Title: Important constituents of heavy water-containing solution for cold storage and subsequent reperfusion on isolated perfused rat liver

Authors: Moto Fukai [a], Sodai Sakamoto [a], Kengo Shibata [a], Takahisa Ishikawa [a], Norio Kawamura [b], Masato Fujiyoshi [a], Sunao Fujiyoshi [a], Kosei Nakamura [a], Hiroki Bochimoto [c], Shingo Shimada [d], Tsuyoshi Shimamura [e], Akinobu Taketomi [a b]

Affiliations:

[a] Gastroenterological Surgery 1, Hokkaido University Graduate School of Medicine, Sapporo, Japan

[b] Department of Transplant Surgery, Hokkaido University Graduate School of Medicine, Sapporo, Japan

[c] Department of Cell Physiology, The Jikei University, School of Medicine, Tokyo, Japan

[d] Division of Hepato-Biliary-Pancreatic and Transplant Surgery, Asahikawa Medical University, Asahikawa, Japan

[e] Division of Organ Transplantation, Hokkaido University Hospital, Sapporo, Japan

Abstract: The University of Wisconsin (UW) solution is the most effective preservation solution currently used; however, to safely utilize expanded-criteria donor grafts, a new cold storage solution that alleviates graft injury more effectively is required. We prepared a heavy water (D_2O)-containing buffer, Dsol, and observed strong protective effects during extended cold storage of rat hearts and livers. In the current study, we modified Dsol (mDsol) and tested its efficacy. The aim of the present study was to determine whether mDsol could protect the rat liver more effectively than the UW solution and to clarify the roles of D_2O and deferoxamine (DFX). Rat livers were subjected to cold storage for 48 h in tests solutions: UW, mDsol, mDsol without D_2O or DFX [mDsol-D_2O(-), mDsol-DFX(-)], and subsequently reperfused on an isolated perfused rat liver for 90 min at 37°C. In the UW group, the liver was dehydrated during cold storage and rapidly expanded during reperfusion. Accordingly, the cumulative weight change was the highest in the UW group, together with augmented portal veinous resistance and ALT leakage and decreased oxygen consumption rate and bile production. These changes were significantly suppressed in the mDsol-treated group. In the mDsol-D_2O(-) and mDsol-DFX(-) groups offered partial protection. In conclusion, mDsol appeared to be superior to the UW solution for simple cold storage of the rat liver, presumably due to improved microcirculation in the early phase of reperfusion. Both heavy water and deferoxamine are essential for alleviating seamless organ swelling that occurs during cold storage and subsequent reperfusion.

Highlights

- Previously, we reported the effects of a heavy water-containing buffer, Dsol, as a cold storage solution. In this study, we modified Dsol and named it mDsol. The protective potential of mDsol was evaluated during extended cold storage and subsequent reperfusion in an isolated perfused rat liver (IPRL).
- mDsol showed better protective effects compared to the UW solution, as indicated by the lower ALT leakage and higher bile production, presumably due to the inhibition of the cumulative graft weight gain from the end of cold storage to the end of normothermic perfusion and reduction of portal venous resistance.
- Both heavy water and deferoxamine were essential for these protective effects of mDsol.

Introduction

The critical shortage of brain-dead donors has prompted clinicians to explore alternate methods to expand the potential donor pool. Although the University of Wisconsin (UW) solution provides the best preservation quality, simple cold storage (SCS) remains insufficient for expanded criteria donor (ECD) grafts, thus leading to a growing focus on machine perfusion (MP) [1]. Regardless, this does not negate the need for improved cold-storage techniques. Although machine perfusion has been demonstrated to be superior to SCS, SCS remains more cost-effective [2]. In the case of mechanical faults during MP, an

