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ORIGINAL ARTICLE

Prediction of renal function upon reperfusion by *ex situ* controlled oxygenated rewarming

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ABSTRACT

Background Post-transplant function of suboptimal kidney grafts can be improved but not accurately predicted by hypothermic machine perfusion. Therefore, a new concept of *ex situ* pre-implantation machine perfusion with controlled rewarming up to subnormothermic temperatures was developed and evaluated.

Materials and methods Porcine kidneys (n = 6/group) were retrieved before or 30 min after cardiac arrest of the donor and subjected to 18 h of static cold storage. In some cases, 90 min of machine-controlled oxygenated rewarming (COR) was added thereafter. Functional integrity was evaluated in all kidneys by subsequent normothermic reperfusion *in vitro*. After supplementation of the preservation solution (Custodiol-N solution + 5 g/L dextran 40) with 10 mg/dL creatinine, *ex situ* renal function was assessed by monitoring urine output, urinary creatinine and creatinine clearance at 20 °C. Functional integrity was evaluated in all kidneys by normothermic reperfusion.

Results COR resulted in a more than twofold improvement of postreperfusion creatinine clearance, oxygen consumption and enzyme release upon reperfusion, when compared with static cold storage. Predictive discrimination between kidneys with good or impaired function upon reperfusion based on parameters during perfusion at 4 °C was only moderate. This improved significantly at 20 °C. Correlation with renal clearance upon reperfusion was weak for vascular resistance at 8° (r^2 = 0·2) and 20 °C (r^2 = 0·41). Best correlation was found for clearance measurements at 20° (r^2 = 0·81).

Conclusions Reconditioning by controlled oxygenated rewarming up to 20 °C improves renal function after reperfusion and can be utilized to assess graft integrity of predamaged donor kidneys.

Keywords Graft evaluation, graft function, machine perfusion, organ preservation, transplantation.

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Introduction

The prevailing shortage of organ donors for transplantation has opened the door for an increasing acceptance of grafts originating from extended criteria donors up to and including organs retrieved after cardiac arrest of the donor [1]. However, these grafts are often conflicted with a reduced functional reserve and, hence, less resilient against preservation and reperfusion injury [2,3].

New efforts were to be undertaken in order to mitigate preservation-associated injury and eventually reduce if not prevent primary dys- or even nonfunction of extended criteria organs after transplantation. These include short cold ischaemia times [4], the improvement of renal preservation by continuous hypothermic machine perfusion and novel approaches to recondition cold stored organs prior to transplantation [5,6].

In every day practice, however, over-optimistic use of marginal organs decreases primary function and graft survival, while precautious discard of grafts corroborates organ shortage.

Thus, a more safe extension of donor organ criteria has to comply with two interrelated goals: (i) improvement of pretransplant integrity of the graft, and (ii) development of evaluative measures, helping to decide whether a marginal graft should actually be used for transplantation or rather be discarded.

Ex situ perfusion with cold [7] or warm [8] perfusates allows for graft reconditioning prior to transplantation along with a variable degree of testing the organ in question.

Recent experiments are suggesting that the protective effect of hypothermic machine perfusion can be further enhanced by a controlled oxygenated rewarming (COR) [9] and, after

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preceding cold preservation, the controlled rewarming resulted in significantly better organ recovery than abrupt perfusion with warm perfusate [10,11].

In the present study, we investigated the potential of COR in predamaged porcine kidneys that were donated after cardiac arrest of the donor, to delineate the putative value of subnormothermic machine perfusion as a tool to assess renal function for prediction of later recovery upon warm reperfusion.

Materials and methods

All experiments were performed in accordance with the federal law regarding the protection of animals. The principles of laboratory animal care (NIH publication no. 85-23, revised 1985) were followed.

Deep general anaesthesia was induced in female German Landrace pigs weighing between 25 and 30 kg with intravenous application of 0·5 mg/kg midazolam and 12·5 μg/kg fentanyl and maintained by mechanical ventilation with isoflurane in air/oxygen and fentanyl perfused intravenously at 0·05 mg/kg/h. The abdomen was opened by midline incision, and cardio-circulatory arrest was induced by exsanguination of the animal through incision of the vena cava.

After a period of 30 min, the kidneys were removed from the abdominal cavity. The renal artery was cannulated, and the kidneys flushed by 100-cm gravity with 100 mL of HTK solution at 4 °C and then preserved by standard cold storage at 4 °C submerged in HTK preservation solution during 18 h in a cryothermostat. Untreated organs (n = 6) received no further treatment. Treated organs (n = 6) were procured and stored as described but additionally subjected to controlled oxygenated rewarming (COR) after the 18 h of static preservation in HTK as described earlier [10].

