Effect of Hepatic Preconditioning with the Use of Methylene Blue on the Liver of Wistar Rats Submitted to Ischemia and Reperfusion

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ABSTRACT

Background. The liver may be injured in situations where it is submitted to ischemia, such as partial hepatectomy and liver transplantation. In all cases, ischemia is followed by reperfusion and, although it is essential for the reestablishment of tissue function, reperfusion may cause greater damage than ischemia, an injury characterized as ischemia-reperfusion (I/R) damage. The aim of this work was to analyze the effect of ischemic preconditioning with the use of methylene blue (MB; 15 mg/kg) 5 or 15 minutes before I/R (IRMB5′ and IRMB15′, respectively) on the hepatic injury occurring after I/R.

Methods. Twenty-eight male Wistar rats were used, and liver samples submitted to partial ischemia (IR) or not (NI) were obtained from the same animal. The samples were divided into 7 groups. Data were analyzed statistically by means of the nonparametric Mann-Whitney test and Wilcoxon Matched test, with the level of significance set at 5% ($P < .05$).

Results. The rate of oxygen consumption by state 3 mitochondria was inhibited in all ischemic groups compared with the sham group (SH vs IR: $P = .0052$; SH vs IRMB5′: $P = .0006$; SH vs IRMB15′: $P = .0048$), which did not occur in the nonischemic contralateral portion of the same liver (SH vs NI: $P = .7652$; SH vs NIMB5′: $P = .059$; SH vs NIMB15′: $P = .3153$). The inhibition of the rate of oxygen consumption by state 3 mitochondria was maintained in the presence of MB (IR vs IRMB5′: $P = .4563$; IR vs IRMB15′: $P = .9021$). The respiratory control ratio was reduced in all ischemic groups compared with the sham group, owing to the inhibition of oxygen consumption in state 3 (SH vs IR: $P = .0151$; SH vs IRMB5′: $P = .005$; SH vs IRMB15′: $P = .0007$).

Conclusions. Methylene blue had no effect on the mitochondrial respiratory parameters studied, but was able to reduce lipid peroxidation, preventing the production of reactive oxygen species (SH vs IRMB15′: $P = .0210$).

THE LIVER may be injured in situations of ischemia, such as those induced in surgery for partial hepatectomy and liver transplantation. In all cases, ischemia is followed by reperfusion and, even though it is essential to reestablish tissue function, reperfusion usually causes more severe damage than ischemia in proportion to the same period of time [1]. When blood flow is restored, a new aggression is imposed on the liver owing to the initial metabolic imbalance caused by the sudden supply of nutrients, oxygen in particular, with consequent worsening of damage. This phenomenon is known as ischemia and reperfusion (I/R) injury and involves complex mechanisms and metabolic cell pathways [2].

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Hepatic ischemia causes damage owing to microvascular changes. At first there is an increase in reactive oxygen species (ROS) mainly originating from the mitochondria, such as xanthine oxidase and uncoupled nitric oxide synthase [3]. Next, reperfusion affects the process of oxidative phosphorylation, eventually causing changes in the permeability of the inner mitochondrial membrane because of the ROS, resulting in less efficient oxidative phosphorylation, which may induce rupture of the mitochondrial membrane [4]. After reperfusion, there is an increase of adhesion and activation of leukocytes and Kupffer cells, which also produce ROS and increase tissue injury [3]. Intracellular calcium is also released in cells submitted to I/R and, together with ROS and activation of the immune system, results in tissue injury with consequent apoptosis/necrosis [2].

ROS and nitrogen reactive species are generated in the mitochondria during ischemia. Hypoxia inhibits oxidative phosphorylation of the cells and obstructs the production of adenosine triphosphate (ATP), causing disorders in mitochondrial Ca^{2+}, Na^{+}, and H^{+} ions that may lead to changes in the permeability of the mitochondrial membrane. Oxidative stress plays an important role in the injury caused by reperfusion. Many highly reactive species, such as superoxide anion, hydroxyl radical, and hydrogen peroxide, are formed and react with proteins, enzymes, nuclei acids, cytoskeleton, and lipids, leading to lipid peroxidation and mitochondrial dysfunction. In addition, some studies have considered the role of ROS in cellular modulation, signaling redox-dependent processes [5].

