©) was mixed with the bioimplants to improve their handling.

2.2.3 Biphasic Calcium Phosphate (BCP)

Biphasic calcium phosphate (BCP) as an osteoconductive biomaterial may be differentiated from its osteoinductive counterpart by its inability to induce new bone formation. It assists bone formation by providing a microstructural scaffold that supports bone growth throughout its structure. Osteoconductive scaffolds allow chemotactic, circulating proteins and cells (e.g. mesenchymal stem cells, osteoinductive growth factors) to migrate and adhere, and within which progenitor cells can differentiate into functioning osteoblasts (Paderni et al., 2009). Such scaffolds chemically bind and integrate with bone, restoring contour and providing strength and support. It is also considered biodegradable and eventually is replaced with host bone (Schmitz et al., 1999).

BCP particles used in this study are comprised of both hydroxyapatite (HA) and beta polymorph of tricalcium phosphate (beta-TCP) at 60:40 ratios by volume. Microporosity and micropore size of BCP particles have a strong impact on their protein adsorption characteristics (Zhang et al., 2010). Porous implants have the potential for ingrowth of bacteria that can be introduced at the time of surgery or post-operatively, due to tissue breakdown. This occurs when the pore size is less than 1 micron. Human host defences including macrophages require a pore size of > 50 microns to enter and engulf bacteria that have infected the implant. Therefore, the ideal porous implant would have pores smaller than 1 micron to avoid bacterial innoculation or >50 microns to allow macrophages to engulf the bacteria (Cohen et al., 1999).

In study IV, a commercial product (Starumann Bone Ceramic ©) was used. Each glass vial contained 0.5g with 400 – 700 μm microporosity. The material was mixed with either autogenous blood or poloxamer 407 to improve handling.

2.3 Growth Factors

Exogenous recombinant growth factors such as bone morphogenic proteins (BMPs), play an important role in driving bone regeneration, and give more consistent results than the previous bone substitutes. These factors modulate cellular activity and provide stimuli to cells to differentiate and produce new bony tissue.

Growth factors include BMPs, fibroblast growth factor (FGF), insulin-like growth factor (IGF), platelet-derived growth factor (PDGF), transforming growth factor (TGF), and vascular endothelial growth factor (VEGF) (Carter et al., 2000). These factors are secreted by the cells involved in the repair process. In the same time, bone itself also

constitutes a large reservoir for many growth factors (Colnot et al., 2003). Factors are constantly synthesized and stored until remodelling or trauma initiates their release. Once released, the growth factors modulate the healing response and mediate the remodelling process. Stem cells can be stimulated by growth factors such TG•-1, BMPs or VEGF to guide the differentiation and growth of the cells (Sándor and Suuronen, 2008).

2.3.1 Transforming Growth Factor (TGF)

The transforming growth factor superfamily members are related to each other by relative degrees of sequence similarities. Diverse biological functions are observed (Schmitt et al., 1999). TGF-• release from platelet's alpha granule degradation was extensively studied and proved to be osteoinductive in multiple applications including sinus augmentation procedures (Marx et al., 1998). The application of TGF-• to implant sites was found to increase the amount of bone healing and significantly improves the implant-bone surface contact (Clokie and Bell, 2003).

2.3.2 Bone Morphogenic Proteins (BMPs)

BMP is a subfamily of TGF-• is made of seven conserved cysteine residues at the mature carboxy terminal (Wozney et al., 1999). They are low molecular weight proteins (19 to 30 kDa) with a pH of 4.9 to 5.1 (Moghadam et al., 2001; Urist et al., 1975). BMPs stimulate mesenchymal stem cells to differentiate into osteoblasts during development and bone healing (Ducy et al., 1997; Schmitt et al., 1999). Being present in most tissues, they play an important role in remodeling of the adult skeleton. When osteoclasts resorb bone matrix, BMP is released providing recruitment and differentiation of stem cell precursors to form new bone (Dragoo et al., 2003).

BMP-7 is alsp known as osteogenic protein 1 (OP-1), was first used to aid the healing of maxillary Lefort I osteotomy by Warnke in 2003 (Warnke and Coren, 2003). It is approved for human use in sinus elevation procedures as well in socket preservation techniques. The recombinant human form is derived from a recombinant Chinese hamster ovary cell line. Laboratory as well as human studies data has shown superior results in reconstructing critical-sized defects (Moghadam et al., 2001; Moghadam et al., 2004). Clokie and Sándor were first to report the use of OP-1 in post-resection defects. They reported 10 cases of post-resection mandibular defects successfully reconstructed with BMP-7 (OP-1) in DBM suspended in a reverse phase medium. An uneventful postoperative course was reported with dental rehabilitation achieved 1 year post-reconstruction (Clokie and Sándor, 2008).

2.3.3 Vascular Endothelial Growth Factor (VEGF)

Angiogenic signals are crucial to establish new vascular networks, which provide the nutrients for tissue growth and homeostasis. Vascular endothelial growth factor (VEGF) is one of the most important in bone repair. Recent studies have shown that VEGF also influences osteogenesis and has a direct effect on osteoblasts (Kaku et al., 2001). Angiogenesis is a prerequisite for bone regeneration. VEGF also found crucial for vascular invasion because it stimulates osteoclastic resorption and chondroclastic

activities (Engsig et al., 2000; Gerber et al., 1999; Olsen et al., 2000). In particular, VEGF may help in patients where the vasculature has been compromised following radiotherapy (David et al., 2001). In these patients, the use of hyperbaric oxygen (HBO) therapy is necessary to provide enough oxygen to the bone and prevent osteoradionecrosis following tooth extraction or bone grafting (Marx et al., 1990). HBO therapy enhances wound healing through increased oxygen tension leading to vascular proliferation (Shirely and Ross, 2001).

2.4 Animal Model

The calvarial critical-sized defect model in rabbit parietal bones has been used by a number of authors (Tuusa et al., 2008). This model has proven to be reproducible and reliable (Moghadam et al., 2004). Ease of handling was reported by multiple investigators (Clokie et al., 2002; Haddad et al., 2006; Moghadam et al., 2004). It provides sufficient defect volume that is surrounded by both cortical and cancellous bone resembling maxillofacial bone healing module. The one difference compared to other anatomical sites is the presence of a pulsatile dural layer in the base of calvarial full thickness defects, which is not present in the maxillofacial skeleton for example. (Ozerdem et al., 2003)

A Critical-sized defect is best defined as the smallest osseous wound that will not heal spontaneously during the experimental period or the life of the animal (Hollinger et al., 1994). Host systems induce healing via fibrous union instead. The critical-sized defect exceeds the body's ability to regenerate bone. The quantity of bone regenerated in the critical-sized defects is influenced by the animal species, age, anatomic location of the defect, size of the defect and finally intactness of periosteum (Schmitz and Hollinger, 1986).

Calvarial critical-sized defects receive their blood supply from the pericranium and the dura, unlike long bone defects, which have nutrient canals. Intact periosteum as well as dura is essential for bone regeneration in full thickness calvarial defects (Ozerdem et al., 2003).

2.5 Hyperbaric Oxygen Therapy

Hyperbaric oxygen therapy is defined as intermittent exposure to 100% oxygen under pressures greater than 1 absolute atmosphere (ATA). The concentration of oxygen in the atmosphere is 21%. At 1 ATA, the oxygen in blood is almost entirely carried by hemoglobin. 97% of oxygen carried in the arterial blood is chemically bound to haemoglobin while only 3% is dissolved in plasma. 1 gram of haemoglobin carries a maximum of 1.34 ml of oxygen. Fully saturated haemoglobin (100%) in 100ml of blood carries approximately 20 ml of oxygen. Hemoglobin that is saturated to 97% carries 19.5ml of oxygen in 100 ml of blood. This amount is reduced to 5ml of oxygen while

passing through capillaries. Increasing the oxygen-carrying capacity of blood by increasing hemoglobin saturation is not possible.

At the sea level, gas pressure is 760 mmHg or 1 Atmosphere Absolute (ATA). Arterial haemoglobin saturation is 97%, while venous haemoglobin saturation is 70%. Inhalation of HBO increases the quantity of oxygen dissolved in plasma. At 1 ATA, the amount of dissolved oxygen in 100 ml of plasma is 0.449 ml. When inhaling 100% oxygen at 1 ATA, O2 concentration increases to 1.5ml / 100 ml of plasma. When inhaling 100% oxygen at 3 ATA, the amount of dissolved oxygen in 100 ml of plasma increases to 6.422 ml / 100ml of plasma, which is enough to meet the basic metabolic needs of healing tissues in the human body.

The driving force for oxygen diffusion from the capillaries to tissues can be estimated by the difference between the partial pressure of oxygen on the arterial side and the venous side of the capillaries. The difference in the partial pressure of oxygen from the arterial side to the venous side of the capillary system is approximately 37 times greater when breathing 100% oxygen at 3 ATA than air at 1 ATA.

Hyperoxia causes a rapid and significant vasoconstrictive effect. (van Golde et al., 1999). Breathing 100% oxygen at 3 ATM leads to a reduction in perfusion of up to 25% in the brain. This leads to neurotoxic activities in 10% of the population (Gelfand et al., 2006). Reduction in perfusion also occurs in other tissues, although to a lesser extent.

Increasing abnormally low tissue oxygen concentrations has been shown to accelerate healing. Fibroblast synthesis of collagen requires tissue oxygen tensions of 30-40 mm Hg. HBO therapy has the potential to achieve these levels in hypoxic or poorly perfused tissues. It was shown in *in vitro* studies that exposure to HBO for 30 minute and 60 minute periods at 2.5 ATA enhances fibroblast cell growth. On the other hand, 120 minute exposure to HBO at 2.5 ATA exerts a marked proapoptotic effect (Conconi et al., 2003).

Hyperbaric oxygen (HBO) has been used to aid in the healing of hypoxic or compromised wounds (Brown et al., 1998; David et al., 2001; Feldmeier, 2003) such as hypoperfused grafts, radiation-induced side effects (Bui et al., 2004) and necrotizing anaerobic bacterial infections (Larson et al., 2002). Hyperbaric oxygen therapy has proven to stimulate osteoblastic proliferation and differentiation *in vitro* (Wu et al., 2007). Marx and Ehler have shown angiogenic effects of HBO therapy at 2.4 ATA with repeated exposures for 90 minutes (Marx et al., 1990).

2.5.1 History of Hyperbaric Oxygen Therapy

The development of hyperbaric medicine is closely linked to the history of diving medicine. In the first documented use of hyperbaric therapy, the British physician Henshaw used compressed air for medical purposes in 1662. In 1775, Joseph Priestly was credited with having discovered oxygen. A system for treating diving accident victims using HBO was proposed by Drager in 1917, but it was not until 1937 that Behnke and Shaw used HBO to treat decompression sickness (Severinghaus, 2003).

Since the 1930s, HBO therapy has been widely used in diving medicine and for numerous other medical conditions. The accredited use of HBO is regulated by the Undersea and Hyperbaric Medicine Society which update their guidelines periodically.

Unfortunately, unproven claims and speculation have made HBO's role, if any, in the treatment of some of these illnesses unclear. Tissues treated with HBO have increased levels of oxygen, which in turn have a negative effect on anaerobic bacteria (Fenton et al., 2004) and a positive effect on blood flow to the area (Marx et al., 1990). HBO has positive effects on osteoblastic activity (Muhonen et al., 2004). HBO reverses hypoxia, hypocellularity, and hypovascularity of irradiated tissues (Brown et al., 1998).

2.5.2 Physics of Hyperbaric Oxygen

In order to understand the physics of hyperbaric oxygen, one should realize that the normal pathway of oxygenation is ventilation through pulmonary alveoli followed by transport through the vascular system. There is an orderly arrangement of alveolar capillaries, the pulmonary venous system, the left atrium, the left ventricle, the systemic arterial system and tissue capillaries which finally not only bring blood and nutrients to the interstitial space but remove waste metabolites. Pressure gradients govern oxygen diffusion in the interstitial space. Partial pressure of oxygen varies from arterial and venous circulations. The partial pressure of oxygen in the alveoli (PAO2) equals 104 mmHg. While the partial pressure of oxygen in arterial blood (PaO2) equals 90 mmHg and in the venous blood (PvO2) it equals 40 mmHg. The oxyhemoglobin dissociation curve determines the dissociation of oxygen from the hemoglobin molecules as the blood components reach the tissues.

The air we breathe, which is room air, consists of 21% oxygen, 79% Nitrogen and 0.04% carbon dioxide. Air pressure at sea level equals 780 mmHg which is represented by one Absolute Atmosphere (ATA). Dalton's law calculates the total pressure exerted by a gaseous mixture as being the sum of the partial pressures of each individual component in a gaseous mixture. According to Dalton's law the partial pressure of oxygen in room air equals 160 mmHg at the sea level.

PO2= 780 X 21/100 = 160 mmHg

The pressure of gas in fluids, such as plasma for example, is calculated by Henry's Law

Gas concentration = pressure X solubility coefficient

The solubility coefficient is directly proportional to the temperature of the tissue.

2.5.3 Administration of Hyperbaric Oxygen

HBO can be administered through either a mono-place chamber or multi place chamber. Both have been used with comparable success in different HBO facilities.

Hyperbaric oxygen therapy is costly. In the United States, Medicare pays approximately 400 US dollars per dive for facilities fee and 125 US dollars professional fee, resulting in an expense of 80,000 dollars for a course of 40 treatments (Attinger et al., 2008). The potential cost of a prolonged course of HBO therapy must be weighed against savings that may be achieved from improved tissue healing and reduction of

amputations and complicated outcomes. Facilities are often scarce. In 1996, 259 hyperbaric facilities were reported to exist in the United States. Only eleven were reported in Canada. In Finland, accredited facilities are available only in larger cities with medical centers or academic health science centers.

Monoplace Chamber

Monoplace chamber contains compressed oxygen and designed to treat individual patients one at a time (Figure 1). This method is less expensive than multiplace chamber. The disadvantage is its limited access to patients.

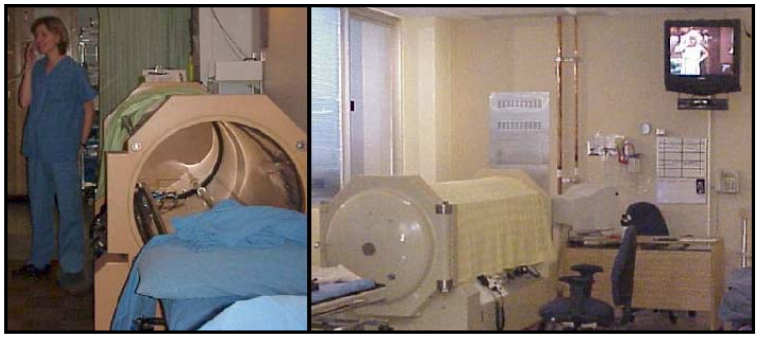

Figure 1: Monoplace chamber can only be utilized to treat one patient at a time. It contains compressed oxygen and is designed to treat individual patients one at a time

Multiplace Chamber

Multi-place Chamber (Figure 2) contains compressed air while patients are breathing 100% compressed oxygen through hoods or masks. Multiple patients can be treated at the same time while a trained attendant is monitoring patients in the chamber. Although this method is more expensive and needs a full time trained attendant, it is more cost effective and can accommodate up to six individual at a time according to the size of the chamber.

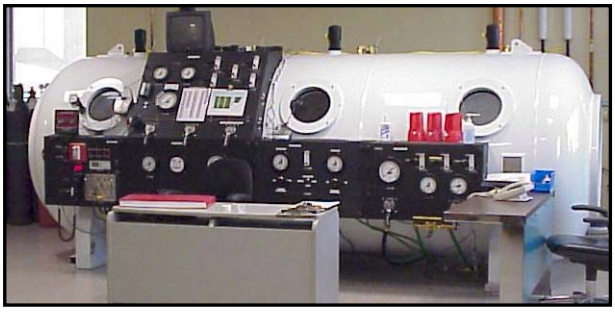

Figure 2: Multi-place chamber contains compressed room air. Multiple patients can be treated simultaneously while a trained attendant is monitoring the patients in the chamber. Patients breath 100% compressed oxygen through hoods or masks while sitting in the chamber.

2.5.4 Indications of Hyperbaric Oxygen Therapy

The undersea and hyperbaric medicine society (UHMS) has approved the following medical indications for HBO therapy (Broussard, 2004):

1. Air or gas embolism
2. Carbon monoxide poisoning with or without Cyanide poisoning
3. Clostridial myositis and myonecrosis (gas gangrene)
4. Crush injury and compartment syndrome
5. Decompression sickness
6. Enhancement of healing in problem wounds
7. Exceptional blood loss (anemia)
8. Intracranial abscess
9. Necrotizing soft tissue infections
10. Refractory osteomyelitis
11. Radiation induced necrosis
12. Compromised skin grafts and flaps
13. Thermal burns

Hyperbaric oxygen (HBO) has been used to aid in the healing of hypoxic or compromised wounds (Brown et al., 1998; David et al., 2001; Feldmeier, 2003) such as hypoperfused grafts, radiation-induced side effects (Bui et al., 2004) and necrotizing anaerobic bacterial infections (Larson et al., 2002). Hyperbaric oxygen therapy has proven to stimulate osteoblastic proliferation and differentiation in vitro (Wu et al., 2007). Oxygen content in the tissues can be boosted to 81% ±5% of normal tissue after 20 sessions of HBO (Marx, 1984). Marx and Ehler have shown 9 fold increases in neoangiogenesis with HBO therapy at 2.4 ATA for 90 minutes a day for 20 days (Marx et al., 1990).

2.5.5 Osteoradionecrosis (ORN) of the Mandible

One of the most widely accepted and extensively documented indications for HBO therapy is its application in the treatment and prevention of osteoradionecrosis (ORN) of the mandible. Marx developed the Wilfred-Hall staging Algorithm for classifying and treatment mandibular (ORN) and for prophylaxis prior to teeth extraction. (Marx et al., 1985)

Stage I ORN

Only cortical bone is exposed by a small mucosal ulcer that is commonly necrotic. Exposed bone is without other signs and symptoms. Those are treated by 30 HBO therapy sessions with no debridement or only minor bony debridement. If the patient progresses favourably, give 10 additional HBO_2 treatments.

Stage II ORN

Localized ORN with both the cortical and a portion of the underlying medullary bone are necrotic. Stage II ORN is usually a non resolving stage I ORN. Those are treated by 30 HBO therapy sessions followed by surgical debridement and 10 more post-operative HBO therapy sessions. Debridement for Stage II ORN ideally maintains mandibular continuity.

Stage III ORN

Diffuse ORN of the mandible. Full thickness of the mandible is necrotic. Stage III ORN is usually a non resolving stage I or II. Mandibular continuity can not be maintained. Those are treated by a reconstruction protocol. 30 HBO therapy sessions are instituted followed by mandibular resection eradicating all necrotic bone followed by 10 post-resection HBO therapy sessions. Reconstruction follows in delayed fashion. The number of HBO sessions varies according to the severity of the condition.

Although there has been controversy regarding the usefulness and the application of hyperbaric medicine as an adjunct to bone healing, laboratory data has always been convincing. HBO therapy demonstrated an elevation of alkaline phosphatase levels in cells derived from alveolar bone. Similar results were also obtained from cells derived from irradiated mandibles (Muhonen et al., 2004; Muhonen et al., 2002a). HBO has also demonstrated an eight to nine fold increased vascular density over normobaric oxygen and air-breathing controls in the irradiated mandible model (Marx et al., 1990). Other in-vivo studies demonstrated a significant increase in bone formation in the rabbit bone harvest chamber (Nilsson et al., 1988). In-vitro studies on the other hand showed stimulated osteoblastic proliferation, enhanced bone nodule formation, calcium deposition, and alkaline phosphatase activity with daily exposure of HBO to osteoblasts in-vitro (Wu et al., 2007). Clinically, Implant failure in irradiated bone has also been a clinical challenge in rehabilitating cancer patients (Granström, 2006). HBO therapy demonstrated a significant improvement in osseointegrated implant survival in irradiated mandibles (Granström, 2003).

3 Aims of the study

The purpose of this study is to expand the body of knowledge associated with the osseous reconstruction of the maxillofacial skeleton and the use of hyperbaric oxygen therapy (HBOT). Therefore the specific aims of the study are:

1. To test the effects of hyperbaric oxygen therapy on the healing of osseous critical-sized and supracritical-sized defects in the rabbit calvarial model.

2. To study the effects of hyperbaric oxygen treatment on the expression of vascular endothelial growth factor (VEGF) in a healing osseous wound in the rabbit calvarial model.

3. To test the effects of hyperbaric oxygen therapy on the healing of autogenous bone grafts in osseous critical-sized defects in the rabbit calvarial model.

4. To test the effects of hyperbaric oxygen therapy on the healing of osseous critical-sized defects treated with bone substitutes in the rabbit calvarial model.

4 Methods and Materials

4.1 Subjects

This research project was performed involving a total of 70 rabbits in which the healing of their calvarial critical-sized or supracritical-sized defects were the focuses of these studies. The work was divided into four studies. The numbers of subjects are listed in Table 1.

Table 1. Number of the subjects included in the four studies.

Study	Number of subjects	Calvarial Defects
I (Hyperbaric oxygen results in an increase in rabbit calvarial critical sized defects)	20	40
II (Hyperbaric oxygen results in increased vascular endothelial growth factor (VEGF) protein expression in rabbit calvarial critical-sized defects)	20 (from Study I)	40 (from Study I)
III (Effect of hyperbaric oxygen on grafted and nongrafted calvarial critical-sized defects)	10	20
IV (Effect of hyperbaric oxygen on demineralized bone matrix and biphasic calcium phosphate bone substitutes)	20	40
Total	50	100

The protocols to conduct these four studies including anesthesia, surgery, HBO administration, sacrifice, data collection and analysis were approved by the animal care and research ethics committees of the University of Toronto, Toronto, Canada (Protocol Reference Number: 20005155) prior to the commencement of the studies.

4.2 Methods

4.2.1 Study I: Critical-sized and Supracritical-sized Defects and Hyperbaric Oxygen Exposure

An animal trial of 12 weeks duration was conducted using 20 New Zealand white rabbits, which were randomly divided into 2 groups of 10 animals each. Calvarial defects were created in the parietal bones of each animal bilaterally (Figure 3). Defects were critical-sized, 15 mm on one side and supra critical-sized, 18 mm on the contra lateral side. Group1 received 90 minutes HBO treatment sessions in a chamber specifically designed for animal use (Figure 4)at 2.4 ATA per day for 20 consecutive days. Group 2 served as a control without any HBO treatment sessions. Five animals in each group were sacrificed at 6 and 12 weeks. Data analysis included qualitative assessment of the calvarial specimens, post sacrifice radiographs and histological sections. Quantitative analysis included radiomorphometrics and histomorphometrics to compute the amount of regenerated bone within the defects. ANOVA and paired sample t-test were used for statistical analysis.

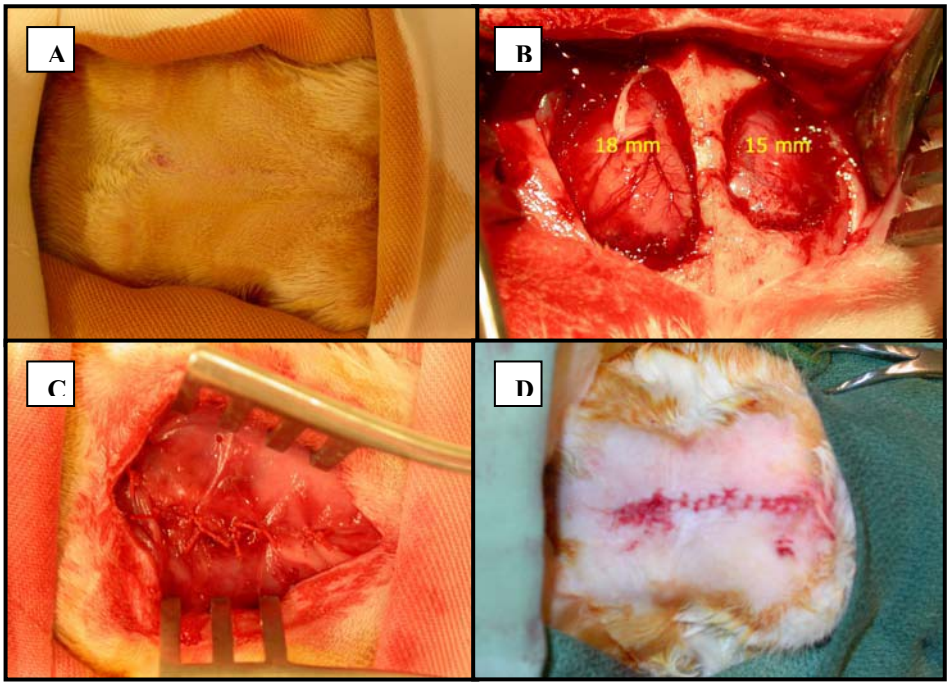

Figure 3: Study I surgical protocol. A; Animal is in the prone position, prepped and draped. B; Full thickness osseous defect measures 15 mm (critical-sized) on one side and 18 mm (supracritical-sized) on the contralateral side. C; Pericranium is closed with 4-0 vicryl. D; Water tight closure for the skin.

Figure 4: Hyperbaric oxygen chamber specially designed for animal research. Rear end glass window permits animal monitoring using the mirror behind the chamber.

4.2.2 Study II VEGF Expression and Hyperbaric Oxygen Exposure

This investigation used archived tissue from the previous study from the 20 New Zealand white rabbits used in that study.

Qualitative Analysis: Histology
Following fixation and decalcification, the midpoint of the defect region was identified and served as the coronal reference plane of section prior to embedding in paraffin. Multiple 6μm sections were cut and stained with hematoxylin-eosin (H&E) for conventional light-microscopy. The defect region was visualized in all samples and the appearance of new bony regenerate was noted.

Quantitative Analysis: Immunohistochemical analysis
Multiple 6 μm sections cut from the same paraffin block were used in the histological analysis. These sections were incubated with mouse mono-clonal anti-human $VEGF_{121}$ antibody (clone JH-21, Lab Vision Corp, Fermont, CA, USA), with known rabbit cross reactivity, as a primary antibody. Then an avidin-biotin complex (Lab Vision Corp.) was incubated to label the primary antibody, and a color reagent was added at the end to allow the horse-radish peroxidase reaction to take place.

Analysis of VEGF expression
Using the image capturing software Image Pro Plus 4.1 for Windows® (Media Cybernetics, Carlsbad, CA, USA), 6 random fields from each section were captured at 40x magnification using an RT Color digital camera (Diagnostic Instruments Inc, Sterling Heights, MI, USA). A total of 3 sections were used for each defect resulting in a total of 18 random images for each right and 18 of each left defect. The area stained for VEGF in each field was measured, by setting a threshold intensity above which a pixel is counted using the Image Pro Plus software.

To determine whether there was any difference in the VEGF expression in the center of the defects compared to the margins of the defects two images were taken from the central one third of the defect with the other 4 being from the margins and their VEGF staining measured.

4.2.3 Study III Autogenous Bone Grafts and Hyperbaric Oxygen Exposure

An animal trial of 6 weeks duration was conducted using 10 New Zealand white rabbits, which were randomly divided into 2 groups of 5 animals each. 15 mm critical-sized calvarial defects were created in the parietal bones of each animal bilaterally. One defect was left void. The contralateral defect was grafted with particulate non-vascularized autogenous bone graft (Figure 5). Group1 received a 90 minute HBO treatment sessions at 2.4 ATA per day for 20 consecutive days. Group 2 served as a control without any HBO treatment sessions. The animals in each group were sacrificed at 6 weeks. Data analysis included micro-CT assessment of calvarial specimens, as well as histomorphometric analysis to compute the amount of regenerated bone within the defects.

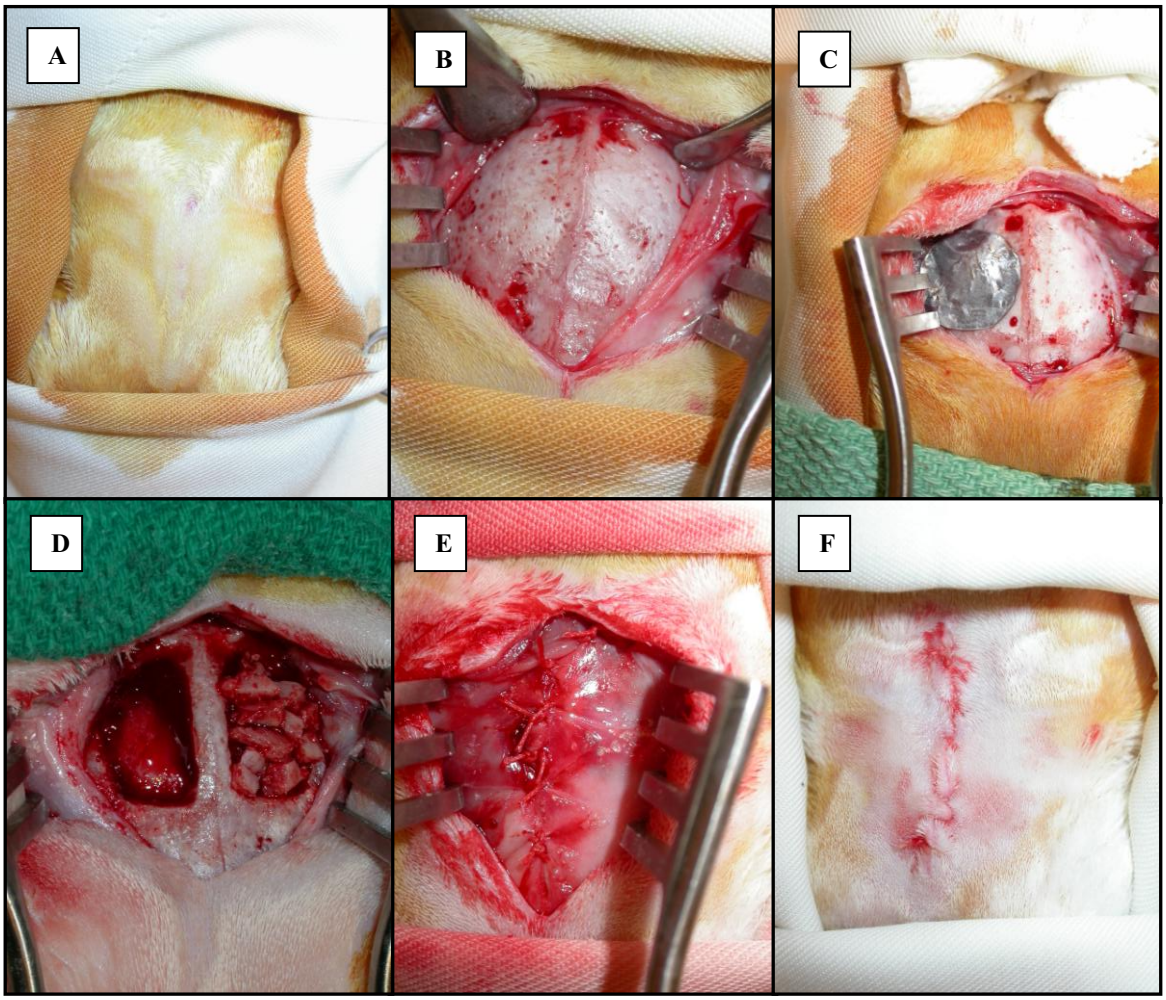

Figure 5: The surgical protocol for Study III. A; Animal is in the prone position, prepped and draped. B; Full thickness flap elevated and retracted by self retaining retractor. C; A surgical template measured 15x15mm was used to guide the osteotomy. D; Full thickness osseous defects created. One side filled with particulate autogenous bone graft. E; pericranium closed. F; Water tight closure for the skin.

