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Development of Transplant Surgeon-Innovated Ex Vivo Machine Perfusion: Validation in a Porcine Donation after Circulatory Death-Simulated Liver Transplant Model

Mun Chae Choi[1,2], Yuri Cho[3], Yu Seol Lee[4,5], Sat Byol Lee[3], Ji Yun Bang[4,5], Eun-Ki Min[3,6], Deok-Gie Kim[3,6], Jae Geun Lee[3,6], Myoung Soo Kim[3,6], Soo Han Bae[4,5], and Dong Jin Joo[3,6]

[1]Department of Surgery, Armed Forces Capital Hospital, Seongnam;
[2]Department of Surgery, Graduate School, Yonsei University College of Medicine, Seoul;
[3]The Research Institute for Transplantation, Yonsei University College of Medicine, Seoul;
[4]Department of Biomedical Sciences, Yonsei University College of Medicine, Seoul;
[5]Graduate School of Medical Science, BK21 PLUS Project, Yonsei University College of Medicine, Seoul;
[6]Department of Surgery, Yonsei University College of Medicine, Seoul, Korea.

Purpose: Ex vivo machine perfusion (EVMP) is increasingly recognized as a promising technique for enhancing the preservation and viability of donor organs, particularly in donation after circulatory death (DCD) liver transplantation (LT). This study validates a transplant surgeon-innovated EVMP protocol by assessing its efficacy in preserving liver function and reducing ischemia-reperfusion injury (IRI) in a porcine DCD-simulated liver transplant (DCD sLT) model.

Materials and Methods: Twenty Yorkshire pigs were used to compare static cold storage (SCS) and EVMP. In Model 1, the SCS group (n=5) underwent 5 hours of cold storage, while the EVMP group (n=9) had 1 hour of cold storage followed by 4 hours of EVMP. In Model 2, the SCS group (n=3) underwent 6 hours of cold storage, while the EVMP group (n=3) had 2 hours of cold storage followed by 4 hours of EVMP. Hemodynamic stability during perfusion, laboratory findings, and apoptosis (via TUNEL assay) after reperfusion were evaluated.

Results: The EVMP system was successfully used all 12 cases without technical complications. Hemodynamic parameters remained stable throughout perfusion. In Model 2, alanine aminotransferase levels were significantly lower in the EVMP group compared to the SCS group (e.g., 134.3±27.0 U/L vs. 48.0±6.2 U/L, p=0.006 at 3 hours post-reperfusion). TUNEL staining revealed significantly reduced hepatic apoptosis in the EVMP group compared to the SCS group at 2 and 3 hours post-reperfusion in both models.

Conclusion: This study successfully demonstrated the stability of the transplant surgeon-innovated normothermic EVMP protocol, validating its efficacy in improving organ preservation and reducing IRI in a porcine DCD sLT model.

Key Words: Perfusion, warm ischemia, liver transplantation, reperfusion injury, experimental animal models

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Co-corresponding authors: Dong Jin Joo, MD, PhD, Department of Surgery, The Research Institute for Transplantation, Yonsei University College of Medicine, 50-1 Yonsei-ro, Seodaemun-gu, Seoul 03722, Korea.
E-mail: DJJOO@yuhs.ac and
Soo Han Bae, PhD, Department of Biomedical Sciences, Graduate School of Medical Science, BK21 PLUS Project, Yonsei University College of Medicine, 50-1 Yonsei-ro, Seodaemun-gu, Seoul 03722, Korea.
E-mail: soohanbae@yuhs.ac

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INTRODUCTION

Liver transplantation (LT) remains the only definitive treatment for patients with end-stage liver disease.[1] However, due to the limited availability of donor organs, a growing shortage increasingly challenges the feasibility of LT. To address this organ shortage, many Western countries have expanded their donor pools by incorporating organs donated after circulatory death (DCD).[2,3] Unlike donation after brain death, DCD organs are subject to prolonged warm ischemic times, which heighten their susceptibility to ischemic injury and increase the risk of ischemia-reperfusion injury (IRI) and early allograft dysfunction.[4]

To mitigate these challenges, ex vivo machine perfusion (EVMP) has gained recognition as a promising alternative to traditional static cold storage (SCS) for preserving and enhancing the viability of donor organs.[5] EVMP mitigates IRI through various mechanisms, primarily by maintaining a continuous supply of oxygenated blood to the liver, which supports metabolic homeostasis and prevents the accumulation of anaerobic metabolites. Additionally, EVMP modulates the inflammatory response following reperfusion by minimizing oxidative stress and cytokine release, thereby reducing hepatocellular injury and improving post-transplant graft function.[5] Randomized trials, regardless of the specific EVMP protocol used, consistently demonstrate advantages over SCS in reducing acute allograft injury, lowering early allograft dysfunction rates, and minimizing histological signs of graft injury.[6-10]

Animal models are essential for advancing transplant research and enabling the translation of novel clinical technologies. Large animal models, particularly pigs, provide physiological conditions closely resembling those of humans, offering critical insights into responses that small animal models cannot adequately simulate. Over the past two decades, research on EVMP and LT in large animal models has built a robust foundation for the clinical adoption of this technology.[11] However, in the Republic of Korea, the lack of established large animal models for DCD LT and the absence of commercially available EVMP devices have limited the progress of such studies. This gap highlights the need for further development of experimental infrastructure to facilitate cutting-edge research in EVMP and its applications within Korea's unique clinical and regulatory environment.

