

# Effect of cold ischemia/reperfusion injury and/or shear stress with portal hypertension on the expression of matrix metalloproteinase-9

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## Abstract

**Background** Matrix metalloproteinase (MMP)-9 plays an important role in liver regeneration after liver surgery. MMP-9 behavior is complicated in cold ischemia/warm reperfusion injury (CIWRI) and/or shear stress with portal hypertension. Small-for-size grafts (SFSGs) are also an issue.

**Materials and Methods** We used a rat model to examine MMP-9 expression 6 h after laparotomy, a temporal clamp (Pringle maneuver), orthotopic liver transplantation (OLT) with a whole-liver graft (100% OLT), partial hepatectomy without the Pringle maneuver (60% hepatectomy) and split orthotopic liver transplantation (SOLT) with a SFSG (40% SOLT) were investigated. Four liver samples were collected in each group.

**Results** The normalized ratio of MMP-9 was not significantly different with a temporal clamp ( $P = 0.1963$ ), 100% OLT ( $P = 0.1781$ ) and 60% hepatectomy ( $P = 0.2367$ ), but it was significantly higher with 40% SOLT compared to that with laparotomy ( $P = 0.0159$ ).

**Conclusion** Forty percent SOLT is accompanied by not only CIWRI but also shear stress. This fatal damage results in increased MMP-9 expression.

**Keywords** extended hepatectomy, Pringle maneuver, remnant liver, split liver transplantation, small-for-size graft

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Conflict of Interest: None

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## Introduction

The extracellular matrix (ECM) has important effects on inflammation, carcinogenesis and regeneration [1-3]. The major components of the ECM include structural proteins (such as collagen and elastin), specialized proteins (such as fibrillin, fibronectin, and laminin) and proteoglycans [3]. There are diverse types of proteases that control ECM remodeling, trigger liver regeneration and drive tumor progression [1-3]. Among them, serine proteases are involved in the breakdown of multiple ECM proteins during various physiopathological situations [4]. Matrix metalloproteinases (MMPs) are a family of enzymes capable of degrading the constituents of the ECM and the basement-membrane. Currently, a total of 28 MMPs have been identified [3]. Since almost every MMP has an alias, the names of individual MMPs are confusing [3]. MMPs have been intensively studied and have been demonstrated to play key roles in inflammation, carcinogenesis and regeneration [1,2,5-8]. MMPs are also involved in cell signaling and are

capable of activating specific cell receptors and growth factors or liberating them from the ECM, thereby regulating various cell behaviors, such as cell growth, differentiation, apoptosis, and migration [9].

MMP-9 is important for liver regeneration, and many studies have focused on the role of MMP-9 after liver surgery [1,2,7-10]. Postoperative liver damage has been confirmed from the early postoperative period, and subsequently, progressive necrosis occurs [11,12]. In the current study, we investigated expression of MMPs at the early postoperative period in various conditions, such as temporal inflow occlusion (Pringle maneuver), orthotopic liver transplantation (OLT), partial hepatectomy without the Pringle maneuver and split orthotopic liver transplantation (SOLT) with small-for-size grafts (SFSGs).

## Materials and Methods

### Animals

Male Lewis rats (RT-1<sup>l</sup>), 8-16 weeks old, were used for experiments. The experimental protocols were approved by the institutional Ethical Committee in accordance with the Declaration of Helsinki. Rats were cared for in accordance with the Institutional Guidelines for Animal Welfare.

### Temporal clamp of hepatic inflow (Pringle maneuver)

After general anesthesia and subsequent laparotomy, the portal vein and common hepatic artery were skeletonized. Clinically, in our institutional criteria, the maximal duration of the Pringle maneuver is 15 min during hepatectomy. Therefore, in this study, hepatic inflow was temporarily obstructed for 15 min by an atraumatic vessel clamp [13]. Hepatectomy models with or without Pringle maneuver were performed to clarify the effects of temporal occlusion.

### OLT with a 100% syngeneic graft (100% OLT)

Comprehensive techniques of the surgical procedures for rat OLT in our institution have been previously described in detailed elsewhere [14]. In brief, syngeneic grafts had a cold ischemic time of 4 h at 4°C in histidine-tryptophan-ketoglutarate solution. To avoid any irrelevant signaling, the hepatic artery was reconstructed by ultra-microsurgery in this study [14,15]. In the OLT/SOLT model, we previously demonstrated the importance of a shortened anhepatic phase and exclusion of unreliable samples based on autopsy findings [14,15]. In this study, the anhepatic phase was maintained within 20 min in each case, and no surgical complications were confirmed with sampling at autopsy.

