Transplant International

ORIGINAL ARTICLE

An oxygenated perfluorocarbon emulsion improves liver graft preservation evaluated in DCD livers of male sprague dawley rats

Guoyi Wu[1,2], Yu Liu[3], Chen Rui[4], Shanshan Zhan[2], Jun Wang[3], Shizhen Cai[4], Xiaolei Shi[2] & Yitao Ding[2]

1 Shanghai Public Health Clinical Center, Fudan University, Shanghai, China
2 Department of Hepatobiliary Surgery, The Affiliated Drum Tower Hospital of Nanjing University Medical School, Nan-jing, China
3 Department of Pharmaceutics, School of Pharmacy, Fudan University & Key Laboratory of Smart Drug Delivery (Fudan University), Ministry of Education, Shanghai, China
4 MediTrans Inc., Nanjing, China

Correspondence
Yitao Ding, The Affiliated Drum Tower Hospital of Nanjing University Medical School, 321 Zhongshan Road, Nanjing, Jiangsu 210008, China.
Tel.: +86 256 818 2806;
fax: +86 256 818 2805;
e-mail: m18936151218@163.com
Xiaolei Shi, The Affiliated Drum Tower Hospital of Nanjing University Medical School, 321 Zhongshan Road, Nanjing, Jiangsu 210008, China.
Tel.: +86 256 818 2806;
fax: +86 256 818 2805;
e-mail: njsxl2000@163.com;

*Equally contributing to this work.

SUMMARY

The application of perfluorocarbons, which can carry large quantities of oxygen, in organ preservation was limited by their poor solubility in water. A stable form of perfluorocarbon dispersed in suitable buffers is urgently needed. Perfluorocarbon emulsion was designed and characterized with respect to size distribution, rheology, stability, and oxygen-carrying capacity. The state of DCD rat donor livers preserved by the oxygenated perfluorocarbon emulsion was studied after ex vivo reperfusion by using biochemistry, pathology, and immunohistochemistry methods. Perfluorocarbon emulsion was successfully prepared by high-pressure homogenization. Optimized perfluorocarbon emulsion showed nanoscale size distribution, good stability, and higher oxygen loading capacity than that of HTK solution or water. The state of preserved livers after cardiac death rat liver was improved significantly after static cold storage for 48 hours in this oxygenated perfluorocarbon emulsion. The ATP content and down-regulation of HIF-1a expression after preservation of the liver graft by the oxygenated perfluorocarbon emulsion suggested the advantage of adequate oxygen supply for adequate time. This perfluorocarbon emulsion reported here might be considered a promising system for oxygenated donor liver storage by attenuation of hypoxia.

Transplant International 2021; 34: 2087–2097

Key words
emulsion, liver transplantation, oxygenated preservation, perfluorocarbon

Received: 16 April 2021; Revision requested: 23 June 2021; Accepted: 12 July 2021; Published online: 8 September 2021

Introduction

Although liver transplantation has improved by improvements of the transplantation management including candidates and donor selection, liver procurement and preservation, post-transplantation care, as well as immunosuppression, shortage of organs is still one of the major issues in liver transplantation worldwide. In the USA, more than 1000 patients died or were removed from the transplant waiting list due to this shortage every

doi:10.1111/tri.13996

year during their waiting for the donor livers [1]. In China, the ratio of patients on the waiting list was 30 times that of transplant recipients [2]. Despite the shortage of donor livers worldwide, an increasing number of donor livers has to be discarded due to the limited maximum safe preservation time. The mainstay technology for liver graft preservation is hypothermia for lower metabolic requirements by static cold storage [3,4]. Improved oxygen loading by oxygenated ex situ machine perfusion of donor livers has been proven to benefit DCD liver preservation as an alternative for static cold preservation. Recently, a multicenter controlled trial which randomly assigned patients who were undergoing transplantation of a liver obtained from a donor after circulatory death to receive that liver either after hypothermic oxygenated machine perfusion or after conventional static cold storage alone found that hypothermic oxygenated machine perfusion led to a lower risk of nonanastomotic biliary strictures following the transplantation of livers obtained from donors after circulatory death than conventional static cold storage [5]. Studies on normothermic machine perfusion which perfuses organs at physiological temperature (35–38 °C) and maintains the metabolic rate at normal physiological level further showed encouraging results with respect to reduced preservation injury and improved graft viability [6]. It is worth noting that normothermic machine perfusion required higher oxygen (O2) demands than during HMP. Clinical trials are currently using artificial oxygen carriers such as hemoglobin-based oxygen carrier 201 (HBOC-201) in the machine perfusion of livers and kidneys [7]. Therefore, it is important to develop oxygenated organ preservation solutions for organ preservation at different temperatures.

Perfluorocarbons are capable of absorbing large quantities of oxygen [8], but they are insoluble in water. Perfluorocarbon emulsion (PFCE) stabilized with surfactants has been reported for oxygenation of tissues [9]. Several research groups have tried to employ fluorocarbon as an oxygen carrier to improve the outcomes of liver transplantations, but no oxygen loading and releasing behavior have been characterized in these studies [10]. But emulsions are thermodynamically unstable in nature and prone to coalescence and Ostwald ripening in which the molecules in small droplets dissolve into the surrounding and then diffuse to larger droplets, finally causing larger droplets to grow. Preparation of perfluorocarbon emulsions is particularly difficult because of the complicated composition for an organ preservation solution including electrolytes, impermeants, buffers, antioxidants, and energy precursors [11]. For example, histidine–tryptophan–ketoglutarate (HTK) solution, which is widely used for organ transplantation by perfusion and flushing of donor organs prior to removal from the donor and preserving organs during hypothermic storage and transport to the recipient [12], is of a complex composition and presents a great challenge for the stability of perfluorocarbon emulsion based on HTK solution composition, because various ions influence the stability of diffused double layers surrounding emulsion droplets.

Therefore, our goals were to develop a PFCE with the characters mentioned above and determine how such oxygenated PFCE preserves liver graft. Accordingly, PFCE was designed based on an oil phase composed of perfluoroctyl bromide and perfluorodecyl bromide, an aqueous phase containing similar ions as histidine–tryptophan–ketoglutarate solution and a safe emulsifier egg yolk lecithin. The optimized PFCE, PFCplus, was of good stability, lower viscosity, appropriate concentration of salts, nutrients, and buffers as well as physiological osmotic pressure using extensively approved egg yolk lecithin as the only emulsifier. The physical characteristics, rheology, stability, and oxygen-carrying capacity of this PFCE were evaluated. The improvement of DCD rat donor livers preserved by our oxygenated PFCE solution was studied *ex vivo* to prove its better preservation capacity including HIF-1a pathway activation.