This study aims to validate a surgeon-innovated EVMP protocol and assess its effectiveness in preserving liver function and reducing IRI in a porcine DCD-simulated liver transplant (DCD sLT) model.

MATERIALS AND METHODS

Animals

From September 2020 to October 2024, 20 Yorkshire pigs (XP Bio Inc., Anseong, Korea) were utilized as our experimental animal model. The pigs were 12-week-old females, weighing 40–50 kg. Prior to the experiment, the animals underwent a 1-week acclimatization period.

This study was conducted in accordance with the institutional guidelines on the use of live animals for research, and the experimental protocol (2023–0230) was approved by the Institutional Animal Care and Use Committee of Yonsei University Health System.

For anesthesia, the pigs were initially injected with a combination of anesthetic agents and muscle relaxants (Alfaxan 10 mg/kg, medetomidine 0.02 mg/kg, azaperone 2 mg/kg). Following this, the animals were intubated by a veterinarian. Anesthesia was maintained using 2% isoflurane inhalation. Throughout the procedure, the animals' vital signs were continuously monitored to ensure stability.

Porcine DCD model

A long midline incision was made, followed by dissection of the liver ligaments and isolation of hilar structures [hepatic artery (HA), portal vein (PV), and common bile duct]. Both suprahepatic and infrahepatic inferior vena cava (IVC) were dissected. To simulate DCD, a 1-hour warm ischemic injury period was established. The HA, PV, and infrahepatic IVC were clamped prior to liver extraction to induce warm ischemic injury. Heparin (200 IU/kg) was administered intravenously 3 minutes before clamping. Porcine whole blood samples were then collected after cannulating the intrahepatic IVC and PV. The liver was extracted, and the HA, PV, and common bile duct were individually cannulated. After the 1-hour warm ischemic period, the liver was flushed with 1.0 L of Histidine-Tryptophan-Ketoglutarate solution for organ preservation.

Experimental study design

For liver preservation, we compared two methods: SCS and EVMP. The diagram of the experimental study design is detailed in Fig. 1.

The first protocol (Model 1) was conducted from September 2020 to December 2023. After 1 hour of warm ischemic time, the SCS group (n=5) underwent 5 hours of cold storage, while the EVMP group (n=9) had 1 hour of cold storage followed by 4 hours of EVMP preservation. After preservation, we performed ex vivo reperfusion and observed it for 2 hours. We set the PV flow at 1000 mL/min and the HA pressure at 100 mm Hg.

The second protocol (Model 2), conducted from March 2024 to October 2024, followed the same procedure with a 1-hour warm ischemic time. However, the SCS group (n=3) underwent 6 hours of cold storage, while the EVMP group (n=3) had 2 hours of cold storage followed by 4 hours of EVMP preservation. We then performed ex vivo reperfusion and observed it for 3 hours. In contrast to the previous protocol, we reduced the portal flow to 800 mL/min and lowered the hepatic arterial pressure to 80 mm Hg.

How to innovate normothermic ex-vivo machine perfusion

The normothermic EVMP system was composed of a reservoir, centrifugal pump, pulsatile pump, oxygenator, and heater-cooler system, with the heater-cooler system set to 37°C (Fig. 2). The liver was placed in a custom-designed metal reservoir, where perfusate from the IVC was collected and circulated through the oxygenator and heater-cooler system after passing through a disposable centrifugal pump. Flow was regulated by a precision centrifugal pump, which delivered perfusate to the PV catheter, while a pulsatile pump was used to supply perfusate to the HA catheter, allowing for continuous HA pressure monitoring. Detailed information about the equipment used, including the name, manufacturer, and country of origin, is provided in the Supplementary Table 1 (only online).

The perfusate consisted of 400–600 mL of red blood cells isolated from porcine whole blood via centrifugation, diluted with normal saline at a 1:2 ratio, resulting in a total perfusate volume of 1200–1800 mL. During perfusion, bicarbonate supplements were administered to the perfusate as needed to correct acidosis, aiming to maintain the pH close to 7.4.

Ex vivo reperfusion

To simulate reperfusion, we employed an EVMP system using porcine whole blood. A total volume of 1600 mL was prepared by diluting 800 mL of porcine whole blood with normal saline in a 1:1 ratio. Ex vivo reperfusion was then performed with this solution. After initiating reperfusion, observations were conducted for 2 hours in Model 1 and 3 hours in Model 2, comparing outcomes between the SCS group and the EVMP group.

Blood samples

Blood samples were sequentially collected at the following time points: baseline, every hour during the 4-hour machine perfusion period, immediately after reperfusion, and then every hour up to 3 hours after reperfusion.