Technique of controlled oxygenated rewarming (COR)

After 18 h of static preservation at 4 °C, grafts were put on a machine perfusion circuit and 500 mL of Custodiol-N solution including 5 g/dL of dextran 40 were recirculated through the renal artery at a pulsatile pressure of 30/20 mmHg, similar to the way described earlier [9]. This time, however, the perfusion time was reduced to 90 min and the perfusate was also supplemented with 10 mg/dL of creatinine in order to allow for evaluation of renal clearance during the subnormothermic machine perfusion period (from 60 to 90 min). The solution was pumped through a hollow fibre oxygenator (Hilite LT 1000, Medos, Stolberg, Germany), fed with 100% oxygen, to allow for sufficient tissue aerobiosis during the rewarming process [12]. Oxygen partial pressures, measured with a temperature-compensated fibre optic oxygen meter (Fibox 3 LCD, PreSens precision sensing, Regensburg, Germany) at the venous effluent, did always exceed 200 mmHg. Urine production was followed at 30-min intervals, and samples were taken for determination of urinary creatinine.

During the first 30 min, temperature was kept hypothermic and was then gradually increased as represented in Fig. 1 by means of a programmable, external circulating cryothermostat connected to the integrated heat exchanger of the oxygenator.

Reperfusion model

Functional recovery of the grafts upon normothermic reperfusion was tested using an established isolated *in vitro* model as detailed previously [13]. In brief, kidneys were put into a moist chamber and perfused at 37 °C with 1000 mL Krebs–Henseleit buffer to which were added 2·2% bovine serum albumin and 20 mL of concentrated amino acid solution (RPMI 1640-50x). Prior to reperfusion, all grafts were left unperfused at room temperature for 20 min so as to simulate the surgical implantation *in vivo*.

Perfusate was oxygenated with a mixture of 95% oxygen and 5% carbon dioxide by a hollow fibre oxygenator (Hilite LT 1000, Medos) and supplemented with 10 mg/dL of creatinine and 100 mg/dL of urea to allow calculation of renal clearances. Cannulation of the ureter was performed with PE-tubing for urine collection throughout the reperfusion period.

Kidney perfusion pressure was set at 90 mmHg and automatically maintained by a servo-controlled roller pump, connected to a pressure sensor placed in the inflow line immediately prior to the renal artery.

After 30, 60 and 90 min of perfusion, urine and perfusate samples were taken for biochemical evaluation. Every 30 min,

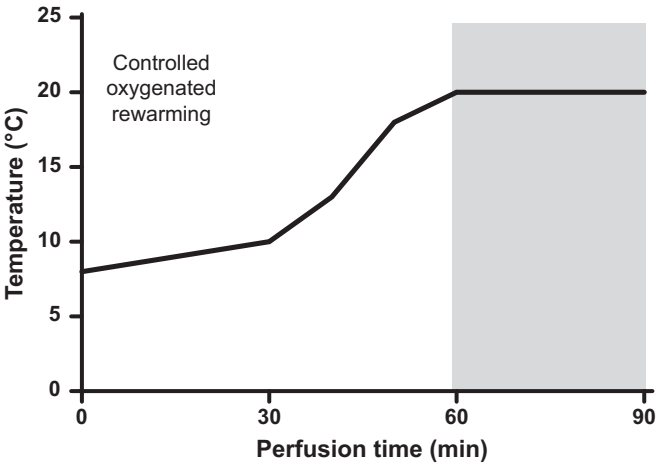

Figure 1 Controlled oxygenated rewarming: Gentle elevation of graft temperature upon extracorporeal oxygenated machine perfusion prior to warm reperfusion. During constant perfusion at 20 °C (grey zone), functional parameters (urine production and clearance of creatinine) are evaluated.

urine loss was replaced by adding equal amounts of balanced salt solution to the perfusion medium. Concentrations of urea and creatinine were determined in perfusate and corresponding urine samples in a routine fashion at the Laboratory centre of the University Hospital.

Clearances were calculated for the respective intervals as urinary creatinine (Urea) × urine flow (mL/min)/perfusate creatinine (urea).

Evaluation of graft function during COR

In a second set of experiments, the potential of COR should be tested as an evaluative tool for prediction of ulterior graft outcome upon reperfusion.