Materials and methods

Materials

Perfluoroctyl bromide and perfluorodecyl bromide were provided by J&K Scientific Ltd. (Beijing, China). Polyclonal anti-HIF-1a and polyclonal anti-caspase 3 were purchased from Abcam PLC (Cambridge, UK). ATP, SOD, and lipid hydroperoxide (LPO) assay kits were provided by Solarbio Life Science (Beijing, China). All other chemicals were of analytical grade, purchased from Sinopharm Reagent Ltd. Co. (Shanghai, China) and used as received. Male Sprague Dawley Rats (10–12 weeks old, weighing 250–300 g) were used for this study.

Preparation and characterization of perfluorocarbon emulsions

Four different PFC emulsions with consistent concentrations of perfluoroctyl bromide and perfluorodecyl bromide and different concentrations of buffer compositions according to HTK solution-based composition [13] (as listed in Table 1) were prepared by high-pressure homogenization. Briefly, appropriate amounts

Transplant International 2021; 34: 2087–2097

of ingredients listed in Table 1 were mixed at room temperature to generate a homogenous suspension, cycled for 8 min at 10,000 PSI through a microfluidizer (Microfluidics model 110Y), and chilled to 8-10 °C to get PFCE. The morphology of PFCE droplets was evaluated using transmission electron microscopy (TEM) on a JEM-2100F microscope (JEOL Co., Tokyo, Japan). The surface charge (z-potential) and the size distribution were determined by dynamic light scattering (DLS, Zetasizer ZS-10-82, Malvern Instruments Ltd. Co., Malvern, UK). The viscosity of the PFCE was determined by a rotatory rheometer (Bohlin Gemini Ⅱ, Malvern Instruments Ltd. Co., Malvern, UK) at 4 °C, 25 °C, and 37 °C in the viscometry mode. The osmotic pressure of PFCE was determined by an osmometer (Gonotec Co., Berlin, Germany). The pH of the microemulsions was determined by a pH meter.

To test the storage stability of PFCE at different temperatures, PFCEs were stored for 3 weeks at 4 °C, 25 °C, or 37 °C in the dark. At predetermined time points, PFCEs were redispersed by gentle inversion upside down for 10 times before size analysis by dynamic light scattering (DLS, Zetasizer ZS-10-82, Malvern Instruments Ltd. Co., Malvern, UK).

As an accelerated condition, centrifugation was performed on freshly prepared PFCEs at 4000 rpm for 10 min at room temperature. Thermal stress stability testing was conducted on the PFCE at 121 °C for 20 min. The influence of oxygen loading on the size distribution of the PFCE was also evaluated. For the above three conditions, microemulsion stability was evaluated by comparing the size distribution of microemulsions before and after treatment.

Measurements of the oxygen loading and offloading properties of the PFCplus

To characterize the oxygen loading and offloading properties of the optimized PFCE, PFCplus, the oxygen contents were tested by natural oxygen release in hypoxic solutions. Briefly, the O_2 concentration was reduced to 0.4 mg/l by bubbling N_2 to produce a severely hypoxic (O_2 0.4 mg/l) saline solution [14]. A total of 1 ml of oxygen-loaded PFCE were introduced into 500 ml of the hypoxic saline solution. Pure water and the HTK solution were used as the control. The total oxygen content in each liquid was monitored using an oximeter (HQ30d, HACH, Loveland, CO, USA) 30 min after adding solutions to the hypoxic saline solution. The tests were performed at different temperatures, and the oxygen-releasing kinetics were monitored every 10 min for each sample. The oxygen retained in each sample was tested under two different conditions to test the oxygen-retaining capacity in normal air or an oxygen-saturated prefilled surrounding at 4 °C in a 95% O_2 and 5% CO_2 prefilled chamber or in normal air for 72 h. The retained O_2 contents in the PFCplus emulsions were measured using the same method of total

Table 1. Composition and physical characterization of tested microemulsions.

Formulation Number	PFC-1	PFC −2	PFC −3	PFC plus
Composition				
Total Perfluorocarbon (g/1000 ml)	20%	20%	20%	20%
Sodium Chloride (mol/l)	0.015	0.015	0.015	0.015
Potassium Chloride(mol/l)	0.009	0.009	0.009	0.009
Magnesium Chloride(mol/l)	0.004	0.002	0.001	0.002
Calcium Chloride(mol/l)	0.000015	0.000008	0.000004	0.000008
Histidine Hydrochloride(mol/l)	0.018	0.009	0.0045	0.009
Potassium Hydrogen Ketoglutarate(mol/l)	0.0044	0.0022	0.0011	0.0022
Histidine(mol/l)	0.18	0.09	0.045	0.09
Tryptophan(mol/l)	0.002	0.001	0.0005	0.001
Mannitol(mol/l)	0.03	0.015	0.0075	0.015
Lecithin(mol/l)	0.053	0.053	0.053	0.053
Glycerol(mol/l)	0	0	0	0.152
Physical characterization				
Mean size (nm)/PDI				
Freshly prepared	1028/0.79	196.1/0.25	783.3/0.32	190.6/0.22
After 2 months	N.A.	198.3/0.25	1772/1.000	197.6/0.264
zeta-potential (mV)	−1.16 ± 8.89	−6.63 ± 4.56	−7.42 ± 8.49	−2.26 ± 4.79
Osmotic pressure (mmol/l)	305.7 ± 3.1	150.5 ± 4.5	137.3 ± 2.5	305.3 ± 3.5
pH	6.94 ± 0.04	6.94 ± 0.04	6.96 ± 0.05	6.95 ± 0.06

oxygen content testing at 0, 24, 48, and 72 h after storage.

Graft Preservation and Reperfusion

All animals were housed in a specific pathogen-free animal facility at Nanjing Drum Tower Hospital under the following conditions: 50% ± 10% relative humidity, 12 h/12 h light-dark cycle, and 24 ± 2 °C. Rats were fed a standard chow diet with free access to tap water. Livers were procured from donors after cardiac death (DCD) and were placed into 3 groups ($n = 6$ for each group): a group preserved with HTK solution for 3 h and two other groups that were preserved with HTK or the preoxygenated PFCplus emulsion for 48 h.

Inhalation anesthesia with isoflurane and oxygen was used before and during the procurement (2%–3% isoflurane). A donor rat abdomen was opened, and the liver was mobilized. After 1000 IU/kg heparin–sodium was infused, for developing a DCD donation, cardiac arrest was induced by external compression of the heart (exogenous tamponade) until contractions ceased. Subsequently, the descending aorta was clamped to ensure a complete block of the liver blood flow, and the rat was kept at 37 °C for 30 min.

