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主論文

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Title page

Hydrogen Flush After Cold Storage (HyFACS), as a new end-ischemic *ex vivo* treatment for liver grafts against ischemia/reperfusion injury

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Keywords: liver transplantation; organ preservation; isolated perfused rat liver; extracorporeal therapy

Footnote page

Abbreviations

ALT, alanine aminotransferase; ANOVA, analysis of variance; AST, aspartate aminotransferase; CEACAM1, carcinoembryonic antigen related cell adhesion molecule 1; CS, cold storage; DAMPs, Damage-associated molecular patterns; DCD, donation after cardiac death; ER, endoplasmic reticula; GLDH, Glutamate dehydrogenase; GSH, glutathione-SH; GSSG, glutathione disulfide; HA, hepatic artery; HMGB-1, high mobility group box protein-1; HyFACS, hydrogen flush after cold storage; IPRL, isolated perfused rat liver; H_2, molecular hydrogen; IRI, ischemia/reperfusion injury; KHB, Krebs-Henselite buffer; LDH, lactate dehydrogenase; MP, machine perfusion; SECs, sinusoidal endothelial cells; LT, liver transplantation; PV, portal vein; PVP, portal vein pressure; ROS, reactive oxygen species; SEM, scanning electron microscope; TBARS, thiobarbituric acid reactive substance; TEM, transmission electron microscope; UWS, University of Wisconsin solution; 8-OHdG, 8-hydroxy-2-deoxyguanosine

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Abstract

Cold storage (CS) remains the gold standard for organ preservation worldwide, though it is inevitably associated with cold-ischemia/warm-reperfusion injury (IRI). Molecular hydrogen (H_2) is well-known to have anti-oxidative properties, however, its unfavorable features, i.e. inflammability, low solubility, and high tissue/substance permeability, have hampered its clinical application. To overcome such obstacles, we developed a novel reconditioning method for donor organs, named HyFACS (hydrogen flush after cold storage), just an end-ischemic H_2-flush directly to donor organs *ex vivo*, and herein report its therapeutic impact against hepatic IRI.

Whole liver grafts were retrieved from Wistar rats. After 24-hour CS in UW solution, livers were cold-flushed with H_2-solution (1.0ppm) via portal vein (HyFACS-PV), or hepatic artery (HyFACS-HA), or both (HyFACS-PV+HA). Functional integrity and morphological damages were then evaluated by 2-hour oxygenated reperfusion at 37°C.

HyFACS significantly lowered portal-venous pressure, transaminase, and HMGB-1 release compared to vehicle-treated controls (P<0.01). Hyaluronic-acid clearance was significantly higher in HyFACS-PV and -PV+HA than in the others (P<0.01), demonstrating the efficacy of PV route to maintain sinusoidal endothelia. In contrast, bile production and

LDH leakage therein were both significantly improved in HyFACS-HA and -PV+HA (P<0.01), representing the superiority of arterial route to attenuate biliary damage. Consistently, electron microscopy revealed sinusoidal ultra-structures were well-maintained by portal HyFACS, while microvilli in bile canaliculi were well-preserved by arterial flush. As an underlying mechanism, HyFACS significantly lowered oxidative damages, thus improving GSH/GSSG ratio in liver tissue.

Conclusion: HyFACS significantly protected liver grafts from IRI by ameliorating oxidative damages upon reperfusion, in the characteristic manner with its route of administration. Given its safety, simplicity, and cost-effectiveness, end-ischemic HyFACS may be a novel pre-transplant conditioning for cold-stored donor organs.

INTRODUCTION

Ischemia/reperfusion injury (IRI) remains one of *"unmet medical needs"* in solid organ transplantation. For over half a century, the gold standard for organ preservation has long been static cold storage (CS). Although numbers of investigations have been attempted so far to develop effective strategies against this unavoidable process in organ transplantation, few of these have been applied into clinical practice. One of such successful developments may be machine perfusion (MP) preservation. The use of such modern but complexed

methods is, however, still limited to only some leading institutions in several industrialized countries. In fact, there are still many obstacles disturbing widespread popularization of such novel techniques, including high medical costs, special equipment/machines, and a number of professional technicians (4). Thus, the universal standard for organ preservation remains CS in the world. To expand the donor pools available, and to alleviate the critical shortage of donor organs, there has been a definite need for developing new strategies to improve CS.

Molecular hydrogen (H_2) is ubiquitous gas, well-known to possess anti-oxidative properties. Since the first report in 2007 (5), accumulating evidence has demonstrated its therapeutic potential against IRI, and the underlying mechanisms therein. Currently, H_2 is well-known to have three definitive effects: anti-oxidant (5-13), anti-inflammatory (10, 14-16), and anti-apoptosis (8, 17). Though some experimental animal studies have shown its therapeutic potential in organ preservation, e.g. lung (11), heart (18), kidney (6), intestine (7), and liver (19, 20), its clinical translation is yet to be applied due to its unfavorable features, as mentioned below.

Besides its remarkable therapeutic potential, H_2 has several characteristic burdens that need to be overcome before clinical application: inflammability (21); low solubility (22); and high tissue/substance permeability (22, 23). First, gaseous H_2 is potentially inflammable,

and 10% seems to be the safety limit for preventing explosion (24). Ideally, less than 4% seems preferable to secure patients' as well as medical staffs' safety (22, 25). Although aqueous H_2 solution is safe and convenient for handling (21), its solubility is very low, and the maximal concentration under 1atm is just 1.6 ppm (26). In addition, high tissue/substance permeability of H_2 is another hurdle. H_2 easily diffuses and is rapidly lost by spreading across any substance, just except for aluminum. Moreover, a recent report demonstrated that neither intravenous, per-oral, nor inhalational administration resulted in sufficient H_2 delivery to the liver (23), because of its easy diffusion to upstream organs (gut) or surrounding tissues before reaching the liver. Such molecular, as well as biological characteristics of H_2 have hampered its clinical application, and development of some innovative, game-changing approach in H_2 delivery is necessary to apply its remarkable therapeutic potential into clinical practice.

Donor organs for transplantation are inevitably exposed to *ex vivo* environment between procurement from donors and implantation to recipients, consequently providing an unique therapeutic window for extracorporeal treatment of organs. We thus intended to take advantage of this timing, and hypothesized that *ex vivo* administration of H_2 solution directly to donor organs may solve the difficulties of H_2 delivery to the liver. This study was thus designed to investigate the therapeutic potential of Hydrogen Flush After Cold Storage

(HyFACS), as a new *ex vivo* treatment for liver grafts, with special interest to its route of administration via portal vein (PV), hepatic artery (HA) or from the both.

MATERIALS AND METHODS

Animals

All experimental protocols were approved by the animal research committee of Kyoto University, and all animals received humane care according to the Guide for the Care and Use of Laboratory animals (National Institutes of Health Publication 86-23, revised 1985). Male Wistar rats (270–320g, 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 were allowed free access to tap water and standard chow pellets *ad libitum*.

Preparation of H_2 solution

H_2 was dissolved in normal saline by the non-destructive hydrogen dissolving method, as detailed previously (22). Briefly, a solution bag (polyethylene) and hydrogen-generating agent ($Ca[OH]_2$ mixed with Aluminum powder; MiZ Co., Ltd.,

Kamakura, Japan) were put together in an aluminum pouch and sealed tightly (Fig.1-A and -B). Importantly, H_2 gradually permeates the solution bag, dissolved into the solution inside, then reaching the plateau concentration of 1.0 ppm by 24 hours. This easy and simple method enables us to prepare H_2 solution, safely, aseptically, and inexpensively (< three US dollars/bag) (22). We prepared H_2-solution each time, and its concentration was measured via an electrochemical gas sensor (model DHD1-1, DKK-TOA Corp., Tokyo, Japan), which was strictly maintained at 1 ppm (0.95 ± 0.02 ppm) throughout the experiments.

