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Ischemic versus pharmacologic hepatic preconditioning

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ABSTRACT

Background: Hepatic ischemia–reperfusion injury has a significant impact on liver resection and transplantation. Many strategies have been developed to reduce the effects of ischemia–reperfusion injury, including pharmacologic and ischemic preconditioning; however, studies comparing these two methods are lacking.

Material and methods: An experimental study was performed in a swine model. Eighteen swine were randomly assigned to three different groups: an ischemic preconditioning (IschPC) group, a pharmacologic preconditioning (PharmPC) group, and a control group. All animals underwent a 40-min liver ischemia, followed by 40 min of reperfusion. The IschPC group received a short period of ischemia (10 min) and a short period of reperfusion (15 min) before prolonged ischemia. The PharmPC group received inhaled sevoflurane for 30 min before prolonged ischemia. The control group did not receive any intervention before prolonged ischemia. Blood samples and liver tissue were obtained after ischemic and reperfusion periods. Injury was evaluated by measure of DNA damage (using COMET assay) and serum biochemical markers (transaminases, alkaline phosphatase, amylase, bilirubin, and C-reactive protein [CRP]).

Results: No significant difference was found in serum biochemical markers, except for the C-reactive protein level that was lower in the PharmPC group than in the control group soon after hepatic ischemia. Soon after prolonged ischemia, DNA damage index, both in blood samples and in liver tissue samples, was similar among the groups. However, an increase in DNA damage after reperfusion was higher in the control group than in the PharmPC group ($P < 0.05$). The increase in DNA damage in the IschPC group was half of that observed in the control, but this difference was not statistically significant.

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Conclusions: Our results suggest an early protective effect of PharmPC (lower levels of C-reactive protein soon after ischemia). The protective effect observed after reperfusion was higher with PharmPC than with ischemic preconditioning. The simultaneous use of both methods could potentiate protection for ischemia–reperfusion.

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1. Introduction

Hepatic ischemia occurs in a variety of conditions in liver surgery [1–7]. It is mainly associated with the use of inflow occlusion during hepatectomy (to prevent blood loss) and in the context of liver transplantation, when a period of cold ischemia is needed [8–10]. The reperfusion of the liver after a long ischemic period induces a number of hepatic lesions, resulting in ischemia–reperfusion (IR) syndrome. This syndrome was described in 1975 by Toledo-Pereyra et al. [11] but its pathophysiology is not entirely understood yet.

Several interventions have been developed to minimize IR injury [12]. Ischemic preconditioning (IschPC) consists of a short period of ischemia followed by a short period of reperfusion, before a prolonged ischemia and reperfusion. Its protective effects were first described in renal surgery [13], and later in an experimental study on myocardial ischemia [14]. Currently, IschPC is a strategy to reduce morbidity after liver resection. Pharmacologic preconditioning (PharmPC), mainly through the use of inhaled anesthetics, has also been used with the same goal. The mechanism through which protection occurs is not yet well established; however, some studies suggest that this is due to the activation of potassium channels, whereas others suggest that protection occurs through anti-inflammatory pathways [15]. Thus, these two types of intervention are used to “prepare” the liver for a prolonged ischemic period.

Most studies have evaluated the ischemic technique in liver preconditioning, and data on PharmPC are scarce. Despite the probable different mechanisms of ischemic and PharmPC, to the best of our knowledge, no previous study has been designed to compare these two methods. Moreover, almost all experimental studies have used small animal models [16–28], whereas our study compared the effect of ischemic versus pharmacologic hepatic preconditioning in medium-size animals (swine).

2. Methods

2.1. Animals and study design

Eighteen male swine weighing 20–25 kg, aged 3–4 mo, were fed with standard chow until 12 h before the surgical procedure. They were randomized in the following three groups (Fig. 1): an IschPC group ($n = 6$), a PharmPC group ($n = 6$), and a control group ($n = 6$). All groups were submitted to a prolonged ischemic period (40 min) followed by a reperfusion period (40 min). The IschPC group received 10 min of ischemia followed by 15 min of reperfusion, before the prolonged ischemia. The PharmPC group received an inhaled anesthetic (sevoflurane) for 30 min, before prolonged ischemia. In the

control group, no protective strategy was used. Blood samples from the suprahepatic superior vena cava and tissue samples from the liver were taken immediately after the prolonged ischemia and also within 40 min of reperfusion. After the reperfusion period, liver and blood samples were collected before animals were sacrificed.