The hepatectomy was performed by ligation of the splenic vein, mesenteric artery, and mesenteric vein and by cannulation of the celiac trunk. After clamping of the infrahepatic inferior vena cava and the portal vein, the latter was cannulated. After immediate in situ perfusion of the liver with 5 ml of saline (37°C) via the portal vein cannula, the liver was flushed with 5 ml of saline (4°C) via the portal vein cannula. The liver was then removed and flushed with an additional 20 ml and 5 ml of cold saline (4°C) via the portal vein and the hepatic artery (celiac trunk cannula), respectively. A 14-gauge catheter-made cuff attached to the portal vein. The liver grafts were then preserved in a small tank for 3 h or 48 h at 4 °C in 20 ml oxygenated preservation solutions. After preservation, livers were re-perfused using an oxygenated perfusion fluid *ex vivo* [14] via the portal vein at 37 °C for 120 min in a closed circuit. The reperfusion solution was continuously ventilated with a 95% O_2 and 5% CO_2 gas mixture.

Histological Study

Formalin-fixed, paraffin-embedded liver parenchyma was sectioned and stained with hematoxylin–eosin (H&E). Each section was evaluated blindly examined by a pathologist, and the severity was graded on a scale of 0-3 (none, mild, moderate, and severe) according to the modified Suzuki's criteria [15]. To detect apoptosis and hypoxia indicator HIF-1a, tissue sections were immunostained using the polyclonal anti-caspase 3 and the polyclonal anti-HIF-1a, respectively.

Biochemistry

The reperfusion perfusate was sampled after 120 min of reperfusion and immediately centrifuged to measure AST, ALT, and LDH as standard indicators of hepatocellular injury by a standard spectrophotometric method (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) with an automated analyzer (SPARK, Tecan Group Ltd., Männedorf, Switzerland) as well as ATP, SOD, and lipid hydroperoxide (LPO) assays by commercialized kits.

Statistical Analysis

All data were expressed as the mean ± the standard deviation. ANOVA tests were used for comparison among three or more groups. Student's *t*-test was used for comparison between each two groups. All statistical analyses were performed using SPSS software (SPSS 13.0; SPSS Inc., Chicago, IL, USA) and Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA). $P < 0.05$ was considered statistically significant.

Results

Preparation and characterization of Perfluorocarbon emulsions

A half concentration of histidine/histidine hydrochloride in the HTK solution (PFC-2 in Table 1) as the aqueous phase could yield homogeneous, ivory, and translucent liquid, different from PFC-1 or PFC-3 which appeared heterogeneous (Figure 1a). Glycerol (0.152 mol/l) was added to the outer phase of PFC-2 to obtain the optimized formulation, PFCplus, with a physiological osmotic pressure (305.3 ± 3.5 mmol/l) and a mean size less than 200 nm (Figures 1b,c).

The viscosity of both PFC-2 and PFCplus did not exceed that of HTK (Figure 1d), allowing for easy tissue perfusion with low resistance. The size distribution of PFCplus remained unchanged after storage for at least 21 days (Figure 1e). Its macroscopic homogeneity and size distribution also did not change after centrifugation at 4000 rpm for 10 min (Figures 2a,b), autoclaving (Figures 2c,d), and oxygen loading (Figures 2e,f).

Transplant International 2021; 34: 2087–2097

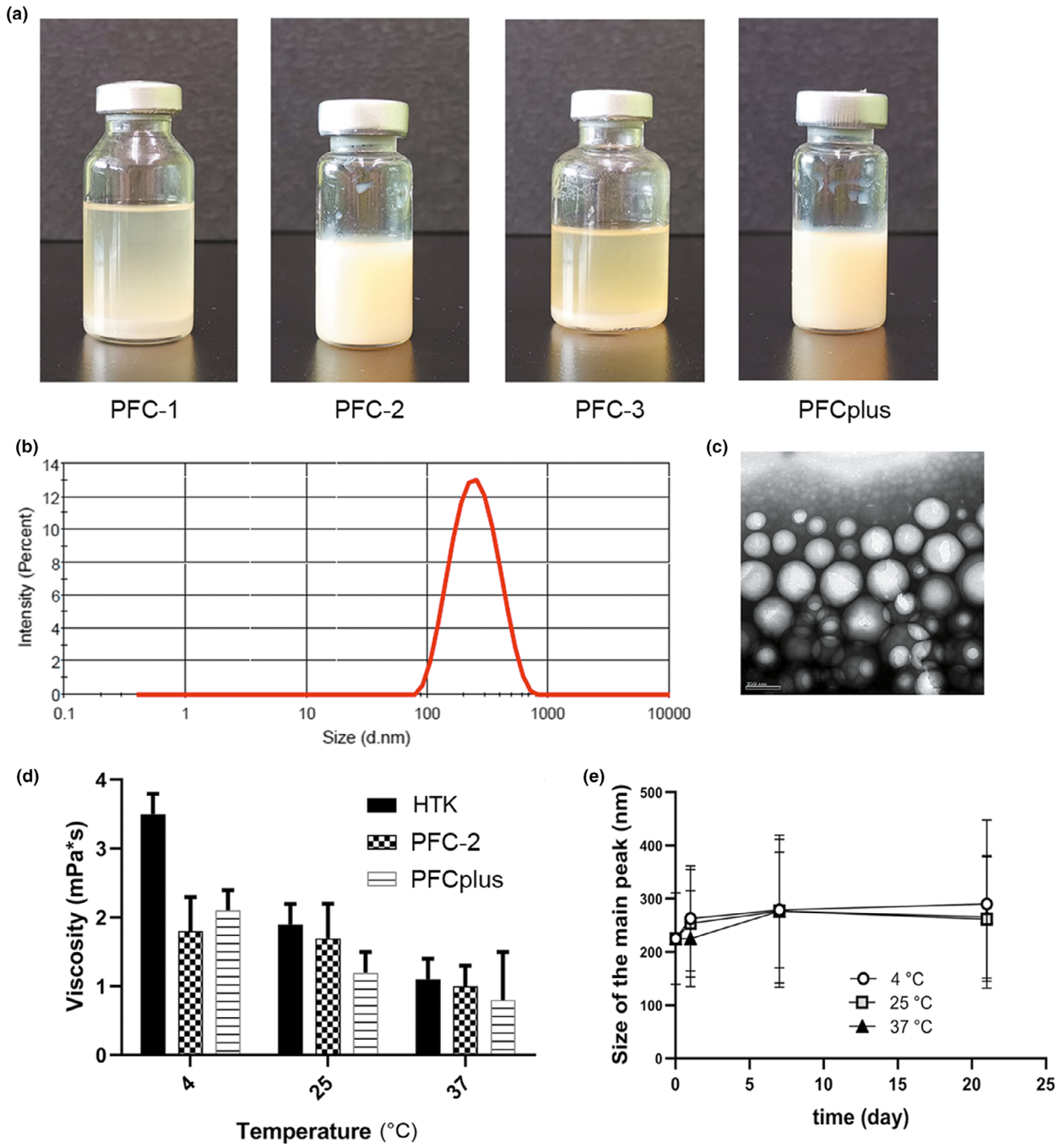

Figure 1 Physical characterization of PFCE (perfluorocarbon emulsion). (a) Appearance of PFC-1, PFC-2, PFC-3, and PFCplus. Both PFC-2 and PFCplus yielded homogeneous, ivory and translucent liquid, but PFC-1 or PFC-3 appeared heterogeneous. (b) Typical size distribution of PFCplus. (c) Morphology of PFCplus droplets shown by TEM (bar=200 nm). (d) Viscosity of PFC-2 and PFCplus in comparison with that of an HTK solution at 4 °C, 25 °C, and 30 °C. (e) Change in size distribution of PFCplus over time when stored at 4 °C, 25 °C, and 37 °C (mean ± std dev of the main peak) measured by DLS. The size distribution of PFCplus remained unchanged after storage for at least 21 days.

