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# Effects of Hyperbaric Oxygen Therapy on the Increase of iNOS and NFKB Expressions and the Acceleration of Wound Healing Process during Inflammation and Proliferation Phases

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## ABSTRACT

Wound is one of the most common causes of morbidity in developing countries. Hyperbaric Oxygen Therapy (HBO), which can improve tissue oxygenation and stimulate the formation of H2O2 as a secondary messenger to Nuclear Factor Kappa Beta (NFKB) Phosporylation, has increasingly been used in wound treatment. Principal mechanism of HBO is based on intracellular generation of Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS), including Nitric Oxide (NO). In the wound area, moreover, the great amount of NO is mostly generated by the enzyme inducible Nitric Oxide Synthase (iNOS) through NFKB pathway. The beneficial effects of NO in wound repair may be attributed to its functional influences on angiogenesis, inflammation, cell proliferation, matrix deposition and remodeling.Accordingly, this research aims to determine whether Hyperbaric Oxygen Therapy (HBO) can improve iNOS and NFKB expressions and wound healing process. This research used a 'Randomized control group pre test-post test design' with twenty-eight wistar rats. These rats were divided into 4 groups (random allocation), namely treatment group 1 receiving 5 sessions of HBO theraphy, treatment group 2 receiving 10 sessions of HBO theraphy, and two control groups, each of which receives the same number of session as each of the two treatment groups yet without HBO theraphy. The result showed Hyperbaric oxygen therapy can improve iNOS expression (p=0.001), NFKB expression (p=0.04) and wound healing process (p=0.002) significantly in the treatment group receiving the five sesions of HBO theraphy at 2.4 ATA for 3x30 minutes. On the other hand, in the treatment group receiving the ten-session HBO theraphy, only iNOS expression (p=0.002) and NFKB expression (p=0.03) were significantly increased, while wound healing process cannot be improved. Therefore, further researches are required to focus on both the repetition time interval after provision of five-session hyperbaric oxygen therapy (during recovery period) and the use of HBO as adjuvant therapy for various types of wounds.
**KEYWORDS**: HBO, iNOS, NO, NFKB, wound healing

## INTRODUCTION

Wound healing process is a normal process with a highly complex mechanism. This process occurs progressively through *overlapping* phases, namely hemostasis phase, inflammatory phase, proliferative phase, contraction phase, and *tissue remodeling* phase [1, 2, 3]. The healing process can end with fibrosis or scar tissue even under optimal condition, thus resulting in multiple disabilities [4]. Acute wounds actually can turn into chronic wounds. The problem of chronic wounds now poses a challenge for modern society due its significant medical, economic, and social impacts.

Chronic wound is one of the most common causes of morbidity in developing countries [4, 5, 6]. It is estimated that 1.5% of the population suffers injuries at any point in time [7]. Laceration/wound is very often encountered daily (estimated at 20 million cases each year) as a result of being cut. Meanwhile, post-operative wound is the biggest cause of skin trauma. Overall, there are more than 110 million surgical incisions every year, and not all can recover completely due to various causes. The wound is then developed into chronic wounds [8]. Hence, the size of the population and the burden of costs require serious attention on early detection, prevention, diagnosis, and treatment of chronic wounds [4].

In addition, hyperbaric oxygen therapy as adjuvant therapy in wound healing will result in an increase in RNS, mainly NO through iNOS and also an increase in ROS, especially H2O2. Excessive doses of RNS and ROS, however, are a threat to the survival of the cell, while HBO theraphy at the therapeutic doses (2.4 ATA) will provide positive benefits for wound healing [2, 6]. In clinical use, HBO theraphy is restricted from 2-2.5

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ATA to a maximum of 3 ATA for 60-120 minutes. HBO therapy actually varies between 5-30 times [3, 5, 9, 10, 11]. The provision of 100% oxygen at a pressure of 2 ATA can improve tissue oxygenation from 30-40 mmHg in normal circumstances to 250-300 mmHg. Meanwhile, the provision of 100% oxygen at a pressure of 3 ATA can improve tissue oxygenation 10-15 times [5].