4.2.4 Study IV Bone Substitutes and Hyperbaric Oxygen Exposure

An animal trial of 6 weeks duration was conducted using 20 New Zealand white rabbits, which were randomly divided into 4 groups of 5 animals each. 15 mm critical-sized calvarial defects were created in the parietal bones of each animal bilaterally. Group1 received 90 minute HBO treatment sessions at 2.4 ATA per day for 20 consecutive days and the defects were grafted with allogeneic demineralized bone matrix in either a gel or putty state (Figure 6) mixed with resorbable Pluronic F-127 (F127; poloxamer 407, BASF Canada Inc., Toronto, Canada). The animals in Group 2 were grafted with the same graft substitute materials but without any HBO treatment sessions. The defects in the animals in Group 3 were grafted with a bone ceramic mixed with either autologous blood or with a combination of the bone ceramic and a resorbable liquid poloxamer gel (Figure 7). The animals received 90 minute HBO treatment sessions at 2.4 ATA per day for 20 consecutive days. The animals in Group 4 were grafted with the same graft substitute materials as the animals in Group 3 but without any HBO treatment sessions. The animals in each group were sacrificed at 6 weeks. Data analysis included micro-CT assessment of calvarial specimens, as well as histomorphometric analysis to compute the amount of regenerated bone within the defects.

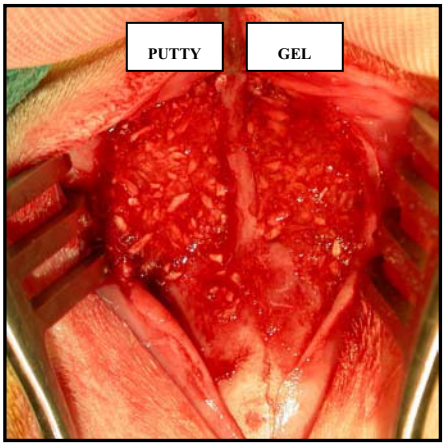

Figure 6: Defects grafted with demineralised bone matrix (DBM) in a putty state or gel state.

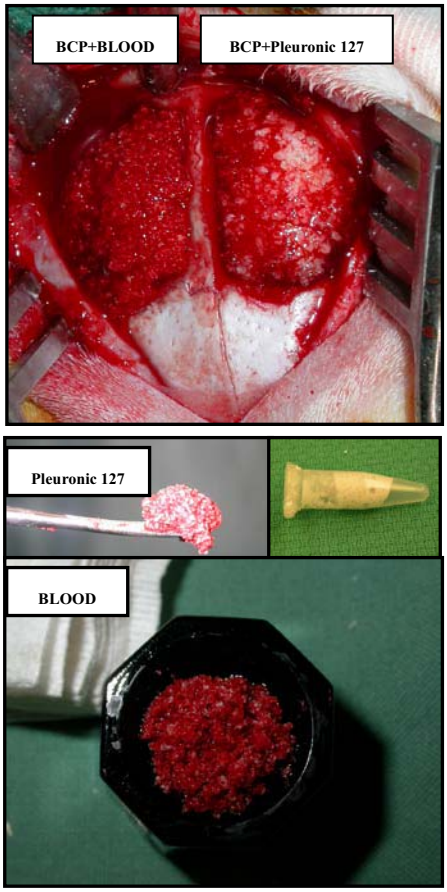

Figure 7: Defects grafted with biphasic calcium phosphate mixed with either autologous blood or poloxamer 407 gel.

4.2.5 Plain Radiography and Radiomorphometrics

Radiographs of the specimens of all the studies were taken using a cephalostat machine standardized for 1:1 magnification on a D speed film at 9mA, 60 KVP for 0.2 seconds. The films were processed in an automatic developer and examined for radiopacities, which represented new bone formation. Fixation followed in 10% formalin for 48 hours. Specimen containers were labelled with a separate coding to allow for blinded analysis.

Radiographs of specimens of study I were digitized. An investigator blinded to the HBO status of the animals traced the areas of radiopacities within the defects. The percentages of radiopacities were calculated via Image Pro Plus 4.1 software for Windows (Media Cybernetics, Carlsbad, CA).

4.2.6 Micro-Computed Tomography (mCT) Evaluation and Bone Analysis

48 hours after fixation of specimens of studies III and IV, micro-computed tomography (mCT) followed. This study utilized an Explore Locus SP© mCT scanner (GE medical systems, London, Ontario, Canada). This scanner was designed to scan specimens that measure at most 25 x 30 mm, which corresponds to a bilateral parietal bone specimen containing both defects. It was impractical to scan the entire calvarium. Prior to scanning the specimens, a calibration scan was performed using a synthetic bone sample, a water sample and an air sample. Calvarial specimens were scanned using the fast mode utilizing 0.05mm sections. Each specimen took 120 minutes to be fully scanned. Reconstruction of scanned images (Figure 8) was done using Microview software (GE Medical Systems, London, Ontario, Canada) after calibrating the program using the bone, water and air standard values. The reconstructed 3D image was then traced in 3 dimensions to the circumference of the original defect margins. This allowed the creation of a 3D reconstruction of the defect, which was referred to as the region of interest (ROI).

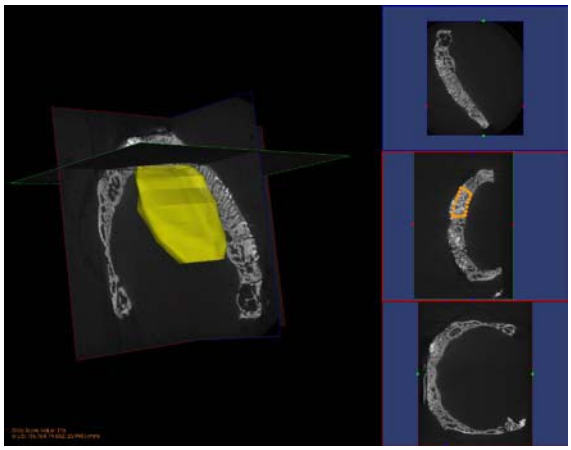

Figure 8: Micro Computed Tomography Coronal sections represent a defect traced in multiple sections. Tracings were interpolated generating a region of interest (ROI) which is shaded in yellow. The ROI was analyzed for total volume, bone volume, bone mineral content, and tissue mineral content.

A reconstructed intact specimen was used to set up the bone value threshold, which was analyzed by the same software. A normal distribution curve had determined the bone value threshold to be at 1300 at a 95% confidence interval. The bone value was used to differentiate bone from non-bone tissues within the defect and to guide the software in

measuring tissue mineral content. The region of interest was analyzed using the following parameters:

1- Total Defect Volume (TV) in (mm3)

2- Bone Volume (BV) in (mm3): Tissues that matched bone value threshold of 1300 at 95% confidence interval.

3- Bone Volume Fraction (BVF): Percentage of bone volume by total defect volume (BV/TV)

4- Bone mineral content (BMC) mg/mm3): Measurement of bone mass in the organic matrix.

5- Bone Mineral Density (BMD): Percentage of bone mineral content in the total defect volume.

6- Tissue Mineral Content (TMC) in (mg/mm3): bone mineral content of voxels within the ROI.

7- Tissue Mineral Density (TMD): Percentage of tissue mineral content in the total defect volume.

4.2.5 Histological Evaluation and Histomorphometrics

Upon completion of the fixation period and radiography, decalcification followed using 45% formic acid and 20% sodium citrate for 4 weeks. Testing for adequate decalcification was accomplished by attempting to cut through a contingency intact specimen with a number 10 scalpel. Adequate decalcification was achieved in 4 weeks in all of the specimens. Each specimen was sectioned into two portions: an anterior and posterior portion. Each half was washed in distilled water for 10 minutes three times. Dehydration was accomplished by using a series of alcohol immersions starting with 70% ethanol for 15 minutes twice followed by 95% ethanol for 15 minutes twice followed by 100% ethanol for 30 minutes twice. Each specimen was then immersed in methylbenzoate overnight. Immersion in Toluene followed for 30 minutes. Specimens were then infiltrated with paraffin embedded under vacuum pressure. Paraffin blocks were then sectioned into 7μm sections, prepared and stained with hematoxylin and eosin. Sections in the middle of the defects, representing the greatest dimension (15 mm or 18 mm) were examined under the light microscope by a blinded investigator in an attempt to prevent bias.

Ten sections within the middle of the each of the defects were digitized. Digital images were captured by a CCD digital camera (RT Color; Diagnostic Instruments Inc., Sterling Heights, MI) attached to the microscope on 4X magnification with 100% zoom to ensure proper focus. To create a single image from each slide the digital images were merged using Adobe Photoshop Elements 2.0 (Adobe Systems Inc., San Jose, CA). The reparative tissues in each defect waer traced and measured by a blinded investigator in an attempt to prevent bias. The amount of new bone was extracted and expressed as the percentage of the total area of the defect. Calibration was achieved using a millimeter grid.

4.2.6 Statistics

Simple statistical methods are routinely used in studies involving bone regeneration basic research. These methods include descriptive statistics such as the mean and its standard deviation for continuous variables, and percentage and sample size for categorical variables. Analytical tests such as ANOVA are useful for comparing means

with multiple variables, whereas t-tests are most commonly useful for a single variable. Paired sample t-test was also useful in comparing defects within individual cohort defects.

The percentage of new bone and the percentage of radiopacities were analyzed in relation to either (1) inspired oxygen (HBO vs. NBO), (2) defect size (15 mm vs. 18 mm), or (3) healing time (6 weeks vs. 12 weeks). The p values below .05 were considered to be statistically significant. Results were compared across groups using 1 and 2 way ANOVA with Student Newman Keuls (SNK) post hoc testing for normal data of equal variance or Holm-Sidak post hoc testing for non-normal/non equal variance data. All statistical analyses were performed using either SPSS 10.0 for Windows (SPSS Inc., Chicago, IL) or SigmaStat v3.5 (Systat Software San Jose CA). Statistical significance was considered to be at $p<.05$.

In study I, analysis of variance (ANOVA) and paired sample t tests were applied in SPSS 10.0 for Windows (SPSS Inc., Chicago, IL), and used to calculate statistical differences between the means of new bone formation based on histomorphometrics. Means of the radiopacities within the defects were also analyzed. The percentage of new bone and the percentage of radiopacities were analyzed in relation to either (1) inspired oxygen (HBO vs. NBO), (2) defect size (15 mm vs. 18 mm), or (3) healing time (6 weeks vs. 12 weeks). The p values below .05 were considered to be statistically significant.

In study II One-way ANOVA was employed for analysis within and between the groups. When differences were found, the Student-Newman- Keuls method was used as a post hoc test to determine which groups were significantly different. Comparisons of VEGF staining between the 15-mm and 18-mm defects in the same rabbits, and between the margins and central regions within each defect, were made using the paired t test. Statistical significance was set at $p<.05$. Statistical analyses were performed using Sigma Stat software (v3.0, SYSTAT, San Jose, CA).

In Studies III and IV, All data was tested for normality and equal variance. Results were compared across all groups using 1 and 2 way ANOVA with Student Newman Keuls (SNK) post hoc testing for normal data of equal variance or Holm-Sidak post hoc testing for non-normal/non equal variance data. Paired t-tests were used to compare the bilateral defects within animals. As there was bone graft in only two groups in study III, a t-test was used to compare the amount of residual graft between the HBO and NBO defects which had been grafted with autogenous bone. Statistical analyses were performed using SigmaStat v3.5 (Systat Software San Jose CA). Statistical significance was considered to be at $p<.05$.

5 Results

5.1 *Critical-sized and Supracritical-sized Defects and Hyperbaric Oxygen Exposure*

The Normobaric Room Air Oxygen (NBO) group defects were uniformly occupied by a thin membrane of flexible tissue. No dehiscence was noted. The junction between this thin flexible membrane and the native bone was clearly demarcated by palpation. The dural and the pericranial layers were intact. Appreciable difference was noted in the size of the 15 and 18 mm defects as expected.

The Hyperbaric Oxygen (HBO) group defects were occupied by firmer tissues that resemble the hardness of bone. The junction between native bone and the defect contents were not palpable. The dural and the pericranial layers demonstrated similar appearances as the NBO counterparts

The radiographs of the NBO defects showed a well demarcated radiolucency with noncorticated borders (Figure 9-A). Very small areas of radiopacities were often seen within 2 mm from the defect margin, which indicated modest bone formation within 1-2 mm of the defect margin. No difference was grossly noted between the critical and the supracritical-sized defects. HBO defects showed islands of radiopacities around the defect margin extending more than 5 mm toward the center of the defect in the 6 week samples, while in the 12 week samples, radiopacities almost approached the center of the defect (Figure 9-B). These islands presented with variable densities. Defect margins were still identifiable.

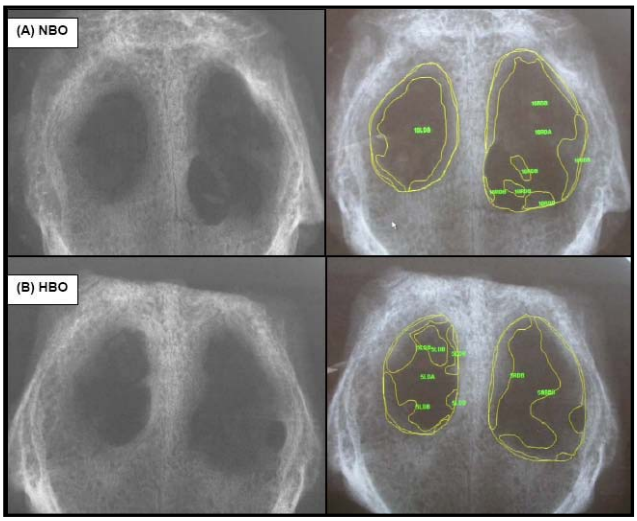

Figure 9: Postsacrifice radiographs of the parietal bones of A, Normobaric (NBO) group animal and B, HBO treated group animal. Tracing of apparent radiopacities indicates bone formation within defects.

Histologically, NBO defects were predominantly filled with fibrous tissue that contained an occasional blood vessel. Defect margins were readily identified (Figure 10-A). New bone was mostly restricted to the defect margins although small islands of woven bone were occasionally seen in the fibrous tissue more centrally. The HBO defects were completely bridged with bony tissue (Figure 10-B). This bridge consisted of newly formed cortical bone with vascular marrow. Defect margins were readily identified.

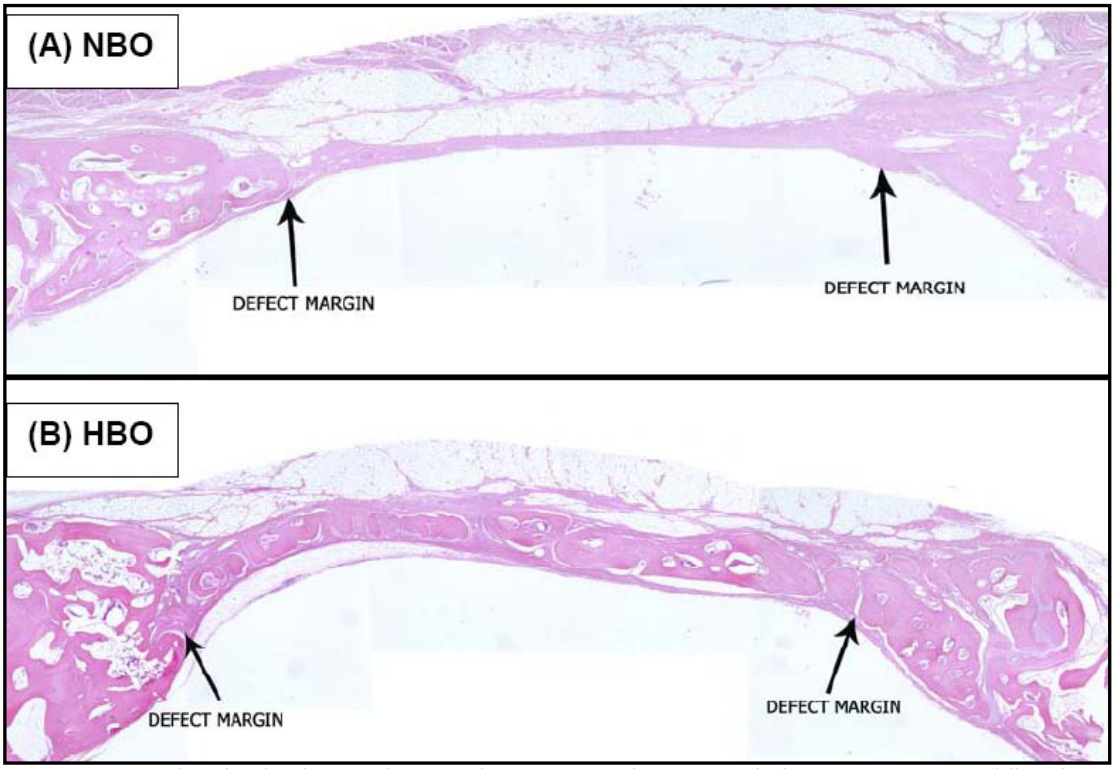

Figure 10: Histological Sections of HBO and NBO defects. 4X magnification hematoxylin and eosin stained sections. A, A normobaric (NBO) control group specimen healed primarily with fibrous band of tissue. B, An HBO treated animal sample showing bony healing of the defect.

(HBO = hyperbaric oxygen, NBO = normobaric room air oxygen).

5.2 *VEGF Expression and Hyperbaric Oxygen Exposure*

5.2.1 Gross appearance and histological evaluation

Gross analysis of the post-mortem defect size showed the defects of the HBO treated groups to be smaller than those of the NBO groups with complete union at 12 weeks in all subjects of the HBO therapy group. Histological analysis revealed that healing of the defects in the NBO group was mainly by scar formation with only a few bony islands scattered along the defect margins. By comparison, the defects from the HBO-treated animals contained significant amounts of bone and marrow that completely bridged the defects by 12 weeks. The bone in the 6-week HBO defects was predominantly woven, but by 12 weeks it tended to be more lamellar in nature (Figure 11).

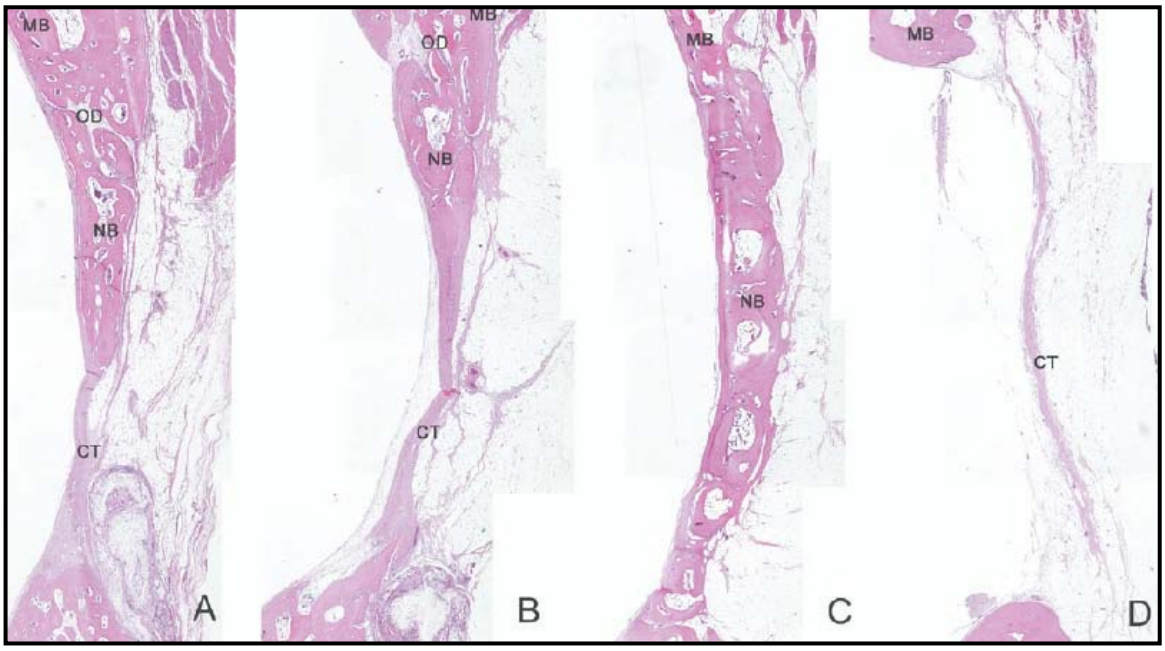

Figure 11. Histological appearance of HBO and NBO defects at 6 and 12 weeks. A, HBO 6-weeks group. A significant amount of new bone is seen within the defect. B, NBO 6 weeks group. Although new bone is present within the defect, it is much less than in the group treated with HBO. C, HBO 12-weeks group. The entire defect is bridged with new bone. D, NBO 12-weeks group. The defects were filled predominantly with dense fibrous tissue. CT, connective tissue; MB, mature bone; NB, new bone; OD, margin of original defect. (Hematoxylin-eosin stain; magnification x4.)

5.2.2 Vascular Endothelial Growth Factor (VEGF) staining

VEGF expression in all groups occurred throughout the fibrous tissue and marrow, with more intense staining often seen near bone. When the extent of VEGF staining was compared between the 15-mm and 18-mm defects, no differences were detected in any group (Table 2) or when considering all the samples as a whole. Consequently, the results for the two defects in each animal were averaged to provide one result per animal for further analysis.

Table 2: Comparison of VEGF staining between 15- and 18-mm defects.

Sacrifice time	6 WEEKS				12 WEEKS			
Oxygen	HBO		NBO		HBO		NBO	
Defect Size	15 mm	18 mm	15 mm	18 mm	15 mm	18 mm	HBO	NBO
Mean VEGF Expression	413	571	222	250	167	104	163	145
+/- SD	171	474	112	137	140	40	71	47
p value (15 vs 18 mm)	.430		.141		.371		.651	

All values are group means +/- SD of the subject means of stained area (μm2) within a field of view. Each subject mean represented the average of 6 fields per slide, 3 slides per defect. Each rabbit had one 15-mm and one 18-mm defect. *p* values were calculated using the paired t test.

HBO, hyperbaric oxygen; NBO, normobaric oxygen; VEGF, vascular endothelial growth factor.

The means and standard deviations for the area stained by VEGF for each group are reported in Table 3 and displayed in Figures 12,13 and 14. Comparison of the areas stained for VEGF between the HBO and NBO groups 6 weeks post-surgery showed that there was a significantly greater area staining for VEGF in the samples from HBO-treated rabbits ($p = .012$). Comparison of VEGF staining between the animals sacrificed at 6 and 12 weeks postsurgery showed that there was no difference between the two NBO groups; however, there was a significant decline in VEGF staining between the 6- and 12-week HBO samples ($p = .008$).

The 12-week HBO and NBO samples had similar amounts of VEGF staining (Fig. 5). We also investigated whether there was any detectable difference in the amount of VEGF staining between the center and margins of the defect in the 6-week samples. However, no differences were detected.

Table 3. Comparison of VEGF staining between HBO and NBO groups.

	Sacrifice time	6 WEEKS	12 WEEKS	*p* value (6 vs 12 weeks)
Mean VEGF Expression	HBO	520 +/- 287	147 +/- 38	.008
	NBO	240 +/- 121	149 +/- 76	.630
p value (HBO vs NBO)		.012	.987	

All values are group means +/- SD of the subject means of stained area (μm2) within a field of view. Each subject mean represented the average of 6 fields per slide, 3 slides per defect, 2 defects per subject (36 fields measured per subject). Results of the 15- and 18-mm defects were combined in this analysis. P values were determined using the Student-Newman-Keuls post hoc test following analysis of variance.

HBO, hyperbaric oxygen; NBO, normobaric oxygen; VEGF, vascular endothelial growth factor.

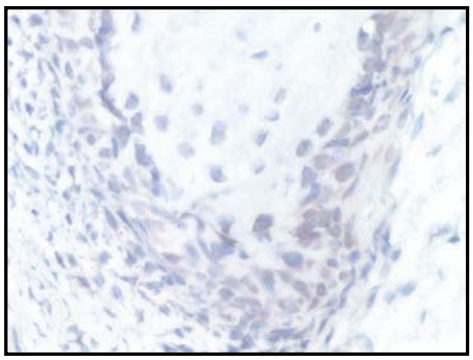

Figure 12. VEGF-stained HBO-treated defects at 6 weeks. Rabbits had been exposed to HBO therapy (90 minutes at 2.4 atmospheres with 100% oxygen) 5 days a week for 4 weeks. Cells stained for VEGF were seen throughout the defect. However the most intense VEGF staining was seen near areas of bone.
(Magnification x200.)

Figure 13. VEGF-stained NBO defects at 6 weeks. Only low levels of VEGF staining were seen throughout the defect. Very few cells were seen that stained strongly.
(Magnification x200)

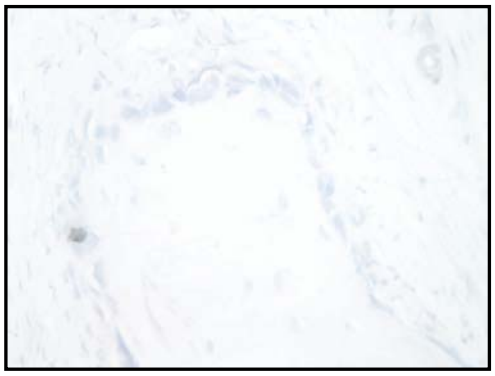

Figure 14. VEGF-stained HBO defects at 12 weeks. Limited VEGF staining was observed, even near areas of bone.
(Magnification x200.)

5.3 *Autogenous Bone Grafts and Hyperbaric Oxygen Exposure*

5.3.1 Non-Grafted Defects

The Non-Grafted defects were uniformly occupied by a thin membrane of flexible tissue. No dehiscence was noted. The junction between this thin flexible membrane and the native bone was clearly demarcated by palpation. The dural and the pericranial layers were intact. The HBO Non-Grafted defects demonstrated similar gross morphological features to their Non-Grafted NBO counterparts.

The radiographs of the NBO Non-Grafted defects showed a well demarcated radiolucency with noncorticated borders. Very small areas of radiopacities were often seen within 2 mm from the defect margin, which indicated modest bone formation within 1-2 mm of the defect margin. The HBO Non-Grafted defects were occupied with a homogeneous radiopaque shadow. This shadow was denser than what appeared in the NBO Non-Grafted defects but still less dense than the grafted defects. Margins of the defects were poorly demarcated.

Upon histological examination, the Non-Grafted NBO defects were predominantly filled with fibrous tissue that contained an occasional blood vessel. Defect margins were readily identified. New bone was mostly restricted to the defect margins although small islands of woven bone were occasionally seen in the fibrous tissue more centrally (Figure 15). On the other hand, it was difficult to distinguish the defect margin in the HBO Non-Grafted treated defects. The defects were completely bridged with new bone, which tended to thin towards the centre of the defects, with the dural and periosteal edges being composed of denser fibrous tissue. The new bone contained marrow elements which were not as extensive as in the grafted defects

5.3.2 Grafted Defects

The NBO grafted defects were uniformly occupied by firmer tissues that resembled the hardness of bone. The junction between native bone and the defect contents were not palpable. The dural and the pericranial layers demonstrated similar appearances as the Non-Grafted counterparts. The HBO grafted defects demonstrated similar gross morphological features to their Grafted NBO counterparts

Radiographically, particles of autogenous bone were clearly visualized within the defects with variable densities (Figure 15). NBO Defects margins were still identifiable. HBO Defects appeared to be more homogenously radiopaque with less demarcated defect borders. Bone graft particles were blended together and less recognizable with more homogenous radiopacity. Non-grafted defect was mostly radiolucent in the NBO group and mostly radiopaque in the HBO group. Nevertheless, grafted defects showed higher degree of radiopacity regardless of the HBO therapy.

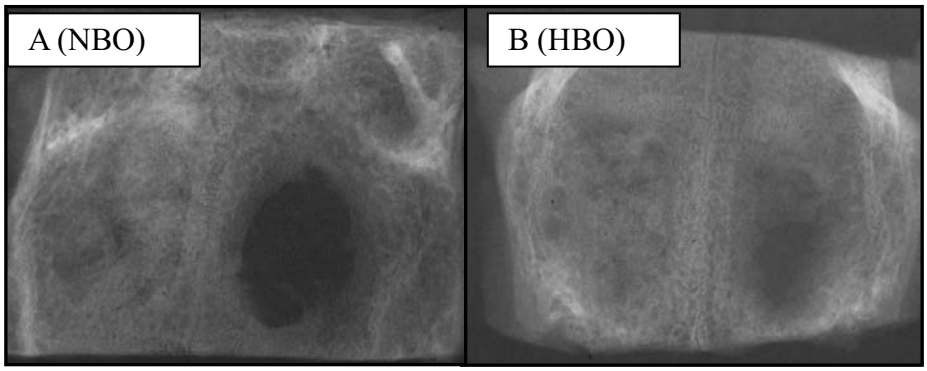

Figure 15: Plain radiographs of study III specimens showing more radiopacities within grafted defects. The HBO non-grafted defect demonstrated denser radiopacity than its NBO counterpart.

HBO, hyperbaric oxygen; NBO, normobaric oxygen

Histologically, the NBO grafted defects were completely bridged with bony tissue. This bridge was a mixture of non-vital cortical bone from the graft, newly formed bone and vascular marrow (Figure 16). Defect margins were readily identified. Meanwhile, margins of the defect were difficult to identify in the HBO grafted defects. These defects were completely bridged with extensive amounts of new bone and marrow. Minimal devitalized remnants of the graft were still visible.

