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Effect of HMGB1/NF-κB in hyperbaric oxygen treatment on decreasing injury caused by skin flap grafts in rats

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Abstract. – BACKGROUND: Skin flap grafting (SFG) is a common surgical operation, and hyperbaric oxygen treatment (HBOT) is an important strategy for restoring the grafted skin flap. Thus, we employed a rat skin flap grafting model treated with HBO, and expression levels of high mobility group protein 1 (HMGB1) and NF-kappaB (nuclear factor-kappaB) were characterized.

MATERIALS AND METHODS: Forty rats were randomly assigned to 5 groups: (1) sham-operation (SH), (2) ischemia followed by reperfusion 3 days after operation (IR3d), (3) ischemia followed by reperfusion 5 days after operation (IR5d), (4) ischemia followed by reperfusion and HBOT 3 days after operation (HBO3d), and (5) ischemia followed by reperfusion and HBOT 5 days after operation (HBO5d). Elevated pedicled skin flaps were designed (size, 9 cm × 6 cm), and feeding vessels were clamped. The microvascular clamp was removed 3 h later and flow was restored. In the HBO3d and HBO5d groups, rats received 1 h of hyperbaric oxygen (HBO) starting immediately after surgery for 3 days and 5 days, respectively. Upon completion of animal experiments, rats were euthanized by general anesthesia, and blood samples were taken for testing. The tissues were sectioned for western blotting and immunohistochemical staining.

RESULTS: Expression of HMGB1 and NF-κB proteins in the HBO groups was lower than in the IR groups.

CONCLUSIONS: The results suggest that HBOT can be used to reduce ischemia-reperfusion (IR) injury of skin flap grafts.

Key Words:
Hyperbaric oxygen treatment, Skin flap, HMGB1, NF-B.

Introduction

The skin flap graft is a form of transplantation used in reconstructive surgery. Skin flap graft surgery is successful in 90-95% of cases; however, some cases may result in partial or complete flap loss. Skin flap injury is a major consequence of ischemia-reperfusion (IR) injury[1-3]. Major mechanisms for IR injury are thought to include oxygen free radical formation and leukocyte-mediated inflammation[4]. Previous studies of hyperbaric oxygen (HBO) treatment have revealed its beneficial effects for treating IR injuries such as ischemic stroke, myocardial infarction, and transplantation[5-7]. Kayvan et al[8] isolated neutrophils from rat gracilis muscle flaps and incubated the neutrophils with HBO on cover slips[8]. The results showed that hyperbaric oxygen significantly reduced neutrophil adhesion. However, further investigation is needed to characterize the molecular mechanisms and signaling pathways contributing to the HBO effect in IR injury.

Several recent studies have shown that HMGB1 (high mobility group box 1) and NF-κB (nuclear factor-kappaB) are major protagonists of IR pathogenesis. NF-κB is a common transcriptional activation factor; its activation can induce the release of tumor necrosis factor (TNF) and interleukin (IL)-1)/IL-6, followed by the inflammatory process. Chen et al[9] found mutual promotion between NF-κB and IL-1 expression in rats during global cerebral ischemia-reperfusion (GCIR). HMGB1 acts as a late-acting distal inflammatory mediator during injury[10]; active release of HMGB1 occurs later than that of the early cytokines IL-1 and TNF. Active release of HMGB1 from monocytes can subsequently activate the release of several different cytokines, producing a cascade effect and increasing the severity of IR injury[11-13].

We explored the molecular mechanisms of the HBOT effect on IR injury through the initiating factor NF-κB and later the inflammatory mediator-HMGB1, as well as the relationship between these molecules. We examined whether HBOT can prevent release of HMGB1 and NF-κB.

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Materials and Methods

Experimental Animals

All experiments were performed in accordance with the ethical guidelines by the Committee for the Control and Supervision of Experiments on animals at Capital Medical University (Beijing, China). Healthy adult male Sprague-Dawley rats (250-300 g at the beginning of the study) were used. Rats were maintained at 25°C ± 1.0°C with a 12-h dark/light cycle and given food and water *ad libitum*.

Experimental Groups

Forty rats were randomly assigned to 1 of the following 5 groups and subjected to the following treatments: sham-operation (SH; 21% O_2 at 1.0 ATA: atmosphere absolute) (n = 8), ischemia followed by reperfusion 3 days after operation (IR3d; 21% O_2 at 1.0 ATA) (n = 8), ischemia followed by reperfusion 5 days after operation (IR5d; 21% O_2 at 1.0 ATA) (n = 8), ischemia followed by reperfusion and hyperbaric oxygen treatment 3 days after operation (HBO3d; 100% O_2 at 2.0 ATA) (n = 8), or ischemia followed by reperfusion and hyperbaric oxygen treatment 5 days after operation (HBO5d; 100% O_2 at 2.0 ATA) (n = 8).

The Epigastric Pedicle Skin Flap Model

All procedures were performed aseptically under anesthesia using intraperitoneal injections of 10% chloral hydrate at a dose of 350 mg/kg. The rats were fixed on wooden shelves after shaving and washing the abdomen. Single inferior epigastric vessel pedicled skin flaps were designed and elevated (9 cm × 6 cm). Skeletonization of the right inferior epigastric artery and vein pedicle was performed while the contralateral inferior epigastric vessel was suture-ligated; feeding vessels were clamped using a microvascular clamp to achieve ischemia. For reperfusion, the microvascular clamp was removed 3 h later and flow was restored. The flaps were repositioned above a silicone sheet (on the same area as the flap) to prevent vascular supply other than the pedicle using continuous 5-0 monofilament nylon sutures. The sham-operated group underwent the same operation but was not exposed to ischemia. All rats received a single dose of an intramuscular injection of 0.8 mg/g penicillin sodium postoperatively.

