

Controlled Oxygenated Rewarming Compensates for Cold Storage–induced Dysfunction in Kidney Grafts

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Background. Normothermic machine perfusion (NMP) provides a promising strategy for preservation and conditioning of marginal organ grafts. However, at present, high logistic effort limits normothermic renal perfusion to a short, postponed machine perfusion at site of the recipient transplant center. Thus, organ preservation during transportation still takes place under hypothermic conditions, leading to significantly reduced efficacy of NMP. Recently, it was shown that gentle and controlled warming up of cold stored kidneys compensates for hypothermic induced damage in comparison to end ischemic NMP. This study aims to compare controlled oxygenated rewarming (COR) with continuous upfront normothermic perfusion in a porcine model of transplantation. **Methods.** Following exposure to 30 min of warm ischemia, kidneys (n = 6/group) were removed and either cold stored for 8 h (cold storage [CS]), cold stored for 6 h with subsequent controlled rewarming up to 35 °C for 2 h (COR), or directly subjected to 8 h of continuous NMP. Kidney function was evaluated using a preclinical autotransplant model with follow-up for 7 d. **Results.** NMP and COR both improved renal function in comparison to CS and displayed similar serum creatinine and urea levels during follow-up. COR resulted in less tenascin C expression in the tissue compared with CS, indicating reduced proinflammatory upregulation in the graft by gentle rewarming. **Conclusions.** COR seems to be a potential alternative in clinical application of NMP, thereby providing logistic ease and usability.

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INTRODUCTION

Extensive research and technical refinement of devices for normothermic machine perfusion (NMP) of organ grafts have succeeded in making NMP a clinical alternative for the preservation and conditioning of extended criteria donor organs.^{1–3}

In contrast to static cold storage (CS), NMP allows for maintenance of physiological cellular metabolism and provides an optimized milieu for possible repair processes in the injured tissue. However, clinical usage of normothermic renal perfusion has so far been limited to a short, postponed reconditioning phase, performed only at the implantation clinic after conventional transport of the organs by classic CS.⁴

At present, the logistic effort of machine trafficking for usage of normothermic perfusion techniques from retrieval to implantation appears to be a rather cumbersome and expensive drawback of these methods. Although “in house” renal graft reconditioning by 1 or 2 h of NMP after preceding CS improved early graft function after transplantation,^{4,5} accumulated experimental evidence is indicating that the protective efficiency of NMP significantly decreases along with the extension of the preceding period of static CS.⁶

Similarly, Reddy et al⁷ have found that even a short period of cold ischemia before NMP is significantly deleterious to the function of porcine liver grafts.

It might hence be conjectured, that irreversible tissue injury is associated with the preceding period of presumably inferior preservation by CS and that this damage could no longer be made up for by postponed machine perfusion.⁶

On the other hand, the abrupt shift in temperature upon warm reperfusion of the cold organ can in itself constitute a genuine trigger for significant impairments in the

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respiratory control ratio and subsequent graft dysfunction after transplantation (temperature paradox).⁸ The deleterious impact of cellular injury by simple rewarming occurs after several hours of hypothermia and increases with the duration of the preceding cooling period.⁹

However, a strategy to circumvent or at least alleviate the temperature paradox upon rewarming after cold preservation was recently investigated by our group, using a gentle and adapted rise in the perfusion temperature from hypothermia to normothermia upon transfer of the kidney to the machine.¹⁰

It could actually be shown in isolated porcine kidneys that the benefit of NMP after preceding 18 h of CS could be significantly enhanced, if a sudden exposure of the cold stored graft to normothermic temperature was avoided by controlled oxygenated rewarming (COR) of the graft on the machine during the first 90 min of perfusion.¹⁰ Interestingly, COR-treated kidneys also displayed a significantly better metabolic efficiency than kidneys after abrupt NMP, as the ratio of oxygen consumption and the major work load of the kidney, the reabsorption of sodium was far less impaired upon reperfusion.^{8,10}

Based on these observations, it is tempting to ask, up to which extent the circumvention of rewarming injury by controlled warming up after CS would be able to compete with the results of a continuous normothermic perfusion right from the beginning.

