The Angiogenic Effects of Ischemic Conditioning in Experimental Critical Limb Ischemia

R. Karakoyun a,*, C. Koksoy b, T.U. Yilmaz c, H. Altun a, O. Banli a, A. Albayrak d, M. Alper d, Z. Şener e

a Department of Surgery, Etlik İhtisas Training and Research Hospital, Ankara, Turkey
b Division of Vascular Surgery, Department of General Surgery, Ankara University, Faculty of Medicine, Ankara, Turkey
c Department of General Surgery, Gazi University, Faculty of Medicine, Ankara, Turkey
d Department of Pathology, Döşkepöl Training and Research Hospital, Ankara, Turkey
e Department of Surgery, Diyarbakör Training and Research Hospital, Diyarbakör, Turkey

WHAT THIS PAPER ADDS

Remote ischemia conditioning is associated with angiogenic promotion in the ischemic tissue. This study provides preliminary data showing that repeated short ischemic stimuli may reduce critical ischemic injury by promoting angiogenesis.

Objectives: Ischemic conditioning (IC) is a method of angiogenic stimulus for limb ischemia. Here, we aimed to investigate the effects of short-term repeated ischemic stimulus on critical lower limb ischemic injury.

Methods: Rats were divided into four groups consisting of 40 animals in each group: sham, ischemia, local IC, and remote IC groups. Right-leg critical limb ischemia was achieved through ligation of the iliac artery and vein in male Sprague–Dawley rats except the sham group. Repeated transient ischemia using the tourniquet method was used for IC of lower extremities in the local and remote groups. IC was performed on the right leg for the local group and on the left leg for the remote group. Ten rats in each group were sacrificed for evaluation on days 1, 7, 14, and 30. Endothelial progenitor cell (EPC) counts were measured. Gastrocnemius muscles were evaluated for the degree of ischemia. Laser Doppler blood flow measurements were performed in order to make comparison between the blood flows of the limbs of the groups.

Results: The blood flow in the right limb of rats in the sham (1.65 perfusion units [PU]) and local IC (1.67 PU) groups was significantly higher than the ischemic group (1.17 PU) (p = .001 and p = .022 respectively). The levels of EPCs in the ischemia (1.09 ± 0.5) and remote IC groups (1.36 ± 0.8) were significantly higher than the sham (0.38 ± 0.2) group on day 7 (p = .026 and p = .002 respectively). Remote IC and local IC groups exhibited increased histopathological ischemia on day 7 when compared with sham group (p = .001, p = .01 respectively). The angiogenic scores on the 7th, 14th and 30th days for local IC and remote IC groups were significantly higher than sham and ischemia groups.

Conclusions: IC seems to be the potent activator of angiogenesis in ischemic tissue. This study provides preliminary data showing that repeated short ischemic stimuli may reduce critical ischemic injury by promoting angiogenesis.

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INTRODUCTION

Standard treatments for critical limb ischemia are surgical or endovascular revascularizations. When surgical or endovascular treatment is not an option, the clinical manifestations of critical limb ischemia are dependent on the balance between the rapidity and extent of “natural” collateral vessel growth versus the progression of occlusive arterial disease. On the other hand, direct revascularization may be unsuccessful in some cases due to the anatomic extent and distribution of arterial occlusive disease.1 The deficiency in effective treatment options for non-reconstructable critical limb ischemia has led to research on cellular therapy as an alternative. As a result, alternative methods, such as gene therapy, stem cell therapy, and angiogenic stimulators have been investigated,2 and much of these have concentrated on the concept of therapeutic angiogenesis.

