

6 – ORIGINAL ARTICLE MODELS, BIOLOGICAL

Study of hepatocellular function in the murine model following hepatic artery selective clamping¹

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ABSTRACT

PURPOSE: To investigate the impact of selective hepatic artery clamping (SHAC) in hepatocellular function.

METHODS: Three groups of Wistar male rats were subjected to SHAC ischemia period of 60min: Group A continuous SHAC were subjected to SHAC ischemia period of 60min, Group B intermittent SHAC of 30min with 5min of reperfusion and Group C intermittent SHAC of 15min with 5min of reperfusion. Animals without SHAC were included-Group D. To evaluate hepatocellular function blood markers and hepatic extraction function (HEF) using ^{99m}Tc-mebrofenin were performed before and after surgery. Flow cytometry was used to analyze oxidative stress and cell viability.

RESULTS: A mortality rate of 7.6% in Group A was observed. HEF maintained normal values between the groups. Flow cytometry demonstrated no significant differences between the groups in viability, type of cell death as well as in the production of reactive oxygen species.

CONCLUSIONS: The selective hepatic artery clamping compared to other clamping techniques results on increased cell viability and decreased hepatocyte death. The SHAC is a potential alternative to decrease per-operative bleeding while maintaining hepatocellular function.

Key words: Liver. Hepatic Artery. Constriction. Radionuclide Imaging. Rats.

Introduction

Surgical treatment of primary or metastatic liver tumors remains the only therapeutic alternative with curative potential. Several clinical factors have been reported as predictive of survival without disease after curative surgical treatment. Intraoperative blood loss and transfusion are major factors influencing morbidity, mortality and survival following liver resection¹. Therefore, all care should be taken to reduce per-operative bleeding and transfusion. Hepatic vascular inflow occlusion, called Pringle maneuver, associated with low central venous pressure are used to prevent bleeding during transection of hepatic parenchyma. However, this maneuver causes ischemia and reperfusion injury²⁻⁴. Two protective strategies have been proposed to enable longer duration of ischemia, namely intermittent clamping and ischemia preconditioning. Intermittent Pringle maneuver consists of successive cycles of 15 minutes of inflow occlusion alternated by periods of five minutes of reperfusion.

Very long duration periods of ischemia have been reported using intermittent Pringle maneuver. However, this technique has inherent disadvantages such as back flow bleeding from the hepatic veins, bleeding during reperfusion intervals as well as a time limit usually around 60 minutes⁴⁻⁷.

With the advent of liver resection by laparoscopy and the effects of pneumoperitoneum on the portal vein flow⁸, the selective hepatic artery clamping (SHAC) has been advocated. In fact, the increased intraperitoneal pressure necessary to perform laparoscopic operations reduces the portal venous flow^{8,9}. The extent of the volume flow reduction is related to the level of intraperitoneal pressure.

The aim of this experimental study was to estimate the impact of different duration of SHAC on the hepatocellular function, viability or death of hepatocytes in an animal model with normal liver.

Methods

All the procedures were performed in accordance with the Coimbra University Institution Animal Care and were approved by the local animal ethics committee.

Two months old male Wistar rats were used in all experiments. The animals were fed a laboratory diet with water and food *ad libitum* until surgery and were kept under constant environment conditions with 12-hour light-dark cycle.

Four groups of animals were studied. The control group consisted of animals subjected to 60 minutes laparotomy without

SHAC - Group D; two experimental groups (C and B) received respectively intermittent SHAC of 15 or 30 minutes followed by five minutes of reperfusion. Finally the experimental Group A was submitted to 60 minutes of continuous SHAC.

Operative procedures

All animals were anesthetized with xylazine (Bayer, Leverkusen, Germany) and ketamine (Rhône Mérieux, Lyon, France). The abdomen was opened through a midline incision and liver was free from its ligaments. The portal triad was dissected and a microvascular clamp (Aesculap, San Francisco, CA) was placed in order to induce SHAC. At the end of SHAC period a hepatic biopsy of the left lobe was performed. Finally, the survival of the animals for 24 hours in each group was monitored.

