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Research Article

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Posted Date: December 1st, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-1078044/v1>

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EFFECTS OF PORTAL PRECONDITIONING IN LOW-VOLUME LIVER REMNANTS: STUDY IN RATS

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Abstract:

The increase of liver surgical indications, the expansion of the margins in hepatic resections and the lack of organ donors led to the use of more split livers from cadaver and living donors and smaller liver remnants in post-operative patients. The use of increasingly smaller grafts associated with hepatic resections broadened the spectrum for observation of small-for size syndrome, caused by significant inflammation and early hepatic fibrosis. The small-for-size syndrome is manifested clinically by prolonged cholestasis, refractory ascites and progressive hepatic dysfunction (encephalopathy and coagulopathy). In the search for mechanisms to reduce liver damage, preconditioning is presented as a possibility of protecting the low weight remnant in experimental works. **Objective:** Study the hepatic tissue measuring the impact of portal preconditioning in small hepatic remnant in Wistar rats **Methods:** Rats weighing approximately 250g were divided in 4 groups with 7 members each. Group 1, Control group requiring only collection of the material, blood laboratory analysis and liver biopsy for pathology and immunohistochemistry; Group 2, Sham, were operated with simple laparotomy, 48 hours later they were subjected to another surgery with sample collection to do blood laboratory analysis and liver biopsy for pathology and immunohistochemistry. Group 3, hepatectomy with preconditioning. In this group was made the preconditioning procedure before the resection of 70% of the liver, 48 hours later they underwent another surgery for sample collection to do blood laboratory analysis and liver biopsy for pathology and immunohistochemistry. Group 4, hepatectomy without preconditioning. In this group the members were operated with resection of 70% of the liver, 48 hours later were reoperated with sample collection to do blood laboratory analysis and liver biopsy for pathology and immunohistochemistry. We studied and compared the impacts in morphology, laboratory, histology, immunohistochemistry.

Results: There was no intraoperative mortality in the model used, there was no statistically significant difference in histological and laboratory parameters between the groups with and

without preconditioning, there was an increase in the expression of PCNA with statistical significance in the hepatic remnant of the group submitted to preconditioning.

Conclusion: Liver preconditioning can provide an increase in cell proliferation in small volume liver remnant.

Key-words: Hepatic preconditioning, Ischemia/reperfusion, small for size syndrome, post hepatectomy liver failure.

Introduction:

Liver resection is the main curative treatment for primary and metastatic liver tumors. Several specialized liver department centers perform more than one hundred hepatectomies, with low operative mortality. Even including extreme resections and critically ill patients, operative mortality rates are 4% reported in 2006, 0.9% in 2011 [1] and 0.3% when there is a predominance of minor resections [2].

However, after majors hepatectomies, remnants of a volume smaller than 35% of the estimated ideal volume start to be at risk for the development of postoperative liver failure, being tolerable at most between 20 and 30% in non-hepatopathic patients [3].

The patients with hepatic small volume remnant are subject to the development of acute liver failure in the postoperative period, characterized by jaundice, coagulopathy, encephalopathy and refractory ascites [4]. This syndrome is the main cause of death after hepatectomy, often associated with sepsis and ischemia/reperfusion injury [5][6].

To deal with this challenging scenario, organ preconditioning has been the subject of studies and is pointed out as a way to reduce the damage caused on livers after major hepatectomies [7]. Organ preconditioning is defined by the induction of ischemia of the organ for a short period of time, followed by a period of reperfusion before a longer duration of ischemia in the intraoperative period of liver resections [8].

Preconditioning is supposed to protect the liver from the deleterious effects of ischemia/reperfusion injury. However, it is still necessary to clarify how this procedure influences the liver regeneration process [9].

In this study we examined the effects of portal preconditioning before a 70% resection of the liver parenchyma in rats.

Materials and Methods:

The research was approved by the Animal Use Ethics Committee of Universidade Federal do Rio de Janeiro (UFRJ) in accordance with Brazilian legislation and international guidelines. The study was carried out at the Center of Experimental Surgery, School of Medicine – UFRJ.

Twenty-eight adults male Wistar rats (*Rattus norvegicus*) weighing between 220g and 290g were used. The animals were kept in individual cages, under temperature control and a 12 hours light/dark cycle at the Center of Experimental Surgery, School of Medicine – UFRJ. They received free water, standard feeding and hygiene care.

