Impact of Subnormothermic Machine Perfusion Preservation in Severely Steatotic

Rat Livers: A Detailed Assessment in an Isolated Setting

Y. Okamura, K. Hata, H. Tanaka, H. Hirao, T. Kubota, O. Inamoto, S. Kageyama, I.

Tamaki, N. Yermek, J. Yoshikawa, and S. Uemoto

Division of Hepato-Biliary-Pancreatic Surgery and Transplantation, Department of

Surgery, Graduate School of Medicine, Kyoto University, Kyoto, Japan

Corresponding author

Koichiro Hata, M.D., Ph.D. khata@kuhp.kyoto-u.ac.jp

Running title

Subnormothermic MP for Macro-steatotic Livers

Abbreviations

ALT, alanine aminotransferase; ANOVA, analysis of variance; AST, aspartate

aminotransferase; ATP, adenosine triphosphate; CS, cold storage; DCDs, donors after

cardiac death; ECD, extended-criteria donor; G, gauge; GLDH, glutamate

dehydrogenase; HA, hyaluronic acid; HMGB-1, high-mobility group box protein-1;

HMP, hypothermic machine perfusion; HTK, histidine-tryptophan-ketoglutarate; IPRL,

isolated perfused rat liver; I/R, ischemia/reperfusion; KHB, Krebs-Henselite buffer;

LDH, lactate dehydrogenase; LT, liver transplantation; MP, machine perfusion; NMP,

normothermic machine perfusion; PEG, polyethylene glycol; PNF, primary non-

function; PV, portal vein; PVP, portal venous pressure; ROS, reactive oxygen species;

SECs, sinusoidal endothelial cells; SEM, scanning electron microscope; SMP,

subnormothermic machine perfusion; TBARS, thiobarbituric acid reactive substances;

TEM, transmission electron microscope

Abstract

Current drastic shortage of donor organs has led to acceptance of extended-criteria

donors for transplantation, despite higher risk of primary non-function. Here, we report

the impact of subnormothermic machine perfusion (SMP) preservation on the protection

of >50% macro-steatotic livers. Dietary hepatic steatosis was induced in Wistar rats via

2-day fasting and subsequent 3-day re-feeding with a fat-free, carbohydrate-rich diet.

This protocol induces 50-60% macrovesicular steatosis, which should be discarded

when preserved via cold storage (CS). The fatty livers were retrieved and preserved for

4 hours using either CS in histidine-tryptophan-ketoglutarate (HTK) or SMP in Polysol

solution. Graft functional integrity was evaluated via oxygenated *ex vivo* reperfusion for

2 hours at 37°C. SMP resulted in significant reductions in not only parenchymal (ALT:

$p < 0.001$) but also mitochondrial (GLDH: $p < 0.001$) enzyme release. Moreover, portal

venous pressure ($p = 0.047$), tissue adenosine triphosphate ($p = 0.001$), bile production

($p < 0.001$), HMGB-1 ($p < 0.001$), lipid peroxidation and tissue glutathione were all

significantly improved by SMP. Electron microscopy revealed that SMP alleviated

deleterious alterations of sinusoidal microvasculature and hepatocellular mitochondria,

both of which are characteristic disadvantages associated with steatosis. SMP could

protect 50-60% macro-steatotic livers from preservation/reperfusion injury, and may

thus represent a new means for expanding available donor pools.

Introduction

Liver transplantation (LT) has emerged as a life-saving option for end-stage liver disease, and widely spread over the world in the last 50 years. However, such increasing and worldwide prevalence of organ transplantation has led to chronic shortages of donor organs (1), currently necessitating the potential use of extended-criteria donor (ECD) livers, such as elderly donors, donors after cardiac death (DCDs), and steatotic livers (2). In particular, much attention has recently been focused on steatotic livers, in parallel to increasing prevalence of non-alcoholic fatty liver disease (3, 4). Hepatic steatosis is currently estimated up to 50% of deceased organ donors (5). However, the use of steatotic livers is associated with high incidence of primary nonfunction (PNF) or dysfunction, resulting in worse patient and graft survival, concomitant with prolonged ICU/hospital stays and high medical costs. The degree of steatosis is strongly correlated with the incidence of PNF and graft loss (3, 6-8), therefore, livers exhibiting severe macrovesicular steatosis (>60%) are usually discarded, and the adequacy of moderate (30-60%) macro-steatosis still remains controversial (3). Moreover, hepatic steatosis has been identified as a negative prognostic factor for HCV recurrence (7), as well as an independent risk factor for postoperative biliary complications (9).

Steatotic livers are particularly vulnerable to ischemia/reperfusion (I/R) injury

(10) (11), which is inevitable in the gold-standard organ preservation, cold storage (CS).

Thus far, microcirculatory disturbance (12, 13), mitochondrial dysfunction (14),

increased oxidative stress (15, 16), increased endoplasmic stress (17), and impaired

apoptotic cascade (18) have been reported as the mechanisms underlying the high

susceptibility of steatotic livers to I/R injury.

To successfully protect vulnerable fatty liver grafts from I/R injury, and thus to

expand available donor pools, development of new preservation techniques is needed.

Until recently, there had been no remarkable progress in allograft preservation (19),

however, normothermic (32-37°C) machine perfusion (NMP) preservation has currently

attracted attention as a possible method capable of overcoming the inherent

disadvantages of CS by avoiding cold I/R injury (20). Theoretically, NMP enables

continuous delivery of oxygen and nutrients, removal of waste products, as well as

viability assessments during preservation (20). These advantages are reported to

improve recipient survival in animal DCD models (21, 22). However, NMP requires a

complex temperature-controlled perfusion system, as well as specific perfusates

containing erythrocytes or artificial oxygen carriers to maintain liver aerobic

metabolism, which has hindered its translation into clinical practice widely. Recently,

Tolboom *et al*. reported that oxygen carriers were not necessary in subnormothermic (20-25°C) machine perfusion (SMP) preservation for 5 hours (23). Also, Berendsen *et al*. demonstrated that rat DCD livers could be successfully preserved using temperature-uncontrolled SMP without oxygen carriers for 3 hours, and could eventually be transplanted with good survival (24). Moreover, SMP could effectively resuscitate discarded human liver grafts by maintaining liver function with minimal injury, and by improving various hepatobiliary parameters during *ex vivo* preservation (25).

Although there have been few studies applying SMP to preserve marginal steatotic livers (26), the numerous abovementioned advantages of SMP led us to hypothesize that SMP might be able not only to overcome the vulnerability of steatotic livers but also to make more precise assessment on remaining potential of marginal organs based on various functional parameters using simpler systems than NMP. This study was thus designed to investigate whether SMP preservation successfully protects >50% macro-steatotic rat livers from preservation/reperfusion injury compared with the gold-standard, CS.

Materials and Methods

Animals

Male Wistar rats (250–300 g) (Japan SLC, Inc., Shizuoka, Japan) were housed under specific pathogen-free conditions in a temperature- and humidity-controlled environment with a 12-hour light-dark cycle, and allowed free access to tap water and standard chow pellets. All experiments were conducted in accordance with the Animal Research Committee of Kyoto University, and all animals received humane care, in accordance with "Guide for the Care and Use of Laboratory Animals" published by the National Institutes of Health (NIH Publication No. 86-23, revised 1985).

Dietary induction of hepatic steatosis

Hepatic steatosis was induced according to the modified protocol by Delzenne *et al*. (27). The rats were fasted for 2 days while having free access to water, followed by refeeding with a fat-free, carbohydrate-rich diet (CLEA Japan, Inc., Tokyo, Japan) for 3 days thereafter. The diet comprised following components: saccharose (38%), starch (38%), casein (16%), and a mineral and vitamin mix (8%). Resulting fatty livers exhibited diffuse hepatomegaly, and liver/body-weight ratios were significantly higher than normal livers ($5.45\pm0.09\%$ *vs.* $4.03\pm0.08\%$, $p<0.001$, Figure-1A and B), as well as significantly increased hepatic triglyceride content (0.157 ± 0.006 *vs.* 0.042 ± 0.001 g/g-

protein, $p<0.001$, Figure-1B). This protocol induced moderate-to-severe macrovesicular

steatosis with hepatocellular enlargement and subsequent disturbances in the trabecular

architecture cords, as detailed elsewhere (27, 28). Steatosis induction was confirmed

histologically not only by hematoxylin-eosin but also by Oil-Red O staining (Figure-

1C).

