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Consequences of head injury and static cold storage on hepatic function: ex vivo experiments using a model of isolated perfused rat liver

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Abstract

The purpose of the study was to evaluate the effect of head injury (HI) on the metabolic and energy functions of the liver and its consequences after cold storage. In male SD rats with HI, livers were isolated 4 days after injury and perfused either immediately (HI) or after 24 hours of cold preservation. Livers isolated from healthy rats were treated identically. The hepatic functions were explored with an isolated perfused liver model. Head injury induced a liver atrophy without significant difference in the intrahepatic energy level versus healthy rats. After cold storage, hepatic adenosine triphosphate and glycogen contents in HI rats were similar to those of healthy rats. The livers of the HI group that underwent cold preservation had a lower protein catabolism. The portal flow rate at the time of reperfusion was significantly increased in the HI group. In conclusion, static cold storage of livers harvested from HI rats revealed a net protein catabolism reduction and a modification of hepatic microcirculation.

1. Introduction

The objectives of liver transplantation are to increase the survival and quality of life of patients with acute liver disease or end-stage chronic liver disease. In the last decades, with the development of new immunosuppressive regimen and molecules, the improvement of surgical technology, and the treatment of postoperative rejection and infection, patient and graft survival has significantly increased [1,2].

Despite this progress, early mortality immediately after orthotopic liver transplantation remains important. The most common cause is a primary graft nonfunction (PGNF), which occurs in 1.3% to 6% of patients, [3-5]. PGNF is defined as an impairment of early graft function that is not due to postoperative complications such as vascular thrombosis or acute rejection. It leads either to immediate retransplantation or to patient death. Over the last years, a number of experimental studies have been carried out with the objective of understanding the physiopathology of the PGNF and limiting its incidence. Until now, only a few risk factors have been associated with the occurrence of primary dysfunction; the exact causes are not yet known. Among risk factors, donor-related factors such as old age, unstable vital signs, use of vasoactive drugs, macrovesicular steatosis, long periods of preservation, and the nutritional status have been identified [6-10].

Liver donors are generally victims of a severe head injury (HI) evolving toward brain death. The series of events preceding liver harvesting (ie, hypercatabolism associated with HI [11], brain death [12]) affects liver metabolism. In a recent experimental study, we observed a decrease in liver weight and hepatic protein content after HI [13]. These alterations linked to HI-related hypercatabolism are responsible for an impairment of adenosine triphosphate (ATP) and glycogen homeostasis during the initial phase of trauma with a decrease in hepatic glycogen and ATP content that is neither anorexia related nor the result of a decreased ability to produce ATP [14].

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We hypothesize that inflammatory response associated with HI is responsible for overconsumption of ATP linked to the enhancement of gluconeogenesis and synthesis of inflammatory proteins, generating the ATP depletion observed beforehand and influencing the graft function. Indeed, liver glycogen has been suggested to be a source of ATP production during both the cold storage and the initial reperfusion periods [15]. In addition, Lanir et al [16] demonstrated a direct correlation between high ATP content and a successful posttransplant outcome.

These liver metabolism modifications after HI could influence the liver function but could also increase the sensitivity of the liver to preservation/reperfusion injury and consequently play a role in the PGNF. Indeed, brain death induces alterations in the donor liver that make it more sensitive to preservation/reperfusion injury and decrease the donor liver viability [17]. This could be partly due to the induction of cytokines with up-regulation of inducible nitric oxide synthase (iNOS) and to nitric oxide (NO) production after severe HI and during brain death [18].

The objectives of this study were thus 2-fold: (1) to characterize the hepatic metabolic and energy changes after HI and (2) to evaluate the resistance of the liver from HI rats to cold preservation and reperfusion.

For the dynamic study of hepatic metabolism, we used the isolated perfused rat liver model. One of the main advantages of this ex vivo model is the preservation of hepatic organization. Cooperation between different cell types (Kupffer cells and hepatocytes) and between the 2 populations of hepatocytes (periportal and perivenous cells) that play an important role in hepatic metabolism is therefore preserved.

