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ORIGINAL ARTICLE

Subnormothermic machine perfusion for preservation of porcine kidneys in a donation after circulatory death model

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Conflicts of interest
U.R. is a consultant of Dr. Franz Köhler Chemie, Bensheim, Germany, and one of the inventors of Custodiol-N. All other authors have no conflict of interest to disclose as described by *Transplant International*.

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Summary

Machine perfusion for preservation led to compelling success for the outcome of renal transplantation. Further refinements of methods to decrease preservation injury remain an issue of high interest. This study investigates functional and morphological aspects of kidneys preserved by subnormothermic (20 °C) machine perfusion (SNTM) compared with oxygenated hypothermic machine perfusion (HMPox) and cold storage (CS) in a donation after circulatory death (DCD) model. After 30 min of warm ischaemia, porcine kidneys were randomly assigned to preservation for 7 h by CS, HMPox or SNTM. Afterwards, kidneys were reperfused for 2 h with autologous blood *in vitro* for assessment of function and integrity. Application of SNTM for preservation led to significantly higher blood flow and urine output compared with both other groups. SNTM led to a twofold increased creatinine clearance compared with HMPox and 10-fold increased creatinine clearance compared with CS. Structural integrity was best preserved by SNTM. In conclusion, this is the first study on SNTM for kidneys from DCD donors. SNTM seems to be a promising preservation method with the potential to improve functional parameters of kidneys during reperfusion.

Introduction

Hypothermic machine perfusion (HMP) was recently re-established, providing an improved preservation method for kidneys before kidney transplantation. Interestingly, application of this preservation method showed a strong clinical benefit when compared with cold storage (CS). Rates of delayed graft function (DGF) and 1- and 3-year graft survival showed far better results in favour of HMP [1–7].

These compelling results were not completely transferable to kidneys from donation after circulatory death (DCD) donors. Here, a significant reduction of the DGF rate was observed without impacting the 1-year graft survival [8]. Absolute numbers of DGF in this study demonstrated still high incidences of this relevant complication in more than 50% of recipients in spite of the improvement by HMP. High discard rates of DCD kidneys ranging from 20% to 30% (OPTN, 1999–2011) [9] further underscore the need of improvement of preservation methods for this pool of donated organs.

Up to date, no studies have been carried out systematically analyzing the influence of the preservation temperature during kidney machine perfusion. Adapting the

temperature out of the hypothermic zone into the subnormothermic range potentially enables utilization of established materials and methods (e.g. perfusion solutions) while decreasing cold-induced organ injury. This hypothermia-induced component of the preservation injury is mediated by highly reactive oxygen species formed as a consequence of release of free iron ions, leading to mitochondrial injury and lipid peroxidation [10–15]. Furthermore, microtubules and the actin cytoskeleton are degraded under hypothermic conditions [16–19] contributing to endothelial barrier failure after CS [16,17]. The cold-induced alterations lead, together with hypoxia-induced alterations, to the reperfusion injury [15], leading to damaging of the allograft [20,21] mediated by an inflammatory reaction including the TNF-α signalling pathway [22]. By avoidance of the cold-induced organ injury, ultimately the reperfusion injury and therefore the overall organ injury might be decreased.

Minimization of the cold-induced organ injury might be especially favourable for organs from DCD donors, which obligatory carry the burden of extensive ischaemic organ injury.

Accordingly, aim of the present study was to analyze functional and morphological aspects of porcine kidneys from DCD donors preserved by subnormothermic machine perfusion (SNTM) compared with the established preservation methods HMP and CS.

Methods

Investigations were carried out with female Landrace pigs weighing approximately 30 kg. All animal procedures were approved by the respective authorities (Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen) and controlled by the animal welfare officers of the University of Duisburg-Essen (certificate AZ 84-02.05.20.12.187). The anaesthetic protocol was standardized and can be read elsewhere [23].