Oxygen Loading and Releasing

Since the air flow rate influences the oxygenation of solutions, the dissolved oxygen concentration before, during, and after air bubbles were pumped into the PFCplus emulsion at different temperatures with 3 different flow rates is shown in Figure 3. The dissolved oxygen in the PFCplus emulsion at 4 °C, which initially had a concentration of 7.5 mg/l, rapidly increased to above 300 mg/l in 15 min by pumping oxygen at a flow rate of 1 L/

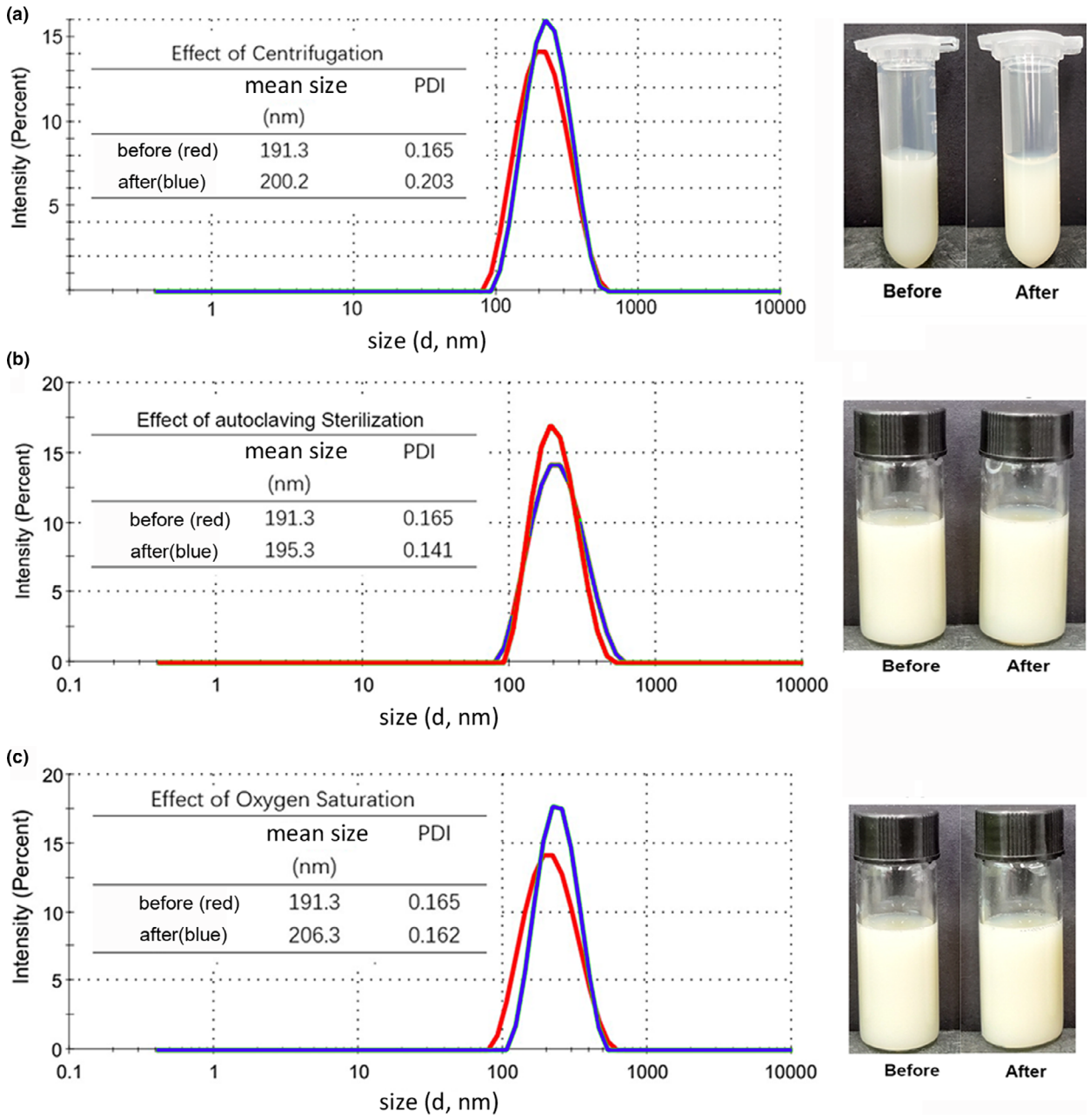

Figure 2 Size distribution and macroscopic homogeneity of PFCplus. (a) Influence of centrifugation (4000 rpm, 10 min). (b) Influence of autoclaving sterilization. (c) Influence of oxygen saturation. The macroscopic homogeneity and size distribution of PFCplus also did not change after centrifugation at 4000 rpm for 10 min, autoclaving or oxygen loading.

minute. The oxygen concentration remained constant after reaching the maximum value of approximately 350 mg/l during bubbling at a flow rate of 1 l/minute (Figure 3a). Bubbling with a lower oxygen flow rate at 0.2 l/minute or 0.04 l/minute was shown to more slowly increase the dissolved oxygen. A slower air flow rate did not generate oxygenated PFCplus emulsions as high as the 350 mg/l flow rate after 30 min at 4 °C. The initial dissolved oxygen concentrations of the PFCplus emulsion at 21 °C and 37 °C were approximately 6.6 and 5.8 mg/l, respectively. The oxygen saturating curve after bubbling was similar to that of the PFCplus emulsion prepared at 4 °C. However, the maximum dissolved oxygen (DO) level was lower than that of the PFCplus emulsion prepared at 4 °C (Figures 3b,c).