Ischemic–reperfusion injury was evaluated through analysis of the liver tissue and the blood cells, and through analysis of serum levels of hepatocellular and systemic inflammatory markers.

Animals were housed and sacrificed according to institutional animal care policies. The study was approved by our institution's ethics committee for experimental studies.

2.2. Standard surgical procedure and sampling

After sedation with intramuscular ketamine at 5 mg/kg and midazolam at 0.3 mg/kg, each animal was placed on a proper operating table and electrocardiographic monitoring was initiated. Peripheral venous access was obtained and tracheotomy performed under local anesthesia with lidocaine. Immediately after a definitive airway was obtained, endovenous anesthesia was performed using fentanyl at 0.05 mg/kg, midazolam at 0.3 mg/kg, and pancuronium at 0.1 mg/kg (with reinfusion as needed). All animals were mechanically ventilated. A carotid artery was cannulated for continuous arterial pressure monitoring.

A median laparotomy was performed and access to the upper abdomen aided by a Balfour retractor (Edlo, Canoas, Brazil). The lesser omentum was opened and an umbilical tape used to encircle the hepatic pedicle. Pringle maneuver was used to perform hepatic ischemia. Blood samples were

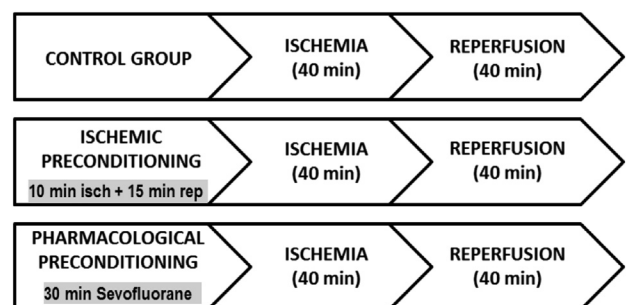


Fig. 1 – Experimental protocol. Animals were randomized into three groups ($n = 6$ per group). They underwent 40 min of ischemia followed by 40 min of reperfusion. Control group did not receive any intervention. IschPC group received a short period of ischemia (10 min) and a short period of reperfusion (15 min). PharmPC group received inhaled sevoflurane for 30 min before prolonged ischemia and reperfusion.

obtained by punctuation in the junction of hepatic veins and inferior vena cava. Hepatic tissue samples were obtained with deep parenchymal biopsy through a hepatotomy.

2.3. Measurement of DNA damage

COMET assay (Single Cell Gel Electrophoresis assay) was used to evaluate DNA damage. This is a rapid, sensitive, and relatively simple method for detecting DNA damage at the level of individual cells.

A single suspension of the cells of interest (blood or hepatic tissue) was suspended in low-melting agarose and layered onto slides precoated with agarose. This process was performed three times for each sample. Lysis of the cells, through high-salt concentration (2.5 M of NaCl, 100 mM of EDTA, 10 mM of Tris, 1% Triton X-100, and 20% Dimethyl Sulfoxide—pH 10.0) for a duration of 2 h, was then carried out to remove cellular proteins and release the damaged DNA. The released DNA was subjected to unwinding under alkaline and/or neutral conditions to allow DNA supercoils to relax and express DNA single-strand breaks and alkali labile sites. Electrophoresis was then carried out under neutral and/or highly alkaline conditions (0.3 M of NaOH and 1 mM of EDTA, pH > 13) for a duration of 15 min, which allowed the broken ends to migrate under the effect of an electric field, toward the anode.

