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# Development of a Large Animal Model of Ischemia-free Liver Transplantation in Pigs

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**Background.** In organ transplantation, ischemia, and reperfusion injury (IRI) is considered as an inevitable event and the major contributor to graft failure. Ischemia-free liver transplantation (IFLT) is a novel transplant procedure that can prevent IRI and provide better transplant outcomes. However, a large animal model of IFLT has not been reported. Therefore, we develop a new, reproducible, and stable model of IFLT in pigs for investigating mechanisms of IFLT in IRI. **Methods.** Ten pigs were subjected to IFLT or conventional liver transplantation (CLT). Donor livers in IFLT underwent 6-h continuous normothermic machine perfusion (NMP) throughout graft procurement, preservation, and implantation, whereas livers in CLT were subjected to 6-h cold storage before implantation. The early reperfusion injury was compared between the 2 groups. **Results.** Continuous bile production, low lactate, and liver enzyme levels were observed during NMP in IFLT. All animals survived after liver transplantation. The posttransplant graft function was improved with IFLT when compared with CLT. Minimal histologic changes, fewer apoptotic hepatocytes, less sinusoidal endothelial cell injury, and proinflammatory cytokine (interleukin [IL]-1β, IL-6, and tumor necrosis factor-α) release after graft revascularization were documented in the IFLT group versus the CLT group. **Conclusions.** We report that the concept of IFLT is achievable in pigs. This innovation provides a potential strategy to investigate the mechanisms of IRI and provide better transplant outcomes for clinical practice.

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Ischemia and reperfusion injury (IRI) denotes the exacerbated injury of organs after the restoration of organ oxygen following initial ischemia.[1] IRI is a profound proinflammatory process initiated by the innate immune system, involving neutrophil and macrophage infiltration, which can lead to graft loss because of primary nonfunction (PNF) or allograft rejection.[2] Therefore, IRI is considered a major limiting factor of graft survival. For decades, attempts have been made to ameliorate adverse consequences of IRI, including application of ischemia preconditioning, protective gases, and stem cell or gene therapy.[3] However, few methods were translated into clinical practice. Notably, none of the reported methods was able to prevent IRI because the initial ischemia could not be avoided.

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*X.H., Z.G., Q.Z., and Y.T. contributed to the conceptualization and trial design. X.H., Z.G., Q.Z., Y.T., J.L., and T.W. managed the samples and participated in performance of relevant experiments. Y.T. J.L., T.W., Z.Z., S.H., Z.Z., and L.W. contributed to data collection. X.H., Z.G., Q.Z., Y.T., J.L., and T.W. analyzed and interpreted the data. Y.T., J.L., and T.W. drafted the article, which was critically reviewed and revised by X.H., Z.G., and Q.Z. All authors reviewed and approved the final article.*

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In the last decade, great progress in ex vivo machine perfusion of organs has been made. In particular, normothermic machine perfusion (NMP) is able to provide oxygen and nutrient supply for procured organs, which has been shown to be advantageous over static cold storage (SCS) by ameliorating IRI, improving hemodynamics of organs,[4] and repairing grafts[5] with cold and warm ischemia injury.[6,7] It has been shown that ideal liver preservation happens when a liver is connected to the NMP device immediately upon procurement. Even under these circumstances, a short period of cold ischemia time leads to significant sinusoidal endothelial cells (SECs) dysfunction and Kupffer cell activation in livers.[8] In the current NMP practice, organs still suffer from ischemia and cold storage injury during procurement and preservation before (cold flush, back-table preparation, and/or preservation on ice) and after (cold flush again) NMP.[9-11] Therefore, the organ actually suffers a "double-hit" IRI during the whole procedure.

We hypothesize that IRI is avoidable if we can procure, preserve, and implant liver grafts without stopping the blood and oxygen supply for liver grafts. We named the novel transplant procedure as ischemia-free liver transplantation (IFLT). Based on previous successful establishment of IFLT, we had already conducted a series of clinical studies, which suggested that IFLT could largely alleviate and even entirely prevent IRI.[12] The successful transplantation of an 85%–90% macrovesicular steatotic liver graft was the first report confirming feasibility of the IFLT.[13] We also showed that IFLT could provide a larger benefit for the reduction of post-LT hepatocellular carcinoma recurrence compared with conventional liver transplantation (CLT).[14] In particular, in a recent randomized controlled trial, we demonstrated that IFLT significantly reduced complications related to IRI.[15] Therefore, here we share our experience on large animals of IFLT in pigs for investigating the protective mechanisms of IFLT in IRI, hepatocellular carcinoma recurrence, immune rejection, and the promotion and potential for application of IFLT at other centers.

In this study, we aimed to develop a new model of IFLT in pigs in a reproducible, stable manner for research purposes; we further compare the transplant outcomes and graft IRI severity in pigs between IFLT and CLT.

## MATERIALS AND METHODS

### Animals

Healthy Wuzhishan miniature pigs, older than 13 mo, were purchased from Guangzhou Feed Research Institute (Guangzhou, China). Animals were housed and fed in accordance with the relevant national and international guidelines. Ten pigs (25–35 kg) served as liver donors, and 10 pigs (25–35 kg) served as liver transplant recipients. Another 5 pigs (50–60 kg) served as blood donors for NMP. They were randomized into the following 2 groups: (1) CLT group (n = 5)—livers were procured using conventional procedure, underwent 6-h SCS, and then implanted, (2) IFLT group (n = 5)—livers were procured, preserved and implanted under continuous 6-h NMP. The study protocol was approved by the ethical committee of animal experiments of The First Affiliated Hospital of Sun Yat-Sen University.

Animals were given access to food and water ad libitum and were restricted from food but not water 12 h before the operation. After slight sedation by intramuscular injection of ketamine, all animals were anesthetized by spontaneous mask ventilation. Once the pharyngotracheal reflex was abolished, tracheal intubation was performed and general anesthesia was prolonged with controlled ventilation. One side of internal jugular venous was catheterized to allow fluid and anesthetic drug administration. Blood pressure was continuously monitored and maintained above 80/50 mm Hg, and vasoactive drugs were used if necessary. Controlled ventilation was maintained throughout the surgical procedure; the fraction of inspired oxygen was maintained above 60%, and $O_2$ saturation on pulse oximetry was maintained above 98%. The operative analgesia was obtained by intravenous remifentanil infusion until the end of surgery. All operations were performed under general anesthesia, and all efforts were made to minimize suffering.

