OPEN

Brief Bubble and Intermittent Surface Oxygenation Is a Simple and Effective Alternative for Membrane Oxygenation During Hypothermic Machine Perfusion in Kidneys

Tom Darius, MD,[1,2] Martial Vergauwen, BSc,[2] Matteo Mueller, MD,[3] Selda Aydin, MD, PhD,[4] Philipp Dutkowski, MD,[4] Pierre Gianello, MD, PhD,[2] and Michel Mourad, MD, PhD[1,2]

Background. The aim of this feasibility study was to determine an alternative oxygenation technique (easy, cheap, and compatible with air transport) for membrane oxygenation during hypothermic machine perfusion (HMP) to improve early graft function in a porcine ischemia-reperfusion autotransplant model. **Methods.** The left kidney of a ±40-kg pig was exposed to 30 minutes of warm ischemia before 22 hours of preservation and autotransplantation. In the experimental group, oxygenation of the perfusate during HMP was obtained by direct bubble and 30-minute surface oxygenation at start and 1-hour end ischemic (n = 4) and outcome measures compared with historical HMP without active oxygenation (n = 6), 22-hour continuous oxygenated HMP ($HMPO_2$) (n = 8), and 2-hour $HMPO_2$ + 20-hour HMP (n = 6) using membrane oxygenation in both historical oxygenated control groups. **Results.** Brief bubble and 30-minute surface oxygenation of the perfusate effectively maintained supraphysiological P_{O_2} levels during the first 2 hours of HMP with improved flow dynamics. Although the metabolic profile of the perfusate (ie, flavin mononucleotide) and tissue (ie, glutamate, ATP) after brief O_2 uploading at the start of HMP seemed to be slightly better with the use of a membrane oxygenator compared with bubble and interrupted surface oxygenation, both techniques yielded similar, superior early graft function when compared with HMP without active oxygenation. **Conclusions.** The data presented in this feasibility study support the conclusion that brief bubble and intermittent surface oxygenation could be an alternative oxygenation technique during HMP to achieve an improved kidney graft function compared with HMP without active oxygenation and similar functional outcome when compared with membrane $HMPO_2$.

(*Transplantation Direct* 2020;6: e571; doi: 10.1097/TXD.0000000000001016. Published online 11 June, 2020.)

The protective effect of supplemental oxygenation (continuous or end ischemic) during hypothermic machine perfusion (HMP) has been demonstrated in preclinical studies.[1-6] In addition, aerobic mechanisms were better supported under high oxygen concentrations during kidney machine perfusion.[1,7-9] Our group demonstrated in an ischemia-reperfusion porcine kidney autotransplant model[6] that brief O_2 uploading by a membrane oxygenator at the start of HMP during 2 hours is an effective preservation strategy for protecting mitochondria.[10] Such a preservation strategy yielded superior early graft function compared with standard, non-oxygenated HMP (non-$HMPO_2$) or briefly administrating oxygen at the end of HMP preservation. Brief O_2 uploading resulted in similar early graft function as observed with continuous oxygenation during HMP.[10] These oxygenated

Received 22 April 2020. Revision received 27 April 2020.

Accepted 28 April 2020.

[1] *Department of Surgery, Surgery and Abdominal Transplant Unit, University Clinics Saint-Luc, Université Catholique de Louvain, Brussels, Belgium.*

[2] *Institut de Recherche Expérimentale et Clinique (IREC), Pôle de Chirurgie Expérimentale et Transplantation, Université Catholique de Louvain, Brussels, Belgium.*

[3] *Department of Surgery and Transplantation, Swiss Hepato-Pancreato-Biliary Center, University Hospital Zurich, Zurich, Switzerland.*

[4] *Department of Pathology, University Clinics Saint Luc, Université Catholique de Louvain, Brussels, Belgium.*

T.D., M.M., and P.G. designed research. T.D. and M.V, performed research. T.D., S.A., P.D., and M.M. analyzed data. T.D. wrote the manuscript. All authors reviewed the manuscript before submission.

The LifePort Kidney Transporter, the perfusion circuits, and the oxygenators were supplied by Organ Recovery Systems (Diegem, Belgium). T.D. received a grant from the "Fondation Saint Luc," the "Fondation Recherche Clinique," and Astellas Pharma BV Belgium and logistic support from Organ Recovery Systems. The other authors declare no conflicts of interest.

Correspondence: Tom Darius, MD, Département de chirurgie et services associés, Chirurgie et Transplantation Abdominale, Clinique Universitaire Saint Luc, Ave Hippocrate 10, 1200 Brussels, Belgium. (tom.darius@uclouvain.be).

Copyright © 2020 The Author(s). Transplantation Direct. Published by Wolters Kluwer Health, Inc.This is an open-access article distributed under the terms of the Creative Commons Attribution-Non Commercial-No Derivatives License 4.0 (CCBY-NC-ND), where it is permissible to download and share the work provided it is properly cited. The work cannot be changed in any way or used commercially without permission from the journal.

ISSN: 2373-8731

DOI: 10.1097/TXD.0000000000001016

preservation strategies could help to decrease the harmful effects of ischemia-reperfusion injury, especially in an era of frequent high-risk donor organ utilization, with associated morbidity.[11-14]

However, the optimal administration technique for oxygen during HMP has not yet been explored. Eliminating a membrane oxygenator as well as an oxygen source during organ transport could reduce the cost of $HMPO_2$ and facilitate air transport.

We therefore evaluate the use of direct bubble and intermittent surface oxygenation during continuous HMP as alternative method for brief O_2 uploading by a membrane oxygenator on early graft function in a pig kidney ischemia-reperfusion autotransplant model. We further measure renal flow, resistance, and concentration of Po_2 in the perfusate during machine perfusion and assess tubular function. Biomarkers and metabolic profiles are obtained from perfusate and renal biopsies.

MATERIALS AND METHODS

Animals

Female Belgian Landrace pigs of approximately 40 kg were used. Experiments were performed according to international guidelines, after approval by the local Ethical Committee for Animal Care. The animals were cared according to Belgian laws on experimental animal welfare.

Study Design

A standardized ischemia-reperfusion porcine kidney autotransplant model was used.[6]

Anesthetic and Surgical Protocol

Anesthetic and surgical protocols were described previously.[6,9,10] The left kidney was removed after 30 minutes of warm ischemia induced by vascular clamping and subsequently flushed with 200 mL of Kidney Perfusion Solution-1 (Organ Recovery Systems, Diegem, Belgium). In the experimental group, oxygenation of the perfusate during HMP was obtained by direct bubble and 30-minute surface oxygenation at the start and 1-hour end ischemic (22-hour HMP + interrupted surface oxygenation) (n = 4). Historical control groups were 22-hour standard HMP (n = 6) with no additional oxygenation, 22-hour continuous $HMPO_2$, and 2-hour $HMPO_2$ + 20-hour HMP (n = 6).[10] Perfusate oxygenation was obtained by membrane oxygenation in both historical oxygenated control groups. Following preservation, the left kidney was autotransplanted into the right renal bed after right nephrectomy.[6]

The surviving animals were euthanized on days 7 and 13 after transplantation by a lethal intravenous injection of T61 (Intervet International; MSD Animal Health, Boxmeer, The Netherlands) under general anesthesia, in the experimental and the historical control groups, respectively.

Machine Perfusion

The LifePort Kidney Transporter (Organ Recovery Systems, Diegem, Belgium) was used for all machine perfusion groups. The Kidney Perfusion Solution-1 was pumped through the kidney at a pressure of 30 mm Hg. The temperature of the kidney and the circulating perfusate was maintained at 2°C to 8°C.

