Ischemic preconditioning modifies mortality and inflammatory response[1]

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ABSTRACT

PURPOSE: To evaluate the effect of ischemic preconditioning on mortality, inflammatory mediators and oxidative stress after intestinal ischemia and reperfusion.

METHODS: Male Wistar rats were allocated according to the period of ischemia with or without ischemic preconditioning which consist on clamping the superior mesenteric artery for 10 minutes followed by reperfusion for 10 minutes before the sustained ischemia period. Mortality was assessed in Phase 1 study, and the CINC-1, CINC-2 and MDA levels in the lungs were analyzed in Phase 2.

RESULTS: Mortality was lower in the ischemic preconditioning group subjected to 90 minutes of ischemia compared to the group without ischemic preconditioning (I-90: 50% and IPC-90: 15%, p=0.018), and it was lower in the ischemic preconditioning group as a whole compared to the groups without ischemic preconditioning (IPC-14% and I=30%, p=0.006). Lower levels of MDA, CINC-1, and CINC-2 were observed in the animals that were subjected to ischemic preconditioning compared to the animals that were not (MDA: I-45=1.23 nmol/mg protein, and IPC-45=0.62 nmol/mg protein, p=0.0333; CINC-1: I-45=0.82 ng/mL and IPC-45=0.67 ng/mL, p=0.041; CINC-2: I-45=0.52 ng/mL and IPC-45=0.35 ng/mL, p=0.032).

CONCLUSION: Ischemic preconditioning reduces mortality, inflammatory process and oxidative stress in rats subjected to intestinal ischemia and reperfusion.

Key words: Ischemic Preconditioning. Reperfusion. Mortality. Oxidative Stress. Rats.

Introduction

Intestinal ischemia/reperfusion (I/R) resulting from the superior mesenteric artery (SMA) occlusion can lead to intestinal mucosal injury which in turn releases inflammatory mediators, resulting in a systemic inflammatory response. The consequence may be multiple organ damage, especially acute lung injury that may culminate into multiple organs dysfunction and failure[1-4]. The mechanism underlying these processes is highly complex and involves the action and interaction of cellular mediators, such as macrophages, neutrophils and endothelium, as well as humoral molecules, such as lipopolysaccharide, superoxide radicals and cytokines. Lipid peroxidation also occurs and there is an increase of malondialdehyde levels, leukocyte-endothelial adhesion, accumulation of polymorphonuclear neutrophils in the lungs alveoli, and polymorphonuclear neutrophils apoptosis[4,5]. Interleukin-8 (and its homologous CINC-1 and CINC-2 in rats) plays an important role in the pathogenesis of inflammatory bowel disease induced by intestinal I/R[5-7]. Among the different models of intestinal I/R in rats, the model involving 45 minutes of ischemia has been considered to be free of mortality[7-10]. The models having ischemia periods longer than 45 minutes have varying mortality rates that have not been clearly determined for these ischemia periods[10,11].

Intestinal preconditioning, compared to hypotermia and modulation of inflammatory mediators[11], IPC, first described by Murry *et al*.[12], consists of one or multiple short periods of ischemia followed by the same periods of reperfusion, applied prior to prolonged or appropriate ischemia[13-16] was found to be the most promising strategy to improve intestinal tolerance to I/R injury, and to protect other organs such as lungs, liver, brain, kidneys, pancreas by reducing I/R damage[16-19].

Hotter *et al*.[13] described the first rat model of IPC preceding 90-min of SMA occlusion. Others reported beneficial effects of IPC in intestinal transplantation[14,19,20]. The attenuation of the increased permeability of the mucosa by adenosine deaminase in an intestinal I/R model suggested that adenosine may be a mediator of IPC[18]. Overall, the studies of the mechanism of IPC in animal models of intestinal I/R have shown that the protective effect of IPC is attributable to the production of various inflammatory mediators[20-22]. Thus, IPC can be considered a potentially promising strategy to minimize reperfusion injury, and further studies are needed to clarify this strategy[22,23].

The effect of IPC on mortality in rats subjected to intestinal I/R has been described in the literature, but with inconclusive results[9,10] and the mortality rates after various periods intestinal I/R have not been clearly established. Moreover, although IPC has been shown to decrease the severity of intestinal I/R injuries, its effect on the mortality of the animals in these experiments has not been clearly determined.