The definition of “ischemic conditioning” (IC) is the application of a series of alternating intervals of brief ischemia and reperfusion in the setting of prolonged ischemia causing tissue necrosis. The conditioning stimulus can be applied before (ischemic preconditioning, IPC),

* Corresponding author. R. Karakoyun, SB Etlik İhtisas Hastanesi, Department of General Surgery, Etlik, Ankara, Turkey.
E-mail address: drrobin@hotmail.com (R. Karakoyun).
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during (preconditioning), or after (post-conditioning) the major ischemic event. All three methods of conditioning are associated with tissue protection not only in normal physiology, but in both animal models and in humans with ischemia–reperfusion syndromes. Several studies have shown the beneficial effects of preconditioning and post-conditioning in different ischemia–reperfusion models. However, ischemia itself is a stimulus for angiogenesis. Also, IC itself may be a stimulus for angiogenesis in the ischemic tissue, and it has been shown that remote IPC would lead to the activation of new pathways and the release of biochemical messengers, including angiogenic ones. More recently, in a liver ischemia model, it has been reported that IC seems to be a potent activator of angiogenic genes. Despite the substantial amount of research on IC in acute ischemia models, the impact of IC on critical ischemic injury has been less well studied.

Endothelium which is in the quiescent state in the vascular tree starts to proliferate in response to injury, growth factors, or neovascularization. Sprouting of endothelium is driven by the recruitment of circulating endothelial progenitor cells (EPCs). The EPC population contained in the CD34/CD45 cell fraction develops into endothelial colony-forming cells and they display the characteristics of mature endothelial cells, have a high proliferative capacity, and are able to form capillary-like structures. EPCs lose typical progenitor markers and acquire endothelial markers and two important receptors (VEGF and CD34) which recruit circulating EPC to damaged or ischemic microcirculation beds. Ischemic preconditioning might be a promising and important method in the treatment of critical limb ischemia by increasing new vessel formation, and the creation of angiogenic stimulators. We hypothesized that short-term repeated ischemic stimulus could reduce critical ischemic injury because of its angiogenic effect. To test this hypothesis, we aimed to investigate the effects of short-term repeated ischemic stimulus on critical ischemic injury of the remote organ by determining angiogenesis with measurements of EPC levels, by determining necrosis with histopathological analysis and by measuring limb perfusion with laser Doppler imaging.

MATERIALS AND METHODS

Animal model

Male Sprague–Dawley rats weighing 180–250 g were housed under cycles of 12 h of light and 12 h of dark in individual cages, and were allowed free access to standard rat chow and water. All experiments were performed with rats that had fasted for 12 h before surgery. Animal housing, care, and the application of experimental procedures were all done in accordance with The Guidelines for Care and Use of Laboratory Animals, published by the National society for Medical Research and the National Institute of Health. All of the animal experiments described herein were approved by the Institutional Review Board.

Critical limb ischemia model

The rats were divided into four groups consisting of 40 animals each: sham group, ischemia group, local IC group, and remote IC group (Fig. 1). Right-leg critical limb ischemia was created by iliac artery and vein ligation, as described previously. Briefly, the rats were anesthetized with 100 mg/kg intraperitoneal (ip) ketamine (Ketalar, Parke-Davis). A midline incision was made in the abdomen, and the right common iliac artery and vein were circumferentially exposed. The artery and vein were doubly ligated with a 6/0 polyglactin suture (coated Vicryl Ethicon, Somerville, NJ, USA) and divided proximal to the internal iliac artery. During the procedure, to avoid influences arising from major fluid loss or drying of the liver, the abdominal cavity was covered with wetting gauze. Following ligation, the abdominal wall was closed with interrupted sutures. After the creation of ischemia in the right limb of rats, rats in the local and remote groups were taken in order to perform conditioning stimulus. In pilot studies, we tested the presence of ischemia in this model using laser Doppler imaging. Also, in all experiments, critical ischemia in the right limb was observed by visual inspection.

