# Pulmonary effects after partial liver ischemia and reperfusion - experimental model

## *Repercussões pulmonares após isquemia hepática parcial e reperfusão – modelo experimental*

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A B S T R A C T

**Objective:** To describe an experimental model of hepatic ischemia/reperfusion injury with systemic manifestations, represented by pulmonary involvement, which may be used by those who intend to comprehend this phenomenon. **Methods:** Fourteen Male Wistar rats (200-250g) were allocated to two groups, G1 with eight rats submitted only to laparotomy and G2, six rats submitted to hepatic ischemia and reperfusion. Hepatic (serum aminotransferases, mitochondrial respiration, histology) and pulmonary (Evans blue test) functions were analyzed. **Results:** There was a statistically significant difference ($p < 0.05$) between G1 and G2 comparing values of AST (24,3 ± 108 and 5406 ± 2263), ALT (88,5 ± 28,5 and 5169 ± 2690), respiratory control ratio (3,41 ± 0,17 and 1,91 ± 0,55) and ADP/O relation (1,93 ± 0,03 and 1,45 ± 0,27), histological lesions (necrosis, inflammatory cells, hemorrhage, microsteatosis) and Evans blue test (194,31 ± 53 and 491,8 ± 141). **Conclusion:** The model has proven useful to study hepatic I/R injury.

**Key words:** Models, animal. Liver. Ischemia. Reperfusion. Rats, Wistar.

## INTRODUCTION

During transplant, the removal of the donor liver and its revascularization in the recipient leads to ischemia / reperfusion (I/R). Ischemia produces tissue damage and a pro-inflammatory state that, with the subsequent establishment of blood flow, leads to another deleterious effect: reperfusion injury.

I/R injury is the major determinant of liver dysfunction after hepatectomy, is also the most important cause of primary dysfunction of liver grafts [1,2] and is the main cause of re-transplantation in the first two postoperative weeks [3].

Despite advances in the study of causes and consequences of ischemia and reperfusion, there is still no effective treatment clinically proved. However, many experimental models have been developed in order to test interventions to reduce their harm and provide knowledge about the pathophysiological events [4,5] and interaction of platelets, complement activation [6, 7] reactive oxygen species and nitric oxide [8] .

The objective of this study is to demonstrate a model of ischemia and reperfusion with systemic manifestations, represented by lung injury, which may be useful for those wishing to understand injury mechanisms of I/R or develop possible therapeutic actions.

## METHODS

Fourteen male, adult Wistar rats weighing between 200g and 250g were used and housed in a controlled environment at 23° C, with light / dark cycles of 12 hours and fed with commercial chow and water *ad libitum*. All animals were treated according to the International Protection of Animals Rules. The protocol was approved by the local Ethics Committee, complied with all regulations for research involving laboratory animals and was ego steered under number 281/03.

The animals were divided into two groups: Group 1 (G1) – six individuals, undergoing laparotomy without clamping of the vascular pedicle; and Group 2 (G2) – eight animals, submitted to ischemia and reperfusion and receiving 0.9% saline solution intravenously.

Anesthesia was induced with intraperitoneal injection of ketamine (Ketalar ®) and xylazine (Rompum ®) in a 4:1 ratio. On the operating table, the rats were set in supine position and had the four extremities and the

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upper jaw immobilized. A 14 gauge intravenous catheter (Jelco ®) was adapted and used as a tracheal tube, which, after insertion, was tested. A Mechanical ventilator (Harvard inc., Holliston, MA) was coupled and adjusted to 60 breaths per minute with a volume of 0.08 ml per gram weight. A rectal thermometer (YSI inc., Dayton, Ohio) was placed.

After shaving the abdominal wall and applying local antisepsis with povidone iodine, we performed a median incision of approximately 5 cm, with upper limit on the xiphoid process, followed by opening of the abdominal cavity and exposure of the operative field. The falciform, triangular, left and inter-lobar ligaments were sectioned, followed by identification and recognition of the hepatic pedicle elements and clamping with a microvascular instrument (Figure 1) to stop blood flow. Once confirmed the color change of the ischemic liver lobes, by a transition line with the non-ischemic lobes, the abdominal wall was closed with 4-0 Mononylon ® to minimize heat and water loss.

