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THERAPY ARTICLES

Expression of inducible nitric oxide synthase in muscle flaps treated with ischemic postconditioning

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Abstract
Background/Objective Preconditioning has been considered promising for the treatment of ischemic flaps. In this study, the therapeutic effect of postconditioning was compared with that of preconditioning during ischemia/reperfusion (I/R) injury, and a role of inducible nitric oxide synthase (iNOS) in postconditioning treatment was also explored.
Methods Sixty rats were randomly divided into four groups with 15 rats in each group. Ischemic injury was induced in a rat's gracilis muscle flap model. Preconditioning and postconditioning were performed respectively on the flaps in the pre-con group and the post-con group. No treatment was given to the flaps in the control group, and flaps without I/R injury were used as a sham control. Muscle viability ratio, histology, and gene expression of iNOS were examined at different time intervals (3, 12, and 18 h).
Results A significantly higher survival ratio was observed in both the pre-con group (78.98±3.39, 62.74±3.7, and 54.42±4.45 %) and the post-con group (77.42±4.14, 59.74±6.67, and 49.52±4.13 %) than the control group (45.22±3.69, 42.44±3.76, and 33.2±3.29 %) at 3, 12, and 18 h postoperatively ($P<0.05$). There was no statistical difference between the pre-con group and the post-con group ($P>0.05$). Histological examination showed delayed and attenuated tissue damage in both the pre-con group and the post-con group when compared to that of the control group. A higher expression of iNOS was observed in both the pre-con group and the post-con group than the control group and the sham group ($P<0.05$).
Conclusions Significant improvement of flap survival could be achieved by both preconditioning and postconditioning treatments; however, better protection could be provided by preconditioning. The higher expression of iNOS may play an important role in the therapeutic effect of postconditioning during I/R injury.

Keywords Ischemic postconditioning · Ischemic preconditioning · Ischemia/reperfusion (I/R) injury · Inducible nitric oxide synthase

Introduction

Muscle flaps have been widely used for reconstruction of large and/or deep anatomic defects for their good blood supply and versatility to provide good bulk of tissue. However, when used as a free flap, it is associated with 5–10 % failure rate, mainly caused by ischemic injury secondary to vascular occlusion after the operation [6]. Even when some salvage measures have been adopted, there is still a failure rate of 10–72 % during salvage surgery, which is caused by both previous ischemic injury and subsequent reperfusion injury [9].

Many studies have been performed to attenuate ischemia/reperfusion (I/R) injury and to increase the viability of the flap. However, these methods have remained limited either by limited practical effects or by many side effects [9, 16, 21]. Preconditioning has been associated with protective effect during I/R injury and is considered promising in the treatment of ischemic flaps [2]. Postconditioning has

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initially been used for the treatment of myocardial ischemia [22]. In contrast to preconditioning, which exerts its effects primarily during ischemic injury, postconditioning exerts its effect during the reperfusion period, and therefore, postconditioning was considered more realistic for clinical application for the "after-injury strategy" [15]. According to recent studies, postconditioning was found to have protective effects on the ischemic flap and could improve survival after I/R injury [7, 19]. However, the mechanism remains unclear.

Inducible nitric oxide synthase (iNOS) belongs to a family of enzymes that catalyze the production of nitric oxide (NO) from L-arginine. It is involved in many physiological and pathological processes. Low levels of iNOS expression have been proven to play an important role in protecting flaps from I/R injury [2, 12, 13].

In this study, the therapeutic effect of postconditioning on ischemic flaps was evaluated by comparing it with preconditioning. The mechanism of postconditioning was also explored with a focus on the role of iNOS.

Materials and Methods

Sixty male Sprague–Dawley rats weighing between 260 and 280 g were used in this study. All of the experimental animals were handled in compliance with the guidelines of the National Research Council for the care and use of laboratory animals. The rats were anesthetized using general anesthesia by inhalation of a mixture of isoflurane and oxygen.

Surgical Procedures

Following induction of general anesthesia, the animals were placed in a supine position. The surgical procedure was performed as described before [19] (Fig. 1a). After preparation of the surgical area, a 5-cm transverse incision was made on the groin region to expose the gracilis muscle. Under a microscope, the vessels from the inguinal ligament were dissected and skeletonized to the distal area. The saphenous vessels and the muscular branches were ligated with 9-0 nylon suture. The gracilis muscle flap was elevated and dissected with blood supply from only the femoral muscular branch. The flap was returned in situ after treatment and the incision was closed with 4-0 silk suture.

