Oxidative stress on ischemia/reperfusion injury in mice with non-alcoholic hepatic steatosis or steatohepatitis

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Abstract

Purpose: To evaluate the oxidative stress, resulting from ischemia and hepatic reperfusion, in mice with non-alcoholic hepatic steatosis and steatohepatitis.

Methods: C57BL/6 male mice were used. Part of them were ob/ob mice, and the other part was fed with standard or MCD diets – this last used to develop steatohepatitis. The animals – MCD-I/R, ob/ob-I/R and I/R groups – were submitted to 30 minutes of partial hepatic ischemia, followed by reperfusion for 24 hours. The blood was collected, for biochemical analysis of AST, and the liver removed for assessment of TBARS and nitrite, and of histology.

Results: After the I/R, the animal fed with MCD diet presented higher AST levels (MCD-I/R: 967±349 U/L / ob/ob-I/R: 606±18 U/L / I/R: 311±172 U/L), TBARS (MCD-I/R: 7±1 nM/mg protein / ob/ob-I/R: 3±1 nM/mg protein / I/R: 3±1 nM/mg protein) and nitrite (MCD-I/R: 614±87 µg/mL / ob/ob-I/R: 512±81 µg/mL / I/R: 459±29 µg/mL) than the ob/ob mice, when both groups were compared to animals fed with standard diet. Regarding histology, the steatosis level (azonal macrovesicular steatosis of level 3 - >66%) and hepatic fibrosis (periportal and perisinusoidal of level 2) was also more intense, but both animal models presented lobular inflammation of level 3 (>66%).

Conclusions: The murine model fed with MCD diet is suitable for the assessment of oxidative stress in hepatic I/R injury associated with the nonalcoholic fatty liver disease. Although both murine models showed inflammatory infiltrate and macro and micro vesicular steatosis.

Introduction

Steatosis is one of the most common forms of hepatic disease and is broadly associated to a bad prognosis after surgery in liver, due to the increasing probability of developing primary dysfunction after transplant when compared with non-steatosis livers.

The liver with steatosis is not very tolerant to ischemic injury and reperfusion, what may result in hepatic failure and death. For example, in orthotopic transplants, moderate and severe steatosis in donated liver is related to the increase in the number of patients with graft dysfunction. It is believed that the causes of vulnerability in liver with steatosis and submitted to ischemia and reperfusion are associated to injuries caused by oxidative stress.

The intracellular accumulation of free fatty acids leads to the development of an inflammatory process and mitochondrial dysfunction by formatting Reactive Oxygen Species (ROS), which impairs cell function and, consequently, results in cell death. The reoxygenation during the reperfusion leads to oxidative burst followed by increase in ROS production, which are released by activated Kupffer cells, neutrophils, and T CD4+ cells; therefore, causing destruction of hepatic tissue.

To assess the effects of ROS production on the liver with steatosis, different experimental models are used to allow a better comprehension of this disease. In studies referring to Nonalcoholic Fatty Liver Disease (NAFLD), genetically modified mice are frequently used to develop obesity (ob/ob), and in the analysis of Nonalcoholic Steatohepatitis (NASH) its induction is achieved by the use of a specific diet.

The genetically obese animals, such as ob/ob mice, present natural occurrence of a mutation of a spontaneous point in Leptin gene (Lep, ob/ob), leading to absolute deficiency of this hormone derived from adipocytes, which results in hyperphagia and obesity followed by a syndrome that is similar to type 2 diabetes, where the intolerance to glucose and hyperglycemia is observed. In addition, these mice present elevated levels of insulin and amylin, associated to insulin resistance. The ob/ob mice develop hepatic steatosis, which may worsen with age, from a syndrome similar to NAFLD up to NASH, presenting inflammation and fibrosis. The deficiency of adiponectin hormone is the main reason for the development of steatosis in these animals.

Another model commonly used in the development study of NASH in mice is the administration of a methionine choline-deficient (MCD) diet for rodents. The animals submitted to MCD diet loose around 25% of their weight in two weeks and reduce size. In addition, develop inflammation – mainly with lymphocytes and neutrophils – severe steatosis, steatohepatitis, and fibrosis. The oxidative stress, present in this model, is considered an important factor for the progression of steatosis condition to steatohepatitis.

