HYPERBARIC OXYGEN (HBO) AND ITS INFLUENCE ON CRITICAL SIZE DEFECTS IN RABBIT CALVARIA

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ABSTRACT

Objectives: 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 bone of each animal bilaterally. Defects were critical sized, 15mm on one side and supra critical sized, 18mm on the contralateral side. Group 1 received 90 minutes HBO treatment sessions 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 as well as histomorphometric analysis to compute the amount of regenerated bone within the defects. Two way ANOVA was used for statistical analysis.

Results: Both radiographic analysis and histomorphometric analysis demonstrated that HBO treated animals had significantly more new bone within defects compared with the control group. There was no statistically significant difference between the percentage of new bone forming in the 15mm and 18mm HBO treated defects. There was no difference between the 6 week and the 12 week HBO treated groups.

Conclusion: HBO is effective in enhancing the bone healing of full thickness critical sized as well as supra-critical sized defects in the rabbit calvarial model. Bone regeneration was significantly greater in the HBO treated animals regardless the defect size. HBO may have increased the diameter of the rabbit critical sized calvarial defect to more than 18mm.

RESUME

Objet: Cette étude a été entreprise afin d'évaluer si les effets de la thérapie d'oxygène hyperbare peut altérer le défaut osseux de taille critique dans le crâne des lapins lors de la guérison spontanée.

Etude : Des études d'une durée de 12 semaines, ont été conduites sur 20 lapins blancs de la Nouvelle Zélande, séparés au hasard dans 2 groupes, chacun composé de 10 animaux. Des défauts crâniens ont été créés dans les os pariétaux bilatéraux de chaque animal. Les défauts étaient de taille critique, 15mm d'un côté et supercritique, 18mm du côté contrelatéral. Le premier groupe a reçu des sessions de 90 minutes de traitement d'oxygène hyperbare à 2.4 ATA, pendant 20 jours consécutifs. Le second groupe a été utilisé comme contrôle sans avoir reçu aucun traitement d'oxygène hyperbare. Cinq animaux dans chaque groupe furent sacrifiés à 6 et 12 semaines. Les analyses des donnés incluant des analyses qualitatives des spécimens crâniens, les radiographies post-sacrifice aussi bien que les analyses histomorphométriques furent utiliséés afin de calculer la régénération osseuse à l'intérieur du défaut. ANOVA furent utilisés pour les analyses statistiques.

Résultats : Les analyses radiographiques aussi bien que les analyses histomorphométriques ont demontré que les animaux traités à l'oxygène hyperbare ont eu une croissance signifiante de nouveau os à l'intérieur du défaut comparativement avec le groupe de contrôle. Il n'y a pas eu statistiquement une différence signifiante entre le pourcentage de formation du nouveau os dans les défauts de 15mm et 18mm traités à l'oxygène hyperbare. Il n'y a pas eu de différence entre les groupes traités 6 semaines ou 12 semaines à l'oxygène hyperbare. Les traitements d'oxygène hyperbare se sont avérés efficaces pour augmenter la guérison osseuse des défauts de taille critique ou supercritique dans les crânes de lapins.

Conclusion : La régénération osseuse, sans tenir compte de la grandeur du défaut, a été significativement plus élévée parmi les animaux traités à l'oxygène hyperbare.

L'oxygène hyperbare peut avoir augmenté le diamètre du défaut de taille critique dans les crânes de lapins, plus de 18mm.

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INTRODUCTION

Reconstructive cranio-maxillofacial surgeons are faced with managing large skeletal defects caused by trauma, oncologic resections, congenital defects, infectious sources and other pathologic etiologies. Autogenous bone grafting techniques, both vascularized and nonvascularized grafts, have been widely used since they provide predictable results and longterm stability. The drawbacks of these techniques are the second surgical site for graft harvest and the donor site morbidity. Bone substitutes have been studied for this application but they have not been able to replace the traditional autogenous graft.

Hyperbaric oxygen (HBO) has been studied extensively for the management of compromised wounds such as irradiated tissue, necrotizing anaerobic infections, and poorly perfused skin grafts and flaps. It is also considered an adjunct to traditional grafting techniques in compromised wounds (Mainous 1973). In the present study, hyperbaric oxygen was investigated as a possible substitute to conventional reconstructive surgery techniques since we are investigating the potential for HBO to allow complete healing of a critical sized defect. A critical sized defect is defined as an osseous defect that would not heal spontaneously during the lifetime of an animal (Schmitz 1986). Since HBO has been described as having angiogenic (Marx 1984) and osteogenic potential (Nilsson 1988), we further hypothesized that it may also allow a supracritical sized defect to heal.

The following research was designed to test the effects of hyperbaric oxygen therapy on the healing of critical (15mm) and supracritical (18mm) sized calvarial defects in mature New Zealand white rabbits. The effects of HBO were evaluated using clinical, histologic and radiographic methods to analyze osseous regeneration in the calvarial defect after 6 and 12 weeks of healing. Osseous healing was compared to control animals which were exposed to room air and did not receive HBO therapy.

LITERATURE REVIEW

BONE BIOLOGY

Intramembranous and Endochondral ossification

There are two mechanisms of bone formation, intramembranous and endochondral. Endochondral ossification has a cartilaginous matrix which then gets replaced by bone through vascular invasion. Blood vessels deposit mesenchymal cells (osteoblast precursors) which transform into osteoblasts and produce osteoid. Initially woven bone is produced, followed by compact lamellar bone. In contrast, intramembranous ossification does not require a cartilaginous stage. This bone develops from mesenchymal cells which differentiate into osteoblasts, which then lay down osteoid (Manson 1994). The repair process of endochondral bone is by formation of a cartilage model, as opposed to intramembranous bone which is via direct bone formation by osteoblasts. Both these mechanisms of bone formation demonstrate that the osteoblast is derived from pluripotent mesenchymal cells (Bassett 1962). Their origin can be from an endosteal, a periosteal, or other induced source (Burwell 1965, Friedlaender 1987, Mulliken 1984).

Examples of endochondral bones are the ribs, the vertebrae, skeletal long bones, and the base of skull. Membranous bones are the cranial vault, the clavicles and most of the facial bones. Certain bones such as the mandible, the sphenoid, the occipital, and the temporal bones have both mechanisms of bone formation (Manson 1994).

Fracture Healing

Bone can heal by primary or secondary intention. For bone to heal by primary intention it has to have almost perfect reduction, no mobility and good vascularity. If bone healing has occurred spontaneously and without rigid fixation then it is by secondary intention.

This type of healing occurs due to an intermediate step which is development of a fibrous scar in the gap, which is then replaced by bone.

Secondary bone healing has four stages: initial stage, cartilaginous callus, bony callus, and the remodeling stage. During the initial stage (0 to 5 days), there is disruption of blood vessels at the fracture site which leads to hypoxia and cell death. There is then an inflammatory response due to necrosis at the bone ends. This induces release of vasoactive angiogenic factors which cause vasodilation in the first few hours of injury (Rhinelander 1968). Hematoma formation occurs from the damaged vessels of the endosteum, periosteum and haversian system. Within the hematoma, there is granulation tissue as well as fragments of bone and muscle. Small nonvital pieces of bone will be lysed within 5-10 days, and pedicled, vascularized muscle will undergo fibrosis (Simmons 1985). Small bone fragments may act as a matrix for surface deposition of bone by migrating periosteal cells (Feinberg 1997). During this inflammatory stage there is the process of cellular proliferation which is happening simultaneously. Within 8-12 hours, the cells of the cambium layer of the periosteum start synthesizing DNA (Knize 1974). The periosteum of the entire injured bone is involved initially, but after a few days this process involves only the periosteum in the area of the fracture. These pluripotential cells will form osteoblasts, fibroblasts and chondroblasts (Kernek 1973). These pluripotential cells exist on all free bone surfaces endosteal as well as periosteal (McKibbin 1978). The fibroblasts begin to lay down collagen and capillary ingrowth follows. The collagen and the capillaries form granulation tissue. There is a low oxygen tension and a low pH which is thought to trigger formation of hyaline cartilage within the hematoma (Brighton 1991). The next stage is formation of the cartilaginous callus. There is an external and an internal callus being formed simultaneously. The external callus is formed of nodules of cartilage separated by fibrous tissue. With increased vascularity of the fibrous tissue there is reversal of hypoxemia and the following occurs. There is further calcification of the cartilage and trapping of chondroblasts and then conversion to chondrocytes. Also, osteoblasts will become more numerous and osteoclasts will become visible. Between the fractured ends, the internal callus is also forming. Since there is more vascularity, there is less hypoxemia and therefore less necrosis. Osteoblasts from

the endosteum form a bony callus directly, without fibrocartilage formation. The callus allows for initial stabilization of the fractured bone ends during healing. The cartilaginous callus is complete three weeks post fracture. Formation of the bony callus is the next stage. This step is similar to endochondral ossification of long bones during growth where the cartilage undergoes calcification into woven bone. Further vascular ingrowth in the spaces between the cartilage increases the oxygen tension in the environment and promotes osteoblast formation (Feinberg 1997). The osteoblasts are derived locally from the endosteum as well as from pluripotential osteoblast precursors (McKibbin 1978). Osteoblasts deposit osteoid on the cartilage, and then it becomes calcified to form woven bone. This process starts at the periphery and eventually causes a calcification of the entire cartilagenous callus (Kernek 1973). The final stage which is the remodeling stage (25-50 days post fracture), will change the woven bone into a more organized lamellar bone. Osteoclasts are responsible for the bone turnover. Factors such as BMP (bone morphogenic protein) are released locally and cause differentiation of mesenchymal cells to bone forming cells (Feinberg 1997). In humans, the time for remodeling is between 3 to 6 months. This time period is referred to as sigma (Hollinger 1999).

Primary bone healing occurs when there is enough vascularity, rigidity and fracture reduction so that new bone is laid down without a cartilaginous callus (Hutzschenreuter 1969). There are two ways that this can occur: gap healing or contact healing. Gap healing is an example of rigid fixation of the bone fragments without perfect anatomic reduction. In some areas, there are still small gaps that exist. Within a few days after the fracture, blood vessels from the periosteum, endosteum and haversian system bring mesenchymal osteoblast precursors. Bone deposition occurs directly on the surface of the fracture ends without intermediate cartilage formation. If the gap is less than 0.3mm, lamellar bone is laid down directly. If the gap is from 0.3mm up to 0.5 to 1.0mm, woven bone is laid down and is subsequently replaced by lamellar bone. The lamellar bone is formed over 6 weeks. It then takes several months to orient the lamellar bundles along the long axis of the repaired bone.

Contact healing occurs when the gap between fragments is zero. Since there is no gap, then vascular and cellular ingrowth cannot occur via the same mechanism. It requires a BMU (bone metabolizing unit) for bone healing to occur (Feinberg 1997). A BMU is an advancing group of osteoclasts which make space for blood vessels and cells to then differentiate into osteoblasts and form new bone. The osteoclast advancing cone proceeds at 20-40 um per day for a variable distance of 2-6mm. It produces a core which is 200um in diameter allowing place for formation of new bone and vessel ingrowth. Since osteon formation is at a slower rate than resorption, this creates a transient porosity in the bone (Parfitt 1994, 1983).

The role of oxygen in fracture healing was studied by Brighton (1972) in his rabbit fibula model. He used oxygen microelectrodes implanted in the rabbit fibulas to measure oxygen tension during the healing process. The oxygen tension ranged from very low in the hematoma (6.25 mmHg) to the highest in diaphyseal bone (89.5 to 109.1 mmHg). Cartilage exhibited a low oxygen tension throughout the experiment (28.9 to 37.2 mmHg), whereas newly formed fiber bone was low initially (22.1 to 33.3 mmHg) and increased to the level of diaphyseal bone (87.5 mmHg) by the thirty-fifth day. The low oxygen tensions during healing of a fracture callus are similar to those of the bone-cartilage junction of the epiphyseal growth plate (Brighton 1971). The callus appears to possess a more anaerobic metabolism than normal bone. The low oxygen tension may be a result of increased cellular proliferation and limited vascular oxygen supply. The newly-formed capillaries may not be in sufficient proximity to all these new cells for adequate oxygenation. When applied to Brighton's study (1972), restoration of diaphyseal oxygen tensions corresponded to fracture healing and reconstitution of medullary space, which may have resulted due to equal distribution between vascularity and cellularity. The role of increased oxygen consumption, may be another factor which cannot be ruled out from his study. Heppenstall et al (1975) conducted a study on healing of large segmental defects in the canine model using a tissue tonometer system to measure variables such a pO2 and pCO2 during the various stages of bone healing. The results of the study demonstrate that new bone was formed under hypoxic conditions, and there was no associated increase in oxygen consumption. This signifies the

importance of anaerobic metabolism with respect to healing osseous defects. When the cellularity equaled the vascularity, hypoxia no longer persisted. Deren JA and Kaplan FS, in a study with Brighton CT (1990), tried to disclose the mechanism by which hypoxia affects bone healing. They measured the concentration of alkaline phosphatase (osteoblastic enzyme) production by periosteal cell cultures, when exposed to varying oxygen concentrations. They were able to show varying amounts of alkaline phosphatase at different oxygen levels, but unable to determine the mechanism by which gene expression is regulated.

Angiogenesis

As depicted in fracture healing, microvasculature appears to be necessary for bone healing. Blood flow studies have shown this steep increase in vasculature during fracture healing, indicating a direct correlation between the two (Grundnes 1992). Traumatized bone goes through an inflammatory stage, with cell death and subsequent formation of granulation tissue prior to the osteogenic stage. Vascularity is essential for healing of all tissues except cartilage (Hulth 1990). Angiogenesis brings endothelial cells in the area of ischemia to bridge the gap and set up an environment for repair. Trueta et al (1963) have demonstrated that endothelial cells and pericytes from blood vessels act as osteoprogenitor cells. Although others have proposed that endothelial cells may not be linked to osteogenesis (Oni 1993), it is still undisputed that microvasculature is essential for ossification (Hansen 1988). A gap that is too large for angiogenesis and in turn diffusion of osteogenic agents may be considered a critical sized defect (Schmitz 1986).

Angiogenesis in wound repair and bone healing occurs harmoniously due to promoting and inhibiting factors. The process of angiogenesis is thought to be initiated by a certain growth factor bFGF (basic fibroblast growth factor) (Glowacki 1998). This factor has been tested in various models to examine its effects on osseous healing. Yamada et al (1997) tested basic fibroblast growth factor on calvarial bone healing in the rabbit model and found complete regeneration 12 weeks after implantation. Lack of bFGF produced fibrous tissue ingrowth rather than bone formation. Wang et al (1996) demonstrated that

bFGF promoted tissue ingrowth in porous hydroxyapatite, and at certain doses it leads to bone ingrowth. Some evidence also exists for recombinant ECGF (Endothelial cell growth factor) to be an angiogenesis and osteogenesis growth factor (Chang 1997). Osteoblasts and chondrocytes can produce angiogenic factors as well. Prostaglandin E2 (PGE2) which is known to stimulate osteogenesis has increased VEGF (Vascular endothelial growth factor) production by an osteoblastlike cell line RCT-3 (Harada 1995). Another osteoblastlike cell line has also been shown to produce VEGF, when stimulated by IGF-I (insulin-like growth factor I) (Goad 1996). VEGF is a potent angiogenic factor with mitogenic effects on endothelial cells. There is extensive literature on the action of VEGF to promote vascularization (Mulhauser 1995, Ozaki 1997, Tsurumi 1996)). The regulation of VEGF appears to be mediated by oxygen tension in a healing wound. Experimental data on malignant as well as normal cultured cell have shown hypoxia to be a potent inducer of VEGF. The mechanism seems to be related to oxygen sensing ability mediated by a heme-containing protein (Minchenko 1994, Steinbrech 2000). There is also evidence that FGF-2 induces VEGF expression in vascular endothelial cells (Seghezzi 1998).

Autogenous bone graft healing

Autogenous bone grafting is important to understanding the mechanism of bone healing since it is the gold standard in grafting techniques. Ideal grafting materials must have three essential elements to promote maximum graft healing. First, they must be osteoconductive which describes that the graft acts as a scaffold to allow vascular invasion, resorption, and new bone formation to occur. This process of vascular invasion, bringing osteoblasts that deposit new bone is called creeping substitution (Burchardt 1983). This property is inherent to other graft materials, including allografts and alloplasts. The osteoinductive property describes the ability to form new osteoblasts by inducing differentiation of osteoblast precursors. Portions of the bone graft can act as the inducer of undifferentiated cells in the recipient bed to form osteoblasts that eventually produce new bone (Manson 1994). Autogenous bone grafts are also osteogenic, which describes their ability to lay down new bone. This is possible since viable osteoblasts

exist within the autogenous graft. Both cancellous and cortical grafts possess these three elements, but cancellous bone offers a greater proportion of each (Ludwig 1999). The following description of graft healing will also provide a comparison between cancellous and cortical grafts.

Cancellous bone grafts are highly osteogenic and easily vascularized compared to cortical bone grafts. The rapid revascularization occurs within hours after grafting and can be complete by two weeks. There is some evidence that vascular anastamosis can occur from host vessels to graft vessels, but this mechanism does not replace the gradual vascular ingrowth by host capillaries. During initial healing following a cancellous graft, there is a hemorrhagic and inflammatory stage. Most of the autotransplanted cells die, especially the osteocytes. The surface osteoblasts survive and allow for deposition of new osteoid which eventually surrounds a central core of dead bone. The porosity of cancellous bone allows peripheral host vessels, osteoblasts and osteoblast precursors to infiltrate the graft toward the center as early as day two post surgery. Osteoclast precursors are also deposited by the ingrowth of blood vessels. These osteoclasts start the remodeling phase, where necrotic bone and new host bone are resorbed and host osteoblasts synthesize new bone (Burchardt 1983, Stevenson 1999). Resorption of first phase bone releases BMP (bone morphogenic protein) which stimulates formation of second phase bone (Axhausen 1956, Marx 1996). The remodeling phase may last several months. The initial bone from surviving endosteal osteoblasts and marrow mesenchymal cells which forms first phase bone, is analogous to the initial randomized, disorganized bone formation in the fractured callus. Second phase bone is the more organized lamellar bone that replaces it (Burchardt 1983, Stevenson 1999).

Cortical autografts initially provide more structural support, but are less osteogenic and have a slower revascularization. Cortical grafts have a more delayed rate of revascularization, since the blood vessels start to penetrate the graft only on the sixth day. A one to two month period of revascularization is necessary since vascular penetration into cortical bone requires peripheral osteoclastic resorption and infiltration of Volkmann's and haversion canals. The appositional phase starts only after this resorptive

phase. This delayed revascularization is due to a structural difference as well as the decreased number of endosteal cells to form vascular anastamosis, previously described with the cancellous grafts. Revascularization of the interior portion of the cortical graft proceeds more quickly due to the osseous tissue invading along already developed vascular channels. Another histologic difference is that the repair of cortical grafts is a result of osteoclastic function, as opposed to osteoblastic function in the initial period of healing. Radiographically, cortical bone will appear less radiodense than cancellous bone. Depending on the graft size, it may take several months to years to remodel to resemble host bone in strength. There may also be areas of necrotic bone present for long periods of time (Burchardt 1983, Stevenson 1999).

