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Original Article

Effects of hyperbaric oxygen and N-acetylcysteine in survival of random pattern skin flaps in rats

Fernando P. da Rocha, Djalma J. Fagundes[1], Jefferson A. Pires[2], Fernanda Salim T. da Rocha[3]

Department of Surgery, Catholic University of Pelotas, Pelotas - RS, [1]Division of Operative Technique and Experimental Surgery, Department of Surgery, UNIFESP, [2]President of Academy League of Plastic Surgery Federal University of Pelotas, Pelotas - RS, [3]Academic of Medicine of University Federal of Pelotas, Brazil

Address for correspondence: Prof. Fernando Passos da Rocha, Rua 15 de Novembro, 1597, Centro, Pelotas, RS-CEP 96015-000, Brazil. E-mail: fprocha.sul@terra.com.br

ABSTRACT

Objective: Our aim is to investigate the role of HBO (hyperbaric oxygen), NAC (N-acetylcysteine), and HBO plus NAC on the necrosis area of random rat's skin flaps of a modified McFarlane flap design. **Materials and Methods:** Thirty-two male Wistar rats were randomly divided into four groups: G-S (sham: *n* = 8), G-NAC (NAC: *n* = 8), G-HBO (HBO: *n* = 8), and G-HN (HBO plus NAC: *n* = 8). A rectangular skin flap (2 × 8 cm^2) was dissected from the muscular dorsal layer, preserving the cranial pedicle. Polyethylene film was placed over the muscular layer and an interrupted 3.0 nylon suture was employed to fix the flap into the original place. On the eighth day, full-thickness biopsy samples (2 × 1 cm^2) were collected from the proximal, middle, and cranial areas of the skin flap, and in a site away from the flap labelled as the control area. **Results:** The measurements of necrotic areas in the groups were 18.3% in G-S, 24.3% in G-NAC, 12.6% in G-HBO, and 14.9% in G-HN. Significant difference was observed between the groups G-HBO and G-HN as well as G-NAC. **Conclusion:** HBO is associated with reduced area of necrosis of skin flap. The G-NAC group was associated with poor results when examined in isolation. The association between HBO and NAC did not produce favourable results with respect to the use of HBO alone. These findings suggest that the diffusion of oxygen through the interstitial space was the determining factor of more favourable results of HBO.

KEY WORDS

Acetylcysteine; hyperbaric oxygenation; necrosis; surgical flaps

INTRODUCTION

Advances in plastic surgery have allowed reconstruction of extensive wounds defects. These defects are frequently corrected by the random pattern flaps. The limiting factor in these flaps is the unpredictable blood supply, which, if insufficient, may produce irreversible damages to the microcirculation.

This damage results in partial or complete flap necrosis[1] and renders the wound more susceptible to infection, thereby causing further healing impairment[2]; thus, improving distal blood supply in the random skin flap is an important goal.[1,3]

The cellular damage that occurs during tissue reperfusion after ischemia is the result of a cascade of events involving free-radical oxygen and inflammatory mediators.[4,5] Molecular oxygen plays a central role in the reparative

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healing process and is one of the critical nutrients of the wound.[4] Hyperbaric oxygen (HBO) increases the tolerance of tissue to ischemia and enhances free-radical formation; however, hyperoxia can increase the biochemical defence mechanisms against free radicals[6] and improve the survival probability of ischemic tissue.[2,7]

The HBO has a protective effect on microcirculation possibly by interfering with the deleterious action of activated neutrophils on microvascular endothelium.[8] This killing ability of neutrophils stimulates angiogenesis and enhances fibroblasts activity and collagen synthesis.[4,9]

Mammals have a complex antioxidant system to protect themselves from such stress. One of the most important components of the intracellular antioxidant system is glutathione, a powerful active radical scavenger that is depleted in ischemia-reperfusion (IR) injury.[10] N-acetylcysteine (NAC) is a prodrug that supplies bioavailable cysteine for glutathione replenishment.[9] In the presence of overwhelming active oxygen species (ROS), glutathione levels in the cell decrease. The antioxidant NAC prevents some of these deleterious effects, indicating an involvement of oxidative stress during HBO exposure.[11]

The effects of HBO in improving the survival of a random skin flap are already known. Thus, it is important to study its combination with other substances, such as enzymes, NAC, and so on, to potentiate their effect to be applied to patients. Thus, in this study, our aim is to investigate the role of HBO, NAC, and HBO plus NAC on the necrosis area of random rat's skin flaps of a modified McFarlane[12] flap design.

