Ischemic preconditioning in different times and its effect on bacterial translocation induced by intestinal ischemia and reperfusion in rats

Pré-condicionamento isquêmico em diferentes tempos e seu efeito na translocação bacteriana induzida por isquemia e reperfusão intestinal em ratos

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Objective: To evaluate the effects of different times of ischemic preconditioning (IPC) on intestinal bacterial translocation (BT). Methods: Thirty Wistar rats weighing 280 ± 27g were divided into five groups. In the IR group (n = 6), laparotomy was performed and the superior mesenteric artery was occluded by an atraumatic microclamp for 30 minutes. In the four preconditioning groups (n = 6 each) before the 30 minutes of ischemia-reperfusion (I/R) rats underwent IPC for two, five, ten and 15 minutes, followed by the same time of reperfusion. In order to assess whether the time of preconditioning influenced the onset of bacterial translocation, samples of mesenteric lymph nodes, liver and spleen were collected in sterile conditions twenty-four hours after the procedures for quantification of bacterial colony forming units per gram of tissue (CFU/g). Blood was collected for measurement of cytokines. Results: In the I/R group, the total CFU/g in mesenteric lymph nodes, spleen, liver, as well as the serum TNF-α, IL-1α and IL-6 were significantly higher than in the other groups (p <0.05). Preconditioning for 15 minutes significantly attenuated BT and serum cytokines when compared to other periods of preconditioning (p <0.05). Conclusion: Our data suggest preconditioning as a key factor to reduce bacterial translocation in intestinal I/R. On a scale of two to 15 minutes, the best time of ischemic preconditioning for the attenuation of bacterial translocation was 15 minutes.

Key words: Bacterial translocation. Ischemia. Reperfusion. Intestines. Rats.

INTRODUCTION

Interruption and restoration of blood flow to organs and tissues triggers a cascade of events known as ischemia and reperfusion syndrome, as occurs in organ transplantation or complex trauma. The generation of free radicals during these events causes changes in cell membrane, resulting in cell death.

The tissue damage caused by ischemia and reperfusion in the intestine is an important factor associated with high morbidity and mortality in surgical patients. This is important in situations where there is an interruption of blood flow, such as during abdominal aortic aneurysm, cardiopulmonary bypass, strangulated hernias, neonatal necrotizing enterocolitis and organ transplantation.

The intestine is probably the most sensitive organ to ischemia and reperfusion syndrome. In an attempt to mitigate this damage, several treatment modalities have been applied successfully in animal models of ischemia-reperfusion, such as hypothermia, antioxidants, ischemic preconditioning (IPC), modulation of inflammatory mediators and adhesion molecules. Among these, ischemic preconditioning seems to be the most promising strategy against reperfusion injury by increasing bowel tolerance against damage caused by ischemia followed by reperfusion.

Sola et al. used this experimental model in the study of intestinal transplantation in rats, performing preconditioning with an interval of ten minutes of ischemia followed by reperfusion of also ten minutes. Abraham et al. also used this same time of preconditioning when...
studying the effects of ischemic preconditioning in the intestinal tissue injury.

Bacterial translocation (BT) was originally defined and described by Berg and Garlington\(^7\) as the passage of viable bacteria through the intestinal mucosa to mesenteric lymph nodes and other tissues and organs. Based on the fact that ischemic preconditioning attenuates the tissue injury caused by ischemia-reperfusion, Aksoyek et al.\(^10\) demonstrated in an experimental model, in which rats were subjected to ischemic preconditioning ten minutes prior to sustained ischemia, that BT was lower than in those animals that had not undergone preconditioning. Although most reports in the literature using the time of ten minutes in ischemic preconditioning of the small intestine of rats, the optimal protocol of intestinal preconditioning in these animals, including the short ischemia time, is not yet well established\(^{5,11}\). The damage to the intestinal mucosal barrier can induce bacterial translocation, the gastrointestinal tract having been defined as the “motor” of multiple organ failure. Given this, ischemic preconditioning has been growing in importance in recent years\(^{12,13}\).

