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Normothermic Machine Perfusion of Donor Livers Without the Need for Human Blood Products

Alix P. M. Matton,[1,2] Laura C. Burlage,[1,2] Rianne van Rijn,[1,2] Yvonne de Vries,[1,2] Shanice A. Karangwa,[1,2] Maarten W. Nijsten,[3] Annette S. H. Gouw,[4] Janneke Wiersema-Buist,[1] Jelle Adelmeijer,[1] Andrie C. Westerkamp,[1,2] Ton Lisman,[1] and Robert J. Porte[2]

[1]Surgical Research Laboratory; [2]Section of Hepatobiliary Surgery and Liver Transplantation, Departments of Surgery; [3]Critical Care; [4]Pathology, University of Groningen, University Medical Center Groningen, Groningen, the Netherlands

Normothermic machine perfusion (NMP) enables viability assessment of donor livers prior to transplantation. NMP is frequently performed by using human blood products including red blood cells (RBCs) and fresh frozen plasma (FFP). Our aim was to examine the efficacy of a novel machine perfusion solution based on polymerized bovine hemoglobin-based oxygen carrier (HBOC)-201. Twenty-four livers declined for transplantation were transported by using static cold storage. Upon arrival, livers underwent NMP for 6 hours using pressure-controlled portal and arterial perfusion. A total of 12 livers were perfused using a solution based on RBCs and FFPs (historical cohort), 6 livers with HBOC-201 and FFPs, and another 6 livers with HBOC-201 and gelofusine, a gelatin-based colloid solution. Compared with RBC + FFP perfused livers, livers perfused with HBOC-201 had significantly higher hepatic adenosine triphosphate content, cumulative bile production, and portal and arterial flows. Biliary secretion of bicarbonate, bilirubin, bile salts, and phospholipids was similar in all 3 groups. The alanine aminotransferase concentration in perfusate was lower in the HBOC-201–perfused groups. In conclusion, NMP of human donor livers can be performed effectively using HBOC-201 and gelofusine, eliminating the need for human blood products. Perfusing livers with HBOC-201 is at least similar to perfusion with RBCs and FFP. Some of the biomarkers of liver function and injury even suggest a possible superiority of an HBOC-201–based perfusion solution and opens a perspective for further optimization of machine perfusion techniques.

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Liver transplantation is the only curative treatment option for end-stage liver disease. Unfortunately, a global discrepancy exists between the availability and need for human donor livers, resulting in substantial wait-list mortality.[1] Over the past decades, machine perfusion has been gaining interest as a promising tool for expanding the human donor liver pool.[2]

Normothermic machine perfusion (NMP) is a technique whereby human donor livers are perfused ex situ at 37°C. This technique can be used for the entire period of preservation, as is currently being evaluated in a clinical trial by Friend et al. in Oxford,[3] and for viability assessment of the organ prior to transplantation.[4-6] In this manner, only well-functioning organs are transplanted, including those that initially may have been declined for transplantation. Furthermore, NMP has the potential to allow for the resuscitation of donor livers.

NMP is generally performed using a perfusion solution based on packed red blood cells (RBCs).[3,7-9] The NMP solution requires an adequate oxygen carrier to deliver oxygen throughout the organ, as well as physiological osmolarity and oncotic pressure. Previous NMP perfusions at our center were performed using matched packed RBCs and fresh frozen plasma (FFP) obtained from the blood bank, with the addition of nutrients and

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; ATP, adenosine triphosphate; BMI, body mass index; CVA, cerebrovascular accident; CV, central vein; DBD, donation after brain death; DCD, donation after circulatory death; ELISA, enzyme-linked immunosorbent assay; FFP, fresh frozen plasma; GGT, gamma-glutamyltransferase; Hb, hemoglobin; HBOC, hemoglobin-based oxygen carrier; H & E, hematoxylin-eosin; HTK, histidine-tryptophan-ketoglutarate; IQR, interquartile range; metHb, methemoglobin; NMP, normothermic machine perfusion; PCO_2, partial pressure of carbon dioxide; PO_2, partial pressure of oxygen; PT, portal triad; RBC, red blood cell; SO_2, oxygen saturation; TBB, anti-tublin beta; UW, University of Wisconsin.

antibiotics.[7] Other centers have performed NMP with RBCs and gelofusine[3,8] or Steen solution,[9] and 1 previous study has also performed hemoglobin-based oxygen carrier (HBOC)-201 and gelofusine.[10]

The use of human blood products is expensive and logistically challenging due to their short preservation time and need for matching. Furthermore, human blood products are scarce and carry the risk of transmitting blood borne infections. For these ethical, financial, and logistical reasons it would be favorable to avoid the use of RBCs and FFPs for NMP. Consequently, the aim of the current study was to design a perfusion solution for NMP that circumvents the use of human blood products. We did this by replacing RBCs with HBOC-201 (Hemopure, HbO_2 Therapeutics LLC, Souderton, PA), a bovine-derived free hemoglobin (Hb) oxygen carrier, and FFPs with gelofusine, a widely used commercially available colloid solution.

Patients and Methods

ORGAN PROCUREMENT

The present study was performed at the University Medical Center Groningen, Groningen, the Netherlands, and was approved by the medical ethical committee of the institute. Between July 2012 and July 2015, 24 human donor livers that were declined for transplantation were included after consent for research had been obtained from relatives. All donor livers were procured using the standard technique of in situ cooling and flush out with ice-cold preservation solution (University of Wisconsin [UW] or histidine-tryptophan-ketoglutarate [HTK] solution, in line with the national organ procurement protocol), as has previously been described.[11] Livers were packed in ice-cold preservation solution (UW or HTK), stored on ice, and transported to our center. Upon arrival, an experienced liver surgeon performed the back-table preparation and cannulated the portal vein, supratruncal aorta, and bile duct for machine perfusion. Meanwhile, the machine perfusion device was set up and primed, and machine perfusion was commenced as soon as possible.