The present study was therefore undertaken to compare both methods, that is, up front NMP and a short period of controlled oxygenated rewarming following CS in a systematic preclinical transplant model in the pig.

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.

German landrace pigs weighing between 25 and 30 kg were used for the study. All animals had free access to tap water and standard pellet food. Solid food was withdrawn 20 h before beginning of the experiments. A porcine autotransplantation model was used as previously established in our laboratory and detailed elsewhere.^{11,12} In brief, the right internal jugular vein was cannulated under general anesthesia, with polyethylene tubing for infusion and daily collection of blood samples. The renal artery of the left kidney was clamped to induce 30 min of warm ischemia. After left nephrectomy, the kidney was flushed on the back-table with HTK solution from a height 100 cm till the effluent was clear. Kidneys (n = 6/group) were randomly assigned to 1 of the following groups (cf. Figure 1).

1. Direct connection to a pulsatile NMP for 8 h. NMP was effectuated based on the technique described by Kathis et al⁶ at 75 mm Hg with Steen solution (XVivo, Göteborg, Sweden) (300 mL, diluted 1/1 with Ringer's solution¹⁰), supplemented with 2.2 mL calcium gluconate 10%, 6.6 mL sodium bicarbonate 8.4%, 4 mg dexamethasone, and 0.33 g/L cefazolin. Amino acid solution (aminomel nephro) was constantly infused by a rate of 1 mL/h. Perfusate was oxygenated with 1 L/min of 95% oxygen and 5% carbon dioxide by means of a

hollow fiber oxygenator placed in the arterial line of the circuit. Venous perfusate pO₂ thus always remained above 200 mmHg and adequate oxygen delivery to the graft was guaranteed. Verapamil was given as a single dose as required to maintain renal flow.

2. Kidneys were exposed to hypothermic storage at 4°C for 6 h, followed by end-ischemic machine perfusion for 2 h with diluted STEEN solution.

The initial perfusion temperature was set to 8°C but later on a COR of the perfusate and the organ graft was initiated in a hyperbolic pattern by rising the temperature to 10°C, 17°C, 30°C, and 35°C after 30, 60, 75, and 90 min, respectively. The rise in temperature was accompanied by an adapted increase in perfusion pressure from 30 to 75 mm Hg. Details of this protocol have been described previously.^{10,13}

3. Kidneys were exposed to 8 h hypothermic storage at 4°C without additional treatment (control group [CS]).

After 8 h of preservation, kidneys were flushed with cold HTK solution and autotransplanted subsequent to removal of the native contralateral kidney. Vascular anastomoses were performed end to side (renal vein-vena cava) and end to end (left renal artery-right renal artery), respectively. At the time of reperfusion, 20 mL of glucose 40% was infused to induce osmotic diuresis. No other diuretics were given. The ureter was cannulated with polyethylene tubing, which was tunneled through the abdominal wall, allowing continuous visual inspection of urine production.

Renal tissue perfusion was assessed noninvasively 10 min after reperfusion as mean cortical erythrocyte flux, determined by LASER Doppler flowmetry as detailed previously.^{14,15} To account for temporal variations in blood flow, we calculated the mean flux value over 10 s of recording and to eliminate the influence of spatial heterogeneity, we performed measurements on 4 distinct places of the renal surface. All flux measurements were taken as percent variation from the baseline values obtained from the non-ischemic native kidneys.

Pigs were transferred back to the care station. Follow-up of animals was 7 d. To reduce the risk of infection, perioperative antibiotics with amoxicillin (Duphamox, 15 mg/kg i.m.) was applied at the onset of experiment. Pigs were supplied with analgesics (Carprieve, 4 mg/kg i.v.) for the first 3 d posttransplantation. After 1 wk, animals were euthanized in deep anesthesia. The transplanted kidney was removed, and tissue samples were collected.