Oxygenated Machine Perfusion

Carbogen (95% O_2/5% CO_2) at 500 mL/min was used for oxygen supplementation at 90% in the experimental group to have identical conditions when compared with the historical oxygenated groups. Two different oxygen administration techniques were compared.

Oxygenation via Membrane Oxygenator

The perfusate circuit of the LifePort Kidney Transporter was modified to include a membrane oxygenator (Dideco Kids D100 neonatal oxygenator; Sorin Group, Mirandola, Italy).[6,9,10]

Bubble and Surface Oxygenation

Oxygen uploading of the perfusate was obtained by bubble oxygenation in the reservoir during the circuit setup (wash phase) before connecting the kidney to the device. This was realized by insufflating the carbogen directly to perfusion solution in the reservoir via the submerged perforated O_2 tubing segment (**Figure S1, SDC**, http://links.lww.com/TXD/A255). Bubble oxygenation was switched to surface oxygenation at the start of renal perfusion. Surface oxygenation was only applied during the first 30 minutes of kidney perfusion to mimic the clinical setting of the perfused kidney leaving the donor hospital. Surface oxygenation was restarted at the end of the machine perfusion during 1 hour before autotransplantation to mimic the clinical setting of the perfused kidney arriving in the transplant center.

Outcome Measures

The primary outcome measure was early graft function (measured as daily serum creatinine). The secondary outcome measures were tubular function (measured by urinary fractional excretion of sodium and protein creatinine ratio), serum markers (lactate dehydrogenase [LDH] and aspartate transaminase [AST]), renal flow and resistance during HMP, Po_2 during machine perfusion, metabolic perfusate and tissue profile, and graft histology.

Samples and Analyses

Renal Flow and Resistance Measurement During HMP and Perfusate Analyses

The machine perfusion device continuously registers renal flow and resistance. Perfusate samples were taken at start, 15 minutes, 30 minutes, 60 minutes, 120 minutes, and at the end of machine perfusion. Perfusate Po_2 was continuously measured during perfusion at 4°C by a microfiber oxygen transmitter (Oxy-4 micro; Precision Sensing GmbH, Regensburg, Germany). In the historical 22-hour $HMPO_2$, perfusate samples and Po_2 measurement were only performed at start and end perfusion. Glucose, AST, and LDH analyses were performed by a dry chemistry analyzer Fuji Dri-Chem NX500 (Fujifilm Corporation, Tokyo, Japan). Perfusate samples were analyzed by fluorescence at 525 nm to detect the amount of flavin mononucleotide (FMN) release.

Blood and Urine Analysis

Venous blood samples and urine samples were collected daily during follow-up for biochemical analysis (eg, creatinine, urea, sodium, glucose, AST, LDH, and proteinuria) determined with methods of the clinical routine.

Tissue Sampling and Analysis

Kidney biopsies were taken at 5 time points. Wedge biopsies were taken before preservation, after 22 hours of preservation, and before euthanasia. Needle biopsies were taken after 5 and 45 minutes reperfusion. Biopsies were stored both fresh at −80°C pending metabolic analysis and also fixed in acidified formal alcohol, dehydrated, and embedded in paraffin wax for histology.

Metabolites (lactate, succinate, glutamate, AMP, ADP, ATP and oxidized and reduced nicotinamide adenine dinucleotide [oxidized nicotinamide adenine dinucleotide and reduced nicotinamide adenine dinucleotide, respectively]) were extracted from frozen kidney biopsies by methanol-chloroform extraction and analyzed by liquid chromatography coupled to electrospray ionization mass spectrometry using a method based on Coulier et al.[15] Metabolite quantification was performed as described by Veiga-da-Cunha et al.[16]

Sections of 4 µm were cut and stained with hematoxylin and eosin for evaluation using light microscopy. Sections were scored blindly assessing changes according to the histopathologic consensus criteria for preimplantation kidney biopsies[17] and an acute tubular injury score described by Hosgood et al[18] to assess changes in 4 morphological categories: tubular dilatation, tubular debris, vacuolation, and infiltration.

Statistical Methods

The study follows a parallel design with 4 active groups. No imputation was done so all analyses were based on the observed cases only.

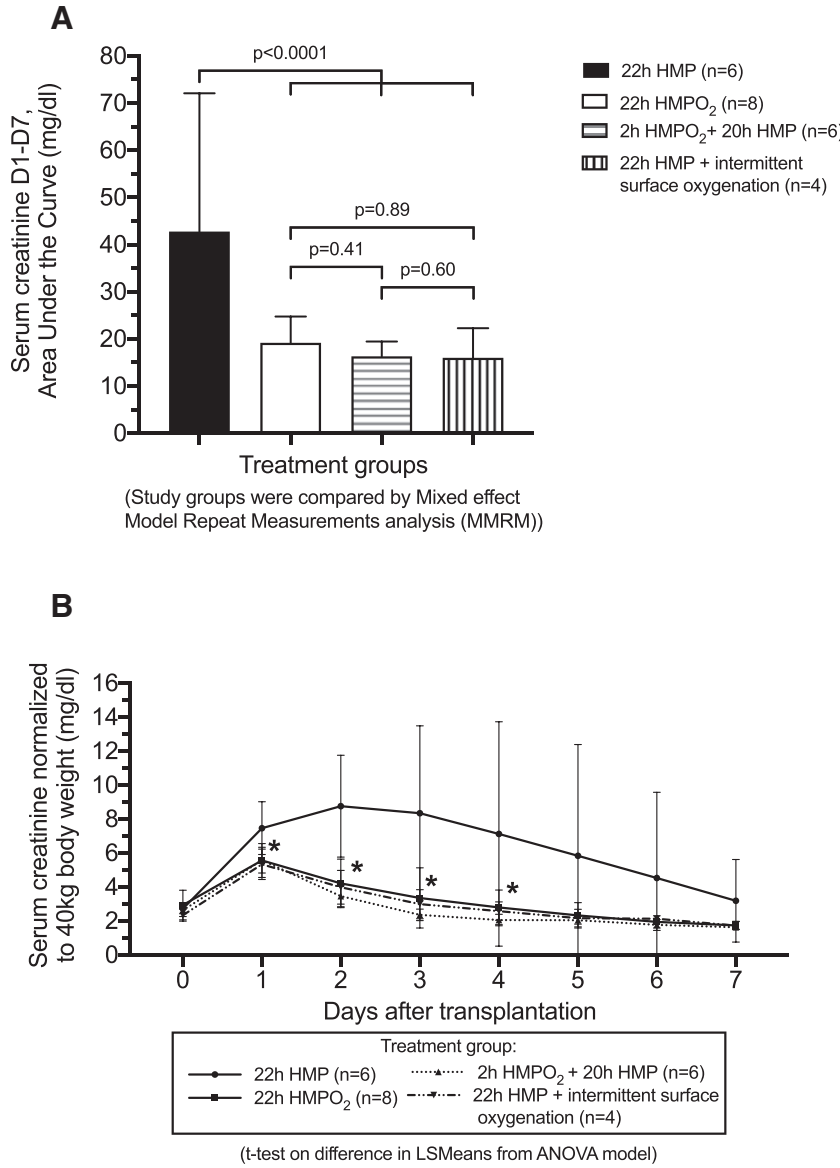

FIGURE 1. Evolution of serum creatinine. Area under the curve analysis of serum creatine from days 1 until 7 after transplantation according to the study group demonstrating that all oxygenated HMP groups, independent of oxygen administration technique and duration of O_2 supply during machine perfusion, are superior to standard, nonoxygenated HMP (A). Evolution of daily serum creatinine demonstrating superiority of all oxygenated HMP groups especially during the first 4 days after transplantation compared with the standard, nonoxygenated HMP groups (B). *Significance of $P < 0.05$ between the 22-h $HMPO_2$, the 2-h $HMPO_2$ + 20-h HMP, and the 22-h HMP + intermittent surface oxygenation group compared with standard 22-h HMP. HMP, hypothermic machine perfusion; $HMPO_2$, oxygenated hypothermic machine perfusion.