To this purpose, another six kidneys were retrieved without prior warm ischaemia *in situ* as to represent an appropriate collective for optimal procured grafts. These grafts also underwent 18 h of static cold storage n HTK, and the described controlled oxygenated rewarming protocol and allowed for an expansion of the range of functional integrity to be investigated. Functional data obtained during controlled rewarming were thus analysed in a total of 12 kidneys for their ability to discriminate between optimal and marginal donor kidneys, as well as for their correlation with kidney function upon warm reperfusion.

Mitochondrial function. Oxygen consumption was calculated from the pO_2 differences between arterial and venous sites, measured in a pH-blood gas analyser (ABL 500 acid-base laboratory, Radiometer, Copenhagen) and expressed as mL/min/g according to transrenal flow and kidney mass.

Renal tubular cell injury. The intracellular carrier protein L-type fatty acid binding protein (LFABP), predominantly expressed in liver tissue as well as in proximal tubular cells of kidney, was analysed to evaluate structural tubular damage [14]. Measurements were made with a commercialized ELISA kit (USCN life science, Wuhan, China) according to the instructions of the manufacturer on a fluorescence microplate reader (Tecan, Crailsheim, Germany).

Inflammatory activation upon ischaemia/reperfusion. Real-time Quantitative Taqman reverse transcriptase–polymerase chain reaction (RT-PCR) analysis of TNF-alpha, monocyte chemotactic protein 1 (MCP1), toll-like receptor 4 (TLR4) and E-Selectin gene expression was performed to detect proinflammatory activation reflecting endothelial injury. All analyses were performed on tissue samples taken at the end of reperfusion. Total RNA was extracted from snap-frozen tissue using TRI reagent (Applied Biosystems, Darmstadt, Germany). First-strand cDNA synthesis, RT-PCR and primer synthesis were essentially the same as described previously [10]. All samples were assayed in triplicate. The amount of specific mRNA in the tissue was expressed in arbitrary units after normalization for the respective individual quantities of transcripts of ribosomal protein L19 (RPL19), which was analysed as house-keeping gene. All primers were purchased from Applied Biosystems.

Statistics. All values were expressed as means ± SD unless otherwise indicated. After proving the assumption of normality, differences between two groups were tested by parametric comparison of the means using INSTAT 3.01 (GraphPad software Inc., San Diego, CA, USA). Areas under the curve were calculated using PRISM6 (GraphPad software Inc.). Linear regression curves, correlation coefficients and ROC curve data were calculated using SIGMAPLOT 13.0 (Systat Software Inc., San Jose, CA, USA). Statistical significance was set at $P < 0{\cdot}05$.

Results

Study 1: effect of COR on renal reperfusion injury in DCD kidneys

In DCD kidneys, renal clearances of creatinine as well as urea were significantly improved, when controlled oxygenated rewarming was performed prior to reperfusion (Fig. 2). While untreated kidneys showed limited and stable clearance values, COR led to an improved functional recovery with even increasing tendency over time. Similar patterns were observed with respect to urea, ending with clearance values of 3·4 ± 1·3 vs. 0·8 ± 0·3 mL/min (COR vs. untreated, $P < 0{\cdot}05$).

Metabolic activity as evaluated by the recovery of renal oxygen consumption was significantly improved to

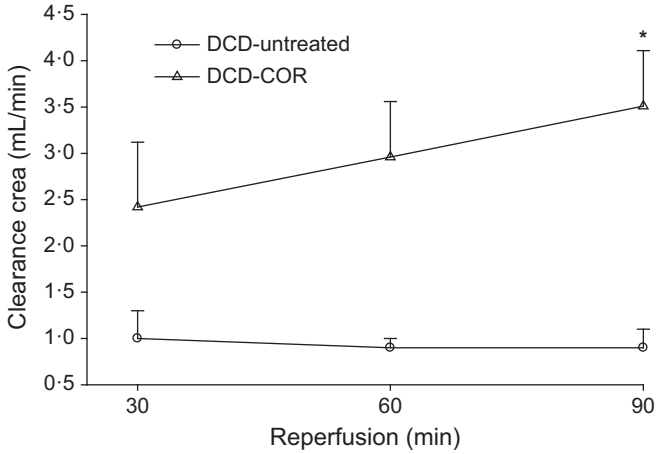

Figure 2 Renal clearances of creatinine upon reperfusion after 18 h of cold preservation with or without 90 min of controlled oxygenated rewarming (COR) prior to reperfusion. Data as mean ± SEM of $N = 6$ per group [$*P < 0{\cdot}05$ (area under the curve, AUC) vs. untreated].