Experimental Design

The rats were randomly divided into four groups. (1) Sham group (n=15): the gracilis muscle flap was elevated and was returned in situ without any treatment. (2) Control group (n=15): ischemic injury was induced by clamping the pedicle of the flap for 4 h. The clamp was then for released unlimited reperfusion. (3) Preconditioning (pre-con) group (n=15): preconditioning procedure was performed before the start of 4 h of ischemia as described previously [4]. The protocol involved two cycles of 15 min ischemia and 15 min reperfusion periods. (4) Postconditioning (post-con) group (n=15): after 4 h of ischemia, the postconditioning procedure was started immediately. A cycle of 15 s of full reperfusion, followed by 15 s of complete reocclusion was repeated six times (six cycles, total intervention time is 3 min). Unlimited reperfusion was allowed after that [19]. Five rats in the sham group, control group, pre-con group, and post-con group were randomly sacrificed respectively at 3, 12, and 18 h postoperatively and sampled for the examination of flap viability, histology, and the expression of iNOS.

Flap Viability Assessment (Nitro Blue Tetrazolium Assay)

Five samples were collected from each group for the examination of flap viability using the nitro blue tetrazolium (NBT) assay as previously described [1]. Specifically, the samples were sectioned transversely into 1-mm-thick slices and incubated at 37°C for 30 min in a 0.005 % solution of nitro blue tetrazolium (MP Biomedicals, Solon, OH, USA) in 0.1 M phosphate buffer. The dark stained area was considered viable tissue, while the unstained area was considered nonviable. The viability of the muscle was calculated by the ratio of dark stained area to total area, which was performed by two independent researchers blinded to the treatment group with Image Pro Plus Software (version 6.0, Media Cybernetics).

Histology

Three samples measuring 0.5×0.5 cm were collected from each group at 3 and 18 h, respectively. Samples were first fixed with 10 % formalin and were then dehydrated in 0.1 M phosphate buffer with 5 % sucrose. After preparation, tissues were embedded in OCT compound (Sakura Finetek USA, Inc) and sectioned into 9 μm slices. Slices were stained with hematoxylin and eosin (H&E) and the histological characteristic of the tissue was evaluated under a microscope.

Reverse Transcriptase-Polymerase Chain Reaction Assay

Reverse transcriptase-polymerase chain reaction (RT-PCR) assay was performed to examine the expression of iNOS. The muscle tissue was homogenized in Trizol (Invitrogen). Total RNA was isolated and the concentration of RNA was examined by Nanodrop (Thermo Scientific). The RNA was transcribed into cDNA with ImProm-II™ Reverse Transcription System (Promega, USA) and the semiquantitative

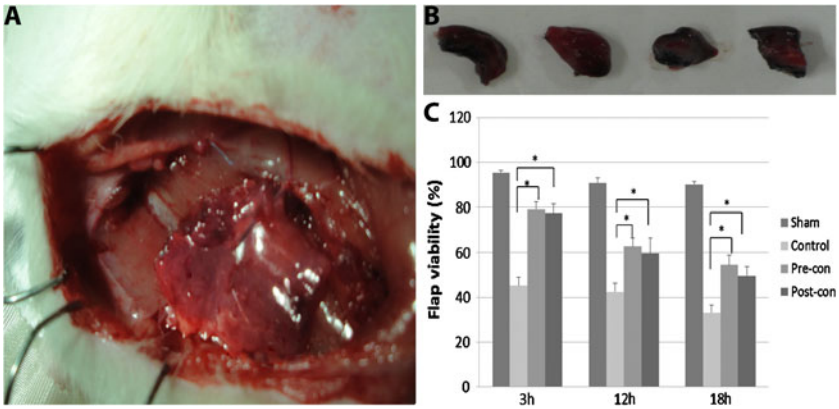

Fig. 1 **a** Gracilis flap model. **b** Representative NBT staining results at 18 h postoperatively (from *left* to *right*: sham group, control group, pre-con group, and post-con group). **c** Statistical analysis of NBT results. The survival rates in the sham group were significantly higher than the other three groups at 3, 12, and 18 h postoperatively ($P<0.05$). Higher survival was observed in both the pre-con group and the post-con group than the control group at 3, 12, and 18 h postoperatively ($P<0.05$). There was no statistical difference between the pre-con group and the post-con group ($P>0.05$)