After clamping the pedicle, the animals underwent an hour of warm ischemia. During this period they were kept anesthetized, mechanically ventilated, with temperature controlled between 36.5 to 37° C using a 45W/127V halogen lamp. After 45 minutes of warm ischemia, one milliliter of 0.9% saline solution was injected into the dorsal penile vein. After the period of warm ischemia, the incision was opened and the vascular clamp removed, initiating the period of reperfusion, certified by color change. The abdominal wall was closed with simple stitches in two layers with 4.0 Mononylon ® suture.

The rats were extubated after recovery of spontaneous respiration and reflexes to stimuli in lower limbs and subsequently housed in individual cages with water *ad libitum*. After four hours of reperfusion the animals were anesthetized with 2 ml of ketamine / xylazine and placed back on the surgical table. Injection of 0.5 mL of Evans blue was performed in the dorsal penile vein. After 15 minutes a thoraco-laparotomy as carried out, with collection of 1 ml of blood by cardiac puncture and section of inferior vena cava.

The liver was removed and divided in two parts: one ischemic and one non-ischemic. The intention was the removal of a portion of each fragment for histological analysis and the assessment of mitochondrial respiration. The lungs were removed and sent to evaluation of optical density with Evans blue.

Aminotransferases were used as markers of liver injury. The mitochondrial function was assessed using the methods described by Coelho *et al.* [9]. According with method of Bielecki *et al.* [10], after sacrifice, fragments of ischemic and non-ischemic liver lobes were removed and immersed in homogenization solution. Oxygen consumption was determined [11] by using a 5/6 oxymeter (Gilson Medical Electronics, Inc.) with an $O_2$ electrode (Clark, Yellow Springs Instruments Co., Yellow Springs, Ohio) at 28°C. Mitochondria received potassium succinate as

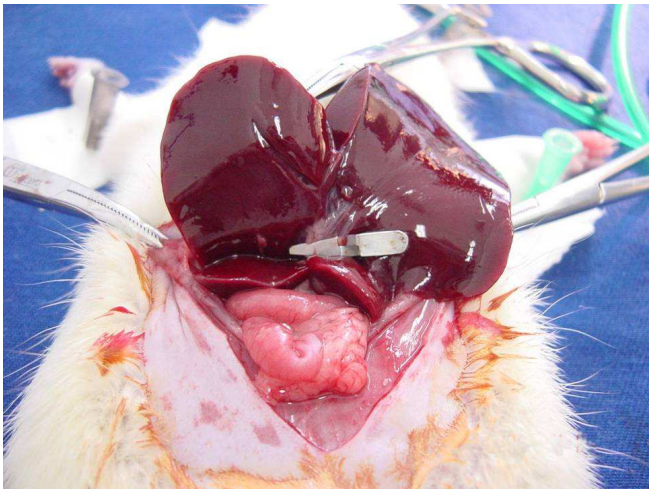

***Figure 1*** – *Clamping of the vascular pedicle common to the median and left-lateral lobes.*

energetic substrate to determine the state 4 (S4) of respiration (baseline). The third state (S3) of respiration (active state) was induced by addition of adenosine diphosphate (ADP, Sigma Chemical Company, St. Louis, Missouri). After complete phosphorylation of the ADP into ATP, S4 was measured again.

The respiratory control ratio (RCR) evaluates the coupling reactions of mitochondrial S3/S4 ratio, i.e., oxygen consumption (basal rate of oxygen consumption in the presence of ADP) and the final use of ADP.

ADP/O represents the ratio of ADP used for phosphorylation of oxygen consumed in the reaction. RCR and ADP/O were calculated as oxidative functions of mitochondria and phosphorylation [12].

S3 and S4 are expressed in nanograms of oxygen atoms per mg of mitochondrial protein per minute, as determined by the method of Lowry *et al.* [13].

Histological changes were evaluated by a blinded pathologist in relation to group allocation. The material was fixed in 2% formaldehyde and stained with hematoxylin and eosin.

The Evans blue was administered 15 minutes before sacrifice and evaluated as described by Jancar *et al.* [14].

The outcome was expressed as averages and standard deviations. For the analysis of histological findings, we used the Kruskal-Wallis test. When statistical differences were observed, the Mann-Whitney test was applied. To determine the significance of other results, analysis of variance (ANOVA) and the Newman-Keuls test were used. A $p<0.05$ was considered significant.

## RESULTS

### Aminotransferases

Serum levels of AST and ALT increased significantly in G2 ($p<0.001$) when compared to G1 (Table 1).

### Mitochondrial respiration

We analyzed the values of RCR and ADP/O. The RCR decreased significantly (p<0.001) in G2 (Table 2). The ADP/O ratio was significantly lower (p<0.001) in G2 (Table 3).