Experimental groups

Steatotic livers were preserved for 4 hours either via CS or SMP. For CS, the

liver was flushed with 20 mL of 4°C histidine-tryptophan-ketoglutarate (HTK, Dr.

Franz Köhler Chemie, Alsbach-Hähnlein, Germany) solution and immersed in 30 mL of

4°C HTK in a sterile cup. For SMP, the liver was connected to a recirculating perfusion

system containing 300 mL of Polysol solution at room temperature. After preservation,

functional viability of liver graft was assessed by *ex vivo* normothermic (37°C)

reperfusion using the isolated perfused rat liver (IPRL) system.

Liver procurement

The rats were weighed and anesthetized with isoflurane (Escain, Mylan, Osaka,

Japan). After midline laparotomy followed by bilateral subcostal incisions, the liver was

carefully mobilized from all ligamentous attachments. The animal was subsequently

heparinized with 300 IU of heparin (Mochida Pharmaceutical Co., Ltd., Tokyo, Japan).

The common bile duct was cannulated with a 24-gauge (G) polyethylene tube

(TERUMO, Tokyo, Japan), allowing for continuous bile collection. The celiac trunk

was cut sharply at its root for later cannulation, whereas its tributaries, namely, left

gastric, splenic and gastroduodenal arteries were ligated and divided. A 14-G catheter

(Argyle^{TM}, COVIDIEN, Tokyo, Japan) was inserted into the portal vein (PV) trunk,

followed immediately by a blood washout with 50 mL of the appropriate preservation

solution. Just before the flushout *in situ*, abdominal vena cava was incised and bled to

avoid hepatic outflow blockages induced by high venous pressure. The liver was then

removed, weighed, and transferred into a back-table on ice. A 20-G catheter (Argyle^{TM},

COVIDIEN, Tokyo, Japan) was inserted into the celiac trunk for arterial perfusion, and

the suprahepatic caval vein was cannulated with a 14-G short stent. These back-table

preparations were completed during a 10-minute cold ischemic period before initiating

perfusion.

SMP preservation

The machine perfusion system consisted of an organ chamber, a reservoir, and

2 independent circulations for PV and arterial perfusion, both of which comprised a

peristaltic pump (Masterflex L/S, Cole Palmer, USA), a membrane oxygenator and a

bubble trap. The liver was perfused both from PV (1 mL/g-liver/minute) and from

hepatic artery (0.1 mL/g-liver/minute) at a controlled flow rate. The perfusion pressures

of both circuits were continuously monitored using pressure transducer systems (BP-

608, OMRON COLIN Co., Ltd., Tokyo, Japan). The total volume of perfusion solution

was 300mL. Carbogen ($95\%O_2+5\%CO_2$) was used for oxygenation, and the inflow pO_2

was continuously maintained >500mmHg throughout preservation (29). The solution

returned freely to a reservoir from the suprahepatic caval vein cannula and was

recirculated by the pump. The perfusate temperature was uncontrolled but constantly

maintained within a range of 20–24°C. Bile was collected throughout the preservation

and used to assess liver graft function. Perfusate samples were collected from both PV

inflow and SHVC outflow after 10, 30, 60, 120, 180 and 240 minutes of preservation to

determine hepatic oxygen consumption and transaminase release.

Regarding the preservation solution, there is no accepted, standard solution for

SMP of steatotic livers to date. Therefore, based on the previous studies (28, 29), we

employed Polysol solution (pH 7.4, 330mOsmol/kg) in this study, in corporation with

AY PHARMACEUTICALS CO., LTD. (Tokyo, Japan). The solution was originally

developed for hypothermic machine perfusion (HMP), containing 1 g% polyethylene

glycol (PEG) as a colloid; glucose; various amino acids, such as glutamine, histidine,

tryptophan and arginine; vitamins, such as ascorbic acid and alpha-tocopherol; and

buffers, such as HEPES. Bessems *et al.* reported that HMP using Polysol resulted in better quality of fatty liver preservation (30). Moreover, even in CS of macro-steatotic livers, Polysol enabled significantly better graft function and integrity than HTK (31).

Ex vivo normothermic oxygenated reperfusion

Before connection of the liver grafts, the system was rinsed with Krebs-Henselite buffer (KHB; K3753, Sigma-Aldrich Inc., St Louis, USA). To account for slow rewarming process upon liver implantation *in vivo*, all the livers were transferred and immersed into saline at room temperature for 15 minutes immediately before reperfusion. Prior to connection to the circuit, the livers were reflushed from PV with 20 mL of saline. Reperfusion was subsequently performed for 120 minutes at a constant flow of 3 mL/g-liver/minute via PV with 150mL of oxygenated KHB at 37°C, as described previously (32). According to the standardized conditions in IPRL (25, 32), Carbogen was used for buffering of perfusate and oxygenation, and the prehepatic pO_2 was continuously maintained above 500mmHg (32). Although carbogen seems to exert vasodilatory effect mainly in arterial vessels (33), its impact on the portal circulation is unclear. Hyaluronic acid (HA) (CAS.9067-32-7, Wako Ltd., Japan) was added to KHB solution (1mg/L) to assess HA clearance.

Both hepatic influent and effluent were sampled at 5, 15, 30, 60, 90, and 120

minutes after reperfusion for analysis of hepatic oxygen consumption, transaminase release and HA clearance. Bile production was monitored throughout reperfusion and used to assess liver graft function after preservation. Portal venous pressure (PVP) was continuously measured by a pressure transducer system, which was connected to the PV inflow catheter throughout reperfusion.

Transaminase release

Aspartate-aminotransferase (AST) and alanine-aminotransferase (ALT) levels in the hepatic effluent were analyzed using the standard spectrophotometric method with an automated clinical analyzer (JCA-BM9030, JEOL Ltd., Tokyo, Japan). Glutamate-dehydrogenase (GLDH) was also measured as an index of mitochondrial damage during reperfusion using a GLDH activity colorimetric assay kit (BioVision Inc., Milpitas, CA, USA), and lactate-dehydrogenase (LDH) in bile served as an index of cholangiocellular damage (34).

HMGB-1 release

To quantify comprehensive tissue damage after 2 hours of oxygenated reperfusion, the release of high-mobility group box protein-1 (HMGB-1), a damage-associated molecular pattern (DAMP), into perfusate was determined using a specific ELISA kit (Shino-Test Corporation, Tokyo, Japan).

HA clearance

Liver sinusoidal endothelial cells (SECs) are the main site of HA uptake and

degradation (35); therefore, SEC damage results in increased serum HA levels. Thus,

HA clearance is an excellent parameter for sinusoidal endothelial function, especially in

the IPRL setting, because HA production by other tissues is completely excluded (32).

HA concentrations in perfusate were measured using an enzyme-linked protein assay

(Corgenix Inc., Colorado, USA), according to the manufacture's protocol.

Hepatic oxygen consumption

Samples were collected both from the inflow and from the outflow of the liver

and immediately analyzed using a blood gas analyzer (Rapid Point 405, Siemens,

Tokyo, Japan). The oxygen consumption rates during SMP and reperfusion were

calculated using the following equation:

Oxygen consumption rate (μL/minute/g-liver) = portal flow $\times$ S $\times$ ($pO_{2inflow}$ −

$pO_{2outflow}$)/g-liver (S, solubility constant of oxygen in water at 37°C =

0.031μL/mL/mmHg).

Tissue ATP concentrations

At the end of oxygenated reperfusion, liver tissue samples were snap-frozen in

liquid nitrogen and preserved below -80°C until analysis. Tissue adenosine triphosphate

(ATP) concentrations were determined by the luciferin-luciferase method with an ATP

bioluminescence assay kit (TOYO B-NET CO., LTD., Tokyo, Japan). The results were

normalized to protein concentrations, measured by a BCA protein assay kit (Thermo

Fisher Scientific K.K., Yokohama, Japan).

Oxidative stress

After 2 hours of reperfusion, lipid peroxidation was analyzed by measuring the

tissue concentrations of thiobarbituric acid reactive substances (TBARS) using a

commercially available Colorimetric TBARS Microplate Assay Kit (Oxford Biomedical

Research, Inc. USA). Total glutathione levels in liver tissue, an index of antioxidative

potential, were determined using a commercially available Total Glutathione

Quantification Kit (Dojindo Molecular Technologies, Inc. USA).

