Transplantation Publish Ahead of Print

DOI: 10.1097/TP.0000000000003051

Influence of different partial pressures of oxygen during continuous hypothermic machine perfusion in a pig kidney ischemia-reperfusion autotransplant model.

Tom Darius, MD[1,2], Martial Vergauwen, BSc[2], Thomas B. Smith, MRes[3], Kamlesh Patel, MBChB[4], Julie Craps, PhD[5], Virginie Joris, Res[6], Selda Aydin, MD, PhD[7], Benoît Ury, Res[8], Antoine Buemi, MD[1,2], Martine De Meyer, MD[1], Jay Nath, MD, PhD[4], Christian Ludwig, PhD[3], Chantal Dessy, PhD[6], Marie-Christine Many, PhD[5], Pierre Gianello, MD, PhD[2], Michel Mourad, MD, PhD[1,2]

[1]Surgery and Abdominal Transplant Unit, University Clinics Saint Luc, Université catholique de Louvain, Brussels, Belgium,

[2]Pôle de Chirurgie Expérimentale et Transplantation, Université catholique de Louvain, Brussels, Belgium,

[3]The Institute of Metabolism and Systems Research (IMSR), University of Birmingham, United Kingdom.

[4]Department of Renal Surgery, Queen Elizabeth Hospital Birmingham, United Kingdom.

[5]Department of Morphology, Experimental and Clinical Research Institute, Université catholique de Louvain, Brussels, Belgium.

[6]Department of pharmacology and therapeutics, Experimental and Clinical Research Institute, Université catholique de Louvain, Brussels, Belgium.

[7]Department of Pathology, University Clinics Saint Luc, Université catholique de Louvain, Brussels, Belgium.

[8]Walloon Excellence in Life Sciences and Biotechnology (WELBIO), Université catholique de Louvain, Brussels, Belgium.

Correspondence: Tom Darius, MD, Physical mailing address: Dr. Tom Darius, Chirurgie et Transplantation Abdominale, Clinique Universitaire Saint Luc, Avenue Hippocrate 10, 1200 Brussels, Belgium. Email address: tom.darius@uclouvain.be

Clinical trial notation: not applicable.

Authorship page

Authorship:

TD, MM, PG designed research.

TD, MV, MM, TS, VJ, JC, BU performed research.

TD, TS, KP, SA, JN, CL, VJ, JC, BU analyzed data.

TD wrote the manuscript.

All authors reviewed the manuscript prior to submission.

Disclosure: The authors of this manuscript have conflicts of interest to disclose as described by Transplantation. The LifePort Kidney Transporter, the Perfusion Circuits and the oxygenators were supplied by Organ Recovery Systems (Diegem, Belgium). Several authors (TD, MV, TS, KP, AB, JN, CL, PG, MM) have ongoing research in part funded by Organ Recovery Systems.

Funding: Tom Darius received a grant from the "Fondation Saint Luc", the "Fondation Recherche Clinique" and Astellas Belgium. Benoît Ury received funding from "Fonds pour la formation à la Recherche dans l'Industrie et dans l'Agriculture (FRIA)".

Abbreviations page

^{1}H-NMR: Proton Nuclear Magnetic Resonance

ANOVA: Analysis of variance

AST: Aspartate Transaminase

ATP: Adenosine triphosphate

AUC: Area under the curve

DCD: Donation after Cardiocirculatory Death

HO-1: Heme Oxygenase 1

HIF-1α: Hypoxia-Inducible Factor 1α

HMP: Hypothermic Machine Perfusion

$HMPO_2$: Oxygenated Hypothermic Machine Perfusion

$HMPO_2$low: Low Oxygenated Hypothermic Machine Perfusion

$HMPO_2$high: High Oxygenated Hypothermic Machine Perfusion

KPS-1: Kidney Perfusion Solution-1

KT: Kidney Transplantation

LC-ESI-MS: Liquid Chromatography Electrospray Ionization Mass Spectrometry

LDH: Lactate dehydrogenase

MMRM: Mixed model for Repeated Measures

NAD^+: Oxidized Nicotinamide Adenine Dinucleotide

NADH: Reduced Nicotinamide Adenine Dinucleotide

NADPH oxidase: Nicotinamide Adenine Dinucleotide Phosphate Oxidase

NMR: Nuclear Magnetic Resonance

NOX2: NAPDH oxidase 2

NOX4: NADPH oxidase 4

pO_2: Partial pressure of oxygen

Prdx1: Peroxiredoxin 1

ROS: Reactive oxygen species

SCS: Static cold storage

SOD1: Superoxide Dismutase 1

Abstract

Background

The optimal perfusate partial oxygen pressure (pO_2) during hypothermic machine perfusion (HMP) is unknown. The aims of the study were to determine the functional, metabolic, structural and flow dynamic effects of low and high perfusate pO_2 during continuous HMP in a pig kidney ischemia-reperfusion autotransplant model.

Methods

The left kidneys of a ±40 kg pigs were exposed to 30 minutes of warm ischemia and randomized to receive 22h HMP with either low perfusate pO_2 (30% oxygen, $HMPO_2$low)(n=8) or high perfusate pO_2 (90% oxygen, $HMPO_2$high)(n=8), prior to autotransplantation. Kidneys stored in 22h standard HMP (n=6) and 22h static cold storage (SCS)(n=6) conditions served as controls. The follow-up after autotransplantation was 13 days.

Results

High pO_2 resulted in a 3- and 10-fold increase in perfusate pO_2 compared with $HMPO_2$low and standard HMP, respectively. Both oxygenated HMP groups were associated with superior graft recovery compared with the control groups. Oxygenation was associated with a more rapid and sustained decrease in renal resistance. Whilst there was no difference in functional outcomes between both oxygenated HMP groups, there were clear metabolic differences with an inverse correlation between oxygen provision and the concentration of major central metabolites in the perfusion fluid but no differences were observed by oxidative stress and metabolic evaluation on preimplantation biopsies.

Conclusions:

Whilst this animal study does not demonstrate any advantages for early graft function for high perfusate pO_2, compared with low perfusate pO_2, perfusate metabolic profile analysis suggests that aerobic mechanism is better supported under high perfusate pO_2 conditions.

Introduction

Machine preservation of kidneys during the organ preservation period is a subject of great clinical interest. More effective perfusion strategies could minimize the harmful effects of ischemia-reperfusion injury, hence improving both early and late allograft function. This is relevant in an era of high-risk donor organs, which are associated with increased rates of primary nonfunction, delayed graft function and increased rates of early graft lost.[1]

The optimal perfusion condition for kidneys has not yet been established. Important perfusion variables, such as storage temperature, active oxygen supplementation, perfusion fluid composition and start time of perfusion (initial, continuous or end ischemic) are under scrutiny. Our group has recently compared various normothermic and hypothermic preservation techniques in a porcine ischemia-reperfusion model.[2] Continuous oxygenated hypothermic machine perfusion (HMP) applied from the time of kidney procurement until transplant demonstrated to be the best preservation strategy to improve early graft function compared with static cold storage (SCS) alone or continuous standard HMP and compared with different end-ischemic preservation strategies (including hypothermic oxygenated perfusion and normothermic perfusion).[2] Although several preclinical studies have similarly shown a beneficial effect of supplemental oxygenation (continuous or end ischemic) for early graft function,[3-7] there is evidence signifying the promotion of aerobic metabolism and restoration of cellular adenosine triphosphate (ATP) only occurs at higher oxygen tension.[8,9]

The optimal perfusate partial oxygen pressure (pO_2) during HMP is unknown. We therefore aim to evaluate the impact of different perfusate oxygen concentrations during continuous HMP on early graft function, tubular function, serum biomarkers, renal flow, resistance and pO_2 during machine perfusion, perfusate metabolic profile and

oxidative stress, metabolic evaluation and graft histology of preimplantation biopsies in a pig kidney ischemia-reperfusion autotransplant model.

Methods

Animals

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

Study design

A standardized ischemia-reperfusion pig kidney autotransplant model was used (Figure 1).

Anesthetic and surgical protocol

Both protocols were already described in detail before.[2] Briefly, 30 minutes warm ischemia was induced in the left kidney by vascular clamping of the renal vessels. Thereafter the kidney was removed and flushed with 200 ml of Kidney Perfusion Solution (KPS-1, Organ Recovery Systems, Diegem, Belgium) at 4°C at a hydrostatic pressure of 100 cm H_20. The kidney was stored for a period of approximately 22 hours by 1 of the 4 studied preservation strategies: 1) 22-hour SCS (n=6), 2) 22-hour standard (no active oxygen supply) HMP (n=6), 3) 22-hour active oxygenated HMP (30% oxygen, $HMPO_2$low)(n=8), and 4) 22-hour active oxygenated HMP (90% oxygen, $HMPO_2$high)(n=8)(Figure 1). Following preservation, the left kidney was auto-transplanted into the right renal bed after a right nephrectomy. Postoperative care was identical as previously described.[2]

The surviving animals were euthanized on the 13th day after transplantation by a lethal intravenous injection of T61® (Intervet International, MDS Animal Health, Boxmeer, The Netherlands) under general anesthesia after surgical biopsies of the transplanted kidney.

