

Does hyperbaric oxygenation have a protective effect on random skin flaps? An immunohistochemical study of cellular apoptosis and vascular endothelial growth factor

Existe efeito protetor da oxigenação hiperbárica em retalhos cutâneos randômicos? Um estudo imuno-histoquímico de apoptose celular e fator de crescimento vascular endotelial

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ABSTRACT

Background: Hyperbaric oxygenation (HBO) therapy aims to increase oxygen tissue pressure by at least 10 times and decrease the adverse effects of ischemia. The aim of this study was to investigate the effect of HBO on the immunohistochemical expressions of caspase 3 and vascular endothelial growth factor (VEGF) in random flaps in rats. **Methods:** Thirty-two male Wistar rats were randomly divided into 4 groups: the sham group (GS), the *N*-acetylcysteine group (GNAC), the HBO group, and the HBO + *N*-acetylcysteine group. A rectangular skin flap (2 × 8 cm) was dissected from the dorsal muscle layer, and a cranial pedicle was preserved. A polyethylene film was placed on the muscle layer, and the flap was fixed in the original site. On the eighth day, biopsy samples (2 × 1 cm) of the entire thickness of the proximal, medial, and cranial areas as well as of the site outside the flap, which was used as a control, were collected. **Results:** VEGF expression in the skin layers and vessels was not significantly different between the groups. The number of apoptotic cells was significantly increased in the area of the flap in all groups. The highest increase was noted in the GS and GNAC groups. HBO significantly reduced the number of caspase 3-positive cells in the skin layers and vessels of the 3 areas. **Conclusions:** HBO was associated with decreased apoptosis. VEGF expression in the skin layers and vessels did not differ significantly. The results suggest that oxygen diffusion through the interstitial space was the determining factor for the positive effect of HBO on the decrease in apoptosis.

Keywords: Hyperbaric oxygenation. Caspase 3. Endothelial growth factors. Surgical flaps.

RESUMO

Introdução: A oxigenação hiperbárica (OHB) tem por objetivo aumentar em pelo menos 10 vezes a pressão tecidual de oxigênio, diminuindo os efeitos deletérios da isquemia. O objetivo deste estudo é investigar o papel da oxigenação hiperbárica na expressão imuno-histoquímica da caspase 3 e do fator de crescimento endotelial vascular (VEGF) em retalhos randômicos em ratos. **Método:** Trinta e dois ratos Wistar machos foram divididos

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aleatoriamente em quatro grupos: grupo *sham* (GS), grupo N-acetilcisteína (GNAC), grupo OHB (GOHB) e grupo OHB + N-acetilcisteína (GHN). Um retalho de pele retangular (2 cm x 8 cm) foi dissecado a partir da camada muscular dorsal, preservando um pedículo cranial. Uma lâmina de polietileno foi colocada sobre a camada muscular e fixou-se o retalho no local original. No 8º dia, foram coletadas biópsias (2 cm x 1 cm) de espessura total das áreas proximal, média e cranial e de um local fora do retalho, que serviu como área de controle. **Resultados:** A expressão de VEGF nas camadas da pele e nos vasos não apresentou diferenças significativas entre os grupos. As células apoptóticas estavam significativamente aumentadas na área central do retalho em todos os grupos. O maior aumento ocorreu nos grupos GS e GNAC. A OHB diminuiu significativamente o número de células caspase 3 positivas nas camadas da pele e nos vasos das três áreas. **Conclusões:** A OHB foi associada a expressão reduzida de apoptose. A expressão de VEGF em camadas da pele e vasos não demonstrou diferença significativa. Os resultados sugerem que a difusão do oxigênio através do espaço intersticial foi o fator determinante para os resultados mais favoráveis da OHB na diminuição da expressão de apoptose.

Descritores: Oxigenação hiperbárica. Caspase 3. Fatores de crescimento endotelial. Retalhos cirúrgicos.

INTRODUCTION

Hyperbaric oxygenation (HBO) is a form of therapy in which 100% medicinal oxygen is supplied under pressure to increase the oxygen pressure in tissues by at least 10 times and reduce the adverse effects of ischemia. This type of treatment has been used since 1968, when its clinical indications were defined. In plastic surgery, HBO is indicated for the treatment of patients with severe burns and for ischemic flaps because it provides hyperoxygenation to tissues, activates fibroblasts, modulates inflammatory response, accelerates growth factors, and has antibacterial activity¹⁻⁵.