Liver procurement

Whole liver grafts were retrieved, as detailed elsewhere (27, 28). Briefly, rats were anesthetized with isoflurane (Escain, Mylan, Osaka, Japan) via a small animal anesthetizer (MK- A110; Muromachi Kikai Co., Ltd., Tokyo, Japan). After heparinization (200 IU/rat; Mochida Pharmaceutical Co., Ltd., Tokyo, Japan), common bile duct was cannulated with a 24-gauge (G) polyethylene tube (TERUMO, Tokyo, Japan). A 14-G catheter (Argyle, COVIDIEN, Tokyo, Japan) was inserted into the PV trunk, followed by a blood washout with 40mL of ice-cold University of Wisconsin (UW) solution (Viaspan, Astellas, Tokyo, Japan). A 24-G polyethylene tube (TERUMO, Tokyo, Japan) was inserted into the hepatic artery

through the celiac trunk. The suprahepatic caval vein was cannulated with a 14-G short stent. The liver was flushed with 20mL of ice-cold UW immediately, and then preserved for 24 hours at 4°C in 40mL of UW. Sham-operated rats underwent the same procedure but not cold-stored, and directly subjected to isolated *ex vivo* perfusion for reference.

Experimental groups

After 24-hour CS, the livers were randomly assigned into four groups, and flushed with the respective solution at 4°C through an infusion tube (polyvinyl chloride, 35cm in length), as follows (n=10, each):

Control: flushed with 30mL of saline solution (vehicle) via both PV and HA

HyFACS-PV: flushed with 30mL H_2-solution via PV and 30mL vehicle via HA

HyFACS-HA: flushed with 30mL vehicle via PV and 30mL H_2-solution via HA

HyFACS-PV+HA: flushed with 30mL H_2-solution via both PV and HA

Sham livers were also provided for reference, all of which were not cold-stored but immediately subjected to cold flush with 30mL of vehicle via both PV and HA. For PV flushing, the pressure was kept less than 10mmHg by gravity, whereas for HA flushing, < 50mmHg (less than half of physiological arterial pressure) was maintained, monitored by a transducer system (BP-608; Omron Colin Co., Ltd., Tokyo, Japan).

Ex vivo oxygenated warm reperfusion

The isolated perfused rat liver (IPRL) system was used to assess liver damages and their functional integrity, according to the standardized conditions (28, 29). Briefly, oxygenated reperfusion was performed for 120 minutes via PV at a constant flow of 3 mL/g-liver/minute with 150mL of oxygenated Krebs-Henselite buffer (KHB; K3753, Sigma-Aldrich Inc., St Louis, USA) at 37°C. Carbogen (95% O_2 + 5% CO_2) was used for oxygenation, and the prehepatic pO_2 was continuously maintained above 500mmHg. Hyaluronic acid (CAS.9067-32-7, Wako Pure Chemical Industries, Ltd., Japan) was supplemented into KHB (1 mg/L), and perfusate was taken to evaluate transaminase release, oxygen consumption, and hyaluronic-acid clearance. Total bile production and portal-venous perfusion pressure (PVP) was measured throughout reperfusion (28).

Liver enzymes

Hepatic effluent was withdrawn at 10, 30, 60, 90, and 120 minutes of reperfusion, and analyzed for aspartate-aminotransferase (AST), alanine-aminotransferase (ALT), and lactate-dehydrogenase (LDH) levels with a standard spectrophotometric method with an automated clinical analyzer (JCA-BM9030; JEOL, Ltd., Tokyo, Japan).

Glutamate-dehydrogenase (GLDH) was also measured as a parameter for mitochondrial injury upon reperfusion using a GLDH activity colorimetric assay kit (BioVision Inc., Milipitas, CA). LDH leakage into bile was determined as an index for biliary damage (30).

Hepatic oxygen consumption

Perfusate samples were collected from both the inflow and the outflow of the liver, and then immediately analyzed using a blood gas analyzer (Rapid Point 405, Siemens, Tokyo, Japan). The oxygen consumption rates were calculated by the difference between the both samples, given as μL/g-liver/minute according to the transhepatic flow and liver mass (28).

Oxidative stress

Thiobarbituric-acid reactive substance (TBARS) in liver tissue was measured as an index for lipid peroxidation (Colorimetric TBARS Microplate Assay Kit, Oxford Biomedical Research, Inc. USA)(28). Total and oxidized glutathione (GSSG) in liver tissue were both determined (GSSG/GSH quantification kit, Dojindo Molecular Technologies, Inc. USA). Reduced glutathione (GSH) was calculated by subtracting GSSG from total glutathione. These results were normalized with protein concentration, determined by a BCA protein assay (Thermo Fisher Scientific, Yokohama, Japan).

Eight-hydroxy-2-deoxyguanosine (8-OHdG) release into the perfusate was also measured by an enzyme-linked immunosorbent assay (8-OHdG check, Nikken seil Co., Ltd, Shizuoka, Japan).

HMGB-1 release

To quantify comprehensive tissue damage after 2-hour oxygenated reperfusion, high mobility group box protein-1 (HMGB-1), one of the most hazardous DAMPs (damage-associated molecular patterns), into perfusate was determined using a HMGB-1 ELISA Kit II (Shino-Test, Sagamihara, Japan).

Hyaluronic acid clearance

To evaluate the functional integrity of sinusoidal endothelial cells (SECs), hyaluronic-acid clearance was determined. SECs are the main site for uptaking and degrading hyaluronic-acid (31, 32), and in the current isolated setting, hyaluronic-acid production from other organs is completely excluded. Thus, its clearance in IPRL is one of the best indicators for estimating SECs function. Hyaluronic acid was measured with an enzyme-linked protein assay (Corgenix Inc., Colorado, USA), and the clearance rate was calculated from the difference between the inflow and outflow, provided as ng/g-liver/hour.

Tissue ATP contents

At the end of oxygenated reperfusion, liver samples were snap-frozen and stored in liquid nitrogen. Tissue adenosine triphosphate (ATP) contents were determined by Luciferin-luciferase reaction with an ATP bioluminescence kit (Toyo B-Net CO., Ltd., Tokyo, Japan). The results were normalized to protein concentration, measured by a BCA protein assay kit (Thermo Fischer Scientific).

Immunohistochemistry for CEACAM-1

Immunohistochemical staining for carcinoembryonic antigen-related cell adhesion molecule-1 (CEACAM-1) was performed to assess the integrity of bile canaliculi (33, 34). As described previously, CEACAM-1 is highly-expressed in bile canaliculi in the liver of rodents and human, and recognized as one of the most sensitive parameters for early diagnosis of cholangiopathy in various pathological conditions (34). After antigen retrieval, quenching, and blocking, the paraffin sections were incubated with the primary mouse anti-Rat CEACAM-1 antibody (clone11-1H, Merck, Darmstadt, Germany), followed by incubation with the second antibody. The stains were visualized with DAB (3,3'-diaminobenzidine tetrahydrochloride) solution, counterstained with hematoxylin, and

observed with a BZ-9000 microscope (Keyence, Osaka, Japan). Negative controls were incubated with non-immunized first antibody. The stained area was quantified by using Image J software (NIH, USA).

Electron microscopy

At the end of reperfusion, additional livers were perfused though PV with 2% glutaraldehyde/4% paraformaldehyde in 0.1M phosphate buffer, pH 7.4. After fixation, the samples were cut into 2mm cubes for transmission electron microscope (TEM), and 1mm slices for scanning electron microscope (SEM) analysis. TEM samples were postfixed with OsO_4 (osmic acid fixative), dehydrated, embedded in epoxy resin, and heat-polymerized at 60°C before observation with H-7650 TEM (Hitachi High-Technologies, Tokyo, Japan). After postfixation with OsO_4, SEM samples were dehydrated, substituted, freeze-dried with t-butyl-alcohol, ion-sputter-coated, and then examined by two independent pathologists in a blind fashon with S-4700 SEM (Hitachi High-Technologies).

Statistical analysis

All statistical analyses were performed among the four groups (Control, HyFACS-PV, -HA, and –PV+HA). Sham-group was prepared just for reference to estimate the inhibitory/recovery rate caused by prolonged CS, or by HyFACS treatments. All results are expressed as the mean ± standard error of the mean (SEM), unless otherwise indicated. Comparisons among the four groups were performed with one-way analysis of variance (ANOVA). Two-way repeated-measures ANOVA followed by Bonferroni's post-test was used to assess time-dependent alterations and differences among the groups at each time point. *P*-value <0.05 was considered statistically significant. All calculations were performed with Prism 6 (Graph Pad Software, Inc., San Diego, CA, USA).

