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Research Article

Procurement and *Ex-situ* Perfusion of Isolated Slaughterhouse-derived Livers as a Model of Donors after Circulatory Death

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Abstract

Ex-situ machine perfusion (MP) techniques are increasingly used in clinical settings, especially on grafts derived from donors after cardiac death (DCD). However, comprehension of biological effects elicited during MP are largely unknown and a substantial number of animal studies are presently focused on this topic. The aim of the present study was to describe a model of DCD based on ex-situ perfusion of liver grafts derived from animals dedicated to food production.

Procurement took place within a slaughterhouse facility. A clinically fashioned closed circuit normothermic MP (NMP) was built up. Autologous blood-enriched perfusion fluid was adopted. Perfusate and tissue samples were collected to asses NMP functionality. Grafts were classified as transplantable (LT-G) or not (n-LT) according to clinical criteria, while histopathological analysis was used to confirm graft viability.

After cold storage, the liver grafts were connected to the NMP. During the rewarming phase, temperature and flows were progressively increased to reach target values. At the end of NMP, 4 grafts were classified as LT-G and 3 nLT-G. Histology confirmed absence of major damage in LT-G, while diffuse necrosis appeared in nLT-G. Interestingly, in nLT-G an early impairment of hepatocyte respiratory chain, leading to cell necrosis and graft non-viability, was documented for the first time. These parameters, together with indocyanine-green dye and citrate clearance could contribute to graft evaluation in clinical settings.

In conclusion, this model provides a promising and reproducible method to replace dedicated experimental animals in DCD and MP research, in line with the 3Rs principles.

1 Introduction

Over the last few years, the increased number of patients requiring organ transplantation and the relatively stable number of suitable donors caused a challenging organ shortage. In 2015, according to the annual report of the Scientific Registry of Transplant Recipients (SRTR), in the USA 1334 patients died on the waiting lists while 703 liver grafts were not transplanted (Kim et al., 2019).

To face this difficulty, liver grafts from high risk donors (also called extended criteria donors, ECD) are increasingly used although ECD grafts generally show a poorer outcome after liver transplantation (LT) (Vodkin and Kuo, 2017). Indeed, an increased risk of primary non-function (PNF), initial poor function (IPF), and long-term worse outcome are associated with ECD liver grafts (López-Navidad and Caballero, 2003; Feng et al., 2006; Dondossola et al., 2017a). Among ECDs, donors after cardiocirculatory death (DCD), in particular type II DCD - unexpected cardiac arrest with unsuccessful resuscitation (Thuong et al., 2016) - represent a major organ source with a potential significant increase in the number of donations (Blackstock and Ray, 2014; Manyalich et al., 2018). However, DCD can exert detrimental effects on post-transplant survival and quality of life. For this reason, there is a high discard rate of these grafts. The DCD-related problems mainly depend on prolonged warm ischemia time (WIT) that reduces the tissue energetic pool leading to cell death (Merlen et al., 2019). However, although the clinical impact of WIT is generally accepted, only few data on the subcellular mechanisms underlying its consequences are available and the potential therapeutic targets are still unclear.

In an attempt to face the ECD detrimental impact and to reduce the discard rate of DCD, ex situ perfusion systems were re-introduced in clinical settings (Guarrera et al., 2010). These procedures consist of extracorporeal perfusion devices that allow a continuous and dynamic perfusion of organs at different temperature using different perfusion fluids. The aims of ex-situ perfusion were different, including improvement of graft preservation, reduction of ischemia-reperfusion injury, and viability evaluation of marginal grafts (Hessheimer and Fondevila, 2017; Selten et al., 2017). Following the demonstration of a clinical advantage of normothermic ex-situ

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perfusion (NMP) in selected grafts (Nasralla et al., 2018), concerns were raised on metabolic and biomolecular mechanisms elicited during these dynamic perfusions. Indeed, although NMP allows graft evaluation before transplantation, increasing safety and acceptance rate, its full potential is presently underestimated due to uncertainty in viability criteria.

Machine perfusion and DCD represent a significant emerging field of experimental and clinical research as demonstrated by approximately 500 publications over the last 10 years. Several groups explored this subject using different methods and techniques. However, the lack of standardized models and the presence of great variability in both procurement and perfusion variables, negatively affect the translational impact of the results. In addition, in-vitro studies cannot be used in this setting due to the absence of physiological cell-to-cell contact and environment (Daniel et al., 2018). For this reason, in-vivo models with dedicated experimental animals are used to study NMP and DCDs. Only few investigations tried to exploit commercial animals as a source of experimental liver grafts (Grosse-Siestrup et al., 2001, 2002b). Further, in these works description of procurement procedures was not provided in detail and technique reproducibility had some bias.

As further development of preclinical studies on NMP and type II DCD is mandatory, standardized easy accessible, unexpansive (in terms of life and economical resources) translational models should be implemented. Large animal organs are preferred to study organ preservation, physiology and pharmacology. Indeed, a standardized model of NMP could be an effective connection between in-vitro and in-vivo experiments. On the other hand, use of dedicated experimental animals to set-up the perfusion protocols does not appear justified according to the 3R principles.

Based on our experience in clinical and preclinical ex-situ perfusions (Bassani et al., 2016; Lonati et al., 2018; Roffia et al., 2018; Dondossola et al., 2018), we designed a study aimed to improve and standardize an ethical and cost-effective procurement/perfusion model using slaughterhouse-derived liver grafts. This model could help characterization of liver metabolism and viability during ex-situ perfusion and provide data that could effectively be translated to clinical settings.

2 Animals, material and methods

Animals

Liver grafts were obtained from Landrance pigs weighing 40-60 kg (n=8). The animals were slaughterhouse pigs used for commercial purposes. Animals received adequate husbandry, and necessary measures were taken to minimize pain, distress, and suffering during the slaughtering process in accordance with the Italian and EU laws (EC, 1099/2009). Pigs were killed by sudden induction of brain haemorrhage followed by terminal exsanguination according to Veterinary and Food Security Department regulations. After sacrifice, the pigs were exsanguinated and immersed into hot water (70°C) to remove hair and to clean the surface.

Materials and instruments

Organ procurement took place in a slaughterhouse located 30 km away from our Preclinical Facility. Surgical instruments, perfusion sets and fluids, ice and organ boxes were transported to the slaughterhouse and kept sterile until procurement (Tab. S1,S2[1]). Personal protection devices consisted in disposable coveralls, surgical gloves and protective glasses. Dialysis fluid and perfusion fluid were stored at 4°C.

