

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 ($5.45 \pm 0.09\%$ vs. $4.03 \pm 0.08\%$, $p < 0.001$, 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™, 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™, 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%O₂+5%CO₂) was used for oxygenation, and the inflow pO₂ 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 pO₂ 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 IPR 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 ($\mu\text{L}/\text{minute}/\text{g-liver}$) = portal flow $\times$ S $\times$ ($\text{pO}_{2\text{inflow}} - \text{pO}_{2\text{outflow}}$)/g-liver (S, solubility constant of oxygen in water at 37°C = 0.031 $\mu\text{L}/\text{mL}/\text{mmHg}$).

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- μ m 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 postfixated with OsO₄ (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 postfixated with OsO₄, 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 $\pm$ 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 $22.5\pm 1^{\circ}\text{C}$ 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 ($3.3\pm 0.1\text{ }\mu\text{L/g-liver/hour}$).

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. $149.7\pm 20.8\text{ ng/mL}$,

$p<0.001$, 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 $\mu\text{L/g-liver/2 hours}$, $p<0.001$, 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 $\mu\text{mol/g-protein}$, $p=0.001$, 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 $\mu\text{mol/g-protein}$, $p=0.037$, 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 $\mu\text{mol/g-protein}$, $p=0.011$, 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 ± 1.4 mmHg 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

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

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 alloreaactions, 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

The authors thank AY PHARMACEUTICALS CO., LTD. (Tokyo, Japan) for their skillful cooperation in preparing the Polysol solution and are grateful for the support of the Center for Anatomical, Pathological, and Forensic Medical Research, Graduate School of Medicine, Kyoto University. This work was supported by Grants-in-Aid for Scientific Research B (K.H. and S.U., No. 26293287) and Challenging Exploratory Research (K.H. and S.U., No.25670555) from the Japan Society for the Promotion of Science, Tokyo, Japan.

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 μ m.

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 ($^{\circ}$ 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

outflow; (D) Perfusate pO₂ (mm Hg) in both the inflow and the outflow; (E) Oxygen consumption rate ($\mu\text{L}/\text{minute}/\text{g-liver weight}$), 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 $\pm$ 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 $\pm$ 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 $\pm$ 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 $\pm$ 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 $\pm$ 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 $\pm$ 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 $\pm$ 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 $\pm$ 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 $\pm$ 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 assessments 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 $\times 4,000$.

(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 $\times 4,000$.

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, Wsóllová 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 antegrade 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 Ca^{2+} -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

