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A Pig Model of Hemivascular Liver Occlusion for The Study of Ischemia-Reperfusion Injury: Use of an Infrared System for Detecting Ischemic Areas

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Abstract

Aim: Different animals are used as experimental models for the hepatic Ischemia- Reperfusion (IR) injury investigations and for each one of these animal models, many different surgical approaches have been performed. The aim of our study was to establish a new surgical pig model in which a hemi-liver is used to study the pathophysiology of hepatic IR injury. Contro-lateral hemi-liver is used as an internal control in the same animal.

Methods: Liver ischemia was performed in six pigs by clamping the hepatic artery and vein and the portal vein to isolate the left hepatic lobe. Four hours of warm ischemia were followed by 4-hourrs of reperfusion. Biochemical and hematological analyses were performed throughout the experiments. Needle biopsies were obtained prior to ischemia and then hourly during the reperfusion for evaluation of tissue damage. To assess local temperature gradients on the liver surface a focal plane array detector camera was used.

Results: Four hours ischemia induced mild signs of hepatic damage on the left ischemic lobe while more dramatic changes were evidenced after 2-hours reperfusion. Absence of tissue damage was detected on the right lobe. The liver functional test reached their maximum value at 2-4 hours after reperfusion.

Conclusion: Our model is easy to perform, feasible and reproducible. This surgical model minimizes biases dependent on the individual response of different animals under the same conditions. In this IR model the new technology of an infrared thermocamera was used to control temperature changes and provide clinically important real-time information during surgery.

Abbreviations: IR: Ischemia/Reperfusion; IV: Intravenous; ALP: Alkaline Phosphatase; ALT: Serum Alanine Aminotrasferase; AST: Aspartate Aminotransferase; LDH: Lactate Dehydrogenase; ROI: Region of Interest

Keywords: Animal model; Infrared imaging; Liver ischemia reperfusion injury, Liver transplantation

Introduction

Hepatic Ischemia-Reperfusion (IR) injury still represents the Achilles' heel in liver transplantation, especially when sub-optimal donors are used, and liver surgery. IR injury is a complex process that involves a variety of pathophysiological mechanisms resulting from a prolonged ischemic insult followed by restoration of blood perfusion. The importance of IR injury is related to its severe consequences in terms of morbidity and mortality [1]. The transient deprivation of blood flow and oxygen followed by their return after reperfusion [2], involves the release of Oxygen-Free Radicals (OFR), cytokines/chemokines, and up-regulation of adhesion molecules with consequent microcirculatory failure, oxidative stress, inflammatory cell infiltration, necrosis and cell death [3-8]. A large number of studies on IR injury published in the past decade have pointed out that, in liver transplantation, IR injury is an antigen-independent event of preservation influencing hepatic graft outcomes. It may cause up to 10% of early organ failure and can lead to high incidence of both acute and chronic rejection [9-11].

On the other hand extreme hepatic resections are performed under portal pedicle clamping (Pringle manouver) for reducing blood loss. This thechnique may isitates in an IR injury, especially in steatotic or cirrotic livers. Because its complexity, *in vitro* experimental procedures are elusive and only partially satisfactory to fully comprehend its pathogenesis. Consequently, a large number of experimental animal models have been proposed to better clarify the mechanisms of this process. The literature reports different animal species used as experimental model (dog, mouse, rat, rabbit) for liver IR injury investigations and for each of these animal models, many different surgical approaches have been applied (segmental vascular exclusion, ischemic preconditioning, major hepatectomy, and portal blood bypass) [12-20]. However these models are often technically demanding, time consuming and are only partially clarifying pathophysiology of hepatic IR injury. Here we describe an easy reproducible IR model in the pig with controlateral hemi-liver used as an internal control within the same animal.

Interestingly in this model it is not necessary to perform a venous-venous shunt to sustain blood pressure and blood flow and both ischemic and non ischemic lobes are subjected to the same anesthesiological, pharmacological and stress conditions. To investigate the relationship between changes in thermal gradients and surgical phases an array detector camera (ThermaCam™ P25, Flir System) was used. We believe that this surgical model may be an useful tool to study the pathophysiology of IR injury and to investigate strategies to prevent the alteration observed in liver IR.