RESULTS

Confirmation of H_2 concentration in the flushing solution

First, we determined H_2 concentration in the flushing solution just before and after the liver (inflow and outflow, respectively). Fig.1-C shows the H_2 concentration in both the inflow and the outflow in HyFACS-PV group, indicating 1) the H_2 loss before reaching the liver was minimal; and 2) almost all H_2 delivered by HyFACS was absorbed by liver tissues.

Hepatocellular damage upon reperfusion

AST release was significantly lower in all the HyFACS groups than that in Group-Control (P <0.001, Fig.2-A). ALT (P <0.01, Fig.2-B), as well as LDH (P <0.01, Fig.2-C) also showed significant decrease throughout reperfusion, indicating significantly less hepatocellular damage, achieved by any route of HyFACS.

Cumulative HMGB-1 release during oxygenated reperfusion was significantly reduced in all the HyFACS groups than in Control (P <0.001, Fig.2-D), demonstrating significantly-less comprehensive tissue damage achieved by HyFACS.

Mitochondrial integrity and ATP restoration upon reperfusion

Tissue ATP concentration after 2-hour oxygenated reperfusion was significantly higher in HyFACS-PV+HA than in Group-Control (P <0.05, Fig.3-A). However, oxygen consumption rate did not differ among the 4 groups (Fig.3-B), indicating that HyFACS-PV+HA produced significantly better ATP charge upon reperfusion with an equal amount of oxygen uptake, compared with untreated controls. Intra-mitochondrial enzyme, GLDH release, was also significantly lowered in all the HyFACS groups compared with that in the control (P <0.01, Fig.3-C), representing significantly less mitochondrial damage upon

reperfusion. Ultrastructual observation with TEM demonstrated mitochondrial swelling with less electron density in the control livers. Endoplasmic reticula (ER) were also disrupted. In contrast, such deleterious alterations were significantly alleviated in all the HyFACS groups (Fig.3-E to -G).

Functional/Morphological integrity of sinusoidal endothelia

End-ischemic HyFACS significantly lowered PVP, an index for total vascular resistance, in all the treated livers, which was maintained throughout reperfusion (P <0.001, Fig.4-A).

Regarding the functional integrity of sinusoidal endothelia, hyaluronic-acid clearance was more than 7-times higher in HyFACS-PV (880.4 ± 138.6 ng/g-liver/hour) and -PV+HA (928.4 ± 370.5) than in Control (119.1 ± 63.4, P <0.01, Fig.4-B). Of interest, HyFACS-HA (188.0 ± 117.0) could not efficiently protect SECs function, indicating HyFACS via PV route maintained the viability of SECs more effectively rather than via HA.

Consistently, SEM observation revealed apparent damages of fused and enlarged fenestrae on SECs in control livers, whereas sinusoidal linings and fenestrae therein were well maintained in all the HyFACS groups, particularly in HyFACS-PV and –PV+HA (Fig.4-C to -F).

Bile production and biliary damage upon reperfusion

In contrast to hyaluronic-acid clearance, arterial HyFACS (-HA and -PV+HA) significantly increased bile production during 2 hours of reperfusion than in the others (P <0.001, Fig.5-A).

In line, arterial HyFACS significantly lowered LDH leakage into bile compared to those in portal HyFACS and the control (P <0.01, Fig.5-B), demonstrating arterial HyFACS, i.e., direct H_2 infusion to biliary plexus, significantly protects intrahepatic bile ducts from IRI.

As shown in Fig.5-C, SEM observation clearly manifested that only sparse microvilli were left in bile canaliculi, and their height was also very lowered. In contrast, microvilli were well-preserved by arterial HyFACS, where both density and height looked

almost intact (Fig.5-E and -F). Portal HyFACS also better maintained microvilli than in the controls, but worse compared to arterial HyFACS (Fig.5-D).

Immunohistochemical staining for CEACAM1 showed specific staining along with bile canaliculi. In particular, arterial HyFACS (-HA and -HA+PV) maintained dense and thick staining, appearing like "chicken-wire" (Fig.6-C and -D, respectively). In the livers treated by HyFACS-PV, CEACAM1 staining was better preserved than in the controls, but worse than in arterial HyFACS (Fig.6-B). The positively-stained area was significantly larger in HyFACS-HA and -PV+HA than in control livers (P <0.001, Fig.6-F), demonstrating significantly better preserved bile canaliculi by arterial HyFACS.

Oxidative stress and redox status

Though all the HyFACS groups exhibited lower trend of TBARS contents, only HyFACS-HA and -PV+HA showed statistically-significant improvement than in the controls (P <0.01, Fig.7-A). Similarly, 8-OHdG release was significantly lowered by any route of HyFACS than in Control (P <0.01, Fig.7-B).

Total tissue glutathione was significantly reduced in all the HyFACS groups compared with Control (P <0.01, Fig.7-C). Thus, GSH/GSSG ratio (mol/mol) was significantly higher in all the HyFACS groups than in Control (P <0.01, Fig.7-D).

DISCUSSIONS

Molecular hydrogen (H_2) is a ubiquitous but quite characteristic molecule, which effectively scavenges ROS, just yielding harmless H_2O (5, 35). Moreover, H_2 selectively erases hydroxyl-radicals and peroxynitrites, both of which are the most harmful ROS generated upon tissue reoxygenation after ischemia (5, 36). From this perspective, H_2 is likely one of the most efficient, safe, and economical ROS scavengers that exist in nature, without any possible adverse effects to treated cells, tissues, and organs. Hence, numbers of studies have tried its application into organ preservation (7, 18, 37), most of which, however, adopted H_2 dissolving into preservation solution (6, 18, 37) or inhalation to recipients (7, 19, 35). As aforementioned, H_2 easily penetrates any substances, and disappears instantly. Continuous H_2 dissolution or production by water electrolysis (6, 37) during whole preservation period may possibly solve such problems, however, which, in turn, inevitably faces explosion risk or needs some special equipment to prevent easy diffusion of H_2. Moreover, the liver is the largest solid organ in the human body, deeply located in the right

subphrenic space; therefore, sufficient H_2 delivery to transplanted livers *in situ* seems rather difficult compared with other organs (23). Thus, we finally conceived of the direct injection of aqueous H_2 solution into liver grafts *ex vivo*, named HyFACS (Hydrogen Flush after Cold Storage). This single flush with H_2 solution into cold-stored organs eliminates the explosion risk of gaseous H_2, and approximately 1.0ppm concentration (less than the saturated concentration of 1.6ppm at 1atm) could prevent a potential risk for gas embolism within hepatic microvasculature. Furthermore, direct administration into donor organs *ex vivo* could solve the difficulty of delivering sufficient amount of H_2, even in deeply located organs, such as the liver. In fact, almost all H_2 delivered by HyFACS was successfully absorbed in liver tissues, demonstrating the superiority and feasibility of HyFACS, as a new delivering method for applying H_2 in liver transplantation.

Now, the last question arises; when is the suitable timing for this novel treatment? We focused on ROS burst upon blood reflow, and hypothesized that oxidative stress during the first several hours upon reperfusion should have a substantial impact on the viability of transplanted organs thereafter. As expected, we demonstrated for the first time that end-ischemic HyFACS significantly decreased oxidative tissue damage, such as TBARS and 8-OHdG upon reperfusion, thereby maintaining morphological, as well as functional integrity

of liver grafts. Considering that control livers consumed an equal amount of oxygen while exhibiting less ATP restoration upon reperfusion, HyFACS enabled efficient oxygen utilization to restore ATP charge, whereas in controls, substantial amount of oxygen was consumed to promote ROS production. Significant decrease of GLDH release, as well as better-maintained mitochondrial ultra-structure directly reflects significant protection of hepatocellular mitochondria achieved by HyFACS, undoubtedly contributing to better ATP restoration. These results suggest a remarkable potential of end-ischemic HyFACS as a novel therapeutic strategy to reduce initial oxidative damages to transplanted organs upon reperfusion. Although hepatocytes possess certain amount of antioxidants, including superoxide dismutase, glutathione, and glutathione peroxidase, as "self-defense systems" against oxidative stresses, we should take into account the characteristic feature in hepatic IRI, i.e. KCs activation early upon reperfusion initiated from sinusoidal space. Thus, HyFACS, direct H_2 delivering into sinusoidal space, significantly attenuated reperfusion injury of liver grafts by neutralizing ROS burst from KCs.

