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Chapter 9

Impact of red blood cells on function and metabolism of porcine deceased donor kidneys during normothermic machine perfusion

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**Abstract**

Normothermic machine perfusion (NMP) protocols using blood-based solutions are increasingly used for assessing kidney function prior to transplantation. This procedure is limited by blood availability and warrants the search for alternatives. We compared a blood-based solution to a serum-like preservation solution (Aqix) enriched with colloids, with and without red blood cells (RBCs). Porcine kidneys retrieved from an abattoir were subjected to 30 min of warm ischemia, followed by 3h of hypothermic oxygenated machine perfusion at 4°C. Subsequently, kidneys (n = 6 per group) were evaluated with NMP for 4h with five different solutions: Diluted blood, Aqix with bovine serum albumin (BSA) +/- RBCs; or Aqix with Dextran 40 +/- RBCs. Throughout NMP, markers of renal function and tubular metabolism were favorable in RBCs groups. The addition of RBCs resulted in 4- to 6-fold higher oxygen consumption rates. Controls had significantly higher ATP levels post-NMP, exhibited decreased production of oxidative stress markers, and had the highest creatinine clearance. In conclusion, this study shows that the addition of RBCs during NMP reduced renal injury, improved function, and increased renal metabolism. Although the RBC-BSA-supplemented Aqix solution was also able to support metabolism and renal function, a blood-based perfusion solution remains superior.

**Introduction**

Kidney transplantation is currently the standard treatment for patients suffering from end-stage renal disease. Unfortunately, renal transplantation is drastically limited due to the shortage of available and suitable donor kidneys. To overcome this persistent shortage, most transplant centers nowadays accept older and higher risk donor kidneys from donation after circulatory death (DCD) donors and extended criteria donors (ECD). However, this often comes with a consequence for graft function, and survival since many of these kidneys are of inferior quality compared to standard criteria donor kidneys.

Ex vivo normothermic machine perfusion (NMP) is a technique to perfuse kidneys at 37°C with an oxygenated blood-based perfusion solution. NMP can be used to evaluate organ quality by assessing kidney function and other parameters prior to transplantation. NMP appears especially relevant in donor kidneys with questionable quality, such as kidneys from DCD and ECD donors. In the past years, the NMP technique has been further developed, and the first clinical results have been published, providing evidence that a brief period of NMP after static cold storage preservation allows successful transplantation of kidneys that were initially declined.

Currently, most perfusion strategies use blood-based solutions with red blood cells (RBCs) as oxygen carriers for clinical NMP of donor organs. RBC-based solutions provide a more ‘physiological’ environment than artificial (or “purely synthetic”) solutions. However, the usage of blood products for NMP also comes with some serious drawbacks. Amongst these, hemolysis is associated with the development of oxidative stress and acute kidney injury, endothelial and platelet activation, and adverse immune responses. In addition, blood products are precious, expensive, and have a limited shelf-life. Therefore, identifying alternatives to RBC-containing perfusion solutions for NMP is desirable, particularly if NMP is to become the new standard of care for assessing the viability of higher-risk donor organs.

AQIX® RS-I (Aqix) is a non-phosphate buffered solution that aims to resemble the human interstitial fluid. It has been used to store biological tissue samples and could serve as an alternative to a blood-based perfusate. The osmolarity (286 mOsmol/L) is comparable to human serum, and its ionic conductivity (12.6 mS/cm) results in an iso-osmotic solution. Under both hypothermic and normothermic conditions, it has been shown that static storage of porcine kidneys using Aqix, maintains the acid-base homeostasis and leads to improved recovery of cellular function. According to Starling principle, the colloid osmotic pressure (COP) is essential for maintaining the...
fluent balance between the intravascular and extravascular compartments. Furthermore, the Starling principle states that fluid movements between blood and tissues are determined by differences in hydrostatic and colloid oncotic pressures between micro-vessels and surrounding interstitial fluid. Reduced COP leads to interstitial fluid overload and edema formation, which increases the diffusion distance for oxygen and nutrients, potentially compromising cellular metabolism. Therefore, we decided to supplement AQIX® RS-I solution with either bovine serum albumin (BSA) or dextran 40 (Dex). Albumin was chosen since it is the most abundant plasma protein and dextran 40 because it has been shown to be beneficial during hypothermic machine perfusion (HMP) of porcine kidneys. As a simple aqueous solution, Aqix will only carry oxygen in a dissolved state. Thus, the question remains whether dissolved oxygen will allow for an oxidative metabolic state during NMP conditions.

