Topical hepatic hypothermia associated with ischemic preconditioning. Histopathological and biochemical analysis of ischemia reperfusion damage in a 24 hour model[1]

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Abstract

Purpose: To develop a new 24 hour extended liver ischemia and reperfusion (LIR) model analyzing the late biochemical and histopathological results of the isolated and combined application of recognized hepatoprotective mechanisms. In addition, we used a new stratification with zoning to classify the histological lesion.

Methods: A modified animal model of severe hepatic damage produced through 90 minutes of segmental ischemia (70% of the organ) and posterior observation for 24 hours of reperfusion, submitted to ischemic preconditioning (IPC) and topical hypothermia (TH) at 26ºC, in isolation or in combination, during the procedure. Data from intraoperative biometric parameters, besides of late biochemical markers and histopathological findings, both at 24 hours evolution time, were compared with control (C) and normothermic ischemia (NI) groups.

Results: All groups were homogeneous with respect to intraoperative physiological parameters. There were no losses once the model was stablished. Animals subjected to NI and IPC had worse biochemical (gamma-glutamyl transpeptidase, alkaline phosphatase, lactate dehydrogenase, aspartate aminotransferase, alanine aminotransferase, direct bilirubin, and total bilirubin) and histopathological scores (modified Suzuki score) compared to those of control groups and groups with isolated or associated TH (p < 0.05).

Conclusion: The new extended model demonstrates liver ischemia and reperfusion at 24 hour of evolution and, in this extreme scenario, only the groups subjected to topical hypothermia, combined with ischemic preconditioning or alone, had better outcomes than those subjected to only ischemic preconditioning and normothermic ischemia, reaching similar biochemical and histopathological scores to those of the control group.

Key words: Ischemic Preconditioning. Ischemia. Reperfusion. Liver. Hypothermia. Rats.

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Topical hepatic hypothermia associated with ischemic preconditioning. Histopathological and biochemical analysis of ischemia reperfusion damage in a 24 hour model
Gabiatti G *et al.*

■ Introduction

Liver ischemia and reperfusion (LIR) has been the subject of intense study in recent decades. LIR is involved in many healthcare scenarios, including trauma, hemorrhagic shock and resuscitation, septic shock, cardiac failure, respiratory insufficiency, hepatectomy and liver transplantation[1]. With the introduction of surgical control techniques of the hepatic vascular pedicle, today eventually still necessary during hepatic surgical procedures[2], ischemia and reperfusion have been considered key elements in postoperative morbidity and mortality since severe complications such as liver dysfunction and/or failure can result from this reperfusion process and injure other organs and systems[3]. The major mechanisms of such injuries are the generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS)[4] together with the infiltration of polymorphonuclear leukocytes among other mechanisms of cellular injury[5]. In normothermic conditions, the liver can tolerate clamping of the vessels of its pedicle for approximately 1 hour when the liver parenchyma is normal, whereas additional protection is required with diseased livers[6].

To minimize the effects of LIR, several methods have been studied[7-9], including ischemic preconditioning (IPC)[10,11] and topical hypothermia (TH)[12]. The reduction of oxidative stress with the application of IPC is multifactorial[13,14], with the activation of specific genes, production of antioxidant enzymes and production of nitric oxide, the latter being a potent vasodilator with important antagonist effects during the ischemia phase[15,16]. Hypothermia achieves its objectives by inducing hypometabolism via suppressing mitochondrial activity, as an essential part of its cytoprotective effect[17,18].

The aim of this study was to compare the association of IPC with TH compared to either method alone in an LIR model with extreme injury, monitoring intraoperative physiological parameters[19] and their possible histopathological[20] and biochemical[21] consequences measured at 24 hours of reperfusion after ischemic insult. The application of TH, alone or in conjunction with IPC, has already been evaluated by our group in an acute animal model with partial 90-minute liver ischemia followed by 120 minutes of reperfusion and immediate euthanasia, with the demonstration of its protective effects through the reduction of inflammatory markers such as tumor necrosis factor-α (TNF-α) and interleukin (IL)-1, IL-6, and IL-12; an increase in anti-inflammatory cytokines such as IL-10; increased levels of nitric oxide; and elevated expression of Nrf2, Keap1, and NQO1 genes; all are related to antioxidant effects[22-24].