PCR assay was performed subsequently using PCR kit from SABioscience (USA). The primers were purchased from Invitrogen. Glyceraldehyde-3-phosphate dehydrogenase (GADPH) was used an internal control. The sense and antisense sequences used for these analyses were as follows: GADPH, sense: 5′-TGCCCCATGTTTGTGATG-3′, antisense: 3′-TGGTGGTGCAGGATGCATT-5′; iNOS, sense: 5′-GGCAACATCAGGTCGGCCATTACTG-3′, antisense: 5′-GGAACCACTCGTACTTGGGATGCTC-3′, 307 bp.

The reaction was performed at 25°C for 5 min, 42°C for 1 h, and 70°C for 15 min for the RT reaction and followed by 40 cycles of 95°C for 10 min, 95°C for 10 s, 57°C for 30 s, and 72°C for 1 min for the PCR process. The expression of iNOS was assessed by the density of bands on 10 % polyacrylamide gel electrophoresis, which was scanned and analyzed by two independent researchers blinded to the treatment group with Quality One software package (PDI Inc. USA). iNOS gene expression was quantitatively expressed as a ratio to GADPH gene expression.

Statistical Analysis

All data were expressed as the mean ± standard error. Statistical analyses were performed with SPSS software version 14.0 (SPSS, USA). One-way ANOVA was used to determine the statistical difference among the groups. A P value of less than 0.05 was considered statistically significant.

Results

All of the rats survived during the procedure. The viability of the flaps was examined by NBT staining. The survival ratios in the sham group were 95.44±1.07, 90.86±2.12, and 90.22±1.10 % at 3, 12, and 18 h postoperatively, which were significantly higher than the other three groups ($P<0.05$). Higher survival was observed in both the pre-con group (78.98±3.39, 62.74±3.7, and 54.42±4.45 %) and the post-con group (77.42±4.14, 59.74±6.67, and 49.52±4.13 %) than the control group (45.22±3.69, 42.44±3.76, and 33.2±3.29 %) at 3, 12, and 18 h postoperatively ($P<0.05$). However, there was no statistical difference between the pre-con group and the post-con group ($P>0.05$).

Histology

Severe inflammation exhibited by infiltration of a large number of inflammatory cells was observed in the control group at 3 h postoperatively (Fig. 2b). Even more serious damage was observed in the control group at 18 h after the operation, evidenced by deformation of muscular cells, severe edema in mesenchymal tissue, keryorrhexis, and karyolysis, accompanied by vasodilation, congestion, and hemorrhage (Fig. 2f). Most tissue underwent myonecrosis and was surrounded by many inflammatory cells. However, tissue from the pre-con and post-con groups remained intact 3 h postoperatively (Fig. 2c, d). Edema was found in the tissue from both the pre-con and post-con groups 18 h after the operation (Fig. 2g, h). There was no myonecrosis in the pre-con group, but slight myonecrosis was observed in the post-con group (Fig. 2h).

iNOS mRNA Expression

Gene expression was examined at 3, 12, and 18 h postoperatively. The expression of iNOS was higher at 3 h after the operation. Its expression decreased and remained stable at 12 and 18 h postoperatively. The lowest expression of iNOS was observed in the control group at different time points (P

