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ORIGINAL ARTICLE

Mesenchymal stromal cell treatment of donor kidneys during ex vivo normothermic machine perfusion: A porcine renal autotransplantation study

Stine Lohmann[1,2] | Merel B. F. Pool[3] | Kaithlyn M. Rozenberg[4] | Anna K. Keller[1,5] | Cyril Moers[3] | Ulla Møldrup[5] | Bjarne K. Møller[6] | Stina J. M. Lignell[1,2] | Søren Krag[7] | Jesus M. Sierra-Parraga[8] | Maria L. Lo Faro[4] | James Hunter[4] | Martin J. Hoogduijn[8] | Carla C. Baan[8] | Henri G. D. Leuvenink[3] | Rutger J. Ploeg[3,4] | Marco Eijken[2,6] | Bente Jespersen[1,2]

[1]Department of Clinical Medicine, Aarhus University, Aarhus, Denmark

[2]Department of Renal Medicine, Aarhus University Hospital, Aarhus, Denmark

[3]Department of Surgery – Organ Donation and Transplantation, University Medical Center Groningen, University of Groningen, Groningen, the Netherlands

[4]Nuffield Department of Surgical Sciences, University of Oxford, Oxford, UK

[5]Department of Urology, Aarhus University Hospital, Aarhus, Denmark

[6]Department of Clinical Immunology, Aarhus University Hospital, Aarhus, Denmark

[7]Department of Pathology, Aarhus University Hospital, Aarhus, Denmark

[8]Department of Internal Medicine, Nephrology and Transplantation, Erasmus MC, University Medical Center, Rotterdam, the Netherlands

Correspondence
Stine Lohmann, Department of Clinical Medicine, Aarhus University, Aarhus, Denmark.
Email: stiloh@clin.au.dk

Normothermic machine perfusion (NMP) of injured kidneys offers the opportunity for interventions to metabolically active organs prior to transplantation. Mesenchymal stromal cells (MSCs) can exert regenerative and anti-inflammatory effects in ischemia-reperfusion injury. The aims of this study were to evaluate the safety and feasibility of MSC treatment of kidneys during NMP using a porcine autotransplantation model, and examine potential MSC treatment-associated kidney improvements up to 14 days posttransplant. After 75 min of kidney warm ischemia, four experimental groups of $n = 7$ underwent 14 h of oxygenated hypothermic machine perfusion. In three groups this was followed by 240 min of NMP with infusion of vehicle, 10 million porcine, or 10 million human adipose-derived MSCs. All kidneys were autotransplanted after contralateral nephrectomy. MSC treatment did not affect perfusion hemodynamics during NMP or cause adverse effects at reperfusion, with 100% animal survival. MSCs did not affect plasma creatinine, glomerular filtration rate, neutrophil gelatinase-associated lipocalin concentrations or kidney damage assessed by histology during the 14 days, and MSCs retention was demonstrated in renal cortex. Infusing MSCs during ex vivo NMP of porcine kidneys was safe and feasible. Within the short post-transplant follow-up period, no beneficial effects of ex vivo MSC therapy could be demonstrated.

Abbreviations: DCD, donation after circulatory death; ECD, extended criteria donor; FBS, fetal bovine serum; FFPE, formalin fixed paraffin embedded; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GFR, glomerular filtration rate; HGF, hepatocyte growth factor; HMP, hypothermic machine perfusion; hMSC, human mesenchymal stromal cell; IL, interleukin; LCN2, lipocalin-2; MAP, mean arterial pressure; MEM-α, minimal essential medium-α; MSC, mesenchymal stromal cells; NMP, normothermic machine perfusion; NOX4, nicotinamide adenine dinucleotide phosphate oxidase 4; oxHMP, oxygenated hypothermic machine perfusion; PBS, phosphate-buffered saline; p-creatinine, plasma creatinine; p-NGAL, plasma neutrophil gelatinase-associated lipocalin; pMSC, porcine mesenchymal stromal cell; qPCR, quantitative real-time polymerase chain reaction; SCS, static cold storage; 99Tc-DTPA, 99Tc-diethylenetriamine pentaacetic acid; TGF-β, transforming growth factor beta; UW-CSS, University of Wisconsin cold storage solution; UW-MPS, University of Wisconsin machine perfusion solution; VEGF, vascular endothelial growth factor.

S. Lohmann and M. B. F. Pool contributed equally and share first authorship.

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basic (laboratory) research/science, kidney transplantation/nephrology, organ procurement and allocation, regenerative medicine, donors and donation: donation after circulatory death (DCD), ischemia reperfusion injury (IRI), organ perfusion and preservation, stem cells

1 | INTRODUCTION

Due to a persistent shortage of donor organs, many transplant centers currently use higher risk donor kidneys such as those recovered from donation after circulatory death (DCD) and from extended criteria donors (ECDs).[1-3] These types of donor kidneys are more prone to ischemia-reperfusion injury.[4] Most European transplant centers still use conventional static cold storage (SCS) for the preservation of donor organs, but with increased numbers of DCD and ECD kidneys a growing need for improved preservation techniques has emerged. Hypothermic machine perfusion (HMP) is superior to SCS, reducing delayed graft function and significantly improving long-term graft survival[5] but there is only limited evidence that it could improve the quality of older and higher risk donor kidneys.[6,7] Normothermic machine perfusion (NMP) of donor organs has been recognized as an opportunity to assess organ viability and allow therapeutic interventions prior to transplantation. This offers the possibility of optimizing the quality of donor kidneys.[8-10]

A potential therapeutic intervention during NMP is the administration of mesenchymal stromal cells (MSCs). MSCs are multipotent cells with the ability to modulate innate as well as adaptive immune responses at the site of injury.[11,12] In addition, MSCs possess regenerative properties and a number of animal studies have reported a beneficial effect on ischemia-reperfusion injury.[13,14] MSCs most likely achieve this via interaction with host immune and progenitor cells, leading to anti-inflammatory, antifibrotic, antiapoptotic, and proangiogenic effects. In addition, MSCs have the potential to suppress oxidative stress and trigger tubular cell proliferation which may minimize tissue injury and stimulate repair.[15,16]

To date, research has mainly focused on MSC therapy *after* kidney transplantation, but not *prior* to transplantation.[12,17] Intravenous infusion (IV) of MSCs to transplant recipients appears to be safe.[11,18-20] With this mode of systemic administration, however, the cells will most likely never reach the kidney as intravenously infused MSCs are largely trapped in the lungs.[21,22] In contrast, the administration of MSCs to donor kidneys in an ex vivo isolated organ perfusion system will deliver cells directly to the injured organ. Also, targeted ex vivo delivery may enhance the possibility of achieving therapeutic effects with relatively low numbers of MSCs. Only a few attempts have evaluated MSC treatment in combination with ex vivo NMP, mainly focusing on traceability and dosing. None of these studies incorporated transplantation after cellular therapy, leaving questions on possible posttransplant effects unanswered.[23-25]

The primary purpose of this study was to determine the safety and feasibility of MSC administration to injured porcine kidneys during NMP. Following autotransplantation of these kidneys, we furthermore investigated whether MSCs had beneficial effects on kidney function and damage within 14 days posttransplant.