Hyperbaric oxygen therapy, moreover, not only increases tissue oxygenation, but also stimulates the formation of H2O2 as a *secondary messenger* for the phosphorylation of *Nuclear Factor Kappa Beta* (NF-kB). *Nuclear Factor Kappa Beta / Rel Proteins* play an important role in quick transcription process on a wide variety of genes in response to extracellular stimuli. Many aspects of the inflammatory response is also governed by NF-kB, including the production of *serum amyloid A* (SAA), angiotensinogen, *complement cascade* factors, and *proinflammatory cytokines* induction, such as IL1 and TNF α. *Nuclear Factor Kappa Beta* also sets the advanced phase of the inflammatory response by mediating the induction of *genes encoding leukocyte adhesion molecule* (*E-Selectin*, VCAM-1 and ICAM-1) which is expressed by vascular endothelial cells as a response to proinflammatory cytokines and bacterial lipopolysaccharide (LPS) [12]. Besides, the angiogenic response of intact venules where blood can freely flow to the injured area is greatly increased by hyperoxia [11].

Hyperbaric oxygen therapy, consequently, can suppress the inflammatory response that may prevent chronic wounds from occurring [13]. Supplementary oxygen through the blood stream will accelerate angiogenesis in ischemic wound, thus accelerating the healing time [5]. The results of our previous studies showed that a five-session provision of HBO theraphy at 2.4 ATA for 3x30 minutes can significantly increase the expression of iNOS ($p = 0.001$) and wound healing process ($p = 0.002$) for five days in a row. On the other hand, the ten-session provision of HBO theraphy at 2.4 ATA for 3x30 minutes can significantly enhance the expression of iNOS ($p = 0.002$) for 10 days in a row, yet fail to significantly improve wound healing process ($p = 0.3$). Accordingly, this research aims to determine whether Hyperbaric Oxygen Therapy (HBO) can improve iNOS and NFKB expressions and wound healing process during inflammatory and proliferative phases.

## METHODS

This research used a 'Randomized control group pre test-post test design' with twenty eight wistar rats. They were divided into four groups (random allocation), each consisting of seven mice. Treatment group 1 (KP1) received 5 sessions of HBO theraphy at 2.4 ATA for 3x30 minutes. Treatment group 2 (KP2) received 10 sessions of HBO theraphy at 2.4 ATA for 3x30 minutes. Meanwhile, two control groups (KK1 and KK2), each of which receives the same number of session as each of the two treatment groups yet without HBO theraphy.

First, those twenty-eight rats were given a full-thickness excisional wound sized 1x1cm. Second, immunohistochemical examination was conducted to measure the expressions of NFkB and iNOS [12]. Third, the size of the wound was also measured to determine the progress of the wound healing process. The immunohistochemical examination and the measurement of the size of the wound in treatment group 1 (KP1) and control group 1 (KK1) were conducted on day 0 (pre-HBO theraphy) and day 5 (during HBO theraphy). Meanwhile, the immunohistochemical examination and the measurement of the size of the wound in treatment group 2 (KP2) and control group 2 (KK2) were conducted on day 0 (pre-HBO theraphy) and day 10 (during HBO theraphy).

Fourth, normality test was conducted using Shapiro Wilks test [12]. The results of the test showed that the data were not normally distributed. Fifth, the expressions of iNOS and NFkB and the progress of the wound healing process in the treatment groups were compared to those in the control groups using Mann Whitney U to measure the changing/delta variables and to compare variables of iNOS and NFkB expressions as well as wound healing process before and after HBO theraphy with 1-sample Wilcoxon test. Finally, the correlation between the increasing of iNOS and NFkB expressions and the improving of wound healing process was analyzed using Spearman rank correlation test.

## RESULTS AND DISCUSSION

This research used twenty-eight samples divided into four groups, each of which consisted of seven samples. Those four groups were treatment group 1 treated with HBO therapy at 2.4 ATA for 3x30 minutes in five sessions (KP1), treatment group 2 treated with HBO therapy at 2.4 ATA for 3x30 minutes in ten sessions (KP2), control group 1 treated without HBO therapy at 2.4 ATA for 3x30 minutes in five sessions (KK1), and control group 2 treated without HBO therapy at 2.4 ATA for 3x30 minutes in ten sessions (KK2). On day 0, the mean and standard deviation of variables of iNOS and NFkB expressions before HBO theraphy (pre HBO) in those two treatment groups were the same as those in those two control groups. The significant level of the five-session theraphy groups before HBO theraphy (pre HBO) was 0.654 ($p>0.05$), while the significant level of the ten-session theraphy groups before HBO theraphy (pre HBO) were 0.798 ($p>0.05$). The data obtained were then analyzed with

Mann Whitney U test. Similarly, the size of the wound area before HBO theraphy (pre HBO) in those two treatment groups were the same as those in those two control groups, approximately 1x1cm (100 $mm^2$).