### Extended hepatectomy with a 40% liver remnant (60% hepatectomy)

The rat hepatectomy model has been well established, and our surgical procedures have been described in detail elsewhere [16]. In brief, partial hepatectomy with a 40% liver remnant was performed without any temporal clamp of hepatic inflow. To maintain hepatic venous flow of the hepatic remnant, the left median and lateral segments remained [14,15]. Inflow occlusions were not performed during surgery to clarify the effects of shear stress with portal hypertension.

### SOLT with a 40% syngeneic SFSG (40% SOLT)

Surgical procedures for rat SOLT in our institution have been previously described [15]. In brief, syngeneic grafts had a cold ischemic time of 4 h at 4°C in histidine-tryptophan-ketoglutarate solution. Forty percent SFSGs were made by the left median and lateral segments at the back table. To avoid any irrelevant signaling, the hepatic artery was reconstructed by ultra-microsurgery in this study [14,15]. The anhepatic phase was maintained within 20 min in each case, and no surgical complications were confirmed with sampling at autopsy.

### Postoperative care

Each rat was kept separately after surgery, and body temperature was maintained by a heating pad. Postoperative observation was performed every 30 min until 6 h after surgery, and 1.0 mL of warm lactate Ringer's solution was routinely administered every 1 h.

### Study design

Recipient rats were divided into five groups according to surgical treatments: (i) laparotomy; (ii) Pringle maneuver (temporal clamp); (iii) 100% OLT; (iv) 60% hepatectomy without inflow occlusion; (v) 60% hepatectomy with inflow occlusion (Pringle maneuver); and (vi) 40% SOLT (Table 1). Serum samples for AST measurements and liver samples for western blotting were collected 6 h after surgery ( $n = 5$  in each group).







### Biochemical assay and coagulation profile

Aspartate aminotransferase (AST) was measured. Serum level of AST was assessed by commercial kit (SGOT reagent, Biotron, Hemet, CA).

### Western blotting

A primary antibody for MMP-9 (anti-MMP-9, clone EP1254, rabbit monoclonal; Millipore, Billerica, MA) was used.

**Table 1** Study design

| Group   | Surgery                             | Hepatic volume       | Inflow occlusion | Cold ischemia<br>Warm reperfusion | Shear stress<br>Portal hypertension |
|---|-------------------------------------|----------------------|------------------|-----------------------------------|-------------------------------------|
|  | Laparotomy                          | 100%-native liver    | -                | -                                 | -                                   |
|  | Pringle maneuver                    | 100%-native liver    | +                | -                                 | -                                   |
|  | 100%-OLT                            | 100%-syngeneic graft | -                | +                                 | -                                   |
|  | 60%-hepatectomy                     | 40%-native liver     | -                | -                                 | +                                   |
|  | 60%-hepatectomy<br>Pringle maneuver | 40%-native liver     | +                | -                                 | +                                   |
|  | 40%-SOLT                            | 40%-syngeneic graft  | -                | +                                 | +                                   |

OLT, orthotopic liver transplantation; SOLT, split orthotopic liver transplantation

Liver samples were collected, homogenized, and centrifuged at high speed for 10 min at 4°C. The supernatant was then collected and used for BCA protein determination (BCA Protein Assay Reagent, Thermo Fisher Scientific, Rockford, IL) and western blot analysis. Forty µg of protein were run on 4-20% tris-glycine gels and transferred onto 0.45 µm nitrocellulose membranes. The membranes were then blocked with 5% nonfat milk made up in a Tris-buffered saline solution. After blocking the membranes, they were incubated at 4°C overnight with the primary antibody. The next day, the membranes were washed three times for 10 min with Tris-buffered saline solution and then incubated with a peroxidase-conjugated secondary for 1 h, with shaking at room temperature. After incubation, the membranes were once again washed three times for 10 min with Tris-buffered saline solution and then developed using chemiluminescence. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as a control. Signals were quantified by using ImageQuant 5.0 software (Molecular Dynamics, Sunnyvale, CA).