Of interest, this simple *ex vivo* treatment results in characteristic protection depending on its route of administration. In addition to hepatocellular protection, HyFACS via PV predominantly protects SECs, whereas that via HA effectively preserves biliary

systems. Such characteristic protection depending on the administration route, however, seems to be reasonable, considering the anatomical feature of the liver: PV directly flows into sinusoidal microcirculation, while only HA enters the biliary plexus to feed the whole biliary system in the liver (38, 39).

Recently, critical shortage of donor organs has led to acceptance of extended-criteria donors (ECD) world-widely, despite higher risk for primary non-function (PNF). Donation after circulatory death (DCD) organs are the primary target to expand potential donor pools, and in fact, DCD-LT has recently increased in Western countries (40), coupled with current remarkable developments of MP techniques (1, 41). In DCD-LT, however, ischemic cholangiopathy is one of the main causes of graft loss (42, 43). Arterial HyFACS successfully maintained microvilli formation in bile canaliculi even after prolonged CS as long as 24 hours. Though not yet tested, arterial HyFACS may be a promising strategy to reduce biliary damage in DCD-LT, particularly in a large majority of transplant centers in the world, where MP is not always available.

As a limitation of this study, we adopted an *ex vivo* reperfusion model, so called IPRL. However, this isolated setting enables to avoid interferences from immunological allo-reactions or from technical dispersions, thereby facilitating a precise and detailed

assessment of organ preservation itself (29). However, long-term observation of this novel technique in a liver transplant model is prerequisite before its clinical application.

In conclusion, just single end-ischemic flush of H_2 solution significantly protected liver grafts from IRI by alleviating oxidative damages, in the characteristic manner with its route of administration: Portal HyFACS predominantly protects SECs, while arterial HyFACS effectively preserves intrahepatic bile ducts. Given its safety, simplicity, and cost-effectiveness, HyFACS may provide a major advance in cells, tissues, and organ transplantation/preservations.

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References

1. 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):764-71.

2. Bruinsma BG, Sridharan GV, Weeder PD, Avruch JH, Saeidi N, Ozer S, et al. Metabolic profiling during ex vivo machine perfusion of the human liver. Sci Rep. 2016;6:22415.

3. Selzner M, Goldaracena N, Echeverri J, Kaths JM, Linares I, Selzner N, et al. Normothermic ex vivo liver perfusion using steen solution as perfusate for human liver transplantation: First North American results. Liver Transpl. 2016;22(11):1501-8.

4. Ravikumar R, Leuvenink H, Friend PJ. Normothermic liver preservation: a new paradigm? Transpl Int. 2015;28(6):690-9.

5. Ohsawa I, Ishikawa M, Takahashi K, Watanabe M, Nishimaki K, Yamagata K, et al. Hydrogen acts as a therapeutic antioxidant by selectively reducing cytotoxic oxygen radicals. Nat Med. 2007;13(6):688-94.

6. Abe T, Li XK, Yazawa K, Hatayama N, Xie L, Sato B, et al. Hydrogen-rich University of Wisconsin solution attenuates renal cold ischemia-reperfusion injury. Transplantation. 2012;94(1):14-21.

7. Buchholz BM, Kaczorowski DJ, Sugimoto R, Yang R, Wang Y, Billiar TR, et al. Hydrogen inhalation ameliorates oxidative stress in transplantation induced intestinal graft injury. Am J Transplant. 2008;8(10):2015-24.

8. Diao M, Zhang S, Wu L, Huan L, Huang F, Cui Y, et al. Hydrogen Gas Inhalation Attenuates Seawater Instillation-Induced Acute Lung Injury via the Nrf2 Pathway in Rabbits. Inflammation. 2016.

9. Huang T, Wang W, Tu C, Yang Z, Bramwell D, Sun X. Hydrogen-rich saline attenuates ischemia-reperfusion injury in skeletal muscle. J Surg Res. 2015;194(2):471-80.

10. Iuchi K, Imoto A, Kamimura N, Nishimaki K, Ichimiya H, Yokota T, et al. Molecular hydrogen regulates gene expression by modifying the free radical chain reaction-dependent generation of oxidized phospholipid mediators. Sci Rep. 2016;6:18971.

11. Kawamura T, Huang CS, Peng X, Masutani K, Shigemura N, Billiar TR, et al. The effect of donor treatment with hydrogen on lung allograft function in rats. Surgery. 2011;150(2):240-9.

12. Shigeta T, Sakamoto S, Li XK, Cai S, Liu C, Kurokawa R, et al. Luminal injection

of hydrogen-rich solution attenuates intestinal ischemia-reperfusion injury in rats. Transplantation. 2015;99(3):500-7.

13. Yu J, Zhang W, Zhang R, Jiang G, Tang H, Ruan X, et al. Molecular hydrogen attenuates hypoxia/reoxygenation injury of intrahepatic cholangiocytes by activating Nrf2 expression. Toxicol Lett. 2015;238(3):11-9.

14. Tao B, Liu L, Wang N, Tong D, Wang W, Zhang J. Hydrogen-Rich Saline Attenuates Lipopolysaccharide-Induced Heart Dysfunction by Restoring Fatty Acid Oxidation in Rats by Mitigating C-Jun N-Terminal Kinase Activation. Shock. 2015;44(6):593-600.

15. Liu L, Xie K, Chen H, Dong X, Li Y, Yu Y, et al. Inhalation of hydrogen gas attenuates brain injury in mice with cecal ligation and puncture via inhibiting neuroinflammation, oxidative stress and neuronal apoptosis. Brain Res. 2014;1589:78-92.

16. Tao B, Liu L, Wang N, Wang W, Jiang J, Zhang J. Effects of hydrogen-rich saline on aquaporin 1, 5 in septic rat lungs. J Surg Res. 2016;202(2):291-8.

17. Du H, Sheng M, Wu L, Zhang Y, Shi D, Weng Y, et al. Hydrogen-Rich Saline Attenuates Acute Kidney Injury After Liver Transplantation via Activating p53-Mediated Autophagy. Transplantation. 2016;100(3):563-70.

18. Tan M, Sun X, Guo L, Su C, Sun X, Xu Z. Hydrogen as additive of HTK solution fortifies myocardial preservation in grafts with prolonged cold ischemia. Int J Cardiol. 2013;167(2):383-90.

19. Li H, Chen O, Ye Z, Zhang R, Hu H, Zhang N, et al. Inhalation of high concentrations of hydrogen ameliorates liver ischemia/reperfusion injury through A2A receptor mediated PI3K-Akt pathway. Biochem Pharmacol. 2017;130:83-92.

20. Shimada S, Wakayama K, Fukai M, Shimamura T, Ishikawa T, Fukumori D, et al. Hydrogen Gas Ameliorates Hepatic Reperfusion Injury After Prolonged Cold Preservation in Isolated Perfused Rat Liver. Artif Organs. 2016.

21. Seo T, Kurokawa R, Sato B. A convenient method for determining the concentration of hydrogen in water: use of methylene blue with colloidal platinum. Med Gas Res. 2012;2:1.

22. Kurokawa R, Seo T, Sato B, Hirano S, Sato F. Convenient methods for ingestion of molecular hydrogen: drinking, injection, and inhalation. Med Gas Res. 2015;5:13.

23. Liu C, Kurokawa R, Fujino M, Hirano S, Sato B, Li XK. Estimation of the hydrogen concentration in rat tissue using an airtight tube following the administration of hydrogen via various routes. Sci Rep. 2014;4:5485.

24. Thomas GO. Flame acceleration and the development of detonation in fuel-oxygen

mixtures at elevated temperatures and pressures. J Hazard Mater. 2009;163(2-3):783-94.

25. Kawamura T, Wakabayashi N, Shigemura N, Huang CS, Masutani K, Tanaka Y, et al. Hydrogen gas reduces hyperoxic lung injury via the Nrf2 pathway in vivo. Am J Physiol Lung Cell Mol Physiol. 2013;304(10):L646-56.

26. Asada R, Kageyama K, Tanaka H, Matsui H, Kimura M, Saitoh Y, et al. Antitumor effects of nano-bubble hydrogen-dissolved water are enhanced by coexistent platinum colloid and the combined hyperthermia with apoptosis-like cell death. Oncol Rep. 2010;24(6):1463-70.