Isolated Organ Perfusion Systems

The liver perfusion circuit consisted of an organ chamber, two roller pumps, a centrifugal pump, a reservoir, a membrane lung, and silicon tubing. This was a closed circuit of cannulated vena cava with portal (PVP), arterial (HAP) and cava (CVP) pressure set at 8 mmHg, 75 mmHg and 1 mmHg, respectively.

The membrane lung and reservoir were equipped with a heat exchanger to control the perfusate temperature. Graft temperature during perfusion was set at 38°C. Three syringe pumps were connected to enable infusion of drugs or fluids.

Perfusion fluid analysis

Inflow and outflow perfusate composition was monitored every 15 min over the first hour and every 30 min thereafter (total perfusion time 240 min). Perfusion fluid gas analyses and blood tests were performed.

Mean perfusion fluid hemodynamic and metabolic measurements

During the whole experiment, resistance in the venous system was calculated as mean PVP minus CVP (mmHg) divided by blood flow in the portal vein (litres per minutes), while resistance in the hepatic artery was calculated as mean HAP minus CVP divided by blood flow in the hepatic artery. Oxygen consumption ($\dot{V}O_2$) was measured using the modified Fick equation. Pre-liver perfusate samples, collected after membrane lung, were intended as O_2 enriched perfusate (arterial blood of the Fick equation), whereas post-liver perfusate samples, collected directly from the IVC, were used for calculation of venous oxygen content in the Fick equation. Oxygen delivery (DO_2) was likewise measured (Banan et al., 2016)

$$[1.34 \times Hb\ (g/dL)_{\text{PRE-LIVER}} \times HbO_2(\%)] + 0.003 \times P_{\text{PRE-LIVER}}O_2(mmHg) = O_2 \text{ content of pre-liver perfusate } (C_{PRE}O_2)$$

$$[1.34 \times Hb\ (g/dL)_{\text{POST-LIVER}} \times HbO_2(\%)] + 0.003 \times P_{\text{POST-LIVER}}O_2\ (mmHg) = O_2 \text{ content of post-liver perfusate } (C_{POST}O_2)$$

$$C_{\text{PRE}}O_2 - C_{\text{POST}}O_2 = \Delta_{PRE\text{-}POST}$$

$$\Delta_{\text{PRE-POST}} \times \text{Pump flow (mL/min)} \times 10 = \dot{V}O_2$$

$$C_{\text{PRE}}O_2 \times \text{Pump flow (mL/min)} \times 10 = DO_2$$

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Viability assessment of the liver
Graft viability was assessed after 180 min of evaluation phase. It was based on the currently used clinical viability criteria (Mergental et al., 2016). The main criteria were: lactate level < 2.5 mmol/L and active bile production. Minor criteria were: perfusate pH> 7.30, arterial flow > 150 mL, portal venous flow > 500 mL per min, and homogeneous graft perfusion with soft consistency. According to these parameters, grafts were divided into viable/transplantable (LT-G) and non-viable/non-transplantable grafts (nLT-G).

Evaluation of liver metabolism
Samples were taken from hepatic artery, portal and cava vein every 15 min for the first 60 min and every 30 min thereafter. Perfusion fluid samples were immediately analyzed to assess acid-base metabolism. pH, partial pressure of oxygen (pO_2), partial pressure of carbon dioxide (pCO_2), HCO_3^-, base excess, glucose, lactate, Na^+, K^+, and Cl^- were recorded. At baseline and every 60 min, alanine aminotransaminase (ALT) and lactic- dehydrogenases (LDH) were measured as markers of hepatocellular injury, BUN as a marker of synthetic function, and alkaline phosphatases (ALP) to asses cholangiocyte injury. Lactate, glucose and potassium absolute concentration and release ratio [$(C_{start} - C_{end})/C_{start}$] were used as markers of graft metabolic recovery.

Sodium citrate was added to blood at the time of collection to obtain adequate anticoagulation taking advantage of its ability to chelate calcium. Sodium citrate is mainly metabolized by the liver in zone 3 (Quistrorff et al., 1992; Kramer et al., 2003). There is a direct correlation between calcium release and metabolic activity. Therefore we used i-Ca concentration to evaluate liver metabolic ability. Indocyanine green dye (ICG) clearance test is clinically used to estimate liver function before liver surgery. Indeed, ICG is cleared by hepatocyte and secreted into bile by cholangiocyte. For this reason we decided to use ICG clearance to estimate metabolic and secretive function of hepatocyte and cholangiocyte, respectively. Because this parameter was never tested before during NMP, we decided to use it only in LT-G in order to obtain reference values. ICG dose was calculated as 0.50 mg/Kg (pig weight). Perfusion fluid samples were collected before ICG administration and 1,2,5,10,15 and 30 min after. Bile samples (n=3) were analyzed after 15 and 30 min. Eight-hundred five-nm spectrophotometry was used to analyze the samples after subtraction of baseline values collected before ICG administration.

Histology
Tissue biopsies were performed 1) at the end of back table preparation, 2) at the beginning of NMP, and 3) at the end of NMP (240 min). The samples were fixed in 4% formalin or de-hydrated to assess wet-to-dry ratio (W/D). Formalin-fixed-paraffin-embedded samples were stained with hematoxylin-eosin, Masson's trichrome and reticulin histochemical staining, and CD31 immunohistochemical staining. Thirty random fields per slide were investigated to determine the necrosis area.
To further reduce the number of animals used we elected to use an histopathological outcome instead of performing a liver transplant. Indeed, the histological samples were scored according to Brockman and colleagues (Brockmann et al., 2009) who demonstrated concordance among NMP results, histopathological analyses and liver viability.

To determine wet-to-dry ratio (W/D), all the specimens were weighed with an analytical balance and dried in an oven at 50℃ for 48h (Dondossola et al., 2017b). W/D was calculated and used as an index of tissue edema. Liver specimens from other experiments (healthy livers with similar age) were used as controls for W/D ratio (n=7).

Statistical analysis
All results are presented as mean ± standard error of the mean (SEM). Statistical analysis was performed using Mann-Witney test or one-way analysis of variance (ANOVA) for repeated measures, followed by Tukey's multiple comparison test to evaluate differences at each time points. A probability value <0.05 was considered significant. Data were analysed using SPSS Statistics 25 dedicated software (SPSS, Inc., Chicago, US).