Materials and Methods

Animals

Six female crossbreed pigs (Landrace x Large White) weighing between 14 and 20 kg (16,2 ± 2,2 kg) were used. All animals were housed individually at a steady temperature, fed commercial dry food and water ad libitum. All experimental procedures had to comply with the Law for the Care and Use of Laboratory Animals in force in Italy (D.L. n.116, 27/01/1992) and were approved by the Veterinary Committee of Padua University, Padua, Italy.

Animal Preparation and Anesthesia

Following a fasting period of 24 hours with free access to water, pigs were pre-medicated with atipamezole (Stresnil®, Janssen Cilag Spa, Cologno Monzese, Italy) 4 mg/kg intramuscularly. An intravenous line was placed and buprenorphine (Temgesic®, Schering Plough Spa, Segrate, Milano, Italy) (0.01 mg/kg) was given intravenously (IV) to obtain pre-emptive analgesia. General anesthesia was induced with propofol (Rapinovet®, Intervet Srl, Segrate, Milano, Italy) at the dose of 3-4 mg/kg IV and, after orotracheal intubation, maintained with isoflurane (Forane®, Abbott, Campoverde di Aprilia, LT, Italy) and oxygen by intermittent positive pressure mechanical ventilation. Isotonic Saline or Ringer's solution (10 ml/kg/h) was given for basal fluid requirement and replacement of peri-operative fluid loss. When necessary additional fluid therapy and dobutamine infusion were used to treat hypotension.

The pigs were placed on a heating pad in dorsal recumbence and connected to the monitoring machines (Datex Homeda). During anesthesia, Heart Rate (HR), external electrocardiogram, Invasive Arterial Blood Pressure (IBP), Percutaneous Pulse Oxymetry (SpO2), Respiratory Rate (RR), Inspired and Expired Gases (Fi/Et gas) and esophageal temperature (T°C) were constantly monitored and recorded every 5 minutes. Vascular access (right external jugular vein and common carotid artery) were surgically isolated by right lateral cervical incision and cannulated for drugs and fluid administration, blood collection and blood pressure monitoring (central venous and arterial pressure).

Surgical Procedure

A midline xyphopubic laparotomy was performed. The intestine was gently shifted to the left caudal abdominal cavity to better expose the porta hepatis. The hepatoduodenal ligament and its components were isolated. The common bile duct was isolated just above the level of the pancreas. A silicon cannula was then inserted into the common bile duct up to its bifurcation: at this level the hepatic duct was ligated around the cannula, while its distal portion was sectioned. During each phase of the surgical procedure, bile was collected and its production (ml/hour) mea-

sured in each experimental phase as a parameter of liver function. The hepatic artery and its branches were exposed: the branches to the left medial lobe and to the left lateral lobe were carefully isolated and a vessel loop was placed around each of them. The portal vein and its principal branches (the right and the left portal vein) were scheletonized and exposed. The left hepatic lobes (left lateral lobe and left medial lobe) were mobilized by section of their ligaments (falciform ligament, left coronary ligament and left triangular ligament). This manoeuvre was necessary to expose the left hepatic vein. At the level of portal bifurcation, a small incision was performed on the dorsal part of the common portal vein and a 5F Fogarty endovascular catheter was inserted and advanced for 3-5 cm into the left portal branch. The catheter was secured to the portal vein by a non-absorbable suture. Prior to insertion, the Fogarty catheter balloon was tested.

Induction of Ischemia: A bolus of heparin (300 UI/kg) was infused intravenously before the induction of ischemia. Segmental hepatic warm ischemia was performed by the total vascular exclusion of the left lateral and the left medial lobes. The left portal vein was occluded by the Fogarty catheter-balloon inflation; the left artery branches were clumped by tightening the vessel loops and placing vascular clamps if necessary. An atraumatic vascular clamp was placed across the left hepatic vein, at the level of its drainage into the inferior vena cava. The ischemic period lasted four hours.