Machine perfusion

The LifePort Kidney Transporter® (Organ Recovery Systems, Diegem, Belgium) was used for all machine perfusion groups. The circuit was modified to include an external oxygenator (Dideco Perfusion Tubing Systems, Sorin Group, Mirandola, Italy) and heat exchanger in both active oxygenated groups. The KPS-1 solution was pumped through the kidney at a pressure of 30 mmHg. Carbogen (95% O_2/5% CO_2) was used for oxygen supplementation at either 30% or 90% in the low- and high-oxygenated group, respectively. The temperature of kidney and circulating perfusate was maintained at 2° to 8° C.

Outcome Measures

The primary endpoint was early graft function (measured as daily serum creatinine). The secondary endpoints were tubular function (measured by fractional excretion of sodium and protein creatinine ratio in the urine), serum biomarkers (Lactate dehydrogenase (LDH) and Aspartate transaminase (AST)), renal flow and resistance during machine perfusion, perfusate pO_2 levels, perfusate metabolic profile and oxidative stress, metabolic evaluation and graft histology on preimplantation biopsy.

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 and at the end of the machine perfusion. Perfusate pO_2 was measured at start and end of all machine perfusion groups using the I-

STATS®1 (Abbott Point of Care Inc, Princeton, NJ, USA). Glucose, AST and LDH analysis on perfusate were performed by a dry chemistry analyzer Fui Dri-Chem NX500 (Fujifilm Corporation, Tokyo, Japan). For metabolite identification and quantification using Nuclear Magnetic Resonance (NMR) spectroscopy, neat perfusate samples were mixed with a concentrated NMR buffer, granting end concentrations of 0.5 mM DSS, 0.1 M phosphate buffer, 25% D_20 and 2mM imidazole for each sample. Proton Nuclear Magnetic Resonance (^{1}H-NMR) spectra were acquired on a 600-MHz Bruker Avance III NMR spectrometer in 5 mm NMR tubes. The spectra were phase and baseline corrected with Metabolab software.[10] Metabolite identification and quantification was performed using Chenomx NMR Suite (Chenomx, Edmonton, Alberta, Canada). The endpoint perfusate metabolic profile was assessed, with acetate, adenine, alanine, aspartate, formate, gluconate, glutamate, glutathione, glycine, hypoxanthine, isoleucine, lactate, leucine, mannitol, myo-inositol and succinate quantified.

Blood and urine analysis

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

Tissue sampling and analysis

Wedge biopsies were taken at 3 time-points. Firstly, baseline samples were taken before the onset of preservation after ex-vivo blood flush with KPS-1. A second preimplantation sample was taken after 22-hours of preservation with a third end-point sample taken before euthanasia. Biopsies were stored fresh at -80°C until metabolic analysis and also fixed in acidified formal alcohol, dehydrated and embedded in paraffin wax for histology. Protein levels were determined by western blotting using

preimplantation samples of frozen kidney biopsies as previously described.[11] Levels of oxidative enzymes such as Nicotinamide Adenine Dinucleotide Phosphate Oxidase (NADHP) 2 and 4 (NOX2 and NOX4)(Abcam ab31092, Lifespan Biosciences, LS-C313066), Heme Oxygenase 1 (HO-1)(Abcam ab13243), antioxidant enzymes such as Superoxide Dismutase 1 (SOD1)(Abcam ab13498) and Peroxiredoxin 1 (Prdx1) and Reactive oxygen species (ROS) sensitive protein such as Hypoxia-Inducible Factor 1α (HIF-1α)(BD transduction, BD 610959), have been detected and normalized using the levels of β-actin. Metabolites (lactate, succinate, glutamate, adenosine monophosphate (AMP), adenosine diphosphate (ADP), ATP and oxidized and reduced nicotinamide adenine dinucleotide (NAD^+ and NADH, respectively)) were extracted from frozen kidney biopsies by methanol-chloroform extraction and analyzed by Liquid Chromatography Electrospray Ionization Mass Spectrometry (LC-ESI-MS) based on the description by Coulier L et al.[12] Metabolite quantification was performed as described by Veiga-da-Cunha M et al.[13]

Sections of 4 μm were cut and stained with haematoxylin and eosin for evaluation using light microscopy. Sections were scored blindly assessing changes according to the histopathological consensus criteria for preimplantation kidney biopsies[14] and an acute tubular injury score described by SA Hosgood[15] assessing changes in 4 morphological variable: tubular dilatation, tubular debris, vacuolation, and infiltration.

Statistical Methods

The trial follows a parallel design with 4 active groups. No explicit imputation was foreseen, so all analyses were conducted on the observed cases only.

The primary endpoint of early graft function was assessed through analysis of serum creatinine over time using generalized linear model techniques. Serum creatinine values were normalized to 40kg animal body weight prior to use in the analysis to account for

differences due to animal and organ size. Serum creatinine was analyzed using a longitudinal mixed model for repeated measurements (MMRM) with main factors treatment, timepoint and interaction term to test for overall difference in graft function over time between treatment groups. Individual comparison of groups were conducted using contrast analyses within the main MMRM model testing the null hypothesis that the difference in least squares means between 2 groups under investigation was zero (no mean difference between groups). For the contrast analysis, p-values (based on t-test of least squares means) were reported along with a graphical Mean (SE) plot where deemed appropriate. All secondary endpoint variables were similarly modeled to the primary endpoint. In case of variables without longitudinal structure (e.g. area under the curve (AUC)) the MMRM model was reduced to a basis analysis of variance (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 SAS9.2 or 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

Thirty-two autotransplants were performed. Four were excluded from the analysis for death in relation to complications (sudden death [n=1], bleeding central line damage [n=1], necrotizing anal prolapse [n=1], massive bleeding after reperfusion [n=1]). The operative data of the 28 animals are reported in Table 1.

Graft function

The AUC analysis of the serum creatinine from day 1 until 13 after transplantation is shown in Figure 2a. Comparison of the study groups based on the main MMRM demonstrated a significant difference in favor of both oxygenated hypothermic machine

perfusion ($HMPO_2$) groups compared with SCS ($p<0.0001$) and standard HMP ($p<0.0001$). No difference could be demonstrated between both $HMPO_2$ groups ($p=0.51$). The evolution of serum creatinine after transplantation is shown in Figure 2b. No statistically significant difference was observed between the $HMPO_2$high and $HMPO_2$low groups during the follow-up period. During the first 3 days after autotransplantation the serum creatinine in these 2 groups was significantly lower ($p<0.05$) compared with standard HMP and SCS alone.

Tubular reabsorption of sodium was transiently disturbed in all study groups after transplantation but demonstrated a faster decrease to baseline values during the first 3 postoperative days in all machine perfusion strategies compared with the SCS group (Figure 3a). The AUC analysis of the fractional excretion of sodium demonstrated a significant difference between the SCS group and the $HMPO_2$high group (Figure 3b). The same findings were observed for the urinary protein creatinine ratio (Figure 3c and 3d).

Evolution of postoperative serum LDH and AST

In all study groups, the peak of serum LDH was observed at day 1 after transplantation and dropped immediately afterwards, normalizing after 6 days. No significant difference was observed in the postoperative LDH profile after autotransplantation between all the study groups (Figure 4a). The same findings were seen in relation to the postoperative AST profile (Figure 4b).

Renal flow and resistance during machine perfusion

Renal flow during machine perfusion was significantly higher in both $HMPO_2$ groups compared with standard HMP during the entire period of machine perfusion (Figure 5a). No significant difference in renal flow was observed between both $HMPO_2$ groups during the whole period of machine perfusion. The same findings were seen for renal

resistance (Figure 5b).

Perfusate pO_2 levels

High pO_2 resulted in a 3- and 10-fold increase in perfusate pO_2 compared with $HMPO_2$low and standard HMP, respectively (Table 1). The perfusate pO_2 at the start and the end of machine perfusion was 170±4 mmHg and 70±5 mmHg, 217±9 mmHg and 222±8 mmHg and 726±41 mmHg and 718±40 mmHg, respectively for the standard HMP, the $HMPO_2$low and $HMPO_2$high group measured at 37°C.

Perfusate glucose, lactate dehydrogenase and aspartate transaminase

No difference was found in perfusate glucose between the different machine perfusion groups. LDH was comparable between the HMP group and the $HMPO_2$low group, however was significantly lower at the end of machine perfusion in the $HMPO_2$high group compared with the $HMPO_2$low group (p=0.02). Perfusate AST was significantly higher at the end of machine perfusion in the $HMPO_2$low group compared with the $HMPO_2$high (p=0.0007) and HMP group (p=0.0007) (Figure 6).

^{1}H-NMR: Quantification of perfusate metabolites concentrations

^{1}H-NMR analysis was used to determine the concentration of metabolites within the circulating perfusate at the end of perfusion (Table S1, SDC, http://links.lww.com/TP/B842). A significantly lower concentration of major central metabolites lactate and formate was observed in the $HMPO_2$high group compared with the standard HMP and the $HMPO_2$low groups. Significantly reduced concentrations of succinate and acetate were observed in both active oxygenated groups compared to the standard HMP group (Figure 7).