Posttransplant pigs were observed and monitored continuously for 7 d and received parenteral nutrition. Remifentanil was used to relieve pain in pigs during observation after transplantation and was dynamically regulated to achieve pain relief. The main parameters included vital signs, mental states, and laboratory test results of the posttransplant pigs. If predetermined animal suffering criteria were met before the end of the intended survival period, pigs were killed under supervision of our veterinarian staff to avoid animal suffering. Pigs were killed at the end of experiments or if defined humane endpoints were met.

### Blood Collection

After anesthesia, the blood donor pigs were laid on the operating table in a supine position. A midline laparotomy was performed. Following cannulation of abdominal aorta (AA), the animals were exsanguinated. Thousand to thousand two hundred milliliters of blood was collected and fully heparinized for preparing NMP perfusate.

### Liver Procurement

In CLT group, after anesthesia, the pigs were laid on the operating table in a supine position. A midline laparotomy was performed. The AA, portal vein (PV), and inferior vena cava (IVC) were dissected and cannulated after heparin (3 mg/kg IV) was administered. The livers were flushed with 3 L of 4°C kidney preservation solution and 1 L of 4°C University of Wisconsin (UW) solution via the AA and PV cannula. The cannula within the IVC was used for venous outflow. The liver grafts were procured using a standard procedure,[16] and then placed in 4°C UW solution.

In the IFLT group, we used a new technique to procure the donor liver under NMP (Figure 1A). The liver was dissected free from the ligaments and the cystic duct was ligated. The celiac artery (CA) and its branches, IVC, and PV were fully dissected. A 3-cm long segment of IVC from the blood donor was anastomosed end-to-side to the PV of liver donors, serving as an access point for PV cannulation while still permitting native blood flow through the PV. A tube was placed in the common bile duct for bile collection. An 8-Fr arterial cannula was inserted into the gastroduodenal artery or splenic artery without interruption of arterial supply for the liver from CA. A 30-Fr venous cannula was placed in the infrahepatic IVC for outflow and connected to the organ reservoir of liver assist (Organ Assist, Groningen, The Netherlands). A straight 24-Fr cannula connecting to the PV perfusion line

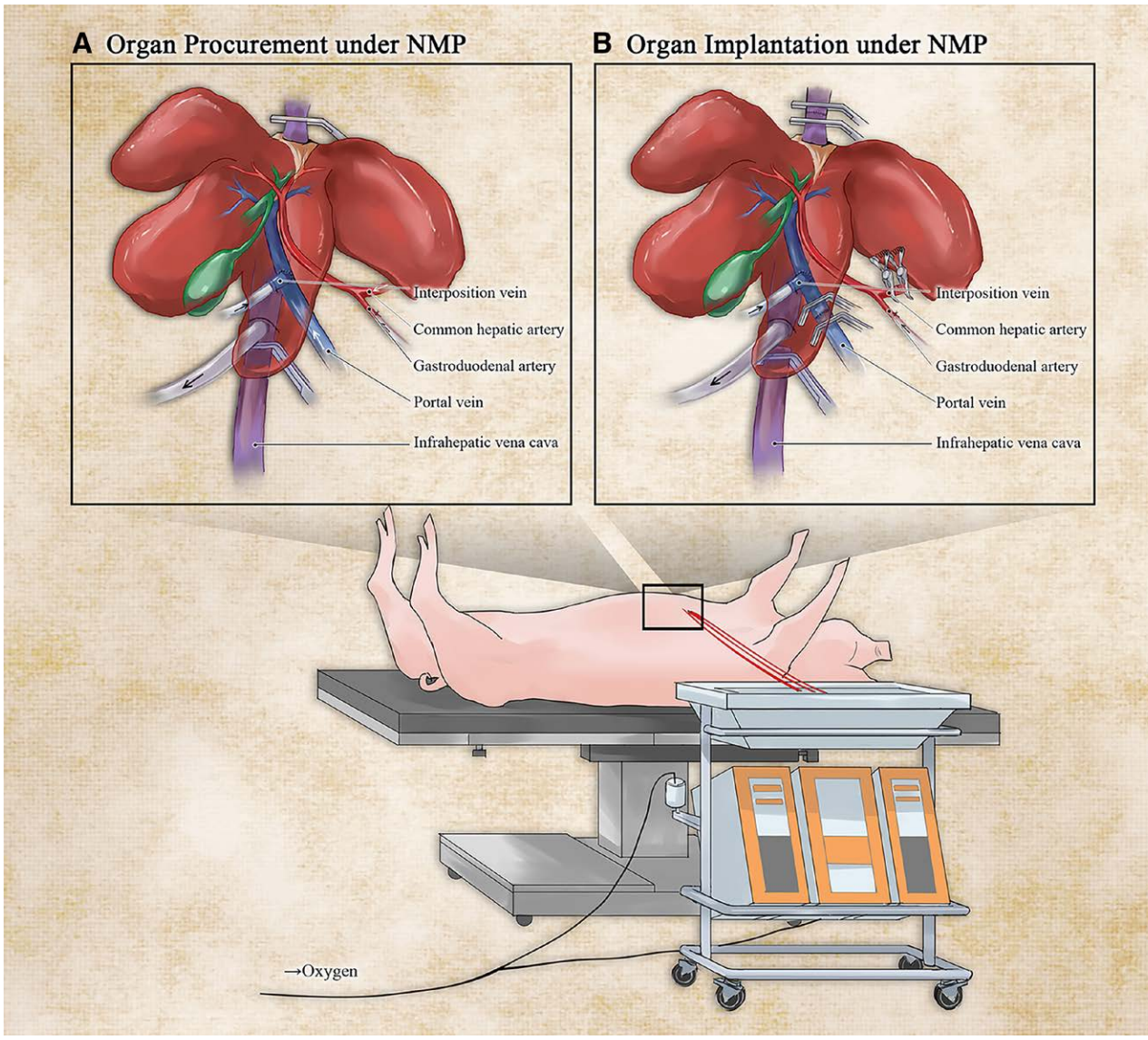


**FIGURE 1.** Ischemia-free liver transplant procedure. A and B, The diagram shows procurement and implantation of the donor liver under normothermic machine perfusion (NMP) using liver assist with cannulation of the infrahepatic vena cava, portal vein, and gastroduodenal artery.

of liver assist was inserted into the PV via the interposition vein. The venous drainage of the suprahepatic IVC to the right atrium was blocked with a vascular clamp. The arterial cannula was then connected to the hepatic artery (HA) perfusion line of liver assist. After the in situ NMP circuit for the livers was established, the livers were procured and moved to the organ reservoir under continuous NMP.

