# Expression of TNF-α, IL-1β, and Macrophages in Intermittent Hypobaric Hypoxia Exposure in Post-Tooth Extraction Socket Healing Process in Rats

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## Abstract

The aim of this study was to investigate expression of TNF-α, IL-1β, and macrophages in intermittent hypobaric hypoxia exposure in post tooth extraction socket healing process in rats.

Forty-five healthy adult male Sprague Dawley rats were used in this study. Tooth extraction was performed on the maxillary left first molar, then randomly divided into 9 groups, namely the intermittent hypobaric hypoxia (IHH) group which was given exposure to hypobaric hypoxia (HH) for 30 minutes every day in the Hypobaric Chamber at an altitude of 18. 000 feet for 1 time HH, 3 times HH, 5 times HH, and 7 times HH, and the normoxia group that was not exposed to HH, and the control group were terminated on days 0, 1, 3, 5 and 7. Molecular changes in mRNA expression were measured by RT-PCR extracted from socket tissue after rat tooth extraction to evaluate the expression of TNF-α mRNA and IL-1β mRNA, as well as histological changes by Hematoxylin and Eosin (H&E) staining to evaluate macrophages. Molecular and histological parameters were calculated at the end of each experiment on days 0,1,3,5, and 7 after tooth extraction as repair phase of the socket healing process.

In this study, it was found that the expression of TNF-α mRNA, IL-1β mRNA and the number of macrophages, began to increase significantly after 1 time exposure to HH on day 1, and reached the highest peak after 3 times exposures to HH on day 3, this increase was higher than the control group and the normoxia group, then after 5 times exposures to HH on day 5 there was a decrease, and further decreased after 7 times exposures to HH on day 7, this decrease also occurred faster approaching the control group than the normoxia group. Repeated or intermittent exposure to hypobaric hypoxic conditions induces a protective response, can make cells adapt to hypoxia. Several inflammatory cytokines and inflammatory processes, such as inflammatory cell migration and monocyte differentiation into macrophages, are modulated under hypoxic conditions. TNF-α and IL-1β are proinflammatory cytokines produced by macrophages in early wounds, and play an important role in inflammation and immune responses in wound healing.

The results showed that changes in TNF-α and IL-1β mRNA expression were in line with changes in the number of macrophages in the socket after tooth extraction under hypobaric hypoxic conditions, and there was a gradual mechanism of cell adaptation under intermittent hypobaric hypoxic conditions which could affect the wound healing process after tooth extraction.

**Experimental article (J Int Dent Med Res 2023; 16(2): 549-559)**

**Keywords:** Intermittent hypobaric hypoxia, TNF-α, IL-1β, Macrophage, tooth extraction.

**Received date:** 23 January 2023 **Accept date:** 22 February 2023

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## Introduction

The post-tooth extraction wound healing process is time-based, following the same pattern as the wound healing process in general with the inclusion of socket and bone healing process.[1] Physiological changes in the post-tooth extraction wound healing process occur in four

phases, namely haemostasis and coagulation phases, inflammatory phase, proliferative phase, and alveolar bone tissue modeling as well as remodeling phase.[2]

After tooth extraction, the empty socket is immediately filled with blood from the reaction of haemostasis and inflammation in the alveolar socket, the result of the dynamic interaction of platelets and connective tissue collagen, as well as the balance between coagulation and fibrinolysis, giving rise to the formation of stable blood clots embeddd in fibrin tissue.[1,2,3,4,] A few minutes after the tooth is removed, the blood vessels will experience vasoconstriction as a result of platelet aggregation, causing disruption of oxygen delivery resulting in tissue hypoxia, increased glycolysis and decreased Ph to be responded to by vasodilation, then there is a migration of leukocyte and platelet cells to wound tissue.[1,3] Tissue hypoxia serves as a signal that stimulates many aspects of the wound healing process.[4,5] Hypoxia is a state of reduced oxygen supply to the cellular level that is not sufficient to maintain cellular function.[6]

Changes in oxygen concentration due to hypoxia will modulate cell function by stabilizing Hypoxia Induced Factor-1α (HIF-1α) which is a transcription factor for many genes that regulate adaptive responses to hypoxia.[7,8] Wound healing involves multiple processes including inflammation, angiogenesis, vasculogenesis and arteriogenesis, all of which are regulated by the HIF-1α target gene.[9] Stabilization of HIF-1α as the main regulator of oxygen homeostasis and determinant of wound healing outcomes through activation of multiple target genes of HIF-1α.[8,10] HIF-1α is also upregulated under inflammatory conditions, suggesting their role in maintaining homeostasis and protecting against cellular inflammation in soft and hard tissues after tooth extraction.[9] Some cytokines and inflammatory processes, such as migration of inflammatory cells (monocytes and macrophages) and differentiation of monocytes into macrophages, modulated by HIF-1α under hypoxic conditions.[11]