MATERIALS AND METHODS

Ethical committee

The experimental protocol (#1431/03) was approved by the Ethics Committee of the Federal University of São Paulo (UNIFESP). All the procedures strictly followed the existing regulations about animal experimentation (Brazilian College on Animal Experimentation, COBEA).

Animal housing and groups

Thirty-two male Wistar rats weighing 280-300 g were kept in individual cages in acoustically isolated rooms at 25°C, with artificial illumination as well as chow and water *ad libitum*. The animals were randomly divided into four groups: G-S (Sham group, $n = 8$), G-NAC (NAC, $n = 8$), G-HBO (HBO, $n = 8$), and G-HN (HBO plus NAC, $n = 8$).

Anaesthesia

After 6 h of fasting without solid diet and 4 h without liquid diet, the animals received 5 mg/kg of acepromazin i.m. (Acepran™ 0.2%, Vetnil Indústria e Comércio de Produtos Veterinários Ltd., São Paulo, Brazil). Subsequently, after 10 min, they received a combination of 50 mg/kg of ketamin (Ketalar™, Medical Division of Pfizer do Brazil, São Paulo, Brazil) and 10 mg/kg of xylazin i.m. (Rompum™, Bayer, São Paulo, Brazil).

Surgical procedure

Under general anaesthesia, the dorsal regions were shaved and the animals were fixed in the prone position. A rectangular area (2 × 8 cm) was longitudinally marked with ink, based on 7th cervical vertebra and running to caudal position with the spine as a central landmark. The caudal and lateral marks were incised with scalpel (No. 15), and a flap skin was displaced from the muscular dorsal layer and the cranial portion was preserved from incision [Figure 1]. A polyethylene film was placed over the muscular region, covering all the wound area and acting as a barrier between the skin and muscles [Figure 2]. An interrupted 3.0 nylon suture (Mononylon™, Ethicon, São Paulo, Brazil) was employed to fix the flap into the original place.

NAC and distilled water administration procedures

A dose of 300 mg/kg of NAC (Fluimucil™ acetylcysteine 300 mg/3 mL, Zambon Laboratório Farmacêutico Ltd., São Paulo, Brazil) was intraperitoneally injected into the G-NAC or G-HN groups after the elevation of skin flap, and consecutively for every 24 h for 7 days. On the other hand, distilled water of 1 mL (Distilled water, Isofarma, São Paulo, Brazil) was intraperitoneally injected into the G-S and G-HBO groups after the elevation of skin flap, and consecutively for every 24 h for 7 days.

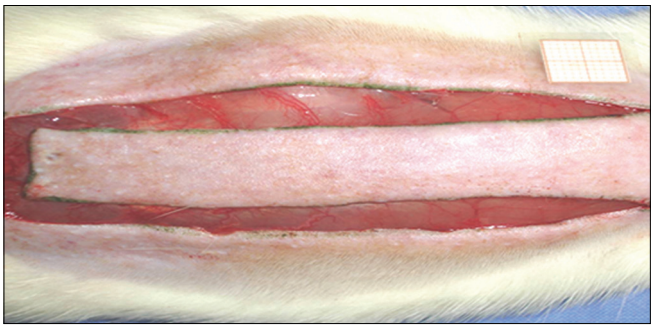

Figure 1: A random skin flap of a modified McFarlane flap design was executed. The caudal and lateral marks were incised with scalpel (No. 15), and a flap skin was displaced from the muscular dorsal layer and the cranial portion was preserved from incision

HBO procedure

HBO procedure was carried out in a hyperbaric chamber for experimental animals[13] of University Regional do Alto Uruguai Campus Erechim (URI). Before pressurization, 100% medical oxygen was flushed through the chamber for 5 min to displace the room air. The oxygen pressure was then increased at a constant rate to reach a pressure of 2.4 Atmosphere. The oxygen concentration was monitored with a calibrated oximeter. The animals were placed at the hyperbaric chamber in random groups. All the animals of G-HBO and G-HN groups were exposed to 100% oxygen at 2.4 ATA for 2 h (once a day), 15 min after flap fixation, and every 24 h for the consecutive 7 days.