### Ex Vivo Perfusion in the IFLT Group

The cannula within the IVC was removed after the livers were placed in the organ reservoir. The device was primed with approximately 2 L of perfusate. The perfusate contained about 1000 mL whole blood, 800 mL NaCl 0.9% solution, 100 mL polygeline, 50 mL sterile $H_2O$, 40 mL 10% calcium gluconate, 20 mL 5% sodium bicarbonate, 5 mL multivitamins, 5 mL trace elements, 1.5 g cefoperazone sodium and sulbactam sodium, 500 mg methylprednisolone, and 125 000 U heparin. Boluses of sodium bicarbonate were added to maintain the perfusate pH values between 7.3 and 7.5. Heparin was given at a rate of 5000 U/h. A mixture of 20 mL of 10% glucose and 30 mL of amino acid solution was supplemented and infused into the reservoir at 15 mL/min throughout the experiment, to provide nutritional support. The perfusate was warmed up to 39°C, and the oxygenator was supplied with a mixture of 95% oxygen and 5% carbon dioxide at a rate of 200 mL/min, with the flow rate adjusted according to the $Po_2$, $Pco_2$, and pH values. NMP was started once the organs were connected to liver assist, with a set HA pressure of 60 mm Hg, and a set PV pressure of 8–10 mm Hg.

Before NMP and every 30 minutes during NMP, samples from the perfusate were collected for analysis of the blood gas parameters (including $Po_2$, $Pco_2$, pH, and lactate). Perfusate samples were also collected and centrifuged, with the supernatant stored at −80°C for liver function tests, such as aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin (Tbil), gamma-glutamyl transferase (GGT), and alkaline phosphatase (ALP), and lactate dehydrogenase

(LDH) using standard biochemical methods. Bile production was collected every 60 minutes from the biliary draining tube for bile production calculation and detection of $HCO_3^-$ and bilirubin levels of the bile.

### Organ Implantation

In the CLT group, after 6-h SCS, the liver grafts were flushed with 1 L cold 0.9% NaCl solution and implanted into the recipient pig using a bicaval procedure. The anhepatic phase was maintained below 30 minutes, and neither veno-venous bypass nor vasoactive substances were used.

In the IFLT group, before organ implantation, the donor infrahepatic IVC was recannulated and the suprahepatic IVC was blocked. Then the donor liver was moved from the reservoir and placed in situ into the recipient's abdominal cavity. The donor suprahepatic IVC, PV, and HA were anastomosed to the recipient counterparts in an end-to-end fashion using 4–0, 6–0, and 8–0 prolene, respectively. At this stage, caution was taken to prevent twisting of the perfusion line. Thanks to the native branches on the CA and the interposition vein on the PV, all these anastomoses were finished under continuous NMP. After that, the clamps on the PV and HA were released, so that the native dual blood supply for the livers was reestablished. In the meantime, NMP was stopped with removal of the HA and PV cannula, followed by release of the clamp on the suprahepatic IVC. The anhepatic phase was finished. Then the cannula within infrahepatic IVC was removed and around 200 mL of perfusate within the liver was flushed out. The donor infrahepatic IVC was anastomosed to the recipient infrahepatic IVC. The donor common bile duct was end-to-end anastomosed to the recipient common bile duct after withdrawal of the draining tube. Figure 1B shows the vascular cannulations in IFLT.

### Postoperative Management

The administration of intravenous fluids was standard, including 500 mL of Ringer's solution during hepatectomy and after reperfusion, 500 mL of colloid during the anhepatic phase, and 500 mL of 5% glucose and sodium chloride post-transplantation. 500 mL Ringer's solution and 500 mL glucose were used daily posttransplantation. 500 mg methylprednisolone was used intraoperatively, and 250 mg methylprednisolone was used daily posttransplantation to prevent rejection. The recipient was followed intensively for up to 7 d. Blood samples were collected daily posttransplantation for arterial blood gas analysis and liver function tests.

### Histology Studies

Tissue samples for the assessment of hepatocellular injury were collected before procurement, at the end of preservation, and after postrevascularization. All liver samples were fixed in 10% buffered formalin, embedded in paraffin, sectioned (5 mm), and stained with hematoxylin and eosin (HE) for histologic analyses. Injury to the liver parenchyma was evaluated via light microscopy with a semiquantitative scoring system as previously reported.[17] The severity of liver IRI was blindly graded by transplant pathologists.

### vWF Immunohistochemistry and Apoptosis Assay

Formalin-fixed, paraffin-embedded liver tissue slides were deparaffinized using xylene and graded ethyl alcohol and then rinsed in water. Antigen retrieval was performed by boiling the slides in 0.01 M citrate buffer in a microwave oven for 10 minutes and cooling at room temperature. The slides were then incubated with 0.05% Triton-X100 in PBS for 5 minutes, followed by sequential treatment in a humidified chamber after quenching endogenous peroxides with 3% $H_2O_2$ in MeOH. Primary antibody was incubated for 2 h at 37°C after 20 minutes of blocking with serum. Then the slides were incubated with secondary antibody for 30 minutes at room temperature. They were washed in PBS 3 times with 5 minutes of shaking. Followed by DAB coloration, the slides were stained by HE for 3 minutes. The stained slides were then costained with HE and coverslipped. Von Willebrand Factor (vWF) antibody was purchased from Servicebio (GB11020). Apoptosis was detected by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL). In Situ Cell Death Detection Kit (fluorescein) was purchased from Roche (11684817910), and the detection was conducted according to the instructions.