The DO content in different solutions after bubbling oxygen at 1 L/minute for 30 min at different temperatures was analyzed using the same method. The DO

Transplant International 2021; 34: 2087–2097

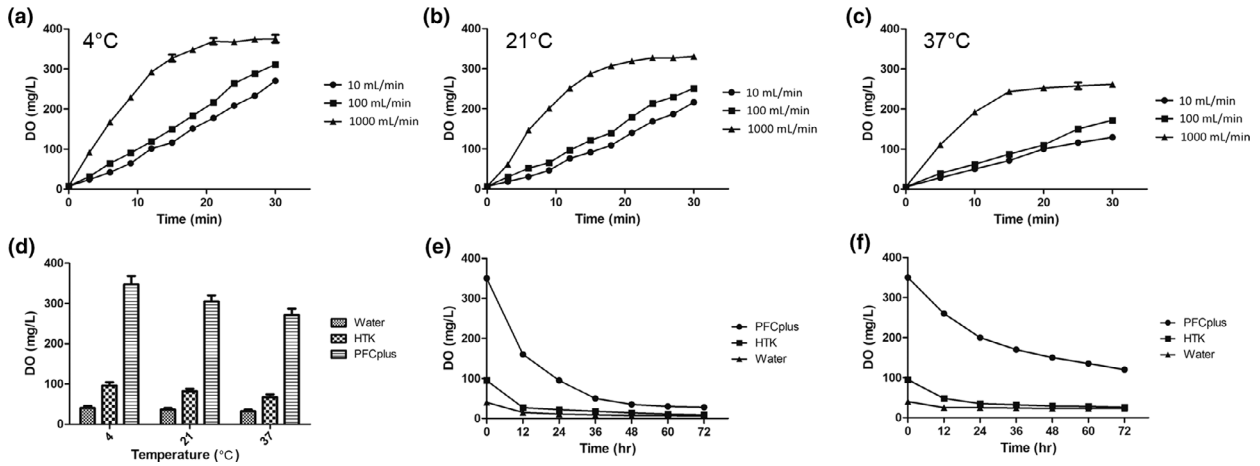

Figure 3 Oxygen loading and offloading characteristics of PFCplus: The increase in oxygen concentration in PFCplus over the course of 30 min with constant oxygen flow at varying rates at 4°C (a), 21°C (b), and 37°C (c), respectively. (d) Final oxygen content after bubbling oxygen at 1000 ml/min for 30 min in different liquids. Oxygen offloading of preoxygenated liquids in atmosphere (e) or oxygen (f) filled chamber at 4 °C. Different oxygen content in PFCplus after bubbling indicated that 30 minutes high oxygen flow were needed for the higher final oxygen content, and the oxygen can retaining in the PFCplus for 3 days under the oxygen-filled condition.

concentrations in the PFCplus emulsion were 8.7, 8.5, and 8.5 times higher than the DO content in tap water at 4, 21, and 37 °C, respectively. The DO level in HTK was higher than that in tap water but significantly lower than that in the PFCplus emulsion (Figure 3d).

The oxygen-saturated PFCplus emulsions were kept in a refrigerator in the general atmosphere (Figure 3e) or in a hypoxia modular incubator chamber filled with mixed 95% O_2/5% CO_2 gas at one atm (Figure 3f) at 4 °C. The preservation of the donor organ at 21 or 37 °C would be combined with the perfusion machine, and oxygen could be added during the circulation. Therefore, the oxygen-retaining capacity of the PFCplus emulsions at 21 or 37 °C was not analyzed.

To quantify the oxygen concentration during 72 h of organ preservation, DO in the solutions was measured every 12 h. During this period, DO in the PFCplus emulsion gradually decreased over time in the general atmosphere at 4 °C, regardless of the high DO concentration of the saturated PFCplus emulsion (Figure 3e). Nevertheless, the DO concentration in the PFCplus emulsion was much higher than that in the oxygen-saturated HTK solution or tap water (Figure 3e). By storing the oxygenated PFCplus emulsion in the modular incubator chamber in a 95% O_2/5% CO_2 hyperoxic surrounding, the DO concentration decreased slower than that of the corresponding solution in general atmosphere (Figure 3f). After 72 h, the final DO concentration in the PFCplus emulsion at this condition was 3 times higher than the DO concentration in the PFCplus emulsion stored under normal surroundings at 4 °C.

3 Preservation of Liver Grafts with an Oxygenated PFCplus Emulsion

Liver injury after preservation was analyzed by detecting ALT, AST, and LDH levels in the perfusates. After 48 h of static cold storage and 120 min of normothermic reperfusion, ALT and AST levels in the perfusates were significantly lower in the PFCplus emulsion group compared with those of the HTK 48-hour group ($P < 0.01$; $n = 6$ per group) but significantly higher when compared with those of the HTK 3-h group ($P < 0.01$; $n = 6$/group) (Figure 4b,c). The LDH level in the perfusates in the PFCplus group was similar to that of the HTK group but significantly lower than that of the HTK 48-h group ($P < 0.01$; $n = 6$ per group) (Figure 4d). The extent of apoptosis in liver grafts was examined by immunohistochemical staining with caspase 3 to further present the liver quality after preservation. There were significantly more caspase 3-positive cells after 48 h of HTK solution preservation when compared with those of the hepatic parenchyma preserved in HTK for only 3 h or in PFCplus for 48 h (Figure 4e). Quantitative analysis of caspase 3-positive cells/field revealed that oxygenated PFC emulsion preservation was associated with significantly less apoptotic cells than those of the other groups ($P < 0.01$; $n = 6$/group) (Figure 4f).