Considering the specificities of each model in the ischemia lesion and hepatic reperfusion, the purpose of this work was to comparatively assess the effect of oxidative stress after the ischemia and reperfusion in ob/ob mice and in animals submitted to MCD diet.

Methods

The study was developed after the approval by the Ethics Committee of Faculdade de Medicina da Universidade de
São Paulo - FMUSP (Process number 243/13). All animals were handled according to the “Ethical Principles of Animal Experimentation of International Union of Animal Protection” and Brazilian Law No. 11.794, from October 8, 2008.

Animals were housed in appropriate cage of dimensions 40x30x25cm in groups of three animals, and it was kept with specific chow according to the group and water ad libitum. The circadian rhythm of the animals was respected maintaining the adequate sanitary conditions. Before the experiments, the animals had a solid food restriction of four hours.

It was used twenty-four male mice, eight weeks old, C57BL/6 and genetically modified obese (ob/ob), both strains were purchased from Central Animal Facilities of the Medical School – University of São Paulo. (São Paulo, Brasil). The animals were distributed in the following experimental groups (N=6 each group): Control = mice C57BL/6 fed with standard diet; I/R = C57BL/6 mice fed with standard diet and submitted to ischemia and reperfusion; MCD-I/R = C57BL/6 mice fed with methionine-choline deficient diet (MCD diet – purchase from Rhoster Ind. Com. LTDA – Araçoiaba da Serra – SP- Brasil) and submitted to ischemia and reperfusion; ob/ob-I/R = mice genetically modified to develop steatosis fed with standard diet and submitted to ischemia and reperfusion. The standard and MCD diets were administrated to the proper groups for a one-month period.

Surgical procedure

The animals were sedated with an association of xylazine (10mg/Kg) and ketamine (70mg/kg) drugs, via intramuscular, and submitted to ischemia during 30 minutes by clamping of portal triad for left and middle liver lobes, afterwards removed removing the clamps and allowing the reperfusion for 24 hours. The abdominal wall of the mice was sutured with polypropylene thread 7-0, and they were maintained in cages with water and ad libitum food. The animals were sedated again, after the reperfusion, and re-operated to collect blood and to surgically remove lateral left and median lobes, which were divided in three fragments: one for histology, fixed in formalin 10%, and two for nitrite dosage and MDA, which were frozen in liquid nitrogen and maintained in freezer -80°C. Euthanasia was performed through exsanguination, while mice were still sedated.

Histologic and biochemical analysis

The liver fragment fixed in formalin 10% was embedded in paraffin. Afterwards, histologic cuts were made 4 µm thick, colored by H/E method. The tissue cuts were assessed according to histological parameters of Kleiner et al.15 by an examiner using a blinding technique for the groups.

The blood of the animals was centrifuged at 3500g for 15 minutes, and the serum was collected and stored in freezer at -80°C for further analysis of aspartate aminotransferase (AST). This analysis was realized by the Biochemistry Laboratory of the FMUSP.

Detection of lipid peroxidation

For the analysis of malondialdehyde (MDA) in liver, the fragments were then unfrozen, diluted at 100mg/mL in phosphate buffer (pH 7.2), homogenized, centrifuged at 10,000g for 15 minutes, and the supernatant collected. Initially, the amount of total proteins was verified in the liver. The hepatic tissue was diluted in a proportion of 1:5 in distilled
water, and 20µL of this solution was added to 1mL of Bradford Reagent. The absorbance was read in spectrophotometer Model TP-Reader (ThermoPlate-China), with 595nm wave length. Afterwards, the samples were diluted in the same proportion used for the quantification of total proteins and mixed to 250µL of Trichloroacetic acid (TCA) at 17.5% and 250µL of Thiobarbituric acid (TBA) at 0.6% (pH 2.0). The samples were placed in water-bath for 15 minutes at 95°C, cooled in ice for 15 minutes, and 250µL of TCA at 70% was then added, and then incubated for 20 minutes at 4°C and centrifuged at 1609g for 15 minutes. The absorbance was read in spectrophotometer Model TP-Reader (ThermoPlate – China), with 534nm wave length. The MDA concentration was provided in nmol of MDA per mg of protein.