Sequential daily procedures

The sequence of procedures in each group is summarized in Figure 3. The animals in G-S (n = 8) received distilled water intraperitoneally 15 min after flap elevation for 7 consecutive days; those in G-NAC (n = 8) received 300 mg/kg of NAC intraperitoneally after flap elevation for 7 consecutive days; those in G-HBO (n = 8) were exposed to 100% oxygen at 2.4 ATA for 15 min following the delay procedure and for 2 h a day each for 7 consecutive days in a hyperbaric animal experimental chamber flushed with 100% oxygen, and distilled water was intraperitoneally injected; and those in G-HN (n = 8) received the combination of NAC and HBO for 7 consecutive days.

Seven days follow-up

Every day (twice a day), all the animals were examined and the occurrence of fever, incision infection, and liquid stools or refuse of the chow or drinking water were recorded. Once any sign of severe suffering was identified, the veterinarian interrupted the research and the animals were euthanized.

On the eighth day, the animals were anaesthetized and fixed in the prone position. The dorsal area was photographed from a standard distance by a 7.2-mega pixel digital camera (Sony™ P-200, Sony, Japan), and the images were saved in JPEG format.

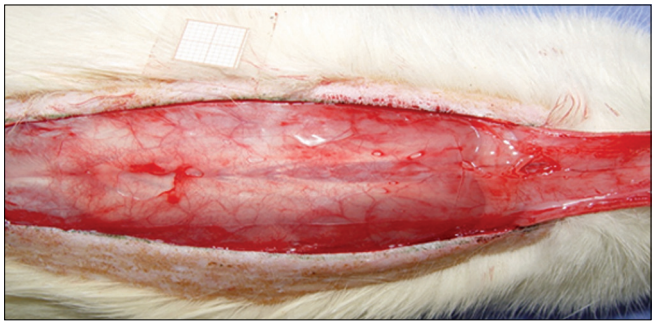

Figure 2: A polyethylene film was placed over the muscular region, covering all the wound area and acting as a barrier between the skin and muscles

Euthanasia

Under anaesthesia and after the collection of samples, the animals were put in a chamber and flushed with CO_2 until cardiorespiratory arrest.

Determination of flap necrosis area

On the eighth postoperative day, the flap area was photographed and compared with that recorded on the first day of the experiment. Flap necrosis was defined by dark colour and eschar formation. The photographs were captured by the computer software Image Pro Plus 4.5™. Mean flap necrosis area was then assessed for all groups. All the results are represented as mean and standard deviation.

Statistical analysis

The areas of flap necrosis were expressed as mean and standard deviation (SPSS 11.0 version). Significance

