Immunohistochemical expression of apoptosis and VEGF expression on random skin flaps in rats treated with hyperbaric oxygen and N-acetylcysteine

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ABSTRACT
Objective: We sought to investigate the role of hyperbaric oxygen (HBO₂), N-acetylcysteine (NAC), and HBO₂ plus NAC (HN) on the immunohistochemical expression of caspase-3 and the vascular endothelial growing factor (VEGF) on random skin flaps of rats (modified McFarlane design).

Methods: Thirty-two male Wistar rats were randomly divided into four groups: GS (sham – n=8); GNAC (N-acetylcysteine – n=8); GHBO₂ (hyperbaric oxygen – n=8); and GHN (HBO₂ plus NAC – n=8).

A rectangular skin flap (2 x 8 cm²) was dissected from the muscular dorsal layer, preserving a cranial pedicle. Polyethylene film was placed over the muscular layer, and an interrupted 3.0 nylon suture fixed the flap into the original place. On the eighth day, full-thickness biopsies (2 x 1 cm²) were collected from the proximal, middle and cranial areas of the skin flap, and in a site away from the flap labeled the control area.

Results: The expression of VEGF in the skin layers (epidermis, dermis, subcutaneous muscles) and vessels showed no significant difference among the groups. Apoptotic cells were significantly increased in the middle area of the flap in all groups. HBO₂ significantly decreased cleaved caspase-3-positive cell numbers in the skin layers and vessels of the three areas.

Conclusions: HBO₂ showed a protective effect in the ischemic skin flap that was associated with reduced expression of apoptosis. GNAC and GHN were not associated with lower expression of apoptosis, and poor results were observed in GNAC. HBO₂ significantly decreased cleaved caspase-3-positive cell numbers in the skin layers and vessels of the three areas.

INTRODUCTION
Advances in reconstructive surgery have led to an increasing use of extensive skin flaps for the closure of surgical defects. Axial flaps are widely used for this purpose. However, ischemic tissue necrosis remains a troublesome complication of skin flap surgery, and unexpected tissue loss may produce significant cosmetic and functional deficits [1,2].

Hyperbaric oxygen (HBO₂) [3] and the specialty antioxidant drug N-acetylcysteine (NAC) [4] are widely used in experimental studies to improve random skin flap survival.

HBO₂ delivers oxygen dissolved in plasma in proportion to its partial pressure according to Henry’s Law. The dissolved gas enhances oxygen transport to hypoxic tissue, promoting oxygen diffusion through tissue inter-
strial fluids [5]. Numerous studies have shown that HBO₂ simultaneously provides a beneficial effect directed at many of the components responsible for ischemic injury, such as neutrophils, endothelium, inflammatory mediators, lipid peroxidation, cellular energetics and microvascular blood flow [5-9].

Mammals have a complex system that protects them from oxidant stress. One of the most important components of the intracellular antioxidant system is glutathione, a powerful active radical scavenger that is depleted in ischemic injury [10]. NAC is a prodrug that supplies bioavailable cysteine for glutathione replenishment in the presence of overwhelming reactive oxygen species (ROS) [11,12]. Acetylcysteine, a well-known antioxidant substance with low toxicity, has been used in the protection of random skin flaps with favorable results [4].

HBO₂ increases the tolerance of tissue to ischemia and enhances free radical formation. However, hyperoxia can increase the biochemical defense mechanisms against free radicals [6] and improve the survival probability of ischemic tissue [3]. In conditions of high oxygen concentrations, the hypoxia-inducible factor-1α (HIF-1α) is degraded by an oxygen-dependent prolyl hydroxylase. The downregulation of HIF-1α and the subsequent target gene expression promote the attenuation of cell apoptosis, the reduction of inflammation and the presence of vascular endothelial growth factor (VEGF) [3]. VEGF, a potent angiogenic, mitogenic and vascular permeability-enhancing protein, improves the survival of ischemic flaps independent of the route of exogenous administration [13-16].