### Statistical analysis

The results are presented as mean ± standard deviation. The non-parametric test, (Kruskal-Wallis test) was used for the comparison of unpaired continuous variables between groups. Statistical calculations were performed using SPSS Software Version 16.0 (SPSS Inc., Chicago, IL). A *p* value <0.05 was considered statistically significant.

## Results

### Serum level of aspartate aminotransferase

The serum levels of AST were shown in Figure 1. There were significant differences in the serum levels of AST

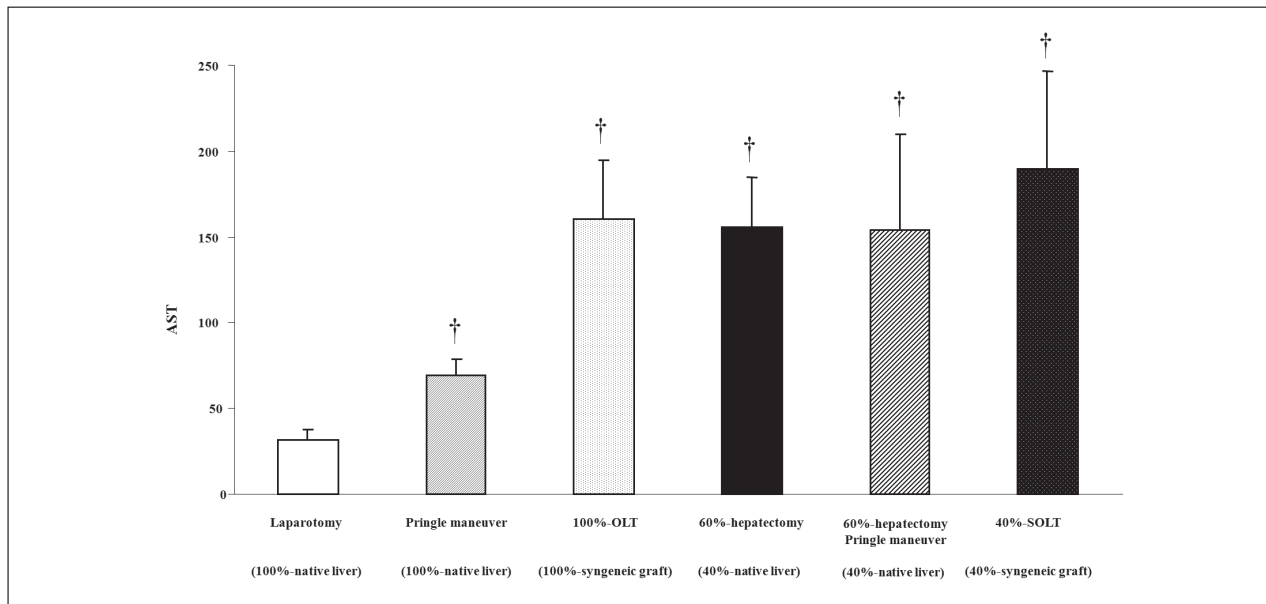
with Pringle maneuver ( $69.8 \pm 8.8$  U/L,  $P = 0.0352$ ), 100% OLT ( $160.8 \pm 34.2$  U/L,  $P = 0.0172$ ) and 60% hepatectomy without Pringle maneuver ( $155.2 \pm 29.4$  U/L,  $P = 0.0212$ ), 60% hepatectomy with Pringle maneuver ( $154.0 \pm 55.5$  U/L,  $P = 0.0251$ ) and 40% SOLT ( $190.0 \pm 56.6$  U/L,  $P = 0.0153$ ) compared with that with laparotomy ( $32.0 \pm 6.0$  U/L).

### MMP-9 expression in the liver

The ratio of MMP-9/GAPDH is shown in Figure 2. There was no significant differences in the ratio of MMP-9/GAPDH with Pringle maneuver ( $0.916 \pm 0.052$ ,  $P = 0.2324$ ), 100% OLT ( $2.164 \pm 1.323$ ,  $P = 0.2514$ ), 60% hepatectomy without Pringle maneuver ( $1.868 \pm 1.133$ ,  $P = 0.3428$ ) and 60% hepatectomy with Pringle maneuver ( $1.880 \pm 1.006$ ,  $P = 0.3329$ ), but it was significantly higher with 40% SOLT ( $3.251 \pm 1.166$ ,  $P = 0.0077$ ) compared with that with laparotomy ( $1.000 \pm 0.052$ ).