Experimental design

Three groups were assigned, and kidneys were randomly distributed. Number of kidneys in each group was $n = 5$. All kidneys were exposed to 30 min of warm ischaemia for simulation of DCD conditions. Kidneys in the first group were subjected to CS in ice for 7 h (CS-group), serving as negative control. Kidneys in the second group were subjected to oxygenated hypothermic machine perfusion (HMPox) for 7 h (HMPox-group). In the final group, kidneys were exposed to 7 h of SNTM (SNTM-group). All kidneys were reperfused for 2 h in an isolated organ perfusion system for assessment of renal function and integrity.

Surgical technique

Details of surgical techniques have been described in detail recently [23]. After ligation of the vessels, kidneys were left free in the abdominal cavity. After a period of 30 min, the kidneys were removed from the abdomen. The renal artery was immediately cannulated, and the kidneys were flushed by gravity (100 cm H_2O) with 100 ml Histidine-Tryptophan-Ketoglutarate (HTK) solution (Dr. F. Köhler Chemie GmbH, Bensheim, Germany) cooled to 4 °C. The distal aorta was punctured, and 1000 ml of blood was collected in commercial whole blood bags for the *in vitro* reperfusions.

Hypothermic machine perfusion (HMPox) and SNTM

Perfusion solution used for HMPox, and SNTM was Custodiol-N supplemented with dextran 40 [24], which has been evaluated for HMP before [23]. Custodiol-N (Dr. F. Köhler Chemie GmbH) was supplemented with 50 g/l pyrogen-free dextran 40 (MW approximately 40 000 g/mol, CAS No. 9004-54-0; AppliChem, Darmstadt, Germany); the completed solution was sterilized by filtration [0.22-μm filter (Steritop-GP Filtereinheit; Milipore, Schwalbach, Germany)]. The interposition of a hollow fiber oxygenator (Minimax; Medtronics; Minneapolis, MN, USA) into the perfusion circuit allowed for oxygenation of the perfusate during the preservation period.

HMPox was carried out by pressure-controlled perfusion with a perfusion pressure of 30 mmHg throughout the 7 h, while SNTM was carried out with a perfusion pressure of 40 mmHg. This pressure was determined by preliminary investigations demonstrating that a pressure of 40 mmHg is sufficient to achieve a partial pressure of oxygen of >150 mmHg (critical partial pressure without oxygen carriers) in the venous effluent. Higher pressures in turn led to occurrence of severe oedema.

The oxygenator was supplied with 100% oxygen at approximately 500 ml/min. During machine perfusion, kidneys were submerged in the preservation solution at 4 or 20 °C, respectively. Temperature control in the oxygenator was achieved by a temperature-controlled water bath that supplied the heat exchanger circuit with temperature-adjusted water resulting in 4 or 20 °C at the oxygenator.

Perfusion parameters were recorded 30, 60 and 120 min after beginning of the perfusion and at the end of HMPox and SNTM. At the end of the preservation, aliquots for measurement of lactate dehydrogenase (LDH) activity were taken from the perfusate.

Kidney reperfusion circuit

After the preservation period, integrity and function of the kidneys were tested by isolated reperfusion *in vitro*