McFarlane et al.⁶ developed an experimental model to assess the viability of random flaps in rats, which had been used in various previous studies. The authors observed that the biochemical and morphological changes were directly related to the distance from the extremity of the flap and the size of its pedicle⁷⁻⁹.

Random flaps are exposed to tissue damage resulting from postischemic reperfusion, in which reintroduction of molecular oxygen facilitates the production of critical levels of energy, increasing the inflammatory response and causing toxicity in endothelial cells. *N*-Acetylcysteine (NAC) is a prodrug that regulates the glutathione levels in the body when they are reduced in the presence of toxic oxygen radicals (TORs). Glutathione is one of the most potent free radical scavengers, and its cellular levels are quickly depleted during damage caused by the ischemia-reperfusion phenomenon^{6,10-12}.

The aim of this study was to assess the effect of HBO, NAC, and their association on random flaps in rats by using an apoptosis marker (cleaved caspase 3) and an endothelial vascular growth marker (VEGF).

METHODS

Ethical Considerations

The experimental protocol (0321/06) was approved by the ethics committee of the Federal University of São Paulo. All procedures were conducted in strict accordance with the existing regulations on animal experimentation of the Colégio Brasileiro de Experimentação Animal.

Samples and Groups

Thirty-two male Wistar rats weighing 280 to 300 g were kept in individual cages in a sound-attenuated room maintained at 25°C under artificial lighting and given food and water *ad libitum*. The animals were randomly divided into 4 groups as follows:

- GS – the sham group, including 8 animals;
- GNAC – the NAC group, including 8 animals;
- GHBO – the HBO group, including 8 animals;
- GHN – the HBO + NAC group, including 8 animals.

Anesthesia Procedure

After 6 h of solid food fasting and 4 h of liquid fasting, the animals received 5 mg/kg of 0.2% acepromazine, intramuscular. Ten minutes later, the animals received a combination of 50 mg/kg⁻¹ of ketamine hydrochloride and 10 mg/kg⁻¹ xylazine hydrochloride, intramuscular.

Surgical Procedure

Under general anesthesia, trichotomy of dorsal regions was performed, and the animals were then fixed in the prone position. After demarcation of a rectangular patch (2 cm x 8 cm) of upper base, incision with a scalpel (blade No.

15) was performed, then the flap was dissected, including epidermis and dermis (Figure 1). A polyethylene film was interposed between the flap and the muscle layer, covering the entire area of the wound and acting as a barrier between the skin and muscles (Figure 2). Then, flap synthesis with polyamide thread 3.0 was performed, fixing the flap in its original position.

Administration Procedures

Animals in the GNAC and GHN groups were intraperitoneally injected with 300 mg/kg of NAC (Fluimucil®; Zambon Laboratório Farmacêutico Ltda., São Paulo, SP, Brazil) after flap elevation and then every 24 hours for 7 days.

The animals in the GS and GHBO groups were intraperitoneally injected with 1 ml of distilled water (Isofarma, São Paulo, SP, Brazil) after flap elevation and then every 24 hours for 7 days.

HBO Procedures

A hyperbaric chamber for several animals was used. Before pressurization, the inside of the chamber was cleaned with 100% medicinal oxygen for 5 minutes. The chamber was then pressurized until a pressure of 2.4 atmosphere absolute (ATA). The oxygen concentration was monitored using a calibrated oximeter. All animals in the GHBO and GHN groups were exposed to 100% of oxygen at a pressure of 2.4 ATA for 2 hours (once/day), starting 15 minutes after the surgery and then every 24 hours for 7 consecutive days.

Treatment

The animals were randomly divided into the following groups:

- the GS group (n = 8), received distilled water intraperitoneally 15 minutes after flap elevation and then for 7 consecutive days;
- the GNAC group (n = 8), received 300 mg/kg⁻¹ NAC intraperitoneally after flap elevation and then for 7 consecutive days;

- the GHBO group (n = 8), received 100% oxygen at 2.4 ATA in a hyperbaric environment for 7 consecutive days for 2 hours;
- the GHN group (n = 8), received a combination of oxygen in a hyperbaric environment and NAC intraperitoneally for 7 consecutive days.

Sample Collection

On the eighth day of the experiment, the rats were once again anesthetized for collection of incisional biopsy samples (2 × 1 cm), which included the entire thickness of the proximal, medial, and distal areas of the flap. As a control, another biopsy sample was collected from outside the area of the flap (Figure 3).

Euthanasia

Under anesthesia, the animals were euthanized in a carbon dioxide chamber after sample collection.