The antioxidant NAC prevents some of the deleterious effects indicated by an involvement of oxidative stress during reperfusion injury [4,17]. Our aim in this study was to investigate the role of HBO₂, NAC and the combination of HBO₂ and NAC on the immunohistochemical expression of apoptosis and VEGF on the random skin flaps of rats in a modified McFarlane flap design [18].

MATERIALS AND METHODS
Ethics Committee
The experimental protocol (0321/06) was approved by the Ethics Committee of the Federal University of São Paulo (UNIFESP). All procedures strictly followed the existing regulations on animal experimentation of the Brazilian College on Animal Experimentation (COBEA).

Samples and groups
Thirty-two male Wistar rats weighing 280-300 g were kept in individual cages in acoustically isolated rooms, at 25ºC, under artificial illumination, with food and water provided ad libitum. The animals were randomly divided into four groups:
• GS (sham – n = 8);
• GNAC (N-acetylcysteine – n = 8);
• GHBO₂ (hyperbaric oxygen – n = 8); and
• GHN (HBO₂ plus NAC – n = 8).

Anesthetic procedure
After six hours of fasting for those on a solid diet and four hours for those on a liquid diet, the animals received 5 mg/kg intramuscular (i.m.) acepromazin (Acepran 0.2%). Ten minutes later, they received a combination of 50 mg/kg i.m. ketamine (Ketalar®) and 10 mg/kg i.m. of xylazine (Rompun®).

Surgical procedure
Under general anesthesia, the dorsal regions were shaved, and the animals were fixed in the prone position. A rectangular area (2 x 8 cm²) was longitudinally marked with ink based on the seventh cervical vertebra and running to the caudal position, with the spine as a central landmark. The caudal and lateral marks were incised with a #15 scalpel, a skin flap was dissected from the muscular dorsal layer, and the cranial portion was preserved from incision (Figure 1, facing page). A sheet of polyethylene film was placed over the muscular layer covering the entire wound area and acting as a barrier between the skin and the muscles (Figure 2, facing page). An interrupted 3.0 nylon suture (Mononylon®) fixed the flap into the original place.

NAC administration procedure
A dose of 300 mg/kg NAC (Flumucil™ acetylcysteine 300 mg/3 mL; Zambon Pharmaceuticals Laboratory Ltd., São Paulo, Brazil) on GNAC or GHN was injected intraperitoneally after the elevation of the skin flap and consecutively every 24 hours for seven days.

GS and GHBO₂
One milliliter of distilled water (Isofarma, São Paulo, Brazil) on GS and GHBO₂ was injected intraperitoneally after the elevation of the skin flap and consecutively every 24 hours for seven days.

HBO₂ procedure
HBO₂ was delivered in a hyperbaric chamber for experimental animals [19] at the University Regional do Alto Urugui – Campus Erechim (URI) (Figure 3, facing page). Before pressurization, 100% medical oxygen
FIGURE 1 – Caudal and lateral marks

The caudal and lateral marks were incised with a scalpel, a skin flap was dissected from the muscular dorsal layer, and the cranial portion was preserved from incision (dorsal view).

FIGURE 2 – Caudal and lateral marks with film

Polyethylene film was placed over the muscular covering the entire wound area and acted as a barrier between the skin and muscles (dorsal view).

FIGURE 3 – The chamber

HBO₂ was delivered in a hyperbaric chamber for experimental animals of the University Regional do Alto Uruguai - Campus Erechim.

was flushed through the chamber for five minutes to displace the room air. Oxygen pressure was then increased at a constant rate to reach a pressure of 2.4 ATA. The oxygen concentration was monitored with a calibrated oxymeter. The animals were placed in the hyperbaric chamber in accordance with the random groups. All animals in GHBO₂ and GHN were exposed to 100% oxygen at 2.4 ATA for two hours (once a day) starting 15 minutes after flap fixation and every 24 hours for seven consecutive days.