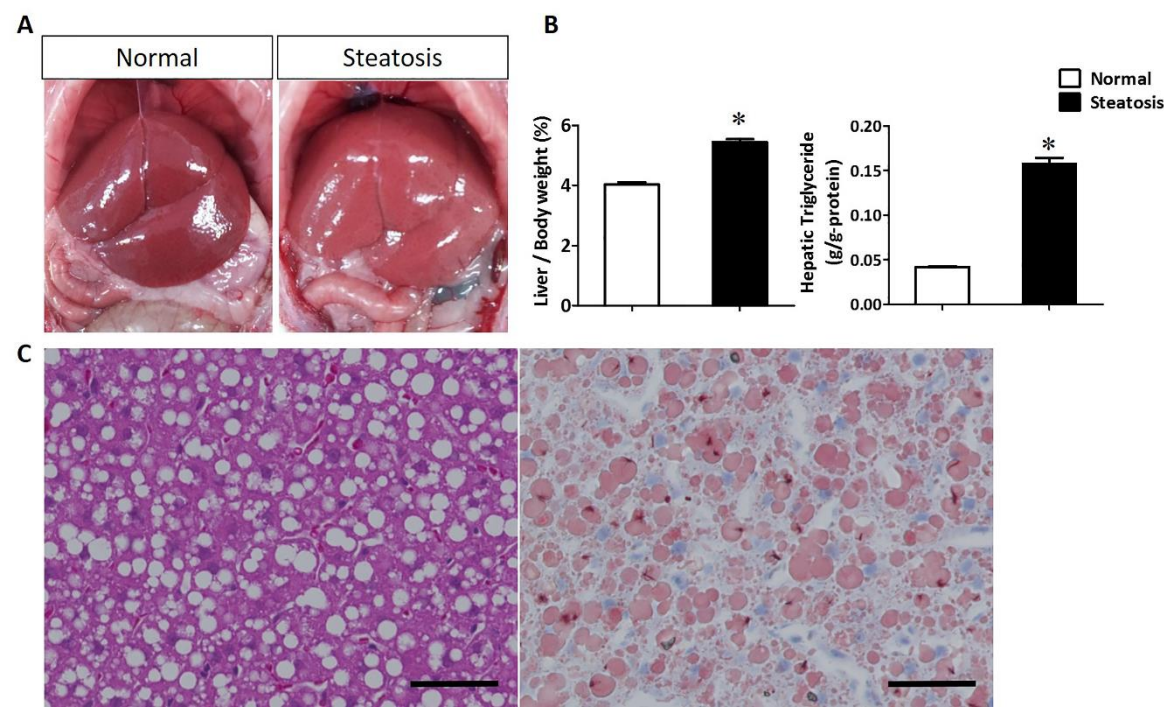


Figure 2

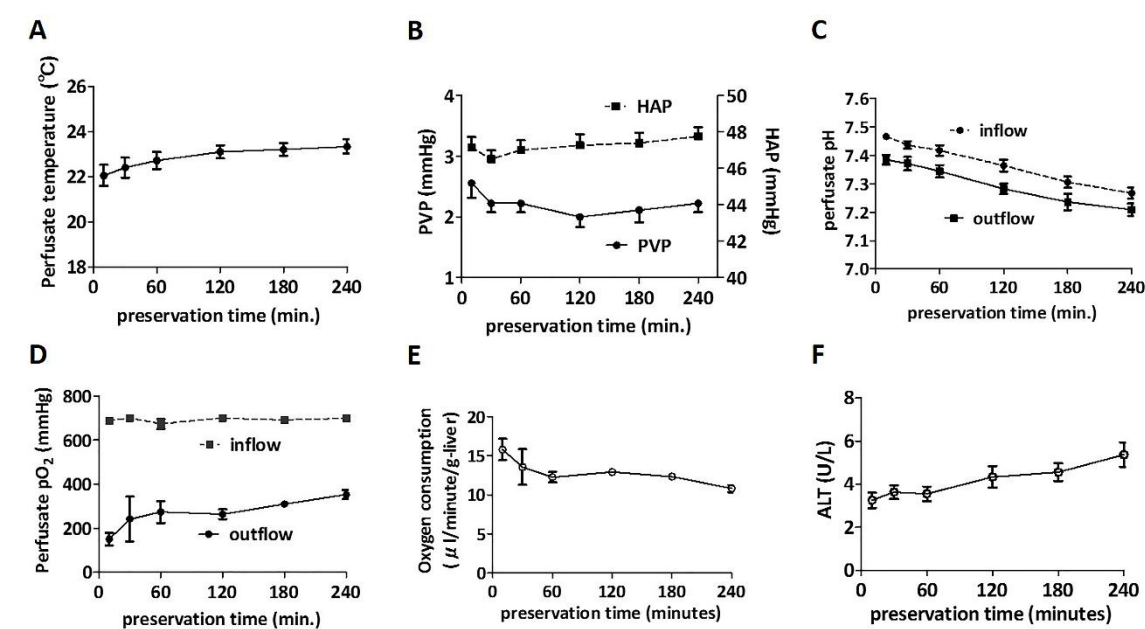


Figure 3