for 120 min. Details of the perfusion system have been described previously [23]. Briefly, renal artery and ureter were cannulated. The perfusion medium consisted of heparinized autologous blood that was collected during the surgical procedures. The blood was diluted with Sterofundin® in a ratio of 1:1 resulting in a haematocrit of 20% approximately. Papaverine was used as an adjunct in a concentration of 1 mg/dl. Creatinine (Sigma-Aldrich, Steinheim, Germany; 0.1 g/l) was added to the perfusate for assessment of renal clearance. The perfusion pressure was set to 85 mmHg. Before beginning of reperfusion, all kidneys were left at room temperature for 20 min for simulation of the surgical anastomosis time. After an equilibration period of 30 min, specimen of serum and urine were taken every 30 min for biochemical assays. The amount of diuresis was replaced by adding equal volumes of Sterofundin® in constant time intervals (every 30 min). Concentrations of creatinine and sodium, LDH and gamma-glutamyl transpeptidase (γGT) activity were determined at the laboratory centre of the University Hospital Essen. For enzyme activities, blank values of the diluted blood were subtracted from the values measured during reperfusion. In an isolated organ reperfusion model, LDH represents the overall cell injury of the respective organ. Release of γGT into urine normalized to the amount of urine was used as a marker of proximal tubulus injury [25,26] in this isolated organ model due to the high concentrations of this enzyme in the proximal tubulus. Creatinine clearance (urinary creatinine × urinary flow/plasma creatinine) and fractional excretion of sodium (FENa) [(urinary sodium × urinary flow)/(glomerular filtration rate × plasma sodium) × 100] were calculated. Blood gases were measured (inflow and outflow) at the same time intervals for assessment of oxygen consumption indicating the metabolic activity of the grafts. Oxygen consumption was calculated from the differences between arterial and venous sites and expressed as ml/min/g according to transrenal flow and kidney mass. Respective oxygen contents of arterial and venous blood were calculated as $O2 = (1.34 \times \text{haemoglobin (g/dl)} \times \text{oxygen saturation (\%)} \times 0.01) + (0.024 \times \text{partial pressure of oxygen})$. Renal blood flow and urine production were documented throughout reperfusion.

Histology

Specimens from the kidneys were taken including cortex and medulla at the end of the reperfusion and fixed in 4% buffered formalin, dehydrated in an alcohol series and embedded in paraffin. Slides with a thickness of 4 μm were cut using a microtome and stained with haematoxylin and eosin for light microscopic evaluation. Renal injury was scored by an experienced pathologist blinded for the groups. Assessment was carried out following Torras *et al.* [27]: In each slide, 10 visual fields were investigated assessing six morphological parameters indicating renal parenchyma injury (tubular dilatation, epithelial vacuolation, epithelial shedding, epithelial necrosis, interstitial oedema and inflammation). A 5-point scale was applied for each parameter: 0 = no damage; 1 = lesions affecting <10% of the field; 2 = 10%–25%; 3 = 25%–50%; 4 = 50%–75% and 5 > 75%.

Western blots

Amounts of TNF-α and cleaved caspase-3 in tissue homogenates were analyzed by western blot analysis. Briefly, specimens of renal cortex were quick-frozen in liquid nitrogen immediately at the end of the reperfusion and stored at −80 °C until further procedures. Homogenization was accomplished by standard procedures [28,29]. Equal amounts of protein (80 μg total protein) from all samples were separated on SDS-polyacrylamide gels and transferred onto nitrocellulose membranes (0.2 μm; BioRad, München, Germany). Primary antibodies TNF-α (1:1000; Thermo Scientific, Waltham, MA, USA), or against cleaved capase-3 (1:1000; Cell Signaling Technology, Danvers, MA, USA), were incubated overnight at room temperature, and the antibody against β-actin (1:200; Santa Cruz Biotechnology Inc., Dallas, TX, USA) was incubated 30 min at room temperature. Immunodetection was accomplished with an anti-mouse IgG, HRP-linked (Cell Signaling Technology) or an anti-rabbit IgG, HRP-linked (Novus Biologicals, Littleton, CO, USA) secondary antibody, respectively, for 2 h at room temperature and visualized by chemiluminescence (BioRad). To confirm comparable quantities of transferred protein, β-actin was used as internal reference. IMAGEJ (version 1.46; NIH, Bethesda, MD, USA) was used for densitometry and semi quantitative analysis.

Statistics

Values are reported as means and standard error of the mean (SEM). Continuous variables were plotted over time, and the area under the curve (AUC) was calculated. Urinary γGT values were adjusted for the amount of urine production during the respective time period. Differences between groups were compared using the Kruskal–Wallis test with Dunn's post test. Western Blot results were compared by ANOVA followed by Tukey's HSD test. The level of significance was set to $P < 0.05$. Statistical analyzes were carried out using EXCEL® (Microsoft Corporation, Redmond, WA, USA) and JMP (version 10.0.0 SAS; SAS Institute Inc., Cary, NC, USA).