Nitrite quantification

The nitrite, byproduct of the nitric oxide (NO) degradation, was quantified in the liver parenchyma using Griess reagent technique. Liver fragments were unfrozen, diluted at 100mg/mL in phosphate buffer (pH 7.2), homogenized, centrifuged at 10,000g for 15 minutes, and the supernatants collected. In a plate with 96 wells, 50 µL of each sample and from standard curve dilutions with 50µL of Griess reagent, previously prepared with volumes equal to the component A (N-(1-naphthyl) ethylenediaminedihydrochloride) and the component B (sulphanilic acid) was mixed. The photometric reference sample was prepared mixing 50µL of Griess Reagent and 50µL of deionized water. The absorbance of samples was measured in spectrophotometer Model TP-Reader (ThermoPlate - China), with 548nm wave length.

Statistical analysis

The values of AST, TBARS and nitrite were presented in Mean ± Standard Deviation. For statistical analysis of results purposes, the test ANOVA one way and the post-test of Bonferroni, using the level of significance in 5% (p<0.05) was applied.

Results

Histopathologic and biochemical analysis

From the histopathologic analysis, it was possible to observe that the liver of animals from the control group maintained the normal architecture (Figure 1A), while the animals submitted the ischemia and reperfusion (group I/R) present steatosis micro vesicular level 2 (<33%), without the presence of inflammatory infiltrate (Figure 1B).

After the ischemia and reperfusion procedure, the animals fed with DMC diet present a more severe injury than the ones found in ob/ob mice, in respect to the steatosis level in hepatic tissue, being observed the predominance of steatosis macro vesicular azonal level 3 (<66%) (Figure 1C) and level 2 periportal and perisinusoidal hepatic fibroses areas. In ob/ob animals, in turn, was found macro steatosis and micro vesicular level 2 (<33%) (Figure 1D) and level 1 periportal hepatic fibrosis. Regarding inflammation, both animal models presented level 3 lobular inflammation (Figure 1 C-D).
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Figure 1 – Photomicrographs of hepatic tissue sections stained with H/E of (A) mice from control group, presenting normal architecture, of (B) mice from I/R group, presenting micro vesicular steatosis (arrow heads), of (C) mice from MCD-I/R group, which presented inflammatory infiltrate (circled area) and predominance of macro vesicular steatosis (arrows), and (D) mice from ob/ob-I/R group, in which macro vesicular steatosis (arrows) and micro vesicular (arrow heads) and presence of inflammatory infiltrate (circled area) are observed. Images represented with x40 magnification. PT = portal tract; CV = central vein.

Regarding the serological analysis (Figure 2A), after the I/R, the mice fed with DMC diet presented higher levels of AST (967 ± 349 U/L) than the ob/ob animals (606 ± 18 U/L), when compared to the animals fed with standard diet and submitted to I/R (311 ± 172 U/L) was observed.

Figure 2 – Assessment of hepatic injury. (A) AST Levels, (B) of TBARS and (C) of nitrite in liver of mice fed with standard diet (Control group), submitted to I/R (group I/R), fed with MCD diet associated to I/R (MCD-I/R), and ob/ob mice submitted to I/R (ob/ob). The asterisks represent the statistical significance of results, where *=p<0.05; **=p<0.01 and ***=p<0.001.
Evaluation of lipid peroxidation and nitrite

Additionally, the evaluation of oxidative stress in hepatic tissue (Figure 2B and 2C) showed an increase in TBARS and nitrite levels, after the I/R, was greater in the animals fed with MCD diet (TBARS: 7±1 nM/mg protein / nitrite: 614 ± 87 µg/mL) than in ob/ob animals (TBARS: 3±1 nM/mg protein / nitrite: 512 ± 81 µg/mL), when compared to the animals fed with conventional diet and submitted to I/R (TBARS: 3 ± 1 nM/mg protein / nitrite: 459 ± 29 µg/mL).