Oxygen gradients also provide the mechanism for graft healing, as described in fracture repair. The graft is inherently hypoxic and the surrounding tissue normoxic (50-55 mmHg), creating an oxygen gradient greater than 30mmHg (usually 35-50 mmHg). This gradient is above the level necessary to trigger macrophage chemotaxis and release of MDAG (macrophage derived angiogenic factor) and MDGF (macrophage derived growth factor). The platelets within the graft also secrete PDGF (platelet derived growth factor). The net result is angiogenesis of the surrounding capillaries and mitogenesis of the transferred osteocompetent cells. When the oxygen gradient decreases there is a feedback mechanism to prevent continued angiogenesis (Marx 1996).

HYPERBARIC OXYGEN (HBO)

Definition: The term hyperbaric oxygen (HBO) refers to the administration of 100% oxygen at greater than one atmosphere pressure absolute (ATA). In order to achieve these elevated pressures, the patients are placed in pressurized chambers. The Undersea and Hyperbaric Medical Society has developed HBO protocols and has outlined a list of approved medical indications for HBO therapy (Broussard 2003).

Approved medical indications for HBO therapy

(Broussard 2003)

1. Air or gas embolism
2. Carbon monoxide/Cyanide poisoning
3. Clostridial myositis and myonecrosis (gas gangrene)
4. Crush injury, compartment syndrome, and other acute traumatic ischemias
5. Decompression sickness
6. Enhancement of healing in selected problem wounds
7. Exceptional blood loss (anemia)
8. Intracranial abscess
9. Necrotizing soft tissue infections
10. Refractory osteomyelitis
11. Soft tissue/ bone radiation necrosis
12. Compromised skin grafts and flaps
13. Thermal burns

The mechanism of action of HBO encompasses mechanical effects, bacteriostatic effects, treatment of poisoning, and treatment of hypoxia. The following will elucidate the involvement of HBO in wound healing and hypoxia.

HBO Protocols

For prevention of osteoradionecrosis, the accepted protocol for HBO prior to surgery in tissues exposed to 6000 cGy or more of radiation is the following: 100% oxygen at 2.4 ATA for 90 minutes each session, 20 sessions prior to surgery and 10 sessions after surgery. HBO is administered intermittently providing alternating hyperoxia and hypoxia, and therefore maintaining the stimulus for healing (Marx 2003). This protocol yields the longterm effects of HBO described in the literature. In tissues irradiated with 5000 cGy or more, HBO induces angiogenesis and cellular fibroplasia. In this application, the mechanism of action is establishment of a steep oxygen gradient between the irradiated tissue and the nearby nonirradiated tissue. The oxygen gradient has been shown to stimulate macrophages to secrete macrophage-derived angiogenesis factor (MDAF) and macrophage-derived growth factor (MDGF) (Knighton 1983).

The effects of varying the partial pressure of oxygen, the oxygen saturation and duration and frequency of exposure of HBO on wound healing has been studied in vitro by Tompach (1997). He cultured endothelial cells and fibroblasts and labeled cellular proliferation with H-labeled thymidine. Increasing partial pressure of oxygen in the form of HBO enhanced proliferation of the cell cultures. Providing two exposures per day did not increase proliferation, nor did increasing the pressure from 2.4 to 4 ATA. There was also evidence that enhanced oxygenation occurred at lower pressures than 2.4 ATA. The endothelial cells required only 15min of HBO to initiate proliferation, whereas the fibroblasts required 120min. A 50% increase in H-labeled thymidine occurred with both cell types 18-24h after exposures of 15-120min. This may be evidence of induction of gene expression. This study provides valuable information for developing the most cost-effective protocol with reduced exposures and decreased secondary effects.

HBO physiology

Understanding the role of oxygen and the effects of pressure in wound healing will further help to define the mechanism of HBO in wound healing.

The solubility of oxygen in plasma at 37 degrees celcius is:

0.0214ml O2/ml plasma/atm pO2 (Bassett 1977)

Hyperbaric therapy involves barometric pressures greater than those found at sea level. The optimum pressure effect is seen at 2 to 3 atmospheres absolute (ATA), whereby it increases the solubility of oxygen in the plasma. Hyperbaric therapy affects oxygen delivery to the tissues in several ways. At the above-mentioned pressures, the partial pressure of oxygen dissolved in the plasma is 1500mmHg to 2200mmHg. This is equivalent to 4.5 to 6.6 volume % in comparison to 5 volume % at normobaric levels, which allows more oxygen to be delivered to the tissues. A second effect is that hemoglobin becomes fully saturated. The tissue oxygen tension never increases to plasma levels due to diffusion limitations. An advantage of these elevated oxygen levels is the distance of oxygen diffusion reaching 10 to 15 times further into the tissues. (Evans 2002)

Stimulation of new vessel formation has been linked to chemical messengers derived from different sources. Platelet derived growth factor and macrophage derived factors cause replication of capillary endothelial cells and formation of new capillaries. Knighton et al (1981, 1983, 1984, 1991) have discovered a macrophage derived angiogenic factor whose production is stimulated by hypoxic environments. Normal angiogenesis requires the establishment of a steep oxygen gradient as described by Marx (2003). In irradiated tissue, due to the scatter of the radiation beam there is no abrupt change in oxygen tension as would be present at the margin of lacerated tissue. The macrophage needs a minimum oxygen gradient of 20 mm oxygen per cm for chemotaxis and secretion of the angiogenic factor. Once a certain amount of repair has diminished this oxygen gradient, new capillary budding ceases to continue (Knighton 1983). The role of HBO is to magnify the oxygen gradient to levels which trigger macrophage angiogenic factor.

Knighton et al (1983) also concluded from their studies that secretion of macrophage mitogens is not increased by hypoxic growth conditions. It seems to be regulated independently from angiogenesis factor. Other studies (Jensen 1986) have demonstrated that lactate can also stimulate angiogenesis by stimulating the macrophage to secrete angiogenesis factors. Lactate is a result of anaerobic metabolism. Aside from stimulating capillary budding, lactate attracts fibroblasts into the wound which in turn produce the collagenous matrix that supports the vascularity to form granulation tissue. Collagen formation is also oxygen dependant with a pO2 of 150mm Hg being the optimum level. Sufficient collagen formation is essential for establishing wound strength (Evans 2002).

Oxygen is critical for energy production, collagen synthesis and cellular proliferation. In a healing wound, vascular ingrowth will only occur if these elements are present (Broussard 2003). Cellular energy production is dependent on ATP which requires oxygenation. Anaerobic metabolism can exist, but it requires increased glucose consumption to provide an equal level of ATP and eventually leads to lactic acidosis (Vaes 1962). This translates to inadequate ATP in the hypoxic state. Another function of oxygen is hydroxylation of proline and lysine during collagen synthesis. In the absence of oxygen, collagen synthesis cannot take place. Cellular proliferation seems to be dependent on oxygen as well since fibroblasts cannot proliferate in tissue culture without oxygen (Broussard 2003).

Environments which are severely oxygen deficient (ischemia) can undergo further damage to adjacent tissue by initiating a cascade which leads to leukocyte activation. Many of the above listed medical indications for HBO are linked to prevention of leukocyte activation. The pathway starts with production of an oxidative stress. Oxygen acts as an electron acceptor in many biochemical reactions. The lack of oxygen inhibits oxidation/reduction (redox) reactions. An example is the production of hypoxanthine in an ischemic environment rather than the high energy ATP. The body reacts with this compound and produces superoxide radical and reactive hydroxyl radicals. Through a complex cascade of biochemical reactions, there is expression of the B2 leukocyte cell wall adhesion receptor which causes adherence of the leukocyte to the endothelium. This

process of leukocyte activation causes damage to the adjacent tissue which is further affected by the superoxide and hydroxyl radicals. HBO prevents leukocyte activation by inhibiting the B2 integrin receptor. (Evans 2002)

The following list outlines the effects of HBO on wound healing:

1. Decreased local tissue edema through vasoconstriction
2. Improved cellular energy metabolism
3. Improved local tissue oxygenation
4. Improved leukocyte-killing ability
5. Increased effectiveness of antibiotics
6. Enhancd uptake of PDGF
7. Promotion of collagen deposition
8. Promotion of neoangiogenesis
9. Enhanced epithelial migration

(Broussard 2003)

If oxygen is necessary for wound healing, then is it possible to improve healing by administering oxygen? The following literature may help to answer this question. The accepted normal range of extracellular oxygen levels to support a resting cell is thought to be 5 to 10 mmHg. When the environment of a fibroblast has a pO2 less than approximately 10 mmHg, the cell cannot function; divide, synthesize collagen, migrate. So the distance the fibroblast can migrate from the nearest capillary is limited by the oxygen tension in the extracellular fluid. As new capillaries develop into the fibrous matrix, the oxygen supply to the more distal fibroblasts increases and normal function resumes. The rate at which new vascularity develops is affected by oxygen supply. If this hypothesis is valid, administering more oxygen may allow more fibroblasts to function adequately and new capillaries can vascularize the healing wound (Hunt 1969).

The pO2 of normal healing tissue is 30-50 mmHg, while nonhealing tissue usually has a pO2 less than 20mmHg. Nonhealing wounds have a nonstimulatory level of hypoxia, or have an increased consumption that may be due to increased metabolic demand or

infection. Normal healing has mild levels of hypoxia and lactate levels which drive the process of collagen synthesis and macrophage stimulation for angiogenesis. (Tompach 1997)

The oxygen cascade describes the delivery of oxygen from ambient air to the cardiovascular system and ultimately to the mitochondria. The alveolar partial pressure (PaO2) is 102mmHg while breathing air at atmospheric pressure. At this exposure of oxygen, the wound tissue has a pO2 of 5-20mmHg. This is contrasted to an alveolar partial pressure of 1813mmHg while breathing 100% oxygen at 2.5 ATA, providing a wound pO2 of 800-1100mmHg. (Tompach 1997)

A healing wound requires increased oxygen tension, since the local environment of wounded tissue develops a hypoxic state. Disruption of the microcirculation is the initial step of wound hypoxia. By day three post injury, the pO2 of the tissues falls below 10mmHg. There is also an increase in hydrogen ion and carbon dioxide since the compromised vasculature cannot transport these byproducts out of the wound. As healing occurs and the vasculature improves, the pO2 of the wound can increase to 30-50mmHg. This has been demonstrated in experimental data using Teflon coils permeable to oxygen, which were implanted under the skin of rabbits. Fluid with known values of pO2 was perfused through the coils and the levels of oxygen measured. Utilizing a technique of implanted electrodes, the response of wound oxygen tension to atmospheric oxygen levels has been studied. In the initial few weeks of wound healing, the rise in oxygen tension is slow. Even when exposed to 600mmHg of oxygen, the pO2 only rises from 10 to 100mmHg. In more mature wounds, the pO2 can rise from 30 to 400 mmHg in about 6 minutes. It appears that early on, the wound may require more oxygen. This demonstrates that when a wound is exposed to normal oxygen levels, it is not receiving the maximum level of oxygen that it can utilize (Hunt 1969). Silver (1965) has shown how the oxygen tension is inversely proportional to the distance from the nearest capillary. During respiration the gradient gets steeper since the pO2 near the capillary rises significantly more than the pO2 at a more distant site in the wound. He has also demonstrated that the cells will utilize more oxygen if it is available.

HBO and Osteoradionecrosis (ORN)

Much of what we know about HBO and bone healing originates from the literature regarding the management of osteoradionecrosis (ORN). Osteoradionecrosis of the mandible is a nonhealing mucosal or skin wound with exposed devitalized bone more than 1cm in diameter. This suggests a clinical diagnosis for this disease (Brown 1998). The pathophysiology of osteoradionecrosis has been described by Marx (1983, p283-288) as radiation induced damage to tissues (mainly endothelial cells and fibroblasts) which subsequently becomes hypoxic, hypocellular and hypovascular. This leads to tissue breakdown and a chronic nonhealing wound. Perfusion studies using transcutaneous oxymetry have shown that the center of an irradiated field has an oxygen tension as low as 5 to 10 mm Hg. As one moves farther away from the center of the radiation beam, the oxygen tension increases by 5mm Hg for each centimeter. Anything less than the normal venous pressure of 40mmHg is considered abnormal (Brown 1998).

In 1984 Marx published the Marx-Wilford Hall USAF protocol for ORN. In his study he also measured transcutaneous oxygen tension values to demonstrate that the maximum value after HBO is 81 $\pm$ 5%, even with additional HBO. After 6-8 exposures, the oxygen tension in the hypoxic wound was only 30 $\pm$ 4% of the control. This initial period was termed the lag phase. This occurs since collagen synthesis needs to precede endothelial cell proliferation. Subsequently, there was an exponential rise in oxygen tension from day 8-18 to a plateau of 81$\pm$ 5% where they achieved the maximum level of oxygenation induced by HBO. Collagen formation was thought to have induced endothelial cell proliferation as well as provide a scaffold for vessel ingrowth. Since the control (nonirradiated left second intercostal space) maintained a constant level of oxygen tension, it was concluded that HBO does not influence uncompromised tissue. HBO is only useful if applied intermittently (90min/day), otherwise it will remove the stimulus for healing (hypoxia, lactate and acidosis). Oxygen has been found to be the most important cofactor for hydroxylation of proline and lysine in collagen synthesis. Hydroxylation enzymes proline and lysine hydroxylase require an oxygen tension no less than 20 torr (mmHg). In Marx's study (1984), the oxygen level in irradiated tissue was

30% that of normal tissue for an oxygen level of only15 torr. This study redefines the pathogenesis of ORN as an imbalance of collagen synthesis and breakdown.

Development of ORN is usually seen in tissues that have received radiation doses above 50cGy. The incidence of ORN post dental extractions reported in the literature is quite varied, ranging from 2 to 84% (Lambert 1997, Clayman 1997). Marx has disputed the previous theories of trauma induced osteoradionecrosis and subsequent sepsis. It has been reported that osteoradionecrosis is a type of osteomyelitis in which tissues are subjected to trauma and then lead to a path of entry for bacterial contamination. One such article by Meyer (1970) makes such claims but never proves through tissue cultures the spread of osteomyelitis. The role of trauma is also challenged since there are reports of spontaneous osteoradionecrosis related to basic tissue damage from radiation exposure (Daly 1972). Marx (1983, p283-288) developed a theory on the pathophysiology of osteoradionecrosis in his clinical study on 26 patients with mandibular osteoradionecrosis. In the 12 specimens that required mandibular resections, he cultured the specimens to see if there were microorganisms in the deep sections of bone. All deep specimens had no microbial growth. The only microorganisms cultured were from superficial contamination. Also, only 35% of the patients had a history of trauma. Clinical studies, including the one by Marx, have demonstrated that osteoradionecrosis is related to a basic tissue damage from radiation exposure, with higher radiation doses playing a crucial role. The radiation induced damage was shown on histologic examination of the endothelium, periosteum, bone, and connective tissue. There was endothelial death, hyalinization, and thrombosis of vessels. The periosteum became fibrotic, and bone osteoblasts and osteocytes became necrotic with fibrosis of marrow spaces. Mucosa and skin also demonstrated fibrosis and decreased cellularity and vascularity of connective tissue. Trauma may be associated with osteonecrosis, but only because trauma increases the metabolic demands on tissue which has a compromised mechanism of repair. In another study by Marx (1987), he enrolled 536 patients with osteoradionecrosis of the mandible and analyzed different parameters. He studied the epidemiology of the disease and was able to demonstrate that higher doses of radiation and implanted sources of radiation were implicated in osteonecrosis to a greater degree. He also showed that 39% were spontaneous osteonecrosis, and of the

trauma induced osteonecrosis, 84% was due to tooth removal. These percentages were supported by his previous study (Marx 1983, p283-288). He further differentiated trauma induced osteonecrosis and spontaneous osteonecrosis relative to time and level of radiation injuries. The majority of cases of spontaneous osteonecrosis presented between 6 and 24 months after radiation treatment. The early occurrence of spontaneous osteonecrosis is explained by the radiation induced damage having been sufficient enough not to allow tissue recovery. Ultimately, the cells have been killed by the radiation damage. Trauma-induced osteonecrosis had a bimodal peak with the first occurrence being at 3 months, due to surgical trauma shortly before or during radiation treatment. This was followed by the second peak at 2 to 5 years. Trauma-induced osteonecrosis is explained as cellular injury rather than cell death. Some cells eventually die due to mutations, some have abnormal function and others die due to the progressive hypovascularity and fibrosis. So when the cells are subjected to surgical trauma they cannot recover. His study showed that in the longterm, osteonecrosis can continue to be a persistent threat. Teeth that had been removed prior to radiation treatment caused osteonecrosis when a healing period of two weeks post extraction was not met. When there was 21 or more days prior to radiation treatment post extraction, there were no reported cases of osteonecrosis. The tissue perfusion portion of the study demonstrated that there was decreased capillary perfusion with time after radiation treatment. This supports the idea that the risk of osteonecrosis does not decrease with time. 50 patients in the study also underwent hyperbaric oxygen treatment (20 sessions at 2.4 ATA for 90min). These patients demonstrated angiogenesis of the irradiated tissue lowering the risk of osteonecrosis. Tissue biopsies were also done as another component of their study. They demonstrated post radiation treatment that there was a sequence of events that lead to hypovascularity and fibrosis, even in the patients that didn't develop osteonecrosis. Early during radiation treatment and in the initial 6 months there was hyperemia and endarteritis. Vascular thrombi appeared later during the radiation treatment, followed by fibrous thrombi which developed in the years post radiation. Decreased cellularity began post radiation and worsened in the years that followed. Hypovascularity and fibrosis began at about 6 months post radiation and progressively worsened over time. In the 50 patients subjected to HBO treatment, the effects of radiation damage were decreased

since the biopsies demonstrated fibroplasia and angiogenesis. The effects of hyperbaric oxygen in the management of 58 cases of refractory osteoradionecrosis are well documented by Marx (1983, p351-357). In this article, a protocol is presented for the use of HBO as an adjunct to surgical treatment. He advocates a combined approach to treatment since HBO alone healed only 15% of the patients. He theorizes that the degree of tissue injury is variable amongst patients depending on the initial radiation injury. He also states that HBO can reverse some of the effects of radiation damage, but it cannot completely restore oxygen levels back to normal. Marx also believes that hypovascularity of the tissue does not allow necrotic bone to be entirely resorbed, therefore surgical debridement becomes essential. In Marx' study, 14% of patients required HBO and some necrotic bone removal, and 71% of patients required mandibular resections, for a total of 85% of patients requiring surgery for complete resolution of osteoradionecrosis.

The following table outlines the protocol advocated by Marx (2003) for the treatment of osteoradionecrosis. There is an increase in the number dives: 30 sessions of HBO at 2.4 ATA for 90 treatment minutes of 100% oxygen, followed by ten sessions after local or more extensive surgical debridement.