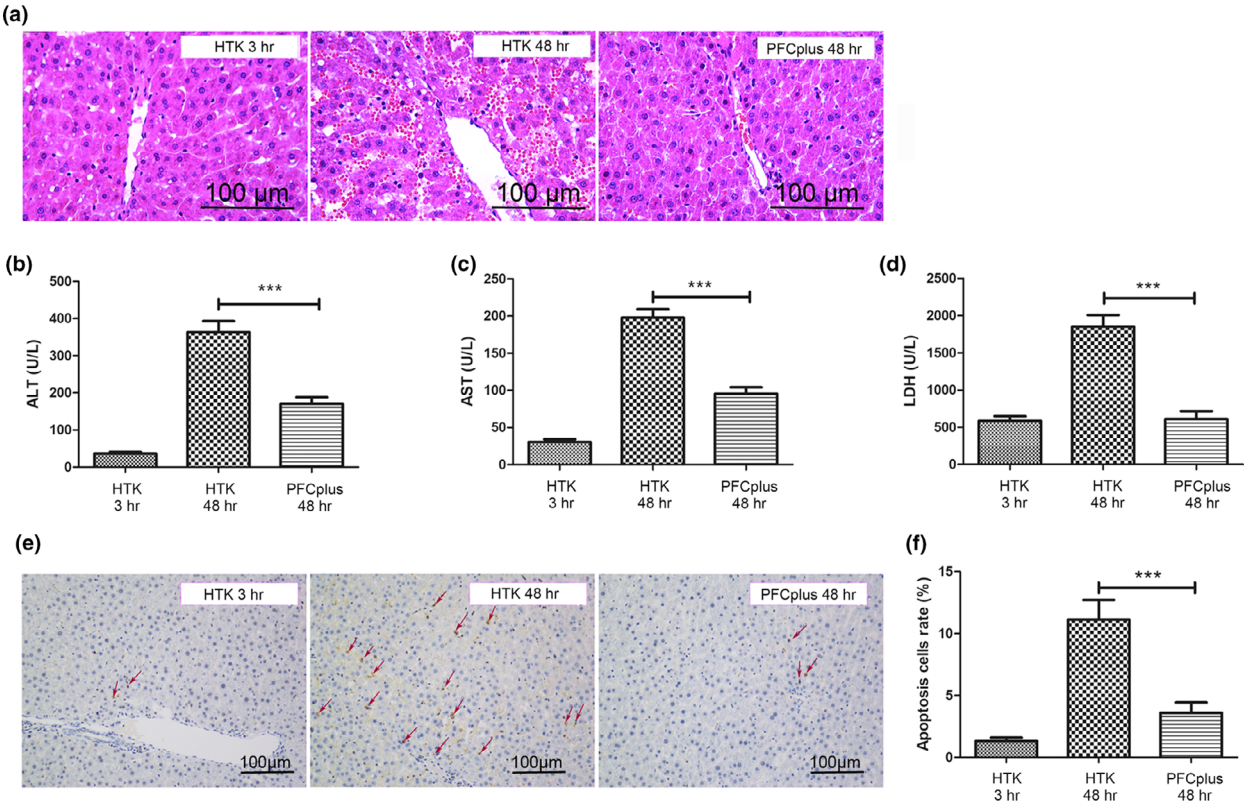

Figure 4 Oxygenated PFCplus provides better protection of liver grafts. (a) H&E-stained section of HTK- and oxygenated PFCplus-preserved liver grafts. (b), (c), and (d) relative change in perfusate concentrations of AST, ALT, and LDH after preservation. (e) and (f) immunoblotting analysis of caspase 3 in different preserved liver grafts and their quantification. *** indicates $P < 0.001$.

Relation of Oxygen Supplementation to PFCplus Emulsion Preservation

As shown in Figure 5a, the intracellular ATP concentration in the DCD graft liver after 3 h of HTK preservation was more than 30 μmol/g protein, whereas much lower ATP levels (10 μmol/g prot) were observed after 48 h of preservation with HTK in static cold storage. Moreover, liver storage in PFCplus for 48 h significantly increased ATP levels compared with those of the other two groups ($P < 0.05$). As we can see from the results above, exhausted ATP stores in the DCD donor liver were shown after warm ischemia and 3 h of HTK solution storage. In contrast, donor livers preserved in oxygenated PFCplus for 48 h exhibited dramatically increased ATP energy stores.

The liver tissue preserved in HTK for 48 h showed a significant decrease in liver antioxidant SOD activities compared with those of the HTK 3-hour group ($P < 0.05$). It was also found that SOD activities in the liver tissue were restored to similar levels to those of the HTK 3-hour group in the oxygenated PFCplus preservation group compared with those of the HTK 48-hour preservation group ($P < 0.05$) (Figure 5b). Additionally, it was observed that tissue LPO at the end of reperfusion was similar in the HTK 3-hour and PFCplus 48-hour groups (9.3 ± 3.3 and 9.5 ± 4.2 nmol/mg, respectively), while significantly higher values were measured in the HTK 48-hour group (27.9 ± 7.8 nmol/mg; $P < 0.05$ vs. HTK 3-hour).

As shown in Figure 5d, HIF-1a-positive cell rates were limited in the liver parenchyma in the HTK 3-hour group, but almost all cells were positive in the liver tissue preserved with HTK after 48 h, and the translocation of HIF-1a into the nucleus was found in some of these cells. The oxygenated PFCplus emulsion-preserved liver grafts had only a few HIF-1a-positive cells. The rates of HIF-1a-positive cells are presented in Figure 5e. The half-life of HIF-1a is as short as only several minutes. [12]

Discussion

In the current study, a stable perfluorocarbon emulsion (PFCE) as a novel organ preservation solution with low viscosity, appropriate salts, nutrients, buffers and

Transplant International 2021; 34: 2087–2097

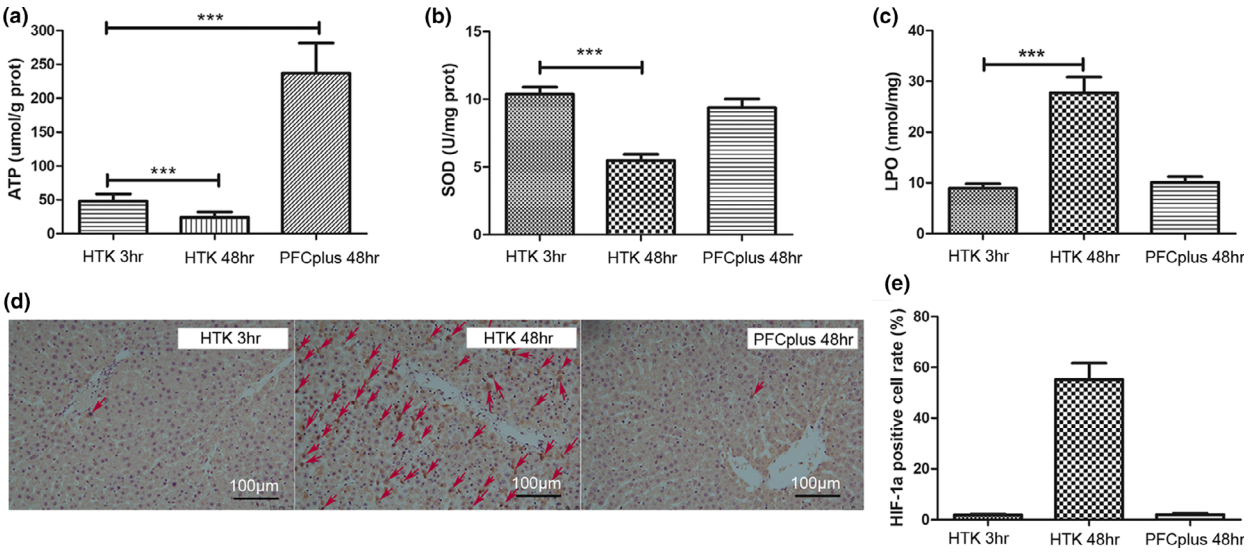

Figure 5 Oxygenated PFCplus preservation supplies liver grafts with energy and reducing peroxidation by attenuation of hypoxia. (a) ATP content in the liver grafts after preservation. (b) SOD and (c) LPO levels after preservation indicated the tissue lipid peroxidation and antioxidant status in the liver tissue. (d) and (e) immunoblotting analysis of HIF-1a in liver grafts after preservation and quantification. *** indicates $P < 0.001$.

osmotic pressure, potent oxygen loading, and releasing property was successfully prepared by high shear liquid processing, containing an oil phase composed of perfluoroctyl bromide and perfluorodecyl bromide, an aqueous phase containing similar ions as the histidine–tryptophan–ketoglutarate (HTK) solution as well as a safe emulsifier egg yolk lecithin [13]. Such perfluorocarbon emulsion has a droplet size smaller than 200 nm, which was approximately that of a commercial PFCE (Oxycyte^{TM}, Oxygen Biotherapeutics, Morrisville, NC). Most importantly, egg lecithin, an ingredient widely found in biological membranes and applied in many intravenous formulations, was used as the sole emulsifying agent, ensuring safety and translational perspectives. The liver preservation capability of this optimized PFCE, PFCplus, was evaluated *ex vivo* and proven with its future potential.

