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Authorship

LHV: Participated in research design, performance of the experiments, participated in data analysis and wrote the paper.

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Abbreviations

ASAT, aspartate amino transferase

ADP, adenosine monophosphate

ADP, adenosine diphosphate

ATP, adenosine triphosphate

AUC, area under curve

CS, cold storage

DBD, donation after brain death

DCD, donation after circulatory death

DGF, delayed graft function

Hb, hemoglobin

H&E, haemotoxylin and eosin

HMP, hypothermic machine perfusion

LDH, lactate dehydrogenase

NAG, N-Acetyl-β-D-glucosaminidase

NMP, normothermic machine perfusion

ROS, reactive oxygen species

SCD, standard criteria donor

TBARS, thio barbituric acid reactive substances

UW MPS, university of wisconsin machine perfusion solution

WIT, warm ischemic time

Abstract

Background:

Hypothermic machine perfusion (HMP) has become standard care in many center's to preserve kidneys donated after circulatory death (DCD). Despite a significant reduction in metabolism at low temperatures, remaining cellular activity requires oxygen. Since the role and safety of oxygen during HMP has not been fully clarified, its supply during HMP is not standard yet. This study investigates the effect of administering oxygen during HMP on renal function in a porcine DCD model.

Methods:

After 30 minutes of warm ischemia, porcine slaughterhouse kidneys were preserved for 24 hours by means of cold storage (CS), or HMP with Belzer Machine Perfusion Solution (UW-MPS) supplemented with no oxygen, 21% or 100% oxygen. Next, kidneys were reperfused for 4 hours in a normothermic machine perfusion (NMP) setup.

Results:

HMP resulted in significantly better kidney function during NMP. Thiobarbituric acid-reactive substances (TBARS), markers of oxidative stress, were significantly lower in HMP preserved kidneys. HMP preserved kidneys showed significantly lower ASAT and LDH levels compared to kidneys preserved by CS. No differences were found between the HMP groups subjected to different oxygen concentrations. ATP levels significantly improved during HMP when active oxygenation was applied.

Conclusion:

This study showed that preservation of DCD kidneys with HMP is superior to CS. Although the addition of oxygen to HMP did not result in significantly improved renal function, beneficial effects were found in terms of reduced oxidative stress and energy status. Oxygen addition proofed to be safe and did not show detrimental effects.

Introduction

Persistent organ shortage in transplantation results in the use of sub-optimal quality organs. In addition, the number of donations after brain death (DBD) from donors younger than 50 without comorbidities or so-called standard criteria donors (SCD) is decreasing.[1] As a result, allografts from donors deceased due to a circulatory arrest are increasing in a number of countries.[1] Kidneys retrieved from such donors are more prone to ischemia-reperfusion injuries and are associated with a significantly higher incidence of delayed graft function (DGF).[2] DGF results in the necessity of dialysis after transplantation until the kidney recovers its function.[3] DGF, therefore, affects the quality of life of the recipient and it also appears to be a risk factor for acute cellular rejection and poorer long-term outcomes.[4] Hypothermic machine perfusion (HMP) as preservation modality has already been proven to reduce the incidence and duration of DGF compared to cold storage(CS).[2,3,5,6]

Both CS and HMP are based on the suppression of metabolism due to hypothermia. Notably, even at 4 degrees Celsius, approximately 10% of physiological metabolic rate remains. This suggests that oxygen continues to be consumed and its addition during preservation might be beneficial to support ongoing metabolism. HMP offers the possibility to provide the kidney with oxygen during the preservation period. However, oxygen supply during preservation is not standard of care and kidneys are usually perfused without oxygen.

Next to limited experimental and preclinical evidence, clinical proof of the need of oxygen during HMP is also still lacking.[5,7–9] Therefore, two international, double blinded, randomized controlled trials are currently ongoing to assess the efficacy of 100% oxygen addition during HMP of older donation after circulatory death (DCD) kidneys and in expanded criteria donor (ECD) kidneys.[10,11] Furthermore, safety of the addition of oxygen at whatever level during preservation, remains a matter of concern. In addition, the work that has been performed until now focused mainly on cold storage versus HMP with 100%

oxygen or on HMP with different oxygen concentrations only. A study comparing CS with HMP with different oxygen concentrations using a clinically approved preservation solutions is still lacking. We, therefore, combined all strategies. Our aim was to provide a comprehensive evaluation about the effects of different oxygen concentrations during HMP of porcine kidneys compared to CS preservation. Besides early renal function, we want to address the safety of different oxygen concentrations by means of the release of reactive oxygen species (ROS), and the effect of oxygen during HMP on the metabolism of the kidney. In order to avoid the use of laboratory animals we used a porcine DCD kidney slaughterhouse model as previously developed in our lab.

Materials and methods

Animal model

Porcine kidneys were obtained from two abattoirs. Pigs were slaughtered by a standardized procedure of sedative electric shock followed by exsanguination. Immediately, 1 liter of blood was collected in a container containing 25.000 IU of heparin (LEO Pharma A/S, Ballerup, Denmark). Since we made use of slaughterhouse waste material as our organ and blood source, no animal ethics committee approval was needed.

Experimental design

Warm ischemic time (WIT) of thirty minutes was chosen to induce ischemic injury. The four different preservation techniques used were applied for 24 hours: cold storage (CS), non oxygenated hypothermic machine perfusion ($HMP_{0\%}$), hypothermic machine perfusion with 21% oxygen ($HMP_{21\%}$) or 100% oxygen addition ($HMP_{100\%}$). All kidneys were subsequently reperfused in an ex vivo normothermic machine perfusion (NMP) setup for a total duration of 4 hours. Every group contained six kidneys.