Hyperbaric Oxygen Treatment

In the HBO3d and HBO5d groups, rats were placed into a custom-made pressure chamber of transparent acrylic plastic (701 Space Research Institute, Beijing, China) immediately after surgery and received 1 h of HBO therapy at 2.0 ATA with 100% O_2 twice per day (at 8-h intervals) for 3 days and then daily for 2 consecutive days. Compressed air was supplied at a rate of 1 kg/cm^2/min to 2.0 ATA/100% oxygen and maintained for 60 min. The chamber was flushed with 100% oxygen at a rate of 5 L/min to avoid carbon dioxide accumulation. Decompression was performed at 0.2 kg/cm^2/min. During HBO exposure, oxygen and carbon dioxide contents were monitored continuously and maintained at ≥ 98% and at ≤ 0.03%, respectively. The chamber temperature was maintained between 22°C and 25°C. To minimize the effects of diurnal variation, all HBO exposures were started at around 8:00 AM and 4:00 PM. For the SH, IR3d, and IR5d groups, the rats were treated postoperatively with normobaric air at 1.0 ATA in 21% oxygen at an ambient temperature of 22-25°C.

Flap Measurements

Flaps were evaluated on postoperative days 3 and 5. The surviving area of the flap was determined grossly based on its appearance, color, and texture. Outlines of viable and nonviable areas were traced using transparent film; the film was subsequently scanned. Using Image Pro Plus Software (version 6.0, Media Cybernetics LP, Silver Spring, MD, USA) the viability of each flap was calculated. Results are expressed as a percentage relative to the total flap surface area.

Histologic Analysis

Each flap was evaluated 3 days and 5 days after operation. For each flap, 3-4-μm sectioned tissue blocks from the viable region were fixed in a standard manner in 10% formalin and embedded in paraffin for hematoxylin-eosin staining. Next, images were obtained using an Olympus BX51 microscope with a 40× objective (Tokyo, Japan). According to Marty Zdichavsky's score for skin injury[41] and Rongione's histological score for acute pancreatitis[42], the degree of microscopic injury was scored based on the following histological changes: congestion, epidermis edema, and leukocyte infiltration. Injury severity was graded for each variable: no injury = 0; injury to 25% of the field = 1; injury to 55% of the field = 2; injury to 75% of the field = 3; and diffuse injury = 4. All evaluations were performed in a double-blinded manner.

Immunohistochemistry Staining

Histological sections of tissues, 3-4-μm thick, were obtained, fixed in 10% formalin, and paraffin-embedded. Sections were deparaffinized in xylene and rehydrated in ethanol, and endogenous peroxidase was blocked by immersion in methanol containing 0.3% hydrogen peroxidase for 20 min. Before incubation, the sections were permeabilized and blocked with normal goat serum. The sections were incubated overnight at 4°C with their respective primary antibodies (Histostain-Plus Kit, Sunbio, Beijing, China). On the following day, sections were incubated with secondary antibodies and horseradish enzyme markers for 10-15 min, followed by staining with diaminobenzidine. The slides were examined using a Nikon i50 microscope (Tokyo, Japan). The proportion of positively stained cells was calculated as the number of positive cells divided by total cell number.

Protein Preparation

Flap tissues were frozen in liquid nitrogen and stored at –80°C until analysis. The tissue was homogenized in ice-cold isolation solution containing 250 mmol/L sucrose, 10 mmol/L triethanolamine, 1 μg/mL leupeptin, and 0.1 mg/mL phenylmethylsulfonyl fluoride. Homogenates were centrifuged at 12,000 rpm for 10 min at 4°C to separate incompletely homogenized tissue. The supernatants were collected and protein concentrations were measured using a protein assay kit (Sunbio, Beijing, China). For deglycosylation of proteins, an N-glycosidase F Deglycosylation Kit (Roche, Mannheim, Germany) was used.

Western Blotting

Total proteins (50 μg/sample) were diluted in 5× loading buffer (0.25 mol/L Tris-HCl, pH 6.8; 10% sodium dodecyl sulfate; 0.5% bromophenol blue; 50% glycerol; 0.5 mol/L dithiothreitol) and boiled for 5 min. Sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) electrophoresis was carried out on 12% gradient gels. The proteins were electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes that had been pre-treated with methanol; membranes were blocked for 1 h at room temperature in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) containing 5% nonfat dry milk. Membranes were incubated overnight at 4°C with anti-HMGB1 antibody (1:100, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and anti-NF-κB antibody (1:500, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) in TBS-T containing 5% nonfat dry milk. After washing in TBS-T, the membranes were incubated with horseradish peroxidase (HRP)-labeled anti-rabbit antibody (1:3,000, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) for 2-3 h at room temperature. The blots were developed using enhanced chemiluminescence agents (ECL Plus, Sunbio, Beijing, China) before exposure to X-rays. To confirm equivalent sample loading, the same membranes were incubated with anti-β-actin antibody (1:300, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and visualized using enhanced chemiluminescence as described. For quantification, films of the western blots were scanned using a Minolta scanner and Adobe Photoshop software. Labeling density was quantitated using Lab Works software (UVP, Upland, CA, USA). The value of the relative densities of HMGB1 and NF-κB bands was normalized to the density of actin to represent the amounts of HMGB1 and NF-κB protein.

Statistical Analysis

Statistical analysis was performed using the SPSS 15.0 (SPSS Inc., Chicago, IL, USA) statistical program. All quantitative data were expressed as mean ± SD (standard deviation). One-way analysis of variance was used to test the differences in HMGB1 and NF-κB western blots and survival area. A value of $p < 0.05$ was considered statistically significant. Relationships between skin injury scores and expression of HMGB1 or NF-κB were analyzed by calculating Pearson product-moment correlation coefficients. A value of $p < 0.05$ was considered statistically significant.