The primary outcome measure of early graft function was assessed through analysis of serum creatinine over time using generalized linear model techniques. Serum creatinine values were normalized to 40 kg animal body weight before use in the analysis to correct for differences in animal and organ size. Serum creatinine was analyzed using mixed model for repeated measurements (MMRM) with factors treatment, time point, and interaction term to test for overall difference in graft function over time between groups. Individual comparison of groups was conducted using contrast analyses within the main MMRM model testing the null hypothesis that the least squares mean difference between 2 groups was zero (no mean difference). For the contrast analysis, *P* values (based on *t* test of least squares means) were reported along with a mean (SD) graph where appropriate. All secondary end point variables were similarly modeled to the primary end point. In case of variables without repeated measurement (eg, area under the curve [AUC]), the MMRM model was reduced to an ANOVA model with factor treatment. As this was a preclinical exploratory trial, no formal sample size calculations were performed, and no correction for multiplicity was foreseen. All analyses were performed using SAS 9.4 (SAS Institute, Cary, NC) and Prism 8.2.0 (GraphPad Software, San Diego, CA).

RESULTS

Operative Data, Major Complications, and Animal Survival

Twenty-eight autotransplantations were performed, and 4 were excluded from the analysis due to early death or graft-interfering complications (sudden death at day 2 [n = 1], hydronephrosis of the right kidney diagnosed during the autotransplant procedure because of mechanical obstruction caused by the balloon of the bladder catheter [n = 1], respiratory arrest 30 min after extubation on day 0 [n = 1], arterial thrombosis at day 3 necessitating euthanasia [n = 1]). Operative data of the 24 autotransplantations are presented in **Table S1, SDC**, http://links.lww.com/TXD/A255. The use of historical control groups resulted in a slightly longer anastomosis time in 2 of these 3 groups compared with the experimental study group.

Graft Function

The AUC of the serum creatinine within 7 days after autotransplantation is shown in Figure 1A. All oxygenated study groups, independent of the O_2 administration technique and duration of O_2 supply during machine perfusion, were superior to HMP without active oxygenation. No differences were observed between all oxygenated study groups.

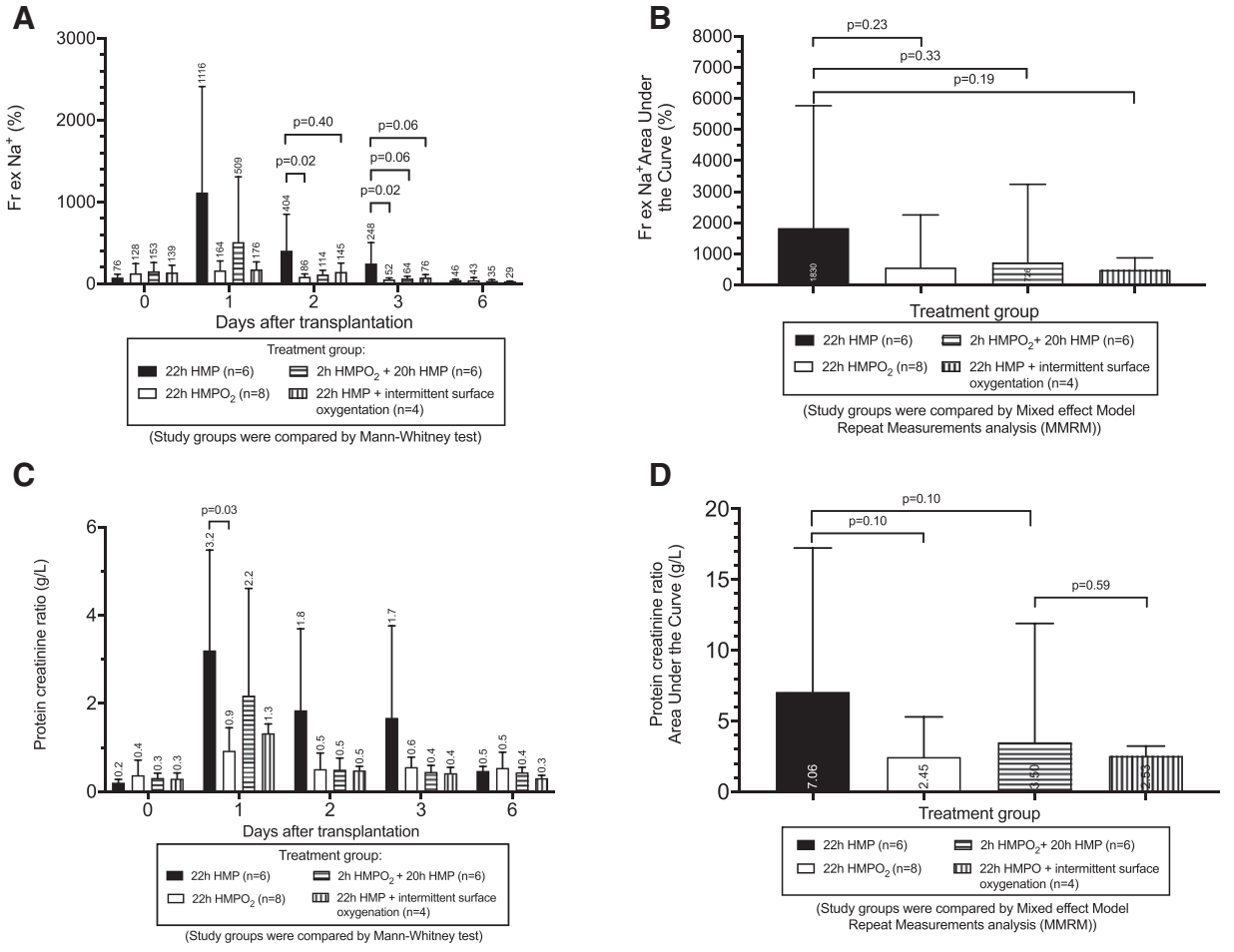

FIGURE 2. Evolution of tubular function. The daily evolution and the area under the curve analysis of the urinary fractional excretion of sodium and protein creatinine ratio according to the study groups (A and B and C and D, respectively). Fr ex Na^+, fractional excretion of sodium; HMP, hypothermic machine perfusion; $HMPO_2$, oxygenated hypothermic machine perfusion.

All oxygenated study groups demonstrated significantly lower serum creatinine ($P < 0.05$) compared with the standard, non-$HMPO_2$ group during the first 4 days after transplantation (Figure 1B). This early functional benefit was comparable in all oxygenated study groups.

Tubular reabsorption of sodium was transiently disturbed in all study groups early after transplantation but demonstrated a faster decrease to baseline values, especially during the first 3 postoperative days in all oxygenated machine groups compared with the standard non-$HMPO_2$ (Figure 2A). The AUC of the fractional excretion of sodium demonstrated no significant differences between the study groups (Figure 2B). Similar findings were observed for the urinary protein creatinine ratio (Figure 2C and D).

Evolution of Postoperative Serum AST and LDH

In all study groups, the peak of serum AST was observed at day 1 and dropped immediately afterward showing no differences between the study groups (Figure 3A). The peak of serum LDH in the study groups was observed at day 1 and dropped afterward to a normal range 6 days after transplantation, except for the 22-hour experimental group where serum LDH peaked again at day 6 to drop down the day after (Figure 3B). No significant differences in serum AST and LDH were observed between the study groups.