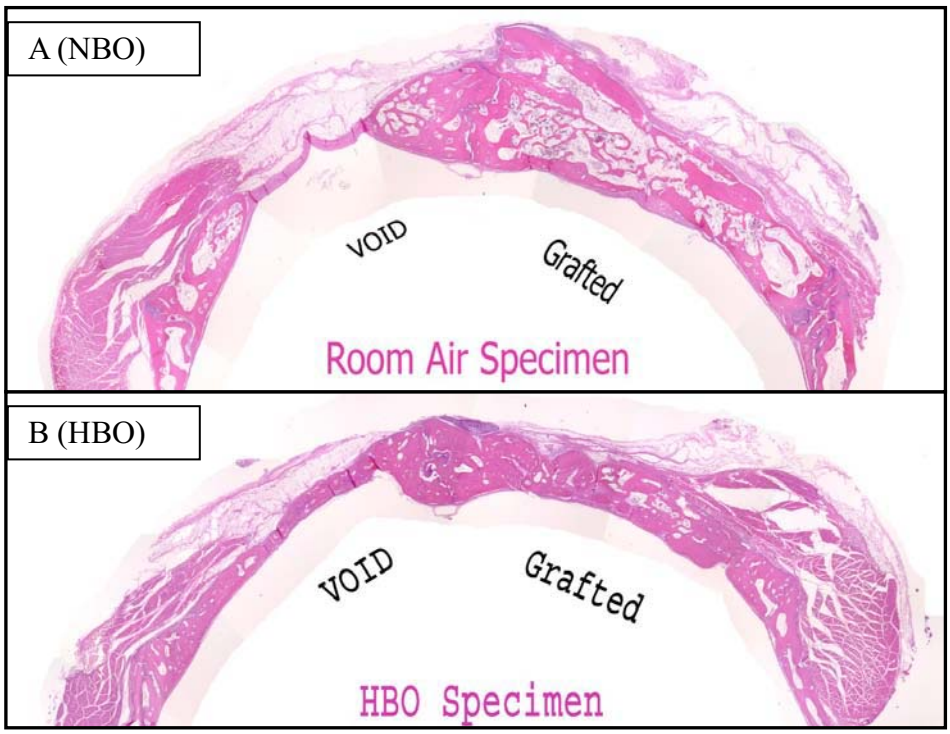

Figure 16: Histological Sections of HBO and NBO grafted and non-grafted defects. 4X magnification hematoxylin and eosin stained sections. A, normobaric (NBO) non-grafted control group specimen healed primarily with fibrous band of tissue. The NBO grafted defects healed with bony bridge. B, An HBO treated animal sample showing bony healing of both the non-grafted and the grafted defects.

HBO, hyperbaric oxygen; NBO, normobaric oxygen

5.4 *Bone Substitutes and Hyperbaric Oxygen Exposure*

5.3.1 Demineralized Bone Matrix

Demineralized bone matrix (DBM) grafted defects were uniformly occupied by firm tissues that resembled the hardness of bone. The junction between native bone and the defect contents were not palpable. The dural and the pericranial layers demonstrated a smooth surface with shadow of residual DBM particles. The HBO-DBM grafted defects demonstrated similar gross morphological features to their NBO-DBM grafted counterparts. No difference was noticed between the DBM gel or putty grafted defects in either the HBO or NBO groups.

Radiographically, evidence of radiopacity was clearly visualized within the defects with variable densities. NBO Defects margins were still identifiable. HBO Defects appeared to be homogenously radiopaque with less demarcated defect borders. DBM particles were blended together and less recognizable with more homogenous radiopacity (Figure 17).

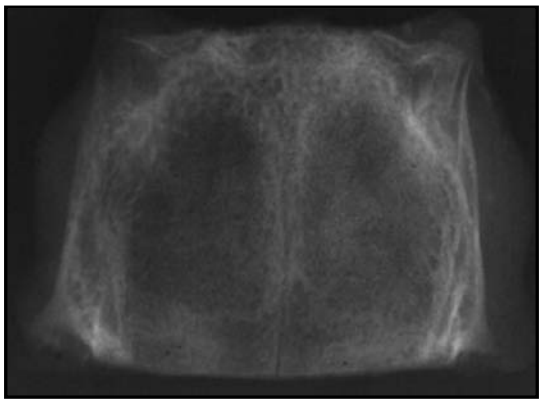

Figure 17: Plain radiograph of a study IV specimen grafted with DBM putty in the left side and DBM gel in the right side. Radiopacities are noted within grafted defects with variable density.

Histologically, the grafted defects were completely bridged with bony tissue. This bridge was a mainly vital corticocancellous bone with minimal areas of non-vital residual bone matrix. Newly formed bone and vascular marrow were observed through out the defect (Figure 18). Defect margins were difficult to identify in the grafted defects.

No difference was noticed between the DBM gel or putty grafted defects in either the HBO or NBO groups.

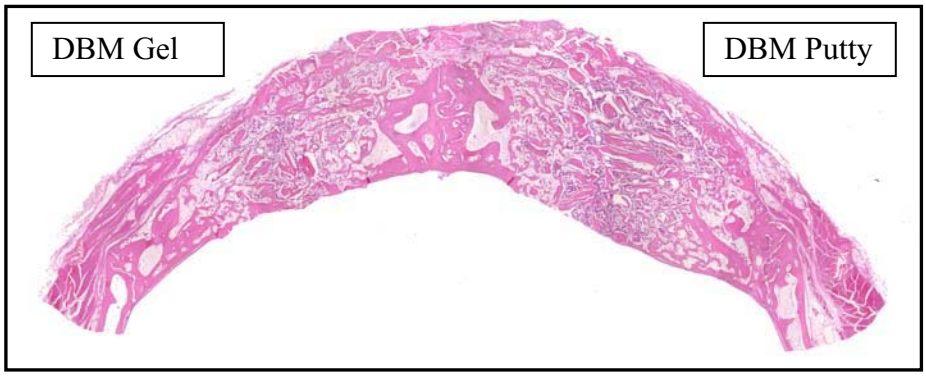

Figure 18: Histological Sections of bilateral parietal bony defects grafted with DBM gel and putty. Bone bridges the entire defect resulting in complete union.
(Hematoxylin and eosin, [H&E], original magnification x4.)

5.3.2 Biphasic Calcium Phosphate

Biphasic Calcium Phosphate (BCP) grafted defects were uniformly occupied by firm tissues that resembled the hardness of bone. The junction between native bone and the defect contents were not palpable. The dural and the pericranial layers demonstrated a smooth surface with shadow of residual BCP particles. The HBO-BCP grafted defects demonstrated similar gross morphological features to their NBO-BCP grafted counterparts.

Radiographically, evidence of radiopacity was clearly visualized within the defects with intense densities. NBO Defects margins were still identifiable. BCP particles were blended together and less recognizable around the defect margins (Figure 19).

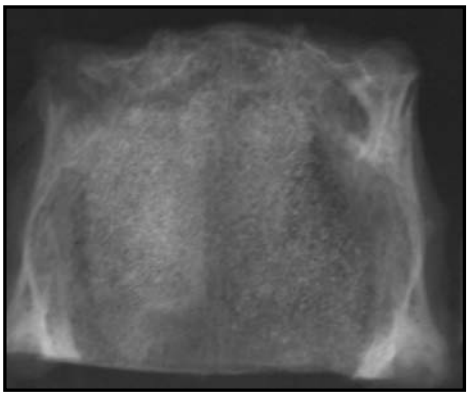

Figure 19: Plain radiograph of a study IV specimen grafted with Biphasic Calcium Phosphate (BCP) in the right side and BCP putty in the left side. Radiopacities are noted within grafted defects with relatively higher density than DBM grafted defects (Figure 17).

Histologically, the grafted defects were completely bridged with bony tissue. This bridge was a mixture of vital corticocancellous bone with significant areas of non-vital residual BCP. Newly formed bone and vascular marrow were observed through out the defect (Figure 20). Defect margins were readily identifiable in the grafted defects.

No difference was noticed between the BCP or BCP putty grafted defects in either the HBO or NBO groups.

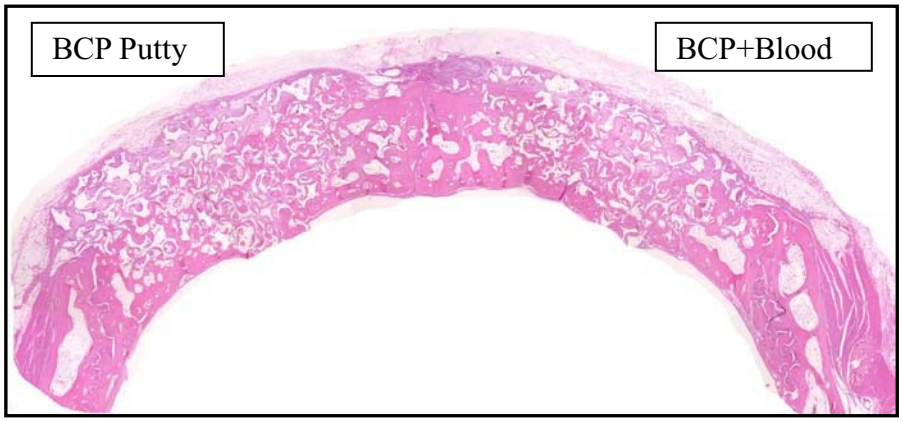

Figure 20: Histological Section of bilateral parietal bony defects grafted with BCP and BCP putty. Because of decalcification, the residual graft has been removed and so appears as voids in the defect. The defect is completely bridged by bone along its dural aspect. New bone and marrow elements are most common at the margins and dural aspect, with the tissue along the periosteal aspect being predominantly fibrous in nature.
(H&E, original magnification of each photomicrograph x4.)

5.5 *Radiomorphometrics, mCT Evaluation, and Histomorphometrics.*

5.5.1 Radiomorphometrics

Radiomorphometrics were utilized in study I. Findings are presented in table 4 and graphically shown in figure 21. More islands of radiopacities in the HBO group compared to the control group in both the 6- and 12-week specimens (p<.001). There were fewer radiopaque foci within the margins of the defects in the control group. In the control group the radiopacities tended to blend with the margins of the defects, whereas in the HBO group more radiopaque areas were evident both along the margins as well as in the center of the defects (Figure 9). No differences were noted between the 15-mm and 18-mm defects (p=.688). The percentage of radiopacities were greater in HBO samples at 12 weeks when compared to those at 6 weeks (p=.019).

Table 4: Percentages of radiopacities at 6 and 12 weeks of Critical-sized (15 mm) and Supracritical-sized (18 mm) defects

Sacrifice time	6 WEEKS				12 WEEKS			
Defect size	15 mm		18 mm		15 mm		18 mm	
Oxygen	HBO	NBO	HBO	NBO	HBO	NBO	HBO	NBO
Percent radiopacities within defects	52.96	38.31	29.73	29.86	79.51	47.29	95.50	20.39
	27.92	29.46	61.34	41.22	65.41	30.85	76.97	23.16
	60.18	45.72	65.25	43.67	95.13	2.10	92.24	39.02
	57.85	47.39	44.22	26.60	90.35	30.50	98.74	54.07
	44.98	35.84	51.55	29.62	67.03	54.67	74.94	65.88
Mean	48.78	39.34	34.19	50.42	79.49	33.08	87.68	40.50
SD	13.03	7.35	14.20	7.69	13.38	20.24	10.97	19.59

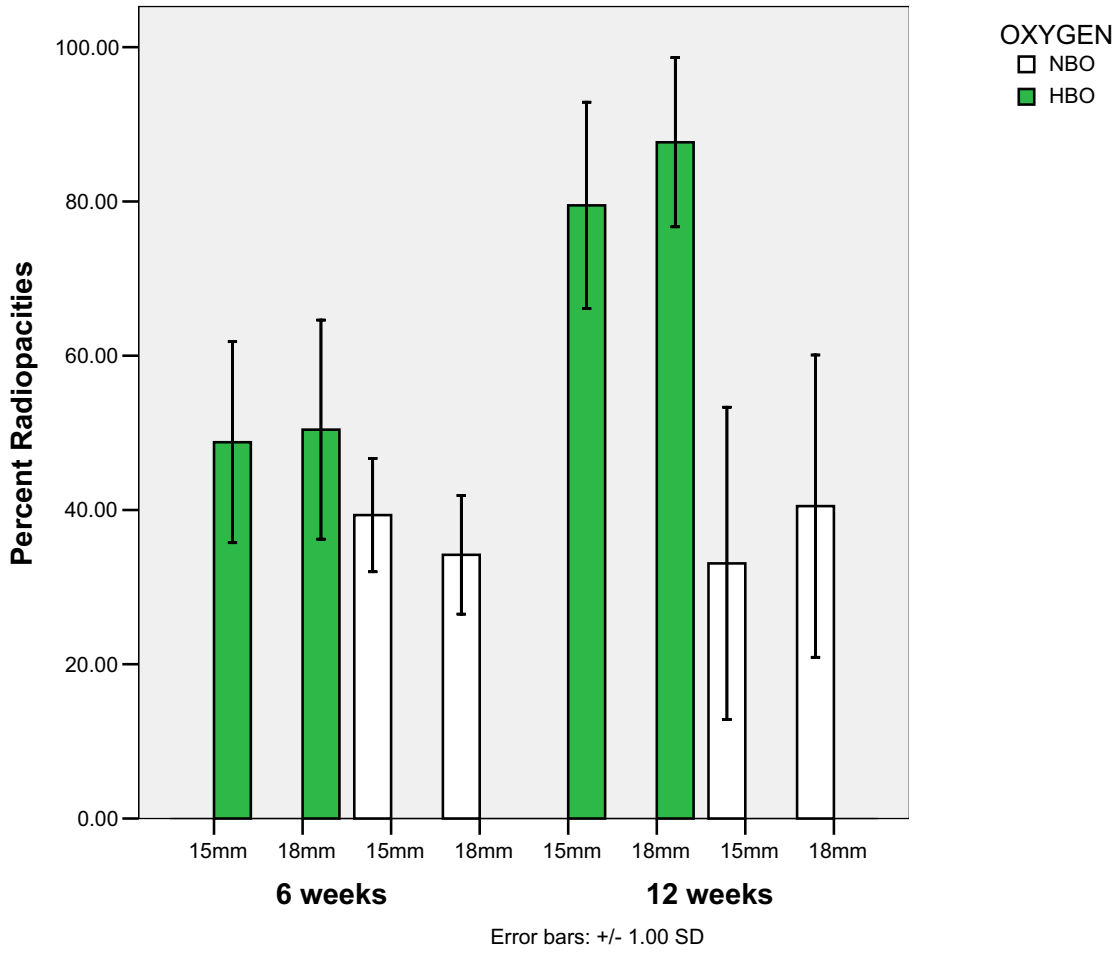

Figure 21: Bar chart, Radiomorphometrics of study II. More mean percent radiopacities within the 15 mm and 18mm defects in the HBO group of phase I study ($p<.001$). More radiopacities were evident at the 12 weeks samples in the HBO group ($p=.019$).

(HBO = hyperbaric oxygen, NBO = normobaric room air oxygen)

Data is plotted as mean±SD.

5.5.2 Quantitative Micro-Computed Tomography (mCT)

Micro-Computed Tomography and bone analysis were utilized in study III and IV.

In study III (Table 5 and Figures 22, 23, 24 and 25) statistical analysis indicated that the presence of the autogenous graft resulted in increased BV, BMC, BMD and BVF compared to Non-Grafted defects under both NBO and HBO ($p<.05$). Comparisons between the HBO and NBO autogenous bone grafted defects revealed that the HBO grafted defects had significantly lower BV ($p=0.03$) and BMC ($p<.05$). However, the reductions in BVF and BMD did not reach significance ($p=0.123$ and 0.078 respectively). No significant differences between the HBO and NBO Non-Grafted defects were seen in any of the parameters measured.

Table 5: Micro CT analysis is demonstrated among the study groups and statistically analyzed using 1-Way ANOVA

	NBO		HBO		1-Way ANOVA
	Non-Grafted	ABG	Non-Grafted	ABG	p value between groups
TV(mm^3)	233±37	263±55	191±27	189±66	0.076
BV(mm^3)	41±7	140±22	39±17	87±36	<0.001
BVF (%)	17.6±2.1	53.9±6.4	20.0±7.3	46.2±11.0	<0.001
BMC (mg)	31.2±6.3	118.0±21.3	32.4±13.0	76.0±27.6	0.002[a]
BMD (mg/mm^3)	134±20	451±25	167±53	403±52	<0.001
TMC (mg)	25.6±4.5	90.3±13.5	23.8±11.3	53.9±24.1	<0.001
TMD (mg/mm^3)	627±27	645±20	602±26	612±32	0.096

[a] Equal variance test failed – *p* calculated using 1 way ANOVA on Ranks

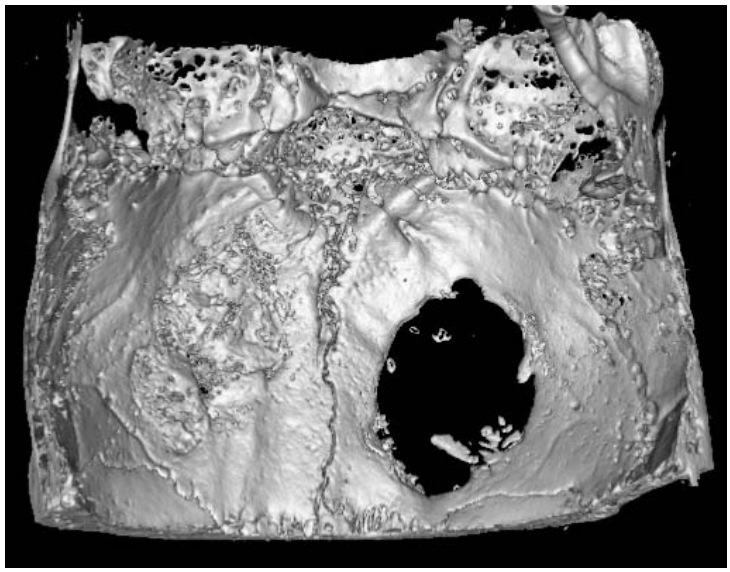

Figure 22 : Micro CT 3D reconstruct showing the cranial side of the parietal bone defects. The right side is left to heal without grafting and demonstrates minimal bone formation at the edges at 6 weeks. The left side id grafted with autogenous bone graft and found to be completely bridged with bone.

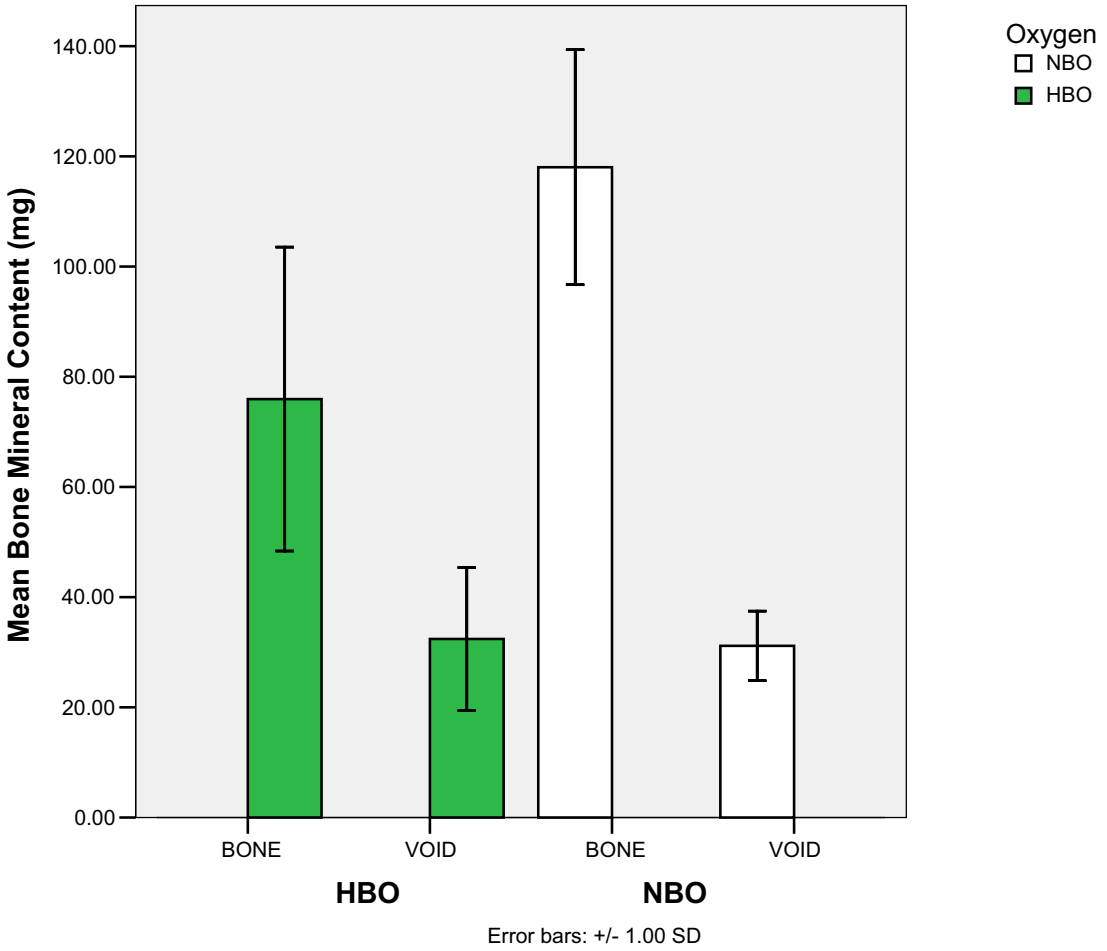

Figure 23: Bar Chart, Micro CT Results. Bone Mineral Content (BMC). The defects containing autogenous bone grafts have significantly higher BMC than the non-grafted defects ($p<.05$). Comparisons between the HBO and NBO autogenous bone grafted defects revealed that the HBO grafted defects had significantly lower BMC ($p<.05$).
Data is plotted as mean±SD.

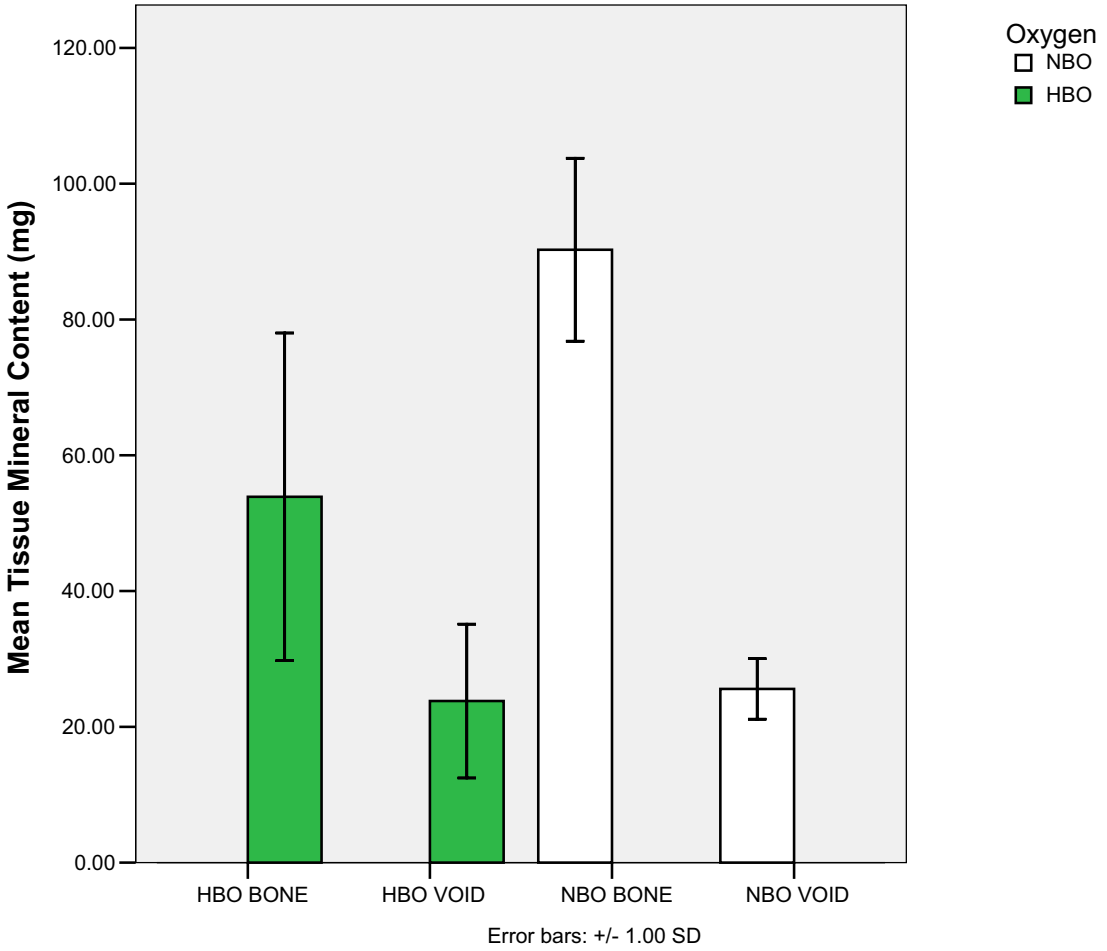

Figure 24: Bar Chart, Micro CT Results. Tissue Mineral Content (TMC). The defects containing autogenous bone grafts have significantly higher TMC than the non-grafted defects ($p<.05$). Comparisons between the HBO and NBO autogenous bone grafted defects revealed that the HBO grafted defects had significantly lower TMC ($p=.002$)

Data is plotted as mean±SD.

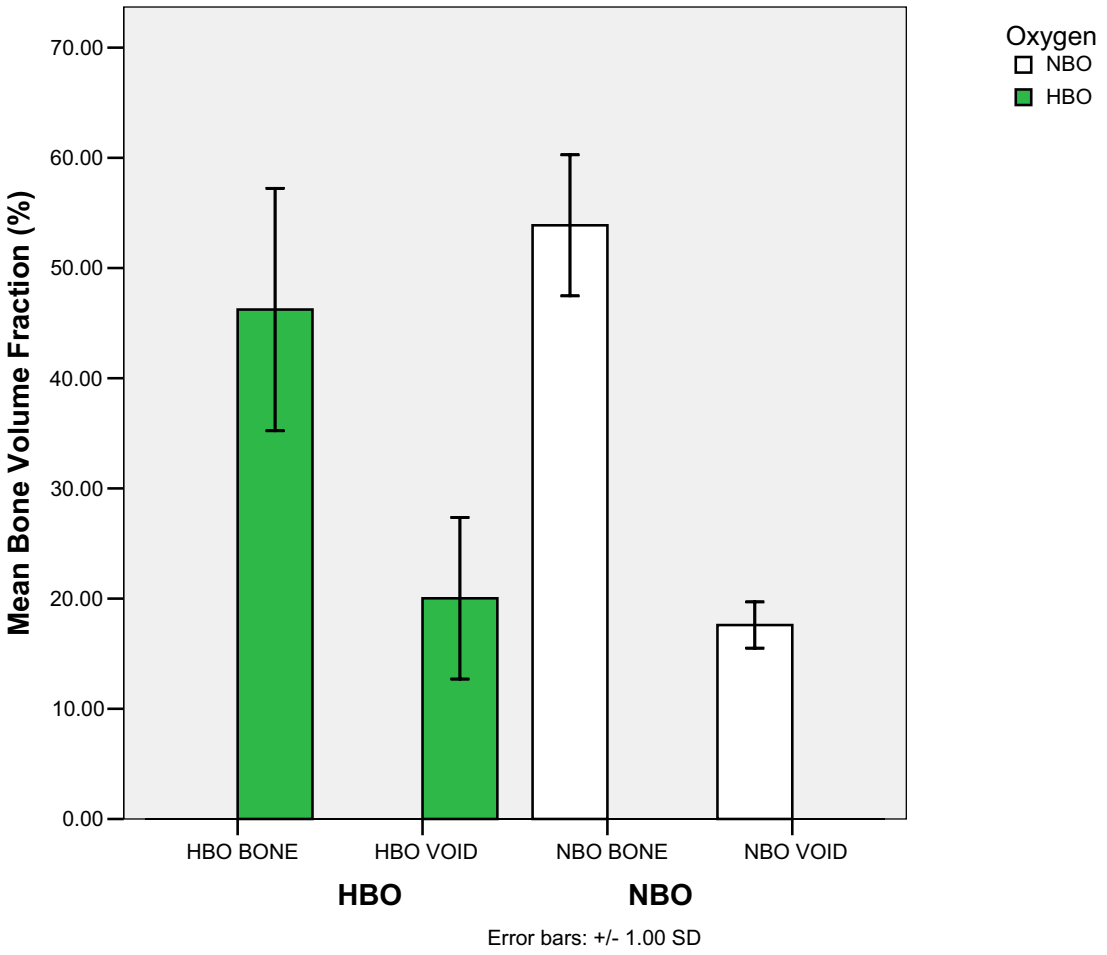

Figure 25: Bar Chart, Micro CT Results. Bone Volume Fraction (BVF). The defects containing autogenous bone grafts have significantly higher BVF than the non-grafted defects ($p<.05$). Comparisons between the HBO and NBO autogenous bone grafted defects revealed reductions in BVF in HBO grafted defects, however, this did not reach statistical significance ($p=0.123$). No significant differences between the HBO and NBO Non-Grafted defects were seen in this parameter.

Data is plotted as mean±SD.

In Study IV (Table 6 and Figures 26 and 27), All parameters were examined for significant differences between the different groups using ANOVA (Table 6). Four different experimental conditions were compared in this study: BCP versus DBM, HBO versus NBO, and two different formulation comparisons of blood versus F127 putty within the BCP group and F127 gel versus F127 putty within the DBM group.

Table 6. MicroCT analysis of study IV specimens

	DBM				BCP				ANOVA *p*
	GEL		PUTTY		BLOOD		PUTTY		
	NBO	HBO	NBO	HBO	NBO	HBO	NBO	HBO	
Vol (mm3)	186±103	161±42	177±95	179±18	162±56	180±30	143±14	128±28	.096
BV (mm3)	67± 36	62±8	64± 36	65±10	103±33	111±29	91±9	72± 21	.017
BVF (%)	35±7	40±7	37±8	34±7	64±4	61±6	64±3	56±8	>.001
BMC (mg)	46± 23	38±7	41± 22	41±5	110±36	116±27	84±7	66±19	>.001*
TMC (mg)	29± 16	25±4	27±16	26±5	107±35	113±29	80± 7	61±19	>.001*
BMD (mg/mL)	223±34	244±28	232±36	233±27	682±43	641±81	594±52	512±72	>.001
TMD (mg/mL) 416	416 ± 30	410 ± 20	410±4	401±11	563±23	547±21	530±23	506±10	>.001

DBM, demineralized bone matrix; BCP, biphasic calcium phosphate; None, the BCP granules were mixed with blood; GEL, the DBM granules were mixed with Pluronic F127 at 40% DBM to 60% F127 (vol/vol); PUTTY, the DBM or BCP granules were mixed with F127 at 70% DBM/BCP to 30% F127 (vol/vol); NBO, normobaric oxygen; HBO, hyperbaric oxygen; BV, bone volume; BVF, bone volume fraction; BMC, bone mineral content; TMC, tissue mineral content; BMD, bone mineral density; TMD, tissue mineral density.

*Data were not normally distributed and groups were compared using analysis of variance (ANOVA) on Ranks.

