Delayed rearterialization unlikely leads to nonanastomotic stricture but causes temporary injury on bile duct after liver transplantation

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Summary
Nonanastomotic strictures (NAS) are common biliary complications after liver transplantation (LT). Delayed rearterialization induces biliary injury in several hours. However, whether this injury can be prolonged remains unknown. The correlation of this injury with NAS occurrence remains obscure. Different delayed rearterialization times were compared using a porcine LT model. Morphological and functional changes in bile canaliculus were evaluated by transmission electron microscopy and real-time PCR. Immunohistochemistry and TUNEL were performed to validate intrahepatic bile duct injury. Three months after LT was performed, biliary duct stricture was determined by cholangiography; the tissue of common bile duct was detected by real-time PCR. Bile canaliculi were impaired in early postoperative stage and then exacerbated as delayed rearterialization time was prolonged. Nevertheless, damaged bile canaliculi could fully recover in subsequent months. TNF-α and TGF-β expressions and apoptosis cell ratio increased in the intrahepatic bile duct only during early postoperative period in a time-dependent manner. No abnormality was observed by cholangiography and common bile duct examination after 3 months. Delayed rearterialization caused temporary injury to bile canaliculi and intrahepatic bile duct in a time-dependent manner. Injury could be fully treated in succeeding months. Solo delayed rearterialization cannot induce NAS after LT.

Introduction
Biliary complications are frequently observed after liver transplantation (LT) and sometimes lead to fatal consequences. Anastomotic complications become rare as the surgical–technical field improves. Nonanastomotic complications, mostly nonanastomotic strictures (NAS) or ischemic-type biliary lesion, have become the main type of biliary complication in recent years [1]. The reasons behind the NAS are complicated and involve several factors. These factors include the use of UW-solution (versus HTK solution), Roux-en-Y reconstruction, postoperative cytomegalovirus infection, prolonged cold ischemia time, repeated rejection episodes, positive lymphocyte crossmatch, and poor HLA match [2–4].

The hepatic artery supplies blood to both intrahepatic and extrahepatic ducts. The common bile duct is supplied via two main arteries, which variably arise from the...
Materials and methods

Animals

Thirty male Bama miniature pigs aged 3–4 months were obtained from the laboratory animal center of the Third Military Medical University in China. All pigs were given access to water ad libitum. Diet was formulated according to the recommended nutrient allowances for this pig breed. The study had obtained an agreement with the Ethics Committee of Xi’an Jiaotong University School of Medicine and performed in accordance with the Guide for the Care and Use of Laboratory Animals from Chinese National Institutes of Health.

Animal model building

All the operations were performed under general anesthesia (propofol with fentanyl proposal), and a tent incision was made in the upper abdominal region. The ligaments around the liver were disconnected. The liver was fully separated from abdominal cavity except for the kinds of ducts into and from the liver. Both gastroduodenal artery and vein were intubated for infusion and ligated the proximal part. Blood inflow and outflow were interrupted with a clamp; the common bile duct was also interrupted near the duodenum. After 5 min of warm ischemia, lactated Ringer’s solution of 500 ml (4 °C, containing heparin 12.5 U/ml) infused the liver from the gastroduodenal artery and vein, respectively. The anterior wall of the infrahepatic artery and vein was split as an outflow tract. The color of the liver then turned sallow. The tubes were released after perfusion, the gastroduodenal artery and vena gastroduodenalis were ligated, and the infrahepatic vena cava was repaired. After the liver was blocked for 15 min, the clamps on the portal vein, suprahepatic vena cava, infrahepatic vena cava and common bile duct were released, and the liver was reperfused. The hepatic artery with different groups was loosened.

Groups

All the animals were separated randomly into three groups, with 10 pigs in each group, according to the time interval between portal venous and hepatic arterial reperfusion. The pigs in group A were simultaneously reperfused through the portal vein and the hepatic artery after cold perfusion. The pigs in groups B and C were rearterialized 1 and 2 h after portal venous reperfusion, respectively.

Specimen collection

A small piece of hepatic tissue as control was resected immediately at the edge of the liver after laparotomy, and another hepatic tissue was resected 2 h after reperfusion. Reoperations were performed under general anesthesia 2 weeks and 1, 2, or 3 months after the model was built. During the reoperation, a subcostal incision of about 4 cm was made and a slice liver tissue was resected. Blood samples were collected preoperatively and postoperatively at 1, 3, 5, 7, 9, 11, 13, 15, 18, 21, 24, and 27 days and 1, 1.5, 2, 2.5, and 3 months after operation via precava vein puncture. All the animals were sacrificed after 3 months, and the liver was removed as a whole. The common bile duct was disconnected near the duodenal, and a small piece of common bile duct was selected for further examination. Common bile duct specimens were also collected as normal control in three animals, whose livers were used for LT graft in another experiment.