Therefore, the aim of this study was to evaluate, in rats, the influence of different times of ischemic preconditioning in bacterial translocation in order to contribute to the understanding of the time period most appropriate for your best protective effect against bacterial translocation.

**METHODS**

We used 30 male, Wistar rats (Rattus norvegicus Albinus) aged approximately four months, with average weight 280 ± 27g, from the vivarium of the Center for Health Sciences, Federal University of Rio Grande do Norte (UFRN). The animals were kept in adjustment period for seven days at the Center of Experimental Surgery-UFRN, in individual cages, with food and water ad libitum, subjected to light-dark cycle of 12 hours, with humidity, noise and brightness control. The protocol was examined and approved by the Ethics Committee for Institutional Research (Protocol 02.09/ animal research) and experiments were performed in accordance with Law 11,794 (Lei Arouca).

The animals were randomized into five groups containing six animals each: Group ischemia and reperfusion (IR): animals submitted only to the period of ischemia and reperfusion; IPC Group 2: animals undergoing a period of ischemic preconditioning (IPC) for two minutes; IPC Group 5: animals submitted to IPC for five minutes; IPC Group 10: animals subjected to ten minutes of IPC; and IPC Group 15: animals undergoing IPC for 15 minutes.

After an overnight fasting period of 12 hours, the animals were anesthetized with an intramuscular injection of ketamine (50mg/kg) and xylazine (7mg/kg). Once suitable anesthesia was evidenced by the absence of interdigital pain reflex and relaxation, they were positioned on the surgical table. After trichotomy and antisepsis of the abdomen with 70% alcohol, a median celiotomy 4cm from the xiphoid process was held. We extracted of the abdominal viscera, wrapped then in gauze soaked in saline solution heated to 37º C, disected, identified and isolated the cranial mesenteric artery.

In IR Group – observation of the animal for 30 minutes, clamping of the mesenteric artery with vascular microclamp for 30 minutes.

In IPC Group 2 – observation of the animal for 26 minutes, mesenteric artery clamping for two minutes and two minutes reperfusion (IPC), followed by new clamping for 30 minutes.

In IPC Group 5 – observation of the animal for 20 minutes, clamping of the mesenteric artery for five minutes and five minutes reperfusion, followed by further clamping for 30 minutes.

In IPC Group 10 – observation of the animal for ten minutes, clamping of the mesenteric artery for ten minutes and ten minutes reperfusion, followed by further clamping for 30 minutes.

In IPC Group 15 – clamping of the mesenteric artery for 15 minutes and reperfusion for 15 minutes, followed by further clamping for 30 minutes.

In all animals subjected to 30 minutes of ischemia, after that time, the clamp was removed and reperfusion of the mesenteric artery was confirmed by the return of pulsation in the mesenteric arcade. After the procedures, all animals had their abdomen closed with nylon 4-0 and were observed in individual cages.

After 24 hours of observation, the animals were killed by an overdose of anesthetic (thiopental 100mg/kg). Under aseptic conditions, a new laparotomy was performed, blood drawn by intracardiac puncture and removal of samples from the liver, spleen and mesenteric lymph nodes for microbiological examination. The specimens were placed in a sterile container and immersed in 2 ml saline, weighed in a precision scale and homogenized. The samples were plated on McConkey Agar culture medium for the detection of Gram-negative bacteria and Blood Agar for Gram-positive ones, incubated at 37º C under aerobic conditions and examined after 24 and 48 hours to evaluate bacterial growth. The identification of bacteria species was performed by standard microbiological methods. Colonization was expressed as number of colony forming units per gram of homogenate (CFU / g).

From the blood collected by cardiac puncture, serum was separated by centrifugation at 2000 rpm and stored at -40º C for subsequent doses. The cytokines TNF-α, IL1-α and IL-6 were measured by ELISA (enzyme-linked-immunosorbent-assay), analytical method based on antigen-antibody interaction to determine specific amounts of proteins in samples of tissue and body fluids. The reagents used were from PeproTech, NJ, USA, dosages being processed according to the manufacturer’s recommendations.
For data analysis we used ANOVA and Bonferroni, considering the differences significant at p < 0.05.