Serum enzymes analysis

Serum levels of aspartate-aminotransferase (AST), alanine-aminotransferase (ALT), alkaline-phosphatase (AP), gamma-glutamyl-transpeptidase (GGT), total-bilirubin (TB), lactic-acid-dehydrogenase (LDH) were used as blood markers of hepatic injury. Blood samples were obtained three days before laparotomy and one hour after surgery. Blood cells were pelleted by immediate centrifugation at 10 000 g for eight minutes at 4°C. Enzyme levels were measured using a serum multiple biochemical analyzer as described¹⁰.

Hepatic extraction fraction (HEF) evaluation by radioisotopic methods

For the HEF calculation, an IDA derivative, 3-bromo-2,4,6-trimethylacetanilidoiminodiacetic acid labelled with technetium-99m (^{99m}Tc-mebrofenin) was used. The labeling procedure was performed by adding ^{99m}Tc freshly eluted to a mebrofenin kit (Bridatec, GE-Amersham Health, Eindhoven, the Netherlands) constituted by 40.0 mg of mebrofenin as sodium salt associated with 0.3 mg of stannous chloride dehydrate. Quality control performed according to the manufacturer was always carried out before animal administration. The ^{99m}Tc-mebrofenin complex formed is stable for at least 6 hours after reconstitution. Related to dosimetry, the target organ which has the highest estimated absorbed dose is the large bowel (9.2 E-02 mGy/MBq) according to Medical Internal Radiation Dose Committee (MIRD) guidelines.

Data acquisition

^{99m}Tc-Mebrofenin scintigraphy was performed in all animals after fasting for at least six hours. Data acquisition was performed by a single detector gamma camera (GE Maxicamera 400 AC, Milwaukee, USA) coupled with a low energy high resolution parallel hole collimator, controlled by a GenieAcq computer. Data were dynamically collected after the bolus injection of 185 MBq ^{99m}Tc-Mebrofenin into an animal tail vein, for 128*128 matrix, every second during the first minute, followed by 59 frames of 60 seconds each. After dynamic acquisition, static images, anterior, 256*256 were performed with an individual duration of 67 seconds for decay correction. The animals' position was with the upper abdomen and cardiac area included in the gamma camera field of view. Energy discrimination was centered on 140 keV with a 20% energy window. After acquisition the data were transferred to a work station Xeleris for processing.

Data processing

On the dynamic sequence of images, regions of interest (ROIs) were flagged over the cardiac region and right hepatic lobe, avoiding the gall-bladder and main intrahepatic biliary ducts. With the total counts, time-activity curves were generated from the ROIs. The HEF was calculated through deconvolution analysis of the first pass curve. Additionally, the time of maximum liver activity (Tmax) and half time of the normalized liver curve (T1/2) were calculated. Values of 98.5±2.7% for HEF, 18±4.6 min for Tmax and 20.9±14.6 min for T1/2 were considered normal¹¹.

Liver cell isolation

Liver cells were isolated from liver biopsy obtained at the end of surgery by using collagenase (Collagenase Hepatocyte Qualified – Gibco / BRL) as described¹².

Flow cytometry assays

After liver cell isolation, viability and death, oxidative stress and mitochondrial membrane potential were investigated by flow cytometry. Flow cytometry analysis was performed using a six-parameter, four-color FACSCalibur™ flow cytometer (Becton Dickinson, San Jose, CA) equipped with a 15 nW argon laser. For each assay 10⁶ cells were used and at least 10 000 events were collected by acquisition using CellQuest software (Becton Dickinson) and analyzed using Paint-a-gate software (Becton

Dickinson).