3 Results

After the slaughterhouse procedures, 8 thoraco-abdominal blocks were successfully procured. One liver did not undergo NMP because of poor macroscopic appearance after cold flush (large non-perfused area, and irregular appearance). The ex vivo procedure was performed in 7 livers. Liver surgical outcomes and functional parameters are the subject of the present research and are described in detail in this article. Together with liver grafts, lungs were procured and were subjected to successful ex-vivo perfusion. Detailed data on procurement and perfusion techniques are not the focus of this publication, however, surgical details are given to fully describe the surgical technique. All materials, instruments and manufacturers are listed in Tab. S1, S2[1].

3.1 Surgical Procedure
En-bloc procurement
The procurement procedure was performed within the slaughterhouse facility in clean (but not sterile) conditions, while perfusion solution, tubing and drugs were kept sterile (Tab. S1, S2[1]). The procurement took place during slaughterhouse work and without interfering with food production. In the first four cases, we adopted the surgical technique incompletely described by Grosse-Siestrup and colleagues (2001). The technique was subsequently modified due to the poor graft viability during ex-situ perfusion. Indeed, optimization of the experimental protocol was the aim of this study. Briefly, after sacrifice, a midline thoraco-laparotomy was performed and the thoracic/abdominal organs were procured en-bloc by slaughterhouse technician. A special attention should be payed to avoid injuries of the liver parenchyma and of the inferior lung lobe during sternotomy and phrenectomy. Thoraco-abdominal block was then laid in a box containing 30°C saline solution, the trachea was cannulated and manual ventilation was started (FiO_2 21%). Thoracic organs were separated from the abdominal ones through the section of the inferior pulmonary ligaments, oesophagus (after its ligation cranially to the diaphragm), aorta and cava vein. The thoracic and abdominal block were place separately in two different boxes (30°C saline solution). The trachea should be procured as long as possible to secure a correct ventilation (the right upper bronchus

has a tracheal bronchus). Moreover, aorta must be retrieved undamaged in its posterior partition to avoid injuries to the celiac and renal arteries. The thoracic organs were then cold flushed and used for experiments that are not a focus in this research.

Liver cold perfusion

At the outset, the abdominal block was inspected. The aorta was identified and celiac trunk was accessed through section of aorta posterior wall. A 12 Ch urinary catheter was placed in the celiac trunk (it was secured with a 0 Polysorb tie, placed at the origin of the celiac trunk) and a cold flush (4°C) was started (4 litres of dialysis solution followed by 3 litres of Celsior). During cold flush, the liver was dissected from the other abdominal organs.

The first step consisted of access to the portal vein through dissection of hepato-duodenal ligament (at the upper border of pancreas) and section of the pancreas. The portal vein was isolated for 1 cm. A 14 Ch urinary catheter was inserted and the catheter balloon inflated (5 ml) to enable portal perfusion (1 litre of Celsior). The portal vein dissection must be particularly careful and as distal as possible to avoid injuries to the origin of the common hepatic artery. The surgical dissection was then completed with the detachment of gut, stomach, spleen, left kidney and right kidney. A careful attention must be paid to a possible twist of the celiac pedicle due to the en-bloc procurement: there may be injuries of the celiac trunk and hepatic artery. The main celiac and mesenteric vessels must be ligated to avoid spillage of perfusion fluid. It is suggested not to dissect the hepatic artery in this phase due to its small calibre. We flushed at the slaughterhouse the bile duct (500cc), but we did not open the gallbladder to avoid bleeding during NMP. Cold perfusion must take place as soon as possible to minimize WIT. If WIT is prolonged for experimental purposes, it should be done inside the animal body.

Maintaining further 1000 cc of cold (4°C) Celsior infusion, the graft was placed in a plastic bag. At the end of perfusion, the graft was placed in an ice box and transferred to our Preclinical Facility.

Surgical outcome

The warm ischemia time (from death induction to cold perfusion) was 29 ± 7 min. Liver dissection lasted 20 ± 3 min. The time from death induction to ice storage was 63 ± 10 min for liver grafts. No major surgical damages were registered during ex-vivo dissection, while we have no data on the number of grafts discarded due to butcher damage during en-bloc procurement (a median of 3 graft/day were inspected to obtain an adequate organs). Liver graft weight was 1050 ± 104 g.

Blood collection

Blood was collected from the same animal during exsanguination (autologous blood). To avoid clotting a close collecting system was connected to a 2 litres PVC bag containing heparin (25.000 UI) and sodium-citrate (30 ml). Blood contamination was reduced by adding ceftriaxone 1 g. Blood bags were kept and transported at 24°C. Once arrived in our lab, the blood was separate by precipitation and then the concentrated red blood cells were transferred to smaller blood bags through a 40 µm filter. The total amount of blood collected/animal was 1450 ± 200 ml.

Back table preparation

Before grafts ex-situ perfusion, back table preparation was performed to prepare liver vessels for cannulation. Small veins and arteries were tied to avoid spillage during ex-situ perfusion. Cannulas were inserted and 1 litre of Ringer lactate was infused through hepatic artery. Bile duct was washed with 500 ml of Ringer lactate (4°C) and a 14 G venous cannula was placed in the gallbladder after closure of the cystic duct.

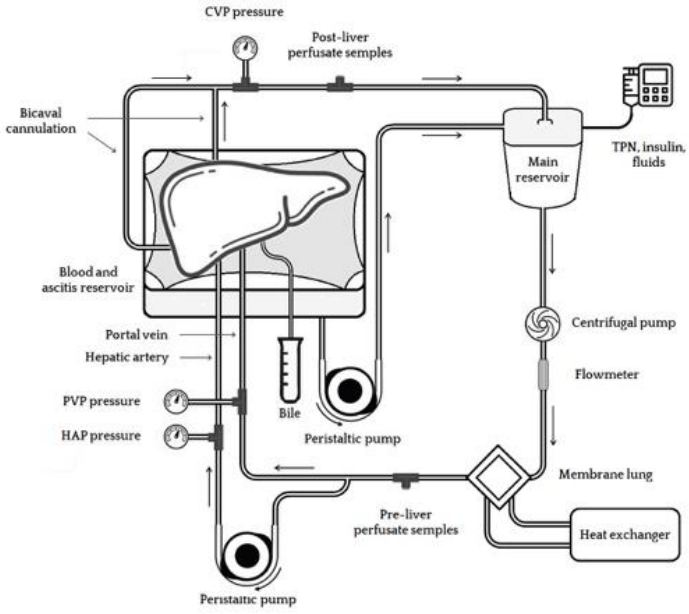

Fig. 1: Schematic representation of normothermic machine perfusion system used in our experiments
Close circuit with cannulated inferior and superior vena cava and portal/hepatic artery perfusion.

