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Original Research Article

Evaluation of Mandibular Osteogenesis Under Hypobaric Hypoxia Exposure: Immunoexpression of HIF-1*α*, VEGF, and Micro-CT Analysis

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ABSTRACT

Intermittent hypobaric hypoxia (IHH) is critical in osteogenesis and angiogenesis via the HIF-1α and VEGF signaling pathways. However, its specific effect on mandibular bone regeneration remains underexplored. This study aims to evaluate the impact of IHH on the expression of Hypoxia Inducible Factor-1 alpha (HIF-1α), Vascular Endothelial Growth Factor (VEGF), immature bone formation, and changes in mandibular bone microarchitecture. This true experimental study was conducted on 27 male Sprague Dawley rats, randomly divided into three groups: acute bone defect (ABD), bone defect (BD), and hypoxic bone defect (HBD). The HBD group received IHH exposure in a hypobaric chamber simulating 25,000 feet altitude for 5 minutes daily over five consecutive days. HIF-1α and VEGF expression were evaluated using immunohistochemistry. Bone matrix formation was assessed using Masson's Trichrome staining, and bone microarchitecture was analyzed by Micro-Computed Tomography (Micro-CT), including Bone Volume/Total Volume (BV/TV), Bone Surface/Total Volume (BS/TV), Trabecular Thickness (Tb.Th), Trabecular Separation (Tb.Sp), and Trabecular Number (Tb.N). Statistical analysis was conducted using ANOVA and path analysis with a significance level of $p < 0.05$. Results showed that the HBD group had significantly higher expression of HIF-1α and VEGF ($p < 0.05$) and increased BV/TV, BS/TV, and Tb.Th, without significant differences in Tb.Sp and Tb.N. These findings demonstrate that IHH enhances osteogenesis and angiogenesis by upregulating HIF-1α and VEGF, improving bone volume and structure. This supports IHH as a promising non-invasive strategy for mandibular bone regeneration, especially in trauma-induced defects.

Keywords: Intermittent Hypobaric Hypoxia, HIF-1α, VEGF, Bone Regeneration, Micro-CT

Introduction

Bone regeneration is a crucial aspect of medicine, particularly in addressing mandibular bone defects caused by trauma, infection, or surgical procedures such as tumor resection. [1] The healing of large bone defects presents significant challenges due to limited blood and oxygen supply to the affected area, which can hinder osteogenesis and slow healing.[2] The key determinant of successful bone regeneration is the balance between osteogenesis and angiogenesis, where the formation of new blood vessels is essential to ensure that osteoblasts receive sufficient oxygen and nutrients to form new bone tissue. [3] However, hypoxic conditions can cause bone defects, which are considered to inhibit the healing process, therefore requiring a therapeutic strategy to optimize the bone regenerative response. [4] The selection of 25,000 feet for IHH exposure is based on its classification as a "critical zone" where oxygen saturation drops significantly, activating hypoxia-responsive pathways such as HIF-1α and VEGF.

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The 5-day duration is supported by previous studies showing that repeated sublethal exposure at this altitude effectively stimulates osteogenic responses and tissue regeneration without causing permanent damage. [5] Hypoxia regulates bone regeneration by activating various molecular pathways that promote osteoblast differentiation and angiogenesis. One of the primary transcription factors induced by hypoxia is hypoxia-inducible factor-1 alpha (HIF-1α), which acts as a key regulator in cellular adaptation to low-oxygen environments. [6] HIF-1α plays a crucial role in activating growth factors such as vascular endothelial growth factor (VEGF), which is a key component in angiogenesis and supports the formation of new bone tissue. [7] VEGF promotes the formation of new blood vessels in the affected area, ultimately enhancing the oxygen and nutrient supply required for osteogenesis. [8] The upregulation of VEGF mediated by HIF-1α has been reported to accelerate and enhance mineralization in defective bone tissue. [9]

Intermittent hypobaric hypoxia (IHH) is one of the approaches that can stimulate bone regeneration through upregulating HIF-1α and VEGF expression. [10] Previous studies have demonstrated that controlled hypoxia exposure can enhance osteoblast proliferation and differentiation by activating various molecular pathways, including the HIF-1α-VEGF pathway [7]. Moreover, hypoxic conditions have been linked to the increased expression of bone morphogenetic proteins (BMPs), which play a role in stimulating the differentiation of osteogenic progenitor cells into mature osteoblasts. [11] Animal model studies have shown that IHH can enhance bone volume and trabecular thickness in defective bone tissue, indicating improved bone regeneration. [12]