2. Materials and methods

2.1. Experimental design

2.1.1. Animals

Thirty-seven male Sprague-Dawley rats (346 ± 25 g; Charles River, Saint-Aubin-Lès-Elbeuf, France) were acclimatized for 1 week at our animal facility and maintained on a standard chow diet (A04; Safe, Augy, France) with water available ad libitum. They were randomized into 4 groups: 2 groups of rats with HI whose livers were isolated 4 days after injury and perfused either immediately after withdrawal (HI group) or after 24 hours of cold preservation (HIP group) (n = 9 in each group), and 2 groups of healthy rats that were fed ad libitum and whose livers were treated identically (AL group, n = 10; ALP group, n = 9).

The experiments complied with our institution's guidelines for animal care and were approved by the Regional Ethical Committee (no. P2.CM.001.04). Two of the authors (CC and CM) are officially authorized (no. 75456 and no. 75522) by the French Ministry of Agriculture and Forestry to perform surgical experimental research on rats.

2.1.2. Head injury

Eighteen rats (HI and HIP groups) were anesthetized with chloral hydrate (400 mg/kg, IP; Prolabo, Fontenay-sous-Bois, France) and placed on a stereotaxic frame. During surgery, animals were positioned on a heating blanket (Harvard, Les Ulis, France) to maintain operative normothermia (37.5°C ± 0.5°C). Head injury was induced by fluid percussion using the protocol described previously [19]. In brief, the scalp was incised; and a 3-mm craniotomy was performed lateral to the right temporoparietal cortex (stereotaxic coordinates: 3.5 mm anterior and 6 mm above the interaural line) using a dental drill, taking care to leave the dura mater intact. A 3-mm polyethylene tube was placed over the dura mater, fixed securely into the craniotomy site using dental cement (Paladur, Hanau, Germany), and connected to a solenoid valve (Danfoss, Nordborg, Denmark). The opposite end of the valve was connected to a high-performance liquid chromatography pump (Gilson, Roissy, France). The system was filled with sterile water, providing a calibrated outflow pressure of 1.6 to 1.8 bar. The solenoid valve controlled with a timer (Omron, Kyoto, Japan) and opening for 20 milliseconds triggered the percussion directly onto the dura mater. The cortical pressure applied was measured extracranially using a pressure transducer (Emka Technologies, Paris, France) connected to an oscilloscope (DSO 400; Gould, Les Ulis, France). Immediately after fluid percussion, the tube was removed; the scalp was sutured, and the animals were placed at 26°C to 28°C to recover from the anesthesia. The animals received 10 mL of saline intraperitoneally to prevent the acute renal failure that occurs after such trauma [13], as currently practiced in other models of stress [20]. The rats were maintained at 26°C to 28°C for 4 hours before being transferred to cages with standard chow diet (A04, Safe) and water available ad libitum.

2.1.3. Isolated perfused rat liver

Isolated perfused liver was performed 4 days after HI because the hypercatabolic state induced by the trauma was maximal at this time [13]. Before the surgery, the rats were fasted for 12 hours. The livers were prepared according to the technique described by Miller [21] under controlled inhalation anaesthesia (isoflurane 4% with oxygen at 2 L/min) associated to subcutaneous injection of analgesia ([buprenorphine] Temgésic, 25 μg/100 g body weight; Schering-Plough). After laparotomy, the bile duct was cannulated with a catheter; then, after intravenous injection into the caval vein of 1 mL of saline containing heparin (500 UI, Choay; Sanofi-Winthrop, Gentilly, France), a polyethylene tube was inserted into the portal vein. The liver was rinsed in situ at constant flow (10 mL/min) by 100 mL Krebs-Henseleit buffer at 37°C without oxygenation and removed without stopping the perfusion. For each animal, the same small lobe was totally excised after careful tight binding (to avoid any leaking during the perfusion), freeze clamped, and stored at −80°C until measurement of adenine nucleotide and glycogen contents.

After the rinse, the livers were perfused at constant pressure (13 cm H_2O) through the portal vein in a recirculating system in a thermostatically controlled cabinet (+37°C) for 90 minutes [22,23]. The perfusate was Krebs-Henseleit buffer (150 mL, pH 7.4) saturated with 95% O_2/5% CO_2 containing bovine serum albumin (30 g/L), glucose (8.5 mmol/L), and calcium (2.4 mmol/L). The perfusate flowed freely back into the reservoir via the hepatic veins.