To determine whether AQIX® RS-I combined with a colloid and RBC could serve as a preferred substitute to a blood-based perfusion solution during ex-situ NMP of porcine kidneys, we have studied the effect of different perfusion solutions on renal metabolism, renal function, and injury.

Materials and methods

Animal model
Porcine kidneys were retrieved from the local abattoir after a highly standardized slaughtering process simulating DCD conditions, as previously described. Briefly, pigs were sedated by an electrical shock and immediately exsanguinated. The blood was collected in a beaker containing 25,000 IU of heparin (LEO Pharma A/S, Ballerup, Denmark). No approval from the animal ethics committee was required because organs of animals slaughtered for meat consumption were used and were thus considered as ‘waste material’.

Experimental design
To induce ischemic injury, kidneys were subjected to 30 minutes of warm ischemic time (WIT) starting after exsanguination and ending when the cold flush has started. All kidneys were preserved with oxygenated HMP for three hours. Afterwards, the kidneys were perfused in the ex vivo NMP set-up for four hours with AQIX® RS-I supplemented with a colloid (BSA or Dex), supplemented with or without the addition of RBCs. Kidneys that were perfused with an autologous blood-based perfusion solution served as controls. Six kidneys were included in each experimental group. For histology assessment, additional kidney tissue samples were taken from kidneys (n=5) that were derived immediately after procurement (WIT of approximately 15 min) and that did not undergo HMP and subsequent NMP.

Kidney preservation
After warm ischemia, all kidneys were flushed with 180 ml of saline solution at 4°C (Baxter BV, Utrecht, The Netherlands) without the addition of heparin. Immediately after the initial flush-out, a cortical needle biopsy (Invivo, Best, The Netherlands) was taken from the upper pole of the kidney and was stored in a solution containing 0.372 g EDTA (0.744 g/L) in 130 ml H2O and NaOH (pH 10.9) + 370 ml 96% ethanol. Thereafter, kidneys were prepared for HMP by cannulating the renal artery and were connected to a Kidney Assist Transporter device (Organ Assist, Groningen, The Netherlands). HMP was performed for three hours at a mean pressure set at 25 mmHg at 4°C using 100% oxygenated (100 ml/min) University of Wisconsin machine perfusion solution (Belzer MPS, Bridge to Life Ltd., London, United Kingdom).

Ex vivo normothermic machine perfusion
After the preservation period, organ quality was assessed during 240 minutes of ex vivo NMP with the different perfusion solutions. Control kidneys were perfused with an autologous blood-based solution. The experimental groups were perfused with Aqix (AQIX® RS-I, AQIX Ltd, London, UK) in combination with a colloid, i.e., either bovine serum albumin (Sigma-Aldrich, St Louis, USA) or Dextran 40 (Sigma-Aldrich, St Louis, USA), supplemented with or without autologous porcine red blood cells that had been washed in phosphate-buffered saline. Before starting NMP, a second biopsy was taken, and the kidney was weighed. The kidneys were placed in an organ chamber and perfused for four hours at 37°C using a pressure-controlled pulsatile pump set at a mean pressure of 75 mmHg (Kidney Assist Transport, Organ Assist, Groningen, The Netherlands). A carbogen mixture (95% O2, 5% CO2) with a fixed flow of 500 ml/min was used to oxygenate the perfusate. After 30 minutes of NMP, another biopsy was taken and snap frozen. At the end of perfusion, the kidneys were weighed, and a cortical biopsy was snap-frozen in liquid nitrogen. Perfusate and urine samples were collected 15, 60, 120, 180, and 240 minutes after the start of NMP. Arterial and venous blood gas analyses were performed at the same time points using an ABL90 FLEX (Radiometer, Brønshøj, Denmark).

