

Evaluation of liver regeneration by modulation with ischemic preconditioning after ischemia and reperfusion and partial hepatectomy

Avaliação da regeneração hepática com modulação pelo pré-condicionamento isquêmico após isquemia e reperfusão e hepatectomia parcial

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A B S T R A C T

Objective: To evaluate liver regeneration modulated by ischemic preconditioning after ischemia, reperfusion and partial hepatectomy. **Methods:** We used 24 Wistar rats of 12 weeks of age, which were randomly assigned into four groups: control (SHAM), hepatectomy (HEP), ischemia and reperfusion (IRG) and Ischemic Preconditioning (SRG). Analyses were made on liver enzymes ALT and AST, assessment of regeneration through the initial and final weight of the liver and the proliferation of hepatocytes by immunohistochemical analysis with Proliferating Cell Nuclear Antigen (PCNA). **Results:** In all groups there was liver regeneration, with no statistically significant difference between them. There were significant differences in ALT and AST between groups HEP and SHAM, PRE and GIR, GIR and SHAM and PRE and SHAM ($p < 0.05$). There were also significant differences in the PCNA labeling of the SHAM group as compared to other groups ($p < 0.05$). **Conclusion:** The ischemic preconditioning decreased liver injury, but did not influence the regeneration up to 48 hours.

Key words: Hepatic regeneration. Ischemic preconditioning. Ischemia. Reperfusion. Hepatectomy.

INTRODUCTION

The regeneration capacity of the liver has been widely recognized over time. This process appears in response to surgical resection, trauma, infections or drug intoxication, which results in loss of parenchyma¹.

In the treatment of liver lesions, resection of segments or lobes is, in most cases, the procedure used. During the surgical procedure bleeding control is of great importance² by total or partial exclusion of the blood flow to the liver during resection^{1,2,3}. This procedure leads to deprivation of oxygen supply to the remaining tissue, causing tissue injury, which is called ischemia and reperfusion (IR)^{4,5,6}.

In 1908, aiming to control liver bleeding, Pringle⁷ created a clamping technique of the portal triad with induction of hepatic ischemia⁸. This technique has

been one of the procedures used in operations for hepatic injury and hepatic resections in order to reduce blood loss⁹. However, the Pringle maneuver induces IR lesion on the remaining liver, which is associated with increased morbidity and mortality¹⁰.

During IR injury, changes occur, such as hepatic microcirculation disorders, elevation of serum aminotransferases and lactic dehydrogenase, mitochondrial dysfunction and lipid peroxidation, related mostly to the duration of ischemia^{1,2,11}.

Ischemic preconditioning (IPC) is one of the procedures used to protect the liver from IR injury. It operates in the period leading to ischemia and consists in inducing a short period of ischemia followed by a short period of reperfusion before longer periods of ischemia^{2,12,13}.

In early 2008 a systematic review¹⁴ of a hepatobiliary surgery group of the Cochrane Foundation

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with clinical trials failed to gain evidence to support or refute the use of IPC in liver surgery. Additional studies were recommended to evaluate the role of IPC in hepatectomy involving the period of normothermic ischemia.

The objective of this study was to analyze the influence of IPC on liver regeneration after partial hepatectomy and IR.

METHODS

This study was approved by the Ethics Committee on Animal Experiments of UFF (Registration No. 049/08).

Were used 24 rats of the species *Rattus norvegicus*, Wistar, 12 weeks of age, provided by Center for Animal Breeding Laboratory of UFF. The animals were maintained in the Animal Laboratory of Experimental Nutrition of FFU until completing the ideal age for surgery. They were randomly divided into four surgical groups: Control Group – SHAM (n=6) – animals subjected to laparotomy, surgical simulation and observation under anesthesia for 60 minutes, a period corresponding to the other groups; Group Hepatectomy – HEP (n= 6) – animals that underwent partial hepatectomy after 55 minutes of the opening of the cavity; Group Ischemia and Reperfusion – GIR (n=6) – animals subjected to ischemia for 30 minutes and partial hepatectomy afterwards; Group Preconditioning – PRE (n=6) – animals subjected to IPC 10/10 minutes followed by 30 minutes of ischemia and subsequent partial hepatectomy.

We used surgical microscope in increases of six to 16 times and microsurgical instruments throughout the surgical procedures. All animals underwent laparotomy, starting at the xiphoid process, with 5 cm long. We performed section of falciform and left coronary ligaments and the identification and isolation of the hepatic pedicle, involving the bile duct, hepatic artery and portal vein. In groups HEP, GIR and PRE we performed a 70% partial hepatectomy by removal of the medial and lateral lobes. The resected livers were weighed.