Results

HBO Therapy Increases Skin Graft Survival

The average area of flap necrosis was 23.5% in group SH and 56.2% in group IR3d. Group IR5d had an average flap necrosis of 66.8%; average necrotic areas in the HBO3d and HBO5d groups were 45.7% and 36.8%, respectively. Groups HBO3d and HBO5d had significantly lower average areas of flap necrosis than groups IR3d and IR5d, respectively ($p < 0.05$) (Figure 1). This shows that HBOT effectively decreased the necrosis rate of skin flaps after grafting.

Figure 1. Flap necrosis area was assessed on postoperative days 3 and 5 using ipp6.0 software to estimate the ratio of the necrotic (brown) and total flap areas in the photograph. Necrosis area is expressed as a percentage of the total flap area.

HBO Therapy Attenuates Ischemia Reperfusion Injury after Skin Flap Graft

After grafting, the skin flaps from IR groups showed edema, effusion, and necrosis; the color of skin flap became dark purple. However, skin flaps of the HBO groups were dry and showed low levels of effusion. The color of the skin flap became light purple or pink. Histological examination of the skin flap sections from the IR groups showed marked edema and congestion (Figure 2). Injury scores, including scores for congestion, epidermis edema, and leukocyte in-

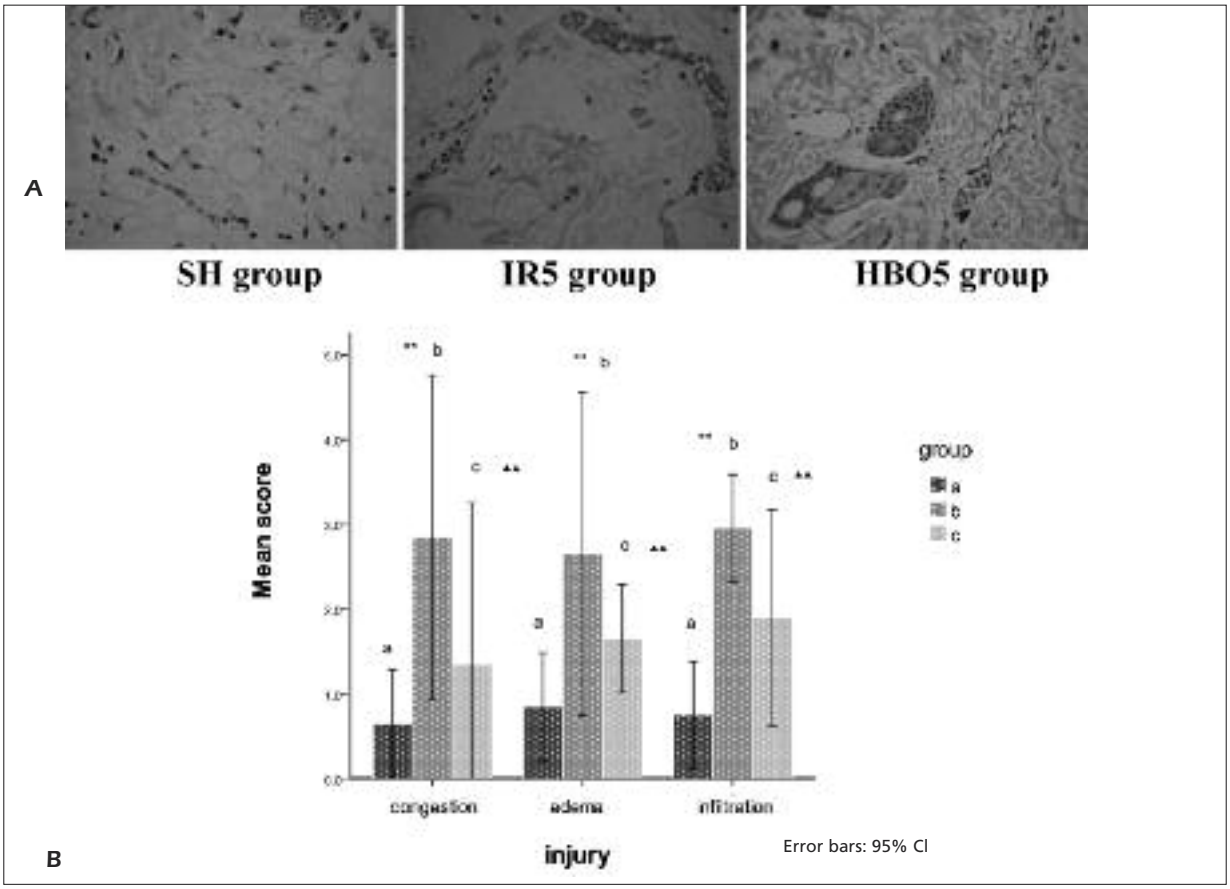

Figure 2. ***A,*** Illustrative skin flap microscopic pictures: SH group rats (*left*), IR5 group rats (middle), HBO5 group rats (*right*). ***B,*** Value of IR injury for SH group (a; n = 8), IR5 groups (b; n = 8), HBO5 groups (c; n = 8). Data are presented as mean ± SD, $^{**}p < 0.05$ or $▲▲p < 0.01$ for SH versus IR5 group or SH versus HBO5 group or IR5 group versus HBO5 group.

filtration, were significantly increased in the IR groups compared with values in the SH groups. Compared with the IR groups, injury scores for animals in the HBO groups were significantly lower. These results indicate that HBO treatment (HBOT) can attenuate IR injury of skin flaps after grafting.

HBOT Attenuates Expression of HMGB1 or NF-κB in Skin Flaps

Both western blotting (Figure 3) and immunohistochemical staining (Figures 4 and 5) showed that expression of NF-κB and HMGB1 in the skin flap tissue in IR groups was significantly higher than in the SH group ($p < 0.01$). A significant difference was observed in expression of HMGB1 and NF-κB proteins between the IR groups and corresponding HBO groups. Levels of HMGB1 and NF-κB proteins were decreased in the HBO groups compared with the corresponding IR groups ($p < 0.01$).