immediate transition to SCS is necessary, as ischemic damage may occur at temperatures above those of cold storage. Therefore, hypothermic machine perfusion (HMP) [3] or hypothermic oxygenated perfusion (HOPE) [4] is considered a methodology possessing a broad safety range. Furthermore, improved preservation should enhance the effectiveness of MP [4]. Previously, we reported the efficacy of a new preservation solution (Dsol) in rat livers [5] and hearts [6]. Dsol is a modified UW solution comprised of reduced potassium and lactobionate, with mannitol and sucrose instead of raffinose, and 30% heavy water (D_2O) as the solvent [5,6]. Cold storage in Dsol and post-reperfusion hydrogen gas treatment provided additional protection for rat livers subjected to extended cold preservation and subsequent reperfusion [7]. In these studies, heavy water appeared to facilitate mitochondrial protection, prevent cytoplasmic calcium overload, and reduce portal vein resistance, thereby suppressing cellular injury [5-7]. Here, we modified Dsol (termed mDsol) by exchanging the buffer component, adding an antioxidant, and increasing the amino acid content. The aim of the present study was to screen for the efficacy of mDsol compared to that of UW solution and to clarify the important components of mDsol in rat livers subjected to an extended SCS and subsequent reperfusion on an isolated perfused rat liver (IPRL) apparatus.

Materials and Methods

Animals and operation

Animal experiments were approved by the Institutional Review Board of Hokkaido University (approval

no. 17-0032). Male Wistar rats (8–10 weeks old) were purchased from Sankyo Labo Service Co. Inc. (Tokyo, Japan). The animals were fed a standard laboratory diet (Oriental Yeast, Tokyo, Japan) for at least 3 days prior to initiation of the experiment. The rats were anesthetized via isoflurane inhalation, and the liver was flushed with ice-chilled saline, cannulated in the common bile duct and the portal vein, excised, and immersed in Belzer UW® cold storage solution (Bridge to Life, Northbrook, IL) or test solutions for 48 h at 4 °C as previously described [7]. All reagents were of analytical grade or higher and were purchased from Wako unless otherwise noted.

IPRL

The originally constructed IPRL system consisted of an AD converter and integrator PowerLab 15T®, a BP-amplifier FE117®, a pump controller IN175, the integration software PowerLab® (ADInstruments, Dunedin, New Zealand), a MINIPULSE III MP-2® peristaltic pump (Gilson, Madison, WI, USA), and a PC along with a monitor. The IPRL conditions were established as previously described [5]. The perfusate was oxygenated Krebs-Henseleit bicarbonate buffer (300 mL) supplemented with sodium taurocholate. The graft was perfused for 90 min in a closed circuit at a constant perfusion pressure of 8 cm H_2O at 37 °C.

Experimental groups

Grafts were subjected to 48 h cold storage in test solutions such as UW solution and mDsol, where Dsol is a modified UW solution with 30% D_2O, supplemented mannitol and sucrose instead of raffinose, and

lacks hydroxy ethyl starch (HES) [6]. Dsol was further modified by exchanging the buffer with a HEPES-based buffer, adding deferoxamine mesylate as an antioxidant, and adding amino acids. Heavy water deleted mDsol (mDsol-$D_2O(-)$) and deferoxamine deleted mDsol (mDsol-DFX(−)) groups, respectively, ($n \geq 6$) were prepared. Grafts that were immediately re-perfused on the IPRL without SCS were designated as the normal group.

Assessment

Liver injury was evaluated by measuring ALT activity of the perfusate at 90 min of reperfusion (R90). Furthermore, the progression of reperfusion injury was evaluated according to the increase in ALT activity from 30–90 min of reperfusion and was designated as delta ALT/LW (R30-R90). The value was standardized to the liver weight before SCS and expressed as IU/L/g liver. Cumulative bile production during the 90 min of reperfusion was measured and expressed as μL/g of liver tissue. Portal venous pressure (PVP) was monitored throughout the experiment, and portal venous resistance (PVR) was calculated as PVP divided by portal flow. Graft weight was measured at the end of 48 h of SCS and R90 and expressed as % versus pre. The cumulative weight change from the end of SCS to the end of reperfusion (R90) was calculated and expressed as a percentage weight change versus the initial liver weight and was designated as the maximum graft weight change. The oxygen concentration in the perfusate was determined using a SIEMENS RAPIDLAB 348EX blood gas system® (Munich, Germany). The oxygen consumption rate (OCR) was calculated by subtracting the oxygen concentration

of the hepatic inflow and outflow and expressed as μmol O_2/min/g liver [5].