Morphological analysis

Light microscopy

At the end of reperfusion, liver tissue samples were immediately obtained from

the left lobe, preserved in 4% paraformaldehyde, paraffinized, and cut into 4-μm

sections. After hematoxylin-eosin staining, the sections were evaluated via light

microscopy.

Electron microscopy

At the end of reperfusion, additional livers from the both groups were perfused

through PV with 2%-glutaraldehyde/4%-paraformaldehyde in 0.1M phosphate buffer,

pH 7.4. After fixation, the samples were cut into 2-mm cubes for transmission electron

microscope (TEM) and 1-mm slices for scanning electron microscope (SEM) analysis.

For TEM analysis, the samples were postfixed with OsO_4 (osmic acid fixative),

dehydrated, embedded in epoxy resin, and heat-polymerized at 60°C, then examined

with H-7650 (Hitachi High-Technologies, Tokyo, Japan). For SEM analysis, the

samples were postfixed with OsO_4, dehydrated, substituted and freeze-dried with t-

butyl-alcohol, and ion-sputter-coated, then examined with S-4700 (Hitachi High-

Technologies, Tokyo, Japan).

Statistics

All results are expressed as the mean ± standard error of the mean (SEM).

Comparisons between the 2 groups were performed with two-sided Student's *t* tests, and

two-way repeated-measures analysis of variance (ANOVA) with Bonferroni's post-test

was used to assess time-dependent changes and differences between the groups at each

time point. *P*-value <0.05 was considered statistically significant. All statistical data

were generated using Prism 5 (Graph Pad Software, Inc., La Jolla, CA, USA).

Results

Liver graft viability during SMP preservation

As shown in Figure-2A, the perfusate temperature was maintained at $22.5\pm1°C$

throughout preservation, the PVP was kept below 3mmHg throughout SMP, and the

arterial perfusion pressure was maintained at approximately 47mmHg (Figure-2B),

although the pH gradually acidulated with time (Figure-2C). The outflow pO_2 was

maintained above 150mmHg, indicating that oxygen supply was sufficient to meet the

metabolic demands of the livers (Figure-2D). Hepatic oxygen consumption remained

stable during SMP (Figure-2E), and ALT release remained at low level throughout SMP

(Figure-2F). Bile was secreted throughout SMP (3.3 ± 0.1 µL/g-liver/hour).

Liver graft damage and functional recovery upon reperfusion

As shown in Figure-3A, AST and ALT releases were both significantly lower

in SMP-preserved steatotic livers than those in CS ($p<0.001$, Figure-3A and -B),

indicating significantly less hepatocellular damage by SMP. Mitochondrial damage was

quantified based on GLDH release. CS-preserved livers exhibited time-dependent

increase in GLDH, whereas SMP significantly attenuated GLDH leakage ($p<0.001$,

Figure-3C). HMGB-1 release from CS-preserved livers after 2 hours of oxygenated

reperfusion was more than 15-fold higher than in SMP (9.5 ± 2.3 *vs*. 149.7 ± 20.8 ng/mL,

p<0.001, Figure-3D), indicating significantly less tissue damage achieved by SMP.

Regarding liver graft functional parameters, we measured bile production throughout the reperfusion period. SMP-preserved livers exhibited significantly higher bile production level than in CS (46.2±5.5 *vs*. 18.0±0.7 μL/g-liver/2 hours, *p*<0.001, Figure-4A). LDH leakage in the bile of SMP-preserved livers was significantly lower than that in CS (29.1±8.9 *vs*. 275.6±65.7 IU/L, *p*=0.018, Figure-4B), indicating less cholangiocellular damage via SMP.

Oxygen consumption and energy charge

As a parameter for functional recovery of the mitochondria and resultant tissue integrity upon oxygenated reperfusion, we measured hepatic oxygen consumption and tissue ATP concentrations. CS-preserved livers exhibited significantly higher oxygen consumption than in SMP (*p*=0.026, Figure-5A). Paradoxically, SMP-preserved livers exhibited significantly higher ATP levels than CS-preserved livers (0.057±0.002 *vs.* 0.041±0.003 μmol/g-protein, *p*=0.001, Figure-5B), indicating that SMP produced significantly better energy charge with less oxygen consumption than by CS.

Oxidative stress

We determined TBARS levels in liver tissue to assess lipid peroxidation by reactive oxygen species (ROS) upon oxygenated reperfusion. SMP significantly

lowered TBARS than by CS (0.928±0.207 *vs.* 1.584±0.077 µmol/g-protein, *p*=0.037,

Figure-5C). Consistent with these findings, total tissue glutathione, served as an index

of antioxidative potential, was significantly higher in SMP-preserved livers than in CS

(0.503±0.002 *vs.* 0.353±0.002 µmol/g-protein, *p*=0.011, Figure-5D). Given that CS-

preserved livers consumed more oxygen while exhibiting less ATP restoration upon

reperfusion (Figure-5A and B), these results indicate that steatotic livers utilize

delivered oxygen to promote ROS production rather than to restore energy charge when

preserved via CS.

Vascular resistance and sinusoidal function upon reperfusion

During reperfusion, PVP, served as an index of total vascular resistance of liver

grafts, exhibited a time-dependent increase up to 9.4±1.4mmHg in CS. In contrast, PVP

in SMP-preserved livers remained at significantly lower level even at the end of

reperfusion (4.7±0.4 mmHg, *p*=0.047, Figure-6A).

Regarding the functional integrity of sinusoidal endothelium, HA clearance in

SMP was significantly higher than in CS (3637±253 *vs.* 2891±232 ng/g-liver/hour,

p=0.029, Figure-6B).

Morphological integrity upon reperfusion

Light microscopy

19

Consistent with the transaminase and HMGB-1 release, hematoxylin and eosin staining demonstrated vacuolization, and sinusoidal obstruction, as well as upstream dilatation around the pericentral acinar zone, in CS (Figure-7A and C). However, these changes were significantly alleviated in SMP (Figure-7B and D).

Electron microscopy

To evaluate tissue damage at the ultrastructural level, especially focusing on hepatocellular mitochondria as well as sinusoidal endothelial lining, we performed electron microscopy.

TEM-based ultrastructural analysis demonstrated mitochondrial swelling with less electron density and cytoplasmic vacuolization after reperfusion in CS-preserved livers (Figure-8A). These damages were, however, significantly mitigated by SMP (Figure-8B). These findings were strongly correlated with lower GLDH release and higher ATP restoration in SMP-preserved livers.

Figure-9 depicts representative SEM observations of the sinusoidal microvasculature, demonstrating that the fenestrae were fused and enlarged and that the SECs were disrupted in CS (Figure-9A). In contrast, the sinusoidal lining and pores therein were both well maintained by SMP (Figure-9B), consistent with better HA clearance in SMP-preserved livers (Figure-6B).

Discussion

Hepatic steatosis is a common clinical problem in industrialized countries currently affecting up to 50% of deceased donor livers (5). Because >30% macrovesicular steatosis is a well-known risk factor for PNF (8), considerable numbers of steatotic livers have been discarded without utilization, further exacerbating the critical organ shortage. The decision regarding "transplantable or not" is normally based on the macroscopic (surgeon's decision) or microscopic (pathologist's decision) appearance of steatotic livers (36). However, this decision is not always correct, therefore, new methods for assessing liver transplantability based on their damage and function have long been desired.

Based on these backgrounds, machine perfusion (MP) preservation has recently become an area of intense investigation. MP preservation is generally classified into the following 3 categories: hypo- (0-10°C, HMP), subnormo- (20-25°C, SMP), and normothermic (32-38°C, NMP) preservation, according to the target range of tissue temperature (29, 37). Several investigations have demonstrated the usefulness of HMP for ECD livers both in clinical and in animal models (38-42), however, the usefulness of SMP or NMP for steatotic livers has not yet been determined. In contrast to HMP, SMP and NMP can maintain tissue metabolism during preservation, thereby enabling *ex vivo*

assessments of the potential liver functions, such as bile production and oxygen

consumption after organ harvesting (43). In fact, the first case of a human LT using a

DCD liver preserved by NMP was recently reported. In this case, liver function and

viability were assessed more precisely than before, and a marginal liver graft was

successfully salvaged and transplanted (44, 45). In contrast to HMP and NMP, SMP

does not require complex temperature control and may thus be more translatable to

clinical practice than the others.