Reperfusion Period: After 4 hours of ischemia, reperfusion was obtained by removing the vascular clamp across the left hepatic vein; deflating the Fogarty catheter-balloon in the left portal vein; and removing the vessel loops and the vascular clamps around the arterial branches.The reperfusion observation period lasted 4 hours then the animals were euthanized by enbutramide/mebezonio iodide/tetracaine IV administration (Tanax® Intervet Srl, Segrate, MI, Italia). During the experimental surgical procedures there were no severe complication and vital signs were within normal values.

Tissue Sampling and Histological Findings

A 14-gauge Tru-cut needle biopsy was performed on each liver lobe prior and immediately after ischemia, and hourly, after reperfusion. Liver specimens were fixed in 10% buffered formalin and embedded in paraffin. Sections (4 μm thick) were myimrade and stained with hematoxylin and eosin. The histological assessment of edema, congestion, inflammatory cell infiltration and necrosis was done by a semi-quantitative histological examination. Liver damage was expressed as a percentage of the liver parenchyma affected by edema, congestion, inflammatory cell infiltration and necrosis (Table 1). Histological examination of the left ischemic and right non ischemic lobes was performed by the same pathologist (FC) who carried out a blind analysis.

SCORE	DAMAGE GRADE	LIVER PARENCHYMA INVOLVED (%)
0	NULL	0%
1	MILD	<30%
2	MODERATE	30-50%
3	SEVERE	>50%

Hepatocellular Function

At the same time-points of the liver biopsies, blood samples were drown from the jugular vein and centrifuged for 10 minutes at 3000 rpm. Serum aliquots were stored at -80˚C to assess hepatocellular function in a hepatic warm IR injury model. Alkaline Phosphatase (ALP), Serum Alanine Aminotrasferase (ALT), Aspartate Aminotransferase (AST) and Lactate Dehydrogenase (LDH) were Measured as Liver Functional Tests (LFTs).

Measurement of Bile Production

Bile was collected during the three different experimental phases: from the common bile duct cannulation until the beginning of the ischemia (T0); during the 4 hours of ischemic phase (T1) and for 4 hours after reperfusion (T2). The bile volume was measured and production expressed as ml/hour.

Infrared Imaging

To assess local temperature gradients on the liver surface a focal plane array detector camera (ThermaCam™ P25, Flir System) was used. This method allows a real-time intraoperative infrared imaging of the liver. As infrared emission at the measured wavelength (3-5 μm) is directly proportional to the temperature, the camera was calibrated in units of temperature. An uncooled microbolometer detects temperature differences as small as 0.08°C. A camera was placed 50-60 cm above the liver surface to achieve a field of view to fit the entire visible liver surface (~20 x 25 cm). Infrared images (~130 images per 10 h trial per animal) were taken by passive acquisition of spontaneous infrared emission from exposed tissue hourly from the beginning of the experimental procedure to the end of the reperfusion period. The camera produces outstanding noise-free, high resolution images (320 x 240 pixels) offering more than 76.000 individual measurement points per image at a refresh rate of 50/60 Hz. On-line visual analysis of the thermal images was followed by an off-line evaluation. To investigate the relationship between changes in thermal gradients and surgical phases, Region-of-Interest (ROI) was established for the right and left liver lobes. The same ROI was selected from each infrared image to obtain a temperature profile of each lobe.

Statistical Analysis

Data were expressed as mean ± Standard Deviation (SD). Levels of significant differences between data sets were determined by ANOVA analysis of variance and Student's t-test. Values of $p<0.05$ were considered statistically significant.

Results

In all animals the IR protocol was completed and uneventful. Images of the surgical procedure and the macroscopic view of the liver following warm ischemia are shown in (Figure 1).