Macrophages in wounds, mostly derived from circulating monocytes recruited to the wound site, and these monocytes will migrate to the site of wound inflammation during the early stage of injury, then these macrophages control wound cellularity through their capacity to induce apoptosis and phagocytize various injured cells including neutrophils during the inflammatory phase of repair and macrophages being the predominant inflammatory cells.[12,13] Macrophages are critical in the wound healing process and provide useful therapeutic targets for wound healing disorders, contributing to the inflammation required to kill potential pathogens, resolve inflammation after pathogens have been cleared, coordinate tissue repair, and initiate and sustains tissue remodeling and regeneration.[14] Macrophages recruited into the wound at the onset of injury, contribute to the release of proinflammatory cytokines such as Tumor Necrosis Factor-α (TNF-α) and Interleukin (IL)-1β, IL-6, IL-12 and IL-23 which stimulate inflammatory cell proliferation and promote angiogenesis through endothelial cell replication.[12,15] Angiogenesis, stimulated by TNF-α, characterized by migration of endothelial cells and formation of capillaries to the wound bed is critical for proper wound healing.[9] Macrophage depletion during the early inflammatory phase of the wound results in delayed wound closure, decreased granulation tissue formation and angiogenesis, decreased collagen synthesis, and decreased growth factor levels, whereas excessive macrophages can lead to scarring or fibrotic tissue. [13,14] Tissue hypoxia in early wounds also triggers inflammation as hypoxia stimulates various cell types, including macrophages, to produce mediators important for inflammation.[13]

Hypoxia can occur in high-altitude environments with low atmospheric pressure, and the subsequent proportional decrease in the partial oxygen pressure (PO2) in air rapidly with increasing altitude, resulting in decreased bioavailability of oxygen in organs, tissues, and cells is known as hypobaric hypoxia (HH).[16,17,18,19,20] Hypobaric is a condition of environmental change that occurs during altitude including changes in air pressure, temperature, and oxygen supply.[21] There is a correlation between altitude and atmospheric pressure. The higher the altitude, the lower the atmospheric pressure or the lower the partial pressure of oxygen which becomes a stressful condition and affects gas exchange at the cellular level. Cells that are not responsive to these stressful conditions become alarmingly dysfunctional.[22] The cellular response to hypobaric hypoxia is very complex and is characterized by changes in the expression of a number of genes including proteins to maintain homeostasis.[23]

It is accepted that the hypobaric effects of hypoxia begin to manifest at an altitude of 10,000 feet (3,048 m), at this altitude the effects of hypoxia on the human body are obvious and easily recognized.[17,21,19] Previous studies of human volunteers ascending to high altitude (3860 m) and rats exposed to hypoxia in a hypobaric chamber (5000 m), there were increased levels of proinflammatory factors TNF-α, IL-1β, IL-6 in human plasma and rat brain, suggesting that induction of hypobaric hypoxia leaves a spaceimmune-inflammatory response in humans and rats.[24]

Hypoxia does not always cause damage to cells and does not always have a negative impact, but exposure to mild hypoxia with tolerable levels and timeframes provides a protective effect, enhancing the adaptive and protective response so that injuries from subsequent exposure to harmful stimulus are reduced.[6, 25] Hypobaric hypoxia adaptation with Intermittent Hypobaric Hypoxia (IHH) efforts performed has a number of effects on tolerance to subsequent hypoxic exposure, which has the effect of preventing damage to cells by reducing the oxidative stress and inhibiting the apoptotic cascade. [26]

Air Force pilots routinely undergo hypobaric hypoxia training to identify hypoxic conditions and allow their bodies to adapt to hypoxia. They can experience intermittent hypobaric hypoxia in both training and assignment. Several studies have suggested that post IHH treatment showed an increase in protection against brain nerve cells damage, increased cardiac endurance, cardiac adaptation to hypoxic conditions, can provide cardiovascular and femoral artery protective responses, there is a protective effect on rat kidney tissue, and can promote bone repair.[26,27,28,29,30,31,32] To the best of our knowledge, no studies have been conducted to analyze the effect of intermittent hypobaric hypoxia exposure on post-extraction socket healing tooth. How the post-extraction molecular changes, especially the expression of TNF-α, IL-1β and macrophages in the post-extraction socket after intermittent hypobaric hypoxia exposure is still unclear. Therefore, this study wanted to analyze the molecular changes of TNF-α, IL-1β and macrophages in the post-extraction socket healing process after intermittent exposure to hypobaric hypoxia.

## Materials and methods

### Study Design

This research is a true experimental study with a Randomized Post-Test Only Control Group Design research design, using animal models of healthy adult male rats Sprague Dawley conducted at the Integrated Research Laboratory of the Faculty of Dentistry, Padjadjaran University, the Molecular Genetics Laboratory of the Faculty of Medicine, Padjadjaran University, and the Aerophysiology Department Laboratory of Aerospace Medicine Lakespra dr.Saryanto Indonesian Air Force.

The total number of rats in this study was 45 male Sprague Dawley rats, 2-3 months old weighing 200-400 gm and in good health, obtained from the Animal Breeding Laboratory of PT Biomedical Technology Indonesia, Bogor, West Java, Indonesia. Rats were housed in the laboratory for one week to adapt before the hypoxia experiment. Animals were well maintained under constant conditions in an air-conditioned room (22±3°C) with light cycle lighting (06.00-18.00) and fed ad libitum food and drink water every day.