In the present study, we demonstrated that SMP could maintain the

morphological architecture of severe macro-steatotic livers both in microscopic and in

ultrastructural observation. Electron microscopy demonstrated that SMP could

remarkably attenuate the most characteristic vulnerabilities of severe steatotic livers,

namely, mitochondrial deterioration and microcirculatory impairment. This was

followed by significantly better viability of usually non-functioning fatty livers, as

evidenced by lower vascular resistance, better-preserved microcirculation, higher

mitochondrial function and resultant higher energy charge, and more bile production.

Among such various benefits by SMP, it is very noteworthy that SMP protected

hepatocellular mitochondria from preservation/reperfusion injury, thereby facilitating

efficient ATP restoration and preserving liver tissues integrity. Mitochondria are the

primary cellular sources of ROS, which bursts upon reperfusion/reoxygenation (16). In this study, 4-hour CS provoked stronger oxidative stress in steatotic livers (Figure-5C), coupled with lower ATP restoration (Figure-5B) despite consuming more oxygen than SMP (Figure-5A). These seemingly-paradoxical results suggest that CS-preserved fatty livers use oxygen to generate more ROS rather than ATP. Because mitochondrial oxidative phosphorylation is essential for restoring ATP, SMP seemed to maintain mitochondrial viability by continuous oxygen supply (46). Mitochondrial swelling (TEM observation) and destruction (increased GLDH release) were both evident in CS, however, SMP could attenuate such deleterious alterations in hepatocellular mitochondria. Mitochondrial ROS formation induces mitochondrial permeability transition after anoxia/reperfusion, resulting in mitochondrial swelling (47). Decreased oxidative stress by SMP, therefore, certainly contributed to protect mitochondria from preservation-reperfusion injury.

Another characteristic advantage via SMP was found in the protection of hepatic microcirculation. Macrovesicular steatosis is inherently characterized by the narrowed sinusoidal lumen and resultant increases in intrahepatic vascular resistance as a result of hepatocyte swelling (12, 48). Moreover, cold ischemia persisting for several hours exacerbates cell/tissue swelling, further increasing the risk of microcirculatory

23

perfusion failure. Additionally, hypothermia harms SECs to a greater extent than it

harms parenchymal hepatocytes (49, 50). Fukumori *et al.* reported that steatotic livers

from obese rats are more susceptible to cold preservation injury than lean livers because

of SEC deterioration (11). Consequently, steatotic liver grafts preserved by CS

inevitably develop devastating microcirculatory disorders those are strongly correlated

with the type and severity of steatosis (13). In this study, CS-preserved steatotic livers

exhibited SEC destruction, concomitant with significantly lower HA clearance. Time-

dependent increases in PVP presumably reflect not only progressive cell/tissue edema

but also sinusoidal disruption upon reperfusion in CS. Parenchymal damage in zone-3

seems to be a consequence of upstream sinusoidal microcirculatory failure. In contrast,

SMP successfully preserved SEC morphology and function, most likely by avoiding

cold ischemia during preservation.

Regarding the perfusate composition, blood-based oxygen carriers seem to be

necessary in NMP setting (52), however, the necessity in SMP remains controversial

(23, 24, 53). Moreover, the ideal regimens or compositions of preservation solution for

SMP have not been established yet. In this study, we used Polysol containing PEG for

keeping colloid osmotic pressure, various vitamins and amino acids, similar to culture

media (30).

With all these benefits by SMP, HMGB-1 release, widely known as one of the most hazardous DAMPs, from steatotic liver grafts was significantly suppressed down to one-fifteenth (6.3%) of the value observed in CS. Because HMGB-1 release provokes substantial inflammatory response locally and systemically (54), marked attenuation of such inflammatory cascades by SMP undoubtedly contributes not only to reduce the risk of PNF, but also to improve critically-ill conditions during perioperative period.

This study employed an *ex vivo* reperfusion model. Performing the study in an isolated setting enabled us to avoid interferences from immunological alloreactions, thereby allowing us to assess organ preservation quality more precisely. However, validation of this novel preservation technique using a liver transplant model is necessary before translating into clinical practice.

In conclusion, SMP preservation successfully protected >50% macro-steatotic livers from preservation/reperfusion injury. Although further investigations are required before clinical translation, this new technique may provide clinicians with the means to successfully protect large amounts of discarded fatty livers from preservation/reperfusion injury and thus to expand the available donor pools.

Acknowledgments

Disclosure

The authors of this manuscript have no conflicts of interest to disclose, as described by the *American Journal of Transplantation*.

Figure Legends

Figure 1: Confirmation of hepatic macrovesicular steatosis

(A) Macroscopic appearance of normal and steatotic livers: as shown on the right, the steatotic liver appears whitish and yellowish, exhibiting diffuse hepatomegaly.

(B) The liver/body weight ratios of steatotic livers were significantly higher than those of normal livers (5.45 ± 0.09 % *vs.* 4.03 ± 0.08 %, *: $p<0.001$). Steatotic livers also exhibited significantly increased hepatic triglyceride content (0.157 ± 0.006 *vs.* 0.042 ± 0.001 g/g-protein, *: $p<0.001$) compared with normal livers.

(C) Photomicrographs of representative tissue sections visualized by hematoxylin-eosin (Left) and Oil Red O staining (Right). Moderate-to-severe macrovesicular steatosis (>50-60%) was confirmed histologically. The original magnification was x 400 for both images. The scale bar in each panel represents 50 μm.

Figure 2: Function and damage monitoring during SMP preservation of steatotic liver grafts.

Chronological alterations in various parameters over 4 hours of SMP preservation: (A) Perfusate temperature (°C); (B) PVP and HAP (mm Hg), the former served as an index of parenchymal vascular resistance; (C) Perfusate pH in both the inflow and the

27

outflow; (D) Perfusate pO$_2$ (mm Hg) in both the inflow and the outflow; (E) Oxygen

consumption rate (μL/minute/g-liver weight), one of the most reliable indices of liver

graft functional integrity; and (F) ALT release (IU/L) into the perfusate, which served as

an index of hepatocellular damage during preservation.

All data are presented as the mean ± standard error of the mean, SEM (*n*=8 each). SMP,

subnormothermic machine perfusion; PVP, portal venous pressure; HAP, hepatic arterial

pressure; ALT, alanine aminotransferase.

Figure 3: Transaminase and HMGB-1 release upon reperfusion

(A, B) ALT and AST release into the perfusate, as indices of hepatocellular damage

upon reperfusion. (C) GLDH release into the perfusate, as an index of hepatic

mitochondrial damage. All differences between the groups were assessed via 2-way

repeated-measures ANOVA: *p*<0.001. Time-point assessments were performed by

Bonferroni's post-test: *: *p*<0.001 *vs.* CS. Error bars are sometimes invisible due to

small SEMs. (D) HMGB-1 release into the perfusate, which served as an index of

cellular and nuclear disruption after 120 minutes of reperfusion. All data are presented

as the mean ± SEM (*n*=8 each). *: *p*<0.001 *vs.* CS. CS, cold storage; SMP,

subnormothermic machine perfusion; HMGB-1, high mobility group box protein-1;

ALT, alanine aminotransferase; AST, aspartate aminotransferase; GLDH, glutamate

dehydrogenase; ANOVA, analysis of variance.

Figure 4: Bile production and cholangiocellular damage

(A) Cumulative bile production over 120 minutes of oxygenated reperfusion, one of the

most credible indices of liver graft function. Differences between the two groups were

assessed by 2-way repeated-measures ANOVA: $p<0.001$. Time-point assessments were

performed by Bonferroni's post-test: †**:** $p<0.01$ and *: $p<0.001$ *vs.* CS. Error bars are

sometimes invisible due to small SEMs. (B) LDH leakage into bile as a marker of

cholangiocellular damage (mean ± SEM, $n=8$ each). ‡: $p=0.018$ *vs.* CS. CS, cold

storage; SMP, subnormothermic machine perfusion; LDH, lactate dehydrogenase;

ANOVA, analysis of variance.