Results

Preservation: CS, HMP, SNTM

Intrarenal resistance showed similar values at the beginning of the preservation period in both machine perfusion groups. In the SNTM-group, lower values were observed with 0.9 ± 0.1 mmHg/ml/min after 30 min decreasing to 0.46 ± 0.03 mmHg/ml/min at the end of the preservation period. The HMPox-group showed an intrarenal resistance of 1.5 ± 0.2 mmHg/ml/min after 30 min that decreased to 1.08 ± 0.2 mmHg/ml/min at the end. Comparison of the AUCs demonstrated significantly lower values over time in the SNTM-group compared with the HMPox-group ($P < 0.001$; Fig. 1b). Release of LDH into the preservation solution during machine perfusion was not different between both groups ($P = 0.87$; data not shown).

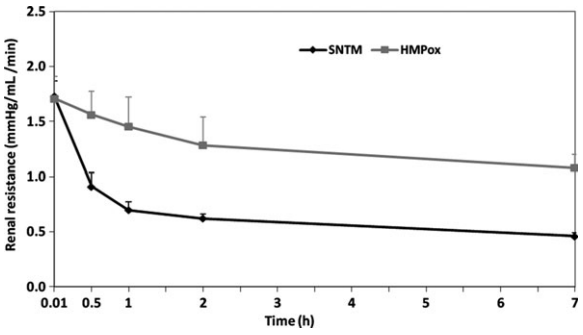

Figure 1 Intrarenal resistance during the preservation period with different preservation temperatures.

Isolated kidney reperfusion

Application of different preservation methods resulted in different patterns of kidney function during the reperfusion period (Fig. 2). SNTM for 7 h led to significantly higher renal blood flow when compared to the HMPox ($P = 0.007$) and the CS-group ($P < 0.001$). The HMPox-group and the CS-group did not differ significantly ($P = 0.348$).

Urine output in the SNTM-group was statistically higher than in the HMPox-group ($P = 0.025$) and the CS-group ($P < 0.001$). The HMPox-group showed significantly higher total urine output than the CS-group ($P = 0.005$).

Oxygen consumption during reperfusion was significantly higher in the SNTM-group compared with the CS-group ($P = 0.002$) but not statistically different to the