Moreover, the number of iNOS and NFkB expressions before the HBO theraphy (pre-HBO on Day 0 in skin preparations was small. In unwounded skin, the number of iNOS and NFkB expressions was small with mild staining in the epithelial cells, endothelial cells, and smooth muscle. However, in wounded skin, the number of iNOS and NFkB expressions was high. iNOS was expressed by macrophages, fibroblasts, and PMN. All of these cells have a specific function in the complex process of wound healing, and NO acts as a mediator. PMN and macrophages are required for the debridement of the wound area. Besides, those cells can attract other cells needed in the wound healing process. Neutrophils in humans create NO, regulating migration. In addition, fibroblasts are responsible for the formation of collagen in granulation tissue, which is essential for tissue repair. *Nitric oxide* regulates synthesis of collagen in fibroblasts in the wound area. Semiquantitative analysis shows that the expression of iNOS cells is significantly more positive and in wounded skin than that in unwounded skin [1].

Subsequently, HBO theraphy at 2.4 ATA was administered for 3x30 minutes to the treatment group 1 for five sessions. The expressions of iNOS and NFkB as well as the size of the wound in treatment group 1 (KP1) and control group 1 (KK1) then were examined. Afterwards, the results of the analysis were tested with Mann Whitney U test. The test results showed that there were significant differences of iNOS expression between treatment group 1 and control group 1 with a significant level of $p = 0.02$ ($p<0.05$). Similarly, the size of wound in treatment group 1 was also significantly different from control group 1 with a significant level of $p = 0.02$ ($p<0.05$).

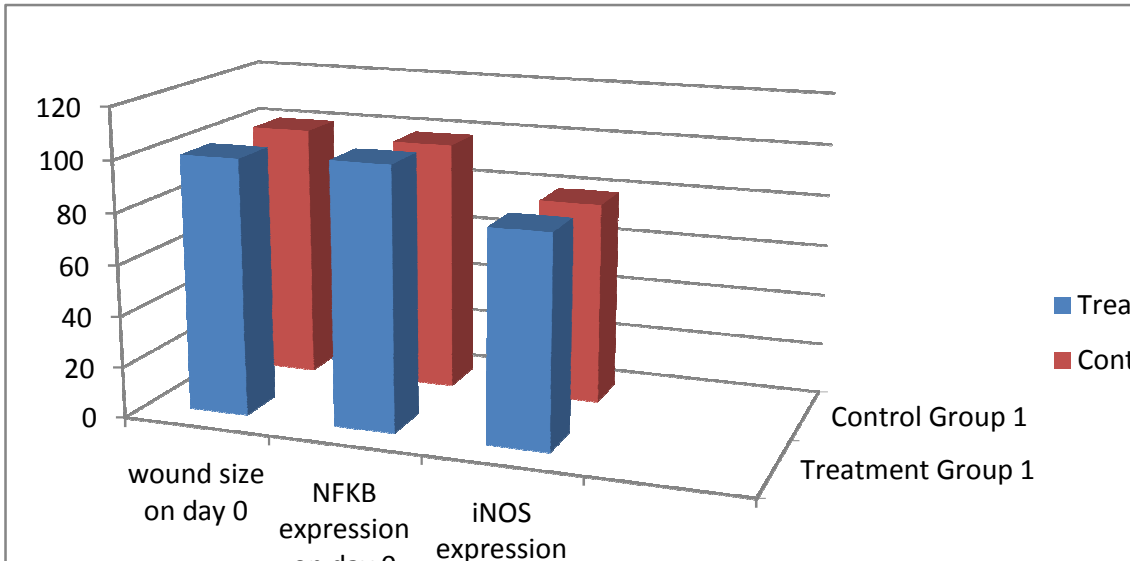


**Figure 1. Data of Wound Size, NFkB Expressions, and iNOS Expressions on day 0**

The result of the examination showed that the expression of iNOS in treatment group 1 treated with the five sessions of HBO theraphy (KP1) was higher than control group 1 (KK1). It also revealedthat there was a strong and direct correlation between iNOS expression and wound healing process.