Figure 5: Oxygen consumption, ATP restoration and oxidative stress upon

reperfusion

(A) Hepatic oxygen consumption rates were calculated from the inflow and outflow

pO_2, the transhepatic flow, and the liver mass (mean ± SEM, $n=8$ each). Inter-group

differences were assessed by 2-way ANOVA: $p=0.026$. (B) Hepatic tissue ATP

concentrations after 2 hours of reperfusion, which served as an index of hepatic

mitochondrial function recovery, as well as microcirculatory perfusion (§: $p=0.001$ by

two-sided Student's t test, mean ± SEM, $n=7$ each). Interestingly, SMP yielded

significantly better energy charge with less oxygen consumption than CS. (C) TBARS

levels in liver tissue, which served as an index of lipid peroxidation, upon reperfusion.

‖: $p=0.037$ by two-sided Student's t test, mean ± SEM, $n=7$ each (D) Total glutathione

levels in liver tissue, which served as an index of liver tissue antioxidative potential.

Inter-group differences were assessed by two-sided Student's t test, ¶: $p=0.011$ (mean ±

SEM, $n=7$ each). CS, cold storage; SMP, subnormothermic machine perfusion; ATP,

adenosine triphosphate; TBARS, thiobarbituric acid reactive substances; ANOVA,

analysis of variance.

Figure 6: Vascular resistance and sinusoidal function upon reperfusion

(A) PVP time-course over 120 minutes of reperfusion, as an index of total vascular

resistance upon reperfusion. PVP was continuously monitored by a pressure transducer

system (BP-608, OMRON COLIN Co., Ltd., Tokyo, Japan), which was connected to

the portal vein inflow catheter throughout reperfusion. All data are presented as the

mean ± SEM in 8 livers per group. Differences between the groups were assessed by 2-

way repeated-measures ANOVA: $p=0.047$.

(B) HA clearance, which served as an index of preserved SEC function. **: $p=0.029$ by

two-sided Student's t test (mean ± SEM, $n=7$ each). CS, cold storage; SMP,

subnormothermic machine perfusion; PVP, portal venous pressure, IPRL, isolated

perfused rat liver; HA, hyaluronic acid, SECs, sinusoidal endothelial cells, ANOVA,

analysis of variance.

Figure 7: Light microscopic assessmets of steatotic liver grafts after preservation

and reperfusion.

Representative tissue sections stained with hematoxylin-eosin after 2 hours of

oxygenated reperfusion.

A and **B:** At lower magnification (x 40), the steatotic livers preserved by CS exhibited

sinusoidal dilatation and deleterious vacuolization around pericentral areas (**A**), whereas

SMP-preserved livers exhibited significantly attenuated damage (**B**). A scale bar in each

panel represents 500 μm.

C and **D:** At high magnification (x 200), hepatocellular vacuolization was evident in

CS-preserved livers (**C**), whereas SMP-preserved livers exhibited significantly

attenuated alterations (**D**). Please note that hepatocellular vacuolization was clearly

improved by SMP; however, macrovesicular fatty droplets were present. The scale bar

in each panel represents 100 μm. CS, cold storage; SMP, subnormothermic machine

perfusion; IPRL, isolated perfused rat liver; CV, centrilobular venule.

Figure 8: Ultrastructural observations using a TEM

Photomicrographs of representative tissue sections, which were taken using a TEM. The

original magnification was x 4,000.

(A) Steatotic livers exhibited mitochondrial swelling with low electron densities

(arrowhead) and cytoplasmic vacuolization after 4 hours of CS followed by 2 hours of

oxygenated-reperfusion. (B) In contrast, SMP preserves mitochondrial structure in

severe macrovesicular fatty livers. CS, cold storage; SMP, subnormothermic machine

perfusion; FD, fat droplet; N, nucleus.

Figure 9: Ultrastructural investigation of sinusoidal microvasculature

Photomicrographs of representative tissue sections, which were taken using by an SEM.

The original magnification was x 4,000.

In steatotic livers preserved by CS, the fenestrae were fused and enlarged, and the

sinusoidal lining was markedly disrupted (A). However, the sinusoidal wall structures

and pores were well maintained by SMP (B). Taken together, these findings indicate that

SMP significantly attenuated microcirculatory impairment, one of the most

characteristic findings associated with hepatic steatotic. CS, cold storage; SMP,

subnormothermic machine perfusion.

References

1.   Wolfe RA, Roys EC, Merion RM. Trends in organ donation and transplantation in the United States, 1999-2008. Am J Transplant 2010;10(4 Pt 2):961-972.

2.   Durand F, Renz JF, Alkofer B, Burra P, Clavien PA, Porte RJ et al. Report of the Paris consensus meeting on expanded criteria donors in liver transplantation. Liver Transpl 2008;14(12):1694-1707.

3.   McCormack L, Dutkowski P, El-Badry AM, Clavien PA. Liver transplantation using fatty livers: always feasible? J Hepatol 2011;54(5):1055-1062.

4.   Eguchi Y, Hyogo H, Ono M, Mizuta T, Ono N, Fujimoto K et al. Prevalence and associated metabolic factors of nonalcoholic fatty liver disease in the general population from 2009 to 2010 in Japan: a multicenter large retrospective study. J Gastroenterol 2012;47(5):586-595.

5.   Angulo P. Nonalcoholic fatty liver disease and liver transplantation. Liver Transpl 2006;12(4):523-534.

6.   Spitzer AL, Lao OB, Dick AA, Bakthavatsalam R, Halldorson JB, Yeh MM et al. The biopsied donor liver: incorporating macrosteatosis into high-risk donor assessment. Liver Transpl 2010;16(7):874-884.

7.   Verran D, Kusyk T, Painter D, Fisher J, Koorey D, Strasser S et al. Clinical

experience gained from the use of 120 steatotic donor livers for orthotopic liver transplantation. Liver Transpl 2003;9(5):500-505.

8.   Dutkowski P, Schlegel A, Slankamenac K, Oberkofler CE, Adam R, Burroughs AK et al. The use of fatty liver grafts in modern allocation systems: risk assessment by the balance of risk (BAR) score. Ann Surg 2012;256(5):861-868; discussion 868-869.

9.   Baccarani U, Isola M, Adani GL, Avellini C, Lorenzin D, Rossetto A et al. Steatosis of the hepatic graft as a risk factor for post-transplant biliary complications. Clin Transplant 2010;24(5):631-635.

10.   Tashiro H, Kuroda S, Mikuriya Y, Ohdan H. Ischemia–reperfusion injury in patients with fatty liver and the clinical impact of steatotic liver on hepatic surgery. Surg Today 2014;44(9):1611-1625.

11.   Fukumori T, Ohkohchi N, Tsukamoto S, Satomi S. The mechanism of injury in a steatotic liver graft during cold preservation. Transplantation 1999;67(2):195-200.

12.   Francque S, Laleman W, Verbeke L, Van Steenkiste C, Casteleyn C, Kwanten W et al. Increased intrahepatic resistance in severe steatosis: endothelial dysfunction, vasoconstrictor overproduction and altered microvascular architecture. Lab Invest 2012;92(10):1428-1439.

13.   Selzner N, Selzner M, Jochum W, Amann-Vesti B, Graf R, Clavien PA. Mouse

livers with macrosteatosis are more susceptible to normothermic ischemic injury than those with microsteatosis. J Hepatol 2006;44(4):694-701.

14. Caraceni P, Bianchi C, Domenicali M, Maria Pertosa A, Maiolini E, Parenti Castelli G et al. Impairment of mitochondrial oxidative phosphorylation in rat fatty liver exposed to preservation-reperfusion injury. J Hepatol 2004;41(1):82-88.

15. Soltys K, Dikdan G, Koneru B. Oxidative stress in fatty livers of obese Zucker rats: rapid amelioration and improved tolerance to warm ischemia with tocopherol. Hepatology 2001;34(1):13-18.

16. Nardo B, Caraceni P, Pasini P, Domenicali M, Catena F, Cavallari G et al. Increased generation of reactive oxygen species in isolated rat fatty liver during postischemic reoxygenation. Transplantation 2001;71(12):1816-1820.

17. Dara L, Ji C, Kaplowitz N. The contribution of endoplasmic reticulum stress to liver diseases. Hepatology 2011;53(5):1752-1763.

18. Selzner M, Rüdiger HA, Sindram D, Madden J, Clavien PA. Mechanisms of ischemic injury are different in the steatotic and normal rat liver. Hepatology 2000;32(6):1280-1288.