Cholangiography

A catheter was inserted into the common bile duct of the removed liver, 76% diatrizoate was injected into the duct, and X-ray was then obtained.

Serum enzyme analyses

Serum alkaline phosphatase (ALP) and γ-glutamyl transpeptidase (γ-GT) were measured in the serum in duplicate using commercial kits, according to the manufacturers'
Liver fragments of approximately 2 mm³ were fixed in Transmission electron microscopy (Olympus Corporation, Japan).

Transmission electron microscopy
Liver fragments of approximately 2 mm³ were fixed in 2.5% glutaraldehyde for 3 h, rinsed in PBS, and then post-fixed in 1% osmium tetroxide for 2 h. Samples were dehydrated in graded alcohols, embedded in Epon 812, cut on an ultramicrotome, and then stained with uranyl acetate and lead citrate. Ultrathin sections were viewed using transmission electron microscopy (Hitachi 7650, Hitachi High-Technologies Company, Japan). A total of 10 bile canaliculi per sample were randomly selected. Area percentage of microvilli within bile canaliculi was analyzed using Image-Pro Plus 6.0 software (Media Cybernetics, Inc., USA).

Immunohistochemical staining and evaluation
Specimens were fixed in 4% paraformaldehyde, embedded in paraffin, sliced to a thickness of 4 mm, and mounted on glass slides. The sections were stained using α-smooth muscle actin (α-SMA) with a rabbit polyclonal antibody (ab5694, Abcam Trading (Shanghai) Company, Shanghai, China), tumor necrosis factor-α (TNF-α) with a goat polyclonal antibody (sc-1351, Santa Cruz Biotechnology, Inc., Dallas, TX, USA), and transforming growth factor-β (TGF-β)1 with a rabbit polyclonal antibody (sc-146, Santa Cruz Biotechnology, Inc., Dallas, TX, USA). This procedure was followed by a second reaction with biotin-labeled anti-rabbit IgG for α-SMA and TGF-β1, and anti-goat IgG for TNF-α (Zhongshan Goldenbrige Biomedical Technology, Beijing, China). An avidin–biotin coupling reaction was performed on the sections by using SP kit (Zhongshan Goldenbrige Biotechnology Co., Ltd.). All the slide images were captured on the Leica SCN400 slide scanner, and the density was analyzed by using Image-Pro Plus 6.0 software. Ten intrahepatic bile ducts per slice were randomly selected. The specific measurement area and optical density value per area in the bile duct were calculated.

Terminal deoxynucleotidyl transferase-mediated nick-end labeling assay
The apoptosis of intrahepatic bile duct epithelial cells was identified by detecting DNA fragmentation in situ. DNA fragmentation was detected by terminal deoxynucleotidyl transferase-mediated nick-end labeling (TUNEL) staining, which was performed on deparaffinized and dehydrated sections using the In Situ Cell Death Detection kit (Zhongshan Goldenbrige Biomedical Technology Co.) according to the manufacturer’s instructions. TUNEL-positive cholangiocytes displayed a characteristic morphology of apoptosis, including chromatin condensation, cell fragmentation, and apoptotic bodies. The apoptotic cells were examined in 10 randomly selected intrahepatic bile ducts per section. The apoptotic index was calculated as the percentage of apoptotic cells in the total number of cholangiocytes.

RNA extraction and real-time quantitative polymerase chain reaction
Total RNA was extracted by using Trizol reagent (Invitrogen, Life Technologies Corporation, USA). RNA was reverse-transcribed to cDNA by using a reverse transcriptase kit (PrimeScript RT Reagent Kit; Takara Biotechnology (Dalian) Co., Dalian, China). The relative abundance of each mRNA sample was quantitated by qPCR with specific primers and SYBR Premix Ex Taq (TaKaRa). Primers for cofilin (forward 5'-CAAGAAGGACCTGGTGTTC-3'; reverse 5'-TTGGAGCTGGCAGTATCATTT-3'), TNF-α (forward 5'-CATGTGTTCCGCGAACATTAA-3'; reverse 5'-TCACCTATTCAATTAGGGCGACCG-3'), α-SMA, (forward 5'-AGGAGGACCTCTATGCTAAAT-3'; reverse 5'-AACACATAAGGTAAACGATCGAGC-3'), TGF-β1 (forward 5'-GAAAGGCAACCAACCACCT-3'; reverse 5'-GGGACTTGAGGGCGAAA-3'), and β-actin (forward 5'-CAGTCACTCCATTCG-3'; reverse 5'-CGTGGTATGGCGTACGGT-3') were designed and synthesized by TaKaRa Biotechnology. Real-time PCRs were conducted using an iQ Multicolor Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). Cycle threshold values were obtained from the Bio-Rad iQ5 2.0 Standard Edition optical System software (Bio-Rad). Data were analyzed using the ΔΔCt method and β-actin served as an internal control.