Cell death and viability analysis

Hepatocytes isolated from hepatectomy specimens and were stained simultaneously with Annexin-V, labelled with the fluorescent probe, fluorescein isothiocyanate (FITC), and propidium iodide, according to manufacturer's recommended protocol (Immunotech Kit). This assay discriminates among intact cells (AV-/PI-), early apoptotic cells (AV+/PI-) and late apoptotic or necrotic cells (AV+/PI+)^{13,14}. Briefly, cells were washed with ice-cold phosphate buffer solution (PBS) by centrifugation at 500g for five min, resuspended in 100µL of binding buffer and incubated with 1µL of AV-FITC and 5 µL of PI solutions for 10 min on ice in the dark. After incubation time, cells were diluted in 400 µL of ice-cold binding buffer, and analyzed by flow cytometry.

Evaluation of apoptotic proteins expression by flow cytometry

To evaluate the levels of apoptotic proteins modulators we analyze by flow cytometry the expression of the proapoptotic protein BAX, the antiapoptotic BCL-2 and the ligand of Tumor Necrosis Factor (TNF) family, TRAIL. The levels of cellular fluorescence are proportional to the concentration of apoptotic proteins in each cell. After surgery, 10⁶ of liver cells were centrifuged and incubated with monoclonal antibodies anti-BAX, BCL-2 and TRAIL ligand, according with manufactured protocols. The results are presented as Mean Fluorescence Intensity (MFI) arbitrary units. This value represents the medium of fluorescence intensity detected in the cells, which is proportional to the number of molecules labeled by the antibody.

Reactive oxygen species production

The accumulation of reactive oxygen species (ROS) in hepatocytes, namely peroxides, was determined using 2',7'-dichlorodihydrofluorescein diacetate (DCFH₂-DA) (Invitrogen), a stable nonfluorescent lipid permeable compound that is converted to DCFH₂ by intracellular esterases. Then, DCFH₂ is oxidized by intracellular ROS to form the impermeable fluorescent compound DCF that emits green fluorescence, upon excitation at 488 nm, proportionally to intracellular ROS levels¹⁵.

Hepatocytes were treated with 5 µM DCFH₂-DA for 30 minutes, at 37°C in the dark. Cells were then washed twice with PBS, resuspended in PBS, and DCF was detected by flow

cytometry. Results are presented as Mean Fluorescence Intensity (MFI) values¹⁶.

Mitochondrial membrane potential measurement

The integrity of the inner mitochondrial membrane can be measured by the gradient potential across this membrane, the mitochondrial membrane potential, using the dye 5,5',6,6'-tetrachloro-1,1',3,3'-tethraethylbenzimidazolcarbo cyanine iodide (JC-1). This lipophilic cationic probe exists in a monomeric form emitting at 527 nm (green fluorescence) and is able to reverse form aggregates, which are associated with a large shift in the emission (590 nm, greenish-orange fluorescence) as the mitochondrial membrane becomes more polarized.

Mitochondrial membrane potential (J_{mit}) in hepatocytes was measured using JC1 (Molecular Probes), as described by others (16). Briefly, cells were washed with PBS through centrifugation at 300g during five min and incubated with JC-1 at final concentration of 5 $\mu\text{g}/\text{mL}$ for 15 min at 37°C, in the dark. At the end of the incubation period, the cells were washed twice in PBS, resuspended in a total volume of 500 μL and analyzed by flow cytometry. The results are presented as monomer/aggregate ratio.

Statistical analysis

Statistical analysis was performed using Kruskal-Wallis test and Mann-Whitney U test for multiple comparisons with Bonferroni correction whenever statistical significance was obtained in Kruskal-Wallis test. Analysis was carried out in SPSS, version 17.0, and evaluated at 5% significant level.

Results

There was no operative mortality. However, the postoperative mortality was 7.6% in animals that were subjected to 60 min of continuous SHAC (Group A) and 0% in other groups. Therefore, we demonstrated an increased mortality in Group A, however, this did not reach a statistical significance (ns).