Cold storage and hypothermic machine perfusion

After warm ischemia the kidney was flushed with 180 ml saline at 4° Celsius (Baxter BV, Utrecht, The Netherlands). A cortical biopsy was taken (Invivo, Best, The Netherlands) and stored in sonification solution (SONOP containing 0.372 g EDTA in 130 mL H_2O and NaOH (ph 10.9) + 370 mL 96% ethanol) and 4% buffered formaldehyde for further analysis. In the CS group the kidneys were stored in a bag, submerged in 500 mL University of Wisconsin solution (Belzers CS, Bridge to life Ltd., London, United Kingdom) and stored on melting ice. In the HMP groups the kidneys were cannulated to connect the renal artery to the HMP device (Kidney Assist Transport, Organ Assist, Groningen, The Netherlands). A total of 500 mL University of Wisconsin machine perfusion solution (Belzers MP, Bridge to life Ltd., London, United Kingdom) was used as perfusion solution. Preservation was performed at 4°C with a pulsatile pressure-controlled perfusion with a mean arterial pressure of 25 mmHg. Either, no oxygen, 21% or 100% oxygen was supplied to the oxygenator (Hilite LT 1000, Medos Medizin technik AG, Stolberg, Germany) with a fixed flow rate of 100 ml/min. Perfusion solution samples were taken after 15, 60 minutes and 24 hours. Perfusion parameters, such as pressure, temperature and flow rates were monitored continuously.

Ex vivo normothermic machine perfusion to assess renal function

After 24 hours of preservation, renal function was assessed in an isolated ex vivo normothermic machine perfusion setup. The renal artery and ureter were cannulated with a 12 and 8 French cannula, respectively. The kidneys were flushed with 50 ml of saline (4°) to remove remaining preservation solution. Afterwards the kidneys were weighed and another biopsy was taken and stored as described above.

The kidney was placed in an organ chamber and perfused at 37°C for 4 hours with a pressure-controlled pulsatile pump at a mean pressure of 75 mmHg (Kidney Assist Transport, Organ Assist, Groningen, The Netherlands). The perfusate was oxygenated with a mixture of 95%

O_2 and 5% CO_2 through the oxygenator (Hilite LT 1000, Medos Medizin technik AG, Stolberg, Germany) with a fixed flow rate of 500 ml/min. The setup was surrounded by a heating cabinet with a feedback system, keeping the ambient temperature at 37°C. The perfusion medium consisted of 500 ml heparinized, leukocyte-depleted autologous whole blood. Leukocyte-depletion was carried out with a leukocyte filter (Bio R O_2 plus, Fresenius Kabi, Zeist, The Netherlands). The blood was diluted with 300 ml of lactated Ringer's (Baxter BV, Utrecht, The Netherlands), containing 6 mg Mannitol (Sigma-Aldrich, St Louis, USA), 6 mg Dexamethasone (Centrafarm, Etten-Leur, The Netherlands) 10 ml 8,4% sodium bicarbonate (B Braun Melsungen AG, Melsungen, Germany), 90 mg creatinine (Sigma-Aldrich, St Louis, USA), 1000mg/200mg Amoxicilline /Clavulanic acid (Sandoz BV, Almere, The Netherlands), and 100 µl 20 mg/ml sodium nitroprusside (Sigma-Aldrich, St Louis, USA). Furthermore, a continuous supply of nutrients consisting of 10% Aminoplasmal (Braun Melsungen AG, Melsungen, Germany), 2.5 ml 8,4% sodium bicarbonate, and 17 IU Novorapid, (Novo Nordisk, Bagsvaerd, Denmark) was added to the perfusion circuit at a rate of 20 ml/h. 5% glucose (Baxter BV, Utrecht, The Netherlands) was administered when glucose levels dropped below 5 mmol/L.

Evaluation of renal function

During the testing period, renal flow rate and urine production were measured every 15 minutes. Blood and urine samples were taken after 15, 60, 120, 180 and 240 minutes. At these same time points arterial and venous blood samples were taken for blood gas analysis (ABL90 FLEX, Radiometer, Zoetermeer, The Netherlands).

Concentrations of creatinine and sodium were determined in blood and urine, using routine procedures at the clinical chemistry lab of the University Medical Center Groningen (UMCG). Creatinine clearance served as the primary functional endpoint of this study. Tubular and glomerular integrity were used as secondary functional endpoints and were

assessed using fractional sodium excretion and urine protein content, respectively. Proteins in the urine were measured in a standardized manner at the clinical chemistry lab of the UMCG.

Metabolic activity

Calculating the renal oxygen consumption (QO2) approximated metabolic activity of the kidneys. The difference between the venous and arterial dissolved and bound oxygen was calculated by using the following formula:

$$Oxygen\ consumption\left(\frac{\frac{mlO_2}{min}}{100gr}\right) =$$

$$((((Hb * 2{,}4794) + (pO_{2arterial} * K)) - ((0{,}024794 * Hb * SO_{2\ venous}) + (pO_{2\ venous} * K))) * Q)/g) * 100$$

Where Hb is the perfusates hemoglobin content in mmol/L, pO_2 is the partial oxygen pressure arterial or venous in kPa, K is the solubility constant of oxygen in water at 37°C and equals 0.0225 (mL O_2 per kPa), SO_2 is the saturation in %, Q is the renal blood flow in L/min and g is the kidney weight in grams.

