

Graft protection of the liver by hypothermic machine perfusion involves recovery of graft regeneration in rats

Journal of International Medical Research

2019, Vol. 47(1) 427–437

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DOI: 10.1177/0300060518787726

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Abstract

Objective: This study was performed to evaluate the impact and underlying mechanisms of hypothermic machine perfusion (HMP) on half-size liver graft regeneration.

Methods: Forty rats were randomly assigned to five groups: two *in vitro* groups (static cold storage [SCS] and HMP) and three *in vivo* groups (orthotopic liver transplantation, SCS, and HMP). Perfusates and plasma samples were collected for analysis of hepatic enzymes. Liver tissue was obtained for evaluation of histology, immunohistochemistry (Ki67 and proliferating cell nuclear antigen [PCNA]), and the regeneration rate. Cell cycle genes were analyzed by quantitative real-time polymerase chain reaction, and cyclin D1 and cyclin E1 were semiquantified by western blot.

Results: HMP improved histopathological outcomes and decreased hepatic enzyme release. The expression of Ki67 and PCNA demonstrated a greater proliferation activity in the HMP than SCS group, and the expression of almost all cell cycle genes was elevated following HMP. Western blot results showed higher protein levels of cyclin D1 and cyclin E1 in the HMP than SCS group.

Conclusions: Our findings suggest for the first time that half-size liver graft protection by HMP involves recovery of graft regeneration.

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Keywords

Hypothermic machine perfusion, half-size liver transplantation, liver regeneration, static cold storage, rats, histopathology

Date received: 23 March 2018; accepted: 18 June 2018

Introduction

With the growing demand for donor organs for transplantation, machine perfusion (MP) promises to be a beneficial alternative to static cold storage (SCS) for donor livers, particularly extended criteria donor liver grafts with advanced donor age, significant macrosteatosis, and donation after cardiac death. MP can be performed at different temperatures: hypothermic MP (HMP) is performed at 4°C to 6°C, subnormothermic MP is performed at 20°C, and normothermic MP is performed at 32°C to 37°C.¹

HMP is widely used to preserve kidney grafts for transplantation with improved results over SCS; however, HMP remains investigational in clinical liver transplantation. Nevertheless, incredible advances in HMP have been made in both numerous experimental studies and limited clinical studies, and most research has verified that HMP effectively improves or sustains various post-ischemia hepatobiliary parameters and maintains liver function with minimal injury.^{1–3} However, the underlying mechanisms of protection by HMP remain unclear.

Numerous studies have been performed to investigate how HMP protects liver grafts by focusing on mechanisms of injury following ischemia or ischemia–reperfusion (I/R). These studies have implicated upregulation of shear stress-sensitive protective genes,⁴ reduction in glutathione depletion and reactive oxygen species release,^{5,6} and unique decreases in damage-associated molecular patterns.^{7,8} Enhancement of liver cell proliferation may facilitate better recovery after cold ischemic injury and allow the

transplantation of partial liver grafts from cadaveric or living donors.⁹ The benefit of HMP in decreasing ischemia or I/R injury could be associated with maintenance of liver regeneration. However, this mechanism has yet to be demonstrated and represents the objective of our study.

This study was performed to evaluate the impact of HMP on the liver in an *in vitro* 6-hour preservation and half-size liver transplantation (HSLT) model. We hypothesized that rat livers undergoing HMP will sustain less injury and exhibit greater regeneration than those preserved by SCS.

Materials and methods

Animals and experimental design

All experimental protocols in our study were approved by the Ethics Committee for the Use of Experimental Animals of Zhejiang University and were conducted in accordance with the Consensus Author Guidelines on Animal Ethics and Welfare for Veterinary Journals. Adult male Sprague–Dawley rats (250–300 g) were used in this study. All rats were kept in a temperature-controlled (26°C–28°C) and humidity-controlled environment and provided a standard diet with water *ad libitum*.

