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Orthotopic liver transplantation from cardiac death donors in the mouse: a new model and evaluation of cardiac death time

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ABSTRACT

Objective(s): The goal of this research was to develop a mouse orthotopic liver transplantation (LTx) model from donor-after-cardiac-death (DCD) grafts.

Materials and Methods: Mice were randomly assigned to the experimental group or the sham group. The mice in the experimental group were divided into three groups according to the warm ischemia time (WIT) of liver graft: normal LTx, WIT 30 minute (min) +LTx and WIT 45 min +LTx. The descending aorta was clamped using a miniature aortic clamp to simulate cardiac arrest in the DCD grafts. Subsequently, the grafts were orthotopically transplanted into C57BL/6 mice. The 7-day survival rate, serum alanine aminotransferase (ALT), inducible nitric oxide synthase (iNOS), interleukin-6 (IL-6) mRNA level, tumor necrosis factor-alpha (TNF-α) mRNA level, as well as hepatic pathologic alterations were observed.

Results: The 7-day survival rate was markedly lower in the WIT 45 min+LTx group than that in the normal LTx group (25% versus 100%, *P*-value<0.05), with no significant difference between the WIT 30 min +LTx and normal LTx group (75% versus 100%, *P*-value>0.05). Serum ALT level of WIT 45 min+LTx group was markedly higher than that of normal LTx and WIT 30 min+LTx group (*P*-value<0.01). There were significant differences in necrosis and apoptosis among the three groups (*P*-value<0.05). The expression of iNOS, IL-6 mRNA and TNF-α mRNA in WIT 45 min +LTx group all increased significantly compared with the normal LTx and WIT 30 min+LTx group.

Conclusion: The DCD LTx model is feasible in the mouse and would provide many advantages for biomedical research on LTx from DCD grafts.

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Introduction

Currently, the growing imbalance between the demand and the availability of liver donors has become one of the greatest challenges to liver transplantation (LTx) (1, 2). The number of demanded donor livers is 2–3 times higher than the number of obtained liver every year (3). Donation-after-brain-death (DBD) grafts are currently the largest source of liver grafts (4), with donation-after-cardiac-death (DCD) grafts accounting for merely 4.4% or so of the entire donor liver pool in the USA (5). However, cerebrovascular death represents only a small fraction of all-cause mortality, whereas cardiovascular death comprises a majority of all-cause mortality and could widely expand the overall pool of donor organs (4, 6). Therefore, the severe shortage of organs has led to a significant increase in the use of DCD grafts (5, 7). However, the use of DCD liver grafts for transplantation comes with an increased incidence of complications such as ischemic cholangiopathy (IC) and graft failure compared with DBD grafts (8, 9). Moreover, there is a great deal of controversy regarding the outcomes between DBD and DCD liver grafts (10, 11). Therefore, more research on DCD grafts is urgently needed. To address these issues fully and rapidly, several models of LTx from DCD grafts have been established. Although the rapid development of research on LTx from DCD grafts has benefited from experiments in large animals such as pigs (12-14), these models are not widely applicable in the biomedical field. In addition, the fact that rodent models of LTx have acted an extremely important role in the biomedical field, due of their easy popularization (15), cannot be ignored. Numerous studies have investigated LTx with DCD grafts in rats (13), in which the grafts were mostly retrieved after a certain period of warm ischemia

