TITLE PAGE: Role of heavy water in modified University of Wisconsin solution for extended cold storage of rat liver

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Grant information:

This work was supported in part by a Grant-in-Aid for Scientific Research (KAKENHI) from the Japan Society for the Promotion of Science (JSPS). Grant numbers: 20K08974 (Fukai) and 20H03737 (Shimamura)

Key words: liver, cold storage, preservation, isolated perfused rat liver, IPRL, heavy water, D_2O

Abbreviations: IPRL (isolated perfused rat liver) OCR (oxygen consumption rate) PVR (Portal vein resistance)

Highlights

- We previously reported using a buffer containing heavy water (D_2O), Dsol, as a cold storage solution. In this study, we modified a University of Wisconsin (UW) solution by removing the hydroxyethyl starch (HES) and adding 30% D_2O and named the resulting product UWD. The protective potential of UWD was evaluated during extended cold storage and subsequent reperfusion of isolated perfused rat liver.
- UWD showed partially better protective effects than the UW solution as indicated by lower portal vein resistance and higher oxygen consumption rate and bile production after reperfusion, whereas organ expansion and liver enzyme leakage were equivalently high in both groups.
- Modification of the UW solution by the removal of HES and addition of 30% D_2O confers partial protection to liver subjected to extended cold storage. Factors that synergize with D_2O are also required.

Abstract

To resolve the critical donor shortage worldwide, enlarging the potential donor pool to include expanded criteria donors is necessary. Despite numerous attempts to establish new preservation solutions, no dramatic innovation has occurred since University of Wisconsin (UW) solution displaced Euro Collins' solution; UW solution remains the global gold standard. We previously developed a heavy water (D_2O)–containing organ storage solution, Dsol, which is effective for livers subjected to extended cold storage (CS), and reported its effectiveness. Dsol is a modified UW solution; however, the substances or conditions that exhibit a synergistic or additive effect with D_2O are unclear. Here we made UWD solution by removing hydroxyethyl starch (HES) from and adding 30%-D_2O to UW solution, and compared the effects of these solutions. After 48 h of CS, the livers were reperfused at 37°C on an isolated perfused rat liver apparatus, and their perfusion kinetics, functions, and injuries were compared. In the UW group, portal vein resistance significantly increased and the oxygen consumption rate and bile production decreased; in contrast, these changes were suppressed in the UWD group. Organ expansion and liver damage progressed in both groups. These results confirmed that the removal of HES from and addition of D_2O to the UW solution reduced CS-induced cellular function impairments and microcirculatory disorders. However, to reduce injury during reperfusion after CS, it is necessary to provide conditions that inhibit injury progression after reperfusion.

Title: Role of heavy water in modified University of Wisconsin solution for extended cold storage of rat liver

Introduction

To expand the potential donor pool for expanded criteria donors, alternative methods of cold storage (CS) at the University of Wisconsin (UW) are required [1]. We previously reported the promising properties of heavy water (D_2O)-containing buffer, Dsol, for extended CS of rat hearts [2] and livers [3]. Because D_2O counteracts liver swelling during CS [4] and hydroxyethyl starch (HES) provokes microcirculatory disorders owing to its high viscosity and promotion of blood aggregation [5], Dsol was prepared as follows: 30% D_2O (v/v), mannitol, sucrose, and potassium-reduced and sodium-increased compositions, while HES and raffinose were excluded [2]. This study tested the efficacy of the UWD solution, which is a modified UW solution without HES but with 30% D_2O.

D_2O exhibits unique biological effects, such as inhibiting organ swelling during CS [4], inhibiting cytoplasmic calcium overload, inhibiting calcium ion–induced protease activation, and preventing mitochondrial disorders and apoptosis [2,3] through biochemical reactions and quantum mechanical processes, including atom tunneling and the isotope effect of hydrogen [6]. However, the factors to be included in D_2O-containing buffers for extended CS of the liver remain unclear. We previously performed an isolated perfused rat liver (IPRL) to assess the efficacy of preservation solutions [3]. The present study aimed to evaluate the efficacy of the UWD versus the UW solution and clarify the important constituents

of D_2O-containing buffer for use in extended CS of the rat liver.