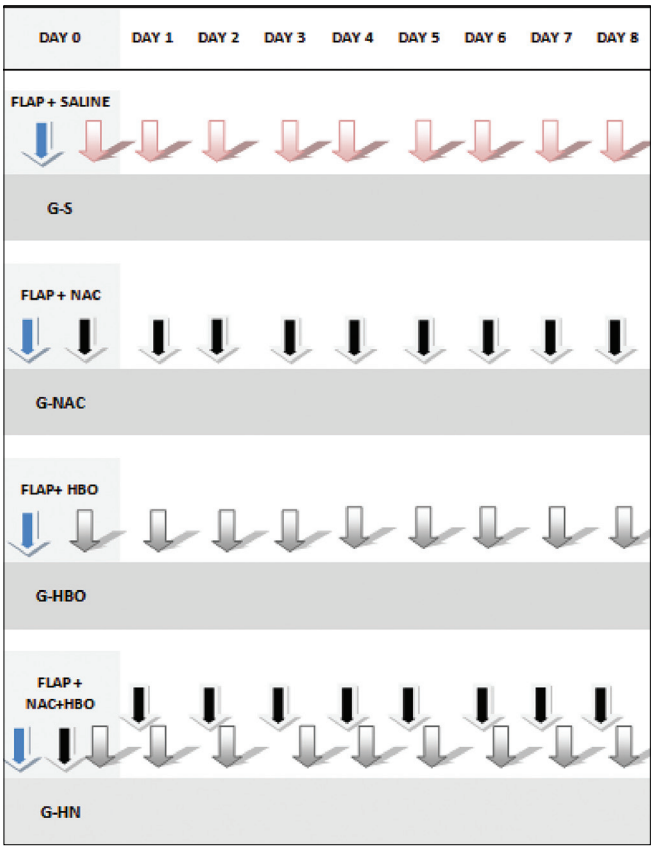

Figure 3: Flowchart of procedures. G-S: Flap proceedings and eight daily intraperitoneal saline injections. G-NAC: Flap proceedings and eight daily NAC intraperitoneal injections. G-HBO: Flap proceedings and eight daily HBO sessions. G-NH: Flap proceedings and eight daily injection of NAC, followed by HBO

of differences in necrosis was determined by one-way analysis of variance (ANOVA), applying post-hoc test of Bonferroni. A P value of 5% ($P < 0.05$) was considered as statistically significant.

RESULTS

In Figure 4, the mean of necrosis (%) is shown by groups. The average flap necrosis area was 18.3% in G-S, 24.3% in G-NAC, 12.6% in G-HBO, and 14.9% in G-HN group.

In Table 1, the results of macroscopic evaluation (area in mm^2) for each group are presented.

G-HBO group showed a smaller area of flap necrosis, when compared with G-S, but was not significant ($P = 0.12$); however, when compared with the G-NAC group, a significant difference was noted ($P < 0.01$). The flap survival in G-HBO was the same as that noted in G-HN, and when compared with the G-NAC, the G-HN group showed significant difference ($P < 0.01$).

In this study, HBO therapy alone led to better survival of skin flaps. However, the combination of NAC and HBO, in contrast to the expectations, had no better survival, when compared with administration of HBO alone.

Table 1: Means, standard deviation, and range of necrosis area (mm^2) in the skin flaps of G-S, G-NAC, G-HBO, and G-HN on day 8

Group	*N*	Mean	Standard deviation	Range
G-S	8	0.18	0.05	0.11-0.28
G-NAC	8	0.24[a, b]	0.05	0.18-0.32
G-HBO	8	0.13[a]	0.04	0.07-0.20
G-HN	8	0.15[b]	0.04	0.07-0.22
Total	32	0.18	0.06	0.07-0.32

ANOVA test [a]G-HBO<G-NAC (P<0.01) [b]G-HN<G-NAC (P=0.002)

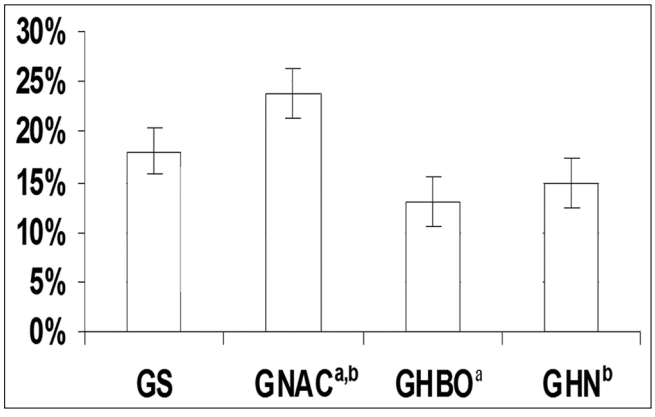

Figure 4: Means and standard deviation of the percentage of necrosis on the random skin flaps in G-S, G-NAC, G-HBO, and G-HN groups. The means of necrosis in all the experimental groups were lesser than those of the G-S group. There was a significant difference between G-HBO < G-NAC ($P < 0.01$) and G-HN < G-NAC ($P < 0.001$) (ANOVA test)

DISCUSSION

There has been much controversy over the angiogenic properties of HBO. In skin wounds, HBO was found to increase the breaking strength and stimulate angiogenesis histologically.[2,3] The angiogenic properties induced by HBO are derived from the increase in oxygen tension that may persist for several hours after HBO.[6,8] Comparison of G-S and G-NAC groups revealed that repeated "on-off" exposures produce a favourable environment in random flaps.