4·1 ± 0·6 mL/min/100 g) after controlled rewarming compared to only 2·3 ± 1·2 mL/min/100 g in the untreated grafts.

The impact of COR at the end of ischaemic preservation on later tubular cell integrity could be evidenced by significant differences between the groups concerning the release of fatty acid binding protein. In untreated kidneys, L-FABP release increased on average to values twice as high as those observed after COR treatment (0·37 ± 0·16 ng/mL vs. 0·11 ± 0·10 ng/mL, $P < 0·05$).

The analysis of molecular expressions of selected genes, pertinent to inflammation or endothelial activation, showed that COR significantly ($P < 0·05$, resp.) reduced renal expression of TNF-alpha (0·09 ± 0·06 vs. 0·33 ± 0·12), MCP1 (0·07 ± 0·04 vs. 0·14 ± 0·02) as well as the endothelial surface receptors e-selectin (0·25 ± 0·32 vs. 1·66 ± 1·48) and TLR4 (0·44 ± 0·23 vs. 1·09 ± 0·34; a.U, COR vs. untreated, resp.).

Study 2: use of COR to predict organ function from grafts harvested prior or after circulatory arrest of the donor

Functional recovery upon reperfusion differed significantly between kidneys retrieved before or 30 min after cardiac arrest of the donor. In kidneys retrieved without warm ischaemic alterations, terminal clearance values of creatinine upon reperfusion averaged 6·8 ± 1·4 mL/min compared to only 3·4 ± 1·3 mL/min in the grafts procured after circulatory standstill.

Defining good functioning kidneys as those, that recovered renal clearance during reperfusion to values above 5 mL/min and marginal kidneys as those that did not, ROC curve analysis showed clearance of creatinine during COR to be significantly discriminative between the two groups (Fig. 3). Slightly less accurate results could be obtained for urine production and vascular resistance at subnormothermia. Of note, all parameters showed better discrimination at subnormothermia than during the initial hypothermic perfusion period, where vascular resistance did not quite reach significance and urine production was virtually absent in the kidneys retrieved after cardiac arrest.

The correlations of pump parameters with ulterior renal function upon reperfusion are depicted in Fig. 4. Vascular resistance during machine perfusion at 8 °C exhibited an only weak correlation with ulterior recovery of renal clearance of creatinine. Interestingly, better and significant values were