Materials and Methods

Animals and surgeries

Animal experiments were approved by the Institutional Review Board of Hokkaido University (no. 17-0032) according to the “Fundamental Guidelines for Proper Conduct of Animal Experiments and Related Activities in Academic Research Institutions” (notice no. 71; 2006), under the jurisdiction of the Ministry of Education, Culture, Sports, Science, and Technology of Japan. Male Wistar rats (8–10-weeks-old) were purchased from Sankyo Labo Service Co. Inc. (Tokyo, Japan) and fed a standard laboratory diet (MF, Oriental Yeast, Tokyo, Japan) for at least three days before the experiment. General anesthesia with isoflurane inhalation was initiated, and the liver was flushed with cold saline. After cannulation of the common bile duct and portal vein, the liver graft was excised and immersed in Belzer UW® CS solution (Bridge to Life, Northbrook, IL, USA) or UWD solution for 48 h at 4 °C and reperfused thereafter, as previously described [3]. The UWD solution was a modified UW solution containing 30% D_2O (v/v) as the solvent; HES was omitted, and the other constituents were the same as those in the UW solution.

Isolated perfused rat liver (IPRL)

The IPRL system comprised a PowerLab 15T®AD converter and integrator (ADInstruments, Dunedin, New Zealand), an IN175 pump controller (ADInstruments), PowerLab® integration software

(ADInstruments), and a MINIPULSE III MP-2® peristaltic pump (Gilson, Madison, WI, USA). IPRL conditions were set as described previously [3]. The perfusate used was Krebs-Henseleit bicarbonate buffer (300 mL) with oxygenation ($450<pO_2<550$ mmHg) at 37 °C with sodium taurocholate (0.3 mM final concentration). After 2 min of initial high flow at 3 mL/min/g to recover the collapsed vascular bed, the portal vein flow was gradually reduced to decrease portal vein pressure (PVP) during the 30-min equilibration period. PVP was maintained at 8 cm H_2O thereafter. The PVP and portal vein flow were continuously monitored, and the portal vein resistance (PVR) was calculated and monitored.

Experimental groups

Grafts subjected to 48 h of CS in UW and UWD solutions, which were subsequently reperfused for 90 min, were defined as the UW and UWD groups, respectively. The liver immediately reperfused without CS after excision was defined as the control (CT) group.

Assessments

Hepatic microcirculation was evaluated primarily by PVR, in which the PVP was divided by portal vein flow. Liver function was evaluated based on bile production and the oxygen consumption rate (OCR). Cumulative bile production during 90 min of reperfusion was expressed as microliters per gram of liver tissue. Oxygen concentration in the perfusate was determined using a RAPIDLAB 348EX blood gas system (Siemens, Munich, Germany). The OCR was calculated as the difference in oxygen concentration between the hepatic inflow and outflow and is expressed as micromole of O_2 per minute per gram of liver

[3].

Graft weight was measured at the end of 48 h of CS and at the end of reperfusion (R90), and expressed as a percentage of the pre-storage weight. The weight change from the end of the CS period to R90 was calculated and expressed as a percentage of the initial liver weight, which was designated as the "maximum graft weight change." Liver injury was evaluated by measuring the alanine aminotransferase (ALT) activity of the perfusate at 30 and 90 min after reperfusion (R30 and R90, respectively). The progression of reperfusion injury was evaluated by the increase in ALT or aspartate aminotransferase (AST) activity from R30 to R90, designated as ΔALT or ΔAST, respectively. The amount of enzyme released during the latter 2/3rd of the time, ΔALT and ΔAST, is expressed as a proportion of the total ALT or AST released up to R90, respectively, to compare the progression of reperfusion injury.

Statistical analysis

Data are presented as the mean ± standard deviation. Non-parametric multiple comparisons (Steel–Dwass test) were performed using JMP ® version 16 software (SAS Institute Japan, Tokyo, Japan). Results were considered statistically significant at $p < 0.05$.

Results

Hepatic circulation

Portal vein flow was reduced from 3 mL/min/g according to the protocol and adjusted to 8 cm H_2O after 30 min. PVP was adjusted to 8 cm H_2O in all groups (Figure 1A). As a result of flow regulation, PVR varied

depending on the condition of the graft. The lower PVR in the UWD group compared to that in the UW group indicated the effect of CS with heavy water (Figure 1B). The PVR values at R30 in the CT, UW, and UWD groups were 3.14±0.66, 8.12±2.65, and 2.40±0.47 (cm H_2O/(mL/min/g)): CT vs. UW ($p<0.0001$); CT vs. UWD ($p=0.0295$); and UW vs. UWD ($p=0.0012$). The PVR values at R90 in the CT, UW, and UWD groups were 3.42±0.59, 7.44±3.39, and 2.74±0.27(cm H_2O/(mL/min/g)): CT vs. UW ($p<0.0001$); CT vs. UWD ($p=0.0125$); and UW vs. UWD ($p=0.0012$). The mean PVR was the highest in the UW group, whereas it was significantly suppressed in the UWD group (Figure 1B).