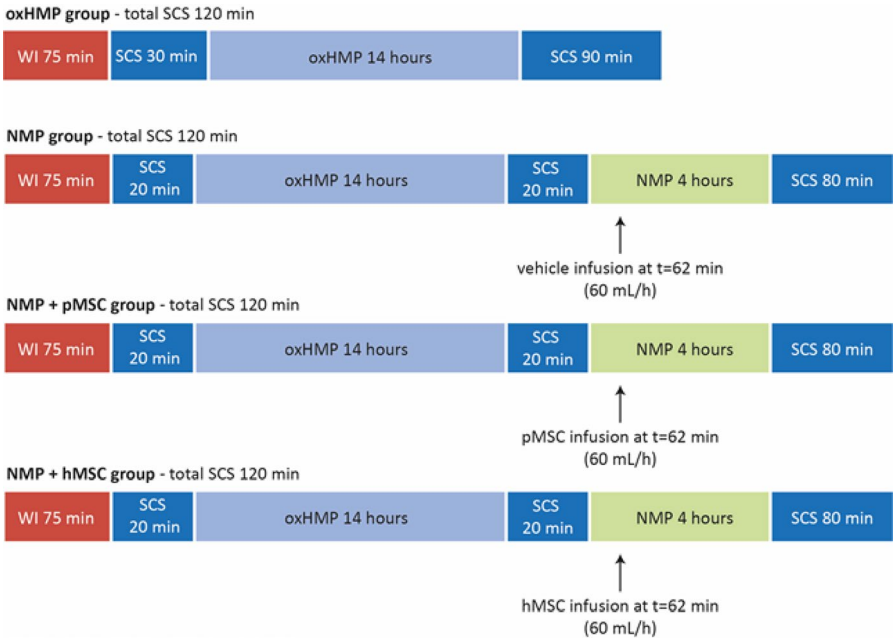

FIGURE 1 Preservation strategies of the four experimental groups. hMSC, human mesenchymal stromal cells; NMP, normothermic machine perfusion; oxHMP, oxygenated hypothermic machine perfusion; pMSC, porcine mesenchymal stromal cells; SCS, static cold storage; WI, warm ischemia [Color figure can be viewed at wileyonlinelibrary.com]

2 | MATERIALS AND METHODS

2.1 | Study design

Kidneys were randomly assigned to four experimental groups of $n = 7$. After 75 min of warm ischemia, kidneys were subjected to different preservation strategies. Control group kidneys underwent oxygenated HMP (oxHMP) for 14 h followed by autotransplantation. In the other three groups, oxHMP was followed by 240 min of NMP with an intra-arterial infusion after 62 min of either vehicle (NMP+vehicle), 10 million porcine adipose tissue-derived MSCs (NMP+pMSC), or 10 million human adipose tissue-derived MSCs (NMP+hMSC) followed by autotransplantation (Figure 1). All researchers involved were blinded to the NMP regimen, except for two individuals handling the MSCs, who had no other involvement in the experiments or posttransplant animal care. Due to technical problems, one kidney suffered anoxia during NMP and was excluded. This experiment was repeated to complete the groups.

2.2 | Ethics and animals

Female laboratory pigs (Danish Landrace and Yorkshire crossbreed) weighing approximately 50 kg were used for autotransplantation while the weight of blood donor pigs was 60–80 kg. All animal care and procedures followed guidelines by the European Union (directive 2010/63/EU) and local regulations. The study was approved by Danish Animal Experimentation Council (reference-number 2016-15-0201-01145). All personnel involved had Federation of European Laboratory Animal Science Associations licenses. Our animal experiment refinements have been reported.[26,27]

2.3 | Surgical procedure

Operative procedures were previously described.[26] Briefly, after induction, anesthesia was maintained by sevoflurane and intravenous remifentanil (0.03–0.06 mg/kg/h).

After midline incision, a left retroperitoneal nephrectomy (day −1) was performed and the kidney was exposed to 75 min of warm ischemia. The midline incision was closed and the pig returned to the pen. After warm ischemia, the graft was flushed with 500 ml cold Belzer UW® Cold Storage Solution (UW-CSS, Bridge to Life) and was subjected to either oxHMP or oxHMP plus NMP.

After ex vivo perfusion (day 0), the kidneys undergoing NMP were disconnected from the device, flushed and cooled with 500 ml cold Belzer UW-CSS. Following right nephrectomy, the left kidney graft, wrapped in a gauze containing ice, was autotransplanted end-to-end to the native right renal artery, vein, and ureter. The midline incision was closed and the pig was returned to the stables for 14 days of observation.

On day 14, pigs were anesthetized and during 3 h with serial blood and urine collections, glomerular filtration rate (GFR) was measured using the urinary clearance of ^{99}Tc-diethylenetriamine pentaacetic acid (99Tc-DTPA) with a priming dose of 75 MBq and maintenance dose of 37.5 MBq/h. Next, a graft nephrectomy was performed and the pigs were euthanized under general anesthesia.

2.4 | Collection of allogeneic erythrocytes

All experimental animals and blood donor pigs were of blood type A (typed using Ortho BioVue™ Card, Ortho Clinical Diagnostics). Under sterile conditions, blood was collected in quadruple-blood bags (Macopharma) from anesthetized pigs. Following blood fractionation (Baxter Optipress II Blood Component Separator, Baxter), the leucocyte-filtered erythrocytes were stored in bags containing the storage solution saline-adenine-glucose-mannitol for a maximum of 14 days at 4°C. Erythrocytes were washed twice in Dulbecco's phosphate-buffered saline (Thermo Fisher) 1 h prior to the start of NMP.