The animals were randomly distributed in four equal groups: Group I - control, Group II - Sham, Group III - Hepatectomy with preconditioning and Group IV - Hepatectomy without preconditioning.

Study steps and surgical procedure:

The animals were induced and anesthetized with an intraperitoneal injection of 0.1ml of xylazine + 0.4ml of ketamine. They were placed in dorsal decubitus, submitted to trichotomy and antisepsis.

In group 1 (control): Median laparotomy was performed and material was collected: blood and liver tissue.

In group 2 (Sham): Median laparotomy was performed with subsequent lysis of the hepatic ligaments, mobilization of the pedicles. After 48 hours, a new surgery was performed with collection of liver tissue and blood from the inferior vena cava.

In group 3 (preconditioning + hepatectomy): Median laparotomy was performed with mobilization of the pedicles, clamping of the portal vein for five minutes and reperfusion made for ten minutes followed by hepatectomy of 70% of the liver total volume: left lateral lobe (34%), left median lobe (10%) and right median lobe (26%).

In group 4 (hepatectomy with no preconditioning): Median laparotomy was performed with mobilization of the pedicles, 70% of the total volume of the liver was resected without doing the preconditioning: left lateral lobe (34%), left median lobe (10%) and right median lobe (26%).

The collected material was stored in vials with 10% formaldehyde solution, processed by the pathology for histopathological evaluation in hematoxylin-eosin and processed for PCNA evaluation in immunohistochemistry. Blood was collected directly from the vena cava, immediately stored in biochemistry and hemogram bottles and sent to the laboratory.

Histological Processing:

Livers were cut into slices, after being collected, fixed in 10% formaldehyde, processed and stained with hematoxylin-eosin. They were analyzed for pathology in a high-magnification light microscope equivalent to 400X. Histological analysis evaluated and quantified the degree of steatosis, the presence of mitosis, the inflammatory infiltrate, the size and morphism of the nucleus and the architecture of the liver parenchyma, later these data were statistically analyzed.

Immunohistochemistry:

Immunohistochemistry was performed in paraffin sections. Antibody against proliferating cell nuclear antigen (PCNA, monoclonal mouse, clone PC10, cat. M089, Dako, USA) was used. After dewaxing sections were hydrated in graded alcohol solutions and distilled water. Then endogenous peroxidase was quenched with a 3% hydrogen peroxide solution in methanol (15 min), followed by heat-mediated antigen retrieval in microwave using 0.01 M Na-citrate buffer (pH 6.0) for 5 min. After reaching room temperature, sections were incubated with a blocking solution for 1 hour, then primary antibody (1:200 dilution in phosphate saline buffer –PBS, pH 7.4) was incubated (16 hours, in a humid chamber, at 4°C). Then, sections were washed with PBS and the secondary antibody (Nichirei-Histofine® Simple Stain conjugated to peroxidase for rat tissue – anti-mouse, cat. 414171F) was incubated onto the sections (1 hour, at room temperature), washed with PBS and revealed with diaminobenzidine (Liquid DAB, DAKO). After washing with PBS and distilled water, sections were counterstained with hematoxyline.

PCNA labeling index:

Twenty high quality images (2048 × 1536 pixel buffer) were randomly obtained from liver sections stained with PCNA using the 40x objective lens. A computer-assisted image

analysis system comprising a Nikon Eclipse E-800 microscope connected to a digital camera (Evolution VF, Media Cybernetics Inc., MD, USA) and to a computer was used. The graphical interface software was Q-Capture 2.95.0, version 2.0.5 (Silicon Graphic Inc., Milpitas, CA). The percentage of PCNA+ nuclei in the fields represents the PCNA labeling index.

Blood analysis:

The blood collected was sent to the veterinary laboratory, where alanine aminotransferase and aspartate aminotransferase were measured and analyzed on the same day.

Statistical assessment:

The data collected were typed in excel® and later exported to the Statistical Package for the Social Sciences (v. 14). A comparison was made between the means of the different groups analyzed, using the paired t test.

The results were considered statistically significant when faced with a p-value lower than 5%.