Impact of Oxygen Delivery Technique on Perfusate Po_2, Renal Flow, and Resistance During Machine Perfusion

During the perfusion circuit setup, perfusate oxygen concentrations of >400 and 500 mm Hg at 4°C were obtained by bubble oxygenation after 20 and 30 minutes, respectively (Figure 4A). Continuous Po_2 measurements during HMP are shown in Figure 4B. Thirty minutes of surface at the start was able to maintain supraphysiological Po_2 levels during the first 2 hours of HMP. The effect of 1-hour surface oxygenation of the perfusate at the end of HMP demonstrated a slight increase of perfusate Po_2. In contrast, prolonged oxygenation for 2 hours at the start via a membrane oxygenator kept perfusate Po_2 levels more stable when compared with shorter surface oxygenation.

Active oxygenation independent of the administration technique results in a significantly higher renal flow during machine perfusion compared with standard HMP, without additional oxygenation (**Figure S2A, SDC**, http://links.lww.com/TXD/A255). Accordingly, the inverse findings were observed for renal resistance (**Figure S2B, SDC**, http://links.lww.com/TXD/A255).

Perfusate Glucose, Lactate Dehydrogenase, and AST

No differences in perfusate glucose, LDH, and AST were detected at the end of machine perfusion between the study groups ($P = 0.16$; $P = 0.95$, $P = 0.15$, respectively) (**Figure S3, SDC**, http://links.lww.com/TXD/A255).

Perfusate FMN

FMN release during machine perfusion was significantly lower during the first 2 hours of machine perfusion in the group with 2-hour oxygenation at the start of HMP oxygenated by a membrane oxygenator compared with standard non-$HMPO_2$ and the bubble and intermittent surface oxygenation groups (Figure 5).

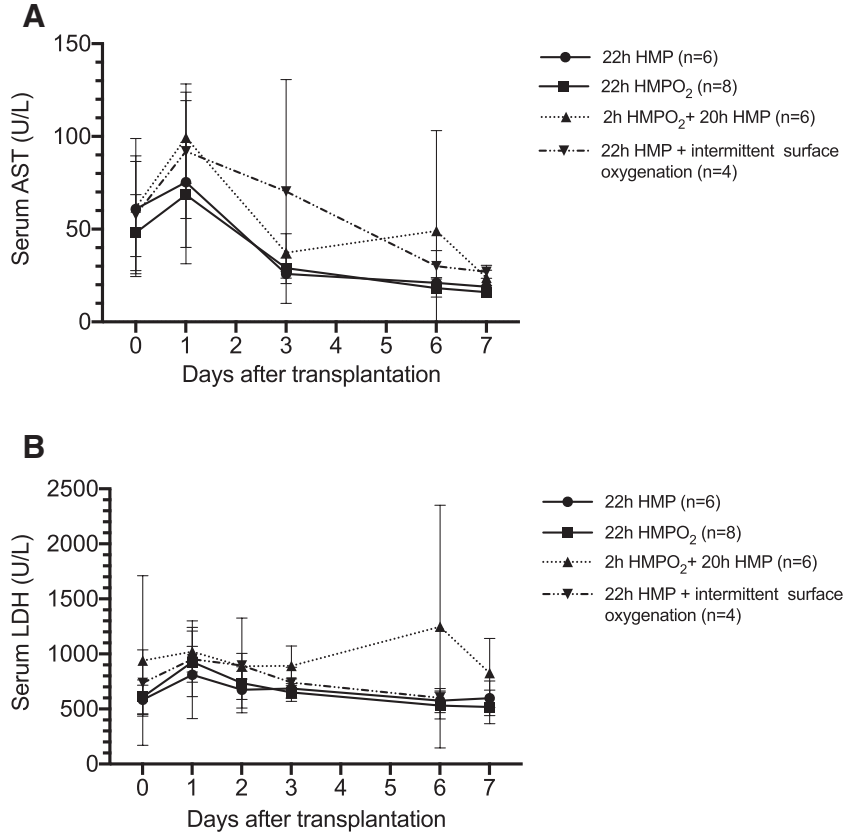

FIGURE 3. Evolution of serum AST and LDH. Evolution of serum AST (A) and LDH (B) after transplantation in all study groups. AST, aspartate aminotransaminase; HMP, hypothermic machine perfusion; $HMPO_2$, oxygenated hypothermic machine perfusion; LDH, lactate dehydrogenase.

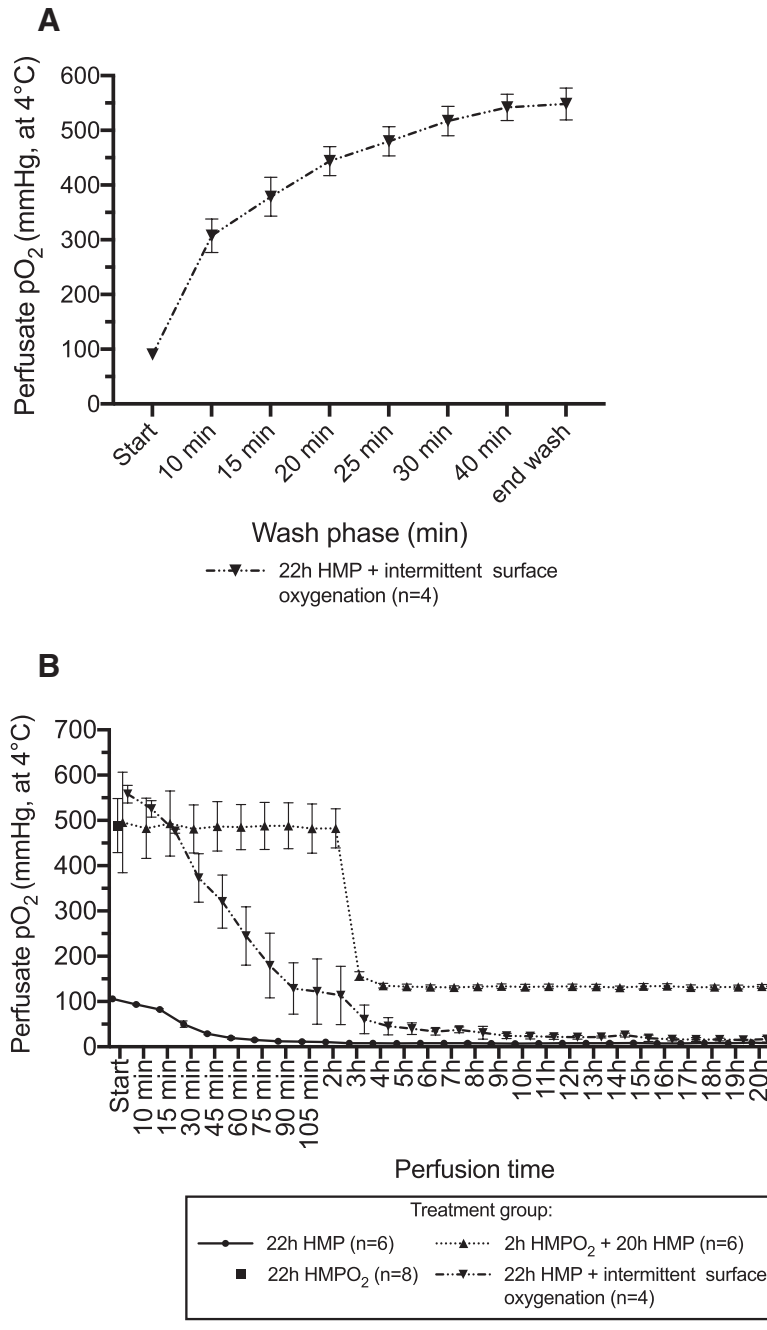

FIGURE 4. Perfusate Po_2 during machine perfusion. Direct perfusate oxygenation by bubble oxygenation during the perfusion circuit setup demonstrates Po_2 concentrations of >400 and 500 mm Hg at 4°C after 20 and 30 min, respectively (A). Continuous Po_2 measurements during kidney perfusion shows that 30 min of surface oxygenation at the start of HMP was able to maintain supraphysiological Po_2 levels during the first 2 h of machine perfusion and 1 h surface oxygenation at the end of HMP demonstrates a slight increase of perfusate Po_2 (B). In contrast, perfusate Po_2 levels using a membrane oxygenator are much more static during HMP. HMP, hypothermic machine perfusion; $HMPO_2$, oxygenated hypothermic machine perfusion.