The sample size in this study was calculated using the Federer formula as follows:

$$(n - 1) (k - 1) \geq 15$$

where :
n = number of samples per group
k = number of groups

$$(n-1) (9-1) \geq 15$$
$$8n - 8 \geq 15$$
$$n \geq 2,9$$

The minimum number of samples per group is 2.9 rounded to 3. To anticipate drop out, we added animal into 5 (n = 5), meaning that the number of each group is 5 rats, so the total number of Sprague Dawley rats is 45 rats. The 45 rats were then randomly divided into 9 experimental groups, each group consisted of 5 rats including:

- The IHH group (n=20) consists of animals that are given exposure to hypobaric hypoxia in the Hypobaric Chamber, namely group P1 (1 time exposure to HH, termination day 1), group P2 (3 times exposure to HH, termination day 3), group P3 (5 times exposure to HH, termination day 5), and group P4 (7 times exposure to HH, termination day 7).

- The normoxia group (n=20) consisted of animals kept under normoxia conditions, and placed in the same room as sea level, namely group K1 (termination day 1), group K2 (termination day 3), group K3 (termination day 5), and group K4 (termination day 7).
- The control group (n=5) consisted of animals maintained under normoxic conditions, and housed in the same chamber as sea level, namely group K0 (termination day 0).

All experimental animals were anesthetized according to body weight, intra peritoneally with a combination of 80 mg/kg BW HCL ketamine and 5 mg/kg BW xylazine premedication. After anesthetized, the molar teeth were extracted using sterile dental and arterial clamps as special pulling forceps with unidirectional movements and being careful to avoid tooth fracture and the teeth were completely extracted, then the post-extraction socket was cleaned with sterile gauze and allowed to heal. natural, and no stitches were done.

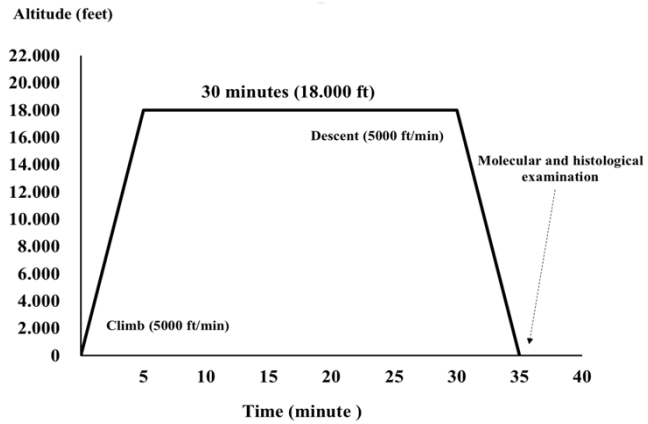


**Figure 1**. Hypobaric hypoxic procedures using a Hypobaric Chamber, each procedure at 18,000 feet for 30 minutes.

Post-tooth extraction hypobaric hypoxia exposure in the IHH group (groups P1, P2, P3, and P4) was carried out by placing experimental animals into the Hypobaric Chamber at an altitude of 18,000 feet for 30 minutes and the air temperature was maintained at around 28$^0$C with humidity around of about 58%. The hypobaric hypoxic procedure is designed based on special training for Indonesian Air Force Soldiers.[33] Hypobaric hypoxia exposure was repeated every day for intermittent hypobaric hypoxia exposure, namely 1 time, 3 times, 5 times and 7 times exposure to HH. A simulation of the hypobaric hypoxic profile procedure from this study is shown in Figure 1.

Termination of several experimental animals for sampling was carried out on days 0, 1, 3, 5, and 7 after tooth extraction. The IHH group was terminated immediately after exposure to hypobaric hypoxia when it arrived at ground level, and the normoxic group was terminated at the end of each experiment, whereas the control group was terminated immediately on the day after tooth extraction. Before termination, the experimental animals were anesthetized intraperitoneally with a combination of 80 mg/kg BW ketamine HCL and 5 mg/kg BW xylazine premedication. The upper jaw was cut with a scalpel, the post-extraction socket tissue was extracted and divided in two with a separating disk drill. Half socket tissue per animal of about 20–30 mg was rapidly placed in microtube RNA centrifuge tubes and then stored at −80°C until use for RT-PCR, and the remaining tissue was fixed in formalin and processed for histological analysis. The experimental design in this study received ethical approval from the Research Ethics Commission of Padjadjaran University with Ethical Approval Number: 419/UN6.KEP/EC/2021 on 15 May 2021.

**Measurement of TNF-α and IL-1β mRNA expression**

Messenger Ribonucleic Acid (mRNA) expression was extracted from wound socket tissue samples after rat tooth extraction using the RNeasy Mini Kit reagent from Qiagen. Real time polymerase chain reaction (RT-PCR) was carried out using the Rotor-Gene Q real-time PCR system from Qiagen with the Sensifast SYBR No-ROX one-Step Kit for TNF-α and IL-1β, mixing the ingredients into a pcr-tube with the composition SensiFAST SYBR (2X) 10 µl, Primer F 0.8 µl , Primer R 0.8 µl, Reverse Transcriptase Enzyme 0.2 µl, Ribosafe RNAse Inhibitor 0.4 µl, Nuclease Free Water 5.8 µl, and RNA Extract 2 µl. Insert the PCR-tube then set on the RT-PCR cycle starting with the first incubation at 45°C for 10 minutes, followed by the second incubation at 95°C for 2 minutes, then Denaturation 40 cycles at 95°C for 5 seconds, and extension at 60° C for 20 seconds. Instructions as per manufacturer's protocol were strictly followed.