2.5 | Machine perfusion

During both oxHMP and NMP, kidneys were perfused with a pressure-controlled pulsatile perfusion system using a custom-made electronic interface and software (LabVIEW Software, National Instruments Netherlands BV). The sterile perfusion circuit consisted of an oxygenator (Terumo Denmark), a centrifugal pump and unit (Medos Deltastream DP2 and Pumpdrive DP2, Medos Medizintechnik AG), and a LifePort® organ chamber with a 3-mm disposable straight cannula (Organ Recovery Systems), where pressure was measured directly (TruWave disposable pressure transducer, Edwards Lifesciences). Flow was monitored using an ultrasonic clamp-on flow probe (Transonic Systems Europe BV).

The oxHMP circuit was primed with 500 ml cold University of Wisconsin machine perfusion solution (UW-MPS, Bridge to Life Ltd.). Temperature was kept between 4 and 7°C, oxygenated with 0.1 L/min 100% oxygen and mean arterial pressure (MAP) was set to 25 mm Hg.

The NMP circuit was primed with a perfusion solution consisting of 250 ml 5% human albumin (Alburex 50 g/L, CSL Behring) and 170 ml allogeneic erythrocytes to acquire a hematocrit of 25%–30%. Additionally, 5 ml sodium bicarbonate 8.4% (B. Braun), 6 ml glucose 5% (B. Braun), 5 IU insulin (Novo Nordisk A/S), 3 ml calcium gluconate 10% (B. Braun), 10 mg mannitol (Sigma Aldrich), and 1000 μmol/L creatinine (Sigma Aldrich) were added. The perfusate was supplemented with a bolus of 300 mg amoxicillin-clavulanate (Bowmed) at baseline with additional doses of 60 mg every 60 min during NMP. Furthermore, a bolus dose of 1.25 mg verapamil (Orion Pharma) was given at baseline supplemented with a continuous infusion during NMP (0.25 mg/h). Perfusate temperature was kept at 37°C and oxygenated with 0.5 L/min carbogen (95% O_2/5% CO_2). After oxHMP, the kidney was flushed with 50 ml cold Ringer's acetate (Fresenius Kabi) to remove UW-MPS solution, connected to the circuit and

perfused for 240 min at a MAP of 70 mm Hg. Gas analysis was carried out every 30 min (ABL90 FLEX, Radiometer). Hourly blood and urine samples were centrifuged and stored at −80°C. Urine was recirculated while Ringer's acetate replaced sample volume.

2.6 | Tissue collection

A 3 mm cortical punch biopsy from the upper kidney pole was collected at the end of oxHMP, end of NMP, and 1 h after graft reperfusion. In addition, both a punch biopsy and 100 mg tissue were collected from the contralateral (nontransplanted) kidney directly after its nephrectomy. All tissue samples were stored at −20°C in RNAlater (Thermo Fisher) prior to DNA and RNA isolation.

On day 14, graft nephrectomy was performed directly after GFR measurements. For histology, tissue samples containing both medulla and cortex from upper and lower pole were collected and put immediately in 4% formalin. Cortical tissue from the lateral side, upper and lower pole for DNA and RNA analysis were stored as described above.

2.7 | Blood and urine and observations during NMP

Blood and urine were collected as previously described.[26] During NMP, perfusion characteristics including renal blood flow, renal resistance, and urine production were monitored. Oxygen consumption was calculated as previously described[28,29] and the equation can be found in Data S1 together with the equations regarding fractional excretion of sodium and creatinine clearance.

2.8 | MSC expansion, characterization, and preparation

Male porcine adipose tissue-derived MSCs (pMSCs) from three donors (80 kg) were isolated, expanded until passage three, and characterized as described previously.[30] Human MSCs (hMSCs) were isolated, expanded, and characterized from male and female perirenal fat with consent from donors as described previously.[23] Cryopreservation and characterization were identical to pMSCs. Batches were >95% positive for CD29, CD44, CD90 and negative for CD31 and CD45. Prior to infusion, 10 million cryopreserved MSCs were thawed and resuspended in 20 ml culture medium followed by centrifugation (440 g, 5 min). Cell pellet was resuspended in 10 ml perfusate and filtered through a 70-µm cell strainer. A small aliquot of cells was used for a control counting and inspection under the microscope to confirm a single cell solution.

2.9 | Detection of pMSCs in kidney tissue

DNA was isolated from cortical tissue using a NucleoSpin Tissue DNA isolation kit (Macherey-Nagel) according to the manufacturer's protocol. In total, 10 mg of tissue was used for day 14 samples and approximately 1–2 mg was used for day 0 samples. Y-chromosome was detected by quantitative real-time polymerase chain reaction (qPCR) using primers directed to the male-specific repeat (MSR) located on the porcine Y-chromosome and primers for the porcine S100C gene for detection of total pig DNA as described previously.[30] Relative Y-chromosome levels are expressed as 2^(Ct value S100C−Ct value MSR) × 1000.

2.10 | Immunohistochemical staining and histomorphological assessment

Tissue samples were fixed in 4% formalin for a total of 24 h before storage in phosphate-buffered saline (PBS) until dehydrated and embedded in paraffin (FFPE). Two-micrometer thick sections of FFPE-harvested tissue were stained with hematoxylin and eosin, Masson's trichrome as well as periodic acid–Schiff, and slides were analyzed by an experienced renal pathologist, blinded to the intervention and cases. Samples were compared to healthy kidney tissue[27] to assess evidence of inflammation, tubular atrophy, fibrosis, tubular and glomerular damage. Each parameter was scored semi-quantitatively on a 0–4 scale based on the degree of injury: 0 if <2% was affected, 1 if 2%–5% was affected, 2 if 6%–25% was affected, 3 if 25%–50% was affected, and 4 if 51%–100% was affected.

2.11 | Gene expression analysis

For day 14 samples, RNA was isolated from approximately 20 mg tissue using a GeneJET RNA purification kit (Thermo Fisher) and for day 0 samples, the RNA was isolated from approximately 2–5 mg tissue using a Nucleospin RNA XS Kit (Machery Nagel). Gene expression for inflammatory cytokines, growth, and injury markers were analyzed; see Table S1. In total, 1 µg (day 14 samples) or 500 ng (day 0 samples) of total RNA was reverse transcribed using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher). qPCR was performed using Power SYBR™ Green PCR Master Mix (Thermo Fisher), 0.4 µmol/L primers and a QuantStudio™ 3 qPCR system. Gene expression was presented relative to the housekeeping gene Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) using the formula 2^(Ct value GAPDH−Ct value gene of interest) × 1000. For day 14 samples, mRNA levels for each kidney were calculated by averaging the levels from cortical kidney tissue collected at the lateral, upper, and lower pole regions.