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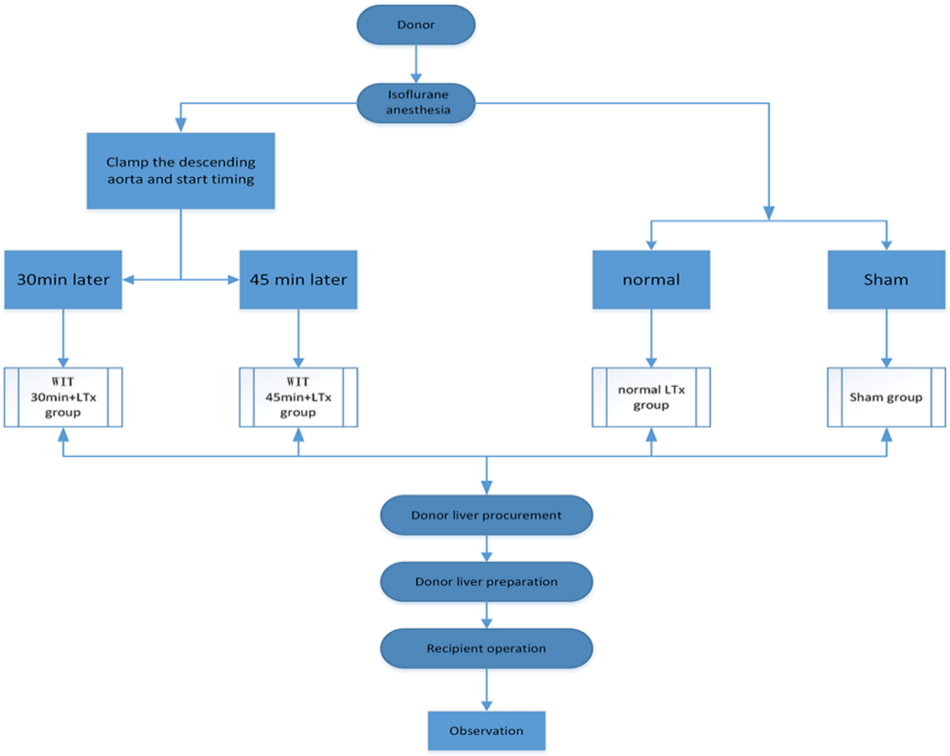

Figure 1. The workflow of liver transplantation in this study

induced by incision of the diaphragm (13, 16-19). The period of cardiac arrest could be approximately 10 min after the incision, which would induce respiratory and subsequent cardiac arrest (16, 17). However, few studies on LTx with DCD grafts have been reported in mice (20). Moreover, many studies have suggested that mouse models are closer to humans compared with rat or other rodent models, owing to the major histocompatibility complex (MHC) and the presence of a gallbladder in mice (21). Therefore, our study aimed to develop a mouse model of LTx from DCD grafts and to estimate the security time of the warm ischemia of DCD grafts.

Materials and Methods

Study design

Male C57BL/6 mice (8-12 weeks, 23-28 g) were assigned to the sham or experimental group randomly. The mice of experimental group were sorted as either donors or recipients. The experimental group were divided into three groups according to the warm ischemia time (WIT) of liver graft : normal LTx (LTx), 30 min warm ischemia *in situ* LTx (WIT 30 min + LTx) and 45 min warm ischemia *in situ* LTx (WIT 45 min + LTx). The workflow of LTx was shown in Figure 1. The weight of the recipient was similar to (no more than 5 g) that of the donor. Before the operation, all the mice were fasted for 12 hr but had free access to water. To study of survival after LTx, mice were monitored for one week. In addition, serum alanine aminotransferase (ALT) levels, hepatic histopathology, apoptosis and necrosis were observed at 6 hr after LTx. In the survival experiment, 8 mice per group were assigned, while 6 mice per group were allocated in all the other experiments.

Surgical procedure

Except for donor liver procurement, the remnant surgical procedures have been described previously (21, 22). The hepatic artery in all groups was reconstructed by stent (23). Isoflurane anesthesia was used during the surgery and all the operations were as far as possible to reduce suffering. To mimic liver donation after cardiac death, heparin (40 IU) in 0.1 ml of lactated Ringer's solution was injected into the inferior vena cava, and the thorax was opened 5 min later under isoflurane anesthesia. In this study, the descending aorta was clamped with a miniature aortic clamp to simulate cardiac arrest for the DCD grafts. The period of warm ischemia *in situ* started when the descending aorta was clamped. Warm ischemia *in situ* was performed for 30 and 45 min in the WIT 30 min and WIT 45 min groups, respectively. Livers were covered and liver temperature was monitored (30 ± 0.75 °C) during the ischemic period. After either 30 or 45 min of warm ischemia *in situ*, the livers were then excised and stored in 0-4°C UW (the University of Wisconsin solution. Subsequently, the liver grafts were orthotopically transplanted into C57BL/6 mice.