19. Guarrera JV, Karim NA. Liver preservation: is there anything new yet? Curr Opin Organ Transplant 2008;13(2):148-154.

20.   Vogel T, Brockmann JG, Coussios C, Friend PJ. The role of normothermic extracorporeal perfusion in minimizing ischemia reperfusion injury. Transplant Rev (Orlando) 2012;26(2):156-162.

21.   Tolboom H, Pouw RE, Izamis ML, Milwid JM, Sharma N, Soto-Gutierrez A et al. Recovery of warm ischemic rat liver grafts by normothermic extracorporeal perfusion. Transplantation 2009;87(2):170-177.

22.   Brockmann J, Reddy S, Coussios C, Pigott D, Guirriero D, Hughes D et al. Normothermic perfusion: a new paradigm for organ preservation. Ann Surg 2009;250(1):1-6.

23.   Tolboom H, Izamis ML, Sharma N, Milwid JM, Uygun B, Berthiaume F et al. Subnormothermic machine perfusion at both 20°C and 30°C recovers ischemic rat livers for successful transplantation. J Surg Res 2012;175(1):149-156.

24.   Berendsen TA, Bruinsma BG, Lee J, D'Andrea V, Liu Q, Izamis ML et al. A simplified subnormothermic machine perfusion system restores ischemically damaged liver grafts in a rat model of orthotopic liver transplantation. Transplant Res 2012;1(1):6.

25.   Bruinsma BG, Yeh H, Ozer S, Martins PN, Farmer A, Wu W et al. Subnormothermic machine perfusion for ex vivo preservation and recovery of the human liver for transplantation. Am J Transplant 2014;14(6):1400-1409.

26.   Vairetti M, Ferrigno A, Carlucci F, Tabucchi A, Rizzo V, Boncompagni E et al. Subnormothermic machine perfusion protects steatotic livers against preservation injury: a potential for donor pool increase? Liver Transpl 2009;15(1):20-29.

27.   Delzenne NM, Hernaux NA, Taper HS. A new model of acute liver steatosis induced in rats by fasting followed by refeeding a high carbohydrate-fat free diet. Biochemical and morphological analysis. J Hepatol 1997;26(4):880-885.

28.   Minor T, Akbar S, Tolba R, Dombrowski F. Cold preservation of fatty liver grafts: prevention of functional and ultrastructural impairments by venous oxygen persufflation. J Hepatol 2000;32(1):105-111.

29.   Karangwa SA, Dutkowski P, Fontes P, Friend PJ, Guarrera JV, Markmann JF et al. Machine Perfusion of Donor Livers for Transplantation: A Proposal for Standardized Nomenclature and Reporting Guidelines. Am J Transplant 2016.

30.   Bessems M, Doorschodt BM, van Vliet AK, van Gulik TM. Improved rat liver preservation by hypothermic continuous machine perfusion using polysol, a new, enriched preservation solution. Liver Transpl 2005;11(5):539-546.

31.   Hata K, Tolba RH, Wei L, Doorschodt BM, Büttner R, Yamamoto Y et al. Impact of polysol, a newly developed preservation solution, on cold storage of steatotic rat livers. Liver Transpl 2007;13(1):114-121.

32.   Bessems M, 't Hart NA, Tolba R, Doorschodt BM, Leuvenink HG, Ploeg RJ et al. The isolated perfused rat liver: standardization of a time-honoured model. Lab Anim 2006;40(3):236-246.

33.   Ashkanian M, Borghammer P, Gjedde A, Ostergaard L, Vafaee M. Improvement of brain tissue oxygenation by inhalation of carbogen. Neuroscience 2008;156(4):932-938.

34.   Vajdová K, Smreková R, Kukan M, Lutterová M, Wsólová L. Bile analysis as a tool for assessing integrity of biliary epithelial cells after cold ischemia--reperfusion of rat livers. Cryobiology 2000;41(2):145-152.

35.   Eriksson S, Fraser JR, Laurent TC, Pertoft H, Smedsrød B. Endothelial cells are a site of uptake and degradation of hyaluronic acid in the liver. Exp Cell Res 1983;144(1):223-228.

36.   Imber CJ, St Peter SD, Handa A, Friend PJ. Hepatic steatosis and its relationship to transplantation. Liver Transpl 2002;8(5):415-423.

37.   Graham JA, Guarrera JV. "Resuscitation" of marginal liver allografts for transplantation with machine perfusion technology. J Hepatol 2014;61(2):418-431.

38.   Guarrera JV, Henry SD, Samstein B, Odeh-Ramadan R, Kinkhabwala M, Goldstein MJ et al. Hypothermic machine preservation in human liver transplantation:

the first clinical series. Am J Transplant 2010;10(2):372-381.

39.   Dutkowski P, Polak WG, Muiesan P, Schlegel A, Verhoeven CJ, Scalera I et al. First Comparison of Hypothermic Oxygenated PErfusion Versus Static Cold Storage of Human Donation After Cardiac Death Liver Transplants: An International-matched Case Analysis. Ann Surg 2015;262(5):764-771.

40.   Bessems M, Doorschodt BM, Kolkert JL, Vetelainen RL, van Vliet AK, Vreeling H et al. Preservation of steatotic livers: a comparison between cold storage and machine perfusion preservation. Liver Transpl 2007;13(4):497-504.

41.   Dutkowski P, Furrer K, Tian Y, Graf R, Clavien PA. Novel short-term hypothermic oxygenated perfusion (HOPE) system prevents injury in rat liver graft from non-heart beating donor. Ann Surg 2006;244(6):968-976; discussion 976-967.

42.   Schlegel A, Kron P, Graf R, Dutkowski P, Clavien PA. Warm vs. cold perfusion techniques to rescue rodent liver grafts. J Hepatol 2014;61(6):1267-1275.

43.   Sutton ME, op den Dries S, Karimian N, Weeder PD, de Boer MT, Wiersema-Buist J et al. Criteria for viability assessment of discarded human donor livers during ex vivo normothermic machine perfusion. PLoS One 2014;9(11):e110642.

44.   Mergental H. Normothermic perfusion of the liver. Liver Transpl 2015;21 Suppl 1:S6-7.

45.   Perera T, Mergental H, Stephenson B, Roll GR, Cilliers H, Liang R et al. First human liver transplantation using a marginal allograft resuscitated by normothermic machine perfusion. Liver Transpl 2016;22(1):120-124.

46.   Pottecher J, Santelmo N, Noll E, Charles AL, Benahmed M, Canuet M et al. Cold ischemia with selective anterograde in situ pulmonary perfusion preserves gas exchange and mitochondrial homeostasis and curbs inflammation in an experimental model of donation after cardiac death. Transpl Int 2013;26(10):1027-1037.

47.   Kim JS, Wang JH, Lemasters JJ. Mitochondrial permeability transition in rat hepatocytes after anoxia/reoxygenation: role of Ca2+-dependent mitochondrial formation of reactive oxygen species. Am J Physiol Gastrointest Liver Physiol 2012;302(7):G723-731.

48.   Seifalian AM, Piasecki C, Agarwal A, Davidson BR. The effect of graded steatosis on flow in the hepatic parenchymal microcirculation. Transplantation 1999;68(6):780-784.

49.   Ikeda T, Yanaga K, Kishikawa K, Kakizoe S, Shimada M, Sugimachi K. Ischemic injury in liver transplantation: difference in injury sites between warm and cold ischemia in rats. Hepatology 1992;16(2):454-461.

50.   Caldwell-Kenkel JC, Currin RT, Tanaka Y, Thurman RG, Lemasters JJ.

Reperfusion injury to endothelial cells following cold ischemic storage of rat livers. Hepatology 1989;10(3):292-299.

51.   Dutkowski P, de Rougemont O, Clavien PA. Machine perfusion for 'marginal' liver grafts. Am J Transplant 2008;8(5):917-924.

52.   Liu Q, Nassar A, Farias K, Buccini L, Mangino MJ, Baldwin W et al. Comparing Normothermic Machine Perfusion Preservation With Different Perfusates on Porcine Livers From Donors After Circulatory Death. Am J Transplant 2015.

53.   Fontes P, Lopez R, van der Plaats A, Vodovotz Y, Minervini M, Scott V et al. Liver preservation with machine perfusion and a newly developed cell-free oxygen carrier solution under subnormothermic conditions. Am J Transplant 2015;15(2):381-394.