27. Hata K, Tolba RH, Wei L, Doorschodt BM, Buttner 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):114-21.

28. Okamura Y, Hata K, Tanaka H, Hirao H, Kubota T, Inamoto O, et al. Impact of Subnormothermic Machine Perfusion Preservation in Severely Steatotic Rat Livers: A Detailed Assessment in an Isolated Setting. Am J Transplant. 2017;17(5):1204-15.

29. Bessems M, 't Hart NA, Tolba R, Doorschodt BM, Leuvenink HG, RJ P. standardization of a time-honoured model. Lab Anim 2006;40(3):236-46.

30. Vajdova K, Smrekova R, Kukan M, Lutterova M, Wsolova L. Bile analysis as a tool for assessing integrity of biliary epithelial cells after cold ischemia--reperfusion of rat livers. Cryobiology. 2000;41(2):145-52.

31. McCourt PA, Smedsrod BH, Melkko J, Johansson S. Characterization of a hyaluronan receptor on rat sinusoidal liver endothelial cells and its functional relationship to scavenger receptors. Hepatology. 1999;30(5):1276-86.

32. Sano N, Tamura T, Toriyabe N, Nowatari T, Nakayama K, Tanoi T, et al. New drug delivery system for liver sinusoidal endothelial cells for ischemia-reperfusion injury. World J Gastroenterol. 2015;21(45):12778-86.

33. Sundberg U, Obrink B. CEACAM1 isoforms with different cytoplasmic domains show different localization, organization and adhesive properties in polarized epithelial cells. J Cell Sci. 2002;115(Pt 6):1273-84.

34. Miyao M, Ozeki M, Abiru H, Manabe S, Kotani H, Tsuruyama T, et al. Bile canalicular abnormalities in the early phase of a mouse model of sclerosing cholangitis. Dig Liver Dis. 2013;45(3):216-25.

35. Fukuda K, Asoh S, Ishikawa M, Yamamoto Y, Ohsawa I, Ohta S. Inhalation of hydrogen gas suppresses hepatic injury caused by ischemia/reperfusion through reducing oxidative stress. Biochem Biophys Res Commun. 2007;361(3):670-4.

36. Jaeschke H, Woolbright BL. Current strategies to minimize hepatic

ischemia-reperfusion injury by targeting reactive oxygen species. Transplant Rev (Orlando). 2012;26(2):103-14.

37. Yamada T, Uchida K, Onuma K, Kuzuno J, Ujihira M, Inoue G, et al. Hydrogen supplementation of preservation solution improves viability of osteochondral grafts. ScientificWorldJournal. 2014;2014:109876.

38. Strazzabosco M, Fabris L. Functional anatomy of normal bile ducts. Anat Rec (Hoboken). 2008;291(6):653-60.

39. Gaudio E, Franchitto A, Pannarale L, Carpino G, Alpini G, Francis H, et al. Cholangiocytes and blood supply. World J Gastroenterol. 2006;12(22):3546-52.

40. Johnson RJ, Bradbury LL, Martin K, Neuberger J, Registry UT. Organ donation and transplantation in the UK-the last decade: a report from the UK national transplant registry. Transplantation. 2014;97 Suppl 1:S1-S27.

41. 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):120-4.

42. Jay CL, Lyuksemburg V, Ladner DP, Wang E, Caicedo JC, Holl JL, et al. Ischemic cholangiopathy after controlled donation after cardiac death liver transplantation: a meta-analysis. Ann Surg. 2011;253(2):259-64.

43. Mallik M, Callaghan CJ, Hope M, Gibbs P, Davies S, Gimson AE, et al. Comparison of liver transplantation outcomes from adult split liver and circulatory death donors. Br J Surg. 2012;99(6):839-47.

FIGURE LEGENDS

Figure 1. ***Non-destructive hydrogen dissolving method***

(A and B) Hydrogen generating agent ($Ca[OH]_2$ mixed with Aluminum powder; MiZ Co., Ltd., Kamakura, Japan) wrapped in non-woven fabric is moistened with water. Then a solution bag and two pieces of hydrogen generating agents are put together into an aluminum pouch and sealed tightly without dead space. Pure hydrogen gas is generated within the aluminum pouch, gradually permeates through plastic bag, dissolved into solution inside, then reaching the plateau concentration of 1.0 ppm by 24 hours. This easy and simple method enables to prepare H_2 solution, maximally 7.0 ppm, safely, aseptically, and inexpensively (< three US dollars/bag).

We prepared H_2-solution each time, and its concentration was measured via the methylene blue platinum colloid reagent-based titration method (Dissolved hydrogen concentration measuring reagent; MiZ Co., Ltd.), as well as verified by an electrochemical gas sensor (model DHD1-1, DKK-TOA Corp., Tokyo, Japan). The H_2 concentration was strictly maintained at 1 ppm (0.95 ± 0.02 ppm) throughout the experiments.

(C) The H_2 concentration in both the inflow and the outflow of the liver (just before and after the liver, respectively) was determined, and summarized in Fig.1-C, indicating 1) the H_2 loss before reaching the liver was minimal; and 2) almost all H_2 delivered by HyFACS was absorbed by liver tissues.

Figure 2. ***Transaminase and HMGB-1 release upon reperfusion***

(A, B, C) AST, ALT and LDH release into the perfusate, as indices for hepatocellular damage upon reperfusion. All differences among the groups were assessed via two-way repeated-measures ANOVA (AST: $P < 0.001$, ALT: $P < 0.01$, LDH: $P < 0.01$). Time-point assessments were performed by Bonferroni's posttest (*: $P < 0.05$, †: $P < 0.01$; ‡: $P < 0.001$ *vs*. Control group). Error bars are sometimes invisible due to small SEMs ($n = 10$ each).

(D) HMGB-1 release into the perfusate, served as an index for comprehensive tissue damage after 2 hours of oxygenated reperfusion, as well as for a hazardous pro-inflammatory signal thereafter. All data are presented as the mean ± SEM ($n = 10$ each). All differences among the groups were assessed via one-way ANOVA ($P < 0.001$), followed by Bonferroni's posttest. *: $P < 0.05$ *vs.* other groups.

Abbreviations: AST, aspartate aminotransferase; ALT, alanine aminotransferase; LDH, lactate dehydrogenase; ANOVA, analysis of variance; SEM, standard error of the mean; HMGB-1, high-mobility group box protein-1.

Figure 3. ***Mitochondrial integrity and ATP restoration upon reperfusion***

(A) Tissue ATP concentration after 24 hours of static cold storage and subsequent 2 hours of oxygenated reperfusion, served as a parameter for microcirculatory integrity, as well as for mitochondrial viability. HyFACS-PV+HA produced significantly better ATP charge upon reperfusion with equal amount of oxygen uptake, compared with untreated livers (Mean ± SEM, n = 10 each). Inter-group difference by one-way ANOVA: P <0.05, and Bonferroni's posttest: *: P <0.05 *vs*. Control group.

(B) Oxygen consumption rates of liver grafts at 120 minutes of oxygenated reperfusion. There was no difference among the groups (One-way ANOVA: P =0.14).

(C) GLDH release into the perfusate, as a parameter for hepatic mitochondrial injury. (Mean ± SEM, n = 10 each). All differences among the groups were assessed via one-way ANOVA (P <0.001), followed by Bonferroni's posttest. *: P <0.05 *vs.* other groups.

(D-H) Ultrastructural photomicrographs of representative tissue sections observed with

transmission electron microscopy (TEM). The original magnification was X 8,000. The scale bar in each panel represents 500nm.

D: In control livers, hepatocellular mitochondria (Mt) showed severe swelling with less electron density. Endoplasmic Reticula (ER) were also disrupted.

E, F, and G: In contrast, such deleterious alterations were significantly alleviated in HyFACS-treated livers (E; -PV, F; -HA and G; -PV+HA).

H: Ultra-structural observation of hepatocytes in sham livers, not cold-stored but subjected to 2-hour oxygenated reperfusion, as a reference for baseline comparison with the four experimental groups. Glycogen granules (G) were observed in non cold-stored livers.