Oxidative stress and metabolic evaluation on preimplantation biopsies

HIF-1α expression on preimplantation biopsies by western blotting was significantly lower in both $HMPO_2$ groups compared with the SCS group (Figure 8). NOX-2 expression was significantly lower in the standard HMP and $HMPO_2$low group compared with standard SCS (p=0.02 and p=0.03, respectively). No significant difference in NOX4, HO-1, SOD1 and Prdx1 protein expressions have been observed between all study groups.

Metabolic quantification by LC-ESI-MS demonstrated that central metabolites succinate and glutamate at the end of the preservation were significantly lower in both HMPO2 groups compared with the SCS and standard HMP groups (Figure 9a-b). No differences were observed in lactate, ATP, ADP, AMP and NADH and NAD^+ concentrations between all study groups (Figure 9c-f)(data not shown for NADH and NAD^+). Although no significant difference between the groups in terms of ATP production was observed at the end of the preservation, ATP was very low in the SCS group compared with all HMP groups.

Histology

Acute tubular injury assessed by the Banff score was significantly lower on the preimplantation biopsies in favor of the SCS group compared to all machine perfusion groups independent of oxygen supply (p<0.0001), nevertheless no differences were observed between all machine perfusion groups. Assessment of the same preimplantation biopsies by the Hosgood score demonstrated only a significantly lower score in favor of the SCS groups compared to the HMP and $HMPO_2$high groups (p=<0.0001 and p=0.05, respectively). No difference was observed between the SCS and $HMPO_2$low group and also no differences were observed between all machine perfusion groups. No differences were observed between all groups for all other Banff

criteria (e.g. 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 end biopsies (data not shown).

Discussion

This preclinical animal study adds to the body of evidence evaluating the role of oxygenation during HMP. Active perfusate oxygenation ($HMPO_2$low and $HMPO_2$high) resulted in improved early graft function and ex vivo renal flow during machine perfusion compared with SCS alone and standard HMP. Lactate and succinate perfusate analysis by ^{1}H-NMR analysis demonstrated significantly lower concentrations in favor of the $HMPO_2$high group at the end of machine perfusion. Preimplantation biopsies demonstrated a significant reduction of central metabolites succinate and glutamate in both $HMPO_2$ groups compared with standard HMP and SCS alone. No difference could be observed in preimplantation ATP levels between all groups. We found, as expected, that the pO_2 within the acellular perfusion fluid (with no designated oxygen carrier) can be dramatically increased during active oxygenation and is dependent on the percentage of oxygen concentration delivered during HMP. In contrast, during standard HMP, the perfusion fluid oxygen was rapidly diminished, resulting in a relatively hypoxic perfusion environment at the end of the machine perfusion period thus hindering the oxygen dependent processes that occur during HMP. However no significant difference in pro- or anti-oxidants expression (NOX4, HO-1, SOD-1, Pdrx1) was observed between all groups on preimplantation biopsies. In contrast, HIF-1α expression was significantly lower in both $HMPO_2$ groups compared with SCS alone. No important structural differences on the preimplantation and the end biopsies were observed by light microscopy between all machine perfusion groups.

An overview of the literature of preclinical porcine kidney studies investigating different oxygen concentrations during continuous HMP and their effect on early graft function and ex vivo renal flow, using an ex vivo normothermic reperfusion or an autotransplant model is summarized in Table 2 and shows conflicting results.[4,5,7,16] Interestingly, in autotransplant models, the functional benefits of oxygen appear to correlate with the ischemic insult, with comparable functional benefits reported following 30 or 60 minutes of preceding warm ischemia before preservation. In the absence of preceding warm ischemia no benefit of active oxygenation was observed.[7] Despite the improved flow and function demonstrated by oxygenation in this study, we found no difference in functional outcome measures between the low and high perfusate pO_2 conditions.

Chouchani et al demonstrated that mitochondrial succinate accumulation (due to reversed electron transport) originated from the citric acid cycle during ischemia is responsible for the major part of the ROS production during ischemia-reperfusion, independent of the type of organ in the human body.[17,18] In addition, Dutkowski P et al recently described that end-ischemic oxygenated hypothermic liver perfusion results in forward electron transport at the level of the mitochondrial respiratory chain during HMP.[19] This results in a decrease in succinate and the increase of ATP at the end of HMP and subsequent improved graft function. Our study results are in line with these observations demonstrating a significant decrease of central metabolites succinate and glutamate on preimplantation biopsies and an improved initial graft function in the presence of supraphysiological perfusate pO_2 compared with standard HMP or SCS alone. The SCS alone group demonstrated the lowest ATP level at the end of the preservation compared with all HMP groups. The high variability of ATP measurements was probably the main reason that our study was not able to detect a

significant difference between all study groups and could have been introduced during the samples acquisition, storage or extraction process.

Whilst this study does not demonstrate functional differences between the 2 oxygenated conditions, there are clear metabolic consequences of supraphysiological perfusate oxygenation. We found that the perfusate concentration of major central metabolites like lactate, succinate, formate and acetate at the end of machine perfusion inversely correlated with the oxygen concentration added during machine perfusion. Such a phenomenon cannot be explained by the washout of metabolites from the kidney, as this would cause the converse (higher concentrations in high flow oxygenated conditions). One possible explanation could be that in oxygen abundant conditions, the efficiency of cellular metabolism improves and therefore overall anaerobic metabolic activity is diminished. This is supported by previous studies demonstrating that whilst the oxygen dependent Krebs cycle is upregulated in oxygen rich environments, other pathways become less active resulting in an overall metabolic 'quiescence'.[8] This relationship appears to be oxygen dose dependent with maximal amount of Krebs cycle activity and corresponding increase in cellular ATP levels occurring with high flow oxygen**.** Interestingly, whilst Krebs related activity increases, the absolute concentration of the Krebs intermediates themselves, such as succinate and citrate were diminished in the high oxygen groups, highlighting the limitations of isolated metabolite quantification. We are aware that the origin of perfusate succinate is multifactorial, with higher perfusate concentrations indicating any combination of upregulated manufacture, down regulated utilization, increased export or increased release under necrosis.

Cell membrane damage may be inferred by increased LDH level detected in the perfusate.[20,21] Higher levels of LDH may coincide with increased leakage of cellular metabolites into the perfusate. We observed higher levels of perfusate LDH in the standard HMP and $HMPO_2$low groups when compared with the $HMPO_2$high group. Therefore, improvements in cell membrane integrity under high oxygenated HMP may help to explain the reductions in perfusate lactate and succinate seen in this group. To determine the significance of perfusate metabolites, advanced analytical tools are required. Tracer studies using isotopically labeled metabolic substrates are a useful tool, which can elucidate whether a detected metabolite has been produced de novo. Using a combination of NMR and Mass spectrometry and an ex vivo DCD porcine model of HMP, Patel K et al recently demonstrated that perfusate supplementation with high concentration of oxygen (95%) results in a greater degree of aerobic metabolism at the end of machine perfusion versus active aeration (21%).[8] In contrast, we did not add the universally labeled glucose ([U-^{13}C] glucose) to the perfusate fluid in our study protocol. However, in line with their results, we observed in our study that the perfusate lactate concentration by ^{1}H-NMR analysis was significantly lower at the end of machine perfusion in the highly oxygenated compared with the low oxygenated and the nonactive oxygenated HMP group. Lactate is considered as a universal marker of anaerobic metabolism. Therefore, this result can be interpreted that supraphysiological levels of oxygen in the perfusate more efficiently promote a metabolic switch to aerobic metabolism during cold machine perfusion compared to low perfusate oxygen levels.

Hypoxia inducible factors are the universal cellular oxygen-sensitive transcription factors that activate a number of hypoxia responsive genes, including vascular endothelial growth factor, erythropoietin, epidermal growth factor receptor and platelet-derived growth factor.[22] HIF-1α normally accumulates during ischemia but

was significantly decreased in this study in the presence of active oxygenation during HMP.[23] Therefore, HIF-1α expression could play a major role in early and late allograft function after $HMPO_2$, as also previously described.[22] NOX2 is involved in the pathogenesis of ischemia reperfusion injury.[24] NOX2 protein expression is weakly expressed in standard HMP and $HMPO_2$low suggesting that oxidative stress is lower in these groups. NOX2 activation requires subunits interaction (mainly p47-phox and p22-phox) in order to rule on its activation state.