As previously described [24-27], this medium was supplemented at the start of the perfusion with 8 anti-proteolytic amino acids (AAs) (ie, AAs that prevent excessive nonphysiologic proteolysis) at twice their physiologic concentration. After the first 30 minutes of perfusion, corresponding to the equilibration phase, a balanced mixture of AAs (MEM 7145 and BME 6766; Sigma-Aldrich, Saint-Quentin Fallavier, France) was added to the perfusate to allow the measurement of AA exchanges under physiologic conditions [25]. The period between 35 and 90 minutes represented the study phase. Perfusate samples were taken from the portal inflow and the venous effluent at 5, 10, 15, 30, 35, 60 and 90 minutes and stored at −80°C until analysis. Throughout the perfusion, the bile was collected in preweighed vials.

At the end of the perfusion, the livers were weighed; and a piece of tissue was removed by freeze clamp to measure adenine nucleotide and glycogen contents.

2.1.4. Cold preservation

Two series of livers (HIP and ALP) were stored for 24 hours. These livers were prepared as described above except for rinse that was performed using Ringer lactate followed by University of Wisconsin (Dupont-de-Nemours, Paris, France) solution (50 mL, +4°C each). Afterward, the livers were immersed in 100 mL of University of Wisconsin solution (reference solution for cold preservation of liver during 10-24 hours [28]) and stored at +4°C for 24 hours.

At the end of the storage period, a small lobe was carefully removed as described above to measure intrahepatic adenine nucleotide and glycogen contents. Samples of the preservation solution were collected for the measurement of glucose, urea, AA, and enzyme release. The livers were rinsed with Ringer lactate (50 mL, 20°C, at constant flow) and perfused through the portal vein in the recirculating system described above.

2.2. Procedures and analytical methods

2.2.1. Measurement of portal and bile flows

The bile was collected over three 30-minute periods, and the bile flow was estimated gravimetrically assuming a specific mass of 1 g/mL. Portal flow was measured at 5, 10, 15, 30, 35, 60, and 90 minutes by measuring the liquid flowing out of the liver in 1 minute. These 2 parameters are expressed in microliters per minute per gram of liver and milliliters per minute per gram of liver, respectively.

2.2.2. Substrate measurements in perfusate and preservation medium

Glucose and urea concentrations were measured by the glucose oxidase and urease methods, respectively, using an AU600 apparatus (Olympus, Rungis, France). Enzyme activities (aspartate aminotransferase [AST], alanine aminotransferase [ALT], creatine phosphokinase [CK], and lactate dehydrogenase [LDH]) were measured using an AU600 apparatus (Olympus) [29]. After deproteinization of the samples with sulfosalicylic acid (30 mg/mL), AAs were determined by ion exchange chromatography on an AminoTac JLC-500/V analyzer (Jeol, Tokyo, Japan) [30].

2.2.3. Intrahepatic adenine nucleotides and glycogen content determination

After storage at −80°C, the liver samples were weighed and homogenized with an Ultra-Turrax T25 (Ika-Labotechnik, Staufer, Germany) with perchloric acid (0.5 N) or perchloric acid–EDTA (4%, 0.5 mmol/L) for adenine nucleotide and glycogen determination, respectively.

Measurements were made in the spun supernatant either directly or after neutralization with KOH (for adenine nucleotides). Adenine nucleotides were separated by reversed-phase high-performance liquid chromatography with spectrophotometric detection (254 nm) [27]. Glycogen was measured by anthrone reagent with spectrophotometric detection (620 nm) [31]. Results are expressed as micromoles per gram of liver weight for ATP and milligrams per gram of liver weight for glycogen.

2.3. Calculations

Release (R) of enzymes and metabolites by the livers during preservation was calculated as:

$$R = (C \times V)/W,$$

where C was the concentration in the preservation medium, V was the medium volume, and W was the liver wet weight measured at the end of storage. Glucose and urea are expressed as millimoles per gram of liver weight; AA, as nanomoles per gram of liver weight; and enzyme, as international units per gram of liver weight.

Glucose, urea, and AA hepatic fluxes (F) during recirculating perfusion of livers were calculated as:

$$F = (C2 \times V2) - (C1 \times V1)/(T2 - T1) \times W',$$

where $C1$ and $C2$ were the concentrations in the perfusion medium at times $T1$ and $T2$, $V1$ and $V2$ were the

Table 1
Total body weight and liver weight on the day of liver withdrawal

	AL + ALP	HI + HIP
Body weight (g)	361 ± 5	289 ± 5*
Liver weight (g)	10.4 ± 0.3	8.3 ± 0.2*

Results are given as means ± SEM and expressed in grams. Groups are defined in the "Materials and methods" section.
Mann-Whitney test: *$P < .001$ vs AL + ALP.