Results:

The 14 animals in the hepatectomies groups survived the first surgery and recovered well for the second surgery. The 7 animals in the Sham group survived the first surgery and recovered well for the second surgery. The 7 animals in the control group had no complications.

During clamping the intestines were congested and dilated as shown in the figure 1.

Figure 1 Aspects of the intestines at the end of preconditioning.

The liver in the control group and in the Sham group had a normal appearance, as seen in figure 2.

Figure 2 Health normal liver.

All the remnant livers in the groups undergoing hepatectomy presented an aspect of steatosis and hypertrophy, as shown in Figure 3.

Figure 3 Remnant liver with an aspect of steatosis and hypertrophy.

Blood analysis:

Alanine aminotransferase (U/L): The mean of Alanine aminotransferase in the Hepatectomy with preconditioning group was 350.643 with a standard deviation of 219,938; while in the Hepatectomy without preconditioning group was 481,229 with a standard deviation of 344.7691; in the paired t test between the two groups, the value of -130.5857 was obtained with a p value of 0.357; this difference being not statistically significant ($p > 0.05$).

Graphic 1

Graphic 1: Graphic with the means of Alanine transferase in the groups

Aspartate aminotransferase (U/L): The mean Aspartate aminotransferase in the Hepatectomy with preconditioning was 450.650 with a standard deviation of 384.1711; while in the Hepatectomy without preconditioning group it was 696.600 with a standard deviation of 223.8700; in the paired t test between the two groups, the value of -245.9500 was obtained with a p value of 0.669; this difference being not statistically significant ($p > 0.05$).

Graphic 2

Graphic 2: Graphic with the means of Aspartate Aminotransferase in the groups

The group of hepatectomie with preconditioning had means of alanine aminotransferase and aspartate aminotransferase lower than the means of the group hepatectomie without preconditioning, however the results had no statistical significance.

Histological Results:

The degree of lipid vacuolization: The mean of the degree of lipid vacuolization in the group of hepatectomy with preconditioning was 2.50 with a standard deviation of 0.837; while in the group of hepatectomy without preconditioning was 3.00 with a standard deviation of 0; in the paired t test between the two groups, a value of -0.5 was obtained with a p value of 0.203; this difference being not statistically significant ($p > 0.05$).

Mitosis: The mean of Mitosis found in 10 fields evaluated in the group of hepatectomy with preconditioning was 9.00 with a standard deviation of 5.888; while in the group of hepatectomy without preconditioning was 10.14 with a standard deviation of 10.383; in the paired t test between the two groups, the value of -1.143 was obtained with a p value of 0.839; this difference being not statistically significant ($p > 0.05$).

Large lipid vacuoles and mitotic figures can be seen in the Figure 4. These findings represent the degree of steatosis and the increase in cell proliferation in the remnant liver 48 hours after hepatectomy in the group with preconditioning and in the group without preconditioning.

The congestion of the hepatic remnant can be verified, microscopically, by evaluating the portal triad. In Figure 5, it is possible to verify the accumulation of red blood cells in the portal vein, representing hepatic congestion in the remnant liver 48 hours after hepatectomy in the group with preconditioning and in the group without preconditioning.

Figure 4

Figure 4: Hematoxylin-eosin stain in light microscope: Mitosis and steatosis can be seen in liver remnant.

Figure 5

Figure 5: Hematoxylin-eosin stain in light microscope: Portal triad seen in liver remnant.

PCNA immunohistochemistry samples:

The groups showed several differences when comparing the values of all groups in relation to the PCNA. The control group (0.99 ± 0.4) was different from the Sham group (2.3 ± 1.0 , $p < 0.03$), from hepatectomy with preconditioning group (86.0 ± 2.1 , $p < 0.0001$) and those without preconditioning group (77.8 ± 2.9 , $p < 0.0001$). The mean of the Sham group (2.3 ± 1.0) was also statistically different from the hepatectomy with preconditioning group (86.0 ± 2.1 , $p < 0.0001$) and without preconditioning (77.8 ± 2.9 , $p < 0.0001$). Finally, we also have that the mean of the hepatectomy with preconditioning group (86.0 ± 2.1) differed from those without preconditioning (77.8 ± 2.9 , $p < 0.0001$), and the hepatectomy without preconditioning group presented value of 85.9296 with a standard deviation of 2.06499 and the Hepatectomy without preconditioning group presented a value of 77.7739 with a standard deviation of 2.88258. The paired t-test revealed a value of 8.15575 with a p value < 0.0001 ; this difference is statistically significant ($p < 0.05$).