A detailed analysis of bone microarchitecture is essential to assess the effectiveness of hypoxia-based therapy in supporting bone regeneration. The quality and quantity of newly formed bone after IHH exposure can be evaluated using parameters such as bone volume/total volume (BV/TV), bone surface/total volume (BS/TV), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp), and trabecular number (Tb.N). [13] These parameters can be analyzed using Micro-CT technology, which enables high-resolution visualization of bone microstructure. Previous studies have indicated that an increase in BV/TV and BS/TV correlates with improved bone regeneration, while increased Tb. This reflects enhanced trabecular thickness, strengthening bone strength. [14]

Based on this scientific evidence, this study aims to evaluate the effect of intermittent hypobaric hypoxia on mandibular bone regeneration by analyzing HIF-1α and VEGF expression, as well as changes in bone microarchitecture as indicators of osteogenesis. The findings of this study are expected to provide a deeper understanding of the molecular mechanisms underlying bone regeneration under hypoxic conditions and open opportunities for the development of oxygen modulation-based regenerative therapies. Thus, intermittent hypobaric hypoxia may serve as a promising alternative for enhancing the healing of bone defects and accelerating bone tissue repair in clinical cases.

Material and Methods

The present study employed a true experimental design with a post-test-only control group approach, involving 27 male *Sprague Dawley* rats aged 8–12 weeks weighing 200–250 grams. The animals were randomly allocated into three groups: the Acute Bone Defect (ABD) group, which underwent mandibular bone defect induction and was sacrificed on day 7 without receiving any treatment; the Bone Defect (BD) group, which received no exposure to intermittent hypobaric hypoxia (IHH) and was sacrificed at a later healing phase; and the Hypoxic Bone Defect (HBD) group, which was exposed to IHH for five consecutive days following defect induction. All animals were acclimatized for one week under standardized environmental conditions, including a temperature range of 22–24°C, relative humidity of 40–60%, and a 12-hour light/dark cycle. The rats were housed in standard plastic cages lined with wood shavings, with a maximum of three rats per cage to minimize stress. Commercial pellet feed and drinking water were provided *ad libitum* throughout the study. Health assessments were routinely performed by a licensed veterinarian to ensure the well-being of the animals and to exclude any individuals unfit for experimentation. All experimental protocols were conducted following established ethical guidelines and were approved by the Animal Ethics Committee of the Faculty of Veterinary Medicine, IPB University, Indonesia (Approval No. 158/KEH/SKE/I/2024), adhering to recognized principles of animal welfare and scientific integrity.

Mandibular Bone Defect Induction

The mandibular bone defect procedure was performed on the first day of the study. Anesthesia was induced with an intramuscular injection of ketamine (95 mg/kg BW) and xylazine (5 mg/kg BW). Once the animals reached an adequate anesthetic state, the mandibular area was cleaned with 10% povidone-iodine, and a 5 mm incision was made in the gingival mucosa using a sterile scalpel. A bone defect measuring 3×1×1 mm was created on the lateral side of the right mandibular bone using an HP7 Round Bur (Meisinger GmbH, Neuss, Germany) attached to a high-speed handpiece (30,000 rpm) under continuous irrigation with 0.9% NaCl solution to prevent thermal damage. After the procedure, the incision was sutured using 4.0 silk sutures, and the animals received oral meloxicam (1 mg/kg BW) for pain relief and intramuscular enrofloxacin (10 mg/kg BW) for three consecutive days to prevent infection. [15]

Intermittent Hypobaric Hypoxia (IHH) Treatment

Intermittent hypobaric hypoxia (IHH) exposure was applied to the HBD group using a simulated flight Hypobaric chamber (Environics Tectonic Corporation, Pennsylvania, USA) to simulate an altitude of 25,000 feet (7,620 meters). The ABD group did not receive hypoxia exposure and was euthanized immediately after bone defect induction. The BD group was not exposed to IHH and was euthanized on day 11 post-defect induction. The HBD group underwent hypoxia exposure for 5 minutes per day over five consecutive days (days 7 to 11) and was euthanized on day 11. During the hypoxia treatment, the animals were placed in the hypobaric chamber under controlled temperature and humidity conditions, with oxygen levels adjusted to achieve the targeted hypoxic state [16]. The altitude of 25,000 feet corresponds to a barometric pressure of approximately 282 mmHg (37.6 kPa) inside the hypobaric chamber. This pressure was consistently maintained during each exposure session to simulate high-altitude hypoxic conditions.

