CHAPTER 7

The addition of 6-chromanol SUL-138 during hypothermic machine perfusion does not affect short-term renal function and injury of porcine donation after circulatory death kidneys

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ABSTRACT

Introduction

Diminishing ischemia-reperfusion injury (IRI) by improving kidney preservation techniques offers great beneficial value for kidney transplant recipients. Mitochondria play an important role in the pathogenesis of IRI and are therefore interesting targets for pharmacological interventions. Hypothermic machine perfusion (HMP), as a preservation strategy, offers the possibility to provide mitochondrial-targeted therapies. This study focuses on the addition of a mitochondrial protective agent SUL-138 during HMP and assesses its effect on kidney function and injury during normothermic reperfusion.

Methods

Thirty minutes of warm ischemia was applied to porcine slaughterhouse kidneys before 24 hours of non-oxygenated HMP with or without the addition of SUL-138. Functional assessment was performed by 4 hours normothermic autologous blood reperfusion.

Results

No differences in renal function or perfusion parameters were found between both groups. ATP levels were lower after 30 minutes of warm ischemia in the SUL-138 group (n.s, p=0.67) but restored significantly during 24 hours of HMP in combination with SUL-138. Aspartate aminotransferase (ASAT) levels were significantly lower for the SUL-138 group.

Conclusion

SUL-138 does not influence renal function in this model. Restoration of ATP levels during 24 hours of HMP with the addition of SUL in combination with lower ASAT levels could be an indication of improved mitochondrial function.

INTRODUCTION

The global observatory on donation and transplantation calculated that less than 10% of the global need for donor organs is met.[1] This shortage has led to the use of sub-optimal quality organs donated from expanded criteria donors (ECD) and donation after circulatory death (DCD) donors.[2] Especially, kidneys donated from DCD donors are more susceptible to ischemia-reperfusion injury (IRI)[3] than donation after brain death (DBD) donors, and this is reflected by higher incidences of delayed graft function (DGF).[4–6] DGF has far-reaching consequences for the recipients of these kidneys since it requires compulsory return of the patients to undergo haemodialysis until recovery of kidney function. Furthermore, the chance of acute cellular rejection and poorer long-term outcomes increases.[7,8]

Preventing or diminishing IRI during the donation- and transplantation setting would be of great beneficial value by decreasing DGF rates and increasing kidney quality. Hypothermic machine perfusion (HMP), instead of static cold storage (SCS), as a preservation technique has already proven to be superior to SCS in terms of better-preserved kidneys in terms of reduced duration and incidence of DGF[4,9–11], and is therefore one of the several strategies that could be applied to reduce IRI.

Mitochondria play a pivotal role in the pathogenesis of IRI[12]. Mitochondrial reactive oxygen species (ROS) production is a fundamental early driver of IRI and is a nonspecific effect of the interaction of oxygen present during reperfusion with dysfunctional mitochondrial respiratory chains.[13] Since the proximal tubule compartments of kidneys contain large numbers of mitochondria, they are especially vulnerable to hypoxia.[14,15] Considering their crucial role, the prevention of mitochondrial injury during kidney preservation seems a logical approach.

In this study we combined HMP with SUL-138, a compound shown to have a protective effect on cells during hypothermia.[16] This 6-chromanol might be beneficial for maintaining mitochondrial homeostasis during kidney preservation.[17] To resemble potential clinical application we explored the effect of SUL-138 during 24 hours HMP of porcine DCD kidneys followed by assessment of kidney function in a normothermic reperfusion model.

MATERIALS AND METHODS

Animal model

Porcine kidneys were retrieved from two local abattoirs after the pigs were killed according to the standardized procedure of a sedative electrical shock followed by exsanguination. Blood was immediately collected in a container containing 25.000 IU of heparin (LEO Pharma A/S, Ballerup, Denmark). No animal ethics committee approval was necessary since slaughterhouse waste material was used for these experiments.