Several modifications have been made to the original partial ischemia model, especially related to the anesthesia method, surgical incision, and intra- and post-operative care, thus allowing an experimental model with extended observation time of 24 hours of reperfusion, enabling the analysis of the late effects of different cytoprotective methods (IPC and TH). The combined application of IPC and TH in a model with this time duration, both with ischemia and reperfusion, has not been described to date; however, it is fully justified considering the therapeutic plateau reached regarding the pursuit of proven effective options related to the control of experimental LIR. It should be noted that it carries a greater likelihood of clinical applicability, possibly the critical point of the current studies related to LIR, namely the application and reproduction of experimental studies in clinical care models[25-27].

■ Methods

This study, identified by number 08486, was approved by the Bioethical Committee

Topical hepatic hypothermia associated with ischemic preconditioning. Histopathological and
biochemical analysis of ischemia reperfusion damage in a 24 hour model
Gabiatti G *et al.*

Acta Cir Bras. 2018;33(10):924-934

and funded by the Post-Graduation Group of the HCPA-UFRGS, which adheres to the rules of the Council for International Organization of Medical Sciences (CIOMS).

Thirty-three male Wistar rats (mean weight, 279g; mean age, 65 days) were divided into five groups: control (C; n = 5), normothermic ischemia (NI = 7), topical hypothermia at 26ºC (TH; n = 7), ischemic preconditioning (IPC; n = 7), and ischemic preconditioning + topical hypothermia at 26ºC (IPC+TH; n = 7).

Anesthesia protocol

The animals were subjected to inhalation anesthesia administered through a facial cannula connected to a respirator, which maintained a constant oxygen flow of 0.5 L per minute and a mixture of anesthesia with isoflurane 1–2%. The upper portion of the abdomen and the right cervical region were trichotomized and prepared with aqueous iodine solution.

Monitoring was performed through a catheter installed in the right carotid artery (PE-50; Portex, Kent, England) to control the mean blood pressure (MBP) during the entire procedure. In addition, oximetry was performed through an external sensor applied to the animal`s tail, that enabled also the recording of heart rate (HR). Saline solution (NaCl 0.9%, 2 mL/kg/h) was continuously infused into the carotid artery to replace the basal needs, compensate for hydroelectrolytic losses and maintain catheter patency. The body temperature was monitored by a rectal digital thermometer and maintained at 36–37.5ºC with the aid of a heating device of the surgical table and by external lamp heating.

Surgical procedure

Access to the abdominal cavity was attained through a median laparotomy. In group C, the hepatic lobes were isolated without any additional procedure. In the groups with ischemia (NI, IPC, TH, and IPC+TH), the liver was isolated in the operative field and an atraumatic neurosurgical clip was used to interrupt the portal and arterial blood flow to the left and middle lobes (70% of the hepatic mass) for 90 minutes, while the right and caudate lateral lobes remained uninterrupted. In the groups with IPC, the left and median lobes of the liver were isolated in the operative field and submitted to 10 minutes of ischemia, followed by reperfusion for another 10 minutes before the final clamping for 90 minutes.

In the hypothermia groups, the hepatic lobes were isolated in the operative field using a concha-like thermo-insulating styrofoam device and cooled with cold physiological solution dripped onto the surface of the ischemic lobes during the 90-minute ischemia period. The excess liquid was removed by aspirators, preventing the freezing liquid from penetrating the abdominal cavity and causing systemic hypothermia in the animals, similar to the model developed by Biberthaler *et al.*[28]. A flexible microprobe (TI-23; Braintree Scientifics) was implanted into the left lobe and the intrahepatic temperature (IHT) was monitored continuously to reach 26ºC locally (Thermalert Monitoring Thermometer TH-5; Boston Scientifics). In the other groups, IHT was also recorded at the same insertion point and with the same measurement duration.