Evaluation of renal metabolism
To assess transport-related renal metabolism, renal oxygen consumption, fractional sodium excretion, and total sodium reabsorption were calculated with the equations presented in Table 2. Creatinine and sodium concentrations were measured in both perfusate and urine samples according to routine clinical procedures at the clinical chemistry lab of the University Medical Center Groningen (UMCG). ATP levels were measured in the biopsies collected after warm ischemia, after HMP, after 30 minutes of NMP, and at the end of NMP (NMP240). ATP was analyzed as previously described, and was normalized to the amount of protein being present in that biopsy.
Evaluation of renal function, urine production, and edema formation

The flow rate during HMP was recorded every five minutes up to 30 minutes, and thereafter every 30 minutes. The flow rate during NMP was recorded every 15 minutes. Creatinine clearance was calculated according to the equation given in Table 2. Urine production was measured every 15 minutes during perfusion. All parameters were normalized to kidney weight measured before NMP. The variation in renal weight was calculated by subtracting the weight measured before NMP to the weight measured after NMP and was expressed as relative difference (%).

Kidney injury, oxidative stress markers and histological assessment

Lactate dehydrogenase (LDH) and aspartate aminotransferase (ASAT) were analyzed by the clinical chemistry lab of the UMCG according to standard procedures. Urinary N-acetyl-beta-D-glucosaminidase (uNAG) was determined as previously described26,27. Reactive oxygen species (ROS) induced injury was assessed by measuring thiobarbituric acid-reactive substances (TBARS) in both urine and perfusate28. TBARS production was calculated at every sampling time point (Table 2).

Kidney biopsies taken after NMP were fixed by immersion in 4% buffered paraformaldehyde, embedded in paraffin and sections were cut at 4 µm. These were subsequently stained with Periodic Acid-Schiff (PAS) and was scored blindly by a pathologist. Glomerular and vascular damage was absent in all groups. In the tubular interstitium varying degree of tubular damage was observed ranging from loss of the tubular brush border integrity to loss of adherence of tubular cells to the tubular basement membrane. Damage was scored when excess cell and/or tubular cell debris was present within the luminal tubular area since this indicates damage to that particular segment. For that purpose, percentages of tubulo-interstitial damage were given to each section: 0%, <1%, 1-10%, 10-25%, 25-50%, 50-75% or 75-100%.

Statistical analysis

All data are expressed as mean with standard error of the mean (SEM), except histology, which are reported as median with interquartile range. The area under the curve (AUC) was calculated using the trapezoid rule for all parameters with multiple measurements over time. For total sodium reabsorption rate, the function subtracts negative areas. The AUC of LDH in the control group was corrected for baseline LDH level. Differences across all experimental groups were assessed by ANOVA. Subsequently, Dunnett’s post-hoc test was used to compare the blood group (reference) with the other experimental groups. As an exploratory analysis, all other pairwise comparisons were made with unpaired Student’s t-tests of which the P-values were corrected using the Benjamini-Yekutieli procedure to control the false discovery rate (set at 5%)29. All statistical tests were two-tailed, and a \( p \leq 0.05 \) was considered statistically significant. Data were analyzed with R version 3.6.1 (R Foundation for Statistical Computing, Vienna, Austria).