Bile production

Bile production during 90 min of reperfusion was highest in the CT group (103.9±23.8 μL/g) and lowest in the UW group (21.4±12.1 μL/g): UW vs. CT ($p=0.0012$) (Figure 2A). The decrease in bile production was significantly suppressed in the UWD group (49.3±14.3 μL/g): CT vs. UW ($p<0.0001$); CT vs. UWD ($p=0.0023$); UW vs. UWD ($p=0.0041$).

Oxygen consumption rate

The OCR values at R30 in the CT and UW groups were 1.13±0.27 and 0.63±0.28 (μmol O_2/min/g), respectively: CT vs. UW ($p=0.0004$). The value was 1.23±0.28 (μmol O_2/min/g liver) in the UWD group, which surpassed the CT level: CT vs. UWD ($p=0.4878$); UW vs. UWD ($p=0.0102$) (Figure 2B). The mean OCR at R90 in the CT and UW groups was 1.28±0.18 and 0.66±0.25 (μmol O_2/min/g liver), respectively: CT vs. UW ($p<0.0001$). The mean value in the UWD group was 1.40±0.28 (μmol O_2/min/g

liver), which surpassed the CT level: CT vs. UWD (p=0.0025); UW vs. UWD (p=0.6267) (Figure 2B).

Graft weight change

Mean liver graft weight decreased at the end of 48 h of CS in the UW group (-13.4±8.3%; n=17) and the UWD group (10.4±3.3%; n=6) (Figure 3A). The maximum weight change was observed during the 90 min of reperfusion from the end of CS to the end of reperfusion (R90). The values in the CT and UW groups were -0.77±7.3% (n=16), whereas those in the UW and the UWD groups were 20.9±8.9% (n=17) and 21.7±12.5 % (n=6), respectively: CT vs. UW (p<0.0001); CT vs. UWD (p=0.0030); UW vs. UWD (p=0.9467) (Figure 3B).

Liver injury

Mean ALT levels in the perfusate at R90 in the CT, UW, and UWD groups were 0.42±0.17, 2.83±2.21, and 3.01±1.18 IU/L/g liver, respectively: CT vs. UW (p=0.0005); CT vs. UWD (p=0.0039); UW vs. UWD (p=0.7605). AST levels in the perfusate at R90 in the CT, UW, and UWD groups were 2.51±1.39, 5.77±3.62, and 3.01±1.18 IU/L/g liver, respectively: CT vs. UW (p=0.0121); CT vs. UWD (p=0.8770); UW vs. UWD (p=0.1836) (Figure 4A).

The increase in ALT in the perfusate from R30 to R90 was defined as ΔALT and assessed. The mean ΔALT was lowest in the CT group (0.13±0.09 IU/L/g liver; n=10) but significantly higher in the UW (1.70±1.96 IU/L/g liver; n=10) and UWD (1.19±0.63 IU/L/g liver; n=6) groups: CT vs. UW (p=0.0010); CT vs. UWD (p=0.0039); UW vs. UWD (p=0.9603). AST exhibited a similar trend. The mean ΔAST was

the lowest in the CT group (1.06±0.71 IU/L/g liver; n=10) and significantly higher in the UW (3.64±3.23 IU/L/g liver; n=10) and UWD (3.64±1.55 IU/L/g liver; n=6) groups: CT vs. UW (p=0.0097); CT vs. UWD (p=0.8770); UW vs. UWD (p=00614) (Figure 4B).

The amount of enzyme released during the latter 2/3rd of reperfusion, evidenced as ΔALT and ΔAST, is expressed as the proportion of the total ALT or AST released, respectively. The mean ΔALT in the CT (n=10), UW (n=10), and UWD (n=6) groups accounted for 29.8±19.3%, 48.2±18.9%, and 37.9±10.6%, respectively. The mean ΔAST in the CT, UW, and UWD groups accounted for 39.3±13.4%, 56.9±14.7%, and 49.1±10.1%, respectively. The mean value was lowest in the CT group and highest in the UW group, whereas the increase in the UW group was suppressed in the UWD (not significant [N.S.]) (Figure 4C).