There was an overall increase of postoperative values of AST, ALT, GGT, AP, total bilirubin and LDH as compared to the preoperative values. However, no differences between Groups A, B, C and D were observed.

The study of hepatocellular function through the evaluation of hepatocyte extraction fraction using ^{99m}Tc-mebrofenin remained normal without differences between the four groups.

To evaluate the impact of different types of SHAC in hepatocytes viability, cells were stained with annexin V and propidium iodide and analysed by flow cytometry as described above (Figure 1). As we can observe rat hepatocytes submitted to 15 minutes intermittent SHAC (Group C) show higher cell viability than other groups. On the other hand, the hepatocytes of rats submitted to 60 minutes of continuous SHAC (Group A) and to intermittent SHAC of 30 minutes followed by five minutes of reperfusion (Group B), show a decrease in cell viability and an increase in cell death, in particular by late apoptosis and necrosis (Figure 1A) but without statistical differences between groups.

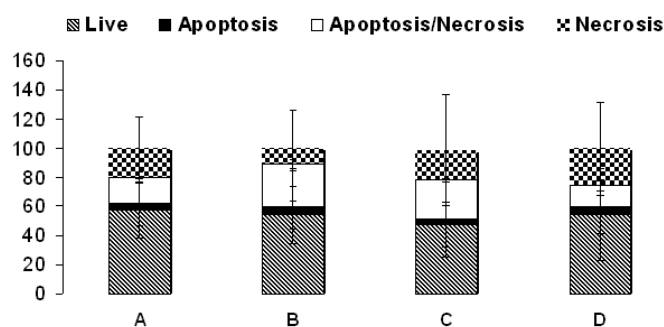


FIGURE 1 – Evaluation of cellular viability and death by flow cytometry. Hepatocytes were extracted from rats subjected to 60 minutes laparotomy without hepatic artery inflow occlusion: (D) continuous inflow occlusion; intermittent SHAC of 15 followed by five minutes of reperfusion (C); intermittent SHAC of 30 followed by five minutes of reperfusion (B); 60 minutes of continuous SHAC (A). Cell death was detected by annexin V and propidium iodide staining and analyzed by flow cytometry. Results are expressed in % \pm SD.

In Figure 2A we can observe the effect of different types of SHAC in hepatocytes peroxides levels. As we can see hepatic cells submitted to 60 minutes of continuous SHAC (Group A) present a decreased in ROS production when compared to control group (Group D). On the other hand, the hepatocytes isolated from rats submitted to intermittent SHAC of 30 minutes followed by five minutes of reperfusion (Group B) and to intermittent SHAC of 15 minutes followed by five minutes of reperfusion (Group C) showed higher ROS levels. However, no statistical differences were found between rats submitted or not to SHAC.

We also evaluated the repercussion of SHAC in mitochondria function by determining the mitochondrial membrane potential through the JC1 monomers/aggregates (M/A) ratio (Figure 2B). Besides the variations observed in JC1 M/A ratio in the different rat groups no statistical differences were found between rats who had and had not SHAC.