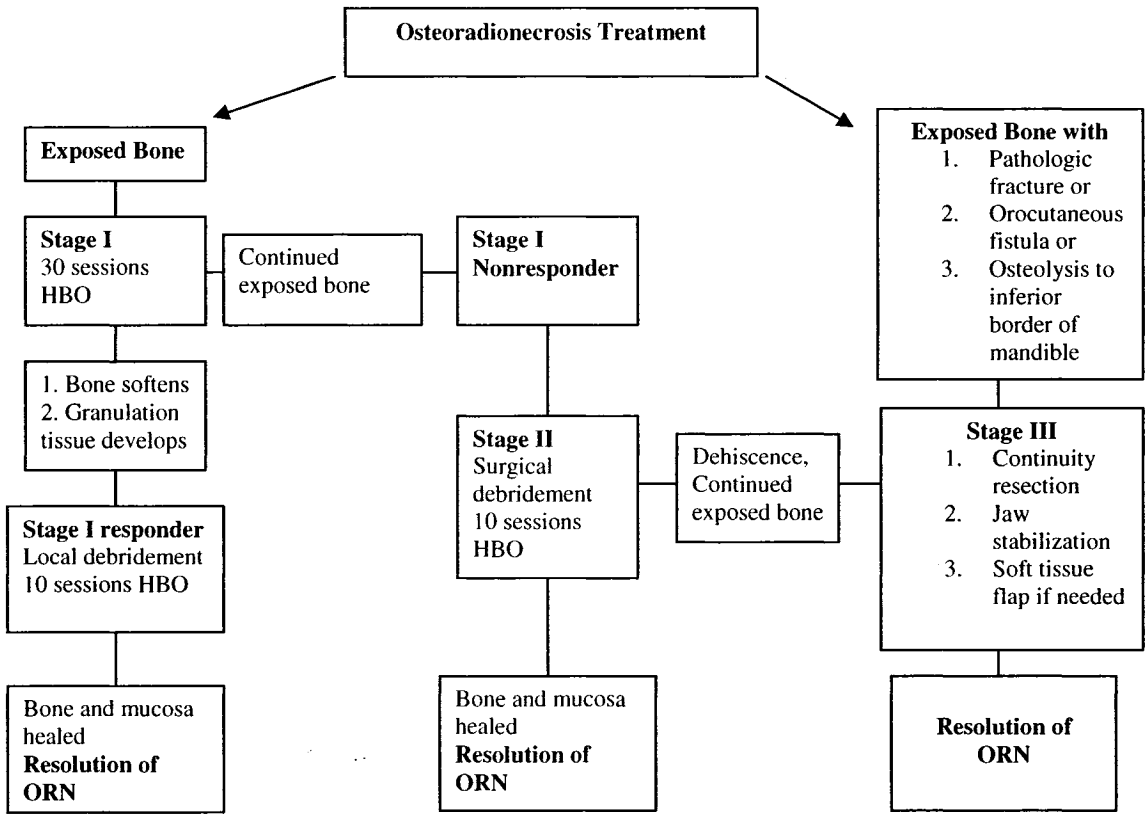

Table I. Marx's protocol for the treatment of ORN.

The use of HBO for the treatment of osteonecrosis has been described by Marx and others (Hart 1976, Marx 1983, p351-357, David 2001). Mainous et al (1975) have reported on the successful management of ORN using HBO as early as the 1970's. The use of HBO for prevention of osteonecrosis is another aspect that has been presented by Marx (1985) in his paper comparing the effects of HBO versus Penicillin following dental extractions in high risk patients. His study was a prospective randomized trial which included 74 patients that required dental extractions post radiation treatment. One of the inclusion criteria was that the patients received a documented absorbed radiation dose of 6,000 rads or greater. One study group consisted of patients exposed to 20 sessions of HBO (breathing 100% humidified oxygen at 2.4ATA for 90 min) prior to dental extractions and 10 sessions of HBO post dental extractions. The second group of patients received Penicillin pre-op and for 10 days after surgery. The results of the study

indicate that HBO, when used prophylactically to prevent osteonecrosis, decreases the risk to a greater extent than antibiotics. There was a 5.4% incidence of ORN with the HBO, and a 29.9% incidence with the antibiotics. This supports the previous literature that the pathogenesis of ORN is not of bacterial origin and is more likely associated with radiation damage. The fact that 5.4% of patients exposed to HBO still developed ORN is evidence that HBO does not restore the tissues to normal. Marx speculates that since HBO does not seem to eliminate spontaneous ORN, perhaps the radiation damage has been lethal to a population of cells with a reduced host response to HBO. It is also unknown if HBO can prevent clinical ORN at a later time (Marx 1985). HBO can bring the oxygen content in the tissues to 81% $\pm$ 5% of normal tissue after 20 sessions of HBO (Marx 1984). This oxygen tension represents 35 to 40mmHg, compared to 5 to 15mmHg in irradiated tissue (Lambert 1997). During HBO therapy, oxygen tensions can intermittently reach up to 100 to 250 mmHg, but then decrease to 35 to 40 mmHg (Marx 1983, p351-357). The vascularization studies show that this level cannot be increased with more HBO. Also, the maximum level is achieved after 30 hours of HBO. When patients were examined up to three years following HBO treatment, the tissue oxygen levels were within 90% of the value immediately after the HBO sessions. This becomes important when clinicians are faced with whether or not to readminister HBO prior to more surgery in the future. There are some authors that report successful results without the use of HBO prior to dental extractions or dental implants. (Maxymiw 1991, Makkonen 1987, Marciani 1986). Since ORN can present any time after radiation treatment, the limitation in these studies is the short follow up period. Others advocate decreasing the burden of increased cost to the medical system, without adequately estimating the costs incurred for treatment of ORN. Marx et al (1985) estimated that the cost for managing patients who develop ORN was significantly greater than the cost that would be required for prophylactic HBO prior to surgery.

There is a recent randomized, placebo-controlled, double-blind trial which compares the effects of the current HBO protocol for ORN to no HBO. The findings of this study propose that no HBO provides a better outcome for treatment of ORN, when severe forms of ORN were excluded. One must consider that the patients were only followed for

1 year, which may miss the peak incidence for spontaneous ORN occurring at 2-5 years. Another important factor is that they modified the protocol by administering HBO twice daily to decrease treatment time (Annane 2004).

HBO AND BONE HEALING STUDIES

There are many examples in the literature of HBO enhancing bone healing, either by increasing the amount of calcification or decreasing the amount of time required for bone healing to occur. In the clinical setting we are accustomed to scenarios where HBO is used in the irradiated patient to change the hypoxic environment created by radiation treatment. There is increasing evidence that HBO may also be useful for bone healing in nonirradiated tissues.

In 1962 Bassett demonstrated that cultures of multipotent mesenchymal cells can differentiate into bone or cartilage depending on the oxygen tension. The cells were also subjected to either tension or compression, so unfortunately the two variables, oxygen and compression or tension were not isolated. At increased oxygen tension with compression, there was enhanced bone formation, whereas reduced oxygen tensions produced cartilaginous cells. The results of Aoki (1997) give us some insight on the effects of hyperbaric exposure without varying the oxygen tension directly. He exposed mice to 2 ATA and then analyzed bone formation using magnification radiography and uptake of a radioactive marker. He concluded that increasing atmospheric pressure promoted bone calcification as compared to the controls at 1 ATA. Shaw (1967) wanted to show the effects of varying oxygen concentrations on osseous and cartilaginous cells of organ-cultures. At 5% oxygen there was very little osteogenesis and chondrogenesis, at 35% oxygen there was maximum amounts, and at 95% oxygen there was less osteogenesis and chondrogenesis than at 35%, and resorption was very prominent. Duration of incubation was thought to be responsible for the adverse effect of high oxygen tensions such as 95%. Tuncay et al (1994) also observed increased osteogenesis with hyperoxia (90% O2) verses hypoxia (10% O2).

Some of the earlier studies used the rat model to test the effects of HBO on fracture healing. Coulson et al (1966) were able to prove that fractured rat femurs treated with HBO (2 hours daily with 100% oxygen at 3 ATA) encorporated increased calcium and had higher breaking strengths at 1, 2, and 3 weeks post fracture than the nonHBO group. Yablon et al (1968) showed histologically and by microradiographs that fractured rat femurs had improved osseous healing when exposed to HBO (1 hour, twice daily with 100% oxygen at 3 ATA). There was an earlier and increased amount of new bone formed. There was also more cartilage, and an accelerated rate of conversion from cartilage to bone in the fracture callus. Penttinen et al (1972) studied fracture healing in rat tibias exposed to HBO (2 hours, twice daily, with 100% oxygen at 2.5 ATA). Tensile strength and mineral content was assessed. This study did not show an increase in mechanical strength with HBO, but did result in improved healing reflected by more cartilage and bone formation. In another study conducted by Niinikoski, Penttinen, and Kulonen (1970) using a similar model as described above, they found an increase in mineral content in the HBO group.

Other animal models have been used in HBO studies. A study by Nilsson et al (1988) uses a bone harvest chamber inserted in rabbit tibias to analyze bone healing with and without HBO. The results of the study showed significant improvement of bone healing in this titanium chamber after exposure to HBO, demonstrated by increased bone formation. Sawai et al (1996) have demonstrated in their animal study that HBO has accelerated the union of autogenous bone grafts. Corticocancellous blocks were harvested from the ilium of Japanese White rabbits and grafted to same-size mandibular bone defects. Union of the grafted bone was observed in the HBO group after a 2 week period, as opposed to a 4 week period in the non HBO group. Some surgeons have already applied this concept to clinical practice. In 1973, Mainous et al had a patient with a mandibular continuity defect which had to be reconstructed. A cadavaric mandibular crib was used to contain a particulate graft of cancellous bone from the iliac crest. This graft was secured to the patient's mandible with a metallic mesh. Postoperatively the patient was exposed to HBO, at 2.0 ATA of 100% oxygen, for 1 hour per day, for a total of 30

days. The patient experienced no postoperative complications and the treatment was successful in restoring mandibular continuity.

Continuity defects of the mandible have also been reconstructed by distraction osteogenesis. Distraction osteogenesis (DO) refers to the application of a mechanical force to regenerate new bone formation at a site of bone disruption. It also has the advantage in reconstructive surgery of expanding the soft tissue envelope (Aronson 1990). Muhonen et al (2002, 2002, 2002, 2004) in many of their reports demonstrated that in the irradiated rabbit mandible, osteogenesis and angiogenesis in distracted bone has increased with HBO. Osteogenesis was evaluated in terms of mineralization and overall osteoblastic activity, by histomorphometry and PET (Fluoride positron emission tomography) scans respectively. Ueng et al (1998) have studied the effects of HBO with regard to DO in nonirradiated tissues. DO was performed on the tibias of 12 rabbits, half of which were exposed to HBO. The results of the study show that HBO enhanced tibial lengthening demonstrated by an increased bone mineral density and an increase in tortional strength.

There isn't much debate when it comes to accepting that HBO is advantageous in irradiated tissue. Another accepted indication is for the placement of osseointegrated dental implants in irradiated tissues. Granstrom et al (1992) have demonstrated the need for HBO to decrease the failure rate of osseointegrated implants in irradiated bone. In his study of 125 implants placed in irradiated bone, 38.4% were lost, as opposed to 17% in nonirradiated bone. In the group of irradiated patients that received HBO, none of the implants were lost. Larsen et al (1993) demonstrated improved osseointegration in rabbit tibias and Niimi et al (1997) in their clinical study demonstrated the advantage of HBO for maxillary implants. Others have extended the application of HBO for implants in nonirradiated bone and demonstrated promising results. Sawai et al (1998) conducted a study where implants were placed in bone grafted from the iliac crest to bilateral mandible on 40 Japanese White rabbits. In the group subjected to HBO there was more bone-to-implant contact. The host bone did not show a difference between the HBO and nonHBO groups. They were able to conclude that HBO treatment was beneficial in

implant osseointegration when implants were placed immediately in free autogenous bone grafts. Chen et al (1999) in their study on rats, showed improved HA–bone contact in both irradiated and nonirradiated bones. There was also a slight increase in bone formation in irradiated rats, as well as an increased rate of bone remodeling in the nonirradiated. Johnsson et al (1993) in their rabbit experiments wanted to assess the biomechanical force necessary to remove an implant placed in irradiated and nonirradiated tibias and femurs, with the use of postop HBO. HBO increased the force necessary to unscrew titanium implants by 44% in irradiated bone compared to 22% in nonirradiated bone.

Others have explored the effects of HBO on healing of osteotomies. Wilcox et al (1976) have reported on the effects of HBO post maxillary and mandibular osteotomies in 38 cases in clinical practice. They describe that HBO augments clinical bone healing when assessed by mobility at the osteotomy sites. Nilsson et al (1987) have demonstrated in the rat mandibular model reduced tissue damage at osteotomy sites exposed to HBO. He also showed an increased vascular bed which was measured by radioactive isotopes. Increasing vascularity may have been the biologic basis for improved bone healing with HBO.

HBO has also been applied to accentuate the effects of osteoinductive growth factors. In 2001, Okubo et al demonstrated that HBO can accelerate the activity and increase the effect of osteoinduction of rhBMP-2 on differentiation of mesenchymal cells to osteoblasts. This was measured radiographically, histologically, as well as biochemically by reporting an increased production of ALP and calcium. HBO has also been combined with osteopromotive (polytetrafluoroethylene) membranes to enhance bone healing of mandibular defects in the rat model (Dahlin 1993). Once again proving that HBO can be combined to accentuate the effect of another osteopromotive technique.

The mechanism whereby HBO affects bone healing has been addressed in some of the above-mentioned literature. Some of the studies demonstrated an increased rate, while

others showed a quantitative increase in bone formation. In our study, we want to further quantify bone healing relative to critical size defects.

ANIMAL MODEL AND CRITICAL SIZE DEFECTS (CSD)

This study has used critical sized defects (CSD) to test the effect of HBO on bone healing. Schmitz and Hollinger (1986) identified critical sized defects as defects of a size that will not heal spontaneously during the lifetime of the animal. Hollinger and Kleinschmidt (1990) further classified the CSD as a defect which had less than 10% bone regeneration during the lifetime of the animal. If this did not occur during one year, then it was unlikely to ever occur. Critical sized defects are used as a model in bone research to test bone repair materials and to study continuity defects and nonunions. Bony nonunion is classified as a combination of fibrous and bony healing which will not restore continuity and function. Schmitz and Hollinger (1986) created a model which healed with osseous union only when bone repair materials were used. The dimensions of critical size defects have been determined for the various animal models at specific sites. The CSD of the calvarium for the rat is 8mm, for the rabbit 15mm and for the dog 20mm in diameter (Frame 1980, Schmitz 1986).

There are five factors which influence bone healing: animal species (Prolo 1982), animal age (Prolo 1982), anatomic site of the defect (Najjar 1977), the size of the defect and the intactness of the periosteum (Hjorting-Hansen and Andreasen 1971). Skeletal maturity is more accurately assessed utilizing radiographic imaging of closure of the epiphyseal growth plate of long bones rather than standardized weight charts. If animals have not reached maturity there is more rapid bone healing and therefore one cannot assess if defects would have healed spontaneously (Marx 1994). Reid et al (1981) have shown that the regenerative ability of calvarial defects of newborn rabbits exceeds that of the adult, especially if the periosteum and dura are preserved. Also low-order phylogenetic species have a more active bone repair mechanism so it is difficult to compare experimental results among species (Schmitz 1986). The anatomic site of the defect influences the pattern of bone healing since there are varying external forces exerted by the

musculature. Najjar (1977) demonstrated the slower healing and remodeling of the maxilla and skull in the rabbit and canine models as compared to the mandible or tibia, due to the smaller magnitude of muscle tension.

Frame (1980) has described the criteria of an ideal animal model for testing bone substitutes for mandibular defects. The animal should be inexpensive, easy to handle and readily available. It should allow us to create CSD with minimal risk of fracture. The test site should consist of both cortical and cancellous bone and should be similar to the jaw bone embryologically and physiologically; It should be of membranous origin and have the same healing pattern as the mandible. It should also allow for accurate histologic and radiographic assessment.

The calvarial model was selected due to many similarities with the maxillofacial area. Embryologically, the calvarium develops from a membranous origin, in contrast to long bones which develop from endochondral bone formation. Also in contrast to long bones which have a primary nutrient artery, there isn't a central nutrient artery in the calvarium. It receives its blood supply from the periphery; periosteum, dura and small muscle insertions (Cutting 1984). Since the human calvarium has fewer muscle insertions than other mammals, it therefore has a poorer blood supply and a decreased regenerative capacity than experimental animals (Sirola 1960). Similar to the mandible is the anatomical configuration of the calvarium, consisting of two cortical plates with intervening cancellous bone (Frame 1980). The calvarium has been described to have a similar healing pattern to the mandible. Hjorting-Hansen et al (1971) in their study on dog mandibles demonstrated that defects healed mainly from the endosteal surface and marrow, with a minor role of the periosteum. However, the effect of elevating the periosteum may have contributed to their result. Kramer et al (1968) demonstrated that cranial bone healing occurred due to the osteogenic cells at the defect margins and some from the endocranium, with the pericranium playing little part in bone regeneration. Ozerdem et al (2003) in their rat study have shown the contribution of the periosteum and dura to assist in osteogenesis of cranial defects. In Reid's study (1981), the dura seemed to promote even more osteogenesis than the periosteum. Thaller (1989) and Sirola

(1960), have shown that the dura, the periosteum and the bony margins all contribute to regeneration of bone. The calvarium as an experimental model offers other advantages. Since a large defect can be created without the risk of fracture, the site takes longer to heal and during this period of time we can study the healing process more thoroughly. Another advantage is that the thin bones of the calvarium allow better radiographic imaging and a more accurate correlation with the histological specimens (Kramer1968). Since the calvarium is minimally influenced by musculature, it has the added advantage of minimizing these external factors. The rabbit calvarium was preferable to the rat because two defects could be made in each animal. In Kramer's (1968) study on calvarial bone healing of subcritical sized defects, he demonstrated that bridging could occur in a two week period of time, but in most cases it took 8 weeks. The advantage that in the rabbit bone repair is 3-4 times faster than in humans, abbreviates the duration of the investigative period (Bruce 1941). Sigma for the rabbit is only 6 weeks (Hollinger 1999).

MATERIALS AND METHODS

EXPERIMENTAL DESIGN

Twenty adult, skeletally mature, male New Zealand White rabbits weighing 3-4 kg were randomly divided into 2 groups of 10 animals. Each animal had bilateral full thickness calvarial defects created in its parietal bones. Defects were allocated randomly as critical sized, 15mm on one side and supracritical sized, 18mm on the contralateral side. Group one was treated with HBO, while group two served as a control and did not receive any supplemental oxygen. Five animals from each group were sacrificed at 6 and 12 weeks.