Statistical analysis

Statistical analysis was performed using the software JMP® version 16 (SAS Institute Japan, Tokyo, Japan). Data are presented as average ± standard deviation. Non-parametric multiple comparisons such as the Steel-Dwass or Dunn's test were performed. Results exhibiting $p < 0.05$ were considered significant.

Results

Liver injury

ALT leakage at the end of reperfusion (R90) was the lowest in the normal group (0.43 ± 0.20 IU/L/g), and it was significantly higher (2.31 ± 0.71 IU/L/g) in the UW group († p = 0.0284 vs. Normal; Dunn's test) (Figure 1A). This value tended to decrease in the mDsol group (1.43 ± 0.35 IU/L/g; p = 1.000 vs. Normal), whereas the values remained high in the mDsol-DFX(−) group (2.46 ± 1.07 IU/L/g; † p = 0.0229 vs. Normal) and the mDsol-D_2O(−) group (2.41 ± 0.88 IU/L/g; † p = 0.0464 vs. Normal). Notably, the values were significantly different in all groups compared to those of the normal group and not only in the mDsol group.

Ongoing liver injury was evaluated based on the increase in ALT activity (per liver weight) from R30 to R90 and was designated as delta ALT/LW (R30-R90) (Figure 1B). The value was the lowest in the normal group (0.11 ± 0.10 IU/L/g), and it was significantly higher (1.13 ± 0.65 IU/L/g) in the UW group († p =

0.0135 vs. Normal) (Figure 2A). The value tended to decrease in the mDsol group (0.57 ± 0.37 IU/L/g; p = 0.8170 vs. normal). These values remained high in the mDsol-DFX(−) group (0.91 ± 0.52 IU/L/g; p = 0.0800 vs. Normal). The value in the mDsol-D_2O(−) group (0.61 ± 0.29 IU/L/g; p = 0.6840 vs. normal) was equivalent to those in the mDsol group.

Bile production

Cumulative bile production during 90 min of reperfusion was the highest in the Normal group (102.3 ± 29.4 μL/g), whereas it was the lowest in UW group (20.7 ± 13.7 μL/g; † p = 0.0012 vs. Normal ; Steel Dwass test) (Figure 2A). The decrease in bile production was significantly suppressed in the mDsol group (54.3 ± 24.3 μL/g; † p = 0.0431 vs. Normal; *p = 0.0400 vs. UW). The efficacy of mDsol remained unchanged in the mDsol-DFX(−) group (43.8 ± 14.7 μL/g; † p = 0.0047 vs. Normal; *p = 0.0396 vs. UW) and in the mDsol-D_2O(−) group (42.4 ± 18.2 μL/g; † p = 0.0226 vs. Normal; p = 0.1981 vs. UW).

OCR

The OCR at R30 was the highest in the Normal group (1.05 ± 0.31 μmol O_2/min/g liver) and the lowest in the UW group (0.59 ± 0.21 μmol O_2/min/g liver; p = 0.0033, Normal vs. UW; Dunn's test) (Figure 2B). This decrease in OCR was significantly suppressed in the mDsol group (1.10 ± 0.36 μmol O_2/min/g liver; *p = 0.0237 vs. UW). The values in the mDsol-DFX(−) and mDsol-D_2O(−) groups were 0.76 ± 0.25 and 0.78 ± 0.24 (μmol O_2/min/g liver), respectively, and exhibited approximately half recovery compared to the ability observed in the mDsol group. The OCR at R90 exhibited a similar trend. The values in the Normal

and UW groups were 1.13 ± 0.10 and 0.52 ± 0.17 μmol O_2/min/g liver, respectively (*p = 0.007 vs. UW). This decrease in OCR was significantly suppressed in the mDsol group (1.10 ± 0.36 μmol O_2/min/g liver; *p < 0.0001 vs. UW). Although the value in the mDsol-DFX(−) group remained almost unchanged compared to that at R30, the value was increased in the mDsol-D_2O(−) group (0.98 ± 0.31 μmol O_2/min/g liver; p = 0.7782 vs. Normal; p = 0.4123 vs. UW; p = 1.000 vs. mDsol)