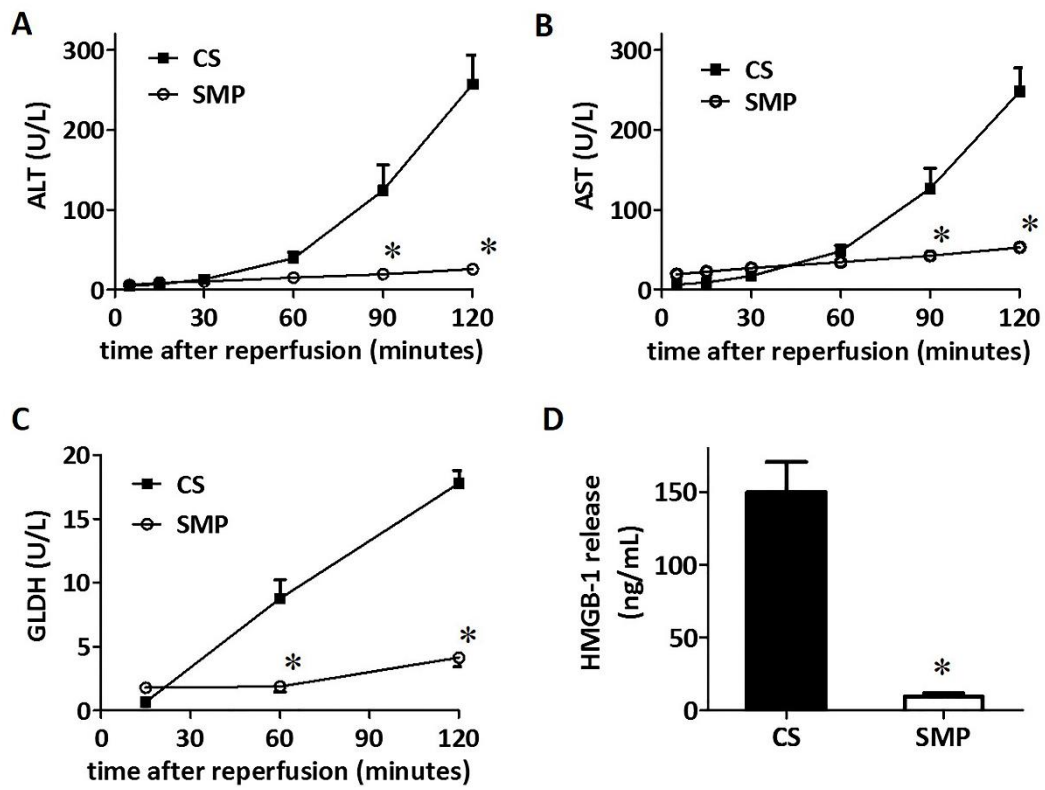


Figure 4

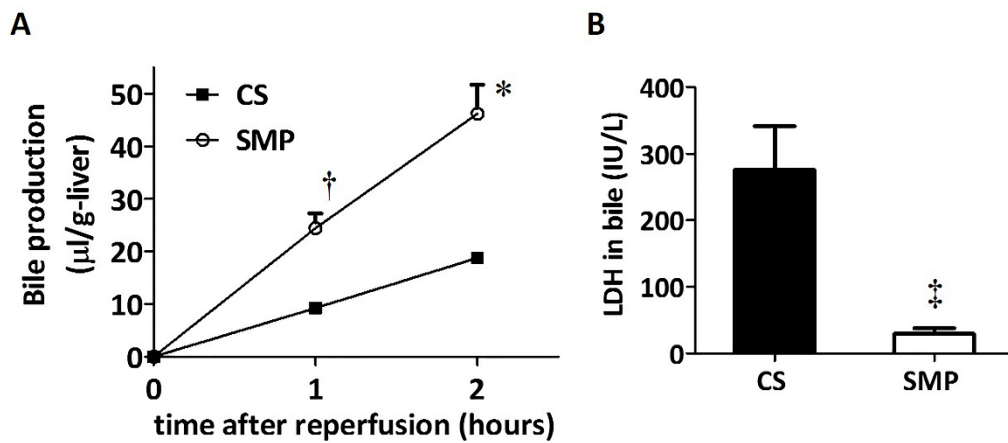


Figure 5