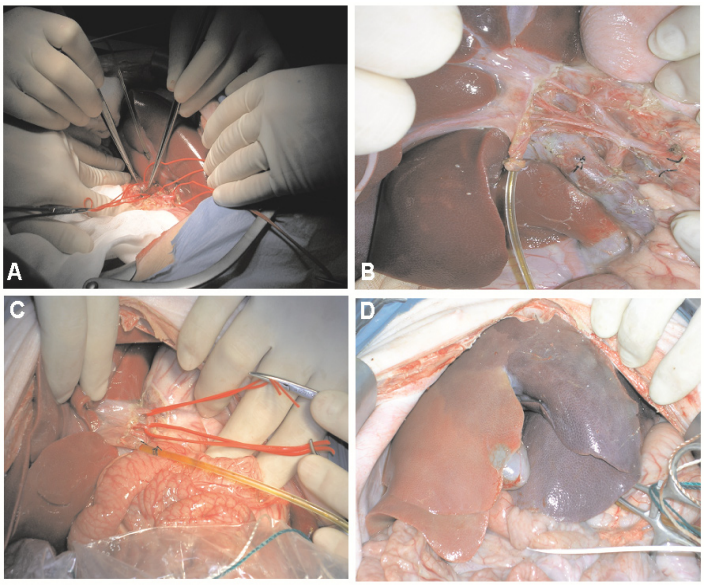

Figure 1: Images of surgical procedure. **(A):** Hepatic/liver hilum preparation/isolation; **(B):** Hepatic hilum isolation; **(C):** Common bile duct cannulation and hepatic artery isolation; **(D):** Ischemia period: demarcation between the right liver lobes (non-ischemic) and the left liver lobes (ischemic lobes).

Histopathological Assessments

Tissue injury was evaluated, as described in methods section, in the left ischemic and the right non-ischemic lobes at equivalent time-points and compared. In Figure 2, typical histological outcomes following ischemia and reperfusion periods can be seen.

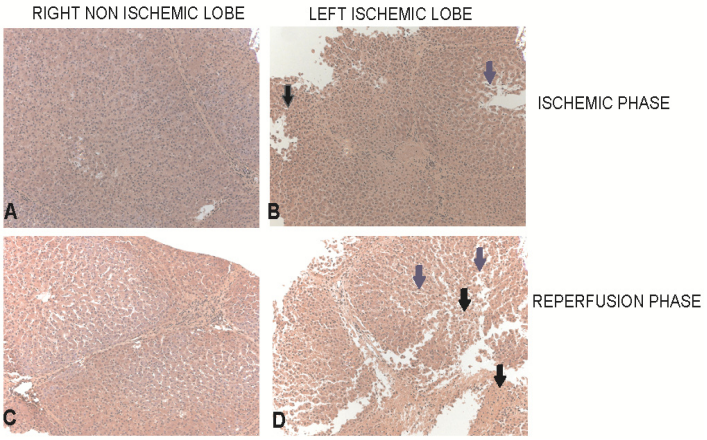

Figure 2: Hematoxylin- eosin staining of right **(A,C)** and left **(B,D)** lobes following 4 hr warm ischemia or 4 hr reperfusion. Blue and black arrows indicate edema and necrotic areas respectively. Hematoxylin- eosin staining, original magnification x 20

- **Edema -** In the ischemic lobes, edema was present after 4 hr-ischemia and progressively increased and involved almost 50% of the liver parenchyma at the end of the reperfusion period. The right non ischemic lobe parenchyma showed a 10% edema throughout the experiment (Figure 3 panel A). The ischemic lobe was significantly more edematous than the contralateral one in each phase considered ($p<0.05$).

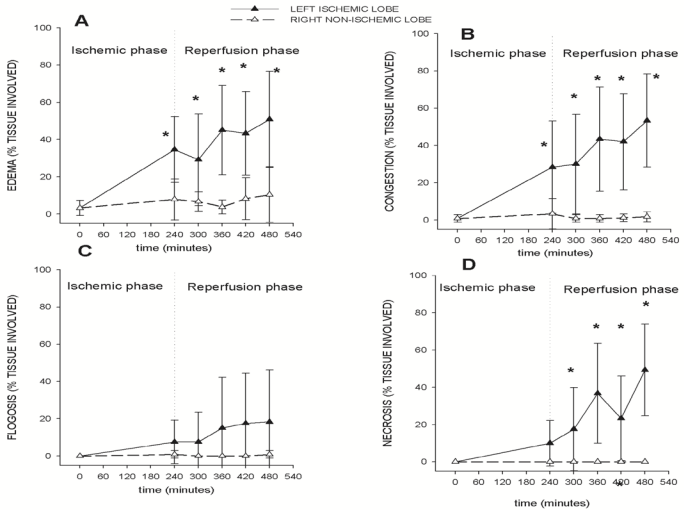

Figure 3: Semi-quantitative analysis of liver damage. Edema **(A):** Congestion **(B):** Inflammation **(C):** Necrosis **(D):** Were analyzed in right (ρ) and left (π) liver lobes and expressed as percentage of tissue involved. Data are presented as mean values (n=6)± SD (*$p<0.05$ left ischemic lobe vs right lobe).