Metabolic Evaluation of Preservation and Reperfusion Biopsies

No differences in lactate level were observed in biopsies obtained during preservation and after reperfusion comparing both brief oxygenation groups and the standard HMP group by liquid chromatography coupled to electrospray ionization mass spectrometry analysis (Figure 6A). Succinate and glutamate levels measured at the end of the cold preservation were significantly lower when oxygen was added for 2 hours by a membrane oxygenator when compared with bubble and intermittent surface oxygenation during HMP and standard non-$HMPO_2$ ($P = 0.02$ and $P = 0.04$ and $P = 0.04$ and $P = 0.01$, respectively, for succinate and glutamate) (Figure 6B and C). No differences in succinate and glutamate were observed between the HMP group without active oxygenation and the bubble and surface oxygenated group. The ATP levels, detected at the end of cold perfusion, were only significantly higher in kidneys receiving oxygen for 2 hours at start of HMP by membrane oxygenator compared with the 22-hour standard non-$HMPO_2$ group. Restoration of ATP

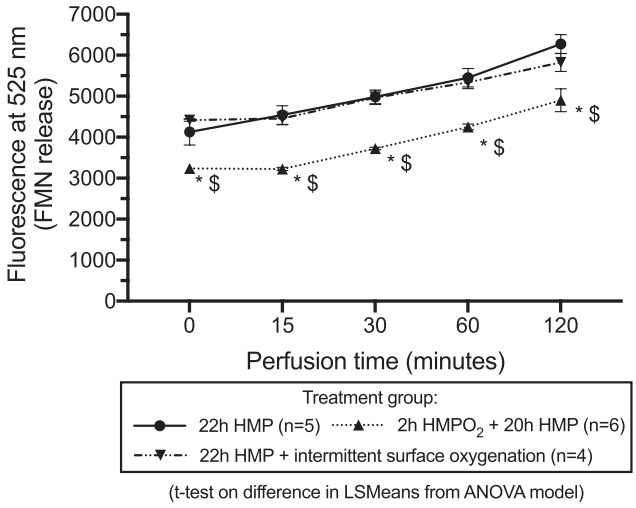

FIGURE 5. FMN detection in perfusate. FMN detection in the perfusate by fluorescence demonstrating significantly lower values during the first 2 h of machine perfusion in favor of the membrane oxygenator group compared with standard, nonoxygenated HMP or brief bubble and intermittent surface oxygenation. *Significance of $P < 0.05$ between standard 22-h HMP and 2-h $HMPO_2$ + 20-h HMP group. $^{\$}$Significance of $P < 0.05$ between 22-h HMP + intermittent surface oxygenation and 2-h $HMPO_2$ + 20-h HMP. FMN, flavin mononucleotide; HMP, hypothermic machine perfusion; $HMPO_2$, oxygenated hypothermic machine perfusion.

levels was observed in both brief $HMPO_2$ groups independent of oxygenation technique. No differences in ATP levels at the end of machine perfusion were observed between both oxygenated groups ($P = 0.25$). No significant differences were observed between both $HMPO_2$ groups and the standard HMP group regarding ADP, AMP, and reduced nicotinamide adenine dinucleotide levels at the end of cold perfusion (Figure 6E–H). At 5 and 45 minutes after in situ reperfusion, oxidized nicotinamide adenine dinucleotide was significantly higher in kidneys treated with oxygen initially independent of O_2 administration technique when compared with standard, non-$HMPO_2$ (Figure 6H).

Histology: Light Microscopy

Acute tubular injury at the end of cold preservation assessed by the Hosgood score demonstrated a significant difference between all groups ($P = 0.038$) compared with no observed difference using the Banff criteria (Figure 7). No differences were observed between all groups for all other Banff criteria (eg, interstitial fibrosis, tubular atrophy, interstitial inflammation, arterial intimal fibrosis, arteriolar hyalinosis, and glomerular thrombi). No significant differences were observed between all groups in the baseline and the biopsy taken before euthanasia (data not shown).

DISCUSSION

This study, using an ischemia-reperfusion porcine kidney autotransplant model, evaluates the impact of brief bubble and intermittent surface oxygenation on early graft recovery compared with membrane oxygenation during HMP. The aim was to determine an alternative oxygenation technique (easy, cheap, and compatible with air transport) for membrane oxygenation during HMP. All oxygenated study groups, independent of oxygen administration technique and duration of oxygenation, demonstrated superior early graft function when compared with 22-hour HMP without additional oxygenation. Brief bubble and surface oxygenation at the start of perfusion was effective to maintain supraphysiological Po_2 levels during the first 2 hours of HMP yielding improved flow dynamics. Both brief O_2 uploading oxygenation techniques demonstrated similar graft function compared with continuous membrane $HMPO_2$. Although the metabolic profile obtained in perfusate (ie, FMN) and kidney tissues (ie, glutamate and ATP) suggests that brief O_2 uploading for 2 hours at the start of HMP might be slightly better to protect mitochondria using a membrane oxygenator compared with bubble and intermittent surface oxygenation, both brief oxygenation techniques demonstrated similar graft function as seen in continuous $HMPO_2$. Bubble and intermittent surface oxygenation might be an easy alternative for membrane oxygenation eliminating the need for a membrane oxygenator and therefore reducing the total costs of oxygenated machine perfusion and making air transport easier.

A bubble oxygenator was incorporated in cardiopulmonary bypass technology during the first successful open heart surgery in 1953[19] and was popular until the end 1970s because of its low cost and applied for short cardiac surgery.[19,20] The idea of a protective membrane between blood and air to decrease the problem of blood trauma resulted in the development of membrane oxygenators, commercially available from 1960.[19,20] From the beginning of the 1980s, hollow fiber membrane oxygenators, which allowed even more optimal gas exchange, replaced bubble oxygenators as extracorporeal oxygenators in most clinical applications.[19,20] For this reason, a neonatal hollow fiber membrane oxygenator was used in our previous research projects to explore the effect of oxygen in different normothermic and HMP strategies.[6,9,10] The rationale for omitting the neonatal membrane oxygenator was based on its oxygen transfer capacity being significantly higher than what is needed for sustaining aerobic metabolism of a single kidney at ±4°C. The average O_2 transfer of 14.8 mL O_2/min of the Dideco kids D100 neonatal oxygenator[21] far exceeds the O_2 consumption of a 100–200 g kidney in the cold which is being estimated at 0.68 mL O_2/min.[22]

Today, machine preservation solutions are acellular without oxygen transporter and oxygen must reach the organ tissue by diffusion. To make organ oxygenation during HMP logistically easier, we had renewed interest in bubble and surface oxygenation to raise the dissolved perfusate O_2 concentration. This technique is based on 4 principles. First, bubble oxygenation is directly proportional to the oxygen volume and inversely proportional to the bubble size. This results in a highly effective O_2 transfer.[23] Although continuing bubble oxygenation of the perfusate during kidney perfusion would have been highly effective to maintain supraphysiological Po_2 levels, it was halted because it could cause massive foam formation due to protein degradation during kidney perfusion. Second, the solubility of oxygen increases as temperature decreases.[24] Third, according to Henry's law, oxygen will slowly diffuse across the surface of the perfusate from the gaseous compartment on the top of it. The amount of O_2 diffusing into the perfusate will be proportional to the percent (=partial pressure) of O_2 above its surface. And fourth, the efficiency of surface oxygenation was enhanced during the regularly scheduled wash cycles (every 10 min) during machine perfusion resulting in breaking the perfusate's surface layer. The