During the entire procedure, at 15-minute intervals, vital physiological parameters such as HR, MBP, respiratory rate (RR), systemic temperature (ST), and intrahepatic temperature (IHT) were recorded. In all groups, the same parameters were recorded 1 minute after declamping of the pedicle (91-minute procedure). The percentage of isoflurane in the inhalational anesthetic mixture was also recorded during all procedures, as was the temperature of the surgical room.

Acta Cir Bras. 2018;33(10):924-934

Topical hepatic hypothermia associated with ischemic preconditioning. Histopathological and biochemical analysis of ischemia reperfusion damage in a 24 hour model
Gabiatti G *et al.*

Post-anesthesia procedures and sampling

After 90 minutes of ischemia, the metal clips of the pedicle were removed, and the organ was reperfused. The abdominal wall was closed, the surgical incision infiltrated with 0.1 mL of 0.5% bupivacaine solution in 0.9 mL of saline solution, and the animal was transferred to an incubator for anesthesia recovery.

At the end of 24 hours, the rats were anesthetized again with isoflurane inhalation as described in the previous procedure. The abdomen was opened and the animals were sacrificed by cardiac puncture and exsanguinating blood aspiration. The liver was removed and stored; a fraction was fixed in formalin and another fraction was frozen in nitrogen and kept in a freezer at -80ºC. The blood was immediately centrifuged and the plasma obtained frozen in liquid nitrogen and stored in a freezer at -80ºC. The liver samples were identified from the area in which they originated in each experiment: ischemic (IL), belonging to the intervention zone (left lobe and median), and non-ischemic (NIL), belonging to the hepatic segment that was protected against ischemia by pedicular clamping and the application of hypothermia (right lobe).

Plasma analysis

Serum biochemistry of the stored material was performed by the certified and specialized laboratory of the Faculty of Veterinary Medicine of the UFRGS using a WIENER-CM200 with imported reagents specific for the animal used (WIENER and RANDOX). The levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), ammonia (AMO), total bilirubin (TB) and direct bilirubin (DB), alkaline phosphatase (AP), gamma-glutamyl transpeptidase (GGT), glucose (GLU), lactate (LAC), and lactate dehydrogenase (LDH) were assessed.

Histopathological analysis

The liver samples, divided into IL and NIL from the same animal, were fixed in 10% formalin and stained with hematoxylin-eosin. The blinded evaluation of each specimen was performed by the pathology service at the Hospital de Clínicas de Porto Alegre using an optical microscope and assigning a value to the morphological changes observed following the original model proposed by Suzuki *et al.*[29] to examine the findings of sinusoidal congestion (SC), hydropic degeneration (HD), neutrophilic leukocyte infiltrate (NLI), and hepatocellular necrosis. The rating of steatosis (S) was added to these parameters and the analysis in the necrosis area was improved (Modified Histopathological Classification of Suzuki [MHCS]), performed in a stratified way using liver zoning (Figure 1)[30], in which each lobe is divided into three zones: zone 1 (NZ1), starting from the area next to the triad portal; Zone 2 (NZ2), passing by the sinusoidal intermediate region; and Zone 3 (NZ3), in the region near the central lobular vein (Table 1).
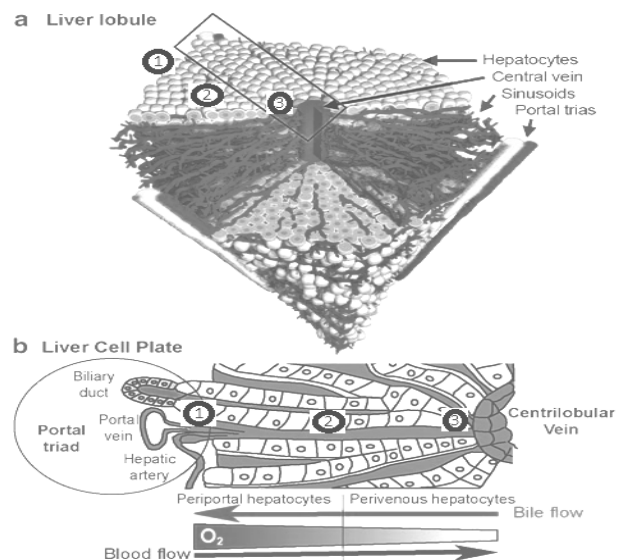