- **Congestion -** In the ischemic lobe, congestion showed the same trend as the edema; it was present after the 4-hour ischemia period; it progressively and significantly increased after reperfusion in all time points considered. (Figure 3 panel B, $p<0.05$). In one animal, 20% congestion was observed also in the non-ischemic lobe exclusively in the biopsy taken at the end of ischemia and was not detected at the following time points.
- **Inflammatory cell infiltration** - At the end of the ischemic period, inflammatory cell infiltration was detected only in the left lobes of animals (affecting between 5 and 30% of the liver lobe; mean 15 ± 13 %) and increased during the reperfusion phase (from 10 to 70%; mean 37 ± 35%) (Figure 3 panel C). In the other animals no cellular infiltration was detected.
- **Necrosis -** In the ischemic lobe, necrosis ranged from 5 to 30% (mean 10 ± 12%) after the ischemia period and progressively increased with time up to 70% (mean 49 ± 24%) at the end of the reperfusion period. Necrosis was not observed in the non-ischemic lobes (Figure 3, panel D, $p<0.05$ in all time points considered).

Bile Production Measurements

Changes in bile production were measured during the different phases of the experimental procedure (Table 2). In the 4-hour ischemic period, in all animal bile production was significantly reduced by 51% and did not recover following reperfusion.

	Basal - T0	Ischemia period	Reperfusion period
Bile production (ml/h)	12,4±4	6.4±3*	7.8±1*

Table 2: Mean bile production (ml/h) ± standard deviation during the experimental procedure. * $p<0.05$ *vs* T0, Student's t test.

Clinical Parameters and Laboratory Values Assessment

After vascular exclusion of the left lobes, no alteration in hemodyinamic parameters was detected. At reperfusion, no remarkable hemodinamic alterations were present; the acidemia was rapidly corrected by sodium bicarbonate infusion and no alteration in electrolytes was detected. During all the experimental period a low blood pressure was evidenced despite fluid therapy and dobutamine infusion; a progressive increase in heart rate was associated with dobutamine administration (Table 3).

	Baseline values	Pre-ischemic period	Ischemia period	Reperfusion period
Heart rate (bpm)	109.2±29	99.9±28	133.7±37	148.7±38
MAP (mmHg)	58.8±6	57.6±10	53.5±8	52.3±12

Table 3: Mean heart rat and invasive Mean Arterial Pressure (MAP) ± standard deviation during the experimental procedure: at the beginning of anaesthesia (baseline values), during surgical preparation (pre-ischemic period), during ischemia and reperfusion period.

Following the ischemia period, AST and ALT levels showed an increase when compared to baseline values. A further increase in these hepatic enzymes was detected following reperfusion. No relevant alteration was recorded for ALP (Figure 4). Pigs naturally have high LDH serum concentration and in our study the values increased throughout all the experimental phases (Figure 4). The liver function tests at 4 hours post-reperfusion confirmed the tissue damage detected by histological analysis.