2.12 | IL-6, IL-10, and NGAL measurements

Interleukin (IL) 6 was measured using a porcine IL6 ELISA (Thermo Fisher, ESIL6) and IL-10 using a porcine IL10 ELISA (Thermo Fisher, KSC0101) according to the manufacturer protocols. Levels of neutrophil gelatinase-associated lipocalin (NGAL) were determined in

perfusate, urine, and plasma using an ELISA assay (pig NGAL ELISA kit 044, BioPorto). For creatinine measurements, i-STAT (Abbot Diagnostics) analyzer and assays were used.

2.13 | Statistical analyses

STATA software version 15.1 (StataCorp) was used for analysis and GraphPad Prism Version 8 (GraphPad Software Inc.) for graphics. Differences between groups, in case of continuous variables, were tested using one-way analysis of variance. Data not normally distributed were log-transformed, and if still failing normality, Kruskal–Wallis with multiple comparison tests were used. For continuous, repeated variables, a mixed model was used to test for significant differences between groups with time and group as factors. Small group sizes were taken into account in the analysis as well as unequal standard deviations in the groups if it occurred. One pig in the NMP+vehicle group was terminated on posttransplant day 6, and data from this animal were included in all analyses until its death. Data are presented as mean ± SD or median [95% confidence interval (CI)] depending on normal distribution. Two-sided significance level of .05 was chosen.

3 | RESULTS

3.1 | Animal characteristics, basic information, and perioperative data

The duration of oxHMP, NMP, cold and warm ischemia were similar between groups. Kidney weight at baseline was lower in the oxHMP group compared to NMP+pMSC (Table 1). Postoperative graft survival across groups was similar; only one pig was terminated (NMP+vehicle) on day 6 because of uremic discomfort with delayed graft function.

3.2 | HMP and NMP perfusion characteristics

During oxHMP, all kidneys showed similar flow patterns with starting values ranging between 11 and 27 ml/min/100 g and an increase over time to a maximum of 39 ml/min/100 g at the end of oxHMP. There were no significant differences in flow progression or resistance over time between groups (p = .104 [flow] and p = .329 [renal resistance]; Figure 2A,B). MSC administration did not have a significant effect on renal blood flow (Figure 2A). However, post hoc comparisons revealed that kidneys perfused in the NMP+pMSC group had significantly lower renal blood flow values and higher renal resistance at the start of NMP before MSCs were administrated (Table S2) compared to NMP+vehicle. During NMP, concentrations of electrolytes and acid–base balance remained stable and within physiological range (data not shown).

3.3 | Metabolic activity, creatinine clearance, and NGAL during NMP

All kidneys produced urine during NMP without differences in cumulative urine output (p = .484, Figure 2C). All kidneys consumed oxygen to the same extent with group mean values ranging from 0.26 to 2.33 ml O_2/min per 100 g (p = .613, Figure 2D). Fractional excretion of sodium improved in all groups during NMP and were similar (p = .529, Figure 2E). Creatinine clearance was very low and similar with end values of 0.97 ± 0.83 ml/min (NMP+vehicle), 0.65 ± 0.60 ml/min (NMP+pMSC), and 0.95 ± 0.96 ml/min (NMP+hMSC) (p = .74,

TABLE 1 Animal characteristics, selected perioperative data, and plasma parameters

	oxHMP (n = 7)	NMP (n = 7)	NMP + pMSC (n = 7)	NMP + hMSC (n = 7)	p-value
Body weight, baseline (kg)	51.6 ± 3.4	51.7 ± 2.9	52.4 ± 2.0	54.4 ± 1.7	.167
Body weight gain, peak-baseline (kg)	3.9 ± 2.2	6.0 ± 2.1	7.0 ± 3.1	6.3 ± 3.9	.256
Kidney weight (g)	155 ± 20	174 ± 20	188 ± 21	175 ± 19	.035[a]
WIT (min)	75.0 ± 0.0	75.1 ± 0.4	75.0 ± 0.0	75 ± 0.0	.926
Total CIT (hh:mm)	16:00 ± 00:11	16:02 ± 0:08	16:06 ± 0:11	16:00 ± 0:02	.812
Time on HMP (hh:mm)	14:04 ± 0:12	13:59 ± 0:01	14:00 ± 0:01	13:59 ± 0:01	.126
Time on NMP (hh:mm)	N/A	04:00 ± 00:00	04:00 ± 00:00	04:01 ± 00:01	.838
Total storage time (hh:mm)	16:00 ± 00:11	20:02 ± 0:08	20:06 ± 0:11	20:00 ± 0:02	.001[b]
Anastomosis duration, day 0 (hh:min)	0.53 ± 0.09	1.01 ± 0.18	0.59 ± 0.10	0.53 ± 0.10	.484
Perioperative fluid (iv), total (L)	5.4 ± 0.4	5.3 ± 0.7	5.7 ± 0.8	5.8 ± 1.2	.745
Plasma creatinine, baseline (μmol/L)	132 ± 21	116 ± 8	115 ± 12	127 ± 23	.244
Plasma creatinine, peak (μmol/L)	1041 ± 358	1261 ± 334	1234 ± 246	1076 ± 194	.403

[a]oxHMP significantly lower than NMP+pMSC.

[b]oxHMP significantly lower than NMP+vehicle, NMP+pMSC, and NMP+hMSC.