Immunohistochemical Analysis

After completion of treatment, rats were euthanized with an intraperitoneal overdose of ketamine (250 mg/kg BW) and xylazine (25 mg/kg BW). The mandibles were harvested and fixed in 10% buffered normal formalin (BNF) for 24 hours. The tissues were processed using a tissue processor for dehydration, clearing, and paraffin embedding. The paraffin blocks were sectioned at 5 µm thickness using a microtome, placed on glass slides, and incubated for drying before staining. Immunohistochemical staining was performed to assess the expression of HIF-1α and VEGF in mandibular bone tissue. The sections were deparaffinized and rehydrated, followed by antigen retrieval using citrate buffer (pH 6.0). Samples were incubated with primary antibodies against HIF-1α and VEGF, followed by secondary antibody application and diaminobenzidine (DAB) substrate to visualize immunoreactive proteins as brown-stained regions. The slides were counterstained with hematoxylin and analyzed under a light microscope. [17]

Masson's Trichrome Staining

Masson's Trichrome staining evaluated collagen deposition and immature bone formation. In this staining method, blue coloration indicates type I collagen and mineralizing bone matrix, whereas red or brown coloration represents mature bone structures. The stained sections were examined under a light microscope, and the intensity of the coloration was analyzed to assess the extent of bone regeneration. [18]

Bone Microarchitecture with Micro-CT

The mandibular bone tissue was scanned using the Bruker Micro-CT SkyScan 1173 system to evaluate alterations in trabecular microarchitecture following treatment. The resulting images were calibrated in 16-bit DICOM format to ensure consistent intensity values, and pseudo-color mapping was applied to aid visual interpretation. The trabecular bone was represented in yellow to orange, soft tissue in purple, and voids in black. Quantitative analysis was conducted using Bruker CTAn Analysis Software following standard protocols. Key parameters included bone volume fraction (BV/TV), calculated as BV/TV (%) = (Bone Volume / Total Volume) × 100, which reflects the proportion of mineralized bone within the analyzed volume, and bone surface density (BS/TV), calculated as BS/TV (%) = (Bone Surface Area / Total Volume) × 100, representing the extent of bone surface per total volume, relevant to bone remodeling activity. Additional microstructural parameters assessed included trabecular thickness (Tb.Th), trabecular separation (Tb.Sp), and trabecular number (Tb.N). All measurements were obtained after defining specific Regions of Interest (ROI) and Volumes of Interest (VOI) within the 3D reconstructed datasets to ensure standardized evaluation across samples.. [19]

Statistical Analysis

Statistical analysis in this study was performed using SPSS version 26 and SmartPLS version 3.4.2. Normality was assessed via the Shapiro-Wilk test due to the small sample size. ANOVA followed by Tukey HSD or the Kruskal-Wallis test was used depending on data distribution. Path analysis using SmartPLS evaluated the direct and indirect effects of IHH on HIF-1α, VEGF, and bone microarchitecture parameters (BV/TV, BS/TV, Tb.Th), with statistical significance determined by $t > 1.96$ and $p < 0.05$.