PVR

PVR was the lowest in the normal group and was the highest in the UW group († p ≤ 0.0002 vs. normal; Dunn's test). Augmentation of the PVR in the UW group was completely inhibited in the mDsol group (*p ≤ 0.0098 vs. UW). The values at R90 reached 3.68 ± 0.98, 8.94 ± 4.21, and 4.75 ± 2.53 (cm H_2O/[mL/min/g]) in the Normal, UW, and mDsol groups, respectively (Figure 3A). The effect of mDsol was suppressed in the mDsol-D_2O(−) and mDsol-DFX(−) groups. The PVR in the mDsol-D_2O(−) and mDsol-DFX(−) groups was not significantly different compared to that in the UW group (Figure 3B).

Graft weight change

The graft weight at the end of SCS at 48 h was the lowest in the UW group (−10.8 ± 4.1% vs. pre), whereas weight loss was significantly suppressed in the mDsol group (1.9 ± 1.3% vs. pre; *p = 0.0075 vs. UW), the mDsol-DFX(−) group (−3.5 ± 3.4% vs. pre; *p = 0.0196 vs. UW), and the mDsol-D_2O(−) group (−0.26 ± 2.8% vs. pre; *p = 0.0107 vs. UW), respectively (Figure 4A).

The graft weight at the end of 90 min reperfusion was the lowest in the Normal group (0.28 ± 2.5% vs. pre),

whereas the graft weight was augmented during the 90 min of reperfusion regardless of the preservation solution (Figure 4B) († $p < 0.0239$ vs. Normal). Cumulative graft weight change from the end of SCS to the end of reperfusion (R90) was designated as the maximum graft weight change and was augmented in the UW group (21.9 ± 7.2% vs. pre), the mDsol-DFX(−) group (19.6 ± 6.2% vs. pre), and the mDsol-D_2O(−) group (18.8 ± 6.7% vs. pre), respectively, and it was significantly suppressed in the mDsol group (7.4 ± 6.2% vs. pre; *$p = 0.029$ vs. UW) (Figure 4C).

Discussion

In the present study, we tested the efficacy of a novel preservation solution (mDsol) and confirmed that its composition affects tissue perfusion in the early phase of reperfusion. Furthermore, the initial good flow in the well-preserved grafts led to better-integrated hepatic function as indicated by cumulative bile production. These observations suggest the importance of the present study and highlight the necessity of continuing basic research focused on the SCS despite the research trend shift toward MP.

In the present study, we used SCS and subsequent reperfusion in an IPRL model. Previously, we reported that preservation solutions that were effective in cellular experiments appeared to be ineffective in transplantation models due to viscosity and the formation of blood clots [8]; however, the PVR in the mDsol group was equivalent to that of the normal group. In the context of severe hepatic ischemia and reperfusion injury, endithelin-1 (ET-1) is activated during cold preservation, and PVR exhibits a high

value from the beginning of reperfusion [9]. Furthermore, reduction in PVR by hydrogen gas [7] or inhibition of ET-1 suppresses graft injury [10]. These results are consistent with those of the current study.

In this study, the change in liver weight was measured three times, and the data indicate dehydration during SCS in the UW group. The liver that had initially shrunk by 10.8% during cold storage was swollen by approximately 11.2% beyond the initial value during reperfusion, ultimately resulting in a rapid volume change equivalent to 22.0% of the initial liver weight within 90 min of normothermic reperfusion. As this rapid weight change and increase in PVR were observed prior to other alterations, the importance of the early suppression of these changes was suggested. Hata et al. reported that SCS in Polysol significantly reduced portal venous pressure in an IPRL in constant-flow mode, thus indicating a reduction in PVR together with reduced tissue edema [11]. Tissue swelling during interventions involving temperature changes such as from SCS or HOPE to normothermic perfusion causes subsequent perfusion failure and worsens organ function if the solutions for SCS and/or MP are insufficient [4].

This study had some limitations. First, as the OCR reflects both oxidative phosphorylation and the generation of reactive oxygen species, the evaluation of ATP and oxidative stress markers is necessary. However, the present study focused on screening the effects of preservation solutions on hepatic microcirculation and injury. Second, the reason why both D_2O and DFX are necessary to suppress changes in liver weight remains unclear. Further detailed determinations, particularly histopathology and ultrastructural, are planned in future investigations.