Experimental design

A total duration of thirty minutes warm ischemia (WI) was chosen to induce ischemic injury as a model of DCD donation. Subsequently, the kidneys were preserved for 24 hours by hypothermic machine perfusion with or without SUL-138. After the preservation period the kidneys were reperfused in an *ex vivo* normothermic machine perfusion (NMP) setup for four hours. A total of twelve kidneys were randomized into either the SUL or the vehicle group.

Hypothermic machine perfusion

During the 30 minutes of WI the kidneys were prepared for HMP by removing excess fat surrounding the kidney, ureter, and renal artery. After 30 minutes kidneys were flushed with 180 ml of cold (4˚ Celsius) saline (Baxter BV, Utrecht, The Netherlands). Saline was supplemented with SUL-138 dissolved in dimethylsulfoxide (DMSO) with a final concentration of $1x10^{-4}$ mol/l in the SUL-138 group and with equimolar DMSO only in the vehicle group. After flushing a needle biopsy (23mm) was taken (Invivo, Best, The Netherlands) from the cortex of the kidney and stored in sonification solution (SONOP containing 0.372 g EDTA in 500 ml 71% ethanol (v:v) and NaOH (pH 10.9)) and 4% buffered formaldehyde for further analysis. The renal artery was cannulated to enable connection to the HMP machine (Kidney Assist Transport, Organ Assist, Groningen, The Netherlands). A total volume of 500 ml University of Wisconsin machine perfusion solution (Belzers MP, Bridge to life Ltd., London, United Kingdom) supplemented with SUL-138 (concentration: $1x10^{-4}$ mol/l) or vehicle (equimolar concentration of DMSO) was used as preservation solution in the SUL-138 and vehicle group, respectively. HMP was initiated after the kidney was placed in the machine for a total duration of 24 hours at a temperature of 4˚C. The perfusion was pressure-controlled with a mean arterial pressure of 25 mmHg. Samples of the perfusion solution were taken after 15, 60 minutes and 24 hours. Furthermore, pressure, temperature and flow rates were continuously monitored.

Ex vivo normothermic machine perfusion to assess renal function

Renal function was assessed in an isolated *ex vivo* normothermic machine perfusion (NMP) setup after the preservation time of 24 hours was passed. For that purpose, the renal artery and ureter were cannulated with a 12 and 8 French cannula, respectively. Just prior to attachment of the kidney to the NMP the remaining preservation solution was flushed away with 50 ml cold saline. Kidneys were weighed, and another biopsy was taken and stored as described above. To assess function, kidneys were attached to a specially designed organ chamber and were pressure-controlled perfused with a mean arterial pressure of 75 mmHg (Kidney Assist Transport, Organ Assist, Groningen, The Netherlands) at a temperature of 37˚C for 4 hours. An oxygen mixture of 95% O_2 and 5% CO_2 was supplied to the oxygenator (Hilite LT 1000, Medos Medizin technik AG, Stolberg, Germany) at a fixed rate of 500 ml/min. A blood-based perfusion solution was used during NMP which consisted of 500 ml whole blood depleted of leukocytes by using a leukocyte filter (Bio R O_2 plus, Fresenius Kabi, Zeist, The Netherlands) and 300 ml lactated Ringer's (Baxter BV, Utrecht, The Netherlands), supplemented with 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), 100 µl 20 mg/ml sodium nitroprusside (Sigma-Aldrich, St Louis, USA) and during NMP a constant infusion of an amino acid mixture (10% Aminoplasmal, Braun Melsungen AG, Melsungen, Germany), 2.5 ml 8,4% sodium bicarbonate, and 17 IU Novorapid, (Novo Nordisk, Bagsvaerd, Denmark) was given at 20 ml/h. When glucose levels dropped below 5 mmol/L, levels were correct with 5% glucose (Baxter BV, Utrecht, The Netherlands). At the end of NMP, biopsies were taken and stored as described above for further analysis.

Renal function testing

During NMP, renal flow rate and urine flow were monitored every 15 minutes. Perfusate and urine samples were taken after 15, 60, 120, 180 and 240 minutes for storage and blood gas measurements (ABL90 FLEX, Radiometer, Zoetermeer, The Netherlands).