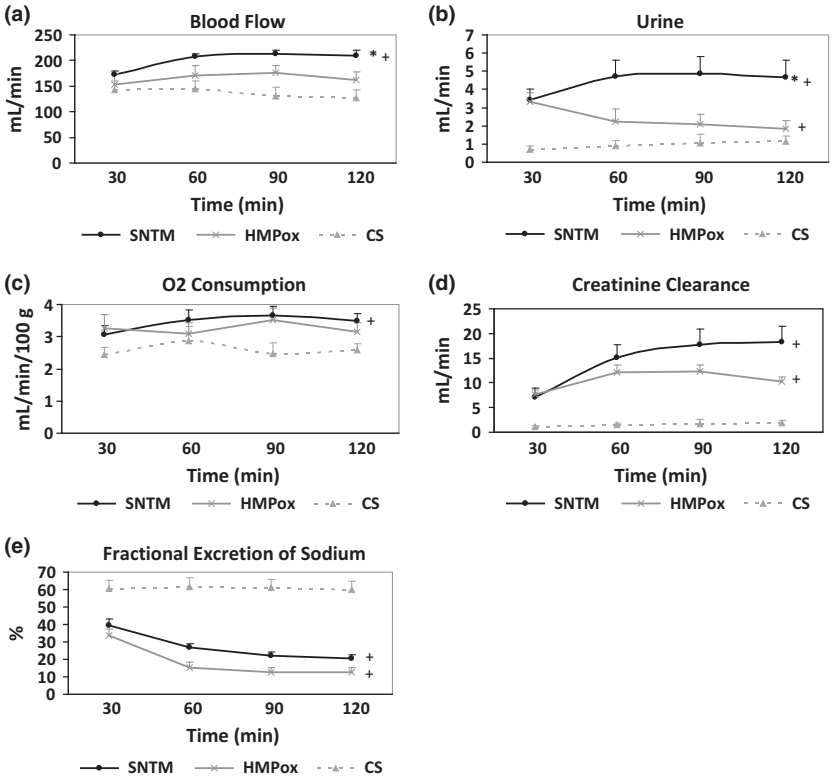

Figure 2 Parameters of kidney function at 30, 60, 90, 120 min after isolated kidney reperfusion. (a) Blood Flow. (b) Urine production. (c) Oxygen consumption. (d) Creatinine clearance. (e) Fractional Excretion of Sodium.

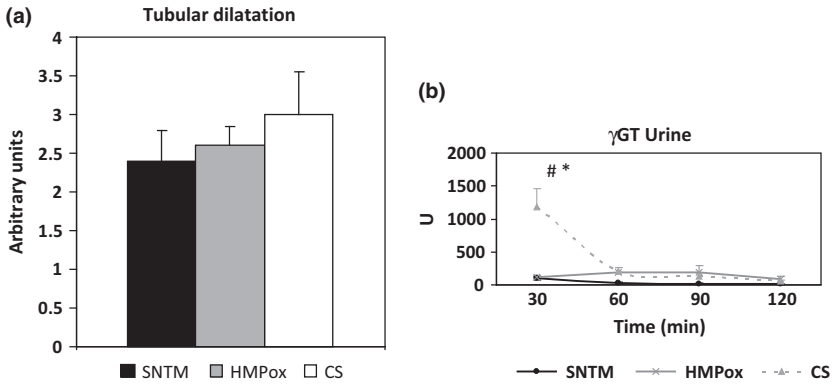

Figure 3 Tubular impairment during and after isolated kidney reperfusion. (a) Semi quantitative histological assessment of tubular dilatation. (b) Release of gamma-glutamyl transpeptidase into the urine during isolated kidney reperfusion.

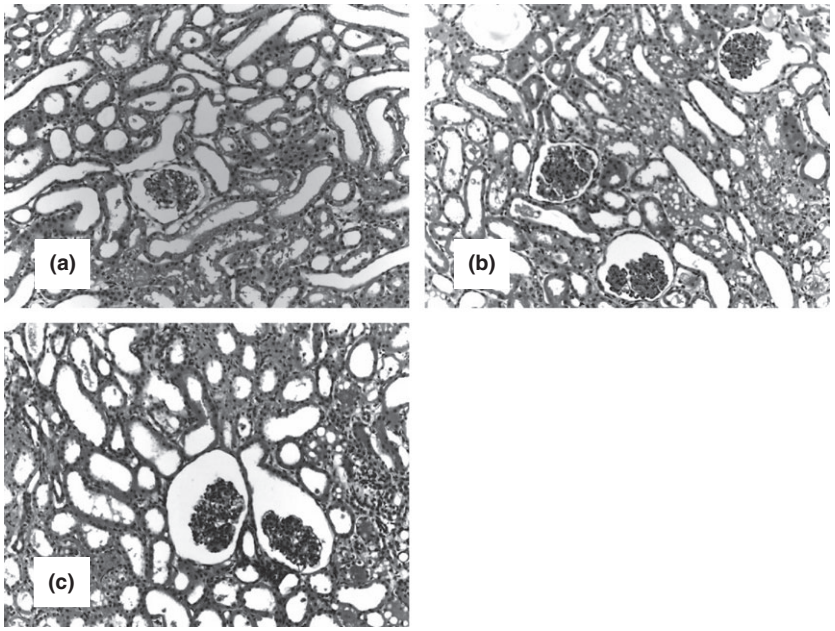

Figure 4 Histopathological changes at the end of reperfusion. H&E stained biopsies at 200× original magnification. (a) Subnormothermic machine perfusion. (b) Oxygenated hypothermic machine perfusion. (c) Cold storage.