Forty rats (including 15 donors) were randomly assigned to five groups: two *in vitro* groups (SCS and HMP; n = 5 in each group) and three *in vivo* groups (orthotopic liver transplantation [OLT], SCS, and HMP; n = 10 in each group, including 5 donors). Each group was subjected to the following procedures.

In vitro: In the SCS group, the half-size liver graft was placed into cold histidine-tryptophan-ketoglutarate solution (HTK) perfusate (0°C–4°C) for 6 hours in accordance with our previous study¹⁰ (we extended the time to 6 hours because we previously evaluated histopathological, functional, and proliferative indicators following 3 hours of HMP or SCS *in vitro* with insignificant results). In the HMP group, the portal vein of the half-size liver graft was connected to a homemade perfusion machine (4°C; nonoxygenated circulating and nonpulsatile perfusion; portal vein velocity, 1.4 mL/minute; perfusate volume, 60 mL; portal vein pressure was monitored in real time and recorded using a computer: mean pressure, 4.61 mmHg)¹⁰ for 6 hours using HTK solution.

In vivo: The OLT group underwent standard orthotopic HSLT following storage in cold HTK perfusate (0°C–4°C) for

45 minutes (the average operative time in our laboratory). The SCS group underwent HSLT following storage in cold HTK perfusate for 3 hours according to our previous study (unpublished data). The HMP group underwent HSLT after the portal vein of the graft had been connected to the perfusion machine for 3 hours with HTK solution (Figure 1).

Rat HSLT model

The HSLT model has been previously described in detail.¹¹ Briefly, the animals were anesthetized with 4% chloral hydrate. Donor half-size liver resection was performed by removing the left lateral lobe and left middle lobe of the donor liver. The right renal vein was ligated and the pulsating abdominal aorta was cannulated with a catheter following injection of 2 mL of physiological saline with 100 U

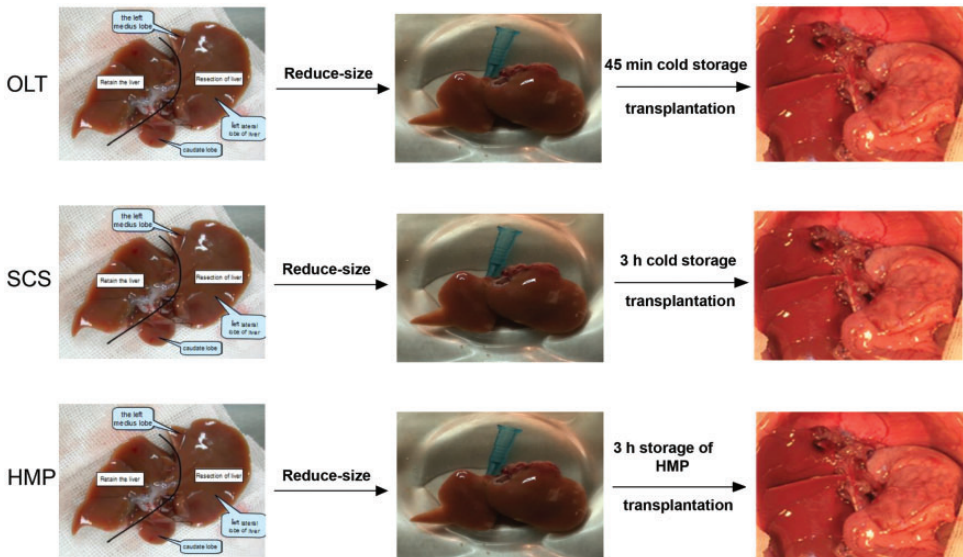


Figure 1. Study design and HSLT model of the study. HSLT rats were divided into three subgroups: the OLT group was subjected to standard orthotopic HSLT, the SCS group underwent HSLT following 3 hours of cold storage, and the HMP group underwent HSLT after 3 hour storage by machine perfusion. HSLT, half-size liver transplantation; OLT, orthotopic liver transplantation; SCS, static cold storage; HMP, hypothermic machine perfusion.