The primar sequence is as follows:
TNF-α: 5'-TGAACTTCGGGGTGATCGGT-3' as forward, and 5'-TCCGCTTGGTGGTTTGCTAC-3' as reverse,

IL-1β: 5'-TCCTCTCCAGTCAGGCTTCCT-3' as forward, and 5'-TCTGGACAGCCCAAGTCAAGG-3' as reverse, β-actin: 5'-CACCCGCGAGTACAACCTTC-3' as forward, and 5'-CCCATACCCACCATCACACC -3' as reverse.

β-actin was used as an internal control. The mRNA expression was analyzed using the Livak 2(-ΔΔCT) method, which compares the Ct value of the treatment group with the control group. ΔΔCT = Ct of target gene - Ct of housekeeping gene. ΔΔCT = ΔCT of treatment - ΔCT of control. Gene expression = $2^{(-\Delta\Delta CT)}$. A gene expression value ≥ 1 indicates increased gene expression compared to the control.

**Histological Analysis**

Termination of experimental animals was carried out on days 0, 1, 3, 5 and 7 after tooth extraction and socket tissue was extracted, then fixed with Formalin combined with Phosphate Buffer Saline (PBS-formalin) solution for 24 hours at 4 °C, then samples decalcified with 10% ethylenediaminetetraacetic acid (EDTA) solution at pH 7.4 and stored at 4°C for 6-8 weeks, depending on the degree of mineralization, with EDTA, updated every 3 days, then trimmed and arranged into tissue cassette and labeled, then dehydrated, which is the stage of immersing the tissue into an alcoholic solution in stages ranging from 70%, 80%, 90%, 95%, 100% and cleaned three times with xylene solution for 60 minutes each cycle, then infiltrated in liquid paraffin in three cycles by immersing the tissues in the molten paraffin for 60 minutes each cycle, blocking until the paraffin freezes. The tissues were cut using a microtome slicer into slide preparations and stained with Hematoxylin and Eosin (H&E), then the preparations were glued using entheses and covered by a cover glass. The dry slides were observed under a 400x magnification binocular lens microscope (Olympus Type CX31) equipped with a camera at 5 different fields of view. Photographs generated by the camera were transferred to a computer and evaluated with Tool image J software. Histological analysis of expressed macrophage cells was counted with a kidney-shaped image having a purple oval nucleus with dark visible cytoplasm and containing small red vacuoles, then tabulated and data analyzed.

**Statistical Analysis**

MegaStat V.10.4 release 3.2.4 Mac Statistical program software was used for the statistical analysis of this research data. The data normality test was carried out using the Chi-Kuadrat test, the data results were all normally distributed. The homogeneity of variance test used the Bartlett test, the results of the data all have homogeneous variance. Data were analyzed using One Way Anova analysis followed by Post hoc t-test to compare the mean of each experimental group with the control group. The difference of statistical significance was determined if the p value < 0.05. If it does not meet the parametric test, a non-parametric test with the Kruskal Wallis Test is carried out followed by the Maan-Whitney test analysis to analyze the difference in expression between groups. The data results are shown as the mean ± standard deviation (SD).

**Results**

All Sprague Dawley rats (n = 45), in a safe condition and without complications after tooth extraction. Differences in the expression of TNF-α mRNA, IL-1β mRNA and macrophages were measured and compared between the experimental group (IHH and normoxia) and the control group. The findings in each group were evaluated and calculated on days 0,1,3,5,7 after tooth extraction. TNF-α is a proinflammatory cytokine produced by macrophages, and plays an important role in inflammation and the immune response involved in the wound healing process. TNF-α mRNA expression was detected in all IHH and normoxia experimental groups and the control group on days 0,1,3,5,7 after tooth extraction.

| TNF-α | day 0 | day 1 | days 3 | days 5 | days 7 |
|---|---|---|---|---|---|
| Control | 1,0 | | | | |
| IHH | | 3,420 ± 0,179*** | 5,719 ± 0,330*** | 1,121 ± 0,344 | 0,866 ± 0,064 |
| Normoxia | | 2,689 ± 0,472** | 3,404 ± 0,521*** | 1,416 ± 0,867 | 1,209 ± 1,303 |

**Table 1.** Differences in the expression of TNF-α mRNA (total n=45) after exposure to IHH, normoxia, and Control.
Values presented are mean ± SD
Significantly different compared to the control group (*p<0.05, One Way Anova Test).

The results of the One Way Anova test showed a significant difference p=0.000000000006 (*** p<0.05), indicating that there was an effect of IHH exposure on changes in TNF-α mRNA expression in the socket after tooth extraction. Post hoc t-test was then performed to test whether there were differences

between each group. Post hoc t-test results showed differences in IL-1β mRNA expression after one exposure to HH (group P1) and IHH exposure (groups P2, P3 and P4) and the normoxia group (group K1, K2, K3, and K4) compared to the group Control (K0), data results are shown as mean ± SD, in Table 1.

In the IHH group, the expression of TNF-α mRNA after one time exposure to HH on day 1 (group P1) showed a very significant increase p=0.00001 (***p<0.05) compared to the control group, then after 3 exposure to HH on day 3 (group P2) increased very significantly p=0.00000000001 (***p<0.05) compared to the control group, then after 5 exposures to HH on day 5 (group P3) the decrease approached the control group with a value of p=0.8028, likewise after 7 exposures to HH on day 7 (group P4) it decreased below the control group with a value of p=0.7823, this indicated that the two groups P3 and P4 did not significantly different from the control group. In the normoxia group, TNF-α mRNA expression was also found in group K1 (day 1) there was a very significant increase p=0.0012 (**p<0.05) and in group K2 (day 3) it increased very significantly p =0.00002 (***p<0.05) compared to the control group, whereas in group K3 (day 5) there was a decrease with a value of p=0.3933 and group K4 (day 7) decreased with a value of p=0.6678 but still above the control group, indicating that both K3 and K4 groups were not significantly different from the control group, shown in Figure 2.