STUDY GROUPS

Twelve donor livers were perfused with RBCs and FFPs (RBC + FFP group). Subsequently, 6 livers were perfused with HBOC-201 and FFPs (HBOC-201 + FFP group), and thereafter, 6 livers were perfused with HBOC-201 and gelofusine (HBOC-201 + gelofusine group). Because of the scarcity of available donor livers at our research center, we used a cohort of livers that had already been perfused and previously published (RBC + FFP group).[11] Perfusions in the 3 study groups were not randomized but instead performed consecutively. All perfusions were performed in the presence of the principal investigator, and after having optimized our perfusion technique extensively before including any of the liver grafts of the present study, no changes were made in perfusion technique.

OXYGEN CARRIER HBOC-201

The HBOC-201 oxygen carrier solution contains polymerized Hb, which is much smaller than a human erythrocyte, is less viscous than RBCs, and has the ability to release oxygen more easily than human Hb.[12] This gives it the ability to perfuse tissues more deeply and oxygenate more remote regions.[12] Because of the extraction and purification process, potential contaminants including plasma proteins, endotoxins, bacteria, viruses, and the prions responsible for bovine spongiform encephalopathy and variant Creutzfeld-Jakob disease are removed, resulting in a sterile, pyrogen-free solution.[13] The in vivo half-life of HBOC-201 is approximately 20 hours.[13] A downside to the use of HBOC-201 is the potential formation of methemoglobin (metHb). However, the small amount

Address reprint requests to Robert J. Porte, M.D., Ph.D., F.E.B., Section of Hepatobiliary Surgery and Liver Transplantation, Department of Surgery, University of Groningen, University Medical Center Groningen, Hanzeplein 1, 9713 GZ Groningen, the Netherlands. Telephone: +31 50 361 2896; FAX: +31 50 361 4873; E-mail: r.j.porte@umcg.nl

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of HBOC-201 that would reach the recipient in a transplantation setting is minimal as the perfusion solution would be washed out prior to transplantation.[13] Lastly, HBOC-201 cannot be spun down and therefore renders the perfusate colored red, which may interfere with spectrophotometric analyses.[14]

MACHINE PERFUSION SOLUTION

The perfusion solutions of the 3 study groups were based on 3 main components:

1. An oxygen carrier, provided by either 3 units of RBCs or 4 units of HBOC-201 (Hemopure, HbO_2 Therapeutics LLC), both with a total of 120 g Hb.
2. A colloid solution, consisting of either 3 units of FFP supplemented with 100 mL 20% human albumin or 500 mL 4% gelofusine (B Braun, Melsungen, Germany) supplemented with 250 mL 20% human albumin.
3. Additional supplements containing nutrients, trace elements, antibiotics, vitamins, insulin, and heparin as described previously.[7]

The total volume of perfusion solution was similar in all 3 groups and approximately 2200 mL. All blood products were supplied by Sanquin, the Dutch blood bank, and were not expired. In each perfusion solution, the colloid oncotic pressure and osmolarity were targeted to reach physiological levels. Prior to connecting the liver, the pH of the perfusion fluid was optimized.

NORMOTHERMIC MACHINE PERFUSION

The Liver Assist (Organ Assist, Groningen, the Netherlands) machine perfusion device was used. It simulates the physiological environment by providing pressure-controlled pulsatile flow to the hepatic artery and continuous flow to the portal vein and gravitational outflow through the vena cava. The hepatic artery and portal vein perfusion circuits are each composed of a rotary perfusion pump, a membrane oxygenator with integrated heat exchanger, and flow and pressure sensors.

The perfusion solution was maintained at 37°C, and NMP was performed for 6 hours. Pressures were set at a mean of 70 mm Hg (systolic and diastolic pressures ±20%) on the arterial and 11 mm Hg on the portal side. Perfusion fluid was oxygenated using a total of 4 L/minute (95% oxygen and 5% carbon dioxide) through the 2 oxygenators. Before NMP and every 30 minutes during NMP, samples of the arterial and venous perfusion fluid, as well as bile samples, were taken for analysis of blood gas parameters (pH, partial pressure of oxygen [PO_2], partial pressure of carbon dioxide [PCO_2], oxygen saturation [SO_2], bicarbonate [HCO_3^-] lactate, glucose, and metHb) using an ABL800 FLEX or ABL90 FLEX analyzer (Radiometer, Brønhøj, Denmark). If needed, sodium bicarbonate (8.4% solution) was added to maintain a pH within the physiological range of 7.35-7.45, as described previously.[7,15] Liver parenchyma wedge biopsies were taken before and every 2 hours during NMP. Biopsies were stored in formalin and embedded in paraffin, or snap-frozen in liquid nitrogen and stored at −80°C. Bile produced by the liver was collected and measured every 30 minutes and stored at −80°C. Perfusion fluid samples were collected every half hour and stored at −80°C (after 5 minutes centrifugation at 2700 rpm at 4°C).