Creatinine clearance and fractional sodium excretion levels were calculated with concentrations of plasma and urine creatinine and sodium that were measured using routine procedures at the clinical chemistry lab of the University Medical Center Groningen (UMCG). Furthermore, the level of proteins in the urine was determined by

the University Medical Center Groningen Department of Laboratory Medicine using standardized protocols on a modular analyser (Roche, Almere, The Netherlands).

Mitochondrial function, integrity, and damage

Renal oxygen consumption (QO_2) was calculated as indication of the metabolic activity of the kidney. Venous and arterial pO_2 and saturation was measured for this purpose. The following formula was used:

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

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

Where Hb is the perfusate's hemoglobin content in mmol/L, pO_2 is the venous or arterial partial oxygen pressure 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.

Total sodium reabsorption (T_{sodium}) was calculated with the following formula:

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

Where CrCl represents creatinine clearance (ml/min), P_{na} perfusate sodium concentration (mmol/l), U_{na} urine sodium concentration (mmol/l), U urine production (ml/min) and g is kidney weight.

Adenosine triphosphate (ATP) was measured in all biopsies that were taken during the experiment (after WI, preservation and NMP) with methods described previously.[18] ATP concentrations were expressed as µmol/g protein.

Kidney injury markers

Lactate dehydrogenase (LDH) was 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.[18,19] ASAT levels were measured as indicator of mitochondrial damage. A standardized protocol by the clinical chemistry lab of the UMCG was used.

Oxidative stress due to active oxygenation

Thiobarbituric acid-reactive substances (TBARS) were analysed in preservation fluid perfusate ($TBARS_{perfusate}$) and urine samples ($TBARS_{urine}$), at all sample moments. The protocol for this analysis has been described in detail before.[18] In brief, the TBARS assay measures the level of products of lipid peroxidation present in the sample. In plasma, these products will consist mainly of malondialdehyde (MDA). TBARS concentrations are expressed in µmol/l. Total TBARS production was calculated with the following formula:

$$\text{TBARS production (IU)} = (TBARS_{urine} * U) + (TBARS_{perfusate} * (P+I))$$

U represents urine production (ml/min), P priming volume of the NMP setup (l) and I is the volume of infusion during NMP (l).

Statistics

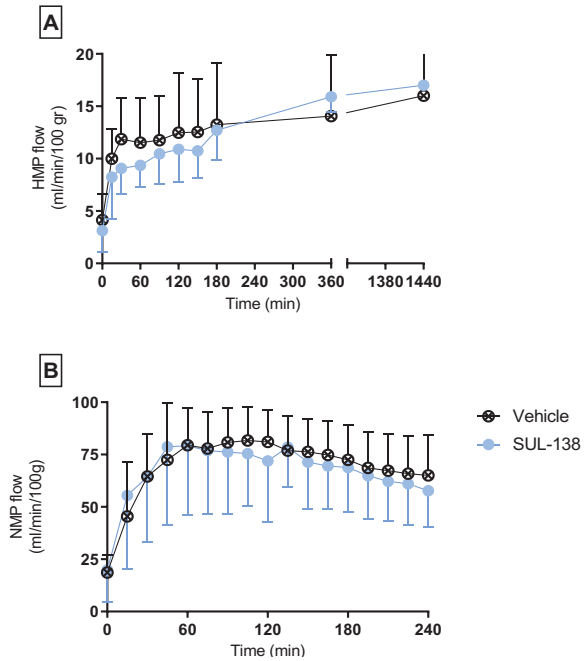
Results are reported as means with standard deviation. Statistical analysis was performed with Graphpad Prism 7.02 (San Diego, CA, USA). The area under the curve (AUC) was calculated for the renal function parameters flow, creatinine clearance, fractional sodium excretion, proteins in urine, oxygen consumption, total sodium reabsorption. The AUC were also calculated for renal injury markers, such as LDH, ASAT and total TBARS production. For uNAG the AUC for the period between 120 and 240 minutes of NMP was calculated since an increase in this tubular injury marker was seen during this period. All values were tested for significance using a Mann-Whitney U test. $P<0.05$ was considered to indicate statistical significance.