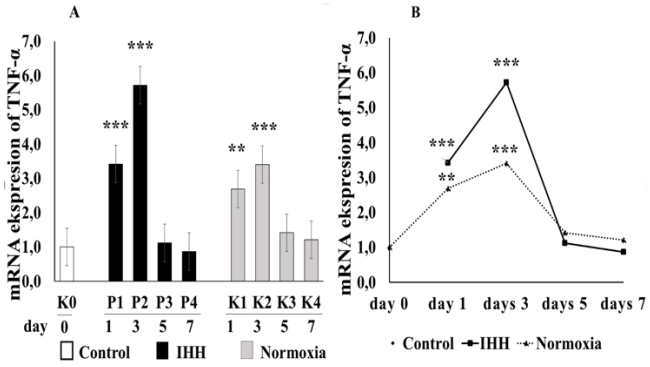


**Figure 2**. Expression of TNF-α mRNA in the socket post-tooth extraction after exposure to intermittent hypobaric hypoxia (IHH) and the normoxia group compared to the control group. **A**: Group K0 (control, normoxia, day 0), hypobaric hypoxic group namely group P1 (one time exposure to HH, day 1), group P2 (three times exposures to HH, day 3), group P3 (five times exposure to HH, day 5), group P4 (seven times exposure to HH, day 7), and normoxic group namely group K1 (normoksia, day 1), group K2 (normoxia, day 3), group K3 (normoxia, day 5), and group K4 (normoxia, day 7). **B**: Differences in TNF-α mRNA expression after exposure to intermittent hypobaric hypoxia and the normoxia group with the control group on days 0,1,3,5, and 7. Significantly different compared to the control group (*p<0.05, One Way Anova Test).

Expression of IL-1β mRNA in sockets after tooth extraction was also measured in this study. IL-1β and TNF-α are two main pro-inflammatory cytokines that work synergistically to strengthen the inflammatory response and have the effect of accelerating wound healing. IL-1β mRNA expression was detected in the entire IHH and normoxia experimental group as well as the control group on day 0,1,3,5,7 post-tooth extraction. The results of the One Way Anova test obtained a significant difference of p = 0.000000002 (***p<0.05), showing the influence of IHH exposure on changes in the expression of IL-1β mRNA in sockets after tooth extraction, then a Post hoc t-test was carried out to test whether there were differences between each group. Post hoc t-test results showed differences in IL-1β mRNA expression after one time exposure to HH (P1 group) and IHH exposure (P2, P3 and P4 groups) as well as normoxia groups (K1, K2, K3, and K4 groups) compared to the Control group (K0), the results are shown in Table 2.

| IL-1β | day 0 | day 1 | days 3 | days 5 | days 7 |
|---|---|---|---|---|---|
| Control | 1,0 | | | | |
| IHH | | 4,004 ± 0,674*** | 2,451 ± 0,101*** | 1,028 ± 0,222 | 0,621 ± 0,164 |
| Normoxia | | 2,069 ± 0,539** | 1,616 ± 0,753 | 1,328 ± 0,645 | 1,212 ± 0,412 |

**Table 2.** Differences in the expression of IL-1β mRNA (total n= 45) after exposure to IHH, normoxia, and Control.
Values presented are mean ± SD
Significantly different compared to the control group (*p<0.05, One Way Anova Test).

We found in the IHH group, that IL-1β mRNA expression in the one time HH exposure group (group P1, day 1) increased significantly p=0.000000002 (***p<0.05) compared to the control group, then after 3 time exposures to HH

(group P2, days 3) it still increased very significantly p=0.0005 (***p<0.05) compared to the control group, but there was a decrease compared to group P1, whereas in the group after 5 time exposures to HH (group P3, day 5) there was a decrease approaching the control group but not significantly different from the value of p=0.9411, likewise in the group after 7 time exposures to HH (group P4, days 7) there was a further decrease below the control group with a value of p=0.3211, but not significantly different from the control group. In this study we also found in the normoxia group, that IL-1β mRNA expression on day 1 post-tooth extraction (group K1) increased significantly p=0.0074 (**p<0.05) compared to the control group, then on days 3 post-tooth extraction (group K2) there was a decrease but not significantly different from the control group with a value of p = 0.1103, and on days 5 (group K3) there was a decrease approaching the control point with a value of p=0.3893 , likewise on days 7 (group K4) it decreased, but was still above the control group with a value of p=0.5772, indicating that on days 3, 5 and 7 it did not differ significantly from the control group, the results are shown in Figure 3.