Also in all animals, after review of hemostasis, closure of the abdominal wall consisted 4-0 interrupted nylon suture for the muscular-aponeurosis and 5-0 nylon for the skin.

After 48 hours of observation, they were relaparotomized to obtain blood sampling by puncture of the infrahepatic vena cava and removal of the remaining lobes, and then euthanized.

The remnant liver was removed and weighed. The percentage of regenerated liver mass was calculated using the formula:

$$\% = [C - (A - B)] / A \times 100\%$$

where:

A = weight estimated at the time of partial hepatectomy; B = weight of the liver resection, (approximately 70% of the total weight of the liver) and C = the weight of the regenerated liver at the time of sacrifice

To evaluate the hepatic injury we performed biochemical analyses of ALT and AST.

For immunohistochemical analysis with PCNA, images were captured and analyzed by a pathologist blinded in relationship to the procedure, counting 1000 cells, recording marked and negative nuclei.

All data were expressed in the form of tables and graphs with the mean values \pm standard-deviation. To analyze the results, tests were applied according to the nature of the variables. We applied the Mann-Whitney test for analyzes of results and comparison among groups. The adopted significance level was 5% ($p < 0.05$).

RESULTS

The final weights of the remaining liver showed regeneration after hepatectomy around 60% in all groups (Table 1) and there was no statistical difference between them (Table 3).

We calculated the mean and standard-deviation of ALT, AST and the percentage of PCNA positivity (Table 2). The GIR group obtained the highest value of ALT and AST (324 and 1442, respectively) and the PRE group had lower ALT and AST (187.5 and 988 respectively) than the GIR. As for transaminases, there were significant differences between the groups HEP and SHAM, GIR and SHAM, PRE and SHAM and PRE and GIR ($p < 0.05$).

In relation to PCNA, the HEP group had the highest percentage (52.25%), whereas the SHAM group displayed the minimum values (5.67%) (Table 2). The SHAM group, when compared to the other groups, showed a significant difference.

DISCUSSION

In this study, ischemic preconditioning was not able, within 48 hours, to increase cell proliferation after ischemia and reperfusion followed by partial hepatectomy. However, it decreased hepatic injury after hepatectomy.

One of the procedures used to protect the liver from IR is IPC, based on the premise that tissue acquires resistance to the effects of IR through previous exposure to brief periods of occlusion¹². The short time interval during IPC ischemia generates oxidative stress, inducing natural defense mechanisms¹³.

In this study, hepatic resections were performed according to the experimental model of liver regeneration in rats already described by Higgins and Anderson in 1931. The partial hepatectomy described by these researchers is

Table 1 - Mean values of total liver weight, initial weight, final weight and weight of the remaining lobe of the SHAM, HEP, GIR and PRE groups.

Grups	Total Weight (A) Initial Weight (B)		Final Weight (C)		Weight Of Regenerated Part(c-(ab)	
	Grams	Grams	Grams	Percentage	Grams	Percentage
SHAM	6.84		6.84			
HEP	6.59	4.62	5.95	90.01%	3.97	60.03%
GIR	6.5	4.55	5.85	89.93%	3.9	60.00%
PRE	6.6	4.62	6.14	93.18%	4.16	63.16%

Table 2 - Comparison of ALT, AST, positivity rate of PCNA and regeneration of hepatocytes in groups SHAM, HEP, GIR AND PRE.

Mann-Whitney	ALT	AST	PCNA	Regeneration
HEP-GIR	0.69	0.07	0.22	0.81
PRE-HEP	0.14	0.81	0.93	0.47
HEP-SHAM	0.005*	0.005*	0.005*	-
PRE-GIR	0.02*	0.02*	0.93	0.23
GIR-SHAM	0.005*	0.005*	0.01*	-
PRE-SHAM	0.005*	0.005*	0.005*	-

Values with * indicate significant differences ($P < 0.05$)

Table 3 - Mean and standard-deviation of ALT, AST and percentage of positive PCNA in groups SHAM, HEP, GIR AND PRE.

Group	Mean (\pm sd)		
	ALT	AST	PCNA
SHAM	41.333 \pm 2.42	216.5 \pm 22.01	5.67 \pm 5.48
HEP	292.5 \pm 133.01	1089 \pm 395.98	52.25 \pm 22.18
PRE	187.5 \pm 35.76	988 \pm 291.5	43.84 \pm 27.42
GIR	324 \pm 119.34	1442 \pm 249.11	38.75 \pm 13.50

considered one of the best models for the study of liver regeneration, since it allows the precise definition regenerating the onset of the stimulus. It consists of resection of the left lateral and median lobes, which represent approximately 70% the total liver mass. The right lateral and the caudate lobes, which correspond to about 24% and 6%, respectively, of the total liver mass, are kept intact.