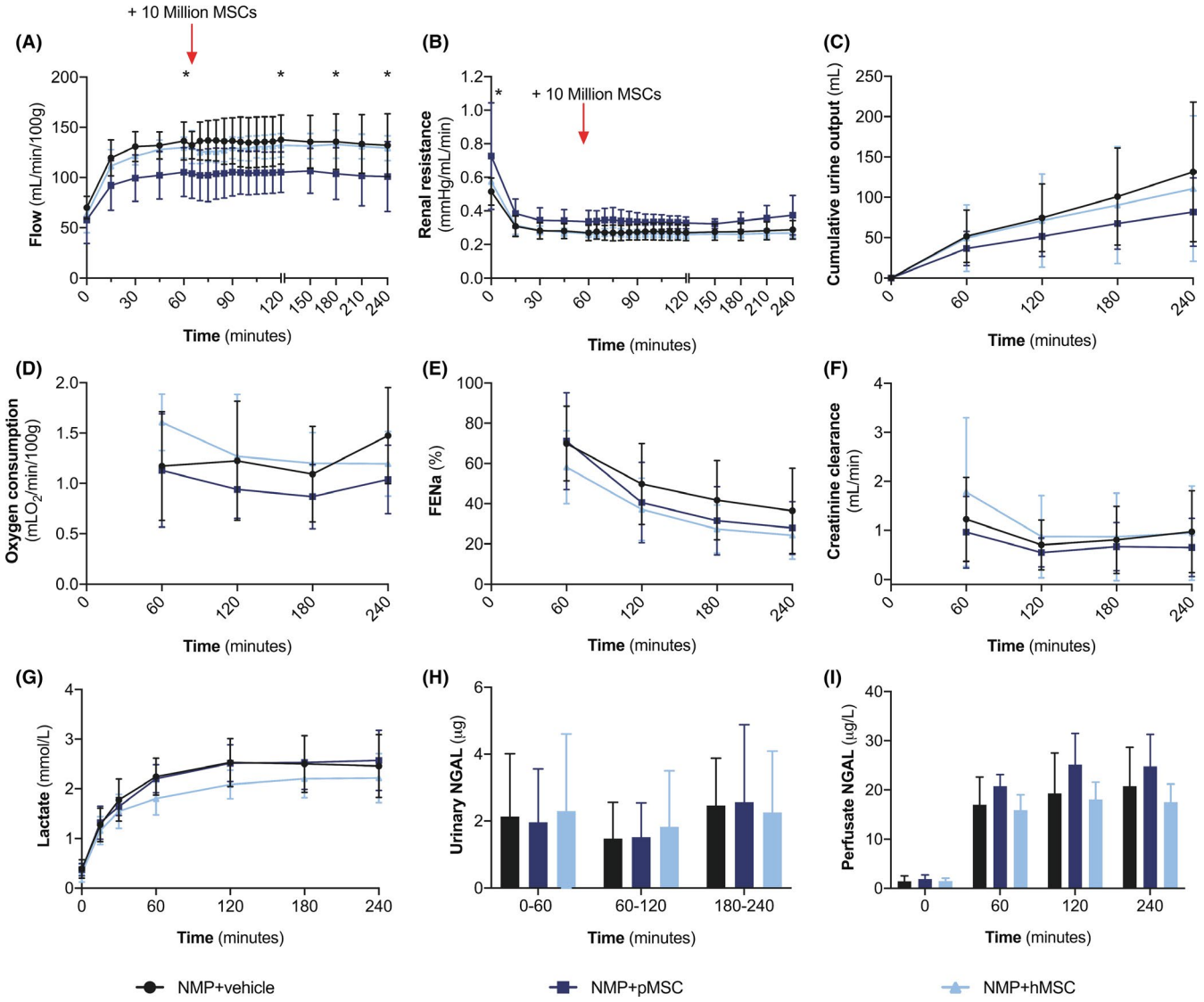

FIGURE 2 Perfusion characteristics, renal function, and injury markers during NMP with administration of vehicle, 10 million porcine MSC, or 10 million human MSC. (A) Flow (ml/min/100 g); (B) renal resistance (mm Hg/flow in ml/min); (C) cumulative urine output (ml); (D) oxygen consumption (ml O_2/min/100 g); (E) fractional excretion of sodium (%); (F) creatinine clearance (ml/min); (G) lactate (mmol/L) and (H) absolute urinary NGAL excretion per hour (µg) and (I) perfusate NGAL (µg/L). Data presented as mean ± SD. NMP+pMSC had significantly lower flow vs. NMP+vehicle, $^*p \leq .018$. Additionally, no other statistical significant differences were detected during NMP. FENa, fractional excretion of sodium; hMSC; human mesenchymal stromal cells; NGAL, neutrophil gelatinase-associated lipocalin; NMP, normothermic machine perfusion; pMSC, porcine mesenchymal stromal cells [Color figure can be viewed at wileyonlinelibrary.com]

Figure 2F), confirming that physiological renal function during NMP after severe ischemia is nearly absent. Perfusate lactate increased in the first 120 min of NMP in all groups and stabilized thereafter with similar mean values at the end of NMP (p = .653, Figure 2G). Urinary and perfusate NGAL were similar between groups during NMP (p =.889 [urine]; p = .304 [perfusate], Figure 2H,I).

3.4 | Renal MSC retention

Y-chromosome levels measured in kidney biopsies obtained after NMP and reperfusion confirmed successful delivery of MSCs during NMP to all porcine MSC-treated grafts. On day 14, Y-chromosome DNA was still detected in the renal cortex, but amounts were reduced approximately 20-fold compared to the amount found directly after NMP and reperfusion (Figure 3).

3.5 | Renal function and injury posttransplant

Pigs developed acute kidney injury posttransplant with 24–48 h of anuria and injury markers increased during the first 72 h. With the exception of the terminated pig, animal welfare scores remained acceptable throughout.

Measured GFR on day 14 was higher in the NMP+vehicle group compared to oxHMP, p = .048. Mean values were 42 ± 12 ml/min

(oxHMP), 55 ± 3 ml/min (NMP+vehicle), 52 ± 8 ml/min (NMP+pMSC), and 48 ± 7 ml/min (NMP+hMSC) (Figure 4A).

Postoperative p-creatinine peaked on day 3 or day 4 with mean levels of 1041 ± 358 μmol/L (oxHMP), 1261 ± 334 μmol/L (NMP+vehicle), 1234 ± 246 μmol/L (NMP+pMSC), and 1076 ± 195 μmol/L (NMP+hMSC) (Figure 4B). During follow-up, the levels of p-creatinine were significantly different between groups (p = .017). Post hoc day-to-day comparisons revealed that on days 4–7, the NMP+vehicle group had significantly higher p-creatinine levels compared to the oxHMP group ($p \leq .011$) and compared to the NMP+hMSC group ($p \leq .032$).

P-NGAL increased in all animals, and during the 14 days of follow-up, the levels were similar between groups, p = .281 (Figure 4C). It peaked on day 2 in all groups with medians of 1557 μg/L (95% CI: 1223–1981) for the oxHMP group, 1871 μg/L (95% CI: 1470–2381, NMP+vehicle), 2002 μg/L (95% CI: 1573–2548, NMP+pMSC), and 2217 μg/L (95% CI: 1742–2821, NMP+hMSC). Despite a considerable decrease in the last week of follow-up, day 14 p-NGAL concentrations remained significantly increased compared to their baseline values in all groups ($p < .0001$).

3.6 | Histology

Histopathological assessment revealed the presence of inflammation, tubular atrophy, and fibrosis in the experimental groups, which was not found in healthy kidneys (Figure 5). Within the experimental groups, there was a tendency of higher scores in the MSC-treated kidneys without reaching statistical significance.

