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

Ahmed Jan, DDS,[a] George K. B. Sándor, MD, DDS, PhD, FRCDC, FRCSC, FACS,[b] Bozidar B. M. Brkovic, DDS, PhD,[c] Sean Peel, PhD,[d] A. Wayne Evans, MD,[e] and Cameron M. L. Clokie, DDS, PhD, FRCDC,[f] Toronto, Canada, Tampere and Oulu, Finland, and Belgrade, Serbia

UNIVERSITY OF TORONTO, THE HOSPITAL FOR SICK CHILDREN, BLOORVIEW KIDS REHAB, REGEA INSTITUTE FOR REGENERATIVE MEDICINE, UNIVERSITY OF OULU, AND UNIVERSITY OF BELGRADE

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

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

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

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

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

[a]Professor and Head of Oral and Maxillofacial Surgery, University of Toronto; Coordinator, Pediatric Oral and Maxillofacial Surgery, The Hospital for Sick Children and Bloorview Kids Rehab, Toronto, Canada; Professor, Regea Institute for Regenerative Medicine, University of Tampere, Tampere, Finland; Dosent, University of Oulu, Oulu, Finland.

[b]Director of Graduate Program in Oral and Maxillofacial Surgery and Anesthesia; Professor, University of Toronto; Coordinator, Pediatric Oral and Maxillofacial Surgery, The Hospital for Sick Children and Bloorview Kids Rehab, Toronto, Canada; Professor, Regea Institute for Regenerative Medicine, University of Tampere, Tampere, Finland; Dosent, University of Oulu, Oulu, Finland.

[c]Stoneman Fellow in Pediatric Oral and Maxillofacial Surgery, The Hospital for Sick Children, University of Toronto, Toronto, Canada; Assistant Professor, Clinic of Oral Surgery, University of Belgrade, Belgrade, Serbia.

[d]Adjunct Professor, Orthobiologics Group, University of Toronto, Toronto, Canada.

[e]Assistant Professor, Hyperbaric Medicine Unit, Department of Anesthesia, Faculty of Medicine, University of Toronto, Toronto, Canada.

[f]Professor of Oral and Maxillofacial Surgery, Director, Orthobiologics Group, University of Toronto, Toronto, Canada.

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

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

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

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

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

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

MATERIALS AND METHODS

Surgical protocol

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

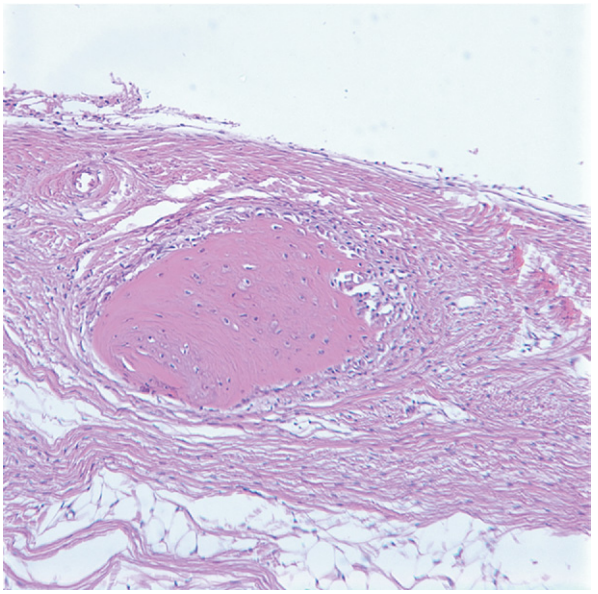

Fig. 1. Unfilled defect from rabbits under normobaric conditions. These defects were mostly filled with fibrous tissue, with the occasional areas of bone formation and very little marrow (hematoxylin and eosin [H&E], original magnification ×20).

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

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

Micro-computed tomography

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

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

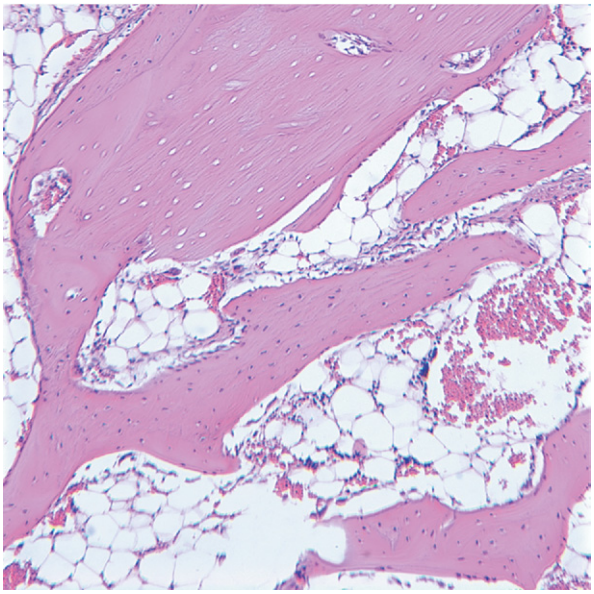

Fig. 2. Autogenous bone–grafted defects under normobaric conditions.Trabeculae of new bone formed around and sometimes incorporated the residual autogenous bone graft (ABG). The ABG could be clearly distinguished from the new bone due to the empty osteocyte lacunae. Extensive marrow is present throughout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