ASSESSMENT OF HEPATOBILIARY FUNCTION AND INJURY

Adenosine triphosphate (ATP) in liver parenchyma biopsies was determined as described previously.[4]

To calculate the peak oxygen extraction, the difference between arterial and venous oxygen content was calculated and corrected for the flow. The following formula was used to calculate the oxygen content:

Oxygen content = $(PO_2 \times K) + (SO_2 \times Hb \times c)$, where PO_2 is the partial pressure of oxygen in kPa, K is a constant (0.0225), SO_2 is the oxygen saturation expressed as a fraction (where 1.00 is 100% saturation), Hb is the concentration in g/dL, and c is the oxygen binding capacity of Hb (1.39 for human Hb; 1.26 for HBOC-201).

Total bilirubin concentration in bile was determined using a competitive enzyme-linked immunosorbent assay (ELISA) kit (Human Total Bilirubin ELISA kit, #MBS756198, MyBioSource, Inc., San Diego, CA) using a monoclonal anti-tublin beta (TBB) antibody and a TBB-HRP conjugate as indicated by the manufacturer. Samples were applied undiluted. Color intensity was measured spectrophotometrically at 450 nm using VersaMax ELISA microplate reader and SoftMax Pro 5.4, and concentrations were calculated.

Total bile salt concentrations in bile were determined by adding 250 μL trisbuffer and 50 μL of the reagent 3α-hydroxysteroid dehydrogenase (H1506-50UN, Sigma-Aldrich) and resazurine (Acros Organics) to 10 μL (diluted 1:100) of each sample.[16] Fluorescence was measured using a PerkinElmer Wallac 1420 Victor3 microplate reader and concentrations were calculated.

Phospholipid concentrations in bile were determined by adding 150 μL of reagent out of a commercially available Phospholipids kit (reference number 15741 9910 930, Diagnostic systems, GmbH, Holzheim, Germany) to 10 μL (diluted 1:9) of each sample. Color intensity was measured spectrophotometrically at a wavelength of 570 nm (VersaMax Molecular devices) in SoftMax Pro 5.4 and concentrations were calculated. In order to calculate the biliary secretion of bicarbonate, bilirubin, total bile salts, and phospholipids, their concentrations were multiplied by the volume of bile produced, corrected for the weight of the liver.

After centrifugation, perfusate samples were diluted 10× and analyzed for alanine aminotransferase (ALT) using routine diagnostic laboratory procedures. Because HBOC-201 Hb is freely suspended in solution and cannot be spun down, ALT concentrations in the HBOC-201 groups were corrected for the 20% hematocrit present in the RBC + FFP group by multiplying ALT values in the HBOC-201 groups by 1.25 (1/0.80 = 1.25).

Paraffin-embedded slides of liver biopsies were prepared for hematoxylin-eosin (H & E) staining and semiquantitatively assessed using the Suzuki liver injury scoring system.[17] All liver slides were examined in a blinded fashion by an expert liver pathologist (A.S.H.G.).

STATISTICS

Continuous variables are presented as median with interquartile range (IQR); categorical variables are presented as absolute numbers. Continuous variables were compared between groups by calculating the area under the curve when indicated and the Kruskal-Wallis H or Mann-Whitney U test with Bonferroni correction. Categorical variables were compared with the Fisher's exact test. The level of significance was set at a P value <0.05. All statistical analyses were performed using SPSS software version 22.0 for Windows (IBM SPSS, Inc., Chicago, IL) and Microsoft Excel 2010 for Windows.

Results

DONOR LIVER CHARACTERISTICS

Table 1 shows the donor liver characteristics in the 3 study groups. There were no significant differences in donor liver characteristics between the groups. Notably, the number of livers discarded due to expected steatosis (based on donor body mass index [BMI], ultrasound, and laboratory results) was 5 in the RBC + FFP group compared with 0 and 1 in the HBOC-201 + FFP and HBOC-201 + gelofusine groups, respectively. However, the level of actual microscopic steatosis, which was only known after the liver had been offered for research, was much lower. Only 2 (17%) livers in the HBOC-201 + FFP group, 0 in the HBOC-201 + FFP group, and 1 (17%) in the HBOC-201 + gelofusine group had a clinically relevant degree of microscopic steatosis (>30%).

NORMOTHERMIC MACHINE PERFUSION

Figure 1 shows photographs of NMP using RBC + FFP (Fig. 1A) and HBOC-201 + gelofusine (Fig. 1B). The color of HBOC-201 is darker than that of human blood. During 1 HBOC-201 + FFP perfusion, there was blood present in the bile and this liver was consequently excluded for biliary analyses, as this would result in the recording of falsely elevated bile production. The fraction of metHb during NMP reached maximally 0.02% in the RBC + FFP group, 0.22% in the HBOC-201 + FFP group, and 0.28% in the HBOC-201 + gelofusine group (healthy human adults range <1%).

HEMODYNAMICS

As shown in Fig. 2A, the portal vein flow increased during the first hour of NMP and thereafter remained stable in all 3 groups. In both HBOC-201 groups, the portal flow was significantly higher at each time point compared with the RBC + FFP group, reaching a median (IQR) of 848 (663-1393) mL/minute/kg liver weight in the RBC + FFP group, 1890 (1530-2173) in the HBOC-201 + FFP group, and 1830 (1713-2030) in the HBOC-201 + gelofusine group at 6 hours of NMP.