Results and Discussion

This study evaluated the effects of intermittent hypobaric hypoxia (HHI) on osteogenesis in rats with defective mandibular by analyzing HIF-1α, VEGF, Micro-CT, and Masson's Trichrome staining. The results demonstrated that IHH significantly increased the expression of HIF-1α and VEGF, which play a crucial role in angiogenesis and bone regeneration. Micro-CT analysis revealed significant BV/TV, BS/TV, and Tb increases.Th in the IHH group, while Masson's Trichrome staining indicated a higher density of immature bone tissue. This study confirms that exposure to IHH has the potential to be a therapeutic approach in bone tissue engineering. Figures 1 and 2 depict the immunohistochemical expression of HIF-1α and VEGF in mandibular bone tissue across three treatment groups: ABD, Bone Defect (BD), and Hypoxic Bone Defect (HBD). Figure 1 presents the expression levels of HIF-1α (A) and VEGF (B) as percentages, with error bars representing standard deviation (SD). Meanwhile, Figure 2 illustrates the immunohistochemical staining of HIF-1α (A, B, C) and VEGF (D, E, F) within the bone tissue. In the ABD group, HIF-1α and VEGF expression levels were low, as indicated by weak immunoreactive staining, reflecting minimal hypoxic response and angiogenesis, which resulted in slower bone regeneration. The HBD group is a BD group exposed to HHI, highlighting both bone defect and hypoxic treatment. Increased HIF-1α and VEGF in the BD group indicate early hypoxic response and angiogenesis contributing to bone formation.

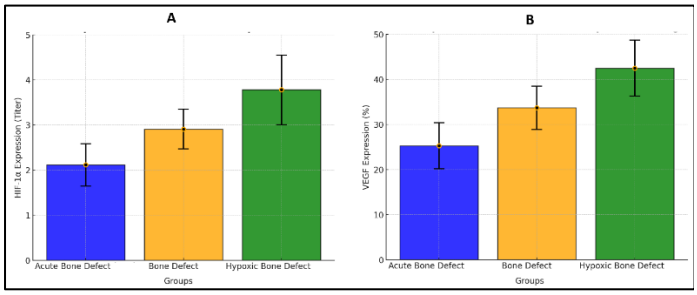

Figure 1. Immunohistochemical expression of HIF-1α and VEGF in mandibular bone defect treatment groups, including Acute Bone Defect (ABD), Bone Defect (BD), and Hypoxic Bone Defect (HBD).

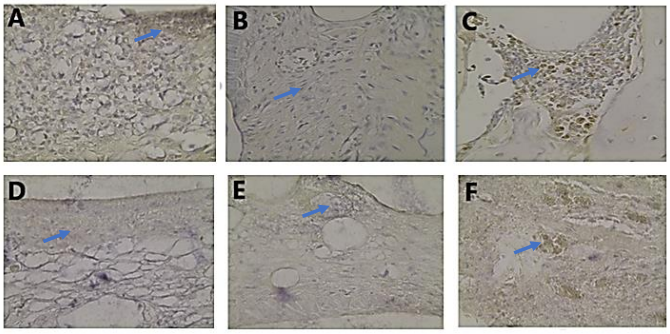

Figure 2: Expression of bone protein. *HIF-1α* (A) Acute Bone Defect (ABD) (B) Bone Defect (BD) (C) Hypoxic Bone Defect (HBD), *VEGF* (D) (A) Acute Bone Defect (ABD) (B) Bone Defect (BD) (C) Hypoxic Bone Defect (HBD). Magnification 400x

The HBD group exhibited the highest expression levels of HIF-1α and VEGF, as evidenced by the more vigorous immunoreactive staining intensity. This confirms that IHH enhances HIF-1α expression, stimulating VEGF and accelerating angiogenesis, osteogenesis, and mandibular bone regeneration. These findings highlight the crucial role of IHH in enhancing HIF-1α and VEGF expression, contributing to osteoblast activation, new bone formation, and increased vascularization in mandibular bone defects. The results support the hypothesis that hypoxia can accelerate bone healing through the immunohistochemical regulation of HIF-1α and VEGF.

The results of this study indicate that intermittent hypobaric hypoxia (IHH) significantly increases the expression of HIF-1α ($p < 0.05$) in the Hypoxic Bone Defect (HBD) group compared to the Bone Defect (BD) and Acute Bone Defect (ABD) groups (Figure 1A). HIF-1α is a key transcription factor in cellular adaptation to hypoxia, activating osteogenesis and angiogenesis pathways to support new bone formation. Previous research demonstrates that HIF-1α plays a role in osteoblast differentiation and the activation of bone progenitor cells, as well as increasing the expression of VEGF and BMPs.[7] Additionally, a study confirms that hypoxia enhances VEGF expression, which is crucial for angiogenesis and improves oxygen and nutrient supply to bone tissue with defects. [11] the HIF-1α regulates the expression of osteogenic genes such as RUNX2 and Osterix, which support the formation of new bone matrix, consistent with previous research reporting that HIF-1α activation increases the expression of ALP (alkaline phosphatase), an early indicator of osteoblastogenesis. [20]