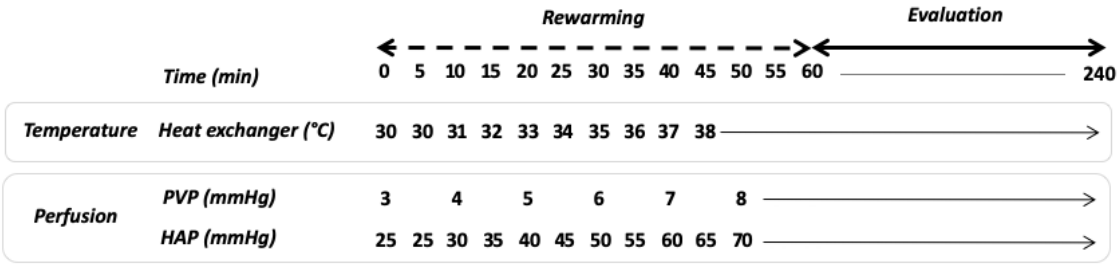

Fig. 2: Schematic overview of normothermic machine perfusion protocol
PVP, portal vein pressure; HAP, hepatic artery pressure.

3.2 *Ex vivo* dynamic perfusion

Perfusate composition

The liver perfusion system consisted of a cellular perfusion fluid as described by Op Den Dries et al. (2013). The solution was freshly prepared in sterile conditions (Tab. S2[1]). Total perfusate volume was 2370 ± 340 ml with a hemoglobin concentration of 7.6 ± 1.4 g/dl. To prevent bacterial contamination, the perfusate was added with Cefrtiaxone 2 g and metronidazole 500 mg. No corticosteroids or other anti-inflammatory drugs were used.

Liver perfusion system

The liver graft was placed onto an organ chamber modified to let the dorsal liver lay on a modelled ad hoc, perforated surface. The portal vein and the hepatic artery were connected to the circuit, together with the superior and inferior vena cava (close circuit). Perfusate oxygenation was guaranteed by a membrane lung. Gas flow was titrated to keep a normal pH, starting with a sweep gas to blood flow ratio of 1:2 to avoid hypocapnic alkalosis during the initial warming period due to low tissue CO2 production. Sweep gas FiO_2 was titrated to keep an arterial saturation (SaO_2) above 92% and avoid hyperoxia. In most of the cases 21% FiO_2 was sufficient. Only during 3 NMP an increase in FiO_2 was required. The chamber was closed with a PVC lid to maintain humidity and temperature. The organ temperature was recorded with a probe placed between liver lobes. In some preliminary experiments a core-graft temperature was measured and it was found equal to the one observed within liver lobes.

In order to obtain a liver temperature of 38℃, the heat-exchanger connected to the membrane lung had to be set at 40℃. A correct priming of the perfusion system is essential to prevent air embolism. To recirculate ascites and perfusate leakage, a further reservoir was placed under the perforated surface and connected to a roller pump. The drained fluids were pumped directly inside the main reservoir. The NMP system is showed in Figure 1.

Liver perfusion protocol

After a static cold storage of 235 ± 47 min, the liver graft was connected to the circuit when a pH of 7.3 was reached through electrolyte adjustment (i.e. $NaHCO_3$ infusion). The overall duration of liver perfusion was 353 ± 138 min and it included 2 steps (Fig. 2). The first 60 min were called "rewarming" and consisted of a progressive increase in graft temperature, pressure and flow. Initial PVP was 3 mmHg, HAP 25 mmHg and heat exchanger temperature 30°C. Pressures and temperature were raised in 6 steps during the 60 min of rewarming to reach target values. No electrolytes or pH adjustments were done during this phase. The following phase was called "evaluation". It started when all parameters reached the 100% of the selected values and ended with the final graft evaluation at 240 min. During this phase, Clinimix N14G30 100 ml/h and insulin 100 UI/h were continuously infused through the portal vein. Portal vein and hepatic artery resistances and flow are showed in Figure 3.

3.3 Graft evaluation

According to Mergental criteria (Mergental et al., 2016), after 240 min NMP 3 (43%) grafts were classified as nLT-G and 4 (67%) as LT-G. The first 3 grafts of our series were all classified as nLT-G (Tab. 1).

Tab. 1: Classification of the seven perfused grafts according to Mergental et al. (2016) criteria

	Liver 1	**Liver 2**	**Liver 3**	**Liver 4**	**Liver 5**	**Liver 6**	**Liver 7**
Major criteria							
Lactate < 2.5 mmol/L	no	no	no	yes	yes	no	yes
Bile production	no	no	no	yes	yes	yes	yes
Score	0/2	0/2	0/2	2/2	2/2	1/2	2/2
Minor criteria							
pH>7.3	yes	yes	no	yes	yes	no	yes
HA flow > 150 mL	yes	yes	yes	yes	yes	yes	no
PV flow > 500 mL	yes	yes	yes	yes	yes	yes	yes
homogenous perfusion	no	yes	no	yes	yes	yes	yes
Score	3/4	4/4	2/4	4/4	4/4	3/4	3/4
Classification	**nLT**	**nLT**	**nLT**	**LT**	**LT**	**LT**	**LT**