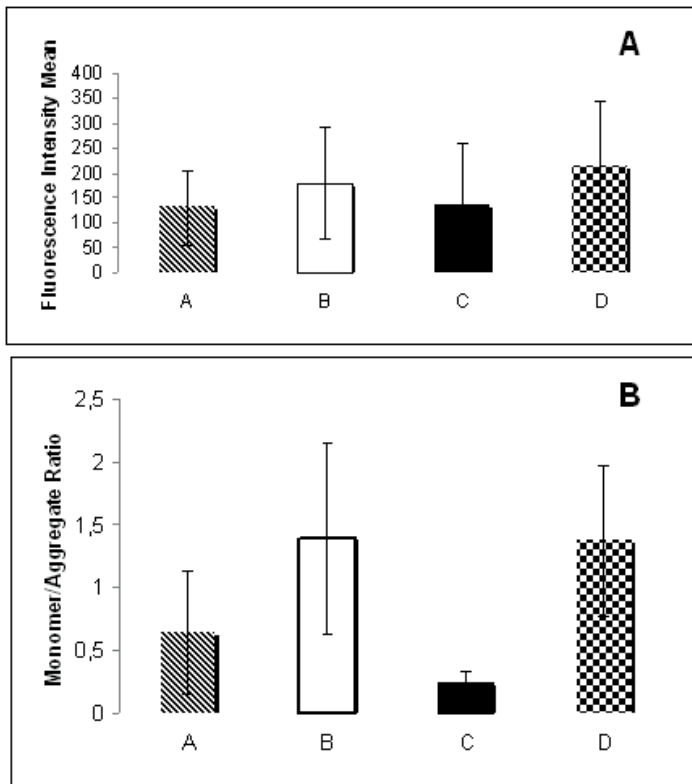


FIGURE 2 – A) Evaluation of peroxides production induced by hepatic artery inflow occlusion. The peroxide levels were analyzed by flow cytometry using the DCFH2-DA fluorescent probe. Hepatocytes were extracted from rats submitted to 60 minutes laparotomy without hepatic artery inflow occlusion: (D) continuous inflow occlusion; intermittent SHAC of 15 followed by five minutes of reperfusion (C); intermittent SHAC of 30 followed by five minutes of reperfusion (B); 60 minutes of continuous SHAC (A). **B) Mitochondrial membrane potential in hepatocytes of rats submitted to different types of SHAC.** Rats were submitted to 60 minutes laparotomy without hepatic artery inflow occlusion: (D) continuous inflow occlusion; intermittent SHAC of 15 followed by five minutes of reperfusion (C); intermittent SHAC of 30 followed by five minutes of reperfusion (B); 60 minutes of continuous SHAC (A). After the surgery, hepatocytes were isolated and stained with the JC-1 probe. JC-1 probe coexist in monomeric (M) or aggregated (A) form depending on the mitochondrial membrane potential. An increase in the M/A ratio indicates a decrease in the mitochondrial membrane potential. Results are expressed in mean±SD of M/A ratio of JC-1. Results are expressed in mean±SD of mean fluorescence intensities (MFI) of DCF.

Finally, we analyzed the expression of the apoptotic protein modulators, BAX, BCL-2 and TRAIL ligand (Figure 3). We observed a decreased expression of these proteins in all groups, when compared to control group (Group D), but the results were not statistically significant. However, hepatocytes obtained from rats submitted to 60 minutes of continuous SHAC (Group A) and to intermittent SHAC of 30 minutes followed by five minutes of reperfusion (Group B) present an increase in BAX/BCL2 ratio compared to control group (Group D). These observations may result from a higher percentage of cell death

observed in the groups, and might implicate the mitochondria in cell death induced by SHAC.