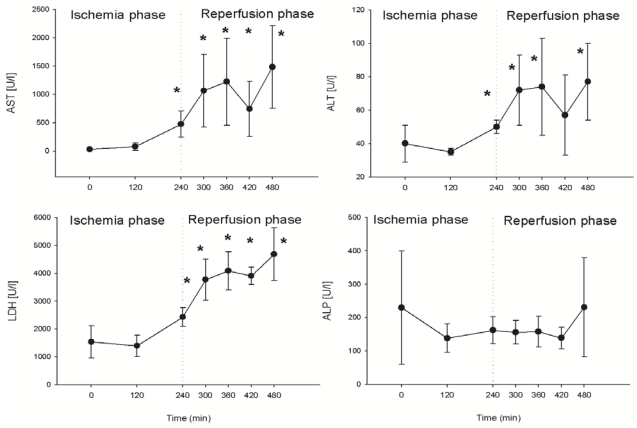

Figure 4: Time course of serum AST **(A):** ALT **(B):** LDH **(C):** ALP **(D):** Enzymes during experimental phases. Points represent mean values (n=6) ± SD (*$p<0.05$ vs baseline levels at 0 hrs).

Infrared Imaging

The real-time visual analysis of the images enabled the evaluation of temperature changes in every anatomical region of the liver during ischemia-reperfusion phases. Regions of interest were established on the right, left lateral and left median lobe of the liver and temperature of the same anatomical areas was evaluated. Digital images taken from the ischemic lobes were compared to those from the non-ischemic lobes of the same liver through the observation period. During the ischemic phases the temperature in the ischemic lobe was significantly lower than in the right non-ischemic lobe, confirming the selective clamp of the blood flow within the left lobe (Figure 5). Hemi-hepatic ischemia influenced the left medial lobe to a lesser extent than the lateral one. In the reperfusion period, the left lobe re-warming did not appear to be homogeneous. Nevertheless the removal of the vascular occlusions determined an increase in the ischemic lobe temperature but after 2 hrs-reperfusion relative hypothermia continue in the left lateral side. In the reperfusion phase, statistical analysis (Wilcoxon Signed Ranks Test, SPSS 16) showed a significant difference between the temperature of left lateral and non ischemic liver lobe ($p<0,05$).

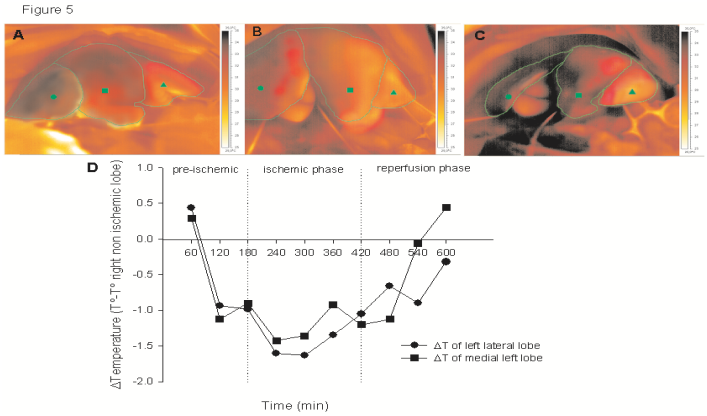

Figure 5: Intraoperative images of liver and its Region of Interest (ROI) temperature profile. Typical images of the liver lobes before the onset of ischemia **(A):** At the end of ischemic phase **(B):** and at 4 hr post reperfusion **(C):** Graph D shows temperature difference profile between median or lateral left lobe and right non ischemic lobe (internal control). Points represent mean values of measures taken in 5 animals.

Discussion

The pathogenesis of liver IR injury represents a very complex series of events leading to cellular and tissue damage which are associated with increased risk of primary graft non function and primary graft dysfunction after liver transplantation [21,22]. Initially, ischemia causes reduction of cellular ATP and a consequent alteration in the active transport mechanisms of the cell membrane resulting in sodium influx and intracellular sodium accumulation with corresponding cell swelling and death [3,23,24]. Subsequently, restoration of blood supply during reperfusion promotes a microcirculation-associated inflammatory response (reflow paradox) that involves the release of oxygen radicals, tumor necrosis factor alpha and interleukin-1, the up-regulation of leukocyte and endothelial adhesion molecules, and the interaction of platelets and leukocytes with the microvascular endothelium. These events culminate with cellular death and organ dysfunction [22, 25-29].