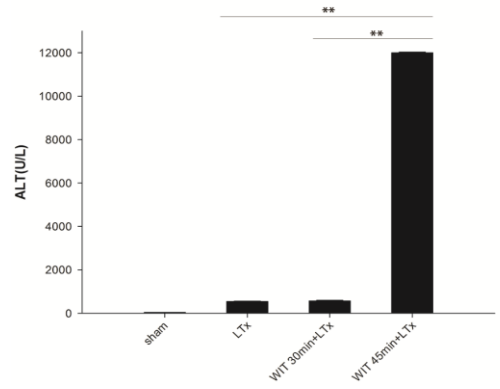

Figure 3. Serum alanine aminotransferase (ALT) level increased significantly after liver transplantation (LTx) in the warm ischemia time (WIT) 45min+LTx group. Blood samples were collected to determine serum ALT level before the mice were sacrificed for histology. The means+SD of six mice each group are given (n=6). ** stands for *P*-value<0.05

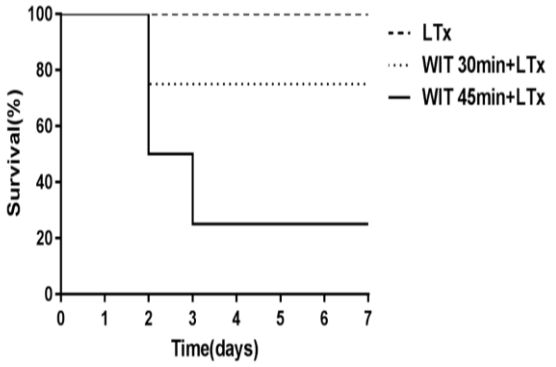

Figure 2. The 7-day survival rate after liver transplantation (LTx). Mice were observed 7 days postoperatively for survival; there were 8 mice in each group(n=8). The 7-day survival rates in each group were 100% (LTx), 75% (WIT 30 min+LTx), and 25% (WIT 45 min+LTx) according to the Kaplan-Meier test (*P*-value<0.05)

Measurement of serum alanine aminotransferase (ALT) levels

Serum ALT levels were measured with analytical kits (Uncoln Park, MI,US) according to themanufacturer's protocols.

Histology and immunohistochemical staining

Liver tissue was collected at 6 hr after transplantation or sham operation. Hematoxylin-eosin (H&E) staining was used to observe the liver histology. Necrotic areas were quantified by image analysis of 10 randomly selected fields in H&E-stained slides using IPlab 3.7v software (BD Biosciences, Rockville, MD) (24). Apoptosis was evaluated by terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) with an *in situ* cell death detection kit as described elsewhere (25).

Detection of TNF-α and IL-6 mRNA levels by quantitative real-time PCR

RNA isolation and cDNA synthesis were performed as described previously. Tumor necrosis factor-α(TNF-α) and interleukin-6(IL-6) mRNA was detected by quantitative real-time PCR, as described elsewhere. mRNA abundance was normalized to hypoxanthine phospho-ribosyl-transferase (HPRT) using the ΔΔCt method.

Western blot for inducible nitric oxide synthase (iNOS)

Liver tissue was collected at 6 hr after transplantation or sham operation, and immunoblotting was performed as described elsewhere (24) using primary antibodies specific for iNOS (BD Biosciences, San Jose, CA) and actin (ICN, Costa Mesa, CA) at 4 °C and concentrations of 1:100 to 1000 for iNOS, and at a concentration of 1:3000 at 4 °C overnight for actin. Horseradish peroxidase-conjugated secondary antibodies were applied, and detection was by chemiluminescence (Pierce Biotechnology, Rockford, IL).