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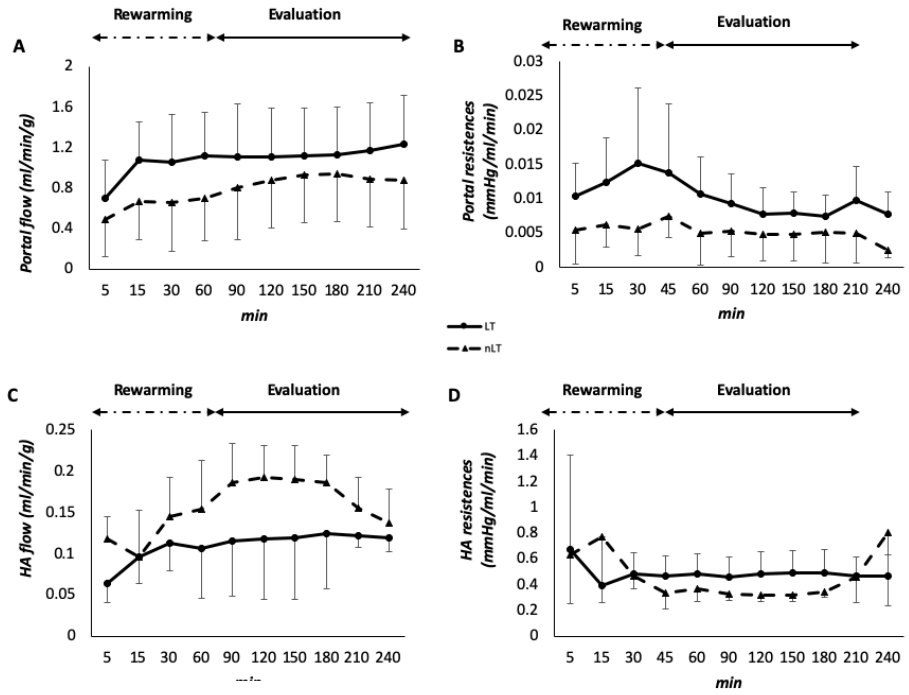

Fig. 3: Portal vein flow (A) and resistances (B) and hepatic artery flow (C) and resistances (D) during normothermic machine perfusion
HA, hepatic artery. LT, n=4; nLT, n=3. Data are expressed as mean ± SEM.

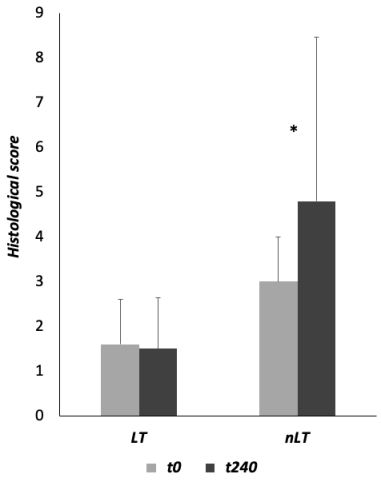

Fig. 4: Histologic scoring of hemorrhage and necrosis of LT-G and nLT-G
Histologic findings were evaluated at the indicated time points in the experimental groups. The severity of these changes was evaluated by the scoring system validated by Brockman et al. LT, n=4; nLT, n=3. Data are expressed as mean ± SEM (*, p=0.042).

Histology after NMP

At the end of the cold storage, histologic evaluation of hepatocyte and sinusoidal integrity was grossly normal without signs of inflammation, steatosis or fibrosis. Conversely, during NMP, a progressive deterioration of hepatocellular and sinusoidal architecture was observed in nLT-G (p=0.042), while only little changes were showed in LT-G (Fig. 4). Although a clear inflammatory response arose during perfusion in both groups, the diffused hepatocellular necrosis of nLT-G resulted in a marked neutrophil recruitment or proliferation. Similarly, biopsies from nLT-G showed diffuse small droplet steatosis deposition (from 10% to 50%). These observations could be considered as a para-physiological attempt of liver grafts to deal with hepatocyte and sinusoidal damage. Indeed, microvescicular steatosis was probably the result of a deficient liver metabolisms and immune cell infiltrate was likely secondary to increased chemotactic signals.

As hepatocyte injury could lead to graft swelling, W/D ratio was assessed. Even if W/D ratio in nLT-G tended to be higher at the end of evaluation phase, the difference was not statistically significant (LT-G 3.013 ± 0.126 vs nLT-G 3.221 ± 0.181, p=0.069). The W/D ratio in the 7 control livers was 2.975 ±. 0.143, significantly lower than in nLT-G (p=0.038).

Ex-situ metabolic evaluation of liver grafts

The main biomarkers measured during NMP are shown in Table 2.

During the rewarming phase (from 0 to 60 min), glucose concentration in the perfusate increased in both LT-G and nLT-G (p=0.001). Glucose release ratio was -0.54 ± 0.18 in LT-G and 0.08 ± 0.11 in nLT-G (p=0.03) (Fig. 5B). Lactate concentration, measured in the cold flush at the end of back table, was 4.3 ± 2.6 mmol/L in LT-G and 4.2 ± 4.1 mmol/L in nLT-G; there was no significant difference between the study groups (p=0.626). Furthermore, lactate in the perfusion fluid before liver graft connection to NMP were 8.9 ± 2.7 mmol/L in LT-G and 3.7 ± 0.5 in nLT-G (p=0.061). Even if LT-G lactate level at the beginning

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Tab. 2: Main characteristics of normothermic machine perfusion during rewarming and evaluation phase
* W/D was compared to native liver ('2.975 ± 0.143, p=0.038). LT-G, transplantable grafts; nLT-G, non-transplantable grafts; n/a, not appliable.

		LT-G (n=4)	nLT-G (n=3)	P (graft)	P (time)
Rewarming phase (0-60 min)					
Glucose, mg/dL	t0	338 ± 183	86 ± 21	0.057	0.001
	t60	516 ± 226	94 ± 25		
Glucose release ratio		-0.35 ± 0.16	-0.08 ± 0.11	0.030	n/a
Lactate, mmol/L	t0	7.55 ± 3.88	4.73 ± 1.19	0.224	<0.001
	t60	3.0 ± 1.3	5.5 ± 3.5		
Lactate release ratio		0.55 ± 0.26	-0.17 ± 0.11	0.022	n/a
Potassium mEq/L	t0	6.75± 4.15	11.30 ± 0.92	0.020	<0.001
	t60	4.5 ± 3.2	13.1 ± 2.7		
Potassium release ratio		-0.46 ± 0.22	0.17 ± 0.64	0.047	n/a
Evaluation phase (60-240 min)					
AST, U/L/g	t60	1.074 ± 1.075	12.008 ± 2.759	< 0.001	< 0.001
	t240	1.141 ± 0.417	22.431 ± 5.118		
LDH, U/L/g	t60	1.301 ± 0.777	9.871 ± 0.395	< 0.001	< 0.001
	t240	2.961 ± 0.962	13.689 ± 1.555		
ALP, U/L/g	t60	0.071 ± 0.032	0.117 ± 0.033	0.001	< 0.001
	t240	0.055 ± 0.061	0.326 ± 0.143		
Bile production, n (%)		3	1	0.237	
Lactate, mmol/L	t60	3.0 ± 1.3	5.5 ± 3.5		
	t240	2.38 ± 0.62	16 ± 2.9	0.024	< 0.001
Lactate release ratio		0.550 ± 0.257	-2.928 ± 0.774	<0.001	n/a
Glucose, mg/dL	t60	516 ± 226	94 ± 25		
	t240	367 ± 243	155 ± 135	0.017	0.001
Glucose release ratio		0.332 ± 0.128	-0.199 ± 0.184	0.031	n/a
i-Ca, mmol/L	t60	0.175 ± 0.115	0.245 ± 0.121	0.147	0.034
	t240	0.328 ± 0.154	0.303 ± 0.257		
i-Ca ratio		1.335 ± 0.582	0.102 ± 0.230	0.010	
W/D ratio*	t240	3.013 ± 0.126	3.221 ± 0.181	0.069	n/a

