Preclinical evaluation of a new organ preservation solution
Schreinemachers, M.C.J.M.

Citation for published version (APA):

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Chapter 6

Hypothermic machine perfusion of kidney grafts: which pressure is preferred?
Hypothermic machine perfusion of kidney grafts: which pressure is preferred?

B.M. Doorschodt¹, M.C.J.M. Schreinemachers², S. Florquin³, R.H. Tolba¹

¹ Institute for Laboratory Animal Science & Experimental Surgery, RTWH-Aachen University, Aachen, Germany
² Department of Surgery, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands
³ Department of Pathology, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands

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Abstract

Objective
The objective of this study was to assess the effect of the perfusion pressure (PP) during machine perfusion (MP) on the preservation quality of kidney grafts. To this end, using a novel MP system for hypothermic pulsatile perfusion, mean PPs of 25 mmHg and 30 mmHg were compared for the preservation of porcine kidney grafts.

Methods
After assessment of the microcirculation, animals underwent left nephrectomy. The kidneys were thereafter washed out, followed by 20 hr of MP at 25 mmHg (MP 25, \(n=7\)) or 30 mmHg (MP 30, \(n=7\)). After MP preservation, the contralateral kidneys were removed and the preserved kidneys heterotopically autotransplanted. Ten minutes after reperfusion, the microcirculation was reassessed. Seven days posttransplant, animals were sacrificed and the kidney grafts removed for histological analysis.

Results
MP using a mean PP of 25 mmHg resulted in higher capillary blood flow at 10 minutes after reperfusion. In the MP 30 group, 6 out of 7 animals survived, whereas in the MP 25 group all animals survived. Overall, improvement in recovery of renal function was seen in the MP 25 group compared with the MP 30 group. Histological evaluation showed better preservation of structural integrity in the MP 25 group.

Conclusion
In a porcine autotransplantation model, better preservation quality of kidney grafts was demonstrated using MP with a mean perfusion pressure of 25 mmHg when compared to a mean perfusion pressure of 30 mmHg.
Introduction

Currently, hypothermic machine perfusion (MP) is gaining ground as the preservation method of choice for kidney grafts since the first large clinical randomized study demonstrated a beneficial effect of MP over cold storage (CS)\(^1\). The benefit of machine perfusion is probably that it ensures a uniform distribution of preservation fluid throughout the organ, which is better than a single flush\(^2\). Although MP provides favorable preservation quality over CS, it remains a more complex and expensive procedure. Therefore, simple CS preservation is still the preservation method of choice. Interestingly, in addition to the favorable results of MP regarding renal function, recently, two studies on the cost-effectiveness of MP as a preservation method for kidney grafts demonstrated that MP is preferable to CS since it adds substantial value, not only from a functional perspective but foremost from a cost-effective perspective\(^3,4\). Therefore, it is expected that MP will gain a wider clinical acceptance in the near future.

Nowadays, three MP systems are commercially available; The Lifeport (Organ Recovery Systems Inc., Des Plaines, IL, USA), the RM3 (Waters Medical Systems LLC, Plymouth, MN, USA) and the Kidney Assist (Organs Assist BV, Groningen, the Netherlands). These systems are used in combination with KPS-1 (Organ Recovery Systems Inc. Ill., USA) which is currently the only commercially available MP solution. In the MP systems, the mean perfusion pressure (PP) with which the perfusion medium is provided to the organ can be altered by the operator. In a survey of 12 organ procurement organizations in the US which used MP for kidney preservation, a variety of PP was described\(^5\).

Although Moers et al. applied a fixed systolic PP of 30 mmHg in the aforementioned clinical trial, other clinical and preclinical investigators applied different mean perfusion pressures. In a study comparing CS and MP for the preservation of kidney grafts using a porcine kidney autotransplantation model, Nicholson et al. applied a mean arterial PP of 60 mmHg\(^6\). Treckmann et al. applied a systolic PP of 40-50 mmHg\(^7\), whereas Maathuis et al. demonstrated that a PP of 30/20 mmHg provided favorable results over 60/40 mmHg\(^8\).

Recently, a new MP system with a gaspressure-driven perfusion pump has been developed for hypothermic pulsatile perfusion of kidney grafts\(^9\). This system is used in combination with POLYSOL, a new solution which has been developed for washout and hypothermic MP and CS preservation of abdominal organs. Polysol demonstrated favorable results in CS kidney preservation compared to the clinical standard, the University of Wisconsin solution, as demonstrated by an improved microcirculatory status and improved graft function\(^10\).