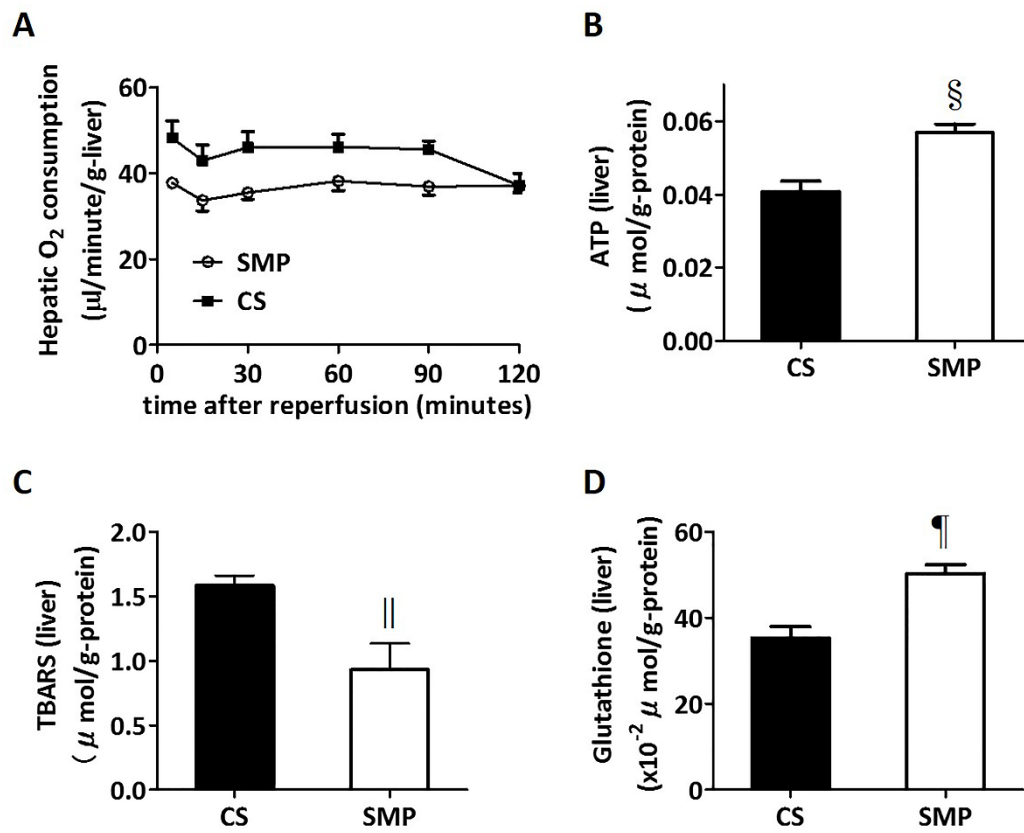


Figure 6

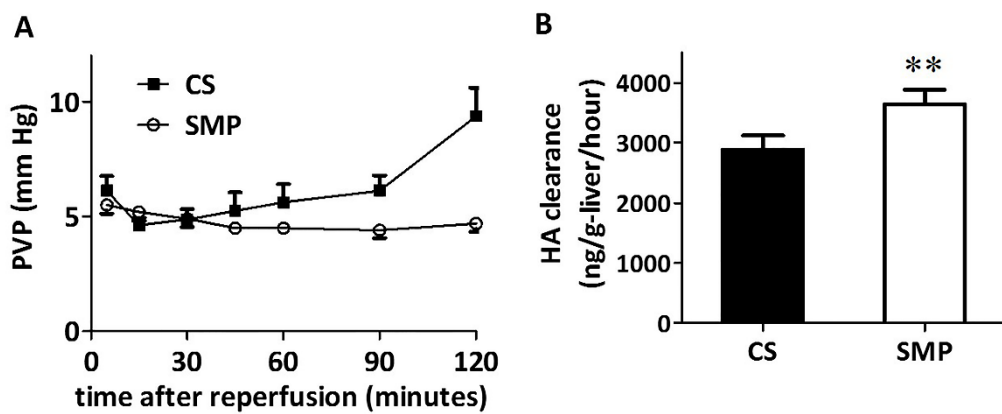


Figure 7