RESULTS

Hypothermic and normothermic perfusion parameters

During HMP no differences in flow rates were observed between the vehicle and SUL-138 group. Flow started with a steep increase during the first 20 minutes and slowly increased thereafter until the end of the 24 hours preservation period (Figure 1A). Renal flow during NMP was comparable between the vehicle and SUL-138 group (Figure 1B). During the first hour flow rates increased to approximately 80 ml/min/100gr and then slowly decreased during 4 hours of NMP.

Figure 1. Flow rates during preservation and testing.



Porcine kidneys were preserved with HMP with SUL-138 (•) and without the addition of SUL-138 (⊗, Vehicle) for 24 hours. (**A**) HMP flow rates during preservation, (**B**) Renal flow rates during normothermic functionality testing. HMP, hypothermic machine perfusion. The data are shown as mean±SD.

Renal functionality during normothermic perfusion

No significant difference was seen between the vehicle and SUL-138 group in terms of glomerular function represented by creatinine clearance. Both groups show a similar trend over time (Figure 2A).

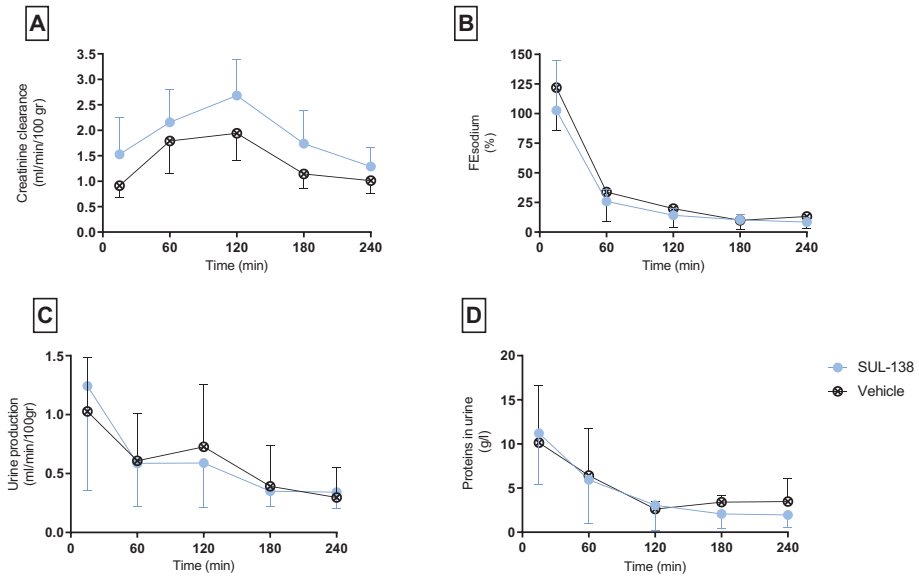
Immediately after start of NMP fractional sodium excretion is 100%, suggesting a total absence of tubular function. Over time both groups restored tubular reabsorption, decreasing FE_{sodium} to approximately 30%. No differences are seen between the vehicle and SUL-138 group (Figure 2B).

Urine production during NMP was also comparable between the two groups. Urine production was the highest the first fifteen minutes and decreased over time (Figure 2C).

Protein levels in the urine were comparable between the vehicle and SUL-138 group (Figure 2D).

As reference, previous experiments showed that when kidneys were preserved with static cold storage for 24 hours and subsequently reperfused in the same way that renal function is significantly lower compared to the kidneys presented here. Creatinine clearance is <0,25 ml/min/100gr, FE_{sodium} stayed around 95% and total protein in urine was approximately 15 g/l at every sample point.[20]

Figure 2. Kidney functionality parameters during normothermic machine perfusion.



Porcine kidneys were tested for kidney function for 4 hours with NMP after 24 hours HMP with (SUL-138 •) and without (Vehicle⊗) SUL-138. (**A**) Creatinine clearance rates, (**B**) Fractional sodium excretion, (**C**) Urine production, (**D**) Protein content in urine. NMP, normothermic machine perfusion; HMP, hypothermic machine perfusion. The data are shown as mean±SD.