Discussion

This study tested the efficacy of the UWD versus UW solution and found that the former significantly suppressed the augmentation of PVR throughout the reperfusion period with a significantly higher bile production and OCR than observed in the UW group. However, the rapid graft weight changes due to organ shrinkage during CS and swelling after reperfusion were not suppressed in the UW and UWD groups. These results highlight the importance of maintaining aerobic metabolism while simultaneously suppressing other pathological mechanisms. Because the UWD was a modified UW solution containing 30% D_2O (v/v) but lacking HES, the influence of HES removal and D_2O mediation was assessed.

Although HES has been used to prevent organ swelling during CS [7], posttransplant survival is

worsened by poor flush-out due to its high viscosity [5]. Furthermore, blood cell aggregation can be prevented by removing HES or substituting HES with polyethylene glycol (PEG) from the UW solution, leading to good perfusion [8]. Therefore, the efficacy of HES for treating hepatic CS remains controversial. In this study, the graft was removed after sufficient *in situ* flushing with CT saline and the preservation solution and stored in the tested solution. Therefore, the increase in PVR in the UW group and decrease in PVR in the UWD group were both unrelated to blood aggregation.

In the present study, we chose a CS duration of 48 h and the endpoint as R90 in the IPRL, causing potential limitations. Although the peak of hepatic CS and reperfusion injury occurs between 6 and 24 h after transplantation [9], in liver CS, the extent of reperfusion injury can be predicted after 60 min of reperfusion in large animal experiments [10], while it can be predicted within 2 h in human hepatic normothermic machine perfusion [11]. Accordingly, we adopted R90 as the endpoint in this study. Regarding the cold ischemic time (CIT), exacerbation of reperfusion injury is dependent on a CIT of 24–36 h in rat liver transplantation [12]. In liver transplants after 40 h of CS, changes in graft survival rate and tissue damage due to treatment have been evaluated [13]. Accordingly, divergent from clinical CIT, we adopted 48 h of CS as the established model [3].

It was unexpected that the PVR in the UWD group was lower than that in the CT group. We speculated that heavy water would inhibit dehydration of the liver during CS, as well as prevent rapid liver expansion during reperfusion, thereby reducing PVR. However, regarding the change in organ weight, UWD was

equivalent to UW. These facts suggest that cold storage with UWD may act to suppress contraction or act to expand sinusoids and veins, and these effects may be maintained during the 90-min perfusion. Since the intracellular D_2O is washed out within 15 min or less [14, 15], it is unlikely that it would continue to act within the organs and cells. The interaction between PEG-35 and the endothelial surface glycocalyx regulates nitric oxide production, decreasing sinusoidal pressure [16]. Although PEG was not included in the UWD solution, it is also speculated that a similar switch was activated during CS to reduce the sinusoidal pressure after reperfusion. In the future, we will plan to evaluate changes in molecules involved in the control of sinusoidal tonus during CS.

In this study, hepatic injury was not ameliorated despite the higher OCR and bile production in the UWD group compared to the UW group. Since sufficient adenosine triphosphate production via aerobic metabolism may cause injury due to oxidative stress, antioxidant therapy at the time of reperfusion may reduce injury [3]. If the difference in the partial pressure of oxygen between the liver inflow and outflow reflects the generation of reactive oxygen species rather than energy production, a high OCR may not contribute to preserving the cellular function through energy production. To clarify the significance of OCR, it is necessary to simultaneously evaluate the energy state and oxidative stress [3]. We have previously found that the ultrastructure of mitochondria during CS and subsequent reperfusion varies depending on the conditions of hypothermic machine perfusion [17]. There may be enhancement of cell death signals, of morphological changes in membranes and intracellular organelles, and of the indices

showing the ongoing functional abnormalities at the end of CS and early reperfusion. Therefore, even if reduction of injury is not achieved as an endpoint, the effects and action points against the harmful cascades are important findings. Evaluation of these parameters mentioned above are planned for future research.

Conclusions

Although the UWD solution failed to reduce overall hepatic ischemia and reperfusion injury, it partially improved post-reperfusion microcirculation and liver function. Heavy water may become a useful tool to preserve functions of liver subjected to extended CS.

Acknowledgements

The authors are grateful to Mr. Masatoshi Horigome for his dedication to animal care. We thank Editage (www.editage.jp) for English language editing.

Funding

This study was supported, in part, by a Grant-in-Aid for Scientific Research (KAKENHI) from the Japan Society for the Promotion of Science (JSPS). Grant numbers:20K08974 (Fukai) and 20H03737 (Shimamura).