54.   Yang H, Wang H, Czura CJ, Tracey KJ. HMGB1 as a cytokine and therapeutic target. J Endotoxin Res 2002;8(6):469-472.
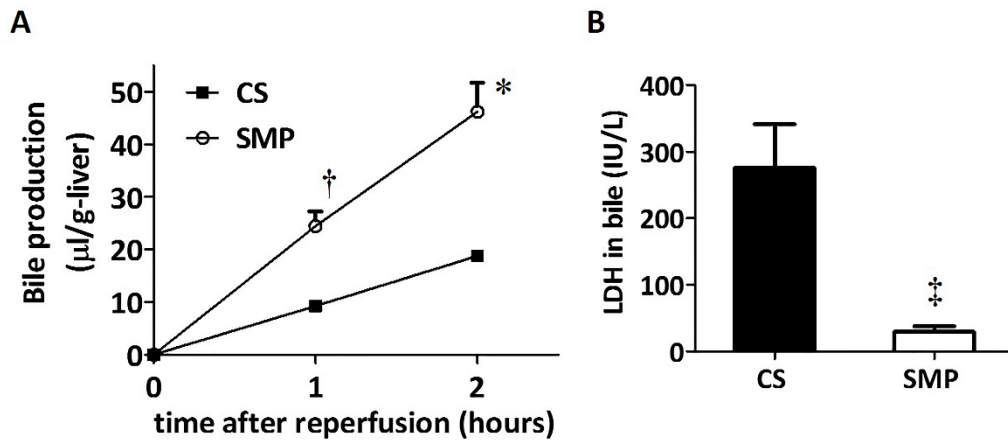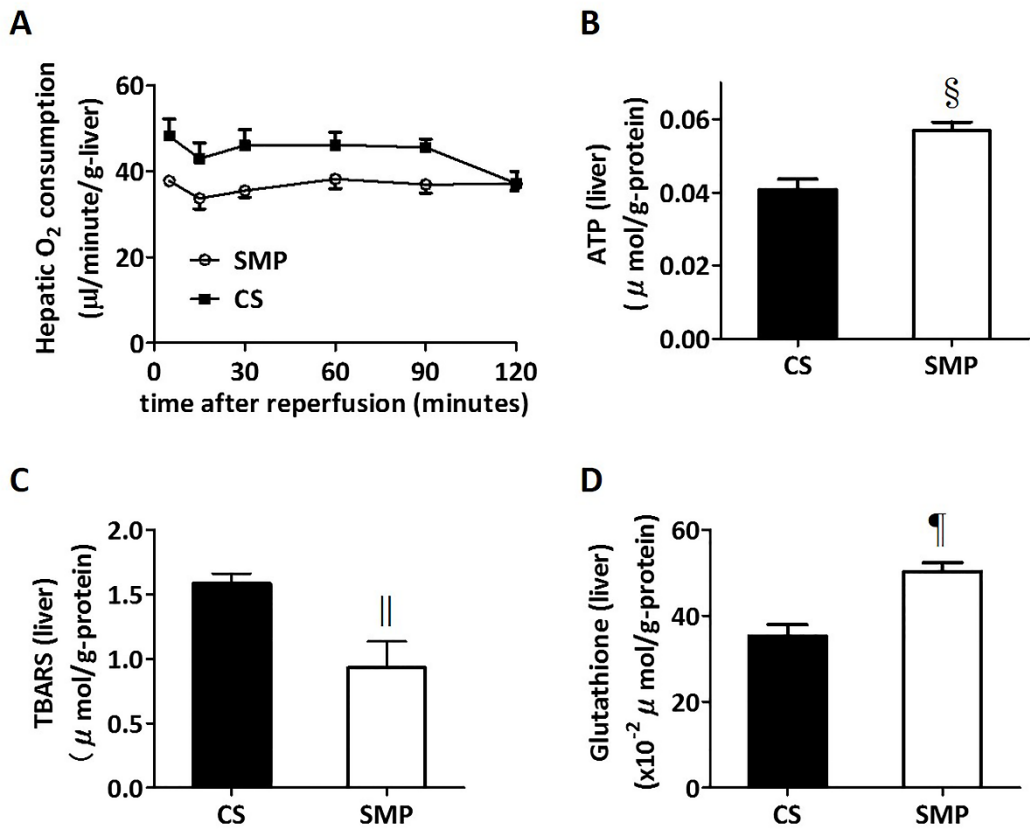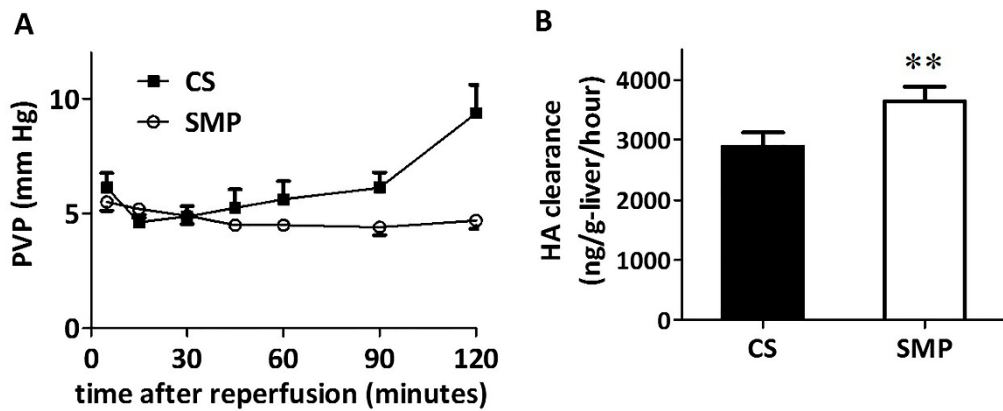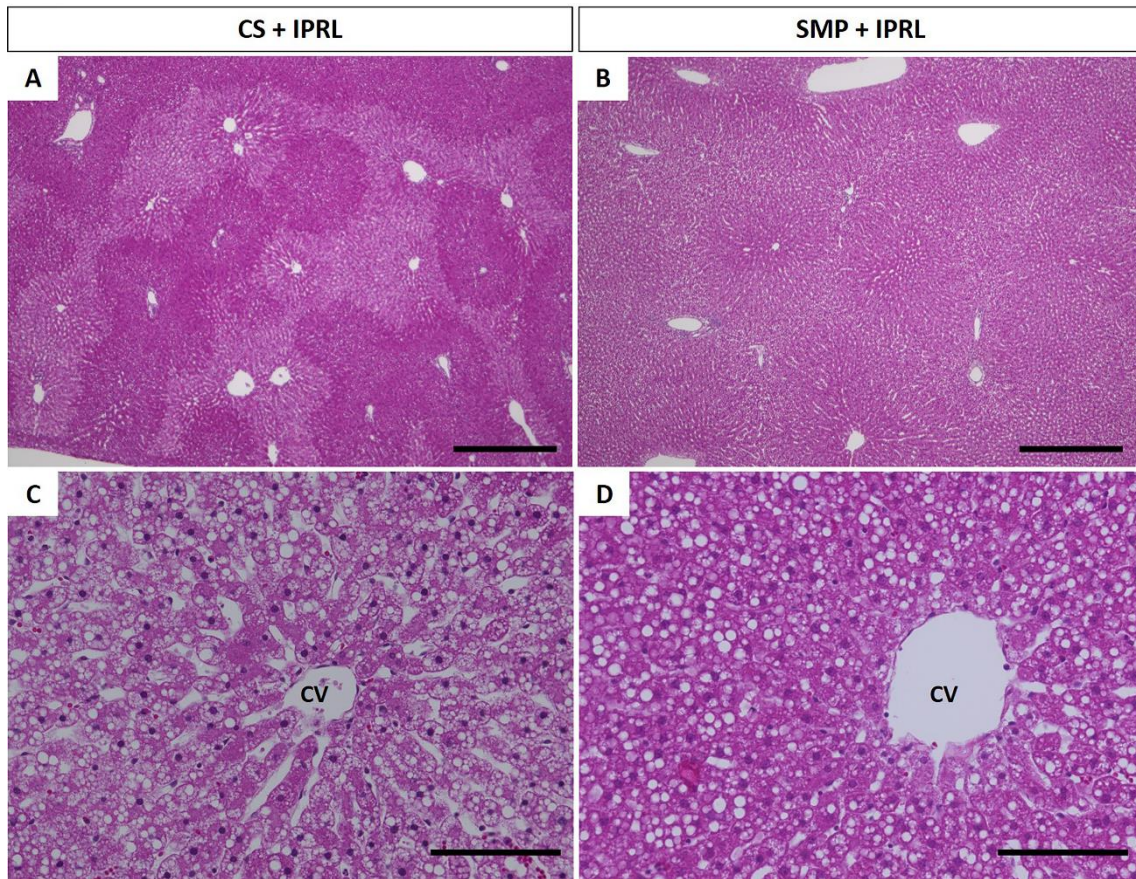