of heparin through the iliac vein. The intrathoracic vena cava was transected and cut to allow rinsing with the flushing solution. Next, the graft was perfused with cold saline containing 25 U/mL of heparin through the abdominal aorta until the liver turned pale; the graft was then placed into HTK (0°C–4°C) solution (in the OLT and SCS groups) or connected to the perfusion machine with HTK solution (in the HMP group). The donor liver graft was removed from the HTK solution ice bath and placed in the orthotopic position of the recipient. After completion of end-to-end anastomosis of the suprahepatic vena cava and insertion of the cuffed portal vein into the recipient portal vein, the liver was reperfused to end the anhepatic phase. The infrahepatic vena cava and common bile duct were anastomosed by the same method. The recipient's abdominal incision was closed with a continuous suture, and 1.5 mL of saline was injected into the recipient's penile vein to complete the operation. During the operation, we ensured that all manipulations were performed lightly and softly to relieve postoperative pain and suffering.

Sample collection

At 0, 1, 3, and 6 hours during the perfusion process in the *in vitro* groups and at 1, 3, and 7 days after HSLT in the *in vivo* groups, 2 mL of perfusate and plasma samples were collected from the *in vitro* and *in vivo* groups, respectively, for analysis of liver function. Six hours after the end of the preservation in the *in vitro* groups and 7 days after HSLT in the *in vivo* groups, liver tissues were obtained and fixed in 10% neutral formalin for subsequent histological and immunohistochemical analyses. The liver was collected for determination of the regeneration rate (RR) (equal to the ratio of the half-size graft weight before HSLT to the half-size graft harvest weight 7 days

after HSLT) in the *in vivo* groups, and the liver tissues of all groups (both *in vitro* and *in vivo*) were stored at –80°C for further experimental analysis.

Histopathologic examination and liver function tests

The excised liver specimens used for histology and immunohistochemistry were fixed in 10% neutral buffered formalin for 24 to 48 hours before the standard procedures of paraffin embedment and sectioning (thickness, 3 µm). After being deparaffinized and hydrated, paraffin sections of the liver specimens were stained with hematoxylin and eosin. Hepatocyte proliferation was measured based on the expression of Ki67 (Abcam, Cambridge, MA, USA) and PCNA (Abcam). Alanine transaminase (ALT) and aspartate aminotransferase (AST) were analyzed using a Hitachi 7600 automatic analyzer (Hitachi, Tokyo, Japan).

Quantitative detection of mRNA expression by quantitative real-time polymerase chain reaction

Total RNA was extracted from liver tissue homogenate using Trizol reagents (Thermo Fisher Scientific, Waltham, MA, USA). Total RNA (1 µg) was isolated and reverse-transcribed to cDNA using a PrimeScript Real-Time Reagent Kit with gDNA Eraser (Takara Bio, Shiga, Japan). The polymerase chain reaction system consisted of 2 µL of template cDNA, 0.4 µL of ROX Reference Dye or Dye II, 0.8 µL of forward primer, 0.8 µL of reverse primer, 10 µL of SYBR Premix Ex TaqII, and 6 µL of distilled water to a total volume of 20 µL. The gene expression of *Cdc25a*, *Cdk1*, *Aldh1a2*, *Cdk2*, *Cdk6*, and *myc* was quantified using an ABI 7500 Fast Real-Time Polymerase Chain Reaction instrument (Applied Biosystems, Foster City, CA, USA) under the following

conditions: 1 cycle at 95°C for 30 seconds, 40 cycles at 95°C for 5 seconds, followed by 1 cycle at 60°C for 30 seconds. Finally, the relative quantification was calculated using comparative Ct.

Western blot analysis for cyclin D1 and cyclin E1

Protein was extracted from the liver tissue using RIPA Lysis Buffer (Beyotime Institute of Biotechnology, Haimen, China). After centrifugation (15,000 × g, 4°C, 20 minutes), the supernatants of the homogenate were collected for protein concentration measurement. After separation by SDS-PAGE gels, the denatured protein was transferred to nitrocellulose membranes. The membranes were then incubated with primary antibodies to cyclin D1 (1:10000; Abcam), cyclin E1 (1:500; Abcam), and glyceraldehyde 3-phosphate dehydrogenase (1:1000; Abcam). The membranes were then incubated with secondary antibodies followed by use of an enhanced

chemiluminescence kit (Pierce Biotechnology, Rockford, IL, USA).