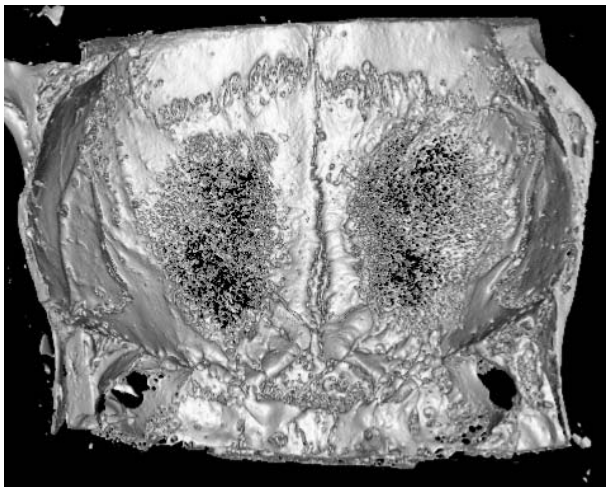

Figure 26: Micro CT 3D reconstruct showing the cranial side of the parietal bone defects grafted with Demineralized Bone Matrix. Both sides show complete bridging with bone formation across the defect with reduced density.

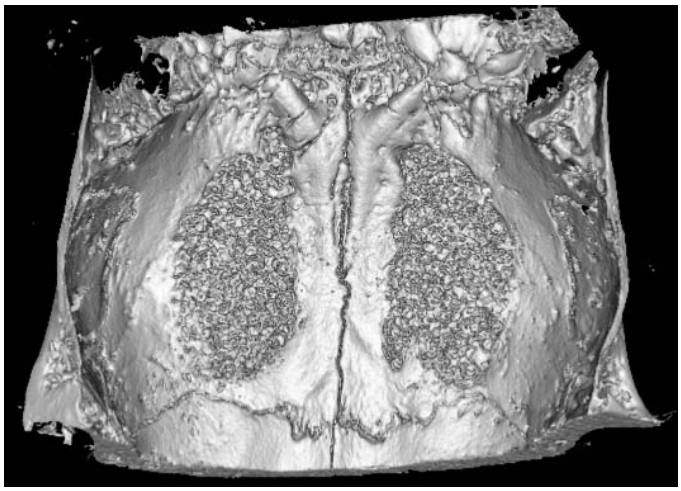

Figure 27: Micro CT 3D reconstruct showing the cranial side of the parietal bone defects grafted with Biphasic Calcium Phosphate. Both sides show complete bridging with bone formation across the defect with greater density.

Following a significant ANOVA result, the matched pairs that were exposed to these experimental conditions were tested for significant differences using a post hoc test. For comparisons of BCP and DBM, the NBO-BCP-putty was compared with the NBO-DBM-putty and the HBO-BCP-putty was compared with the HBO-DBM putty (Table 7).

Table 7. Post hoc P values for mCT parameters by matched groups of study IV.

	Post hoc *p* values					
	BV[1]	BVF	BMC[2]	TMC[2]	BMD	TMD
BCP vs DBM						
Putty-HBO	.638	>.001	>.05	>.05	>.001	>.001
Putty-NBO	.098	>.001	>.05	>.05	>.001	>.001
Blood vs F127 putty						
BCP-HBO	.024	.294	>.05	>.05	>.001	.012
BCP-NBO	.459	.991	>.05	NSD	.026	.045
F127 gel vs putty						
DBM-HBO	.718	.718	NSD	NSD	.729	.506
DBM-NBO	.522	.829	NSD	NSD	.775	.693
NBO vs HBO						
BCP-blood	.661	.671	NSD	NSD	.206	.232
BCP-putty	.249	.152	>.05	>.05	.015	.087
DBM-gel	.661	.611	NSD	NSD	.905	.233
DBM-putty	.896	.896	NSD	NSD	.968	.087

Following a significant result in the 1-way ANOVA, the matchedvgroups within each of the four different experimental conditions (BCP vs DBM, HBO vs NBO and two different formulation comparisons blood versus F127 putty with the BCP group and F127 gel versus F127 putty within the DBM group) were tested for significant differences using a post hoc test.

ANOVA, analysis of variance; BV, bone volume; BVF, bone volume fraction; BMC, bone mineral content; TMC, tissue mineral content; BMD, bone mineral density; TMD, tissue mineral density; BCP, biphasic calcium phosphate; DBM, demineralized bone matrix; NBO, normobaric oxygen; HBO, hyperbaric oxygen; NSD, not significantly different.

[1] Following a significant ANOVA test, none of the group comparisons for the BV were found to be significant by Student-Newman-Keuls post hoc testing. We therefore used Fisher's least significant difference (LSD) method to identify differences.

[2] *p* values were not obtained from post hoc testing of non-normal data and are reported as >.05 if they were found to be significantly different, or NSD if there were not.

ANOVA of the mCT results demonstrated significant differences between the groups for all parameters except total volume (Table 6). Identification of the groups that had significantly different bone volumes (BV) required use of the Fisher least significant differences (LSD) post hoc testing, as SNK was unable to do so. The bone volume was higher in the defects grafted with BCP mixed with blood compared with those mixed with F127 when exposed to HBO ($p<.024$) but not for the matching grafts under NBO. No other significant differences were seen in this parameter between the various matched groups. Defects containing biphasic calcium phosphate had significantly higher BVF, BMC, TMC, BMD, and TMD than those containing demineralized bone matrix under the same experimental conditions (ie, NBO-putty and HBO-putty) ($p<.05$).

5.5.3 Histomorphometrics

Histomorphometrics were utilized in studies I, III and IV.

In Study I (Table 8 and Figure 28), histomorphometric analysis demonstrated more bone formation in the HBO group when compared to the control group ($p<.001$). Both critical sized (15 mm) and supracritical sized (18-mm) defects healed with significantly more bone in the HBO group when compared with the control group. There was no significant difference between the percentage of new bone formed in the 15 mm and the 18 mm defects ($p=.520$), nor between the 6-week and 12-week groups ($p=.309$).

Table 8: Percentages of new bone formation at 6 and 12 weeks of Critical-sized (15 mm) and Supracritical-sized (18 mm) defects

Sacrifice time	6 WEEKS				12 WEEKS			
Defect size	15 mm		18 mm		15 mm		18 mm	
Oxygen	HBO	NBO	HBO	NBO	HBO	NBO	HBO	NBO
Percent new bone formation	64.22	20.63	74.89	30.23	64.02	18.68	58.56	6.84
	49.60	22.77	43.36	45.33	58.06	10.07	52.58	20.03
	67.22	33.26	75.77	46.55	46.67	14.53	57.35	19.90
	53.94	22.64	49.34	24.48	55.39	10.02	50.46	24.16
	47.73	20.01	61.04	22.75	56.67	17.61	39.25	42.26
Mean	56.54	23.86	60.88	33.87	56.16	21.46	51.64	26.83
SD	8.74	5.31	14.65	11.37	6.25	18.20	7.67	15.36

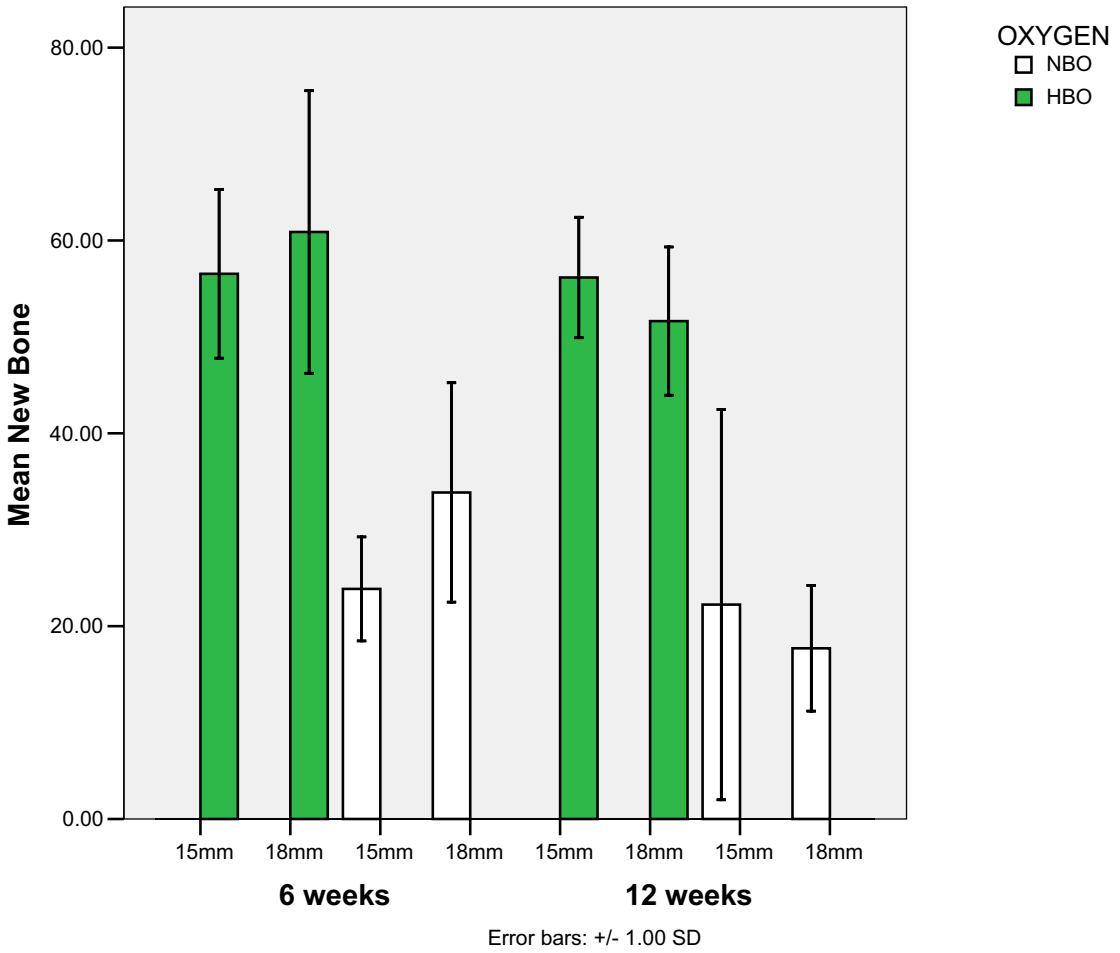

Figure28: Bar chart, Histomorphometrics New bone formation in study I specimens. Differences in the means of new bone formation based on histomorphometric measurements in the study groups are demonstrated. More new bone was evident in the HBO treated defects ($p<.001$).

(HBO = hyperbaric oxygen, NBO = normobaric room air oxygen).
Data is plotted as mean±SD.

In study III (Table 9, Figures 29, 30, 21, 32, and 33), histomorphometric analysis demonstrated that HBO treated Non-Grafted defects had significantly more new bone ($p<.001$) and marrow ($p<.05$) and less fibrous tissue ($p<.05$) than Non-Grafted defects exposed to normobaric air. Defects grafted with autogenous bone showed no statistical differences in any of the parameters measured. Lesser amount of residual graft was present in the HBO compared to HBO defects neared significance ($p=0.085$) (Figure 32). As expected in the normobaric defects, there was significantly more new bone and marrow and less fibrous tissue in the grafted defects ($p<.05$). However comparing the Non-Grafted and autogenous bone grafted defects in the HBO treated animals there was no significant difference in the amount of new bone ($p=0.196$), although there was less marrow and more fibrous tissue in the Non-Grafted defects ($p<.05$).

Table 9: Histomorphometric Analysis is demonstrated among the study III groups and statistically analyzed using 1-Way ANOVA

	NBO		HBO		1-Way ANOVA
Type of Tissue (%)	Non-Grafted	ABG	Non-Grafted	ABG	*p* value between groups
New Bone	19.7±2.6	36.6±8.6	46.7±5.3	41.4±6.9	<0.001
Marrow	6.3±3.6	37.8±9.1	26.7±9.0	38.7±5.7	0.004[a]
B+M	26.0±2.7	74.4±8.1	73.5±12.5	80.1±5.5	0.008[b]
Fibrous	74.0±2.7	6.4±1.8	26.5±12.5	8.8±6.1	<0.001[b]
Graft	N/A	19.1±7.7	N/A	11.2±4.7	0.085[c]

[a] Data not normal, p value generated by 1-way ANOVA on Ranks
[b] Data not equal variance, *p* value generated by 1-way ANOVA on Ranks
[c] *p* value for residual graft was determined by T-test.

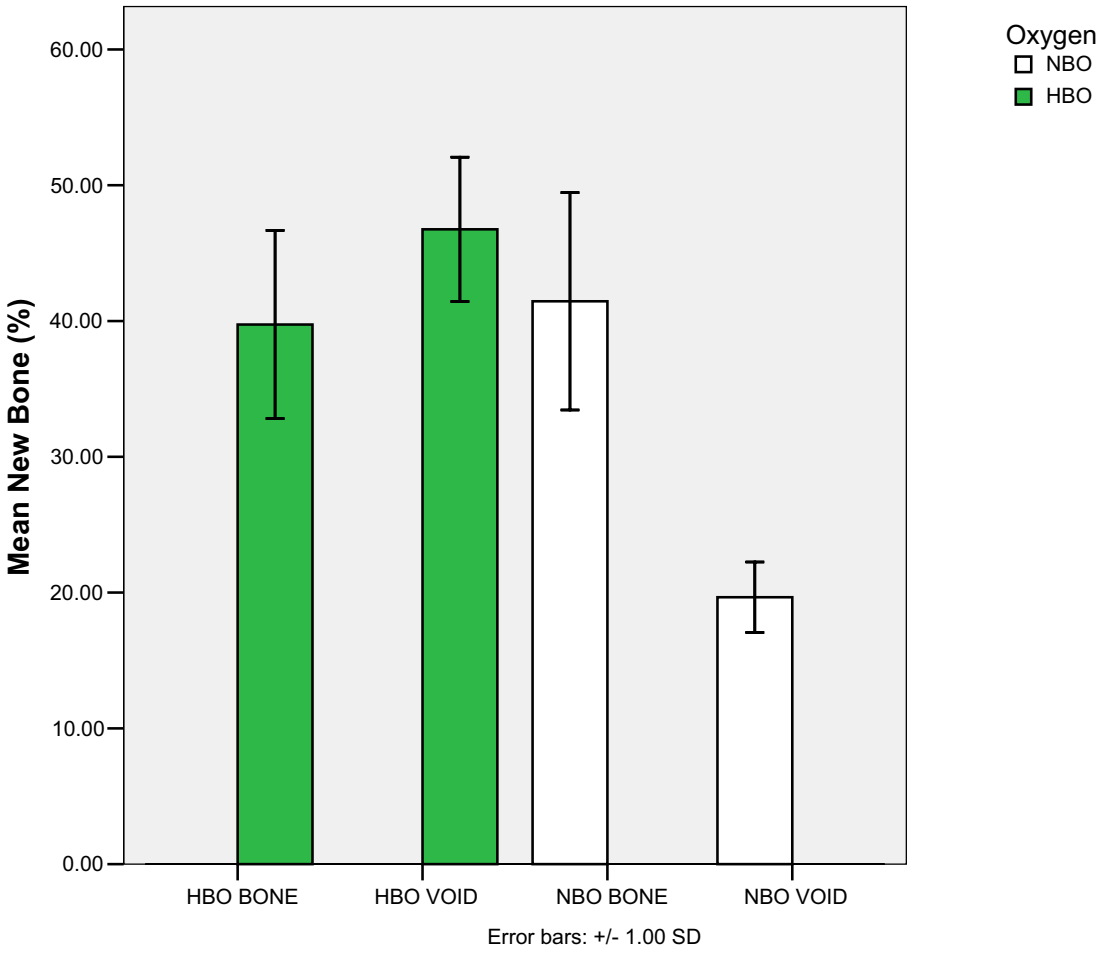

Figure 29: Bar Chart, Histomorphometry, New Bone Formation in study III specimens. HBO treated Non-Grafted defects had significantly more new bone ($p<.001$) than Non-Grafted defects exposed to normobaric air. Comparing the Non-Grafted and Autogenous Bone Grafted defects in the HBO treated animals there was no significant difference in the amount of new bone ($p=0.196$).

Data is plotted as mean±SD

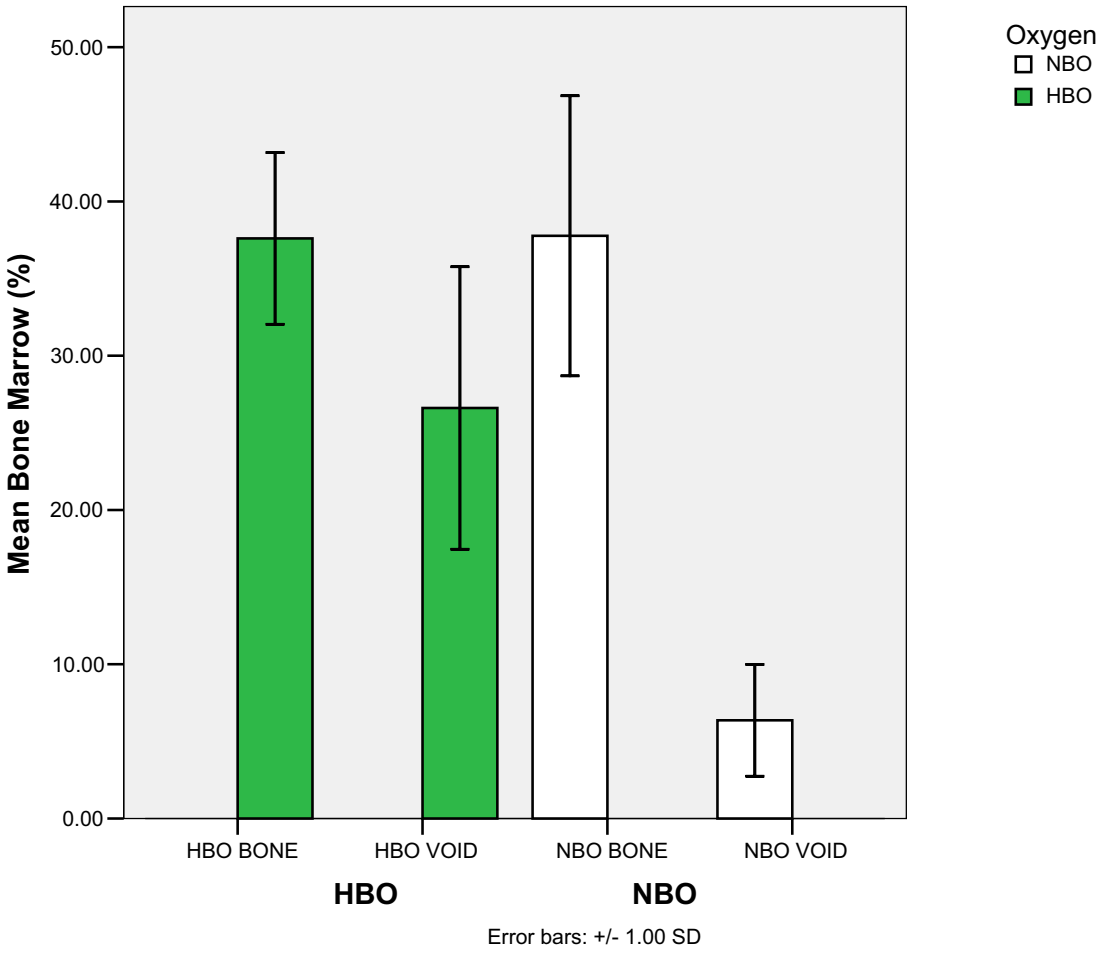

Figure 30: Bar Chart, Histomorphometry, Bone Marrow Formation in study III specimens. HBO treated Non-Grafted defects had significantly more bone marrow ($p<.05$) than Non-Grafted defects exposed to normobaric air. When comparing the Non-Grafted and autogenous bone grafted defects in the HBO treated animals, there was less marrow in the Non-Grafted defects ($p<.05$).

Data is plotted as mean±SD.

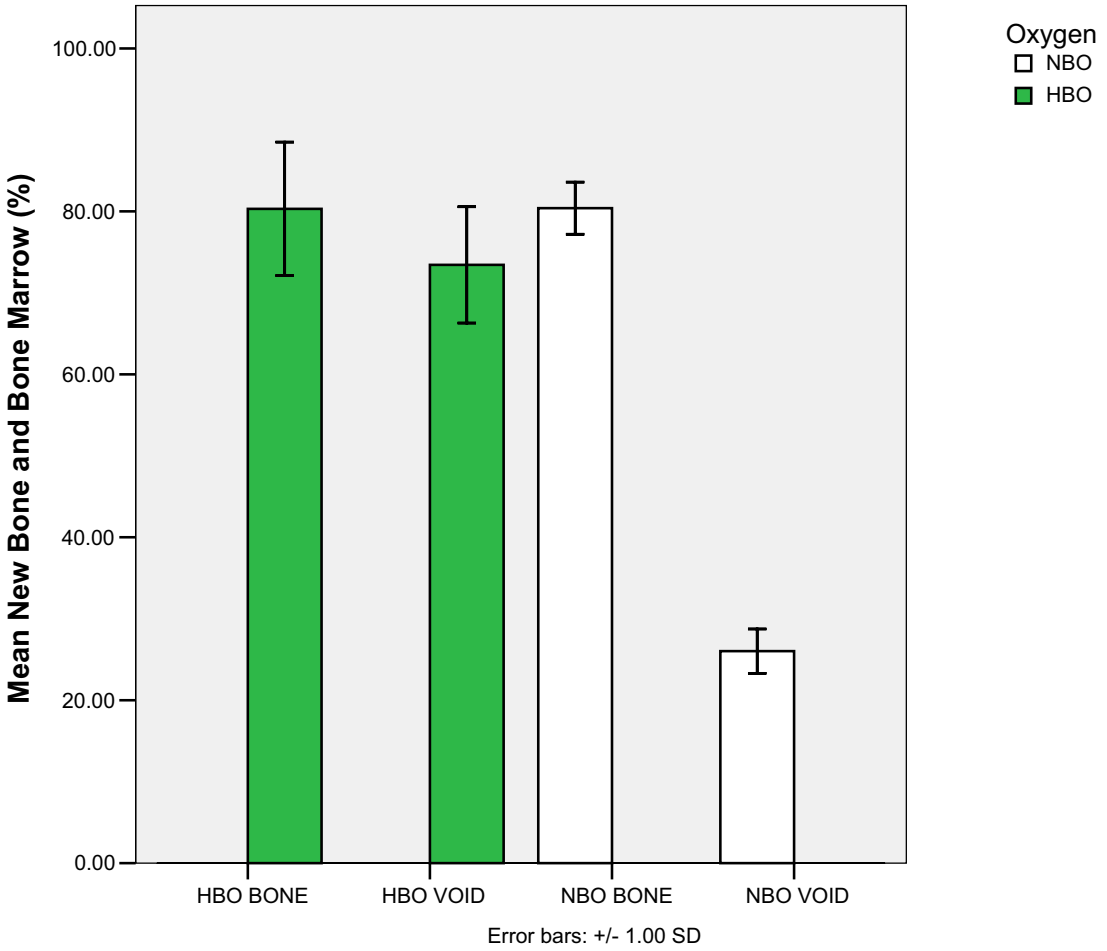

Figure 31: Bar Chart, Histomorphometry, New Bone Formation and New Bone Marrow Formation Combined in study III specimens. Significantly less new bone and marrow were observed in the void defects exposed to normobaric air when compared to the other 3 types of defects (p=0.008). HBO void defects and grafted defects formed comparable amounts of new bone and new marrow.

Data is plotted as mean±SD.

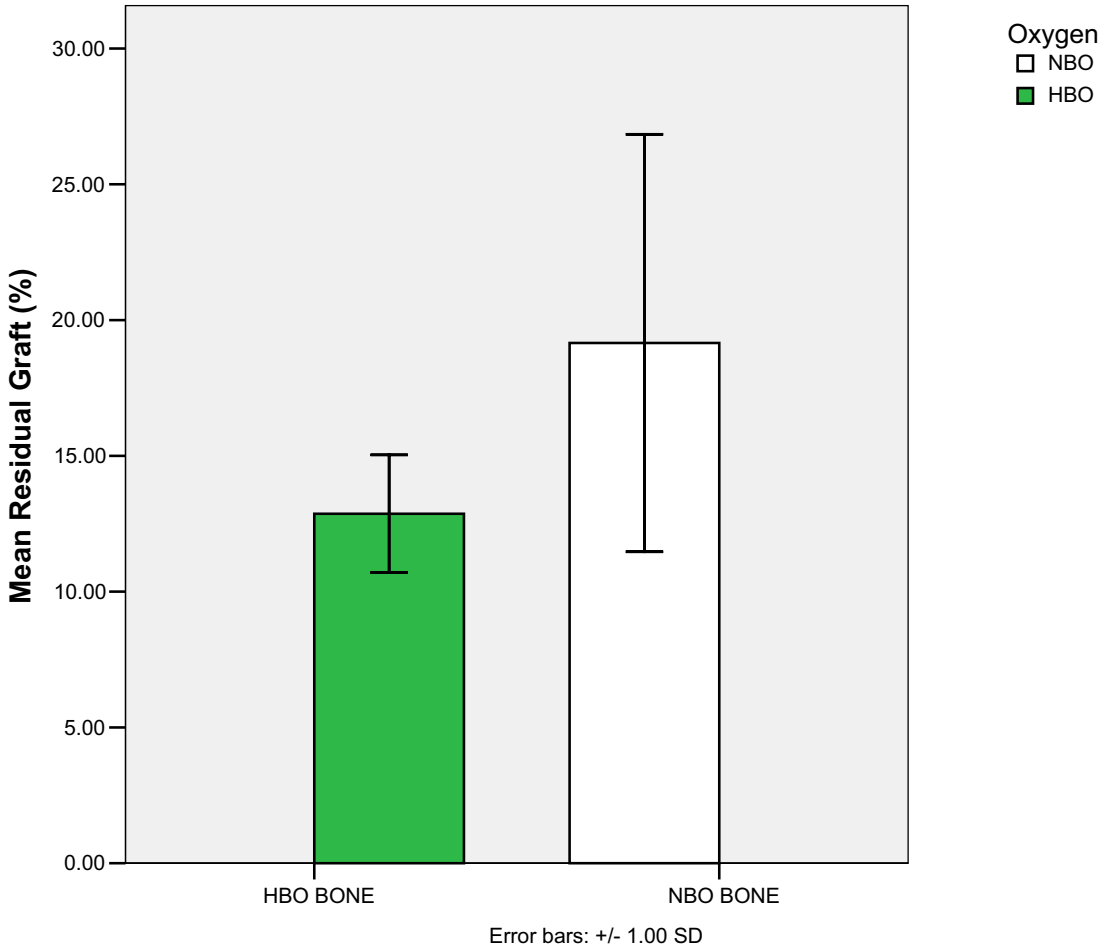

Figure 32: Bar Chart, Histomorphometry, Residual Graft in study III specimens. The lesser amount of residual graft present in the HBO compared to NBO defects neared significance (p=0.085).

Data is plotted as mean±SD.

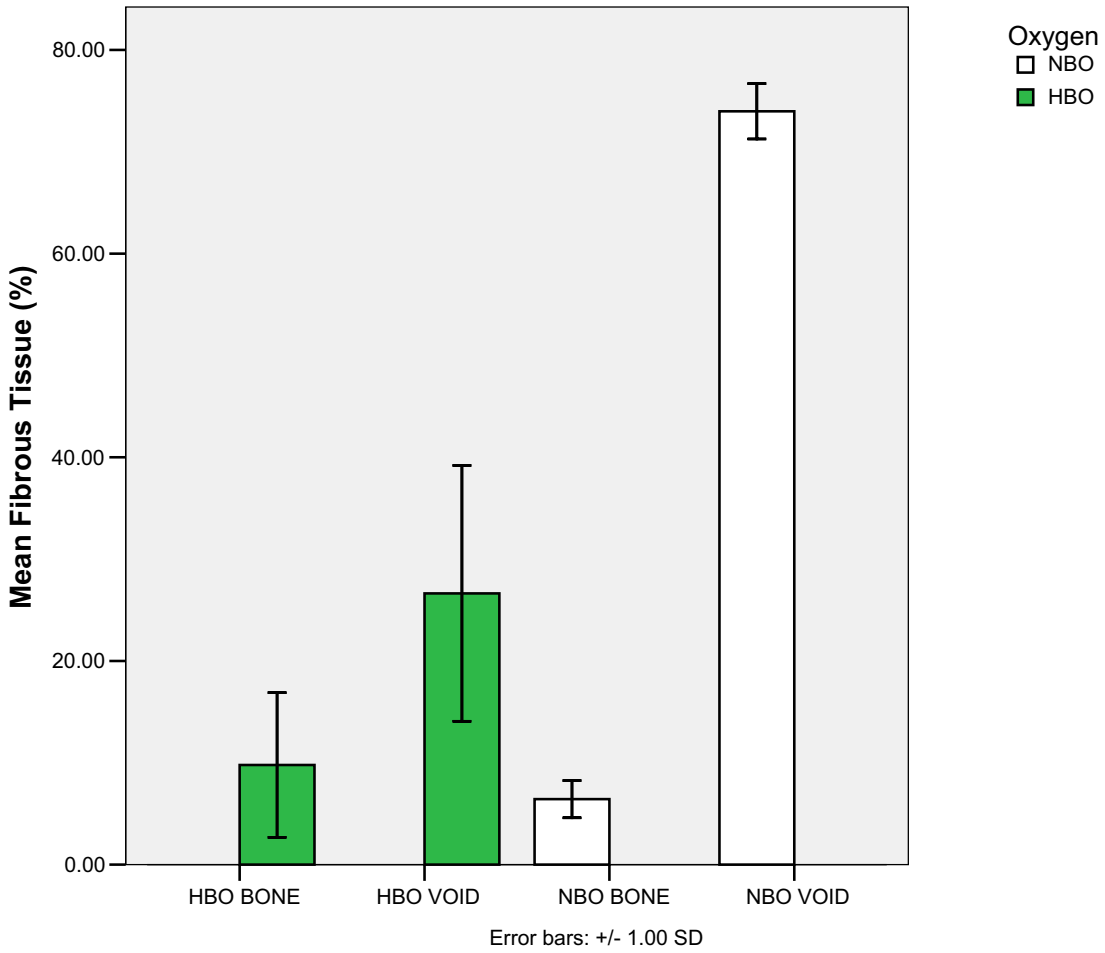

Figure 33: Bar Chart, Histomorphometry, Fibrous Tissue Formation in study III specimens. Significantly less fibrous tissue ($p<.05$) was detected in the HBO treated defects. Void defects exposed to normobaric air healed mostly with fibrous tissue.

Data is plotted as mean±SD.