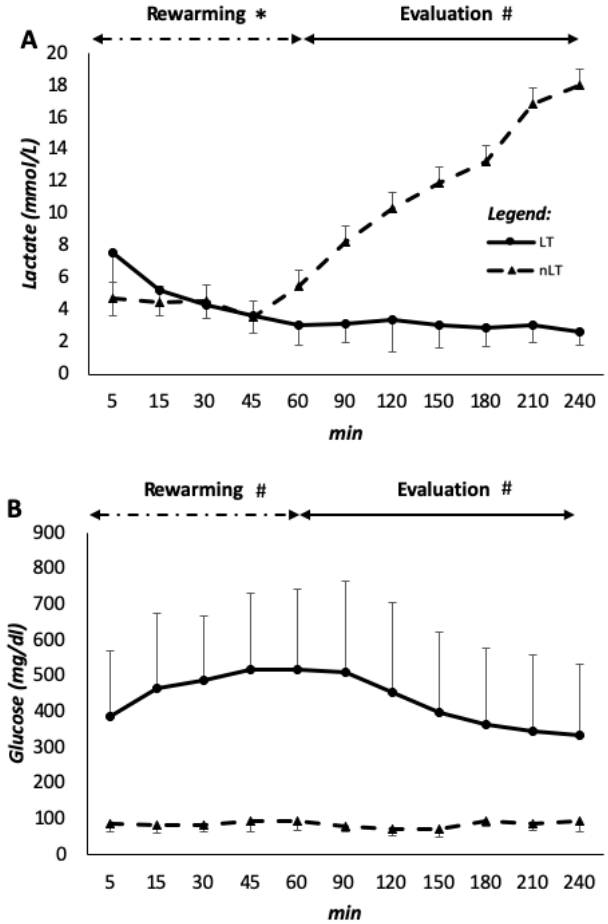

Fig. 5: Lactate *(A)* and glucose *(B)* concentrations in the two study groups during rewarming and evaluation phase of normothermic machine perfusion
LT, n=4; nLT, n=3. Data are expressed as mean ± SEM (*, p=0.02; #, p<0.001).

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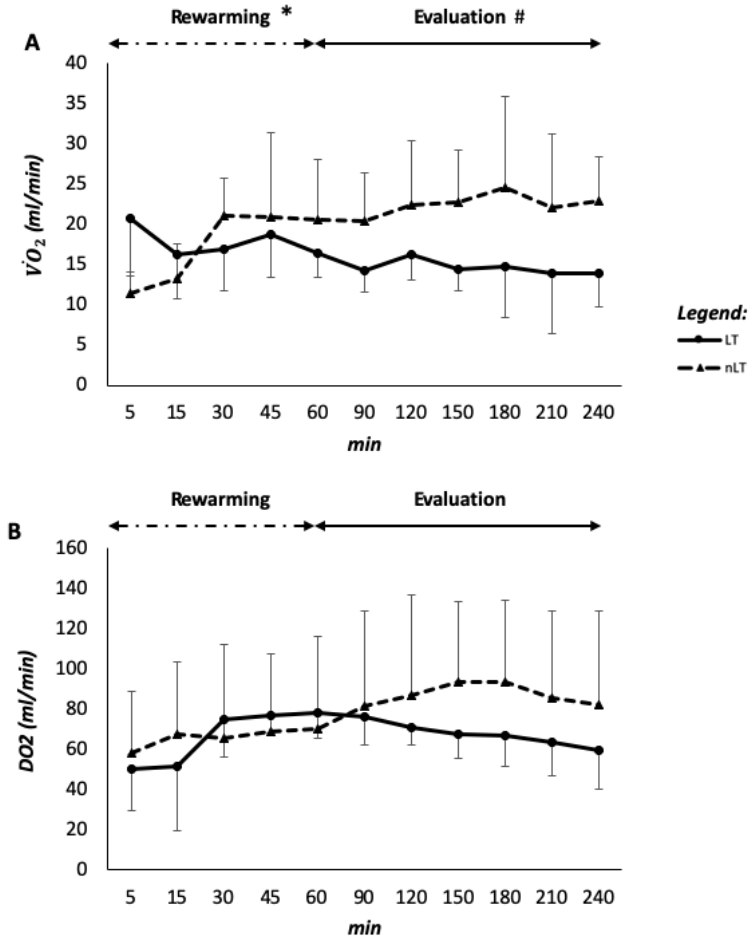

Fig. 6: Oxygen metabolic parameters during normothermic machine perfusion in the two study groups
A, Oxygen consumption ($\dot{V}O_2$) (*, p=0.001 and #, p<0.001). **B**, oxygen delivery (DO_2). LT, n=4; nLT, n=3. Data expressed as mean ± SEM.

of NMP were increased, there was no significant difference in lactate concentration between the two groups (p = 0.224). Conversely, lactate concentration significantly decreased in LT-G, while it was stable in nLT-G (p=0.02) (Fig. 5A). Lactate release ratio was LT-G 0.55 ± 0.26 vs nLT-G -0.17 ± 0.64 (p=0.022). Potassium concentration in the perfusion fluid was consistently higher in nLT-G relative to LT-G (p=0.020). The LT-G had an higher potassium release ratio compared to nLT-G (-0.46 ± 0.22 and 0.17 ± 0.64, respectively LT-G and nLT-G, p= 0.047). Interestingly, potassium and lactate release ratio showed a significant linear correlation (R^2=0.570; p=0.05) (Fig. S1[1]). $\dot{V}O_2$ increased over time in LT-G, while it decreased in nLT after an early burst (p=0.001) (Fig. 6).