Conclusions

For simple cold storage of the rat liver, mDsol conferred stronger protective properties than did the UW solution due to improved microcirculation during reperfusion. Both heavy water and deferoxamine are essential for alleviating the seamless organ swelling that occurs during cold storage and subsequent reperfusion.

Acknowledgements

I am grateful to Mr. Masatoshi Horigome for his dedication to animal care and to Ms. Nozomi Kobayashi for technical assistance. We would like to thank Editage (www.editage.jp) for English language editing.

Funding

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) and the Ministry of Education, Culture, and Technology (MEXT) of Japan. Grant numbers: 20K08974 (Fukai); 20H03737 (Shimamura); 20K17546 (Fujiyoshi); 21K16394 (Ishikawa)

References

1. Widmer J, Eden J, Carvalho MF, Dutkowski P, Schlegel A. Machine Perfusion for Extended

Criteria Donor Livers: What Challenges Remain? *J Clin Med.* 2022;11;5218.

2. Amersi F, Shen XD, Anselmo D, et al. Ex vivo exposure to carbon monoxide prevents hepatic ischemia/reperfusion injury through p38 MAP kinase pathway. *Hepatology.* 2002;35:815-823.

3. Iwata H, Matsuno N, Ishii D, et al. Applicability of the histidine-tryptophan-ketoglutarate solution as a machine perfusion solution for marginal liver grafts. *J Gastroenterol Hepatol.* 2023;38:783-790.

4. Asong-Fontem N, Panisello-Rosello A, Sebagh M, Gonin M, Rosello-Catafau J, Adam R. The Role of IGL-2 Preservation Solution on Rat Livers during SCS and HOPE. *Int J Mol Sci.* 2022;23;12615.

5. Shimada S, Fukai M, Shibata K, et al. Heavy Water (D(2)O) Containing Preservation Solution Reduces Hepatic Cold Preservation and Reperfusion Injury in an Isolated Perfused Rat Liver (IPRL) Model. *J Clin Med.* 2019;8;1818.

6 Wakayama K, Fukai M, Yamashita K, et al. Successful transplantation of rat hearts subjected to extended cold preservation with a novel preservation solution. *Transpl Int.* 2012;25:696-706.

7. Fukai M, Sakamoto S, Shibata K, et al. Combination of Cold Storage in a Heavy Water-Containing Solution and Post-Reperfusion Hydrogen Gas Treatment Reduces Ischemia-Reperfusion Injury in Rat Livers. *Transplant Proc.* 2023;55:1027-1031.

8. Hirokata G, Fukai M, Wakayama K, et al. Does heavy water attenuate cold preservation and

reperfusion injury in canine kidney? *Low Temp Med.* 2012;38:62-68.

9. Shimada S, Wakayama K, Fukai M, et al. Hydrogen Gas Ameliorates Hepatic Reperfusion Injury After Prolonged Cold Preservation in Isolated Perfused Rat Liver. *Artif Organs.* 2016;40:1128-1136.

10. Jin MB, Zhu Y, Zhang S, et al. Attenuation of ischemic liver injury by a non-selective endothelin receptor antagonist. *Transplant Proc.* 1997;29:1335.

11. Hata K, Tolba RH, Wei L, et al. Impact of polysol, a newly developed preservation solution, on cold storage of steatotic rat livers. *Liver Transpl.* 2007;13:114-121.

Figure Legends

Figure 1: Liver injury and function after 48 h of cold storage and subsequent reperfusion. The efficacy of mDsol was compared to that of the UW solution and to that of mDsol without deferoxamine (mDsol-DFX(−)) and without heavy water (mDsol-D_2O(−)). In the normal group, grafts were immediately applied to the IPRL without cold storage. (A) Overall injury: ALT/LW (R90). All groups with the exception of the mDsol group exhibited significantly higher values compared to that of Normal group. (B) Ongoing injury: delta ALT/LW (R30-R90). The UW group exhibited a significantly higher value compared to that of the Normal group. * $p < 0.05$, vs. UW; † $p < 0.05$, vs. Normal. Dunn or Steel-Dwass test. Mean ± SD, $n \geq 6$.