Statistical analysis

Statistical analysis was implemented through the Kaplan-Meier test and ANOVA plus the Student-Newman-Keuls (S-N-K) test or Fisher's least significant difference (LSD) test as appropriate. Data are presented as the mean ± standard deviation. Significance was defined as *P*-value<0.05.

Results

DCD 45 min grafts resulted in a significantly lower 7-day survival rate

The 7-day survival rates in each group were 100% (LTx), 75% (WIT 30min+LTx), and 25% (WIT 45 min+LTx), as shown in Figure 2. There were two mice that died in the second day after the operation in the WIT 30 min+LTx group. There were four mice that died in the second day, and two mice with poor condition (lethargy) were euthanized in the third day after the operation in the WIT 45 min+LTx group. The reason for death was likely primary graft non-function. Acute diffuse peritonitis and bleeding were not found after anatomy. There were significant differences (*P*-value =0.006) on the 7-day survival rates in the three groups. There was no significant difference (*P*-value=0.143) in the 7-day survival rate between the WIT 30 min+LTx and LTx group. There was no significant difference (*P*-value=0.065) in the 7-day survival rate between the WIT 30 min+LTx and WIT 45 min+LTx group. However, there was an obvious significant difference (*P*-value=0.002) between the WIT 45 min+LTx and LTx group. The DCD 45 min grafts resulted in lower survival rates, as low as 25%, than the grafts in the WIT 30 min+LTx group and LTx group.

Serum ALT levels indicated more severe hepatocellular damage in WIT 45 min+LTx mice

Serum ALT level is usually used for evaluation of hepatocellular injury. At 6 hr after transplantation,

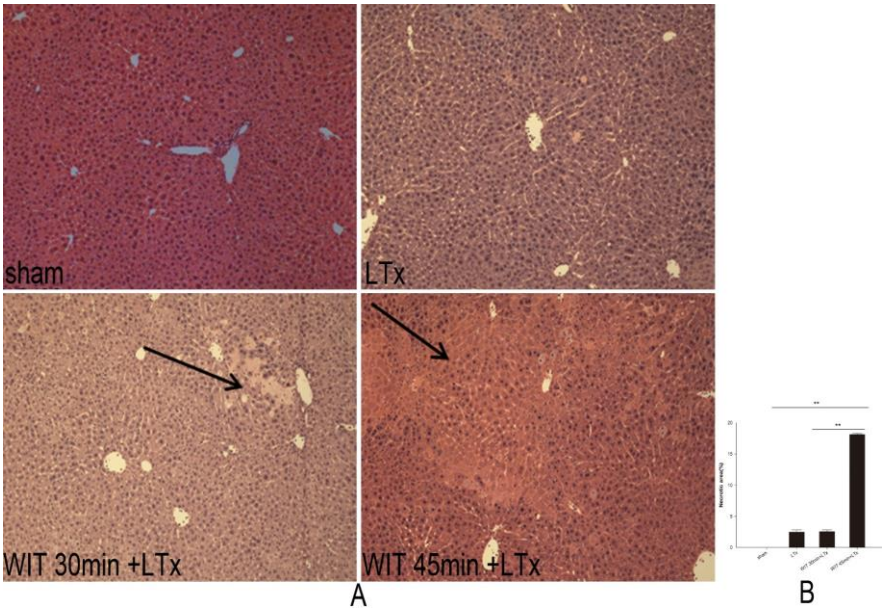

Figure 4. (A) Histology of the grafts at 6 hr after liver transplantation (LTx). H&E-stained liver sections from the grafts of each group at 6 hr after transplantation showing small foci of necrosis (black arrow) (magnification 200×). Marked necrosis was found in the warm ischemia time (WIT) 45 min+LTx group. (B) The necrotic areas of the grafts at 6 hr after LTx. Necrotic areas in 10 random fields per slide were counted on a microscope with a 20× objective using IPlab 3.7v software (BD Biosciences, Rockville, MD). There were 6 mice in each group(n=6). ** stands for *P*-value<0.05