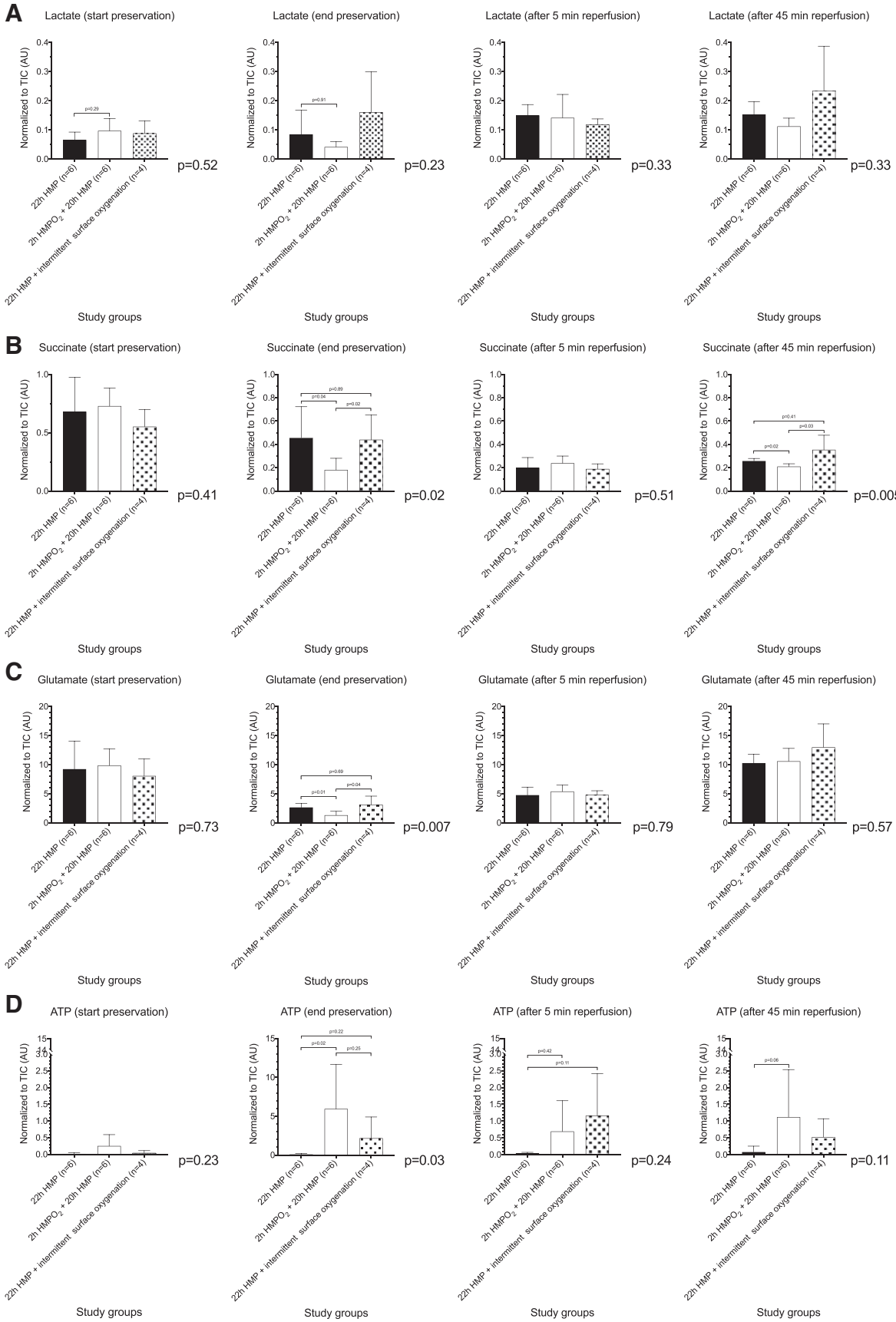

FIGURE 6. Metabolic tissue profile analysis. Metabolic profile analysis [lactate (A), succinate (B), glutamate (C), ATP (D), ADP (E), AMP (F), NADH (G), NAD+ (H)] on preservation and reperfusion biopsies by liquid chromatography electrospray ionization mass spectrometry demonstrates that active oxygenation during HMP realized by a membrane oxygenator seems better to support aerobic metabolism, in particular the central major metabolites succinate, glutamate, and the end product ATP when compared with bubble and intermittent surface oxygenation and standard, nonoxygenated HMP. Statistical analysis was performed using Kruskal-Wallis and Mann-Whitney tests. HMP, hypothermic machine perfusion; $HMPO_2$, oxygenated hypothermic machine perfusion.