Table 1 Composition of perfusion solution per experimental group.

| Group                | Priming                        | Additives                                                                 |
|----------------------|--------------------------------|---------------------------------------------------------------------------|
| Control              | 310 mL Lactated Ringer         | 90 mg creatinine, 1000 mg/200 mg Amoxicillin/Clavulanic acid, 6 mg dexamethasone, 6 mg mannitol, 2 mg sodium nitroprusside, 10 mL 8.4% sodium bicarbonate, 10 mL 5% glucose, 90 mL aminoplasmal, 2.75 mL 8.4% bicarbonate, 18.6 IU insulin |
| Hb: 5.0±1.0 mmol/L   | 500 mL Leukocyte depleted blood |                                                                           |
| Aqix-BSA             | 800 mL Aqix® RS-I              | 90 mg creatinine, 1000mg/200mg Amoxicillin/Clavulanic acid, 6 mg dexamethasone, 6 mg mannitol, 2 mg sodium nitroprusside, 17 IU insulin, No infusion |
|                      | 80 mL Aqix® RS-I               |                                                                           |
|                      | 17.6 gr BSA (2.2%)             |                                                                           |
| Aqix-Dex             | 800 mL Aqix® RS-I              | The same additives as the Aqix-BSA group, No infusion                     |
|                      | 28 gr Dextran 40 (3.5%)        |                                                                           |
| Aqix-BSA-RBC         | 580 mL Aqix® RS-I              | The same additives as the Aqix-BSA group, No infusion                     |
| Hb: 5.4±1.0 mmol/L   | 220 mL RBCs                    |                                                                           |
|                      | 12.8 gr BSA (2.2%)             |                                                                           |
| Aqix-Dex-RBC         | 580 mL Aqix® RS-I              | The same additives as the Aqix-BSA group, No infusion                     |
| Hb: 5.5±0.8 mmol/L   | 220 mL RBCs                    |                                                                           |
|                      | 20.3 gr Dextran 40 (3.5%)      |                                                                           |

Composition of Aqix®RS-I

| Components           | Concentration (mmol/L) | Classification          |
|----------------------|------------------------|-------------------------|
| NaCl                 | 110.00                 | Salts                   |
| KCl                  | 5.00                   |                         |
| CaCl2                | 1.25                   |                         |
| MgCl2                | 0.45                   |                         |
| NaHCO3               | 25.00                  | pH buffer               |
| BES                  | 5.00                   |                         |
| D-glucose            | 10.00                  | Metabolic substrates    |
| Glycerol             | 0.11                   |                         |
| L-Glutamate          | 0.30                   |                         |
| L-glutamine          | 0.40                   |                         |
| L-Aspartate          | 0.02                   |                         |
| L-Carnitine          | 0.05                   |                         |
| Choline Chloride     | 0.01                   |                         |
| Thiamine Pyrophosphate | 40 nmol/L            |                         |
| Human insulin        | 28 mIU                 |                         |

BSA: bovine serum albumin; Dex: dextran 40; RBC: red blood cells.
Results

Energy-demanding processes during normothermic machine perfusion

Compared to the acellular perfusion solution groups (Aqix-BSA and Aqix-Dex), total oxygen consumption during NMP was significantly higher in all perfusion solutions containing RBCs ($p<0.05$) (Figure 1A). The arterial oxygen content was significantly higher in the control group compared to both acellular groups (Aqix-BSA $p=0.045$, Aqix-Dex $p<0.01$), and between Aqix-BSA and Aqix-Dex ($p=0.01$) (Figure S1A).

Fractional sodium excretion ($\text{FE}_{\text{Na}}$) was calculated to estimate tubular function, with a lower level corresponding to an improved tubular function. $\text{FE}_{\text{Na}}$ (Figure 1B) levels ranged between 55 and 165% at the start of NMP. The acellular groups showed higher $\text{FE}_{\text{Na}}$ levels and kept relatively stable at around 75% during NMP. The $\text{FE}_{\text{Na}}$ among cellular groups tended to remain lower and decreased during NMP towards 25%. There was, however, only a significant difference between Aqix-BSA, Aqix-Dex, and the control group ($p=0.02$ for each pair-wise comparison). The Aqix-BSA-RBC group tended to have decreased $\text{FE}_{\text{Na}}$ compared to the acellular groups ($p=0.059$).

