No Protective Function Found in Wistar Rats Submitted to Long Ischemia Time and Reperfusion After Intermittent Clamping of the Total Hepatic Pedicle

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ABSTRACT

Background. Although the intermittent Pringle maneuver is used for major transplant surgery, traumas, and hepatic protection, long ischemia time and reperfusion may limit some protection in Wistar rats. The aim of the study was to evaluate the protection effects of intermittent clamping in the total hepatic pedicle after a long period of ischemia and reperfusion in Wistar rats.

Methods. Forty-two male Wistar rats, weighing ±327.7 g, were anesthetized intravenously with sodium thiopental and given a U-shaped incision in the abdomen. The total hepatic pedicle was isolated and subjected to clamping with a microvascular clamp. Groups included were the continuous group (CG, n = 14, 40 minutes of ischemia/40 minutes of reperfusion); the intermittent group (IG, n = 14, 4 cycles a 10 minute ischemia/reperfusion 10 minutes); and the sham group (SG, n = 14, 80 minutes of observation time). Blood collection for transaminase dosage was carried out, and hepatic biopsy specimens were taken for mitochondrial respiration and histological evaluation.

Results. In groups CG and IG, aspartate aminotransferase and alanine aminotransferase enzymes were elevated in comparison to group SG ($P < .008$); mitochondrias, when stimulated by use of adenosine diphosphate or carbonylcyanide p-trifluoromethoxyphenylhydrazone, had a significant decrease in mitochondrial respiration ($P < .05$), and the respiratory control ratio in the ischemic groups was lower ($P < .03$) when compared with the GS. On histological examination, 100% of the GC had lesions: 33% focal hemorrhagic necrosis, 17% sinuzoidal congestion and/or vacuolization, and 50% venous congestion; in the IG, 100% had lesions: 43% sinusoidal congestion and/or vacuolization and 57% venous congestion.

Conclusions. The intermittent total hepatic pedicle clamping for a long period of time in the Wistar rats had no efficacy in protection of liver injury.

THE INTERRUPTION of the afferent liver blood flow is necessary for the control of bleeding in liver transplants and trauma [1,2].

In organisms submitted to hepatic ischemia followed by reperfusion, ischemia-reperfusion injuries can occur [3–5]. These injuries involve a complex cascade of events that includes energy loss, imbalance of ionic homeostasis, the production of reactive oxygen species, and cellular death [3,5]. In this context, the mitochondria play a critical role through major changes that may contribute to the injury of the organ during the phenomenon of ischemia-reperfusion [6].

The intermittent clamping of the hepatic pedicle is one of the surgical options used in the routine of surgeons in

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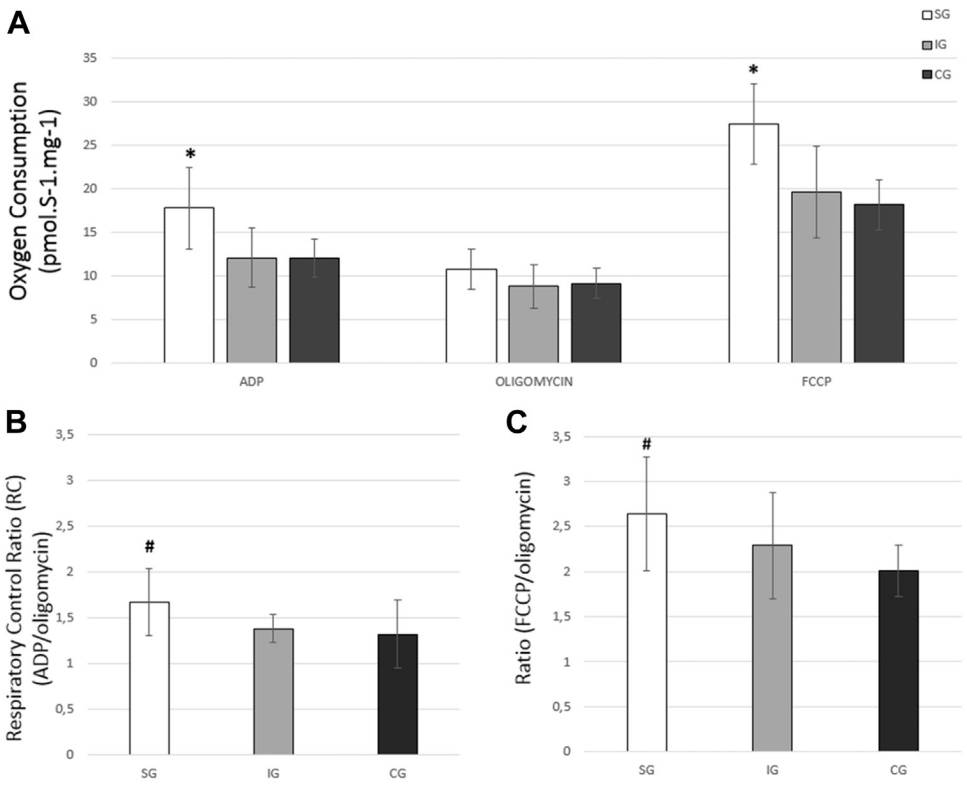