ICs of either right (local) or left (remote) legs were achieved through repeated transient ischemia using the tourniquet method. Under anesthesia with low-dose ip ketamine (30 mg/kg body weight), a tourniquet (MAS rubber band, number 15) was looped six times as proximal as possible to the thigh. Ten minutes of ischemia followed by 10 minutes of reperfusion were performed three times every morning for the assigned period of time. The time period was similar to the study of Weinbrenner et al. Repeated transient ischemia performed after right limb ischemia is a way of post-conditioning. Repeated transient ischemia was performed after the initial rat pads were scanned with a laser Doppler imager (Perimed PIM II, Jar-walle, Sweden) for confirmation of transient ischemia. Rats

![Figure 1. Summary of the study protocol. R (right), L (left), IC (ischemic conditioning), S (tissue sampling), L (laser imager evaluation), gray arrows ischemic conditioning in every day for 1, 7, 14, and 30 days.](image-url)
in the sham group were subjected to anesthesia and laparotomy only. Rats in the ischemia group underwent permanent right limb ischemia only. Rats in the local IC group were subjected to ipsilateral (right limb) repeated transient ischemia after permanent right limb ischemia. Rats in the remote IC group were subjected to contralateral (left limb) repeated transient ischemia after permanent right limb ischemia. Transient ischemia was applied to the rats in the local and remote groups for different time periods: 1, 7, 14, and 30 days. There were 10 rats in each time period study.

After 1-, 7-, 14-, and 30-day transient ischemia periods, 10 rats from each group were killed for evaluation. To exclude the effects of anesthesia that had been given every day to the transient ischemia groups, the same dose of anesthesia was also given to the sham and ischemia groups during the experiment. In order to make the method more understandable, we added a flow chart (Fig. 1).

At the end of the time periods, blood samples were taken from the abdominal aorta for EPC count evaluations. Tissue samples were taken from the gastrocnemius muscle of the critical (right) ischemic limb for the evaluation of the degree of ischemia. Laser Doppler blood flow measurement was performed on day 30 of the experiment. The laser Doppler results of the sham group were regarded as the basal level.

**Endothelial progenitor cells**

Endothelial progenitor cells (EPCs) were measured by the method described by Goldstein et al. EPCs have membrane markers for vascular endothelial growth factor-2 (VEGF-2), CD34, CD117, CD133, CD31, VE-cadherin, and vWF. In this method, multicolor flow cytometry phenotyping was used to demonstrate VEGF-2 and CD34 (Fig. 2). The test depends on the binding of specific monoclonal antibodies, expressed by the leucocytes, to the antigenic markers. In selected cells, analysis was performed based on the differentiation of fluorescent-positive cells (binding to anti-mouse CD34 and VEGF-2 without cross-reaction) as mentioned in a previous study.

**Flow measurement**

Limb perfusion was assessed on day 30 using laser Doppler perfusion imaging, and was quantified using the laser Doppler imager (Fig. 3). Laser Doppler perfusion imaging was performed in a temperature-controlled (28–30 °C) facility with weight-based sedation, and in a dark room to minimize artifacts due to temperature fluctuations and levels of sedation. The limb was defined as all-imaged tissue distal to the inguinal ligament of the rat in prone position and the foot sole in supine position. Signals from the skin were recorded continuously for 4 minutes by fiberoptic probe. The results were given in PU (perfusion units).

**Evaluation of angiogenesis**

In the tissue samples, immunohistopathologic examinations for angiogenesis were performed using CD34 tissue antibodies. CD34 is an important target for therapeutic angiogenesis as reported in several studies.

Cytoplasmic CD34 staining was accepted as positive for angiogenesis, in accordance with the previous studies.

**Determination of muscle necrosis**

The tissue samples from gastrocnemius muscle were kept in 10% formaldehyde. Hematoxylin and eosin microscopic evaluation of ischemia was performed according to the criteria mentioned in the study by Carter et al. A subjective histopathology score was recorded for each muscle group and ranged from 0 to 10: 0 = no abnormal score; 1 = mild localized mononuclear cell infiltration; 3 = moderate generalized mononuclear cell infiltration, occasional necrotic fibers; 10 = massive cell infiltration, and complete loss of tissue architecture. A mean muscle histopathology score (MHS) was recorded as the mean
**Figure 3.** Examples of laser Doppler images of the rats. The decreased blood flow in the right limb as a result of critical limb ischemia is shown (dorsal view). (A) Laser Doppler image of a rat in ischemia group. Decreased blood flow in right limb is shown. (B) Laser Doppler image of a rat in the local ischemic conditioning group. Decreased blood flow in right limb is shown.

