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

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

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

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

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

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

METHODS AND MATERIALS

Experimental design

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

Qualitative analysis: histology

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

Quantitative analysis: immunohistochemical analysis

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

Analysis of VEGF expression

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

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

Statistical analysis

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

Comparisons of VEGF staining between the 15-mm and 18-mm defects in the same rabbits, and between the margins and central regions within each defect, were made using the paired *t* test. Statistical significance was set at $P < .05$. All statistical analyses were performed using Sigma Stat software (v3.0, SYSTAT, San Jose, CA).
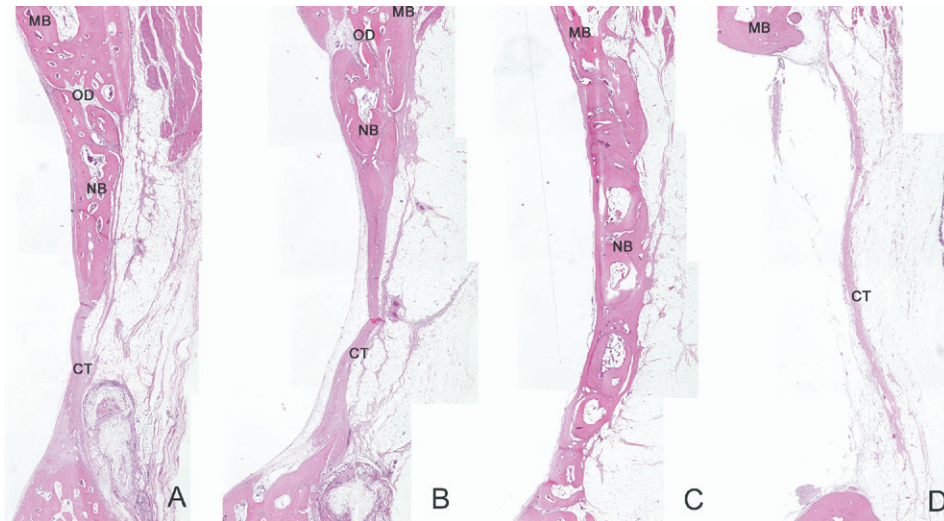
Fig. 1. Histological appearance of HBO and NBO defects at 6 and 12 weeks. **A,** HBO 6-weeks group. A significant amount of new bone is seen within the defect. **B,** NBO 6-weeks group. Although new bone is present within the defect, it is much less than in the group treated with HBO. **C,** HBO 12-weeks group. The entire defect is bridged with new bone. **D,** NBO 12-weeks group. The defects were filled predominantly with dense fibrous tissue. CT, connective tissue; MB, mature bone; NB, new bone; OD, margin of original defect. (Hematoxylin-eosin stain; magnification ×4.)

RESULTS

Gross appearance and histological evaluation

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

VEGF staining

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

Comparison of the areas stained for VEGF between

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

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

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

P values were calculated using the paired *t* test.