Histological evaluation of the preimplantation biopsies using the Banff score[14] and the Hosgood score[15] focusing on the degree of tubular injury did not correlate with initial graft function in our study groups. This could reflect a limitation of our autotransplant model using young healthy pigs without preexisting kidney disease and only 30 minutes of preceding warm ischemia time before procurement. However, 30 minutes of warm ischemia is similar to the mean first warm ischemia time in most clinical controlled DCD kidney transplant programs and is frequently used in preclinical models.[2,4,16] Tubular reabsorption of sodium was transiently disturbed in all study groups but demonstrated a faster decrease to baseline especially during the first 3 days after transplantation in all machine perfusion strategies, independent of oxygen supply compared with SCS alone. In contrast, acute tubular injury assessed by the Banff score[14] was even significantly lower on the preimplantation biopsies in favor of the SCS group compared with all machine perfusion groups independent of oxygen supply. In conclusion, this ischemia-reperfusion porcine autotransplant model demonstrated a significant functional benefit from active supraphysiological oxygenation during HMP on initial graft. Perfusate metabolic profile analysis suggests that aerobic mechanism is better supported under high perfusate pO_2 conditions compared with standard HMP and $HMPO_2$low but no difference between high and low perfusate pO_2 was observed by

oxidative stress and metabolic evaluation on preimplantation biopsies. The next question to be answered is to determine the optimal start time of active supraphysiological oxygen administration during continuous HMP.

Acknowledgments

The authors are grateful to Vergauwen M and Beaurin G regarding the support during the pig experiments, to De Muylder P regarding the scientific collaboration and logistic support, to Druart S regarding the lab analysis and to Jansen B regarding the statistical analysis. Darius T received a grant from the "Fondation Saint Luc", the "Fondation Recherche Clinique" and Astellas Belgium. Benoît Ury received funding from "Fonds pour la formation à la Recherche dans l'Industrie et dans l'Agriculture (FRIA)".

References

1. Hamed MO, Chen Y, Pasea L, et al. Early graft loss after kidney transplantation: risk factors and consequences. *Am J Transplant.* 2015;15(6):1632-1643.

2. 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(3):752-762.

3. Koetting M, Frotscher C, Minor T. Hypothermic reconditioning after cold storage improves postischemic graft function in isolated porcine kidneys. *Transplant Int.* 2010;23(5):538-542.

4. 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(9):944-950.

5. 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(2):1174-1181.

6. 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(5):815-822.

7. Gallinat A, Paul A, Efferz P, et al. Role of oxygenation in hypothermic machine perfusion of kidneys from heart beating donors. *Transplantation.* 2012;94(8):809-813.

8. Patel K, Smith TB, Neil DAH, et al. The Effects of Oxygenation on Ex Vivo Kidneys Undergoing Hypothermic Machine Perfusion. *Transplantation.* 2019;103(2):314-322.

9. Buchs JB, Lazeyras F, Ruttimann R, et al. Oxygenated hypothermic pulsatile perfusion versus cold static storage for kidneys from non heart-beating donors tested by in-line ATP resynthesis to establish a strategy of preservation. *Perfusion.* 2011;26(2):159-165.

10. Ludwig C, Gunther UL. MetaboLab--advanced NMR data processing and analysis for metabolomics. *BMC Bioinformatics.* 2011;12:366.

11. Craps J, Wilvers C, Joris V, et al. Involvement of nitric oxide in iodine deficiency-induced microvascular remodeling in the thyroid gland: role of nitric oxide synthase 3 and ryanodine receptors. *Endocrinology.* 2015;156(2):707-720.

12. 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(18):6573-6582.

13. 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(4):1241-1250.

14. Liapis H, Gaut JP, Klein C, et al. Banff Histopathological Consensus Criteria for Preimplantation Kidney Biopsies. *Am J Transplant.* 2017;17(1):140-150.

15. Hosgood SA, Shah K, Patel M, et al. The effect of prolonged of warm ischaemic injury on renal function in an experimental ex vivo normothermic perfusion system. *J Transl Med.* 2015;13:207.

16. Venema LH, Brat A, Moers C, et al. Effects of Oxygen During Long-term Hypothermic Machine Perfusion in a Porcine Model of Kidney Donation After Circulatory Death. *Transplantation.* 2019;103(10):2057-2064.

17. Chouchani ET, Pell VR, Gaude E, et al. Ischaemic accumulation of succinate controls reperfusion injury through mitochondrial ROS. *Nature.* 2014;515(7527):431-435.

18. Chouchani ET, Pell VR, James AM, et al. A Unifying Mechanism for Mitochondrial Superoxide Production during Ischemia-Reperfusion Injury. *Cell Metab.* 2016;23(2):254-263.

19. Dutkowski P, Guarrera JV, de Jonge J, et al. Evolving Trends in Machine Perfusion for Liver Transplantation. *Gastroenterology.* 2019;156(6):1542-1547.

20. Kumar P, Nagarajan A, Uchil PD. Analysis of Cell Viability by the Lactate Dehydrogenase Assay. *Cold Spring Harb Protoc.* 2018;(6). doi: 10.1101/pdb.prot095497.

21. Chan FK, Moriwaki K, De Rosa MJ. Detection of necrosis by release of lactate dehydrogenase activity. *Methods Mol Biol.* 2013;979:65-70.

22. Akhtar MZ, Sutherland AI, Huang H, et al. The role of hypoxia-inducible factors in organ donation and transplantation: the current perspective and future opportunities. *Am J Transplant.* 2014;14(7):1481-1487.

23. Nangaku M, Eckardt KU. Hypoxia and the HIF system in kidney disease. *J Mol Med.* 2007;85(12):1325-1330.

24. Karim AS, Reese SR, Wilson NA, et al. Nox2 is a mediator of ischemia reperfusion injury. *Am J Transplant.* 2015;15(11):2888-2899.

Tables

Table 1: Operative data for 28 pigs in an ischemia reperfusion autotransplant model according to the study group. HMP, hypothermic machine perfusion; $HMPO_2$low, low oxygenated hypothermic machine perfusion; $HMPO_2$high, high oxygenated hypothermic machine perfusion; SCS, static cold storage.

Table 2: Overview of preclinical pig studies investigating different oxygen concentrations during continuous hypothermic machine preservation of kidneys using an ex vivo normothermic reperfusion or an autotransplant model. HMP, hypothermic machine perfusion; $HMPO_2$, oxygenated hypothermic machine perfusion; NA, not available; pO_2, partial pressure of oxygen; SCS, static cold storage; WI, warm ischemia.

Figure Legends

Figure 1: Study design of an ischemia-reperfusion pig kidney autotransplant model to evaluate the impact of different oxygen pressures during continuous hypothermic machine perfusion on early graft function. HMP, hypothermic machine perfusion; $HMPO_2$, oxygenated hypothermic machine perfusion; KPS-1: Kidney Perfusion Solution-1; KT, Kidney transplantation; SCS, static cold storage.

Figure 2: Area under the curve analysis of serum creatine from day 1 until day 13 after transplantation according to the study group demonstrating that both continuous active oxygenated study groups significantly improved early graft function compared to SCS alone and passive aerated HMP (a). Evolution of daily postoperative serum creatinine in all study groups (b). Significance of $p<0.05$ between HMP and $HMPO_2$low group is indicated with*

Significance of $p<0.05$ between HMP and $HMPO_2$high group is indicated with$^{\Delta}$. HMP, hypothermic machine perfusion; $HMPO_2$, oxygenated hypothermic machine perfusion; SCS, static cold storage.

Figure 3: 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 natrium; HMP, hypothermic machine perfusion; $HMPO_2$, oxygenated hypothermic machine perfusion; SCS, static cold storage.

Figure 4: Evolution of posttransplant serum LDH and AST in the continuous hypothermic machine perfusion groups with or without active oxygenation and the static cold storage group (a and b, respectively). AST, aspartate transaminase; HMP, hypothermic machine perfusion; $HMPO_2$, oxygenated hypothermic machine perfusion; LDH, lactate dehydrogenase; SCS, static cold storage.

Figure 5: Influence of oxygen supply on ex vivo renal flow (a) and renal resistance (b) during continuous hypothermic machine perfusion with or without active oxygenation. HMP, hypothermic machine perfusion; $HMPO_2$, oxygenated hypothermic machine perfusion.

Figure 6: Evolution of perfusate glucose, LDH and AST during continuous hypothermic machine perfusion with and without active oxygenation (a, b, c, respectively). Significance of $p<0.05$ between $HMPO_2$ low and $HMPO_2$ high group is indicated with* and between the $HMPO_2$ low and the HMP group with$^{\Delta}$.

AST, aspartate transaminase; HMP, hypothermic machine perfusion; $HMPO_2$, oxygenated hypothermic machine perfusion; LDH, lactate dehydrogenase.

Figure 7: Concentrations of lactate, succinate, formate and acetate in circulating perfusion fluid at the end of machine perfusion in all machine perfusion groups. HMP, hypothermic machine perfusion; $HMPO_2$, oxygenated hypothermic machine perfusion.

Figure 8: HIF-1α expression on preimplantation biopsies by western blotting was significantly lower in both oxygenated HMP groups compared with the SCS group. NOX-2 expression was significantly lower in the standard HMP and $HMPO_2$low group compared with standard SCS. No significant difference in NOX4, HO-1, SOD1 and Prdx1 protein expressions have been observed between all study groups.

HMP, hypothermic machine perfusion; $HMPO_2$, oxygenated hypothermic machine perfusion; SCS, static cold storage.