HMPox-group ($P = 0.99$). The HMPox-group did not show different values compared with the CS-group ($P = 0.404$).

Preservation by SNTM resulted in the best renal function in terms of creatinine clearance: the SNTM-group showed nearly twofold increased values compared with the HMPox-group though not reaching statistical significance ($P = 0.09$) and 10-fold increased values compared with the CS-group ($P < 0.001$). The HMPox-group demonstrated about fivefold higher creatinine clearance values compared with the CS-group ($P < 0.001$).

Tubular function reflected by FENa showed no difference between the SNTM-group and the HMPox-group ($P = 0.211$), but significantly lower values in both groups compared with the CS-group ($P < 0.001$).

Structural cell injury

As a marker for tubular damage, release of γGT into the urine during reperfusion was analyzed (Fig. 3b). Highest values were observed in the CS-group with resulting statistical significance compared with the HMPox-group ($P = 0.016$) and the SNTM-group ($P < 0.001$). Release of LDH into the serum as a marker of general cell injury was not different between groups (data not shown).

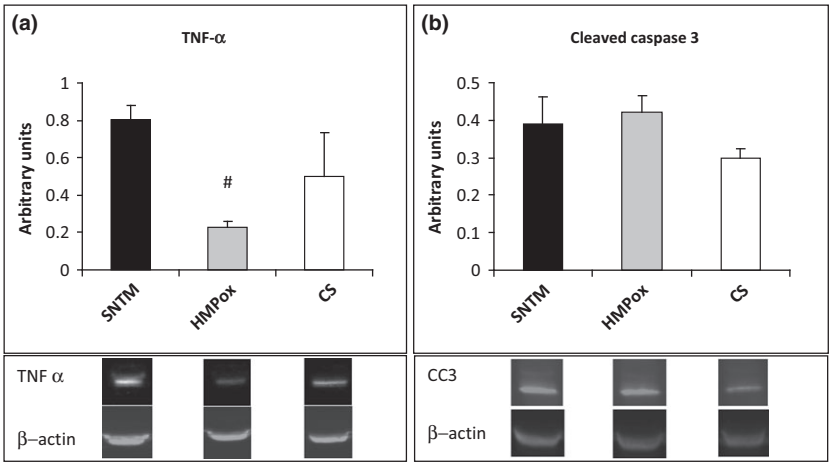

Figure 5 Semi quantitative protein expressions at the end of the isolated kidney reperfusion. (a) TNF α expression in relation to β-actin. (b) Cleaved caspase-3 expression in relation to β-actin.

Histology

Distinct alterations of morphology were observed in terms of tubular dilatation and epithelial vacuolation (Fig. 4). In the CS-group, tubular dilatation showed grade 3–4, while in the HMPox-group, tubular dilatation was observed as grade 2–3 ($P = 0.524$). The SNTM-group showed the lowest rate of tubular dilatation with grade 2–2.5 without statistical difference to any other group ($P = 0.681$ vs. HMPox, $P = 0.402$ vs. CS; Fig. 3a). Epithelial vacuolation was observed in the CS-group, the HMPox-group and the SNTM-group with grade 2–3. Interstitial oedema was present as grade 1 in all specimens in all groups. Changes indicative of irreversible cellular damage such as epithelial shedding, epithelial necrosis or inflammation were not observed.

Western blot

Western blot analysis showed that the expression of TNF-α was lowest in the HMPox-group, while the SNTM-group and the CS-group displayed higher expression patterns. Comparison of the HMPox-group with the SNTM-group showed significant differences ($P < 0.029$). Expression of TNF-α in the CS-group showed intermediate values between the HMPox-group ($P = 0.3808$) and the SNTM-group ($P = 0.2843$) but without statistical significance to any of these (Fig. 5a).