The results of this study also reveal a direct correlation between HIF-1α and VEGF ($p < 0.05$) (Figure 1B and 2). VEGF is a key angiogenic factor in forming new blood vessels that enhance oxygen and nutrient supply, essential for bone regeneration. In the HBD group, VEGF expression was significantly higher compared to the BD and ABD groups, indicating that HIF-1α activation due to hypoxia increases VEGF, thereby enhancing vascularization in the bone defect area. Previous research supports these findings, showing that hypoxia induces VEGF through HIF-1α activation, ultimately promoting osteogenesis and angiogenesis. [21] Similar results found that increased VEGF due to hypoxia enhances microcapillary formation in complex tissue regeneration, accelerating new bone growth in a rat cranial defect model. [22]

Furthermore, a study states that VEGF plays a role in angiogenesis and osteoblast differentiation and proliferation through the PI3K/Akt pathway, supporting the formation of new bone tissue. [23] Increased VEGF under hypoxic conditions is associated with enhanced osteoclast activity, accelerating bone remodeling. [24] The results of this study confirm that IHH enhances bone regeneration by promoting angiogenesis through the HIF-1α-VEGF pathway, as shown in Figure 2, where the HBD group exhibited the highest VEGF expression. These findings align with other research found that hypoxia-induced VEGF regulation enhances osteogenesis by stimulating the expression of osteopontin (OPN) and integrin β1, which are involved in osteogenic cell adhesion.[25]

The Masson's Trichrome staining results presented in Figure 3 provide important insights into the dynamics of immature bone formation across the treatment groups. The Hypoxic Bone Defect (HBD) group exhibited a notably more intense blue staining pattern compared to both the Bone Defect (BD) and Acute Bone Defect (ABD) groups. This blue staining corresponds to increased deposition of type I collagen and the mineralizing matrix, signifying a higher degree of osteogenic activity in the HBD group. These findings are consistent with the Kruskal-Wallis statistical test, which showed a significant difference ($p < 0.05$) among the groups, with the HBD group displaying the highest histological scores. The intense blue coloration in the HBD group reflects early bone matrix formation enhancement, likely facilitated by the activation of the HIF-1α–VEGF signaling axis in response to intermittent hypobaric hypoxia (IHH). This hypoxic stimulus has been shown in previous studies to induce osteoblast proliferation and differentiation and enhance vascularization necessary for nutrient and oxygen delivery in regenerating tissues. [26] The increased presence of immature bone in the HBD group indicates a robust osteogenic response, likely mediated by hypoxia-driven molecular signaling.

In contrast, the ABD group, which was sacrificed earlier without treatment, showed minimal immature bone matrix, while the BD group, although showing some blue staining, exhibited less osteogenic activity than the HBD group. The normal control group (D) displayed mature bone features with a well-organized structure and minimal blue staining, confirming its role as a baseline reference for complete mineralization. The histological findings confirm that IHH exposure promotes accelerated bone matrix formation, aligning with the

molecular findings of increased HIF-1α and VEGF expression in the HBD group. This supports the hypothesis that IHH effectively enhances early bone regeneration by stimulating osteoblast function and angiogenesis.

Table 1 and Figure 4 present the Micro-CT analysis results of mandibular bone microarchitecture across three study groups: Acute Bone Defect (ABD), Bone Defect (BD), and Hypoxic Bone Defect (HBD). The analyzed parameters include Bone Volume/Total Volume (BV/TV), Bone Surface/Total Volume (BS/TV), Trabecular Thickness (Tb.Th), Trabecular Separation (Tb.Sp), and Trabecular Number (Tb.N). Micro-CT analysis was conducted by calibrating the numerical range of image datasets in 16-bit DICOM format to standardize the intensity of scanned objects for accurate descriptions. Pseudo-color was applied to facilitate interpretation, where trabecular bone appears in yellow to orange, soft tissue in purple and void spaces in black.

