The Angiogenic Effects of Ischemic Conditioning in Experimental Critical Limb Ischemia

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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),

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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.[3,4] 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 nerve pathways and the release of biochemical messengers, including angiogenic ones.[5,6] More recently, in a liver ischemia model, it has been reported that IC seems to be a potent activator of angiogenic genes.[7] 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).[8] 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.[9] 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.[10]

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.[11] 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.[12] 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, Jarwalle, Sweden) for confirmation of transient ischemia. Rats

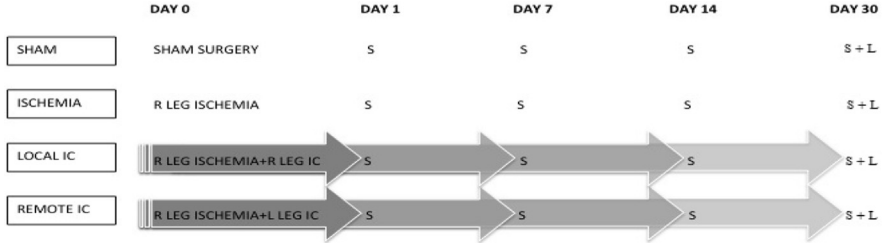

Figure 1. Summary of the study protocol. R (right), L (left), IC (ischemic conditioning), S (tissue sampling), L (laser imager evaluation), gray arrows ischemic conditioning in every day for 1, 7, 14, and 30 days.