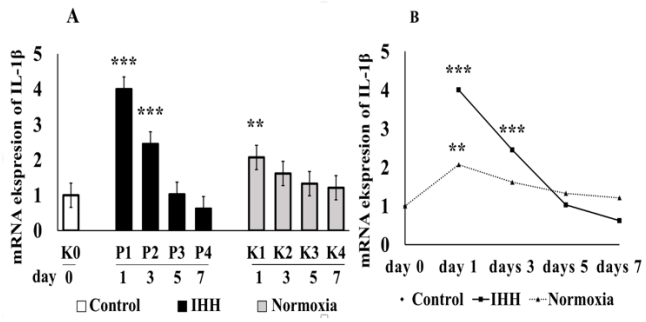


**Figure 3**. Expression of IL-1β mRNA in the socket post-tooth extraction after exposure to intermittent hypobaric hypoxia (IHH) and the normoxia group compared to the control group. **A**: Group K0 (control, normoxia, day 0), hypobaric hypoxic group namely group P1 (one time exposure to HH, day 1), group P2 (three times exposures to HH, day 3), group P3 (five times exposure to HH, day 5), group P4 (seven times exposure to HH, day 7), and normoxic group namely group K1 (normoksia, day 1), group K2 (normoxia, day 3), group K3 (normoxia, day 5), and group K4 (normoxia, day 7). **B**: Differences in IL-1β mRNA expression after exposure to intermittent hypobaric hypoxia and the normoxia group with the control group on days 0,1,3,5, and 7. Significantly different compared to the control group (*p<0.05, One Way Anova Test).

The number of macrophages in the socket post tooth extraction after exposure to hypobaric hypoxia was also measured in this study on days 0,1,3,5,7. Macrophages are mononuclear phagocytic cells that can activate proinflammatory cytokines and growth factors which play an important role in the wound healing process. Macrophages were detected in all IHH and normoxia experimental groups as well as the control group. The results of the Kruskal Wallis Test showed a very significant difference p=0.00064 (***p<0.05), indicating that there was an effect of IHH exposure on changes in the number of macrophages in the socket post tooth extraction, then the Maan-Whitney test was performed to test whether there are differences between each group. The results of the Maan Whitney test showed that there was a difference in the number of macrophages after HH exposure (group P1) and IHH exposure (groups P2, P3 and P4) and the normoxic group (groups K1, K2, K3 and K4) compared to the control group (K0),the results are shown in Figure 4.

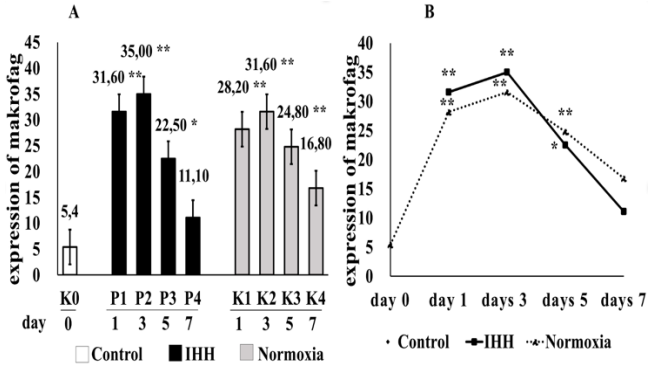


**Figure 4**. The number of macrophages in the socket post-tooth extraction after exposure to intermittent hypobaric hypoxia (IHH) and the normoxia group compared to the control group. **A**: Group K0 (control, normoxia, day 0), hypobaric hypoxic group namely group P1 (one time exposure to HH, day 1), group P2 (three times exposures to HH, day 3), group P3 (five times exposure to HH, day 5), group P4 (seven times exposure to HH, day 7), and normoxic group namely group K1 (normoksia, day 1), group K2 (normoxia, day 3), group K3 (normoxia, day 5), and group K4 (normoxia, day 7). **B**: Differences in the number of macrophages after exposure to intermittent hypobaric hypoxia and

the normoxia group with the control group on days 0,1,3,5, and 7. Significantly different compared to the control group (*p<0.05, One Way Anova Test).

We found the number of macrophages in the IHH group, after one time exposure to HH on day 1 (group P1) there was a very significant increase p=0.0039 (**p<0.05) compared to the control group, then it increased very significantly p=0.0053 (**p<0.05) after three time exposures to HH on day 3 (group P2) compared to the control group, whereas after 5 time exposures to HH on day 5 (group P3) a decrease began to occur the number of macrophages compared to the P2 group but still increased significantly with a value of p=0.0185 (*p <0.05) compared to the control group, then after 7 time exposures to HH on day 7 (group P4) it decreased to the control group, but not significantly different from the value of p=0.0933 compared to the control group. This shows that there were significant differences on days 1, 3 and 5 between the IHH treatment group and the control group.

This study also found the number of macrophages after tooth extraction in the normoxia group, on day 1 (group K1) there was a significant increase p=0.0053 (**p<0.05) compared to the control group, then on day 3 ( group K2) there was a very significant increase p=0.0062 (**p<0.05) compared to the control group, whereas on day 5 (group K3) there was a significant decrease with a value of p=0.0014(** p <0.05) close to the control group, then on day 7 (group K4) there was a decrease that was not significantly different from the value of p=0.1088 and was still above the control group.