SURGICAL PROCEDURE

The surgical protocol for this study was approved by the University of Toronto Animal Care and Ethics Committee (Protocol number 20005145). Each animal was premedicated according to their weight with a mixture of Ketamine (35mg/kg), Xylazine (2mg/kg) and Acepramazine (1mg/kg). After sedation was achieved, an infusion line was started in the marginal ear vein with a 22 gauge catheter and Ringer's lactate infused at 30cc/hr. General anesthesia was induced using intravenous Sodium Thiopental (20mg/kg). After induction, the rabbits were intubated using a 3mm uncuffed endotracheal tube. Anesthesia was maintained with a 1.5% Isoflurane and oxygen mixture using mechanical ventilation. The animals were monitored with pulse oxymetry and the respiration rate was set at 20 breaths per minute with a tidal volume of 10ml/kg.

The animals were placed in a prone position and the scalp was shaved and wiped with 70% EtOH. The surgical site was prepped with a poviodine solution and the animal was draped to provide a sterile field. Local anesthesia was obtained by injecting 1.8cc of 2% xylocaine with 1:100 000 epinephrine for hemostasis. A 5cm incision was made through skin and subcutaneous tissues in the midline between the base of the ears. This exposed the pericranium. The pericranium was then incised in the midline and subperiosteal

dissection exposed the parietal bones. Full-thickness calvarial defects were created in each parietal bone bilaterally. The defect was prepared using a #702 bur on a straight handpiece with copious irrigation. Standardization of the defects was accomplished by using a trimmed template of the desired size. Care was taken to avoid the sagittal sinus and the dura matter. The most medial aspect of the osteotomy was 2mm away from the sagittal suture, which was used as a landmark for identifying the original defect on histological sections. Wound closure was performed in layers. The pericranium was closed with interrupted 4-0 vicryl sutures and the subcuticular layer and skin closed with 4-0 vicryl and 3-0 plain gut sutures. The anesthetic gas was stopped and the animals were extubated after they began breathing spontaneously. Postoperative analgesics consisted of Bupremorphine 0.02mg/kg IM tid for pain. Baytril 2.5mg/kg bid for 48 hours was given as antibiotic coverage. The rabbits were caged individually and fed sterile water and food. They were weighed and examined daily to ensure adequate health throughout the experimental period.

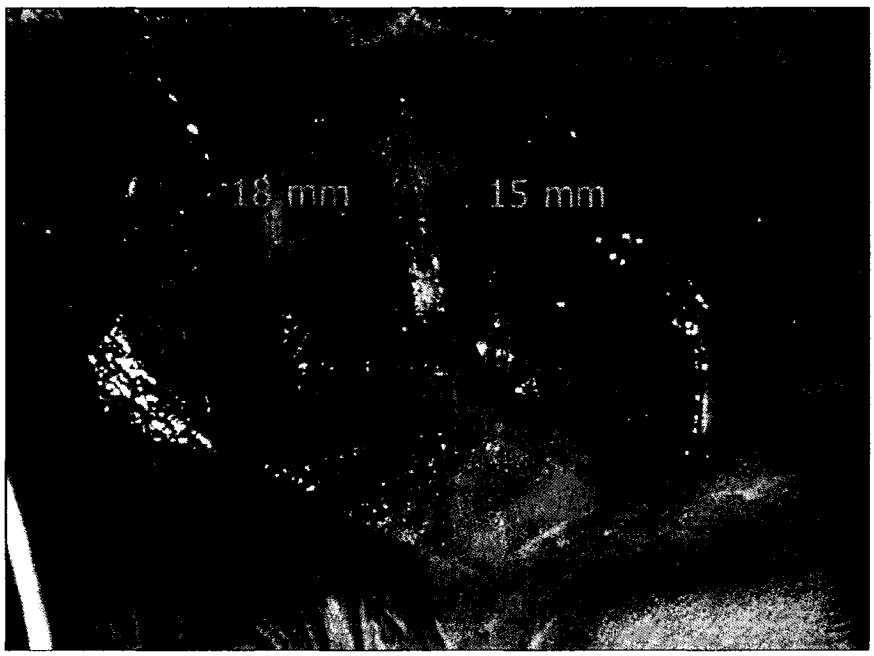

FIGURE I. Critical (15mm) and supracritical (18mm) sized defects created in the rabbit parietal bones.

HYPERBARIC OXYGEN SESSIONS

The 10 animals in group one (n=10) underwent a 90 minute HBO session at 2.4 absolute atmospheric pressure (ATA) per day, 5 days a week, for 4 weeks (20 days total). Pressurization and depressurization was done at a very slow rate (0.2 ATA per minute) in order to avoid barotraumas 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 minutes a 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 minute sessions.

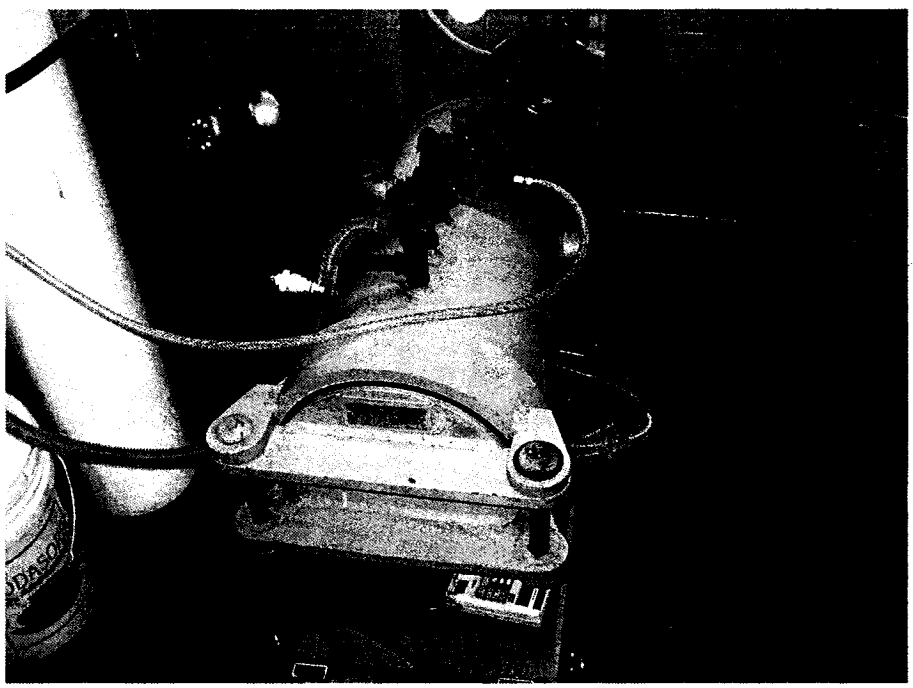

FIGURE II. Animal hyperbaric oxygen chamber.

SACRIFICE AND QUALITATIVE ANALYSIS

Five animals from each group were sacrificed 6 weeks postoperatively and five from each group were sacrificed 12 weeks postoperatively using T-61 administered intravenously. The parietal bones were divided from the rest of the cranium with an oscillating saw, carefully maintaining the pericranium and the dura matter intact. Specimens were examined grossly for signs of inflammation, transilluminated, photographed, radiographed with a cephalostat, and then fixed in 10% buffered formalin solution for 3 days before proceeding with the histological preparations.

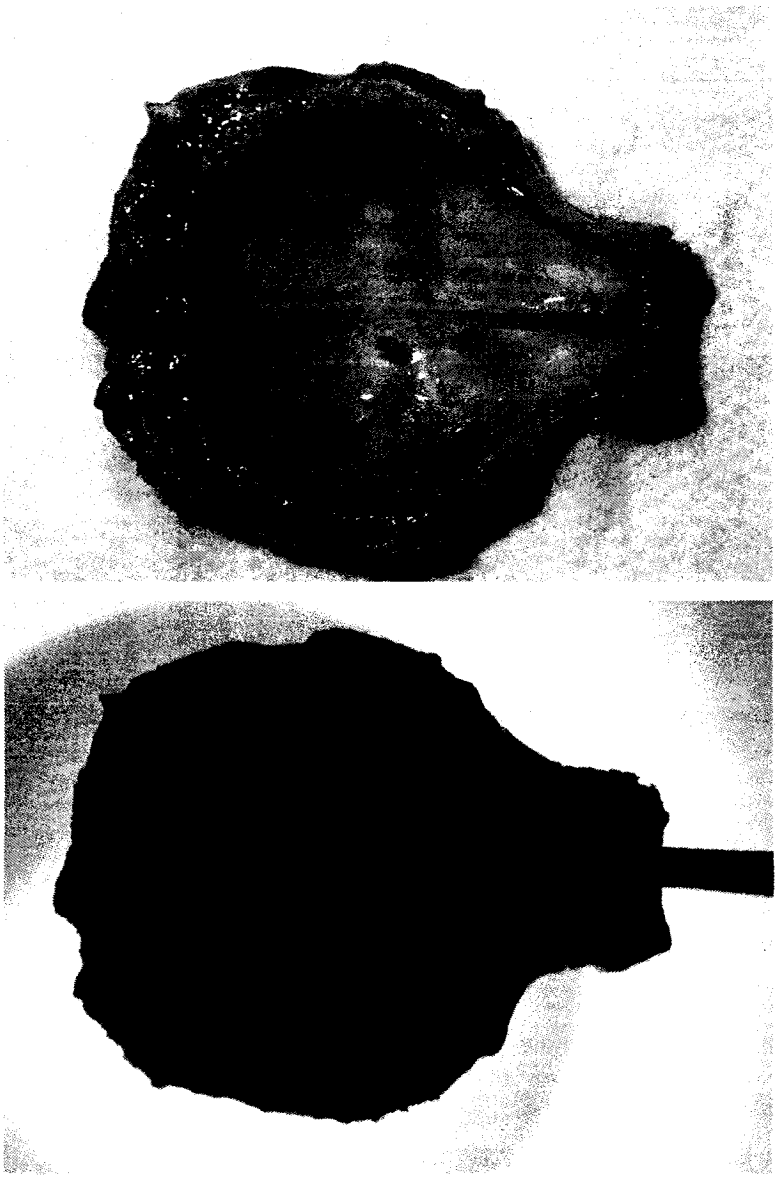

FIGURE III. Gross examination and transillumination of specimens

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).

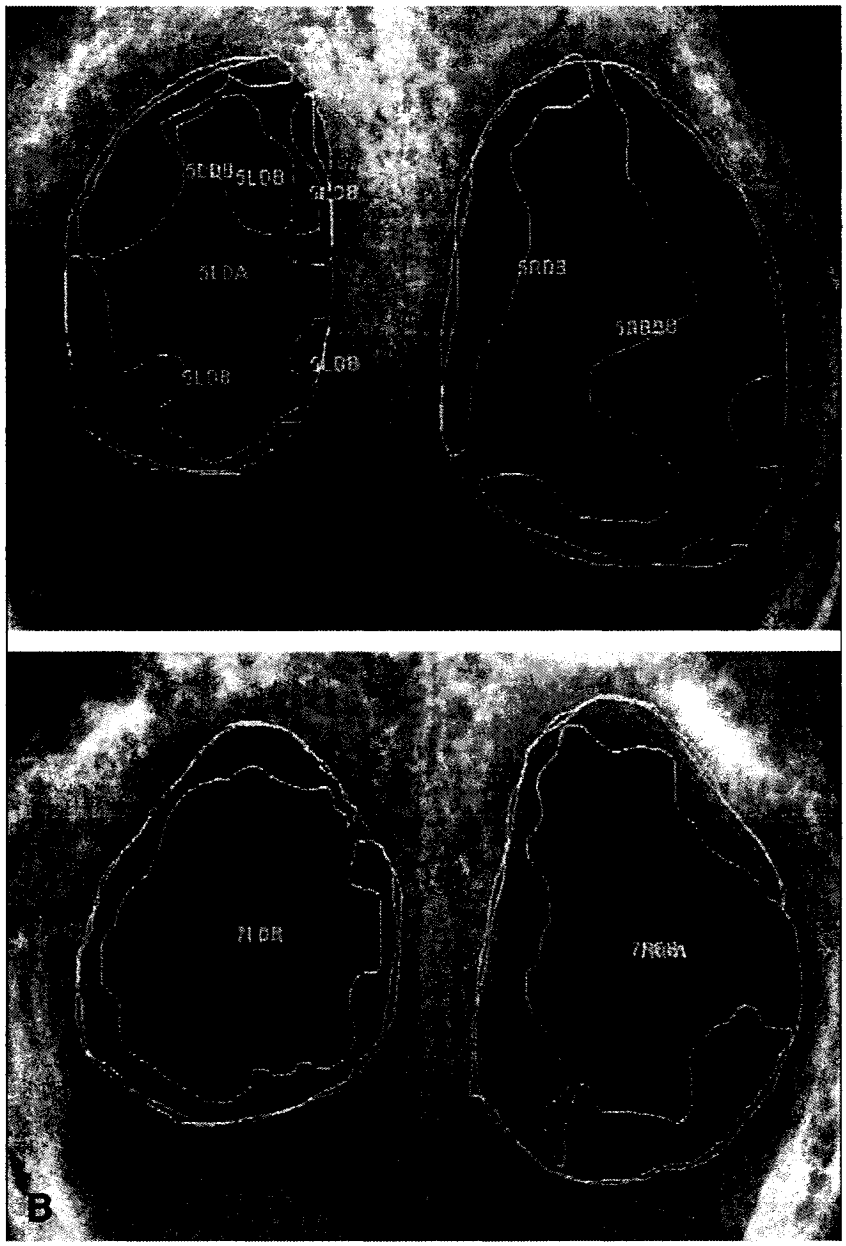

FIGURE IV. Postsacrifice radiograph of the parietal bones of A, HBO-treated group animal and B, control group animal.

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

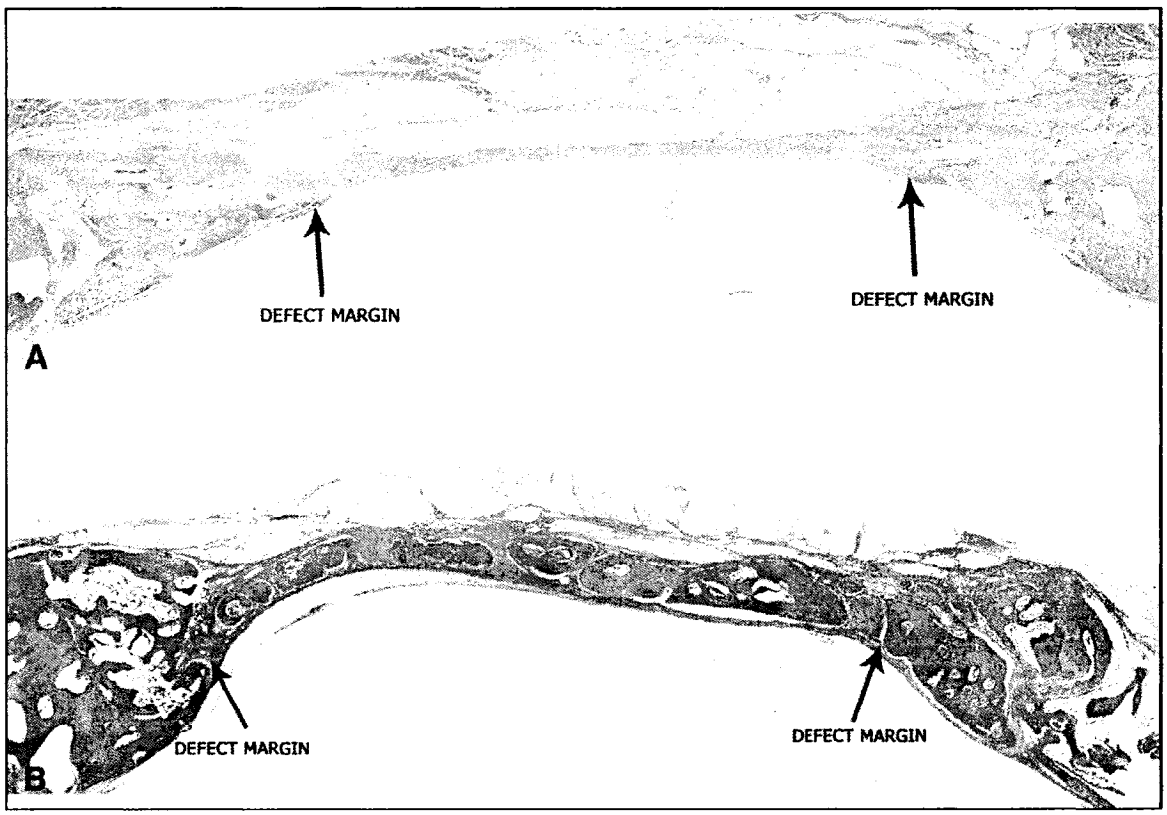

FIGURE V. 4X magnification hematoxylin and eosin stained sections. A, control group specimen healed primarily with fibrous band of tissue. B, HBO-treated animal sample showing bony healing of the defect.

HISTOMORPHOMETRICS

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. The result was a series of images captured for each slide. To create a single image from each slide, the digital images were merged using Adobe Photoshop Elements® 2.0. The images were organized by the software to create the perfect overlap. 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

DATA MED Statistics utilized Two Way ANOVA 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 versus Normobaric Oxygen [NBO]), (2) Defect size (15mm versus 18mm), or (3) Healing time (6 weeks versus 12 weeks). The p values below .05 were considered to be statistically significant (Refer to the appendex).

RESULTS

QUALITATIVE ANALYSIS

All 20 rabbits tolerated the anesthesia and the surgical procedures well and experienced no complications during the experimental period. HBO sessions were uneventful for the test 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 15mm and 18mm defects in terms of signs of inflammation and integrity of the healing wound.

QUANTITATIVE ANALYSIS

Radiomorphometrics

Radiographs demonstrated more islands of radiopacities in the HBO group compared to the control group in both the 6 and 12 week specimens ($p=.0142$, and $p<.0001$ respectively). 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 while in the HBO group more radiopaque areas were evident both along the margins as well as in the center of the defects. No differences were noted between the 15mm and 18mm defects. The percentage of radiopacities were greater in HBO samples at 12 weeks when compared to those at 6 weeks.

TABLE II. Percent radiopacities within the HBO (experimental) and the NBO (control) defects

Sacrifice Time	6 Weeks				12 Weeks			
Defect size	15mm		18mm		15mm		18mm	
Oxygen	HBO	NBO	HBO	NBO	HBO	NBO	HBO	NBO
Percent radiopacities within defect	52.96	38.31	29.73	29.86	79.51	47.29	95.5	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	2.1	92.24	39.02
	57.85	47.39	44.22	26.6	90.35	30.5	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.