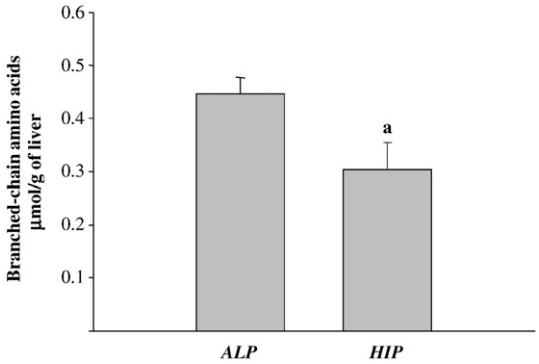

Fig. 1. Branched-chain AA release in preservation solutions over 24 hours of cold storage. At the end of 24 hours of cold storage, the storage media were collected and branched-chain AA (valine, isoleucine, and leucine) concentrations were measured. Data are expressed as means ± SEM. Mann-Whitney test: $^{a}P < .05$ vs ALP.

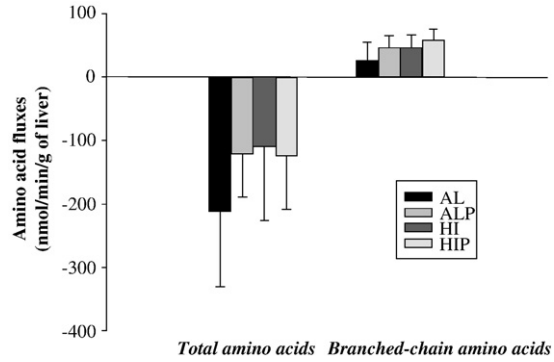

Fig. 3. Amino acid fluxes between 35 and 90 minutes of perfusion. The total AA and the branched-chain AA fluxes between 35 and 90 minutes of perfusion are represented. Data are expressed as means ± SEM. Kruskal-Wallis test. Positive values equal release; negative values equal uptake.

perfusion medium volumes at the same times to allow for variations in volume due to additions and sampling, and *W'* was the liver wet weight measured at the end of perfusion. Amino acid fluxes are expressed as nanomoles per minute per gram of liver weight; and glucose and urea fluxes, as micromoles per minute per gram of liver weight.

2.4. Statistical analysis

All results are given as mean ± SEM. Comparisons between sets of data were made using analysis of variance on repeated measurements followed by the Newman-Keuls test for perfusion flow. A Kruskal-Wallis test was used to analyze bile flow urea, glucose, AA fluxes and enzyme release at each time as well as intrahepatic adenine nucleotide and glycogen contents. For liver and rat weights as well as for AA and enzyme release in the preservation medium, a Mann-Whitney test was used. A P value < .05 was considered to be the threshold for a significant difference. Statistical analysis was performed using Statview 5.0 for Macintosh (SAS Institute Inc., Cary, NC).

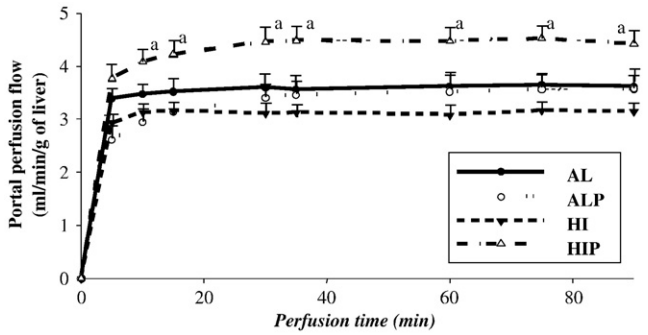

Fig. 2. Portal flow rate in perfused rat livers. Portal flow rate was measured for 90 minutes on isolated perfused rat liver. Data are expressed as mean ± SEM. Analysis of variance + Newman-Keuls test: $^{a}P = .02$, HIP vs 3 other groups.