Fig 1. (**A**) In ischemic groups, both IG and CG promoted inhibition of mitochondrial respiration in the biopsy specimens stimulated by ADP or FCCP in relation to the SG, $P < .05$. Respiration did not differ between IG and CG. (**B**) Respiratory control ratio (ADP/oligomycin) was reduced in the ischemic groups in relation to the SG, $P < .03$. (**C**) The ratio (FCCP/oligomycin) was reduced in the ischemic groups compared with the SG, $P < .03$. Comparison between the IG and CG showed no differences. Statistical analysis: *ANOVA 1-way test; #respectively, $P < .05$ and $P < .03$. ADP, adenosine diphosphate; FCCP, carbonylcyanide p-trifluoromethoxyphenylhydrazone.

long-term interventions [4]. It is important to evaluate hepatic rat tissue through an intermittent long-time ischemia period such as hepatic protectors. Some research has shown that severe liver injury is identified in the postoperative period even in short-time clamping of the hepatic pedicle in rats [7].

In the present study, we used total hepatic ischemia in rats to compare hepatic cell injury through two types of portal triad clamping.

METHODS

This research was approved by the Ethics Committee on Animal Use (CEUA–1646-1).

The surgeries were performed at the Laboratory Unit of Liver Transplantation; the mitochondrial tests were performed at the Laboratory of Bioenergetics; histological analysis was performed at the Laboratory of Pathology at the Experimental Medicine and Surgery (NMCE) and Pathology and Clinical Pathology Department–Unicamp.

In this study, 42 male Wistar rats (*Rattus norvegicus allbinus*, *rodent*, *mammalia*) weighting 327.7 ± 100.0 g were included. The animals were provided by CEMIB (Surgery Vivarium-Unicamp).

The 42 animals were randomly divided into 3 groups. After the anesthetic procedure, trichotomy incision in a U-shape [7] was carried out. Isolation of the hepatic pedicle (hepatic artery, portal vein, and bile duct) with flexible cotton buds was performed in each group before intervention.

In the sham group (SG), the animals were subjected to a total period of 80 minutes of observation, equivalent to the period of ischemia/reperfusion in the other groups. In the continuous group (CG), clamping was continuous for 40 minutes of ischemia followed by 40 minutes of reperfusion. In the intermittent group (IG), clamping was carried out in 4 cycles of 10 minutes of ischemia followed by 10 minutes of reperfusion.

After 40 minutes, 1.0 mL of saline solution (0.9%) in the peritoneum was applied. In the CG and IG, the abdominal wall was maintained closed with 3 vascular clamps during the ischemia period.

After the operative procedure, hepatic biopsies for mitochondrial respiration and histological evaluation were collected. Blood collection for aminotransferase serum levels was performed. At the end of each experiment, the animals were killed by exsanguination under anesthesia.