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used the AI-assisted proofreading tool Paperpal (provided by Cactus Communications Group) solely to improve language. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of this publication.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the author(s) used Chat GPT3.5 / OPEN AI in order to improve language and readability. After using this tool, the author(s) reviewed and edited the content as needed and take full responsibility for the content of this publication.

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Figure Legends

Figure 1: Hepatic perfusion kinetics. The graft was subjected to 48 h of cold storage (CS) in UW (n=17) or 30% D_2O–containing UW (UWD; n=6) solutions and reperfused thereafter. The liver immediately reperfused after excision was defined as the CT group (n=16). (A) Portal vein pressure was adjusted to 8 cm H_2O in all groups. (B) Portal vein resistance (PVR) was the highest in the UW group and

the lowest in the UWD group throughout the experiment. The PVR was significantly lower in the UWD versus UW groups. * vs. UW ($p \leq 0.0025$); † vs. CT ($p \leq 0.0295$). Steel–Dwass test. Mean ± standard deviation.

Figure 2: Liver function. Bile production and oxygen consumption rates (OCR) after 48 h of cold storage (CS) in UW (n=17) and 30% D_2O–containing UW (UWD; n=6) solutions were evaluated at 90 min after reperfusion. The livers immediately reperfused were defined as the CT (n=16) group. (A) Bile production. The CT and UW groups showed the highest and the lowest values, respectively. The decrease by CS was significantly suppressed in the UWD group. CT vs. UW ($p<0.0001$); CT vs. UWD ($p=0.0023$); UW vs. UWD ($p=0.0041$). (B) The OCR in the CT and UW groups were the highest and the lowest, respectively. * vs. UW ($p \leq 0.0102$); † vs. CT ($p \leq 0.0004$). Steel–Dwass test. Mean ± standard deviation.

Figure 3: Changes in graft weight. The graft was subjected to 48 h of cold storage (CS) in UW (n=17) or 30% D_2O–containing UW (UWD; n=6) solutions and reperfused thereafter. The liver immediately reperfused after excision was defined as the CT group (n=15). The changes in graft weight were evaluated. (A) At the end of 48 h of CS, the graft weight was significantly decreased in the UW and UWD groups. (B) The weight change from the end of CS to the end of the 90-min reperfusion was assessed. The weights remained almost unchanged in the CT group but were significantly higher in the UW and UWD groups. † vs. CT ($p \leq 0.0378$,). Steel–Dwass test. Mean ± standard deviation.

Figure 4: Liver injury. The graft was subjected to 48 h of cold storage (CS) in UW (n=10) or 30% D_2O–

containing UW (UWD; n=6) solutions and reperfused thereafter. The liver immediately reperfused was defined as the CT group (n=10). Liver enzymes in the perfusate at 90 min after reperfusion (R90) were determined. (A) Alanine aminotransferase (ALT) levels were significantly higher in UW and UWD groups than that of the CT group: CT vs. UW († p=0.0039); CT vs. UWD († p=0.0005). Aspartate aminotransferase (AST) levels showed a similar trend to ALT. (B) Increase in ALT and AST levels from 30 min after reperfusion (R30) to R90 is defined as ΔALT and ΔAST, respectively. ALT released during the latter 60 min was the lowest in the CT group and significantly higher in the UW and UWD groups: vs. CT († $p \leq 0.039$). The UW and UWD groups exhibited similar values (N.S.). AST levels exhibited a similar trend. (C) The amount of enzyme released during the latter 60 min, ΔALT and ΔAST, are expressed as a proportion of the total ALT or AST released, respectively. The mean values were the lowest in the CT group and highest in the UW group, and the increase in ALT and AST levels in the UW group was suppressed in the UWD group (N.S.). Steel–Dwass test. Mean ± standard deviation.