### Quantitative Real-Time PCR

Real-time quantitative Taqman reverse transcriptase polymerase chain reaction (RT-PCR) analysis of interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α was performed to detect inflammation in serial tissue samples. Total RNA from tissues was isolated with TRIzol reagent (Invitrogen) in accordance with the manufacturer's protocol. cDNA synthesis was performed according to standard procedures using primeScript RT Master kit (Takara Bio Inc). Quantitative RT-PCR was performed by SYBR Green quantitative PCR kit (Takara Bio Inc) using the LC480 Real-Time PCR System (Roche). The primers used in the mRNA levels detection are shown in Table S1 (**SDC**, http://links.lww.com/TXD/A629).

### Statistical Analysis

Data analysis was performed using SPSS (v26.0) software (Chicago, IL). Descriptive statistics used for continuous variables were the mean and SD for parametric variables, median, and range for nonparametric variables. Normality assumptions were demonstrated with histograms and the Kolmogorov/Smirnov test. Differences over the period of the study for the other variables analyzed were assessed with the ANOVA test for repeated measures. Two group comparisons of continuous measures were conducted using the t-test or Wilcoxon rank-sum test. $P < 0.05$ was considered statistically significant.

## RESULTS

### NMP and Allograft Viability in the IFLT Group

The livers were well perfused with a consistent pink color, and the textures were soft during NMP (Figure 2A). Both the arterial and portal flow increased during the first hour of NMP and remained stable thereafter, with a mean arterial flow of 152 ± 20 mL/min and a mean portal flow of 630 ± 80 mL/min at the end of NMP (Figure 2B). The NMP device provided adequate $O_2$ and extraction of $CO_2$ from the perfusion fluid (Figure 2C). Overall, the pH value at the beginning of perfusion was slightly acidotic, but it returned to the normal range and remained stable within the first 1.5 h. The lactate levels dropped quickly from 4.4 ± 1.9 mmol/L to normal, reflecting active metabolism by the liver grafts (Figure 2D). Lactate levels increased during the first 1 h may be caused by the application of whole blood that was neither donor nor recipient. The AST

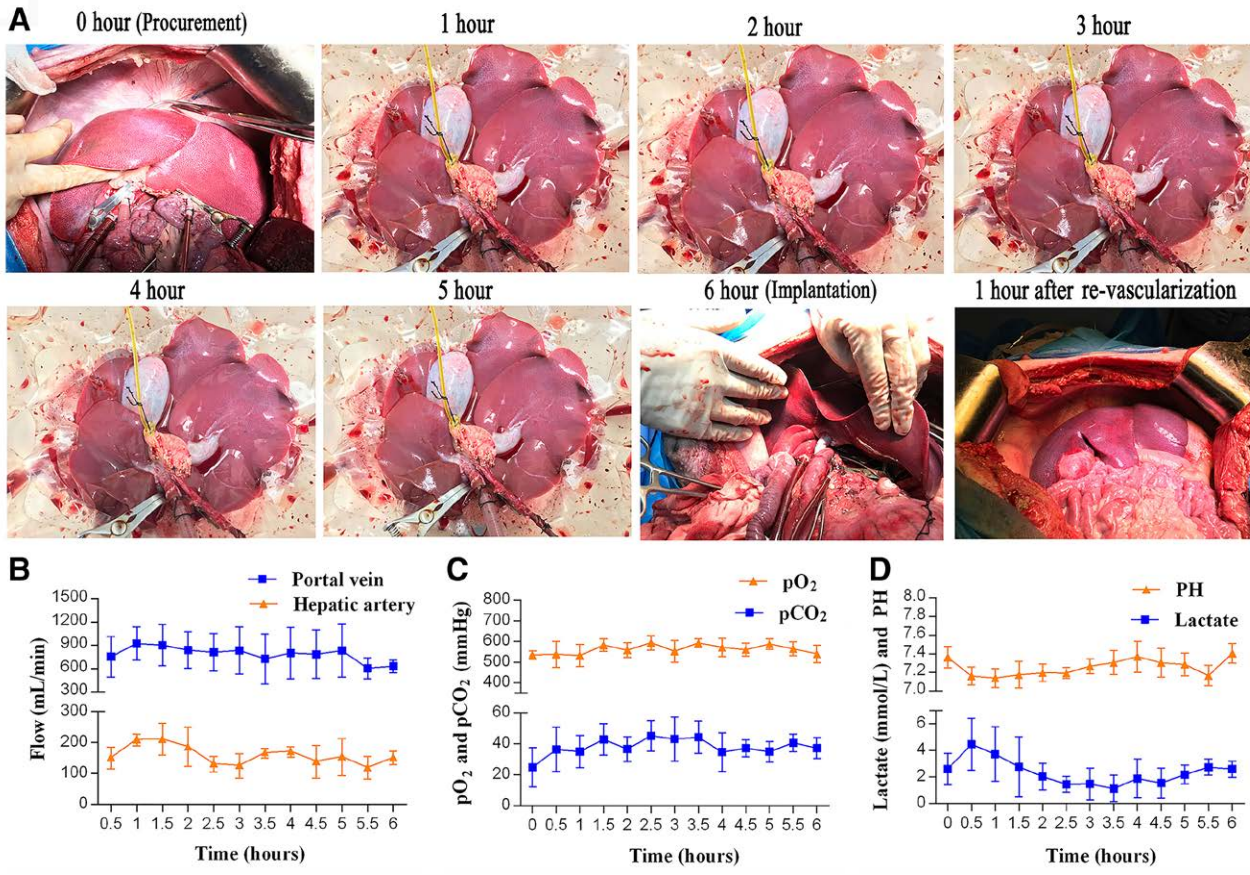


**FIGURE 2.** Normothermic machine perfusion. A, The photos show the liver under ex vivo perfusion. B, The arterial and portal venous flow rates. C and D, The $O_2$ and $CO_2$ tension, pH values, lactate levels in the perfusate.

levels peaked at 124.6 ± 48.9 U/L after 1-h NMP and decreased to 78.8 ± 32.6 U/L at the end of NMP. After a slight rise after 1-h of NMP, the ALT levels remained stably low (Figure 3A). The levels of markers (Tbil, GGT, and ALP) concerning biliary epithelial cell function also remained stable and low (Figure 3B and C). Continuous bile production was observed throughout the whole procedure (Figure 3D), with the peak bile production of 7.5 ± 2.4 mL at 3 h. The pH values of the bile were higher than detected (>8.2). The bicarbonate and Tbil levels indicated good quality of the bile (Figure 3E and F). These results together suggested efficient NMP and excellent organ viability.