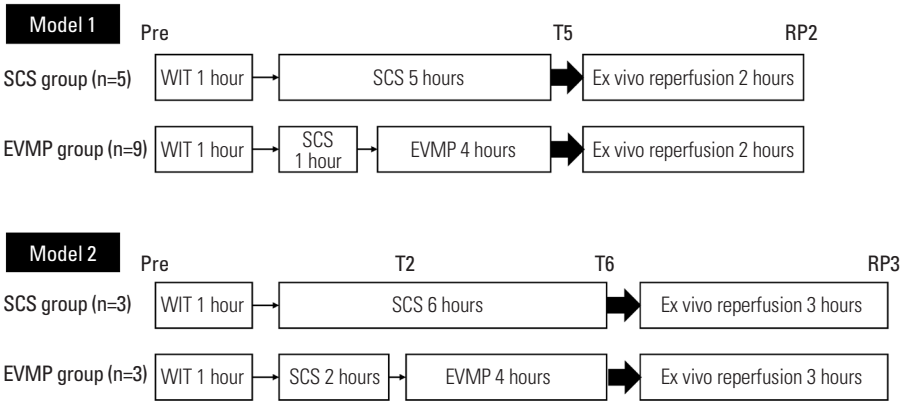

Fig. 1. Experimental study design. Both Model 1 and Model 2 were set with a 1-hour warm ischemic time. The SCS groups underwent cold storage for 5 hours in Model 1 and 6 hours in Model 2 before reperfusion. For the EVMP groups, 4 hours of EVMP was applied in both models, preceded by 1 hour of cold storage in Model 1 and 2 hours in Model 2. SCS, static cold storage; EVMP, ex vivo machine perfusion; WIT, warm ischemic time.

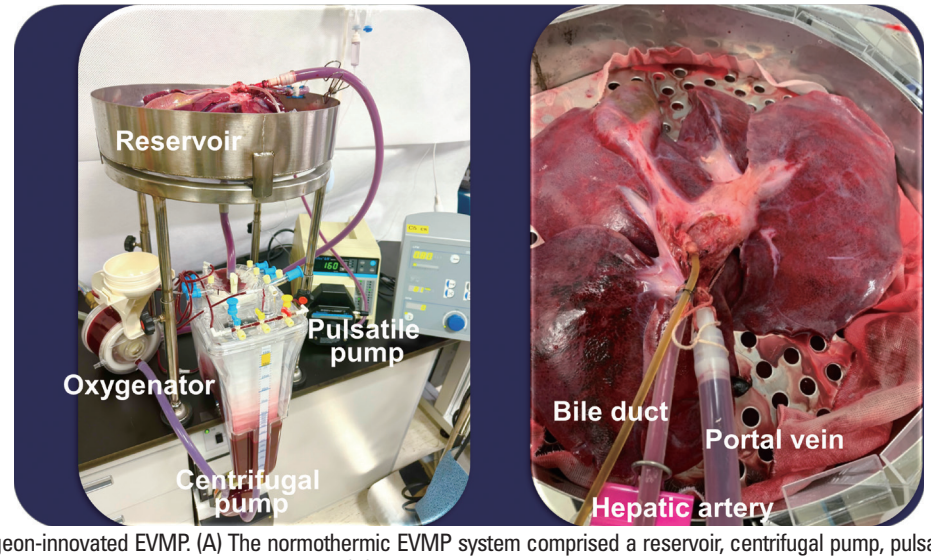

Fig. 2. Transplant surgeon-innovated EVMP. (A) The normothermic EVMP system comprised a reservoir, centrifugal pump, pulsatile pump, oxygenator, and heater-cooler system, with the heater-cooler set to 37°C. (B) Portal vein, hepatic artery, and bile duct of graft liver were each cannulated. EVMP, ex vivo machine perfusion.

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Blood samples were analyzed for hepatocyte injury markers, including aspartate aminotransferase (AST) and alanine aminotransferase (ALT), synthetic liver function via prothrombin time, and the ischemic injury marker lactate dehydrogenase (LDH). Complete blood count tests were also performed. Additionally, arterial blood gas analysis was conducted to measure pH, HCO_3^-, pO_2, and pCO_2. Furthermore, the activated clotting time of the perfusate was monitored.

TUNEL assay

For Model 1, liver biopsies were collected at three time points: baseline, the end of preservation, and 2 hours after reperfusion. For Model 2, liver biopsies were taken at six time points: baseline; 2 hours after preservation; the end of preservation; and 1, 2, and 3 hours after reperfusion.

To detect apoptosis in liver tissues, TUNEL staining was performed on 5-μm-thick paraffin-embedded sections using a TUNEL assay kit (Roche, Mannheim, Germany), following the manufacturer's instructions. Apoptosis was defined as TUNEL-positive cells among hepatocytes, confirmed by the presence of nuclear condensation using DAPI staining (VECTASHIELD h1200, Vector Laboratories Inc., Burlingame, CA, USA).