Figure 9: Metabolic quantification by LC-ESI-MS demonstrated that central metabolites succinate (a) and glutamate (b) at the end of the preservation were significantly lower in both oxygenated HMP groups compared with the SCS and standard HMP groups. No differences were observed of lactate (c), ATP (d), ADP (e) and AMP (f) in all study groups. Although no significant difference between the groups in terms of ATP production was observed at the end of the preservation, ATP was very low in the SCS group compared with all HMP groups.

AU, arbitrary unit; HMP, hypothermic machine perfusion; $HMPO_2$, oxygenated hypothermic machine perfusion; LC-ESI-MS, Liquid Chromatography Electrospray Ionization Mass Spectrometry; SCS, static cold storage; TIC, total ion current.

Supplemental digital content

Table S1.

Table 1: Operative data for 28 pigs in an ischemia-reperfusion autotransplant model according to the study group.

Variable	22 h SCS (n=6)	22 h HMP (n=6)	22 h HMPO_2low (n=8)	22 h HMPO_2high (n=8)	p-value
Weight pig, kg	40.40±5.57	39.23±2.00	43.29±2.91	43.83±7.30	0.29
Left nephrectomy procedure time, h:m	3:41±0:46	3:16±0:31	3:31±0:42	3:31±0:26	0.72
Weight left kidney, mg - before preservation - after preservation	 89±10 80±9	 100±13 151±22	 104±14 154±22	 109±18 149±30	0.08
Perfusate pO_2, mmHg (measured at 37°C) - at start HMP - at end HMP	 	 170±4 70±5	 217±9 222±8	 726±41 718±40	 <0.0001 0.02
Right nephrectomy and autotransplant procedure time, h:m	3:04±0:38	3:52±0:54	3:31±0:56	2:54±0:45	0.72
Cold ischemic time, h:m	21:56±0:37	22:40±0:36	22:41±0:22	20:51±0:57	0.12
Anastomotic time, min	30±5	32±4	28±3	26±3	0.20
Total ischemic time, h:m	22:56±0:35	23:42±0:35	23:20±0:24	23:09±0:29	0.17

Table 2: Overview of preclinical pig studies investigating different oxygen concentrations during continuous hypothermic machine preservation of kidneys using an ex vivo normothermic reperfusion or an autotransplant model.

	Author, year of publication	Model	Study groups (number per group)	PaO2, mmHg at 37°C	Initial graft function (clearance*, serum creatinine**)	Evolution of renal flow during HMP	Renal flow at the end
Ex Vivo Normothermic Reperfusion model	Hoyer DP et al, 2014[4]	30min WI + 21 hr preservation	1. 21 hr HMP 0% O_2 (n=5) 2. 21 hr HMP air (n=5) 3. 21 hr HMP 100% O_2 (n=5)	0 200-220 760-800	*HMP 100% O_2 provides the best initial graft function, followed by HMP 20% O_2 and HMP 0% O_2.	Fastest increase of renal flow using HMP 100% O_2, followed by HMP20% O_2 and HMP0% O_2.	HMP100% O_2 and HMP20% significantly higher than HMP0% O_2.
	Venema LH et al, 2018[16]	30min WI + 24 hr preservation	1. 24 hr SCS (n=6) 2. 24 hr HMP 0% O_2 (n=6) 3. 24 hr HMP 21% O_2 (n=6) 4. 24 hr HMP 100% O_2 (n=6)	- 0 NA NA	*All HMP groups significantly better than SCS group. *No difference between all HMP groups.	NA	NA
Autotransplant model	Gallinat A et al, 2012[17]	No WI + 21 hr preservation	1. 21 hr HMP 0% O_2 (n=5) 2. 21 hr HMP 100% O_2 (n=5)	0 > 500	*Significantly better after HMP0%O2 compared to HMP100% O_2.	NA	NA
	Thuillier R et al, 2013[5]	60min WI + 22 hr preservation	1. 22 hr HMP 0% O_2 (n=4) 2. 22 hr HMP 100% O_2 (n=4)	0 NA	**HMP100% O_2 lower serum creatinine peak and faster return to normal levels compared to HMP 0% O_2.	NA	No difference between HMP100% O_2 and the HMP0% O_2 group.

Abbreviations: HMP, hypothermic machine perfusion; HMPO_2, oxygenated hypothermic machine perfusion; NA, not available; PaO_2, partial oxygen pressure; SCS, static cold storage; WI, warm ischemia.

	Darius T et al, 2019		30min WI + 22 hr preservation	1. 22 hr SCS (n=6) 2. 22 hr standard HMP (n=6) 3. 22 hr $HMPO_2$low (n=8) 4. 22 hr $HMPO_2$high (n=8)	- 65-75 210-230 680-760	*$HMPO_2$low and $HMPO_2$high demonstrated significant better initial graft function compared to standard HMP. *All HMP groups demonstrated significant better initial graft function compared to SCS.	Faster increase in both $HMPO_2$high and $HMPO_2$low groups compared to standard HMP, but no significant difference between $HMPO_2$high and $HMPO_2$low.	