Positive Correlation between Skin Injury Score and HMGB1 or NF-κB Expression

Pearson correlation analysis showed that according to western blot results, there was a positive correlation between acute skin injury scores (epidermis edema, leukocyte, and congestion scores) and HMGB1 or NF-κB expression (Table I). Thus, HMGB1 or NF-κB release increased during IR injury.

Discussion

The use of skin flap grafts in reconstructive surgery has increased significantly. Various studies have shown that HBOT can improve the survival rate of grafted skin flaps after operation[6,14-16]. HBOT can improve oxygen partial pressure and oxygen reserves of the grafted skin flap, as well as relieve the hypoxic state of a grafted skin flap before blood circulation has been established.

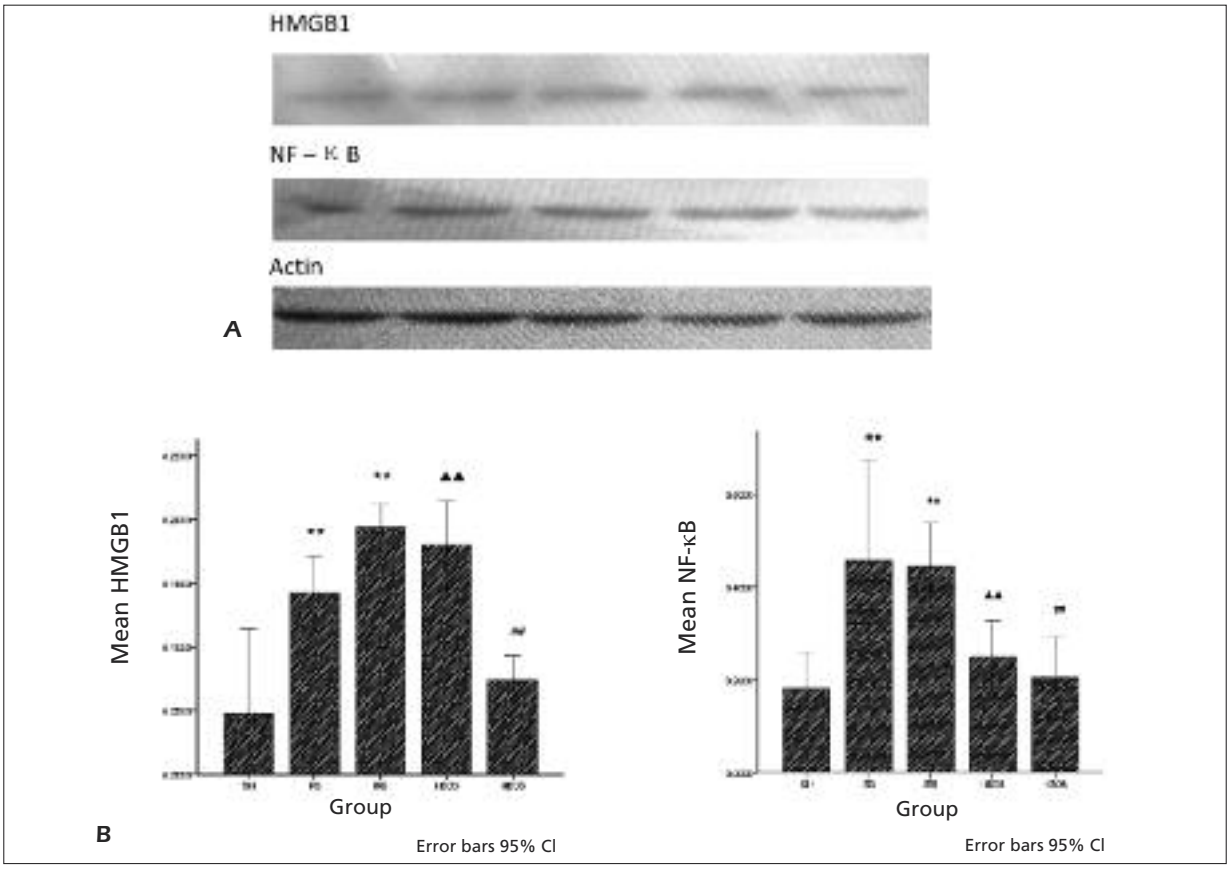

Figure 3. ***A-B,*** Expression of HMGB1 and NF-κB protein in the SH group (n = 7), IR groups (IR3 group, IR5 group, n = 7), and HBO groups (HBO3 group, HBO5 group, n = 7), detected using western blot analysis. Data are presented as mean ± SD, $^{**}p < 0.05$ for SH versus IR3 group or SH versus IR5 group $^{▲▲}p < 0.05$ or $^{\#\#}p < 0.01$ for IR3 group versus HBO3 group or IR5 group versus HBO5 group.