Table 1: Micro-CT Analysis of Mandibular Bone with Different Treatments

Parameter	Group Treatments			*p-value	Scale analyses		
	ABD	BD	HBD				
	Mean ± SD	Mean ± SD	Mean ± SD		ABD	BD	HBD
BV/TV (%)	12.5 ± 1.2	18.4 ± 1.5	25.6 ± 2.0	< 0.05	Low	Moderate	High*
BS/TV (%)	10.3 ± 1.1	15.2 ± 1.3	21.5 ± 1.7	< 0.05	Low	Moderate	High*
Tb.Th (mm)	0.13 ± 0.02	0.18 ± 0.03	0.25 ± 0.04	< 0.05	Thin	Moderate	Thick*
Tb.Sp (mm)	0.50 ± 0.05	0.48 ± 0.04	0.46 ± 0.03	> 0.05	No Difference	No Difference	No Difference
Tb.N (1/mm)	2.1 ± 0.3	2.3 ± 0.4	2.4 ± 0.3	> 0.05	No Difference	No Difference	No Difference

* One Way ANOVA BV/TV (Bone Volume/Total Volume); BS/TV (Bone Surface/Total Volume);Tb.Th (Trabecular Thickness); Tb.Sp (Trabecular Separation); Tb.N (Trabecular Number). Acute Bone Defect (ABD); Bone Defect (BD); Hypoxic Bone Defect (HBD)

The results indicate that the HBD group exhibited significantly higher BV/TV, BS/TV, and Tb. Compared to the other groups ($p < 0.05$), Th values indicate increased bone volume and more active regeneration. The increase in BS/TV suggests a greater bone surface area, which correlates with osteoblast activity and bone mineralization in response to intermittent hypobaric hypoxia (IHH). The greater Tb.Th observed in the HBD group indicates osteoblast stimulation and enhanced vascularization, accelerating osteogenesis and angiogenesis. Conversely, Tb.Sp and Tb.N showed no significant differences among the groups ($p > 0.05$), suggesting that trabeculae's overall distribution and number remained stable despite increased bone volume and trabecular thickness. The significant increase in BV/TV and BS/TV in the HBD group is associated with HIF-1α and VEGF activation, which play crucial roles in osteoblast stimulation and angiogenesis, ultimately accelerating new bone formation.

Micro-CT analysis shows that VEGF significantly contributes to increased Bone Volume/Total Volume (BV/TV), Bone Surface/Total Volume (BS/TV), and Trabecular Thickness (Tb.Th) ($p < 0.05$) in the HBD group compared to the BD and ABD groups (Table 1, Figure 4). This indicates that improved angiogenesis supports the formation of stronger and denser bone through the stimulation of osteoblasts and the differentiation of bone progenitor cells. [27] Previous researchers also state that VEGF increases bone density by stimulating molecular pathways associated with bone growth.[28] VEGF reported enhances vascularization in bone tissue with defects, thereby improving mineralization and new bone formation. [29] These findings are further supported research, which found that increased VEGF under hypoxic conditions facilitates enhanced bone mineralization by activating the ERK/MAPK pathway.[30]

Although VEGF plays a role in increasing trabecular volume and thickness, no significant correlation was found between VEGF and Trabecular Separation (Tb.Sp) or Trabecular Number (Tb.N) ($p > 0.05$) (Table 1). This suggests that while new bone formation is enhanced, the distribution and number of trabeculae do not change significantly. The VEGF is more involved in improving bone quality and density rather than altering the trabecular number, [31] while other research also reported that VEGF increases trabecular thickness without changing their number as existing trabeculae become denser. [32] Micro-CT analysis in Figure 4 shows that the HBD group has higher bone mineral density than the other groups. Still, the trabecular distribution does not change drastically, indicating that VEGF plays a greater role in increasing bone density rather than altering trabecular numbers.