Analytical Procedures

Serum concentrations of creatinine and urea were determined in a routine fashion at the Laboratory center of the University Hospital.

Clearances were calculated for the respective intervals as urinary creatininexurine flow/perfusate creatinine.

Oxygen partial pressure and perfusate concentrations of sodium and glucose were measured in a pH-blood gas analyzer (ABL 815flex acid-base laboratory, Radiometer, Copenhagen).

Oxygen consumption (VO₂) was calculated from the differences between arterial and venous sites and expressed as μmol min⁻¹ g⁻¹ according to transrenal flow and kidney mass.

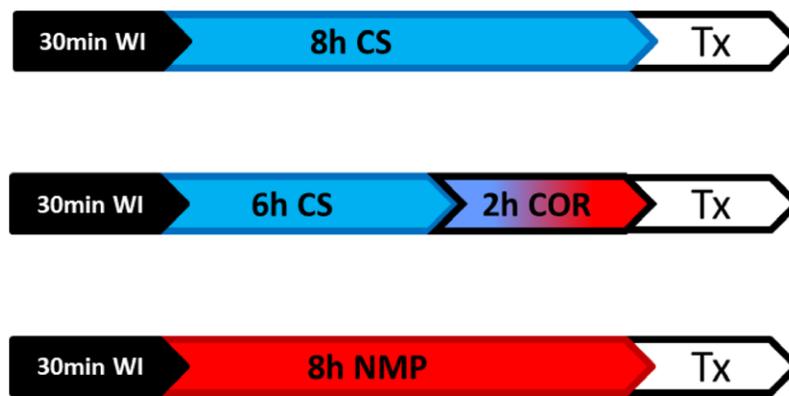


FIGURE 1. Schematic representation of study design and experimental groups. CS, cold storage; COR, controlled oxygenated rewarming; NMP, normothermic machine perfusion; Tx, transplantation; WI, warm ischemia.

Fractional excretion of sodium has been calculated according to:

$$\text{Fractional excretion of sodium} = \frac{\text{Na}_{(\text{urine})} \times \text{Creatinine}_{(\text{perfusate})}}{\text{Na}_{(\text{perfusate})} \times \text{Creatinine}_{(\text{urine})}} \times 100.$$

Concentrations of aspartate aminotransferase were measured by reflectance photometry on a Reflotron Plus point of care unit (Roche Diagnostics, Mannheim, Germany).

Oxygen-free radical-induced tissue injury was approximated by the amount of thiobarbituric acid-reactive substances, breakdown products of lipid peroxidation, released into the circulation. Thiobarbituric acid-reactive substances were evaluated by fluorimetry from deproteinized serum samples using the adduct formation with thiobarbituric acid as detailed elsewhere.¹⁶

Leukocyte infiltration into the tissue was approximated by the content of myeloperoxidase (MPO) that was determined in tissue homogenates using a commercialized Elisa test-kit distribute by LS Bio (Seattle, WA).

Gene Expression Analyses

Total RNA was isolated from snap-frozen samples and analyzed as described previously.¹⁰ The amount of specific mRNA in the tissue was normalized for the respective individual quantities of transcripts of GAPDH, which was analyzed as housekeeping gene. Results are expressed as relative deviation from baseline levels that were analyzed from native cortical kidney samples processed in parallel. All reagents and primers for GAPDH (No. PPS00192A), interleukin 6 (No. PPS00991A), tenascin C (TNC; No. PPS00771A), and tumor necrosis factor (No. PPS00426A) were purchased from Qiagen GmbH (Hilden, Germany).