Caspase-3 cleavage showed higher values in the SNTM and the HMPox-group and lower values in the CS-group without significant intergroup differences (Fig. 5b).

Discussion

The aim of the present study was to analyze the integrity of porcine kidneys from DCD donors preserved by SNTM in comparison to the established preservation methods HMP and CS. The presented data showed for the first time that increase in preservation temperatures into the subnormothermic field during machine perfusion is practicable for DCD kidneys and imply a possible functional benefit. As a next step, transplantation models should be carried out to validate this observation. Indeed, creatinine clearances at the end of the reperfusion showed nearly twofold better values in favour of SNTM compared with HMPox. Other parameters under investigation demonstrated similar patterns (though not always reaching statistical significance due to small groups), suggesting that this form of preservation reduces the cold-induced organ injury.

The higher temperature results in higher metabolic demands for the organs. Accordingly, without complete alteration of the currently used preservation setup (perfusion solutions, oxygen carriers, etc.), approximately 20 °C represents an upper temperature limit.

However, some alterations of the perfusion setup are necessary which, along with some model specifications, demand justification: to serve the higher metabolic demands, active oxygenation and sufficient perfusion flow are inevitable. Therefore, SNTM included active oxygenation. To eliminate effects of active oxygenation during SNTM, oxygenated HMP was chosen as control group, though not representing the current clinical standard. Recent data demonstrated clear benefits of oxygenated HMP [30] (D.P. Hoyer, A. Gallinat, S. Swoboda, J. Wohlschlaeger, U. Rauen, A. Paul, T. Minor, under review), and translation into the clinic is possible in the near future. Additionally, SNTM was carried out with a slightly higher perfusion pressure than HMP. For HMP, 25 mmHg was described as optimal mean perfusion pressure before [31]. During SNTM, higher pressures might

be used without risk of endothelial and vascular impairment as the higher temperature reduces vascular stiffness. A pressure of 40 mmHg demonstrated in preliminary investigations sufficiency to achieve a partial pressure of oxygen of >150 mmHg (critical partial pressure without oxygen carriers) in the venous effluent, while higher pressures led to severe oedema. The preservation solution HTK-N (Custodiol-N; [24,32]), a new preservation solution based on the traditional HTK solution, was chosen for two reasons: first, HTK-N contains two iron chelators, the membrane-permeable LK 614 and the stronger deferoxamine [24,32], which strongly inhibit cold-induced cell injury [33,34], an injury which is mediated by highly reactive oxygen species formed in an iron-dependent way in many cell types [10,11,13–15,33]. This is of particular concern for hypothermic perfusion (as compared to CS) because of the availability of oxygen during perfusion [15,35]. Second, HTK-N draws much of its buffering capacity from the use of *N*-acetylhistidine, a buffer with lower toxicity [36,37], and is thus the solution of choice at higher temperatures, at which the toxic potential of preservation solutions is a concern [36]. HTK-N, which has been evaluated in diverse experimental preservation/transplantation models [23,24,32,35,38] and has recently been evaluated in a clinical study in cardioplegia, is about to enter the first clinical study in kidney preservation in Germany; thus, a future transfer of the setting used here to the clinical situation is not beyond reach. In the present study, the HTK-N variant supplemented with dextran 40 [23,24] was used, which has been shown to provide better preservation of function during HMP of kidneys than the usually employed solution KPS-1 [23].

A DCD model was chosen to establish a new preservation modality for these critical organs. Indeed, the preservation period was restricted to 7 h so that an additional harmful stimulus of longer preservation was avoided. Hence, against the background of DCD, a group of lower risk organs is represented, nonetheless representing clinically relevant situations [39]. We refrained from the re-application of hypothermia after SNTM before the 20 min of simulated anastomosis time, as this would have been incoherent in the concept of subnormothermic preservation in our opinion. In fact, this additional warm ischaemic injury was accepted in this preservation modality and might be considered in the interpretation of the results.