Treatment

The animals were randomly divided. GS (n=8) received distilled water intraperitoneally 15 minutes after flap elevation and for seven consecutive days. GNAC (n=8) received 300 mg/kg intraperitoneally after flap elevation and for seven consecutive days. In GHBO₂ (n=8) all rats were exposed to 100% oxygen at 2.4 ATA absolute for 15 minutes following the delay procedure and the following seven consecutive days of two hours a day. Each was in a hyperbaric animal experimental chamber that was flushed with 100% oxygen. GHN (n=8) received the combination of NAC and HBO₂ for seven consecutive days.

Seven days of follow-up

Every day all the animals were examined, and each occurrence of fever, incision infections, liquid stools, or refusal of food or drinking water was recorded. Once any sign of severe suffering was identified, the veterinary staff interrupted the research, and the animals were sent to be euthanized. None of the groups observed showed signs of suffering or death.

Samples collected

On the eighth day, full-thickness biopsied specimens (2 x 1 cm²) were collected from the proximal, median and cranial areas of the skin flap. Another biopsy was collected out of the flap in the normal skin area called the control area (Figures 4 and 5, Page 170).

Euthanasia

Under anesthesia and after the samples were collected, the animals were put in a chamber and flushed with CO₂ until cardiorespiratory arrest.

Histological procedure

For light microscopy, the biopsied specimens were fixed in 10% formalin, soaked in paraffin, and stained for immunohistochemical expression of apoptosis and determination of VEGF.
VEGF immunohistochemistry
To observe the expression of VEGF, rabbit anti-EGF was obtained from Sigma Chemical (St. Louis, Mo., USA). All steps were done according to the supplier’s instructions.

Apoptosis immunohistochemistry
A standard avidin-biotin complex method with alkaline phosphatase detection was performed by in situ apoptosis detection with the antibody to caspase-3 (Transduction Laboratories, Lexington, Ky., USA). After incubation, slides were treated with biotinylated rabbit antimouse immunoglobulin (1:600 for 30 minutes; Dako Ltd., Ely, UK). All steps were done according to the supplier’s instructions.

VEGF expression
VEGF expression was classified considering the following aspects in the epidermis, dermis, vessels and sub-muscle areas. All biopsies were observed in median amplification (100x and 200x) and then tabulated according to the strength of color. The intensity of cytoplasmatic pattern color for each stratus was observed and classified according to a scale from 0 to 3:

0  without expression (negative reaction);
1  low expression (weakness brown reaction);
2  moderate expression (moderate brown reaction);
3  high expression (strong brown reaction).

Apoptosis expression
The images were captured through a high-resolution camera, AXIOcam MRC software from ZEISS®, and the analysis was conducted in a CARL ZEISS Axilab® optic microscope. The software used was the Axion Vision Rel 4.2 from ZEISS®, which calculated the ratio of brown-colored tissue/no brown-colored tissue. Apoptotic cells were counted in 10 sites of each plate in all samples of all groups.

Statistical analysis
Apoptosis and VEGF were expressed as mean and standard deviation. The significance of differences in necrosis area and histological expression was determined by one-way analysis of variance (ANOVA), applying the post-hoc test of Bonferroni. A p-value of 5% (p<0.05) was considered statistically significant.

RESULTS
The expression of VEGF in the epidermis, dermis, sub-muscles and vessels did not show significant differences on the eighth day among different thirds of full-thickness biopsied specimens (proximal, median and in the normal skin called the control area) (Table 1, facing page). All biopsies collected in cranial areas of all groups had signs of necrosis.

Caspase-3 is a common and reliable apoptotic marker; hence, we next assessed whether HBO₂ decreased the expression of caspase-3. As shown in Figure 6, (Page 172) HBO₂ significantly decreased cleaved caspase-3 expression in the epidermis and dermis, which was confirmed by immunohistochemistry.