Figure 1 - Schematic representation of three-dimensional liver zoning. Zone 1, next to the portal triad; Zone 2, intermediate sinusoidal region; Zone 3, proximal central lobular vein. Modified from Colnot S., 2011.

Topical hepatic hypothermia associated with ischemic preconditioning. Histopathological and biochemical analysis of ischemia reperfusion damage in a 24 hour model
Gabiatti G *et al.*

Acta Cir Bras. 2018;33(10):924-934

Table 1 - Modified Histopathological Classification of Suzuki (MHCS).

Score	Sinusoidal congestion (SC)	Hydropic degeneration (HD)	Neutrophilic infiltrate (NLI)	Steatosis (S)	Zone 1 necrosis (NZ1)	Zone 2 necrosis (NZ2)	Zone 3 necrosis (NZ3)
0	Absent	Absent	Absent	Absent	Absent	Absent	Absent
1	Dilation of the centrilobular vein	Minimal	Minimal	Up to 30%	Isolated cell necrosis	Isolated cell necrosis	Isolated cell necrosis
2	Dilation of the centrilobular vein and sinusoids in some Zone 3 areas	Mild	Mild	30 to 60%	Up to 30%	Up to 30%	Up to 30%
3	Dilation of the centrilobular vein and sinusoids in most Zone 3 areas	Moderate	Moderate	Greater than 60%	30 to 60%	30 to 60%	30 to 60%
4	Dilation of the centrilobular vein and sinusoids in Zones 2 and 3	Severe	Severe		Greater than 60%	Greater than 60%	Greater than 60%

Note: MSS, modified Suzuki score; SC, sinusoidal congestion;
HD: hydropic degeneration; NLI, neutrophilic leukocyte infiltrate; NZ1, necrosis of Zone 1; NZ2, necrosis of Zone 2;
NZ3: necrosis of Zone 3.

The final histological score (Modified Suzuki Score [MSS] was calculated using Formula 1:

$$MSS = SC + HD + NLI + S + \left(\frac{(NZ1 + NZ2 + NZ3)}{3}\right)$$

All data are presented as mean and standard deviation and were analyzed statistically using the Statistical Package for Social Sciences (SPSS) version 17 and Statistical Analysis System (SAS) version 9.4 assuming a significance of 0.05 for the tests employed (mixed-model repeated-measures analysis of variance [ANOVA], generalized linear model with a gamma distribution to evaluate the effect of groups and Kruskal-Wallis test for independent samples).

■　**Results**

Intraoperative biometric data

The analysis of intraoperative vital signs (mixed-model repeated-measures ANOVA) confirmed the homogeneity of the parameters in the groups. At 91 minutes, 1 minute after declamping of the hepatic pedicle, a uniform decrease occurred in the MBP in all groups, while a rapid elevation of the IHT in the groups submitted to hypothermia. There was no statistically significant difference in ST or

Acta Cir Bras. 2018;33(10):924-934

Topical hepatic hypothermia associated with ischemic preconditioning. Histopathological and
biochemical analysis of ischemia reperfusion damage in a 24 hour model
Gabiatti G *et al.*

other parameters between the intraoperative physiological data of the different groups. The percentage of isoflurane anesthetic mixture remained stable between groups in the different assessed moments without intergroup differences. The variables of weight, age, and surgical room temperature also showed no significant intergroup differences.