### Histology

Table 4 shows the histological findings of the two groups. We evaluated necrosis, apoptosis, inflammatory infiltrate, sinusoidal dilatation, bleeding, edema and microsteatosis. The findings were classified according to intensity: absent, mild, moderate or severe. For statistical analysis, when the changes were absent or mild, they were grouped as absent, while in cases where the changes were moderate or severe, they were grouped as present.

### Pulmonary evaluation

The study of the lung optical density of Evans blue showed a significant increase (p <0.01) in G2 (Table 5).

## DISCUSSION

Wistar rats were chosen for easy handling, low cost and availability. In addition, standardization of size, weight and feeding favored the production of more homogeneous groups for the experiment.

Aspects related to the anesthetic care, monitoring vital signs and surgical procedures were considered when developing the model. Most experimental models in animals maintained spontaneous breathing during the operation [15-18]. The pathophysiology of the effects of variations of the partial pressures of gases and pH are well established [19]. It is also known that gas pressure and arterial pH directly influence

**Table 1 –** Determination of serum aminotransferases.

| Group | AST | ALT |
|---|---|---|
| G1 | 24.3 ± 108 | 88.5 ± 28.5 a |
| G2 | 5,406 ± 2,263 | 5,169 ± 2,690 b |

*a,b – p <0,001*

**Table 2 -** Determination of RCR.

| Group | RCR |
|---|---|
| G1 | 3.41 ± 0.17 a |
| G2 | 1.91 ± 0.55 b |

*a,b – p <0,001*

**Table 3 -** Determination of the ADP/O ratio.

| Group | ADP/O |
|---|---|
| G1 | 1.93 ± 0.03 a |
| G2 | 1.45 ± 0.27 b |

*a,b – p <0,001*

**Table 5 -** Evans blue test.

| Group | Evans blue (ug/g in pulmonary tissue) |
|---|---|
| G1 | 194.31 ± 53 a |
| G2 | 491.8 ± 141 b |

*a,b – p < 0,01*

**Table 4 -** Analysis of histological findings.

| Variable | Group | Absent | Present | | Fisher's Test |
|---|---|---|---|---|---|
| Necrosis | G1 | 6 | 0 | a | |
| | G2 | 0 | 8 | b | a,b – p<0.005 |
| Apoptosis | G1 | 6 | 0 | a | |
| | G2 | 4 | 4 | b | a,b – NS |
| Inflammatory | G1 | 6 | 0 | a | |
| Cells | G2 | 0 | 8 | b | a,b – p<0.005 |
| Sinusoidal | G1 | 6 | 0 | a | |
| Dilation | G2 | 7 | 1 | b | a,b – NS |
| Hemorrhage | G1 | 6 | 0 | a | |
| | G2 | 1 | 7 | b | a,b – p<0.05 |
| Swelling | G1 | 4 | 2 | a | |
| | G2 | 8 | 0 | b | a,b – NS |
| Microsteatosis | G1 | 6 | 0 | a | |
| | G2 | 1 | 7 | b | a,b – p <0.05 |

*NS – non-significant difference.*

the hepatic circulation. Hughes *et al* [20] demonstrated that hypercapnia increases portal blood flow and decreases hepatic artery blood flow in dogs, while hypocapnia decreases both flows [21]. Hypoxemia results in decreased arterial flow, with no changes in portal venous flow [22]. These changes in hepatic circulation must be related to potentialization or attenuation of injury in case of I/R. Variations in hepatic blood flow can also justify the changes that occur at the cellular level. The influence of *in vitro* acidosis in hepatocytes and endothelial cells was studied by several authors. [23,24] Heijnen *et al*. [25] demonstrated that the respiratory acidosis worsens the I/R lesion with normal oxygen partial pressure *in vivo*, while in conditions of hypoxia the opposite occurs, i.e., I/R injury is attenuated.

Mechanical ventilation requires adequate muscle relaxation to avoid the interaction of active-breathing, inadequately anesthetized rats with the ventilator. It is noteworthy that rats are hemodynamically unstable animals, increasing the need for anesthetic care in I/R models, since the phenomenon of reperfusion alone can determine cardiac arrhythmias and hypotension [26]. Anesthesia with muscle relaxation suitable for positive pressure ventilation and maintenance of hemodynamic stability was achieved with the combination of xylazine and ketamine. This association is commonly used in many models, even in the absence of ventilatory support [17].