Furthermore, inducible NOS produces NO which has a crucial role in angiogenesis, inflammation, proliferation, as well as collagen tissue and extracellular matrix formation during the wound healing process. Activation of nuclear factor kappa β (NF-KB) with lipopolysaccharide can increase iNOS expression, NO production, prostaglandin E2 production, collagen formation, and wound area healed. Nuclear factor kappa β (NF-KB) can also stimulate the expression of genes that mediate immune response, stress reponse, cell growth, and cell survival. Thus, the activation of NF-KB and $PGE_2$ pathways can be associated with *cross talk* between iNOS and NO expressions in the production of fibroblast collagen and wound healing process.

In addition, the result of the healed-wound size examination showed that the size of wound healed in treatment group 1 receiving five sessions of HBO theraphy (KP1) was higher than in control group 1 (KK1). Next, the correlation between iNOS expression and wound healing process was analyzed using Spearman rank correlation test. The results showed that there was a significant and positive correlation between iNOS expression and wound healing process on day 5 with $p = 0.02$ ($p<0.05$) and with the strength of the correlation approximately 0.749 ($r> 0.5$-$0.75$).

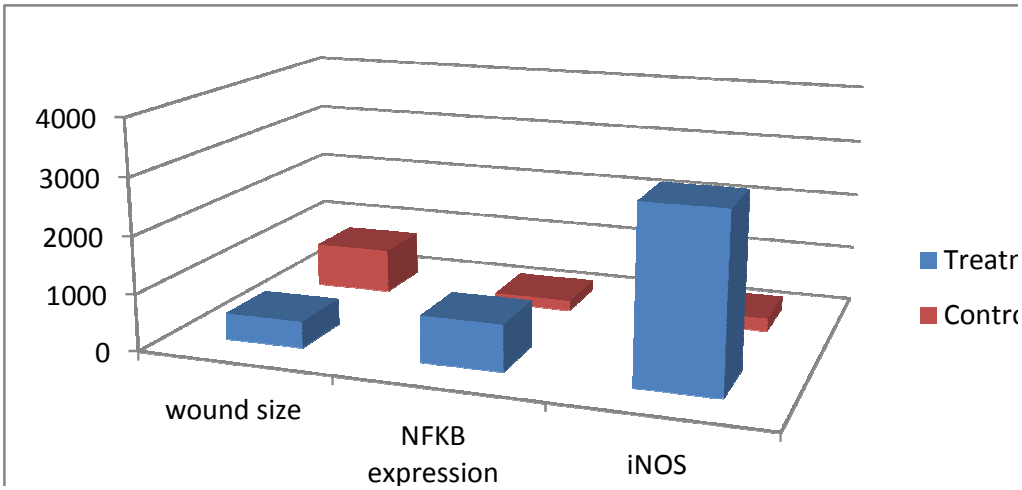


**Figure 2. Data of Wound Size, NFkB Expression, and iNOS Expression on day 5**

Barriers of iNOS activity will inhibit angiogenesis, inflammatory process, collagen production, extracellular matrix formation, wound healing process, and collagen deposition, causing fibrosis. Barriers of iNOS activity will also inhibit wound reepithelialization. Fibroblasts in the wound healing process are phenotypically characterized by the changing in the characteristics of collagen production, proliferation, and cell migration. The cells of the scar tissue can increase the expression of iNOS and the production of NO.

As a matter of fact, oxygen is an important factor in every stage of the wound healing process. In the inflammatory phase, oxygen can even regulate the migration and proliferation of fibroblasts. In acute wounds, control of bacterial infections can be conducted through the mechanism of intracellular oxidative leukocytes. Consequently, molecular oxygen is necessarily required for the production of oxidants. The success of wound healing process, moreover, requires infection prevention. Resistance to infection depends on the level of oxygen in the wound area due to the fact that neutrophils become ineffective in hypoxic conditions. In the phase of proliferation, angiogenesis requires oxygen. In the remodeling phase, the production of collagen and fibroblasts is also highly dependent on oxygen.

Hypoxia, therefore, can impede the wound healing process by inhibiting fibroblast proliferation, collagen production, and capillary angiogenesis as well as increasing the risk of infection [7]. Therefore, the positive effects of HBO therapy on wound healing process is based on the benefits of increased tissue oxygenation pressure, consisted of fixing ischemia, reducing edema, modulating NO production, modifying effects of *growth factors* and cytokines, stimulating cell proliferation, accelerating collagen deposition, stimulating the formation of new capillaries, accelerating *microbial oxidative killing*, inhibiting bacterial proliferation, modulating immune system response, and repairing *ischemia reperfusion injury*. Furthermore, neovascularization holds a central function in wound healing process. In vitro studies indicate that VEGF, the most potent angiogenic protein, is induced by NO. Barriers, as a result, will disrupt NO synthesis of VEGF expression and angiogenesis, which eventually inhibits the wound healing process [5].