The aim of this study was to assess the effect of the PP during hypothermic MP preservation of kidney grafts. To this end, pulsatile perfusion preservation at a mean PP of 25 mmHg was compared to MP at a PP 30 mmHg using a porcine kidney autotransplantation model.
Materials and methods

Animals and experimental protocols
All experiments were performed in accordance with the German legislation governing animal studies following the *Principles of Laboratory Animal Care* (NIH publication. 85-23, revised 1985). Kidneys were retrieved from female German landrace pigs, weighing 24.9 ± 4.1 kg (mean ± SD). This study describes two groups, both representing a different PP during MP, MP at 25 mmHg (MP 25) and MP at 30 mmHg (MP 30).

Surgical Procedures
Animals were premedicated with ketamine (90 mg/kg), xylazine (10 mg/kg) and atropine (0.01 mg/kg) administered intramuscularly (IM). General anesthesia was induced by midazolam (0.5 mg/kg), and fentanyl (12.5 μg/kg), muscle relaxation was achieved by pancuronium (0.2 mg/kg), all administered intravenously (IV) by means of a butterfly cannula inserted into an external marginal ear vein and maintained after intubation by mechanical ventilation with isoflurane. Thereafter, the right internal jugular vein was cannulated with a PE (polyethylene) catheter for infusion and daily collection of blood samples. After the assessment of the microcirculation at 4 pre-defined locations on the renal surface using a combined laser Doppler and flowmetry system (oxygen-to-see, O2C system, LEA Medizintechnik, Giessen, Germany), a left nephrectomy was performed. Immediately thereafter, kidneys were washed out ex vivo using 500 ml of Polysol (Doorzand Polysol B.V., Amsterdam, The Netherlands) at 4ºC at a hydrostatic pressure of 100 cm H₂O followed by weighing of the grafts.

Machine perfusion
Subsequently, kidneys were connected to the MP system (Airdrive, Doorzand Airdrive B.V., Amsterdam, The Netherlands) for a 20 hr period. The features of the Airdrive MP system have been described in detail previously⁹. Oxygen pressure generated by a 2 Liter medical oxygen cylinder is used to propel a pulsatile membrane pump and to actively oxygenate the perfusion medium during perfusion. For this study, the mean PP of the pressure-controlled MP system was preset according to the group assigned, MP 25 or MP 30. The mean PP was calculated by the onboard electronic control system from continuous recordings of the pressure sensor and defined as the mean area under the pressure curve during 10 pump cycles. Perfusion parameters, flow and renal resistance (mmHg/ml/100 gr kidney weight), were continuously monitored. Kidney weights after 20 hr MP were recorded.
**Autotransplantation model**
Twenty hours after left nephrectomy, the contralateral kidney was removed, followed by immediate heterotopic transplantation of the preserved kidney. The renal artery was anastomosed end-to-end to the right renal artery and the renal vein end-to-side to the inferior vena cava. Before completion of the arterial anastomosis, a bolus of 3,000 IU of heparin was injected to prevent vascular thrombosis. Following reperfusion, 250 ml of 20% Glucose was administered intravenously to induce osmotic diuresis. The ureter was cannulated with a PE tube (CH 10) to allow free outflow of urine through an ureterocutaneostomy. Ten minutes after reperfusion, the renal microcirculation was reassessed. Postoperatively, the animals were allowed free access to water and were supplemented with 1L of 0.9% NaCl infusion IV. Ranitidine 50 mg IV was administered daily and postoperative analgesia was provided every 6 to 8 hr with Tramadol (1 mg/kg IM) for up to 72 hr after transplantation. Ampicillin 500 mg was administered IV perioperatively as well as twice daily during follow-up. Also, Aspirin 500 mg IV was given on a daily base for the entire period of follow-up. Animals were observed for seven days after transplantation with daily assessment of renal function. Creatinine clearance was calculated from 24 hr urine production and serum creatinine (urine creatinine x 24 hr volume / (serum creatinine x 24 x 60 min)). At day 7 posttransplant, the transplanted kidney was removed for histological evaluation and animals were sacrificed by injection of T61 IV (Hoechst Roussel Vet, Wiesbaden, Germany).