Total sodium reabsorption was higher in controls than in kidneys perfused with Aqix supplemented with BSA, Dex, and Dex-RBC, respectively ($p=0.02$ for each pairwise comparison). The Aqix-BSA-RBC group showed a peak in sodium reabsorption after 1 hour of NMP but decreased over time.

At the start of HMP, tissue ATP levels were not different between the experimental groups (Figure 1D, HMP0). Thereafter, ATP levels remained similar among experimental groups until at the end of NMP (NMP-240). At this time point, ATP levels were lower in the Aqix-Dex ($p=0.02$) and Aqix-BSA-RBC ($p=0.03$) groups, respectively, when compared to controls.

Lactate levels are presented in Figure S1B, which shows high levels of lactate at the beginning of NMP due to the use of lactated Ringer’s solution. Compared to the control group, the increase in lactate during 4 hours of NMP was significantly higher in the Aqix-Dex-RBC group ($p=0.002$) and tended to be higher in the Aqix-BSA-RBC group ($p=0.068$).

Renal function and the effects of colloids on the kidney

During HMP (Figure 2A, left and right panels, $p=0.61$) and NMP (Figure 2B, $p=0.27$), the flow first increased at the start of both machine perfusion modalities, then remained

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Table 2: Equations for calculating renal metabolic and functional parameters.

| Equation | Abbreviations |
|----------|---------------|
| Arterial oxygen content (mLO$_2$/L) | Hb, hemoglobin content (mmol/L). 2.4794 = arterial oxygen content in mLO$_2$/dl and is calculated by: 1.54 (mLO$_2$/g Hb at 37°C * (Hb (mmol/l) * 1.61) * 100 = SO$_2$ arterial) * 0.01 |
| Venous oxygen content (mLO$_2$/L) | D0.024794 = venous oxygen content in mLO$_2$/dl and is calculated by: 1.54 (mLO$_2$/g Hb at 37°C * (Hb (mmol/l) * 1.61) * SO$_2$, partial oxygen pressure (kPa). K solubility constant of oxygen in water at 37°C (0.0225 mLO$_2$/kPa). SO$_2$, hemoglobin saturation (%). *Bold part of the formula is omitted when no RBC were used |
| Oxygen consumption (mLO$_2$ min$^{-1}$ 100g) | Q, renal blood flow (L/min). g kidney weight (gram) |
| Fractional sodium excretion (%) | $100$ * $\frac{\text{UNa}}{\text{PNa}}$ |
| Total sodium reabsorption (mmol min$^{-1}$ 100g$^{-1}$) | $\frac{\left(\text{UcrCl} * \text{PNa}\right) - \left(\text{UNa} * U\right)}{g} * 100$ |
| Creatinine clearance (mL min$^{-1}$ 100g$^{-1}$) | $\frac{\left(\text{UcrCl} * U\right)}{\text{Pcr}} * 100$ |
| TBARS production (U/L) | TBARS$_{urine}$, concentration of TBARS in urine (µM). U, urine production rate (mL/min). TBARS$_{perfusion}$, concentration of TBARS in the perfusate (µM). P, priming volume of the NMP setup (L). I, volume of infusion during NMP (L).
Porcine kidneys were perfused for 4 hours with blood (control), with Aqix supplemented with a colloid (2.2% BSA or 3.5% Dextran 40), or Aqix with a colloid (BSA or Dextran 40) and RBC.

A. Oxygen consumption rates during 4 hours normothermic perfusion, *p<0.05 AUCs of all RBC-containing groups compared to acellular groups.

B. Fractional sodium excretion levels during 4 hours perfusion, *p<0.02 Aqix-BSA, Aqix-Dex vs. controls (Dunnett’s test), versus Aqix-Dex-RBC group, significantly lower in the Aqix-Dex-RBC group compared to the control group (Bonferroni test).