Adenosine triphosphate (ATP) was analyzed in biopsies that were taken before and after the preservation period, and at the end of reperfusion. ATP content was determined according to a standard protocol and expressed in µmol/g protein.[12]

Metabolic coupling of sodium transport by ATPase in tubular epithelial cells was calculated by dividing transported sodium (T_{Sodium}) with renal oxygen consumption QO_2:

$$T_{Sodium\ \left(\frac{\frac{mmol\ Sodium}{mmol\ O2}}{100gr}\right)} = \frac{\left((Cr_{clearance} * Plasma_{Sodium}) - (Urine\ flow * Urine_{Sodium})\right)}{QO_2}$$

Oxidative stress due to active oxygenation

Thiobarbituric acid-reactive substances (TBARS) were measured as indicator of oxidative stress in the preservation solution, blood and urine at the specified sampling time points. The

protocol for this analysis has been described in detail previously.[12] TBARS concentrations are expressed in µM.

Kidney injury markers

Enzymatic activities of lactate dehydrogenase (LDH), and aspartate aminotransferase (ASAT) were determined at the clinical chemistry lab of the UMCG according to standard procedures. Urinary N-acetyl-beta-D-glucosaminidase(uNAG) was determined following a protocol described previously by our lab.[12,13]

Histological examination and morphology scoring

Kidney biopsies were fixed by immersion in 4% buffered formaldehyde, embedded in paraffin and cut into 4 µm slices. These sections were stained with haematoxylin-eosin (H&E). Ischemia reperfusion injury was scored on the basis of 3 criteria[14]: proximal tubular cell edema, tubular cell vacuolation and proximal tubular cell necrosis. Every item was given a score between 1 and 5, as representing no signs of edema, vacuoles or cell necrosis (score 1), minor (score 2), medium (score 3), severe (score 4) or extreme signs (score 5). The biopsies were randomly assigned to two independent experienced examiners for light microscopy evaluation.

Statistics

Results are reported as means with standard deviations. Statistical analysis was performed with IBM SPSS Statistics 23. Area under the curve (AUC) was calculated for renal flow rates during HMP and NMP, creatinine clearance and oxygen consumption rates. All other markers were tested for significant differences at every time point. Groups were compared using a Kruskal-Wallis test followed by a Mann-Whitney U posthoc test. $P<0.05$ was considered to indicate statistical significance.

Results

Hypothermic and normothermic perfusion parameters

All HMP groups showed similar flow patterns in the cold, starting with a steep increase within the first twenty minutes and a slow increase thereafter until the end of preservation (Figure 1A). No statistical differences in flow rate during HMP was found.

During the period of NMP, renal blood flow increased during the first 120 minutes in every group, and slowly decreased thereafter (Figure 1B). The CS group showed a trend towards a higher mean flow rate ($p=0.072$)) compared to the HMP groups.

Renal function during normothermic perfusion

In terms of creatinine clearance all HMP groups showed significantly higher clearances at every time point in comparison to the CS group. The $HMP_{100\%}$ group presented the highest clearance rate. It was, however, not significantly different from the $HMP_{0\%}$ and $HMP_{21\%}$ groups (Figure 2A). Proteinuria in CS kidneys was significantly higher than in HMP groups (Figure 2B). HMP kidneys had reduced urinary levels of protein, but no differences were observed within the 3 HMP groups. Significant improvement in fractional sodium excretion levels was found when HMP was applied. Again no differences were found when comparing the different oxygen concentrations (Figure 2C). In all groups urine production was the highest during the first fifteen minutes after reperfusion (Figure 2D).

Metabolic activity during normothermic perfusion

Oxygen consumption rates were significantly higher in all HMP groups compared to the CS kidneys. Although renal function in the CS kidneys was almost absent (filtration < 0.1 mL/(min.100g; FENa% > 70%), oxygen consumption was still present (Figure 3A).

After 30 minutes WIT and before preservation was initiated, ATP was almost completely depleted in every group (Figure 3B, timepoint 0.5). CS for 24 hours resulted in complete loss of all ATP. $HMP_{0\%}$ resulted in no additional ATP production during HMP. The addition of 21

or 100% yielded significant higher ATP during 24 hours preservation in these groups (Figure 3B, timepoint 24). 4 hours of NMP resulted in significant lower ATP levels in the $HMP_{0\%}$ group, while the other groups showed similar ATP levels (Figure 3B, timepoint 28). Metabolic coupling ratio was improved in HMP perfused kidneys and was significantly improved after 120 and 180 minutes after start reperfusion in all HMP kidneys compared to CS kidneys (Figure 3C).

Oxidative stress due to active oxygenation

TBARS measured in the preservation solution during HMP were negligible in all groups at every time point (Figure 4A). TBARS in the perfusate were significantly higher after 120 minutes in the CS kidneys compared to the HMP groups, and slowly decreased in the HMP groups over the 4 hours NMP period (Figure 4B). The urinary TBARS showed an immediate increase during the first hour of reperfusion in all HMP groups, but decreased thereafter. The CS group had a gradual increasing concentration of TBARS in the urine and has a higher value (not significant) at the end of 4 hours perfusion (Figure 4C).

Kidney injury markers

LDH values remained stable in the HMP groups while the CS kidneys showed a rise in LDH levels in the (NMP) perfusate over time (Figure S1A, SDC, http://links.lww.com/TP/B723). At the end of 4 hours reperfusion, LDH levels were significantly higher in the CS group. Urinary N-acetyl-beta-D-glucosaminidase (uNAG) remained stable during 4 hours in the HMP groups. The CS kidneys showed a significant increased value from the onset of reperfusion and also a rise over time (Figure S1B, SDC, http://links.lww.com/TP/B723). All 3 HMP groups have a similar trend for ASAT levels; however, there is a significant benefit for the 100% oxygen group in comparison with the $HMP_{0\%}$ and CS groups. Similar for LDH and NAG, the CS group showed an increase over a time and significant higher levels of ASAT compared to all HMP groups (Figure S1C, SDC, http://links.lww.com/TP/B723).