**Noninvasive assessment of renal microcirculation**
The O2C combined Laser Doppler and flowmetry device was used to evaluate the microcirculation noninvasively. At 2 and 8 mm tissue depths, capillary blood flow (flow, arbitrary units, AU) and capillary blood flow velocity (velocity, AU) were simultaneously recorded. At 4 predefined locations on the renal surface, measurements were performed for 30 seconds each, to prevent the influence of regional heterogeneity and temporal blood flow variations.

**Histological analysis**
At sacrifice, renal tissue specimens were fixed in neutral 10% buffered formalin and embedded in paraffin. Tissue injury was assessed on periodic acid-Schiff (PAS) stained 4 µm-thick sections by scoring glomerular damage (shrinking), tubular damage, infiltration of inflammatory cells, interstitial edema and necrosis. Injury of each specimen was graded to the extent of region involvement in 10 randomly chosen, nonoverlapping fields (original magnification, X400). Injury was scored by a pathologist blinded for the groups on a scale from 0 to 5: 0 = no abnormality, 1 = mild, lesions affecting up to 10% of the field, 2 = moderate lesions, affecting 10%-25%, 3 = severe, affecting 25%-50%, 4 = very severe, affecting 50%-75% and 5 = extensive damage, involvement of more than 75% of the field.
Statistics
Data are expressed as mean ± SD. Statistical analysis of kidney weights, microcirculation parameters as well as MP parameters were performed using the repeated measurements analysis of variance (RM ANOVA) followed by the Bonferroni posttest. For renal function, area under the curve (AUC) was calculated individually using the GraphPad Prism 5.0 statistics package (GraphPad Software, San Diego, CA, USA). Statistical significance of differences was assessed using the Mann Whitney U test. A $P$-value < 0.050 was considered statistically significant.

Results
Animal weights did not differ between the groups (MP 25, 23.5 ± 4.6 kg; MP 30, 26.3 ± 3.2 kg; $P = 0.201$). All animals demonstrated normal renal function before the start of the experiments (serum creatinine; MP 25, 1.09 ± 0.14 mg/dL; MP 30, 1.13 ± 0.10 mg/dL; $P = 0.565$). Cold ischemic times (CITs) of the groups were comparable (CIT, MP 25, 20:32 hr ± 0:06; MP 30, 20:28 hr ± 0:17; $P = 0.158$). Also, times required for both anastomoses were comparable between the groups (MP 25, 00:41 ± 0:08 minutes; MP 30, 00:40 ± 0:08 minutes; $P = 0.847$). At the end of the 500 mL washout, all kidneys showed a macroscopically asanguinous effluent.

Kidney weights after washout did not differ between the groups (MP 25, 66.0 ± 11.8 gr; MP 30, 72.3 ± 12.5 gr; $P = 0.369$). Also, repeated measurement analysis of kidney weights after washout and after MP preservation, did not differ between the groups (RM ANOVA kidney weights, $P = 0.209$).

Machine perfusion parameters
Data on perfusion parameters per 100 gr kidney weight are shown in Figure 1. Analysis of perfusion flow showed a trend towards higher flow rates in the MP 30 group, though not significant (RM ANOVA, MP 25 versus MP 30, $P = 0.068$, Fig. 1A). Overall, intravascular resistance did not differ between the groups (RM ANOVA, MP 25 versus MP 30, $P = 0.419$, Fig. 1B). Over time, intravascular resistance in the MP 30 group decreased in the first 6 hr of MP, to stabilize thereafter for a period of 4 hr followed by a slight increase during the final 10 hr MP. In the MP 25 group, however, renal resistance decreased over time for the duration of 20 hr MP.

Renal microcirculation
Overall, cortical microcirculatory flow at 8 mm tissue depth was better in the MP 25 group compared to the MP 25 group (RM ANOVA, capillary blood flow at 8 mm, MP 25 versus MP 30, $P = 0.005$; Bonferroni posttest; preretrieval, MP 25 versus MP 30, $P < 0.050$; postreperfusion, MP 25 versus MP 30, $P < 0.001$, Fig. 2A). After 20 hr MP capillary blood flow at 8 mm tissue depth increased in the MP 25 group, whereas a decline in
blood flow was recorded in the MP 30 group. Capillary blood flow at 2 mm tissue depth did not differ between the groups (RM ANOVA, capillary blood flow at 2 mm, MP 25 versus MP 30, $P = 0.051$). Blood flow velocities were comparable between the groups at both tissue depths (RM ANOVA, at 2 mm, MP 25 versus MP 30, $P = 0.850$; at 8 mm, MP 25 versus MP 30, $P = 0.185$, Fig. 2B). Directly after revascularization, macroscopically, both the MP 25 and the MP 30 grafts showed a homogeneous perfusion.