Biochemical analysis of plasma after 90 minutes of ischemia and 24 hours of reperfusion

The analysis of biochemical data performed through the adjustment of a generalized linear model with a gamma distribution to evaluate the effect of groups (for asymmetric data) revealed significant differences (p<0.05) in serum GGT, AP, LDH, AST, ALT, DB, and TB (Figures 2 and 3) for the IPC and NI groups. AMO, LAC, and GLU levels were not significantly different between groups.

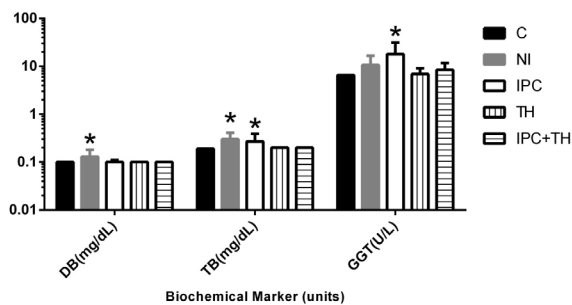Direct Bilirubin (DB), Total Bilirubin (TB), and Gamma-Glutamyl Transpeptidase (GGT)

Figure 2 - Levels of DB, TB and GGT in 24-hour plasma for all groups: Control (C), Normothermic Ischemia (NI), Ischemic Preconditioning (IPC), Topical Hypothermia (TH), and Ischemic Preconditioning + Topical Hypothermia (IPC+TH). Data are presented as mean and standard deviation converted to log 10. *Generalized linear model with gamma distribution to assess the group effect revealed p<0.05 for NI and IPC groups.

Alkaline Phosphatase (AP), Lactate Dehydrogenase (LDH), Aspartate Aminotransferase (AST), Alanine Aminotransferase (ALT)
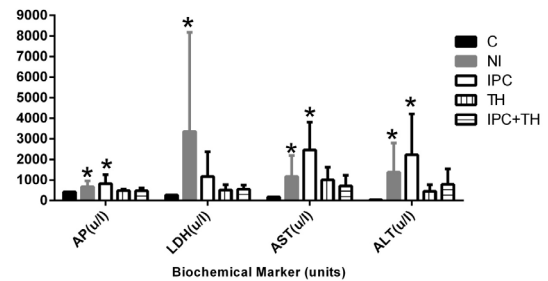
Figure 3 - Levels of AP, LDH, AST and ALT in 24-hour plasma for all groups: Control (C), Normothermic Ischemia (NI), Ischemic Preconditioning (IPC), Topical Hypothermia (TH), and Ischemic Preconditioning + Topical Hypothermia (IPC+TH). Data are presented as mean and standard deviation. *Generalized linear model with gamma distribution to assess the group effect revealed p <0.05 for NI and IPC groups.

Histological analysis after 90 minutes of ischemia and 24 hours of reperfusion

The analysis of the histopathological data (through adjustment of the generalized linear model with a gamma distribution to evaluate the effect of groups) revealed significant differences (p<0.05) for the MSS in IL samples of the NI and IPC groups (Figure 4) compared to those of the other groups. Comparing individual items of the MSS among the groups, using the Kruskal-Wallis test for independent samples, significant differences were observed (p<0.05) for items NZ2 and NZ3 and for NLI in IL samples of the NI group.

The other individual aspects of histopathological scores evaluated (SC, HD, NZ1, and S) showed no significant differences between the IL specimens. Under the point of view of analysis of the method applied in the experiment, the comparative analysis of the NIL of all groups showed no statistically significant differences in any comparisons.

When analyzed pairwise, the MSS between samples of the IL and NIL zone within the same groups demonstrated marked

Topical hepatic hypothermia associated with ischemic preconditioning. Histopathological and
biochemical analysis of ischemia reperfusion damage in a 24 hour model
Gabiatti G *et al.*