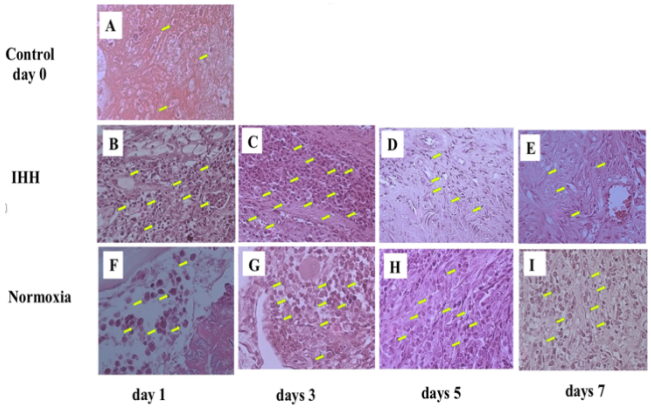


**Figure 5.** Histological test of the number of macrophages in the socket post-tooth extraction after exposure to intermittent hypobaric hypoxia (IHH). (A). Control group (normoxia, day 0), IHH group, namely: (B) one time exposure to HH group, day 1, (C) three times exposure to HH group, day 3, (D) five times exposures to HH group, day 5, (E) seven times exposures to HH group, day 7. The normoxia group, namely: (F) the normoxia group, day 1, (G) normoxia group, day 3, (H) normoxia group, day 5, (I) normoxia group, day 7. Magnification 400X, yellow arrows indicate macrophages. (hematoxylin and eosin [H&E]).

In this study, the histological results of the number of macrophages in the post-extraction socket in the IHH group, the normoxic group, and the control group on days 0, 1, 3, 5, and 7, and stained with hematoxylin and eosin [H&E]), have started to show a lot in day 1, and increased reaching a peak on day 3, then there was a reduction in the number of macrophages on day 5, and decreased on day 7, shown in Figure 5.

## Discussion

In the present study, we explore the direct impact of IHH exposure on post-extraction rats. Flying at an altitude of 18,000 feet (8,330 m) for 30 minutes 1 time, 3 times, 5 times and 7 times exposure to HH on days 1,3,5 and 7 after tooth extraction, we found molecular changes in the socket after tooth extraction in a state of hypobaric hypoxia as a regulator of wound healing. Flying at high altitudes hypobaric hypoxia can activate several target genes in response to hypoxia. Hypoxic wound conditions can cause monocytes circulating to the site of injury to become macrophages releasing inflammatory cytokines such as TNF-α and IL-1β which act synergistically to strengthen the inflammatory response and play an important role in protecting the wound from infection.[12,34]

The results of this study showed that there were significant differences in the expression of TNF-α and IL-1β mRNA in the post-extraction socket after intermittent hypobaric hypoxia exposure on days 1, 3, 5, and 7 after tooth extraction compared to the control group. It was found in this study, there was a very significant increase in the expression of TNF-α mRNA and IL-1β mRNA in the socket after tooth extraction on day 1 after one exposure to hypobaric hypoxia, illustrating that acute hypobaric hypoxic conditions with low oxygen levels lead to

increased expression of TNF-α mRNA and IL-1β mRNA. In this study, it was also found that after giving exposure to IHH there was a very significant increase in TNF-α mRNA expression, the highest after 3 times exposures to HH on day 3, while IL-1β mRNA expression experienced the highest very significant increase after 1 time exposures to HH on day 1, then began to decrease after 3 times exposures to HH on day 3, suggesting that IL-1β is involved in the acute immune response induced by bleeding after tooth extraction. After exposure to hypobaric hypoxia significantly increased TNF-α and IL-1β levels in human plasma and rat brain cortex.[24] Hypoxia can cause platelets and monocytes to release inflammatory cytokines and growth factors that affect wound healing cells.[5,34] TNF-α and IL-1β are pro-inflammatory cytokines produced by macrophages in the initial wound, and play an important role in the inflammatory and immune response in wound healing.[8,35] The expression of TNF-α mRNA and IL-1β mRNA, in this study decreased to that of the control group after 5 exposures to HH on day 5, then decreased further below the control group after 7 exposures to HH on day 7. This suggests that under conditions of intermittent hypobaric hypoxia, there is gradual systemic adaptation at the cellular level in the cells and tissue of the socket wound after tooth extraction, so that it can suppress pro-inflammatory cytokines produced by macrophages in the post-extraction socket, then these inflammatory cytokines are immediately regulated and decrease on day 7 after tooth extraction. Repeated or intermittent exposure to hypobaric hypoxic conditions induces a protective response, possibly adapting cells to hypoxia.[26,36]