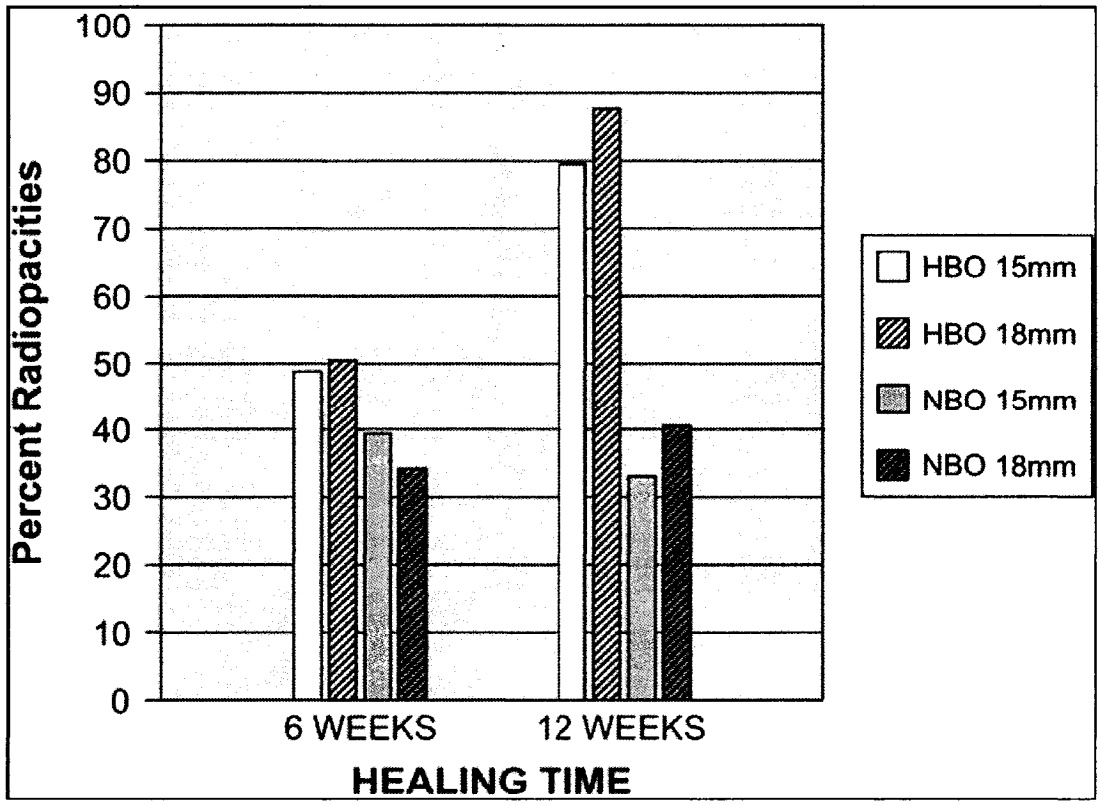

TABLE III. Bar chart showing the differences in the mean radiopacity measurements within defects in the study groups (HBO: hyperbaric oxygen, NBO: normobaric oxygen).

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 during preparation of the calvarial defects. 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 trabecular bone.

Histomorphometrics

Histomorphometric analysis demonstrated more bone formation in the HBO group when compared to the control group ($p<.0001$). Both critical (15mm) and supracritical (18mm) 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 15mm and the 18mm defects, nor between the 6 week and the 12 week groups.

TABLE IV. Percent new bone formation in the HBO (experimental) and the NBO (control) groups

Sacrifice Time	6 Weeks				12 Weeks			
Defect size	15mm		18mm		15mm		18mm	
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
	49.6	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	19.9
	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.

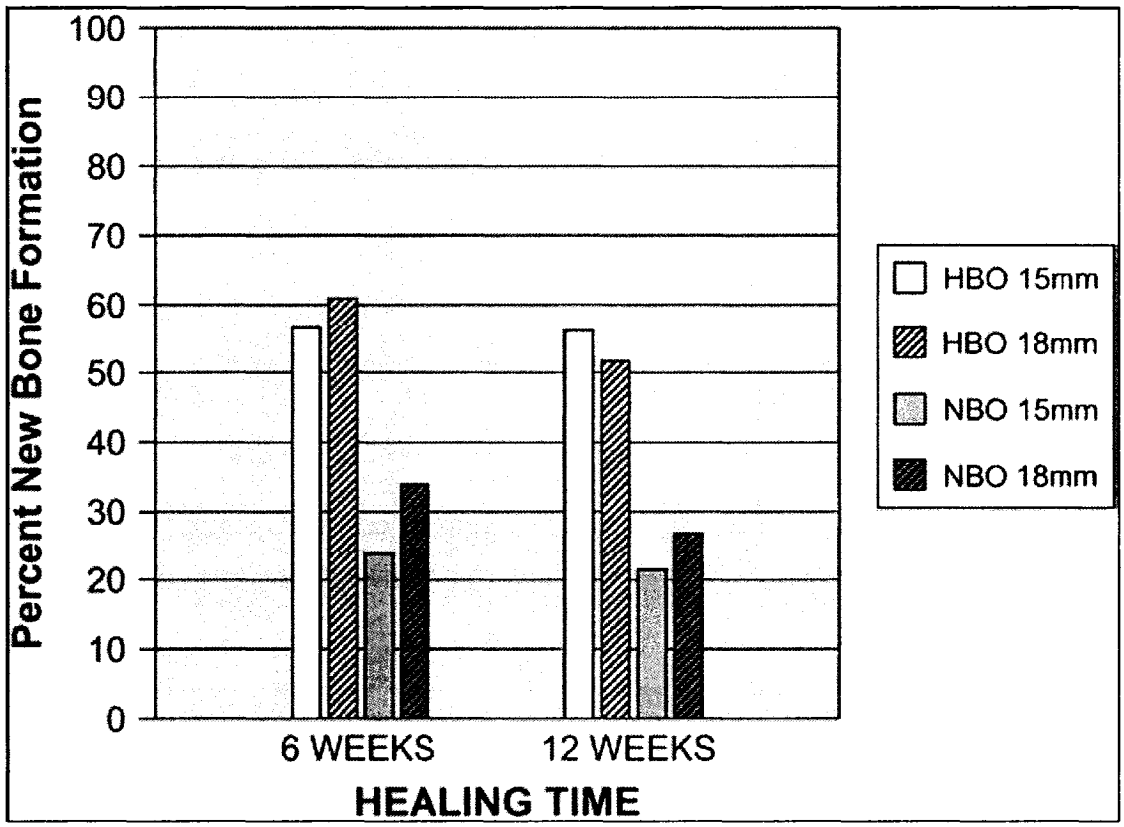

TABLE V. Bar chart showing the differences in the means of new bone formation based on histomorphometric measurements in the study groups (HBO: hyperbaric oxygen, NBO: normobaric oxygen)

DISCUSSION

The reconstruction of skeletal defects has traditionally been dealt with by means of bone grafting techniques, including vascularized and nonvascularized autografts. Recently, there has been much research in the area of minimally invasive techniques for maxillofacial surgery to decrease morbidity associated with surgical procedures. Although HBO may also be considered an adjunct to traditional grafting techniques in compromised wounds (Mainous 1973), in this experiment hyperbaric oxygen was investigated as a possible substitute to reconstructive surgery.

The rabbit calvarial model was selected since, like most craniofacial bones, it heals by intramembranous bone formation and has a periosteal blood supply. The difference with this model is the presence of a dural layer, which does not exist in extra-cranial sites. The critical sized defect, employed in this animal model corresponded to that 15mm defect defined by Schmitz and Hollinger (1986). In 1990 Hollinger and Kleinschmidt further defined such a defect as having a maximum of 10% bony healing over a period of ten years postoperatively. We also created supracritical sized defects of 18mm on the contralateral side. Defect size in this animal model was limited by the midline sagittal sinus, and risk of hemorrhage. These parameters prevented extension across the temporal and frontal bones in order to avoid involvement of the sutures which may have altered healing. Since these bone defects could be created bilaterally, it allowed us to decrease the number of experimental animals by half. The rabbit calvarium has served as a suitable animal model in prior studies that tested bone substitutes for the maxillofacial region (Clokie 2002, Moghadam 2004). To increase the validity of the study, we did not include animals that had not reached maturity (as determined by standardized weight charts) since they would have an increased healing potential (Reid 1981).

Hyperbaric oxygen (HBO) has been applied for various medical indications including healing of compromised wounds, carbon monoxide poisoning and osteomyelitis. The widely used protocol for HBO is exposure to 100% oxygen at 2.4 ATA for 90 min per day, for 20 exposures pre-operatively and 10 exposures post-operatively. There is also an

extended protocol for the treatment of osteoradionecrosis, whereby there are 30 dives of HBO pre-operatively and 10 dives post-operatively. The effects of HBO reach a plateau at these exposures. There are many actions of HBO, but the mechanism whereby HBO improves bone healing is via angiogenesis and collagen synthesis (Marx 2003, Knighton 1981). Since hypoxia and lactate are necessary triggers for promoting osseous regeneration (Knighton 1983, Jensen 1986), it is crucial that HBO application is intermittent (Marx 2003). In irradiated tissue, HBO is required to create an oxygen gradient to stimulate angiogenesis. An oxygen gradient does not inherently exist since the tissue is exposed to different levels of radiation. In the present study, our hypothesis is that HBO is indicated to improve the oxygen gradient that exists in the critical sized defect, to ultimately improve bone healing. All wounds and all osseous defects are inherently hypoxic which drives the healing process. We hypothesized that a critical sized defect may have an oxygen tension too low to stimulate spontaneous wound healing. As postulated by Hunt et al (1969), if more oxygen is made available, fibroblasts will migrate further from the last functioning capillary, laying down collagen and supporting capillaries more quickly and at greater distances. Under hyperbaric conditions, diffusion can be ten to fifteen times further into tissues (Evans 2002). In our study, this was achieved via a HBO protocol of 100% oxygen at 2.4 ATA for 90 min per day for 20 post-operative dives. By applying HBO to the bony defect we predicted an increase in available oxygen to allow further migration of fibroblasts into the defect. This in turn would increase the production of collagen to provide the framework for capillary support. Subsequently, angiogenesis would promote complete bone healing in a defect that wouldn't have otherwise healed. Since the HBO chamber that was used was an animal chamber and therefore with smaller dimensions, it had to be calibrated to equate to 2.4 ATA of pressure. This pressurization was equal to 20 psi.

There is evidence in the literature regarding the effects of HBO on bone healing in irradiated tissue (Marx 1983, Mainous 1975). There is also evidence that HBO is beneficial in nonirradiated tissues. Early in vitro studies by Bassett (1962) showed that when multipotential mesenchymal cells were cultured with increased oxygen levels, there was an elevated amount of bone formation. Other in vitro studies also showed increased

osteogenesis with hyperoxia (Tuncay 1994). Current studies demonstrate an increased calcification or a decreased time for osseous healing. The literature has promising results when HBO is utilized for fracture healing. Coulson et al (1966) studied bone healing in the rat and showed increased calcium incorporation and increased breaking strengths when exposed to HBO. Nilson et al (1988) used a bone harvest chamber implanted in the rabbit tibia to also demonstrate increased bone healing. Its application with distraction osteogenesis and osseointegrated dental implants is also promising (Granstrom 1992, Muhonen 2004). Others have applied this experimental data to clinical practice and used HBO to improve bone healing with osteotomies (Wilcox 1976) as well as bone grafts (Mainous 1973) with successful results. Fracture healing, graft healing and irradiated tissue healing have a requirement for HBO since they are all inherently hypoxic. The hypoxia has been shown to trigger macrophage chemotaxis and release MDAF and MDGF to stimulate angiogenesis of the surrounding capillaries and mitogenesis of osteocompetent cells. A minimum oxygen gradient of 20mmHg must exist to trigger this response (Knighton 1983). There is feedback inhibition when the hypoxia is eliminated from the environment. The effects provided by a total of 30 hours of HBO achieve approximately 80% normality. These results appear to be longterm, so HBO does not have to be readministered (Marx 1984). In our study, the effects of HBO were correlated with the amount of bone formation in the critical and supracritical sized defects. Complete osseous healing (sigma) in the rabbit model has been shown to take 6 weeks (Hollinger 1999). We selected two time intervals (6 and 12 weeks) for studying healing since we expected critical sized defects to require a longer duration of time to heal, especially since these defects are too large for sufficient angiogenesis to occur (Schmitz 1986). There is also a lag period of vascular insufficiency, whereby HBO therapy has a less than optimal effect on osseous healing. Whether this lag period is due to a period of collagen synthesis preceeding endothelial proliferation (Marx 1984) or gene induction (Tompach 1997) is unknown. VEGF is another angiogenic factor that is induced by hypoxia. It has been shown to stimulate endothelial cell proliferation (Mulhauser 1995). An extension of this study could be to measure the amounts of VEGF in the defect during osseous healing. Okubo et al (2001) has already published evidence that HBO can

increase the effect of growth factors, such as rhBMP-2 on differentiation of mesenchymal cells to osteoblasts.

The present study has validated our theory that HBO can enhance bone healing even in the nonirradiated rabbit model. Since critical sized defects are too large for sufficient angiogenesis to occur, osseous healing would therefore not occur without the application of HBO. Histological evaluation demonstrated that the control group which was not exposed to HBO, healed mainly by scar formation with some bone islands at the periphery. The HBO group produced a more confluent bone formation, with large marrow spaces and blood vessels. Mature lamellar bone was present at 12 weeks, whereas immature woven bone was seen at the 6 week interval. The radiomorphometrics demonstrated more radiopacities in the HBO groups, after both 6 and 12 weeks. The healing pattern of the control group was consistent with incomplete osseous healing; the islands of bone being mainly in the periphery. The greater percentage of bone healing, as represented by increased number of radiopaque bone islands at the periphery and in the center of the defect, were attributed to the effects of HBO. At 12 weeks, there was a greater percentage of radiopacities than at 6 weeks, whereas there was no difference between the 15mm and the 18mm defects. When compared to the histomorphometrics, once again the amount of bone formation was increased in the HBO groups with no significant difference between the 15mm and 18mm defects. However, bone formation at 6 and 12 weeks was unchanged. This finding could be explained by the fact that bone that is present histologically will not be evident radiographically unless it is considerably mineralized. Histological specimens demonstrated less trabeculated bone at 6 weeks and more trabeculated bone at 12 weeks, which would be expected to result in an increase in the radiodensity of the bone. Since HBO produced complete healing of supracritical sized defects, it may have changed the definition of critical size defect for this rabbit model to more than 18mm. To evaluate the accuracy of our control group, one may compare the means of new bone formation calculated by histomorphometry, to the definition of a critical sized defect. The means varied from 21.46% to 33.87% new bone formation, as compared to the definition of CSD which should not exceed 10% new bone formation. This represents an elevated level in the control group. An explanation for this finding

could be that some of the animals were still in the developmental stage. Although standardized weight charts are often used to confirm skeletal maturity, radiographic imaging of closure of the epiphyseal growth plates is a more accurate method (Marx 1994). Another contributing factor could be that perhaps the bone filings may not have been debrided adequately prior to closure of the incisions, but it could not be determined with certainty as a source of error. This healing could also have been site specific. Perhaps HBO has improved healing in the rabbit calvarial model and may not have affected another site equally. The calvarium has been shown to heal from pluripotential cells of the periosteum, marrow and dura. There is even some evidence that the dura contributes to more osteogenesis than the periosteum (Reid 1981). This bone healing could have been enhanced by the presence of two highly vascularized soft tissue beds approximating the defect. Our model has still proven to be of value since the difference between groups (HBO vs Control) was statistically significant. One could argue that there was a deficiency in the study, since the control group breathing room air was not placed in the hyperbaric chamber. The group receiving HBO had increased handling and confinement in the HBO chamber and therefore increasing stress which in turn would be expected to adversely affect healing and not improve it. Furthermore, since we acclimatized the HBO group to the chamber for one week before the surgical procedure, this would reduce the discomfort from confinement and thus minimize stress.

Whether or not the application of increased oxygen can predictably improve bone healing, has yet to be determined. The process is multifactorial since the oxygen delivery system may be affected on multiple levels. Pulmonary, cardiac and vasomotor activity are but a few factors which may lead to a varied response to oxygen therapy. The preceeding studies on bone healing are quite consistent in their results by demonstrating a positive response to hyperoxia and HBO therapy. Another aspect which was not analyzed in our study is the effect of HBO or hyperoxia relative to soft tissue healing. Kulonen et al (1967) has shown that under normal barometric conditions, added oxygen can increase healing in normal wounds, demonstrated by an increase in the tensile strength of healing skin wounds. Hunt and Pai (1972) have experimental data that also supports the theory that hyperoxia increases collagen synthesis. The beneficial effect of hyperoxia for soft

tissue healing is prominent in the literature. Although, there is some mention of the reverse finding, where oxygen applied under hyperbaric conditions, inhibits collagen synthesis (Kulonen 1968). Heppenstall et al (1975) speculated that the repair process for soft tissue healing and bone healing may be different. An oxygen gradient may be a trigger for repair in osseous healing, whereas the hypoxia itself may limit the rate of repair in soft tissue healing.

The results of the study are quite promising, but cannot yet be extrapolated to the human model since lower order species have a more active bone repair. The next step would be to study higher order species and then ultimately clinical trials. Eventually, application of these findings can be made to other bones of the maxillofacial complex, but one must consider that there may be some differences. For example, the mandibular vascularity consists of a central vessel and a periosteal blood supply, whereas the calvarium has primarily a periosteal source.

It is already shown in previous studies that HBO can accelerate the healing of osseous defects both quantitatively and qualitatively (Coulson 1966, Penttinen 1972, Kulonen 1970). In this study we wanted to induce bone healing in a defect that would not have otherwise healed on its own (CSD). In clinical practice the application of these results would be directed towards utilizing HBO for restoration of continuity defects alone or in combination with other surgical approaches such as vascularized or nonvascularized grafts. In the nonirradiated patient as in our animal model, HBO could be applied after ablative surgery prior to fibrosis taking place at the defect site. The cost to the medical system if we decide to routinely incorporate HBO in our surgical protocols would be astronomical. There has been an effort to try to find the most cost-effective protocol and to try to reduce exposures to decrease side effects (Tompach 1997, Aitasalo 1998, Barth 1990). Marx (1990) has also revisited the idea that perhaps HBO therapy may be replaced by hyperoxygenation. In his study he demonstrated an 8 to 9 fold increase in vascular density with HBO compared to normobaric oxygen. At this point the results still support the current protocol of 100% oxygen for 90min per day at 2.4ATA for 20 dives pre-op and 10 dives post-op.

CONCLUSION

Hyperbaric oxygen has become a reliable method of treating a long list of medical conditions. Of relevance to the cranio-maxillofacial surgeon is the application of HBO for treatment of compromised wounds. To date the reconstruction of skeletal defects have been treated with autogenous bone grafts, alloplasts and allografts and HBO has not been indicated in these scenarios. There can be increased morbidity and extended hospitalizations as a result of this treatment approach. There may be a role for HBO in the normal bone healing pathway. This has become evident in recent literature.

This thesis presents the effects of hyperbaric oxygen (HBO) on the healing of critical and supracritical sized calvarial defects in mature New Zealand white rabbits. The effects of HBO were analyzed by reporting osseous regeneration in the calvarial defect, assessed both radiographically and by histomorphometry after 6 and 12 weeks of healing. The results are promising since complete osseous healing occurred in both the critical and supracritical sized defects when the rabbits were exposed to HBO. The results of this study may signify that HBO can substitute conventional bone grafting techniques. Another possibility is utilizing HBO as an adjunct to improve the outcome of these techniques or decrease the surgical morbidity associated with these procedures.