Graphic 3

Graphic with PCNA expression means in the groups

The Control group had livers with normal PCNA expression as seen in Figure 6.

The Sham group had livers with normal PCNA expression as seen in Figure 7, with an index very similar to Control group.

The Hepatectomy without preconditioning group had remnants livers with an increase of PCNA expression as seen in Figure 8.

The Hepatectomy with preconditioning group had remnants livers with an increase more prominent of PCNA expression as seen in Figure 9.

Figure 6

Figure 6: Stain of hepatic parenchyma of control group:
Observed little PCNA expression and no steatosis present.

Figure 7

Figure 7: Stain of hepatic parenchyma of Sham group:
Observed little PCNA expression and no steatosis present.

Figure 8

Figure 8: Stain of hepatic remnant of hepatectomy without preconditioning group: Observed an increase of PCNA expression and steatosis in microscopy compared to Sham and control group. Observed less PCNA expression compared to hepatectomy with preconditioning group.

Figure 9

Figure 9: Stain of hepatic remnant of hepatectomy with preconditioning group: Observed an increase of PCNA expression and steatosis in microscopy compared to Sham and control group. Observed more PCNA expression compared to hepatectomy without preconditioning group.

Discussion:

Hepatectomy is the most effective treatment in malignant liver tumors such as hepatocarcinoma, intra-hepatic cholangiocarcinoma and metastatic liver lesions. Patients undergoing resection with disease-free margins have better outcomes [10].

The increase in hemodynamic control, the development of specialized centers with well trained professionals and the technological evolution of instruments allowed the expansion of indications for hepatectomy, resulting in larger hepatectomies. However, this expose patients to the risk of post hepatectomy liver failure [11].

Extensive liver surgeries with small remnants as well as liver graft implants proportionately small to the recipient can lead to postoperative liver failure. These two syndromes known as post-hepatectomy liver failure and “Small for Size” syndrome present similar conditions characterized by hyperbilirubinemia, coagulopathy, encephalopathy and refractory ascites [12][13].

It has been established that the probability of developing this syndrome increases in patients with liver remnant parenchyma below 25% of the total in normal livers, below 30% in steatotic livers and below 40% in cirrhotic livers or exposed to numerous cycles chemotherapy [3][14].

In order to minimize the incidence of liver failure in the postoperative period, the calculation of liver volume is used to verify whether the remaining liver will be sufficient for the patient [15].

To study the characteristics of liver regeneration, models with rats of the Wistar lineage are well consolidated in the literature. This models have great translational capacity. It is possible to perform regulated hepatectomies in segmented livers which have a high correlation with humans hepatectomies [16].

In the literature, studies in models with resection of 90%, 95% and 97% [17], had high postoperative mortality, therefore these models do not correlate with liver failure after hepatectomy in humans [18].

In the present study in experimental surgical model in rats, we were able to study the effects of hepatic preconditioning before the resection of 70% of the liver volume. We compare the results between hepatectomies with preconditioning and without preconditioning. We also include in the study a group Control and a group Sham (simulation) to analyze all the possibilities of cause and effect and to minimize biases. There was no surgical mortality in all groups in the study.

Liver preconditioning is suggested as a way to reduce the damage caused in extensive liver surgery with low volume remnants. Short-term induction of liver ischemia, followed by a period of reperfusion before a major procedure, may increase tolerance to ischemia, resulting in greater postoperative hypertrophy. Preconditioning is supposed to protect the liver from the deleterious effects of ischemia/reperfusion injury [19].

In the presented study, the hepatectomy with preconditioning group had means values of alanine aminotransferase and aspartate aminotransferase lower than the means present in the hepatectomy group without preconditioning, indicating a benefit of the procedure, however the statistical difference was not significant, $p > 0.005$. Others experimental works corroborate with the results found in relation to the comparative decrease of alanine aminotransferase and aspartate aminotransferase in animals submitted to hepatectomy with preconditioning [20].