Acta Cir Bras. 2018;33(10):924-934

statistical significance for groups NI (p = 0.028) and IPC (p = 0.046), but in groups C (p = 1.0), TH (p = 0.75), and IPC+TH (p = 0.470) there were no significant differences between the IL and NIL specimens.
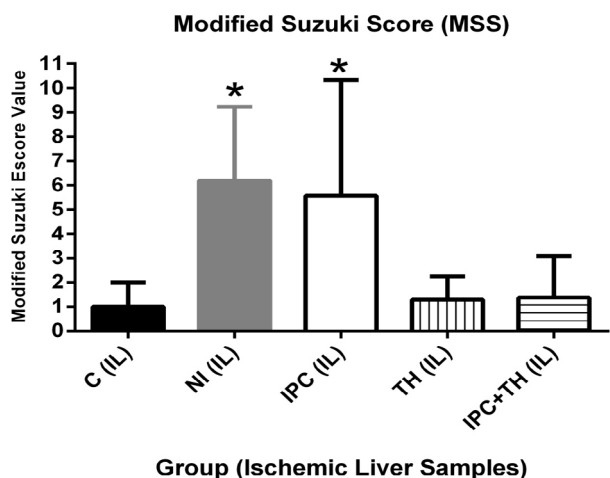

Figure 4 - MSS for ischemic liver samples of all groups: Control (C), Normothermic Ischemia (NI), Ischemic Preconditioning (IPC), Topical Hypothermia (TH), and Ischemic Preconditioning + Topical Hypothermia (IPC+TH). Data are presented as mean and standard deviation. *Generalized linear model with a gamma distribution to assess the group effect revealed p<0.05 for NI and IPC groups.

■ **Discussion**

Here we evaluated the effects of LIR monitored during its implementation through intraoperative physiological parameters and 24 hours after the initial insult with assessments of biochemical and histopathological parameters using different combinations of protective mechanisms (TH, IPC, IPC+TH), associating the principle of zoning in hepatic histopathological analysis (Formula 1, Table 1). Given the already expected serious injury produced by 90 minutes of liver ischemia in this type of model, the obtained biochemical and histopathological

findings indicated that, in this extreme scenario, only the animals subjected to TH, with or without the application of IPC, displayed histopathological findings with obvious cytoprotection compared to those in the other groups (Figure 4). Animals subjected to NI with or without IPC displayed worse results in the MSS compared to the animals subjected to TH, with or without IPC. Considering the findings of biochemical analyses in relation to the findings of the MSS in each group, the ALT, AST, DB, TB, LDH, AP, and GGT levels were different in groups C, TH, and IPC+TH compared to those in groups NI and/or IPC, i.e., higher MSS scores were associated with higher biochemical alterations of these markers.

No references were found in the literature with an LIR model that combined 90 minutes of ischemia associated with the application of hypothermia and/or preconditioning and subsequent evolution of 24 hours during the reperfusion phase. So we can only infer some considerations of our findings and those available in other LIR models in which the ischemia phase and/or the reperfusion phase and/or the protective mechanisms tested were distinct and/or occurred at different times. In an IPC model associated with 90 minutes of IR associated with the combined prior infusion of several substances, Peralta *et al.*[31] reported a protective effect of IPC in the liver and the lungs. In an IPC model consisting of 60 minutes of ischemia and up to 24 hours of reperfusion, Jin *et al.*[32] showed a protective effect of IPC with reduced enzyme levels, a reduction in apoptosis, and improved NLI. In an IPC model consisting of 60 minutes of ischemia and 3 hours of reperfusion, Adam *et al.*[33] also demonstrated a beneficial effect of IPC with elevated levels of hepatic glycogen compared to the group without IPC and concluded that the levels of glycogen would be inversely related to ischemia duration. In a model of pharmacological IPC

Acta Cir Bras. 2018;33(10):924-934

Topical hepatic hypothermia associated with ischemic preconditioning. Histopathological and biochemical analysis of ischemia reperfusion damage in a 24 hour model
Gabiatti G *et al.*

(minocycline) with 90 minutes of ischemia and up to 24 hours of reperfusion, Li *et al.*[34] showed a peak of enzymatic activity (AST, ALT, LDH) at 6 hours of reperfusion, marked histopathological changes from 6 hours of reperfusion, a progressive decline in these markers after 24 hours of evolution and a statistically significant cytoprotective effect in the group subjected to IPC. In our study, biochemical markers including bilirubin (DB and TB), transaminases (ALT and AST), alkaline phosphatase (AP), and GGT indicated significant organ lesions in the normothermic groups (NI and IPC) compared to those of the hypothermic groups (TH and IPC+TH) (Figures 2 and 3).