Data analysis
Data are presented as mean ± SD and processed with SPSS 19.0 statistics software (Chicago, IL, USA). Comparisons were made between groups by Student’s t-test. A value of P < 0.05 was considered statistically significant.

Results
Animal models were successfully constructed, and long-term follow-up study was achieved.

Serum ALP and γ-GT were elevated during the early period with delay rearterialization. The serum total bilirubin of each group was maintained in the normal range during all observation times (data not shown). Serum ALP and γ-GT levels were within normal...
range immediately after the operation but progressively increased during the first few days in all groups. Both levels reached peak values on days 7–9. The levels gradually declined and fully recovered to normal ranges within 1 month. In the following months, ALP and γ-GT were maintained in the normal range. During the entire observation period, the ALP was significantly higher in both delay rearterialization groups than that in group A from days 1 to 24 (P < 0.05), and group C was higher than group B only from days 3 to 18 (P < 0.05) (Fig. 1a). The γ-GT in group C was higher than that in other groups from days 1 to 24 (P < 0.05), and group B was higher than group A from days 1 to 21 (P < 0.05; Fig. 1b).

Rearterialization delay exacerbated bile canalicular damage in early postoperative period

Transmission electron microscopy showed that 2 h after reperfusion, the microvilli exhibited slight edema in group A, whereas some microvilli disappeared and bile thrombus was formed in the bile canaliculi in groups B and C. After 2 weeks, the microvilli also showed edema in group A, the bile thrombus could still be seen, and some of the microvilli were absent in group B; however, almost all the microvilli disappeared in group C. After 1 month, the microvilli recovered to normal status in group A, a few of the microvilli were absent in group B, and some microvilli...
reappeared in group C. By the second and third month, the microvilli in all the groups were in normal state (Fig. 2a). We analyzed the area of percentage microvilli in the bile canaliculi and found a significant difference among the three groups at 2 h and 2 weeks; we also observed a significant difference between groups A and C in 1 month ($P < 0.05$; Fig. 2b).

To further evaluate the function of bile canaliculi, we tested the cofilin, which decomposes the actin microfilament and implies the movement ability of microvilli [9]. The mRNA of cofilin was downregulated 2 h after reperfusion, and the lowest values were obtained on the second week before recovery occurred gradually and reached the preoperative level in 2 months. A significant difference was revealed at 2 h, 2 weeks, and 1 month in groups A and B compared with group C ($P < 0.05$). The mRNA of cofilin was lower in group B than that in group A at 2 weeks or 1 month ($P < 0.05$; Fig. 2c).

Rearterialization delay led to temporary intrahepatic bile duct injury after LT

Similar inflammatory changes were observed in the TNF-$\alpha$ of the intrahepatic bile duct. It exhibited upregulation 2 h after reperfusion and maintained overexpression at 2 weeks. After 1 month, it decreased in all groups (Fig. 3a). The density value per area of the bile duct in each group was higher in group C than that in other groups from the periods of 2 h to 2 months, and group B was higher than group A from 2 h to 1 month ($P < 0.05$; Fig. 3b).

TGF-$\beta$ serves an important function in the pathogenesis of biliary strictures [10]. In contrast to a rapidly ascending TNF-$\alpha$, the TGF-$\beta1$ changed in a slower and longer duration. No significant elevation of TGF-$\beta1$ expression was revealed 2 h after reperfusion. The overexpression of TGF-$\beta1$ was discovered on the second week, which was also the peak time. TGF-$\beta1$ was maintained at a high level on the first month, but began to decline. The normalization of TGF-$\beta1$ appeared on the third month in each group (Fig. 3c). According to density analysis, the image optical density value per area was higher in groups B and C than that in group A at 2 weeks, 1 month, and 2 months ($P < 0.05$), and group C was also higher than group B at 2 weeks and 1 month ($P < 0.05$; Fig. 3d).

