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EFFICACY OF HYPERBARIC OXYGEN THERAPY ON REGENERATION OF MANDIBULAR BONY DEFECTS IN RATS WITH INDUCED DIABETES MELLITUS

Rodina H. Eldisoky[1*] BDS, Salwa A. Younes[2] PhD, Samia S. Omar[2] PhD, Hagar Samir[3] PhD, Tarek A. Tamara[4] MS.

ABSTRACT

INTRODUCTION: Bone repair abnormalities have been associated with diabetes mellitus (DM), including inhibited osteoblastic differentiation, induced osteoblast apoptosis, and compromised angiogenesis. Thus, restoring critical-sized defects is challenging in clinical practice, especially in people with diabetes. Hyperbaric oxygen therapy (HBOT) can be used together with bone grafts to reconstruct these defects.

OBJECTIVES: To evaluate the effect of HBOT on the regeneration of critical-sized defects in rats with induced diabetes mellitus.

MATERIALS AND METHODS: Twelve adult male albino rats were divided into two groups of 6 animals each. Animals in both groups were given a single intraperitoneal injection of streptozotocin to induce DM. Critical-sized defects were created in the posterior mandibles and filled with beta-tricalcium phosphate (β-TCP). The study group was subjected to HBOT at (2.4 ATA) for 5 days per week for 90 minutes each. Animals were euthanized one week postoperatively. Bone regeneration was assessed histologically and histomorphometrically. Angiogenesis was evaluated by immunohistochemistry against vascular endothelial progenitor cell marker (CD34), and the microvessel density (MVD) was calculated.

RESULTS: Histological and immunohistochemical results revealed superior bone regeneration and angiogenesis in the study group. These results were further confirmed by the histomorphometrical analysis which showed higher MVD and new bone surface area in the study group compared to the control group.

CONCLUSIONS: HBOT enhanced bone regeneration, improved the regenerative effect of β-TCP, and increased angiogenesis in the defects.

KEYWORDS: Hyperbaric oxygen therapy, regeneration, mandibular defects, diabetes mellitus.

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1. Demonstrator of Oral Biology -Faculty of Dentistry –Alexandria University
2. Professor of Oral Biology -Faculty of Dentistry –Alexandria University
3. Lecturer of Oral Biology- Faculty of Dentistry - Alexandria University
4. Assistant Professor of Diving Medicine -Naval Hyperbaric Medical institute - Military Medical Academy

Corresponding author:
rodina.eldisoky@gmail.com

INTRODUCTION

There is a constant search for innovative techniques that induce extensive and rapid healing. Hyperbaric oxygen therapy (HBOT) is an effective, non-invasive, and reliable treatment method for different disorders. In this treatment approach, the patients inhale pure oxygen at a pressure greater than 1 atmosphere absolute (ATA), which takes place inside a hyperbaric chamber (1).

The beneficial effect of HBOT on bone healing has been revealed in several studies (2-4). This therapy has been associated with osteoblast proliferation and differentiation, increased bone nodule formation, and alkaline phosphatase activity in vitro (5,6). Moreover, it was demonstrated that HBOT accelerates fracture healing in vivo by increasing bone mineral apposition rate, and bone formation rate (7). Additionally, HBOT enhances the ingrowth of blood vessels which leads to accelerated bone repair (8,9). The literature indicates that this process occurs through different mechanisms, including an increase in the synthesis of various growth factors, which stimulate angiogenesis and granulation tissue formation, as well as the mobilization of blood-circulating endothelial progenitor cells (EPCs) derived from the bone marrow that eventually recruit in wounds (10-13).

However, most studies investigating the effect of HBOT on diabetes mellitus (DM) were achieved on soft tissue healing (14-16). Type I diabetes mellitus (T1DM) is a metabolic disease, resulting from

immune-mediated destruction of pancreatic beta cells that produce insulin. The development of many skeletal system complications has been linked to the chronic hyperglycemia in T1DM (17).

Various studies demonstrated that DM can adversely impact bone health through the unbalancing of several mechanisms, such as bone turnover rate, collagen synthesis, secretion of proinflammatory cytokines, and calcium metabolism (18-20). To explain bone repair abnormalities in diabetics; numerous mechanisms have been postulated, including compromised angiogenic process, inhibition of osteoblastic differentiation, maturation, function, and the induction of their apoptosis (21-23). Therefore, it is essential to research adjunctive therapies to minimize these negative effects of DM on bone regeneration.