The rabbit calvarial critical-sized defect model has been suitable for testing the effect of HBO for cranio-maxillofacial bone repair. Before conducting clinical trials, this research must be tested on higher order species.

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APPENDIX

1. STATISTICAL ANALYSIS
2. ANIMAL PROTOCOL APPROVAL

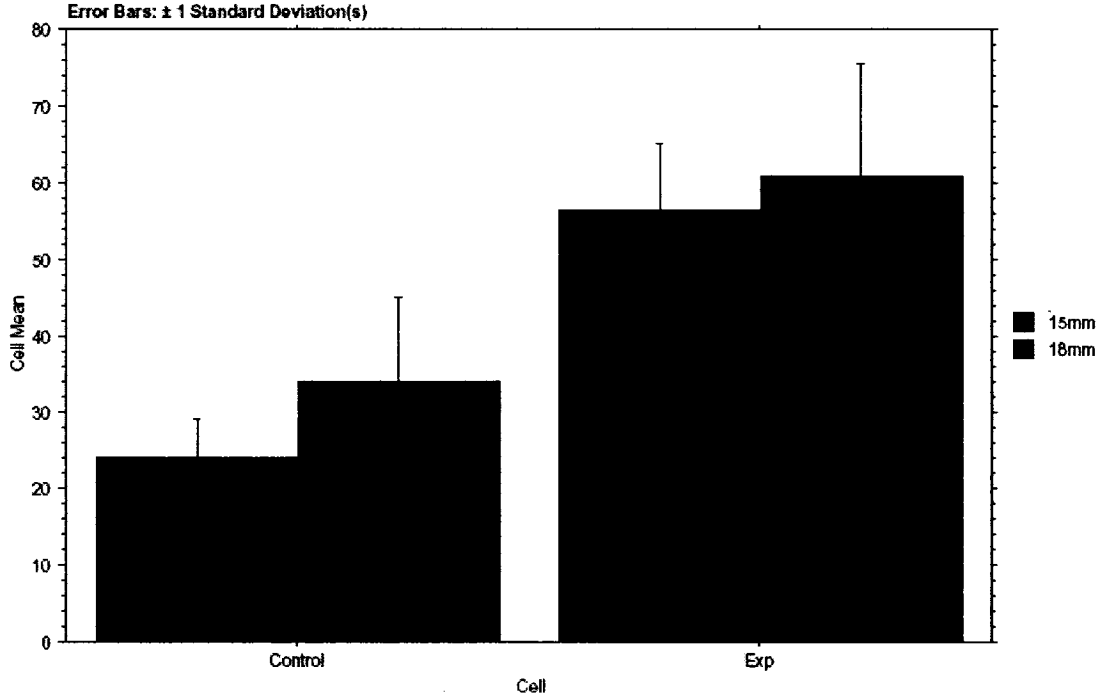
ANOVA Table for Histomorpho 6wks

	DF	Sum of Squares	Mean Square	F-Value	P-Value	Lambda	Power
Group	1	4453.919	4453.919	39.652	<.0001	39.652	1.000
Size	1	257.188	257.188	2.290	.1497	2.290	.283
Group * Size	1	40.158	40.158	.358	.5583	.358	.085
Residual	16	1797.206	112.325				

Means Table for Histomorpho 6wks
Effect: Group * Size

	Count	Mean	Std. Dev.	Std. Err.
Control, 15mm	5	23.862	5.392	2.411
Control, 18mm	5	33.868	11.371	5.085
Exp, 15mm	5	56.542	8.740	3.909
Exp, 18mm	5	60.880	14.647	6.550

Interaction Bar Plot for Histomorpho 6wks
Effect: Group * Size
Error Bars: ± 1 Standard Deviation(s)



Fisher's PLSD for Histomorpho 6wks
Effect: Group
Significance Level: 5 %

	Mean Diff.	Crit. Diff.	P-Value	
Control, Exp	-29.846	10.048	<.0001	S

Fisher's PLSD for Histomorpho 6wks
Effect: Size
Significance Level: 5 %

	Mean Diff.	Crit. Diff.	P-Value
15mm, 18mm	-7.172	10.048	.1497

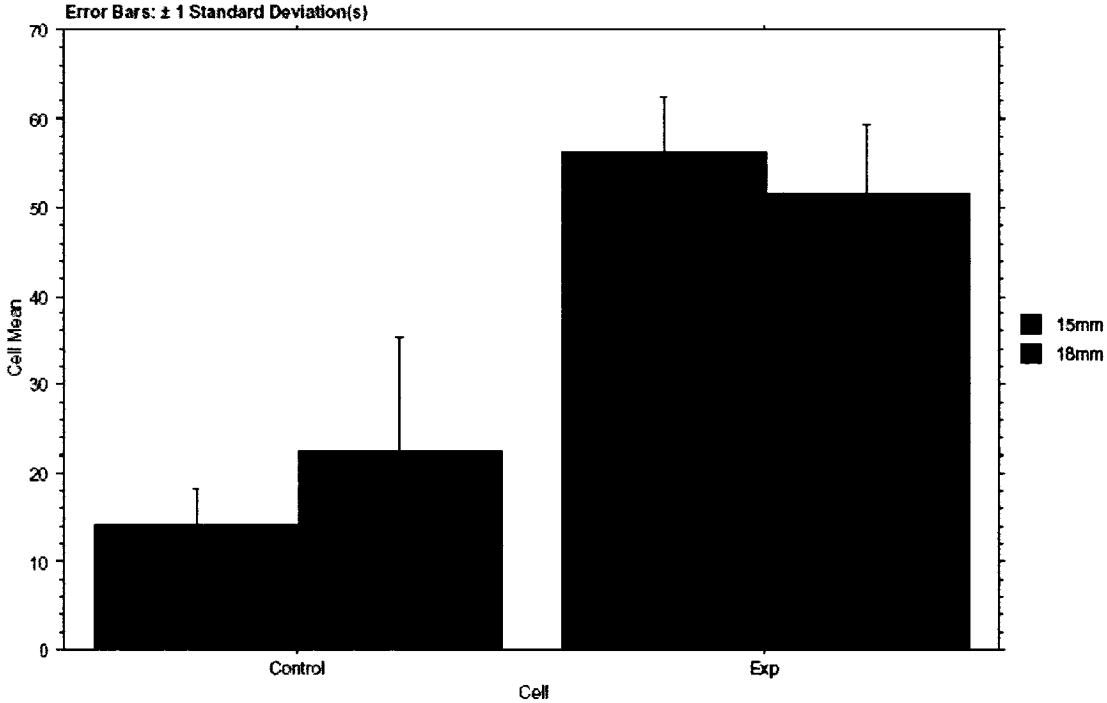
ANOVA Table for Histomorpho 12wks

	DF	Sum of Squares	Mean Square	F-Value	P-Value	Lambda	Power
Group	1	6298.055	6298.055	90.772	<.0001	90.772	1.000
Size	1	19.345	19.345	.279	.6047	.279	.078
Group * Size	1	210.536	210.536	3.034	.1007	3.034	.360
Residual	16	1110.131	69.383				

Means Table for Histomorpho 12wks
Effect: Group * Size

	Count	Mean	Std. Dev.	Std. Err.
Control, 15mm	5	14.182	4.072	1.821
Control, 18mm	5	22.638	12.759	5.706
Exp, 15mm	5	56.162	6.251	2.796
Exp, 18mm	5	51.640	7.686	3.437

Interaction Bar Plot for Histomorpho 12wks
Effect: Group * Size
Error Bars: ± 1 Standard Deviation(s)



Fisher's PLSD for Histomorpho 12wks
Effect: Group
Significance Level: 5 %

	Mean Diff.	Crit. Diff.	P-Value	
Control, Exp	-35.491	7.897	<.0001	S

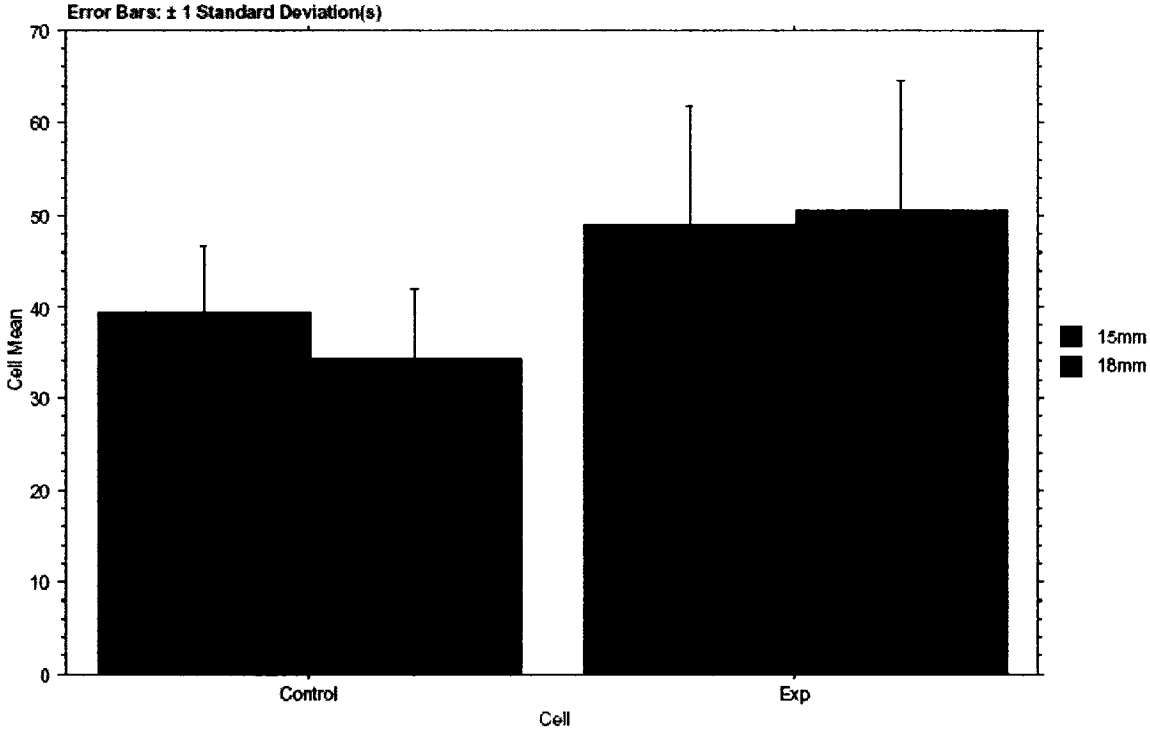
Fisher's PLSD for Histomorpho 12wks
Effect: Size
Significance Level: 5 %

	Mean Diff.	Crit. Diff.	P-Value
15mm, 18mm	-1.967	7.897	.6047

Means Table for Radiomorpho 6wks
Effect: Group * Size

	Count	Mean	Std. Dev.	Std. Err.
Control, 15mm	5	39.344	7.356	3.290
Control, 18mm	5	34.194	7.690	3.439
Exp, 15mm	5	48.778	13.031	5.828
Exp, 18mm	5	50.418	14.204	6.352

Interaction Bar Plot for Radiomorpho 6wks
Effect: Group * Size
Error Bars: ± 1 Standard Deviation(s)



Fisher's PLSD for Radiomorpho 6wks
Effect: Group
Significance Level: 5 %

	Mean Diff.	Crit. Diff.	P-Value	
Control, Exp	-12.829	10.437	.0191	S

Fisher's PLSD for Radiomorpho 6wks
Effect: Size
Significance Level: 5 %

	Mean Diff.	Crit. Diff.	P-Value
15mm, 18mm	1.755	10.437	.7262

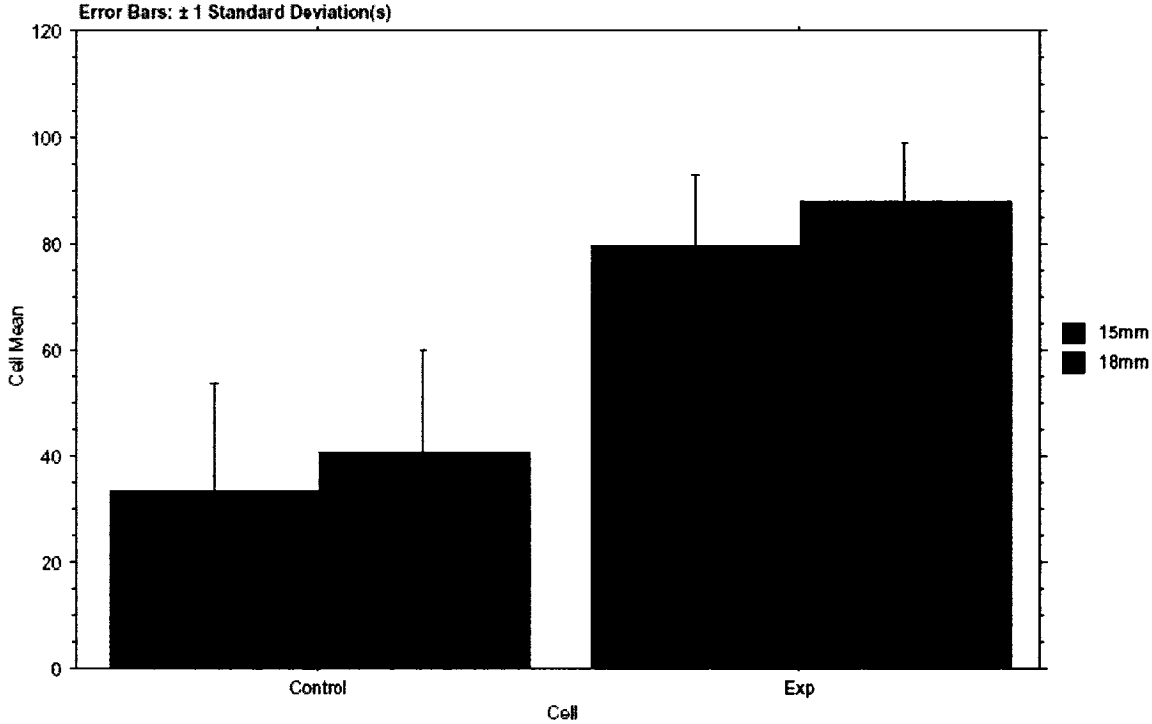
ANOVA Table for Radiomorpho 12wks

	DF	Sum of Squares	Mean Square	F-Value	P-Value	Lambda	Power
Group	1	10946.053	10946.053	40.056	<.0001	40.056	1.000
Size	1	304.746	304.746	1.115	.3066	1.115	.161
Group * Size	1	.741	.741	.003	.9591	.003	.050
Residual	16	4372.338	273.271				

Means Table for Radiomorpho 12wks
Effect: Group * Size

	Count	Mean	Std. Dev.	Std. Err.
Control, 15mm	5	33.082	20.245	9.054
Control, 18mm	5	40.504	19.593	8.762
Exp, 15mm	5	79.486	13.380	5.984
Exp, 18mm	5	87.678	10.969	4.906

Interaction Bar Plot for Radiomorpho 12wks
Effect: Group * Size
Error Bars: ± 1 Standard Deviation(s)



Fisher's PLSD for Radiomorpho 12wks
Effect: Group
Significance Level: 5 %

	Mean Diff.	Crit. Diff.	P-Value	
Control, Exp	-46.789	15.672	<.0001	S

Fisher's PLSD for Radiomorpho 12wks
Effect: Size
Significance Level: 5 %

	Mean Diff.	Crit. Diff.	P-Value
15mm, 18mm	-7.807	15.672	.3066

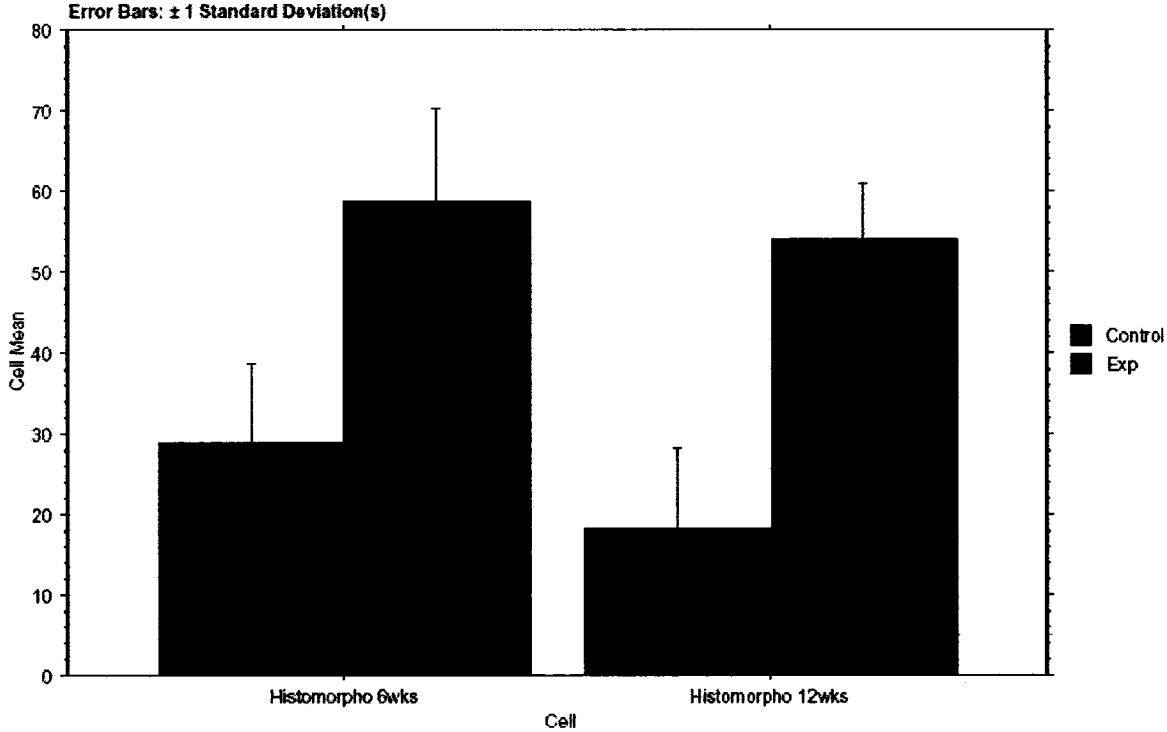
ANOVA Table for Histomorpho

	DF	Sum of Squares	Mean Square	F-Value	P-Value	Lambda	Power
Group	1	10672.309	10672.309	110.039	<.0001	110.039	1.000
Subject(Group)	18	1745.755	96.986				
Category for Histomorpho	1	582.551	582.551	6.209	.0227	6.209	.654
Category for Histomorpho * Group	1	79.665	79.665	.849	.3690	.849	.135
Category for Histomorpho * Subject(Group)	18	1688.809	93.823				

Means Table for Histomorpho
Effect: Category for Histomorpho * Group

	Count	Mean	Std. Dev.	Std. Err.
Control, Histomorpho 6wks	10	28.865	9.909	3.134
Control, Histomorpho 12wks	10	18.410	9.979	3.156
Exp, Histomorpho 6wks	10	58.711	11.599	3.668
Exp, Histomorpho 12wks	10	53.901	7.021	2.220