During the evaluation phase, baseline AST and LDH levels were higher in nLT-G relative to LT-G (AST: 14962 ± 1076 U/l vs 1305 ± 1192 U/l, p= 0.001; LDH 10170 ± 540 vs 1584 ± 946 U/l, p<0.001). During machine perfusion, the hepatocellular damage (AST and LDH) significantly increased in nLT-G (end-NMP: AST 22315 ± 3560 U/l; LDH 14237 ± 1617 U/l), while it was stable in LT-G (end-NMP: AST 1444 ± 800 U/l; LDH 1674 ± 1707 U/L) (p<0.001) (Fig. S2[1]). Consistenly, NMP preserved cholangiocyte function in LT-G, while a progressive bile duct injury was observed in LT-G (p=0.001) (Fig. S3[1]). Bile production was almost absent in the 3 nLT-G and in only 1 LT-G. The mean bile volume/h was 8 ± 5 ml/h. During "evaluation" lactate remained stable in LT-G (t60 min 3.1 ± 1.3 mmol/L; t240 min 2.7 ± 0.8 mmol/L), whereas they increased in LT-G to more than 300% (t60 min 5.4 ± 3.5 mmol/L; t240 min 18.0 ± 2.0) (p<0.001) (Fig. 5A). Lactate release ratio was 0.073 ± 0.310 in LT-G vs -3.749 ± 3.811 in nLT-G (p<0.001). Glucose concentration decreased in LT, while it remained stable in nLT-G (p=0.001) (Fig. 5B). Glucose release ratio was 0.384 ± 0.108 in LT-G vs 0.048 ± 0.429 in nLT-G. A stable i-Ca was observed in nLT-G, whereas it increased in LT-G groups (p=0.005) (i-Ca release ratio: LT-G -1.355 ± 0.582 vs nLT-G -0.107 ± 0.231, p=0.018). Hemoglobin concentration decreased to 2.3 ± 0.9 g/dL in LT-G, while it remained stable in LT-G (0.6 ± 0.9 g/dl) (p=0.009) (Fig. S4[1]). $\dot{V}O_2$ decreased in nLT-G, while it increased in LT-G (p<0.001). Transported oxygen (DO_2) was not different in the two groups (p=0.764) (Fig. 6). In the four LT-G, 25 ± 3 mg of ICG were infused. R15 was 5.4 ± 1.1% in the perfusate and bile concentration of ICG was 131 ± 18% at 15 min and 2049 ± 188% at 30 min (Fig. 7).

4 Discussion

The present research shows that organs retrieved from a slaughterhouse can be used to implement a model of liver DCD donation. Procurement and ex-situ perfusion procedures were optimized and accurately described in order to achieve a NMP model that resembles the clinical scenario. The results should provide significant information to develop an effective preclinical research complying with the 3R ethical issues.

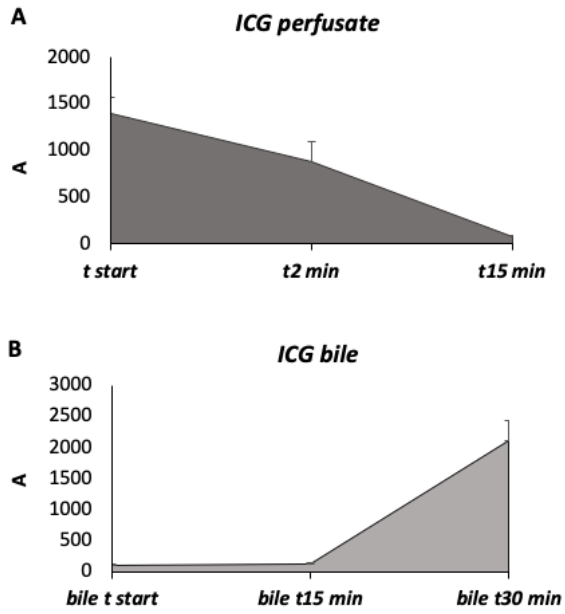

Fig. 7: Indocyanine green dye (ICG) clearance in perfusate *(A)* and secretion in bile *(B)* during normothermic machine perfusion in LT-G
Bile secretion was evaluated only in the 3 of 4 LT grafts that produced bile.

DCD donors are considered a valuable potential resource to increase the number of organs suitable for transplantation (Manyalich et al., 2018). However, the use of liver grafts derived from these donors is burdened by sub-optimal post-LT outcome (Foley et al., 2011). Although several detrimental factors contributing to this end result have been identified (e.g. WIT), the underlying biomolecular mechanisms are still largely unknown. Undoubtedly, these detrimental factors need to be extensively investigated and repaired through committed research.

Recently introduced evaluation and reconditioning strategies demonstrated their capacity to revert the detrimental impact of WIT and IRI (Dutkowski et al., 2015; Hessheimer and Fondevila, 2017). In our experience, machine perfusion was identified as a valuable technology to recondition ECD and DCD liver grafts (Dondossola et al., 2018). Machine perfusion is an emerging approach with technical and biological aspects that are presently explored, adapted, and improved. However, reactions elicited during ex-situ dynamic perfusion and identification of criteria to establish liver graft viability are still ambiguous and their definition appears mandatory (Dutkowski et al., 2019). To this purpose, based on low cost, reproducibility and better understanding of subcellular events rat models have been widely used. However, these models provide observations that have a low translational impact requiring further experiments prior to a reliable clinical application (Bassani et al., 2016). Conversely, the translational potential of pig models is definitely greater. However, pig models have high costs in terms of life and economy and non-experimental animals should be rather used (Daniel et al., 2018; Chung et al., 2019). Unfortunately, among the many studies on DCD and machine perfusion published over the last 20 years, only few used non-dedicated experimental animals (Grosse-Siestrup et al., 2001, 2002a,b). This limitation probably depends on incomplete standardized procurement and perfusion protocols of organs derived from slaughterhouse animals.

Although the use of commercial animals to study specific biomolecular phenomena is still problematic, they can be exploited to refine physiological and technical aspects in accordance to 3R principles. Furthermore, in our setting, the cost connected to a slaughterhouse procurement are lower than a model based on dedicated animals. For this reason, we focused our research on the optimization of all the steps required to standardize a slaughterhouse NMP model. The detailed description of our procurement and perfusion procedures can help researchers unfamiliar with the 3R-rule and contribute to stimulate awareness on this topic with a substantial impact on live animals.