Histological Procedure

The samples were fixed in 10% formalin, embedded in paraffin, and immunohistochemically stained to examine the expression of the apoptosis marker and VEGF.



Figure 1 – Standard random skin flap (2 × 8 cm) by McFarlane et al.6 on the seventh cervical vertebra.



Figure 2 – Polyethylene film placed on the muscle layer, covering the entire area of the wound and acting as a barrier between the skin and the muscle.



Figure 3 – Areas from where biopsy samples were collected.

Immunohistochemical Staining for VEGF

An anti-VEGF marker (Sigma Chemical Company, St. Louis, USA) was used to detect VEGF expression. All procedures were performed according to the manufacturer instructions.

Immunohistochemical Staining for the Apoptosis Marker

The avidin-biotin standard method was used to detect apoptotic cells using a detection kit containing anti-caspase 3 primary antibody (Lexington, USA). After incubation, the specimens were treated with rabbit immunoglobulin/biotinylated antibody (at 1:600 dilution; Dako Ltd., Ely, UK) for 30 min. All procedures were performed according to the manufacturer instructions.

VEGF Expression

Medium-power magnification ($\times 100$ and $\times 200$) was used to examine VEGF expression in the biopsy samples from the epidermis, dermis, vessels, and submuscular area. VEGF expression was classified according to the intensity of the color of the cytoplasm (from 0 to 3):

- 0 – no expression (negative reaction);
- 1 – low expression (weak brown reaction);
- 2 – moderate expression (moderate brown reaction);
- 3 – high expression (strong brown reaction).

Apoptosis Marker Expression

Images were obtained using a high-resolution camera with Axiomcam software (Mission-oriented Resilient Clouds;

Zeiss®) and analyzed using an optical microscope (Carl Zeiss Axiolab®). Axiovision Rel 4.2 software (Zeiss®) was used to calculate the ratio of brown cells (i.e., apoptotic cells) to non-brown cells (i.e., non-apoptotic cells). Apoptotic cells were counted in 10 fields per slide in all the samples for all the groups.

Statistical Analysis

All procedures for histological and immunohistochemical analyses were performed with the examiner blinded to the identity of the groups to which the morphological samples belonged.

For the analysis of the immunohistochemical results, nonparametric tests were applied, taking into consideration the variables under study and the sample size. Two variables were analyzed, a quantitative variable (cleaved caspase 3) and a semiquantitative variable (VEGF).

Cleaved caspase 3 levels were expressed as mean and standard deviation values. VEGF expression was presented as a score, and mean and standard deviation values were obtained for each group. Analysis of variance was performed to determine within-group differences, and the Bonferroni test was used to compare the groups.

The probability of rejecting the null hypothesis was set at 0.05 or 5%, and a P value of <0.05 was considered statistically significant (with a 95% confidence interval).

RESULTS

Regarding VEGF expression, all the biopsy samples collected from the distal third exhibited necrosis. Data on

Table 1 – Mean, standard deviation, and P value for VEGF expression in 10 fields per slide in all the samples collected for each third from all the groups.

Tissue	Third	GS	GNAC	GHBO	GHN	P value*
		Mean \pm SD	Mean \pm SD	Mean \pm SD	Mean \pm SD	
Epidermis	Control	2 \pm 0.6	2.8 \pm 0.3	2.2 \pm 0.9	2.4 \pm 0.5	0.1
	Medial	1.8 \pm 0.8	2.6 \pm 0.7	2.3 \pm 1	1.7 \pm 1	0.2
	Proximal	2.3 \pm 0.7	2.7 \pm 0.7	2.6 \pm 0.5	2.7 \pm 0.5	0.5
Dermis	Control	1.5 \pm 0.5	2 \pm 0.7	2 \pm 0.5	1.4 \pm 0.5	0.06
	Medial	1.9 \pm 0.8	1.7 \pm 0.7	1.6 \pm 0.7	1.5 \pm 0.5	0.7
	Proximal	1.6 \pm 0.5	1.9 \pm 0.8	1.4 \pm 0.7	1.5 \pm 0.5	0.5
Submuscular	Control	1.2 \pm 0.7	2 \pm 0.5	2 \pm 1.2	1 \pm 0.5	0.06
	Medial	2.3 \pm 0.8	2.1 \pm 0.4	1.9 \pm 0.6	2.2 \pm 0.9	0.7
	Proximal	1.6 \pm 0.7	1.3 \pm 1	1.3 \pm 0.7	2 \pm 0.8	0.2
Vessels	Control	1.6 \pm 0.8	2 \pm 0.1	2.4 \pm 0.7	2.1 \pm 0.8	0.1
	Medial	2.3 \pm 0.7	2.1 \pm 0.6	2.3 \pm 0.5	2.3 \pm 0.5	0.8
	Proximal	1.8 \pm 0.6	2.1 \pm 0.6	2 \pm 0.7	2.1 \pm 0.6	0.8

*Analysis of variance.