C. Total sodium reabsorption (\(T_{\text{Na}}\)) during 4 hours perfusion, *p<0.001 control versus Aqix-BSA/Dex and control versus Aqix-Dex-RBC group.

D. ATP content in kidney cortex tissue after 30 minutes warm ischemia and before preservation (HMP0), 3 hours preservation (HMP180), 30 minutes of normothermic machine perfusion (NMP30), and at the end of 240 minutes of normothermic machine perfusion (NMP240), *p<0.03 vs. controls. Every group contains 6 kidneys. BSA: bovine serum albumin; Dex: dextran 40; RBC: red blood cells; HMP: hypothermic machine perfusion; NMP: normothermic machine perfusion. Data are presented as mean ± SEM. Except for urine production and weight gain, area under the curves were compared to assess differences between experimental groups.

Stable afterwards, but did not differ between experimental groups. Irrespective of treatment group, NMP flow (40-75 mL/min/100gr) was 4-7-fold higher than HMP flow (10-13 mL/min/100gr). Creatinine clearance was the highest in the control group (Figure 2C) and was significantly better in comparison with the Aqix-Dex-RBC group (\(p<0.01\)) and the Aqix-Dex group (\(p<0.02\)), respectively, during 4 hours NMP. *p<0.05. Every group contains 6 kidneys. BSA: bovine serum albumin; Dex: dextran 40; RBC: red blood cells; HMP: hypothermic machine perfusion; NMP: normothermic machine perfusion. Data are presented as mean ± SDM. Except for urine production and weight gain, area under the curves were compared to assess differences between experimental groups.
and Figure S2, *p*<0.02). The Aqix-BSA group showed significantly higher urine production compared to Aqix-Dex and Aqix-Dex-RBC (*p*<0.05). Compared with controls, weight gain during NMP was increased in the Aqix-BSA, Aqix-Dex, and Aqix-Dex-RBC groups, respectively (Figure 2D, *p*<0.05).

**Renal injury and oxidative stress during normothermic machine perfusion**

Compared to all other experimental groups, kidneys perfused with Aqix including BSA exhibited significantly increased ASAT and LDH production during NMP (Figure 3A and Figure S3, *p*<0.001). Despite increased release of injury markers, no differences in the specific groups were found as regards urinary NAG or total TBARS production, the latter being a marker for oxidative stress (Figure 3B/D, respectively). Notably, kidneys perfused with Aqix-BSA-RBC and Aqix-Dex-RBC had increased TBARS production compared to the Aqix-Dex group. Also, kidneys perfused with Aqix-Dex-RBC had higher TBARS production compared to controls (Figure 3D, *p*<0.013). Histology examination revealed that kidneys perfused with Aqix-BSA and Aqix-Dex had a significantly higher level of damage per section compared to the negative control samples, indicating that most renal damage occurred in kidneys perfused with acellular solutions (Figure 3C, *p*<0.05). No differences between groups were found, however, the median percentage of damage was lower in the cellular groups compared to the acellular groups. Histology sections can be found in Figure S4.

**Discussion**

The objective of our study was to determine whether acellular AQIX® RS-I (Aqix) in combination with a colloid and RBC as an oxygen carrier could be a viable substitute for blood-based perfusion solution during ex-situ NMP of porcine kidneys. The control group consisted of perfusion with leukocyte-depleted autologous blood diluted with Ringers’ lactate, supplemented with antibiotics, a vasodilator, a corticosteroid, mannitol, and nutrients; this solution has been successfully used as a standard preclinical perfusion solution by our group25. The main finding in this study was that the oxygen consumption rates were 4-to-6-fold lower in the acellular solutions compared to the RBC-containing groups with a significantly higher sodium transport in the control group. In addition, a trend towards improved fractional sodium excretion in both the control and Aqix-BSA-RBC groups was observed. Interestingly, sodium transport and reabsorption were impaired when dextran was added as a colloid.