In study IV (Table 10), histomorphometric analysis demonstrated that DBM-filled defects had significantly more new bone ($p<.008$) and less residual graft ($p<.001$) than matching BCP-filled defects. Although DBM-filled defects exposed to NBO also had increased marrow and reduced fibrous tissue ($p<.001$ for both), when the BCP defects had been exposed to HBO these differences were abolished. These results also correlated with an observed increase in marrow and reduction in fibrous tissue in BCP defects exposed to HBO compared with BCP-grafted defects under NBO conditions. HBO was also seen to lead to a small but significant increase in the amount of new bone in the DBM-grafted defects ($p<.04$). Both marrow and fibrous tissue were reduced in DBM-grafted defects exposed to HBO; however, this did not reach significance for either tissue type. Histomorphometric analysis was unable to note any significant differences between groups grafted with different formulations of BCP (blood versus F127) or DBM (gel versus putty) in any of the measured parameters.

Table 10. Histomorphometric analysis of study IV specimens

	DBM				BCP				ANOVA *p*
	GEL		PUTTY		BLOOD		PUTTY		
	NBO	HBO	NBO	HBO	NBO	HBO	NBO	HBO	
New Bone	$36.3^{\pm}3.0$	$43.7^{\pm}4.9$	$35.1^{\pm}5.5$	$44.3^{\pm}2.9$	$22.0^{\pm}6.9$	$23.9^{\pm}3.7$	$23.6^{\pm}5.7$	$24.8^{\pm}6.6$	>.001
Marrow	$47.0^{\pm}5.2$	$41.7^{\pm}7.8$	$50.6^{\pm}8.7$	$43.6^{\pm}5.6$	$28.6^{\pm}4.9$	$39.1^{\pm}4.1$	$29.4^{\pm}5.5$	$37.4^{\pm}5.1$	>.001
New Bone + Marrow	$83.3^{\pm}6.1$	$85.5^{\pm}3.6$	$85.8^{\pm}4.4$	$87.8^{\pm}4.4$	$50.6^{\pm}8.9$	$63.0^{\pm}4.9$	$53.0^{\pm}5.5$	$62.2^{\pm}7.6$	>.001
Fibrous Tissue	$13.7^{\pm}5.1$	$9.6^{\pm}3.3$	$9.8^{\pm}2.5$	$7.1^{\pm}3.0$	$25.0^{\pm}6.4$	$12.9^{\pm}5.0$	$23.1^{\pm}5.4$	$13^{\pm}4.7$	>.001
Residual Graft	$2.9^{\pm}1.8$	$5.0^{\pm}1.2$	$4.4^{\pm}2.7$	$5.1^{\pm}2.5$	$24.3^{\pm}3.0$	$24.1^{\pm}5.9$	$23.9^{\pm}3.2$	$24.4^{\pm}5.4$	>.001

All results are reported as percentage area of defect occupied by tissue type.

DBM, demineralized bone matrix; BCP, biphasic calcium phosphate; GEL, the DBM granules were mixed with Pluronic F127 at 40% DBM to 60% F127 (vol/vol); PUTTY, the DBM or BCP granules were mixed with F127 at 70% DBM/BCP to 30% F127 (vol/vol); ANOVA, analysis of variance; NBO, normobaric oxygen; HBO, hyperbaric oxygen.

6 Discussion

6.1 *Critical-sized and Supracritical-sized Defects and Hyperbaric Oxygen Exposure*

One of the major problems encountered by surgeons who deal with large craniomaxillofacial defects is the difficulty of maintaining viability within bone graft tissue in order to ensure graft survival and eventual restoration of the defect. Microvascular reconstructive techniques provide one approach by supplying the graft with its own blood supply (Ang et al., 2003). Harvesting such grafts results in significant morbidity of the donor site. Free autogenous bone-grafting techniques are associated with fewer donor-site complications but still have the limitation of the requirement of an adequately vascularized soft tissue bed in order to maintain graft viability. This is a major problem in patients who have received radiotherapy and extensive resection in order to control a malignant or an aggressive infectious condition (Jisander et al., 1999).

HBO has been used with success in treating hypoxic wounds and in anoxic conditions. Nilsson et al. have shown that HBO treatment would significantly increase bone formation in the rabbit bone harvest chamber (Muhonen et al., 2002a; Nilsson et al., 1988; Sawai et al., 1998). The intent of this study was to evaluate the effects of HBO on osseous wound healing and to see if HBO can permit bony repair of critical sized defects.

The rabbit calvarial defect model is analogous in many ways to clinical maxillofacial reconstruction. There is an osseous defect with a periosteal blood supply and there is a membranous pattern of bone repair and healing. One difference, however, is the presence of a pulsatile dural layer in the base of the rabbit calvarial model, which is not present in extracranial maxillofacial wounds.

A 15-mm defect was used in this animal model and defined as a critical-sized defect by Schmitz and Hollinger in 1986 (Schmitz and Hollinger, 1986). This was revised by Hollinger and Kleinschmidt in 1990 to be a defect having at most 10% of bony healing 10 years postoperatively (Hollinger and Kleinschmidt, 1990). Further increases in the size of the defect beyond 18-mm would have required crossing the midline of the cranial vault, which posed a significant risk of lethal hemorrhage by potentially violating the sagittal sinus. Extending the defect to involve the temporal and the frontal bones might have altered the healing due to the involvement of the sutures.

Based on radiomorphometrics, there was a significant difference in the percentage of radiopacities in the HBO group at 6 versus 12 weeks. However, this was not reflected in the histomorphometric measurements, where the amount of bone in the defects was

unchanged between 6 and 12 weeks. This finding can be explained by the fact that actual bone that is demonstrated histologically will not be evident radiographically unless it is considerably mineralized.

Histological evaluation indicated a change in bone from woven to lamellar bone between 6 and 12 weeks, which would be expected to result in an increase in the radiodensity of the bone. It could be argued that the differences observed between the two groups were due to the increased handling of the rabbits in the HBO chamber rather than the actual HBO treatment. This is unlikely as the increased handling and confinement in the HBO chamber would result in increased stress to the HBO treated animals which would in turn be expected to adversely effect healing and not improve it. Further, the process of acclimatizing the HBO group of rabbits to the chamber for one week before the surgical procedure reduced the discomfort of the rabbits to being confined in the chamber and thus minimized stress.

HBO therapy was applied intermittently to minimize the theoretical blockade of hypoxia and lactate induced collagen synthesis and neovascularization (Mainous, 1982), (Tuncay et al., 1994) as well as the differentiation of osteoprogenitor cells in the calvarial bone marrow and in the periosteal layer of the pericranium and the dura matter (Ozerdem et al., 2003). A total of 20 HBO sessions were chosen because neovascularization reaches a plateau by 20 sessions (Marx et al., 1985).

6.2 *VEGF Expression and Hyperbaric Oxygen Exposure*

HBO has been shown to enhance bone repair in bone harvest chambers in the tibia and the mandible (Nilsson, 1989; Nilsson et al., 1988); however, the mechanism by which this occurs has not been elucidated. It has been reported that HBO can increase neovascularization in soft tissue wounds (Broussard, 2004) and the in-growth of new blood vessels into the defect is an essential step in bone repair (Glowacki, 1998). VEGF and basic fibroblast growth factor (FGF) have been identified as the primary growth factors implicated in neovascularization (angiogenesis) in vitro (Klagsbrun and D'Amore, 1991). However, in vivo, the role of FGF in angiogenesis has been questioned.

Nissen and coworkers demonstrated that while FGF levels dramatically rise immediately after injury, they also return to normal within 3 days, several days prior to the onset of angiogenesis (Nissen et al., 1998). Conversely, VEGF increased, peaking at 7 days postinjury, matching the initiation of angiogenic activity that began after 1 week (Denissen and Kalk, 1991). A similar time course for VEGF expression has been reported for fracture healing (Komatsu and Hadjiargyrou, 2004). The current study was unable to address the effect of HBO on VEGF expression prior to 6 weeks, as this was a retrospective study. VEGF expression following trauma in soft tissue and bone has been reported to return to normal within 21 days following trauma (Komatsu and Hadjiargyrou, 2004; Nissen et al., 1998). Consequently, while we expect that the VEGF levels in the NBO-treated animals had been elevated following trauma, they had returned to background levels before 6 weeks, explaining the similarity in levels observed between the 6-week and 12-week defects in the NBO group. Nevertheless, we were able to demonstrate that VEGF levels were elevated 6-weeks following trauma when the rabbits had been exposed to HBO.

It has been shown in a rabbit ischemic ear model that HBO therapy (100% O_2; 2.0 ATA (atmospheres absolute); 90 minutes a day for 14 days) transiently increased tissue oxygen partial pressure in the ischemic tissue from hypoxic levels to significantly above values seen in NBO non-ischemic tissue. However O_2 partial pressure returned to ischemic values within 4 hours (Siddiqui et al., 1997). It is possible in our study that the cycling between hyperbaric and hypoxic conditions caused by the repeated 90-minute treatments of 2.4 atmosphere hyperbaric oxygen exposes the cells of the wound to a normoxic or hyperoxic environment at the injured site, removing the hypoxic stimuli for VEGF synthesis. However, upon return to normal atmosphere the defects re-experienced a hypoxic environment. This change from normoxic to hypoxic conditions may have resulted in the continued and prolonged synthesis of VEGF beyond what would occur in healing under NBO conditions. While this theory must be tested, there are examples where the same stimuli can alter gene expression and tissue formation depending on whether it was applied in a constant or cyclic manner. Examples include differences between constant and cyclic hydrostatic pressure (Suzuki et al., 2006) and cyclic and chronic administration of parathyroid hormone (PTH) (Tam et al., 1982). Sheikh et al., using a different HBO protocol (100% O_2; 2.1 ATA; 90 minutes, twice per day for 7days), demonstrated elevated VEGF levels in a subcutaneous wound cylinder mouse model following 7 days of HBO (Sheikh et al., 2000). However, in contrast to our results, they reported that VEGF levels returned to baseline within three days of termination of treatment. This may have been due to the differences in the HBO protocol, duration of treatment, and tissue and/or species studied.

Another interesting finding was that there was no difference in VEGF expression between the two different defect sizes under either of the treatment conditions. It is possible that in the NBO group differences may have been observed if we had been able to look at earlier stages of healing, prior to VEGF returning to basal levels. In the case of the HBO treatments, time of observation may also have been a factor. However, it is also

possible that as complete union of both the 15 and 18mm defects did occur at 12 weeks, the HBO therapy was able to induce VEGF expression evenly across the whole defect.

As this study was a retrospective study using archived tissue samples, the study was not optimized for quantifying the VEGF expression. Specific shortcomings in the study design include that the sample preparation was not optimized for immunohistochemical analysis, as samples were fixed in 10% neutral formalin and demineralized using formic acid. Second, the time points studied, while useful for studying defect repair, did not permit us to investigate the levels of VEGF earlier at periods previously seen to have elevated VEGF during normoxic healing and when angiogenesis would have been initiated. Third, VEGF exists as multiple isoforms, and there is differential expression of these isoforms during healing (Hofstaetter et al., 2004). The antibody used in this study was raised against one of the most common isoforms, $VEGF^{121}$, however we do not know it's cross-reactivity with the other isoforms.

6.3 *Autogenous Bone Grafts and Hyperbaric Oxygen Exposure*

The current "gold standard" for the treatment of bony defects that are unable to heal spontaneously (critical-sized defects) is the use of autogenous bone grafts. However, the volume of bone available for grafting is limited and the use of large grafts results in significant complications including donor site morbidity, loss of blood, and extended time in surgery. HBO therapy has been shown to promote the healing of unfilled critical-size defects in rabbits, and may be useful as a replacement for autogenous grafts in certain instances and as an adjunct therapy to autogenous bone grafting, minimizing the amount of graft required in others. The aim of this study was to evaluate the effect of HBO on the healing of critical-sized defects in the presence and absence of autogenous bone grafts in comparison to defect healing with grafts under normobaric conditions. This study demonstrated that histologically there was significantly more bone ($p<.001$) and marrow ($p<.05$) in the HBO-treated unfilled defect than the control unfilled defects in untreated animals. These results agree with the only other previous study to investigate the effect of HBO on critical-size defect healing. Interestingly, we were unable to demonstrate this difference by micro-CT, suggesting that the newly formed bone had not yet matured and become fully mineralized. Comparison of the amounts of new bone in the HBO-treated unfilled defect and the normobaric grafted defect indicated more bone in the HBO group that neared significance (46.7 ± 5.3 versus 36.6 ± 8.6; $p<.054$). Conversely, there was significantly less marrow and more fibrous tissue in the HBO non-grafted defect than the NBO grafted defect (marrow: 26.7 ± 9.0 versus 37.8 ± 9.1; $p<.05$; fibrous 26.5 ± 12.5 versus 6.4 ± 1.8; $p<.05$).

When the amount of bone and marrow are considered as a single measure of "reparative tissue" the amounts in the HBO unfilled and NBO-grafted defects were almost identical (73.5 ± 12.5 versus 74.4 ± 8.1), with the a volume equivalent to that occupied by residual graft in the NBO defects being occupied by fibrous tissue in the HBO-treated defects. Histomorphometric comparison of the HBO and NBO defects that contained autogenous grafts revealed there were no significant differences, although the reduction in the amount of residual graft in the HBO group neared significance (11.2 ± 4.7 versus 19.1 ± 7.7; $p=.085$). However, the micro-CT did show that there was a significant reduction in the bone mineral content of the defect ($p<.05$) and a near significant reduction in bone mineral density ($p=.078$). This can be adequately explained by the effect of VEGF on osteoclastic activity within grafted defects. VEGF is found to increase osteoclastic and chondroclastic activity within healing bone tissue as part of its role in inducing neoangiogenesis (Engsig et al., 2000; Sipola et al., 2006). We think this reduction in bone mineral content is only transient and related to increased VEGF levels. Tarkka etal, using adenoviral VEGF-A gene transfer, showed a significant hastening of endochondral bone formation in the rat femur. They have also shown a transient increase in bone mineral content in the VEGF group that normalized within 4 weeks (Tarkka et al., 2003). However, defects were not critical-sized.

The difference between the micro-CT and histomorphometric results may indicate that there is increased resorption and/or demineralization of the residual graft, which would not as easily be detected by histomorphometry. That change, however, was readily detected by micro-CT bone analysis. Sawai et al. investigated the effect of HBO on mandibular defect healing with autogenous bone grafts in rabbits by histology. They reported that HBO increased the amount of bone formed initially and that the graft becomes incorporated into the surrounding bone making it difficult to distinguish the graft from new bone after 4 weeks, although they did not evaluate the effects quantitatively (Sawai et al., 1996). Chen et al demonstrated that HBO increased the rate of union of rabbit spinal fusions in the presence of autogenous grafts (Chen et al., 2002).

6.4 *Bone Substitutes and Hyperbaric Oxygen Exposure*

Bone graft substitutes have been successfully used to treat defects, avoiding some of the limitations associated with autogenous bone including donor site morbidity, blood loss, and extended time in surgery. HBO has been shown to enhance the bony healing of critical-sized defects in rabbits without bone grafting and may be useful as an adjunct to bone graft substitutes in smaller defects (Chen et al., 2002; Muhonen et al., 2004). The use of HBO as a testing modality may also allow the detection of differences between bone and various bone substitute materials that are not evident using other testing methods.

The aim of this study was to evaluate the effect of HBO on the healing of critical-sized defects in the presence of two commonly used types of bone graft substitutes, demineralized matrix (DBM) and biphasic calcium phosphate (BCP). We also investigated whether different preparations of these substitutes affected their performance.

Both bone substitute materials were able to promote healing of the critical-sized defects. HBO did not significantly increase the amount of bone present in the BCP-grafted defects compared with defects in animals exposed to normal air. This result matches our previous observations where We observed promoted healing and bone formation in unfilled defects with HBO, but HBO provided no further increase in bone when used in conjunction with autogenous bone grafts. We did, however, observe a small but significant increase in new bone with DBM-grafted defects exposed to HBO.

Exposure to HBO reduced the amount of fibrous tissue in the BCP-grafted defects, and showed a matching increase in the marrow component of the repair tissue. We have previously demonstrated that HBO treatment results in prolonged increases in vascular endothelial growth factor (VEGF) expression, whereas others have demonstrated VEGF promotes angiogenesis, bone formation, and remodeling, (Chen et al., 2002; Peng et al., 2005; Street et al., 2002) suggesting that that HBO may potentially reduce fibrous tissue formation through promotion of angiogenesis, which could result in increased marrow. Comparison of the amounts of new bone in the DBM-filled defects and the BCP-filled defects indicated more bone and marrow formation in the DBM group irrespective of HBO therapy.

DBM-filled defects also revealed less fibrous tissue formation when compared with BCP-filled defects when treated under NBO conditions. We did not see any differences in the repair of defects by different preparations of DBM (gel versus putty), whereas the different preparations of BCP (blood versus F127) showed some differences in mineralization as assessed by the mCT, but not by histology.

6.5 Future Perspectives

Our main interest for conducting this investigation was to study the influence of Hyperbaric Oxygen (HBO) on the healing of critical sized defects with and without additional grafting. Results of this four phase investigation were interesting in light of the change in the accepted critical size of full thickness calvarial wound model. We were able to demonstrate bony union in critical-sized defects treated with HBO in the absence of bone grafting. HBO increases the critical size of the calvarial defect model in rabbits to more than 20%. Moreover, the regenerate has new bone quantity that is equivalent to autogenous bone grafting

HBO resulted in a reduction of the bone mineral content of the autogenous bone grafted sites. Angiogenesis within the graft may account for the reduction observed during the experimental period of this study. The clinical significance if this reduction in bone mineral density and bone mineral content has yet to be determined. Further studies with higher statistical power and larger sample sizes should be conducted in a load bearing model.

Moreover, testing Demineralized bone matrix and biphasic calcium phosphate as a scaffold for adipose derived mesenchymal cells is one of the future directions.

7 Summary and conclusions

The following main conclusions can be drawn from the results obtained in the present studies:

1. The HBOT protocol described in this study effectively enhanced membranous bone healing in the rabbit calvarial critical sized defect model. HBOT significantly enhanced bone formation within the critical-sized and supracritical-sized defects, even in the absence of an autograft or a bone substitute, possibly minimizing the amount or totally eliminating the bone graft required or bone substitute required to permit healing of the bony calvarial defect.

2. HBOT demonstrates an increase in VEGF expression within critical-sized and supracritical-sized defects at the 6 weeks time point.

3. HBOT enhanced bony healing in non-grafted calvarial critical-sized defects with a bone volume that iwa comparable with autogenous nonvascularized bone grafting. HBOT resulted in a reduction of the bone mineral content of the autogenous bone grafted sites. Angiogenesis within the graft may account for the reduction in BMD observed during the experimental period of this study.

4. HBOT resulted in small increases in new bone formation in defects grafted with Demineralized Bone Matrix. In defects grafted with Biphasic Calcium Phosphate, HBOT resulted in a significant reduction in fibrous tissue and an increase in the replacement of Biphasic Calcium Phosphate with bone marrow.

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9 Role in publications

List of papers:

I. **Jan A**, Sándor GKB, Iera D, Mhawi, A, Peel SA, Clokie CML (2006). *Hyperbaric oxygen results in an increase in rabbit calvarial critical sized defects*. Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology and Endodontology. 101(2): 144-149.
Role: Performed all animal surgeries, collected and analyzed data, statistical analysis of results and wrote paper.

II. Fok, TCO, **Jan A,** Peel SA, Clokie CML, Sándor GKB (2008). *Hyperbaric oxygen results in an increase in vascular endothelial growth factor (VEGF) protein expression in rabbit calvarial critical sized defects*. Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology and Endodontology. 105(4): 417-422. Epub 2008 Jan 16.
Role: Performed all animal surgeries, collected and analyzed data together with TC Fok, performed statistical analysis of results and wrote paper.

III. **Jan A**, Sándor GKB, Brkovic BMB, Peel SA, Evans AW, Clokie CML (2009). *Effects of hyperbaric oxygen on grafted and non-grafted on calvarial critical-sized defects*. Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology and Endodontology. 107(2): 157-163. Epub 2008 September 19.
Role: Performed all animal surgeries, collected and analyzed data, statistical analysis of results and wrote paper.

IV. **Jan A**, Sándor GKB, Brkovic BMB, Peel SA, Kim YD, Xiao WZ, Evans AW, Clokie CML (2010). *Effects of hyperbaric oxygen on demineralized bone matrix and biphasic calcium phosphate bone substitutes.* Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology and Endodontology. 109(1):59-66. Epub 2009 Oct 20.
Role: Performed all animal surgeries, collected and analyzed data, statistical analysis of results and wrote paper.

10 Original Publications

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Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontology

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Hyperbaric oxygen results in an increase in rabbit calvarial critical sized defects

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Objective. This study was undertaken to evaluate whether the effects of hyperbaric oxygen (HBO) therapy could alter the critical size for spontaneous healing of a bone defect in the rabbit calvarial model.
Study design. An animal trial of 12 weeks duration was conducted using 20 New Zealand white rabbits, which were randomly divided into 2 groups of 10 animals each. Calvarial defects were created in the parietal bones of each animal bilaterally. Defects were critical-sized, 15 mm on one side and supra−critical-sized, 18 mm on the contralateral side. Group 1 received a 90-min HBO treatment sessions at 2.4 absolute atmospheric pressure (ATA) per day for 20 consecutive days. Group 2 served as a control without any HBO treatment sessions. Five animals in each group were sacrificed at 6 and 12 weeks. Data analysis included qualitative assessment of the calvarial specimens, post-sacrifice radiographs, as well as histomorphometric analysis to compute the amount of regenerated bone within the defects. ANOVA and paired sample *t* test were used for statistical analysis.
Results. Both radiographic analysis and histomorphometric analysis demonstrated that HBO-treated animals had significantly more new bone within their defects compared with the control group ($P < .001$). There was no statistically significant difference between the percentage of new bone forming in the 15-mm and 18-mm HBO-treated defects. There was no difference between the 6-week and the 12-week HBO-treated groups. HBO is effective in enhancing the bony healing of full thickness critical sized as well as supra−critical-sized defects in the rabbit calvarial model.
Conclusion. Bone regeneration was significantly greater in the HBO-treated animals regardless of the defect size. HBO may have increased the diameter of the rabbit critical-sized calvarial defect to more than 18 mm.
(Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2006;101:144-9)

A critical-sized defect is by definition the smallest full thickness osseous wound that will not heal spontaneously during the lifetime of an animal.[1] Such a defect requires an adjunctive technique to permit its complete bony healing. The rabbit calvarial critical-sized defect model has been used to study the efficacy of a variety of bone substitute materials in promoting defect healing.[2,3]

Hyperbaric oxygen (HBO) has been used to aid in the healing of hypoxic or compromised wounds[4-6] such as hypoperfused grafts, radiation-induced side effects,[7] and necrotizing anaerobic bacterial infections.[8] Further, Muhonen et al.[9-11] have demonstrated that HBO treated rabbits have more osteoblastic activity and osteogenic potential in their irradiated distracted mandibles when

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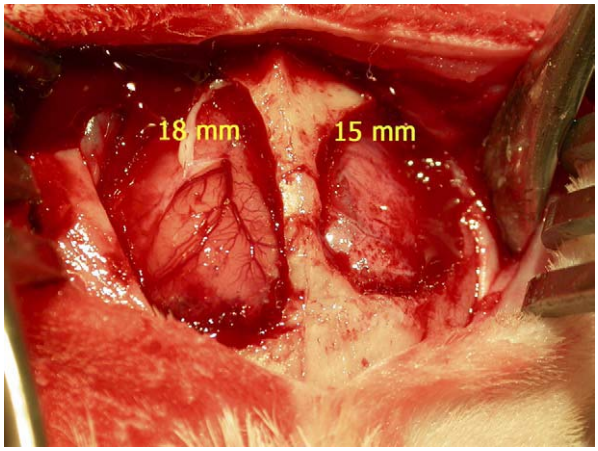

Fig. 1. Critical- (15 mm) and supra−critical- (18 mm) sized defects created in the rabbit parietal bones.

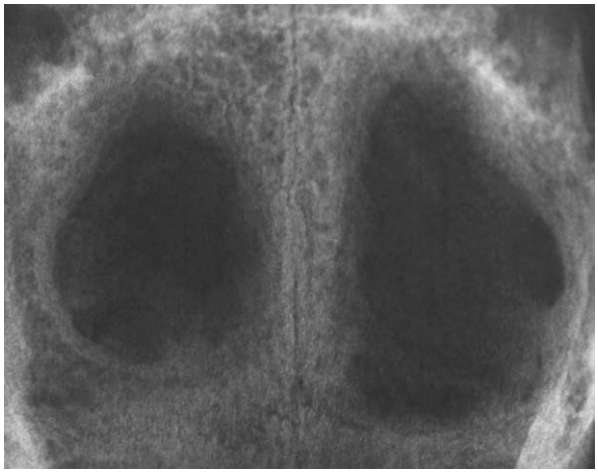

Fig. 2. Postsacrifice radiograph showing defects bilaterally in the parietal bones.

compared to a non-HBO−treated group. Consequently we hypothesized that HBO treatment would promote the healing of a rabbit critical-sized calvarial defect, possibly even allowing a supra-critical sized defect to heal.

MATERIALS AND METHODS

Animals

Twenty adult, skeletally mature, male New Zealand White rabbits weighing 3 to 4 kg were randomly divided into 2 groups of 10 animals. Group 1 was treated with HBO, while group 2 served as a control and did not receive any supplemental oxygen. Five animals from each group were sacrificed at 6 and 12 weeks.

Surgical procedures

The surgical protocol for this study was approved by the University of Toronto Animal Care and Ethics Committee (Protocol number 20005145). The surgical technique and analgesic and antibiotic protocols have been previously described.[2,3] Each animal had bilateral full thickness calvarial defects created in its parietal bones. Defects were allocated randomly as critical-sized, 15 mm on one side and a supra−critical-sized, 18 mm on the contralateral side (Fig. 1). Standardization of defects was accomplished by using a trimmed template of the desired size. Wound closure was performed in layers.

HBO sessions

The 20 animals in group one (n = 10) underwent a 90-min HBO session at 2.4 absolute atmospheric pressure (ATA) per day 5 days a week for 4 weeks (20 days total). Pressurization and depressurization were done at a very slow rate (0.2 ATA per minute) in order to avoid barotrauma and potential discomfort. The control group, group 2 (n = 10) had no HBO sessions, breathing normobaric oxygen (NBO), otherwise referred to as *ambient pressure room air*, during the entire experimental period. HBO treatment sessions were begun 24 hours postoperatively in a monoplace chamber specially designed for small animal use. The test group was acclimatized to the HBO chamber one week preoperatively. This involved the animals being placed in the unpressurized hyperbaric chamber for 90 min/day for 5 days. The chamber had a glass window in its rear allowing the investigator to monitor the rabbits' behavior and comfort throughout the 90-min sessions. Pressurization and depressurization were done at a constant slow rate of 0.2 ATA/min in order to avoid barotrauma and potential discomfort.

Sacrifice and qualitative evaluation

Five animals from each group were sacrificed 6 weeks postoperatively and 5 from each group were sacrificed 12 weeks postoperatively. The parietal bones were divided from the rest of the cranium with an oscillating saw, carefully maintaining the intactness of the pericranium and the dura matter. Specimens were examined grossly for signs of inflammation, transilluminated, photographed, radiographed with a cephalostat (Fig. 2), and then fixed in 10% buffered formalin solution for 3 days before proceeding with the histological preparations.

Radiomorphometrics

Radiographs were digitized. An investigator blinded to the HBO status of the animals traced the areas of radiopacities within the defects. The percentages of radiopacities were calculated via Image Pro Plus 4.1 software for Windows (Media Cybernetics, Carlsbad, CA).

Histological evaluation

The specimens were decalcified using 45% formic acid and 20% sodium citrate for 4 weeks. The right and left parietal bones were separated through the

Table I. Percent radiopacities within the HBO (test) and NBO (control) defects

Sacrifice time	*6 Weeks*				*12 Weeks*			
Defect size	*15 mm*		*18 mm*		*15 mm*		*18 mm*	
Oxygen	*HBO*	*NBO*	*HBO*	*NBO*	*HBO*	*NBO*	*HBO*	*NBO*
Percent radiopacities	52.96	38.31	29.73	29.86	79.51	47.29	95.50	20.39
within defects	27.92	29.46	61.34	41.22	65.41	30.85	76.97	23.16
	60.18	45.72	65.25	43.67	95.13	2.10	92.24	39.02
	57.85	47.39	44.22	26.60	90.35	30.50	98.74	54.07
	44.98	35.84	51.55	29.62	67.03	54.67	74.94	65.88
Mean	48.78	39.34	50.42	34.19	79.49	33.08	87.68	40.50
SD	13.03	7.35	14.20	7.69	13.38	20.24	10.97	19.59
SE	5.83	3.29	6.35	3.44	5.98	9.05	4.90	8.76

Defects measured by calculating areas of radiopacities within the individual defect divided by the total defect area and multiplied by 100, with standard deviation (SD) and standard error (SE).
HBO, hyperbaric oxygen; *NBO,* normobaric oxygen.

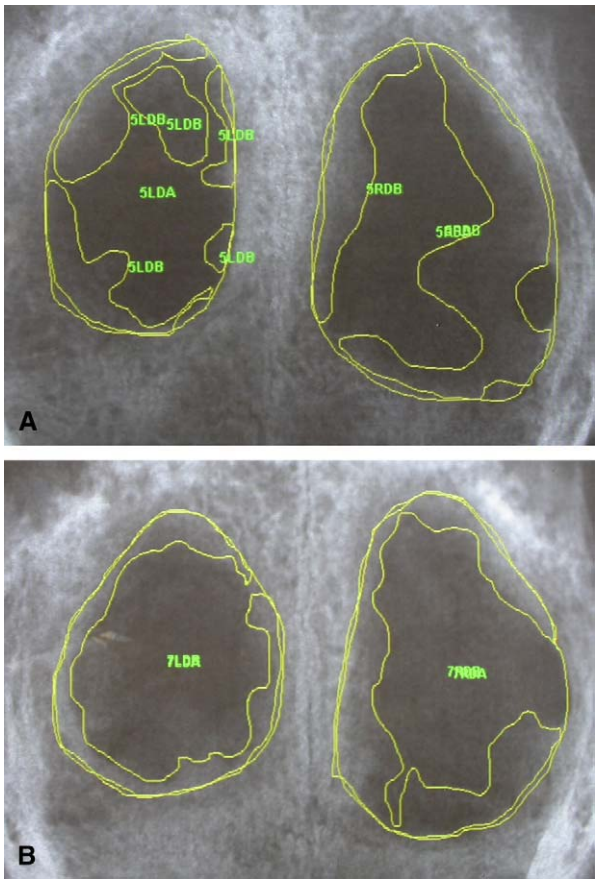

Fig. 3. Postsacrifice radiograph of the parietal bone of **A**, HBO-treated group animal and **B**, control group animal.

midline sagittal suture. Each bone was further sectioned into two portions: an anterior and posterior portion. Both portions were embedded in paraffin blocks. Then 7-μm sections were sliced and stained with hematoxylin and eosin. Sections in the middle of the defects, which corresponded to the greatest defect dimension (15 mm or 18 mm) were examined under the light microscope.