Figure 1

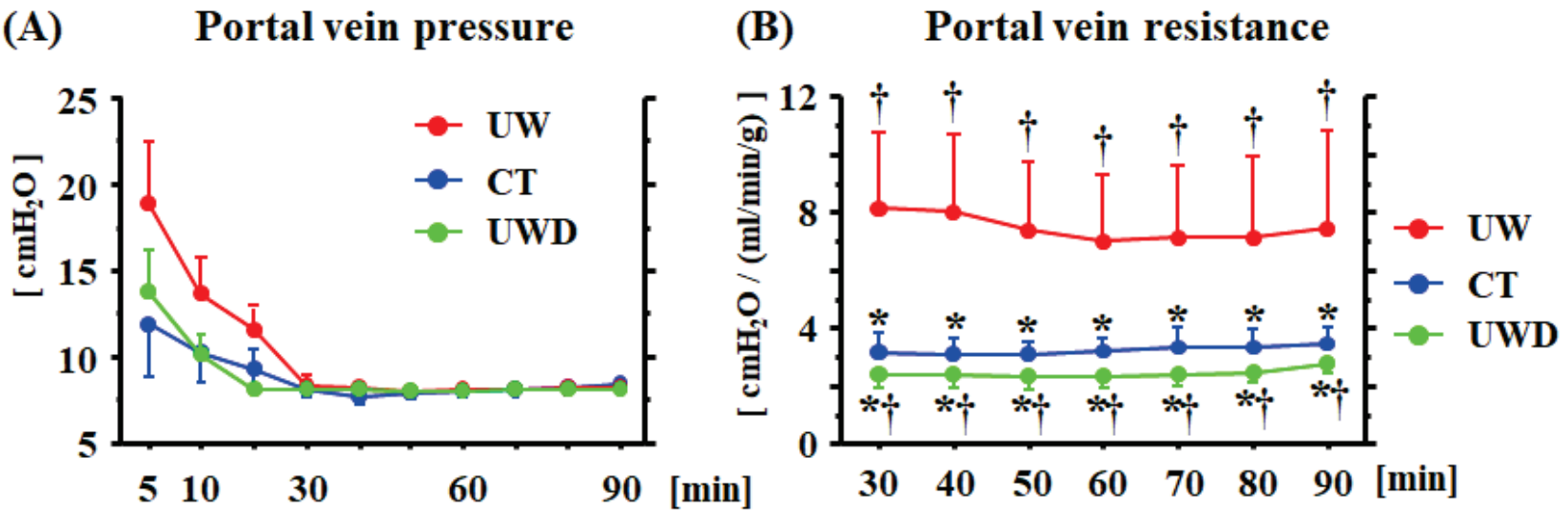

(A)
Portal vein pressure
[cmH2O]
25
20
15
10
5
UW
CT
UWD
5
10
30
60
90
[min]
(B)
Portal vein resistance
[cmH2O / (ml/min/g)]
12
8
4
0
30
40
50
60
70
80
90
[min]
UW
CT
UWD