the mean serum ALT level per group were 38.5 U/l (sham), 542.7 U/L (LTx), 572.2 U/L (WIT 30 min+LTx) and 12001.7 U/L (WIT 45 min+LTx), as shown in Figure 3. There existed obvious differences in serum ALT level among the three experimental groups (*P*-value<0.05). The level in the WIT 45 min+LTx group was markedly higher than the other three groups and showed significant differences compared with the levels in the normal LTx and WIT 30 min+LTx group (*P*-value <0.05). But, the serum ALT level in the WIT 30 min+LTx group showed no significant differences compared with the normal LTx group (*P*-value=0.064).

More severe injury was revealed in the WIT 45 min+LTx group

Necrosis was obvious in the WIT 30 min+LTx and WIT 45 min+LTx group, as shown in Figure 4, and much more serious injury was found in the WIT 45 min+LTx group. The necrotic areas of the grafts displayed significant differences (*P*-value<0.05) among the three experimental groups, and there was no obvious difference between the normal LTx and WIT 30 min+LTx group (*P*-value=0.621) at 6 hr after LTx. However, significant differences in necrotic area was detected between the WIT 45 min+LTx group and WIT 30 min+LTx groups (*P*-value <0.05). There were 6 mice in each group.

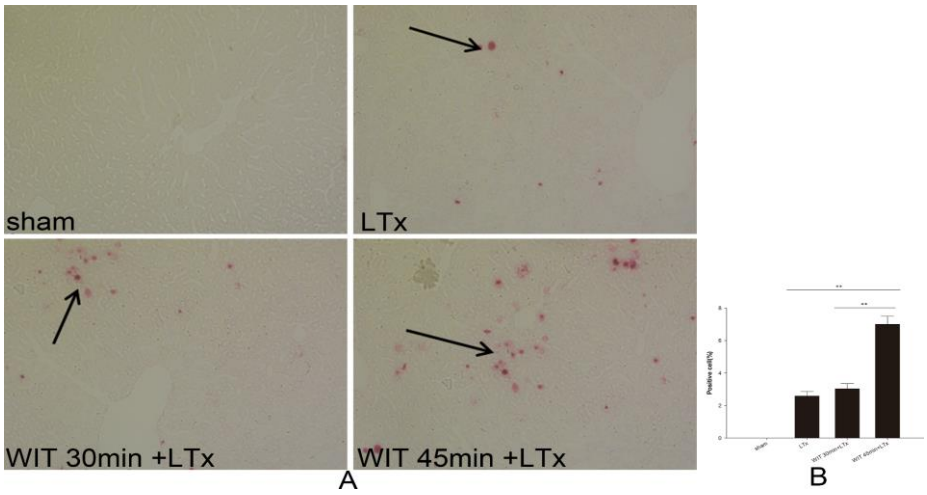

Figure 5. Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining of the liver grafts at 6 hr after liver transplantation (LTx). Apoptotic cells of the liver grafts were detected by immunohistochemical DNA strand break labeling. Ten fields were captured at random under a 10× objective lens. TUNEL-positive and negative cells were counted (n=6 per group). The percentage of TUNEL-positive cells in the warm ischemia time (WIT) 45 min+LTx group was 7.0%, which was significantly higher than that in the normal LTx and WIT 30 min+LTx group (*P*-value<0.05) (n=6). ** stands for *P*-value <0.05