Interaction Bar Plot for Histomorpho
Effect: Category for Histomorpho * Group
Error Bars: ± 1 Standard Deviation(s)



Fisher's PLSD for Histomorpho
Effect: Group
Significance Level: 5 %

	Mean Diff.	Crit. Diff.	P-Value	
Control, Exp	-32.668	6.543	<.0001	S

Fisher's PLSD for Histomorpho
Effect: Category for Histomorpho
Significance Level: 5 %

	Mean Diff.	Crit. Diff.	P-Value	
Histomorpho 6wks, Histomorpho 12wks	7.632	6.435	.0227	S

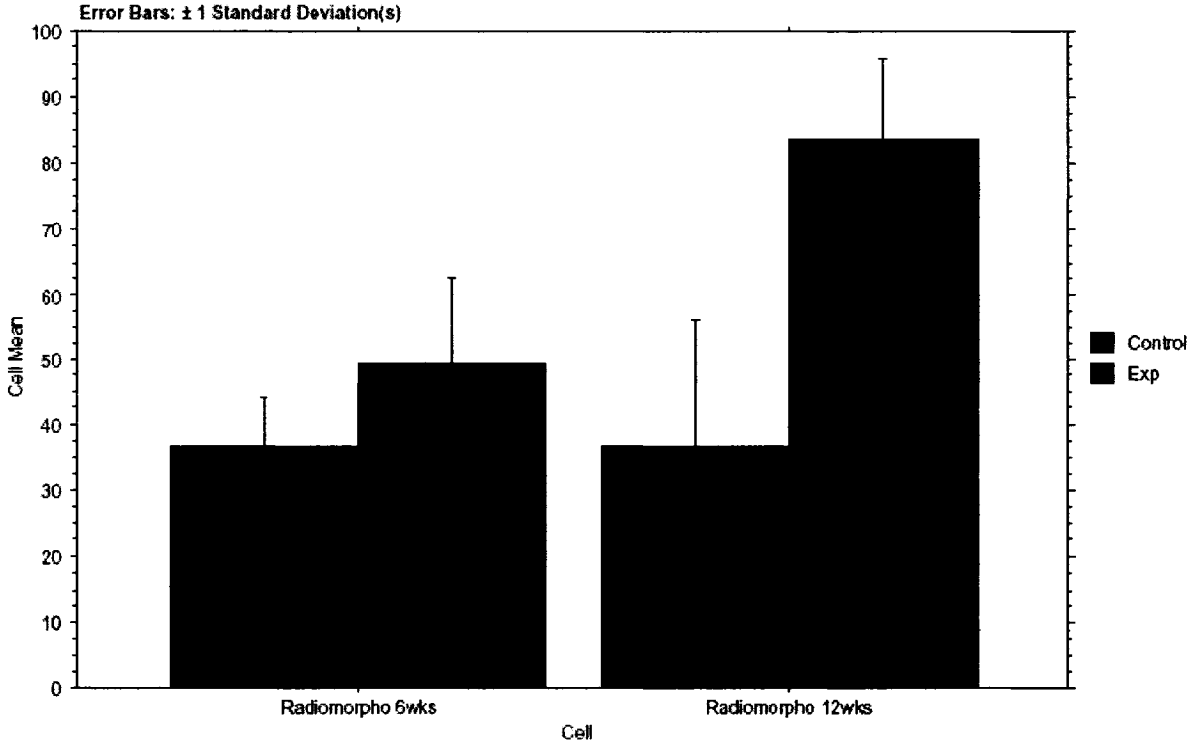
ANOVA Table for Radiomorpho

	DF	Sum of Squares	Mean Square	F-Value	P-Value	Lambda	Power
Group	1	8885.765	8885.765	52.398	<.0001	52.398	1.000
Subject(Group)	18	3052.478	169.582				
Category for Radiomorpho	1	2891.360	2891.360	14.307	.0014	14.307	.960
Category for Radiomorpho * Group	1	2883.204	2883.204	14.267	.0014	14.267	.960
Category for Radiomorpho * Subject(Gro...	18	3637.588	202.088				

Means Table for Radiomorpho
Effect: Category for Radiomorpho * Group

	Count	Mean	Std. Dev.	Std. Err.
Control, Radiomorpho 6wks	10	36.769	7.596	2.402
Control, Radiomorpho 12wks	10	36.793	19.185	6.067
Exp, Radiomorpho 6wks	10	49.598	12.880	4.073
Exp, Radiomorpho 12wks	10	83.582	12.316	3.895

Interaction Bar Plot for Radiomorpho
Effect: Category for Radiomorpho * Group
Error Bars: ± 1 Standard Deviation(s)



Fisher's PLSD for Radiomorpho
Effect: Group
Significance Level: 5 %

	Mean Diff.	Crit. Diff.	P-Value	
Control, Exp	-29.809	8.652	<.0001	S

Fisher's PLSD for Radiomorpho
Effect: Category for Radiomorpho
Significance Level: 5 %

	Mean Diff.	Crit. Diff.	P-Value	
Radiomorpho 6wks, Radiomorpho 12wks	-17.004	9.445	.0014	S

Means Table for Histomorpho 6wks
Effect: Group

	Count	Mean	Std. Dev.	Std. Err.
Control	10	28.865	9.909	3.134
Exp	10	58.711	11.599	3.668

ANOVA Table for Histomorpho 6wks

	DF	Sum of Squares	Mean Square	F-Value	P-Value	Lambda	Power
Group	1	4453.919	4453.919	38.276	<.0001	38.276	1.000
Residual	18	2094.552	116.364				

Means Table for Histomorpho 12wks
Effect: Group

	Count	Mean	Std. Dev.	Std. Err.
Control	10	18.410	9.979	3.156
Exp	10	53.901	7.021	2.220

ANOVA Table for Histomorpho 12wks

	DF	Sum of Squares	Mean Square	F-Value	P-Value	Lambda	Power
Group	1	6298.055	6298.055	84.600	<.0001	84.600	1.000
Residual	18	1340.012	74.445				

Means Table for Radiomorpho 6wks
Effect: Group

	Count	Mean	Std. Dev.	Std. Err.
Control	10	36.769	7.596	2.402
Exp	10	49.598	12.880	4.073

ANOVA Table for Radiomorpho 6wks

	DF	Sum of Squares	Mean Square	F-Value	P-Value	Lambda	Power
Group	1	822.916	822.916	7.361	.0142	7.361	.734
Residual	18	2012.241	111.791				

Means Table for Radiomorpho 12wks
Effect: Group

	Count	Mean	Std. Dev.	Std. Err.
Control	10	36.793	19.185	6.067
Exp	10	83.582	12.316	3.895

ANOVA Table for Radiomorpho 12wks

	DF	Sum of Squares	Mean Square	F-Value	P-Value	Lambda	Power
Group	1	10946.053	10946.053	42.120	<.0001	42.120	1.000
Residual	18	4677.825	259.879				

Means Table for Histomorpho 6wks
Effect: Group
Inclusion criteria: 15mm defect from Sheet1 (imported)

	Count	Mean	Std. Dev.	Std. Err.
Control	5	23.862	5.392	2.411
Exp	5	56.542	8.740	3.909

ANOVA Table for Histomorpho 6wks
Inclusion criteria: 15mm defect from Sheet1 (imported)

	DF	Sum of Squares	Mean Square	F-Value	P-Value	Lambda	Power
Group	1	2669.956	2669.956	50.630	.0001	50.630	1.000
Residual	8	421.876	52.735				

Means Table for Histomorpho 12wks
Effect: Group
Inclusion criteria: 15mm defect from Sheet1 (imported)

	Count	Mean	Std. Dev.	Std. Err.
Control	5	14.182	4.072	1.821
Exp	5	56.162	6.251	2.796

ANOVA Table for Histomorpho 12wks
Inclusion criteria: 15mm defect from Sheet1 (imported)

	DF	Sum of Squares	Mean Square	F-Value	P-Value	Lambda	Power
Group	1	4405.801	4405.801	158.313	<.0001	158.313	1.000
Residual	8	222.638	27.830				

Means Table for Radiomorpho 6wks
Effect: Group
Inclusion criteria: 15mm defect from Sheet1 (imported)

	Count	Mean	Std. Dev.	Std. Err.
Control	5	39.344	7.356	3.290
Exp	5	48.778	13.031	5.828

ANOVA Table for Radiomorpho 6wks
Inclusion criteria: 15mm defect from Sheet1 (imported)

	DF	Sum of Squares	Mean Square	F-Value	P-Value	Lambda	Power
Group	1	222.501	222.501	1.987	.1963	1.987	.229
Residual	8	895.709	111.964				

Means Table for Radiomorpho 12wks
Effect: Group
Inclusion criteria: 15mm defect from Sheet1 (imported)

	Count	Mean	Std. Dev.	Std. Err.
Control	5	33.082	20.245	9.054
Exp	5	79.486	13.380	5.984

ANOVA Table for Radiomorpho 12wks
Inclusion criteria: 15mm defect from Sheet1 (imported)

	DF	Sum of Squares	Mean Square	F-Value	P-Value	Lambda	Power
Group	1	5383.328	5383.328	18.284	.0027	18.284	.969
Residual	8	2355.489	294.436				

Means Table for Histomorpho 6wks
Effect: Group
Inclusion criteria: 18mm defect from Sheet1 (imported)

	Count	Mean	Std. Dev.	Std. Err.
Control	5	33.868	11.371	5.085
Exp	5	60.880	14.647	6.550

ANOVA Table for Histomorpho 6wks
Inclusion criteria: 18mm defect from Sheet1 (imported)

	DF	Sum of Squares	Mean Square	F-Value	P-Value	Lambda	Power
Group	1	1824.120	1824.120	10.611	.0116	10.611	.823
Residual	8	1375.330	171.916				

Means Table for Histomorpho 12wks
Effect: Group
Inclusion criteria: 18mm defect from Sheet1 (imported)

	Count	Mean	Std. Dev.	Std. Err.
Control	5	22.638	12.759	5.706
Exp	5	51.640	7.686	3.437

ANOVA Table for Histomorpho 12wks
Inclusion criteria: 18mm defect from Sheet1 (imported)

	DF	Sum of Squares	Mean Square	F-Value	P-Value	Lambda	Power
Group	1	2102.790	2102.790	18.955	.0024	18.955	.974
Residual	8	887.493	110.937				

Means Table for Radiomorpho 6wks
Effect: Group
Inclusion criteria: 18mm defect from Sheet1 (imported)

	Count	Mean	Std. Dev.	Std. Err.
Control	5	34.194	7.690	3.439
Exp	5	50.418	14.204	6.352

ANOVA Table for Radiomorpho 6wks
Inclusion criteria: 18mm defect from Sheet1 (imported)

	DF	Sum of Squares	Mean Square	F-Value	P-Value	Lambda	Power
Group	1	658.045	658.045	5.045	.0549	5.045	.500
Residual	8	1043.501	130.438				

Means Table for Radiomorpho 12wks
Effect: Group
Inclusion criteria: 18mm defect from Sheet1 (imported)

	Count	Mean	Std. Dev.	Std. Err.
Control	5	40.504	19.593	8.762
Exp	5	87.678	10.969	4.906

ANOVA Table for Radiomorpho 12wks
Inclusion criteria: 18mm defect from Sheet1 (imported)

	DF	Sum of Squares	Mean Square	F-Value	P-Value	Lambda	Power
Group	1	5563.466	5563.466	22.068	.0015	22.068	.988
Residual	8	2016.849	252.106				

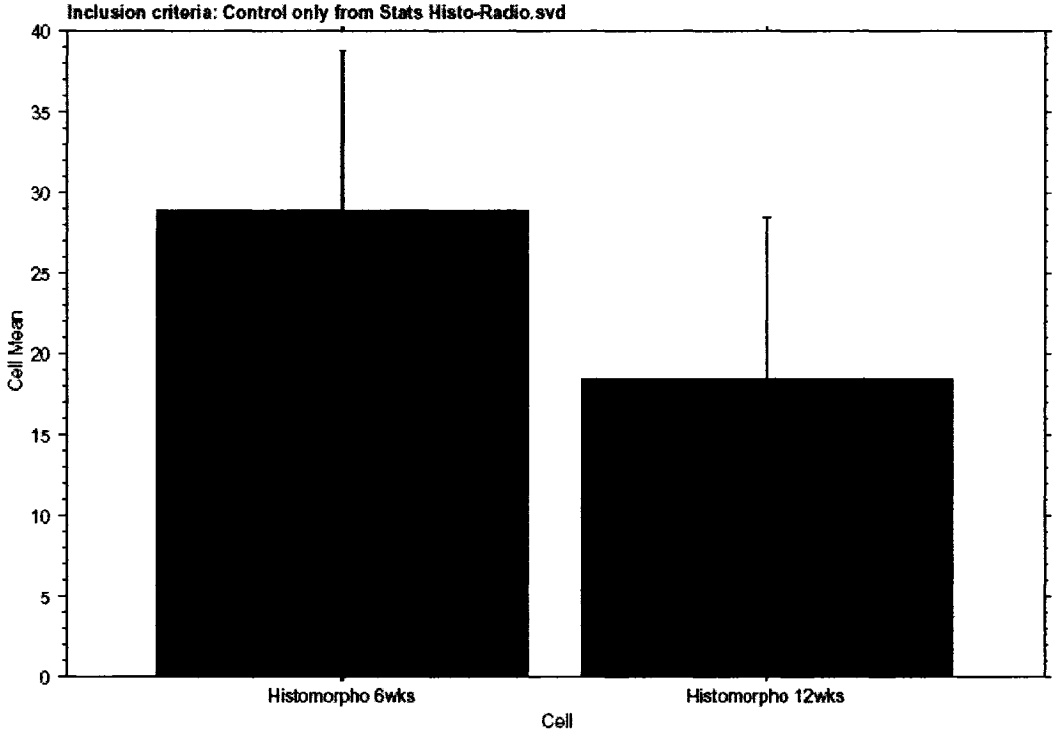
ANOVA Table for Histo
Inclusion criteria: Control only from Stats Histo-Radio.svd

	DF	Sum of Squares	Mean Square	F-Value	P-Value	Lambda	Power
Subject	9	847.088	94.121				
Category for Histo	1	546.535	546.535	5.272	.0473	5.272	.530
Category for Histo * Subject	9	933.003	103.667				

Means Table for Histo
Effect: Category for Histo
Inclusion criteria: Control only from Stats Histo-Radio.svd

	Count	Mean	Std. Dev.	Std. Err.
Histomorpho 6wks	10	28.865	9.909	3.134
Histomorpho 12wks	10	18.410	9.979	3.156

Interaction Bar Plot for Histo
Effect: Category for Histo
Error Bars: ± 1 Standard Deviation(s)
Inclusion criteria: Control only from Stats Histo-Radio.svd



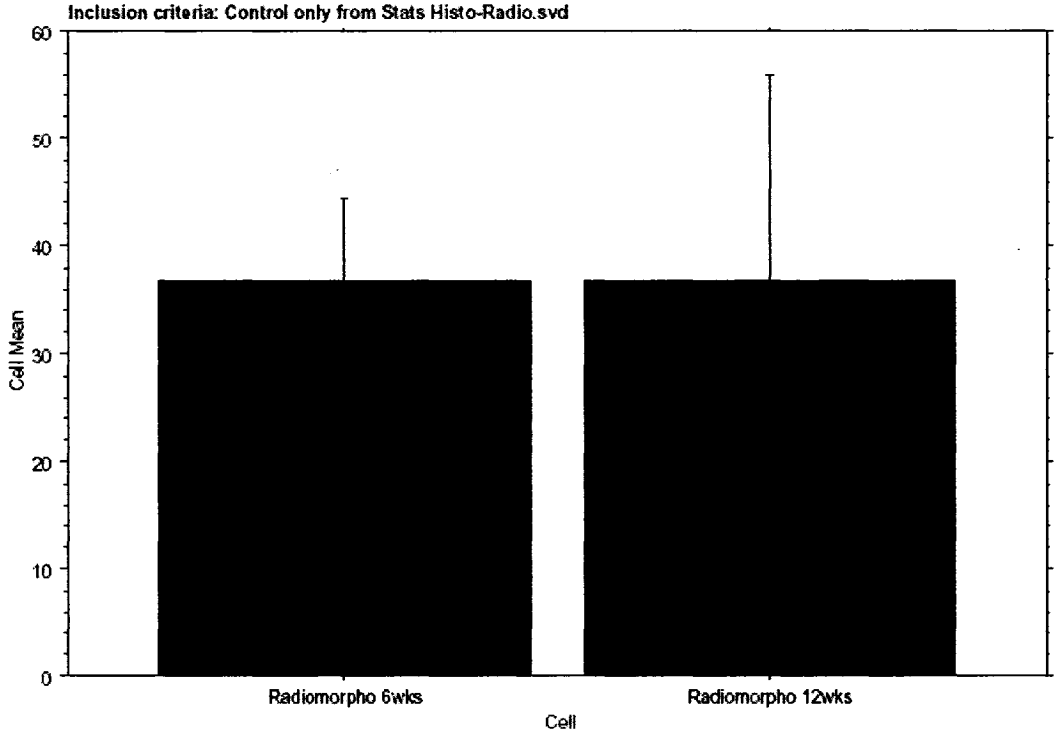
ANOVA Table for Radio
Inclusion criteria: Control only from Stats Histo-Radio.svd

	DF	Sum of Squares	Mean Square	F-Value	P-Value	Lambda	Power
Subject	9	1266.012	140.668				
Category for Radio	1	.003	.003	1.010E-5	.9975	1.010E-5	.050
Category for Radio * Subject	9	2565.984	285.109				

Means Table for Radio
Effect: Category for Radio
Inclusion criteria: Control only from Stats Histo-Radio.svd

	Count	Mean	Std. Dev.	Std. Err.
Radiomorpho 6wks	10	36.769	7.596	2.402
Radiomorpho 12wks	10	36.793	19.185	6.067

Interaction Bar Plot for Radio
Effect: Category for Radio
Error Bars: ± 1 Standard Deviation(s)
Inclusion criteria: Control only from Stats Histo-Radio.svd