Mitochondrial function (integrity) and metabolic activity

Comparable oxygen consumption rates were found for both groups. These rates slowly increased over the four-hour reperfusion time from 1 mlO_2/min/100 gr to 2 mlO_2/min/100 gr (Figure 3A). Total sodium reabsorption was equal between both groups during NMP (Figure 3B).

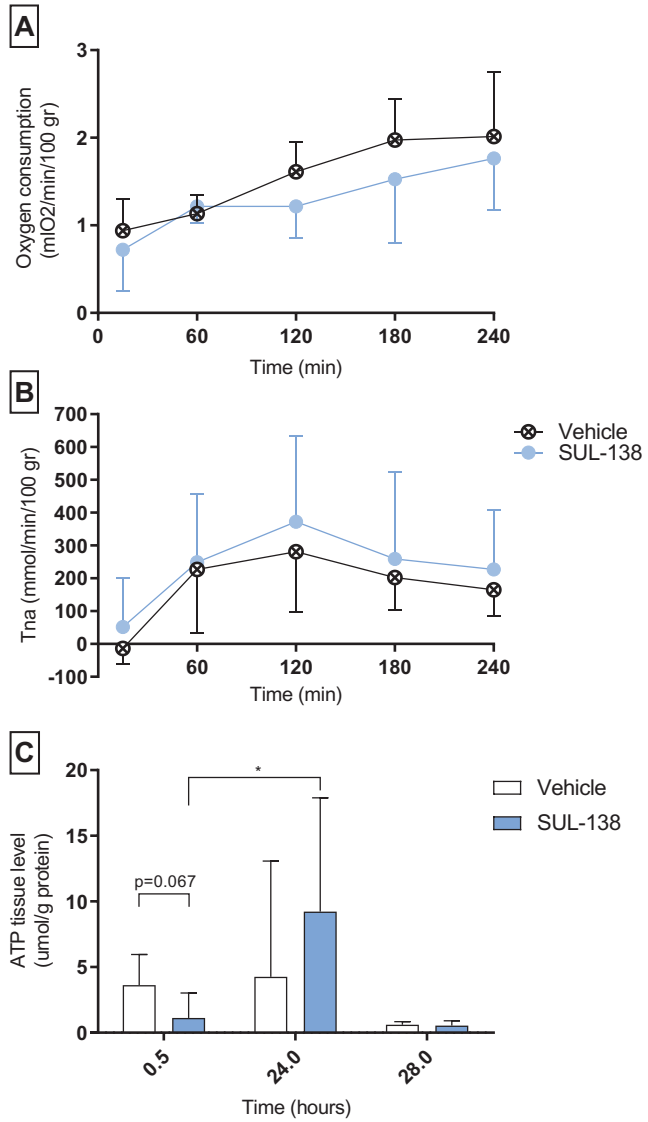
ATP levels measured after warm ischemia (timepoint 0.5) were almost significantly higher in the vehicle group compared to the SUL group (p=0.067). ATP levels significantly increased during 24 hours of preservation in the SUL group. No significant differences between groups were found in ATP values after 24 hours preservation (timepoint 24.0) and after NMP (timepoint 28.0) (Figure 3C).

Kidney injury and oxidative stress markers

The AUC of generic injury LDH and tubular injury marker, urinary N-acetyl-beta-D-glucosaminidase (uNAG), were comparable between the two groups (Figure 4A and B).