SD = standard deviation; GHN = GNAC + GHBO group; GNAC = N-acetylcysteine group; GHBO = hyperbaric oxygenation group; GS = sham group.

VEGF expression in the epidermis, dermis, panniculus carnosus, and vessels showed that HBO did not promote any significant change in both the GS and the GNAC groups (Table 1). Moreover, no significant difference was observed in the GHN group. These findings suggest that OHB, NAC, or the combination of both did not promote changes in VEGF expression in the tissues.

HBO caused a significant reduction in apoptotic cells in all the biopsy samples obtained from the proximal and medial thirds. The combination of HBO and NAC, in addition to not having a protective effect, exerted a negative effect (Figures 4 and 5).

Statistical analysis of the biopsy data for the proximal, medial, and distal thirds of the flaps showed that sham (GS) group had greatest number of apoptotic cells followed by the NAC and GHN groups. The GHBO group presented fewer cells apoptosis in all strata of the epithelium under study (epidermis, dermis, submuscular area, and vessels), as shown in Table 2.

DISCUSSION

The mean oxygen tension in ischemic flaps is only 30 mmHg. Hypoxia occurs as a normal stimulus for wound healing; however, this situation can deteriorate if the local

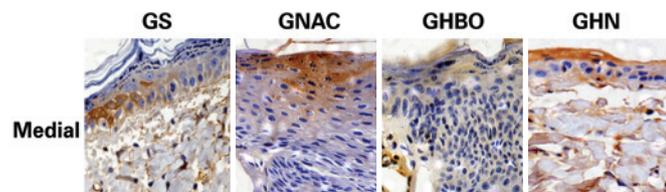


Figure 4 – Photomicrographs of the GS, GNAC, GHBO, and GHN groups, showing caspase 3 expression (brown color) in the hypodermis samples collected from the medial part of the flap (original magnification '200). GHN = GNAC + GHBO group; GNAC = N-acetylcysteine group; GHBO = hyperbaric oxygenation group; GS = sham group.

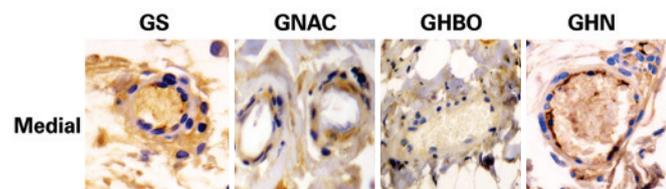


Figure 5 – Photomicrographs of the GS, GNAC, GHBO, and GHN groups, showing caspase 3 expression (brown color) in the vessels collected from the medial part of the flap (original magnification '400). GHN = GNAC + GHBO group; GNAC = N-acetylcysteine group; GHBO = hyperbaric oxygenation group; GS = sham group.

conditions of poor irrigation persist, thus impairing wound healing and leading to tissue necrosis¹³.

During the ischemic period, random flaps are subjected to hypoxia and hypoglycemia. These conditions lead to cellular dysfunction and cell death. When blood flow is restored, a second event known as flow/no-reflow occurs; this phenomenon is the result of the accumulation (plug) of polymorphonuclear cells that adhere to the endothelium and are responsible for the production of TORs, which are associated with the second wave of cellular damage^{1,14}.

TORs are responsible for oxidative damage that leads to mitochondrial changes; adenosine triphosphate depletion; increase in intracellular calcium levels; and protease, phosphatase, lipase, and nuclease activation, which results in the loss of cellular integrity¹⁴.

The phenomenon of ischemia/reperfusion is mediated by TORs. These molecules are highly unstable and extremely toxic to most biological processes by means of their effects of lipid peroxidation of cell membranes, formation of free radicals in a self-propagating cycle, and cell death due to necrosis or apoptosis⁵.