## General Information of the Surgery and Posttransplant Liver Function

General operation information of the pigs from the 2 groups is listed in Table S2 (**SDC**, http://links.lww.com/TXD/A629). A significant difference was observed in the donor operation time between the 2 groups ($P = 0.02$), while no significant differences were found in the other variables (all $P > 0.05$).

All the pigs in both the IFLT group and CLT group survived the transplants with functional grafts. The peak ALT (74.8 ± 20.0 versus 376.6 ± 157.1, $P = 0.002$) and AST levels (567.9 ± 282.9 versus 3916.8 ± 2495.6, $P = 0.017$) within the first 7 d posttransplantation in IFLT group were significantly lower than those in the CLT group (Figure 4A and B). The peak Tbil (24.7 ± 25.7 versus 95.0 ± 23.3, $P = 0.001$), ALP (441.9 ± 128.3 versus 942.1 ± 344.5, $P = 0.016$) and LDH levels (1343.9 ± 398.5 versus 3154.0 ± 933.9, $P = 0.004$) within the first 7 d posttransplantation in the IFLT group were also lower than those in the CLT group (Figure 4C and E). In addition, the peak lactate levels (2.1 ± 0.7 versus 5.1 ± 1.7, $P = 0.008$) within the first 7 d in the IFLT group were lower than that in the CLT group (Figure 4F).

## Histologic Analysis of Liver Tissues

HE staining evaluation of biopsies taken from liver tissues before procurement, at the end of NMP, and after revascularization, showed that the livers in the IFLT group were architecturally intact with no obvious sinusoidal congestion, dilatation, or necrosis. In contrast, livers in the CLT group exhibited widespread sinusoidal congestion and dilatation, foci of hepatocellular injury, and extensive erythrocyte extravasation after revascularization. The semiquantitative scores were significantly lower in the IFLT group than in the CLT group both at the end of preservation (1.0 ± 0.32 versus 2.0 ± 0.42, $P = 0.046$) and postrevascularization (1.20 ± 0.38 versus 3.75 ± 0.50, $P = 0.028$) (Figure 5A). The TUNEL assay showed no significant increase in apoptotic hepatocytes at the end of NMP and postrevascularization in the IFLT group, while a significant increase in apoptotic hepatocytes was observed at the end of preservation (4.00 ± 1.00 cells/HPF versus 32.00 ± 2.64 cells/HPF, $P < 0.001$) and postrevascularization (4.00 ± 1.00 cells/HPF versus 70.33 ± 4.04 cells/HPF, $P < 0.001$) in the CLT group (Figure 5B).

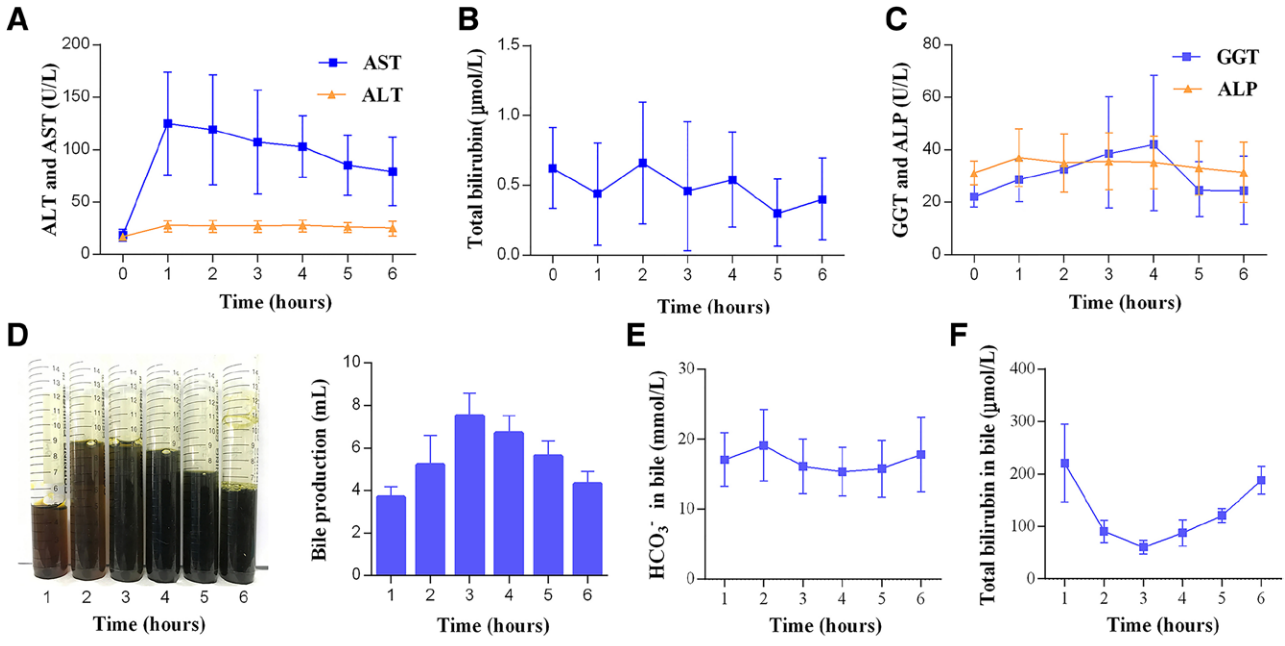


**FIGURE 3.** Assessment of graft function. A, B, and C, The aspartate aminotransferase (AST) and alanine aminotransferase (ALT), total bilirubin (Tbil), gamma-glutamyl transferase (GGT), and alkaline phosphatase (ALP) level in the perfusate. D, E, and F, The bile production, bicarbonate levels, and Tbil of the produced bile.