Histology

Kidney tissue was collected at the conclusion of the experiments, cut into small blocks (3 mm thickness), and fixed by immersion in 4% buffered formalin. The blocks were embedded in paraffin, and 2 to 4 mm tissue slides were prepared using a microtome (SM 2000R, Leica Instruments, Nußloch, Germany). Hematoxylin eosin staining was conducted adherent to in-house standards and used to assess morphological integrity of the parenchyma.

Assessment was carried out in an anonymous fashion in 10 randomly chosen, nonoverlapping fields ($\times 400$ magnification), using a 5-point scale for tubular dilatation,

vacuolization, glomerular damage, and tubular shedding as described previously¹⁷: 0 = no damage; 1 = lesions affecting <10% of the field; 2 = 10% to 25%; 3 = 25%–50%; 4 = 50% to 75%; and 5 > 75%. In each individual kidney, the mean value from the 4 respective parameters was calculated and taken as individual mean histological injury score.

Statistics

All values are expressed as means \pm SD. After proving the assumption of normality, differences between the groups were tested by 1-way ANOVA and post hoc testing with the Student Newman Keuls test (Instat 3.01; Graph Pad software, Inc, San Diego, CA), unless otherwise indicated. Statistical significance was set at $P < 0.05$.

RESULTS

There were no significant differences in the weight of the animals or anastomoses among the groups ($n = 6$).

The weight averages were 27.4 ± 2.5 , 28.4 ± 1.5 , and 27.8 ± 2.4 kg in the CS group, the COR group and the NMP group, respectively. The mean anastomoses times were 29.67 ± 2.7 min in the CS group, 31.00 ± 3.7 min in the COR group, and 28.3 ± 3.5 min after NMP.

Renal Function at the End of Machine Perfusion

Table 1 shows the results obtained upon oxygenated NMP for 8 h or after 6 h of CS and subsequent COR for 2 h.

TABLE 1. Functional data at the end of machine perfusion in the NMP and the COR group.

	NMP	COR
AST (U/L)	392 \pm 342	17.1 \pm 15.6 ^a
Flow (mL/min)	235 \pm 45	195 \pm 63
Urine (mL/30 min)	14.0 \pm 18.7	17.7 \pm 15.8
Clearance	1.5 \pm 1.5	2.2 \pm 2.1
FENa%	0.24 \pm 0.22	0.30 \pm 0.13
VO ₂ (mL/100 g/min)	2.0 \pm 0.9	2.3 \pm 0.5

Values are given as mean and SD of $n = 6$ experiments per observation point.

^a $P < 0.05$ or better.

AST, aspartate aminotransferase; COR, controlled oxygenated rewarming; FENa, fractional excretion of sodium; NMP, normothermic machine perfusion; VO₂, volume of oxygen.

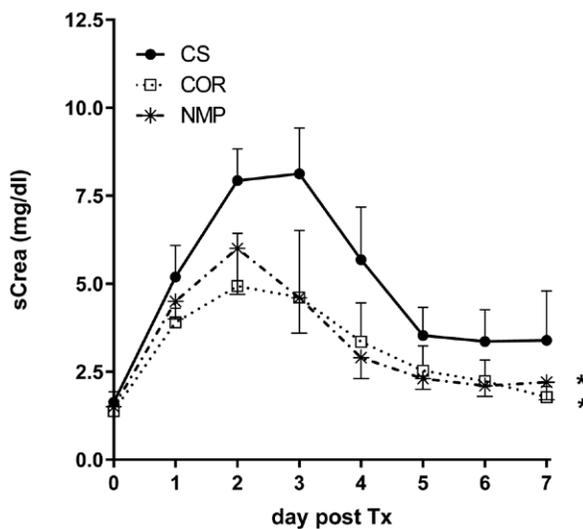


FIGURE 2. Autograft function evaluated by levels of serum creatinine (sCrea) after transplantation in kidneys preserved by cold storage (CS) and 2 h of controlled oxygenated rewarming (COR) or by continuous normothermic machine perfusion (NMP). Values are given as mean \pm SD. Statistical analysis done by comparing areas under the curves and post hoc testing with Student Newman Keuls test. * $P < 0.05$ vs CS and NMP vs CS. Tx, transplantation.