Figure 1

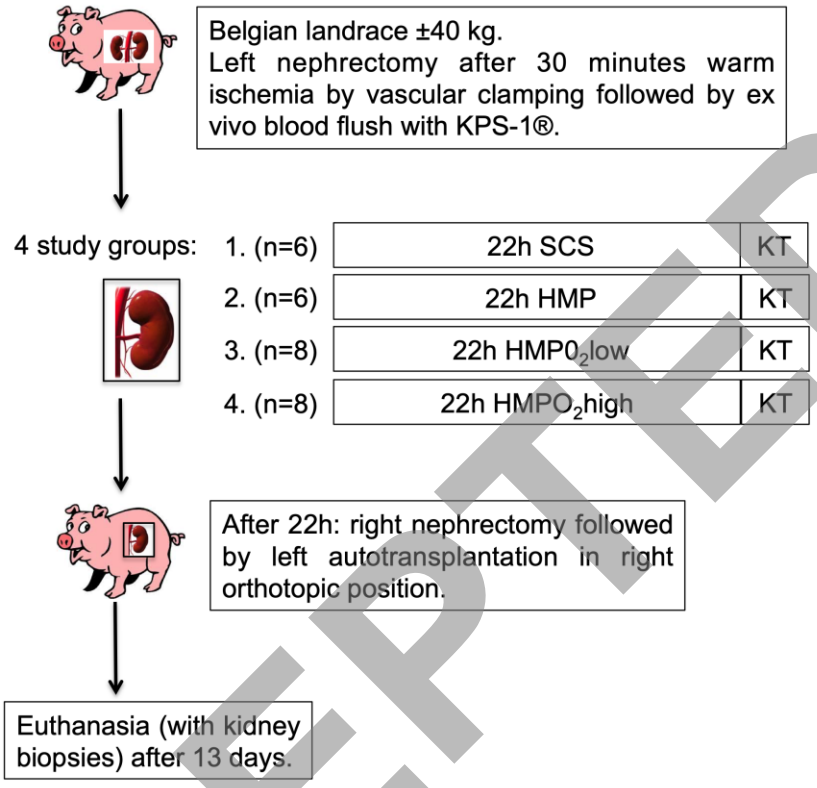

Figure 2

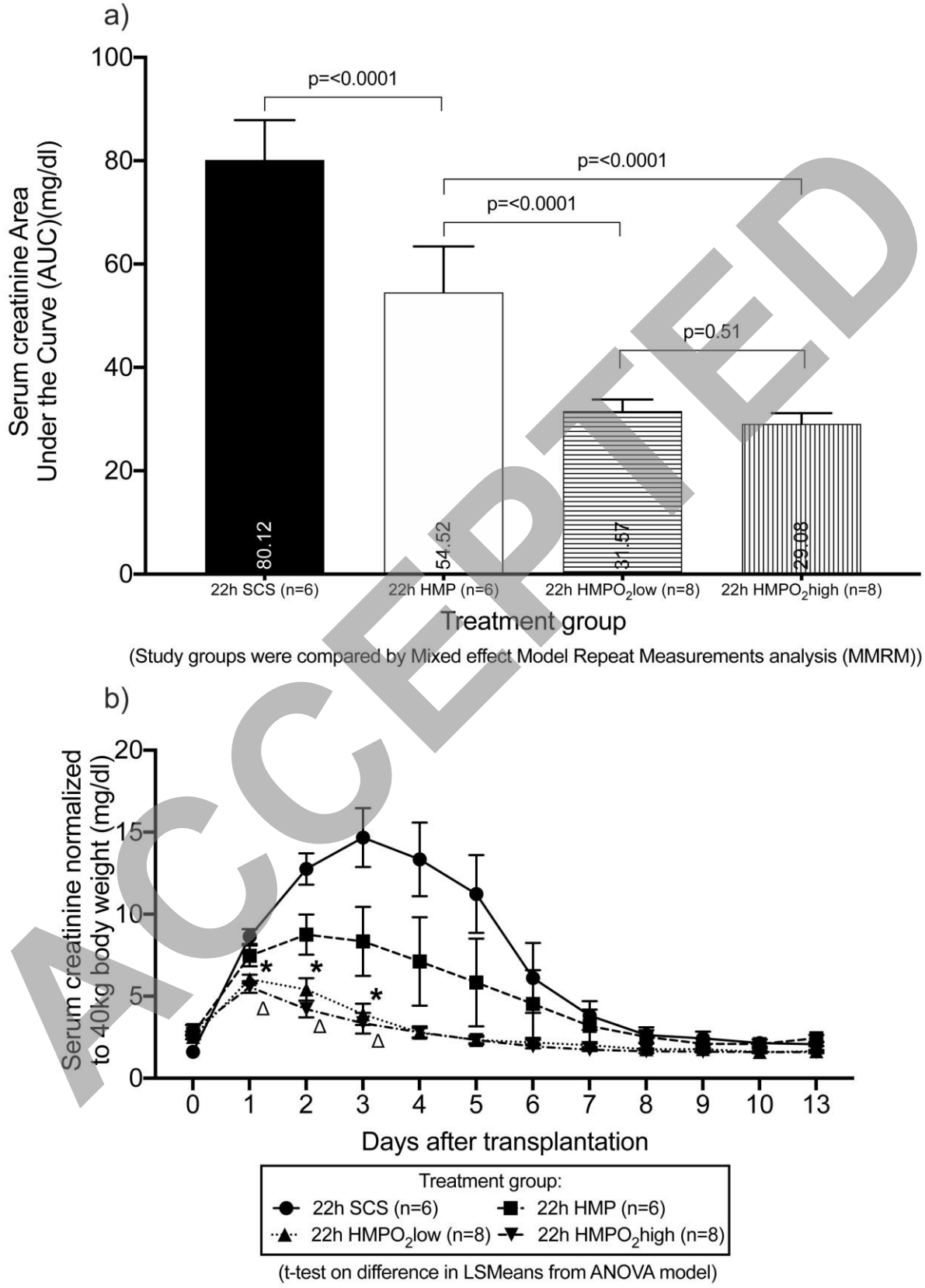

Figure 3

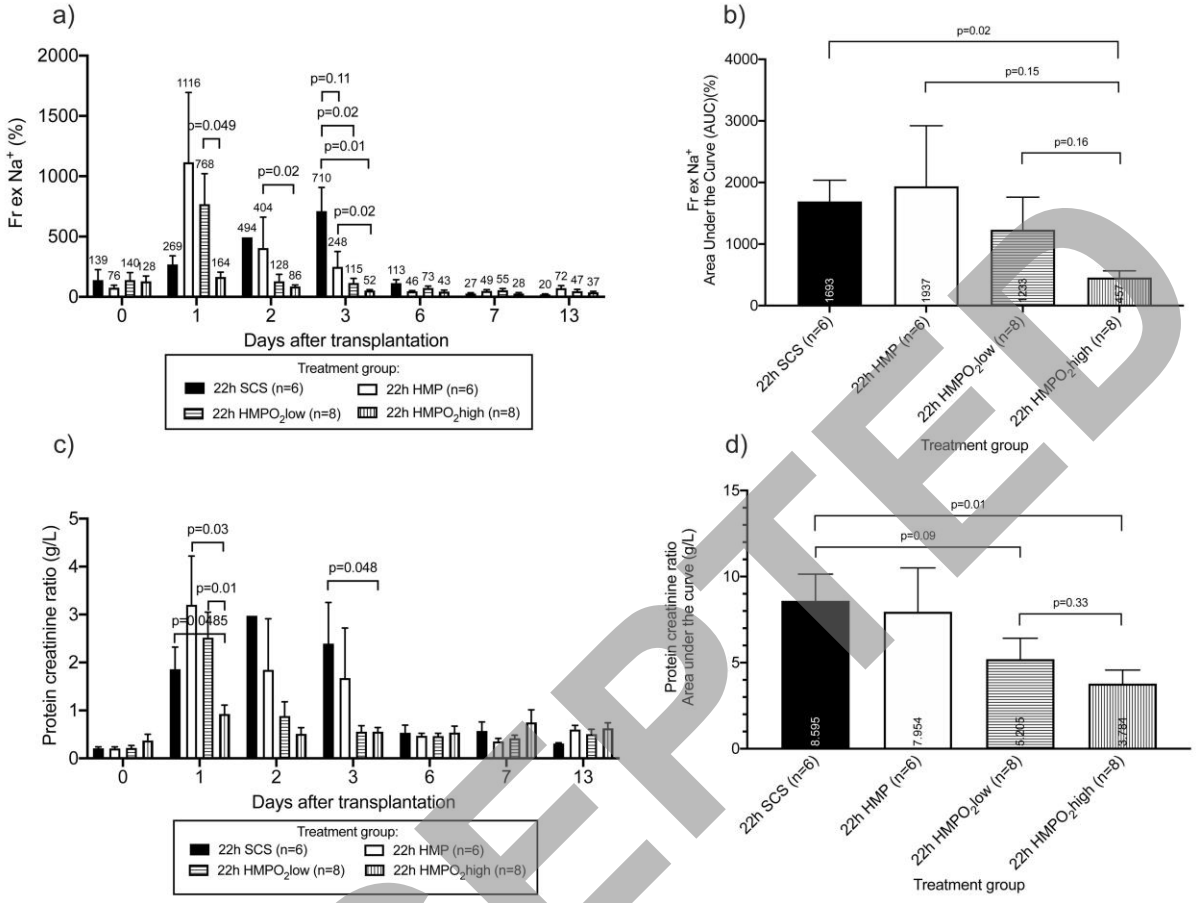