**Comparison with other studies**

NMP is an upcoming technique that has only limitedly been introduced in clinical practice for kidney transplantation. It has been demonstrated that one hour of NMP is feasible and safe using a blood-based electrolyte perfusion solution without adding a colloid7–10. Subsequently, it became evident that perfusions longer than one hour are needed for better graft assessment and potential organ repair29,31. Several studies have shown that NMP with acellular perfusion solutions of donor liver and lungs can be successful. However, there is only limited experience with acellular perfusion solutions for kidney NMP18,32–35. Similar outcomes were found when comparing a synthetic acellular hemoglobin-based oxygen carrier with packed red blood cells during NMP of discarded human kidneys36. The comparison between a cellular and an acellular solution has not been made yet. There is some preclinical data on acellular (re)
perfusions in which renal function of porcine kidneys was evaluated after preservation with various techniques using an ex vivo normothermic reperfusion protocol without supplementation of oxygen carriers. During 90 minutes of perfusion, differences in renal function were observed, providing evidence for the suitability of oxygen carrier-free solutions to assess organ function\textsuperscript{13,37-41}. Our data does not support this finding since the renal function was suppressed in the acellular groups, based on fractional sodium excretion and creatinine clearance. Relatively low quality of the kidneys in our study, due to the standardized slaughtering process and exposure to 30 minutes of warm ischemia, could explain these findings. However, we aimed to evaluate the different solutions used for NMP in donor kidneys that have been injured and require proper assessment. A limitation of this study and the studies that used acellular (re)perfusion techniques to assess renal function is that the donor kidneys were not transplanted. Therefore, it is unknown whether perfusion with oxygen carrier-free solutions may affect safety and long-term organ function. As NMP of donor kidneys is still in its infancy, the exact needs of the organ during NMP are not yet known, and predicting post-transplant outcomes based on kidney function parameters measured during NMP is impossible. It is still unknown which level of renal clearance, tubular function, or oxygen consumption during NMP may be predictive of adequate post-transplant renal function. During normothermia, it is known that cellular metabolism is fully active, and the demand for oxygen is high.\textsuperscript{42} Therefore, the question remains whether sufficient oxygen is present to support cell metabolism during NMP when perfused with acellular solutions.

**Renal metabolism**

Although representing less than 0.5% of the body weight, kidneys use approximately 10% of the whole-body oxygen consumption\textsuperscript{43}. Such oxygen requirements are needed for active sodium reabsorption by the tubular cells\textsuperscript{44}. Additionally, both oxygen and metabolic substrates are necessary to fuel oxidative phosphorylation\textsuperscript{45,46}. All RBC-containing groups showed higher oxygen consumption levels than the acellular groups by 4-6-fold. Nevertheless, the only significant improvement in total sodium reabsorption and ATP levels was seen in the control group. Although AQIX\textsuperscript{®} RS-I appears to have essential components to fuel oxidative phosphorylation, the solution did not support ATP production and subsequent sodium reabsorption at the same level as the control group. The AQIX-BSA-RBC group showed an almost significant decrease in ATP production when comparing 30 and 240 minutes of NMP (p=0.06). Furthermore, this group showed a peak in sodium reabsorption after 1 hour of NMP but decreased over time, indicating that AQIX-BSA-RBC is not favorable to fuel oxidative phosphorylation during NMP for more extended perfusion periods. We have previously reported similar results when comparing static cold-stored kidneys with hypothermic machine perfused kidneys using different oxygen concentrations\textsuperscript{25}. Although cold-stored kidneys consumed oxygen (with a comparable consumption as found in this study), sodium reabsorption was disrupted (FE\textsubscript{Na} 70-100%) because the oxygen consumption resulted in reactive oxygen species formation instead of ATP production. In the present study, we also found higher TBARS levels in the AQIX-Dex-RBC and AQIX-BSA-RBC group compared to the control, suggesting increased oxidative injury.\textsuperscript{47,48} Our data possibly indicate mitochondrial malfunction and/or uncoupling, partially reducing oxygen consumption and production of reactive oxygen species. Higher lactate production during NMP suggests increased anaerobic metabolism in both acellular groups and the AQIX-BSA/Dex-RBC groups, indicating that insufficient oxygen is delivered to the renal cells to perform oxidative phosphorylation. Metabolic tracer studies, such as isotopic labeling of central metabolites, could provide additional insight into renal metabolism during NMP\textsuperscript{49}.