The aim of this study was to determine the mortality rates caused by different periods of intestinal I/R in rats, and to evaluate the effect of IPC on these mortality rates, and also to evaluate the effect of IPC on the levels of the inflammatory mediators and on the marker of oxidative stress.

Methods

This research project was approved by the Ethics Committee for animal care and use for experimental research of the Faculty of Medicine, University of São Paulo (FMUSP), and was conducted at the Laboratory of Surgical Physiopathology (LIM-62) Department of Surgery.

It was used 254 Wistar male rats from the Central Animal Laboratory of the FMUSP. The study was divided into **Phase 1** (mortality) and **Phase 2** (inflammatory and oxidative stress mediators). The animals were weighed and then anesthetized with an intraperitoneal injection of ketamine hydrochloride 5% (Ketalar®, Cristalia) and xylazine 2% (Rompum®, Bayer). A median laparotomy was performed, and the superior mesenteric artery (SMA) was exposed at its origin.

Phase 1 – Mortality. The animals were allocated into the following 11 groups.

Group 1 (n=30) - control group; the animals underwent anesthesia and a median laparotomy with identification and isolation of the SMA without inducing ischemia; **Group 2** (n=26) - 45 minutes of mesenteric ischemia (I-45); the animals underwent 45 minutes of mesenteric ischemia by means of clamping the SMA at its origin;

Group 3 (n=20) - 60 minutes of mesenteric ischemia (I-60);

Group 4 (n=20) - 75 minutes of mesenteric ischemia (I-75);

Group 5 (n=26) - 90 minutes of mesenteric ischemia (I-90);

Group 6 (n=20) - 105 minutes of mesenteric ischemia (I-105);

Group 7 (n=26) - IPC + 45 minutes of mesenteric ischemia (IPC-45); the animals were subjected to IPC by SMA occlusion for 10 minutes followed by reperfusion for 10 minutes once and then 45 minutes of mesenteric ischemia;

Group 8 (n=20) - IPC + 60 minutes of mesenteric ischemia (IPC-60);

Group 9 (n=20) - IPC + 75 minutes of mesenteric ischemia (IPC-75);

Group 10 (n=20) - IPC + 90 minutes of mesenteric ischemia (IPC-90);

Group 11 (n=20) - IPC + 105 minutes of mesenteric ischemia (IPC-105).

After the sustained ischemia, the animals had its abdominal cavity closed and were kept under observation for seven days with free access to water and food to observe their mortality.

Phase 2 – Inflammatory and oxidative stress mediators

The evaluation of the changes in the inflammatory mediators and oxidative stress marker was performed in the following four groups: **Group 2** (I-45), **Group 5** (I-90), **Group 7** (IPC-45) and **Group 10** (IPC-90). These groups were selected after the statistical analysis of the results of the Phase 1 study. The aim of the Phase 2 study was to evaluate the changes in the inflammatory mediators as a function of the variation of the ischemia duration, either in the presence and absence of IPC. A total of 24 animals, 6 from each group (Groups 2, 5, 7 and 10), were employed in the Phase 2 study.

The difference between the initial procedures for the Phase 2 study, compared to the Phase 1 study, was the time of reperfusion that was established as 2 hours for the Phase 2 study. At this point, rats were anesthetized again for reoperation, and both lungs were removed for analysis. The rats were sacrificed by exsanguination under anesthesia.

Inflammatory mediators (CINC-1 and CINC-2)

The levels of the systemic inflammatory mediators, CINC-1 and CINC-2, in the lungs were determined by using ELISA (Enzyme-linked immunosorbent assay method), using commercially available kits and following the manufacturer's instructions (R & D Systems Inc., Minneapolis, MN, USA).

Malondialdehyde

The quantification of malondialdehyde in the lungs was performed using the thiobarbituric acid reactive substances technique as described elsewhere[24]. It is based on a reaction with thiobarbituric acid in low pH and high temperature forming the MDA-TBA (malondialdehyde-thiobarbituric acid) complex, which presents characteristic color and absorption. Those characteristics can be detected through spectrophotometry.

Statistical analysis

For the statistical analysis, the results were organized into tables and graphs. The variables, including initial weight, final weight, and weight gain, were subjected to the Kolmogorov-Smirnov test to assess the normality of the distribution. The initial weight was normally distributed among the 11 groups in the study. Then, we tested whether this variable was uniformly distributed among the various groups using an analysis of variance with a criterion (One-way ANOVA).