**Follow-up after transplantation**

In the MP 30 group, 6 out of 7 animals survived 7 days. One animal in the MP 30 group was sacrificed at postoperative day 2 as it was suffering from renal failure, as demonstrated by rising creatinine levels. In the MP 25 group all animals survived 7 days. Animals surviving the 7 day follow-up after transplantation were included in the postoperative analyses.

**Fig. 1.** Machine perfusion parameters. (A) perfusion flow recorded during 20 hr MP per 100 gr kidney weight. (B) intravascular resistance during 20 hr MP per 100 gr kidney weight.
Renal function
Overall, posttransplant serum creatinine values in the MP 25 group were lower than posttransplant serum creatinine of surviving animals in the MP 30 group (AUC serum creatinine, MP 25 versus MP 30; \( P = 0.035 \), Fig. 3A). Peak serum creatinine levels were comparable between the MP 25 and the MP 30 groups (MP 25, 5.5 ± 5.4 mg/dL; MP 30, 11.1 ± 2.9 mg/dL; \( P = 0.051 \)). Times to peak serum creatinine did not differ between the groups (MP 25, 2.9 ± 2.6 days; MP 30, 5.2 ± 1.3 days; \( P = 0.149 \)). At sacrifice, serum creatinine levels in the MP 25 group were lower compared to the MP 30 group (MP 25, 4.0 ± 5.8 mg/dL; MP 30, 7.4 ± 3.2 mg/dL; \( P = 0.035 \)).
Posttransplant blood urea values in the MP 25 group were lower than posttransplant blood urea values of surviving animals in the MP 30 group (AUC blood urea; MP 25 versus MP 30; \( P = 0.035 \), Fig. 3B). Also, peak blood urea values in the MP 25 group were lower compared to peak blood urea values in the MP 30 group (MP 25, 104 ± 99 mg/dL; MP 30, 241 ± 136 mg/dL; \( P = 0.022 \)). Times to peak blood urea in the MP 25 group were shorter compared to the MP 30 group (MP 25 3.3 ± 2.4 days; MP 30, 5.7 ± 0.8 days; \( P = 0.049 \)). At sacrifice, blood urea values in the MP 25 group were lower than in the MP 30 group (MP 25, 75 ± 109 mg/dL; MP 30, 197 ± 156 mg/dL; \( P = 0.022 \)).

Overall, creatinine clearances rates in the MP 25 group were significantly lower compared to creatinine clearance rates in the MP 30 group (AUC creatinine clearance; MP 25 versus MP 30 , \( P = 0.026 \), Fig. 3C).

**Histological examination**

Overall, tissue injury of grafts in the MP 25 group was significantly less compared to grafts in the MP 30 group (Table 1, Fig. 4).

<table>
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<th>Table 1. Quantification of morphological data</th>
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<td>MP 25</td>
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<tr>
<td><strong>Glomerular shrinking</strong></td>
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Semiquantitative scale: 0 = no injury; 1 = lesions affecting ≤ 10% of the field; 2 = 10%-25%; 3 = 25%-50%; 4 = 50%-75%; and 5 = involvement of > 75% of the field. Data expressed as mean ± SD.
Fig. 3. Renal function. (A) Serum creatinine values posttransplant in the MP 25 and the MP 30 groups (AUC serum creatinine, MP 25 versus MP 30, *P = 0.035). (B) Blood urea levels posttransplant in the MP 25 and the MP 30 groups (AUC blood urea, MP 25 versus MP 30, *P = 0.035). (C) Creatinine clearance rates posttransplant in the MP 25 and MP 30 groups (AUC creatinine clearance rates, MP 25 versus MP 30, *P = 0.026).
Discussion

In the early days of kidney transplantation from deceased donors, machine perfusion was the only preservation method available\textsuperscript{13}. However, for over two decades, the less cumbersome and expensive cold static storage method has been the standard for preservation of kidney grafts. Following recent studies showing the beneficial effect of MP over CS preservation of deceased kidney grafts, MP holds the potential for a true comeback\textsuperscript{1,3,4,14}. In the first large multi-center, prospective, randomized clinical trial performed by Moers et al., it was demonstrated that kidney grafts from standard as well as extended criteria donors benefit from MP as the risk of delayed graft function was significantly reduced\textsuperscript{1}.