The cellular metabolism of donor grafts was slowed by more than 10-fold during static cold storage, but low metabolic levels continued [16]. In graft liver tissue stored at 0 °C, the oxygen consumption was 27% that of fresh liver at 37 °C.[17] It was estimated that the oxygen dissolved in the preservation solution was consumed within the first two hours of organ preservation [18]. To the best of our knowledge, this is the first study determining the effect of oxygen bubbles to increase the DO content in PFCE at different flow rates and temperatures.

The hypoxia-inducible factor (HIF) is a key regulator for adapting and sensing cellular oxygen levels [19]. HIF-1a pathway, one of the pivotal biological oxygen sensors, is influenced by the oxygenated PFCplus emulsions during cold storage. Under normoxic conditions, the HIF-1a gene is constitutively expressed at low levels. Under hypoxia, HIF-1a transcription is often significantly upregulated. HIF-1a-positive cell ratio indicated that the oxygenated PFCplus could decrease the number of HIF-1a-positive cells and improve hypoxia, which revealed that the mechanism of the oxygenated PFCplus reduces energy exhaust and ischemia-reperfusion injury by supplementing oxygen.

Blood as an oxygen carrier exhibits a sigmoidal oxygen dissociation curve [20] because the affinity of hemoglobin for oxygen at 0 °C is 22 times greater than that at 37 °C [21], so hemoglobin is not a good candidate for oxygen carrying at hypothermic or subnormothermic temperatures. In contrast, PFCE showed a linear relationship between oxygen partial pressure and oxygen content, which could be a better oxygen carrier at hypothermic or subnormothermic temperatures than red blood cells. Furthermore, PFC droplets can perfuse through even the tiniest capillaries (4-5 μm in diameter) where red blood cells (approximately 7 to 8 μm) may not be able to flow.

During cold ischemia, ATP depletion was considered crucial for graft function [22]. Oxygen supplementation was reported to generate ATP during graft organ preservation. When oxygen is present, the organ can continuously produce ATP for up to 96 h [23]. Increased ATP was detected in donor livers preserved in oxygenated PFCplus for 48 h, indicating that oxygen

supplementation by PFCplus may support oxidative phosphorylation and the production of ATP during static cold preservation of the liver.

Oxidative stress is one of the main cellular features of reperfusion damage after ischemia and hypoxia [24]. Thus, antioxidant effects were evaluated by measuring superoxide dismutase (SOD). Hypoxia and reperfusion-induced oxidative stress could be the result of decreased antioxidant defense in liver tissue. Depletion of SOD after 48 h of preservation with the oxygenated PFCplus emulsion proved that SOD generated by oxygenated PFCplus emulsion preservation can prevent lipid oxidation from occurring in preserved liver tissues.

Reactive oxygen species-induced lipid peroxidation (LPO) results from the interaction with polyunsaturated fatty acids in the cellular membrane, resulting in the destruction of cellular integrity, which plays a critical role in cell death, including apoptosis and autophagy [25]. We analyzed the LPO level in each donor liver tissue to study whether the oxygen tension in our preservation system induced LPO. High oxygenation might favor the production of oxygen free radicals, which results in impaired liver tissue integrity. The impact of oxygen free radicals on tissue integrity was approximated by analyzing LPO levels in graft liver tissue. ROS induce lipid oxidative deterioration via the lipid peroxidation process, resulting in the generation of LPO as a fundamental constituent. Thus, the LPO level in the tissue is a convenient indicator of oxidative cell damage.

The results of the present study demonstrated that limited injury was observed in livers preserved in an oxygenated PFCplus emulsion. Although previous investigations found that PFCs together with commercialized preservation solutions can be beneficial with preserved organ drafts, there have been no studies to date on the effect of stable PFC emulsion formulation, especially for organ preservation. To the best of our knowledge, this is the first study to investigate the long-term (48 h) outcomes of liver grafts preserved by oxygenated PFCplus emulsions. Oxygenation improved liver preservation because O_2 is essential for energy metabolism,but it also generates toxic reactive oxygen species (ROS). Previous experimental studies reported that the increase in oxygen tension during preservation did not increase harmful ROS formation [26].

Some research groups have tried using two-layer preoxygenated perfluorocarbon plus the UW solution for the static cold preservation of DCD rat livers and resulted in reducing the severity of ischemic tissue damage. However, they also found that the deeper parenchymal tissue may be unequally exposed compared with the liver surface close to the oxygenated solution due to the passive nature of O_2 diffusion. Our results are consistent with several recent rat liver transplant experiments performed by Okumura and Bezinover [27,28]. But these studies were not performed by using PFC emulsion preservation solutions specially designed for organ storage but used the UW solution + 20% Oxycyte™ as the experimental group. There are several defects in these studies because dilution of Oxycyte™ with the UW solution will change the concentrations of the constituents of the UW solution, and the actual perfluoro-tert-butylcyclohexane concentration was 12% because the initial w/v of Oxycyte™ was 60% [29]. The complex composition of the UW solution may cause the instability of the PFC emulsion; thus, the studies using Oxycyte™ + the UW solution had to be mixed right before use. Additionally, the UW solution mixed with Oxycyte™ is not suitable for perfusion preservation due to the high viscosity of the UW solution. Our results indicated that our oxygenated, stable PFCplus reduced liver tissue damage and apoptosis. This suggests that using PFCplus for the extension of preservation time and further testing by combining it with perfusion devices are promising. This work established the groundwork to trial this approach in large animal models. Systematic studies using a miniature pig model to confirm the potential of using PFCplus as the oxygenated perfusate for machine perfusion preservation will be included in our future work.

In summary, data from characterization, stability testing, and evaluation of the oxygen-carrying characteristics demonstrated that the optimized emulsion PFCplus might be a promising solution for organ preservation with low viscosity, appropriate salts, nutrients, buffers and osmotic pressure, potent oxygen loading and releasing property and good translational perspectives.

Authorship

Guoyi Wu and Yu Liu equally performed the writing of the paper, the performance of the research, and data analysis. Chen Rui and Shanshan Zhan conducted the animal experiments. Jun Wang and Shizhen Cai prepared and characterized the perfluorocarbon emulsion. Xiaolei Shi and Yitao Ding designed the work and substantively revised the manuscript.