Restoration of critical-sized bone defects, created by injuries, infections, or resection surgeries, is challenging in clinical practice (24), especially in diabetics for the previously mentioned reasons. Therefore, HBOT can be utilized in conjunction with bone grafts to reconstruct these defects in an impaired osteogenic condition (9). Beta-tricalcium phosphate (β-TCP) is a bioceramic bone grafting material used in the dental and medical fields (25). β-TCP is biocompatible and resorbable with both osteoconductive and osteoinductive properties. Hence, it is considered a good substitute for allografts and autografts for certain maxillofacial grafting procedures (26,27).

Studies available about using HBOT in bone defect regeneration in cases with T1DM are limited. Thus, the present study was carried out to clarify this aspect.

However, the null hypothesis of the current study proposed that HBOT has no significant effect on bone regeneration of mandibular defects in rats with induced type I diabetes mellitus.

MATERIALS AND METHODS

Study design

This study has been approved by the Research Ethics Committee, Faculty of Dentistry, Alexandria University (IORG0008839). The approval number by the ethical committee is (0216-01/2021).

Twelve adult male albino rats weighing 250-280 grams, six months of age, were used in this study. They were kept under the same controlled laboratory conditions in the experimental animal house with access to standard food and water. The cages were cleaned twice daily due to repeated urination in diabetic animals.

Based on calculations of the sample size made in the department of Biomedical Informatics and Medical Statistics, Medical Research Institute, Alexandria University, the number of these animals was estimated. The animals were divided randomly into two groups of 6 animals each, as follows:

Group A (n=6): Control group, diabetic animals with critical-sized bone defects filled with β-TCP bone graft.

Group B (n=6): Study group, diabetic animals with critical-sized bone defects filled with β-TCP bone graft and exposed to hyperbaric oxygen therapy.

Induction of Type 1 Diabetes Mellitus (28)

All rats were kept on 12-hour fasting before inducing T1DM. A single intraperitoneal injection of (50 mg/kg) streptozotocin (STZ), dissolved in 0.1 M citrate buffer (4.5 pH) immediately before the injection, was used to induce DM. After 72 hours, DM was confirmed by blood glucose level analysis using a digital glucometer. Blood samples were collected from each animal's tail vein. Any animal having a blood glucose level of more than (250 mg/dL) was regarded as diabetic.

Surgical procedure

Animals were anesthetized with intramuscular injection of (7mg/ kg) xylazine 2% and (80mg/ kg) ketamine 10% (29). Full mucoperiosteal flaps were reflected, and critical-sized osseous defects of (4 mm) diameter were created in the right side of the posterior mandible of animals using a sterile trephine bur under a water cooling system.(28) The osseous defects were irrigated with sterile saline and filled with β-TCP bone graft. The flaps were repositioned and sutured with resorbable sutures. Rats were monitored for any symptoms, and the operative sites were checked daily. Antibiotics and analgesics were administered as (30 mg/kg) cefazolin sodium and (5 mg/kg) Carprofen, postoperatively and for 3 days (29).

Hyperbaric Oxygen Therapy

Animals in group B were subjected to HBOT, starting 24h postoperatively using a mono-place HBOT chamber at the Naval and Underwater Medical Institute, Alexandria, Egypt. They were placed in custom-made plastic boxes with oxygen administration tubes. During the HBOT sessions, 100% oxygen was given as breathable air (9). The animals were exposed to (2.4 ATA) for 5 consecutive days per week, for 90 minutes each (3,9). To avoid barotrauma and discomfort, the rate of pressurization and depressurization was (0.14 ATA /min). The untreated control rats in group A were placed in the same room outside the hyperbaric chamber.

Animal euthanasia

The rats were euthanized by the end of the 1st week (n= 6/group). They were sedated and euthanized by

intravenous injection with a lethal dose (100 mg/kg) of pentobarbital sodium (30). The mandibles were dissected out and cleaned of soft tissues. The segments of the mandibles with the defects were prepared for histological evaluation, immunohistochemical and histomorphometrical analysis. Disposal of the rats was done by burning.

Histological examination

Specimens were labeled and fixed in (10 %) buffered formalin, washed, then decalcified by (8 %) tri-chloroacetic acid. Paraffin-embedded specimens were cut in 5-μm thick serial sections through the center of the defect (31). Hematoxylin & eosin (H&E) stain was used for the general examination of bone regeneration, whereas collagen formation was evaluated using Gomori trichrome stain. Finally, sections were examined by light microscope for qualitative evaluation of regeneration.