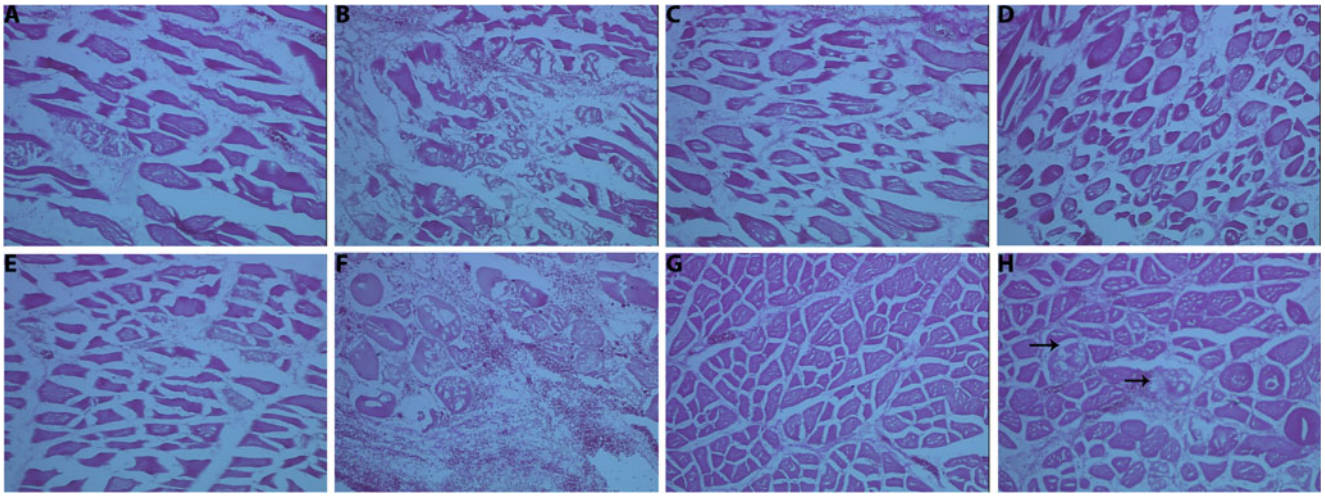

Fig. 2 Histological evaluation with H&E staining. **a**–**d** Samples from the sham group, control group, pre-con group, and the post-con group (*left* to *right*) were collected at 3 h postoperatively. **e**–**h** Samples from the sham group, control group, pre-con group, and the post-con group (*left* to *right*) were collected at 18 h postoperatively. **b**, **f** Severe inflammation and myonecrosis were observed in the control group at both 3 and 18 h postoperatively. **c**, **d**, **g**, **h** Tissue from the pre-con group and the post-con group remained largely intact except for some edema. Slight myonecrosis was observed in the post-con group 18 h after the operation (*black arrows* in **h**) (×100)

<0.05). The expression of iNOS was higher in both the pre-con group and the post-con group than the sham group at 3, 12, and 18 h postoperatively ($P<0.05$). There was no statistical difference between the pre-con group and the post-con group at different time points ($P>0.05$) (Fig. 3).

Discussion

Free muscle flaps have been used widely to reconstruct complex defects. However, flap necrosis following by I/R injury is a common complication which has remained unsolved despite many studies [9].

In 1992, Mounsey et al. initially proved that preconditioning, a treatment for myocardial ischemia, could also exert protective effects on muscular flaps [8]. The effectiveness of this simple technique has been validated in a series of experimental and clinical studies [2, 15, 16]. However, its clinical application remains limited because preconditioning is performed before ischemic injury, which is unpredictable and impractical in the clinical setting [14].

Based on the well-established role of preconditioning and the understanding of I/R injury, a new concept of "postconditioning" was proposed later and gained popularity for its "after-injury strategy" [22, 23]. Its therapeutic effects on muscle flaps have also been validated in several studies in reconstructive surgery [7, 22]. However, some questions