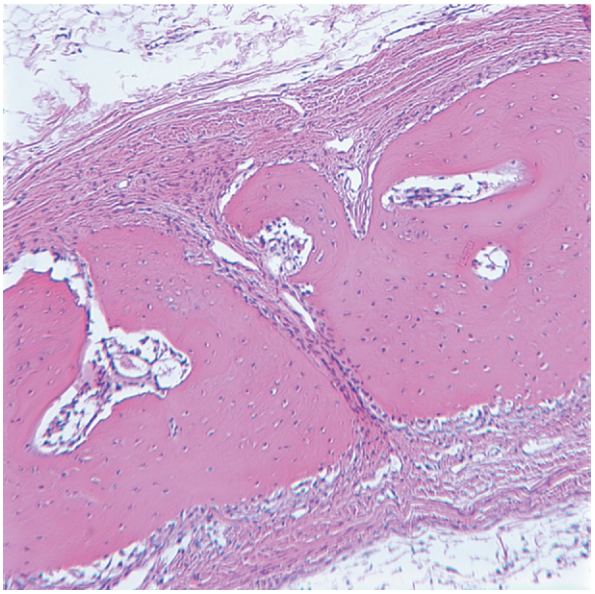

Fig. 3. Unfilled defect exposed to hyperbaric oxygen conditions. Thick blocks of new bone are surrounded by dense fibrous tissue. Only small amounts of marrow are seen, within the new bone (H&E, original magnification ×20).

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

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

Histomorphometry

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

Statistical analysis

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

RESULTS

Histological analysis

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

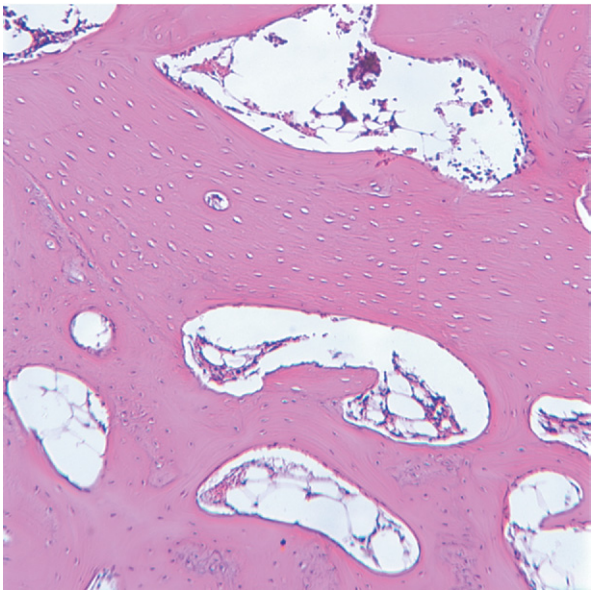

Fig. 4. Autogenous bone–grafted defects under hyperbaric oxygen conditions. New bone forms along with and merges into the residual graft matrix. The remaining space is filled with marrow. No fibrous tissue is visible (H&E, original magnification ×20).