Statistical analysis

Data are expressed as mean ± standard deviation. One-way analysis of variance was used to compare differences between the experimental groups. A P value of <0.05 was considered statistically significant.

Results

Effects of HMP on liver histopathological outcomes and function during preservation *in vitro* and *in vivo*

HMP reduced the congestion around the sinusoids and vacuolation with better histopathological presentations compared with the SCS group (lower Suzuki's scores¹² in the HMP than SCS group) both *in vitro* and *in vivo* (Figure 2) and decreased the ALT and AST release during the 3- to 6-hour period *in vitro* with a significant decrease in AST at 6 hours. Similarly, the

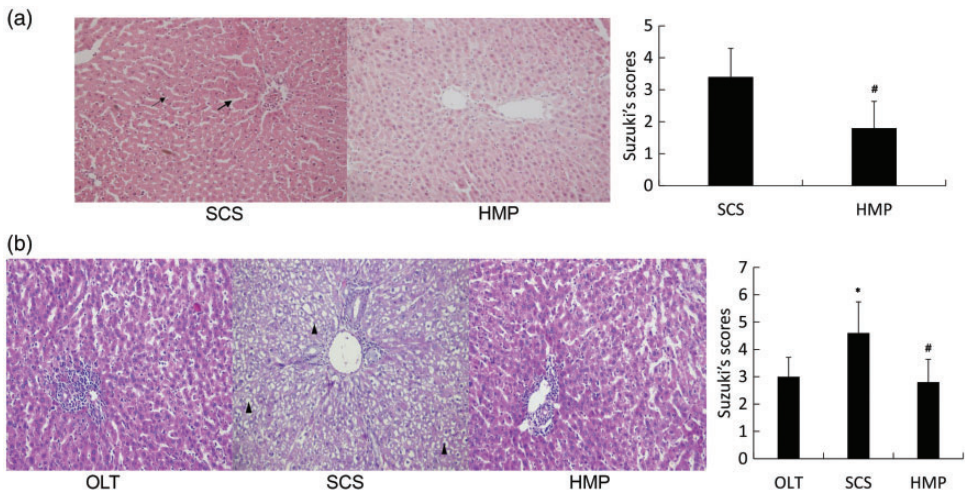


Figure 2. Effects of HMP on liver histopathological outcomes (a) *in vitro* and (b) *in vivo* (original magnification, ×200). Hepatocyte congestion of the sinusoids (arrow) and vacuolation (arrowhead). Data represent mean ± standard deviation for five animals per group. *P < 0.05 vs. OLT group; #P < 0.05 vs. SCS group. OLT, orthotopic liver transplantation; SCS, static cold storage; HMP, hypothermic machine perfusion.

ALT and AST levels decreased with time in the HMP group almost in parallel to the OLT group 7 days after HSLT, whereas these levels inconsistently changed in the SCS group with a significantly higher level 7 days after HSLT ($P < 0.05$) (Figure 3).

Effects of HMP on liver PCNA and Ki67 antigen expression *in vitro* and *in vivo* and on liver RR after HSLT

The immunohistochemical analyses with statistical analysis of cell cycle markers (Ki67 and PCNA) demonstrated obviously greater proliferation activity in the HMP than SCS group at the end of the 6-hour preservation. The same trend was found 7 days after HSLT, with a similar highest RR and expression of the two cell cycle markers in the HMP and OLT groups (Figure 4).