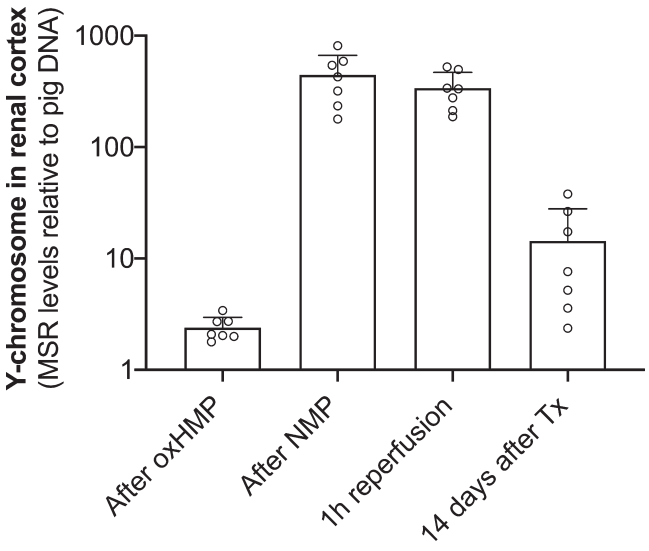

FIGURE 3 Relative Y-chromosome levels of the NMP+pMSC group. Y-chromosome levels in renal cortex biopsies relative to total pig DNA after oxHMP, NMP, 1 h after reperfusion and 14 days posttransplant. Data are presented as mean ± SD. MSR, male-specific repeat; NMP, normothermic machine perfusion; oxHMP, oxygenated hypothermic machine perfusion; pMSC, porcine mesenchymal stromal cells; Tx, transplantation

3.7 | Fibrosis markers

A set of fibrosis-related genes were examined at mRNA level to investigate the potential initiation of fibrosis at day 14 as a supplement to the histological assessment. Collagen1a1, collagen3a1, and fibronectin significantly increased in all experimental groups compared to healthy kidneys (all $p < .0001$). However, NMP with or without MSCs did not change the expression of these markers (Figure 6).

3.8 | IL-6 and IL-10 levels during NMP and posttransplant

During NMP, IL-6 secretion increased in all NMP groups to similar levels (Figure 7A), while plasma IL-6 from baseline to day 7 progressed differently (p = .001); the pairwise post hoc analyses displayed significantly lower levels in the oxHMP group 2 h after transplantation compared to the NMP+vehicle group (p = .002, Figure 7C). IL-10 levels in the perfusate and plasma were similar (Figure 7B,D).

3.9 | Inflammatory, injury, and repair-related gene expression in renal cortex tissue

Renal biopsies taken after oxHMP, NMP, and 1 h after reperfusion were analyzed for mRNA expression of inflammatory cytokines, growth factors, and damage markers (Figure 8). IL-6, IL-8, IL-10, TGF-β, and LCN2 mRNA levels were found to be significantly lower in the oxHMP group 1 h after transplantation compared to those in the NMP+vehicle group (all $p \leq .001$). The levels were similar between NMP+vehicle and NMP+MSC treated groups. The opposite was seen in regard to HGF, which was significantly higher an hour after transplantation in the oxHMP group vs. NMP+vehicle ($p < .0001$). No differences in VEGF and NOX4 expression was found between groups (Figure 8).

4 | DISCUSSION

This study aimed to demonstrate the safety of MSC therapy to ischemically injured donor pig kidneys during ex vivo NMP followed by autotransplantation. In addition, we sought to evaluate any early potential beneficial effects of MSC treatment posttransplant.

The first reports of renal infusions of MSCs during *sub*-NMP and NMP were on human-discarded kidneys by Brasile et al[24] and Thompson et al.[25] Both groups showed beneficial effects of MSCs and multipotent adult progenitor cells. In our study, we took the additional step of transplanting the MSC-treated grafts to analyze the retention of MSCs and local effects. All pigs transplanted with MSC-treated kidneys survived the crucial first postoperative days, suggesting the safety of administration of 10 million MSCs to an isolated donor kidney. Neither NMP, nor the addition of MSCs during NMP improved renal injury or function in the posttransplant phase