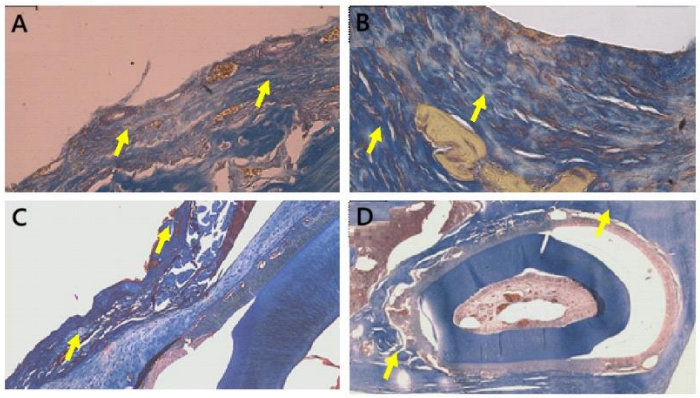

Figure 3: Masson's Trichrome staining of mandibular bone: (A) Acute Bone Defect (ABD), (B) Bone Defect (BD), (C) Hypoxic Bone Defect (HBD), and (D) Normal bone structure. Magnification 400x

Table 2 presents the results of the Path Analysis conducted to evaluate the relationships between hypoxia, HIF-1α expression, VEGF expression, and mandibular bone microarchitecture parameters (BV/TV, BS/TV, Tb.Th, Tb.Sp, and Tb.N). The findings indicate that hypoxia has a direct effect on HIF-1α expression ($p < 0.05$), which subsequently increases VEGF expression ($p < 0.05$) as a key angiogenic factor in bone regeneration. VEGF significantly influences BV/TV, BS/TV, and Tb.Th ($p < 0.05$) suggests enhanced angiogenesis contributes to increased bone volume, surface area, and trabecular thickness. However, no significant relationship was found between VEGF and Tb.Sp or Tb.N ($p > 0.05$), indicating that the Intermittent hypobaric hypoxia (IHH) enhances osteogenesis and angiogenesis by upregulating HIF-1α and VEGF, leading to increased bone volume and thickness (BV/TV, Tb.Th) without significantly affecting trabecular number or spacing (Tb.N, Tb.Sp). [10]

This aligns with existing studies, which suggest that while hypoxia improves bone quality, structural changes in trabecular distribution require longer remodeling or additional stimuli. [24]

The results of the post-hoc Tukey HSD analysis (Table 3) demonstrated significant differences in bone volume/total volume (BV/TV), bone surface/total volume (BS/TV), and trabecular thickness (Tb.Th) among the treatment groups, particularly between the hypoxic bone defect (HBD) group and the acute bone defect (ABD) and bone defect (BD) groups ($p < 0.05$ to $p < 0.01$). These findings indicate that exposure to intermittent hypobaric hypoxia (IHH) significantly enhances bone volume and trabecular thickness, reflecting increased osteogenic activity and more advanced bone maturation in the HBD group. The observed increases in BV/TV and BS/TV suggest enhanced new bone formation and greater bone surface area, which are closely associated with elevated osteoblastic activity and mineralization. Furthermore, the rise in Tb.Th indicates that the trabeculae in the HBD group were thicker, implying improved structural integrity and density of the bone microarchitecture.

Table 2: Path Analysis of Mandibular Bone Regeneration

Relationship	p-value	Conclusion
Hypoxia → HIF-1α	< 0.05	Hypoxia increases HIF-1α expression
HIF-1α → VEGF	< 0.05	HIF-1α influences VEGF expression
VEGF → BV/TV	< 0.05	VEGF increases Bone Volume/Total Volume
VEGF → BS/TV	< 0.05	VEGF increases Bone Surface/Total Volume
VEGF → Tb.Th	< 0.05	VEGF increases Trabecular Thickness
VEGF → Tb.Sp	> 0.05	No significant relationship
VEGF → Tb.N	> 0.05	No significant relationship

Table 3. Post-hoc Tukey HSD Analysis for Multiple Comparisons

Parameter	Tukey HSD		
	ABD vs BD	ABD vs HBD	BD vs HBD
BV/TV (%)	p<0.05 (0.032)	p<0.01 (0.007)	p<0.05 (0.021)
BS/TV (%)	p<0.05 (0.041)	p<0.01 (0.005)	p<0.05 (0.037)
Tb.Th (mm)	p<0.05 (0.028)	p<0.01 (0.003)	p<0.05 (0.026)
Tb.Sp (mm)	p>0.05 (0.075)	p>0.05 (0.092)	p>0.05 (0.078)
Tb.N (1/mm)	p>0.05 (0.084)	p>0.05 (0.091)	p>0.05 (0.08)

Acute Bone Defect (ABD), Bone Defect (BD), Hypoxic Bone Defect (HBD). Tukey HSD multiple comparisons: p<0.05 indicates a significant difference. p<0.01 indicates a highly significant difference.