A recent preclinical study comparing hypothermic MP using either a high or low PP with CS, also showed the advantage of MP over CS for the preservation of porcine kidney grafts. In addition to better survival data when a PP of 30/20 mmHg was applied, less damage to the endothelial cells was observed when compared to a PP of 60/40 mmHg\textsuperscript{8}. The two non-surviving animals in the 60/40 mmHg group revealed diffusely black colored grafts with patent arterial and venous anastomoses suggesting impairment of intrarenal circulation. It was concluded that the PP was of critical importance for transplantation outcome.

Following the aforementioned reports and the favorable results of the clinical trial in which a PP of 30 mmHg was applied, we investigated whether a mean PP of 25 mmHg could be advantageous over a PP of 30 mmHg. The rationale of lowering the PP originates from the common regard that pressure induced endothelial damage is one of the main drawbacks associated with MP\textsuperscript{15-17}. Also, the feasibility of the new perfusion solution Polysol was evaluated. By using a similar experimental model as

\begin{figure}[h]
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\includegraphics[width=\textwidth]{fig4}
\caption{Light microscopy (A) MP 25 group, well preserved glomerulus, moderate tubular dilatation and interstitial inflammation (original magnification X20, PAS stain). (B) MP 30 group, moderate shrinking of glomerulus, simplification of tubular epithelium, few inflammatory cell infiltrates (original magnification X20, PAS stain).}
\end{figure}
Maathuis et al., parameters of microcirculatory integrity, renal function and morphology were assessed. The results of our study confirm earlier findings that lowering of the PP is advantageous since MP using a mean PP of 25 mmHg resulted in a higher capillary blood flow at 10 minutes after reperfusion. Moreover, histological evaluation showed better preservation of structural integrity in the MP 25 group. In the MP 30 group, 6 out of 7 animals survived, whereas in the MP 25 group all animals survived. Overall, improvement in recovery of renal function was seen in the MP 25 group compared with the MP 30 group. The effect of the Polysol solution does not appear to be of importance, since the results of this study resemble the results obtained in similar studies using KPS-1,8,18. Also, Polysol meets the prerequisites for an effective MP solution (the presence of a colloid, impermeants and extracellular electrolyte concentrations), as postulated by Belzer et al.19. Before starting this study, we performed a pilot study using a mean PP of 40 mmHg during pulsatile perfusion of porcine kidney grafts (data not shown). In contrast to the studies by Nicholson et al. using a mean PP of 60 mmHg6 and Maathuis (mean PP approximately 50 mmHg)8, in our pilot study, none of the animals (n=3) survived the intended 7 day follow-up after autotransplantation.

In all three experiments, kidney failure, as demonstrated by a steep rise of creatinine levels, was observed resulting in premature sacrificing of the animals. Histological examination of the grafts showed severe tubular injury and necrosis in all sections which was considered a result of long-term renal hypertension. Although the mean PPs applied were lower than human and porcine physiological pressure levels, endothelial damage is likely to occur even at lower PPs since hypothermia severely increases cell membrane stiffness6. The perfusion time in the studies applying a PP of 60 mmHg was limited to 6 hr followed by 18 hr of CS, whereas in our study, as well as the study by Maathuis, a more clinically relevant perfusion time of 20 hr was chosen. The 6 hr perfusion preservation period was chosen as previous experience had shown that intrarenal resistance fell to a baseline level after 4 to 6 hr, and after this period, the formation of edema increased rapidly. Also, from preclinical studies a time-dependent increase in vascular resistance was observed during prolonged hypothermic pulsatile perfusion15-17.

Our study did not confirm these phenomena since, after an initial reduction, intrarenal resistance remained at a basement level for the duration of the 20 hr perfusion period. A steady or decreasing intrarenal resistance is considered a useful indication of graft viability, suggesting preservation of structural integrity of the endothelium and patency of the vascular bed18,20,21.
Conclusions
In a porcine autotransplantation model, better preservation quality of kidney grafts was demonstrated using MP with a mean perfusion pressure of 25 mmHg when compared to a mean perfusion pressure of 30 mmHg. These results corroborate earlier studies suggesting a direct effect of the perfusion pressure applied during MP on vascular injury and organ viability.

Acknowledgements
The authors would like to thank Mario Sitzia, Wei Lai, Mareike Schulz and Ute Lohmer for their continuous support.
References