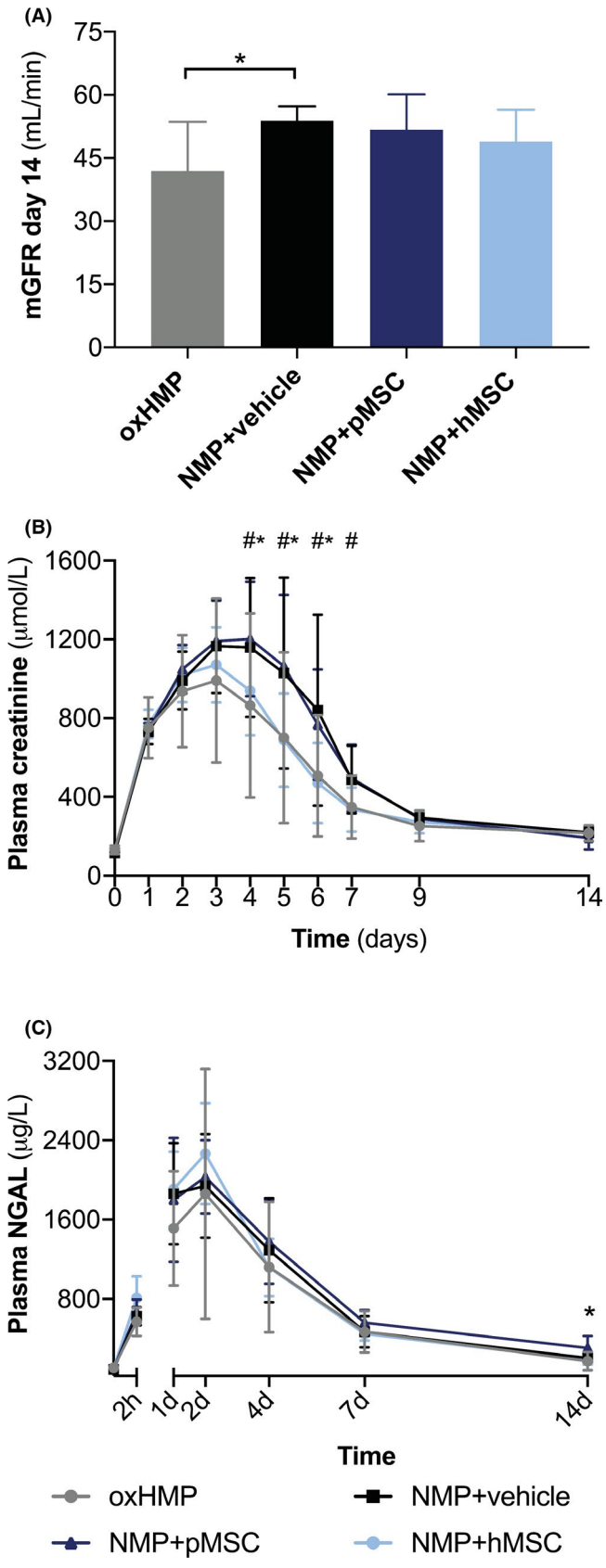

FIGURE 4 Renal function and injury posttransplant. (A) mGFR 14 days after transplantation (ml/min). *p = .048. (B) Postoperative plasma creatinine (μmol/L). NMP+vehicle had significantly higher p-creatinine on days 4–7 vs. oxHMP (*all $p \leq .011$) and NMP+hMSC ($^{\#}$all $p \leq .032$). (C) Postoperative plasma NGAL (μg/L). All groups had significantly higher levels of p-NGAL on day 14 compared to their baseline values, *all $p < .0001$. Data are presented as mean ± SD. mGFR, measured glomerular filtration rate; MSC, mesenchymal stromal cells; NGAL, neutrophil gelatinase-associated lipocalin; NMP, normothermic machine perfusion; oxHMP, oxygenated hypothermic machine perfusion [Color figure can be viewed at wileyonlinelibrary.com]

compared to oxHMP alone. We found a significantly higher GFR in the NMP+vehicle group in comparison with the oxHMP group, but this may be related to the tendency to lower body mass and significantly smaller kidneys in the oxHMP group as also indicated by the slightly lower peak p-creatinine in the oxHMP group. These outcomes are in line with the results published by Darius et al, who demonstrated higher serum creatinine levels after NMP preceded by HMP vs. oxHMP alone.[31] In contrast, a study by Urbanellis et al,[32] demonstrated superiority of 16 h of NMP over both SCS and HMP suggesting that our NMP duration of 240 min might have been too short to demonstrate a significant improvement in function. However, our objective was not to test prolonged NMP but to utilize NMP as a platform to deliver potentially regenerative therapy to injured grafts. Our experiments demonstrate that the addition of MSCs during NMP is safe and does not cause significant alterations to perfusion dynamics or graft survival. Renal arterial administrated MSCs retain in the glomeruli,[23,30] of which the consequences are unknown; however, this study brought new insight to the fate of the MSCs. Tracking porcine MSCs displayed that cortical Y-chromosome levels were similar after NMP and 1 h *after* transplantation. We also investigated whether MSCs elicit a detectable action during NMP and after transplantation but the analysis of biomarkers was not conclusive. Cortical tissue mRNA expression of IL-6, IL-8, IL-10, TGF-β, and LCN2 production increased in all NMP groups. This was not found in the oxHMP group of which levels were significantly lower. This may be related to higher metabolic rates as a result of normothermia; we did not find evidence of a MSC-induced immunoregulatory response, which is in line with the lack of improvement in functional posttransplant results as well as early fibrosis markers and histology.

MSCs are generally short lived[21] and, hence, the smaller fraction of viable cells detected on day 14 was expected. However, potentially beneficial effects exerted by MSCs do not necessarily depend on their long-term viability as the interaction between MSC and host cells may initiate immunoregulatory and regenerative processes that continue after the disappearance of MSCs.[33-35] In this study, donor kidneys were treated with 10 million MSCs during NMP. This dose was based on results from other preclinical studies in pigs and a human study, where renal intra-arterial administration of approx. 10 million MSCs appeared to be therapeutically effective.[36-38] It is possible that the dose or timing could be optimized; dose–response studies are still lacking as are studies with prestimulated MSCs.

MSCs derived from different sources can have different therapeutic potential.[39-41] We chose adipose tissue-derived MSCs as in clinical applications the harvesting approach is less invasive,

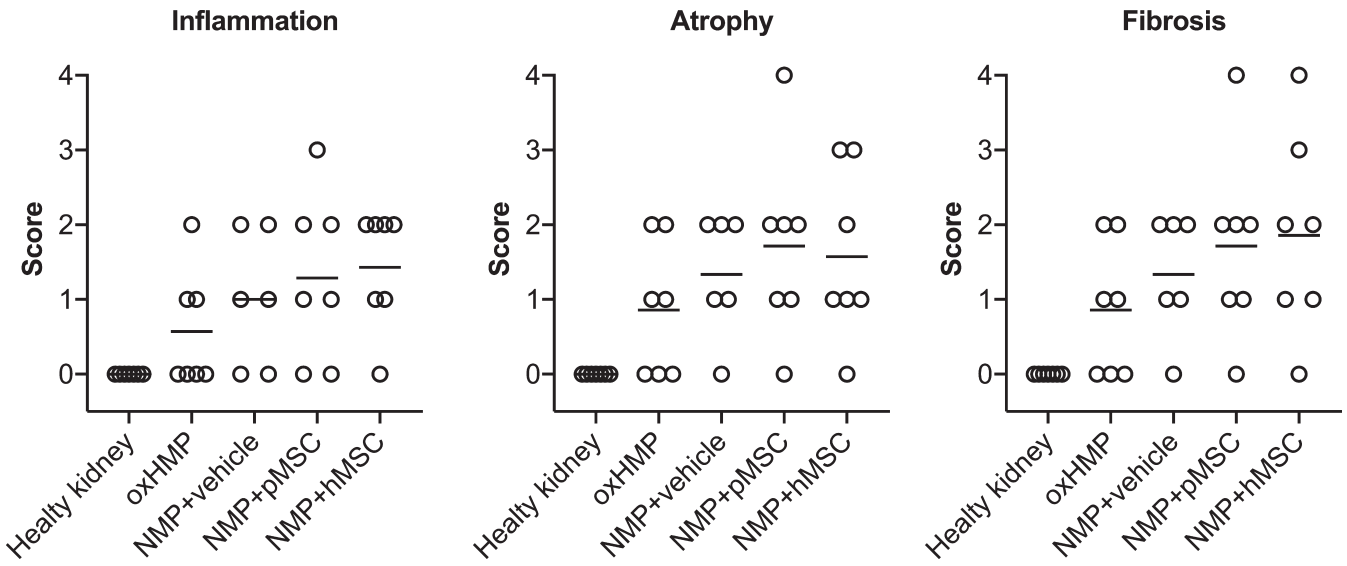

FIGURE 5 Histological scoring of biopsies collected 14 days after transplantation. Individual scores of the four experimental groups. — indicates mean value. MSC, mesenchymal stromal cells; NMP, normothermic machine perfusion; oxHMP, oxygenated hypothermic machine perfusion