Histomorphometric analysis

Computer-assisted histomorphometry using Image J (1.46 software) was performed on H&E stained sections, to measure the percentage of the surface area of the newly formed bone compared to the total surface area of the defects. Measurement was done under the same magnification power (X40) and used for statistical analysis.

Immunohistochemical analysis and microvessel density (MVD)

For quantitative evaluation of intraosseous MVD, immunostaining of the endothelial cells using the endothelial progenitor cell marker CD34 was performed using monoclonal anti-CD34 (32).

Surveying of the immunostained sections was first done at a magnification of (x100), followed by a randomized selection of five vascular hot spots (microscopic areas with the highest density of vessels) per section. In each image, a standardized counting frame was placed over these areas, and the number of microvessels was counted under a magnification of (x400) using Image J (1.46 software). Any brown stained endothelial cells or clusters of endothelial cells, visibly separated from adjacent microvessels or other connective tissue elements, were counted as a single microvessel. MVD was calculated as the mean value of microvessel number per mm^2 according to the established protocol by Weidner et al., (33).

Statistical analysis

Quantitative data from the histomorphometry was subjected to statistical analysis using IBM SPSS software (package version 20.0). These data were expressed as mean values and standard deviations. The mean percentage of new bone surface area and the mean microvessel density among the two groups were compared using Student's *t*-test. The significance of the achieved results was judged at the 5% level.

RESULTS

Histological results

Group A: Control group

The histological picture of the defects of this group showed early events of healing. It included the presence of the characteristic granulation tissue, inflammatory cells, and early signs of woven bone formation. Granulation tissue occupied the major parts of the defects, while the newly formed woven bone was seen in areas near the defects' periphery, or continuous with the defects' borders. The surface of the immature newly formed bone was lined by few active osteoblast cells and accommodated large, trapped osteocytes. Also, osteoclast cells could be traced along many segments of the defect margins. (Figure 1 A-D)

Group B: study group

The healing and regenerative features appeared more advanced in this group. Considerable amounts of newly formed trabeculae were seen extending from the defects' margins towards the center and occupying considerable parts of the defects. They were of greater thickness and better organization than those of the control group. Most of the formed bone trabeculae were lined by a continuous layer of voluminous active osteoblasts and contained numerous osteocytes. Broad areas of dense fibrous tissue of rich cellularity appeared mapping the forming bone trabeculae. Osteoclastic activity was traced at the borders of the new bone and on the defect boundary. An important observation was the large dilated blood vessels and the rich vascularity seen among the regenerating trabeculae. (Figure 2 A-D, Figure 3 A and B).

Immunohistochemical results

In the present immunostaining protocol, endothelial cells acquired yellow-brown color, indicating a positive reaction with the antibodies against the endothelial progenitor cell marker (anti-CD34). The Immunohistochemical results showed higher angiogenesis in the study group, observed as an increase in microvessels, compared to the control group. (Figure 4 A-D)

Histomorphometric results

Newly formed bone surface area in the defects

The percentages of the bone surface area formed in the defects in the control and the study groups after one week of healing are illustrated in (Figure 5).

Morphometric analysis revealed that the percentage of new bone surface area was higher in the study group. The mean value of the percentages of the formed bone in group B was (10.93 ± 2.86 %); this was a statistically significant difference ($P<0.001$) compared

to group A which showed a mean value of (3.11 ± 1.21 %).

Microvessel density (MVD) in the regenerated tissues

The mean values of MVD in the defect area in the control and the study groups are illustrated in (Figure 6). Group B, receiving HBOT, showed a statistically significant difference (P<0.003) in MVD (83.33 ± 9.0 /mm2) compared to group A (52.08 ± 9.92 /mm2).