RESULTS

There was bacterial growth, especially *Escherichia coli* and *Enterobacter sp.* in all groups and organs examined from all animals. In some of them, more specifically in the group without preconditioning, we even observed growth of *Proteus mirabilis*. The quantities of bacterial colony forming units per gram of tissue (CFU/g) found in the organs examined are summarized in Table 1.

The data showed that the pre-conditioning, starting five minutes, resulted in reduction in the total number of CFU/g when compared to IR without preconditioning. In IPC group 15, the quantity of CFU/g was significantly lower than in all other groups in the three organs examined (p < 0.05). Using the ANOVA and Bonferroni with p < 0.05, it was observed that the only comparisons whose values were not statistically significant were between the pre-conditioning of two (IPC group 2) and five minutes (IPC group 5) and between the preconditioning of two minutes (IPC 2) versus without preconditioning (IR). This statistical interpretation was uniformly observed in the mesenteric lymph nodes, the liver and spleen. The lowest presence of translocated bacteria was found in the spleen in all groups.

Therefore, ischemic preconditioning was shown to be protective in bacterial translocation after ischemia and reperfusion in rats and the best result was found in the group with the longest time of IPC (15 minutes), according to this protocol.

Measurement of pro-inflammatory cytokines TNF-á, IL-1â and IL-6 showed a significant reduction in serum (p < 0.05) in IPC 15 (preconditioning with 15 minutes) when compared to animals treated with preconditioning for two, five and ten minutes (p < 0.05). The animals not subjected to preconditioning showed the highest values of serum cytokines, featuring significant difference compared with the other groups (p < 0.05). Decreasing values of cytokines were found as IPC time increased (Table 2).

DISCUSSION

The relation of ischemic preconditioning in the prevention of ischemia and reperfusion injury has been heavily studied for the intestine, as well for the lung, liver, kidney and other organs. Since 1986, we know that repeated brief episodes of ischemia do not have cumulative deleterious effect, as shown by Reimer *et al.*, noting that four ten-minute episodes of myocardial ischemia did not cause necrosis, while a single 40-minute episode of ischemia had this effect [14].

Among the many changes caused by ischemia and reperfusion, there is is bacterial translocation (BT), resulting from the breakdown of the epithelial barrier by inflammatory cytokines, as suggested by some authors [15,16]. These same authors also demonstrated that ischemia and reperfusion cause the release of proinflammatory cytokines and subsequent dysfunction of the epithelial barrier in the intestine, which is associated with increased BT to extraintestinal sites. However, pretreatment with *Lactobacillus plantarum* prevented BT and reduced the

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<th>Table 1</th>
<th>Quantification of CFU/g in lymph nodes, liver and spleen.</th>
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<td>Groups</td>
<td>IPC 2</td>
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<tr>
<td>Organ Nodes</td>
<td>2 min</td>
</tr>
<tr>
<td>Mesentéricos Lymph</td>
<td>1033 ± 186ab</td>
</tr>
<tr>
<td>Liver</td>
<td>952 ± 139ab</td>
</tr>
<tr>
<td>Spleen</td>
<td>835 ± 63ab</td>
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<tr>
<td>Mean ± SD. Values followed by the same letter in the same row do not differ significantly (p&gt; 0.05). IPC: ischemic preconditioning; IR: ischemia and reperfusion.</td>
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<th>Table 2</th>
<th>Values of serum proinflammatory cytokines.</th>
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<td>Cytokines</td>
<td>IPC 2</td>
</tr>
<tr>
<td>Groups</td>
<td>2 min</td>
</tr>
<tr>
<td>TNF-á (pg/mL)</td>
<td>81.7 ± 9.1</td>
</tr>
<tr>
<td>IL-1β (pg/ml)</td>
<td>58.4 ± 8.6</td>
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<tr>
<td>IL-6 (pg/mL)</td>
<td>154 ± 15</td>
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*p<0.05 vs. groups PCI2, PCI5, PCI10 e IR."
release of cytokines, as well as decreased apoptosis of intestinal cells. In the present study it was shown that ischemic preconditioning (IPC) attenuated BT induced by ischemia and reperfusion, and that, between the times of IPC two, five, ten and 15 minutes, the more effective was the 15 minutes. Our results clearly show that the time of IPC is important, since it was detected a relationship between IPC time and BT, ie the longer of IPC, the fewer CFU/g. Microbiological data (Table 1) showed that the IPC reduced BT.