As shown in Figure 7 (Page 172), strongly positive staining for cleaved caspase-3 was seen in tissue vessels in the control group and GNAC. HBO₂ significantly decreased cleaved caspase-3–positive cell numbers in ischemic tissue. The microscopic assessment of tissue viability revealed severe ischemic damage in GS >
TABLE 1

<table>
<thead>
<tr>
<th>Skin layer</th>
<th>Sample</th>
<th>GS Mean(sd)</th>
<th>GNAC Mean(sd)</th>
<th>GHBO Mean(sd)</th>
<th>GHN Mean(sd)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epidermis</td>
<td>Control</td>
<td>2.0 (0.6)</td>
<td>2.8 (0.3)</td>
<td>2.2 (0.9)</td>
<td>2.4 (0.5)</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>Middle</td>
<td>1.8 (0.8)</td>
<td>2.6 (0.7)</td>
<td>2.3 (1.0)</td>
<td>1.7 (1.0)</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>Proximal</td>
<td>2.3 (0.7)</td>
<td>2.7 (0.7)</td>
<td>2.6 (0.5)</td>
<td>2.7 (0.5)</td>
<td>0.5</td>
</tr>
<tr>
<td>Dermis</td>
<td>Control</td>
<td>1.5 (0.5)</td>
<td>2.0 (0.7)</td>
<td>2.0 (0.5)</td>
<td>1.4 (0.5)</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>Middle</td>
<td>1.9 (0.8)</td>
<td>1.7 (0.7)</td>
<td>1.6 (0.7)</td>
<td>1.5 (0.5)</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td>Proximal</td>
<td>1.6 (0.5)</td>
<td>1.9 (0.8)</td>
<td>1.4 (0.7)</td>
<td>1.5 (0.5)</td>
<td>0.5</td>
</tr>
<tr>
<td>Subcutaneous muscle</td>
<td>Control</td>
<td>1.2 (0.7)</td>
<td>2.0 (0.5)</td>
<td>2.0 (1.2)</td>
<td>1.0 (0.5)</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>Middle</td>
<td>2.3 (0.8)</td>
<td>2.1 (0.4)</td>
<td>1.9 (0.6)</td>
<td>2.2 (0.9)</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td>Proximal</td>
<td>1.6 (0.7)</td>
<td>1.3 (1.0)</td>
<td>1.3 (±0.7)</td>
<td>2.0 (0.8)</td>
<td>0.2</td>
</tr>
<tr>
<td>Vessels</td>
<td>Control</td>
<td>1.6 (0.8)</td>
<td>2.0 (0.1)</td>
<td>2.4 (0.7)</td>
<td>2.1 (0.8)</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>Middle</td>
<td>2.3 (0.7)</td>
<td>2.1 (0.6)</td>
<td>2.3 (0.5)</td>
<td>2.3 (0.5)</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>Proximal</td>
<td>1.8 (0.6)</td>
<td>2.1 (0.6)</td>
<td>2.0 (0.7)</td>
<td>2.1 (0.6)</td>
<td>0.8</td>
</tr>
</tbody>
</table>

No statistical significance / (GS = GNAC = GHBO = GHN)
No statistical significance / (Epidermis = Dermis = Subcutaneous muscle = Vessels)
One-way analysis of variance ANOVA (p<0.05)

TABLE 1 – Percentage of area (cm²) of VEGF expression (mean ± sd) on the epidermis, dermis, subcutaneous muscle, and vessels. The samples were collected in the normal skin (control), in the middle and proximal area to the skin flap pedicle in the sham, NAC, HBO2 and HBO2 plus NAC groups.

GNAC > GHN > GHBO2. Flap dissection resulted in an accumulation of apoptotic cells in the middle part of the flap in all groups; the worst was GS in all thirds (proximal, median and control) in the epidermis, dermis, vessels and submuscular tissue (Table 2, Page 173).