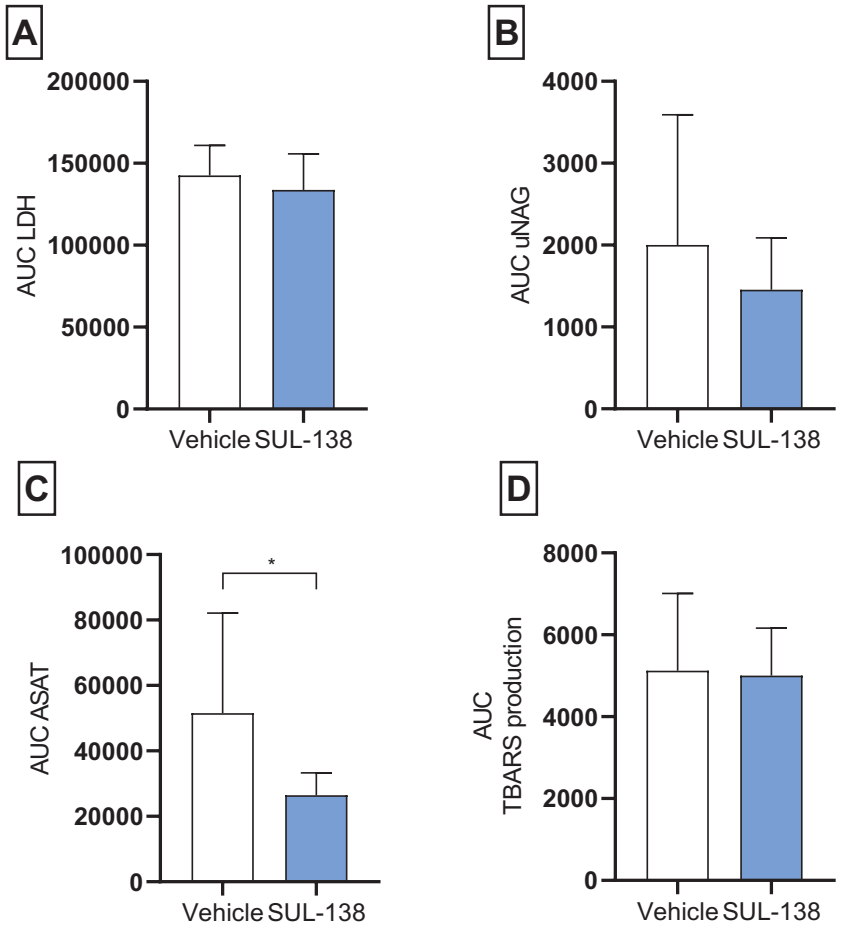
The AUC for ASAT was significantly lower in the SUL-138 group compared to the vehicle. No differences were seen regarding oxidative stress represented by similar total TBARS levels (Figure 4C and D).

Figure 3. Mitochondrial function, damage and metabolic activity.



Mitochondrial function and damage was assessed in porcine kidneys that were preserved with 24 hours of non-oxygenated hypothermic machine perfusion with (SUL-138 •) and without (Vehicle⊗) the addition of SUL-138, (**A**) Oxygen consumption rates during NMP, (**B**) Total sodium reabsorption rates during NMP, (**C**) ATP levels were measured during the experiment after 30 minutes warm ischemia (timepoint 0.5), after 24 hours HMP (timepoint 24) and at the end of 4 hours NMP (timepoint 28), * p<0.05 significant increase in ATP values during 24 hours HMP with the addition of SUL-138 . NMP, normothermic machine perfusion; ATP, adenosine triphosphate; WIT, warm ischemic times; ASAT, Aspartate Aminotransferase; AUC, Area under the curve. The data are shown as mean±SD.

Figure 4. Kidney injury and oxidative stress markers released during 4 hours normothermic machine perfusion.



Porcine kidneys were tested for kidney function for 4 hours with NMP after 24 hours HMP with (SUL-138) and without (Vehicle) SUL-138. (**A**) AUC for LDH during NMP, (**B**) AUC for uNAG between 120 and 240 minutes of NMP, (**C**) AUC for ASAT during NMP, * p<0.05 significant lower ASAT in SUL-138 group, (**D**) AUC for total TBARS levels during NMP. NMP, normothermic machine perfusion; AUC, area under the curve; HMP, hypothermic machine perfusion; LDH, lactate dehydrogenase; uNAG, urinary N-acetyl-beta-D-glucosaminidase; TBARS, thiobarbituric acid-reactive substances. The data are shown as mean±SD.