Histology

During histological examination it became clear that the stage of damage was already beyond the point of vacuolation and that proximal tubular cell necrosis was already detectable. The histological scoring resulted in the following mean necrosis scores: 2.17±0.49, 2.60±0.55, 2.67±0.82 for the HMP 0, 21 and 100% groups, respectively. This was significantly lower than the average necrosis for the CS kidneys (of 4.7±0.49; Figure S2A, SDC, http://links.lww.com/TP/B723).

The average edema score for CS, HMP 0, 21 and 100 were comparable, with scores of 2.0±0, 2.50±0.84, 2.20±0.84, and 2.5±0.58, respectively (Figure S2B, SDC, http://links.lww.com/TP/B723).

Discussion

In this study we evaluated the effect of different oxygen concentrations during HMP on renal function with a clinically approved perfusion solution. We found that HMP is superior to CS in terms of creatinine clearance and fractional sodium excretion. Active oxygenation during preservation did not result in significant advantages with regard to renal function in this porcine DCD model. Only for ASAT and ATP levels, a significant beneficial effect of oxygen was found. No signs of oxidative stress through the addition of oxygen during preservation were observed.

Experimental research on the addition of oxygen during HMP is scarce. There are only two studies that assessed CS versus 100% oxygenated HMP in preclinical porcine (auto) transplantation models. Both were able to show the significant beneficial effects of oxygenated HMP in comparison to CS on renal function.[5,7] This result corresponds with our findings. However, non oxygenated HMP was not included and the addition of oxygen can therefore not be assessed. Furthermore, in another study, short-term effects of oxygen during HMP was addressed in a reperfusion model.[8] This study found beneficial effects of oxygen

during the preservation of DCD kidneys in terms of function and injury. Unfortunately, we were not able to reproduce these findings, however, there are some major differences in setup of these studies. One of these differences is that we used porcine slaughterhouse kidneys. The downside of using these is that not only the conditions of the experiment are less controlled but also baseline quality of these kidneys is not controllable since we are not allowed to take samples at life. Therefore, we cannot elaborate on preexisting injury and the amount of injury induced by warm ischemia that we have chosen in this study. Animal welfare inspired us to develop a slaughterhouse model since ethical considerations concerning the use of animals for scientific research is an important topic in the Netherlands. We believe that slaughterhouse organs can provide us reliable and translational data and we are not the first group believing in slaughterhouse organs for machine perfusion research.[15–18] Nath et al already demonstrated similarity in metabolic processes between human and slaughterhouse pig kidneys, which provides additional confidence in these kidneys for scientific research.[16] One prerequisite to make sure that the slaughtering procedure does not negatively influence the outcome we streamlined the process with our local butchers by explaining them our goals and procedures such as ischemic times and appropriate handling of blood and organs.

The reason for conducting this study was to assess a clinically approved HMP device in combination with a clinically approved perfusion solution. Hoyer et al used an experimental perfusion solution, Histidine-tryptophan-ketoglutarate –N, and their results can therefore not be directly extrapolated to the current clinical situation.[8] In our setup, UW-MP solution was used, which is currently the only approved clinical machine perfusion solution for preservation of kidney grafts. The oxygen carrying capacity of UW-MP solution was not measured in this study but has been measured before. The amount of dissolved oxygen present in UW-MP is approximately 70 kPa, and 21 kPa with the addition of 100 ml/min 100% and 21% oxygen, respectively.[17,19,20] A recent study also showed that oxygen is indeed

delivered to kidneys cells and supports aerobic metabolism, as reflected by both adenosine monophosphate (AMP), adenosine diphosphate (ADP) and ATP levels in both the medulla and cortex of the kidneys when UW-MPS solution is used.[17]

The only other comparable study on different oxygen concentrations during HMP was performed in a porcine DCD auto transplantation model.[9] This group was able to show that animals transplanted with $HMP_{0\%}$ oxygen had significantly higher peak creatinine levels at day 5 post transplant in comparison to pigs that were transplanted with $HMP_{100\%}$ oxygen kidneys. The effect of oxygen was still present at the 3-month follow up, shown by significantly lower serum creatinine levels and a significantly reduced proteinuria. These significant differences first became apparent at day 5 after transplantation. Long-term function cannot be assessed with reperfusion duration of only 4 hours and a (auto) transplantation model is necessary to address chronic injury and long-lasting quality and function. Another possibility would be longer reperfusion times. However, NMP as reperfusion modality also has its limits. NMP up to 24 hours are reported but maintaining a physiological electrolyte content and pH is problematic.[21–23] In our study, we only tested kidney function for four hours. This interval could be too short to find conclusive results concerning active oxygenation in our model. In the end, the ongoing clinical studies need to answer the question of oxygen during HMP is beneficiary for the long-term quality of transplanted kidneys.

Both studies addressed in the prior paragraph, conclude that active oxygenation during HMP of DCD kidneys is beneficial in terms of renal function. We were not able to show this. However, we do see a trend in favor of active oxygenation during long-term HMP. Kidney quality is, however, more than function alone. Therefore, we performed supplementary analyses to address a broad range of other quality and injury markers. With these we can answer some oxygen-specific issues that are fundamental in kidney preservation.

Oxygen consumption during NMP was calculated as indicator of metabolic activity and a significantly lower consumption was found for CS kidneys. Similar oxygen consumption rates were found for the different HMP groups and were in-line with renal function. We think that oxygen consumption could function as a suitable quality marker during NMP. This is supported by studies comparing subnormothermic machine perfusion with HMP and CS, where significant improvements in oxygen consumption were found for kidneys that were better preserved in terms of function.[24,25] Two different formulae are described; the first considering only dissolved oxygen,[26] the second also considering hemoglobin-bound oxygen.[24] In our model, variations in hemoglobin (Hb) and venous saturations were present, which urges us to make use of the more complicated formula.