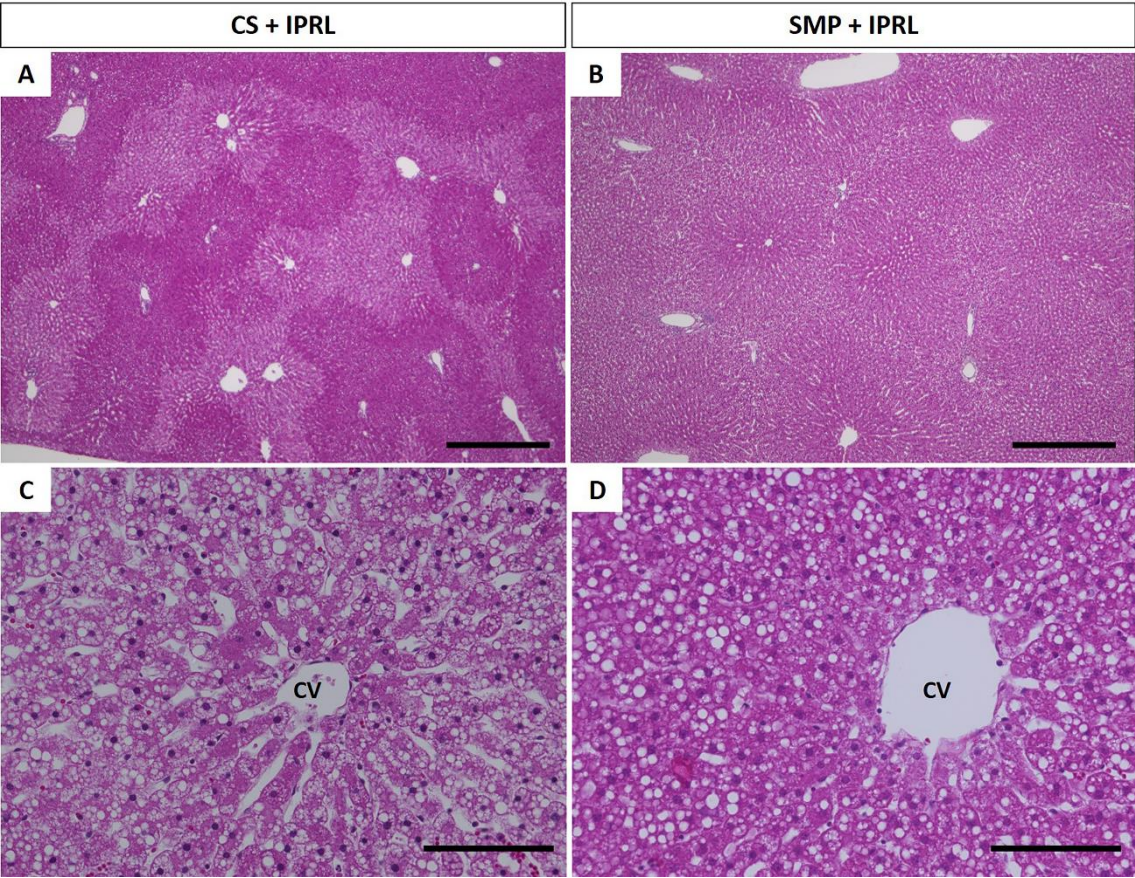


Figure 8

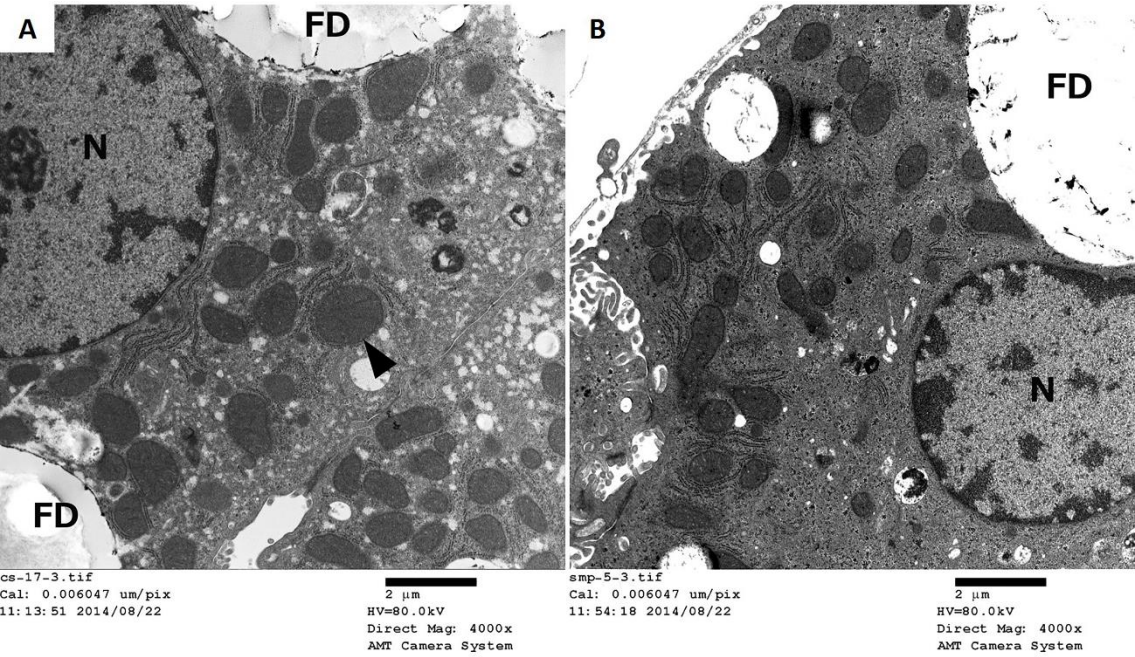


Figure 9