## Discussion

Cold ischemia/warm reperfusion injury in OLT/SOLT and shear stress with portal hypertension in hepatectomy/SOLT trigger a liver regeneration cascade, but they also can cause fatal damage in liver remnant/grfts [11,12]. Cell signaling involving cell proliferation, differentiation and apoptosis is present from the early postoperative period, and subsequently, progressive necrosis is observed [11,12]. MMPs are zinc-dependent endopeptidases primarily involved in ECM degradation and tissue remodeling [1,2]. In light of the critical roles for MMPs in inflammation, carcinogenesis and regeneration, MMPs appear to be ideal targets for investigation [1,2,7-10]. It is recognized that MMPs play complex roles, and it needs to be determined how to effectively target MMPs for therapy. MMP-9 is important for liver regeneration after liver surgery [1,2,7-10], and our results of protein expression also

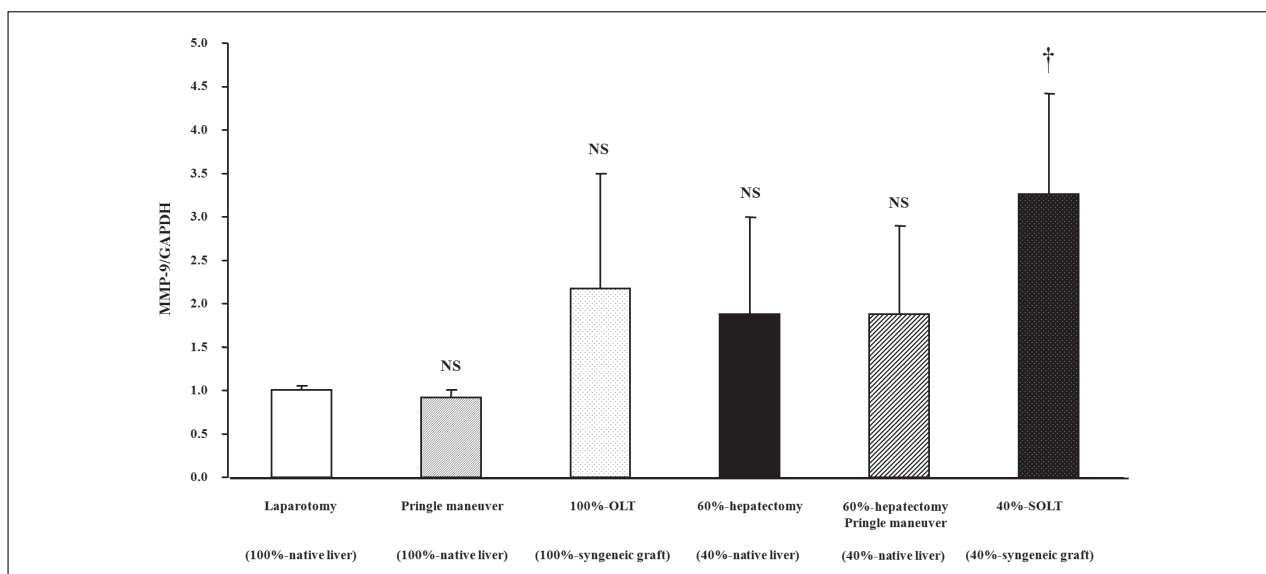


**Figure 1** Serum level of AST. The serum level of AST is shown. There were significant differences in the serum level of AST with Pringle maneuver, 100% OLT, 60% hepatectomy, 60% hepatectomy with Pringle maneuver and 40% SOLT compared with that with laparotomy (†P <0.05) AST, aspartate aminotransferase; OLT, orthotopic liver transplantation; SOLT, split orthotopic liver transplantation

showed that MMP-9 plays an important role from the early postoperative period, especially in SOLT with SFSGs, which are accompanied by the most fatal damage.