Because the weight gain was not normally distributed among the 11 groups in the study, we applied the nonparametric Mann-Whitney test. To evaluate the differences between the groups with IPC and the groups without IPC, all of the IPC groups and the ischemia without IPC groups were matched according to the duration of ischemia (45, 60, 75, 90 and 105 minutes), and then, these groups were compared with the control group.

The analysis of outcome (survival or death) was performed using the measures of association (chi-square test) in the following groups: IPC groups *versus* ischemia without IPC groups (45, 60, 75, 90 and 105 minutes), i.e., the IPC groups (IPC-45, IPC-60, IPC-75, IPC-90 and IPC-105) versus the ischemia without IPC groups (I-45, I-60, I-75, I-90 and I-105) and the control group.

For the qualitative variables (CINC-1, CINC-3 and MDA), we analyzed the median absolute (N) and relative frequencies (%). The results of the measurements of the inflammatory mediators (CINC-1 and CINC-2), as well as those of the marker of oxidative stress (malondialdehyde), which were considered as parametric variables, were evaluated using the ANOVA test. In cases with a significant difference, the Tukey test was used to analyze the pairs. The Graph Pad Prism version 6.0 program was used for statistical analysis, and the significance level was 5% for all of the tests.

Results

Mortality - Phase 1

In this phase, 230 animals were included: 20 in each of groups 2 to 11, and 30 animals in the control group (CTR). The mortality rates observed in the groups are presented in Figure 1. The mortality rate increased as the duration of ischemia increased, and no mortality was observed in the control group (CTR) or in the I-45 and IPC-45 groups (Figure 1). A significant difference was observed between the I-90 and IPC-90 groups (p=0.018). The lower mortality observed in the IPC groups with 60, 75 or 105

minutes of ischemia was not significant. However, the mortality rates observed in all groups with IPC, when taken together, were significantly small when compared with the similar rates of the groups without IPC, also taken together (Figure 2).

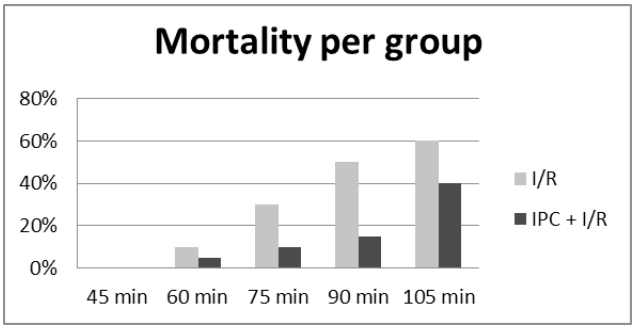

FIGURE 1 - Comparison of the mortality observed in the groups subjected to I/R with those subjected to IPC + I/R in the diverse duration of I/R.

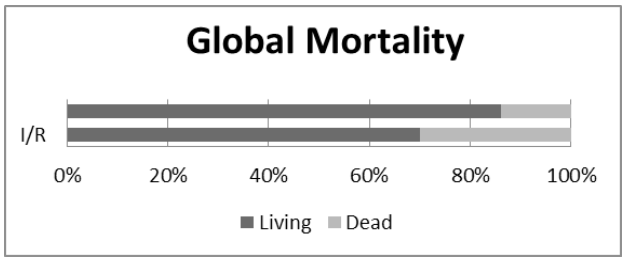

FIGURE 2 - Compares the mortality observed when all the groups undergoing IPC, taken together, were compared with those undergoing only I/R.

Phase 2 – Evaluation of inflammatory mediators

We observed a significant increase in both CINC-1 and CINC-2 levels in IPC-45 compared to I-45 groups. The malondialdehyde levels in the lungs were significantly decreased in IPC-45 compared with I-45 groups. No significant difference was observed in any of the measurements (CINC-1, CINC-2 and malondialdehyde levels) between the I-90 and IPC-90 groups (Figures 3 to 4).