The hepatic artery flow was higher after the first 2 hours of NMP in both HBOC-201 groups compared with the RBC + FFP group, reaching a median (IQR) of 273 (231-327) mL/minute/kg liver weight in the RBC + FFP group, 742 (480-867) mL/minute/kg liver weight in the HBOC-201 + FFP group, and 533 (187-741) mL/minute/kg liver weight in the HBOC-201 + gelofusine group at 6 hours NMP. The arterial flow remained stable in the RBC + FFP group, continued to increase in the HBOC-201 + FFP group, and declined slightly after 3 hours of NMP for unknown reasons in the HBOC-201 + gelofusine

TABLE 1. Donor Liver Characteristics

	RBC + FFP (n = 12)	HBOC-201 + FFP (n = 6)	HBOC-201 + Gelofusine (n = 6)	*P* Value
Age, years	61 (53-63)	54 (39-67)	65 (63-66)	0.22
Sex				0.43
Male	8	4	3	
Female	4	2	3	
BMI, kg/m^2	27 (25-35)	19 (17-29)	25 (24-28)	0.19
Type of donor				1.00
DCD	9	5	5	
DBD	3	1	1	
Warm ischemia time, minutes*	35 (24-39)	31 (25-37)	39 (28-45)	0.56
Cold ischemia time, hours†	9.1 (7.2-10.2)	7.6 (7.1-8.6)	8.0 (7.1-8.4)	0.38
Donor risk index‡	2.8 (2.4-3.2)	2.7 (2.0-3.2)	3.0 (2.6-3.2)	0.86
Cause of death				0.19
Anoxia	5	4	2	
CVA	1	2	2	
Trauma	6	0	2	
Reason for discarding				0.17
Expected steatosis	5§	0	1	
DCD and age > 60 years	5	2	4	
High AST/ALT/GGT	1	3	0	
Other‖	1	1	1	
Preservation solution				0.39
HTK	3	0	0	
UW	9	6	6	

NOTE: Data are presented as median (IQR) and n.
*Time between withdrawal of life support until the aortic cold flush in the donor (DCD only).
†Time between the donor aortic cold flush until the start of NMP.
‡Donor risk index was calculated according to Braat et al.[25] (2012).
§Only 2 of these 5 livers turned out to have microscopic steatosis >30%.
‖RBC + FFP group, unknown; HBOC-201 + FFP group, DCD in combination with 26 minutes between cardiac arrest and aortic cold flush; HBOC-201 + gelofusine group, DCD age 57 in combination with out-of-hospital cardiac arrest.

group (Fig. 2B). The total flow (portal + arterial), however, remained stable in all 3 groups. This is in line with the fact that the portal vein and hepatic artery compete for blood flow (Fig. 2C). There were no significant differences in either portal or arterial flow between the 2 HBOC-201 groups. Furthermore, there were no significant differences in resistance between the 3 groups (data not shown). The higher flow rates,

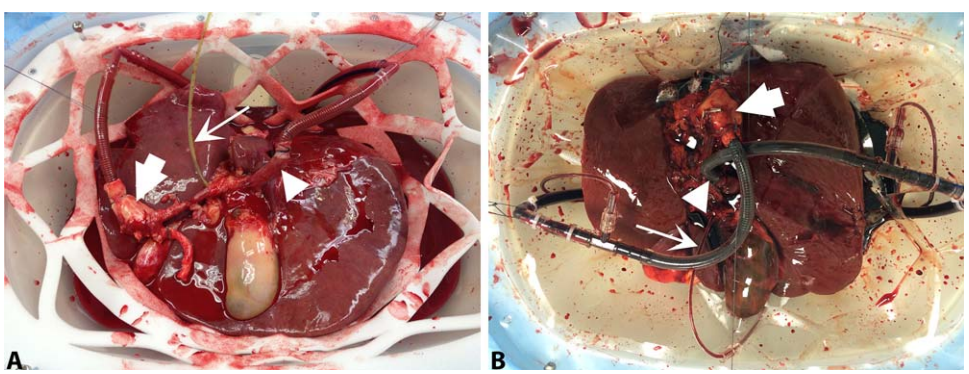

FIG. 1. Photographs of donor livers during NMP. (A) NMP using a perfusion fluid based on RBC + FFP. (B) NMP using a perfusion fluid based on HBOC-201 + gelofusine. The supratruncal hepatic artery (large arrow), portal vein (arrowhead), and bile duct (thin arrow) are cannulated. Note the darker color of the HBOC-201 perfusion solution.

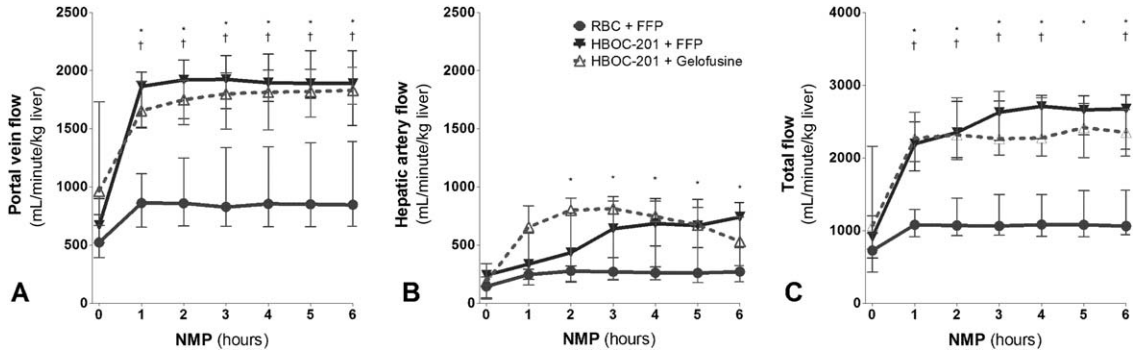

FIG. 2. Portal vein, hepatic artery, and total flow during NMP. (A) The portal vein flow during NMP was significantly higher at each time point after the first hour in both HBOC-201 groups compared with the RBC + FFP group. (B) The hepatic artery flow was significantly higher after the first 2 hours of NMP in the HBOC-201 + FFP group compared with the RBC + FFP group. (C) The total (portal vein + hepatic artery) flow during NMP remained significantly higher at nearly each time point after the first hour in both HBOC-201 groups compared with the RBC + FFP group. There were no significant differences in hepatic or portal vein flow between the 2 HBOC-201 groups. *Significant difference between RBC + FFP and HBOC-201 + FFP; †significant difference between RBC + FFP and HBOC-201 + gelofusine. Median and IQR values are shown.