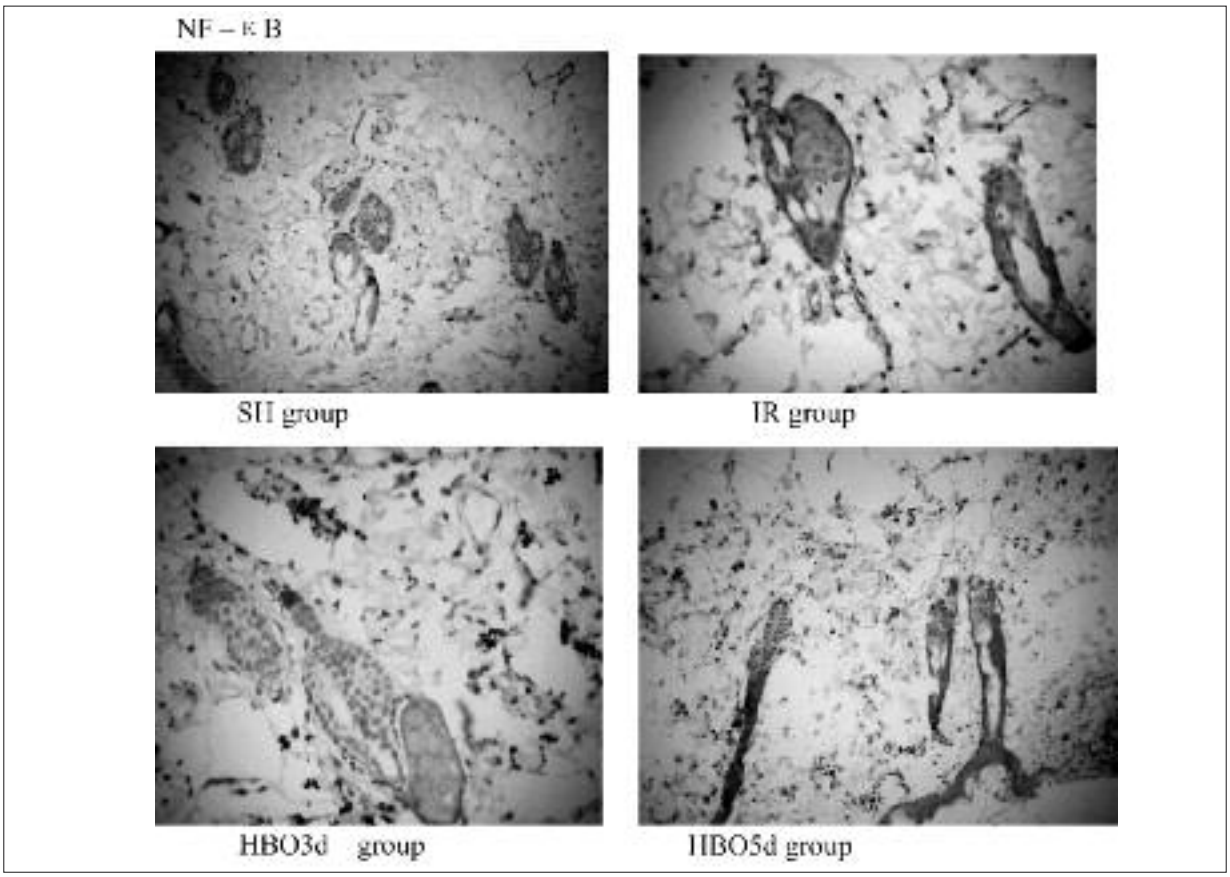

Figure 4. Immunohistochemical localization of NF-κB protein. The percentage of positive staining in the SH group was 15.2%. The percentages of positive staining in the IR3d and IR5d groups were 41.2% and 52.7%, respectively. The percentages of positive staining in the HBO3d and HBO5d groups were 34.5% and 27.2%, respectively.

However, the molecular mechanics of HBOT in grafted skin flaps remain unclear. In this study, we showed HBOT influences IR injury after skin flap graft by altering expression of HMGB1and NF-κB.

IR injury is the main cause of flap loss. This can induce a cascade of pathophysiological changes, namely, neutrophil influx, interstitial edema, and increased permeability, and lead to further tissue damage after surgery, eventually leading to skin flap necrosis[17,18]. In this study, we designed inferior epigastric vessel pedicled skin flaps, in which the feeding vessels were clamped and removed 3 h later, to achieve ischemia/reperfusion. During ischemia after surgery, anaerobic metabolism increases, inducing production of pro-inflammatory cytokines; more importantly, following reperfusion, reactive oxygen species (ROS) are produced[19]. Marieke et al[20,21] found that during ischemia, and particularly during reperfusion, ROS are produced, which initiate IR damage[20,21]. ROS induces cytotoxicity, resulting in increased lipid peroxidation, altering membrane protein function and causing endothelial cell swelling[22,23]. Next, adhesion molecules are activated, leading to leukocyte infiltration[24].

It is known that antioxidants can prevent tissue damage by neutralizing ROS[25,26]. Many previous studies have shown that administration of various antioxidants improves skin flap survival[27,28]; for example, reduced glutathione (GSH) is an endogenous antioxidant that donates electrons to free radicals to prevent tissue damage. Furthermore, Yu et al[29] performed a study to determine whether preconditioning rats with HBO could protect the liver from IR injury by increasing the concentration of the antioxidants GSH and superoxide dismutase (SOD) to prevent membrane lipid peroxidation induced by hydroxyl radicals[29]. HBOT may mitigate IR after surgery by neutralizing ROS and further inhibiting subsequent release of inflammatory mediators and pathways, particularly HMGB1 and NF-κB.