Studies support the hypothesis that oxygen functions as a signaling molecule for a series of events that promote wound healing. The levels of hypoxia-inducible factor alpha (HIF- α) are decreased in HBO-treated tissues. HIF- α is an important regulator of genetic expression involved in the regulation of tissue oxygenation, and its levels increase in the presence of hypoxia. The level of the protein BNip3 (a pro-apoptotic protein located in the mitochondria) increases greatly after the 10th day in ischemic tissues. High levels of oxygen induced by HBO decrease and attenuate the expression of the protein BNip3, thus modulating apoptosis despite the persistent high level of lactic acid^{1,4,5,15}.

Therefore, HBO increases antiapoptotic activity and decreases pro-apoptotic activity; there is a subsequent reduction in the release of pro-apoptotic molecules by mitochondria and attenuation of apoptosis. The molecular basis of the protective effect of HBO on ischemic wound healing is attributable to HIF-1 α modulation. Wounds treated with HBO exhibit reduced levels of HIF-1 α and a consequent reduction in expression of the HIF-1 α gene, as well as reduced apoptosis and inflammation^{1,4,5}.

The process of angiogenesis begins between the first and third days after ischemia and is modulated by growth factors that are present in endothelial cells and in the extracellular matrix. VEGF is the most important stimulator of angiogenesis and its expression is associated with the initiation of vascular neoformation. In addition, it is a reliable marker for monitoring the process of vascular neoformation¹⁵⁻¹⁸.

Caspase 3 is an apoptosis-effector protein. It converts cytoplasmic DNase into an active form. During cell death programming, caspase 3 induces early activation of endonu-

Table 2 – Mean, standard deviation, and P value for the presence of apoptotic cells (caspase 3) in 10 fields per slide in all the samples collected for each third from all the groups.

Tissue	Third	GS	GNAC	GHBO	GHN	P value***
		Mean + SD	Mean + SD	Mean + SD	Mean + SD	
Epidermis	Control	2.1 (0.6)	2.4 (0.7)	1.4* (0.5)	2 (0.8)	< 0.03
	Medial	4.1 (1.3)	3.8 (1.2)	1.9** (0.4)	2.8 [€] (0.7)	< 0.01
	Proximal	3.1 (1.3)	2.9 (0.4)	1.1 [§] (0.6)	1.5 [¥] (0.5)	< 0.01
Dermis	Control	0.8 (0.9)	1.3 (0.5)	0.4 (0.5)	0.8 (0.7)	0.09
	Medial	2.1 (1.1)	1.8 (0.5)	0.5* (0.8)	1.3 (0.9)	< 0.01
	Proximal	1.1 (0.6)	1.5 (0.8)	0.1 [§] (0.4)	1.1 (1)	< 0.01
Submuscular	Control	1.5 (1.2)	1.8 (0.7)	1.1 (0.4)	1.8 (0.7)	0.06
	Medial	3.8 (2)	2.3 (0.9)	1.6* (0.5)	2.4 (1.2)	< 0.01
	Proximal	3 (1.2)	2 (1.1)	1.1** (0.4)	1.8 [¥] (0.5)	< 0.01
Vessels	Control	1.3 (1.3)	1.8 (0.5)	1.1 (0.4)	1.9 (0.6)	0.1
	Medial	3.6 (1.8)	2.4 (0.9)	1.5* (0.5)	2.6 (0.5)	< 0.01
	Proximal	2.9 (0.6)	1.9 (0.8)	1.1** (0.4)	2 [¥] (0.5)	< 0.01

Control – Epidermis/GHBO* < GS

Medial – Epidermis/GHBO** = GHN[€] < GS = GNACProximal – Epidermis/GHBO[§] = GHN[¥] < GNAC

Control – Submuscular/without statistically significant difference

Medial – Submuscular/GHBO* < GS = GNAC

Proximal – Submuscular/GHBO** = GHN[¥] < GS***Analysis of variance, complemented by a *post hoc* Bonferroni test.

GHN = GNAC + GHBO group; GNAC = N-acetylcysteine group; GHBO = hyperbaric oxygenation group; GS = sham group.

Control – Dermis/without statistically significant difference

Medial – Dermis/GHBO* < GS = GNAC

Proximal – Dermis/GHBO[§] < GS = GNAC

Control – Vessels/without statistically significant difference

Medial – Vessels/GHBO* < GS

Proximal – Vessels/GHBO** = GHN[¥] < GS

cleases, causes DNA fragmentation, and impairs enzymatic processes that are vital to cells. Caspase 3 has been used in previous studies on ischemic skin flaps^{5,19}.