The left lobe biopsy specimen was put on 5.0 mL of preservation solution containing 2.77 mmol/L CaK_2 EGTA, K_2EGTA 7.23 mmol/L, 6.65 mmol/L $MgCl_2.6H_2O$, 15 mmol/L creatineNa_2 Pospho, 20 mmol/L imidazole, 0.5 mmol/L of dithiothreitol, and 50 mmol/L 2-(N-Morpholino)ethanesulfonic acid hydrate at 1.0 L (pH 7.1) in a beaker and kept in a cooler at 4°C with crushed ice for mitochondrial analysis. A respirometry Oroborus (Innsbruck, Austria) and a magnetic stirrer with regular temperature at 37°C were used to measure the oxygen consumption.

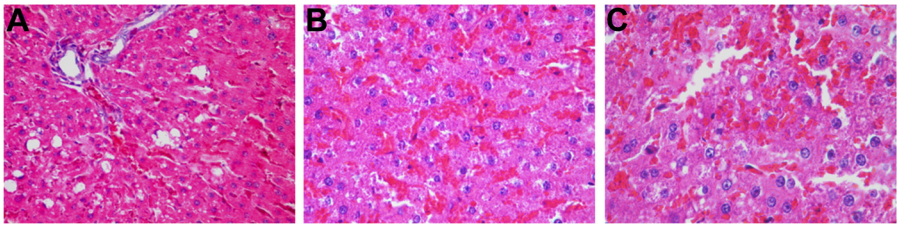

Fig 2. (**A**) Liver parenchyma with preserved lobular structure. Note portal field with habitual aspect and hepatocyte vacuolation associated with intense sinusoidal congestion (hematoxylin and eosin stain; magnification ×400). (**B**) Severe sinusoidal congestion. (hematoxylin and eosin stain; magnification ×500). (**C**) Diffuse sinusoidal congestion with focal area of hemorrhagic necrosis (hematoxylin and eosin stain; magnification ×640).

Two randomly chosen samples were collected (average weight, of 4.0 and 6.0 mg) and inserted into the apparatus tubs with 2.1 mL of MIRO5 reaction (0.5 mmol/L EGTA, 3 mmol/L $MgCl_2$. 6 H_2O, 60 mmol/L K-lactobionate, 20 mmol/L taurine, 10 mmol/L KH_2PO_4, 20 mmol/L HEPES, 110 mmol/L sucrose, and 1 g/L of bovine serum albumin; pH 7.2). Mitochondrial respiratory activity was then analyzed by sequential addition of 20 μL of pyruvate, 20 μL of malate, 20 μL of 42 mmol/L adenosine diphosphate (ADP), 2 μL of a solution of 1 mg/mL of oligomycin, and 0.8 μL of 1 mmol/L solution of carbonylcyanide p-trifluoromethoxyphenylhydrazone (FCCP). The data were determined with the use of the specific software of the apparatus [8–10].

Middle lobe liver biopsy specimens were placed in a solution of 10% formalin for a maximum time of 24 hours. After this period, it was placed in 70% ethanol and dehydrated with alcohol and xylol battery and then embedded in paraffin and stained with hematoxylin and eosin (H&E) and Masson's trichrome [11].

Blood samples were collected (3.0 mL) by means of abdominal aorta catheterization for dosages of aspartate aminotransferase (AST, in IU/L) and alanine aminotransferase (ALT, in IU/L), performed by use of the kinetic enzymatic method with the use of automated equipment (MODULAR P800 EVO Hitachi high, Technologies Corporation, Tokyo, Japan) [11].

Statistical analyses were performed with the use of 1-way ANOVA, Tukey's test, and Student's *t* test for comparison of means and descriptive frequency analysis for ordinal variables and the nonparametric Mann-Whitney test for continuous variables. The level of significance was 5%. The SigmaStat program version 3.5 was used (Systat Software, Chicago, Ill, United States).

RESULTS

All animals survived until the end of the experiment. In the IG and CG, mitochondrial respiration was inhibited in comparison to the SG. There was a reduction in respiratory control ratio ADP/oligomycin and FCCP/oligomycin in ischemic groups when compared with the SG ($P < .05$) (Fig 1).