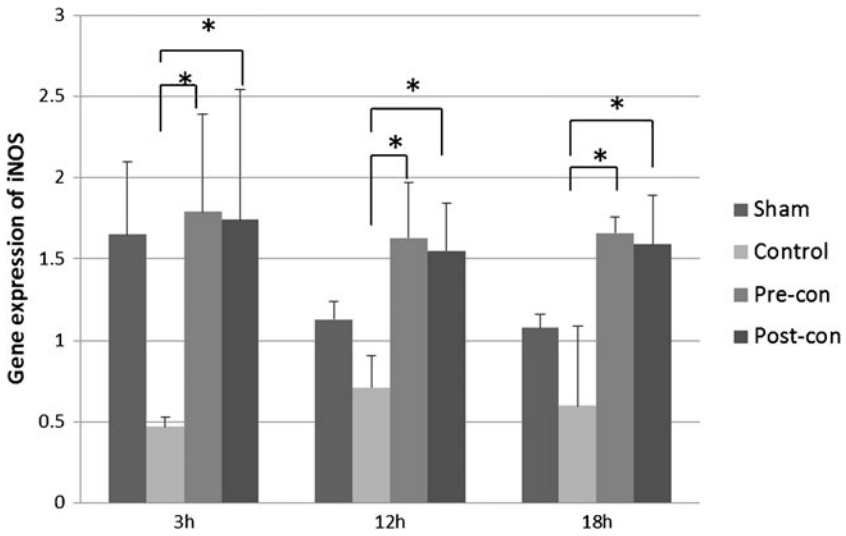

Fig. 3 iNOS gene expression. The lowest expression of iNOS was observed in the control group at different time points ($P<0.05$). The expression of iNOS was higher in both the pre-con group and the post-con group than the sham group at 3, 12, and 18 h postoperatively ($P<0.05$). There was no statistical difference between the pre-con group and the post-con group at different time points ($P>0.05$)

still remain, such as the mechanism of this technique and its efficiency compared to preconditioning.

In this study, both preconditioning and postconditioning were applied on a muscle flap model. The therapeutic effects of both techniques were compared and the mechanism of postconditioning was explored. The results in this study clearly demonstrate that improvement of flap viability could be achieved by both preconditioning and postconditioning during the early period of reperfusion.

Histological examination showed that severe damage of the tissue appeared in the control group as early as 3 h after reperfusion, evidenced by severe inflammation, infiltration of inflammatory cells, and even myonecrosis. However, tissues in the pre-con group and the post-con group remained largely intact at 3 h postoperatively, suggesting that a similar protective effect existed in both the pre-con group and the post-con group. Tissue damage in the control group became even more serious at 18 h after the operation. However, attenuated damage was found in the tissue from both the pre-con group and the post-con group, especially the pre-con group in which only slight edema was observed while slight myonecrosis could be observed in the post-con group. The aforementioned results suggest that delayed and attenuated reperfusion injury was found in the tissue treated by both preconditioning and postconditioning 3 h postoperatively. A significant protective effect was also observed in both groups 18 h after the operation; however, better protection was provided by preconditioning than postconditioning at this time point. This finding is also consistent with recently published investigation results that preconditioning could provide better protection during I/R injury [17, 18, 20].

iNOS can be synthesized by many cell types in response to cytokines and during stress. It is closely related to vascular functions and can produce large amounts of NO as a defense mechanism. It also plays an important role during I/R injury [13]. Expression of iNOS has been found to improve the survival of flaps during I/R injury; however, excessive production of NO can cause damage to the tissue [5]. iNOS has been considered one of the main mediators during preconditioning, but its role in postconditioning has remained largely unclear [3, 10, 11].

The results from this study show that the expression of iNOS was higher in both the pre-con group and the post-con group than the control group at 3, 12, and 18 h postoperatively. The lowest level of iNOS expression could be observed in the control group during IR injury. We therefore hypothesize that the tissue protection in the pre-con and the post-con group might be due to the high level of iNOS expression while tissues in the control group were exposed to more damage due to the lack of iNOS. In this study, the highest expression of iNOS was observed 3 h after the operation in both the pre-con group and the post-con group, which suggested that the greatest protective effect was achieved within this time frame.

Clinically, most flap failures are caused by vascular problems, which can be prevented by the improvement of surgical skills. However, in trauma surgeries such as replantation of an amputated limb, decompression of compartment syndrome, or salvage of a failed flap, when prolonged ischemia has already existed, postconditioning can yield great clinical benefit by protecting tissue from reperfusion injury.

Conclusion

Significant protection of muscle flaps from I/R was achieved by both preconditioning and postconditioning treatment during the early period of reperfusion injury; however, better protection can be provided by preconditioning. A higher level of iNOS was found in both treatments, suggesting an important role of iNOS in the protective effects of both treatments. With feasibility to be adopted when I/R injury has occurred and a crucial protective effect during the early period of reperfusion, postconditioning can still be a good alternative for the ischemic flaps and, therefore, deserved further investigation.

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Conflict of interest The authors have no conflict of interest to declare.

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