Effects of HMP on mRNA level of cell cycle genes *in vitro* and *in vivo*

Expression of the six studied cell cycle genes (Cdc25a, Cdk1, Aldh1a2, Cdk2, Cdk6, and myc) was elevated, with two of them (Cdc25a and Cdk1) reaching statistical significance following HMP compared with the SCS group at the end of the 6-hour preservation ($P < 0.05$). *In vivo*, the mRNA levels of these cell cycle genes (excluding Cdk1) were reduced by the SCS intervention compared with the OLT group; this was reversed by HMP treatment, and Aldh1a2 and myc showed statistical significance ($P < 0.05$) (Figure 5).

Effects of HMP on protein level of cyclin D1 and E1 *in vitro* and *in vivo*

Western blot with statistical analysis showed higher protein levels of cyclin D1

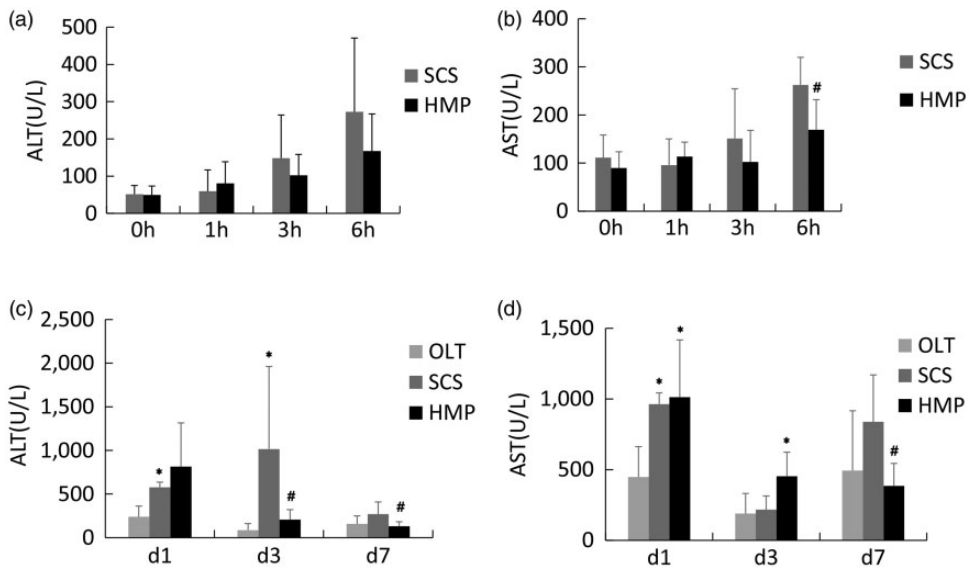


Figure 3. Effects of HMP on liver graft function *in vitro* and *in vivo*. Effects of HMP on ALT and AST, parameters of hepatocellular damage, (a, b) at the end of 6 hours of preservation and (c, d) 7 days after HSLT. Data represent mean \pm standard deviation for five animals per group. * $P < 0.05$ vs. OLT group; # $P < 0.05$ vs. SCS group. HSLT, half-size liver transplantation; OLT, orthotopic liver transplantation; SCS, static cold storage; HMP, hypothermic machine perfusion; ALT, alanine aminotransferase; AST, aspartate aminotransferase.