despite equal pressures and resistance, in the HBOC-201 groups can be explained by the fact that the viscosity of the HBOC-201 perfusion fluid is lower than that of the RBC perfusion fluid.

ATP CONTENT IN LIVER PARENCHYMA

The median ATP content in liver parenchyma was higher in both HBOC-201 groups at each time point during NMP compared with the RBC + FFP group, reaching significance at 2 time points (Fig. 3A). At 6 hours NMP, the median (IQR) ATP content was 24 (14-51) μmol/g protein in the RBC + FFP group, 50 (35-59) μmol/g protein in the HBOC-201 + FFP group, and 79 (50-103) μmol/g protein in the HBOC-201 + gelofusine group. Furthermore, the ATP content in the HBOC-201 + gelofusine group was higher at each time point compared with the HBOC-201 + FFP group. However, this did not reach significance.

The normal value of ATP content in healthy livers using our assay is approximately 60 μmol/g protein, implying that physiological ATP levels were reached during NMP with HBOC-201.

PEAK OXYGEN EXTRACTION

The peak oxygen extraction was higher in the HBOC-201 perfused groups. However, this did not reach statistical significance. The median (IQR) peak oxygen extraction was 0.0014 (0.0010-0.0022) mL O_2/minute/g liver in the RBC + FFP group, 0.0023 (0.0020-0.0024) mL O_2/minute/g liver in the HBOC-201 + FFP group, and 0.0024 (0.0022-0.0033) mL O_2/minute/g liver in the HBOC-201 + gelofusine group.

BILE PRODUCTION

After the second hour of NMP, the cumulative bile production was significantly higher in the HBOC-201 groups compared with the RBC + FFP group, reaching a median (IQR) of 8.2 (6.1-17.7) mL/kg liver weight in the RBC + FFP group, 27.3 (26.6-31.2) mL/kg liver weight in the HBOC-201 + FFP group, and 29.0 (25.6-39.4) mL/kg liver weight in the HBOC-201 + gelofusine group ($P = 0.04$ and $P = 0.03$, respectively) at 6 hours of NMP (Fig. 3B). There were no significant differences between the 2 HBOC-201 groups.

BILIARY COMPOSITION

The biliary secretion of bicarbonate (marker for cholangiocyte function), bile salts, phospholipids, and bilirubin (markers for hepatic function) were not significantly different between the 3 groups (Fig. 3C).

LACTATE AND GLUCOSE IN THE PERFUSION FLUID

As shown in Fig. 4A, the lactate concentration during NMP declined more quickly in the HBOC-201 groups compared with the RBC + FFP group, with