Discussion

In this study, the animals from experimental models of hepatic steatosis and steatohepatitis assessed were submitted to ischemia for 30 minutes followed by reperfusion for 24 hours, with the purpose of observing the effects of I/R procedure over the oxidative stress in hepatic tissue with steatosis. After the I/R procedure, the increase of oxidative stress in mice that developed steatohepatitis due to the ingestion of MCD diet was more intense when compared to that occurred in ob/ob mice, which developed only hepatic steatosis. However, the presence of inflammatory infiltrate was observed in both models of animals evaluated, not occurring difference with respect to this histologic parameter.

The I/R injury is one of the main clinical problems that appear during the hepatic transplant and partial liver resection surgery, being one of the most common causes of hepatic dysfunction, graft rejection and chronic diseases. When the I/R injury is associated with hepatic steatosis, the effects of the inflammatory process is exacerbated, causing an even more intense injury to the liver, which results in poor prognosis in hepatobiliary surgeries, hepatectomies and liver transplantation.

In our study, both animal models of hepatic injury, ob/ob mice and those fed with MCD, showed infiltrate of inflammatory cells in liver submitted to I/R, which may have led to an injury in hepatic tissue, evidenced by the presence of fibrosis.

According to Takahashi et al., the young ob/ob mice only show hepatic inflammation after manipulation of the diet or exposure to toxins; but, Sutter et al. affirms that these mice with approximately 20 weeks of age may present a significant increase of inflammatory activity, with the presence of inflammatory mediators and neutrophils, even when fed with normal diet. Like in ob/ob mice, the animals of C57BL/6 strain, when fed with MCD diet, also present increase of inflammatory response. The studies of Sutter et al. and Yu et al. corroborate with our results, in which was observed the presence of inflammatory infiltrate in steatosis hepatic tissue of both animal models.

The Kupffer cells and CD4+ lymphocytes, when activated by I/R, are the main producers of inflammatory mediators in liver cells, with cytokines and chemokines. Such mediators promote the recruitment of neutrophils, which are responsible for the induction of oxidative stress in liver post-ischemic. The oxidative stress occurs due to the unbalance between ROS and the detoxification capacity of tissue through antioxidant enzymes, causing therefore tissue destruction. In physiological conditions, the synthesis of ROS is balanced with the number of antioxidants present in the organs; however, if the ROS concentration exceeds the normal values and the antioxidants are unable to compensate the excess, these free radicals end up causing lipid damage, proteins, and DNA.

Regarding oxidative stress in liver, the production of TBARS and nitrite after the I/R procedure presented significantly elevated levels only in mice fed with MCD diet. These
develop a steatohepatitis, when compared to ob/ob mice fed with standard diet, which presented only hepatic steatosis. This could be the cause of the difference in production of ROS between the models.

The steatosis causes liver inflammation with ROS production and, as consequence, the hepatocytes develop vulnerability to oxidative stress, which in turn, is the main responsible for progression of NAFLD for a steatosis associated to necroinflammation and fibrosis, denominated NASH\(^{30}\). It is believed that hepatic steatosis causes the obstruction of hepatic microcirculation and mitochondrial dysfunction, which results in the increase of ROS production as subsequent cell death\(^{30}\). In addition, according to Gehrau et al.\(^19\), the lipid peroxidation is also associated to hepatic steatosis and represent an additional source for ROS production, contributing to intensify I/R injury. Our results corroborate with these studies, once the fatty infiltration in liver was severe in animals fed with MCD diet causing the increase in TBARS levels and in nitrite. These, in turn, may have promoted the evolution for NASH in these animals, since steatosis in hepatic tissue and oxidative stress are intimately related.

### Conclusions

The murine model fed with methionine choline-deficient diet is suitable for the assessment of oxidative stress in hepatic I/R injury associated with the nonalcoholic fatty liver disease. Although both murine models showed inflammatory infiltrate and steatosis macrovesicular and microvesicular.

### References

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