To assess the extent of apoptosis, the ratio of TUNEL-positive cells at each time point was calculated relative to baseline levels. These ratios were compared between the SCS and EVMP groups to evaluate differences in apoptotic activity.

Statistical analysis

The data are presented as mean±SD. Statistical comparisons between the SCS and EVMP groups were performed using Student's t-test and two-way analysis of variance, with a p value of less than 0.05 considered statistically significant. Graphing and statistical analyses were conducted using GraphPad Prism (version 10.3.1; GraphPad Software, San Diego, CA, USA).

RESULTS

Stability of EVMP

In all 12 cases in the EVMP group (9 cases in Model 1 and 3 cases in Model 2), perfusion was successfully conducted without any events such as air embolism, tube kinking, hardware malfunction, or failure in pressure or flow monitoring.

The target PV flow and HA pressure were set to 1000 mL/min and 100 mm Hg, respectively, in Model 1, and 800 mL/min and 80 mm Hg in Model 2. During the 4-hour perfusion period, hemodynamic parameters (PV flow and HA pressure) were stably maintained in both models, consistently achieving their respective target values. With the reduced target PV flow in Model 2, the revolutions per minute (RPM) of the PV centrifugal pump was significantly lower compared to Model 1, while remaining relatively stable in both models (Model 1: 570.0±54.8 vs. Model 2: 400.7±77.0 at 4 hours after perfusion, p=0.019) (Fig. 3A). The RPM of the HA pulsatile pump gradually decreased during the perfusion period in both models, suggesting a progressive reduction in HA resistance (Fig. 3B). The pH and bicarbonate levels of the perfusate showed an improvement in acidosis over the perfusion period, and were stably maintained in both models (Fig. 3C and D).

Difference of laboratory finding after reperfusion

In Model 1, no significant differences in AST and ALT levels were observed between the SCS and EVMP groups from immediately after reperfusion up to 2 hours. The INR tended to be lower in the EVMP group, but this difference was not statistically significant. LDH levels also showed no significant difference between the two groups (Fig. 4A).

In Model 2, however, ALT levels were significantly lower in the EVMP group compared to the SCS group from immediately after reperfusion up to 3 hours (immediately after reperfusion: 62.0±19.3 U/L vs. 30.3±3.5 U/L, p=0.049; 3 hours after reperfusion: 134.3±27.0 U/L vs. 48.0±6.2 U/L, p=0.006). AST, INR, and LDH levels also trended lower in the EVMP group, though these differences did not reach statistical significance (Fig. 4B).

Comparison of TUNEL results

In Model 1, TUNEL staining comparisons between the SCS and EVMP groups revealed that, at 2 hours after reperfusion, the SCS group exhibited approximately a 16-fold increase in TUNEL-positive cells compared to baseline liver values. This increase was significantly higher than that observed in the EVMP group, with TUNEL ratios (relative to baseline) of 16.2±3.4 vs. 3.7±3.9, respectively (p<0.001 at 2 hours after reperfusion) (Fig. 5A).

In Model 2, no significant difference in TUNEL-positive cell ratios was observed between the groups up to the end of preservation. However, a significant divergence emerged starting from 2 hours after reperfusion, with the EVMP group demonstrating a markedly lower TUNEL ratio compared to the SCS group (5.0±3.8 vs. 23.1±17.3, p=0.002 at 3 hours after reperfusion) (Fig. 5B). Representative images of TUNEL staining for both the SCS and EVMP groups in Model 2 are presented in Fig. 5C.

DISCUSSION

This study successfully demonstrated the stability and reliability of the transplant surgeon-innovated normothermic EVMP protocol by monitoring hemodynamic parameters during perfusion without technical complications such as air embolism or equipment malfunctions. Additionally, this study validated the efficacy of the EVMP protocol in reducing IRI and improving liver preservation outcomes in a porcine DCD sLT model. In both experimental models, EVMP demonstrated superior performance, particularly in Model 2, where optimization of PV flow and HA pressure settings (800 mL/min and 80 mm Hg, re-