The electrophoresis conditions were 0.7 V/cm for 20 min. Then the slides were washed with neutralizing buffer (0.4 M of Tris, pH 7.5) and distilled water and then placed in an oven to dry. Fixing was performed for 10 min with a solution containing 15% trichloroacetic acid, 5% zinc sulfate, and 5% glycerol. After drying at room temperature, the samples were rehydrated and slides were stained with silver nitrate solution for 35 min. The slides were kept in a humidified slide chamber until they were scored. The analysis of the specimens was made using conventional optical microscopy at 100-fold increase. Two of the three slides prepared were analyzed by counting 50 nucleoids per slide. Each nucleoid was classified visually into five classes according to the damage found: from undamaged (class 0) to maximum damage (class 4) (Fig. 2).

The damage index was calculated by multiplying the number of comets in each class and the denominator numeral of the class (0, 1, 2, 3, or 4). Thus, damage index could vary from zero (100 nucleoids observed \times class 0) to 400 (100 nucleoids observed \times class 4) [29].

2.4. Measurement of biochemical markers

The serum levels of aspartate transaminase, alanine transaminase, alkaline phosphatase, amylase, direct bilirubin, total bilirubin, and C-reactive protein (CRP) were measured with a Biosystems A25 automatic analyzer (Barcelona, Spain).

2.5. Statistical analysis

Data were tabulated in spreadsheets and statistical analysis was performed using the GraphPad Prism 5.0 software (GraphPad Software, La Jolla, USA). Comparison of quantitative variables among groups was done with a Mann–Whitney U-test. A value of $P < 0.05$ was considered significant.

3. Results

3.1. DNA damage

The DNA damage index evaluated through the COMET assay immediately after the prolonged ischemic period showed less cellular damage in blood samples than in liver tissue samples ($P < 0.05$).

3.2. DNA damage after ischemia

The damage index measured immediately after the prolonged ischemic period in the control group, IschPC group, and PharmPC group was 0.5 ($n = 6$), 0.5 ($n = 6$), and 1.3 ($n = 6$) in the blood samples; and 36 ($n = 6$), 78 ($n = 6$), and 63 ($n = 6$) in the liver tissue samples, respectively (Figs. 3 and 4). No significant difference in the damage index was detected among the groups, in the blood samples, and the liver tissue samples.

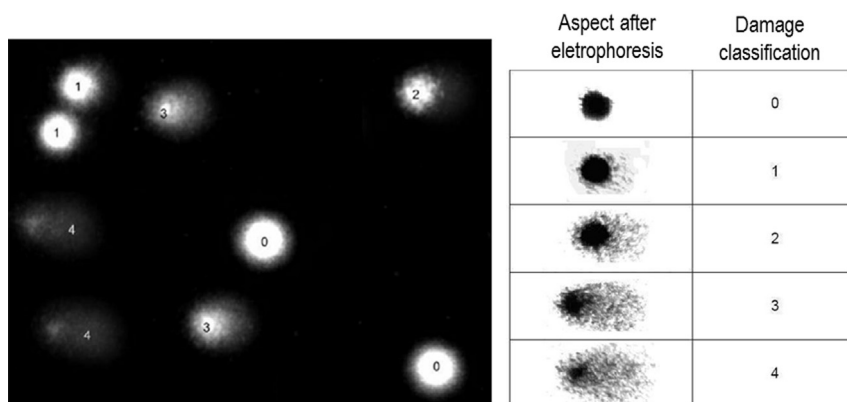


Fig. 2 – Classification of cellular damage on COMET Assay. After electrophoresis each nucleoid is classified from 0 (no damage) to 4 (severe damage). The damage index is calculated by multiplying the number of comets in each class by the respective damage classification (0–4). Photomicrography of a case on the left (numbers indicate the damage class) and schematic representation on the right.