The temperature, important variable in the regulation of vascular contractility and oxygen dissociation curve of red cells [19], was monitored via rectal thermometer. After defining the anesthetic care and monitoring, the surgical procedure needed to be standardized.

The option for ischemia of the median and left-lateral lobes was due to three factors. First, for technical simplicity: after the release of the left triangular and inter-lobar ligaments, exposure allows the isolation of the pedicle common to median and left lobes without much surgical manipulation. Second, the liver volume corresponding to the two lobes is significant, representing 70% of the total. And third, the partial clamping allows the portal venous drainage through the remaining liver segments, allowing prolonged ischemic time and preventing severe mesenteric congestion, which reduces the chances of bacterial translocation [27] and minimizes the interference of inflammatory mediators secondary to mesenteric stasis. The clamping of the pedicle was performed with atraumatic vascular clamps specially adjusted to avoid direct injury to the vascular endothelium.

The rats estimated blood volume corresponds to 5.4 ml/100mg weight [28]. We administered one ml of saline, which is approximately 8% of blood volume in rats. This was done in order to compensate blood loss during the procedure and prevent establishment of hypotension, and also to simulate the injection of drugs.

There are different types of experimental models of hepatic temporary ischemia[4]. The partial interruption of flow through the middle and left lobes characterizes the partial hepatic ischemia model used in this and many other studies [6,7,27,29,30]. By definition, these models are similar. However, the ways of anesthetic care, monitoring of vital signs and performance of surgical procedures provide uniqueness to each one.

Zumbado *et al*.[17] showed that ischemia times lower than 60 minutes do not cause significant tissue damage. Moreover, times greater than 120 minutes originates significant increases in morbidity and mortality. Similar results were observed in the pilot study. Therefore, in order not to unnecessarily increase surgical risk, the determined duration of ischemia was 60 minutes.

The time to reperfusion was also determined by means of a pilot study and the literature. Zumbado *et al*. [17] demonstrated that after 60 minutes of reperfusion it is possible to observe 60% of secondary lesions due to free radicals, and after 24 hours, changes derived from almost all the known mechanisms of injury are present. To determine the time of reperfusion in the present study we analyzed 60 minutes, 24 hours and four hours in preliminary tests. It was demonstrated that the time of four hours is suitable for intense development of lesions. Therefore, four hours was the chosen time of reperfusion.

Canedo *et al*. [5] used this model with success, but instead of saline solution, in a group of rats they applied hydrochloride tirofiban, an antiplatelet agent, and demonstrated its protective action against I/R lesions, not only in the liver, but also in the lung.

The Evans blue was used here because it is a dye that has high affinity for albumin. This feature allows using it to assess vascular permeability, since its concentration in the washed lung tissue is proportional to the leakage of albumin through the injured endothelium.

In conclusion, credit of effectiveness must be given to this model, due to its capability to demonstrate that hepatic injury in I/R associate with pulmonary involvement, with no deaths of the studied animals. Thus, it can be used to study the mechanisms of I/R injury, as well as the ways to mitigate it.

## RESUMO

***Objetivo:*** *Descrever um modelo experimental de lesão de isquemia/reperfusão hepática com manifestações sistêmicas, representadas pelo envolvimento pulmonar, que possa ser utilizado por aqueles que pretendem compreender esse fenômeno.* ***Métodos:*** *Ratos Wistar machos (200-250g) foram usados. Quatorze foram alocados em dois grupos, sendo G1 com oito submetidos somente à laparotomia e G2, seis à isquemia e reperfusão hepática. As funções hepática (aminotransferases séricas, respiração mitocondrial, histologia) e pulmonar (teste do azul de Evans) foram analisadas.* ***Resultados:*** *houve diferença estatística significativa entre G1 e G2 ao se comparar valores de AST (24,3 ± 108 e 5406 ± 2263), ALT (88,5 ± 28,5 e 5169 ± 2690), razão de controle respiratório (3,41 ± 0,17 e 1,91 ± 0,55) e relação ADP/O (1,93 ± 0,03 e 1,45 ± 0,27), lesões histológicas (necrose, células inflamatórias, hemorragia, microesteatose) e teste do azul de Evans (194,31 ± 53 e 491,8 ± 141).* ***Conclusão:*** *O modelo mostrou-se útil para o estudo de lesão de isquemia/reperfusão hepática.*

***Descritores:*** *Modelos animais. Fígado. Isquemia. Reperfusão. Ratos Wistar.*

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