In the CG and IG, 100% of the animals showed histopathological disorders (Fig 2) (GC: 33% focal hemorrhagic necrosis, 17% sinusoidal congestion and/or vacuolization, and 50% of the venous congestion branch; IG: 43% sinusoidal congestion and/or vacuolization, 57% of the venous congestion branch).

We observed a marked increase ($P < .008$) in transaminase in the ischemic groups (IG and CG) when compared with the SG (Table 1).

DISCUSSION

The role of ischemic preconditioning as a liver protector in vascular occlusions has led to major debates about its effect on ischemia and reperfusion for long period of time [12–15].

In the present study, we adopted two modalities of clamping of the hepatic pedicle in Wistar rats, aiming at to evaluate the protective factor in a long period of ischemia and reperfusion.

Many authors report that reperfusion injury is directly associated with mitochondrial dysfunction and increased reactive oxygen species [16,17]; its exact mechanisms are not fully known.

We sought to carry out evaluation of mitochondrial respiration directly in hepatic biopsies, thus avoiding possible damage to its structure in the irregular tubular network induced by mechanical homogenization, and avoiding the loss of soluble proteins and of other molecules from the mitochondrial matrix and degradation of

Table 1. Tests of Liver Profile and Histological Changes Observed in the Sham Group, the Continuous Group, and the Intermittent Group

	SG	CG	IG	*P*
Laboratory tests				
AST (IU/L)	129,23 ± 38,82	2233,20 ± 216,24	1316,80 ± 1193,18	<.008
ALT (IU/L)	34,80 ± 15,58	1726,40 ± 617,25	1143,40 ± 617,25	<.008
Histologic aspects				
Congestion venous branch	Absent	50%	57%	<.05
Sinuzoidal congestion and/or vacuolization	Absent	17%	43%	<.05
Focal hemorrhagic necrosis	Absent	33%	Absent	<.05

Abbreviations: SG, sham group; CG, continuous group; IG, intermittent group; AST, aspartate aminotransferase; ALT, alanine aminotransferase.

mitochondrial matrix proteins by the insidious action of isolation present in the buffer, which can inhibit the activity of the vital functions [8–10].

In our study, mitochondrial respiration demonstrated a significant decrease in the ischemic animals, both in intermittent clamping and in continuous clamping with inhibition of the respiratory state III (capacity of mitochondrial oxidative phosphorylation).

Regarding respiratory status IV, the groups of ischemic animals and control animals were similar to those described in studies that used isolated mitochondria [8–11].

Respiratory control was similar between ischemic animals and lower when compared with control animals. This reduction was 17% and 21%, respectively, in intermittent and continuous groups. Research that used mitochondria isolated from rats also reported lower respiratory control [18].

Some authors have demonstrated in models of ischemia and reperfusion performed in partial small pets that injury may culminate in apoptosis or necrosis, depending on other variables such as the concentrations of ATP or degree of hypoxia, emphasizing that the intermittent method, when compared with the continuous method, demonstrates a marked improvement [11–16].

In the GC, the lesions were more severe, with the presence of venous congestion, sinusoidal congestion, vacuolization, and focal hemorrhagic necrosis. However, in the IG the greater injuries were intense venous and sinusoidal congestion and vacuolization. This occurred as the result of the long ischemia period in this study, although some authors had reported an improvement when the intermittent clamping was used in for a short time [18].

Aminotransferases (AST and ALT) are good markers of hepatic injury and ischemia and are widely used in clinical practice for patient accompaniments, pointing out changes in plasma membrane permeability; their high levels depend on the time of reperfusion [11,15–18].

Our results showed that the ischemic groups had significantly elevated levels of AST and ALT compared with the control group after reperfusion. However, when contrasted with ischemic groups, this difference and protection was not corroborated.

CONCLUSIONS

We conclude that intermittent clamping of the total hepatic pedicle in Wistar rats for long periods of time had no efficacy in protecting liver injury in comparison to the continuous group.

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