Many mechanisms are involved in tissue healing; however, most studies show that the presence of growth factors is associated with the production of energy derived from glycolysis and the Krebs cycle.

HBO increases oxygen tissue pressure by up to 10 times by supplying oxygen dissolved in plasma, according to the laws of Henry and Boyle; in addition, it acts directly on neutrophils and inflammatory mediators responsible for tissue damage.

Humans have a complex antioxidant system. Glutathione is depleted during antioxidation. NAC is a prodrug that replenishes the levels of glutathione when these are depleted during the oxidative process and has been used in many experimental studies because of its low toxicity and protective effect on random flaps.

Data regarding VEGF expression in the epidermis, dermis, panniculus carnosus, and vessels indicate that HBO did not promote any significant change (Table 2). The proposed mechanism to explain the absence of increased VEGF production is that the partial pressure of oxygen does not return to the pre-ischemic levels between hyperoxygenation sessions; therefore, there is no stimulus for VEGF production⁵.

Because VEGF expression is upregulated under ischemic conditions, we did not expect VEGF expression to be upregulated in the groups treated with HBO (GHBO and GHN), in which higher amounts of oxygen were supplied. However, a significant increase should have been observed in the GS group. The most plausible hypothesis to explain the results of this study is the significant reduction in metabolic activity and protein synthesis caused by ischemia, which inhibits VEGF expression²⁰.

Another hypothesis that may explain the results of this study was previously put forward by other authors, who observed significant decreases in metabolic activity and protein synthesis, including the synthesis of VEGF, under ischemic conditions²⁰.

The high concentrations of NAC used in the GNAC group might have been responsible for the inhibition of VEGF production. Previous studies have shown that high concentrations of NAC inhibit angiogenesis and the wound healing response through an oxidant/antioxidant balance that has not yet been clarified¹¹. However, in this study, VEGF expression was not altered in any of the groups, and all groups had a profile similar to the GS group.

The model used in this study, in which a polyethylene film was applied, might have been responsible for the absence of VEGF expression in the groups treated with NAC

or HBO. Because there was no difference between these groups and the control group, we suggest that the stimulus for angiogenesis was dependent on the bed on which the flap was placed after its confection. The growth of new vessels might have been delayed because there was no contact with the bed.

Data regarding the mean number of apoptotic cells in the biopsied strata showed that, in the external part of the random flap, that is, where there was no ischemia, HBO (0.4 ± 0.5) promoted a greater decrease in caspase 3 expression compared with the GS (0.8 ± 0.9), GNAC (1.3 ± 0.5), and GHN (0.8 ± 0.7) groups. These findings allowed us to infer that HBO had a protective effect in the normal tissues by decreasing caspase 3 expression and that hyperoxidation tended to have a protective effect even in the tissues that were not exposed to ischemia; surgical trauma functions as a form of preconditioning.

The overall assessment of apoptosis in the various strata suggests that HBO has a protective effect on the medial and proximal portions of the flaps. HBO group presented reduced caspase 3 expression in the external skin of the flap, indicating that the effect of hyperoxygenation on healthy tissues was similar to that in tissues that only received the surgical stimulus or were treated with NAC. The effect of the use of NAC was not different from that in the control group, and the use of HBO did not produce a synergistic effect, which implies that the use of NAC as an antioxidant did not have a protective effect.

The combination of NAC with HBO yielded worse results than the isolated use of HBO. NAC concentrations greater than 20 mmol/L may reverse the antioxidant effect through pH inversion, cytochrome C activation, and mitochondrial pore opening, and thus lead to cell death¹¹.

The results of this study suggest a clear reduction in apoptosis in the group treated with HBO. The most plausible hypothesis for the protective effect of HBO on cellular apoptosis is the abundant supply of oxygen to the tissues during the initial stage of ischemia, which attenuates the inflammatory response and reduces lipid peroxidation⁴⁻⁶.

CONCLUSIONS

Considering the positive effect of HBO on the reduction of cellular apoptosis, we suggest that this is dependent on the time of exposure or the number of exposures to hyperbaric oxygen. The rationale for using HBO, previously published results, and the results of this study suggest that more prolonged or frequent daily sessions may result in decreased apoptosis. These conclusions still need to be confirmed in further studies in this area.

HBO, NAC, or the combination of both did not affect VEGF levels. This implied that the flap was exclusively de-

pendent on the vascular pedicle when a polyethylene film was applied. HBO and NAC were not effective in increasing VEGF expression, demonstrating the absence of a stimulus for vascular neogenesis during the period under study.

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