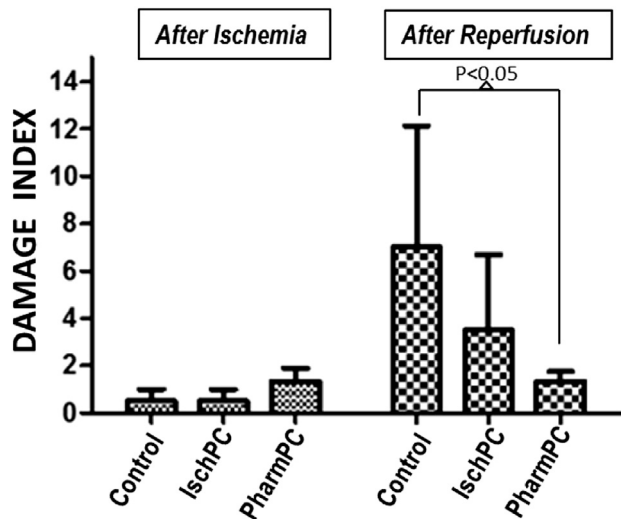


Fig. 3 – Damage index measured on blood cells. Blood was collected from the inferior vena cava in the control, IschPC, and PharmPC groups after 40 min of ischemia and 40 min of reperfusion. After ischemia there was a low damage index in all groups, however after reperfusion there was a greater increase of the damage index in the control group compared with the PharmPC ($P < 0.05$) and the IschPC (P non significant) groups.

However, the damage index immediately after the ischemic period was higher in both the preconditioning groups than in the control group ($P < 0.05$).

3.3. DNA damage after reperfusion

Blood samples—After the reperfusion period of 40 min, there was a significant increase in the detection of DNA damage in

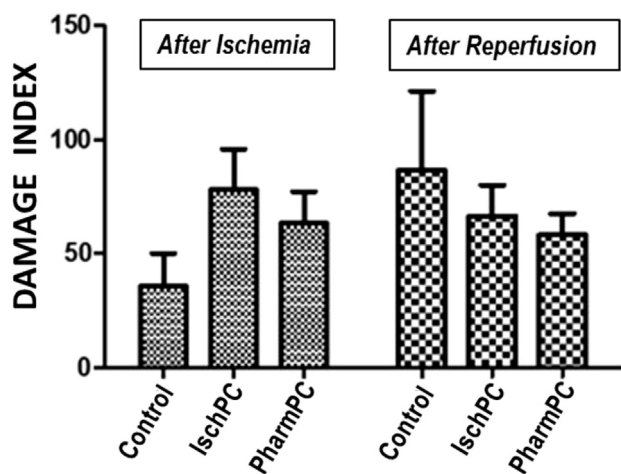


Fig. 4 – Damage index in liver tissue. Damage index is measured on liver cells in the control, IschPC, and PharmPC groups after 40 min of ischemia and 40 min of reperfusion. After reperfusion the damage index in the control group more than doubled, conversely, the damage index after reperfusion in the IschPC and PharmPC groups tended to be lower than before reperfusion (these differences did not reach statistical significance).

the blood samples of the control group (damage index from 0.5–7.0). In the IschPC group, this increase was modest (damage index from 0.5–3.5), and in the PharmPC group no increase in the damage index was observed in blood samples after the reperfusion period. The increase in damage index detected in the blood samples after reperfusion was significantly greater in the control group than in the PharmPC group (increase of 6.5 in the control group versus 0 in the PharmPC group, $P < 0.05$) (Fig. 3).

Tissue samples—In contrast to the findings in blood samples, analysis of hepatic tissue samples demonstrated elevated damage index immediately after prolonged ischemia and also after reperfusion ($P > 0.05$). After the reperfusion period of 40 min, the damage index in hepatic tissue samples in the control group was more than two times greater than that observed before reperfusion. On the other hand, in both the IschPC group and PharmPC group, there was a tendency toward reduction in the damage index measured in hepatic tissue. However, there was no statistically significant difference among the groups (Fig. 4).

3.4. Changes in serum biochemical markers

The serum levels of CRP were significantly lower in the PharmPC group than in the control group after 40 min of ischemia ($P < 0.001$) (Fig. 5). After the reperfusion period, this difference was no longer statistically significant.