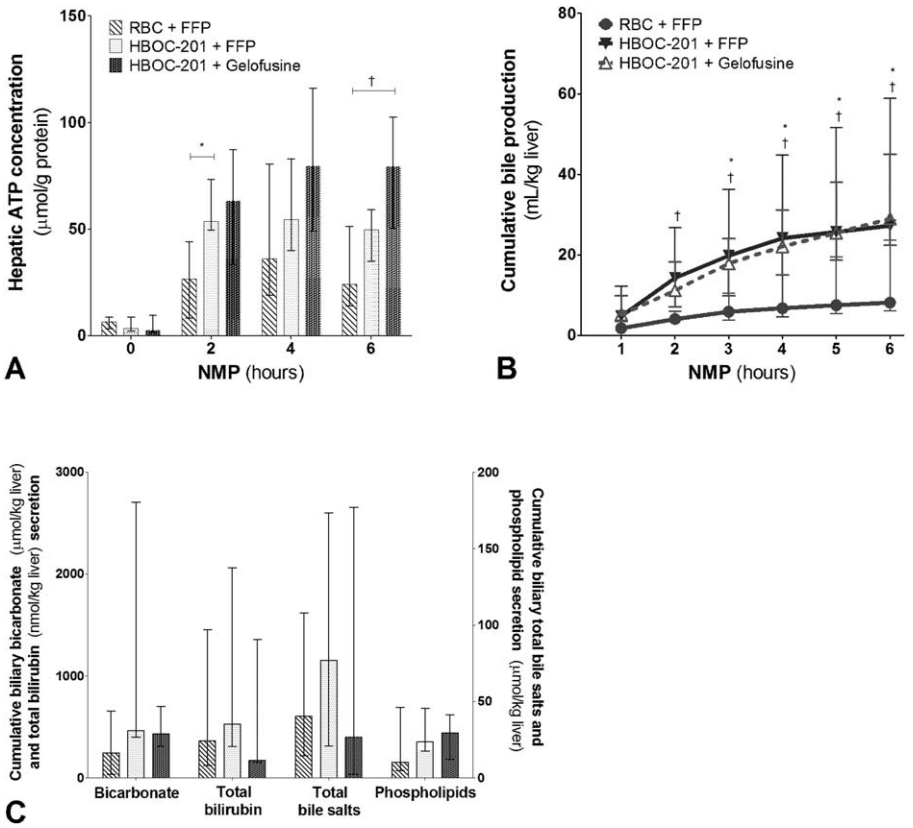

FIG. 3. ATP content in liver parenchyma, cumulative bile production, and cumulative biliary secretion of bicarbonate, bilirubin, bile salts, and phospholipids during 6 hours of NMP. (A) The hepatic ATP content was highest in the HBOC-201 + gelofusine group, followed by the HBOC-201 + FFP group, and lastly the RBC + FFP group at each time point. (B) Cumulative bile production during NMP was significantly higher at each time point in both HBOC-201 groups compared with the RBC + FFP group, after the second hour of NMP. (C) The cumulative secretion of bicarbonate, bilirubin, bile salts, and phospholipids in bile during 6 hours of NMP was not significantly different between the 3 study groups. *Significant difference between RBC + FFP and HBOC-201 + FFP; †Significant difference between RBC + FFP and HBOC-201 + gelofusine. Median and IQR values are shown.

an approximately 2-fold higher median lactate concentration at 2 hours NMP in the RBC + FFP group compared with the HBOC-201 perfused groups (median [IQR] of 6.7 [4.1-10.0] mmol/L in the RBC + FFP group, 3.6 [1.8-10.3] in the HBOC-201 + FFP and 2.6 [0.5-6.0] in the HBOC-201 + gelofusine group at 2 hours NMP). Although the differences did not reach significance, these data could suggest that the HBOC-201 perfused livers have a more adequate aerobic metabolism than the RBC + FFP perfused livers. The glucose concentration also seemed to normalize more rapidly in the HBOC-201 perfused livers compared with the RBC + FFP perfused livers (Fig. 4B), though this did not reach significance.

BUFFERING CAPACITY

The amount of bicarbonate that needed to be added to the perfusion system was not statistically different between the 3 groups. The median (IQR) volume of 8.4% sodium bicarbonate added during NMP was 20 (3-44) mL in the RBC + FFP group, 10 (10-10) mL in the HBOC-201 + FFP group, and 25 (10-40) mL in the HBOC-201 + gelofusine group.

ALT CONCENTRATION IN THE PERFUSATION FLUID

The concentration of ALT in perfusate during NMP was higher in the RBC + FFP group compared with

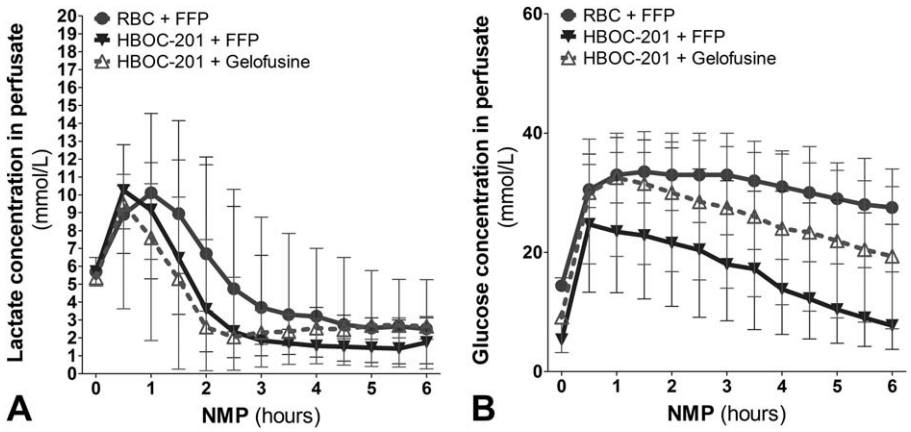

FIG. 4. Lactate and glucose concentrations in perfusion fluid during NMP. (A) The perfusate lactate concentration declined more quickly in the HBOC-201 groups compared with the RBC + FFP group, with an approximately 2-fold higher median lactate concentration at 2 hours NMP in the RBC + FFP group compared with the HBOC-201 perfused groups. There were, however, no significant differences in perfusate lactate concentrations between the 3 groups. (B) Although glucose concentration during NMP normalized more quickly in the HBOC-201 groups compared with the RBC + FFP group, this did not reach statistical significance. Median and IQR values are shown.

both HBOC-201 groups during NMP, nearly reaching significance at 4 hours of NMP (both $P = 0.07$) and at 6 hours of NMP between the RBC + FFP and HBOC-201 + FFP groups ($P = 0.06$; Fig. 5). The median (IQR) ALT concentration at 6 hours NMP was 5817 (2957-14,023) IU/L in the RBC + FFP group, 2550 (942-5562) IU/L in the HBOC-201 + FFP group, and 2418 (1968-3768) IU/L in the HBOC-201 + gelofusine group.

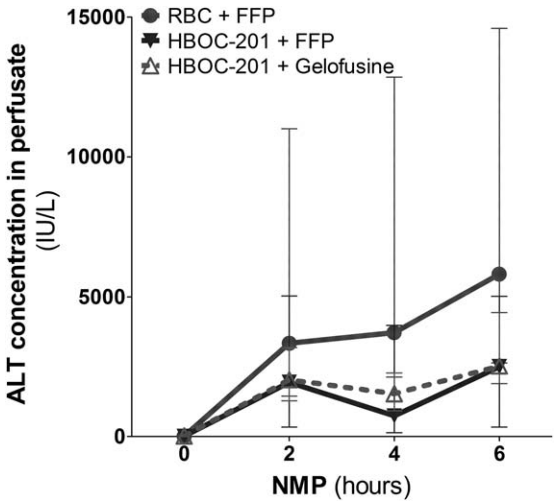

FIG. 5. ALT concentration in perfusion fluid during NMP. The ALT concentration is higher in the RBC + FFP group compared with both HBOC-201 groups during NMP, nearly reaching significance at 4 hours of NMP (both $P = 0.07$) and at 6 hours of NMP between the RBC + FFP and HBOC-201 + FFP groups ($P = 0.06$). Median and IQR values are shown.