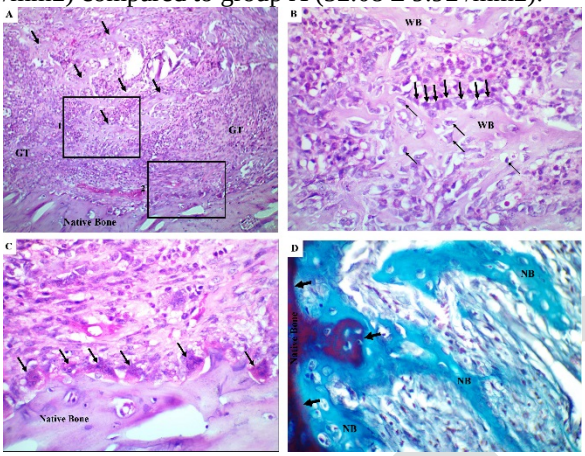

Figure 1: Light micrographs (LM) (Control group): **(A)** Showing the beginning of immature bone formation (arrows). Note the surrounding granulation tissue (GT). H&E stain X100. **(B)** Higher magnification of inset **1** in micrograph **A** revealing spicules of woven bone (WB) with trapped osteocytes (thin arrows). Osteoblasts are seen lining the new bone (thick arrows). H&E stain, X400. **(C)** Higher magnification of inset **2** in micrograph **A** showing many osteoclasts (arrows) at the defect margin. H&E stain, X400. **(D)** showing the early regenerative events in the defect with a line of demarcation (arrows) between the newly formed bone (NB) and the native bone. Trichrome stain, X400.

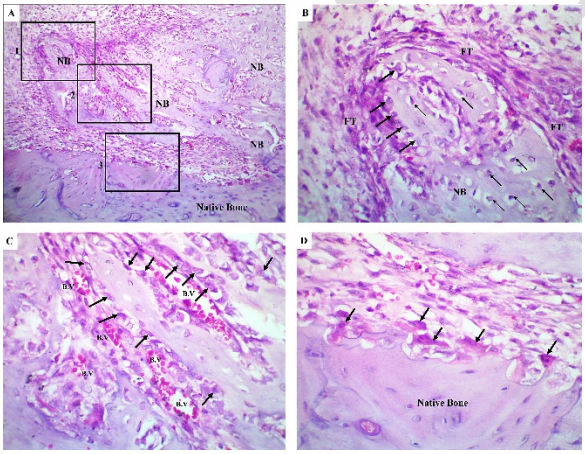

Figure 2: LM (Study group): (A) Showing the newly formed bone (NB) trabeculae starting from the defect's margin towards its center. H&E stain, X100. (B) Higher magnification of inset 1 in micrograph A showing the new bone (NB) outlined by dense fibrous tissue (FT). Note the entrapped osteocytes (thin arrows) and the early trapping of osteoblasts (thick arrows). H&E stain, X400. (C) Higher magnification of inset 2 in micrograph A showing the trabeculae of the new bone lined by active osteoblasts (arrows). Note the rich vascularity (B.V). H&E stain, X400. (D) Higher magnification of inset 3 in micrograph A showing osteoclastic activity (arrows) at the defect margin. H&E stain, X400.

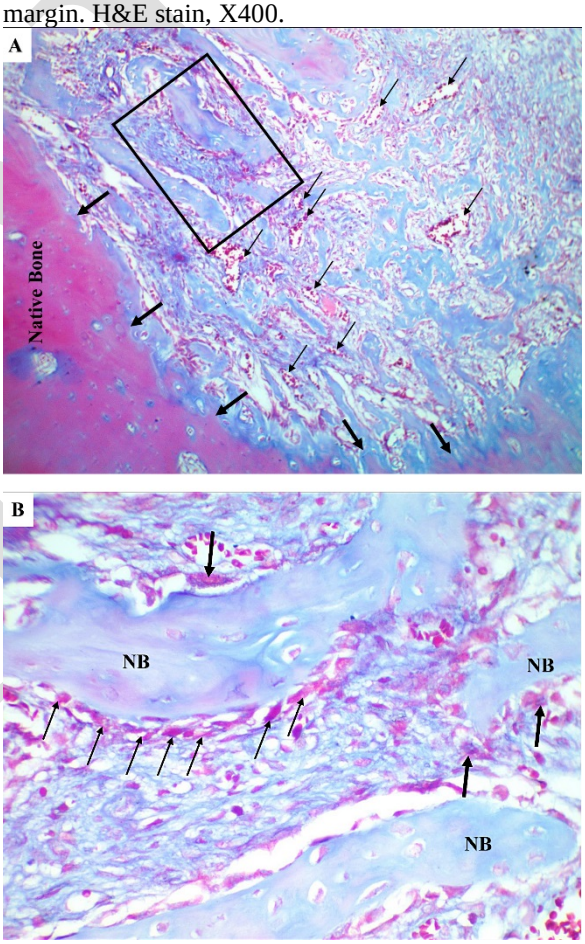

Figure 3: LM (Study group): **(A)** Showing the newly formed bone occupying a large part of the defect with rich vascularity (thin arrows). Note the extensive continuity between the new and the native bone (thick arrows). Trichrome stain, X100. **(B)** Higher magnification of the inset in micrograph **A** showing a continuous layer of active osteoblasts (thin arrows) lining the new bone (NB) and osteoclasts on the opposite side of the trabeculae (thick arrows). Trichrome stain, X400.