Historically, hepatectomy has had significant complications and death resulting from extensive blood loss and the corresponding need for massive blood transfusions

[17,18]. Recent advances in perioperative care [19], and improvements in surgical techniques coupled with the inclusive understanding of the liver's anatomy [20] have considerably altered the outcome [21]. Such improvements included the use and refinement of controlling hepatic inflow (Pringle maneuver) [22]. The Pringle maneuver, applied



**Figure 2** Normalized values of MMP-9. The ratio of MMP-9/GAPDH is shown. There were no significant differences in the ratio of MMP-9/GAPDH with Pringle maneuver, 100% OLT, 60% hepatectomy and 60% hepatectomy with Pringle maneuver, but it was significantly higher with 40% SOLT compared with that with laparotomy (†P <0.05)

MMP, matrix metalloproteinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; OLT, orthotopic liver transplantation; SOLT, split orthotopic liver transplantation

intermittently or continuously, remains the most commonly used technique to control blood loss during hepatectomy. However, these techniques carry the risk of inducing inflow occlusion to the remnant liver and could potentially lead to hepatic failure [23,24]. The risk of damage is considerably higher with the use of continuous occlusion [25,26]. Such adversity is more prominent when continuous occlusion is used in patients with advanced cirrhotic liver [25] and when it is applied for more than 1 h [27]. Therefore, the intermittent approach has been favorably employed as a standard technique for inflow occlusion [25,28]. To clarify the effect of temporal occlusion of inflow and subsequent reperfusion, in the current study, we employed a temporal clamp model with the whole native liver. Our results revealed that temporal occlusion showed no increase in MMP-9 at the early postoperative period. Currently, temporal occlusion (Pringle maneuver) clinically provides an excellent outcome, even in hepatectomy [28-30]. Moreover, recent studies have shown that continuous or intermittent occlusion following in situ liver cooling is useful to improve the outcome [28,31]. Therefore, temporal inflow occlusion does not appear to be a promising topic in future research, although the safety of the Pringle maneuver during more extended hepatectomy has the potential to be investigated.

OLT is an accepted therapy for children and adults with end-stage liver disease, and currently provides long-term survival and a quality lifestyle. However, cold ischemia during organ storage and subsequent reperfusion can severely damage a transplanted liver [32]. During cold ischemic preservation, the parenchymal cells swell and bleb [32], and then Kupffer and endothelial cells trigger the production of free radicals after warm reperfusion [32]. This cold ischemia/warm reperfusion injury (CIWRI) is still a major cause of morbidity and mortality after OLT due to primary graft dysfunction or a non-functioning graft [32]. Warm reperfusion triggers a liver regeneration cascade, but also causes fatal damage in liver grafts. Currently, strategic procedures are required to improve liver tolerance against CIWRI. MMP-9 is a potential target for this strategy after OLT [1,2,9,10]. Proactive strategies that limit graft damage from CIWRI have an advantage for excellent graft function after OLT, and a lot of research has focused on MMP-9 in OLT [1,2,33]. In the current study, we used the OLT model with syngeneic whole-liver grafts, and the expression of MMP-9 appeared to be increased, but this was not statistically significant. Our results are consistent with previous research [1,2], and one possible explanation was a sufficient initial graft volume. Our results suggested that a deceased-donor liver transplantation (DDLT) with a whole-liver graft is safe, and previous reports have shown that whole-liver grafts show excellent outcomes compared with those with SFSGs [34-36]. Therefore, CIWRI in 100% OLT does not appear to be worth further study, although preventive strategies for primary graft dysfunction or a non-functioning graft should be established [32].

Liver resection is considered the standard treatment for primary malignant tumors and liver metastases. Currently,

advanced surgical techniques for hepatectomy and technical improvements in the preoperative evaluation of liver function have resulted in a decline in perioperative morbidity and mortality [19-21]. Extended hepatectomy has an advantage of high curability, but increases morbidity and mortality compared with more limited resections [37]. The volume of the remnant liver is correlated with perioperative morbidity and mortality [37]. Shear stress induced by hepatectomy and subsequent portal hypertension trigger a liver regeneration cascade, and also cause fatal damage in the liver remnant [11,12]. MMP-9 is crucial for liver regeneration after partial hepatectomy [7,8], and many studies have focused on the role of MMP-9 after hepatectomy [7,8]. In the current study, we used a hepatectomy model with a 40% remnant liver, and our results showed that MMP-9 expression was increased but this was not statistically significant. One possible explanation for this finding is that 40% of the remnant liver (native liver) is relatively sufficient after hepatectomy, and a more severe model, such as extended hepatectomy with >70% resection, may show some significant differences.