HISTOLOGICAL ANALYSIS OF LIVER INJURY

The amount of histological injury of liver parenchyma was not significantly different between the 3 groups before or after NMP. The median (IQR) total Suzuki injury score was 2.0 (1.0-3.0) before and 3.0 (2.0-4.3) after NMP in the RBC + FFP group; 1.0 (1.0-1.0) before and 2.0 (2.0-2.0) after NMP in the HBOC-201 + FFP group; and 1.5 (1.0-2.0) before and 2.5 (1.3-4.5) after NMP in the HBOC-201 + gelofusine group. The main factor contributing to the total injury score was the degree of necrosis, with a median increase of 1.0 point in each group, as is shown in representative H & E–stained liver sections in Fig. 6.

Discussion

Machine perfusion is revolutionizing the field of organ transplantation and, as it is rapidly making its way into the clinic, is responsible for increases in the quality and quantity of liver transplants. Finding an alternative to using scarce, expensive, and logistically complex

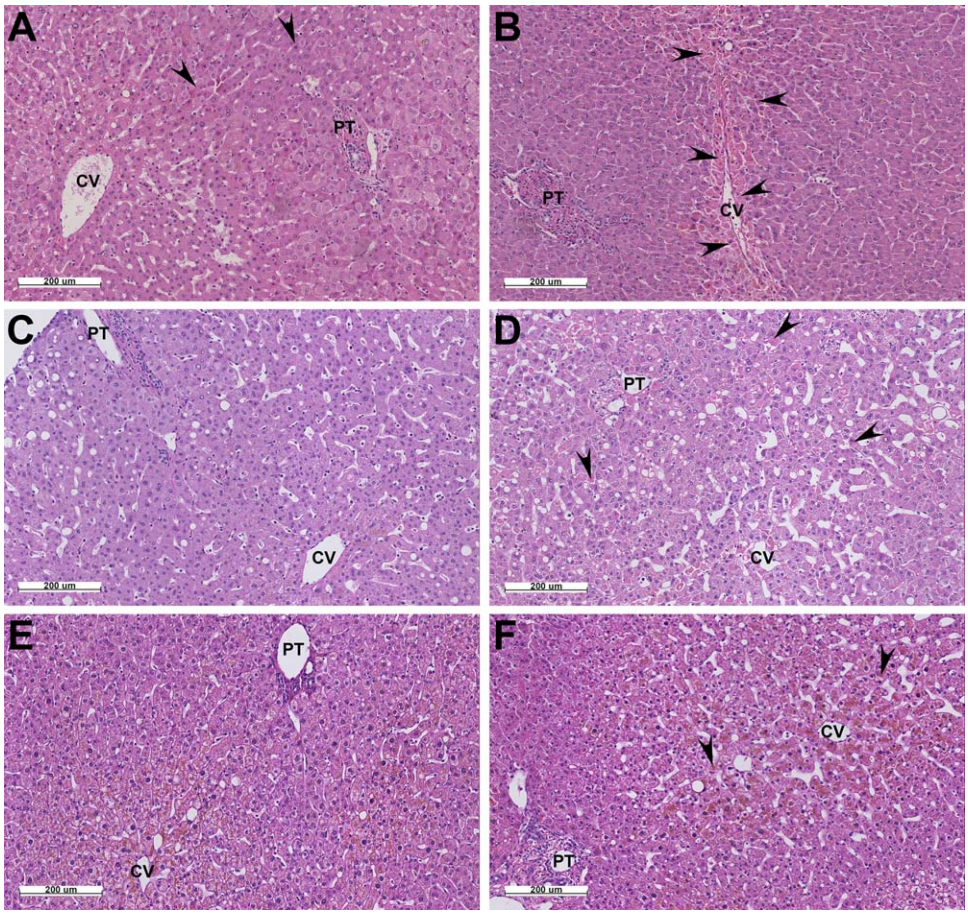

FIG. 6. Histological liver injury. Representative H & E stainings of liver biopsies prior to and after 6 hours NMP in each study group. There were no significant differences in the degree of liver injury between the 3 study groups before or after NMP. Arrowheads indicate necrotic cells. (A) Liver section of an RBC + FFP liver prior to NMP. (B) Liver section of the same RBC + FFP liver after 6 hours NMP. (C) Liver section of an HBOC-201 + FFP liver prior to NMP. (D) Liver section of the same HBOC-201 + FFP liver after 6 hours NMP. (E) Liver section of an HBOC-201 + gelofusine liver prior to NMP. (F) Liver section of the same HBOC-201 + gelofusine liver after 6 hours NMP.

human blood products for NMP is an important step in making NMP more widely applicable and accessible. In this study, we have shown the following:

1. NMP can be effectively performed without the use of human blood products by replacing RBCs with HBOC-201, a polymerized bovine Hb, and FFPs by gelofusine, a widely available colloid solution.
2. That perfusion with HBOC-201 is at least as effective as with RBCs.

Some end points in our study indicate that an HBOC-201–based perfusion fluid may even be superior, as shown by the increased recovery of hepatic ATP content, bile production, and improved glucose and lactate metabolism, as well as lower injury markers (ALT).

