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ORIGINAL ARTICLE

One or 4 h of "in-house" reconditioning by machine perfusion after cold storage improve reperfusion parameters in porcine kidneys

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Summary

In-house machine perfusion after cold storage (hypothermic reconditioning) has been proposed as convenient tool to improve kidney graft function. This study investigated the role of machine perfusion duration for early reperfusion parameters in porcine kidneys. Kidney function after cold preservation (4 °C, 18 h) and subsequent reconditioning by one or 4 h of pulsatile, nonoxygenated hypothermic machine perfusion (HMP) was studied in an isolated kidney perfusion model in pigs (n = 6, respectively) and compared with simply cold-stored grafts (CS). Compared with CS alone, one or 4 h of subsequent HMP similarly and significantly improved renal flow and kidney function (clearance and sodium reabsorption) upon warm reperfusion, along with reduced perfusate concentrations of endothelin-1 and increased vascular release of nitric oxide. Molecular effects of HMP comprised a significant (vs CS) mRNA increase in the endothelial transcription factor KLF2 and lower expression of endothelin that were observed already at the end of one-hour HMP after CS. Reconditioning of cold-stored kidneys is possible, even if clinical logistics only permit one hour of therapy, while limited extension of the overall storage time by in-house machine perfusion might also allow for postponing of transplantation from night to early day work.

Introduction

Early graft dysfunction due to preservation/reperfusion injury still represents a notable issue after kidney transplantation, affecting long-term prognosis of graft viability [1–3]. Any improvement in the preservation of grafts that experienced extended times of preservation or were procured from extended criteria donors will help to enlarge the total number of viable donor organs and to circumvent the need of re-transplantation.

Recent upsurge in more dynamic preservation modalities has brought up experimental as well as clinical evidence that continuous hypothermic machine perfusion significantly improves renal function and survival after transplantation compared with conventional cold storage [4–7].

Since the ischemic period itself in large parts only predisposes the tissue to compromised resumption of cellular function upon warm reperfusion, deleterious priming of the graft during ischemia might still be abrogated prior to transplantation by dynamic revitalization techniques at the end of cold storage.

Meanwhile, brief in-house machine perfusion after preceding cold storage (hypothermic reconditioning) has been proposed as a convenient tool to improve organ graft function in livers and kidneys [8]. Thus, a two-hour period of endischemic reconditioning by pulsatile oxygenated HMP was shown in porcine kidney transplants to be equally

effective than continuous perfusion starting from the time of organ retrieval [9].

Subsequent clinical application of hypothermic machine perfusion after cold storage produced a first clinical evidence for the beneficial effect of the technique [10].

However, the question of the importance of the duration of machine perfusion prior to transplantation still remains open to debate.

While a too short time on the machine could bear the risk of producing inferior benefits, extension of machine perfusion after arrival of the organ at the hospital would possibly interfere with hospital logistics and extend total cold ischemia time.

This study was therefore aimed to compare two protocols of reconditioning, that is, an ultra-short perfusion period with an extended duration of post-cold storage HMP in their ability to improve renal function upon warm reperfusion in vitro as well as the respective influence on underlying pathophysiological mechanisms.

Material and methods

All experiments were performed in accordance with the federal law regarding the protection of animals.

Porcine kidneys were procured from German landrace pigs weighing between 25 and 30 kg. Premedication was performed with ketamine (90 mg/kg), xylazine (10 mg/kg) and atropine (10 μg/kg) 10 min before inducing anesthesia.

Prior to intubation and mechanical ventilation with an air/oxygen mixture (70/30%), midazolam (0.5 mg/kg) and fentanyl (12.5 μg/kg) were given intravenously via the ear vein. An intravenous jugular vein catheter was inserted for continuous infusion of fentanyl (0.004 mg/kg/h) and propofol (10 mg/kg/h) to maintain general anesthesia.

After nephrectomy, the procured organ was flushed on the backtable with approx. 100 ml HTK solution at 4 °C using gravity perfusion (100 cm H20). After static cold storage at 4 °C in HTK for 18 h, the kidneys were randomly assigned to one of the following groups ($n = 6$, respectively):

Group 1: Cold-stored kidneys were used for the experiments without further treatment and served as controls.