When analyzed separately, in this specific model, the mechanism of IPC was ineffective at promoting hepatic cytoprotection since the scores of animals subjected to IPC were similar to those of the NI group, through the histopathological assessment and the intraoperative biochemical and physiological effects. Similar findings were described by Steenks *et al.*[35] in an animal model comparing IPC with intermittent ischemia in steatotic livers, in which the authors described a smaller protective effect of IPC compared to that of intermittent or continued ischemia and argued that such findings might be explained by the negative effects of cytokines and other inflammatory molecules released by the ischemic lobe subjected to IPC onto the untreated lobes. Chu *et al.*[36], in a systematic review, described conflicting findings of some studies that evaluated the effect of IPC on steatotic livers submitted to LIR, suggesting that future experimental studies should attempt to better emulate the usual clinical scenario to provide an accurate understanding of the mechanisms and effects of IPC and identify a particular group of patients that can benefit from IPC. The scenario in which this method is most effective, likely does not include ischemia periods as long as the one used in this study.

It must be also highlighted that randomized studies in humans have failed to demonstrate these beneficial effects of IPC identified in experimental models[37,38].

The MHCS and the MSS, variants developed from the initial proposal of their authors, were used in this project to grade and compare the histopathological findings, permitting, with this expansion of its original indicators, especially those related to the division of necrotic areas in zones from the prior knowledge concerning the functional zoning of this organ, the obtaining of more accurate data in relation to the effects of the LIR and each protective mechanism tested, thus calculating a more comprehensive and more detailed injury score upon separate analysis of the different histological areas of necrosis. Originally, the table of Suzuki *et al.*[29] considered a single value for the item of necrosis and did not include the degree of S. There was no significant finding for NZ1 in all groups, but the findings for NZ2 and NZ3 in isolation are significant for group NI. The same significant finding alone in Group NI is repeated with the NLI item of the score. It is speculated that the lack of significance for the findings of NZ1 in this study was due to the proximity of this zone with the triad portal, a zone with greater tissue perfusion.

The complementary analyses of the intraoperative physiological parameters of different groups (p > 0.05) between the NIL samples of different groups (p > 0.05) and the paired analysis between the IL and NIL samples within each group (p < 0.05) using the MHCS and MSS scores, making each animal as a control of oneself and the others, highlight the methodological rigor employed since the different groups have similar intraoperative physiological parameters. They also show the effectiveness of the experimental model modified from the acute original model and used in this project. The modifications developed from the original technique of the

Topical hepatic hypothermia associated with ischemic preconditioning. Histopathological and
biochemical analysis of ischemia reperfusion damage in a 24 hour model
Gabiatti G *et al.*

Acta Cir Bras. 2018;33(10):924-934

LIR model[28], especially related to the anesthetic method, as already reported by Steenks *et al.*[35], in addition to the surgical incision and intra- and postoperative care, allowed for a viable extended LIR experimental model with 24 hours of evolution after severe original ischemic insult, thus enabling the analysis of the effects of different methods of cytoprotection cited previously compared between themselves and jointly in this scientific project.

■ Conclusions

In this extreme scenario of LIR of this new model, evaluated with 24 hours of evolution after reperfusion, we concluded that only the groups submitted to intraoperative topical hypothermia demonstrated an expected hepatoprotective effect. This LIR model performs well and opens possibilities to test other tools to get better understanding and control of this important pathological process.

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Acta Cir Bras. 2018;33(10):924-934

Topical hepatic hypothermia associated with ischemic preconditioning. Histopathological and
biochemical analysis of ischemia reperfusion damage in a 24 hour model
Gabiatti G *et al.*

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