After having performed perfusions with RBCs and FFPs, we first replaced RBCs with HBOC-201 and kept FFPs, and subsequently also replaced FFPs with gelofusine. The ATP content in liver parenchyma was continuously higher in both HBOC-201 groups compared with the RBC + FFPs group. Previous research has shown that during static cold storage, hepatic ATP levels are depleted and that these levels can be restored during machine perfusion.[11,18] Livers with higher ATP levels show significantly better outcomes after transplantation, as has been validated in several animal and clinical studies,[19-21] holding great promise for future clinical perfusion with HBOC-201.

A possible explanation for the higher ATP content in liver parenchyma in the HBOC-201–perfused livers lies in the properties of HBOC-201. The HBOC-201 molecule has a lower affinity for oxygen than human Hb with a dissociation curve that is shifted to the right, causing HBOC-201 to give off oxygen more readily.[12] In addition, HBOC-201 solution is less viscous and contains free Hb, which is much smaller than erythrocytes, thereby allowing it to penetrate more deeply into the tissue.[12] The peak oxygen extraction

also appeared higher in the HBOC-201 perfused groups than in the RBC + FFP group, although this did not reach significance.

Bile production is an ATP-dependent process. In line with this, the cumulative bile production was also significantly higher in both HBOC-201 groups compared with the RBC + FFP group. According to the "viability criteria" described by Sutton et al., 7 out of 12 livers in the RBC + FFP group, 4 out of 5 in the HBOC-201 + FFP group, and 6 out of 6 livers in the HBOC-201 + gelofusine group would have potentially been transplantable.[4] Similarly, bile production is a transplantation criterion established in a clinically validated group of livers described by the Birmingham group.[22]

The amount of bicarbonate, bile salts, phospholipids, and bilirubin secreted into bile was, however, not significantly different between the 3 groups. Bile flow is mainly driven by the secretion of bile salts, but a significant part is also driven by bile salt–independent factors.[23] It could be possible that the secretion of other molecules, such as HBOC-201 or derivatives thereof, are hypercholeretic and thereby cause higher bile flow with an altered bile composition.

Both the lactate and glucose concentrations in perfusion fluid declined more rapidly in the HBOC-201–perfused livers compared with the RBC + FFP–perfused livers, though this did not reach significance. This may indicate that the HBOC-201–perfused livers were able to metabolize lactate and glucose at least equally well, or perhaps even better, as the RBC-perfused livers, reflecting proper restoration of aerobic metabolism.

The HBOC-201–perfused livers consistently showed significantly higher flows through the portal vein compared with the RBC + FFP–perfused livers. Flow through the hepatic artery was also consistently higher in the HBOC-201–perfused groups, reaching significance between the RBC + FFP and HBOC-201 + gelofusine groups. The increased flow is likely a result of the aforementioned lower viscosity of HBOC-201, compared with human blood and not caused by a difference in intrahepatic resistance between the groups. The size of HBOC-201 is 1×10^{-8} the size of an RBC. This makes an HBOC-201–based perfusion fluid much less viscous than an RBC-based fluid, resulting in higher flows at a given intrahepatic resistance.

Interestingly, the concentration of the liver injury marker, ALT, in perfusion fluid was consistently lower in the HBOC-201 groups compared with the RBC + FFP group, despite no histological differences in the amount of liver parenchyma injury. There were no significant differences in donor parameters between the 3 groups; in fact, the DRI was even slightly higher in the HBOC-201 + gelofusine group. The number of livers declined for transplantation due to expected steatosis was higher in the RBC + FFP group. However, this did not translate into a higher number of livers with microscopically confirmed clinically relevant steatosis and is therefore unlikely to have played a major role in the results of the present study.

Two other studies have reported the use of HBOC-201 in a machine perfusion setting. In the first study, subnormothermic (21°C) machine perfusion was compared with static cold storage using pig donor livers. The investigators noted significantly higher survival, superior graft function, and bile production after liver transplantation in the machine-perfused group compared with static cold-stored livers.[24] The second study compared NMP using RBCs with HBOC-201 and reported similar flows, lactate clearance, and histological findings. They also reported significantly higher oxygen extraction in the HBOC-201–perfused group.[10] The results of these studies are in line with the results of our study and indicate that machine perfusion with HBOC-201 is equal or even superior for the function and quality of liver grafts.

Limitations of this study are relatively small sample sizes in the HBOC-201 study groups, lack of transplant validation, and perfusions in the 3 study groups were performed consecutively rather than after randomization. We do not, however, believe that a potential learning curve could have played a role in the current study as our research team had extensively optimized its NMP technique prior to the perfusion of any of the included liver grafts, after which no changes in perfusion technique were made.

In conclusion, NMP can be performed without the use of RBCs and FFPs by replacing them with HBOC-201 and gelofusine, respectively. This reduces the costs and logistical complexity of NMP and avoids the use of scarce human blood products, which carry the potential to transmit blood-borne infections. The current study indicates that perfusing livers with HBOC-201 is at least similar to perfusion with human RBC. Some of the biomarkers of liver function and injury used in this study even suggest a possible superiority of an HBOC-based perfusion solution. Altogether, this suggests that NMP with HBOC-201 and gelofusine is a favorable method and opens a perspective for further optimization of machine perfusion techniques. Future studies are needed to assess the safety of performing NMP with HBOC-201 and gelofusine in a clinical transplantation setting. For this reason, a clinical trial has recently been

initiated at our center (Dutch Trial Register; www.trialregister.nl, number NTR5972).

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