Figure 2: Liver function after 48 h of cold storage and subsequent reperfusion. The efficacy of mDsol was compared to that of the UW solution and to that of mDsol without deferoxamine (mDsol-DFX(−)) and

without heavy water (mDsol-D_2O(−)). In the normal group, grafts were immediately applied to the IPRL without cold storage. (A) Bile production. The Normal group exhibited significantly a higher value than those of all other groups. The value was the lowest in UW group, whereas it was significantly augmented in the mDsol and mDsol-DFX(−) groups. (B) Oxygen consumption rate (OCR). The value was the highest and the lowest in the Normal and UW groups, respectively. OCR was significantly recovered in the mDsol group and appeared to exhibit half recovery in the mDsol-DFX(−) and mDsol-D_2O(−) groups.*$p < 0.05$, vs. UW; †$p < 0.05$, vs. Normal. Dunn or Steel-Dwass test. Means ± SD, n≥6.

Figure 3: Portal venous resistance (PVR) after 48 h of cold storage and subsequent reperfusion. The effect of the preservation solutions on PVR was assessed. (A) Efficacy of mDsol. PVR was significantly higher in the UW group than that in the Normal group throughout the experiment. Furthermore, PVR was significantly lower in the mDsol group than that in the UW group throughout the experiment. (B) PVR tended to increase in the mDsol without deferoxamine (mDsol-DFX(−)) and mDsol without heavy water (mDsol-D_2O(−)) groups (N.S.). *$p < 0.05$, vs. UW; †$p < 0.05$, vs. Normal. Dunn or Steel-Dwass test. Mean ± SD, n≥6.

Figure 4: Graft weight change after 48 h of cold storage and subsequent reperfusion. The effect of the preservation solutions on graft weight change was assessed. (A) At the end of SCS at 48 h. The graft weight was significantly decreased in the UW group compared to those of the other groups. (B) At the end of

reperfusion for 90 min (R90). In the Normal group, the graft weight was almost unchanged after 90 min of perfusion, whereas the grafts were significantly swollen in all groups regardless of preservation solution. (C) Maximum graft weight change. The value was the highest in the UW group, whereas it was significantly decreased only in the mDsol group. *$p < 0.05$, vs. UW; **$p < 0.05$, vs. mDsol; †$p < 0.05$, vs. Normal. Dunn or Steel-Dwass test. Mean ± SD, $n \geq 6$.