Regeneration of liver hepatocytes is activated by cytokines, particularly to initiate the cell cycle, in which there is activation of genes that cause hepatocytes to go from G0 phase to G1. Several proteins, growth factors and cycle-dependent kinases are involved in this process^{15,16}. The cells progress through the cell cycle, reaching phase S, with DNA synthesis. Phase G2 (replication) follows, with synthesis of proteins required for cell division, and the end stage of mitosis (M) itself¹⁷. After a phase of intense growth and

reconstruction of the hepatic parenchyma, the regenerative process ceases^{16,18}. The hepatocytes are the first to proliferate, constituting about 90% of hepatic mass and 60% of the total number of cells^{12,17}. Therefore, these authors chose to evaluate the cell proliferation of hepatocytes.

For detection of cellular proliferation in various tissues, different methods have been used to provide information about the growth and tissue repair. The technique we used was proliferating cell nuclear antigen (PCNA), which is expressed in the late G1 phase and during phase S¹⁵.

Liver injury was assessed through the levels of liver enzymes, ALT and AST, which indicate a higher than normal liver damage¹⁹. In mild hepatocellular injury, the predominant form in serum is the cytoplasmatic, whereas in severe injury there is release of the mitochondrial enzyme,

raising AST / ALT ratio. In this case, ALT may have been reduced more rapidly because it has first been released in the bloodstream, the elevated AST remaining longer, resulting in a higher AST / ALT ratio²⁰.

In this experiment, the animals undergoing hepatectomy obtained an increase in remaining liver weight of about 60%. This percentage is due to the observation time of 48 hours, since the process of restoring the original mass takes from five to seven days²¹.

The groups submitted to hepatectomy displayed ALT values above normal (considering the values of 26 and 78 IU/L as physiological to the species)¹⁹. A significant difference was found between the groups GIR and PRE, demonstrating reduction of liver damage with IPC. Considering the values of 157 IU/L to 246 IU/L as physiological AST for this species¹⁹, only the SHAM group had values within normal limits. Again, the difference between the GIR and PRE groups reinforces the protection of IPC on the liver, as described by other authors in experimental and clinical studies^{15,22,23,24}.

In respect to the percentage of PCNA positivity, the HEP group had the highest rate, followed by the PRE group, GIR and SHAM, respectively. The SHAM group

differed significantly from the others; however, no significant difference existed when comparing them with one another. This demonstrates that despite the higher cell proliferation of the group with ischemic preconditioning, it did not increase significantly, since it was reduced by IR injury in relation to rats that underwent only the hepatectomy stimulus.

In a prospective study in 2000, Clavien *et al.*²³ showed the effectiveness of IPC in reducing IR lesions, the first clinical study involving the IPC on the liver in patients undergoing hepatectomy. However, they showed some situations for which IPC was ineffective, as in resections greater than 50%. More recently, Azoulay *et al.*²⁴ first reported the use of IPC in clinical transplantation, showing a greater tolerance to IR injury, but they identified the initial worsening of liver function.

This experimental study analyzed only the period of 48 hours after hepatectomy, but it corroborated other clinical studies^{14,23,24}, which do not definitely advocate ischemic preconditioning for extended resections.

In conclusion, ischemic preconditioning decreased liver injury after hepatectomy, but did not improve regeneration after ischemia and reperfusion.

R E S U M O

Objetivo: Avaliar a regeneração hepática com modulação pelo pré-condicionamento isquêmico após isquemia, reperfusão e hepatectomia parcial. **Métodos:** Foram usadas 24 ratas Wistar, de 12 semanas de idade, distribuídas randomicamente em quatro grupos: Grupo Controle (SHAM), Grupo Hepatectomia (HEP), Grupo Isquemia e Reperfusão (GIR) e Grupo Pré-condicionamento Isquêmico (PRE). Foi feita a análise das enzimas hepáticas ALT e AST, avaliação da regeneração através dos pesos inicial e final do fígado e da proliferação dos hepatócitos pela análise imunoistoquímica com o Proliferating Cell Nuclear Antigen (PCNA). **Resultados:** Em todos os grupos ocorreu regeneração do fígado, não havendo significância estatística entre eles. Houve diferenças significativas em relação a ALT e AST entre os grupos HEP-SHAM, GIR-PRE, GIR-SHAM E PRE-SHAM ($p < 0,05$). Também houve diferença significativa em relação à marcação de PCNA do grupo SHAM quando comparado aos demais grupos ($p < 0,05$). **Conclusão:** O pré-condicionamento isquêmico diminuiu a lesão hepática, mas não influenciou na regeneração até 48 horas.

Descritores: Regeneração hepática. Precondicionamento isquêmico. Isquemia. Reperfusão. Hepatectomia.

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