DISCUSSION

In wound healing, endothelial cells may be involved in the process of angiogenesis and are the source of releasing VEGF [15,20]. VEGF induced by HBO2 has also been investigated. In this study, however, we did not find VEGF induced by HBO2 in seven days of treatment for two hours a day at 2.4 ATA.

VEGF is a heparin-binding glycoprotein and a most potent endogenous stimulator of both angiogenesis and vascular permeability [16,20]. Several experimental studies have indicated that administration of exogenous VEGF can induce regional angiogenesis and improve the survival of random extensions of skin flaps [13,14]. A lot of controversy has surrounded the angiogenic properties of HBO2. In skin wounds, HBO2 was found to increase the bursting strength and to stimulate angiogenesis histologically [12, 21]. The angiogenic properties promoted by HBO2 are derived from high oxygen tension and may persist for a few hours after the procedure [22]. In this study, the repeated “on-off” exposures produced an environment favorable to random flaps when compared with GS and GNAC.

NAC is a precursor of glutathione, a potent antioxidant that inhibits the induction of pro-inflammatory cytokines, inducible nitric oxide synthase (iNOS), adhesion molecule 1, as well as vascular cell adhesion molecule 1, and stimulates the production of nitric oxide (NO) [4,10,11].

In this study, the flaps treated with HBO2 alone led to a more improved average survival tissue over those treated with a combination therapy with both HBO2 and NAC. This suggests that these agents did not potentiate
each other [12]. The groups treated with distilled water and NAC alone had the worst results in this experiment.

The doses of NAC (300 mg/kg⁻¹/day) herein utilized were chosen in view of the low toxicity of this drug and the favorable results found in the protection of random skin flaps in rat models. The plastic barrier interposed between the flap and the donor site prevents flap revascularization for the local vessels [4].

Most probably, the right concentration of NAC impairs VEGF production, inhibiting angiogenesis and the wound-healing response, either through an imbalance in the cell redox state or through a yet-to-be-determined mechanism [12].

The negative effect of VEGF expression in GHN and GHBO₂ may be the result of an imbalance in the oxidation-reduction environment in the cell, making the cell more reduced; this is incompatible with the normal wound-healing response [16].

The combination of HBO₂ and antioxidant therapy did not improve survival of tissue above and beyond the effect of HBO₂ alone, suggesting that the potential toxic effects of hyperoxia from ROS are not minimized by antioxidant therapy with NAC. There is an understanding that low concentrations of ROS may play a beneficial role in wound healing [2]. Oxidizing species such as free radicals and hydrogen peroxide may serve as cellular messengers mediating complex redox-sensitive processes such as extracellular matrix formation, cytokine action, angiogenesis and cell motility [1, 20, 21].

The expression of VEGF in the epidermis, dermis,
submuscular tissue, and vessels was not significant in the different treatment groups. Therefore, although hypoxia is an essential sign during normal wound healing, this signaling may be detrimental when the wound is surrounded by an ischemic environment [3].

In GHBO2, the presence of cells undergoing apoptosis was significantly lower in the middle third when compared with GS and GNAC. This study confirms a hypothesis that HBO2 decreases apoptosis and VEGF expression at Day 7, possibly due to a relative increase in tissue oxygen tension (Psco2) in the ischemic region in direct proportion to the cumulative numbers of treatments [7-9,16].

Biopsies obtained in areas considered normal and healthy (control) demonstrated an increased number of apoptosis, and although it cannot exclude the deleterious effect of surgical trauma in normal tissues, some authors consider that ischemia may induce apoptosis through hypoxia-related mitochondrial dysfunction in a body area distant from the random flap [22, 23] or by direct activation of caspase via stress-simulated cytokines [24].

**CONCLUSION**

HBO2 is associated with reduced expression of apoptosis, and although NAC is not associated with the reduction of apoptosis, poor results were observed when only NAC was used. The combination of the two procedures did not produce potentiation of the favorable results of using the two separately. The findings suggest that the diffusion of oxygen through the interstitial might be the determining factor of more favorable results of HBO2.
REFERENCES