Histopathology scores of the evaluated muscle group of the animals. The pathological evaluation was performed by an experienced pathologist who had no idea or information about the groups.

**Statistical analyses**

Data which were normally distributed are presented as mean ± SEM. A computer program (SPSS version 13.00; SPSS Inc. Chicago, IL, USA) was used for statistical analysis. The difference between EPC levels and the number of CD34 stained cells on each day among groups and blood flows was determined by one-way analysis of variance (ANOVA) followed by a post hoc Tukey test. The median MHC results were given among groups because of the non-homogeneous distribution of the results ($p < .05$, Kolmogorov–Smirnov test). Non-parametric statistical evaluation of MHS was performed by the Kruskal–Wallis test. A $p$ value $< .05$ was considered statistically significant.

**RESULTS**

No mortality was observed during the experiment. The results of blood flow obtained from laser Doppler on day 30 are given in Fig. 4. Also, an example of laser Doppler images of the rats is provided in Fig. 3. There was a significant difference between groups ($p = .001$, ANOVA). It can be seen that the blood flows of the right limb of rats in the sham (1.65 ± 0.28) and local IC (1.67 ± 0.4) groups were significantly higher than that of the ischemic group (1.17 ± 0.14) ($p = .001$ and $p = .022$, respectively). Although a significant difference was noted between remote IC (1.28 ± 0.1) and sham groups ($p = .04$), the same could not be observed between local IC and remote IC groups ($p = .35$). There was no significant difference between the flow rate of left limb of the groups ($p > .05$).

The levels of circulating EPCs are given in Fig. 5, and an example from multicolor flow cytometry phenotyping to demonstrate VEGF-2 and CD34 of the rats can be seen in Fig. 2. There was no difference in the levels of EPCs among the groups on the first day of the experiment ($p = .87$, ANOVA). However, significant changes were observed on day 7 ($p = .001$, ANOVA); the levels of EPC in ischemia group on day 7 were significantly higher than that of the sham group on day 7 ($p = .026$). The levels of EPCs in remote IC groups on day 7 were significantly higher than that of the sham group on day 7 ($p = .002$). Furthermore, the levels of EPCs in the remote IC group were significantly higher than that of the local IC group ($p = .013$).

There were no significant differences in the MHS for the gastrocnemius muscle among the time points in the sham group. Ischemia was observed in the study groups. There were significant differences between groups on days 7 and 30. Remote IC and local IC groups exhibited increased MHS on day 7 when compared with that of the sham group ($p = .01$ and $p = .02$ respectively). There was a significant difference between the MHS levels of ischemia and remote IC groups on day 7 ($p = .01$). MHS on day 30 was significantly increased in the ischemia group when compared with that in the sham group ($p = .04$). MHS of the local IC group was significantly increased on day 30 when compared with that of the sham group ($p = .01$). There was a significant increase in MHS scores in the local IC group when compared with the remote IC group on day 30 ($p = .02$). Furthermore, there were significant differences between the MHC scores of local IC and ischemia groups on day 30 ($p = .04$) (Table 1).

The comparison of angiogenic scores is given in Fig. 6. On day 1, there were no significant differences between the angiogenesis scores of the groups ($p = .98$, ANOVA). On
days 7, 14, and 30, there were significant differences between groups ($p = .001$, ANOVA). Local IC and remote IC groups had significantly increased angiogenesis when compared with the sham group ($p < .001$). The remote IC group had significantly increased angiogenesis compared with the ischemia group on day 7 ($p < .001$). On day 14, angiogenesis in the sham group was significantly lower than in the local IC and remote IC groups ($p = .007$ and $p < .001$, respectively). On day 30, angiogenesis in the sham group was significantly lower than the ischemia, local IC and remote IC groups ($p = .041$, $p < .001$, $p < .001$ respectively). Angiogenesis in the ischemia group was significantly lower than in the local IC and remote IC groups ($p < .001$).