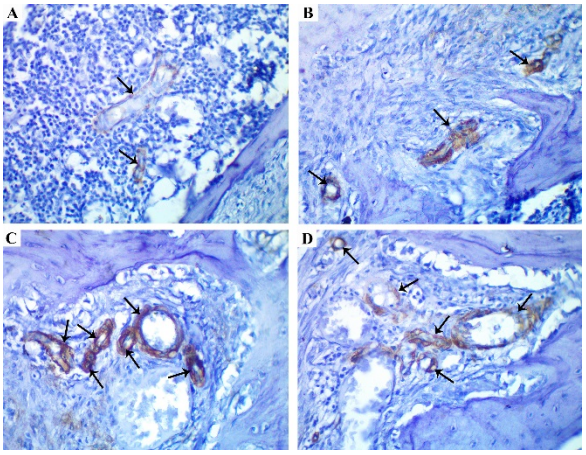

Figure 4: Immunohistochemical micrographs showing the difference in angiogenesis (arrows) between the control group **(A and B)** and the study group **(C and D)**. X400.

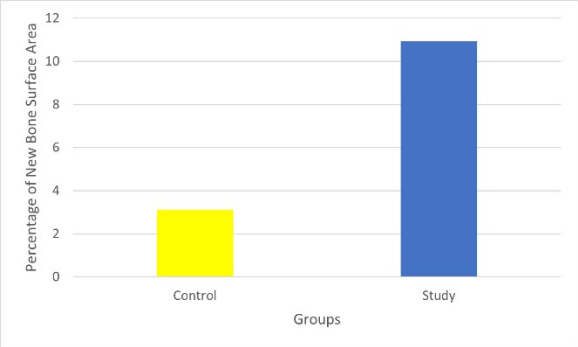

Figure 5: Graph showing the difference between the percentage of the newly formed bone occupying the defect area in the control and the study groups.

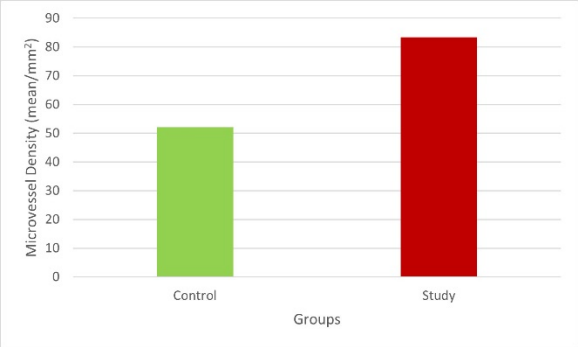

Figure 6: Graph showing the difference between the microvessel density (MVD) of the control and the study groups.

DISCUSSION

Bone regeneration represents a major constituent of the clinical practice aiming at restoring critical-sized defects. Although numerous current strategies can be applied to repair these defects, the incomplete closure or non-union of large defects remains a clinical challenge for orthopedic, reconstructive, and maxillofacial surgeons (34). Hyperbaric oxygen can efficiently encourage the bone healing process through different mechanisms (5-7). Therefore, the present study was conducted to assess the efficacy of HBOT on critical-sized osseous defect regeneration in rats with experimentally induced T1DM. Adult male rats were used in the current study; to avoid any hormonal changes observed in female rats that may affect bone loss (35). Diabetic rat models were used since diabetic patients are more likely to experience impaired postoperative bone healing, as bone is the most affected tissue from hyperglycemia (17). The bone defect model created in the current study is the smallest possible critical-sized osseous defect of (4 mm) diameter; to keep the risk of complication at the lowest level, based on a study by Özkan et al., (28). To the best of our knowledge, the data available regarding the outcome of HBOT on bone regeneration in conjugation with alloplastic bone grafts is limited. Therefore, in the present study, β-TCP was used as a grafting material owing to its bioactive, biocompatible, and biodegradable properties (26,27). Concerning the HBOT protocol in this study, (2.4 ATA) for 90 minutes once daily for 5 times per week was used; this followed the procedure carried out by Jan et al., (3) and Grassmann et al., (9) who proved that this dose significantly increased bone regeneration and enhanced neovascularization.