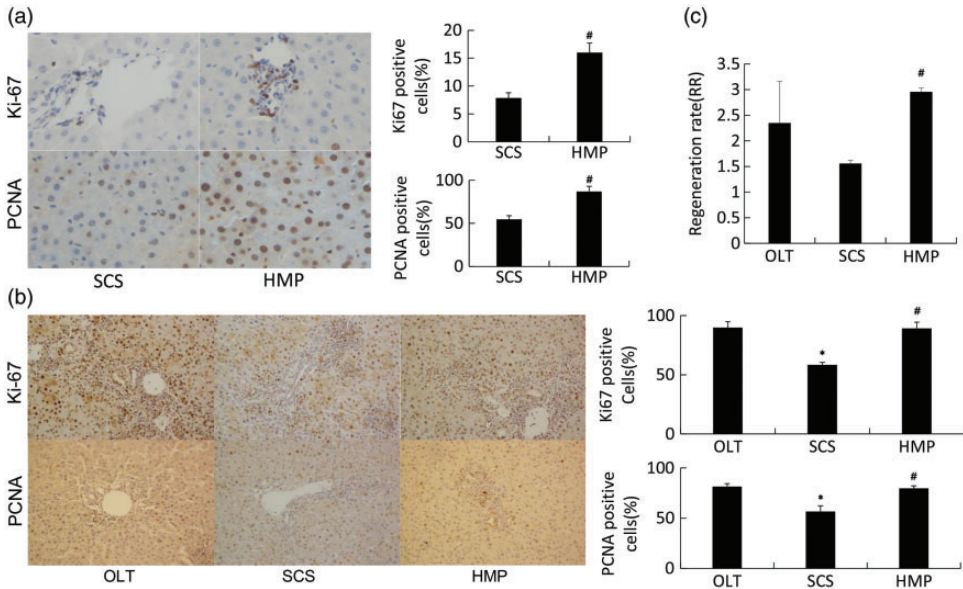


Figure 4. Effects of HMP on liver PCNA and Ki67 antigen expression (a) *in vitro* and (b) *in vivo* (original magnification, $\times 200$) and (c) on the liver regeneration rate after HSLT. Data represent mean \pm standard deviation for five animals per group. * $P < 0.05$ vs. OLT group; [#] $P < 0.05$ vs. SCS group. HSLT, half-size liver transplantation; OLT, orthotopic liver transplantation; SCS, static cold storage; HMP, hypothermic machine perfusion; PCNA, proliferating cell nuclear antigen.

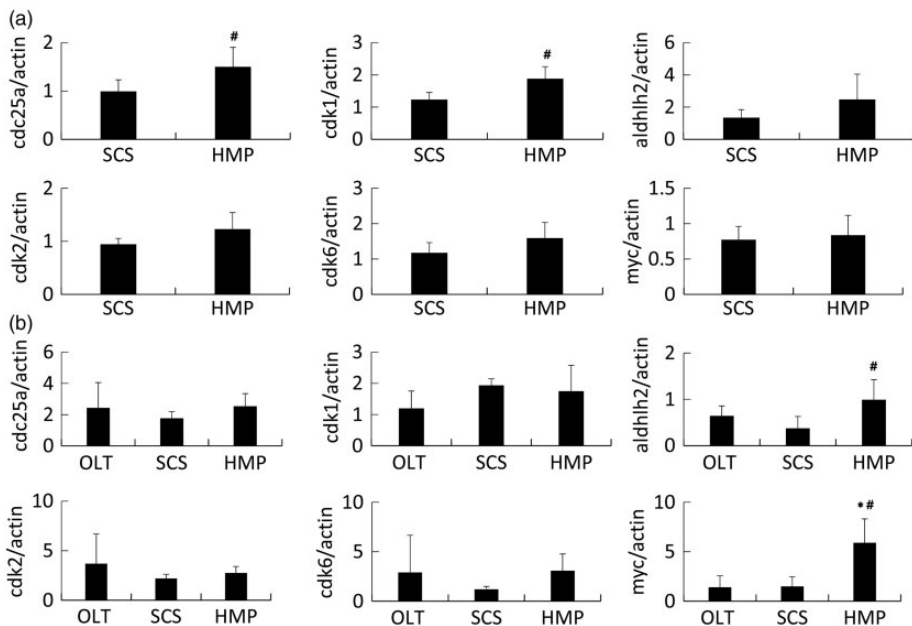


Figure 5. Effects of HMP on mRNA level of cell cycle genes (a) *in vitro* and (b) *in vivo*. Data represent mean \pm standard deviation for five animals per group. * $P < 0.05$ vs. OLT group; [#] $P < 0.05$ vs. SCS group. OLT, orthotopic liver transplantation; SCS, static cold storage; HMP, hypothermic machine perfusion.

and E1 in the HMP than SCS group at the end of the 6-hour preservation (Figure 6). The same trend was found 7 days after HSLT, although the protein levels of cyclin D1 and E1 were lower than in the OLT group.