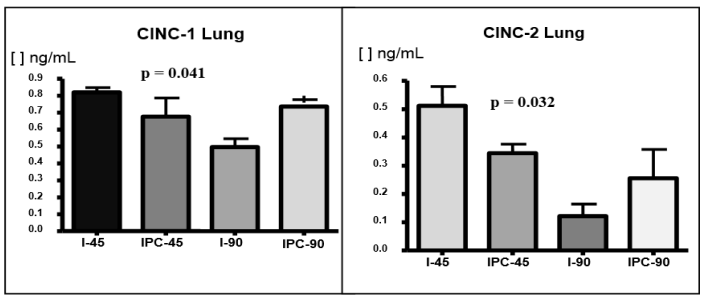

FIGURE 3 - CINC-1 and CINC-2 values in the lung according to the groups.

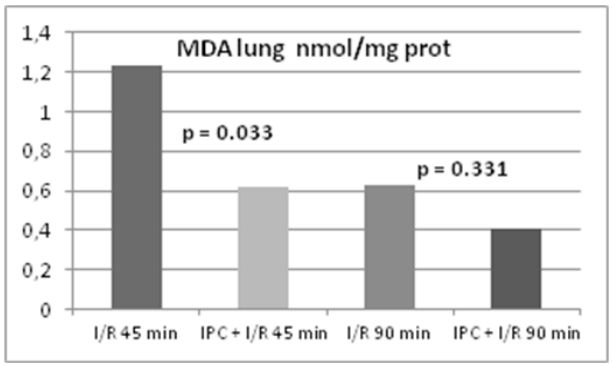

FIGURE 4 - The malondialdehyde (MDA) levels in the lung (groups 45 minutes and 90 minutes of ischemia, followed by 2 hours of reperfusion, with or without ischemic preconditioning (IPC)) in Phase 2. I/R –ischemia and reperfusion.

Discussion

The study of intestinal ischemia/reperfusion (I/R) is of great importance because the deleterious effects of I/R may aggravate the clinical conditions of patients undergoing major surgery (i.e., cardiac, vascular, transplant, strangulated hernias and neonatal necrotizing enterocolitis) and also of patients in certain emergency medical situations, such as severe trauma, extensive burns, hemorrhagic shock and septic shock. Furthermore, in cases of prolonged ischemia, intestinal I/R may cause distant organ damage due to a systemic inflammatory response syndrome[22,23]. Because the intestine is the most perfused organ, it is usually affected by I/R during major cardiac surgery, myocardial infarction and vascular surgery[25], and it is likely the most sensitive to I/R injury[11,26]. Although intestinal ischemia is uncommon in heart surgeries (0.53% to 3.7%); when present, it is associated with high mortality (64% to 100% of the cases)[27].

Kubes *et al*.[28] showed that most of the liver I/R models (including liver transplantation) have some degree of intestinal ischemia, which enhances the liver injury by inducing the release of toxins and intestinal neutrophil recruitment[27,29]. Recently, intestinal transplantation has become the preferred therapy for patients with short bowel syndrome, with intolerance to total parenteral nutrition[14,17,19]. However, the lesion caused by intestinal I/R is a limiting factor for successful intestinal transplantation[19], and it is as important as the proper preservation of the intestinal graft for minimizing the I/R injury[14,17].

Since even short periods of mesenteric ischemia can damage the intestinal mucosa, then intestinal I/R can cause major changes in the mucosal barrier, leading to translocation and release of endotoxins in the bloodstream[22,23], causing systemic inflammatory response and MOSF[2]. Therefore, controlling

intestinal I/R injury is important to achieve successful intestinal transplantation[14,17,22]. Among the several strategies developed to minimize the deleterious effects of intestinal I/R, IPC has great importance because it protects the intestine against I/R injury[20].

The 10 minutes IPC was chose for use in this study because it has been demonstrated to protect organs against I/R injury[3,11,14,19,30]. Based on the statistical analysis of the results of the Phase 1 of this study, we chose the periods of 45 and 90 minutes of ischemia for the Phase 2 because 45 minutes was confirmed as free from mortality, either with or without IPC, and 90 minutes caused significantly less mortality when IPC was applied. Based on the literature supporting that acute intestinal and acute lungs injuries are detectable at two hours after 45 minutes of intestinal ischemia[2], we chose two hours of reperfusion to evaluate the effect of IPC on the levels of the inflammatory mediators CINC-1 and CINC-2, and the marker of oxidative stress malondialdehyde.