A large number of preclinical models are described to study IR injury in both large and small animals. In this work an experimental model of partial liver IR injury was developed in the pig. In particular we perform a segmental vascular exclusion where the left lobes are totally excluded from arterial, portal and venous vascularization, while the right non- ischemic lobes are normally perfused and used as an"internal" control. Compared to whole hepatic ischemia models described in earlier studies [30,31], that require porto- or femoral-jugular, or a cavo-caval and porto-caval shunt to avoid portal consumption and to maintain macro hemodyinamics, this hemi-ischemia liver procedure requires short execution time and fewer surgical skills. The use of large ordinary forceps to obtain a partial hepatic ischemia have been described [32,33], however in our model the use of a more refined hepatic vascular occlusion, by means of Fogarty catheter and atraumatic vascular clamps, allowed a selective occlusion of the left portal vein and hepatic arteries without traumatizing the parenchyma.

In our model, liver IR injury was monitored by measurement of plasma concentration of liver function marker enzymes and by analysis of liver histology. Bile flow was also employed as an indicator of hepatic IR damage [34]. Four hours ischemia induced mild signs of hepatic damage while more dramatic morphological changes were evidenced later on and reached a plateau after two hours reperfusion. The plasma liver functional test started to increase following the 4 hr ischemia and reached their maximum value at 2-4 hours after reperfusion confirming histological findings. In agreement with our observations in a preliminary pilot study, in this partial liver IR model 4 hours was the minimum ischemia time resulting in parenchymal damage and elevated LFTs, detectable during both ischemia but mainly during the subsequent reperfusion phase. These data confirm that the pig liver is remarkably resistant to normotermic ischemia [32,35], and that damage is mostly detectable during recirculation after declamping [9].

As expected, the histopathological evaluations highlighted differences between the ischemic and the non-ischemic lobes with reference to the presence of edema, congestion, inflammatory cell infiltration and necrosis. Furthermore, the absence of tissue damage including necrosis in the non-ischemic lobes suggest that we have developed a model with an internal non-ischemic control exposed to the same experimental condition as the ischemic tissue. In this light, in the same animal, it will be possible to test both the efficacy and the tolerability of new molecules, reducing the numbers of animals needed for experimental purposes and avoiding the inter-animal bias.

Several methods have been developed to measure organ perfusion in the operating room. These include Doppler ultrasound or laser, near infrared spectroscopy and thermo dilution methods [36,37]. Invasive methods or methods requiring direct contact with the parenchyma clearly cause tissue trauma or compression that affect local perfusion. Methods limited to regional assessment require sequential probe positioning and thus are not capable of simultaneous or retrospective analysis of data from unanticipated sites of interest [1,37]. In this IR injury model the new technology of an infrared thermocamera was used to monitor possible temperature changes. This approach can provide clinically important real-time information during surgery. Application of this technology to the exposed organ of interest in our model was found to provide useful and additional informations related to organ perfusion [38]. In a clinical renal transplantation trial, it has been demonstrated that the overall parenchymal perfusion as measured by infrared imaging can be used to predict subsequent allograft recovery [36]. This method allows the quantization of liver lobes perfusion and,

in this model, confirmed the selective blood flow exclusion in the left liver lobe. In the IR surgery parenchymal warming is not homogeneous and this technology could be helpful for evaluation of regional defects during reperfusion, or for performing biopsies in a specific hot or cold spot.

Conclusion

We believe that this surgical animal model is an useful tool to study IR liver injury, its pathophysiological aspects and its clinical implications. The procedure is easily feasible, reproducible and the possibility to have an internal control could minimize the biases dependent on the individual response of different animals under the same conditions (i.e. surgical, anesthesiological and pharmacological conditions, ischemic stress). Furthermore, it allows to reduce the number of animals for the experiment, eliminating the control group. This aspect is very crucial and rapresents, in our opinion, an absolute advantage in terms of less animal sacrifice and lower costs for experiments.

This animal model offers the possibility to test drugs and preservation solutions with potentially protective effects on IR injury. In addition it is useful for studying hepatic regeneration after portal vein occlusion [39] in the era of staged hepatectomies [40,41]. The particular surgical technique used guarantees the accuracy of the selective vascular occlusion without any further mechanical damage of the parenchyma. Finally, it is not necessary to perform an external venous shunt avoiding hypothermic and hemodynamic alterations related to the shunt.

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