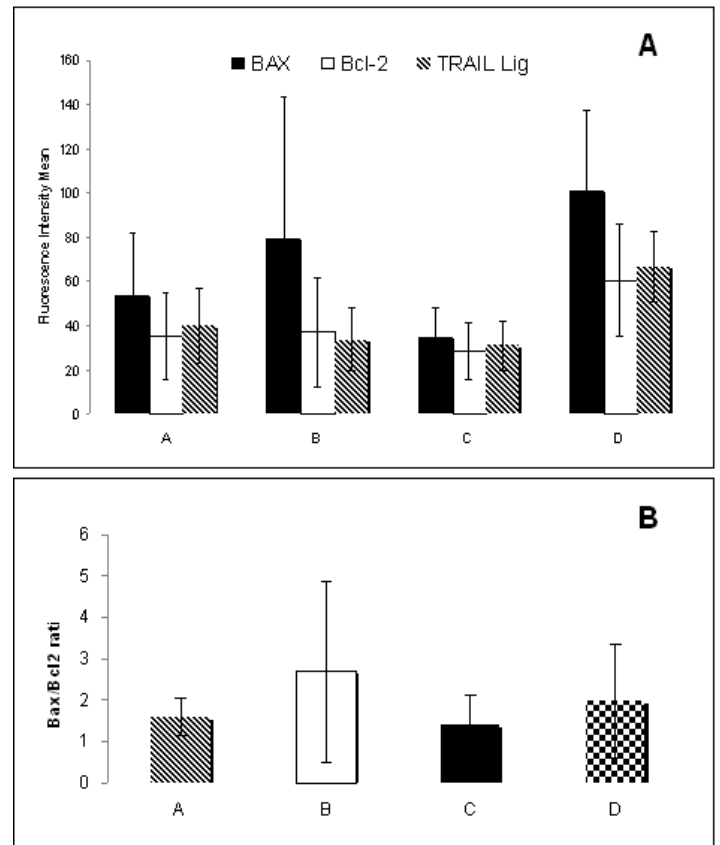


FIGURE 3 – Evaluation of BAX, BCL2 and TRAIL ligand expression in rats hepatocytes submitted to different types of SHAC. **A)** Represents BAX, BCL2 and TRAIL ligand expression and **(B)** the ratio BAX/BCL2 (ratio between pro-apoptotic/anti-apoptotic proteins). After the surgery, hepatocytes were isolated and stained with monoclonal antibodies anti-BAX, anti-BCL2 and anti-TRAIL ligand according with manufacture’s instructions. Rats were submitted to 60 minutes laparotomy without hepatic artery inflow occlusion (D group); intermittent SHAC of 15 followed by five minutes of reperfusion (C group); intermittent SHAC of 30 followed by five minutes of reperfusion (B group); 60 minutes of continuous SHAC (A group). Results are expressed as mean fluorescence intensities (MFI), and represents the mean ±SD of fluorescence intensity detected in the cells. Fluorescence is proportional to the number of molecules labelled with antibody. Bax/Bcl-2 ratio was calculated as the ratio of MCF observed for each molecule.

Discussion

Blood loss and per-operative blood transfusions cause morbidity and mortality after hepatectomy. Different methods of vascular control such as the Pringle maneuver, selective clamping of left or right hepatic arteries or portal vein branches, the supra-selective intrahepatic vascular occlusion or total vascular exclusion are used to reduce bleeding during liver resection. However, all these techniques can lead to ischemia or reperfusion injury.

In addition, activation of Kupffer cells may take

place, leading in turn to production of reactive oxygen species or cytokines such as tumor necrosis factor or interleukin 8 and consequent injury of endothelial cells of liver parenchyma. To date, studies have suggested that ischemia induced liver injury or reperfusion after clamping of the portal triad can promote metastases and consequently lower patients' survival¹⁰. Animal experimental studies submitted to 20 or 30 min of selective lobe ischemia, showed that tumor progression was higher in the lobe selectively clamped⁸. In fact, microvascular ischemia is associated with changes in endothelial cells that increase the adhesion ability of tumor cells to the endothelium, promoting the progression of liver metastases. Another possible mechanism to explain the idea that occlusion of the portal triad stimulates the progression of liver metastases was proposed in an experimental study in a rat model subjected to 45 min of clamping showed that there was a profound change of liver microcirculation leading to cell necrosis due to hypoxia¹⁷. Prolonged hypoxia induces in turn the activation of factor 1 α which in turn promotes activation of several genes and subsequent synthesis of proangiogenic growth factors, proliferation and differentiation, an emerging ideal microenvironment for tumorigenesis¹⁷. However, this correlation between clamping of the hepatic pedicle and the acceleration of tumor growth is observed only after continuous clamping. In fact, same authors verified that intermittent clamping seemed to have a protective effect against metastatic growth because it is associated with absent or less necrosis of the liver parenchyma and hepatocellular function^{18,19}. These results are consistent with experimental and clinical studies conducted by us and others showing that the liver parenchyma tolerates better intermittent hepatic pedicle clamping^{18,19}.