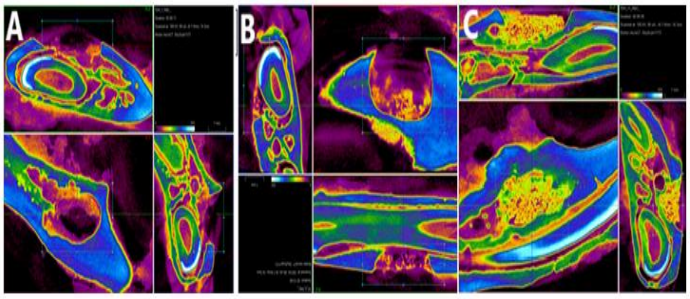

Figure 4: Micro-CT Analysis of Mandibular Bone. (A) Acute Bone Defect (ABD), (B) Bone Defect (BD), (C) Hypoxic Bone Defect (HBD).

Conversely, trabecular separation (Tb.Sp) and trabecular number (Tb.N) did not show significant differences across the groups ($p > 0.05$), indicating that the spatial distribution and quantity of trabeculae remained relatively stable. This finding aligns with previous studies, which reported that repeated hypoxic exposure improves bone quality and density rather than induce substantial changes in trabecular patterning, particularly over short intervention periods.[33] Therefore, these post-hoc results further support the conclusion that IHH significantly improves bone quality through increased trabecular volume and thickness without significantly altering trabecular number or spacing. Clinically, this outcome is relevant, as enhanced bone density and strength are key indicators of successful bone regeneration. These findings are consistent with research by Chu et al. (2025), which shows that IHH increases the expression of osteogenic growth factors such as BMP-2 and RUNX2, which play a role in bone formation and faster recovery of bone defects. [34] A study by Song et al. (2023) states that hypoxia-induced VEGF can enhance osteoblast activity through the PI3K/Akt pathway, accelerating the formation of new bone tissue and increasing trabecular thickness [21]. Tan et al. (2021) also found that hypoxia regulates bone remodeling by increasing osteoclast activity, allowing for more mature and stable bone structures. [35] Further studies by Xue et al. (2020) show that prolonged hypoxia can activate other factors, such as SDF-1 and CXCR4, which also regulate mesenchymal stem cells to accelerate bone healing. [36]

The results of this study demonstrate that Intermittent Hypobaric Hypoxia (IHH) significantly increases the expression of HIF-1α and VEGF, supporting the activation of molecular pathways involved in osteogenesis and angiogenesis. The HBD group exhibited the highest expression levels, consistent with enhanced bone formation as observed in Masson's Trichrome staining and Micro-CT analysis. These findings align with previous studies reporting that hypoxia enhances the expression of osteogenic and angiogenic genes. However, this study has limitations, including potential differences in biological response between rodents and humans and possible confounding factors. Further research is needed to confirm the effectiveness of IHH in mandibular regenerative therapy.

Conclusion

This study demonstrates that intermittent hypobaric hypoxia (IHH) significantly enhances mandibular bone regeneration by activating the HIF-1α and VEGF pathways, accelerating osteogenesis and angiogenesis. The increased expression of HIF-1α and VEGF observed in the HBD group was associated with improved trabecular volume, surface area, and thickness without significantly altering trabecular distribution or number. These findings provide strong evidence that IHH can stimulate biological responses favorable to bone healing. Importantly, this study highlights the potential clinical application of IHH as a non-invasive strategy for mandibular regenerative therapy. The controlled use of IHH could be integrated into treatment protocols for patients with critical-sized bone defects, particularly in military and aerospace medicine, where hypoxic training environments are already utilized. Further clinical studies are needed to validate its safety and efficacy in human applications.

Conflict of interest
The authors declare no conflict of interest.

Author's Declaration
The authors hereby declare that the works presented in this article are original and that any liability for claims relating to the content of this article will be borne by them.

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