In the meantime, the provision of HBO theraphy at 2.4 ATA for 3x30 minutes in ten sessions was conducted in the treatment group 2 (KP2). Next, the expressions of iNOS and NFkB as well as the size of the wound healed in treatment group 2 (KP2) were examined and compared to control group 2 (KK2). The results were then analyzed using Mann Whitney U test. The results showed that there was a significant difference in iNOS expression between treatment group 2 (KP2) and control group 2 (KK2) with a significant level of $p = 0.002$ ($p<0.05$). Meanwhile, there was no significant difference in the size of the wound healed between treatment group 2 (KP2) and control group 2 (KK2) with a significant level of $p = 0.300$ ($p>0.05$).

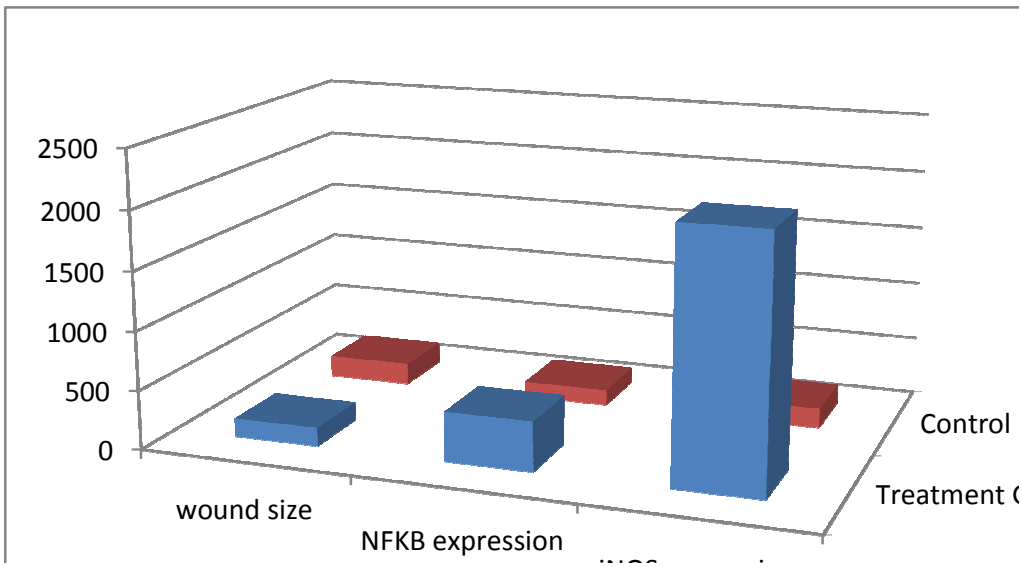


**Figure 3. Data of Wound Size, NFkB Expression, and iNOS Expression on day 10**

The result of the examination showed that the expression of iNOS in treatment group 2 receiving ten sessions of HBO theraphy (KP2) was higher than that in control group 2 (KK2). However, the expressions of iNOS and NFkB on day 10 was lower than on day 5 since on day 10 the peak of inflammatory phase had passed. As a result, negative feedback to the regulation of iNOS and NFkB expressionsstarted to prevent excessive inflammation.

In addition, the result of the healed-wound size examination showed that there was no significant difference in the size of wound healed between treatment group 2 (KP2) and control group 2 (KK2). This is presumably because the wound had entered the phase of remodeling. Next, the correlation between iNOS expression and wound healing process was analyzed using Spearman rank correlation test. The results showed that there was a strong and positive correlation between iNOS expression and wound healing process on day 10 with $p = 0.02$ ($p<0.05$) and with the strength of the correlation around 0.748 ($r>0.5$-$0.75$).