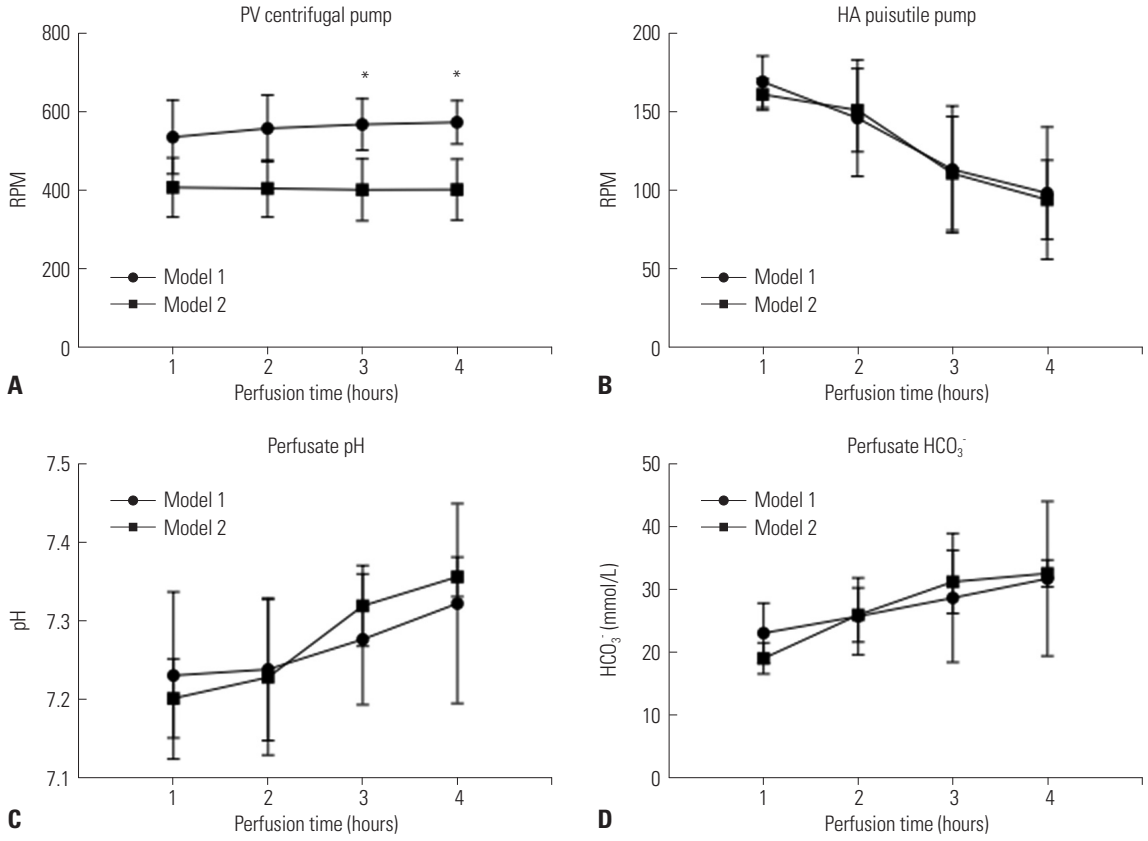

Fig. 3. RPM of the centrifugal and pulsatile pumps (A and B) and the pH and HCO_3^- levels of the perfusate (C and D) during perfusion. At 3 and 4 hours after perfusion, the RPM of the PV centrifugal pump (A) was significantly lower in Model 2 compared to Model 1 (Model 1: 565.0±64.5 vs. Model 2: 400.0±78.1 at 3 hours after perfusion, p=0.028; Model 1: 570.0±54.8 vs. Model 2: 400.7±77.0 at 4 hours after perfusion, p=0.019). The RPM of the HA pulsatile pump (B) exhibited a gradual decrease over the perfusion period in both models, though no significant differences were observed between the two groups. While no significant differences were noted between the models, the pH (C) and HCO_3^- (D) levels of the perfusate showed improvement in acidosis throughout the perfusion period. *p<0.05 at each time point. PV, portal vein; HA, hepatic artery; RPM, revolutions per minute.

spectively) led to significant improvements in liver function markers. ALT levels, in particular, were significantly lower in the EVMP group compared to the SCS group, reflecting reduced hepatocyte injury. Furthermore, TUNEL assay results indicated significantly fewer apoptotic cells in the EVMP group after reperfusion, supporting the hypothesis that EVMP mitigates IRI more effectively than traditional SCS.

While normothermic EVMP devices have been extensively developed and commercialized in the United States and Europe,[5] such devices remain inaccessible in South Korea due to import restrictions. Additionally, current regulations limit the use of DCD donors in Korea,[12] and the relatively short distances between donor and recipient hospitals reduce the demand for prolonged organ preservation. Despite these factors, there is an increasing body of research demonstrating EVMP's benefits in DCD, marginal, and severe fatty liver donors, as well as in studies on IRI mechanisms.[4,5,13,14] This necessity drove the development and experimentation of a custom-made EVMP device by the transplant surgeon's laboratory, setting a foundation for future research and application within South Korea.

The utility of EVMP is increasingly recognized, particularly for DCD donors, due to its potential to reduce IRI and improve LT outcomes, such as lower rates of early allograft dysfunction. Consequently, numerous studies have explored EVMP using animal DCD LT models.[11] This study aimed to validate the efficacy of our newly developed EVMP device using a DCD porcine model, selecting Yorkshire pigs due to their physiological similarities to humans. Regarding the setup for the DCD porcine LT model, we reviewed previous studies which employed warm ischemic times ranging from 30 to 90 minutes, depending on the goal of ischemic injury severity.[14-19] To maximize IRI in our study, we set the warm ischemic time to 60 minutes.