Figure 1
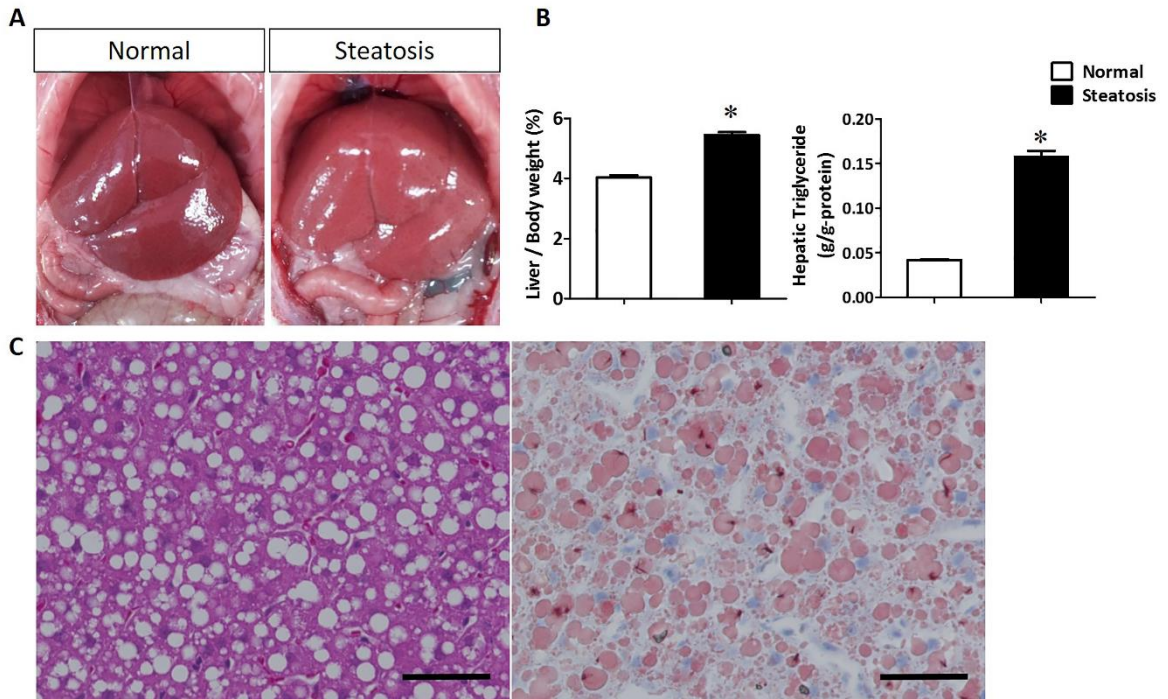
Figure 2
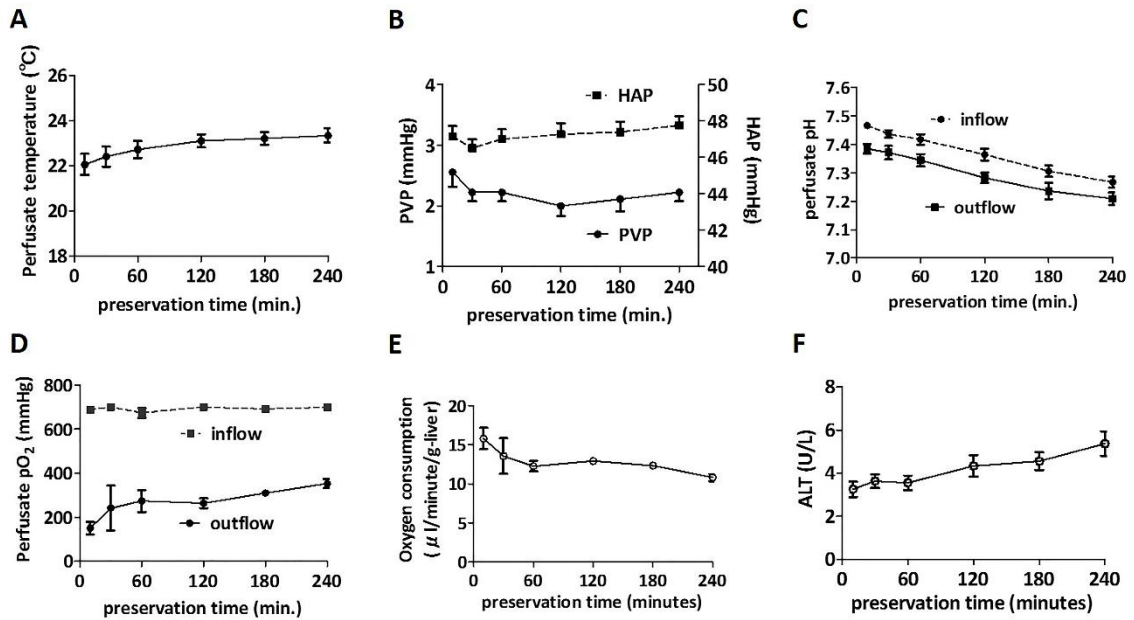
Figure 3
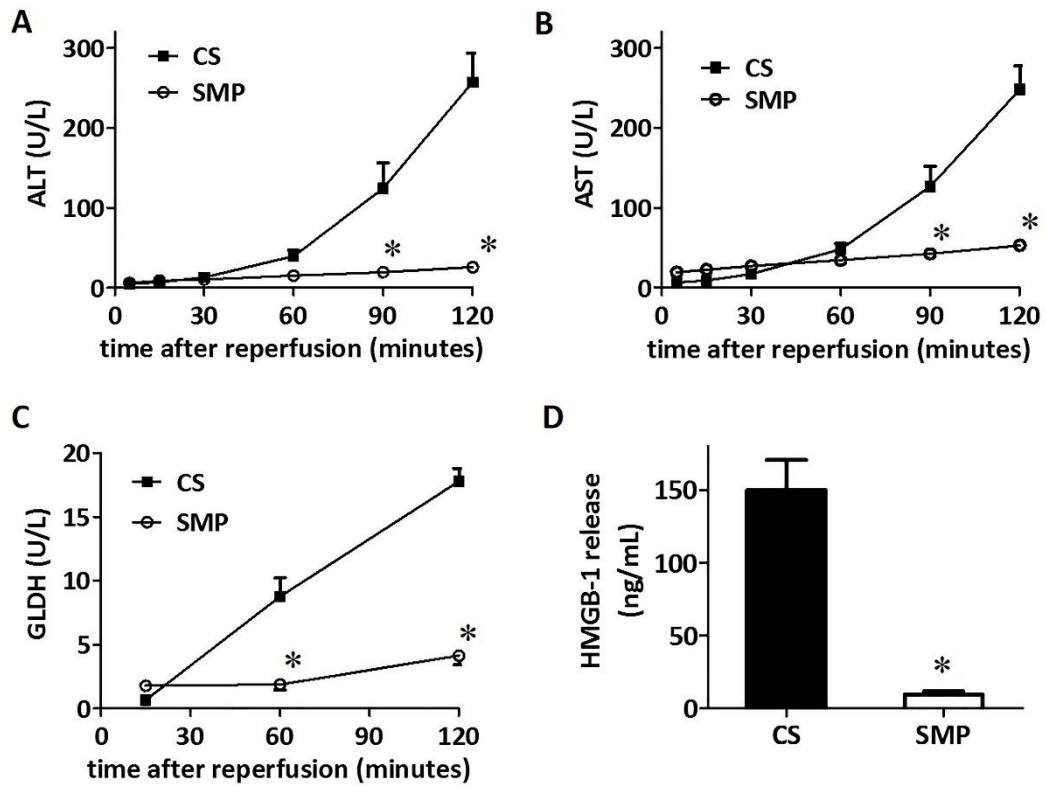
Figure 4

Figure 5

Figure 6

Figure 7

CS + IPRL	SMP + IPRL

Figure 8

Figure 9