**DISCUSSION**

Several studies have shown the beneficial effects of pre-conditioning and post-conditioning in different ischemia—reperfusion models. 3,5,6,20–28 IC already has clinical applications in acute events such as myocardial ischemia and cardiac surgery. The discovery that remote IC can be performed non-invasively using a blood pressure cuff on the upper arm to induce brief episodes of limb ischemia and reperfusion has facilitated the translation of IC into the clinical arena. 29,30 In this study, we tried to investigate the effects of intermittent ischemia on established limb ischemia. For this purpose, we investigated the effects of both local (in situ) and remote repeated intermittent limb ischemia on critical limb ischemia. The most important physiological and morphological finding of the present study is that local and remote ischemic stimuli might significantly increase the blood flow and capillary supply in the ischemic limb. This finding has not been previously reported.

The formation of true new blood vessels, or angiogenesis, and the development of collateral vessels from pre-existing blood vessels, or arteriogenesis, are important in the pathophysiology of vascular disease. Hypoxia or ischemia itself is one of the stronger inducers of angiogenesis. By stimulating these processes, it may be possible to provide an alternative therapeutic strategy for patients with non-reconstructable lower limb ischemia. Research in therapeutic angiogenesis to treat ischemia has been performed for decades. Arteriogenesis, when the lumen of pre-existing vessels increases to form collateral arteries, is a process that can ameliorate the harmful effects of vessel obstruction.

Remote IC is a physiological adaptation mechanism, whereby brief exposure to non-lethal ischemia in one tissue confers protection against a prolonged ischemic insult in a distant tissue. 22,23 Several clinical trials about cardiac ischemia have reported the beneficial effects of remote IC in different clinical settings. 20,24 Also, contralateral hind limb remote IPC prior to ipsilateral cremasteric flap ischemia and reperfusion in a rat model has been observed to improve red blood cell flow and reduce neutrophil adhesion. 21,23,27 Liaw et al. 28 showed that remote IPC (ipsilateral gracilis) reduced muscle necrosis of contralateral muscles by 60%, when compared with non-preconditioned controls. These studies show that remote IPC is associated with better microcirculation, decreased leucocyte endothelial sticking and endothelial dysfunction, as well as better capillary blood flow with terminal arteriole dilation. 25 However, the above-mentioned studies have been performed in acute ischemia—reperfusion injury settings. To our knowledge, the present study is the first to investigate both in situ and remote repeated intermittent limb ischemia in critical limb ischemia.

In the current study, we found that remote IC caused increased angiogenesis in the ischemic tissue. Despite the higher angiogenic scores with remote IC, these findings were not associated with amelioration in tissue injury and blood flow in the critical ischemic limb. On the other hand, local IC in the critical ischemic limb was associated with increased angiogenesis, as well as improved blood flow. However, tissue injury was even higher in this group than that in the ischemic group. It was thought that the blood flow rate in the local IC group was increased because of the inflammation occurring in the ipsilateral limb because of

![Figure 5. The mean EPC levels of groups within days.](image-url)

**Table 1.** The mean muscle histopathology scores for the gastrocnemius muscle of the right limb of rats of all groups are given by $p$ values.