Myofibroblasts are considered an important cause of biliary stenosis and $\alpha$-SMA is its marker [11,12]. Although TGF-$\beta1$ fluctuated after operation, no changes of $\alpha$-SMA expression were observed during the study, particularly in the portal area (Fig. 3e).

Cell apoptosis of the intrahepatic bile duct increased 2 h after reperfusion in all groups and lasted for 2 months. The second week was the peak time point (Fig. 4f). Compared with group A, groups B and C exhibited a significant increase in apoptosis index at 2 h, 2 weeks, 1 month, and 2 months ($P < 0.05$); the apoptosis index in group C was also higher than that in group B ($P < 0.05$) (Fig. 5g).

No difference was revealed in common bile duct 3 months after LT

The common bile duct was detected only after 3 months. The results of $\alpha$-SMA, TNF-$\alpha$, and TGF-$\beta1$ examination showed that all the indices were similar with control, and no difference was revealed in each group (Fig. 4).

Solo delayed rearterialization did not induce NAS 3 months after LT

Figure 5a–c shows that intrahepatic bile duct and extrahepatic bile duct were normal and without any stricture, lesion, or expansion in all animals 3 months after LT by cholangiography.

Discussion

The incidence of NAS is between 1% and 20% after LT, and the median time to presentation was reported to be 3 months after the donors are brain dead and within 30 days in donors after cardiac death [13,14]. Several factors, including old age, donation after cardiac death (DCD) LT, and hepatic artery stenosis, correlated with the occurrence of NAS [15,16].

LT is commonly performed by operation; the portal vein is initially opened, and the hepatic artery is subsequently opened; this procedure may damage the biliary tract that solely depends on the hepatic artery blood supply in the rewarming graft [6]. Wang et al. [7] found that the incidence of NAS was associated with the delay of rearterialization time. If the rearterialization time was less than 60 min, only two of 126 patients developed NAS, whereas the incidence increased to 8 of 34 when it was more than 60 min. By contrast, some researchers showed that the incidence of NAS was 15% in delay arterialization. However, only 2% of patients developed NAS after LT if both portal vein and hepatic artery were released simultaneously [8]. These results indicated that delay rearterialization might serve an important function in postoperative biliary tract injury and NAS. However, another study obtained an opposite result, with neither of these two reperfusion protocols showing any advantages, particularly with respect to the incidence of NAS [6]. Several animal studies have also confirmed that delayed rearterialization could lead to bile duct injury [17]; however, their conclusion was based on a short observation time of several hours and lacked long-term observation. Sheng et al. found that NAS could be induced by clamping the hepatic artery for 2 h or more; however, the artery did...
not infuse and thrombosis may occur in this study [18]. Thus, whether delayed rearterialization will cause long-term biliary injury or further relate to NAS in LT remains unanswered.

To study this problem, we designed an autotransplantation animal model to exclude allograft rejection. This design offered the probability of long-term survival for porcine. Clamping and releasing the vessels were used to simulate disconnection and reconstruction. This approach could avoid potential surgical–technical complications and made the operation easier to perform. As a result, the operations were successful for all the animals and long-term follow-up

Figure 2 Morphological and functional changes in bile canalicular. (a) Morphological changes of bile canalicular by transmission electron microscopy observation (×40 000). (b) Comparison of the area percentage of microvilli in bile canaluli. (c) Cofilin mRNA level by real-time PCR. *: P < 0.05
Figure 3  Morphologically evaluated intrahepatic bile duct after delay rearterialization. (a) Expression of TNF-α in portal area (×200). (b) Comparison of the density value per area of TNF-α in the bile duct. *: P < 0.05. (c) Expression of TGF-β1 in portal area (×200). (d) Comparison of the density value per area of TGF-β in the bile duct. *: P < 0.05. (e) Expression of α-SMA in the portal area after LT (×200). (f) TUNEL staining in each group (×200). (g) Comparison of apoptotic index (percentage of apoptotic cells in the total number of cholangiocytes) between groups. *: P < 0.05.
Figure 3 continued
was ensured in our study. Although conventional LT procedures were simplified, the key sequences of LT, such as graft perfusion, warm ischemia (5 min), cold ischemia (10 min), cold ischemia–reperfusion, warm ischemia–reperfusion, and anhepatic phase, occurred. Thus, this model could adequately imitate conventional LT procedure. Most of the confounding factors, such as age, rejection, perfusion method, and cold ischemia time related to NAS, were excluded or well controlled in this model.