One week postoperatively, the histological results showed that the defect area in the control group was filled with granulation tissue intermingled with minor ingrowth of woven bone lined with few osteoblasts. At the same time, osteoclasts could be traced along many segments of the defect boundary. This is consistent with the experimental and clinical studies which revealed that bone repair is impaired in diabetes due to the deterioration in osteoblast differentiation and bone turnover, decreased production of collagen, osteocalcin, and bone mineralization (19-23). On the other hand, in the current study, the healing and regenerative features were better in the study group subjected to HBOT than those observed in the control group. A considerable amount of newly formed bone trabeculae of a more mature appearance occupied a larger part of the defects. The trabeculae were of greater thickness, better organization, and lined by a continuous layer of active osteoblasts. These results are thought to confirm the positive role of HBOT in enhancing the regenerative effect of β-TCP during the early phase of bone healing.

The current findings coincide with the results of Kawada et al., (7) who created a mouse femur fracture model followed by HBOT and demonstrated

that the fracture callus was remarkably more developed in the HBO group compared to the non-HBO group. They also investigated the influence of HBOT on bone mineral apposition rate (MAR) and bone formation rate (BFR) in vivo. After HBOT, both MAR and BFR were considerably higher in the HBO group. In another study, Dias et al., (36) provided histological proof for accelerated early bone regeneration in femoral defects of diabetic rats after 7 days of treatment with HBO. However, bone grafting material was not utilized, in contrast to the current study.

The histomorphometric analysis of the new bone surface area in the current experiment confirmed the histological observations. Regarding the bone surface area in the defect site, the morphometric results of the study group revealed a superior percentage of newly formed bone than that of the control group with a statistically significant difference. This agrees with the results of Park et al., (37) who created an irradiated rat calvarial bone defect model, where the HBO groups received the treatment for 1 and 3 weeks. Their histomorphometric analysis revealed that new bone formation was significantly higher in both groups treated with HBOT compared to the control groups. They concluded that HBOT was effective for bone regeneration with only one week of exposure, in cases where irradiation reduced the bone regenerative capacity.

Since bone regeneration and neovascularization are closely related, examination of the effect of HBOT on angiogenesis was also studied. It is generally accepted that hyperglycemia is associated with endothelial damage and reduced endothelium-dependent vasodilation, resulting in micro- and macroangiopathy which are well-known vascular complications of diabetes mellitus (38).

In the present study, visualization of endothelial cells inside the regenerating bone using the antibody against the endothelial progenitor cell marker (anti-CD34) was the basis for the analyses of angiogenesis. Angiogenesis was quantified and expressed through MVD. The histomorphometric analysis concerning the MVD at the healing site revealed significantly more positively stained cells in the study group. This finding supports the previous results of Pedersen et al., (39) who provided evidence that HBOT improved bone healing and vascularization of calvarial bone defects in a rat model. The high microvessel density in the HBO-treated group is thought to prove the significant increase in angiogenesis with HBOT which is in accordance with the results presented by Grassmann et al., (9) They proved enhanced angiogenesis with HBOT in rabbit diaphyseal defect model by detecting intraosseous MVD using transglutaminase staining.

Confirming this therapy's beneficial action on angiogenesis; it was documented clinically and in experimental animal models that HBOT stimulates the release of EPCs into the circulation (12,13). HBOT has been proven to increase the ingrowth of blood vessels within the damaged tissues and enhance the availability of oxygen for healing, Marx et al., (40) demonstrated this effect of HBOT using a rabbit mandibular model, in which the bone and the surrounding soft tissues were heavily irradiated. The HBO-treated group showed 80% recovery of blood vessel density.

Finally, all the previous findings in the present study revealed remarkable bone regeneration and angiogenesis in group B, treated with HBO, compared to group A. The results of the current study rejected the null hypothesis proposed before conducting the experiment, as HBOT proved to have a significant beneficial effect on the regeneration of mandibular critical-sized osseous defects in rats with induced type I diabetes mellitus.

CONCLUSION

The current study verifies that HBOT enhances bone regeneration, both qualitatively and quantitively, augments the regenerative properties of β-TCP bone graft, and increases the proliferation of endothelial cells and intraosseous microvessel density. Thus, this therapy can be a valuable adjunctive therapeutic approach for treating critical-sized bone defects.

CONFLICT OF INTEREST

The authors declare having no conflicts of interest.

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