Group 2: Cold-stored kidneys were subsequently subjected to hypothermic reconditioning consisting of 1 hour of hypothermic machine perfusion in a pulsatile manner (30/20 mmHg) at 30 bpm. with the recently developed Custodiol-N solution containing 5 g % of dextran 40 [11]. No active oxygenation was applied during machine perfusion.

Group 3: In this group, hypothermic reconditioning after cold storage was performed like in group 2, but the duration of machine perfusion was extended to 4 h.

Isolated kidney perfusion

Immediately prior to reperfusion, all organs were exposed to no-flow conditions at room temperature for 20 min in order to imitate warm ischemia time in the clinical setting [12].

Renal reperfusion in vitro was performed using an established model according to earlier studies [13,14] with some modifications.

The perfusion medium consisted of 1000 ml freshly prepared Krebs-Henseleit buffer containing 2.2% bovine serum albumin and 20 ml of concentrated amino acid solution (RPMI 1640-50x). Creatinine (0.01 g/l) and urea (1 g/l) were added to the perfusate to allow for calculation of respective renal clearances. The ureter had been cannulated with PE tubing, and urine was collected throughout the reperfusion period.

Kidneys were placed in a moist temperature chamber and perfused at 37 °C. Perfusate was oxygenated in a temperature-controlled hollow fiber oxygenator (Hilite LT 1000, Medos, Stolberg, Germany) with a mixture of 95% oxygen and 5% carbon dioxide. Temperature was regulated by a circulating thermostat, connected to perfusion chamber and oxygenator.

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

Concentrations of creatinine and urea were determined in perfusate and corresponding urine samples in a routine fashion at the Laboratory centre of the University Hospital Bonn.

Clearances for creatinine (and urea, respectively) were calculated for the respective intervals as urinary creatinine x urine flow (ml/min)/perfusate creatinine.

For analysis of endischemic gene expression after cold preservation with or without subsequent machine perfusion for different time periods, additional experiments were carried out without reperfusion.

Total RNA was isolated from snap-frozen samples using TRIreagent (Applied Biosystems, Darmstadt, Germany). Equal amounts of RNA were quantified by Nano Drop (Thermo Fisher, Waltham, MA) complementary DNA by incubation with High Capacity cDNA RT Kit (Applied Biosystems). The PCR mix was prepared using TaqMan GenEx Master Mix (Applied Biosystems). 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 analyzed as house-keeping gene.

Primers for RPL19 (n°Ss03375624_g1), endothelin 1 (n°Ss03392455_m1), endothelial nitric oxide synthase—eNOS (n°Ss03383940_u1), nuclear factor kappa B1

(n°Ss03388575_m1) and interleukin 6—IL6 (n°Ss0338 4604_n1) were purchased from Applied Biosystems.

Sequences of the PCR primers for Krüppel-like Factor 2 (KLF-2), customized by Applied Biosystems (custom Taq-Man Gene Expression Assay, Part Number 4331348), were as follows: sense GCGCTGGGCTTGGC and antisense GCGGCGTGAGGAGACC.

Analytic kits from the following companies were used according to the instructions of the manufacturers to analyze perfusate levels of endothelin 1 (USCN life science, Wuhan, China) and total nitric oxide (R&D Systems, Wiesbaden, Germany).

Perfusate concentrations of caspase-cleaved keratin 18 were assessed photometrically as indicator of renal apoptosis upon reperfusion using a commercialized ELISA kit (M30, Pevia, Stockholm, Sweden). Measurements were done on a fluorescence micro plate reader (Tecan, Grailsheim, Germany).

Statistics

All values were expressed as means ± SEM. After proving the assumption of normality, differences between groups were tested by analysis of variance (ANOVA) and multiple comparison of the means with the Student–Newman–Keuls test, unless otherwise indicated. Statistical significance was set at $P < 0.05$.

Results

Renal function

Glomerular function of the preserved kidneys was estimated by the calculation of renal clearances for creatinine or urea (Fig. 1). This was found to be significantly improved over the controls by 1 h of machine perfusion and just alike by 4 h of machine perfusion subsequent to the cold storage period, both groups showing a mean of 60–80% higher values at the end of the experiments.

The impact postpreservation machine perfusion on tubular cell function was less pronounced than on glomerular filtration, as judged from differences in fractional excretion of sodium (FE Na, Fig. 2).