Figure 1

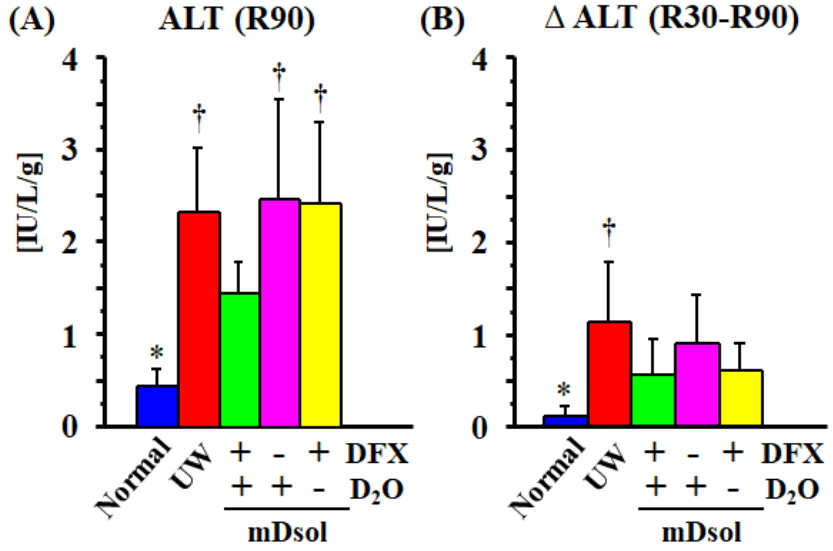

Figure 2

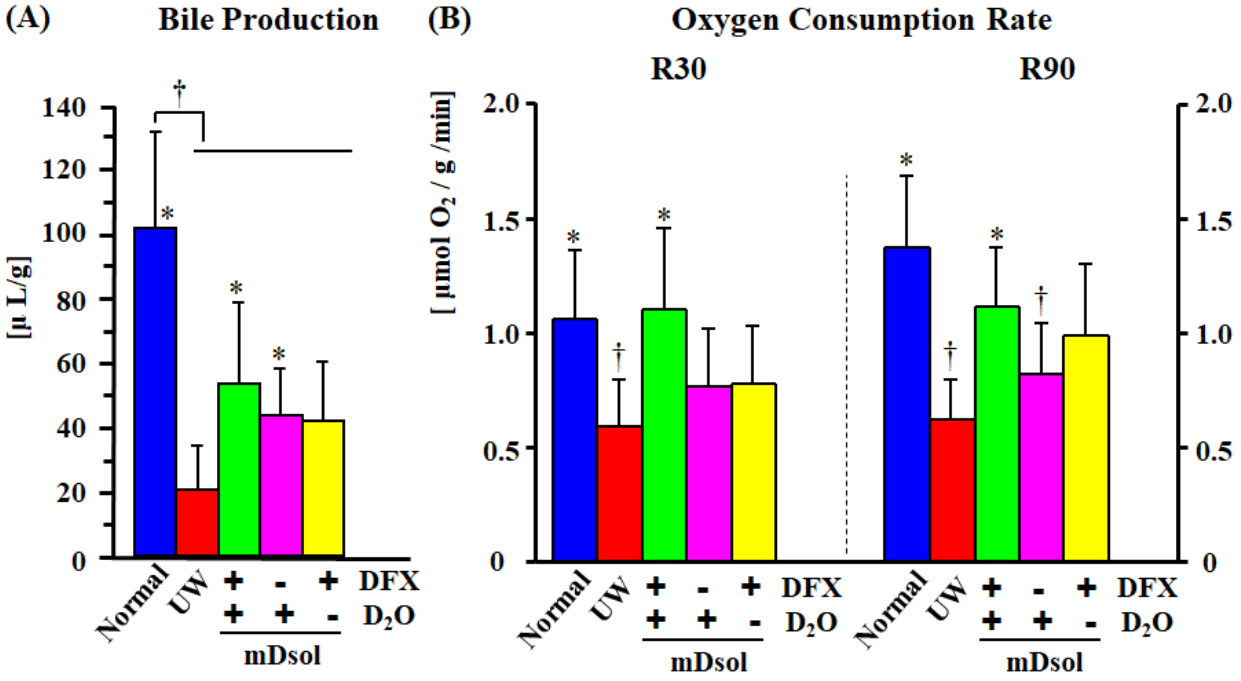

Figure 3

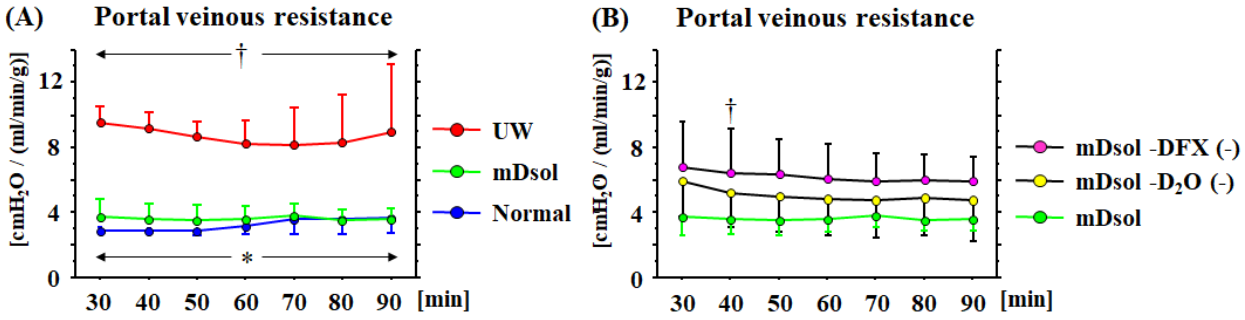

Figure 4