Abbreviations: ANOVA, analysis of variance; ATP, adenosine triphosphate; ER, endoplasmic reticula; GLDH, glutamate dehydrogenase; HyFACS, hydrogen flush after cold storage; Mt, mitochondria; Nu, nucleus; SEM, standard error of the mean; TEM, transmission electron microscope

Figure 4. ***Functional/Morphological integrity of sinusoidal endothelia***

(A) Chronological alteration of portal-venous perfusion pressure (PVP) during 2 hours of oxygenated reperfusion, served as an index for total vascular resistance of liver grafts. All differences among the groups were assessed via 2-way ANOVA (P <0.001), followed by Bonferroni's posttest: ‡: P <0.001 *vs*. Control group. Error bars are sometimes invisible due to small SEMs (mean ± SEM, n =10 each).

(B) Hyaluronic acid clearance, as a parameter for functional integrity of SECs. One-way ANOVA (P <0.01), followed by Bonferroni's posttest. *: P <0.05 *vs*. Control group (Mean ± SEM, n =10 each).

(C-G) Scanning Electron Microscopy (SEM) for Sinusoidal Endothelia. The original magnification was X 4,000. The scale bar in each panel represents 50μm.

C: In control livers, sinusoidal fenestrae were fused and enlarged, and sinusoidal linings were almost disrupted after 24-hour cold preservation followed by 2-hour oxygenated reperfusion.

D, E, and F: In contrast, sinusoidal linings and fenestrae therein were well preserved in all the HyFACS group, particularly in HyFACS-PV (D) and -PV+HA (F), demonstrating significant protection of sinusoidal endothelia by portal HyFACS.

G: Sinusoidal endothelia in sham livers, not cold-stored but subjected to 2-hour oxygenated reperfusion, as a reference for baseline comparison with the four experimental groups

Abbreviations: ANOVA, analysis of variance; HyFACS, hydrogen flush after cold storage; LDH, lactate dehydrogenase; PVP, portal-venous perfusion pressure; SEM, scannning electron microscope; SECs, sinusoid endothelial cells.

Figure 5. ***Bile production and biliary damage upon reperfusion***

(A) Bile production during 2 hours of reperfusion, as a comprehensive parameter for liver graft function. One-way ANOVA: P <0.001, followed by Bonferroni's posttest *: P <0.05 *vs*. Control group (mean ± SEM, n =10 each).

(B) LDH leakage into bile, as an indicator for biliary damage. Inter-group difference by one-way ANOVA: P <0.01, and Bonferroni's posttest *: P <0.05 *vs*. Control group.

(C-G) Ultrastructural Observation for Bile Canaliculi using Scanning Electron Microscopy (SEM). The original magnification was X 13,000. The scale bar in each panel represents 2μm.

C: In the controls, ultrastructural architecture of microvilli formation in bile canaliculi was severely injured and only sparse microvilli were left after prolonged CS and oxygenated reperfusion. Height of microvilli was also lowered.

D, E, and F: In contrast, microvilli formation was well preserved by arterial HyFACS, where both density and height looked almost intact (E and F). Portal HyFACS also better maintained microvilli than in the controls, but worse than in arterial HyFACS (D).

G: Bile canaliculi in sham livers, not cold-stored but subjected to 2-hour oxygenated reperfusion, as a reference for baseline comparison with the four experimental groups

Abbreviations: ANOVA, analysis of variance; CS, cold storage; HyFACS, hydrogen flush after cold storage; SEM, standard error of the mean; SEM, scannning electron microscope

Figure 6. ***Immunohistochemistry for CEACAM1***

Light-microscopic appearance of immunohistochemical staining for CEACAM1. Scale bar in each panel indicates 100μm.

A: Control livers (A) showed weak staining in almost all liver lobules.

B, C, and D: In contrast, arterial HyFACS (C; -HA, D; -PV+HA) exhibited dense and thick staining along with bile canaliculi, appeared like ‘chicken-wire’. Similarly in the SEM

observation (Fig.8), HyFACS-PV resulted in better staining than in the controls (B), but worse than in arterial HyFACS (C and D), demonstrating the superiority of arterial route for H_2 administration to protect biliary epithelia.

E: CEACAM1 staining in sham livers, not cold-stored but subjected to 2-hour oxygenated reperfusion, as a reference for baseline comparison with the four experimental groups

F: Quantification of CEACAM1 stained area by using the Image-J software. All differences among the groups were assessed via one-way ANOVA (P <0.001), followed by Bonferroni's posttest (*P <0.05 *vs*. Control group).

Abbreviations: ANOVA, analysis of variance; CEACAM1, carcinoembryonic antigen related cell adhesion molecule 1

Figure 7. ***Oxidative Stress and Redox Status***

(A, B, and C) TBARS level in liver tissue, 8-OHdG release into the perfusate, and total glutathione contents in liver tissue are served as parameters for tissue oxidative stresses upon reperfusion (Mean ± SEM, n = 10 each). One-way ANOVA (P <0.01 in all three parameters), followed by Bonferroni's posttest. *: P <0.05 *vs.* Control group.

(D) GSH/GSSG ratio (mol/mol), given as an index for cellular redox status. All differences among the groups were assessed via one-way ANOVA (P <0.01), followed by Bonferroni's posttest. *: P <0.05 *vs.* Control group.

Abbreviations: TBARS, thiobarbituric acid reactive substances; 8-OHdG, 8-hydroxy-2-2deoxyguanosine; GSH, glutathione-SH; GSSG, glutathione disulfide; ANOVA, analysis of variance; SEM, standard error of the mean