Figure 4

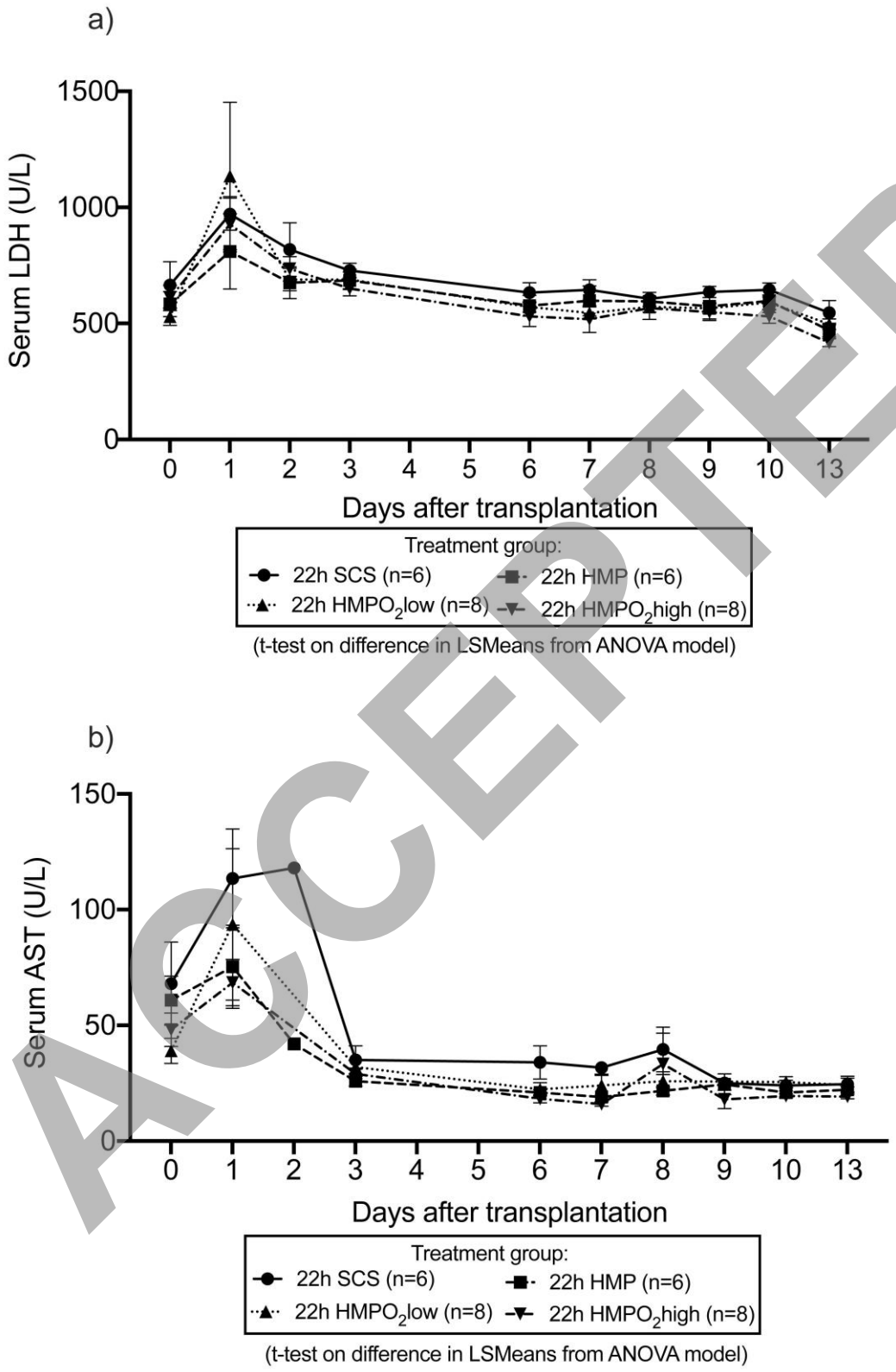

Figure 5

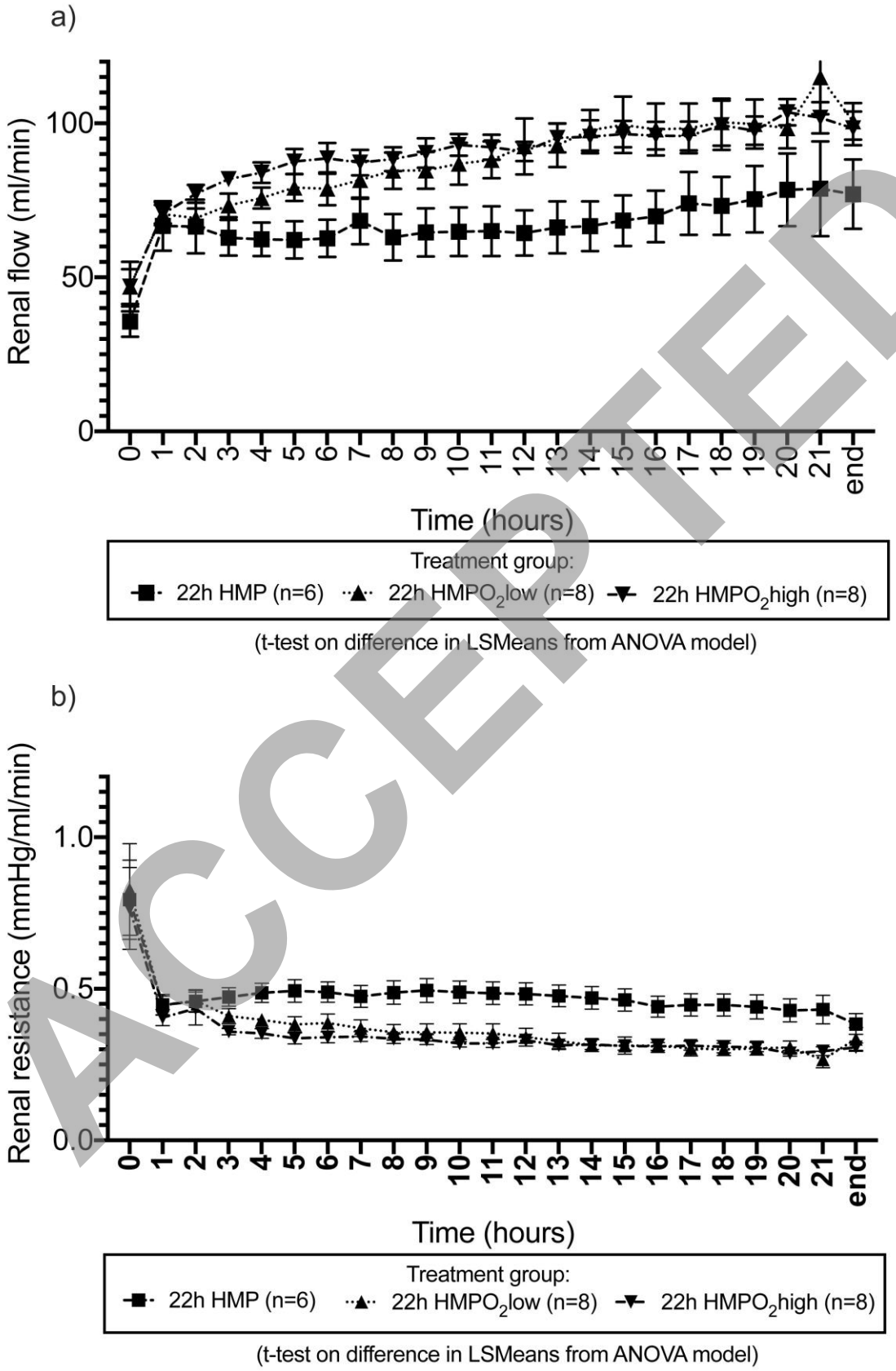

Figure 6

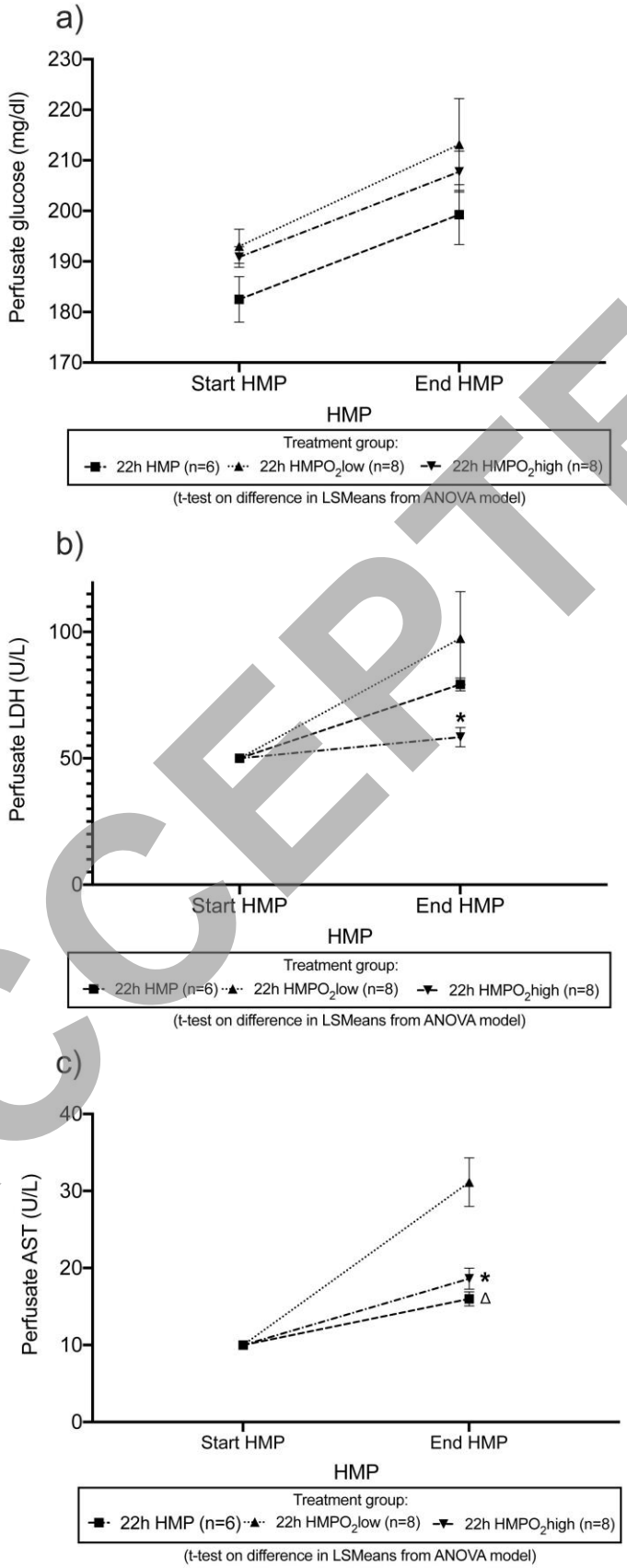

Figure 7