Studies have demonstrated that several substances protect the liver from possible I/R injury. Propofol has antioxidant properties and protects the liver from I/R injury caused by ROS [6]. Methylene blue (MB) can reduce the effects of intestinal I/R in organs such as the liver and has been administered to patients with vasoplegic shock, sepsis, cardiopulmonary bypass, and liver transplantation [7]. It also has a biochemical effect on the mitochondrial respiratory chain and on the blockade of oxidant production through its oxidized and reduced forms [8]. The objective of the present study was to analyze the effect of ischemic preconditioning with the use of MB (15 mg/kg) on hepatic injury due to I/R.

METHODS

Twenty-eight male Wistar rats (200–300 g) were used in the experiment. Samples submitted to partial ischemia (IR) and not submitted to partial ischemia (NI) were obtained from the same animal. The samples were divided into 7 groups: SH, liver samples from rats submitted to surgical and anesthetic stress but receiving no I/R (sham); IR, ischemic liver samples from rats who had received 0.5 mL saline solution and were then submitted 5 minutes later to 60 minutes of ischemia and 15 minutes of reperfusion; NI, nonischemic liver sample from the same rat; IRMB5′, ischemic liver samples from rats who had received 0.5 mL MB (15 mg/kg) and were then submitted 5 minutes later to 60 minutes of ischemia and 15 minutes of reperfusion; NIMB5′, nonischemic liver sample from the same rat; IRMB15′, ischemic liver sample from rats who had received 0.5 mL MB (15 mg/kg) and were then submitted 15 minutes later to 60 minutes of ischemia and 15 minutes of reperfusion; and NIMB15′, nonischemic liver sample from the same rat. The rats were anesthetized with the use of 20 mg/mL xylazine hydrochloride and 50 mg/mL ketamine hydrochloride at a 1:2 ratio and applied at 100 mg/kg. After shaving, the rat's abdomen was exposed by means of a median laparotomy, and partial ischemia of the hepatic pedicle was performed. The animals had free access to water and food and were maintained in the animal facilities at room temperature under a 12-hour light-dark cycle according to the recommendations of the Animal Experimentation Ethics Committee of the University of São Paulo Ribeirão Preto Medical School.

Mitochondrial Preparation

Liver mitochondria were isolated by differential centrifugation at 4°C [9]. The liver was removed immediately after the surgical procedure and washed with the use of physiologic saline solution. Next, it was placed in medium containing 250 mmol/L sucrose, 1 mmol/L EGTA, and 10 mmol/L Hepes-KOH, pH 7.2, and punched and homogenized in a Potter-Elvehjem tissue grinder in 3 cycles of 15 seconds each separated by a 1-minute interval. The homogenate was centrifuged at 770*g* for 5 minutes, and the resulting supernate was centrifuged at 9,800*g* for 10 minutes. The sediment obtained was suspended in 10 mL medium containing 250 mmol/L sucrose, 0.3 mmol/L EGTA, and 10 mmol/L Hepes-KOH, pH 7.2, and centrifuged at 4,500*g* for 15 minutes. The final sediment containing the isolated mitochondria was suspended in 0.5 mL medium containing 250 mmol/L sucrose and 10 mmol/L Hepes-KOH, pH 7.2.

Determination of Mitochondrial Protein

Mitochondrial protein was determined with the use of the Coomassie Plus (Bradford) Assay Kit (Thermo Scientific) at 595 nm and a Versamax (Molecular Devices) microplate reader. The results are reported as mg/mL with the use of bovine serum albumin as standard [10].

Oxygen Consumption by the Mitochondria

Mitochondrial respiration was monitored with the use of a Hansatech-Oxygraph Plus oxygraph equipped with an oxygen electrode and assessed by the rates of oxygen consumption in state 4 (basal respiration) and state 3 (activated respiration) as well as by the respiratory control rate (RCR), which indicates the degree of coupling between oxygen consumption and adenosine diphosphate (ADP) phosphorylation [11].

Determination of Osmotic Mitochondrial Swelling

The permeability transition of the inner mitochondrial membrane induced by 20 μmol/L calcium chloride and 1 mmol/L potassium phosphate was determined at 540 nm with the use of a Beckman DU-640B spectrophotometer based on the reduction of optical density (ΔOD) [12].

Determination of Malondialdehyde

Colorimetric malondialdehyde (MDA) determination based on its reaction with thiobarbituric acid at 532 nm was performed with the use of a Versamax microplate reader and the use of 0–100 μmol/L 1,1,3,3-tetrametoxypropane as standard, and the results obtained are expressed as μmol/L/mg protein [13].