In only cold-stored kidneys, FE Na showed high values during early ex vivo reperfusion, while limited albeit significant protection of tubular sodium reabsorption could be evidenced after 1 as well as 4 hours of additional hypothermic machine perfusion.

The release of caspase-cleaved keratin 18 (ccl K18) was investigated during reperfusion and taken as a readout of cellular apoptosis. Neither one hour nor 4 h of HMP after CS did have a significant influence on renal cleavage of keratin18 (82.1 ± 14.4 vs. 72.2 ± 4.8 vs. 67.6 ± 5.6 U/L; CS vs. HR1 vs. HR4).

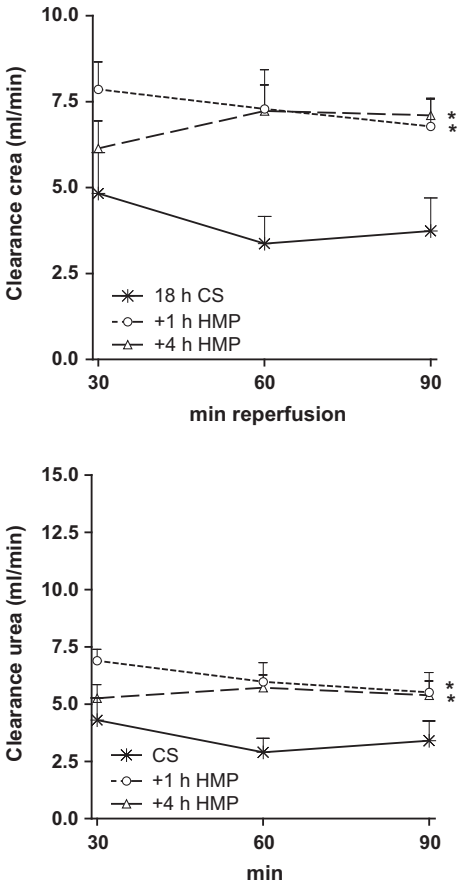

Figure 1 Renal clearances of creatinine and urea upon reperfusion after cold storage (CS), CS + 1 h (+1 h HMP) or CS + 4 h of pulsatile hypothermic machine perfusion (+4 h HMP). $N = 6$ per group (*$P < 0.05$; vs. CS).

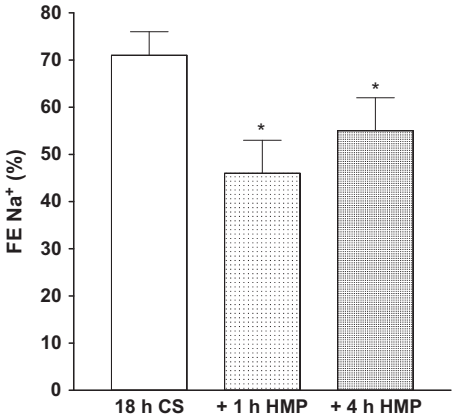

Figure 2 Fractional excretion of sodium (FE Na^+) upon isolated reperfusion in vitro after cold storage (CS), CS + 1 h (+1 h HMP) or CS + 4 h of pulsatile hypothermic machine perfusion (+4 h HMP). $N = 6$ per group (*$P < 0.05$; vs. CS).

Vascular resistance

Renal perfusate flow upon pressure constant reperfusion significantly differed among the groups. While cold preservation resulted in constant flow values around 2.5 ml/g/min, normal postischemic flow patterns were seen in the 1-h as well as the 4-h machine perfusion groups, both showing a progressive rise upon early reperfusion and ending up at stable flow values of approximately 4 ml/g/min after 60 min (Fig. 3).

In line with the rheological data, perfusate concentrations of the endothelial vasodilator nitric oxide were significantly and similarly higher after 1 or 4 h of postpreservation machine perfusion than after cold storage alone (Fig. 4). Conversely, endothelin-1 concentrations in the perfusate, as an indicator of endothelial stress and vasoconstrictive phenotype, were found significantly reduced in both treatment groups.

Molecular triggers of vascular stress and pro-inflammatory activation

The molecular expressions of selected genes pertinent to vascular function are depicted in Table 1.