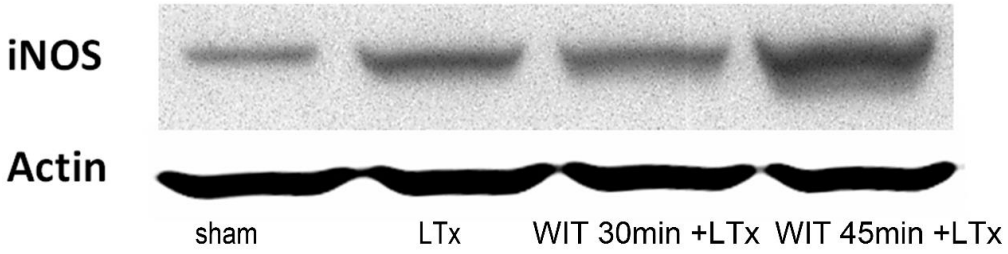

Figure 6. Expression of inducible nitric oxide synthase (iNOS) increased significantly after transplantation in warm ischemia time (WIT) 45min+ liver transplantation (LTx) group. iNOS was detected by immunoblotting and quantified by densitometry. Compared with the normal LTx and WIT 30 min+LTx group, iNOS expression was markedly increased in the WIT 45 min+LTx group. Representative gels of iNOS expression among the four groups were shown

Apoptosis of the liver grafts was obvious in WIT 45 min+LTx group

As shown in Figure 5, few TUNEL-positive cells were found after sham operation. TUNEL staining displayed obvious differences (*P*-value<0.05) in apoptosis among the three experimental groups after LTx. In the normal LTx and WIT 30 min+LTx group, the percentage of TUNEL-positive cells increased to 2.6% and 3.0%, respectively, without significant difference between them (*P*-value=0.053). While, the percentage of TUNEL-positive cells in the WIT 45 min+LTx group was 7.0%, with significant differences compared with the normal LTx and WIT 30 min+LTx group (*P*-value<0.05). There were 6 mice in each group.

Inducible nitric oxide synthase (iNOS) was up-regulated liver grafts in the WIT 45 min+LTx group after transplantation

As shown in Figure 6, iNOS expression increased in the normal LTx group but was obviously increased in the WIT 45 min+LTx group compared with the sham group. However, iNOS expression did not continue to increase in the WIT 30 min+LTx group. iNOS expression was markedly increased in the WIT 45 min+LTx group in comparison with the other two groups.

IL-6 and TNF-α mRNA expression in liver grafts after transplantation also indicated more severe injury in the WIT 45 min+LTx group

Both IL-6 and TNF-α mRNA expression in the liver grafts revealed obvious differences among the sham, normal LTx, WIT 30 min+LTx and WIT 45 min+LTx group at 6 hr after transplantation (*P*-value<0.05). There were no marked differences in IL-6 or TNF-α mRNA expression between the normal LTx and WIT 30 min+LTx group (*P*-value =0.339, *P*-value=0.674). However, both IL-6 and TNF-α mRNA expression of the WIT 45 min+LTx group were obviously higher than that of the normal LTx and WIT 30 min+LTx group (*P*-value<0.05), as shown in Figure 7, indicating that liver inflammation and injury were mediated by many pathways.