In the macroscopy, all the animals in the control group were healthy, with healthy livers, without any changes, the animals in the Sham group tolerated the first surgery well and the macroscopy showed normal livers. The animals underwent to hepatectomies in both groups with and without preconditioning all presented with hypertrophied and congested remnants similar to that expected from a major hepatectomy.

The immunohistochemistry was performed with PCNA evaluation. The percentage of PCNA+ nuclei in the fields represents the PCNA labeling index. The Control and Sham groups had averages of 0.99 and 2.3 respectively of index, showing an ordinary small amount of cell proliferation. The groups that underwent to hepatectomy with preconditioning and without preconditioning had respectively 86 and 77.8 of index showing an increase of cell proliferation, with a P value less than 0.005; therefore the result is significantly statistical.

PCNA plays a crucial role in DNA replication and its expression is related to cell proliferation. Therefore, this increase in PCNA percentage bigger in the hepatectomy with preconditioning group demonstrates that there was a greater cell proliferation with preconditioning.

Larger hepatectomies with small remnants are complex surgeries in which it is necessary to associate adequate margins with a parenchyma capable of hypertrophy [21]. Searching for mechanisms in experimental models that provide greater hypertrophy in the remnant liver is one of the ways to increase the indications for surgery in previously inoperable patients [22].

New studies may help to better understand the role of preconditioning in hepatectomy in order to optimize the recovery of low-volume liver remnants, extending the indications for hepatectomy and preventing postoperative liver failure, a high-lethal complication [23].

Conclusions:

Liver preconditioning can provide an increase in cell proliferation in the remaining liver and does not change the morbidity or mortality of hepatectomy.

Abbreviations:

DNA: deoxyribonucleic acid; PCNA: proliferating cell nuclear antigen; PBS: phosphate saline buffer; UFRJ: Universidade Federal do Rio de Janeiro

Acknowledgements:

The authors would like to recognize the physicians, nurses, and all healthcare workers who are dedicated to the care of patients with liver diseases

Author's contributions:

First author:

Professor AAR,

Collected the data;

Performed the anesthesia and the Surgeries;

Collected the samples;

Analyzed the data;

Wrote the paper;

Wrote the manuscript text;

Co-author 2:

Professor CMT,

Collected the data;

Performed the immunohistochemistry;

Hystological and immunohistochemistry analyses;

Wrote the paper;

Reviewed the manuscript text;

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Professor VNP,

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Hystological analyses;

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Reviewed the manuscript text;

Co-author 4:

Professor MAP,

Analysed the data;

Statistical work;

Wrote the paper;

Reviewed the manuscript text;

Co-author 5:

Professor JRF

Design and conceived the study;

Coordinated the tasks;

Wrote the paper;

Reviewed the manuscript text;

All authors meet the requirements for authorship, approved the final manuscript as submitted and agree

to be accountable for all aspects of the work. All authors read and approved the final manuscript.

Funding:

Resources from the Federal University of Rio de Janeiro

Availability of data and materials:

All data are stored at the Department of General Surgery in Federal University of Rio de Janeiro, located located on Rua Prof. Rodolpho Paulo Rocco, 255 - University City of the Federal University of Rio de Janeiro, Rio de Janeiro - RJ, 21941-617, Brazil.). Alternatively, all authors will have the files. Each author will be available to provide them following a reasonable request.

Ethics approval:

The Committee of Ethics in the Use of Animals (CEUA) in Scientific Experimentation of the Science Center of Health of the Federal University of Rio de Janeiro registered with the National Council for the Control of Animal Experimentation (CONCEA) under process number 01200.001568/2013-87 certifies that the project titled: “Effects of portal preconditioning in low-volume liver remnants. Study in rats.”, protocol No. 058/20, under its responsibility which involves the production, maintenance and/or use of animals for the purposes of scientific research (or teaching) is in accordance with the precepts of the Law No. 11,794, of October 8, 2008, of Decree No. 6,899, of July 15, 2009, and with the published rules by the National Council for the Control of Animal Experimentation (CONCEA), was approved by this commission of ethics, at a meeting on 10/13/2020.

The study is in accordance to ARRIVE guidelines for animal experiments.

Consent for publication:

Not applicable

Competing interests:

None

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