The porcine liver ex-situ perfusion model described in this paper was initially based on the technique described by Grosse-Siestrup (2001). However, the first four grafts retrieved according to that procedure and perfused with a clinical-fashioned circuit resulted in non-viable organs. Consequently, a technical refinement of critical steps (organ procurement, blood collection, and perfusion system) was pursued. A peculiar attention was paid to organ cooling. Indeed, an excessively rapid graft cooling induced an increase in vascular resistances, causing impaired organ perfusion (Tashiro et al., 2014). After protocol refinement, the temperature was progressively decreased and led to an homogenous cold graft perfusion, adequate blood wash-out enabled a reliable and efficient ex-situ normothermic perfusion.

In nLT-G grafts, ischemic areas during NMP represented a major problem that led to a histological progression of liver damage. Conversely, no ischemic areas were observed in LT-G, perfusion parameters improved and our results are comparable to those obtained in other preclinical research based on experimental animals and in clinical series (Ravikumar et al., 2016; Banan et al., 2015). We used the criteria proposed by Mergental et al. (2016) to evaluate graft viability at the end of NMP. The differences in weight and vascular flow between human and pigs could affect our evaluation. Different articles analysed the in-vivo pig splanchnic flow (Iozzo et al., 2007; Slimani et al., 2008; Winterdahl et al., 2011). According to the reported data and our mean graft weight, the estimated in-vivo mean portal flow ranges from 1100 to 1500 ml/min and hepatic artery flow from 150 to 250 ml/min. These values are fully comparable to the human ones and the resulting graft classification showed full concordance with histopathological evaluation.

The Cambridge group demonstrated a significant correlation between transaminase concentration in the perfusion fluid and post-LT graft function (Watson et al., 2017). Consistently, in the present study nLT-G (categorized according to Mergental et al. (2016)) showed higher baseline AST and LDH levels that markedly increased during NMP. These changes matched the pronounced

deterioration of hepatocytes shown by histopathological analysis. Therefore, a concordance between Mergental criteria and Cambridge observations could be determined for the first time. Further, the progressive increase in hepatocellular necrosis markers during NMP is a well-known phenomenon(Verhoeven et al., 2014) that underlines the need for novel strategies addressed to limit the ex-situ reperfusion damage, especially in severely damaged grafts (Boteon, 2019; de Vries et al., 2019).

Although the reconditioning and preservation capacity of NMP was the focus of relevant publications, the metabolic profile of liver grafts during NMP was insufficiently investigated. In particular, the role of the rewarming phase in understanding perfusion results was clearly underestimated. To mend this limitation, an accurate metabolic evaluation of NMP rewarming phase is provided in our study. The oxygen debt, accumulated during ischemia, is restored during reperfusion and analysis of O_2 metabolism could help comprehension of graft viability and evaluation of ischemic damage. Interestingly, in in-vivo studies on acute liver failure, the $\dot{V}O_2$ was directly related to the remnant liver function (Clemmesen et al., 1999; Hart et al., 2003). A low $\dot{V}O_2$ could be seen as a marker of cell necrosis or dysfunction causing increased lactate levels, inadequate glucose metabolism and increased potassium concentration, likely due to cell death and Na-k ATP-dependent pump inactivation. Conversely, LT-G showed higher $\dot{V}O_2$, adequate lactate metabolism, active glucose and potassium absorption. These data suggest that the extent of oxygen debt during warm/cold ischemia exceeds the nLT-G liver graft ability to adequately repair it and to sustain the simultaneous metabolic demand of ex-situ rewarming (Bjerkvig et al., 2016). The consequent microcirculation damage and mitochondrial dysfunction leads to cell necrosis and liver graft non-transplantability, as demonstrated by hepatocellular necrosis and lactate accumulation in nLT-G. The rewarming metabolic parameters in our research (glucose, lactate and potassium release ratio) are in agreement with the evaluation criteria provided by Mergental et al. (2016) (Tab. 3). This observation deserves peculiar emphasis as an early graft evaluation procedure could have considerable clinical and practical implications. Furthermore, in accordance with our recent publication on a NMP rat model (Dondossola et al., 2019), these metabolic parameters, together with citrate clearance, easily measured with i-Ca, and ICG clearance, a well-known in-vivo liver function test, could be a parameter to precisely evaluate graft function and transplantability.

Tab. 3: Main metabolic characteristics of transplantable (LT-G) and non-transplantable (nLT-G) during rewarming (Rew.) and evaluation (Ev.) phase
n/a, not appliable.

	Phase	**Viability criteria**				
		Glucose	***Lactate***	***Potassium***	***i-Ca***	***VO2***
LT-G	*Rew. (0-60min)*	released	uptake	uptake	n/a	increased
	Ev. (60-240 min)	uptake	uptake	n/a	increase	increased
nLT-G	*Rew. (0-60min)*	stable	stable	released	n/a	decreased
	Ev. (60-240 min)	released	released	n/a	stable	decreased

We are aware of issues potentially problematic in our model. First of all, an extended WIT could be common. Indeed, a very short WIT requires a well programmed synergy with slaughterhouse technicians to reduce the interval between sacrifice and organ procurement. Further, we observed a substantial hemolysis in nLT-G that remains unexplained. Of interest, hemolysis was a clinically relevant issue in NMP series (Watson et al., 2017). Lactate levels in the perfusate before NMP graft connection tended to be higher in LT-G. This is probably due to blood composition and collection procedures that cannot be controlled. However, it should be considered that similar changes occur in clinical practice as well and they do not appear to affect graft quality.

In conclusion, the present model with a prolonged no-flow period (WIT > 20 min) shows that slaughterhouse organs could be exploited to study and recondition liver grafts after cardiocirculatory arrest. Indeed, liver grafts obtained from animals with peri-mortem events that resemble DCD donation, demonstrated full viability at the end of the NMP evaluation. The optimized ex-situ perfusion system resembles the physiological in-vivo response to ischemia-reperfusion injury and could be used to test graft viability as well as liver physiology/metabolism. These data represent a substantial advance in translational research that promotes a reduction of dedicated experimental animals.

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Conflict of interest

Authors have no conflicts of interest to declare.

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