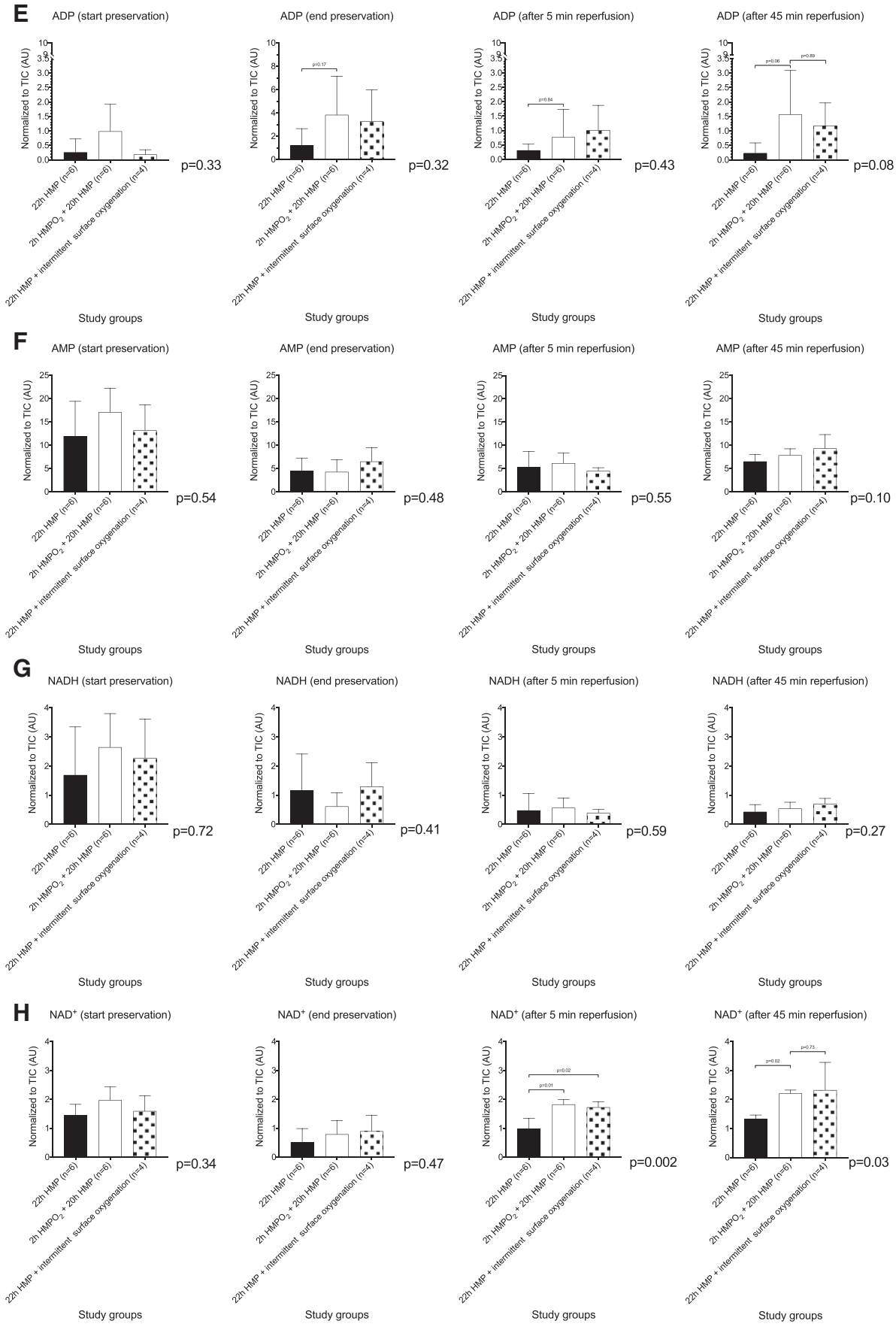

FIGURE 6. Continued.

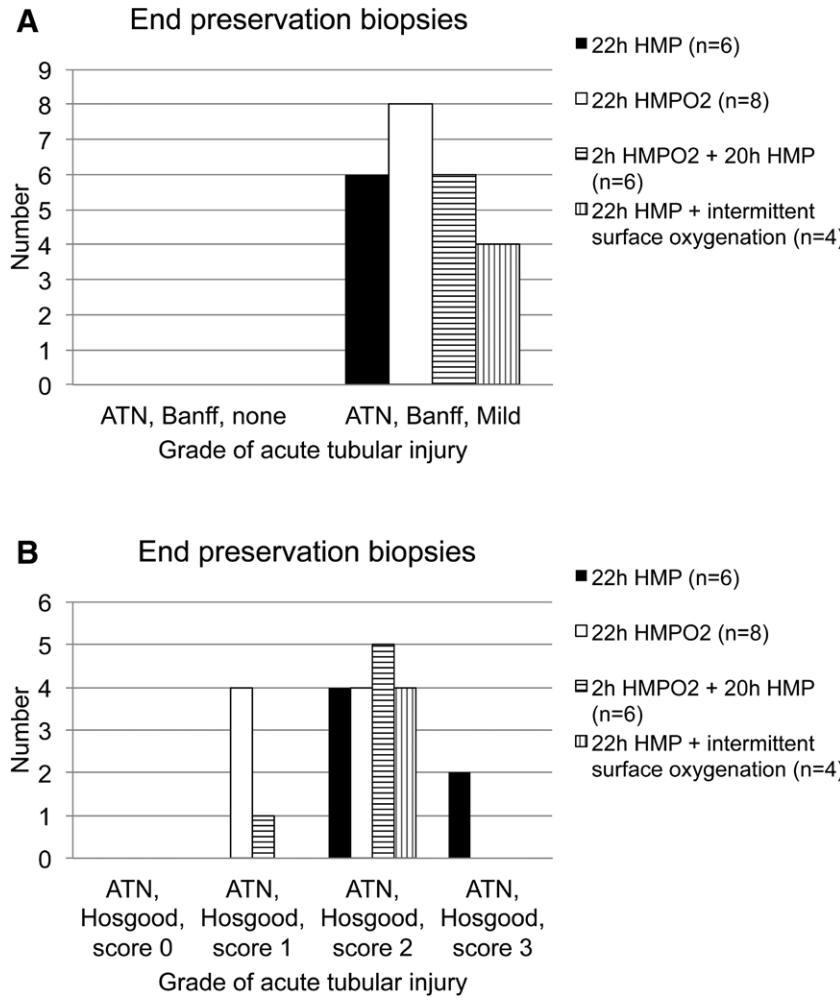

FIGURE 7. Acute tubular injury evaluation on end preservation biopsies. Acute tubular injury at the end of cold preservation assessed by the Banff criteria demonstrated no difference (A) compared with a significant difference between all groups (P = 0.038) using the Hosgood score (B). ATN, acute tubulus necrosis; HMP, hypothermic machine perfusion; $HMPO_2$, oxygenation hypothermic machine perfusion.

stream of perfusate exiting the port of the bypass line during this wash cycles causes an active mixing effect facilitating the entry of oxygen into the perfusate.

A paired international randomized controlled trial demonstrated a clinically relevant improvement in glomerular filtration rate at 12 months when considering the effect of continuous oxygenated perfusion on graft survival (mean {SE} 47.6 [1.9] versus 42.6 [2.0] mL/min/1.73 m^2, P = 0.035). The beneficial effect of oxygenated perfusion may be mediated via the reduction of organ rejection (14% versus 28%, P = 0.014), with a 50% reduction in acute rejection observed in the oxygenated arm. No difference was observed in early kidney function as measured by the occurrence of delayed graft function (38% in both groups).[25] In our study, bubble oxygenation in combination with 30-minute surface oxygenation at the start of HMP demonstrated to be highly effective to raise and maintain perfusate Po_2 concentrations above physiological level (Figure 4A–B). These observations are in line with the results of Lazeyras et al.[7] Although we could have opted to continue surface oxygenation for 2 hours or even throughout the entire perfusion time, we decided to stop active surface oxygenation after 30 minutes of perfusion. We based this decision on previous work showing that 2 hours of active oxygenation at the start of 22-hour HMP was efficient to correct O_2 and ATP debts.[10] The reason to stop surface oxygenation after 30 minutes was also driven by clinical considerations. It is most common that after 30 minutes of perfusion the machine-perfused kidney is about to leave the donor hospital, being sent to the recipient's transplant center. By demonstrating that active oxygenation would no longer be required once oxygen and ATP debts have been fully corrected and perfusate has been fully uploaded with oxygen, one would avoid membrane oxygenation and an external oxygen source during transport while exchanging oxygenated machine-perfused kidneys between centers.

Mitochondrial succinate accumulation originating from the citric acid cycle during ischemia is one of the main contributors for superoxide generation by reverse electron transport during subsequent in vivo reperfusion under normothermic conditions.[26-28] We previously described how oxygen during different $HMPO_2$ strategies resulted in more forward electron transport at the mitochondrial oxidative phosphorylation chain with superior mitochondrial protection at the end of preservation and superior subsequent in vivo early graft function compared with standard, non-$HMPO_2$.[10] In this current study, brief oxygenation at the start of HMP demonstrated that mitochondrial protection might be slightly superior using a membrane oxygenator for 2 hours when compared with brief bubble and intermittent surface oxygenation. This might

be explained by the differences in perfusate Po_2 levels during the first 2 hours of HMP. Membrane oxygenation results in a higher and more constant perfusate Po_2 level when compared with 30 minutes of surface oxygenation demonstrating a faster drop in Po_2 levels in the first 2 hours of machine perfusion. Therefore, forward electron transport at the mitochondrial oxidative phosphorylation chain might be lower during HMP using surface oxygenation compared with membrane oxygenation. In practice, the dissolved oxygen in the perfusate can be increased by 2 strategies. First, according to Henry's law, a higher Po_2 concentration at the surface of the perfusate will increase the perfusate oxygen concentration during HMP. This could be realized by an air pump insufflating continuously room air or carbogen during organ transport. Another way to realize this in clinical practice is to interrupt surface oxygenation only for organ transport and restart surface oxygenation from the moment the machine-perfused kidney arrived in the recipient center until transplantation. Second, a different approach is to further enhance O_2 delivery to increase flow during HMP. This could be achieved by adding vasodilators in the perfusate as demonstrated during liver preservation.[29-31]

We recognize the limitations inherent to this autotransplant model as mentioned previously.[6,9] The use of historical control groups might have introduced a relevant bias due to slightly shorter anastomosis time in the experimental study group compared with 2 of the 3 historical groups but was recommended by the ethical committee to reduce animal numbers and suffering. Because total preservation time was comparable between all study groups, we do not expect to have an important influence on outcome measures. The difference in duration of active oxygenation in the 2 intermittent oxygenated study groups must be taken into account interpreting the metabolic profile analysis. Another limitation is that oxygen consumption during HMP was not measured.