Histomorphometrics

Digital images were captures by a CCD digital camera (RT Color; Diagnostic Instruments Inc., Sterling Heights, MI) attached to the microscope on 4× magnification with 100% zoom to ensure proper focus. To create a single image from each slide the digital images were merged using Adobe Photoshop Elements 2.0 (Adobe Systems Inc., San Jose, CA).

The repair tissue in each defect was measured by a blinded investigator. The amount of new bone was determined as the percentage of the total area of the defect. Calibration was achieved using a millimeter grid. Five randomized sections from each sample were analyzed in an attempt to prevent bias.

Statistical analysis

ANOVA and paired sample *t* tests were applied in SPSS 10.0 for Windows (SPSS Inc., Chicago, IL), and used to calculate statistical differences between the means of new bone formation based on histomorphometrics. Means of the radiopacities within the defects were also analyzed. The percentage of new bone and the percentage of radiopacities were analyzed in relation to either (1) inspired air (HBO vs. NBO), (2) defect size (15 mm vs. 18 mm), or (3) healing time (6 weeks vs. 12 weeks). The *P* values below .05 were considered to be statistically significant.

RESULTS

Qualitative findings

All 20 rabbits tolerated the anesthesia and the surgical procedures well and experienced no complications during the experimental period. HBO sessions were uneventful in the experimental group. Upon sacrifice, gross examination of the dissected specimens of the calvaria showed no difference between the HBO group and the control group or between the 15-mm and 18-mm

Table II. Percent new bone formation in the HBO (test) and NBO (control) groups

Sacrifice time	*6 Weeks*				*12 Weeks*			
Defect size	*15 mm*		*18 mm*		*15 mm*		*18 mm*	
Oxygen	*HBO*	*NBO*	*HBO*	*NBO*	*HBO*	*NBO*	*HBO*	*NBO*
Percent new bone formation	64.22	20.63	74.89	30.23	64.02	18.68	58.56	6.84
	49.60	22.77	43.36	45.33	58.06	10.07	52.58	20.03
	67.22	33.26	75.77	46.55	46.67	14.53	57.35	19.90
	53.94	22.64	49.34	24.48	55.39	10.02	50.46	24.16
	47.73	20.01	61.04	22.75	56.67	17.61	39.25	42.26
Mean	56.54	23.86	60.88	33.87	56.16	21.46	51.64	26.83
SD	8.74	5.31	14.65	11.37	6.25	18.20	7.67	15.36
SE	3.91	2.41	6.55	5.08	2.80	7.43	3.44	6.27

Percent new bone formation measured by calculating areas of new bone formed within the individual defect divided by the total defect area and multiplied by 100, with standard deviation (SD) and standard error (SE).
HBO, hyperbaric oxygen; *NBO,* normobaric oxygen.

defects in terms of signs of inflammation and integrity of the healing wound.

Radiomorphometrics (Table I)

Radiographs demonstrated more islands of radiopacities in the HBO group compared to the control group (Figs. 3 and 4) in both the 6- and 12-week specimens ($P < .001$). There were fewer radiopaque foci within the margins of the defects in the control group. In the control group the radiopacities tended to blend with the margins of the defects, whereas in the HBO group more radiopaque areas were evident both along the margins as well as in the center of the defects (Fig. 3). No differences were noted between the 15-mm and 18-mm defects ($P = .688$). The percentage of radiopacities were greater in HBO samples at 12 weeks when compared to those at 6 weeks ($P = .019$).

Histological evaluation

The healing of the defects in the control group was mainly by scar formation. There were a few bony islands scattered along the defect margins, which might have resulted from bone debris produced by drilling through bone. The healing of the HBO group produced a tissue regenerate with many blood vessels and cellular marrow spaces. The 12-week HBO samples tended to have more mature, trabecular bone whereas the 6 week samples had more woven bone and less trabeculated chunks of bone.

Histomorphometrics (Table II)

Histomorphometric analysis demonstrated more bone formation in the HBO group when compared to the control group ($P < .001$). Both critical- (15 mm) and supra−critical- (18-mm) sized defects healed with significantly more bone in the HBO group when compared with the control group. There was no significant difference between the percentage of new bone formed in the 15-mm and the 18-mm defects ($P = .520$), nor between the 6-week and 12-week groups ($P = .309$). (Figs. 5 and 6).

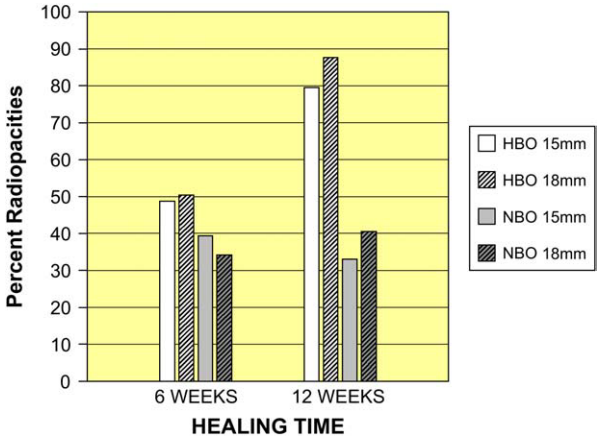

Fig. 4. Bar chart showing the differences in the mean radiopacity measurements within defects in the study groups (HBO = hyperbaric oxygen, NBO = normobaric oxygen).

DISCUSSION

One of the major problems encountered by surgeons who deal with large craniomaxillofacial defects is the difficulty of maintaining viability within bone-grafted tissue in order to ensure graft survival and eventual restoration of the defect. Microvascular reconstructive techniques provide one approach by supplying the graft with its own blood supply.[12] Harvesting such grafts results in significant morbidity of the donor site. Free autogenous bone-grafting techniques are associated with fewer donor-site complications but still have the limitation of the requirement of an adequately vascularized soft tissue bed in order to maintain graft viability. This is a major problem in patients who have received radiotherapy and extensive resection in order to control a malignant or an aggressive infectious condition.[13]

HBO has been used with success in treating hypoxic wounds and in anoxic conditions. Nilsson et al. have shown that HBO treatment would significantly increase

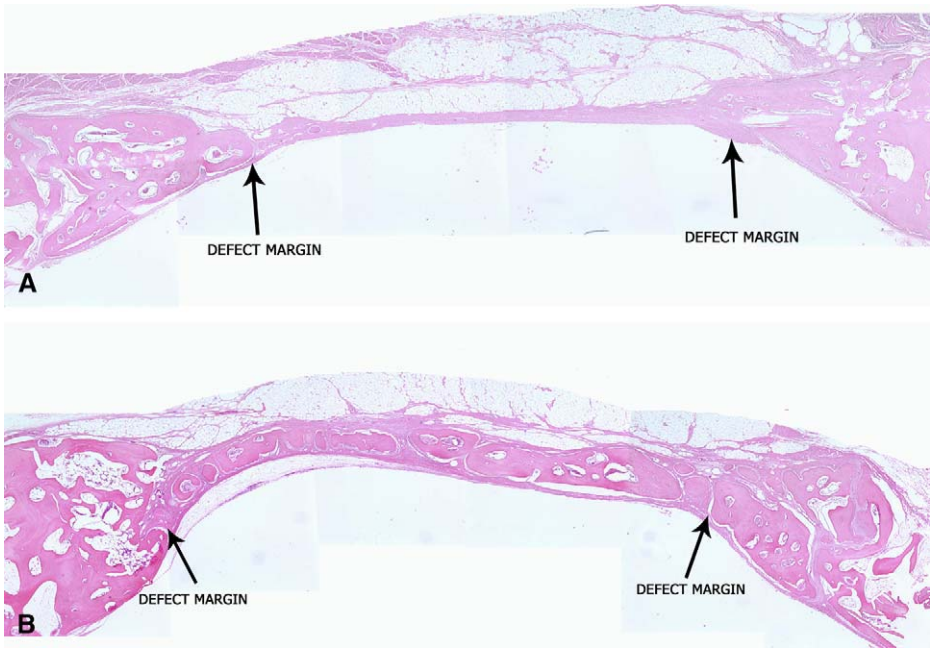

Fig. 5. 4× magnification hematoxylin and eosin stained sections. **A**, A control group specimen healed primarily with fibrous band of tissue. **B**, An HBO-treated animal sample showing bony healing of the defect.

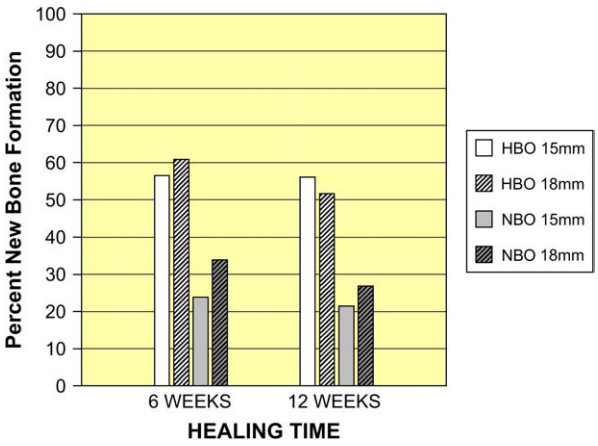

Fig. 6. Bar chart showing the differences in the means of new bone formation based on histomorphometric measurements in the study groups (HBO = hyperbaric oxygen, NBO = normobaric oxygen).

bone formation in the rabbit bone harvest chamber.[11,14,15] The intent of this study was to evaluate the effects of HBO on osseous wound healing and to see if HBO can permit bony repair of critical sized defects.

The rabbit calvarial defect model is analogous in many ways to clinical maxillofacial reconstruction. There is an osseous defect with a periosteal blood supply and there is a membranous pattern of bone repair and healing. One difference, however, is the presence of a pulsatile dural layer in the base of the rabbit calvarial model, which is not present in extracranial maxillofacial wounds.

A 15-mm defect was used in this animal model and defined as a critical-sized defect by Schmitz and Hollinger in 1986.[1] This was revised by Hollinger and Kleinschmidt in 1990 to be a defect having at most 10% of bony healing 10 years postoperatively.[16] Further increases in the size of the defect beyond 18-mm would have required crossing the midline of the cranial vault, which posed a significant risk of lethal hemorrhage by potentially violating the sagittal sinus. Extending the defect to involve the temporal and the frontal bones might have altered the healing due to the involvement of the sutures.

Based on radiomorphometrics, there was a significant difference in the percentage of radiopacities in the HBO group at 6 versus 12 weeks. However, this was not reflected in the histomorphometric measurements, where the amount of bone in the defects was unchanged between 6 and 12 weeks. This finding can be explained by the fact that actual bone that is demonstrated histologically will not be evident radiographically unless it is considerably mineralized. Histological evaluation indicated a change in bone from woven to lamellar bone between 6 and 12 weeks, which would be expected to result in an increase in the radiodensity of the bone.

It could be argued that the differences observed between the two groups were due to the increased handling of the rabbits in the HBO chamber rather than the actual HBO treatment. This is unlikely as the

increased handling and confinement in the HBO chamber would result in increased stress to the HBO treated animals which would in turn be expected to adversely effect healing and not improve it. Further, the process of acclimatizing the HBO group of rabbits to the chamber for one week before the surgical procedure reduced the discomfort of the rabbits to being confined in the chamber and thus minimized stress.

HBO therapy was applied intermittently to minimize the theoretical blockade of hypoxia and lactate induced collagen synthesis and neovascularization[17,18] as well as the differentiation of osteoprogenitor cells in the calvarial bone marrow and in the periosteal layer of the pericranium and the dura matter.[19] A total of 20 HBO sessions were chosen because neovascularization reaches a plateau by 20 sessions.[20]

CONCLUSIONS

The HBO treatment protocol described in this study seems to be an effective measure to enhance membranous bone healing in the rabbit calvarial critical sized defect model. The results reported here suggest that HBO treatment sessions as in the protocol described in this study significantly enhance bone formation within the critical-sized defect, even in the absence of an autograft or a bone substitute, possibly minimizing the amount or eliminating totally the bone graft required or bone substitute required to permit healing of the bony calvarial defect.

HBO may also increase the osteogenic, osteoinductive, and osteoconductive properties of bone regeneration materials indirectly by promoting early angiogenesis or by the genesis of a more viable soft tissue and hard tissue graft recipient wound for such materials. The rabbit calvarial critical-sized defect model is suitable for assessing the effectiveness of HBO therapy on cranio-maxillofacial bone repair and may be suitable for examining the effect of HBO in combination with various grafting materials or bone substitutes in the future.

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Hyperbaric oxygen results in increased vascular endothelial growth factor (VEGF) protein expression in rabbit calvarial critical-sized defects

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Background. Hyperbaric oxygen therapy (HBO) promotes osseous healing, however the mechanism by which this occurs has not been elucidated. HBO may promote angiogenesis, which is vital for bone healing. Vascular endothelial growth factor (VEGF) is one of the key factors that stimulates angiogenesis.
Objective. The objective of this study was to investigate whether HBO altered VEGF expression during bone healing.
Methods and materials. Archived samples from calvarial defects of rabbits exposed to HBO (2.4 ATA, 90 minutes a day, 5 days a week for 4 weeks) and normobaric oxygen controls (NBO) were analyzed by immunohistochemistry.
Results. VEGF expression in 6-week HBO samples was elevated compared to NBO ($P = .012$). Staining of the 12-week HBO samples was reduced compared to 6-week HBO ($P = .008$) and was similar to 6- and 12-week NBO control samples.
Conclusion. HBO therapy resulted in increased VEGF expression in the defects even 2 weeks after the termination of treatment (6 weeks postsurgery). **(Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2008;105:417-22)**

A critical-sized osseous defect is defined as the minimum dimension of a bony lesion that cannot repair itself to its preinjured state without intervention during an individual's life span.[1] In the rabbit calvarium this is defined as a defect 15 mm in diameter. Critical-sized osseous defects may lead to numerous complications including fracture, non-union and pseudo-arthrosis.[2] Surgical treatments prevent further complications, which may involve the use of a fixation device and autogenous bone graft material to bridge the gap in the defect. All such reconstructive procedures that require a second surgical site for the harvesting of tissue are associated with potential morbidity.[3,4] Synthetic biomaterials have been used in place of autogenous bone grafts.[5] Recently, Jan et al.,[6] using the rabbit critical-sized calvarial defect model, showed that 20 treatments of 90 minutes of hyperbaric oxygen (HBO) at 2.4 atmospheres could heal both critical-sized and supra-critical-sized calvarial defects when compared to normobaric oxygen controls (NBO). This suggests that HBO has the potential to augment bony healing.

HBO's mode of action in the treatment of decompression sickness and carbon monoxide poisoning is well understood based on its effects on reducing gas emboli

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and hastening carboxyhemoglobin dissociation.[7] However, it has also demonstrated effectiveness in the treatment of necrotizing soft tissue infections, soft tissue radiation necrosis, diabetic wound healing, and now osseous defect repair where other mechanisms are believed to involved.[8,9] It has been well established that the formation of new blood vessels (angiogenesis) is essential in the process of soft tissue and bone repair.[10,11] Vascular disruption, caused by traumatic injury has been shown to lead to the formation of a hypoxic zone. Wound hypoxia is necessary to stimulate angiogenesis and revascularization. HBO increases the amount of oxygen dissolved in the blood (oxygen tension) which can in turn increase the amount of oxygen delivered to these hypoxic tissues reducing the effects of the hypoxia.[7] While this is helpful in cases of chronic hypoxia, which blunts the repair process, it is not so clear as to how this would stimulate the normal repair process.

Vascular endothelial growth factor (VEGF) has been identified as one of the primary growth factors responsible for neovascularization during wound healing and embryonic development.[12] Oxygen tension is a key regulator of VEGF expression in vitro and in vivo.[13-15] We therefore wished to investigate the effect of HBO on VEGF expression in bone healing.

METHODS AND MATERIALS

Experimental design

This investigation used archived tissue from a previous study.[6] The surgical protocol for the study was approved by the University of Toronto Animal Care and Ethics Committee (Protocol number 20005145). A total of 21 skeletally mature male New Zealand white rabbits were divided into 2 groups (n = 10 for the 6-week group, n = 11 for the 12-week group). Five animals in the 6-week group received hyperbaric oxygen treatment (HBO) and 5 control rabbits were kept in a normobaric environment (NBO). Similarly, 5 rabbits in the 12-week group received hyperbaric treatment (HBO) while 6 control animals were exposed to a normobaric environment (NBO). Critical-sized calvarial defects of 15 mm and supra critical-sized defects of 18 mm were randomly assigned to the right and left parietal bones of the rabbits and created using a straight fissured bur guided by a template. For the HBO treatment group, each rabbit was placed into an animal hyperbaric oxygen chamber and exposed to 100% O_2 under 2.4 atmospheres of pressure, for 90 minutes a day, 5 days a week for 4 weeks (20 treatments). The rabbits were sacrificed 6 weeks or 12 weeks postsurgery and the parietal bones were harvested. These samples were fixed with 10% formalin and decalcified in a solution of 45% formic acid in 0.2 M sodium citrate.

Qualitative analysis: histology

Following fixation and decalcification, the midpoint of the defect region was identified and served as the coronal reference plane of section prior to embedding in paraffin. Multiple 6-μm sections were cut and stained with hematoxylin and eosin (H&E) for conventional light-microscopy. The defect region was visualized in all samples and the appearance of new bony regenerate was noted.

Quantitative analysis: immunohistochemical analysis

Multiple 6-μm sections cut from the same paraffin block were used in the histological analysis. These sections were incubated with mouse monoclonal anti-human $VEGF_{121}$ antibody (clone JH-21, Lab Vision Corp, Fremont, CA), with known rabbit cross-reactivity, as a primary antibody. Then an avidin-biotin complex (Lab Vision Corp) was incubated to label the primary antibody, and a color reagent was added at the end to allow the horseradish peroxidase reaction to take place.

Analysis of VEGF expression

Using the image capturing software Image Pro Plus 4.1 for Windows (Media Cybernetics, Carlsbad, CA), 6 random fields from each section were captured at ×40 magnification using an RT Color digital camera (Diagnostic Instruments Inc, Sterling Heights, MI). A total of 3 sections were used for each defect resulting in a total of 18 random images for each right and 18 of each left defect. The area stained for VEGF in each field was measured by setting a threshold intensity above which a pixel is counted using the Image Pro Plus software.

To determine whether there was any difference in VEGF expression in the center of the defects compared to the margins of the defects, 2 images were taken from the central one third of the defect and 4 images were taken from the margins and their VEGF staining measured.

Statistical analysis

One-way analysis of variance (ANOVA) was employed for analysis within and between the groups. When differences were found, the Student-Newman-Keuls method was used as a post hoc test to determine which groups were significantly different.

Comparisons of VEGF staining between the 15-mm and 18-mm defects in the same rabbits, and between the margins and central regions within each defect, were made using the paired *t* test. Statistical significance was set at $P < .05$. All statistical analyses were performed using Sigma Stat software (v3.0, SYSTAT, San Jose, CA).

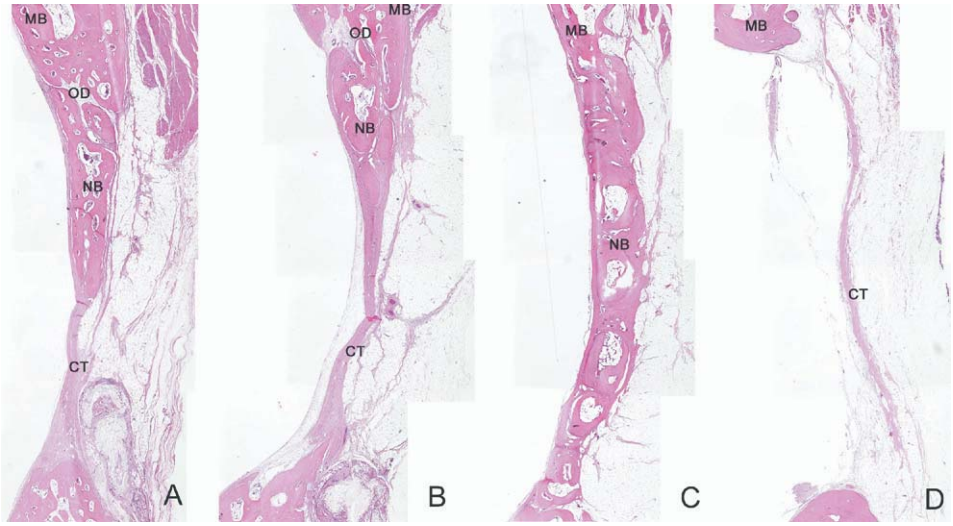

Fig. 1. Histological appearance of HBO and NBO defects at 6 and 12 weeks. **A,** HBO 6-weeks group. A significant amount of new bone is seen within the defect. **B,** NBO 6-weeks group. Although new bone is present within the defect, it is much less than in the group treated with HBO. **C,** HBO 12-weeks group. The entire defect is bridged with new bone. **D,** NBO 12-weeks group. The defects were filled predominantly with dense fibrous tissue. CT, connective tissue; MB, mature bone; NB, new bone; OD, margin of original defect. (Hematoxylin-eosin stain; magnification ×4.)

RESULTS

Gross appearance and histological evaluation

As reported previously, gross analysis of the postmortem defect size showed the defects of the HBO-treated groups to be smaller than those of the NBO groups with complete union at 12 weeks in all subjects of the HBO therapy group. Histological analysis revealed that healing of the defects in the NBO group was mainly by scar formation with only a few bony islands scattered along the defect margins. By comparison, the defects from the HBO-treated animals contained significant amounts of bone and marrow that completely bridged the defects by 12 weeks. The bone in the 6-week HBO defects was predominantly woven, but by 12 weeks it tended to be more lamellar in nature (Fig. 1, *A-D*).

VEGF staining

VEGF expression in all groups occurred throughout the fibrous tissue and marrow, with more intense staining often seen near bone. When the extent of VEGF staining was compared between the 15-mm and 18-mm defects, no differences were detected in any group (Table I) or when considering all the samples as a whole. Consequently, the results for the 2 defects in each animal were averaged to provide 1 result per animal for further analysis. The means and standard deviations for the area stained by VEGF for each group are reported in Table II and displayed in Fig. 2.

Comparison of the areas stained for VEGF between the HBO and NBO groups 6 weeks postsurgery showed that there was a significantly greater area staining for VEGF in the samples from HBO-treated rabbits (P = .012) (Figs. 3 and 4).

Comparison of VEGF staining between the animals killed at 6 and 12 weeks postsurgery showed that there was no difference between the 2 NBO groups; however, there was a significant decline in VEGF staining between the 6- and 12-week HBO samples (P = .008). The 12-week HBO and NBO samples had similar amounts of VEGF staining (Fig. 5).

We also investigated whether there was any detectable difference in the amount of VEGF staining between the center and margins of the defect in the

Table I. Comparison of VEGF staining between 15- and 18-mm defects

	15 mm	*18 mm*	$P_{(15\ mm\ vs\ 18\ mm)}$
HBO, 6 weeks	413 ± 171	571 ± 474	.430
NBO, 6 weeks	222 ± 112	250 ± 137	.141
HBO, 12 weeks	167 ± 140	104 ± 40	.371
NBO, 12 weeks	163 ± 71	145 ± 47	.651

All values are group means ± SD of the subject means of stained area (μm^2) within a field of view. Each subject mean represented the average of 6 fields per slide, 3 slides per defect. Each rabbit had one 15-mm and one 18-mm defect.

P values were calculated using the paired t test.

HBO, hyperbaric oxygen; *NBO,* normobaric oxygen; *VEGF,* vascular endothelial growth factor.

Table II. Comparison of VEGF staining between HBO and NBO groups

	6 wk	*12 wk*	$P_{(6\ wk\ vs\ 12\ wk)}$
HBO	520 ± 287	147 ± 38	.008
NBO	240 ± 121	149 ± 76	.630
$P_{(HBO\ vs\ NBO)}$	.012	.987	

All values are group means ± SD of the subject means of stained area (μm^2) within a field of view. Each subject mean represented the average of 6 fields per slide, 3 slides per defect, 2 defects per subject (36 fields measured per subject).
Results of the 15- and 18-mm defects were combined in this analysis. *P* values were determined using the Student-Newman-Keuls post hoc test following analysis of variance.
HBO, hyperbaric oxygen; *NBO,* normobaric oxygen; *VEGF,* vascular endothelial growth factor.

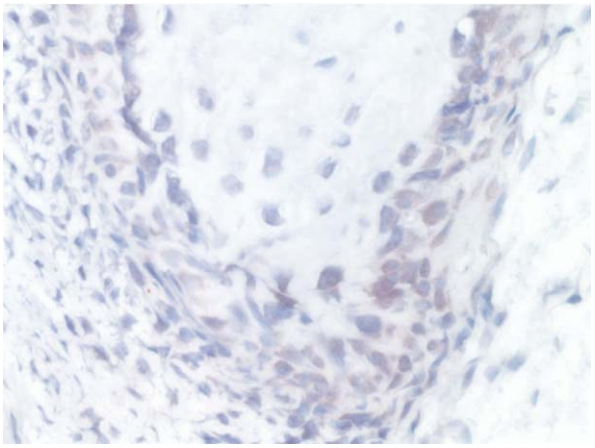

Fig. 2. VEGF-stained HBO-treated defects at 6 weeks. Rabbits had been exposed to HBO therapy (90 minutes at 2.4 atmospheres with 100% oxygen) 5 days a week for 4 weeks. Cells stained for VEGF were seen throughout the defect, however the most intense VEGF staining was seen near areas of bone. (Magnification ×200.)

Fig. 3. VEGF-stained untreated defects at 6 weeks. Only low levels of VEGF staining were seen throughout the defect. Very few cells were seen that stained strongly. (Magnification ×200.)

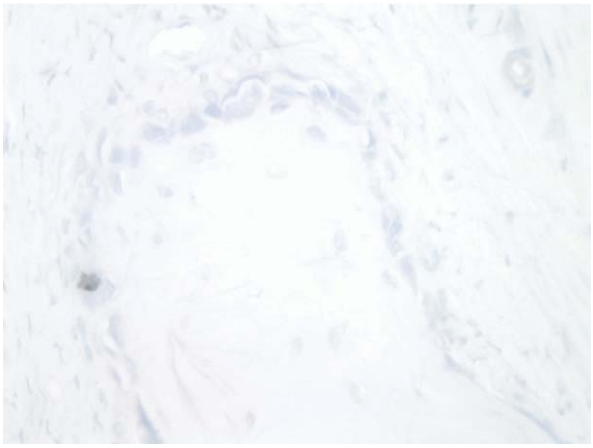

Fig. 4. VEGF-stained HBO-treated defects at 12 weeks. Limited VEGF staining was observed, even near areas of bone. (Magnification ×200.)

6-week samples. However, no differences were detected (Table III).

DISCUSSION

HBO has been shown to enhance bone repair in critical-sized calvarial defects[6]; however, the mechanism by which this occurs has not been elucidated.

It has been reported that HBO can increase neovascularization in soft tissue wounds[16] and the in-growth of new blood vessels into the defect is an essential step in bone repair.[11] VEGF and basic fibroblast growth factor (FGF) have been identified as the primary growth factors implicated in neovascularization (angiogenesis) in vitro.[12] However, in vivo, the role of FGF in angiogenesis has been questioned. Nissen and coworkers[17] demonstrated that while FGF levels dramatically rise immediately after injury, they also return to normal within 3 days, several days prior to the onset of angiogenesis. Conversely, VEGF increased, peaking at 7 days postinjury, matching the initiation of angiogenic activity that began after 1 week.[18] A similar time course for VEGF expression has been reported for fracture healing.[19]

The current study was unable to address the effect of HBO on VEGF expression prior to 6 weeks, as this was a retrospective study. VEGF expression following trauma in soft tissue and bone has been reported to return to normal within 21 days following trauma.[17,19]

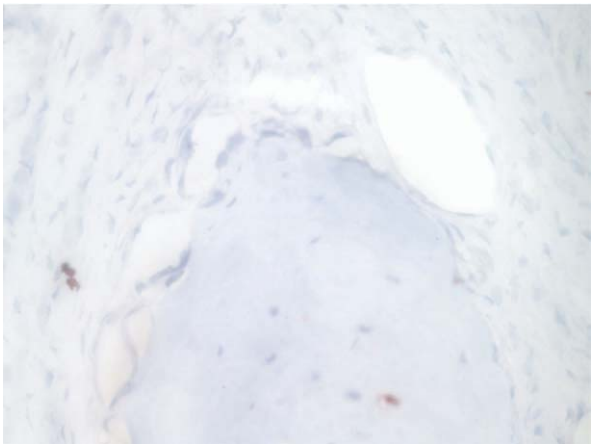

Fig. 5. VEGF-stained untreated defects at 12 weeks. VEGF staining in the untreated defects is similar to that in the HBO defects. (Magnification ×200.)

Table III. Comparison of VEGF staining between the center and margins of the defects

	Center	*Margin*	$P_{(center\ vs\ margin)}$
HBO, 6 weeks	633 ± 462	464 ± 246	.255
NBO, 6 weeks	181 ± 200	269 ± 188	.105

All values are group means ± SD of the subject means of stained area (μm^2) within a field of view.
Two random fields were measured from the central one third of each section and 4 random fields were measured from the margins of the same defect. Three sections were measured per defect.
Results of the 15- and 18-mm defects were considered separately in this analysis.
P values were calculated using the paired *t* test.
HBO, hyperbaric oxygen; *NBO,* normobaric oxygen; *VEGF,* vascular endothelial growth factor.

Consequently, while we expect that the VEGF levels in the NBO-treated animals had been elevated following trauma, they had returned to background levels before 6 weeks, explaining the similarity in levels observed between the 6-week and 12-week defects in the NBO group.

Nevertheless, we were able to demonstrate that VEGF levels were elevated 6-weeks following trauma when the rabbits had been exposed to HBO. It has been shown in a rabbit ischemic ear model that HBO therapy (100% O_2; 2.0 ATA (atmospheres absolute); 90 minutes a day for 14 days) transiently increased tissue oxygen partial pressure in the ischemic tissue from hypoxic levels to significantly above values seen in NBO nonischemic tissue. However O_2 partial pressure returned to ischemic values within 4 hours.[20] It is possible in our study that the cycling between hyperbaric and hypoxic conditions caused by the repeated 90-minute treatments of 2.4 atmosphere hyperbaric oxygen exposes the cells of the wound to a normoxic or hyperoxic environment at the injured site, removing the hypoxic stimuli for VEGF synthesis. However, upon return to normal atmosphere the defects reexperienced a hypoxic environment. This change from normoxic to hypoxic conditions may have resulted in the continued and prolonged synthesis of VEGF beyond what would occur in healing under NBO conditions. While this theory must be tested, there are examples where the same stimuli can alter gene expression and tissue formation depending on whether it was applied in a constant or cyclic manner. Examples include differences between constant and cyclic hydrostatic pressure[21] and cyclic and chronic administration of parathyroid hormone (PTH).[22]

Sheikh et al.,[23] using a different HBO protocol (100% O_2; 2.1 ATA; 90 minutes, twice per day for 7days), demonstrated elevated VEGF levels in a subcutaneous wound cylinder mouse model following 7 days of HBO. However, in contrast to our results, they reported that VEGF levels returned to baseline within 3 days of termination of treatment. This may have been due to the differences in the HBO protocol, duration of treatment, and tissue and/or species studied.