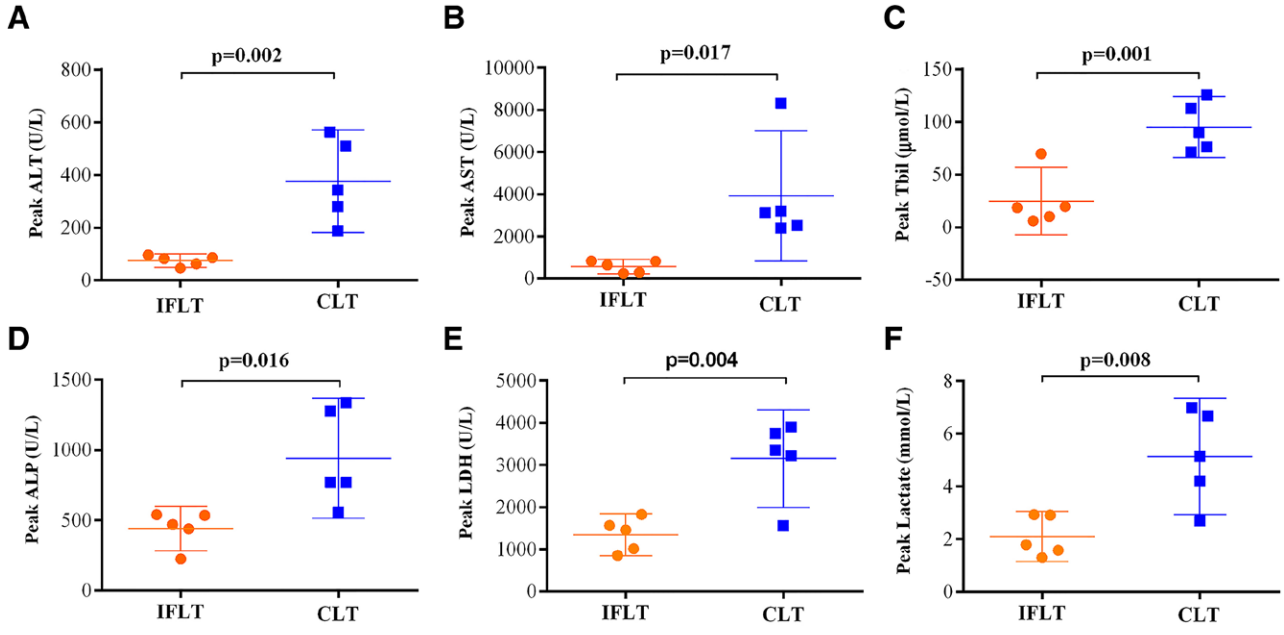


**FIGURE 4.** Comparison of posttransplant liver function within the first 7 d between the ischemia-free liver transplantation (IFLT) and the conventional liver transplantation (CLT) group, including peak alanine aminotransferase (ALT) (A), peak aspartate aminotransferase (AST) (B), peak total bilirubin (Tbil) (C), peak alkaline phosphatase (ALP) (D), peak lactate dehydrogenase (LDH) (E), and peak lactate (F). ALP, alkaline phosphatase.

## SECs Injury and Release of Inflammatory Cytokines After Revascularization

The SECs were well-protected with only slight activation of vWF after revascularization in the IFLT group, while obviously increased activation of vWF at the end of preservation (1.00 ± 0.82 cells/HPF versus 17.00 ± 2.94 cells/HPF, $P < 0.001$) and postrevascularization (7.68 ± 0.82 cells/HPF versus 36.25 ± 2.08 cells/HPF, $P < 0.001$) were documented in the CLT group (Figure 6A). The expression of IL-1b mRNA postrevascularization was significantly higher in the CLT group than in the IFLT group (293.4 ± 163.2 fold change versus 24.0 ± 3.2 fold change, $P < 0.001$) (Figure 6B). Furthermore, we also observed the expression of IL-6 (252.4 ± 29.3 fold change versus 115.3 ± 12.5 fold change, $P < 0.001$) and TNF-α (54.5 ± 49.5 fold change versus 7.5 ± 4.4 fold change, $P < 0.001$) postrevascularization were significantly higher in the CLT group than in the IFLT group (Figure 6C and D).