3. Results

3.1. Body and liver weight

Head injury induces a significant body weight loss at the day of liver harvesting (day 4) that is associated with a significant difference in liver weight between HI and AL rats (Table 1).

3.2. Measurements in preservation solutions

Analysis of the preservation fluid at the end of the cold storage period shows the presence of free AAs and enzymes released by the liver. Activities of AST, ALT, LDH, and CK are similar in both HIP and ALP groups (data not shown). The AAs released are the same in HIP and ALP groups

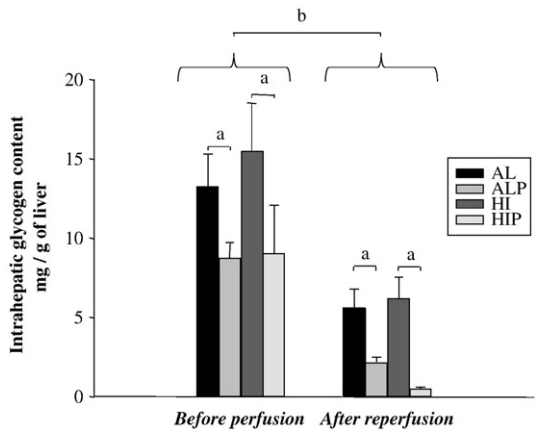

Fig. 4. Intrahepatic glycogen content before and after reperfusion. The glycogen content before reperfusion is measured after storage in the HIP and ALP groups and after the withdrawal in the HI and AL groups. Glycogen is measured again in all groups after reperfusion. Data are expressed as means ± SEM. Kruskal-Wallis test: $^{a}P < .05$, with storage vs without storage; $^{b}P < .05$ vs before perfusion.

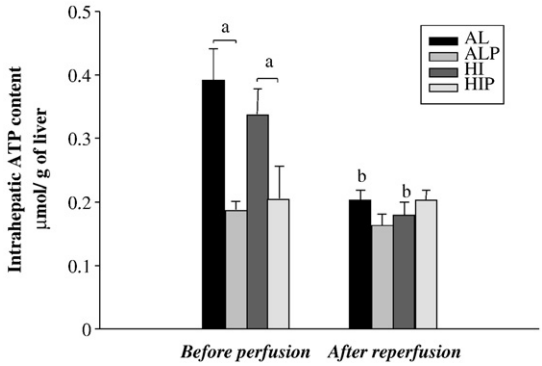

Fig. 5. Intrahepatic ATP content before and after reperfusion. The ATP content before reperfusion is measured after storage in the HIP and ALP groups and after the withdrawal in the HI and AL groups. Adenosine triphosphate is measured again in all groups after reperfusion. Data are expressed as means ± SEM. Kruskal-Wallis test: $^{a}P < .05$, with storage vs without storage; $^{b}P < .05$, after vs before perfusion.

except for the amount of branched-chain AAs whose levels are significantly lower in the HIP group ($P < .05$) (Fig. 1).

3.3. Perfusion

In stored livers (HIP and ALP), the portal flow increases during the first 30 minutes of perfusion; and then it is constant (Fig. 2). At the plateau, the flow rate is similar for livers of HI, AL, and ALP groups, whereas the portal flow in the HIP group is significantly increased ($P = .02$).

In the same way, a decrease in bile production during the first 30 minutes of perfusion is observed in the stored livers (ALP and HIP: 0.25 ± 0.03 vs AL and HI: 0.41 ± 0.04 μL/min per gram of liver, $P = .01$). Afterward, the bile flow rate of stored livers (HIP and ALP) is not statistically different from that of nonstored livers (HI and AL).

The AA, glucose, and urea fluxes are similar in the 4 groups. Head injury, with or without cold storage, does not affect the uptake or the release of AA (Fig. 3).

At the end of the perfusion (T90), the release of CK is decreased in the HIP group (HIP: 329 ± 36 vs AL: 881 ± 132, ALP: 619 ± 134, and HI: 745 ± 82 IU/L; $P < .02$), whereas the release of cytolysis markers (ALT and AST) and LDH does not differ between groups.

3.4. Energy state

The intrahepatic glycogen (Fig. 4) and ATP (Fig. 5) contents 4 days after HI are similar to those of healthy rats. After 24 hours of preservation, the glycogen and ATP contents are significantly decreased but are similar in both HI and AL rats.