In Model 1, the differences in reperfusion laboratory findings between the SCS and EVMP groups were not substantial. This was likely due to the relatively short preservation time and potentially elevated PV flow and HA pressure settings during perfusion. To address these limitations, we made two adjustments in Model 2. First, we extended the preservation time for the SCS group to 6 hours to provide longer ischemic exposure. Second, after reviewing methodologies from previous studies, we noted a lack of consensus on optimal PV flow and HA pressure targets. Reported PV flows varied widely, from 310 to 1600 mL/min,[14,15] and HA pressures ranged from 30 to 100 mm Hg.[14,18,19] Given that our initial settings (PV flow of 1000 mL/min

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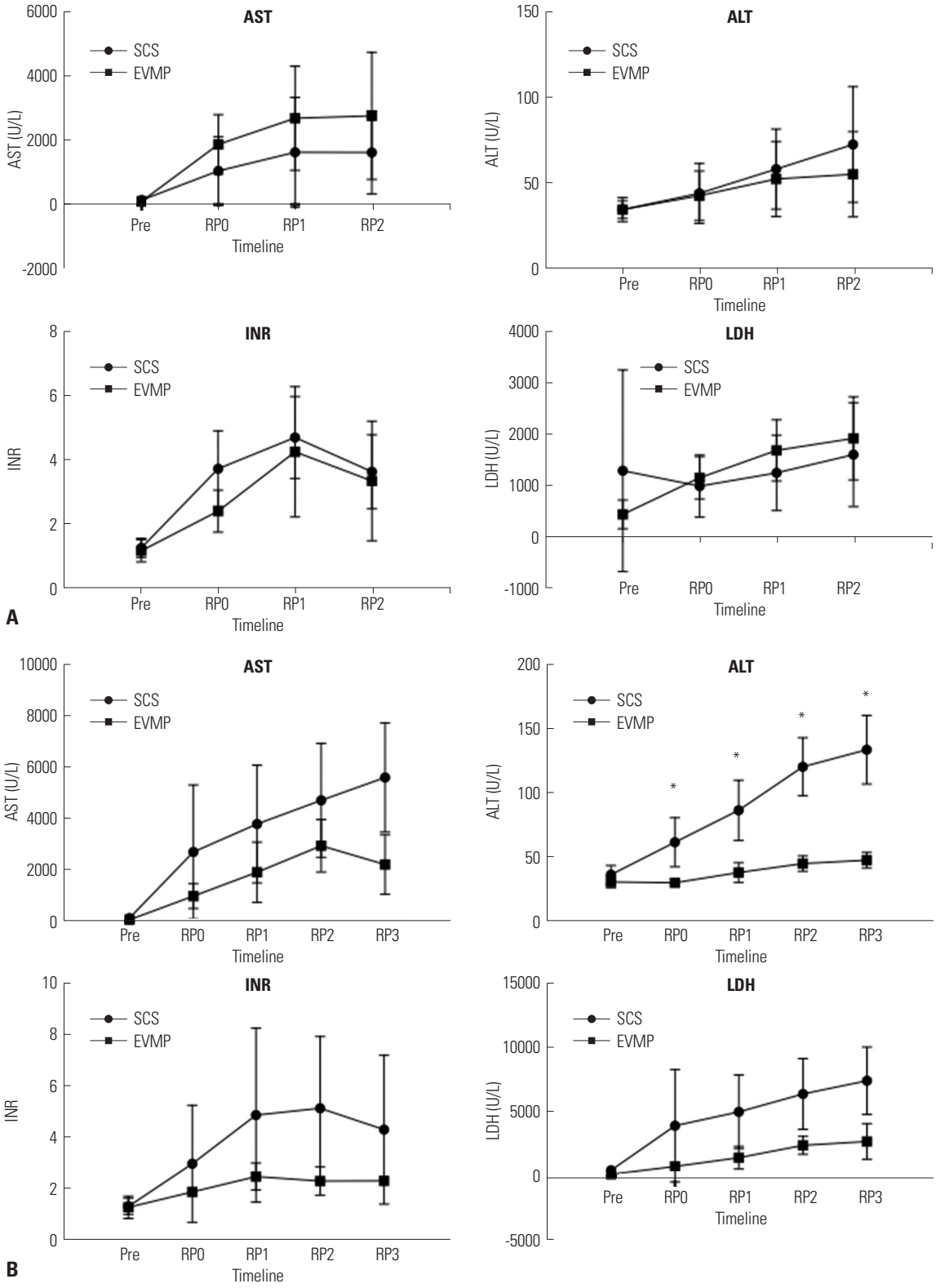

Fig. 4. Levels of AST, ALT, INR, and LDH after perfusion in Model 1 (A) and Model 2 (B). (A) In Model 1, there were no significant differences in AST, ALT, INR, and LDH levels between the SCS and EVMP groups from immediately after reperfusion up to 2 hours. (B) In Model 2, ALT levels were significantly lower in the EVMP group from immediately after reperfusion up to 3 hours (SCS vs. EVMP; RP0: 62.0±19.3 U/L vs. 30.3±3.5 U/L, p=0.049; RP1: 87.0±23.5 U/L vs. 38.3±7.8 U/L, p=0.027; RP2: 121.0±22.7 U/L vs. 45.3±6.0 U/L, p=0.005; RP3: 134.3±27.0 U/L vs. 48.0±6.2 U/L, p=0.006). AST, INR, and LDH levels also trended lower in the EVMP group, though these differences did not reach statistical significance. *p<0.05 at each time point. AST, aspartate aminotransferase; ALT, alanine aminotransferase; INR, international normalized ratio; LDH, lactate dehydrogenase; SCS, static cold storage; EVMP, ex vivo machine perfusion; Pre, baseline; RP0, immediately after reperfusion; RP1, RP2, RP3, 1, 2, and 3 hours after reperfusion.