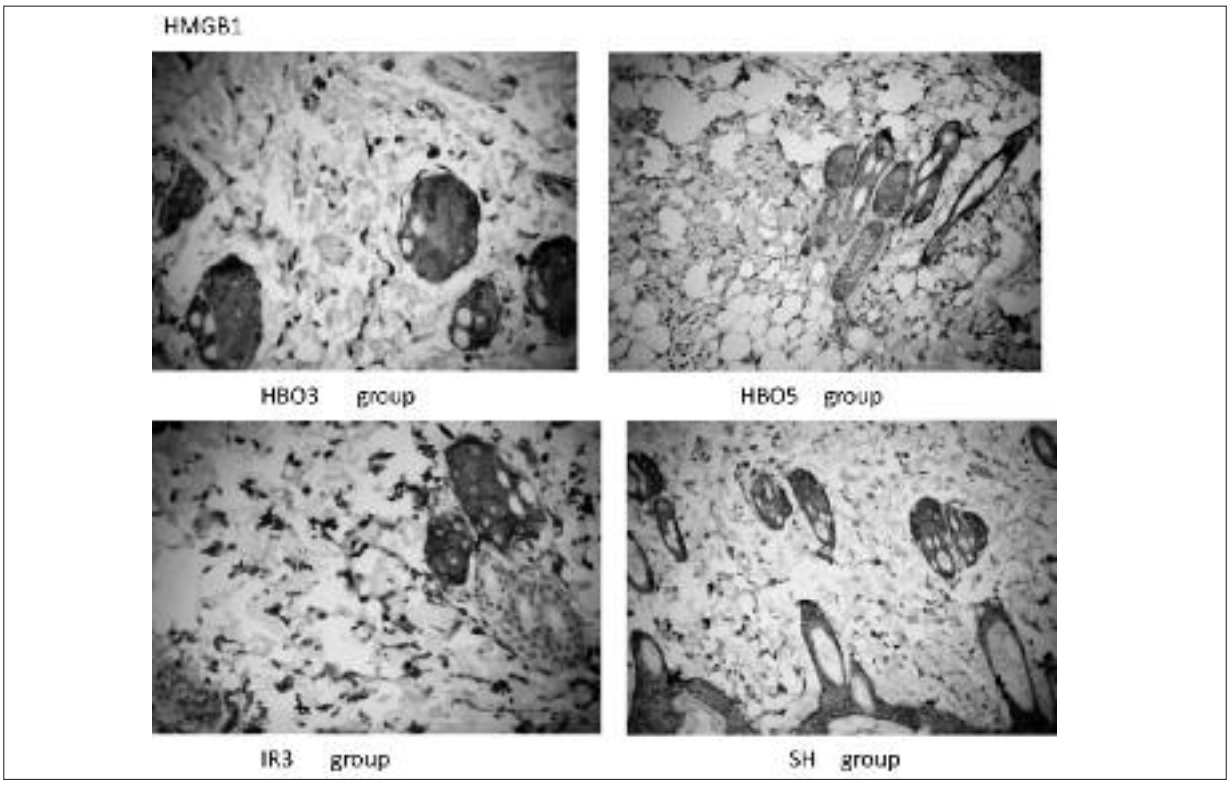

Figure 5. Immunohistochemical localization of HMGB1 protein. The percentage of positive staining in the SH group was 18.5%. The percentages of positive staining in the IR3d and IR5d groups were 43.7% and 50.3%, respectively. The percentages of positive staining in the HBO3d and HBO5d groups were 38.5% and 30.2%, respectively.

It has been demonstrated high HMGB1 expression is associated with severe IR injury. As shown in this study, expression of HMGB1 protein was higher in the IR groups. Pearson correlation analysis revealed a positive correlation between skin flap injury scores and protein expression of HMGB1 according to immunohistochemical staining and western blot analysis.

A previous study reporting the beneficial effects of HBOT examined the decreased levels of HMGB1 in patients with severe cerebral injury[30].

Table I. Pearson correlation analysis between injury scores and HMGB1 and NF-κB protein expression.

	Edema	Congestion	Leukocyte infiltration
HMGB1			
R	0.672[a]	0.515[b]	0.716[a]
P	0.001	0.017	0.009
NF-κB			
R	0.626[a]	0.756[a]	0.687[b]
P	0.000	0.005	0.023

[a]$p < 0.01$; [b]$p < 0.05$.

We also observed that HMGB1 expression in the HBO groups was lower than that in the corresponding IR groups. This indicates that HBOT can significantly relieve IR injury after skin flap grafting by downregulating HMGB1 expression. During the early stage of IR, ischemia and tissue damage lead to the passive release of endogenous HMGB1, which functions as an inflammatory mediator[31]. It is known that HBOT can reduce ROS production, protect membrane proteins from lipid peroxidation, and relieve endothelial cell swelling and necrosis. Therefore, we hypothesized that HBOT may reduce the induction of endothelial cell injury by ROS, thus decreasing the passive release of HMGB1.

Our study also showed that expression of NF-κB in the IR groups was much higher than in the SH group, while it was lower in the HBO groups than in the corresponding IR groups. NF-κB is an initiating inflammatory cytokine related to HMGB1. It is inactive in the cytoplasm due to the presence of its inhibitory protein IκB. When injury occurs, HMGB1 is passively released from endothelial cells, combined with Toll-like receptors (TLRs), and through MyD88-dependent

pathways, it activates NF-κB. Activated NF-κB can translocate from the cytoplasm into the nucleus through nuclear pores. It combines with the K structure area of target genes, resulting in transcription of the corresponding genes encoding inflammatory mediators such as TNF and IL-6[32-34]. HBOT decreases the passive release of HMGB1, inhibits its interaction with TLRs, cuts off signal transduction pathways, and results in decreased activation of NF-κB. Xu et al[35] also demonstrated that HBO can significantly inhibit the expression of NF-κB[35].

Importantly, we also found that HBOT inhibited not only the passive release of HMGB1 but also the active release of HMGB1 during IR injury after skin flap grafting. A previous study showed that levels of HMGB1 increase within 1 h after reperfusion and remain elevated for 72 h due to the active release of HMGB1 from macrophages[36]. We observed in rats that the necrotic area of HBO groups was smaller than that of the corresponding IR group, particularly the HBO5d group. HMGB1 expression in the HBO5 group significantly decreased compared with the HBO3d group. The result was not only consistent with the proposed role of HMGB1 but also demonstrated that HBOT primarily inhibits the active release of HMGB1. HBOT inhibits the activation of NF-κB and decreases the release of the cytokines TNF and IL-6. This prevents active release of HMGB1 from macrophages and monocytes[37-39]. If HMGB1 were actively released into the intravascular space, amplification of the inflammatory response would occur due to the increased release of cytokines and chemokines and interactions with endothelial cells, resulting in positive feedback[40]. Because HBOT downregulates HMGB1 expression, the "waterfall effect" produced by HMGB1 can be prevented.