It is seen that controlled rewarming effectively reconstituted renal function even after preceding CS to the same level as was achieved by continuous NMP. Moreover, cellular enzyme leakage of aspartate aminotransferase was notably reduced, if total time on the machine was restricted to the last 2 h of preservation, but this might be due to the much lower time of accumulation in the perfusate.

Renal Function After Transplantation

Microcirculatory tissue perfusion was investigated in the renal cortex by Laser Doppler flowmetry 10 min after reperfusion. We could disclose a significantly less compromised erythrocyte flux in both machine perfusion groups in comparison with the CS protocol. However, no significant difference could be substantiated between COR and NMP. The detected Flux values averaged 39.9 ± 7.3 in the CS group, $65.0 \pm 21.5^*$ in the COR group and $58.2 \pm 10.0^*$ after continuous NMP, expressed in percent of baseline values obtained from the nonischemic naive kidney before explantation (* $P < 0.05$ versus CS).

Functional Recovery

Serum creatinine levels rose after kidney transplantation in all of the 3 groups (Figure 2), but the increase remained significantly lower in the 2 treatment groups. Thereby, no relevant difference could be seen between COR or NMP treated kidneys. In both treatment groups, near-normal values were reached after approximately 5 d, whereas serum creatinine levels of cold stored kidneys did recover later and in a more incomplete manner during the observation period.

Animals with kidney grafts transplanted after CS preservation showed a peak serum creatinine of 8.3 ± 1.1 mg/dL. In contrast, animals that received kidneys after CS and COR treatment had a significantly lower peak of 5.4 ± 1.3 mg/dL ($P < 0.05$) and were thus quite

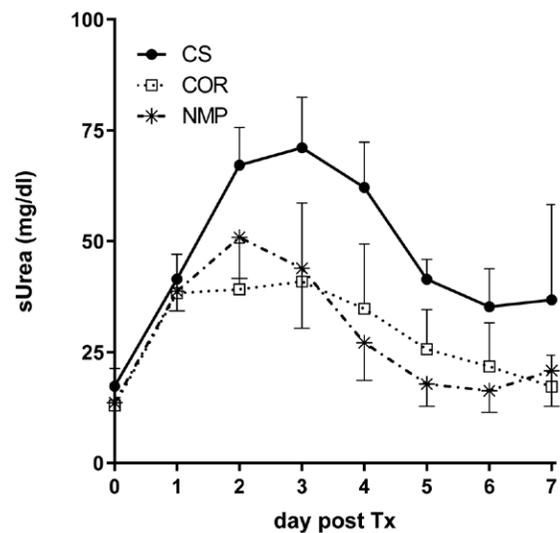


FIGURE 3. Systemic levels of urea after transplantation in kidneys preserved by cold storage (CS), cold storage and 2 h of controlled oxygenated rewarming (COR), or by continuous normothermic machine perfusion (NMP). Values are given as mean \pm SD. Statistical analysis done by comparing areas under the curves and post hoc testing with Student Newman Keuls test. * $P < 0.05$ vs CS and NMP vs CS. Tx, transplantation.

comparable to kidneys after NMP (6.0 ± 2.5 mg/dL; $P < 0.05$ versus CS).

Peak values of serum creatinine were reached on average after 2.83 ± 0.7 d postoperatively in the CS group, whereas in the COR and the NMP group serum creatinine already peaked after 1.80 ± 0.7 and 2.0 ± 0 d following transplantation.

Systemic concentrations of urea followed a similar pattern (Figure 3). COR as well as NMP significantly mitigated posts ischemic rise of serum urea levels in comparison to simple CS. Maximal values of serum urea averaged 75 ± 10 mg/dL in the CS group, but were significantly reduced to 49 ± 11 mg/dL and 49 ± 17 mg/dL in the COR and the NMP group, respectively ($P < 0.05$ COR versus CS and NMP versus CS).