Another interesting finding was that there was no difference in VEGF expression between the 2 different defect sizes under either of the treatment conditions. It is possible that in the NBO group differences may have been observed if we had been able to look at earlier stages of healing, prior to VEGF returning to basal levels. In the case of the HBO treatments, time of observation may also have been a factor. However, it is also possible that as complete union of both the 15- and 18-mm defects did occur at 12 weeks, the HBO therapy was able to induce VEGF expression evenly across the whole defect.

As this study was a retrospective study using archived tissue samples, the study was not optimized for quantifying the VEGF expression. Specific shortcomings in the study design include that the sample preparation was not optimized for immunohistochemical analysis, as samples were fixed in 10% neutral formalin and demineralized using formic acid. Second, the time points studied, while useful for studying defect repair, did not permit us to investigate the levels of VEGF earlier at periods previously seen to have elevated VEGF during normoxic healing and when angiogenesis would have been initiated. Third, VEGF exists as multiple isoforms, and there is differential expression of these isoforms during healing.[24] The antibody used in this study was raised against one of the most common isoforms, $VEGF_{121}$, however we do not know it's cross-reactivity with the other isoforms.

In conclusion, this retrospective study did demonstrate differences in VEGF expression between HBO and NBO and is the first study we are aware of to report such differences during bone repair, or over such an extended period in any tissues.

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Effect of hyperbaric oxygen on grafted and nongrafted calvarial critical-sized defects

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Objectives. This study was undertaken to evaluate the effect of hyperbaric oxygen (HBO) on the repair of critical-sized defects in the presence and absence of a nonvascularized autogenous bone graft.

Study design. Ten New Zealand White rabbits were randomly divided into 2 groups of 5 animals each. Bilateral 15-mm calvarial defects were created in the parietal bones of each animal, resulting in 20 critical-sized defects. Autogenous bone grafts (ABG) were allocated to the left or right defect of each animal. Group 1 received HBO treatment at 2.4 ATA 100% oxygen for 90 minutes per day 5 days a week for 4 weeks. Group 2 served as a normobaric (NBO) control, breathing only room air. The animals in each group were humanely killed at 6 weeks. Calvaria were analyzed by micro-CT and histomorphometry.

Results. Micro-CT analysis indicated that as expected there was a higher bone mineral density (BMD) and bone mineral content (BMC) in ABG than unfilled defects ($P < .05$). However, there was a significant decline in the bone mineral content (BMC) of HBO-treated grafted defects compared to NBO-treated grafted defects ($P < .05$). Histologically complete bridging of the defect was observed in both NBO and HBO ABG grafted defects. Histomorphometic analysis showed that HBO treatment increased new bone and marrow, and reduced fibrous tissue in the defects ($P < .01$ for all). Examination of residual graft showed a near significant reduction in residual graft volume (11.2 ± 4.7 versus 19.1 ± 7.7, HBO versus NBO $P = .085$) in the HBO group. The use of a graft increased new bone and marrow in the NBO group ($P < .001$ for both); however, in the HBO-treated animals the differences between grafted and ungrafted were not significant.

Conclusion. HBO enhances bony healing in ungrafted rabbit calvarial critical-sized defects and may increase the rate of residual graft resorption in autogenous bone–grafted defects. **(Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2009;107:157-163)**

Following trauma there is a vascular disruption that leads to the formation of a hypoxic zone. While hypoxia is necessary to stimulate angiogenesis and revascularization, extended hypoxia will blunt the healing

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process. Hypoxia inhibits fibroblast proliferation, collagen synthesis, and granulation tissue formation.[1] Hyperbaric oxygen (HBO) therapy is the exposure of the patient to 100% oxygen at elevated pressure. HBO has been used to improve the healing of a variety of compromised or hypoxic wounds including diabetic ulcers, radiation-induced tissue damage, gangrene, and necrotizing anaerobic bacterial infections.[2,3]

HBO therapy is thought to increase wound healing by increasing the amount of oxygen dissolved in the blood (oxygen tension), which in turn can increase the amount of oxygen delivered to the hypoxic wound site.[4] HBO can promote angiogenesis[5] and results in an increase in the vessel density in irradiated tissue.[6]

The current standard of practice for the treatment of critical-sized defects is the use of autogenous bone grafts. While for small defects the graft can be obtained intraorally,[7] larger defects require the harvesting of bone extraorally, which requires a second surgical site and results in increased risk of complications.

Studies have demonstrated that HBO increases bone formation in bone harvest chambers in rabbits[8] and elevates alkaline phosphatase activity, a marker of bone formation, in rats following mandibular osteotomy[9] and increased osteoblastic activity and angiogenesis in irradiated mandibles undergoing distraction.[10]

To our knowledge, only one study has investigated the ability of HBO to promote bony healing of critical-sized defects. This study demonstrated that HBO enabled the healing of critical- and supra critical–sized calvarial defects in rabbits,[11] possibly due to the enhanced expression of vascular endothelial growth factor (VEGF).[12] These results suggest that HBO may minimize the amount of graft required to achieve adequate bone healing of such defects.

The aim of the current study was to determine whether the use of HBO in the absence of a graft produces critical-sized defect healing equivalent to that caused by a graft and whether HBO enhances healing of defects that do contain autogenous bone grafts.

MATERIALS AND METHODS

Surgical protocol

Ten adult, skeletally mature, male New Zealand White rabbits weighing 3 to 4 kg were randomly divided into 2 groups of 5 animals. The animals of Group 1 (HBO) underwent HBO treatment using a protocol described in a previous study.[11] Rabbits were placed in a hyperbaric chamber and exposed to 100% oxygen at 2.4 atmospheres absolute for 90 minutes per day for 5 days a week for 4 week (20 days total). The animals of Group 2 served as normobaric controls (NBO) and were left to heal at room air without any further intervention.

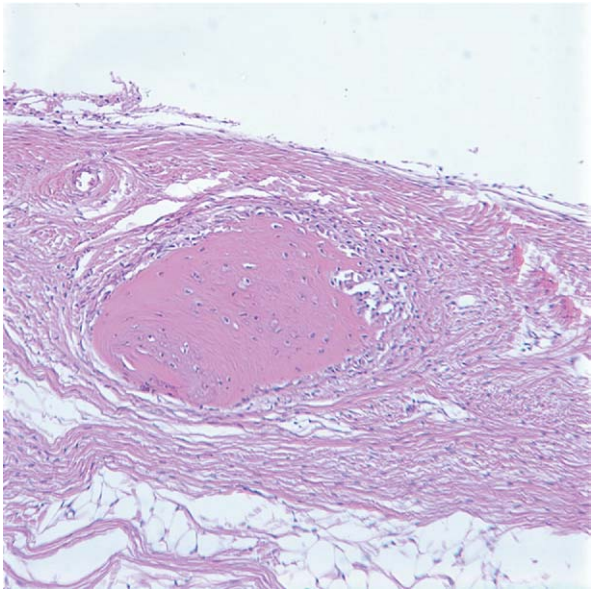

Fig. 1. Unfilled defect from rabbits under normobaric conditions. These defects were mostly filled with fibrous tissue, with the occasional areas of bone formation and very little marrow (hematoxylin and eosin [H&E], original magnification ×20).

Before the HBO treatment sessions, bilateral 15-mm full-thickness osseous defects were created surgically in the parietal bones of all the animals. The surgical procedure was performed in sterile fashion in accordance with the University of Toronto animal research committee protocol number 20005155. Autogenous bone grafts (ABG) were placed randomly on the right or left side of each animal (Fig. 1). The other defect was left unfilled. HBO treatment was initiated 24 hours after surgery.

Rabbits were humanely killed 6 weeks postoperatively. The parietal bones were harvested using an oscillating saw preserving the pericranium over the defects. Care was also taken to preserve the sagittal, coronal, and the lambdoid sutures because they served as a reference to the circumference of defects. The final harvested specimens measured 30 × 25 × 12 mm in the greatest dimensions. Specimens were fixed in 10% formalin prior to analysis by micro-computed tomography (micro-CT).

Micro-computed tomography

This study used an Explore Locus SP micro-CT scanner (GE Medical Systems, London, Ontario, Canada). Prior to scanning the specimens a calibration scan was performed using a synthetic bone, a water, and an air sample.

Calvarial specimens were scanned using the fast mode using 0.05-mm sections. Each specimen took 120

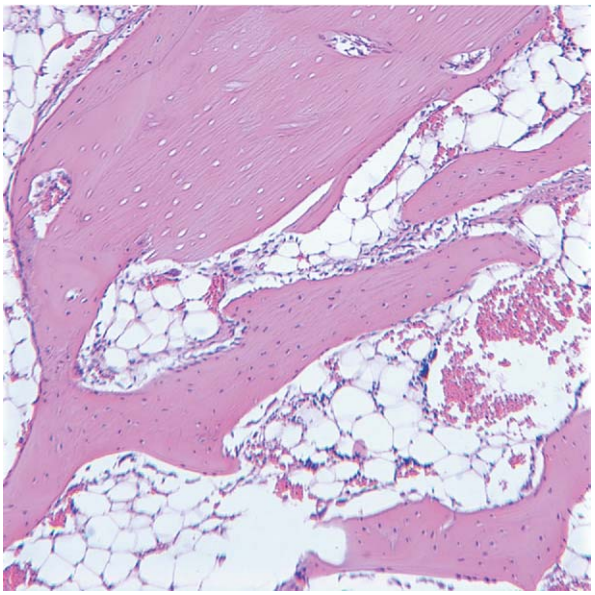

Fig. 2. Autogenous bone–grafted defects under normobaric conditions.Trabeculae of new bone formed around and sometimes incorporated the residual autogenous bone graft (ABG). The ABG could be clearly distinguished from the new bone due to the empty osteocyte lacunae. Extensive marrow is present throughout the defect (H&E, original magnification ×20).

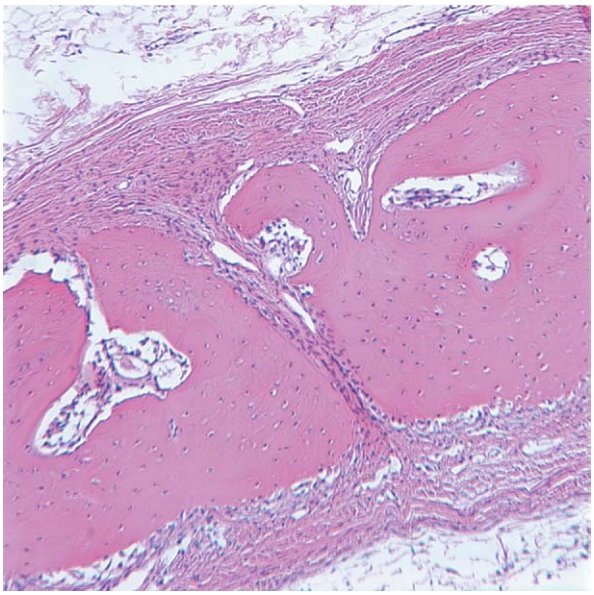

Fig. 3. Unfilled defect exposed to hyperbaric oxygen conditions. Thick blocks of new bone are surrounded by dense fibrous tissue. Only small amounts of marrow are seen, within the new bone (H&E, original magnification ×20).

minutes to be fully scanned. Reconstruction of scanned images (Fig. 2) was done using Microview software (GE Medical Systems) after calibrating using the bone, water, and air standard values. The reconstructed 3-demensional (3D) image was then traced in 3 dimensions to the circumference of the original defect margins. This allowed the creation of a 3D reconstruction of the defect, which was referred to as the region of interest (ROI) (Fig. 3). A threshold level was selected manually based on 25% of the bone standard provided by the manufacturer.

The ROI of each specimen was analyzed for total volume (TV), bone volume (BV), the bone volume fraction (BVF = BV/TV), bone mineral content (BMC), and bone mineral density (BMD). The software also permitted us to measure the mineral content and mineral density of the voxels, which are counted as "bone" based on the threshold setting selected. These measures are called the tissue mineral content (TMC) and tissue mineral density (TMD).

Histomorphometry

Upon completion of micro-CT scanning, specimens were decalcified using 45% formic acid and 20% sodium citrate for 4 weeks. Each bone was sectioned into 2 portions: an anterior and posterior portion. Both portions were embedded in paraffin and 7-μm sections were prepared and stained with hematoxylin and eosin. Sections in the middle of the defects, representing the greatest dimension, were examined under the light microscope. Ten randomized sections within the middle of defects were digitized using a digital camera (RT Color; Diagnostic Instruments Inc., Sterling Heights, MI). A blinded investigator traced the images for new bone formation.

Statistical analysis

All data were tested for normality and equal variance. Results were compared across all groups using 1- and 2-way analysis of variance (ANOVA) with Student Newman Keuls (SNK) post hoc testing for normal data of equal variance or Holm-Sidak post hoc testing for non-normal/non-equal variance data. Paired *t* tests were used to compare the defects within the animals. As there was only graft in 2 groups, a *t* test was used to compare the amount of residual graft between the HBO and NBO defects that had been grafted with ABG. All statistical analyses were performed using SigmaStat v3.5 (Systat Software, San Jose, CA). Statistical significance was considered to be P less than .05.

RESULTS

Histological analysis

The unfilled non-HBO defects were predominantly filled with fibrous tissue that contained an occasional

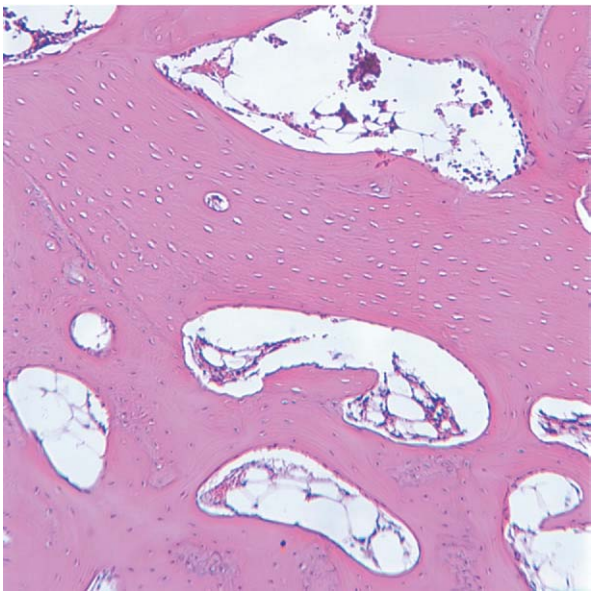

Fig. 4. Autogenous bone–grafted defects under hyperbaric oxygen conditions. New bone forms along with and merges into the residual graft matrix. The remaining space is filled with marrow. No fibrous tissue is visible (H&E, original magnification ×20).

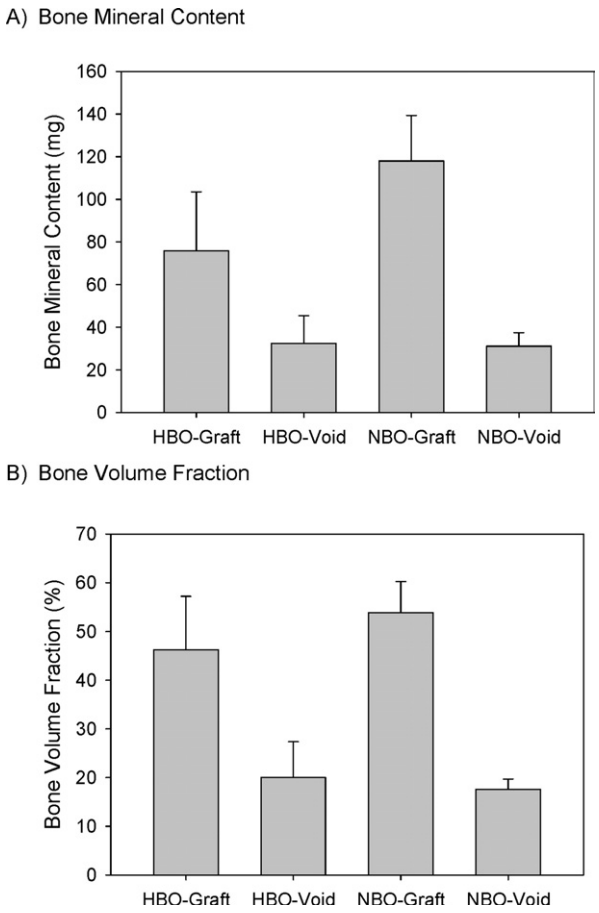

Fig. 5. Micro-CT results. Bone mineral content (BMC) (**A**) and bone volume fraction (BVF) (**B**).The defects containing autogenous bone grafts have significantly higher BMC and BVF than the ungrafted defects ($P < .05$). Comparisons between the HBO and NBO autogenous bone–grafted defects revealed that the HBO-grafted defects had significantly lower BMC ($P < .05$), although the reductions in BVF did not reach significance ($P = .123$). No significant differences between the HBO and NBO unfilled defects were seen in either of these parameters. Data are plotted as mean ± SD.

blood vessel. New bone was mostly restricted to the defect margins, although small islands of woven bone were occasionally seen in the fibrous tissue more centrally (Fig. 1).

The ABG non-HBO defects were completely bridged with bony tissue. The defects were filled with a mixture of nonvital cortical bone from the graft, newly formed bone, and vascular marrow (Fig. 2).

It was difficult to distinguish the defect margin in the unfilled HBO-treated defects. The defects were completely bridged with new bone, which tended to thin toward the center of the defects, with the dural and periosteal edges being composed of denser fibrous tissue. The new bone contained marrow elements (Fig. 3), which were not as extensive as in the grafted defects.

Similar to the HBO void defects the margin of the defect was difficult to identify in the ABG-grafted HBO-treated defects. These defects were completely bridged with extensive amounts of new bone and marrow. Devitalized remnants of the graft were still visible (Fig. 4).

Micro-computed tomography

Statistical analysis of the micro-CT results indicated that the presence of the graft resulted in increased BV, BMC, BMD, and BVF compared to unfilled defects under both NBO and HBO ($P < .05$) (Fig. 5). Comparisons between the HBO and NBO autogenous bone–grafted defects revealed that the HBO-grafted defects had significantly lower BV ($P = .03$) and BMC ($P < .05$), although the reductions in BVF and BMD did not reach significance ($P = .123$ and .078, respectively). No significant differences between the HBO and NBO unfilled defects were seen in any of the parameters measured (Table I).

Histomorphometry

Histological analysis (Table II) demonstrated that HBO-treated unfilled defects had significantly more new bone ($P < .001$) and marrow ($P < .05$) and less fibrous tissue ($P < .05$) than unfilled defects exposed

Table I. Micro-CT analysis

	NBO		*HBO*		*1-way ANOVA*
	Unfilled	*ABG*	*Unfilled*	*ABG*	$P_{between\ groups}$
TV(mm^3)	233 ± 37	263 ± 55	191 ± 27	189 ± 66	.076
BV(mm^3)	41 ± 7	140 ± 22	39 ± 17	87 ± 36	<.001
BVF (%)	17.6 ± 2.1	53.9 ± 6.4	20.0 ± 7.3	46.2 ± 11.0	<.001
BMC (mg)	31.2 ± 6.3	118.0 ± 21.3	32.4 ± 13.0	76.0 ± 27.6	.002*
BMD (mg/mm^3)	134 ± 20	451 ± 25	167 ± 53	403 ± 52	<.001
TMC	25.6 ± 4.5	90.3 ± 13.5	23.8 ± 11.3	53.9 ± 24.1	<.001
TMD	627 ± 27	645 ± 20	602 ± 26	612 ± 32	.096

NBO, normobaric oxygen; HBO, hyperbaric oxygen; ABG, autogenous bone graft; ANOVA, analysis of variance; TV, total volume; BV, bone volume; BVF, bone volume fraction; BMC, bone mineral content; BMD, bone mineral density; TMC, tissue mineral content; TMD, tissue mineral density.
*Equal variance test failed – *P* calculated using 1-way ANOVA on Ranks.

Table II. Histomorphometric analysis

	NBO		*HBO*		*1-way ANOVA*
Type of tissue (%)	*Unfilled*	*ABG*	*Unfilled*	*ABG*	$P_{between\ groups}$
New Bone	19.7 ± 2.6	36.6 ± 8.6	46.7 ± 5.3	41.4 ± 6.9	<.001
Marrow	6.3 ± 3.6	37.8 ± 9.1	26.7 ± 9.0	38.7 ± 5.7	.004*
Bone + Marrow	26.0 ± 2.7	74.4 ± 8.1	73.5 ± 12.5	80.1 ± 5.5	.008†
Fibrous	74.0 ± 2.7	6.4 ± 1.8	26.5 ± 12.5	8.8 ± 6.1	<.001†
Graft	N/A	19.1 ± 7.7	N/A	11.2 ± 4.7	.085‡

NBO, normobaric oxygen; HBO, hyperbaric oxygen; ABG, autogenous bone graft; ANOVA, analysis of variance.
*Data not normal *P* value generated by 1-way ANOVA on Ranks.
†Data not equal variance, *P* value generated by 1-way ANOVA on Ranks.
‡*P* value for residual graft was determined by *t* test.

to normobaric air (Fig. 6). Defects grafted with autogenous bone showed no statistical differences in any of the parameters measured, although the lesser amount of residual graft present in the HBO compared to NBO defects neared significance ($P = .085$) (Fig. 7).

As expected in the normobaric defects there was significantly more new bone and marrow and less fibrous tissue in the grafted defects ($P < .05$). However, comparing the unfilled and autogenous bone–grafted defects in the HBO-treated animals there was no significant difference in the amount of new bone ($P = .196$), although there was less marrow and more fibrous tissue in the unfilled defects ($P < .05$).

DISCUSSION

The current "gold standard" for the treatment of bony defects that are unable to heal spontaneously (critical-sized defects) is the use of autogenous bone grafts. However, the volume of bone available for grafting is limited and the use of large grafts results in significant complications including donor site morbidity, loss of blood, and extended time in surgery. HBO therapy has been shown to promote the healing of unfilled critical-size defects in rabbits, and may be useful as a replacement for autogenous grafts in certain instances and as an adjunct therapy to autogenous bone grafting, minimizing the amount of graft required in others. The aim of this study was to evaluate the effect of HBO on the healing of critical-sized defects in the presence and absence of autogenous bone grafts in comparison to defect healing with grafts under normobaric conditions.

The current study demonstrated that histologically there was significantly more bone ($P < .001$) and marrow ($P < .05$) in the HBO-treated unfilled defect than the control unfilled defects in untreated animals. These results agree with the only other previous study to investigate the effect of HBO on critical-size defect healing.[11] Interestingly, we were unable to demonstrate this difference by micro-CT, suggesting that the newly formed bone had not yet matured and become fully mineralized.

Comparison of the amounts of new bone in the HBO-treated unfilled defect and the normobaric autografted defect indicated more bone in the HBO group that neared significance (46.7 ± 5.3 versus 36.6 ± 8.6; $P = .054$). Conversely, there was significantly less

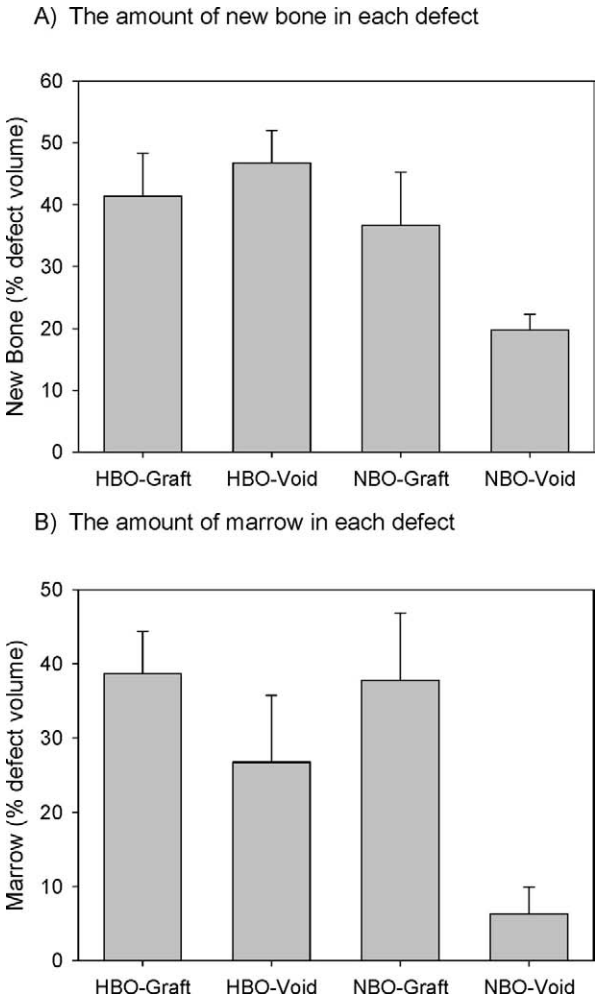

Fig. 6. Histomorphometry results. **A,** Amount of new bone. **B,** The amount of marrow.HBO-treated unfilled defects had significantly more new bone ($P < .001$) and marrow ($P < .05$) than unfilled defects exposed to normobaric air. Comparing the unfilled and autogenous bone–grafted defects in the HBO-treated animals there was no significant difference in the amount of new bone ($P = .196$), although there was less marrow in the unfilled defects ($P < .05$). Data are plotted as mean ± SD.

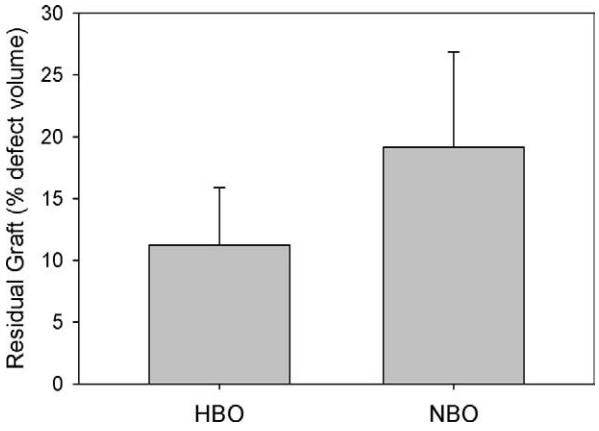

Fig. 7. Amount of residual graft in the grafted defects. The lesser amount of residual graft present in the HBO compared to HBO defects neared significance ($P = .085$). Data are plotted as mean ± SD.

marrow and more fibrous tissue in the HBO unfilled defect than the NBO-grafted defect (marrow: 26.7 ± 9.0 versus 37.8 ± 9.1; $P < .05$; fibrous 26.5 ± 12.5 versus 6.4 ± 1.8; $P < .05$). When the amount of bone and marrow are considered as a single measure of "reparative tissue" the amounts in the HBO unfilled and NBO-grafted defects were almost identical (73.5 ± 12.5 versus 74.4 ± 8.1), with the a volume equivalent to that occupied by residual graft in the NBO defects being occupied by fibrous tissue in the HBO-treated defects.

Histomorphometric comparison of the HBO and NBO defects that contained autogenous grafts revealed there were no significant differences, although the reduction in the amount of residual graft in the HBO group neared significance (11.2 ± 4.7 versus 19.1 ± 7.7; $P = .085$). However, the micro-CT did show that there was a significant reduction in the bone mineral content of the defect ($P < .05$) and a near significant reduction in bone mineral density ($P = .078$). The difference between the micro-CT and histomorphometric results may indicate that there is increased resorption and/or demineralization of the residual graft, which would not as easily be detected by histomorphometry.

Sawai et al.[13] investigated the effect of HBO on mandibular defect healing with autogenous bone grafts in rabbits by histology. They reported that HBO increased the amount of bone formed initially and that the graft becomes incorporated into the surrounding bone making it difficult to distinguish the graft from new bone after 4 weeks, although they did not evaluate the effects quantitatively. Chen et al.[14] demonstrated that HBO increased the rate of union of rabbit spinal fusions in the presence of autogenous grafts.

In conclusion, HBO enhances bony healing in non-grafted rabbit calvarial critical-sized defects with a bone volume that is comparable with autogenous non-vascularized bone grafting. HBO resulted in a reduction of the bone mineral content of the autogenous bone–grafted sites. Angiogenesis within the graft may account for the reduction in BMD observed during the experimental period of this study.

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Effect of hyperbaric oxygen on demineralized bone matrix and biphasic calcium phosphate bone substitutes

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UNIVERSITY OF TORONTO, UNIVERSITY OF TAMPERE, UNIVERSITY OF OULU, UNIVERSITY OF BELGRADE, PUSAN NATIONAL UNIVERSITY, AND SECOND HOSPITAL YUANNAN PROVINCE

Objectives. The aim of this study was to assess the possible effect of hyperbaric oxygen (HBO) on the healing of critical-sized defects that were grafted with demineralized bone matrix (DBM) combined with Pluronic F127 (F127) to form a gel or putty, or a commercially available biphasic calcium phosphate (BCP), mixed either with blood or F127 to form a putty.

Study design. Twenty New Zealand White rabbits were randomly divided into 2 groups of 10 animals each. Bilateral 15-mm calvarial defects were created in the parietal bones of each animal, resulting in 40 critical-sized defects. Group I defects were grafted with either DBM putty or DBM gel. Group II defects were grafted with either BCP or BCP putty. Five animals from each group received HBO treatment (100% oxygen, at 2.4 ATA) for 90 minutes per day 5 days a week for 4 weeks. The other 5 animals in each group served as a normobaric (NBO) controls, breathing only room air. All animals were humanely killed at 6 weeks. The calvariae were removed and analyzed by micro computed tomography (mCT) and histomorphometry.

Results. mCT analysis indicated a higher bone mineral content (BMC, $P < .05$), bone volume fraction (BVF; $P < .001$), and bone mineral density (BMD; $P < .001$) of the defects grafted with BCP rather than DBM. Furthermore, the voxels that were counted as bone had a higher tissue mineral density (TMD) in the BCP- than in the DBM-filled defects ($P < .001$).

Histologically complete bony union over the defects was observed in all specimens. Histomorphometric analysis showed that DBM-filled defects had more new bone ($P < .007$) and marrow ($P < .001$), and reduced fibrous tissue compared with the BCP defects ($P < .001$) under NBO conditions.

HBO treatment reduced the amount of fibrous tissue in BCP filled defects ($P < .05$), approaching levels similar to that in matching DBM-filled defects. HBO also resulted in a small but significant increase in new bone in DBM-grafted defects ($P < .05$).