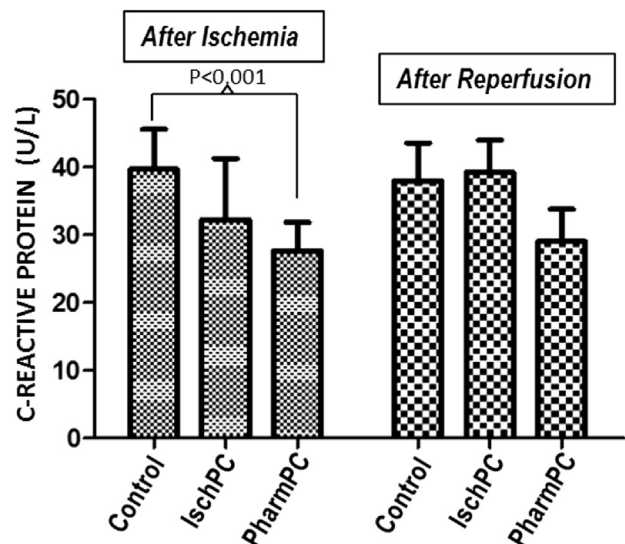


Fig. 5 – Serum levels of CRP protein. The serum levels of CRP protein in the control, IschPC, and PharmPC groups after 40 min of ischemia and 40 min of reperfusion. The serum levels of CRP were significantly lower in the PharmPC group than in the control group after ischemia ($P < 0.001$), but no significant difference was detected after reperfusion.

No differences in serum levels of aspartate transaminase, alanine transaminase, amylase, alkaline phosphatase, direct bilirubin, or total bilirubin were observed among the groups after the ischemic and reperfusion periods.

4. Discussion

Several experimental and clinical trials have attempted to elucidate the benefits of ischemic and PharmPC in liver surgery [16–28]. The clinical studies are limited by the large number of variables that potentially influence the results, requiring a large number of patients to reach solid conclusions [30]. Furthermore, most studies in humans use biochemical endpoints as surrogates of clinical data.

Experimental studies are widely used for evaluation of IR injury in various organs [31,32]. Most of these studies are performed in small animals, with very different life cycles compared with humans, making the findings less helpful in clinical practice [16–28]. We developed a model of IR in animals of medium size (swine), with biology more similar to humans. Although most studies have evaluated the effect of a single method of preconditioning, we used a unique model to compare two preconditioning methods, ischemic and pharmacologic.

The damage index measured in hepatic tissue samples showed no difference among the groups. The protective effect of preconditioning that was clearly demonstrated in blood samples might not have been demonstrated in liver tissue samples due to the precocious damage induced by the hepatic ischemia. A high rate of DNA damage was observed in hepatic tissue immediately after prolonged ischemia (before the reperfusion period). Thus, in the hepatic tissue, the difference between ischemic and reperfusion indices was not as great as in the blood samples. This more evident effect on blood samples than hepatic tissue samples could be explained by the fact that, before reperfusion, a minimal amount of cells with damaged DNA was discharged in the systemic bloodstream; however, ischemia *per se* could be responsible for significant DNA damage detected in liver tissue before reperfusion. Thus, the measurement of DNA damage in central venous blood seems to represent more clearly the damage caused by IR syndrome, and it allows a better evaluation of the effects of different methods of preconditioning. Blood sampling was always performed at the junction of the hepatic veins into the inferior vena cava, meaning minimal dilution of the damaged DNA released from the liver.

The lesser increase of serum CRP after ischemia in the PharmPC group suggests an early protective effect with this strategy, even before reperfusion injury.

IschPC has been widely investigated in the context of hepatic, cardiac, and renal surgery. On the other hand, studies on PharmPC are scarce. As in our study, volatile anesthetics are the commonest agents applied for PharmPC in liver surgery. However, other drugs have been tested to induce organ protection before IR. Preconditioning with steroids alone or combined to hiperoxia was efficacious to protect cardiac function after cardiac IR [33,34].

Despite the numerous studies on liver preconditioning, the exact mechanisms of protection have not yet been completely

explained. The different results found in our study, using ischemic and PharmPC, suggest that stimulation of protective mechanisms may occur by different routes or different time frames by one or the other of these methods. Thus, the concomitant use of two methods of preconditioning, ischemic and pharmacologic, could enhance protective effects. Future studies are warranted to define the role of single and combined preconditioning strategies.

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Disclosure

The authors reported no proprietary or commercial interest in any product mentioned or concept discussed in the article.

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