In conclusion, the data presented in this feasibility study support the conclusion that brief bubble and intermittent surface oxygenation could be an alternative oxygenation technique to achieve improved kidney graft function compared with HMP without active oxygenation and similar functional outcome when compared with membrane $HMPO_2$. This alternative oxygenation technique has the potential to decrease the total costs of $HMPO_2$ by eliminating the membrane oxygenator and making air transport easier. However, translation of these animal results into clinical practice must be done with caution. Further experiments need to determine the optimal duration of surface oxygenation during HMP to improve mitochondrial protection before its introduction into clinical trials.

ACKNOWLEDGMENTS

The authors are grateful to M. Vergauwen and G. Beaurin regarding the support during the pig experiments, to P. De Muylder regarding the scientific collaboration and logistic support, to S. Druart regarding the laboratory analysis, and to B. Jansen regarding the statistical analysis.

REFERENCES

1. Hoyer DP, Gallinat A, Swoboda S, et al. Influence of oxygen concentration during hypothermic machine perfusion on porcine kidneys from donation after circulatory death. *Transplantation.* 2014;98:944–950.
2. Gallinat A, Paul A, Efferz P, et al. Hypothermic reconditioning of porcine kidney grafts by short-term preimplantation machine perfusion. *Transplantation.* 2012;93:787–793.
3. Kron P, Schlegel A, de Rougemont O, et al. Short, cool, and well oxygenated - HOPE for kidney transplantation in a rodent model. *Ann Surg.* 2016;264:815–822.
4. Thuillier R, Allain G, Celhay O, et al. Benefits of active oxygenation during hypothermic machine perfusion of kidneys in a preclinical model of deceased after cardiac death donors. *J Surg Res.* 2013;184:1174–1181.
5. Koetting M, Frotscher C, Minor T. Hypothermic reconditioning after cold storage improves postischemic graft function in isolated porcine kidneys. *Transpl Int.* 2010;23:538–542.
6. Darius T, Gianello P, Vergauwen M, et al. The effect on early renal function of various dynamic preservation strategies in a preclinical pig ischemia-reperfusion autotransplant model. *Am J Transplant.* 2019;19:752–762.
7. Lazeyras F, Buhler L, Vallee JP, et al. Detection of ATP by "in line" 31P magnetic resonance spectroscopy during oxygenated hypothermic pulsatile perfusion of pigs' kidneys. *MAGMA.* 2012;25:391–399.
8. Patel K, Smith TB, Neil DAH, et al. The effects of oxygenation on ex vivo kidneys undergoing hypothermic machine perfusion. *Transplantation.* 2019;103:314–322.
9. Darius T, Vergauwen M, Smith TB, et al. Influence of different partial pressures of oxygen during continuous hypothermic machine perfusion in a pig kidney ischemia-reperfusion autotransplant model. *Transplantation.* 2020;104:731–743.
10. Darius T, Vergauwen M, Smith TB, et al. Brief O2 uploading during continuous hypothermic machine perfusion is simple yet effective oxygenation method to improve initial kidney function in a porcine autotransplant model. *Am J Transplant*. 2020. doi: 10.1111/ajt.15800. [Epub ahead of print].
11. Hamed MO, Chen Y, Pasea L, et al. Early graft loss after kidney transplantation: risk factors and consequences. *Am J Transplant.* 2015;15:1632–1643.
12. Lim WH, Johnson DW, Teixeira-Pinto A, et al. Association between duration of delayed graft function, acute rejection, and allograft outcome after deceased donor kidney transplantation. *Transplantation.* 2019;103:412–419.
13. Gill J, Dong J, Rose C, et al. The risk of allograft failure and the survival benefit of kidney transplantation are complicated by delayed graft function. *Kidney Int.* 2016;89:1331–1336.
14. Schröppel B, Legendre C. Delayed kidney graft function: from mechanism to translation. *Kidney Int.* 2014;86:251–258.
15. Coulier L, Bas R, Jespersen S, et al. Simultaneous quantitative analysis of metabolites using ion-pair liquid chromatography-electrospray ionization mass spectrometry. *Anal Chem.* 2006;78:6573–6582.
16. Veiga-da-Cunha M, Chevalier N, Stephenne X, et al. Failure to eliminate a phosphorylated glucose analog leads to neutropenia in patients with G6PT and G6PC3 deficiency. *Proc Natl Acad Sci U S A*. 2019;116:1241–1250.
17. Liapis H, Gaut JP, Klein C, et al; Banff Working Group. Banff histopathological consensus criteria for preimplantation kidney biopsies. *Am J Transplant.* 2017;17:140–150.
18. Hosgood SA, Barlow AD, Dormer J, et al. The use of ex-vivo normothermic perfusion for the resuscitation and assessment of human kidneys discarded because of inadequate in situ perfusion. *J Transl Med.* 2015;13:329.
19. Cohn LH. Fifty years of open-heart surgery. *Circulation.* 2003;107:2168–2170.
20. Lim MW. The history of extracorporeal oxygenators. *Anaesthesia.* 2006;61:984–995.
21. Lawson DS, Smigla GR, McRobb CM, et al. A clinical evaluation of the Dideco Kids D100 neonatal oxygenator. *Perfusion.* 2008;23:39–42.
22. Epstein FH. Oxygen and renal metabolism. *Kidney Int.* 1997;51:381–385.
23. High KSM, Bashein G, Kurusz M. Principles of oxygenator function: gas exchange, heat transfer, and blood-artificial surface interaction. In: Gravlee GP, Davis RF, Utley JR, eds. *Cardiopulmonary Bypass, Principles and Practice*. Baltimore, MD: Williams & Wilkins; 1993:29–54.
24. Hosgood SA, Nicholson ML. Oxygen supplementation supports energy production during hypothermic machine perfusion in a model of donation after circulatory death donors. *Transplantation.* 2019;103:1980–1981.
25. Heller KM, Apel H, Wach S. Full oral. *Transpl Int*. 2019;32:7–126.

26. Robb EL, Hall AR, Prime TA, et al. Control of mitochondrial superoxide production by reverse electron transport at complex I. *J Biol Chem.* 2018;293:9869–9879.
27. Chouchani ET, Pell VR, Gaude E, et al. Ischaemic accumulation of succinate controls reperfusion injury through mitochondrial ROS. *Nature.* 2014;515:431–435.
28. Chouchani ET, Pell VR, James AM, et al. A unifying mechanism for mitochondrial superoxide production during ischemia-reperfusion injury. *Cell Metab*. 2016;23:254–263.
29. Guarrera JV, Henry SD, Chen SW, et al. Hypothermic machine preservation attenuates ischemia/reperfusion markers after liver transplantation: preliminary results. *J Surg Res.* 2011;167:e365–e373.
30. Guarrera JV, Henry SD, Samstein B, et al. Hypothermic machine preservation facilitates successful transplantation of "orphan" extended criteria donor livers. *Am J Transplant.* 2015;15:161–169.
31. Guarrera JV, Polyak M, O'Mar Arrington B, et al. Pulsatile machine perfusion with Vasosol solution improves early graft function after cadaveric renal transplantation. *Transplantation.* 2004;77:1264–1268.