Endothelial Activation and Inflammation

Thiobarbituric acid reactive substances were measured as a general readout of oxygen free radical mediated tissue injury. We did find 3.8 ± 0.5 nmol/mL in the CS group and 3.0 ± 0.5 nmol/mL versus 3.1 ± 0.8 nmol/mL after COR and NMP, respectively. No significant differences among the groups were established ($P = 0.08$).

Leukocyte infiltration, followed by quantification of MPO in the tissue, did not differ between cold stored kidneys and kidneys that were subjected to the COR protocol (cf. Table 2). However, NMP did result in a significant increase of MPO tissue levels at the completion of the experiment.

Two inflammatory cytokines and 1 danger-associated matrix protein were also studied to examine the influence of the preservation methods on inflammatory activation in the graft.

It was seen that the COR protocol never resulted in higher expression of any of the parameters under investigation in comparison to the NMP group.

Only gene expression of tumor necrosis factor significantly increased after NMP, whereas the expression of the

TABLE 2.

Intragraft inflammatory reaction as evaluated by tissue levels of leukocyte MPO and gene expression of the danger-associated matrix protein TNC and proinflammatory cytokines IL6 and TNF α , expressed in percent of baseline values (%BL).

	CS	COR	NMP
MPO, pg/mL	133 \pm 46	100 \pm 64 ^b	210 \pm 52 ^a
TNC, %BL	4.3 \pm 3.5	0.8 \pm 0.3 ^a	1.8 \pm 1.1
IL6, %BL	38 \pm 34	17 \pm 17	29 \pm 30
TNF, %BL	4.5 \pm 3.7	3.2 \pm 2.1	8.2 \pm 6.1 ^a

^a*P* < 0.05 vs CS.

^b*P* < 0.05 vs NMP.

COR, controlled oxygenated rewarming; CS, cold storage; IL6, interleukin 6; MPO, myeloperoxidase; NMP, normothermic machine perfusion; TNC, tenascin C; TNF α , tumor necrosis factor alpha.

danger signal TNC was found to be significantly reduced after COR with respect to the CS group.

Histological Analysis

Light microscopy performed on tissue samples obtained after conclusion of the experiment, 1 wk after transplantation did not disclose severe alterations in either group. Overall, slight alterations of normal structural appearance were observed in any group, mainly comprising tubular cell damage and inflammatory reactions.

However, least injury could be detected in the COR-treated kidneys, the pathological injury score of which was found significantly reduced in relation both other groups (cf. Figure 4).

DISCUSSION

Recent upsurge of NMP for preservation and conditioning of marginal organ grafts has brought along some logistic inconveniences, as the necessity to bring in the more cumbersome perfusion devices to the donor hospital and to supervise the transport of the perfused organ to the recipient. This study provides first in vivo evidence that postponed NMP, if done by controlled rewarming of the cold stored graft, actually can compete with continuous normothermic perfusion preservation of kidney grafts in respect to early functional recovery after transplantation.

Although postponed NMP has already been applied clinically with obviously superior results than mere preservation by simple CS,⁴ there is experimental evidence, that preceding periods of static cold preservation gradually impede the beneficial effect of NMP thereafter⁶ and that after extended CS, hypothermic machine perfusion (HMP) might be superior to NMP.¹⁸ HMP has often been shown to improve on the results of simple CS and has the advantage over NMP to allow for a comparatively facilitated organ transport. However even optimal preservation by HMP leaves the kidney subject to the rewarming injury upon reperfusion,¹⁹ which, by contrast, can be mitigated by the COR protocol and prevented by up-front continuous NMP.