It was found that cold preservation resulted in an approximately 30% reduction in gene expression of the vasoprotective transcription factor KLF2. Concomitantly, the mRNA content of endothelial nitric oxide synthase was reduced by approx. 50% and the expression of endothelin 1 was threefold higher than under baseline conditions.

By contrast, no influences of the reconditioning procedure were seen with regard pro-inflammatory cell activation, as judged from the expression levels of NF kB1 or IL6.

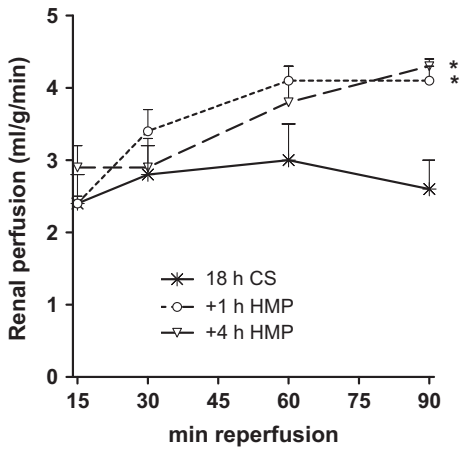

Figure 3 Renal perfusate flow upon reperfusion after cold storage (CS), CS + 1 h (+1 h HMP) or CS + 4 h of pulsatile hypothermic machine perfusion (+4 h HMP). $N = 6$ per group (*$P < 0.05$; vs. CS).

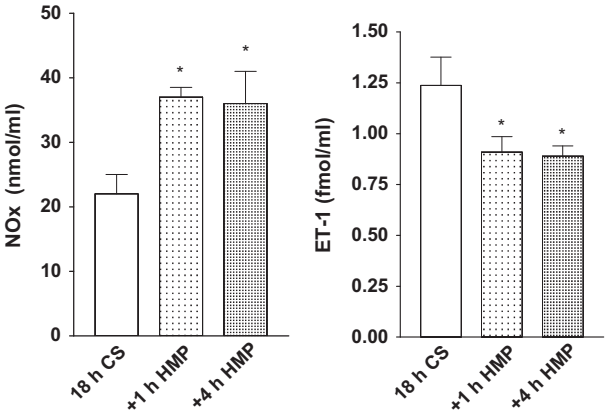

Figure 4 Perfusate concentrations of of nitric oxide (NOx) and Endothelin-1 (ET-1) during isolated ex vivo reperfusion after cold storage (CS), CS + 1 h (+1 h HMP) or CS + 4 h of pulsatile hypothermic machine perfusion (+4 h HMP). $N = 6$ per group (*$P < 0.05$; vs. CS).

Table 1. Gene transcription of Kruppel-like factor 2 (KLF-2), endothelin-1 (ET-1), endothelial nitric oxide synthase (eNOS), nuclear factor kappa B1 (NF kB1) and interleukin 6 (IL6) in renal cortical tissue at the end of cold storage (CS) and after additional hypothermic machine perfusion for 1 h (+1 h HMP) or 4 h (+4 h HMP).

	CS	+1 h HMP	+4 h HMP
KLF-2 (%bl)	0.61 ± 0.06	0.89 ± 0.06*	1.14 ± 0.08*
ET-1 (%bl)	3.08 ± 0.24	1.09 ± 0.19*	1.47 ± 0.14*
eNOS (%bl)	0.48 ± 0.05	0.68 ± 0.07*	0.83 ± 0.14*§
NF kB1 (%bl)	0.52 ± 0.05	0.62 ± 0.06	0.67 ± 0.04
IL6 (%bl)	0.94 ± 0.08	0.89 ± 0.05	0.99 ± 0.12

Data are given as mean and standard error of $n = 6$ experiments; *$P < 0.05$ vs. CS, §$P < 0.05$ vs. HR1.

Only one hour of subsequent hypothermic machine perfusion led to a significant normalization of renal cortical expression of KLF2, as well as endothelial nitric oxide synthase (eNOS) and endothelin-1. After 4 h of machine perfusion, mRNA levels did not differ significantly from those seen after 1 h of machine perfusion, except for eNOS, which was found even higher expressed after 4 h of HMP than after 1 h.