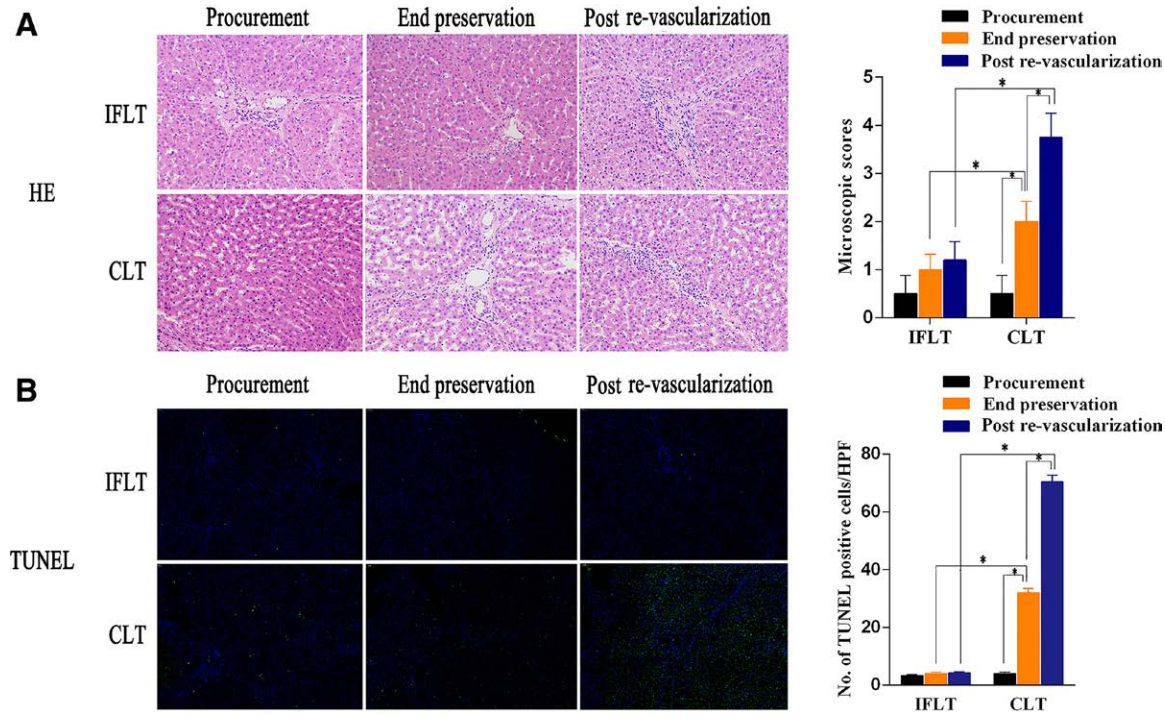


**FIGURE 5.** Histologic analysis of liver tissues. A and B, The hematoxylin and eosin (HE) and TdT-mediated dUTP nick end labeling (TUNEL) staining of donor liver tissue biopsies before procurement, at the end of normothermic perfusion and postreperfusion in the ischemia-free liver transplantation (IFLT) and conventional liver transplantation (CLT). dUTP, deoxyuridine triphosphate; TdT, terminal deoxynucleotidyl transferase.

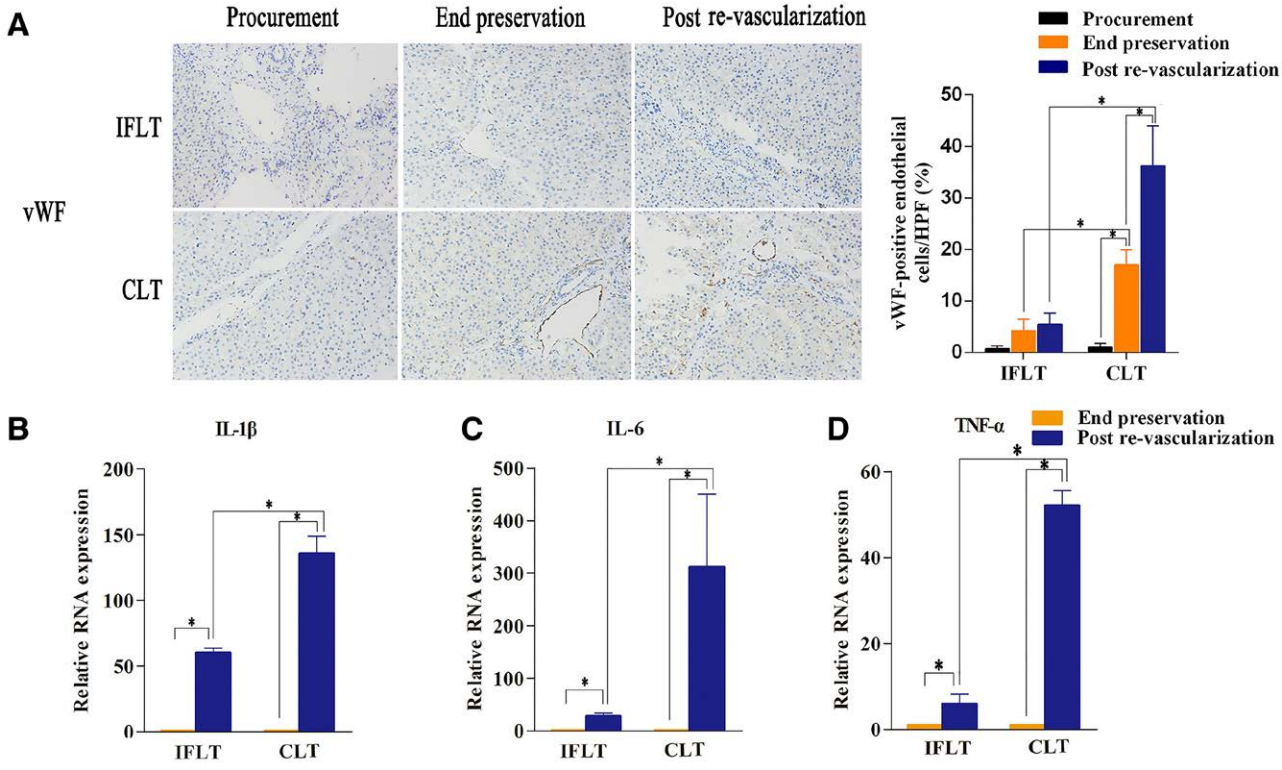


**FIGURE 6.** Sinusoidal endothelial cells injury and release of inflammatory cytokines after revascularization. A, The vWF staining of the donor liver biopsies, at the end of preservation and postrevascularization in the ischemia-free liver transplantation (IFLT) and conventional liver transplantation (CLT). B, C, and D, The inflammatory cytokines (interleukin [IL]-1β, IL-6, and tumor necrosis factor [TNF]-α) levels of the donor livers during end preservation and postrevascularization detected by real-time polymerase chain reaction in IFLT and CLT. vWF, von Willebrand Factor.

## DISCUSSION

During IFLT, we are able to procure, preserve, and implant liver grafts without stoppage of blood supply for the allografts, allowing us to transplant a good quality, live (without ischemia) liver to the recipient and provide better transplant outcomes. There are still few reports on large animal IFLT in pigs and related basic research on the animal model of IFLT. In this study, we successfully establish a reproducible and stable model of IFLT in pigs for IRI research and a series of subsequent studies.

The liver is home to a tightly regulated cytokine network, both under acute and chronic physiologic or pathologic conditions.[18] IRI often causes profound sterile inflammation in reperfused grafts, which could recruit neutrophils and macrophages and produce proinflammatory cytokines and chemokines.[19] Any cytokines might be induced upon IRI. Of the proinflammatory cytokines associated with graft IRI, IL-1β, IL-6, and TNF-α are among the most important.[12,20] IFLT could completely avoid the occurrence of the IRI, which has been shown to significantly reduce activation of the proinflammatory pathway. Therefore, transcription of IL-1β, IL-6, and TNF-α was not increased in the IFLT group, whereas significant increases in the transcription of these cytokines were observed in the CLT group.