Conclusions

Based on the results of this study, HBOT can be used to effectively increase the survival rate of skin flap grafts. Downregulating the initiating factor NF-κB and late inflammatory factors HMGB1 and HBOT influences the entire process of IR injury after skin flap grafting. Whether HBOT applied after grafting is more effective than HBO preconditioning should be examined in future studies.

Conflict of Interest

None.

References

1) Kerrigan CL, Stotland MA. Ischemia reperfusion injury: a review. Microsurgery 1993; 14: 165-175.
2) Siemionow M, Arslan E. Ischemia/reperfusion injury: a review in relation to free tissue transfers. Microsurgery 2004; 24: 468-475.
3) Yoshida WB, Campos EB. Ischemia and reperfusion in skin flaps: effects of mannitol and vitamin C in reducing necrosis area in a rat experimental model. Acta Cir Bras 2005; 20: 358-363.
4) Kuo YR, Wang FS, Jeng SF, Huang HC, Wei FC, Yang KD. Nitrosoglutathione modulation of platelet activation and nitric oxide synthase expression in promotion of flap survival after ischemia/reperfusion injury. J Surg Res 2004; 119: 92-99.
5) Brkic P, Stojiljkovic M, Jovanovic T, Dacic S, Lavrnja I, Savic D, Parabucki A, Bjelobaba I, Rakic L, Pekovic S. Hyperbaric oxygenation improves locomotor ability by enhancing neuroplastic responses after cortical ablation in rats. Brain Inj 2012; 26: 1273-1284.
6) Sterling DL, Thornton JD, Swafford A, Gottlieb SF, Bishop SP, Stanley AW, Downey JM. Hyperbaric oxygen limits infarct size in ischemic rabbit myocardium *in vivo*. Circulation 1993; 88(4 Pt 1): 1931-1936.
7) Cabigas BP, Su J, Hutchins W, Shi Y, Schaefer RB, Recinos RF, Nilakantan V, Kindwall E, Niezgoda JA, Baker JE. Hyperoxic and hyperbaric induced cardioprotection: Role of nitric oxide synthase 3. Cardiovasc Res 2006; 72: 143-151.
8) Khiabani KT, Bellister SA, Skaggs SS, Stephenson LL, Nataraj C, Wang WZ, Zamboni WA. Reperfusion-induced neutrophil CD18 polarization: effect of hyperbaric oxygen. J Surg Res 2008; 150: 11-16.
9) Chen YQ, Liu DH, Yang GT. Expression of nuclear factor kappa B in rats after acute global cerebral ischemia-reperfusion and its significance. J Clin Res 2004; 121: 772-774.
10) Luo L, Xie P, Gong P, Tang XH, Ding Y, Deng LX. Expression of HMGB1 and HMGN2 in gingival tissues, GCFand PICF of periodontitis patients and peri-implantitis. Arch Oral Biol 2011; 56: 1106-1111.
11) Andersson U, Wang H, Palmblad K, Aveberger AC, Bloom O, Erlandsson-Harris H, Janson A, Kokkola R, Zhang M, Yang H, Tracey KJ. High mobility group 1 protein (HMG-1) stimulates pro-inflammatory cytokine synthesis in human monocytes. J Exp Med 2000; 192: 565-570.
12) Yang H, Hreggvidsdottir HS, Palmblad K, Wang H, Ochani M, Li J, Lu B, Chavan S, Rosas- Ballina M, Al-Abed Y, Akira S, Bierhaus A, Erlandsson- Harris H, Andersson U, Tracey KJ. A critical cysteine is required for HMGB1 binding totoll-like receptor 4 and activation of macrophage cytokinerelease. Proc Natl Acad Sci USA 2010; 107: 11942-11947.
13) Pavare J, Grope I, Kalnins I, Gardovska D. High-mobility group box-1 protein, lipopolysaccharide-binding protein, interleukin-6 and C-reactive protein in children with community acquired infections and bacteraemia: a prospective study. BMC Infect Dis 2010; 10: 28.