Concerning glycogen content, this decline continues during the reperfusion and remains in the same range between HIP and ALP groups and between HI and AL groups. The decrease at the end of the reperfusion period is similar whether the liver is preserved or not (Fig. 4).

The ATP concentrations decrease during the reperfusion period in the nonpreserved groups and do not change in the preserved groups (without difference in both HI and AL groups) (Fig. 5).

4. Discussion

The present investigation was conducted to study the consequences of HI on the hepatic metabolism and energy status. Previous results from our laboratory have shown that HI by fluid percussion in rats induced the same hypercatabolic state as the one observed clinically in patients [24,32,33]. This state, as reported in other models of stress (burns, sepsis) [20,34,35], was limited to the early days after trauma (days 1-4) and was characterized by profound anorexia, body weight loss, increased myofibrillar proteolysis associated with a deterioration in nitrogen balance, liver atrophy, and a decrease in hepatic glycogen and ATP content [13,14].

In the present study, we have observed the same perturbations along with whole-body weight decrease and liver atrophy 4 days after HI. During perfusion, in the AL group, bile production, portal flow, AA, glucose, and urea fluxes, enzyme release, and energy status were similar to those previously published [36-38]. Despite the hypercatabolic state induced by HI, we have not observed any significant perturbation of the hepatic metabolism. Glucose and protein metabolisms in livers from HI rats were similar to those of healthy rats. Similarly, no differences were found in portal and bile flows between HI and AL rats during perfusion. Of note, the ATP and glycogen data are not in line with those previously obtained that show an ATP content significantly lowered in HI groups vs AL groups [14]. In the latter work, all rats were killed by beheading and liver samples were immediately removed and frozen, whereas in the present work, hepatic samples for ATP and glycogen measurement were removed after the anaesthesia and surgery period. This period represents an aggression that may generate (1) a hypermetabolism with ATP consumption and (2) a hypotension promoting anaerobic metabolism, which produces only 2 ATP molecules instead of 38. This may explain why ATP content was 4-fold lowered in our work and why such a low basal value could mask the effect of HI (4 days before) on the energy metabolism.

We have observed that the association of HI with cold preservation and reperfusion induces liver metabolism modifications. Firstly, branched-chain AA release in preservation solution is significantly lower in the HIP group. Branched-chain AAs, poorly metabolized by the liver, are considered as markers of hepatic proteolysis [39,40]. Thus, in livers of HI rats, we have found a lower proteolysis. This net protein catabolism reduction in HI rats during preservation could be linked to the synthesis of hepatic acute-phase proteins. In fact, after trauma, the catabolic state is secondary to an increasing imbalance between proteolysis and protein

synthesis [41]. This modification in protein metabolism has been extensively studied at the tissue level. In the liver, an increase in the rate of the synthesis of positive acute-phase proteins (eg, fibrinogen) has been noticed in different models (sepsis, aseptic inflammation with turpentine, trauma) [42-45]. For example, in HI patients, Mansoor et al [46] have shown a similar increased synthesis of albumin and positive acute-phase proteins (fibrinogen), a phenomenon probably mediated by cytokines, glucocorticoids, and other stress hormones.

This protein metabolism disturbance has an impact on the liver function. Indeed, Calmus et al [39] have suggested that the graft outcome is influenced by the importance of the liver cell proteolysis that was significantly higher in patients with primary nonfunctioning liver grafts.

In addition, the metabolic disturbances after liver preservation in HI animals are associated with modifications of hepatic microcirculation. Increased perfusion flow in the HIP group could be induced by the ischemia-reperfusion phenomena [42]. Its pathophysiology involves the release of vasoactive mediators, with modification in the delicate balance between NO and endothelin [42]. Severe HI leading to brain death induces an up-regulation of iNOS messenger RNA in the liver and an increase of iNOS protein content [17]. It is likely that such high iNOS expression leads to the production of NO just after the reperfusion and modification of hepatic microcirculation.

Of note, a limitation of our work is that HI was studied alone and not in combination with brain death (a situation that would be closer to the clinical practice). However, before such a study, it was mandatory to discriminate the consequence of HI alone on metabolic disturbances at all the steps of liver withdrawal, preservation, and reperfusion. The next step of this work will be to combine HI and brain death and to evaluate their effects on hepatic metabolism with the ultimate goal of transplantation.

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