Figure 1

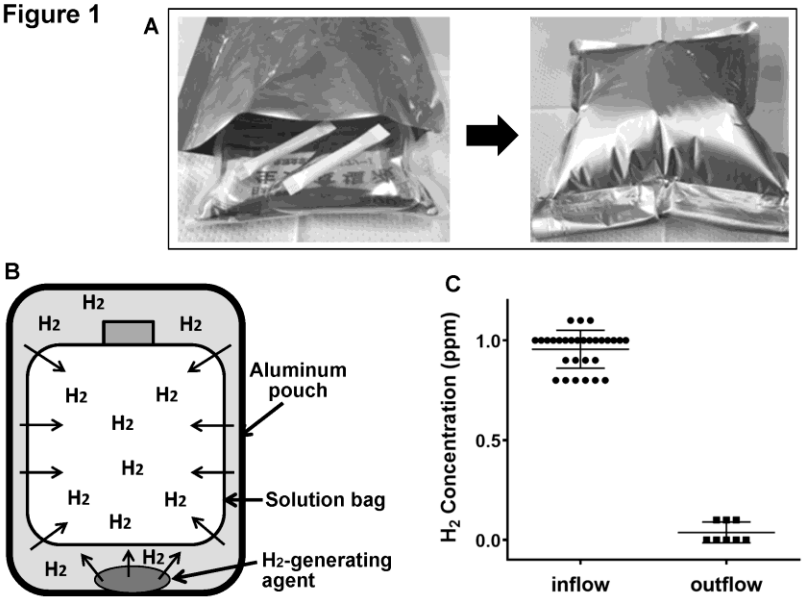

Figure 2

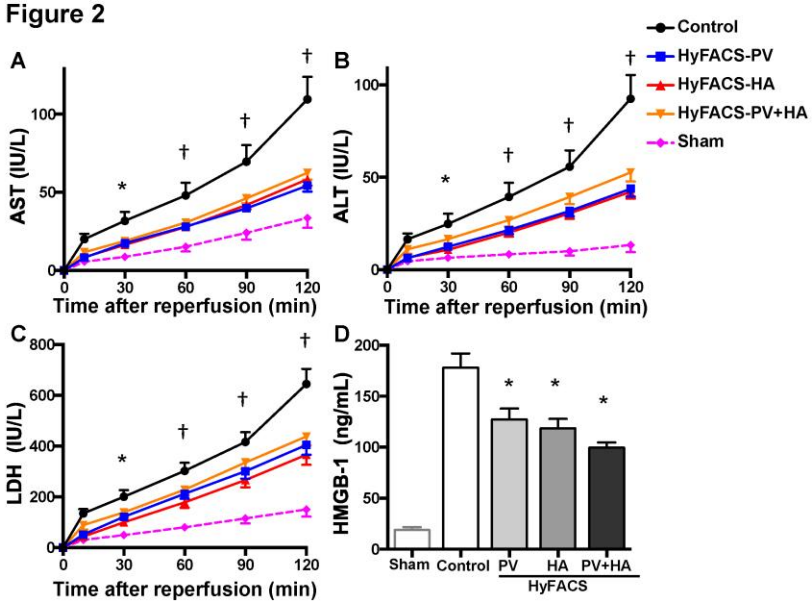

Figure 3

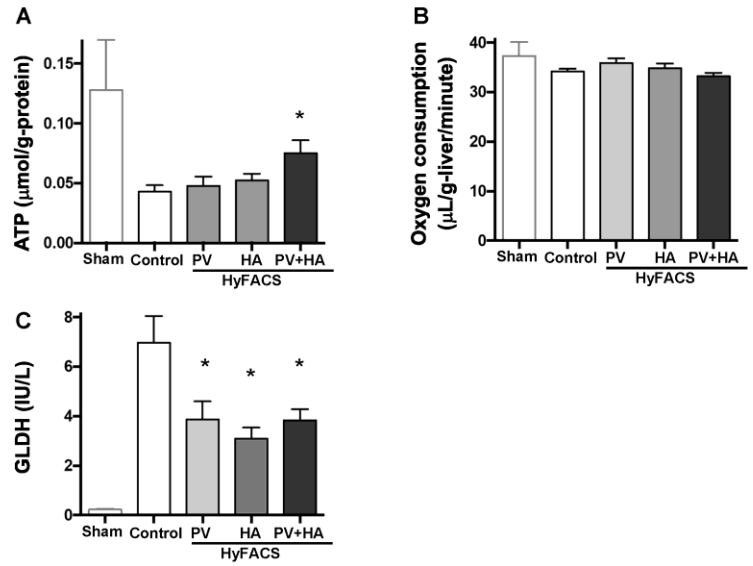

Figure 3

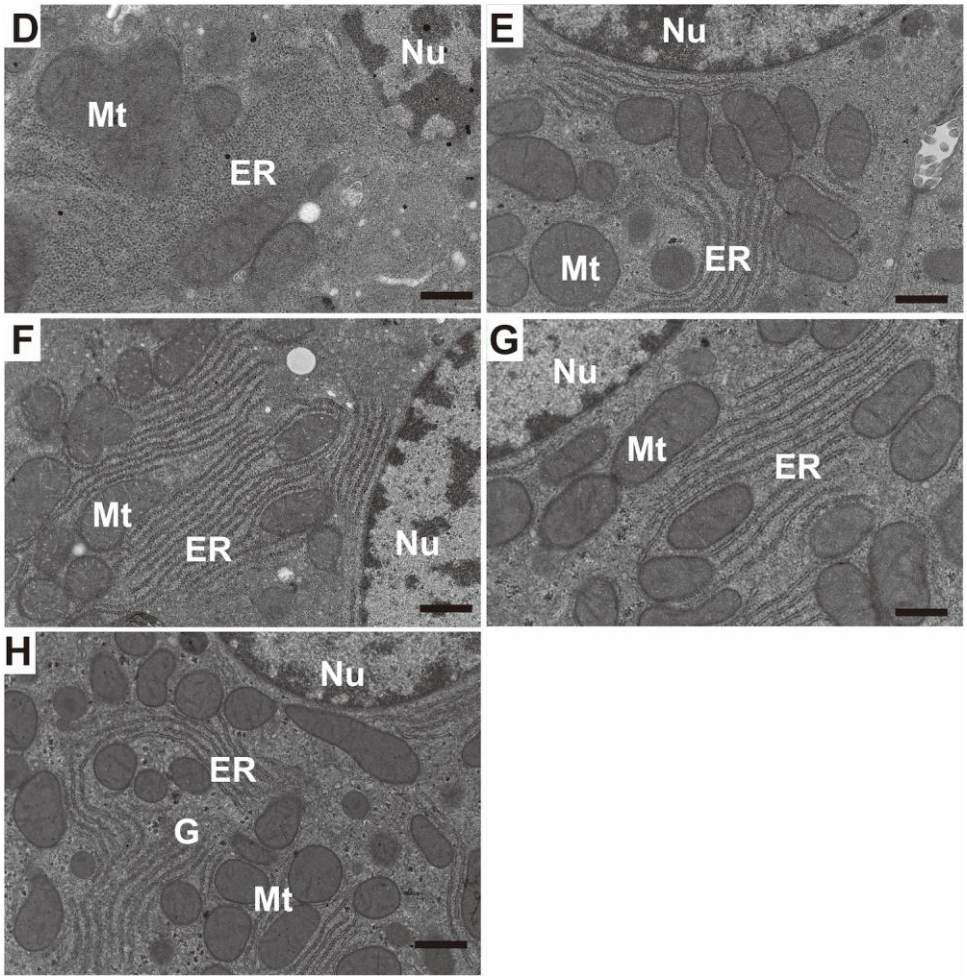

Figure 4

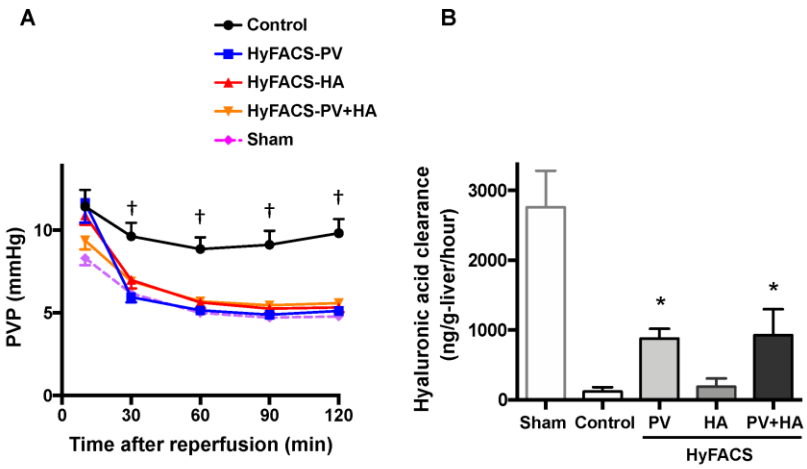