Figure 2

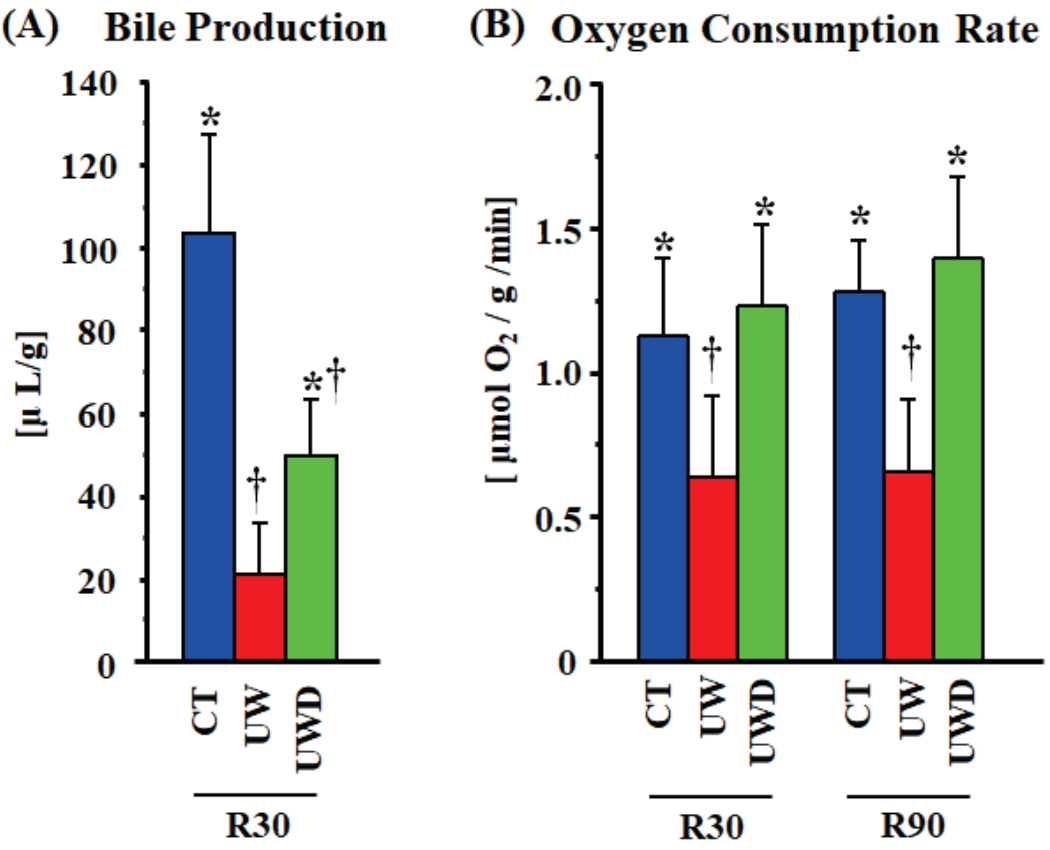

(A) Bile Production
[μ L/g]
140
120
100
80
60
40
20
0
*
†
*†
CT
UW
UWD
R30
(B) Oxygen Consumption Rate
[μmol O2 / g /min]
2.0
1.5
1.0
0.5
0
*
†
*
*
†
*
CT
UW
UWD
CT
UW
UWD
R30
R90