Discussion

Liver transplantation has been confirmed to be an effective solution for most patients with end-stage liver disease. During transplantation, the method of organ preservation is a key factor affecting the prognosis of recipients. Large animal model findings have advanced the knowledge of post-reperfusion events, indicating that normothermic MP may be a beneficial preservation modality that can improve biliary regeneration after a major ischemic event.¹³ Similarly, HMP may therefore enlarge the donor pool by revitalizing liver grafts

before implantation. The present study showed the impact of HMP on rat livers during a 6-hour preservation *in vitro* and an HSLT model *in vivo*. We also demonstrated that HMP improved liver histopathological outcomes and function, and new insights into the role of HMP in increasing hepatocyte proliferation were obtained *in vitro*. Furthermore, this higher mitogenic effect was also observed for the first time in the regenerating liver when rat liver grafts were treated with 3 hours of HMP prior to HSLT compared with the SCS group.

In contrast to conventional SCS, HMP may provide better preservation of donor livers.¹⁴ Once removed from the donor, the organ undergoes a period of ischemia and incurs subsequent damage as the tissue responds in a variety of ways to the hypothermia and lack of oxygen and nutrients. HMP provides continuous

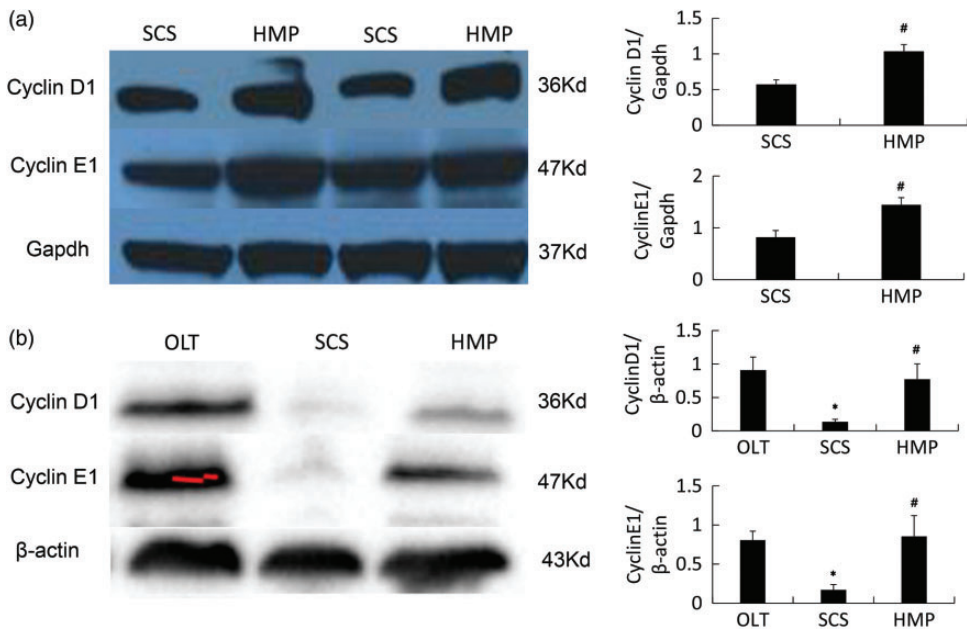


Figure 6. Effects of HMP on protein level of cyclin D1 and E1 (a) *in vitro* and (b) *in vivo*. Data represent mean \pm standard deviation for five animals per group. * $P < 0.05$ vs. OLT group; # $P < 0.05$ vs. SCS group. OLT, orthotopic liver transplantation; SCS, static cold storage; HMP, hypothermic machine perfusion.

delivery of antioxidants and metabolic substrates while flushing cytokines, toxins, and metabolic byproducts from the liver. HMP is supported because of its preservation of the liver by protection of liver function and significant improvement in the prevention of hepatocellular damage compared with SCS.¹⁵ The first clinical application of HMP in human liver transplantation without active oxygenation of the perfusion fluid may attenuate classic biochemical markers of liver preservation injury and improve graft function. Early molecular results indicate a reduction in perfusion injury that may be associated with fewer complications after transplantation.¹⁶ In agreement with the above findings, we found that HMP improved the histopathological outcomes and decreased ALT and AST release *in vitro* and *in vivo*. Although these results are promising, knowledge about the mechanisms through which HMP improves histopathological outcomes and liver graft function is limited.