In addition to oxygen consumption, metabolic coupling was calculated. This provides information regarding efficient use of oxygen for ATP production and subsequent active transport of sodium ions over the tubules. We observed a significant improvement in metabolic coupling at time points t=120 and 180 for all HMP groups. This result is in-line with the fractional sodium excretion. All HMP kidneys are able to transport sodium in comparison to the CS kidneys. This active sodium transport requires ATP as energy source. We found a significantly lower ATP content after reperfusion in the $HMP_{0\%}$ group and it is likely that the total ATP production and usage during NMP is balanced to make active sodium transport possible causing this lower ATP content after reperfusion in the non oxygenated HMP group. The required net ATP content in the oxygenated groups after reperfusion indicates that the addition of oxygen during HMP leads to better mitochondrial function. ATP levels in the CS group, however, indicate a low usage of ATP since metabolic coupling is poor in this group. It is also likely that mitochondrial function is disturbed in the CS group. TBARS levels in the plasma support that there is indeed a distortion in the mitochondrial respiratory chain that resulted in significantly higher TBARS levels in the CS

kidneys. Furthermore, mitochondrial damage can also be assessed with ASAT, which is present in the cytoplasm of mitochondria. In this isolated perfusion system the only source are kidney mitochondria and, therefore, serves as a valuable marker for mitochondrial damage. In-line with the TBARS levels, there are the significantly higher ASAT levels, indicating more mitochondrial damage in the CS compared to the HMP kidneys. In favor of 100% oxygenation during HMP are the significant lower ASAT levels in comparison with $HMP_{0\%}$ and HMP_{21}High oxygen concentrations during HMP resulted in better restoration of tissue ATP content in this study. This given is also supported by Patel et al, showing not only increased levels of AMP, ADP and ATP, but also increased lactate and alanine levels, metabolites of glycolysis, indicating a switch to anaerobic metabolism when insufficient oxygen was supplied to the cells.[17] A short period of oxygenated HMP after CS has been shown to reestablish cellular respiration, resulting in improved preservation of rat kidneys[27] and rat and porcine livers.[28,29] Long-term oxygenated HMP seem to result in improved cellular respiration as well, considering the ATP levels that we and others found.[17] It clearly proves that at low temperatures metabolism is ongoing and should be supported by oxygenation.

In conclusion, cellular energy status significantly improved when active oxygenation was applied during long-term HMP in this slaughterhouse reperfusion model. Although a trend towards preservation benefits was seen during NMP, this never reached a statistical significance. Long-term HMP on itself, significantly improved renal function, tissue integrity and in lower injury compared to CS during NMP.

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Figure legends

Figure 1. *Flow rates during preservation and testing.*

Porcine kidneys were treated with hypothermic machine perfusion with the addition of 0, 21 or 100% oxygen or cold storage. (**A**) Renal flow rates during 24 hours kidney preservation, (**B**) Renal flow rates during functionality testing. CS, cold storage; HMP 0%, hypothermic machine perfusion with no oxygen; HMP 21%, hypothermic machine perfusion oxygenated with air; HMP 100%, hypothermic machine perfusion with 100% oxygen. The data are shown as mean±SD.

Figure 2. *Parameters of renal function at 15, 60, 120, 180 and 240 minutes after normothermic machine perfusion.*

Porcine kidneys underwent 4 hours of normothermic autologous blood perfusion after 24 hours preservation to test renal function (**A**) Creatinine clearance, (**B**) Total protein content in urine, (**C**) Fractional sodium excretion, and (**D**) Urine production. CS, cold storage; HMP 0%, hypothermic machine perfusion with no oxygen; HMP 21%, hypothermic machine perfusion oxygenated with air; HMP 100%, hypothermic machine perfusion with 100% oxygen. The data are shown as mean±SD.* $p<0.05$ significance between CS and all HMP groups.

Figure 3. Parameters of metabolism.

(**A**) Oxygen consumption. * $p<0,05$ significance between CS and all HMP groups, (**B**) ATP content in kidney tissue after thirty minutes warm ischemia (timepoint 0,5), 24 hours preservation (timepoint 24) and at the end of 240 minutes normothermic machine perfusion (Timepoint 28). * $p<0.05$ significance between HMP 100% and all other groups, ** $p<0,05$ significance between HMP 100% and HMP 21 and 0%, # $p<0,05$ significance between HMP0% and all other groups, **(C)** Metabolic coupling. * $p<0,05$ significance between CS and all HMP groups. CS, cold storage; HMP 0%, hypothermic machine perfusion with no

oxygen; HMP 21%, hypothermic machine perfusion oxygenated with air; HMP 100%, hypothermic machine perfusion with 100% oxygen. The data are shown as mean±SD.

Figure 4. *Parameters of oxidative stress during preservation and testing*. Samples were taken during hypothermic and normothermic machine perfusion for oxidative stress analysis (**A**) TBARS concentrations at 15, 60, and 24 hours measured in the UW solution during hypothermic machine perfusion, (**B**) TBARS concentrations in plasma at 15, 60, 120, 180 and 240 minutes after normothermic machine perfusion, *p<0,05 significance between CS and all HMP groups, (**C**) TBARS concentrations in the urine at 15, 60, 120, 180 and 240 minutes after normothermic machine perfusion, ^ p<0,05 significant difference between CS and HMP21 and 100%, @ p<0,05 significance between HMP0 and HMP21%, $ p<0,05 significant difference between CS, HMP0% and HMP21%. TBARS, thiobarbituric acid-reactive substances; CS, cold storage; HMP 0%, hypothermic machine perfusion with no oxygen; HMP 21%, hypothermic machine perfusion oxygenated with air; HMP 100%, hypothermic machine perfusion with 100% oxygen. The data are shown as mean±SD.