Figure 3

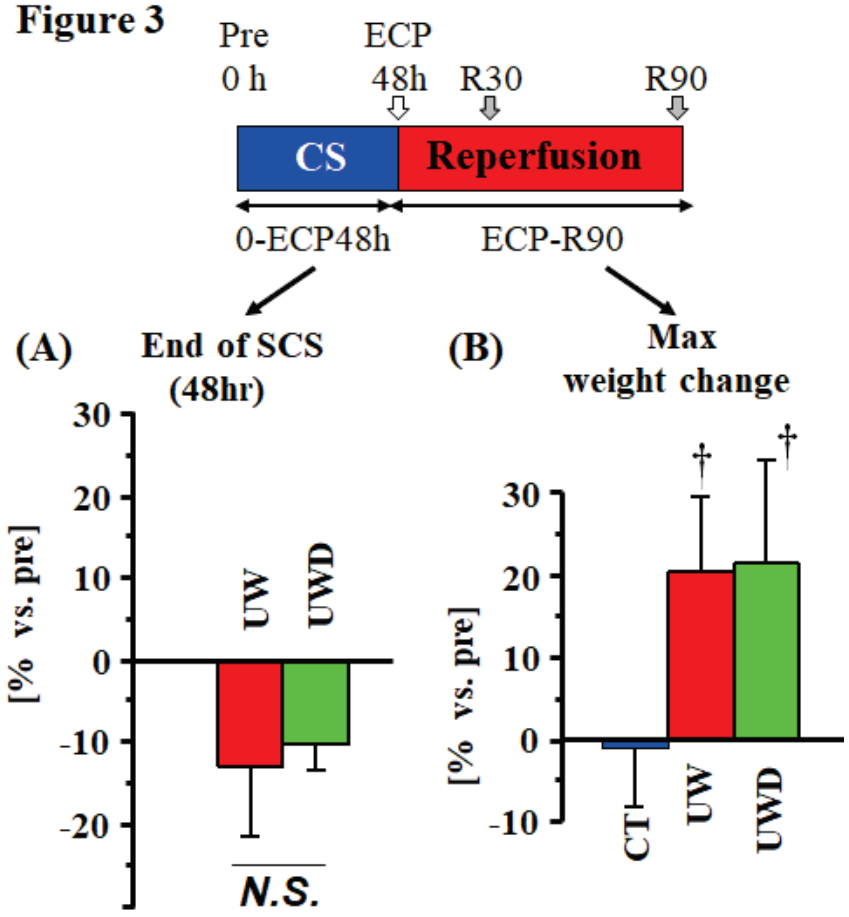
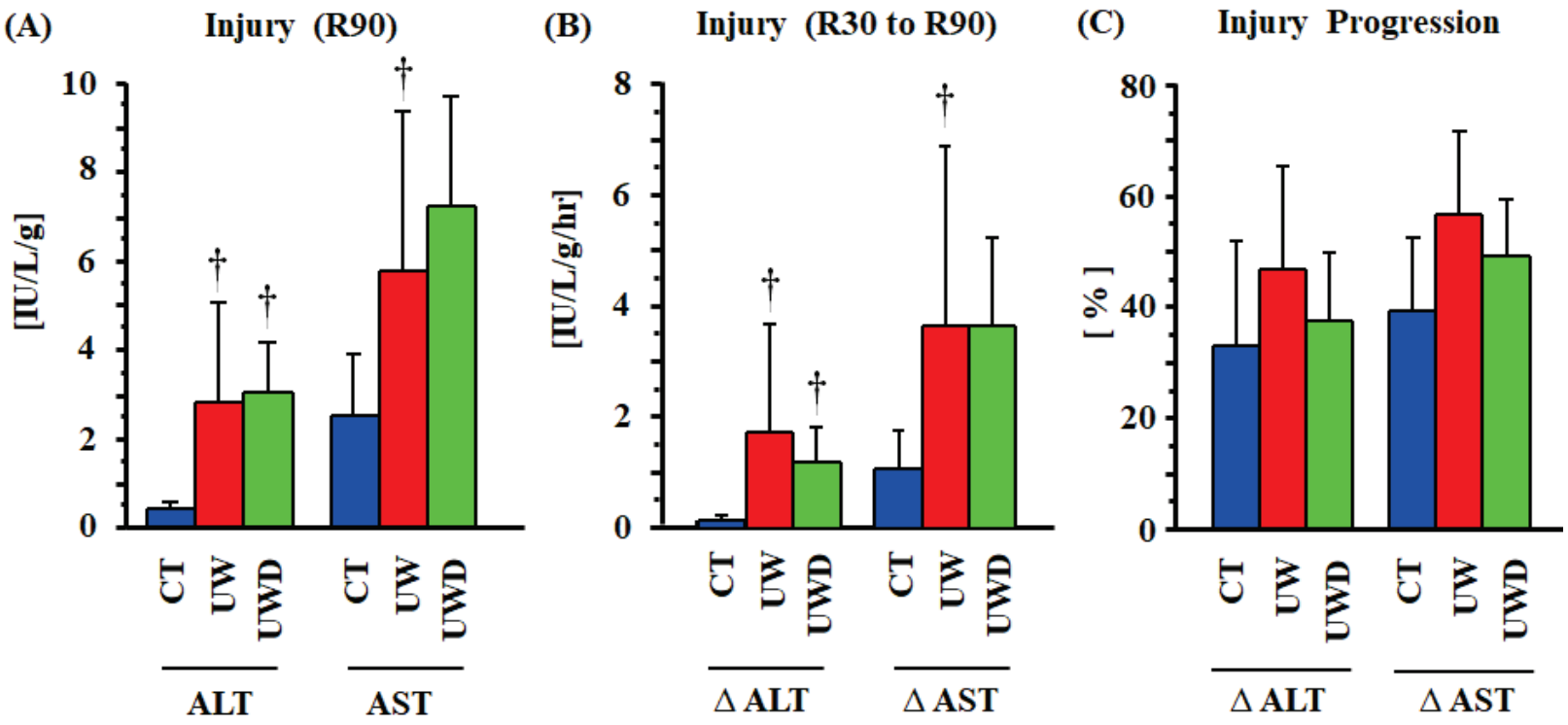

Pre
0 h
ECP
48h
R30
R90
CS
Reperfusion
0-ECP48h
ECP-R90
(A)
End of SCS
(48hr)
[% vs. pre]
30
20
10
0
-10
-20
UW
UWD
N.S.
(B)
Max
weight change
[% vs. pre]
30
20
10
0
-10
CT
UW
UWD
†
†

Figure 4


(A)
Injury (R90)
[IU/L/g]
10
8
6
4
2
0
†
†
†
CT
UW
UWD
CT
UW
UWD
ALT
AST
(B)
Injury (R30 to R90)
[IU/L/g/hr]
8
6
4
2
0
†
†
†
CT
UW
UWD
CT
UW
UWD
Δ ALT
Δ AST
(C)
Injury Progression
[%]
80
60
40
20
0
CT
UW
UWD
CT
UW
UWD
Δ ALT
Δ AST

Declaration of interests

☐ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☒ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Moto Fukai reports financial support was provided by Japan Society for the Promotion of Science. Tsuyoshi Shimamura reports financial support was provided by Japan Society for the Promotion of Science. Moto Fukai reports a relationship with Japan Society for the Promotion of Science that includes: funding grants. Tsuyoshi Shimamura reports a relationship with Japan Society for the Promotion of Science that includes: funding grants. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.