In clinical CLT setting, organ injury starts before organ procurement, due to donor warm ischemia in donation after cardiac death (DCD) or during donation after brain death (DBD). Following this initial hit, donor livers undergo cold flush during procurement and subsequent SCS. Previous studies have indicated that the process of transplant-induced IRI is mainly initiated during the cold preservation period due to the depletion of cellular energy sources.[21,22] Cold preservation can lead to activation of Kupffer cells and SECs damage. During implantation, liver grafts are exposed to rewarming ischemia, further aggravating anaerobic metabolism. The subsequent sudden exposure to normothermic blood and oxygen during reperfusion leads to an immediate release of cytotoxic metabolites, which starts an inflammatory cascade that leads to parenchymal and SEC injury.[23] In this study, by using IFLT, all the procedures leading to IRI, including cold flush, cold preservation, and rewarming implantation, are completely avoided. The grafts were continuously functioning during the whole procedure, resulting in no increase in hepatocyte apoptosis, no/low activation of Kupffer cells or SECs damage, and less release of proinflammatory cytokines. Furthermore, the porcine IFLT recipients had a much better posttransplant graft function as compared with the CLT recipients. These data suggest that IRI is largely avoided in IFLT in pigs like in humans,[12] which proves that the IFLT model established in pigs is feasible and can be applied to the relevant research in the future and may achieve clinical translation.

In the modern era of transplantation, IRI still represents an unavoidable event.[24] In the early stage posttransplantation, IRI is considered the major reason for graft failure and patient mortality by inducing PNF and early allograft dysfunction (EAD).[20] Notably, the growing gap between liver supply and demand has forced professionals to use livers from older, fattier, ischemically damaged, or otherwise extended criteria donors (ECD).[25] Such livers are more sensitive to IRI, thus carrying high risk of PNF and EAD. By using IFLT, more marginal or ECD organs can be used safely, thus expanding the donor pool. Therefore, the reason for our center to develop and apply IFLT technique was mainly to consider both donor and recipient, especially if the surgeons suspected that the donor liver was of poor quality, the recipient was frail to withstand the surgical strike, or the patient has serious complications after transplantation. In addition, logistical reasons preventing immediate transplantation are also a reason for using the IFLT technique. Furthermore, based on the results of the randomized controlled trial, we have reason to believe that IFLT could significantly improve the outcomes and reduce complications such as biliary complications of patients undergone LT.[15] Additionally, IRI can also lead to late graft loss by contributing to ischemia-type biliary lesions (ITBL).[20] ITBL is the major reason for late graft loss in liver transplantation. Since hypoxia and ischemia of the liver grafts were completely avoided in IFLT, the biliary epithelium was well-protected, as shown in the production of good-quality bile and almost normal Tbil levels posttransplantation. Although we did not show that in this study, a much lower incidence of ITBL is expected in IFLT. Also, the profound proinflammatory cascade during IRI is one of the key triggers of allograft rejection.[26,27] It is of great importance to test how the lack of IRI would change the characteristics of immunologic rejection in IFLT.

Implementation of the concept of ischemia-free organ transplantation (IFOT) is based on the NMP technique. NMP has the potential to achieve extended-duration liver preservation and recover compromised livers by providing full metabolic support and oxygenation to the liver grafts.[28,29] Hosgood et al[30] have successfully transplanted 18 ECD kidneys after resuscitation under NMP. In addition, some investigations also indicate normothermic donor heart perfusion provides superior preservation of myocardial function and improves transplant outcomes.[31] Similarly, it has been reported that 21 discarded human lungs were recovered and successfully transplanted after 4-h NMP.[32] These studies have fully demonstrated the feasibility and superiority of NMP in almost all solid organs. Therefore, the concept of IFOT is probably applicable in these organ transplant procedures after the introduction of similar surgical innovations reported in this study. Importantly, it is of great interest to know to what extent IFLT is superior to CLT using NMP, although the "double-hit" IRI in CLT using NMP has been reported to diminish the best benefits of NMP.[29]

Undoubtedly, there is still room for improving our IFLT porcine model. In the animal study of NMP, autologous blood of the livers, instead of blood from a third donor which may lead to liver damage, is most frequently used.[7,33] However, to achieve ischemia-free procurement of liver grafts, only blood from a third donor was used in this study. A previous report has shown whole blood from an allogenous donor would result in high incidence of severe vascular resistance and subsequent worse liver function during NMP.[34] We did encounter frequent episodes of severe vascular resistance and high $PaO_2$ during ex vivo NMP. Therefore, the use of whole blood from a third donor might largely explain the imperfect posttransplant liver function in IFLT. It may be necessary to collect enough donor autologous blood before conducting IFLT on pigs which may be more effective. What's more, liver function may further improve when white blood cells depleted blood is used. Besides, the mean arterial pressure of the miniature pigs we used was 90–110 mm Hg. However, the set arterial perfusion pressure was 60 mm Hg in this study, which is in accordance with the previous clinical study using the same device.[35] The

discrepancy of arterial pressure between in vivo and ex vivo settings would probably result in minimal liver injury. In addition, high perfusion flow is equally harmful by activation of the hepatic Kupffer cells,[36] and hyperoxia in the perfusate during NMP may lead to postreperfusion syndrome in humans.[35] Therefore, the optimal perfusion pressure, flow, and $O_2$ tension are yet to be defined in IFLT to achieve the ideal graft function. The composition of perfusate needs to be adjusted to better adapt to machine perfusion of the liver, such as some trace elements and sodium taurocholate. In addition, IFOT might be challenging for DCD donors. A combination of normothermic regional perfusion in situ and IFOT may prove to be feasible in controlled DCD donors and we have verified that combined technique in pigs successfully. What is more, we have improved the IFLT technique to develop the NMP without a recooling technique.[37] Finally, a portable NMP device is required if the donor and recipient are at different locations.

We have established a new, reproducible, and stable model of IFLT in pigs. Although the model is not perfect at this moment, the model of IFLT in pigs will certainly provide a better research platform for basic research such as IRI, and can provide a good direction for transplant patients to optimize the transplant outcomes.

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