<table>
<thead>
<tr>
<th>Group</th>
<th>Day 1</th>
<th>Day 7</th>
<th>Day 14</th>
<th>Day 30</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>0 (0−2)</td>
<td>0 (0)</td>
<td>0.5 (0−3)</td>
<td>1 (0−1)</td>
</tr>
<tr>
<td>Ischemia</td>
<td>0 (0−1)</td>
<td>0 (0−3)</td>
<td>2 (1−3)</td>
<td>1.5 (0−3)</td>
</tr>
<tr>
<td>Local IC</td>
<td>0 (0−1)</td>
<td>1 (0−4)</td>
<td>2 (1−3)</td>
<td>2.5 (1−5)</td>
</tr>
<tr>
<td>Remote IC</td>
<td>0 (0−1)</td>
<td>1.5 (0−4)</td>
<td>1 (0−3)</td>
<td>1 (0−2)</td>
</tr>
</tbody>
</table>

$p$ Values

- Sham vs. local, IC, $p = .02$
- Sham vs. remote, $p = .04$
- Sham vs. local, IC, $p = .01$
- Ischemia vs. local IC, $p = .04$
- Ischemia vs. local, $p = .01$
- Local IC vs. remote IC, $p = .02$
- Local IC vs. remote IC, $p = .02$
the repeated ischemia created in the ipsilateral limb. Also, the increased ischemia score is probably due to the repeated ischemic trauma in the ipsilateral limb. The higher tissue injury seen in the local IC group may be related to the technique of conditioning. We used IC by wrapping a rubber band under light anesthesia, which can itself cause damage. Local IC is associated with higher flow and MHC value in the ischemic limb. We believe that local IC may cause better conditioning but less tissue protection because of de novo tissue damage in the already injured tissue.

In the experiment, limb perfusion was assessed only on day 30 by using laser Doppler perfusion imaging. Unfortunately, we did not perform laser measurements for all time points, namely day 1, day 7, and day 14, and we did not induce ischemia. There was no difference in the flow of the left leg which was used for control or conditioning on day 30. However, blood flow diminished in the ischemic right leg in animals in the ischemia group. Local IC resulted in an increase in blood flow in the ischemic right leg, but remote IC did not result in increased flow in the ischemic right leg. Amelioration of tissue injury in the remote IC group without any increase in blood flow may be related to other mechanisms of IC.

The results of the present study suggest that IC promotes angiogenesis. Although the actual angiogenic mechanistic pathway underlying IC remains to be elucidated, it might be related to the cascade following ischemic insult. Induction of ischemia has previously been shown to occur in animal models of skeletal muscle ischemia and systemic hypoxia. In response to ischemic insults, most of the tissues in the body have extraordinary capacities to compensate for low levels of oxygen by mechanisms of vasodilation, angiogenesis, arteriogenesis, vascular remodeling, and hematopoiesis. Molecular mechanisms underlying the compensation of oxygen involve induction of a distinct set of gene products that control the transcriptional activation of vascular and hematopoietic modulators. Also, the infiltrating inflammatory cells or, potentially, EPCs that invade in response to acute ischemia may assist in arteriogenesis and blood flow recovery in animal models that rely on ligation or excision. In this study, we used IC for 1, 7, 14, and 30 days after right-leg ischemia in relation to EPCs.

This study has important limitations. First, we used IC by wrapping a rubber band under light anesthesia, which can itself cause damage. The higher tissue injury seen in the local IC group may be related to the technique of conditioning. The second limitation is the aim to investigate the level of angiogenesis, and not arteriogenesis, in the ischemic limb. Arteriogenesis to increase blood flow, is distinct from microvascular angiogenesis. The clinical observation of robust collateral formation allowing asymptomatic limb perfusion and function in many patients confirms the importance of arteriogenesis as a necessary component of revascularization. Therefore, angiogenesis itself may not be good enough for critically ischemic limbs. The absence of amelioration in tissue injury in local and remote IC groups may be related to insufficiency of arteriogenesis, instead of angiogenesis. Finally, we did not measure the functional outcome of ischemic legs in the animals, which is an index of arteriogenesis.

In summary, we have found that remote IC is associated with angiogenic promotion in ischemic tissue. This study provides preliminary data showing that repeated short ischemic stimuli may reduce critical ischemic injury by promoting angiogenesis. Our model can be used in cases in which reconstruction was not possible. However, further studies are required to establish the protective effects and the mechanism of angiogenesis promoted by IC.

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**CONFLICT OF INTEREST**
None.
REFERENCES