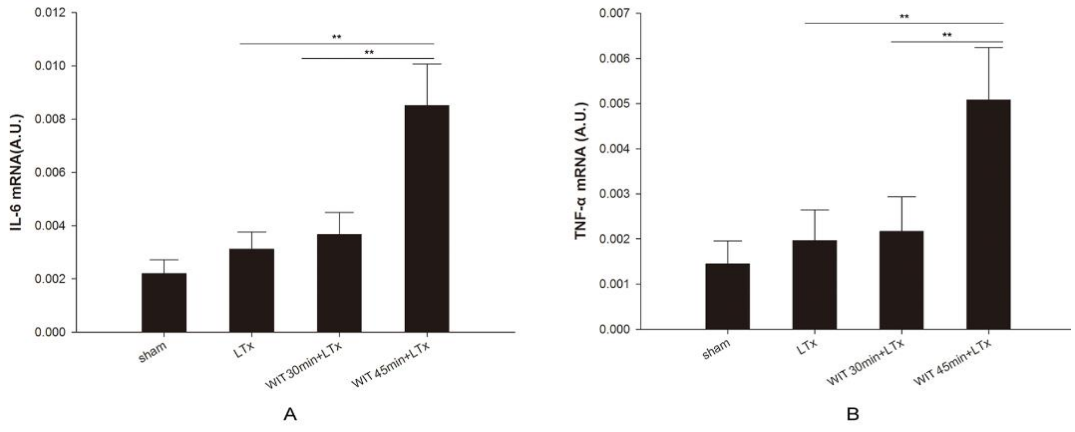

Figure 7. Higher interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) mRNA expression in liver grafts after transplantation in the warm ischemia time (WIT) 45 min+ liver transplantation (LTx) group. IL-6 and TNF-α mRNAs in liver tissue were measured by quantitative real-time PCR.(A.U., arbitrary units) .Both IL-6 and TNF-α mRNA levels of the WIT 45 min+LTx group were significantly higher than those of the other three graft groups at 6 hr post-transplantation. IL-6 mRNA level of the normal LTx and WIT 30 min+LTx group were higher than that of the sham group, but no significant differences were observed. TNF-α mRNA expression revealed similar changes to those in IL-6 mRNA expression between the groups. There were 6 mice in each group (n=6). ** stands for *P*-value<0.05

Discussion

The major cause of the inferior outcomes after DCD LTx is the additional WIT during the process of liver procurement, which impairs organ function (14). Therefore, it is important to establish an excellent animal model of a cardiac-death donor for additional research on DCD LTx. In 2015, our team described a mouse DCD model by clamping the descending aorta with a mini-bulldog vascular clamp (20). However, different period of cardiac arrest might interfere with the research on the warm ischemia injury. Therefore, our study aimed to popularize a mouse model of LTx from DCD grafts. This method could accurately calculate the WIT. Clinically, a cardiac death time of 30 min was associated with consistent long-term recipient survival and minimal graft injury. However, the longer cardiac time of 45 min was followed by higher mortality, a lower survival rate and more severe damage to liver function, findings that remain unclear (1). Moreover, our model has displayed the similar phenomenon, which might help doing some deep research on different WIT DCD grafts. However, the success of the procedure relies on an experienced and talented micro-surgeon. All of these factors led us to elaborate on the development of a mouse model of LTx from DCD grafts and to evaluate the security time of the warm ischemia of mouse DCD grafts.

In this study, the WIT 45 min+LTx group displayed higher mortality, a lower survival rate and more severe damage to liver function than the WIT 30 min+LTx group, which was similar to findings from a previous reports on DCD in humans (1, 26). In the present study, the donor WIT was defined as the time between discontinuation of mechanical ventilation and initiation of aortic perfusion with a cold preservative solution (27). As mentioned in other studies, the longer the WIT, the worse the post-transplantation outcomes (17). Thus, we conclude that WIT > 30 min will lead to worse outcome in DCD LTx.

In the present study, we investigated the iNOS changes in each group. iNOS expression increased slightly after LTx in the WIT 30 min+LTx group, but its expression increased greatly in the WIT 45 min+LTx group. Here, TNF-α and IL-6 also increased greatly in the WIT 45 min+LTx group compared with the WIT 30 min+LTx group. The mechanism of iNOS up-regulation is complicated, but the up-regulation of TNF-α and IL-6 might induce iNOS expression and thus cause liver graft injury (3, 20).

Although the precise mechanisms responsible for liver failure after DCD LTx were not evaluated in depth in this model, the data indicated that a consistent pattern of higher ALT, more necrosis and apoptosis, and higher IL-6, TNF-α and iNOS expression, eventually leaded to animal death, typically 2 to 3 days after surgery. The underlying mechanisms responsible for these changes remain speculative, and it was only found that the longer the WIT is, the more serious injury to hepatocytes may occur. It could not be ignored that the mechanisms of human disease are extremely complicated, and mouse model of various disease might only simulate part of the situation. There are still many risks associated with DCD LTx in clinical practice. Therefore, further research is needed in the future.

Conclusion

Our study developed a mouse model of LTx from DCD grafts and found that WIT exceeding 30 min resulted in worse outcome in DCD LTx. This model would provide many advantages for biomedical research on LTx from DCD grafts.

Acknowledgment

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