Figure 1a

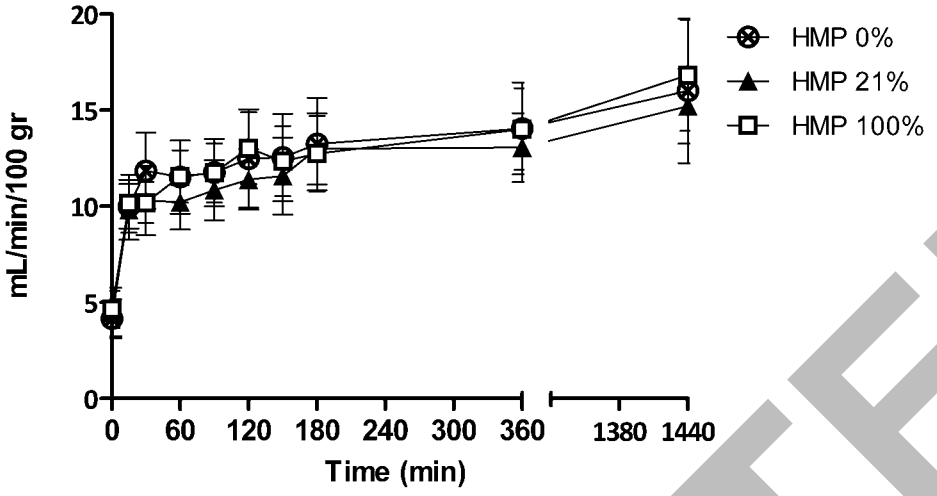


Figure 1b

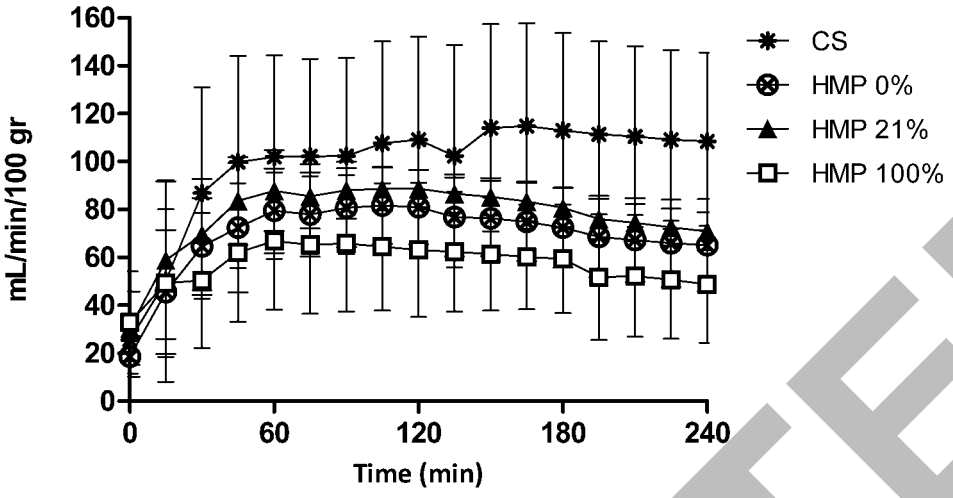
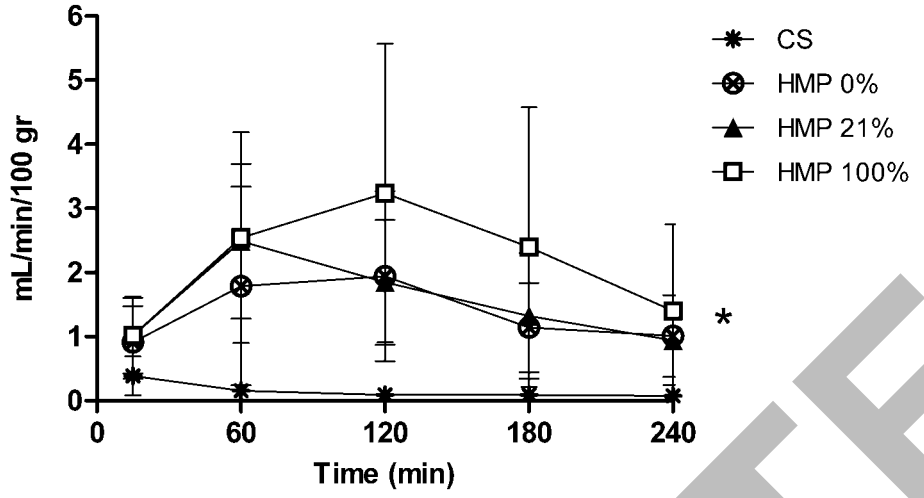