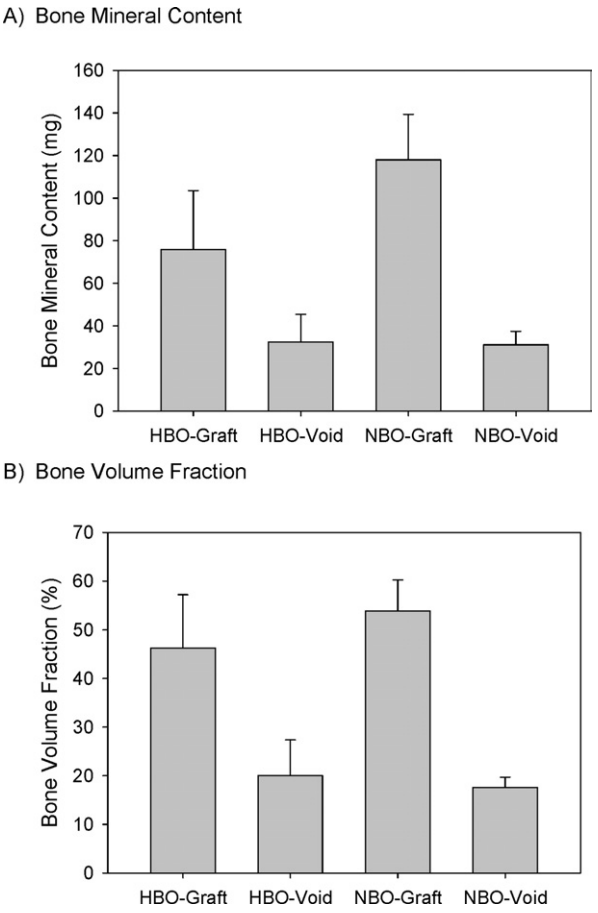

Fig. 5. Micro-CT results. Bone mineral content (BMC) (**A**) and bone volume fraction (BVF) (**B**).The defects containing autogenous bone grafts have significantly higher BMC and BVF than the ungrafted defects ($P < .05$). Comparisons between the HBO and NBO autogenous bone–grafted defects revealed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

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

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

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

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

Micro-computed tomography

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

Histomorphometry

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

Table I. Micro-CT analysis

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

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

Table II. Histomorphometric analysis

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

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

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

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

DISCUSSION

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

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

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

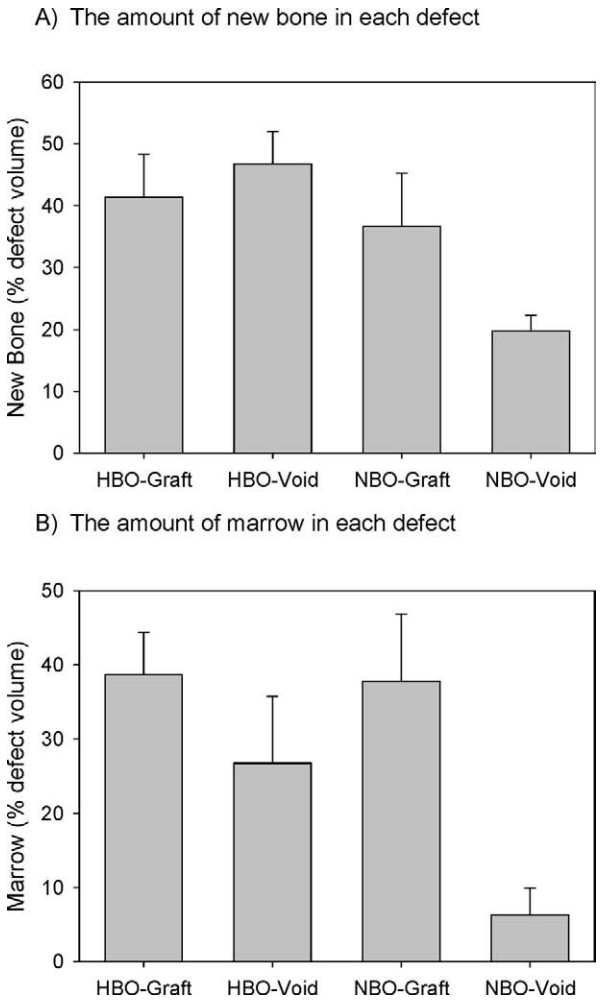

Fig. 6. Histomorphometry results. **A,** Amount of new bone. **B,** The amount of marrow.HBO-treated unfilled defects had significantly more new bone ($P < .001$) and marrow ($P < .05$) than unfilled defects exposed to normobaric air. Comparing the unfilled and autogenous bone–grafted defects in the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

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

Histomorphometric comparison of the HBO and NBO defects that contained autogenous grafts revealed

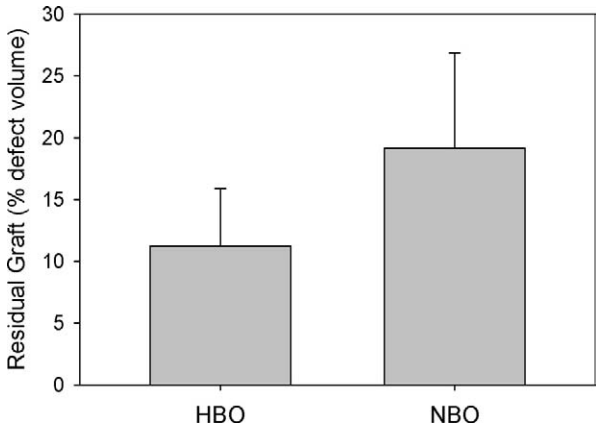

Fig. 7. Amount of residual graft in the grafted defects. The lesser amount of residual graft present in the HBO compared to HBO defects neared significance ($P = .085$). Data are plotted as mean ± SD.

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

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

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

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Reprint requests:

George K. B. Sándor, MD, DDS, PhD
The Hospital for Sick Children
S-525, 555 University Avenue
Toronto, Ontario, Canada M5G 1X8
george.sandor@utoronto.ca