The abrupt return to normothermia after longer times at hypothermia constitutes a noxious process to cellular homeostasis, notably at the mitochondrial level.^{8,10,20} Thus, the organ-protective potential of postponed NMP could be

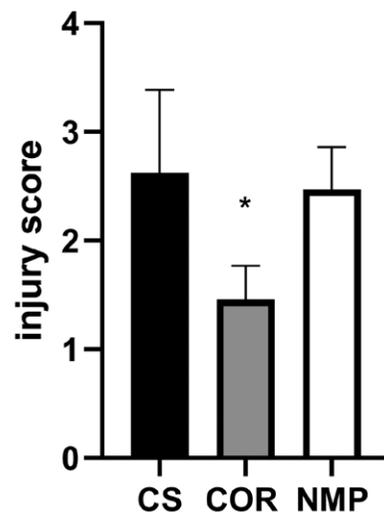


FIGURE 4. Histological injury evaluation by light microscopy 1 wk after transplantation of kidneys preserved by cold storage (CS) and 2h of controlled oxygenated rewarming (COR) or by continuous normothermic machine perfusion (NMP). Sections were scored in a blinded fashion using a semiquantitative scale as detailed in material and methods. Values are given as mean \pm SD (**P* < 0.05 vs CS and NMP).

optimized by gradually re-equilibration of cellular energy and signal homeostasis through controlled restitution of normal temperature and resumption of metabolism.^{8,10}

The main issue of the present study was to delineate the extent, up to which a controlled rewarming could make up for in comparison with the “gold standard” of up-front NMP.

In comparison to static storage alone, NMP and COR equally and significantly enhanced global renal function after transplantation as judged from peak values and time course of serum creatinine and urea levels.

Less consistent, however, were the parameters on inflammatory reaction in the graft.

Several authors have suggested NMP to be operative in minimizing proinflammatory gene expression in livers²¹ or interstitial inflammation in kidney grafts.²² On the other hand, NMP is told to potentially trigger the activation of “danger” signals²³ and experimental studies describe a proinflammatory response with increase of interleukin levels after NMP in kidneys²⁴ or cytokine upregulation during NMP of lungs,²⁵ respectively. Moreover, Scheuermann et al²⁶ reported that subnormothermic machine perfusion had a less priming influence on the NLRP3 inflammasome than was observed after normothermic liver perfusion.

In our case, NMP did not actually reduce signs of inflammation in the grafted kidney either. However, COR after CS resulted in a significant reduction of the expression of tenascin C in comparison to the CS group.

In so far, our results confirm earlier in vitro data also showing a significant attenuation of the expression of danger associated patterns including TNC by controlled rewarming before reperfusion of cold stored kidney grafts.²⁷ Tenascin C is an extracellular matrix glycoprotein that is specifically induced upon injury and functions like a danger signal, normally absent in most healthy tissue.²⁸

It is operative in suppressing the synthesis of anti-inflammatory cytokines and enables proinflammatory early immune responses.²⁹ Beyond that, in promoting interstitial fibroblast

proliferation,³⁰ TNC might also be considered a risk factor for the development of renal fibrosis after transplantation.

However, long-term developments after transplantation were not to be addressed in this study with a follow-up period limited to the evaluation of early graft function after transplantation. Other putative limitations of our study model may be seen in the use of young and healthy animals, the circumvention of immunosuppressive medication and a possible risk of bias owing to the unblinded study design. The implementation of a warm ischemic challenge to the donor organ before preservation has been undertaken to induce a less than optimal graft and mitigate the first aspect. The shortfall of not challenging the implanted organs by an immunosuppressive regimen has been deliberately chosen to minimize confounding variables incurred by interindividual variances in the reaction to the immunosuppression in experimental animals that had not been immunologically standardized. Under the conditions of our study, the combination of simple transport on ice with a postponed controlled warming up perfusion at the implant clinic has shown to be as effective as continuous up front NMP in regard to the early functional recovery of kidney grafts. In preserving the ease of simply putting the graft on CS during retrieval and transportation, the postponed machine perfusion has obvious logistic advantages. Further exploitation of this concept is thus recommended.

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