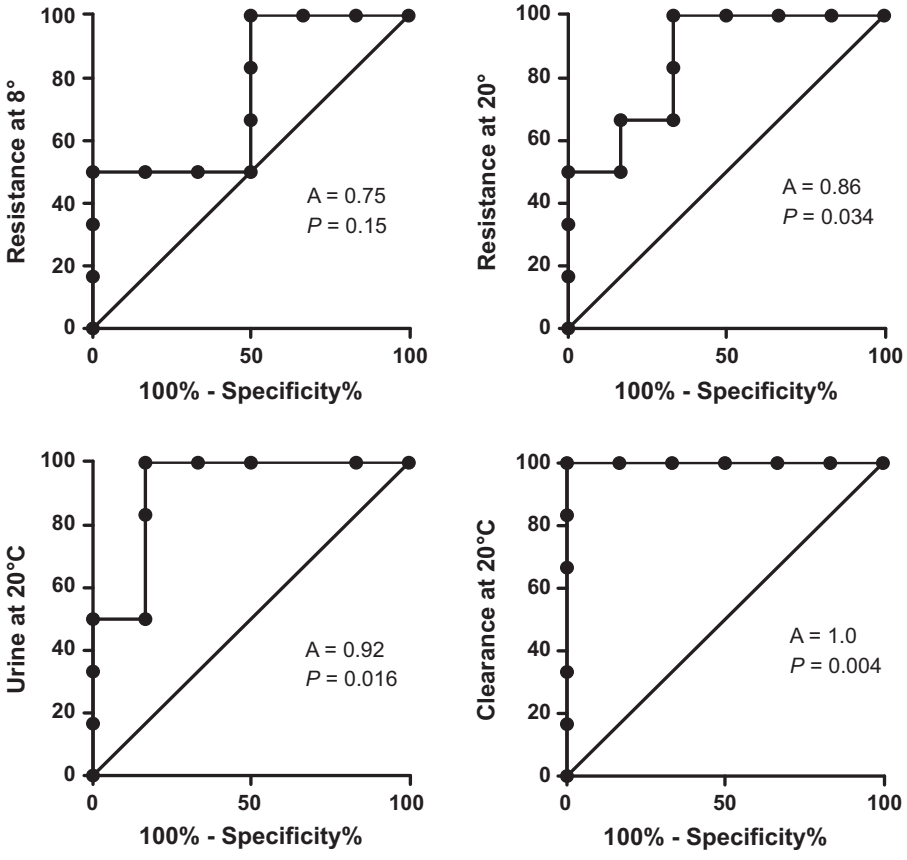

Figure 3 Receiver-operating curves illustrating the accuracy for some perfusion parameters observed during COR to allow for discrimination between kidneys that achieved or did not achieve terminal clearance values for creatinine of more than 5 mL/min upon warm reperfusion. The numbers in brackets indicate the area under the curve for each parameter.

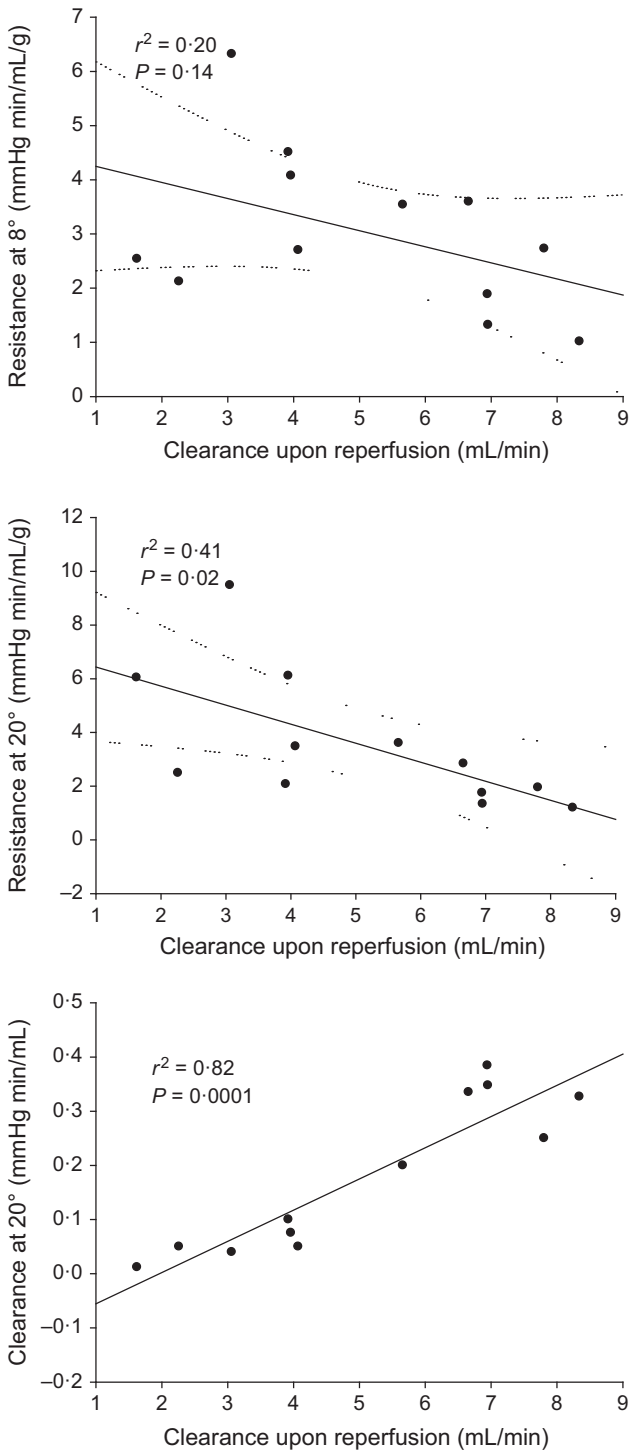

Figure 4 Correlation between vascular resistance values obtained during machine perfusion at 8 °C or at 20 °C, as well as clearance values of creatinine during machine perfusion at 20 °C and ulterior renal function observed upon reperfusion.

obtained when resistance during the later period of machine perfusion at 20° was taken into account (Fig. 4), but the correlation remained moderate with a coefficient of r^2 = 0·41 (P = 0·02).

Actual renal clearance during subnormothermic machine perfusion, however, yielded a much closer and highly significant correlation (r^2 = 0·82; P = 0·0001) with ulterior kidney function upon reperfusion.