Conflict of interest

Declaration of Competing Interest: The authors declare no competing financial interest.

Transplant International 2021; 34: 2087–2097

Acknowledgment

The financial support from the National Key Research and Development Program of China (grant no. 2016YFC1302603), National Natural Science Foundation of China (81573361), and Chuangju Jiangning innovation and entrepreneurship program are gratefully acknowledged.

Statement

All human studies have been reviewed by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in an appropriate version of the 2000 Declaration of Helsinki as well as the Declaration of Istanbul 2008. All persons gave their informed consent prior to their inclusion in the study.

REFERENCES

1. Kim WR, Lake JR, Smith JM, *et al.* OPTN/SRTR 2017 annual data report: Liver. *Am J Transplant* 2019; **19**(S2): 184.
2. Wang Y, Lei T, Wei L, Du S, Girani L, Deng S. Xenotransplantation in China: present status. *Xenotransplantation* 2019; **26**: e12490.
3. Tchilikidi KY. Liver graft preservation methods during cold ischemia phase and normothermic machine perfusion. *World J Gastrointest Surg.* 2019; **11**: 126.
4. Shailendra J, Lee SH, Korneszczuk K, *et al.* Improved preservation of warm ischemic livers by hypothermic machine perfusion with supplemented University of Wisconsin solution. *J Invest Surg* 2008; **21**: 83.
5. van Rijn R, Schurink IJ, de Vries Y, *et al.* Hypothermic Machine Perfusion in Liver Transplantation - A Randomized Trial. *N Engl J Med* 2021; **384**: 1391.
6. Sopden D, Karimian N, Sutton ME, *et al.* Ex vivo normothermic machine perfusion and viability testing of discarded human donor livers. *Am J Transplant* 2013; **13**: 1327.
7. Bodewes SB, van Leeuwen OB, Thorne AM, *et al.* Oxygen transport during ex situ machine perfusion of donor livers using red blood cells or artificial oxygen carriers. *Int J Mol Sci* 2020; **22**: 235.
8. Lambert E, Gorantla VS, Janjic JM. Pharmaceutical design and development of perfluorocarbon nanocolloids for oxygen delivery in regenerative medicine. *Nanomedicine (Lond).* 2019; **14**: 2697.
9. Spiess BD. Perfluorocarbon gas transport: An overview of medical history with yet unrealized potentials. *Shock* 2019; **52**: 7.
10. Martins PN, Berendsen TA, Yeh H, *et al.* Oxygenated UW Solution Decreases ATP Decay and Improves Survival After Transplantation of DCD Liver Grafts. *Transplantation* 2019; **103**: 363.
11. Watrobska-Swietlikowska D, Szlagatys-Sidorkiewicz A, Łuszkiewicz K. Evaluation of physical stability of all in one parenteral admixtures for pediatric home care with high electrolytes concentrations. *Nutr Hosp* 2014; **31**: 236.
12. Aarsaether E, Stenberg TA, Jakobsen Ø, Busund R. Mechanoenergetic function and troponin T release following cardioplegic arrest induced by St Thomas' and histidine-tryptophan-ketoglutarate cardioplegia–an experimental comparative study in pigs. *Interact Cardiovasc Thorac Surg* 2009; **9**: 635.
13. Kumar A, Saw RK, Mandal A. RSM optimization of oil-in-water microemulsion stabilized by synthesized zwitterionic surfactant and its properties evaluation for application in enhanced oil recovery. *Chem Eng Res Desig.* 2019; **147**: 399.
14. Op den Dries S, Karimian N, Westerkamp AC, *et al.* Normothermic machine perfusion reduces bile duct injury and improves biliary epithelial function in rat donor livers. *Liver Transpl* 2016; **27**: 994.
15. Suzuki S, Toledo-Pereyra LH, Rodriguez FJ, *et al.* Neutrophil infiltration as an important factor in liver ischemia and reperfusion injury. Modulating effects of FK506 and cyclosporine. *Transplantation* 1993; **55**: 1265.
16. Reddy SP, Brockmann J, Friend PJ. Normothermic Perfusion: A Mini-Review. *Transplantation* 2009; **87**: 631.
17. Tsukamoto K, Murakami M, Seo Y, *et al.* Energetic recovery from hypothermic preservation in the rat liver. *J Surg Res* 1990; **48**: 46.
18. Steichen C, Giraud S, Bon D, *et al.* Barriers and advances in kidney preservation. *Biomed Res Int* 2018; **9206257**: 1.
19. Choudhry H, Harris AL. Advances in Hypoxia-Inducible Factor Biology. *Cell Metab* 2018; **27**: 281.
20. Leow MK. Configuration of the hemoglobin oxygen dissociation curve demystified: a basic mathematical proof for medical and biological sciences undergraduates. *Adv Physiol Educ* 2007; **31**: 198.
21. Weber RE, Campbell KL. Temperature dependence of haemoglobin-oxygen affinity in heterothermic vertebrates: mechanisms and biological significance. *Acta Physiol (Oxf)* 2011; **202**: 549.
22. Ravaioli M, Baldassare M, Vasuri F, *et al.* Strategies to restore adenosine triphosphate (ATP) level after more than 20 hours of cold ischemia time in human marginal kidney grafts. *Ann Transplant.* 2018; **23**: 34.
23. Fujino Y, Kuroda Y, Suzuki Y, *et al.* Preservation of canine pancreas for 96 hours by a modified two-layer (UW solution/perfluorochemical) cold storage method. *Transplantation* 1991; **51**: 1133.
24. Li F, Yang Z, Stone C, *et al.* Phenothiazines enhance the hypothermic preservation of liver grafts: a pilot in vitro study. *Cell Transplant* 2019; **28**: 318.
25. Clavien PA, Strasberg HPR, Strasberg SM. Preservation and reperfusion injuries in liver allografts. An overview and synthesis of current studies. *Transplantation* 1992; **53**: 957.
26. Patel K, Smith TB, Neil DAH, Thakker A, Tsuchiya Y, Higgs EB. The effects of oxygenation on ex vivo kidneys undergoing hypothermic machine perfusion. *Transplantation* 2019; **103**: 314.
27. Okumura S, Uemura T, Zhao X, *et al.* Liver graft preservation using perfluorocarbon improves the outcomes of simulated donation after cardiac death liver transplantation in rats. *Liver Transpl* 2017; **23**: 1171.
28. Bezinover D, Ramamoorthy S, Postula M. Effect of cold perfusion and perfluorocarbons on liver graft ischemia in a donation after cardiac death model. *J Surg Res* 2014; **188**: 517.
29. Yacoub A, Hajec MC, Stanger R, Wan W, Young H, Mathern BE. Neuroprotective effects of perflurocarbon (oxycyte) after contusive spinal cord injury. *J Neurotrauma* 2014; **31**: 256.