Figure 2a



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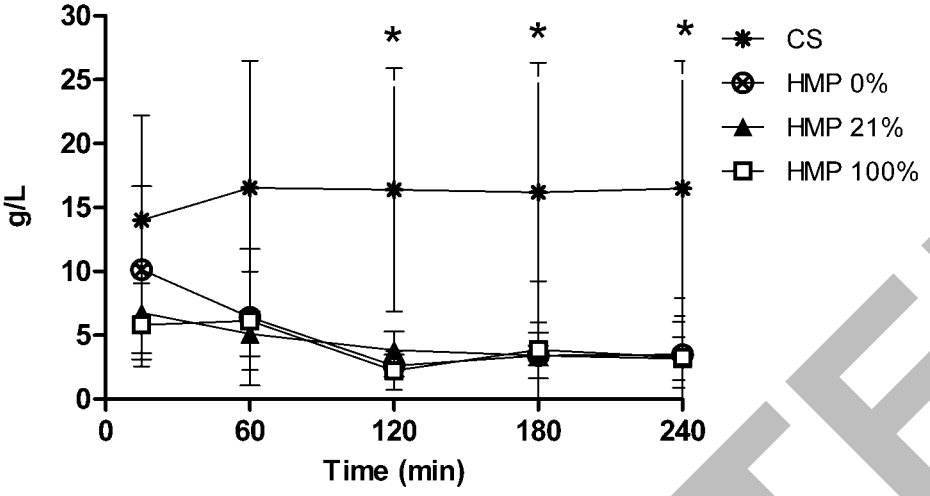
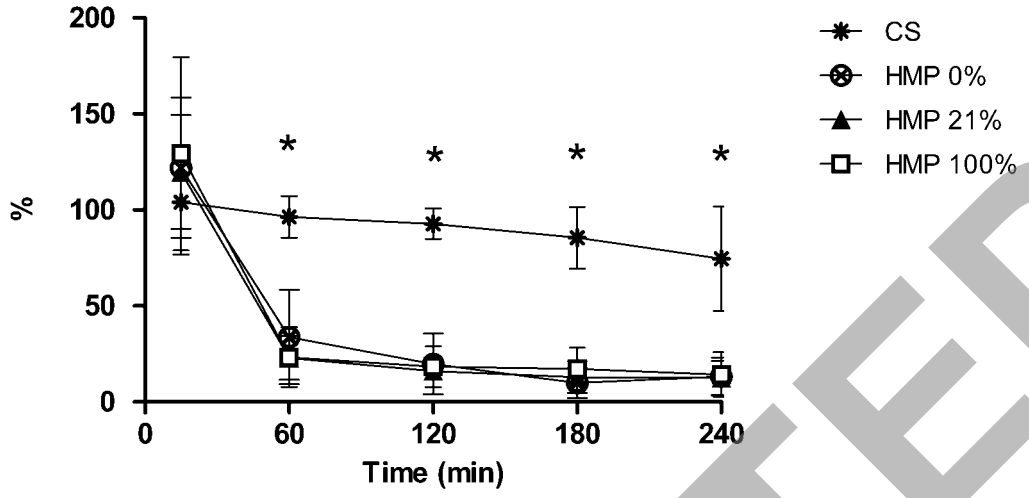


Figure 2c



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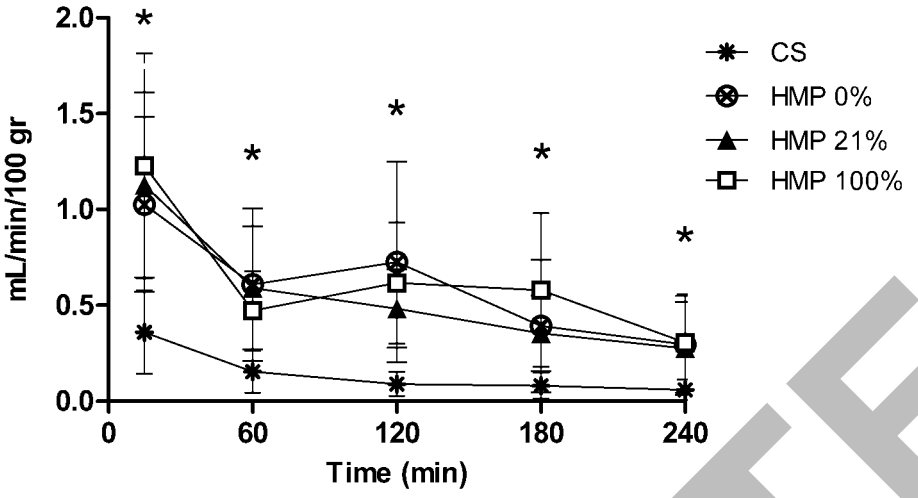

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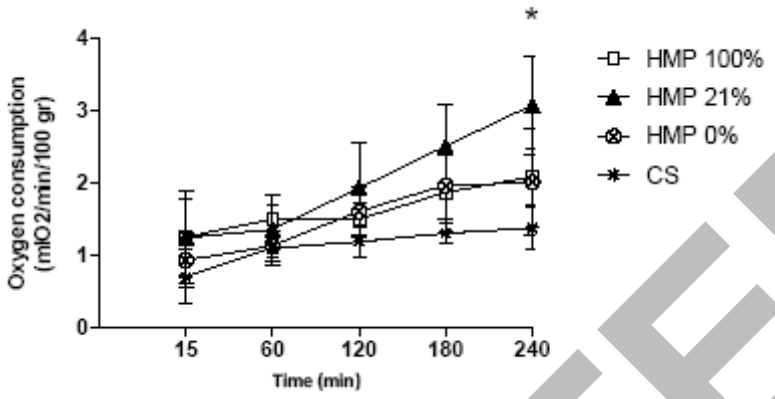
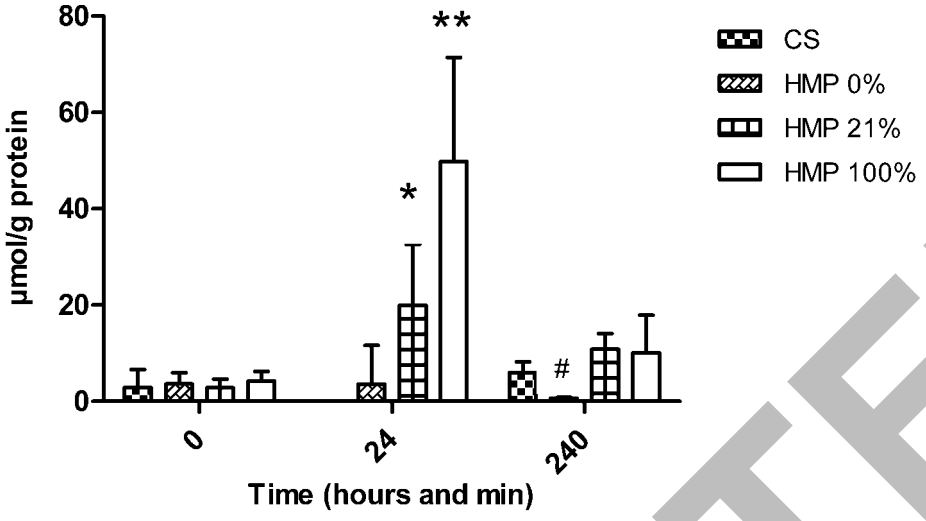