A study of Araujo-Filho et al. showed that α-D-glucan was able to control the release of inflammatory cytokines in injury produced by ischemia and reperfusion, reducing translocation of Escherichia coli. Aksoyek et al. performed intestinal preconditioning for ten minutes and observed a reduction of BT for bloodstream, liver, spleen and mesenteric lymph nodes, corroborating our results. As differential, the model used by us, characterized by a time scale of preconditioning of two, five, ten and 15 minutes, showed that the greatest reduction of serum cytokines and BT occurred at 15 minutes.

It has been shown that levels of TNF-α and IL-6 in the intestine rises after ischemia and reperfusion and that there is a direct relationship between the magnitude of the injury and the release of cytokines. Our results showed that the longer preconditioning, ranging from two to 15 minutes, the smaller the presence of TNF-α, IL-1β and IL-6 in serum, and that in animals not subjected to preconditioning cytokines were present in comparatively very high levels.

Yahweh-Montalvo et al. studied the ischemic preconditioning in the liver and found that periods of ten minutes of ischemia followed by ten minutes of hot reperfusion, with subsequent ischemia maintained for 60 minutes, promoted less elevated liver enzymes and indirect indicators of lipid peroxidation.

A similar alternative as a prevention strategy is the ischemic postconditioning, which has shown promising results. Santos et al. compared the effects of these two mechanisms by submitting their samples to three cycles of ischemia and reperfusion, two minutes each. The preconditioning preceded ischemia, which was followed by reperfusion, and in another group ischemia was caused, then postconditioning and posteriorly reperfusion. Their results showed that both techniques are protective against tissue injury.

Data from the present study have revealed IPC of 15 minutes to have significantly attenuated bacterial translocation to target organs and this is one of the few studies showing that, in an augmenting system from two minutes to 15 minutes of ischemic preconditioning in the small intestine, 15 minutes was the optimal time for prevention of bacterial translocation after intestinal ischemia and reperfusion.

R E S U M O

Objetivo: avaliar os efeitos de diferentes tempos de pré-condicionamento isquêrmico (PCI) intestinal sobre a translocação bacteriana (TB). Métodos: Trinta ratos Wistar pesando 280±27g foram alocados em cinco grupos. No grupo IR (n=6), foi realizada laparotomia e a artéria mesentérica superior foi acluída por microclampe atraumático por 30 minutos. Nos quatro grupos com pré-condicionamento (n=6 cada), antes dos 30 minutos de isquemia-reperfusão (IR) os ratos foram submetidos a PCI de dois, cinco, dez e 15 minutos e, em seguida, ao mesmo tempo de reperfusão. Vinte e quatro horas após, para avaliar se os tempos de pré-condicionamento influenciavam o aparecimento de translocação bacteriana, amostras de linfonodos mesentéricos, fígado e baço foram coletadas em condições estéreis, para quantificação de unidades formadoras de colônias bacterianas por grama de tecido (UFC/g). Sangue foi coletado para dosagem de citocinas. Resultados: No grupo IR, o total de UFC/g em linfonodos mesentéricos, baço, fígado, bem como, a dosagem sérica de TNF-α, IL-1β e IL-6 foram significativamente maiores do que nos demais grupos (p<0,05). Pré-condicionamento de 15 minutos atenuou significativamente a BT e as citocinas séricas, comparando com os outros tempos de pré-condicionamento (p<0,05). Conclusão: Nossos dados sugerem o pré-condicionamento como fator-chave para reduzir translocação bacteriana em IR intestinal. Numa escala de dois a 15 minutos, o melhor tempo de pré-condicionamento isquêrmico para a atenuação da translocação bacteriana foi 15 minutos.


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