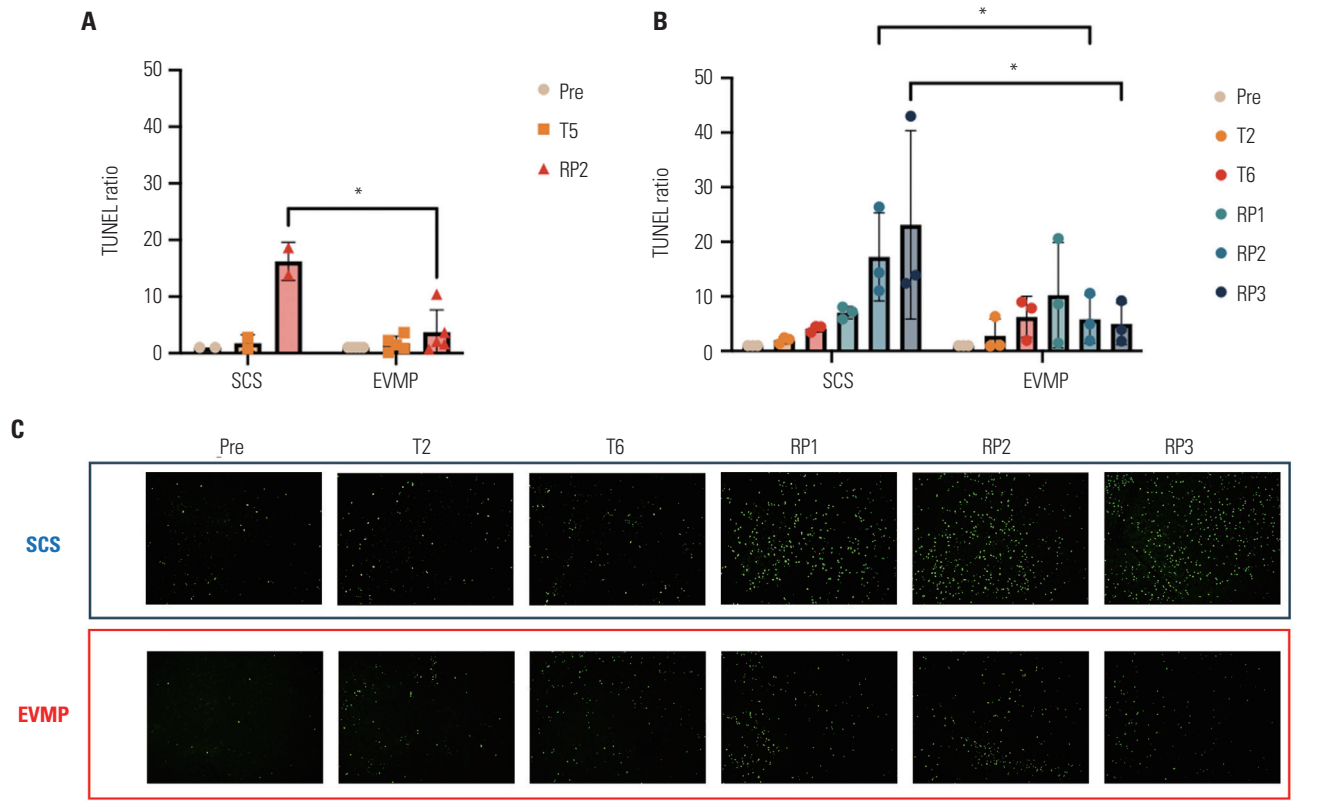

Fig. 5. Comparison of TUNEL results between SCS and EVMP groups. (A) In Model 1, the SCS group showed significantly higher TUNEL-positive cell ratios (relative to baseline liver) compared to the EVMP group at 2 hours after reperfusion (RP2) (16.2±3.4 vs. 3.7±3.9, $p<0.001$). (B) In Model 2, no significant difference in TUNEL-positive cell ratios was observed between the groups up to the end of preservation. However, starting from 2 hours after reperfusion, the EVMP group demonstrated a markedly lower TUNEL ratio compared to the SCS group (5.8±4.4 vs. 17.2±8.1, $p=0.043$ at RP2; 5.0±3.8 vs. 23.1±17.3, $p=0.002$ at RP3). (C) Representative images of TUNEL staining in the SCS and EVMP groups in Model 2 (magnification ×200). *$p<0.05$ at each time point. Pre, baseline; T2, 2 hours into preservation; T6, end of preservation; RP1, RP2, RP3, 1, 2, and 3 hours after reperfusion; SCS, static cold storage; EVMP, ex vivo machine perfusion.

and HA pressure of 100 mm Hg) might have contributed to pressure-related injury, we adjusted these to a PV flow of 800 mL/min and HA pressure of 80 mm Hg in Model 2. These modifications resulted in clearer distinctions between the EVMP and SCS groups, allowing us to validate the efficacy of EVMP more definitively.