We also found expression of TNF-α mRNA and IL-1β mRNA in normoxia without exposure to hypobaric hypoxia on days 1, 3, 5, and 7 after tooth extraction, showing significant differences compared to the control group. The expression of TNF-α mRNA began to increase on day 1 after tooth extraction, and was highest on day 3 after tooth extraction, while IL-1β mRNA expression increased highest on day 1 after tooth extraction, then began to decrease on day 3, demonstrating that IL-1β is also involved in the acute immune response induced by bleeding after tooth extraction under normoxic conditions. On days 5 and 7 after tooth extraction, there was a decrease in the expression of TNF-α and IL-1β mRNA, but still above the control group. These results illustrate that in the initial wound tissue hypoxia occurs, resulting in the release of a number of signaling factors such as migration of inflammatory cells to the wound and differentiation of monocytes into macrophages, then macrophages release proinflammatory cytokines which play an important role in the inflammatory process. Early wound epithelialization begins and is first stimulated by the inflammatory cytokines IL-1 and TNF-α which increase the expression of keratinocyte growth factor genes in fibroblasts.[9] The pro-inflammatory cytokine IL-1β is produced in abundance in the inflamed area of the socket after tooth extraction and there is a decrease in IL-1β expression during the formation of osteoblast cells in alveolar bone regeneration.[37] Previous researchers stated that, three hours after tooth extraction, the levels of most inflammatory cytokines such as TNF-α showed a slight increase compared to controls, while IL-1β expression increased dramatically, then decreased 1 week after tooth extraction.[38] Another study also stated that the expression of TNF-α was not significantly different from the control group after 7 days of tooth extraction during the initial wound healing of the rat tooth socket.[39] In line with this study, the expression of TNF-α mRNA and IL-1β mRNA in the normoxia group on days 5 and 7 after tooth extraction decreased, but in this study it was found to be still above the control group. The expression of TNF-α mRNA, IL-1β mRNA and macrophages on day 7, when compared between the normoxia group and the IHH group, it was found that the normoxia group was still higher than the control group, while the IHH group was below the control group and the normoxia group, this situation illustrates that exposure to IHH after 5 and 7 times exposure to HH on day 5 and day 7 will accelerate these pro-inflammatory cytokines to be regulated immediately and there will be a decrease in TNF-α and IL-1β on day 7 after tooth extraction, thereby accelerating the reduction of inflammation in the wound and entering the healing phase.

The results of histology analysis revealed that the number of macrophages in the socket post tooth extraction showed that the IHH group and the normoxia group began to increase on day 1 and reached the highest peak on day 3, then began to decline on day 5 and further decreased

on day 7, but there were differences between the IHH group with the normoxia group and the control group. On day 1 and days 3, the IHH group after one time and three times exposure to HH, there was a very significant increase in the number of macrophages and a higher increase than the control group and the normoxia group, illustrating that at the beginning of the wound there was already tissue hypoxia plus acute HH exposure with low oxygen levels causes macrophage cells to be activated to a higher degree, resulting in an increase in the number of macrophages higher than the normoxia group and the control group. On days 5 and 7, the IHH group after five times and seven times exposure of HH, there was a decrease in the number of macrophages faster approaching the control group than the normoxia group, illustrating that under intermittent hypobaric hypoxia conditions there is a gradual systemic adaptation at the cellular level in cells and tissues of the socket wound after tooth extraction, so that macrophage cells are activated to a lesser degree.Repeated or intermittent exposure to hypobaric hypoxic conditions induces a protective response, can make cells adapt to hypoxia.[26]

Several inflammatory cytokines and inflammatory processes, such as migration of inflammatory cells and differentiation of monocytes into macrophages, are modulated by HIF-1α under hypoxic conditions.[11] In addition, the cytokine TNF-α produced by macrophages stabilizes and enhances HIF-1α activity, resulting in a positive feedback loop leading to further differentiation of monocytes into macrophages, so that the relationship between hypoxia-induced factors, such as HIF-1α, and inflammatory pathways in macrophages is strengthened.[16,40]After 24 hours under hypobaric hypoxia, cells developed increased HIF-1α intracellular compared with baseline, indicating that hypobaric hypoxic exposure is sufficient to induce the hypoxic-inflammatory pathway.[41] HIF-1α expression increases significantly under hypobaric hypoxic conditions and returns to normal levels after intermittent hypobaric hypoxia exposure, causing cells to adapt to intermittent hypobaric hypoxic conditions.[26,29,42] This increase in macrophage numbers was in line with increased TNF-α mRNA expression and mRNA IL-1β, illustrates that inflammatory cytokines such as TNF-α and IL-1β in hypoxic conditions regulated by macrophages. TNF-α and IL-1 are two major inflammatory cytokines that generally work synergistically to amplify the inflammatory response during wound repair expressed by macrophages.[43] Macrophages begin to disappear from the wound during the resolution of the inflammatory phase of repair, some disappear in the wound and others tend to migrate to the wound. lymph nodes that dry up, then fibroblasts and endothelial cells appear towards the proliferative phase.[12]

## Conclussions

Under the limitations of the present study, we concluded that flying at high altitudes has an impact on changes in mRNA expression and tissue in response to hypobaric hypoxia involved in the wound healing process after tooth extraction. This was evidenced by an increase in the expression of TNF-α mRNA and IL-1β mRNA which was in line with the increase in the number of macrophages under hypobaric hypoxic conditions, as well as a gradual mechanism of cell adaptation under intermittent hypobaric hypoxia conditions in socket tissue after tooth extraction, as proven by a decrease in the proinflammatory cytokine IL-1β and TNF-α and the rapid incline of macrophages, that may reduce inflammation and accelerate wound healing. However, further study is needed to clarify the effect of IHH on tooth extraction socket healing by administering more than seven times exposures to HH.

## Declaration of Interest

The author declares no conflict of interest.

## Acknowledgments

The author would like to thank the Team Aerospace Medicine of the Aviation and Space Health Institute (Lakerspra) dr.Saryanto, Indonesian Air Force, for providing hypobaric chamber facilities, Team of the Integrated Research Laboratory, Dental Faculty, Universitas Padjadjaran, Bandung, Indonesia, Team of the Molecular Genetics Laboratory, Faculty of Medicine, Universitas Padjadjaran, Bandung, Indonesia, Team of the Veterinary Teaching Hospital, IPB University, Bogor, Indonesia, which has provided laboratory facilities, and Center of Military Dentistry Research, Universitas Padjadjaran.

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