Figure 3b



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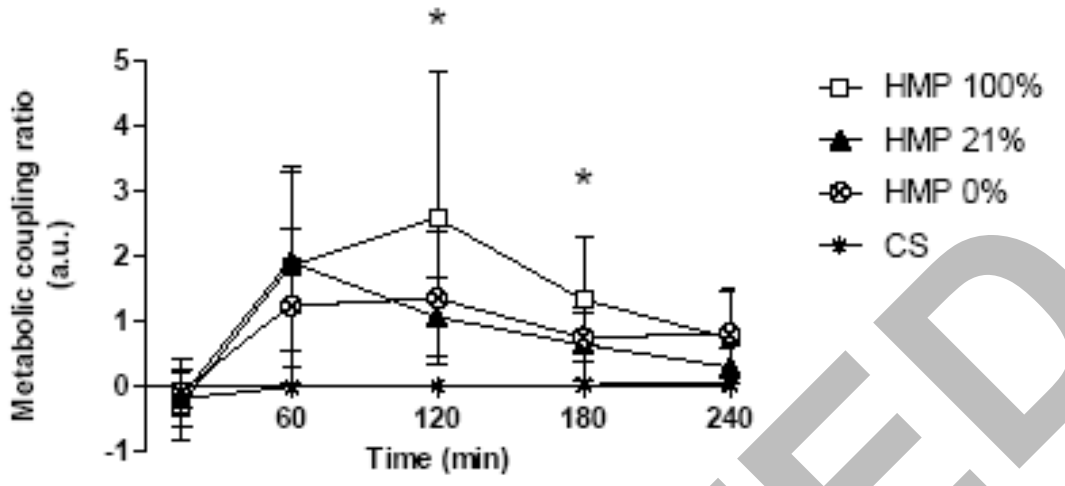

Figure 4a

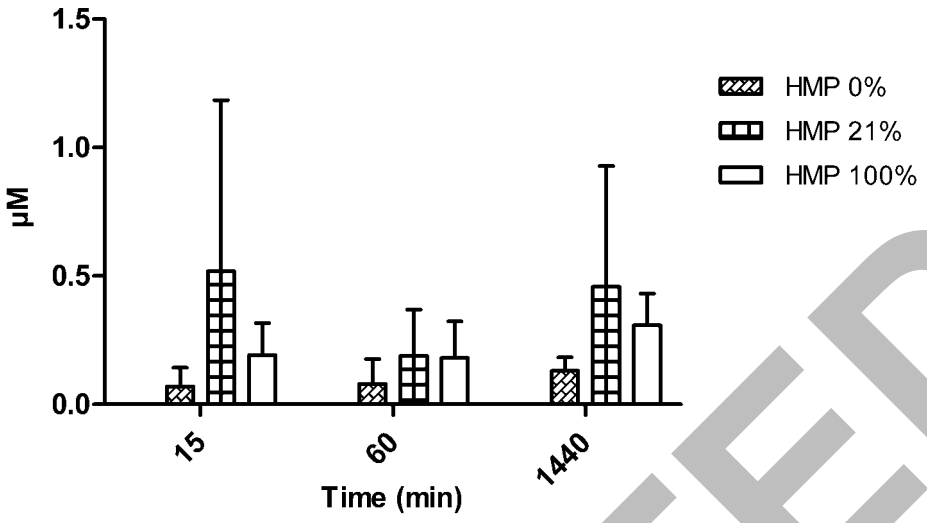

Figure 4b

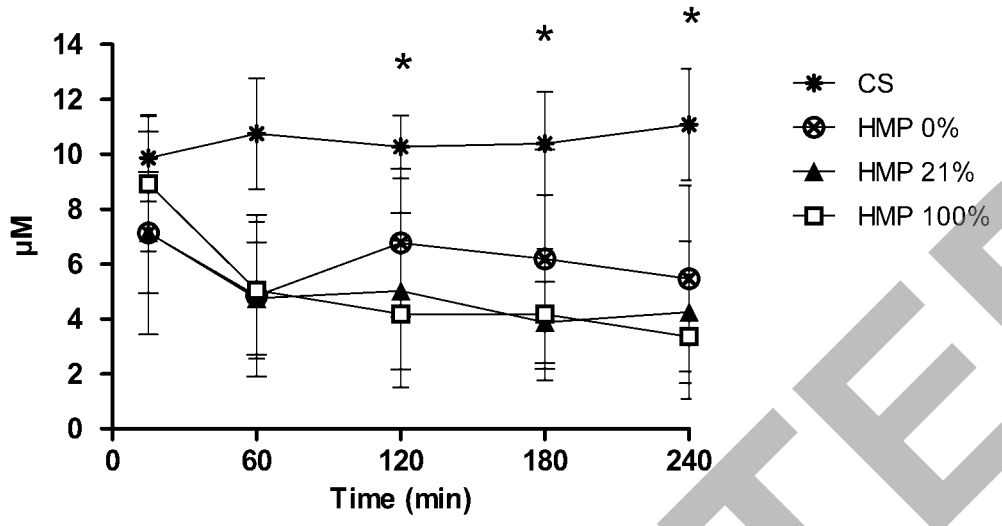

Figure 4c