This study underscores the critical importance of optimizing experimental models, including adjustments to preservation duration and perfusion parameters, to effectively evaluate the efficacy of EVMP in mitigating IRI and improving transplant outcomes. While commercially available EVMP devices are currently in clinical use, their high costs often limit their applicability in experimental research settings. In this study, we successfully demonstrated that a simplified EVMP system, constructed using basic components such as a centrifugal pump, pulsatile pump, oxygenator, and heat-cooler system, can be effectively implemented in laboratory environments. This cost-effective approach offers significant potential for broader adoption in preclinical research.

Moreover, the laboratory-adaptable EVMP system developed in this study provides a versatile platform for diverse research applications. Recently, studies utilizing machine perfusion have explored the delivery of therapeutic agents during perfusion,[20,21] identified biomarkers associated with liver viability,[15] and investigated the molecular mechanisms underlying IRI.[14] Building on this foundation, we plan to conduct further molecular studies using the porcine DCD sLT model established in this research. These follow-up studies will aim to elucidate the mechanisms of IRI and advance our understanding of potential therapeutic strategies.

Despite these promising results, this study has several limitations. First, the sample size in both experimental models was small, which may limit the generalizability of the findings. Larger-scale studies are needed to confirm these results. Second, while this study focused on short-term outcomes, the long-term viability of the preserved grafts was not assessed. Future research should incorporate long-term in vivo transplantation models to evaluate graft function and survival. Lastly, the experimental settings may not fully replicate clinical conditions, and further validation in a clinical setting is necessary before widespread implementation.

In conclusion, this study validates the transplant surgeon-innovated EVMP protocol in a porcine DCD sLT model, demonstrating superior preservation stability and reduced IRI com-

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pared to traditional cold storage. By optimizing perfusion parameters and conducting various large animal experiments, EVMP shows potential not only for improving clinical transplant outcomes but also as a valuable platform for future research.

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AUTHOR CONTRIBUTIONS

Conceptualization: Mun Chae Choi, Yuri Cho, Yu Seol Lee, Sat Byol Lee, Ji Yun Bang, Soo Han Bae, and Dong Jin Joo. Data curation: Mun Chae Choi, Yuri Cho, Yu Seol Lee, Sat Byol Lee, and Ji Yun Bang. Formal analysis: Mun Chae Choi, Yuri Cho, and Yu Seol Lee. Funding acquisition: Soo Han Bae and Dong Jin Joo. Investigation: Mun Chae Choi, Yuri Cho, Yu Seol Lee, Sat Byol Lee, Ji Yun Bang, Soo Han Bae, and Dong Jin Joo. Methodology: Mun Chae Choi, Yuri Cho, Yu Seol Lee, Soo Han Bae, and Dong Jin Joo. Project administration: Mun Chae Choi and Dong Jin Joo. Resources: Eun-Ki Min, Deok-Gie Kim, Jae Geun Lee, Myoung Soo Kim, Soo Han Bae, and Dong Jin Joo. Software: Mun Chae Choi, Yuri Cho, and Yu Seol Lee. Supervision: Eun-Ki Min, Deok-Gie Kim, Jae Geun Lee, Myoung Soo Kim, Soo Han Bae, and Dong Jin Joo. Validation: Soo Han Bae and Dong Jin Joo. Visualization: Mun Chae Choi, Yuri Cho, Yu Seol Lee, Sat Byol Lee, and Ji Yun Bang. Writing—original draft: Mun Chae Choi, Yuri Cho, and Yu Seol Lee. Writing—review & editing: all authors. Approval of final manuscript: all authors.

ORCID iDs

Mun Chae Choi	https://orcid.org/0000-0002-2708-0755
Yuri Cho	https://orcid.org/0000-0001-8805-4975
Yu Seol Lee	https://orcid.org/0009-0004-4731-7838
Sat Byol Lee	https://orcid.org/0000-0002-1668-4756
Ji Yun Bang	https://orcid.org/0009-0000-2123-1703
Eun-Ki Min	https://orcid.org/0000-0003-3255-1942
Deok-Gie Kim	https://orcid.org/0000-0001-9653-926X
Jae Geun Lee	https://orcid.org/0000-0002-6722-0257
Myoung Soo Kim	https://orcid.org/0000-0002-8975-8381
Soo Han Bae	https://orcid.org/0000-0002-8007-2906
Dong Jin Joo	https://orcid.org/0000-0001-8405-1531

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