14) Qi Y, Lin SH, Jiang YH, Zhang GQ. The effect of hyperbaric oxygen therapy in 36 cases of skin flap transplantation. J Rare Uncommon Dis 2009; 16: 30-33.
15) Wong HP, Zamboni WA, Stephenson LL. Effect of hyperbaric oxygen on skeletal muscle necrosis following primary and secondary ischemia in a rat model. Surg Forum 1996; XLVII: 705.
16) Elktzschig HK, Collard CD. Vascular ischemia and reperfusion injury. Br Med Bull 2004; 70: 71-86.
17) Arslan E, Basterzi Y, Aksoy A, Majka C, Unal S, Sari A, Demirkan F. The additive effects of carnitine and ascorbic acid on distally burned dorsal skin flap in rats. Med Sci Monit 2005; 11: 176-180.
18) van den Heuvel MG, Buurman WA, Bast A, van der Hulst RR. Ischaemia-reperfusion injury in flap surgery. Plast Reconstr Aesthet Surg 2009; 62: 721-726.
19) Ozmen S, Ayhan S, Demir Y, Siemionow M, Atabay K. Impact of gradual blood flow increase on ischemia- reperfusion injury in the rat cremaster microcirculation model. J Plast Reconstr Aesthet Surg 2008; 61: 939-948.
20) Aydogan H, Gurlek A, Parlakpinar H, Askar I, Bay-Karabulut A, Aydogan N, Fariz A, Acet A. Beneficial effects of caffeic acid phenethyl ester (CAPE) on the ischaemia-reperfusion injury in rat skin flaps. J Plast Reconstr Aesthet Surg 2007; 60: 563-568.
21) Feng GM, Yang WG, Huan-Tang Chen S, Chu YM, Tsai LM, Chang TM, Mardini S, Chen HC. Periodic alterationsvof jejunal mucosa morphology following free microvascularvtransfer for pharyngoesophageal reconstruction. J Plast Reconstr Aesthet Surg 2006; 59: 1312-1317.
22) Tomur A, Etlik O, Gundogan NU. Hyperbaric oxygenation and antioxidant vitamin combination reduces ischemia-reperfusion injury in a rat epigastric island skin-flap model. J Basic Clin Physiol Pharmacol 2005; 16: 275-285.
23) Reichenberger MA, Heimer S, Schaefer A, Lass U, Gebhard MM, Germann G, Leimer U, Köllensperger E, Mueller W. Adipose derived stem cells protect skin flaps against ischemia-reperfusion injury. Stem Cell Rev 2012; 8: 854-862.
24) Cetin C, Köse AA, Aral E, Colak O, Erçel C, Karaba li Y, Alata O, Eker A. Protective effect of fucoidin (a neutrophil rolling inhibitor) on ischemia reperfusion injury: experimental study in rat epigastric island flaps. Ann Plast Surg 2001; 47: 540-546.
25) Ramires PR, Ji LL. Glutathione supplementation and training increases myocardial resistance to ischemia-reperfusion *in vivo*. Am J Physiol Heart Circ Physiol 2001; 281: 679-88.
26) De Celle T, Heeringa P, Strzelecka AE, Bast A, Smits JF, Janssen BJ. Sustained protective effects of 7-monohydroxyethylrutoside in an *in vivo* model of cardiac ischemia-reperfusion. Eur J Pharmacol 2004; 494: 205-212.
27) Coban YK, Kurutas EB, Ciralik H. Ischemia-reperfusion injury of adipofascial tissue: an experimental study evaluating early histologic and biochemical alterations in rats. Mediators Inflamm 2005; 2005: 304-308.
28) Zaccaria A, Weinzweig N, Yoshitake M, Matsuda T, Cohen M. Vitamin C reduces ischemia-reperfusion injury in a rat epigastric island skin flap model. Ann Plast Surg 1994; 33: 620-623.
29) Yu SY, Chiu JH, Yang SD, Yu HY, Hsieh CC, Chen PJ, Lui WY, Wu CW. Preconditioned hyperbaric oxygenation protects the liver against ischemia-reperfusion injury in rats. J Surgical Res 2005; 28: 28-35.
30) Duan XF, Huang YM, Zhou YQ, Xu JJ, Zhang Q. Study of hyperbaric oxygenation on serum resistin and HMGB1 in patients with severe craniocerebral injury. Chin J Clinicians 2011; 5: 3189-3192.
31) Yang H, Tracey KJ. Targeting HMGB1 in inflammation. Chim Biophys Acta 2010; 1799: 149-156.
32) Chen YQ, Liu DH, Yang GT. Expression of nuclear factor Kappa B in rats after acute global cerebral ischemia-reperfusion and its significance. J Clin Res 2004; 21: 772-774.
33) Qin C, Xiao YB, Zhong QJ, Chen L, Wang XF. Anti-inflammatory effect of erythropoietin pretreatment on cardiomyocytes with hypoxia/reoxygenation Injury and the possible mechanism. Chin J Traumatol 2008; 11: 352-358.
34) Ghosh S, Baltimore D. Activation in vitro of NF-kappa B by phosphorylation of its inhibitor I kappa B. Nature 1990; 344: 678-682.
35) Xu J, Huang XL. Effects of hyperbaric oxygen on the expression of NF-κB and ICAM-1 after focal cerebral ischemia-reperfusion in rats. Chin J Phys Med Rehabil 2005; 27: 259-262.
36) Tsung A, Sahai R, Tanaka H, Nakao A, Fink MP, Lotze MT, Yang H, Li J, Tracey KJ, Geller DA, Billiar TR. The nuclear factor HMGB1 mediates hepatic injury after murine liver ischemia-reperfusion. J Exp Med 2005; 201: 1135-1143.
37) Wang H, Bloom O, Zhang M, Vishnubhakat JM, Ombrellino M, Che J, Frazier A, Yang H, Ivanova S, Borovikova L, Manogue KR, Faist E, Abraham E, Andersson J, Andersson U, Molina PE, Abumrad NN, Sama A, Tracey KJ. HMG-1 as a late mediator of endotoxin lethality in mice. Science 1999; 285(5425): 248-251.
38) Andersson U, Wang H, Palmblad K, Aveberger AC, Bloom O, Erlandsson-Harris H, Janson A, Kokkola R, Zhang M, Yang H, Tracey KJ. HMG-1 stimulates pro-inflammatory cytokine synthesis in human monocytes. J Exp Med 2000; 192: 565-570.
39) Abraham E, Arcaroli J, Carmody A, Wang H, Tracey KJ. HMG-1 as a mediator of acute lung inflammation. J Immunol 2000; 165: 2950-2954.
40) Luan ZG, Zhang H, Yang PT, Ma XC, Zhang C, Guo RX. HMGB1 activates nuclear factor-k B signaling by RAGE and increases the production of TNF-alpha in human umbilical vein endothelial cells. Immunobiology 2010; 215: 956-962.
41) Zdichavsky M, Jones JW, Ustuner ET, Ren X, Edelstein J, Maldonado C, Breidenbach W, Gruber SA, Ray M, Barker JH. Scoring of skin rejection in a swine composite tissue allograft model. J Surg Res 1999; 85: 1-8.
42) Rongione AJ, Kusske AM, Kwan K, Ashley SW, Reber HA, McFadden DW. Interleukin 10 reduces the severity of acute pancreatitis in rats. Gastroenterology 1997; 112: 960-967.