In this study, HBO administered after surgery improved the survival of random skin flaps. This finding is in contrast to the original hypothesis that suggests that extra oxygen increases the production of free radicals that would increase flap necrosis.

Another prominent hypothesis suggests that HBO treatment causes peripheral arteriolar vasoconstriction. Theoretically, this counteracts postischemia reflex vasodilatation, and decreases interstitial fluid and oedema.[3,7]

Hong JP[14] showed that when hypoxic tissue is exposed to HBO, there is an increase in pO_2 in the plasma and a reduction in pO_2, and the rate of diffusion of oxygen increases.

NAC is a precursor of glutathione, a potent antioxidant that inhibits the induction of pro-inflammatory cytokines. Glutathione also induces the production of nitric oxide synthase (iNOS) as well as adhesion molecule 1. This antioxidant also induces the production of a vascular cell adhesion molecule 1[10,15-17] and stimulates the production of nitric oxide (NO).[18]

In this study, the flaps treated with HBO alone led to improvement in the average survival than those treated with a combination therapy with both HBO and NAC, suggesting that these agents do not potentiate one another. The groups treated with distilled water and NAC alone exhibited the worst results in this experiment.

The doses of NAC (300 mg/kg/day) utilized in this study were chosen owing to the reported low toxicity of this

drug and favourable results in the protection of random skin flaps in rat model. The plastic barrier interposed between the flap and donor bed was observed to prevent flap revascularization through bed vessels.[19]

HBO in association with NAC may be responsible for a protective effect on the deleterious outcome of NAC. When NAC is used alone, the measures of necrotic areas were found to be higher in the G-NAC (24%). On the other hand, with the combination of HBO and NAC, the measures of necrotic areas were significantly better (15%) ($P < 0.01$).

Probably, in this study, the correct concentration of NAC could have inhibited angiogenesis and wound-healing response. This inhibition is considered to occur through an imbalance in the cellular redox state or an undetermined mechanism.[9]

It has been demonstrated that HBO can increase the tolerance of tissue to ischemia, diminish metabolic disturbances, improve tissue microcirculation, and reduce platelet aggregation. These characteristics, combined with the ability of plasma to carry dissolved oxygen to areas where red blood cells cannot reach, have been shown to have a beneficial effect on oxygenation of many hypoxic tissues.[2,20]

In the present study, the combination of HBO and antioxidants therapy did not improve survival above and beyond the effect of HBO administered alone, suggesting that the potential toxic effects of hyperoxia from ROS were not minimized by antioxidant therapy with NAC. Low concentrations of ROS are understood to play a beneficial role in wound healing.[6] 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.[9]

Knight *et al*. demonstrated in rabbits that high dose of NAC has no significant difference in the survival of flaps.[21]

Furthermore, Ramon *et al*. showed an increase in the survival of the transverse rectus abdominis myocutaneous flap treated with HBO alone.[22]

Tomur *et al.* demonstrated in rats that HBO associated with vitamin E and C could improve the survival of epigastric island skin flaps,[23] while Rocha *et al.* reported that HBO alone showed a protective effect in the ischemic skin flap, which was associated with reduced expression of apoptosis.[24]

However, despite these results, multicenter prospective clinical studies are clearly needed to compare HBO treatment with other mechanical or pharmacologic interventions, to improve wound healing for grafting or to support flap survival.[25]

CONCLUSION

With the experimental model, it can be concluded that HBO produced an improvement in the distal blood supply of the random skin flap; however, this improvement was not significant.

Furthermore, the effect of combination with NAC was not significantly different on the survival of flap, when compared with the HBO therapy alone.

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