On day 10, the expression of iNOS was significantly increased, yet the wound healing process was not significantly improved for several reasons as follow:

1. The autoregulation mechanism of iNOS through S-nitrosylation process
2. The existence of such an adaptative response through the inhibition of iNOS Carbon monoxide (CO) endogenously produced by Heme Oxygenases (HO). Carbon monoxide can act as an anti-inflammatory, anti-apoptotic and anti-proliferation. Endogenous carbon monoxide produces *negative feedback* on iNOS. Endogenous carbon monoxide inhibits formation of NO from L-Arginine through barriers to the expression of iNOS. The clinical features of the wound are not always in line with the quantitative molecular phenomena.

Hyperbaric Oxygen Therapy (HBOT) can increase in tissue oxygenation through hypoperfusion and infected wounds. Hyperbaric oxygen therapy can also stimulate collagen deposition, reepithelialization, angiogenesis, and bacterial clearance in the wound area through ROS and RNS. Reactive Nitrogen Species are composed of Nitric oxide (NO) and other materials that are formed in the reaction between NO and ROS or its oxidation product. Hyperbaric oxygen therapy, furthermore, can influence both of neovascularization processes, namely angiogenesis and vasculogenesis.

Based on the results of this research, there was a linear relation between the increase of iNOS expression and the wound healing process in the acute wounds. On day 5, the increase of iNOS expression even accelerated the wound healing process. Nitric Oxide (NO) is a signaling molecule that regulates a number of physiological effects. The release of NO by vascular cells plays an important role in the regulation of blood flow by inhibiting vascular tone, proliferation of smooth muscle cells, matrix deposition, platelet aggregation, and leukocyte adhesion. NO is also an important modulator, which functions as cell survivor with a tendency to be pro or anti-apoptotic aptosis, depending on concentration, delivery method (delivery method), cellular redox status and cell type. Proapoptosis activities of NO are mainly mediated by the loss of mitochondrial potential, the release of cytochrome-c, and the activation of caspase. The role of NO as cell protection involves binding of ROS, direct process of S-nitrosylation, as well as barriers to caspase and upregulation of sitoprotective genes.

Moreover, the results of this research also show that the five-session provision of hyperbaric oxygen therapy (HBO) at 2.4 ATA for 3x30 minutes (for 5 consecutive days) can increase iNOS and NFkB expression as

well as accelerate wound healing process significantly. Meanwhile, the ten-session provision of HBO theraphy at 2.4 ATA for 3x30 minutes can also increase iNOS and NFkB expressions, yet fail to accelerate wound healing process. HBO theraphy can accelerate wound healing process through mechanisms of iNOS and NFkB expressions in inflammatory and proliferative phases.In short, the provision of HBO theraphy can potentially be used for post-surgical adjuvant therapy in certain cases; consequently, it is expected to reduce the duration of treatment (Length of Stay/LOS) in hospital. HBO theraphy is also expected to reduce cost and recovery time for post-surgical patients so as to enable them to immediately return to their normal activities. However, further research is required on the effect of hyperbaric oxygen therapy against various types of wounds, including wounds that are conventionally difficult to cure, such as post-trauma wounds and post-surgery wounds.

In addition, the results of this research also show that the long-term provision of HBO do not guarantee significant benefits for wound healing process. Therefore, it is recommended that patients be given time off (recovery time) after the five-session provision of HBO theraphy before continuing with the next session. Further research is required to analyze the effect of the recovery period on the speed of wound healing process or the interval of recovery time before the next session of HBO therapy. Finally, it is also necessary to conduct further research with more dose variation (between 1-5 sessions and between 5-10 sessions) to obtain the optimal dose of hyperbaric oxygen therapy for acute wounds without triggering infection.

## CONCLUSION

In conclusion, the five-session provision of HBO theraphy at 2.4 ATA for 3x30 minutes successfully increases iNOS and NFkB expressions, as well as accelerates wound healing process. Meanwhile, the ten-session provision of HBO theraphy at 2.4 ATA for 3x30 minutes can also increase iNOS and NFkB expressions, yet unable to accelerate wound healing process. This research, moreover, shows that the effect of HBO therapy is to accelerate wound healing process through mechanisms of iNOS and NFkB expressions. Therefore, the provision of HBO theraphy can potentially be used for post-surgical adjuvant therapy for specific cases with the aim to shorten the duration of treatment in hospital and reduce cost and recovery time for patients after surgery, which eventually help patients to return to their normal activities shortly. Nevertheless, further research needs to be conducted to determine the repetition time interval after the provision of five-session hyperbaric oxygen therapy (during recovery period) as well as the use of HBO theraphy as an adjuvant therapy for various types of wound.

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