An *ex vivo* reperfusion model was chosen to reduce biological variability (that occurs to greater extend in transplantation models), therefore emphasizing on the blunt effects of preservation.

Interestingly, a clear and significant delineation of perfusion parameters during the preservation (intrarenal resistance) as well as the reperfusion (blood flow) as a function of the preservation method applied was observed. Though, providing only indirect evidence, it is likely that these primarily descriptive parameters hint vascular and endothelial function, indicating better preservation of these by higher temperature. The intrarenal resistance showed potential for an early assessment of organ quality and function during HMP in several studies [40–42], as a correlation to the function after reperfusion was observed. This is in accordance to the outcomes of the present study, where lowest intrarenal resistances during perfusion correlated with the best function during reperfusion. Against this background, it should be of interest that preservation by SNTM demonstrated a steep decrease of intrarenal resistance already during the first 30–60 min resembling a fast restitution, in contrast to organs preserved by HMPox.

While tubular cells are described to be susceptible to hypoxia, and to cooling [14,43], in the current experimental setup, only minor influence by the preservation temperature (Figs 3 and 4) was observed: Preservation by HMPox and SNTM demonstrated better results than CS, but without differences between the two groups, which showed comparable results for the FENa during reperfusion (Fig. 2e).

The data suggest that – at least in DCD donors – tubular cells are predominantly injured by hypoxia and less susceptible to hypothermia, while vascular structures appear to be more susceptible to hypothermia.

Other studies analyzing preservation of livers by SNTM demonstrated comparable results with best-preserved functions of the organs by application of SNTM. Here, endothelium and vasculature played a pivotal role too [44–48].

The mechanisms underlying the benefits of machine perfusion are not completely understood so far. Major influence is attributed to maintenance of energetic homoeostasis during preservation [49,50]. Additional effects like shear stress in the vessels [51] and control of acidosis [52,53] were demonstrated before. Higher temperature during preservation might optimize the environment for these processes, enhancing the effects of machine perfusion.

As cardinal injury after reperfusion of the allografts results from ischaemia reperfusion injury, pivotal aspects of this complex signalling pathway were analyzed: expressions of TNF-α as a major mediator of the pathological signal and cleaved caspase-3 as a major executor leading finally to apoptosis in this cascade were investigated [22,54–56]. The specific expression patterns suggest that the beneficial functional effects of HMPox are in part mediated by reduced expression of the pro-inflammatory TNF-α. SNTM in turn did not show reduced values of this molecule. Hence, alternative mechanisms might mediate the favourable outcome of an assumed reduced cold-induced injury. Cleaved caspase-3 expressions were not different across groups indicating that the rate of apoptosis was not different at this early time point of reperfusion.

Some limitations of this study should be kept in mind: The ex vivo reperfusion model, though reducing biological variability, might give limited idea of what to expect upon reperfusion in vivo in a transplant model. Clinical relevance of these data can be merely estimated. However, the concurring data of all parameters under investigation and the improving kidney function (e.g. creatinine clearance about twofold better in SNTM vs. HMPox) proposes some relevance. Furthermore, preservation was restricted to 7 h and reperfusion to 120 min; therefore, extrapolation of the data to longer time periods of preservation and reperfusion should be approached with caution.

In conclusion, this is the first study on SNTM for kidneys from DCD donors. SNTM seems to be a promising preservation method with the potential to improve functional parameters of kidneys during reperfusion.

Authorship

DPH, AP and TM: study design. DPH, AG, SS, JW and TM: data collection. DPH and TM: statistical analysis. DPH, JW, UR, AP and TM: data interpretation. DPH, JW, UR, AP and TM: manuscript preparation. DPH, AG, SS, UR and TM: literature search. UR: contribution of important reagents. AP and TM: funds collection.

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