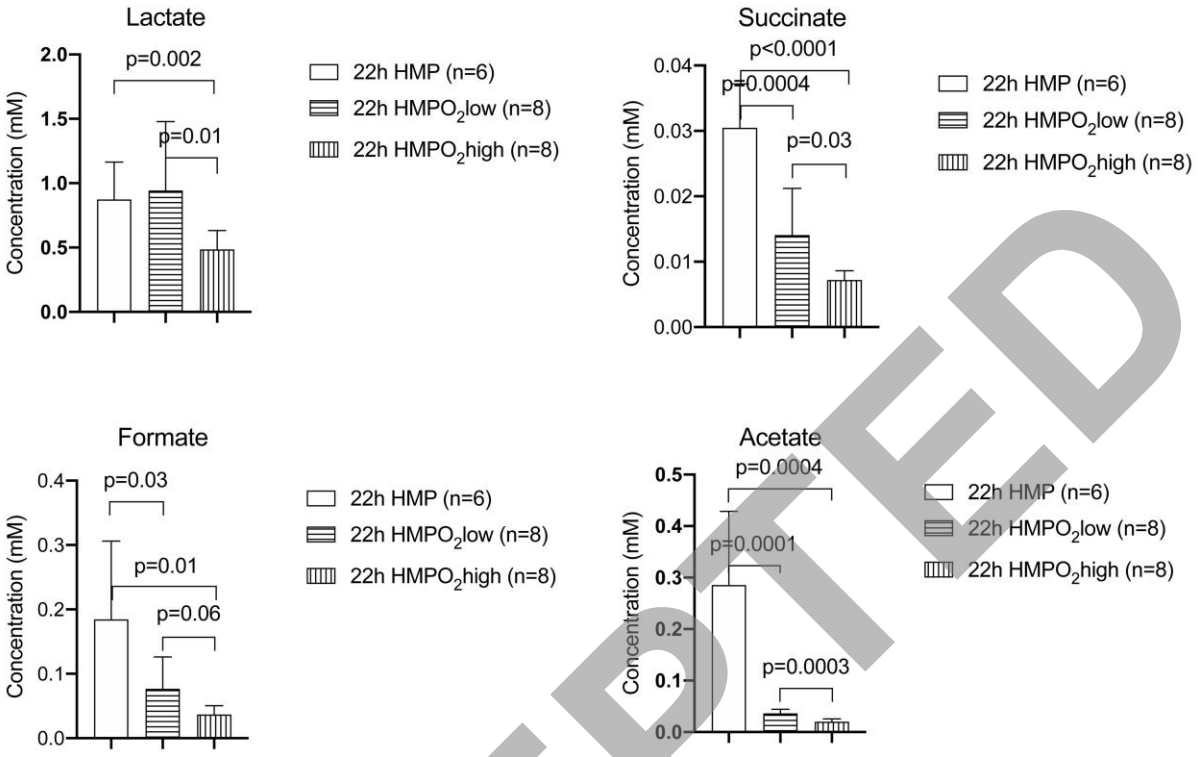

Figure 8

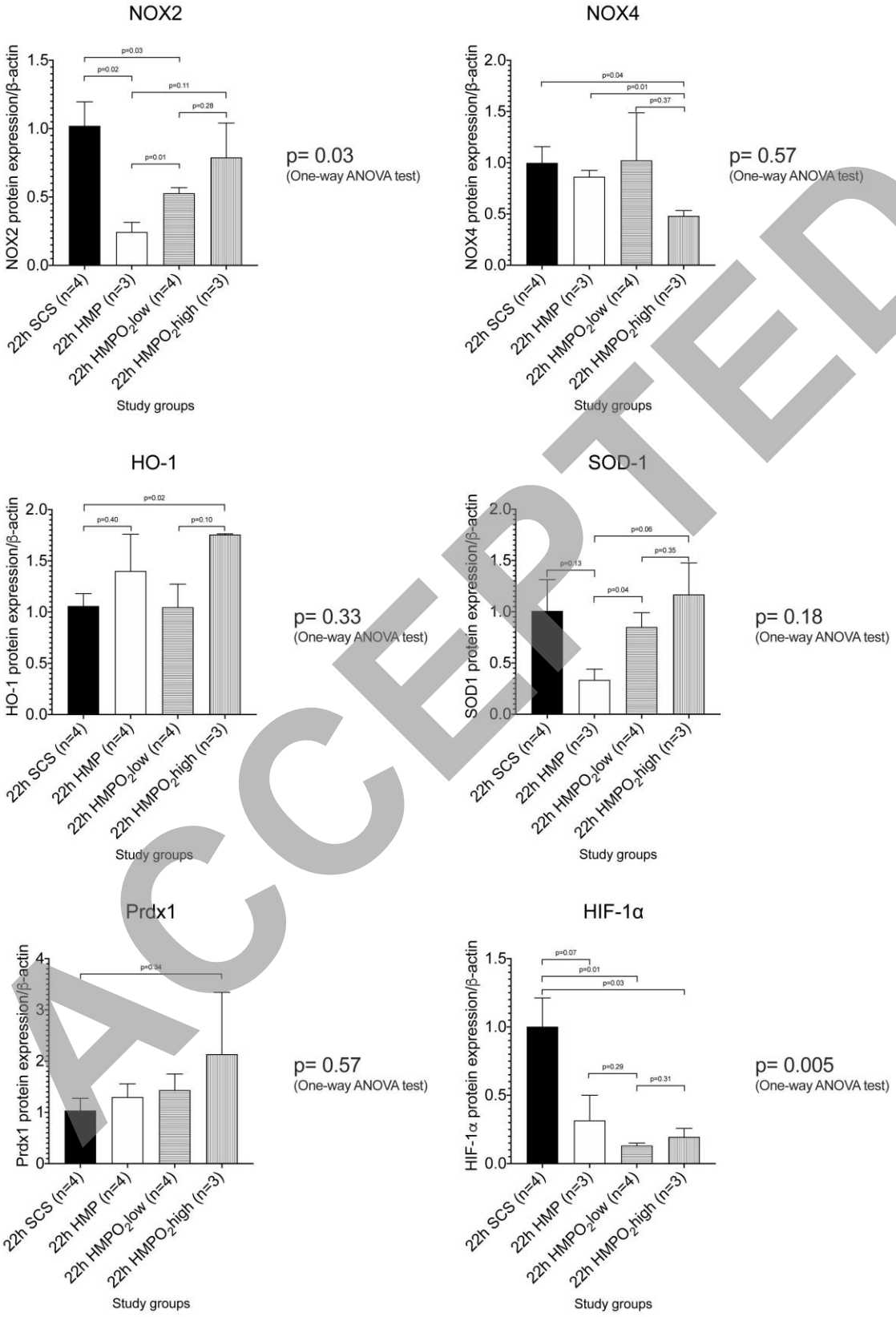

Figure 9A-C

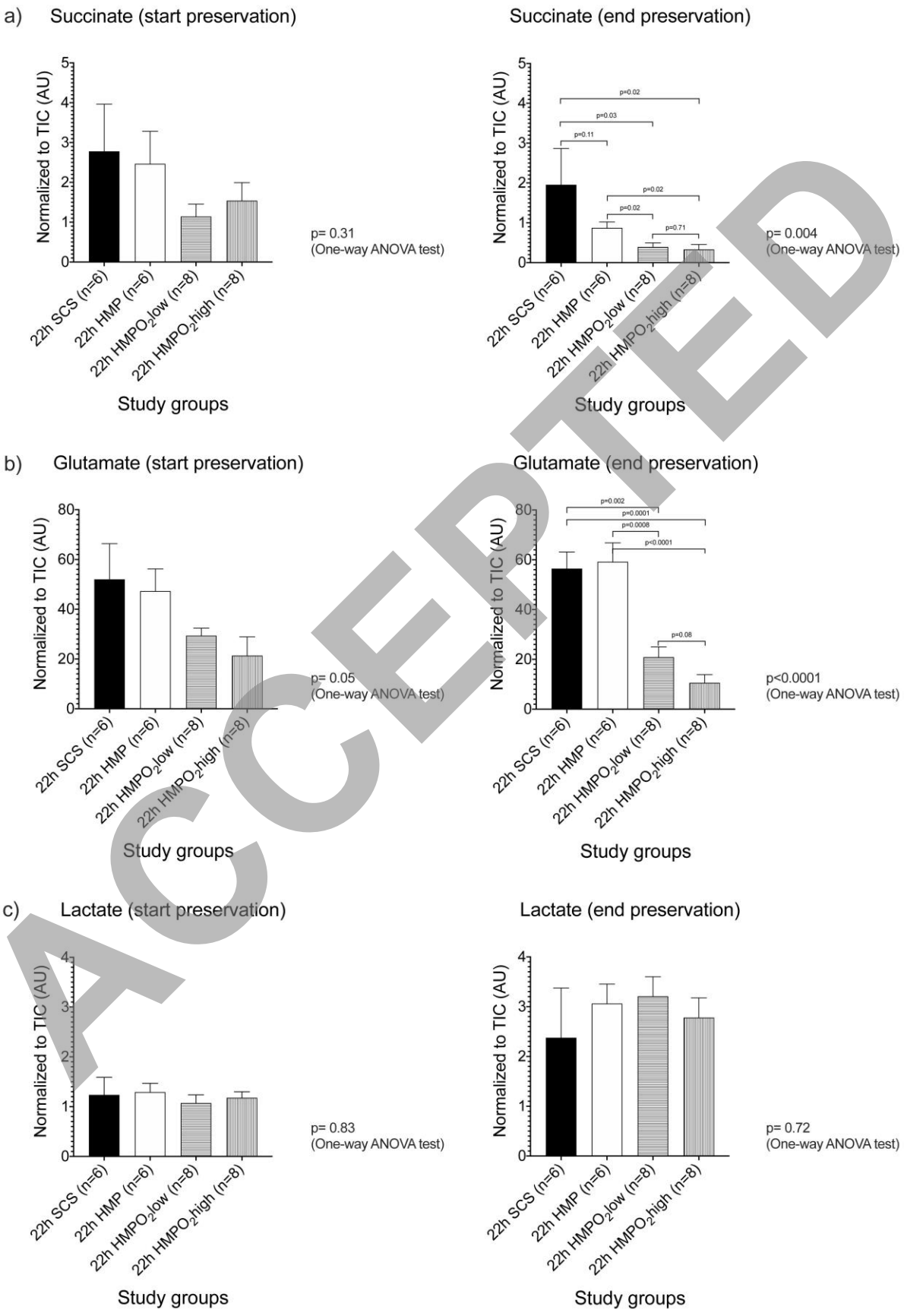

Figure 9D-F