Phase 1 of this study showed increased mortality rates as the duration of ischemia increased. This was expected once longer ischemia periods lead to greater organ damage, with increased injury degrees, with greater chances of occurring systemic inflammatory response, MOSF and death[23]. In both, Phase 1 and Phase 2, no death occurred during the ischemia period. All deaths occurred within the first 24 hours of reperfusion, which is consistent with previous demonstrations that reperfusion is more damaging than ischemia itself[2,4,7].

The type of rats employed, aged between three and four months, receiving food and water ad libitum, usually have a weight gain of 30 grams per week[31]. The weight gain of all the rats subjected to I/R, when allocated into two groups (with IPC, and without IPC), was respectively 7.86g and 5.52g, with a significant difference between the groups ($p=0.014$). Furthermore, the weight gain in each group was significantly lower than that observed in the CTR group ($p<0.001$), These results demonstrate the suppressive effect of I/R on the weight gain, as well as the attenuating effect of IPC on this effect of I/R.

Avgerinos *et al*.[32] reported a significant decrease in plasma malondialdehyde values in rats subjected to IPC followed by 45 minutes of mesenteric I/R, compared to the rats without IPC. Likewise, we observed a significant reduction in the malondialdehyde values in the lungs of rats that were subjected to 45 minutes of mesenteric ischemia preceded by IPC and followed by two hours of reperfusion. In the group of animals that were subjected to IPC plus 90 minutes of ischemia, the decrease in the malondialdehyde value did not reach the level of significance.

Tsuboi *et al*.[6] demonstrated that the CINC-1 levels in the intestinal mucosa increased considerably in rats subjected to 30 minutes of intestinal ischemia followed by 60 minutes of reperfusion. We observed a significant increase of the CINC-1 level in the lungs of the animals subjected to 45 minutes of intestinal ischemia followed by 2 hours of reperfusion, whereas this increase of the CINC-1 level was attenuated in the animals that underwent IPC. Interestingly, we observed a significant reduction of both the CINC-1 and CINC-2 values in the animals subjected to 90 minutes of ischemia followed by reperfusion; however, increased CINC-1 and CINC-2 values were also observed in the animals subjected to IPC followed by 90 minutes of ischemia. The finding of extremely low CINC-1 and CINC-2 values in the animals that were subjected to 90 minutes of ischemia without IPC was interpreted as a lack of response in the animal, which, after 90 minutes of intestinal ischemia, was unable to produce and release the inflammatory mediators. The animals that underwent IPC before 90 minutes of ischemia also had a theoretically greater capacity to provide a response to injury; these animals released greater amounts of inflammatory mediators and, therefore, had higher CINC-1 and CINC-2 values. This hypothesis seems to be supported by the findings of the Phase 1 study: 50% mortality in the animals that underwent 90 minutes of intestinal ischemia without IPC *versus* 15% mortality in the animals in the IPC-90 group.

It was also observed that the CINC-1 and CINC-2 values were both higher in the animals that were subjected to 45 minutes of mesenteric ischemia than in the animals that were subjected to 90 minutes of ischemia. Initially, this result was unexpected because the animals subjected to longer ischemic times have higher mortality and, therefore, should have higher values of pro inflammatory cytokines. This finding seems to reconfirm the hypothesis that the animals subjected to prolonged mesenteric ischemia (for 90 minutes in this study) are in an advanced stage of the disease and bowel inflammation and, therefore, would be unable to produce an adequate amount of pro-inflammatory cytokines. Conversely, the animals subjected to the same period of ischemia preceded by IPC would be able to produce an adequate amount of these cytokines because of the protective effect of IPC.

Sola *et al*.[30,33] observed a decrease in serum malondialdehyde levels caused by the effect of IPC in rats subjected to intestinal I/R. A similar result was observed in the present study, indicated by the reduction of the pulmonary malondialdehyde value in rats subjected to 45 minutes of intestinal ischemia.

As it has been reported, intestinal ischemia also occurs during abdominal surgeries that are not performed directly on the intestine, and also during extra-abdominal surgeries[11,25,26]. However, despite this potentially promising result, further studies are necessary to characterize the possibility of using intestinal IPC in humans.

Conclusion

Ischemic preconditioning reduces mortality, inflammatory process and oxidative stress in rats subjected to intestinal ischemia and reperfusion.

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