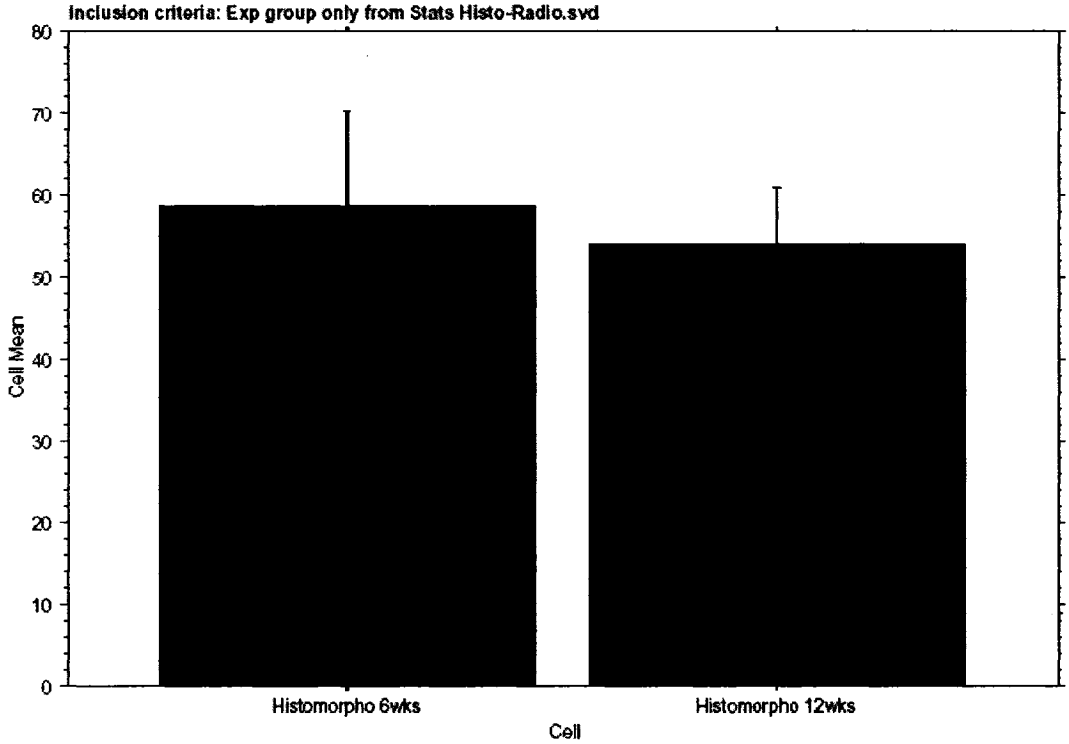
ANOVA Table for Histo
Inclusion criteria: Exp group only from Stats Histo-Radio.svd

	DF	Sum of Squares	Mean Square	F-Value	P-Value	Lambda	Power
Subject	9	898.667	99.852				
Category for Histo	1	115.681	115.681	1.378	.2706	1.378	.176
Category for Histo * Subject	9	755.806	83.979				

Means Table for Histo
Effect: Category for Histo
Inclusion criteria: Exp group only from Stats Histo-Radio.svd

	Count	Mean	Std. Dev.	Std. Err.
Histomorpho 6wks	10	58.711	11.599	3.668
Histomorpho 12wks	10	53.901	7.021	2.220

Interaction Bar Plot for Histo
Effect: Category for Histo
Error Bars: ± 1 Standard Deviation(s)
Inclusion criteria: Exp group only from Stats Histo-Radio.svd



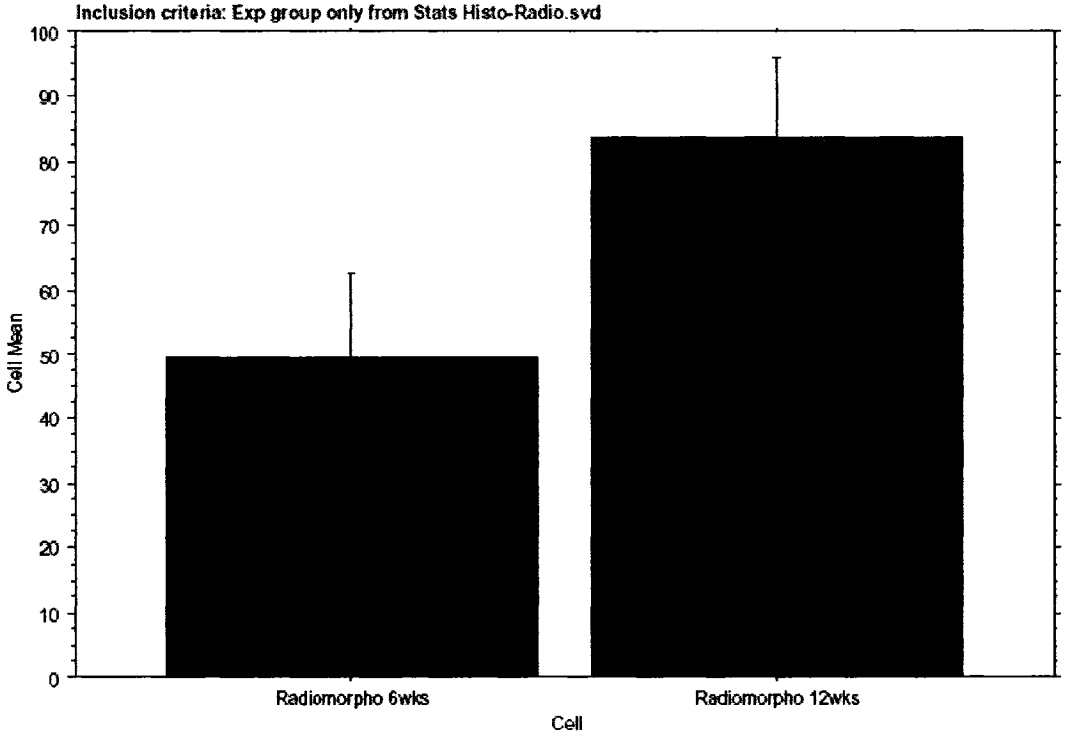
ANOVA Table for Radio
Inclusion criteria: Exp group only from Stats Histo-Radio.svd

	DF	Sum of Squares	Mean Square	F-Value	P-Value	Lambda	Power
Subject	9	1786.466	198.496				
Category for Radio	1	5774.561	5774.561	48.498	<.0001	48.498	1.000
Category for Radio * Subject	9	1071.604	119.067				

Means Table for Radio
Effect: Category for Radio
Inclusion criteria: Exp group only from Stats Histo-Radio.svd

	Count	Mean	Std. Dev.	Std. Err.
Radiomorpho 6wks	10	49.598	12.680	4.073
Radiomorpho 12wks	10	83.582	12.316	3.895

Interaction Bar Plot for Radio
Effect: Category for Radio
Error Bars: ± 1 Standard Deviation(s)
Inclusion criteria: Exp group only from Stats Histo-Radio.svd



University of Toronto Animal Use Protocol

CONFIDENTIAL

FOR OFFICE USE ONLY	
PROTOCOL NO.	20005145
Expiry Date	

1 PRINCIPAL INVESTIGATOR or COURSE DIRECTOR

Surname	First Name	Initial
Sandor	George	ML

Personnel No.	Rank / Position	PI / Co-I / Collaborator (please indicate)
	Associate Professor	Co-I

Department / Faculty
Division of Oral and Maxillofacial Surgery/Faculty of Dentistry

Business Telephone	Laboratory Telephone	Residence Telephone
		.6)

Emergency Telephone	Pager / Cell Phone	E-mail Address
	-	

Mailing Address
Hospital for Sick Children,
Toronto ON M5G 1X8

NAME OF DESIGNATED ALTERNATE FOR EMERGENCIES

Wayne Evans	UHN	Direct.of Hyperb Med	((416)

A. Faculty (mandatory) Name	Rank	Emergency Telephone
Sean Peel	Research Associate	

B. Other (optional) Name	Rank	Emergency Telephone

2 PROJECT

Title (including Course Number if applicable)
Effects of Hyperbaric oxygen on the healing of critical sized defects in rabbit calvarium.

3 FUNDING

External ☑			Internal ☐		
Agencies			Source		
ORDCF					
Status of Grant	Awarded ◉	Pending ○	Status of Grant	Awarded ○	Pending ○
Status of Protocol (choose one)	○	Pending ◉ Non-Peer Reviewed ○	Status of Protocol (choose one)	Peer Reviewed ○	Pending ○ Non-Peer Reviewed ○

4

	Day	Month	Year	OR ongoing?
PROPOSED START DATE OF RESEARCH	01	March	2004	☑
EXPECTED DATE OF COMPLETION	Day	Month	Year	**OR** ongoing? ☑

5

See Instruction Page. Check Applicable Category.

A. ○ B. ○ C. ○ D. ◉ E. ○

6

TYPE OF EXPERIMENT Check All Applicable Boxes

Research ☑ Teaching ☐ Testing ☐ Research/Teaching ☐

SURGICAL		**NON-SURGICAL**	
Acute ○	Survival ◉	Acute ○	Chronic ◉

7 **DESCRIPTION OF PROJECT AND PROCEDURES**

Describe in DETAIL all procedures, techniques to be used; emphasizing those performed on animals. Use flow charts to illustrate procedures as appropriate. Append additional page(s), if necessary. Copies of complete grant applications will not be accepted.

Background:

A critical size defect is the smallest intraosseous wound that will not heal spontaneously with bony union over the lifetime of an animal. Practically, the defect should not heal spontaneously during the experimental period. The literature has demonstrated that hyperbaric oxygen (HBO) therapy is an effective adjunct to improve bone healing postoperatively, even in nonirradiated tissues. Our study evaluates whether the effects of HBO can potentially alter the predicted size of a critical size defect. We will be conducting this study as an extension of our previous study on critical size defects and bone substitutes (Haddad et al). The additional factor in our experimental design will be exposure of the rabbits to HBO.

References:

Haddad AJ, Sandor GKB, Clokie CML. Enhanced bone healing in rabbit calvarium using novel bone substitutes. J Oral and Maxillofac Surg. Sept 2003; 61 (8) Suppl 1:96.

Experimental Design:

20 adult New Zealand male white rabbits will be obtained from Charles River at 3-4 kg. They will be divided into 2 groups of 10 animals. Cranial vault defects will be created in the parietal bones. Group 1 (n=10) will have a 15mm defect on one side (critical size defect), and a 12mm defect on the contralateral side. Group 2 (n=10) will have defects larger than the critical size, 17mm on one side and 20mm on the contralateral side. All animals will undergo a course of HBO treatment using the following protocol: 100% Oxygen, 2.4 ATA, 90 mins/day, 20 dives. Five animals from each group will be sacrificed at 6 weeks and 5 animals at 12 weeks. Results from Haddad et al eliminated the need for a control group that was not subjected to HBO, since we will use the data from this previous study.

Surgery:

The surgical procedures for this investigation will be performed according to recognized techniques approved by the University Health Network. Each animal will be premedicated according to their weight with a mixture of Ketamine (35mg/kg), xylazine (2mg/kg) and acepromazine (1mg/kg). Once sedated an infusion line will be started in the marginal ear vein with a 22 gauge catheter and Ringer's lactate infused at 30cc/hr. General anesthesia will be induced using intravenous sodium thiopental (20mg/kg). After induction a 3mm uncuffed endotracheal tube will be used for intubation. Anesthesia will be maintained with 1.5% isoflurane and oxygen mixture using mechanical ventilation. The animals will be monitored using pulse oxymetry. Respiration rate will be set at 20 breaths per minute with a tidal volume of 10ml/kg.

Continued in attachment...

8 **Shoud the experiment / procedure result in experimentally-induced disease or life-threatening condition, describe the endpoint(s) of the experiment for the animal. As examples, any of the following could call for prompt and humane euthanasia (e.g. ulcerated tumour, weight loss, hypothermia).**

In our previous study by Haddad et al, we had no rabbit death during the experimental period. However, all animals will be monitored and if they show signs that they are not thriving(not eating, weight loss exceeding 20%), they will be euthanized immediately.

9 In **LAY (NON-SCIENTIFIC)** terms, please summarize (A) the primary objective(s) and (B) the benefit(s) expected from the study.

Purpose of the study:
A) Critical size defects are defined as the smallest size bony defect that will not heal spontaneously during the lifetime of the animal. For the rabbit calvarium, the size is 15mm. We would like to derive from the study whether or not HBO therapy (animals subjected to pressurized oxygen within an animal chamber) would increase bone healing, reflected by an increase in size for the "critical size defect" in the rabbit model.
B) The treatment of craniofacial defects arising from trauma or congenital malformations can be challenging. For example,using bone harvested from the patient's own body or different commercial products. There is evidence that hyperbaric oxygen (HBO) treatment increases bone healing, but it has never been quantified relative to critical size defects. If we can demonstrate that HBO has allowed for (Please See Below at #15)

10 RESEARCH STAFF

Name	Department	Professor	Technic / Technol	Post - Doctoral	Graduate Student	Undergrad Student	
Sean AF Peel	Oral Surgery	○	○	◉	○	○	☑
ZhenMing Hu	Oral Surgery	○	○	◉	○	○	☑
Deborah Iera	Oral Surgery	○	○	◉	○	○	☐
Yair Langa	Dentistry	○	○	○	◉	○	☐
Wayne Evans	UHN-Dir.of Hyp.Med.	◉	○	○	○	○	☐
George Sandor	Oral Surgery	◉	○	○	○	○	☐

NOTE University one-day Animal User Training Course is mandatory for new graduate students, research technicians / technologists, research assistants / associates and postdoctoral fellows. *Check if the staff member has completed the University Course or equivalent training. (Please indicate nature of training).

11 ANIMALS TO BE USED

Animal Species (Common Name)	Total Number of Animals	Source of Animals	Location of Animal (Room #, Building)	Location of Experiment (Room #, Building)	Surgical Suite (Room #, Building)
			Dentistry		
New Zealand	20	Charles River	532,534,539	N.A.	550,Dentistry
Adult Rabbits			or 540		

12 **JUSTIFICATION FOR:**

A. Species

In the calvarium of rabbit, it is 15 mm Frame, 1980; Schmitz and Hollinger, 1986)The rabbit calvarium defect is preferable to the rat since it allows for two defects in each animal. An important difference between defects in the calvarium versus those in long bones is that the latter contain primary nutrient arteries where as the main supply to the calvarium is from periosteum and muscle attachments. See below at #13.

B. Number of Animals Used

We chose to use 20 rabbits-10 in each group in our study in order to have the smallest number of animals that would allow us to show a difference between groups, as per our previous study by Haddad et al. Group 1: Will consist of the rabbits having 15mm defects on one side of the skull and 12mm defects on the other side. Group 2: Will consist of the rabbits having 18mm defects on one side of the skull and 21mm defects on the other side.

13 **ALTERNATIVES**

Are non-animal alternatives available for this project? If Yes, explain why they have been rejected. YES ○ NO ◉

CONTINUING 12A. The healing pattern of jaw bones and the calvarium are similar. The rabbit calvarial vault provides a suitable model for testing in the craniofacial area. This model has been used successfully in previous studies in our lab as well as other researchers. The model provides a standard, reproducible method to evaluate bone healing.

14

DRUGS USED FOR ANAESTHESIA / ANALGESIA	DRUG	DOSAGE	ROUTE OF ADMINISTRATION
A. Pre-Anaesthesia	Ketamine/Xylazine	35mg/kg//2mg/kg	Premedicated to weight
Pre-Anaesthesia	Acepromazine	1mg/kg	Intravenous
B. Anaesthetic	Sodium Thiopental	20mg/kg	Intravenous
Anaesthetic	Isoflurane	1.5% Mainten	Oxygen mixture using vent
C. Analgesic	Buprenorphine	.02mg/kg	Intramuscular for pain
Analgesic			

15 EUTHANASIA

- [x] Anaesthetic overdose (Specify Anaesthetic Agent) — T-61 I.V.
- [ ] Cervical Dislocation
- [ ] Exsanguination (under Anaesthesia)
- [ ] Carbon Dioxide
- [ ] Decapitation
- [ ] Pithing
- [ ] Other (Specify)

Please provide justification for use of any physical method of euthanasia (e.g. cervical dislocation, decapitation, etc.) without prior use of anaesthetic:

CONTINUING 9B: bone healing of critical size defects in the rabbit model, we may be reducing the need for reconstructive surgery for defects of this size.

Final disposition of animals if not euthanized:

16 HAZARDOUS AGENTS

Specify **Each** Agent: Not Applicable [x]

- [ ] Biological — Biohazard Committee Approval YES [ ] NO [ ]
- [ ] Chemical
- [ ] Carcinogen
- [ ] Radioisotope / Radiation — R / A Permit No. — Expiration Date

Specify for **Each** Agent:

Amount of agent and dosage

Route of administration

Frequency of administration

How agent is excreted by animal

Time period of excretion

16 (cont'd) HAZARDOUS AGENTS

Specify:

Description of potential health risk(s) to humans or animals

Special animal care requirement(s)

Precautions to be taken by personnel

Special containment requirements (i.e. special storage, waste and animal disposal requirements, emergency procedures)

17 TRAPPING or WILDLIFE LICENCES

Name of Licence Holder ☑ Not Applicable

Permit / Licence Number

Expiration Date

Specify: Method of capture (if a trap is used, indicate type of trap, its injury potential and monitoring frequency)

Transportation and / or housing of animals in the field

Capture of non-target species

Potential injuries or mortality during capture

Potential ecological disruption (type and degree of disruption anticipated)

Disposal of animals (e.g. euthanasia, release to field)

DECLARATION

All animals in this research, teaching or testing proposal will be maintained and used in accordance with the recommendations of the Canadian Council on Animal Care, the requirements under the Animals for Research Act, (Revised Statues of Ontario, 1980, Chapter 22 as amended by 1989, Chapter 72, s6 and Regulations 16, 17, 18, 19. Revised Regulations of Ontario, 1980, March 1990.), and the University of Toronto Animal Care Policies and Guidelines, and other applicable University of Toronto policies and procedures.

Principal Investigator or Course Director Date

APPROVALS

Chairperson, Local Animal Care Committee Date

University Veterinarian Date

Chairperson, University Animal Care Committee Date

NOTE: *FORM CANNOT BE PROCESSED UNLESS ALL SECTIONS COMPLETED.

PROTOCOL IS APPROVED FOR A PERIOD OF ONE YEAR FROM DATE OF APPROVAL BY THE UNIVERSITY ANIMAL CARE COMMITTEE.

A NEW PROTOCOL MUST BE SUBMITTED EACH YEAR.

16 (cont'd) HAZARDOUS AGENTS

Specify:

Description of potential health risk(s) to humans or animals

Special animal care requirement(s)

Precautions to be taken by personnel

Special containment requirements (i.e. special storage, waste and animal disposal requirements, emergency procedures)

17 TRAPPING or WILDLIFE LICENCES

Name of Licence Holder ☑ Not Applicable

Permit / Licence Number

Expiration Date

Specify: Method of capture (if a trap is used, indicate type of trap, its injury potential and monitoring frequency)

Transportation and / or housing of animals in the field

Capture of non-target species

Potential injuries or mortality during capture

Potential ecological disruption (type and degree of disruption anticipated)

Disposal of animals (e.g. euthanasia, release to field)

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Principal Investigator / Course Director — Date: February 9, 2004

APPROVALS

Chairperson, Lo — Date: Apr 28/04

Un — Date: APR 30 2004

Chairpers[illegible] COMMITTEE — Date: May 5/04

NOTE: *FORM CANNOT BE PROCESSED UNLESS ALL SECTIONS COMPLETED.

PROTOCOL IS APPROVED FOR A PERIOD OF ONE YEAR FROM DATE OF APPROVAL BY THE UNIVERSITY ANIMAL CARE COMMITTEE.

A NEW PROTOCOL MUST BE SUBMITTED EACH YEAR.