Figure 4

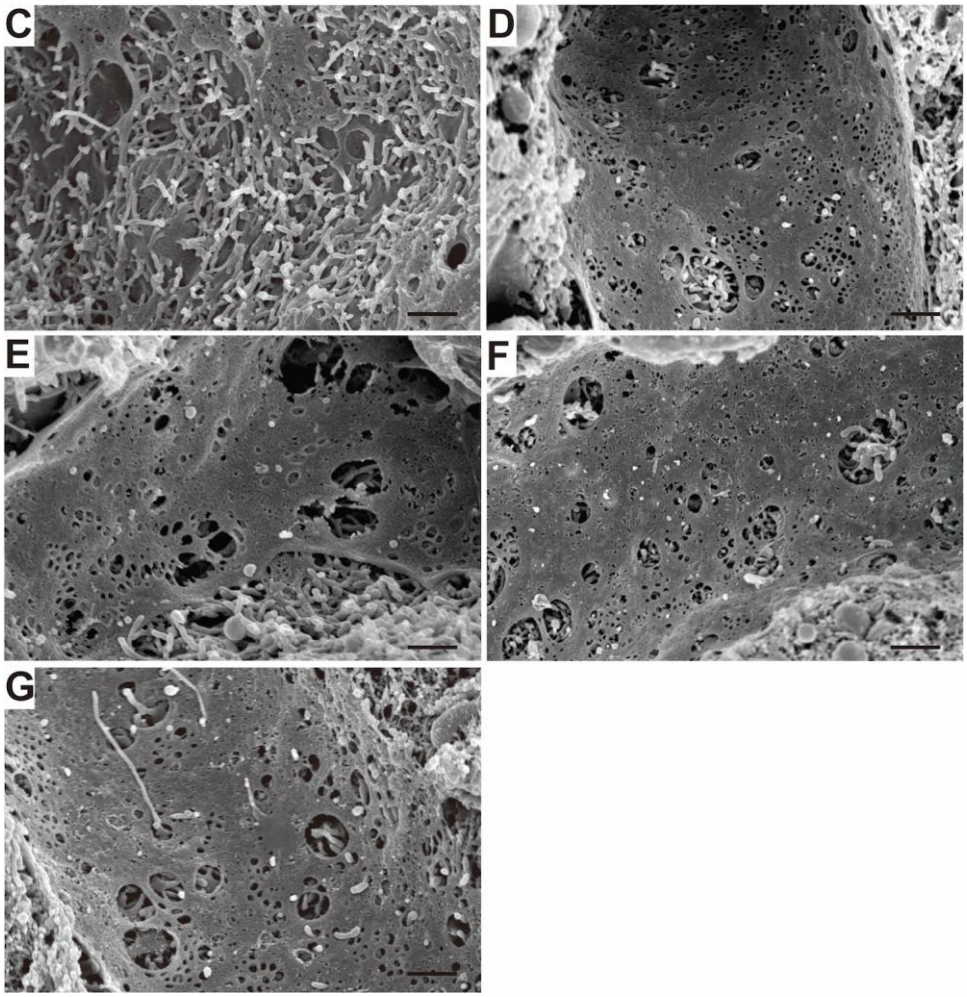

Figure 5

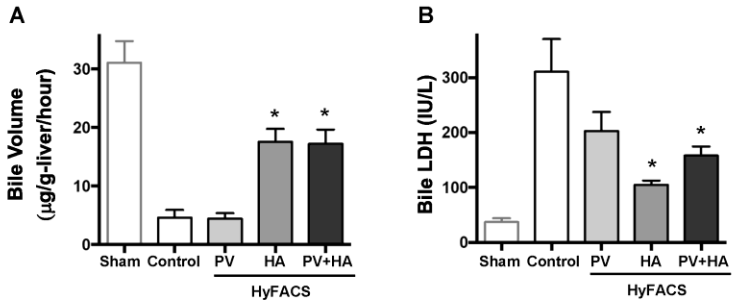

Figure 5

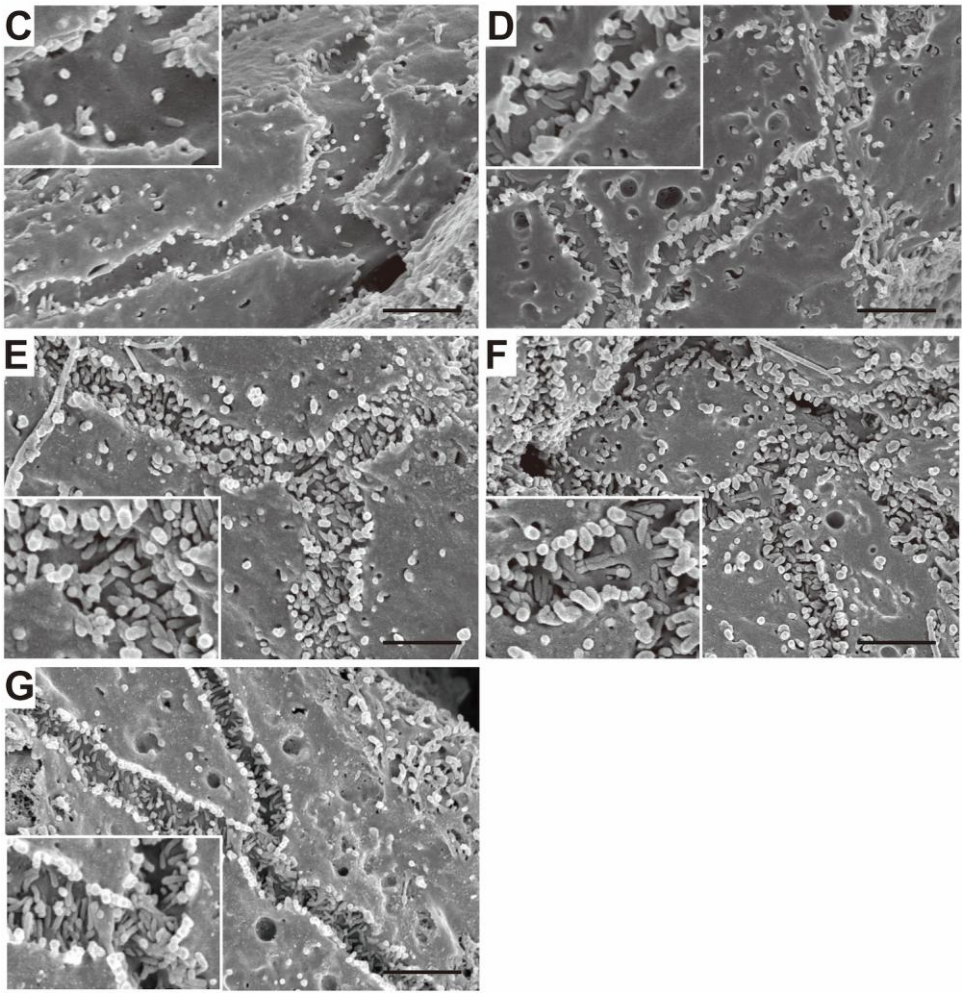

Figure 6

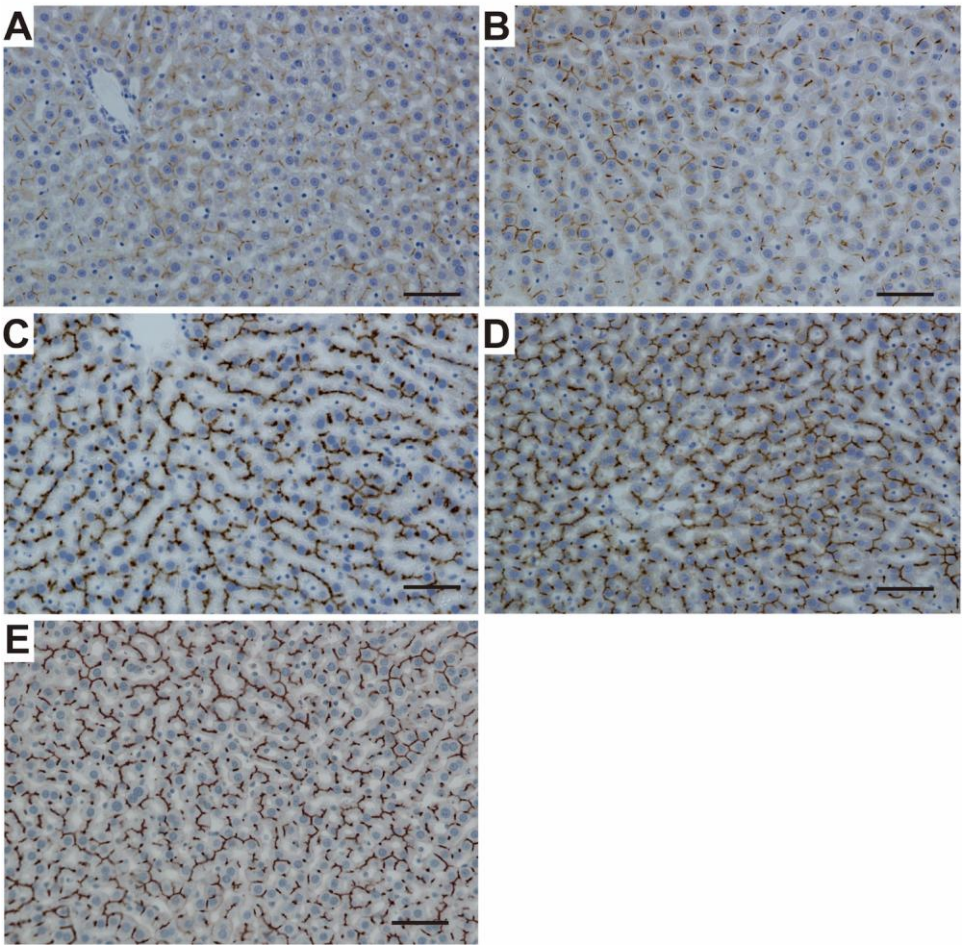

Figure 6

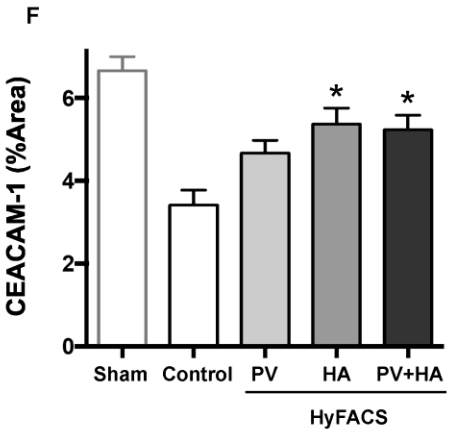

Figure 7