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

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

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

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

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

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

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

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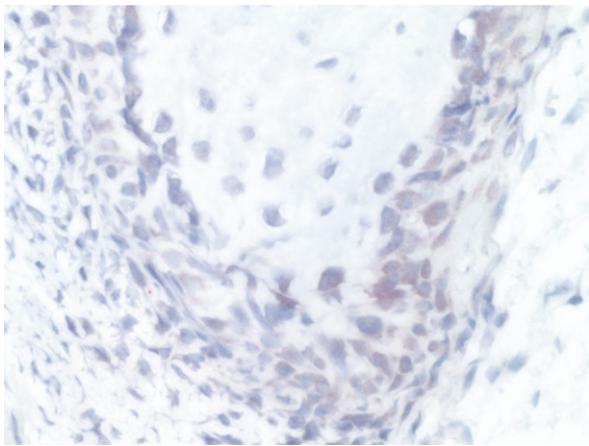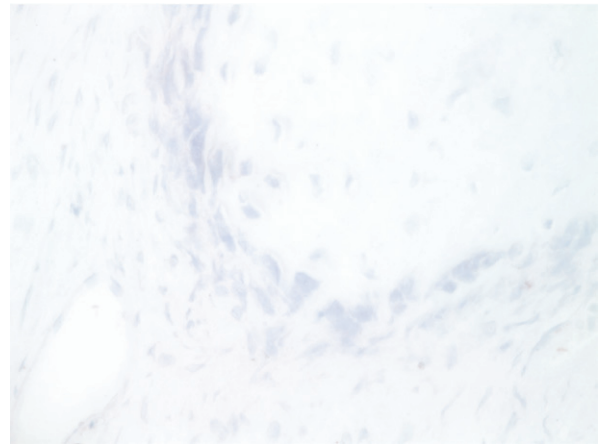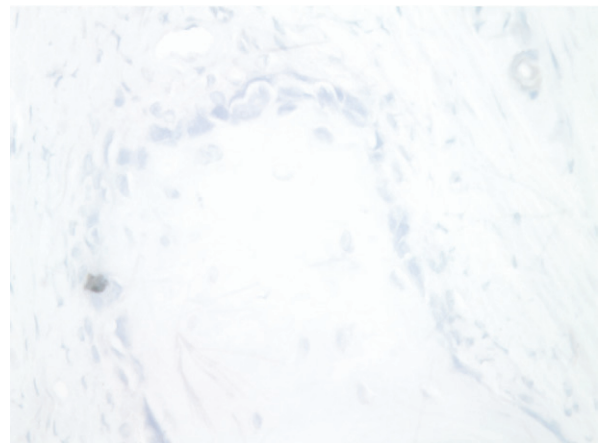

Fig. 2. VEGF-stained HBO-treated defects at 6 weeks. Rabbits had been exposed to HBO therapy (90 minutes at 2.4 atmospheres with 100% oxygen) 5 days a week for 4 weeks. Cells stained for VEGF were seen throughout the defect, however the most intense VEGF staining was seen near areas of bone. (Magnification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

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

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

DISCUSSION

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

It has been reported that HBO can increase neovascularization in soft tissue wounds[16] and the in-growth of new blood vessels into the defect is an essential step in bone repair.[11] VEGF and basic fibroblast growth factor (FGF) have been identified as the primary growth factors implicated in neovascularization (angiogenesis) in vitro.[12] However, in vivo, the role of FGF in angiogenesis has been questioned. Nissen and coworkers[17]

demonstrated that while FGF levels dramatically rise immediately after injury, they also return to normal within 3 days, several days prior to the onset of angiogenesis. Conversely, VEGF increased, peaking at 7 days postinjury, matching the initiation of angiogenic activity that began after 1 week.[18] A similar time course for VEGF expression has been reported for fracture healing.[19]

The current study was unable to address the effect of HBO on VEGF expression prior to 6 weeks, as this was a retrospective study. VEGF expression following trauma in soft tissue and bone has been reported to return to normal within 21 days following trauma.[17,19]
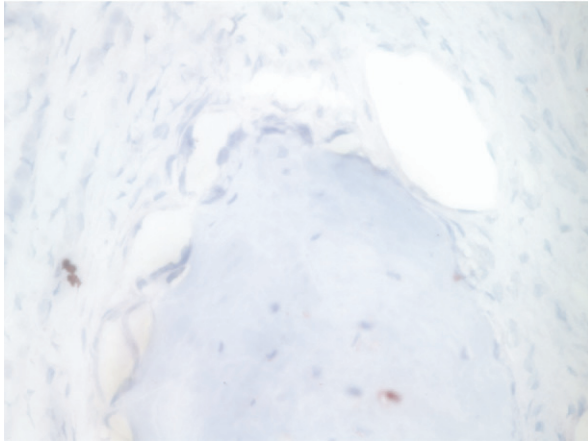
Fig. 5. VEGF-stained untreated defects at 12 weeks. VEGF staining in the untreated defects is similar to that in the HBO defects. (Magnification ×200.)

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

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

All values are group means ± SD of the subject means of stained area (μm^2) within a field of view.

Two random fields were measured from the central one third of each section and 4 random fields were measured from the margins of the same defect. Three sections were measured per defect.

Results of the 15- and 18-mm defects were considered separately in this analysis.

P values were calculated using the paired *t* test.

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

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

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

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

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

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

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

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