The liver is unique in its regenerative potential, recovering lost mass and function after injury from ischemia, resection, and rejection.¹⁷ I/R injury remains one of the obstacles to further development of liver transplantation.¹⁸ Many studies have investigated the underlying mechanisms involved in the probable interpretation of how HMP protects the liver graft from ischemia or I/R injury,⁴⁻⁸ but none of these studies mentioned the probable protective role of recovery of graft regeneration. Enhancement of liver cell proliferation may facilitate better recovery after cold ischemic injury and allow cadaveric or living donor partial liver graft transplantation.⁹ Therefore, we hypothesize that the benefit of HMP in decreasing ischemia or I/R injury occurs through maintaining liver regeneration.

Ki67 is involved in regulation of cell cycle progression, including higher-order chromatin organization, DNA replication, and acting with motor proteins to manage

centrosome separation. Defects in Ki67 expression are regarded as closely associated with defects in the M phase.¹⁹ PCNA, which is commonly used as an indicator of cell proliferation, is a marker of DNA synthesis, encircling dsDNA to act as a scaffold for DNA-related enzymes.²⁰ In the present study, the statistical analysis of Ki67 and PCNA demonstrated obviously greater proliferation activity in the HMP than SCS group at the end of the 6-hour preservation. Additionally, the RR was calculated to estimate the extent of liver regeneration in other experimental models.^{21,22} We observed a higher RR 7 days after HSLT in the HMP than SCS group. The cell cycle status is closely associated with the cellular function: rapid cell cycling is necessary for the expansion of a progenitor population, slow cell cycling is required for self-renewal of primitive stem cells, and withdrawal from the cell cycle is needed for various functions of terminally differentiated cells. We studied six major cell cycle genes: Cdc25a, Cdk1, Aldh1a2, Cdk2, Cdk6, and myc.^{23,24} Several of these genes were significantly higher after HMP than SCS *in vitro* and *in vivo*. We also determined the effects of HMP on protein levels by statistical analysis of two other very important cell cycle regulators (cyclin D1 and E1) *in vitro* and *in vivo*. Cell cycle progression consists of two steps: the transition of hepatocytes from G0 to G1 and entry into subsequent progression through the S phase.²⁵ Cyclin D1 and cyclin E1 were used to monitor these two steps of hepatocellular cell cycle progression.²⁶ Our western blot results showed higher protein levels of cyclin D1 and E1 in the HMP than SCS group at the end of the 6-hour preservation and 7 days after HSLT. Taken together, these results prove our hypothesis. Promisingly, HMP shows results similar to those of OLT, which includes split liver transplantation.

Interestingly, evaluation of the histopathological, functional, and proliferative indicators following 3 hours of HMP or SCS *in vitro* showed no statistically significant results, but this largely changed when the time was extended to 6 hours. We speculate that some cytokines are generated systematically or locally during reperfusion after HMP. More studies are required to verify this hypothesis.

In conclusion, our findings suggest for the first time that liver graft protection by HMP involves recovery of graft regeneration, while the underlying mechanism deserves further investigation. Therapeutic interventions based on these findings could be potentially important strategies for biochemical reconditioning of grafts and may be potentially applicable in clinical practice.

Declaration of conflicting interest

The authors declare that there is no conflict of interest.

Funding

This work was supported by the National Natural Science Foundation of China [grant numbers 81421062 and 81470891]; the Science and Technology Bureau of Zhejiang Province, China [grant number 2016C33145]; the 863 National High Technology Research and Development Program of China for young scientist [grant number 2015AA020923]; the Zhejiang Health Technology Project [grant number 2019RC153]; and the China Postdoctoral Science Foundation [grant number 2017M610374].

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