The choice of a left-side graft is preferred from the viewpoint of greater donor safety and expanded donor candidates in living-donor liver transplantation (LDLT) [34,35]. Guaranteed SOLT with successful outcomes resolves a donor shortage in DDLT [34,36,38]. A SFSG is defined as a ratio of graft weight against standard liver volume <40% [34-36]. Therefore, a 40% SFSG is a current issue that needs to be addressed. An inevitable insufficiency in graft size cannot be avoided in LDLT or SOLT for DDLT. A SFSG in LDLT or DDLT (SOLT) is accompanied by not only CIWRI but also shear stress with portal hypertension. Therefore, a SFSG results in a higher mortality and morbidity after LDLT or DDLT (SOLT) [34-36]. In experimental animal models of SFSGs, the main early pathological changes include sinusoidal congestion, an irregular large gap of sinusoidal lining cells and collapse of the space of Disse [39]. The ECM is critical for the architecture and integrity of hepatic sinusoids [40]. Loss of mechanical support, as a result of MMP-mediated digestion of the ECM in the space of Disse, may directly contribute to the collapse of sinusoids. Changes in the ECM have been reported in studies describing the potential implication of MMPs in the process of CIWRI in human and experimental liver transplantation [2,41-43]. Damage in 40% SFSGs is more fatal than sufficiently sized grafts, and our own results clearly showed that MMP-9 expression was strongly increased from the early postoperative period. Therefore, especially in SOLT with SFSG, MMP-9 is a reasonable target for therapeutic strategies from the early postoperative period.

Because of these clinical situations as described above, many studies in the liver surgery field have mainly focused on shear stress with portal hypertension after hepatectomy/SOLT and CIWRI after OLT/SOLT. The development of clinically relevant hepatectomy/OLT/SOLT models have the advantage of being reliable for research [14,15,44]. However, simulated models of ischemia/reperfusion injury, such as a temporal clamp or simple hepatectomy [45,46], are still used

### Summary Box

#### What is already known:

- Currently, a total of 28 matrix metalloproteinases (MMPs) have been identified. MMP-9 is important for liver regeneration, and many studies have focused on the role of MMP-9 after liver surgery
- Postoperative liver damage has been confirmed from the early postoperative period, and subsequently, progressive necrosis occurs
- MMP-9 behaviors are still controversial

#### What the new findings are:

- In the current study, we investigated expression of MMP-9 at the early postoperative period in various conditions, such as temporal inflow occlusion (Pringle maneuver), orthotopic liver transplantation (OLT), partial hepatectomy without the Pringle maneuver and split orthotopic liver transplantation (SOLT) with small-for-size grafts (SFSGs)
- A SFSG is accompanied by not only cold ischemia/warm reperfusion injury (CIWRI) but also shear stress with portal hypertension
- The 40% SFSG is a good possibility for clinical liver transplantation
- Detailed investigation for MMP-9 behaviors may be a key

experimentally for assessing CIWRI after OLT/SOLT, because of the technical demands of the real models [14,15,44]. Data from pseudo models are clinically irrelevant, and should not be translated into the actual clinical field.

The MMP-9 is produced as an inactive proenzyme. We understand that the measurement of MMP9 by Western blot is not activity, and that the measurement of enzyme activity may differentiate the results. Either an ELISA for MMP-9 activity, or zymography is required, and we now perform these assays to confirm the MMP-9 activities in each group.

Forty percent SFSGs are clinically important in liver regeneration because of the donor shortage of split and pediatric grafts in cadaveric donors in the United States and Europe, and the shift to left-lobe grafts in a living-donor for donor safety in Japan [34,38]. Currently, many transplant centers consider SOLT as a routine operation in DDLT [38], although an ethical policy for SOLT is still not established in some countries. The 40% SFSG is a good possibility for detailed investigation for DDLT with SFSG and LDLT with left-lobe grafts. Although simulated models of CIWRI are not relevant, real SOLT models with hepatic arterial reconstruction, which can provide relevant clinical data without any bias, are now available [14,15].

### Conclusions

The question can be asked: "What direction should liver research for ischemia/reperfusion injury take in the next decade?" We consider that it is important to focus on the 40% SFSG as the next frontier of research in both clinical and experimental studies.

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