Discussion

Ischemic reperfusion injury (IRI) represents one of the main risk factors for acute kidney failure after kidney transplantation as well as long-term graft function.

One primary mechanism contributing to renal ischemic reperfusion injury is described to be microvascular dysfunction, resulting in secondary tissue hypoxia and mitochondrial failure [15–17].

Postischemic vasoconstriction may thus result in acute tubular necrosis (ATN) and functional failure of the graft [18]. By contrast, improvement in renal function could be obtained, when an endothelin receptor antagonist is added to cold perfusate prior to implantation [1]. Cessation of pulsatile shear stress during ischemia has recently been incriminated to induce an antivasodilative phenotype in the vascular endothelial cells. Continuous mechano-stimulation normally upregulates vasoprotective genes in the endothelium [19], which are missing after ischemic no-flow conditions [20].

The physiological vascular phenotype is mainly orchestrated by the mechano-sensitive transcription factor KLF2, which regulates a variety of protective genes including transcriptional control of vascular tone by endothelial synthase of NO (eNOS) and endothelin-1 [21,22]. Laminar shear stress moreover induces the release of mediators from glomerular EC which affect podocyte signalling and behavior, especially podocyte barrier function [22].

Thus, reversal of KLF2 breakdown by continuous pulsatile shear stress and consecutive preservation of adequate expression of vasodilative signal transduction pathways upon reperfusion has emerged as one pivotal mechanism of hypothermic machine perfusion to improve postischemic renal graft function in experimental as well as clinical studies [23,24].

In the present study, it could hence been shown that already 1 h of endischemic machine perfusion significantly improves gene expression of KLF2 prior to warm reperfusion. This effect was slightly more pronounced after 4 h of poststorage machine perfusion, but there was no difference between both treatment groups in respect to immediate endothelium-dependent vascular conductivity and secretion of nitric oxide upon early reperfusion.

Of note, simple reflush of the cold-stored grafts with Custodiol-N solution did not bring up the protection described after pulsatile machine perfusion and failed to provide functional improvements upon reperfusion, for example, creatinine clearance 3.2 ± 0.7 ml/min vs. 3.7 ± 0.9 ml/min after simple CS.

Although it might be conjectured that a short reconditioning period of 1 h presumably is not sufficient to already increase actual transcription of KLF2 or the expression of KLF2-induced vasculoprotective proteins, these data suggest that pulsatile stimulation for only one hour actually promotes swift phosphorylation of, for example, endothelial nitric oxide synthase in a sufficient way as to promote adequate vascular conductance upon initial warm reperfusion.

Indeed, phosphorylation of eNOS under shear stress conditions has been shown to proceed as early as after 20 min in cell culture experiments [25] and is possible to promote via by activation of protein kinase A [26].

The initial improvement of renal circulation by adequate release of NO during early reperfusion is likely to prevent inadequate supply of oxygen and nutrients during the first vulnerable phase of normothermic metabolism and translates into enhanced renal clearance function as well as reabsorption of sodium.

Due to the temporal limitations of the in vitro model, no long-term parameters are available, but it could be speculated from present data that later support of physiological endothelial phenotype would concordantly be improved by actual transcription of the protective proteins, gene expression of which were shown to be increased already after short-term machine perfusion in this pilot study. Final proof of concept will be dependent on further experiments including actual transplantation and follow-up in vivo, which are thus strongly warranted.

The proposed maneuver of short-term in-house reconditioning of kidney grafts, which were previously retrieved and transported using the conventional and simple cold storage technique, could easily be implemented in the logistic routine of any individual transplant center, without interfering with global procurement routines.

To this purpose, the present data suggest already one hour of therapy, which might easily be performed during the time necessary for the preparation of the recipient, will result in a significant reconditioning of the cold-stored graft.

On the other hand, longer periods of HMP after arrival of the organ in the implantation clinic actually compete with the option of immediate transplantation.

Although the extension of the overall cold ischemic time, inherent to longer durations of 'in-house' machine perfusion, might have cut down on the functional results, even the 4-hour protocol still improved ulterior kidney function and might represent a suitable solution to postpone the transplantation procedure from night to early day work.

Authorship

PE: performed research and collected data. AG: designed study and analyzed data. AP: designed the study and revised the paper. TM: designed study, performed research and wrote the paper.

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