In the absence of transport-related processes, the kidney exhibits a low basal oxygen consumption rate of approximately 15% of total renal oxygen consumption of a filtering/reabsorbing kidney\textsuperscript{44,45}. However, oxygen consumption has been shown to increase linearly with glomerular filtration rate which is associated with T\textsubscript{m} through tubuloglomerular feedback\textsuperscript{43,44}. Because sodium reabsorption appeared to be almost absent in the acellular groups, we hypothesize that this is the reason that the tubular injury marker uNAG was not increased in these groups despite insufficient oxygen supply.

**Effect of different colloids during NMP**

A significant increase in weight gain compared to control was observed in the AQIX-BSA, AQIX-Dex, and AQIX-Dex-RBC groups, reflecting tissue swelling and possible edema formation. Edema increases the oxygen diffusion distance and could compromise cellular metabolism, as observed in the AQIX-BSA, AQIX-Dex, and AQIX-Dex-RBC groups\textsuperscript{21,22}. A higher concentration of colloids and a higher COP could lead to less edema formation and so improved cellular metabolism; however, we believe an oxygen carrier is still essential for optimal support of the kidney’s metabolic needs.

Dextran 40 was tested as a potential artificial colloid during NMP\textsuperscript{51}. Based on pilot experiments, we determined that 3.5% dextran 40 was most beneficial for minimizing edema formation when looking at the wet/dry ratio; however, weight gain during NMP was significantly higher in both dextran 40 groups compared to control. Furthermore, sodium transport and reabsorption were impaired in both dextran 40 groups. We did not observe severe renal injury based on injury markers measured in the perfusate (ASAT, LDH) or urine (uNAG) in dextran perfused kidneys. However, histology findings showed significant increased cell damage compared to our negative controls and kidneys that were perfused with Dextran, without RBC, showed the highest median
percentage of cell damage per section scored by the pathologist. This finding is in-line with other studies showing that dextran can cause tubular damage. Little is known about using dextran 40 during machine perfusion, and additional studies should be conducted to determine the pharmacokinetics and toxicity of dextran 40 during ex vivo NMP.

Limitations of the study
Due to the use of porcine kidneys obtained from a slaughterhouse, we could not provide post-transplant outcomes. Furthermore, these pigs were sedated followed by exsanguination and were not exposed to the agonal phase commonly seen in DCD donation. Therefore, functional warm ischemia is not considered in this model. Nonetheless, our model has shown to be a validated technique to assess donor kidney quality after reperfusion. Slaughterhouse kidneys provide the opportunity to test early preclinical hypotheses in organs of large animals without the need of laboratory animals, thereby reducing both costs and animals for research. Ultimately, a transplant model with laboratory DCD porcine donors will be required as a next step towards translation to clinical practice with human kidneys. Another limitation is that we did not include the addition of an acellular synthetic oxygen carrier such as a hemoglobin-based oxygen carrier or other alternatives. We cannot answer the question of whether RBCs are necessary or whether another oxygen carrier could also perform the task of transporting oxygen towards the kidney during NMP.

In conclusion, this study shows that the addition of RBCs to the perfusion solution significantly enhances oxygen consumption of ex vivo perfused kidneys under normothermic conditions. We have demonstrated that RBCs are necessary to support sodium reabsorption during NMP, indicating that to be able to perform this energy-consuming process, sufficient oxygen transportation is mandatory to support basal renal function. The Aqix supplemented with BSA and RBC can partly support renal metabolism and function, but the control blood-based perfusion solution was found to be superior. Further evaluation in the transplant setting is required to fully apprehend these new findings.

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