DISCUSSION

The aim of this study was to evaluate the effect of SUL-138 during 24 hours of hypothermic machine perfusion on kidney function. We found that the addition of SUL-138 during hypothermic preservation did not result in improved renal function during 4 hours of normothermic reperfusion. We did find a significant increase in ATP levels during HMP in the SUL-group and a lower release of ASAT during reperfusion.

Warm and cold ischemic periods are unavoidable during a donation- and transplantation setting, and this results in ischemia-reperfusion injury (IRI). ROS production by mitochondria is a well-known early effect of IRI[13] and preventing this would be a valuable target for diminishing IRI. SUL compounds have shown to be protective against cold-induced ischemia and mitochondrial dysfunction.[17,21] Both SUL compounds 109 and 121 have shown to increase ATP levels after hypothermic preservation followed by a period of rewarming of adipose-derived stem cells and in rat kidneys.[17,22] Furthermore, both compounds reduced the production of ROS in various hypothermia/rewarming and disease models that are characterized by mitochondrial dysfunction. [22–24] SUL-138, the compound used in this study, has already shown to maintain cell growth and morphology during hypothermic storage of various cell lines *in vitro* (3T3-L1, HUVEC, HEK293 and NRK-52E)[16] but has no published data on its effect on mitochondrial integrity.

Hajmousa et al[22] has shown that SUL-109 preserves the mitochondrial membrane potential (MMP) during hypothermic conditions. We were not able to confirm this effect of SUL-138 on isolated mitochondria due to technical issues. Which is very unfortunate because a low MMP was recently linked as independent predictor for DGF.[25] We used oxygen consumption, total sodium reabsorption and ATP levels as surrogate markers for mitochondrial function. In this experiment, we found an almost significant (p=0.067) lower ATP content after 30 minutes of warm ischemia in the SUL-138 group. Since SUL-138 was already present in the first flush out it could be that the observed ATP depletion can be attributed to the addition of the 6-chromanol. Since we did not measure ATP levels directly after death, we cannot be sure about this effect of SUL. However, we compared these ATP levels to a historical cohort of kidneys in which we measured ATP at the same moment. The kidneys flushed with SUL have a significant lower ATP content compared to this historical cohort (n=47, p=0.0003). A positive effect that we found on mitochondrial function was the significant restored ATP content

during 24 hours of HMP in which SUL-138 was present. We cannot elaborate if this is due to improved mitochondrial integrity, but it could be worthwhile exploring in further experiments. Another indirect proof of mitochondrial protection by SUL-138 is presented by the significant lower ASAT levels during NMP in the SUL-group compared to the vehicle group.

Due to the use of porcine slaughterhouse kidneys variability in this model is higher compared to laboratory animals and therefore subtle differences are difficult to show. The model however enables studying effects on a kidneys of similar size and physiology of human kidneys and it decrease the number of laboratory animals needed.[26,27] This model has proven its strength and reproducibility with several research questions.[28–30] For example, we have reported that in the presence of oxygen (21 and 100%) during HMP a significant higher ATP production in kidneys was seen.[30] Others have already shown this same effect, that the addition of oxygen during HMP of both kidneys and livers is beneficial to support cellular respiration and subsequent ATP production.[31–36] The lack of oxygen during HMP in this study very likely leads to low ATP production during HMP. For this experiment we choose not to include oxygen during HMP since non oxygenated HMP is the current clinical standard. The mitochondrial protective capacity of SUL compounds have been shown in a rat model in which oxygen was present.[17] One could therefore assume that the lack of efficacy of the SUL compounds may be due to the absence of oxygen in our model.

In conclusion, we unfortunately did not see any beneficial effects of the addition of SUL-138 during non-oxygenated HMP in terms of renal function during short-term normothermic reperfusion. The ATP levels in combination with significant lower ASAT levels found in this study could be an indication that mitochondria are better preserved in the SUL-group. However, more in-depth assessment on mitochondrial function markers is necessary to support this finding.

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