Conclusion. Use of DBM or BCP promoted healing in these critical-sized defects. Hyperbaric oxygen therapy resulted in a slight increase in new bone in DBM-grafted defects and much larger reduction in fibrous tissue and matching increases in marrow in BCP-grafted defects, possibly through increased promotion of angiogenesis. **(Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2010;109:59-66)**

Vascular disruption and hypoxia are consequences of the creation of bony defects.[1] Although hypoxia has shown to stimulate vascular in-growth, extended hypoxia will blunt the healing process.[2-4] Hyperbaric oxy-

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gen (HBO) has been successfully used to improve the healing of bony defects that will not heal spontaneously (critical-sized defects).[5] The mechanism by which HBO is believed to work is that it increases the amount of oxygen dissolved in the blood (oxygen tension), which in turn can increase the amount of oxygen delivered to the hypoxic wound site, stimulating angiogenesis and osteogenesis.[6,7]

The current standard for the treatment of critical-sized defects is the use of autogenous bone grafts[8]; however, studies have shown that bone graft substitutes can be used to promote bony regeneration in critical-sized defects.[9] Demineralized bone matrix (DBM) has been shown to induce bony in-growth to critical-sized defects; however, this material lacks rigidity because of lack of mineralized components in its matrix. Alternatively, ceramics such as hydroxyapatite have been shown to have good rigidity but they take longer to resorb and be replaced by bone. Ceramics also have a tendency to integrate by fibrous union. HBO has been demonstrated to reduce bone mineral density (BMD) in particulate autogenous bone grafts possibly by activating angiogenesis and resorption of the grafted matrix.[10,11] These results suggest that HBO may promote resorption and minimize fibrous union when ceramics are used.

The aim of this study was to determine if the use of HBO in combination with DBM or bone ceramic enhances osseous healing and whether HBO prevents fibrous union when bone ceramics are used.

MATERIALS AND METHODS

The surgical protocol for this study was approved by the University of Toronto Animal Care and Ethics Committee (Protocol number 20005145). The surgical technique, anesthetics, and prophylactic antibiotics were described in a previous publication.[6] In brief, bilateral 15-mm full thickness osseous defects were created surgically in the parietal bones of 20 adult, skeletally mature, male New Zealand White rabbits weighing 3 to 4 kg. Bone graft substitutes were placed randomly on the right or left side of each animal. Animals were randomly divided into 2 groups of 10 subjects.

Experimental design

Group I (DBM) n = 10. Allogeneic demineralized bone matrix was prepared from rabbit long bones by treatment with 0.6 M HCl for 48 hours followed by extensive washing and lyophilization.

Pluronic F-127 (F127; poloxamer 407, BASF Canada Inc., Toronto, Canada) was prepared by slowly adding 33g F127 to 100 mL MilliQ water held at 4°C. The F127 was then autoclaved for sterility.

Animals of Group 1 were grafted with either DBM putty or DBM gel, both of which were prepared with allogeneic rabbits' long bones. DBM putty was prepared as a mixture of 70% DBM and 30% Pluronic F-127 gel. DBM gel was prepared as a mixture of 40% DBM and 60% Pluronic F-127 gel. The main differences between the gel and the putty are the consistency of the mixture and the amount of DBM in the compound.

Group II (BCP) n = 10. Animals of Group II were grafted with either BCP or BCP putty. Biphasic calcium phosphate (Straumann AG, Basel, Switzerland) is a commercially available synthetic bone substitute. It is made of a mixture of 60% hydroxyapatite and 40% tricalcium phosphate. BCP putty was prepared as a mixture of 70% BCP with 30% Pluronic F-127 gel. In defects where BCP was used without the gel, it was mixed with the animal's own blood.

Hyperbaric oxygen therapy. Five animals from each group underwent HBO. Rabbits were placed in a hyperbaric chamber and exposed to 100% oxygen at 2.4 atmospheres absolute for 90 minutes per day for 5 days a week for 4 weeks (20 treatments total). Five animals in each group served as normobaric controls (NBO) and were left to heal at room air without any further intervention. HBO treatment was initiated 24 hours after surgery.

Rabbits were humanely killed 6 weeks postoperatively. The parietal bones were harvested using an oscillating saw. Care was also taken to preserve the pericranium, the sagittal, coronal, and the lambdoid sutures because they served as a reference to the circumference of the defects. The final harvested specimens measured 30 × 25 × 12 mm in the greatest dimensions. Specimens were fixed in 10% formalin before analysis by micro computed tomography (mCT).

Micro-computed tomography

This study used an Explore Locus SP micro CT scanner (GE medical systems, London, Ontario, Canada). Before scanning the specimens, a calibration scan was performed using the manufacturers' standard, which includes synthetic bone, water, and an air sample.

Calvarial specimens were scanned at a resolution of 28 μm. Reconstruction of scanned images was done using the manufacturer's software after calibration using the bone, water, and air standard values. The reconstructed 3-dimensional image was then analyzed using MicroView software (v2.1.2 GE Medical Systems, London, Ontario, Canada). Before analysis, threshold values must be determined that permit the software to distinguish bone and ceramic from soft tissue. A lower threshold was selected that would count bone + ceramic by identifying areas of bone within the defects tracing regions of interest that incorporated only

Table I. MicroCT analysis

	DBM				*BCP*				
	GEL		*PUTTY*		*Blood*		*PUTTY*		*ANOVA*
	NBO	*HBO*	*NBO*	*HBO*	*NBO*	*HBO*	*NBO*	*HBO*	*P*
Vol (mm^3)	186 ± 103	161 ± 42	177 ± 95	179 ± 18	162 ± 56	180 ± 30	143 ± 14	128 ± 28	.096
BV (mm^3)	67 ± 36	62 ± 8	64 ± 36	65 ± 10	103 ± 33	111 ± 29	91 ± 9	72 ± 21	.017
BVF (%)	35 ± 7	40 ± 7	37 ± 8	34 ± 7	64 ± 4	61 ± 6	64 ± 3	56 ± 8	<.001
BMC (mg)	46 ± 23	38 ± 7	41 ± 22	41 ± 5	110 ± 36	116 ± 27	84 ± 7	66 ± 19	<.001*
TMC (mg)	29 ± 16	25 ± 4	27 ± 16	26 ± 5	107 ± 35	113 ± 29	80 ± 7	61 ± 19	<.001*
BMD (mg/mL)	223 ± 34	244 ± 28	232 ± 36	233 ± 27	682 ± 43	641 ± 81	594 ± 52	512 ± 72	<.001
TMD (mg/mL)	416 ± 30	410 ± 20	410 ± 4	401 ± 11	563 ± 23	547 ± 21	530 ± 23	506 ± 10	<.001

DBM, demineralized bone matrix; *BCP,* biphasic calcium phosphate; *GEL,* the DBM granules were mixed with Pluronic F127 at 40% DBM to 60% F127 (vol/vol); *PUTTY,* the DBM or BCP granules were mixed with F127 at 70% DBM/BCP to 30% F127 (vol/vol); *NBO,* normobaric oxygen; *HBO,* hyperbaric oxygen; *BV,* bone volume; *BVF,* bone volume fraction; *BMC,* bone mineral content; *TMC,* tissue mineral content; *BMD,* bone mineral density; *TMD,* tissue mineral density.
*Data were not normally distributed and groups were compared using analysis of variance (ANOVA) on Ranks.

bone and then determining the bone volume fraction (BVF) at a range of threshold values. The "bone + ceramic" threshold was determined to be the highest threshold value that gave a BVF greater than 0.95 for all bone ROIs (regions of interest).

The threshold value for the "ceramic only" was determined in a similar manner by tracing 8 particles in each defect that could be clearly identified. The highest threshold that resulted in every particle having a BVF (which in this case is the ceramic volume fraction) was greater than 0.95.

After determination of the threshold values, the margins of the defect were traced in 3 dimensions resulting in an ROI that incorporated the entire defect.

The ROI of each specimen was analyzed for total volume (TV), bone volume (BV), bone volume fraction (BVF = BV/TV), bone mineral content (BMC), and bone mineral density (BMD). The software also permitted measurement of the mineral content and mineral density of the voxels. which are counted as "bone" based on the threshold setting selected. These measures are called the tissue mineral content (TMC) and tissue mineral density (TMD). The measurements were made at both the bone + ceramic and ceramic only threshold values.

To correct for the presence of BCP in the defects corrected values for BV, BMC, BMD, TMC, TMD, and BVF were determined by subtracting the values obtained at the ceramic only threshold from those for the bone + ceramic threshold and recalculating.

Histomorphometry

Upon completion of mCT scanning, specimens were decalcified using 45% formic acid and 20% sodium citrate for 4 weeks. Each bone was sectioned into 2 portions: an anterior and posterior portion. Both portions were embedded in paraffin and 7-μm sections were prepared and stained with hematoxylin and eosin. Sections in the middle of the defects, representing the greatest cross-sectional dimension, were examined under the light microscope. Ten randomized sections within the middle of defects were digitized using a digital camera (RT Color; Diagnostic Instruments Inc., Sterling Heights, MI). A blinded investigator analyzed each section for the amount of new bone, marrow, fibrous tissue, and residual graft using Image ProPlus software (Media Cybernectics, Bethesda, MD).

Statistical analysis

Results were tested for normality and equal variance. Each parameter was compared across all groups using 1-way Analysis of Variance (ANOVA) for normal data and Kruskal-Wallis 1-way ANOVA on RANKS for non-normal data, followed by Student-Newman-Keuls (SNK) post hoc testing. Statistical significance was considered to be *P* less than .05. All analyses were done using Sigma Stat v3.5 (Systat Software, San Jose, CA).

RESULTS

Micro-computed tomography

All parameters were examined for significant differences between the different groups using ANOVA (Table I). Four different experimental conditions were compared in this study: BCP versus DBM, HBO versus NBO, and 2 different formulation comparisons of blood versus F127 putty within the BCP group and F127 gel versus F127 putty within the DBM group. Following a significant ANOVA result, the matched pairs that were exposed to these experimental conditions were tested for significant differences using a post hoc test (ie, for comparisons of BCP and DBM, the NBO-BCP-putty was compared with the NBO-DBM-putty and the

Table II. Post hoc *P* values for mCT parameters by matched groups

	Post hoc P values					
	BV[1]	*BVF*	*BMC*[2]	*TMC*[2]	*BMD*	*TMD*
BCP vs DBM						
Putty-HBO	.638	<.001	<.05	<.05	<.001	<.001
Putty-NBO	.098	<.001	<.05	<.05	<.001	<.001
Blood vs F127 putty						
BCP-HBO	.024	.294	<.05	<.05	<.001	.012
BCP-NBO	.459	.991	<.05	NSD	.026	.045
F127 gel vs putty						
DBM-HBO	.718	.718	NSD	NSD	.729	.506
DBM-NBO	.522	.829	NSD	NSD	.775	.693
NBO vs HBO						
BCP-blood	.661	.671	NSD	NSD	.206	.232
BCP-putty	.249	.152	<.05	<.05	.015	.087
DBM-gel	.661	.611	NSD	NSD	.905	.233
DBM-putty	.896	.896	NSD	NSD	.968	.087

Following a significant result in the 1-way ANOVA, the matched groups within each of the 4 different experimental conditions (BCP versus DBM, HBO versus NBO and 2 different formulation comparisons blood versus F127 putty with the BCP group and F127 gel versus F127 putty within the DBM group) were tested for significant differences using a post hoc test.

ANOVA, analysis of variance; *BV,* bone volume; *BVF,* bone volume fraction; *BMC,* bone mineral content; *TMC,* tissue mineral content; *BMD,* bone mineral density; *TMD,* tissue mineral density; *BCP,* biphasic calcium phosphate; *DBM,* demineralized bone matrix; *NBO,* normobaric oxygen; *HBO,* hyperbaric oxygen; *NSD,* not significantly different.

[1]Following a significant ANOVA test, none of the group comparisons for the BV were found to be significant by Student-Newman-Keuls post hoc testing. We therefore used Fisher's least significant difference (LSD) method to identify differences.

[2]*P* values were not obtained from post hoc testing of non-normal data and are reported as <.05 if they were found to be significantly different, or NSD if there were not.

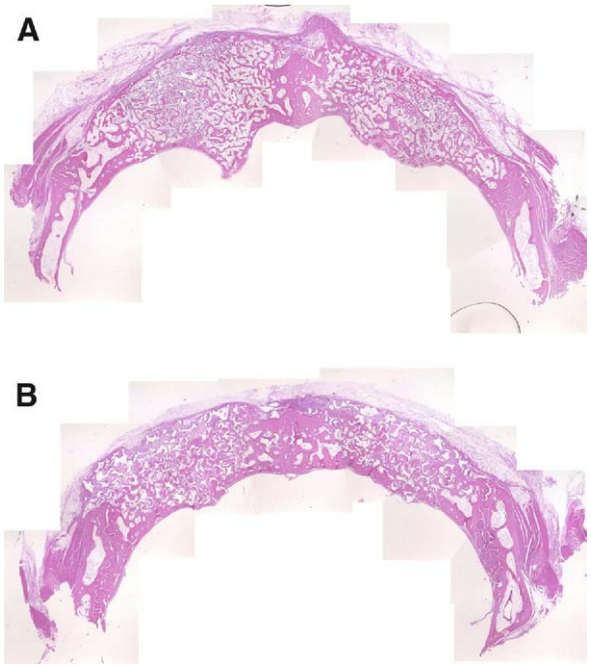

Fig. 1. **A,** Montage of photomicrographs covering an entire calvaria grafted with DBM gel and putty. Rabbit was exposed to HBO treatment. Bone bridges the entire defect resulting in complete union. (Hematoxylin and eosin, [H&E], original magnification of each photomicrograph ×4.) **B,** Montage of photomicrographs covering an entire calvaria grafted with BCP and BCP putty from a rabbit exposed to HBO treatment. Because of decalcification, the residual graft has been removed and so appears as voids in the defect. The defect is completely bridged by bone along its dural aspect. New bone and marrow elements are most common at the margins and dural aspect, with the tissue along the periosteal aspect being predominantly fibrous lighter. (H&E, original magnification of each photomicrograph ×4.)

HBO-BCP-putty was compared with the HBO-DBM-putty) (Table II).

ANOVA of the mCT results demonstrated significant differences between the groups for all parameters except total volume (see Table I). Identification of the groups that had significantly different bone volumes (BV) required use of the Fisher least significant differences (LSD) post hoc testing, as SNK was unable to do so. The bone volume was higher in the defects grafted with BCP mixed with blood compared with those mixed with F127 when exposed to HBO ($P = .024$) but not for the matching grafts under NBO. No other significant differences were seen in this parameter between the various matched groups.

Defects containing biphasic calcium phosphate had significantly higher BVF, BMC, TMC, BMD, and TMD than those containing demineralized bone matrix under the same experimental conditions (ie, NBO-putty and HBO-putty) ($P < .05$).

HBO reduced BMC, TMC, BMD and TMD in the BCP-putty treated defects, but not the defects filled with BCP mixed with blood. HBO did not alter any of the other parameters in any of the other groups.

Defects grafted with BCP mixed with blood compared with BCP mixed with F127 had significantly higher BMC, BMD, and TMD under NBO and HBO conditions. The amount of F127 mixed with DBM had no significant effects on any of the parameters measured.

Histological analysis

The DBM- and BCP-filled defects each had their own unique histological appearance (Fig. 1, *A* and *B*). The DBM gel- and putty-filled defects revealed the same microscopic features. All defects were bridged with a mixture of bone and residual bone substitute (Fig. 1, *A*).

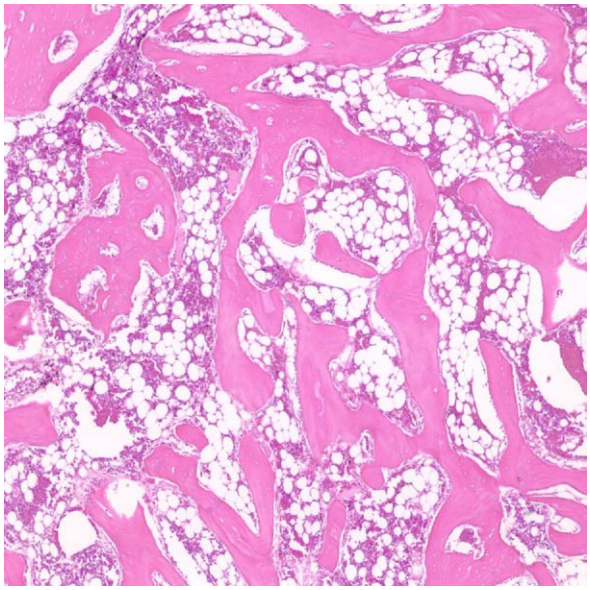

Fig. 2. Photomicrograph of a defect of a rabbit filled with DBM putty from the NBO group. Extensive marrow (M) and new bone (NB), which is often apposed to residual DBM, can be seen throughout the field. (H&E, original magnification ×20.)

DBM-filled defects contained bone, marrow, and some fibrous tissue as well as residual DBM particles (Fig. 2). Residual DBM contained empty lacunae (Fig. 3). Woven bone bounded the residual DBM and was lined by cuboidal cells that appeared morphologically to be osteoblasts (Fig. 3, *A* and *B*). New bone formation was also noted within the DBM particles.

BCP defects revealed similar microscopic features to DBM defects (Fig. 1, *B*), although fibrous tissue was more pronounced and could be found throughout the defect (Figs. 4 and 5, *A* and *B*), while it was limited to the margins in the DBM groups.

Histomorphometry

One-way ANOVA analysis revealed that there were significant differences within all parameters measured (Tables III and IV). Histomorphometric analysis demonstrated that DBM-filled defects had significantly more new bone ($P < .008$) and less residual graft ($P < .001$) than matching BCP-filled defects. Although DBM-filled defects exposed to NBO also had increased marrow and reduced fibrous tissue ($P < .001$ for both), when the BCP defects had been exposed to HBO these differences were abolished. These results also correlated with an observed increase in marrow and reduction in fibrous tissue in BCP defects exposed to HBO compared with BCP-grafted defects under NBO conditions.

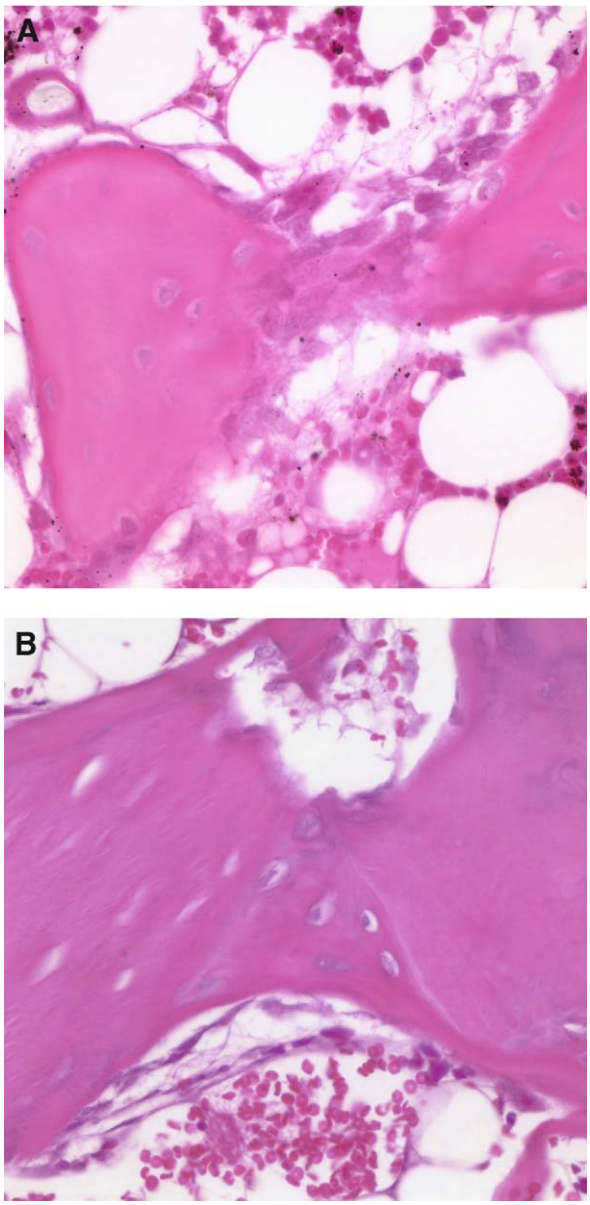

Fig. 3. **A,** Photomicrograph of a defect of a rabbit filled with DBM gel and treated with hyperbaric oxygen. Deposits of new bone are associated with cuboidal osteoblastlike cells some of which appear to be becoming entrapped in the bone matrix. (H&E, original magnification ×40.) **B,** Photomicrograph of a defect from rabbit filled with DBM putty and treated with hyperbaric oxygen. The new bone formed on the residual DBM can clearly be distinguished from the DBM by the presence of larger rounded lacunae that contain cells compared to the elliptical empty lacunae present in the DBM (H&E, original magnification ×40).

HBO was also seen to lead to a small but significant increase in the amount of new bone in the DBM-grafted defects ($P < .04$). Both marrow and fibrous tissue were

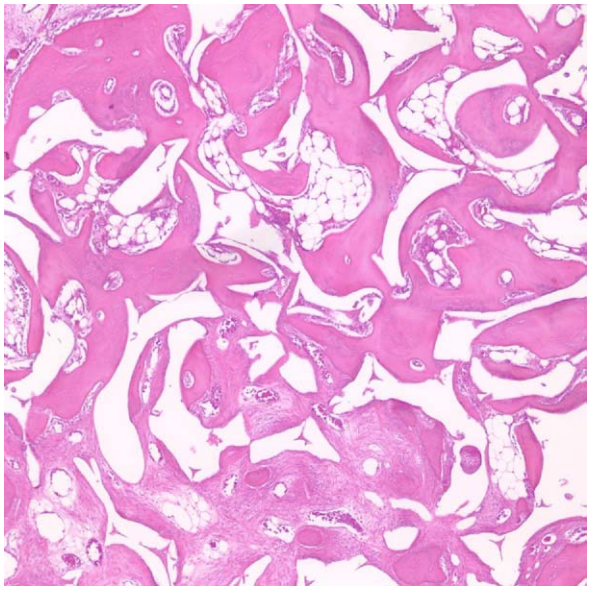

Fig. 4. Photomicrograph of a defect filled with BCP putty from a rabbit in the NBO group. The large empty voids are where the biphasic calcium phosphate particles were present. They were lost during decalcification. New bone, marrow, and fibrous tissue with a thick cellular infiltration can been seen apposed to the BCP particles (H&E, original magnification ×20).

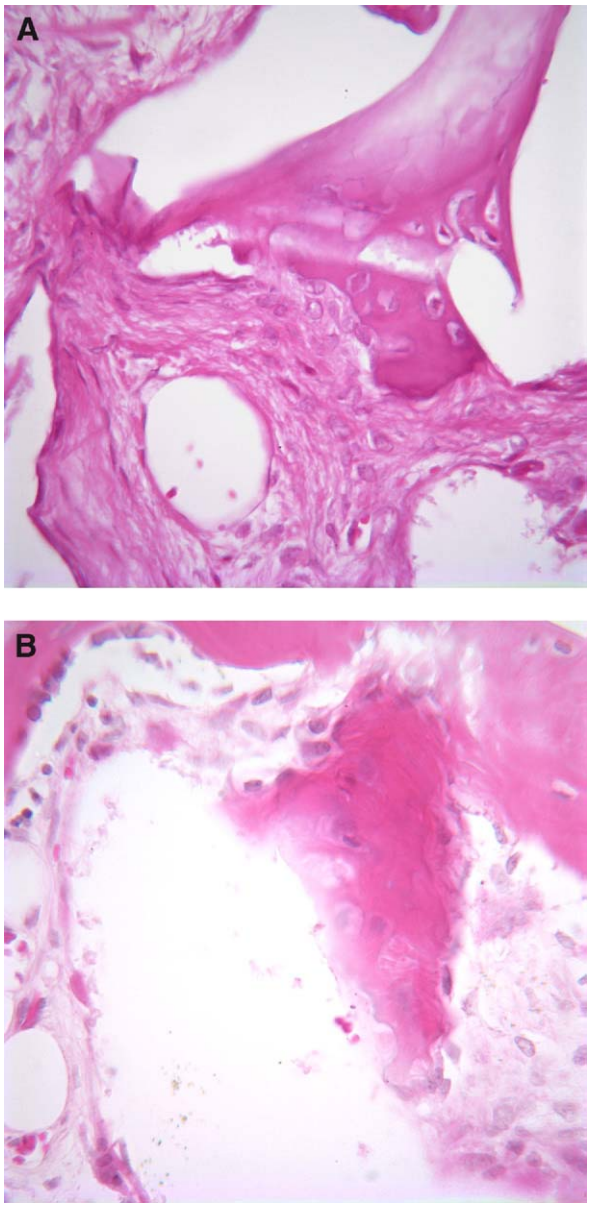

Fig. 5. **A,** Photomicrograph of a defect filled with BCP Putty in rabbits kept under normobaric conditions. A small island of bone can be seen apposed to the missing BCP particle and surrounded by the highly cellular fibrous tissue (H&E, original magnification ×40). **B,** Photomicrograph of a defect filled with BCP Putty in rabbits kept under hyperbaric conditions. A small island of bone can be seen apposed to the missing BCP particle and surrounded by marrow and new bone (H&E, original magnification ×40).

reduced in DBM-grafted defects exposed to HBO; however, this did not reach significance for either tissue type.

Histomorphometry was unable to note any significant differences between groups grafted with different formulations of BCP (blood versus F127) or DBM (gel versus putty) in any of the measured parameters.

DISCUSSION

Bone graft substitutes have been successfully used to treat defects, avoiding some of the limitations associated with autogenous bone including donor site morbidity, blood loss, and extended time in surgery. HBO has been shown to enhance the bony healing of critical-sized defects in rabbits without bone grafting and may be useful as an adjunct to bone graft substitutes in smaller defects.[12,13] The use of HBO as a testing modality may also allow the detection of differences between bone and various bone substitute materials that are not evident using other testing methods.

The aim of this study was to evaluate the effect of HBO on the healing of critical-sized defects in the presence of 2 commonly used types of bone graft substitutes, demineralized matrix (DBM) and biphasic calcium phosphate (BCP). We also investigated whether different preparations of these substitutes affected their performance.

Both bone substitute materials were able to promote healing of the critical-sized defects. HBO did not significantly increase the amount of bone present in the

Table III. Histomorphometric analysis

	DBM				*BCP*				
	GEL		*PUTTY*		*Blood*		*PUTTY*		*ANOVA*
	NBO	*HBO*	*NBO*	*HBO*	*NBO*	*HBO*	*NBO*	*HBO*	*P*
NB	36.3 ± 3.0	43.7 ± 4.9	35.1 ± 5.5	44.3 ± 2.9	22.0 ± 6.9	23.9 ± 3.7	23.6 ± 5.7	24.8 ± 6.6	<.001
M	47.0 ± 5.2	41.7 ± 7.8	50.6 ± 8.7	43.6 ± 5.6	28.6 ± 4.9	39.1 ± 4.1	29.4 ± 5.5	37.4 ± 5.1	<.001
NB+M	83.3 ± 6.1	85.5 ± 3.6	85.8 ± 4.4	87.8 ± 4.4	50.6 ± 8.9	63.0 ± 4.9	53.0 ± 5.5	62.2 ± 7.6	<.001
FT	13.7 ± 5.1	9.6 ± 3.3	9.8 ± 2.5	7.1 ± 3.0	25.0 ± 6.4	12.9 ± 5.0	23.1 ± 5.4	13. ± 4.7	<.001
RG	2.9 ± 1.8	5.0 ± 1.2	4.4 ± 2.7	5.1 ± 2.5	24.3 ± 3.0	24.1 ± 5.9	23.9 ± 3.2	24.4 ± 5.4	<.001

All results are reported as percentage area of defect occupied by tissue type.
DBM, demineralized bone matrix; *BCP,* biphasic calcium phosphate; *GEL,* the DBM granules were mixed with Pluronic F127 at 40% DBM to 60% F127 (vol/vol); *PUTTY,* the DBM or BCP granules were mixed with F127 at 70% DBM/BCP to 30% F127 (vol/vol); *ANOVA,* analysis of variance; *NBO,* normobaric oxygen; *HBO,* hyperbaric oxygen; *NB,* new bone; *M,* marrow; *NB+M,* new + marrow; *FT,* fibrous tissue; *RG,* residual graft.

Table IV. Post hoc *P* values for histomorphometric parameters by matched groups

	Post Hoc P-values				
	NB	*M*	*NB+M*	*FT*	*RG*
BCP vs DBM					
Putty-HBO	<.001	.391	<.001	.223	<.001
Putty-NBO	.007	<.001	<.001	<.001	<.001
Blood vs F127 putty					
BCP-HBO	.782	.666	.843	.868	.991
BCP-NBO	.630	.834	.537	.513	.977
F127 gel vs putty					
DBM-HBO	.877	.633	.642	.415	.978
DBM-NBO	.706	.346	.800	.547	.515
NBO vs HBO					
BCP-blood	.831	.047	.013	.002	.908
BCP-putty	.926	.045	.021	.006	.996
DBM-gel	.030	.365	.577	.611	.637
DBM-putty	.039	.169	.590	.630	.958

Following a significant result in the 1-way ANOVA, the matched groups within each of the 4 different experimental conditions (BCP versus DBM, HBO versus NBO and 2 different formulation comparisons blood versus F127 putty with the BCP group and F127 gel versus F127 putty within the DBM group) were tested for significant differences using a post hoc test.
NB, new bone; *M,* marrow; *NB+M,* new + marrow; *FT,* fibrous tissue; *RG,* residual graft; *BCP,* biphasic calcium phosphate; *DBM,* demineralized bone matrix; *NBO,* normobaric oxygen; *HBO,* hyperbaric oxygen.

BCP-grafted defects compared with defects in animals exposed to normal air. This result matches our previous observations where HBO promoted healing and bone formation in unfilled defects, but provided no further increase in bone when used in conjunction with autogenous bone grafts.[6,10] We did, however, observe a small but significant increase in new bone with DBM-grafted defects exposed to HBO.

Exposure to HBO reduced the amount of fibrous tissue in the BCP-grafted defects, and showed a matching increase in the marrow component of the repair tissue. We have previously demonstrated that HBO treatment results in prolonged increases in vascular endothelial growth factor (VEGF) expression,[7] whereas others have demonstrated VEGF promotes angiogenesis, bone formation, and remodeling,[13-15] suggesting that that HBO may potentially reduce fibrous tissue formation through promotion of angiogenesis, which could result in increased marrow.

Comparison of the amounts of new bone in the DBM-filled defects and the BCP-filled defects indicated more bone and marrow formation in the DBM group irrespective of HBO or NBO therapy. DBM-filled defects also revealed less fibrous tissue formation when compared with BCP-filled defects when treated under NBO conditions. We did not see any differences in the repair of defects by different preparations of DBM (gel versus putty), whereas the different preparations of BCP (blood versus F127) showed some differences in mineralization as assessed by the mCT, but not by histology.

In conclusion, HBO resulted in small increases in new bone formation in defects grafted with DBM. In defects grafted with BCP, HBO resulted in a large reduction in fibrous tissue and an increase in its replacement with marrow.

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