Under normal circumstances, ALP and $\gamma$-GT are bound to the canalicular membranes of hepatocytes and the apical cell surface of cholangiocytes. Increased release of these enzymes occurs under pathologic conditions when cell membranes are injured [19,20]. These two enzymes were increased as delayed rearterialization time was prolonged during early postoperative period in our study. This result implies that delay rearterialization could induce biliary tract injury. However, the serum levels of ALP and $\gamma$-GT are influenced not only by its release, but also by clearance from the circulation; therefore, these enzymes may not always represent actual injury. Thus, we further analyzed the injury in different biliary tract sites.

Bile canaliculi, connected to the canal of Hering and bile ducts, are formed by grooves on some of the lateral faces of hepatocytes [21]. Although hepatocytes share double blood supply, some researchers revealed that delay of rearterialization could aggravate the ischemia–reperfusion injury in hepatocytes. According to Post’s study, even arterial reperfusion at 8 min after portal revascularization could impair the hepatocellular excretory function and its microcirculatory in rats [22]. Through the porcine LT model, the liver allograft also showed less reperfusion injury in rearterialization 20 min before the portal reperfusion group than that in rearterialization 60 min after the portal reperfusion group [23]. Our observation showed
that delay in rearterialization led to the loss of more microvilli of bile canaliculi during the early stage after LT. The microvilli rely on the continual polymerization and depolymerization of the actin microfilament, and coflin serves an important function in it [9]. Detecting the coflin mRNA level revealed that the function of bile canaliculi was also impaired after delay rearterialization in the bile canaliculi. However, the impairment of the microvilli or coflin only limited the early postoperative period; the damage was repaired, leading to full recovery with time.

Graft microcirculatory dysfunction is a major determinant of posts ischemic liver injury [24]. The intrahepatic cholangiocytes are only nourished by PVP, which represent the terminal branches of the hepatic artery [5]. However, PVP communicated with branches of the portal vein, and the portal vein blood may then reflow into PVP during the warm ischemia. Microthrombus may further form in PVP because of the slow flow rate [25]. This phenomenon is assumed as the reason why delay rearterialization leads to NAS. Puhl et al. [26] found that the time interval between portal venous and hepatic arterial reperfusion is significantly correlated with the changes in the microcirculation in liver grafts. By contrast, prolonged delayed rearterialization time also led to decreases in the number of blood vessels and the bile duct surrounding the blood vessels in the portal area [27]. Our research revealed that intrahepatic bile duct injury was positively related to delayed rearterialization time during the early period of LT. These findings are in line with the previous studies. However, the injury was corrected as the observation time extended. The intrahepatic bile duct fully recovered eventually within the months that followed in all of the groups. However, whether the number of blood vessels in portal area can also be recovered as time is extended and whether such recovery can also fully treat the intrahepatic bile duct remain unknown; therefore, further studies should be conducted.

We only collected the common bile duct specimen after the animal was sacrificed, owing to concerns of bile spillage or stenosis. All the indices, whether on α-SMA, TGF-β1, or TNF-α, were normal in each groups after 3 months. Whether common bile duct once suffered a similar experience like intrahepatic biliary tract, which was damaged during the early period and repaired in the following stage, remained undetermined in our research.

Cholangiography was believed as a gold standard for NAS diagnosis [28,29]. After cholangiography was performed on the third month after LT, intrahepatic bile ducts and extrahepatic bile ducts were normal without stricture or expansion in each group. NAS may appear from 3 months to 336 months and usually occur from 3 months to 6 months after transplantation [14,30]; however, with the normalization of all the indices, both intrahepatic bile ducts and extrahepatic bile ducts unlikely exhibit NAS further without any other causative factor. The longest delayed rearterialization time is notably only 2 h in our study because few surgeons reconstruct the hepatic artery more than 2 h after reperfusion of the graft. Whether a longer delayed rearterialization time will induce biliary tract injury at 3 months after LT remains unknown.

In conclusion, rearterialization delay aggravates damage in bile canaliculi and intrahepatic bile duct during the early stage after LT, and this damage is time dependent. Injury possibly healed during the following months. Delay rearterialization does not impact the common bile duct at 3 months after LT at least. Solo delayed rearterialization unlikely caused NAS. Whether combining other factors will lead to NAS, with which factor and to what extent, would be determined in a follow-up research.

Authorship
YL: wrote the paper, collected the data and performed most of the experiments. JW: collected the data and performed most of the experiments. PY, HL and LL: built the animal model. JW: performed some of the experiments. HL: designed the research. YD: performed some of the experiments. JW: analyzed the data. YL: designed the research and revised the paper.

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