Discussion

This study describes first experimental data in support for controlled oxygenated rewarming (COR) to be a valid therapeutic as well as evaluative tool in the preoperative handling of grafts donated after cardiac death. In the isolated kidney setting, COR helped to minimize renal preservation/reperfusion injury and resulted in an impressively improved early recovery of kidney function upon reperfusion.

Abrupt warming up after extended periods of hypothermia has already been incriminated to bear an intrinsic pathogenic potential, possibly triggering mitochondrial permeability pore opening and consecutive perturbation of respiratory chain function during warm reperfusion [15]. Although re-establishment of cellular redox homoeostasis by short-term oxygenated machine perfusion prior to transplantation has shown beneficial effects after cold preservation of livers [16] as well as kidneys [17], gentle rewarming of the graft resulted in even better protection and limited activation of the mitochondrial caspase cascade [9,10].

Actually, studies on mitochondria, isolated from rabbit kidney cortex, suggest that cold preservation affects mitochondrial function, but severe respiratory deficits actually manifested only upon transplantation [18]. Moreover, swelling of mitochondria and activation of the mitochondrial apoptotic pathway is recognized as one leading component that contributes to reduced renal function and survival after transplantation [19]. The present results in predamaged donor kidneys are in line with this, showing substantially improved mitochondrial function after COR while renal oxygen utilization was depressed after simple cold storage. Similarly, injury markers of renal tubular cells, that are known to rely heavily on aerobic energy production [20], were significantly less prominent after COR.

The pivotal challenge, however, when dealing with critical donor organs lies in the prediction of graft quality upon reperfusion.

It is known that warm ischaemic injury in kidneys from deceased donor kidneys is associated with distinct molecular signatures in the graft and could, for instance, be associated with strong tubular immunostaining for HMGB1, while no positive staining was observed in the kidney specimens from living donors [21].

These molecular parameters, however, may not be timely available before transplantation, and the evaluation of biomarkers and functional data during hypothermic machine perfusion has thus been the topic of many experimental and clinical studies [7,22,23].

In a large prospective analysis on 306 patients, the best predictive value of biomarkers was found for perfusate concentrations of glutathione-S-transferase, reaching an area under the curve of 0·67 in a ROC analysis [7]. Similar results were reported from Parikh *et al*.[23] concluding that perfusate biomarkers should not be used in isolation for discard decisions. Functional data on vascular resistance appear to be of slightly better prognostic value, but despite some association with the development of DGF in the recipient, the predictive power of hypothermic resistance values remains only moderate [24].

Likewise, we did not find better correlations of vascular perfusion parameters during long-term HMP nor short-term post-cold storage HMP in previous experimental studies with ulterior function of the kidney upon isolated reperfusion or after porcine autotransplantation [17]. Thus, in a model similar to the present study, vascular resistance during 18-h preservation by continuous pulsatile HMP at 2–4 °C correlated only weakly (r^2 = 0·4) with renal clearance function upon warm reperfusion (unpublished observations).

Our present data on vascular resistance are in also line with the above-mentioned reports, however, indicating that their discriminative value might be improved by increasing the temperature from hypo- to subnormothermia.

This study was aimed to test the hypothesis that even limited elevation of the temperature might increase accuracy of functional markers obtained during *ex situ* machine perfusion.

The present experiments suggest that warming up to 20° could be sufficient for that purpose, and may constitute as the more promising approach to predict ulterior kidney function upon warm reperfusion, but do not rule out that warming up to normothermia might further improve the predictive accuracy of the model.

We already know that, in order to obtain maximal tissue protection, cold stored organs should initially be subjected to hypothermic perfusion and only then be gently warmed up by gradually increasing the perfusate temperature [10]. While warming up only to subnormothermia can be performed with conventional preservation solution and does not require additional substrates or oxygen carriers, warming up to normothermia might possibly produce even more accurate functional evaluation of the kidney. However, cold-to-normothermic perfusion may need further investigations of novel adequate perfusate compositions, including the possible need of oxygen carriers for final perfusion at 37 °C, and will certainly increase the complexity of the approach and make it more cumbersome.

Future studies will surely be needed to optimize technique and temperature for the most meaningful protocol while still keeping the system as safe and simple as possible.

Thus, final conclusions must not be drawn from the present *in vitro* study, but controlled oxygenated rewarming seems, on principle, to be a promising conceptual adjunct for preoperative care of renal grafts that could help to improve and to evaluate graft function and merits further, more complex investigations with longer observation times and actual transplantation.

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Conflict of interests

The authors of this manuscript have no conflict of interests to disclose.

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