Although the intermittent clamping of the hepatic pedicle for 15 min in patients with a normal liver can be applied for more than 60 min, the purpose of this study was to evaluate long continuous selective hepatic artery clamping, results on the same level of protection of liver parenchyma and hepatocellular function. Eventually, this surgical strategy might allow a reduction of intraoperative bleeding during hepatectomy maintaining the portal blood supply, and so reducing the risk of hypoxia of the parenchyma.

A clinical study showed that the Pringle maneuver with clamping duration lasting 15 minutes, followed by five minutes of reperfusion in patients undergoing liver resection, increases the tolerance of the liver parenchyma compared to continuous clamping particularly in patients with chronic liver diseases¹⁹.

In our study, we found that the SHAC was not associated with a statistically significant increase in mortality compared to

animals that were continuously clamped for 15 minutes or not clamped. Our study suggests that this strategy has a relative advantage over the Pringle maneuver as far as the postoperative mortality is concerned.

The study of hepatocyte function has been evaluated by multiple methods. We opted for biochemical evaluation by studying preoperative and postoperative blood parameters (normally used in biological assessment of liver function) and the study of hepatocyte extraction fraction using ^{99m}Tc-mebrofenin. The HEF by the radioisotope methods evaluate the capacity of hepatocytes to uptake the radiopharmaceutical, carry it in the cytoplasm and excrete it into the bile ducts. So, we can take during this study various aspects of the dynamic function of hepatocytes and more accurately assess their function. In our study we observed an overall increase of the postoperative values (compared to preoperative values) of ALT, AST, GGT, AP, total bilirubin and LDH in all animals subjected to different protocols of SHAC but no differences among the four groups. Second, the dynamic evaluation of hepatocellular function through the study of HEF by radioisotope methods showed maintenance of normal levels of HEF and no statistically significant differences between different groups.

In this study we found that different duration periods of SHAC (60 and 30 min) induce significant changes in the viability of hepatocytes isolated from the liver of the rats. The methodology revealed that the percentage of viable hepatocyte cells (not stained with annexin V and propidium iodide) and cells in late apoptosis/necrosis (labelled simultaneously with annexin V and propidium iodide) was not statistically significant between the groups submitted to different SHAC types versus the control group (no clamping done).

To evaluate the mechanisms involved in the viability and death of hepatocytes we analyzed peroxide levels, the expression of apoptotic protein modulators and the role of mitochondria through the change of mitochondrial membrane potential.

We have not found statistically differences in peroxide levels between the animals who were and were not submitted to SHAC. However, in hepatic cells obtained from rat livers submitted to intermitted SHAC of 30 minutes followed by five minutes of reperfusion we observed a decrease in M/A ratio, in agreement with the higher cell viability found in this rat group. Usually, a decrease in monomer/aggregated JC1 ratio indicates an increase in the mitochondrial membrane potential that is related with proliferative capacity.

On the other hand an increase in monomer/aggregated JC1 ratio (M/A ratio) indicates a decrease in the mitochondrial

membrane potential, implicating the mitochondria in the cell death²⁰. The decrease in cell viability observed in rats submitted to 60 minutes of continuous SHAC and to intermittent SHAC of 30 minutes followed by five minutes of reperfusion produces a higher susceptibility of the hepatocytes to death that may be related to the observed increase in the ratio between the pro-apoptotic and anti-apoptotic proteins (BAX/BCL2 ratio). This increase is in agreement with the higher percentage of cell death observed in these groups and might implicate the mitochondria in cell death induced by SHAC.

Conclusions

Selective hepatic artery clamping is associated with an increase in cell viability and a decrease in the cell death compared to Pringle maneuver. This clamping technique may be an alternative to the Pringle maneuver, taking into account the changes of portal blood flow caused by pneumoperitoneum.

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