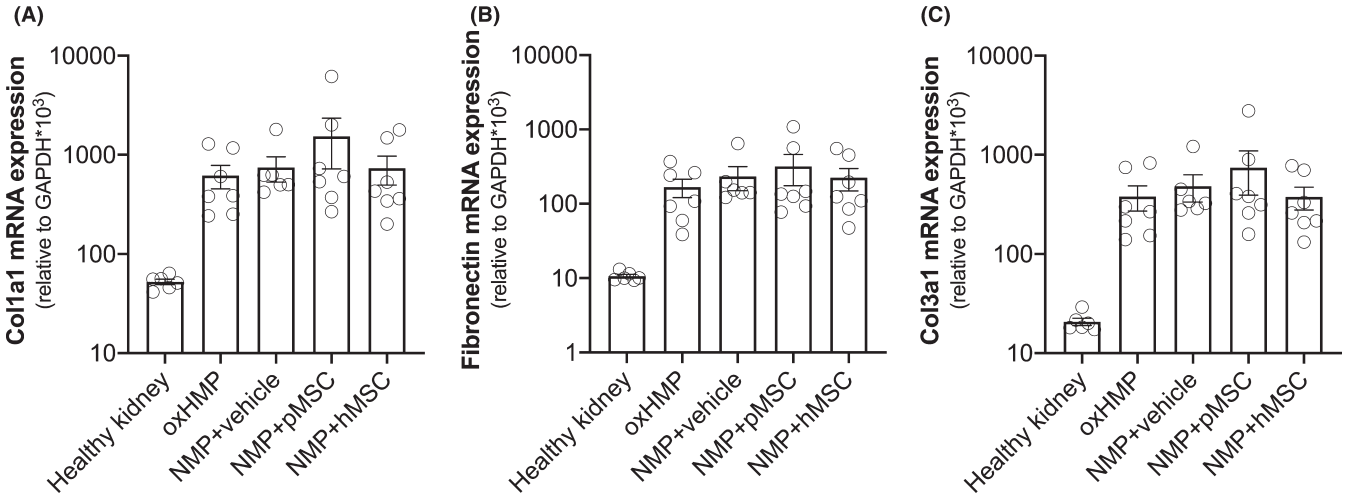

FIGURE 6 Fibrosis markers relative to GAPDH 14 days after transplantation in biopsies from healthy kidneys and kidneys of all experimental groups. (A) Col1a1 mRNA expression; (B) Fibronectin mRNA expression; (C) Col3a1 mRNA expression. Data are presented as mean ± SD. Col1a1, collagen type I alpha chain; Col3a1, collagen type III alpha chain; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MSC, mesenchymal stromal cells; NMP, normothermic machine perfusion; oxHMP, oxygenated hypothermic machine perfusion

a significantly higher number of MSCs can be obtained from the same amount of tissue in comparison with other sources[42] and the immunosuppressive potential may be superior to that of bone marrow-derived MSCs.[39,40] Our study used thawed cryopreserved MSCs as opposed to other experimental studies that used fresh MSCs directly from culture.[21,22,30,43] Although the latter approach may have advantages in terms of MSC activity,[44-48] the method we chose is more in line with clinical reality. For our current study we chose to have an experimental group with human MSCs to see if our porcine model was suitable to test the behavior of a future clinical product, and evidence so far suggests that MSCs do not elicit a substantial immunological xeno-response.[49] It was not our scope to test potential functional differences between porcine and human MSCs and therefore statistical results were not reported.

Our study design was chosen from a clinical perspective based on offering the regenerative MSC therapy upon graft arrival at the transplantation center and we do not believe the minor difference in total storage duration (14 vs. 18 h) significantly impacted our findings. Our setup was similar to another study in which SCS was followed by various periods of NMP leading to a difference in preservation time of up to 16 h.[50]

Summarizing, this study has demonstrated that delivery of 10 million MSCs during ex vivo NMP of porcine kidneys is safe without a negative effect on perfusion characteristics or early transplantation

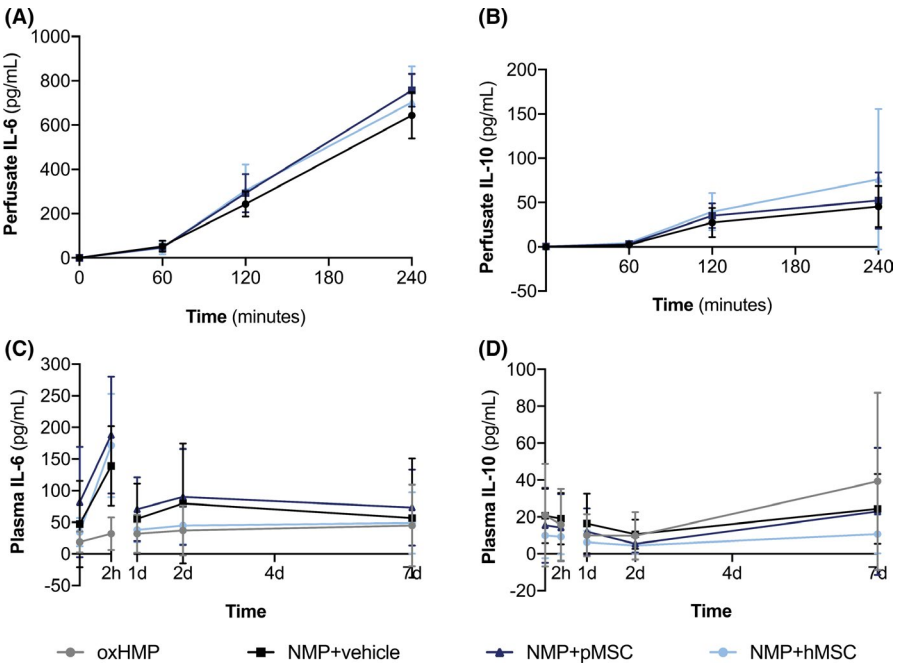

FIGURE 7 Cytokines in the perfusate during NMP and in plasma after transplantation. Perfusate levels of (A) IL-6 (pg/ml) and (B) IL-10 (pg/ml). Plasma levels of (C) IL-6 (pg/mL) and (D) IL-10 (pg/ml). Data are presented as mean ± SD. IL-6, interleukin 6; IL-10, interleukin 10; MSC, mesenchymal stromal cells; NMP, normothermic machine perfusion; oxHMP, oxygenated hypothermic machine perfusion [Color figure can be viewed at wileyonlinelibrary.com]

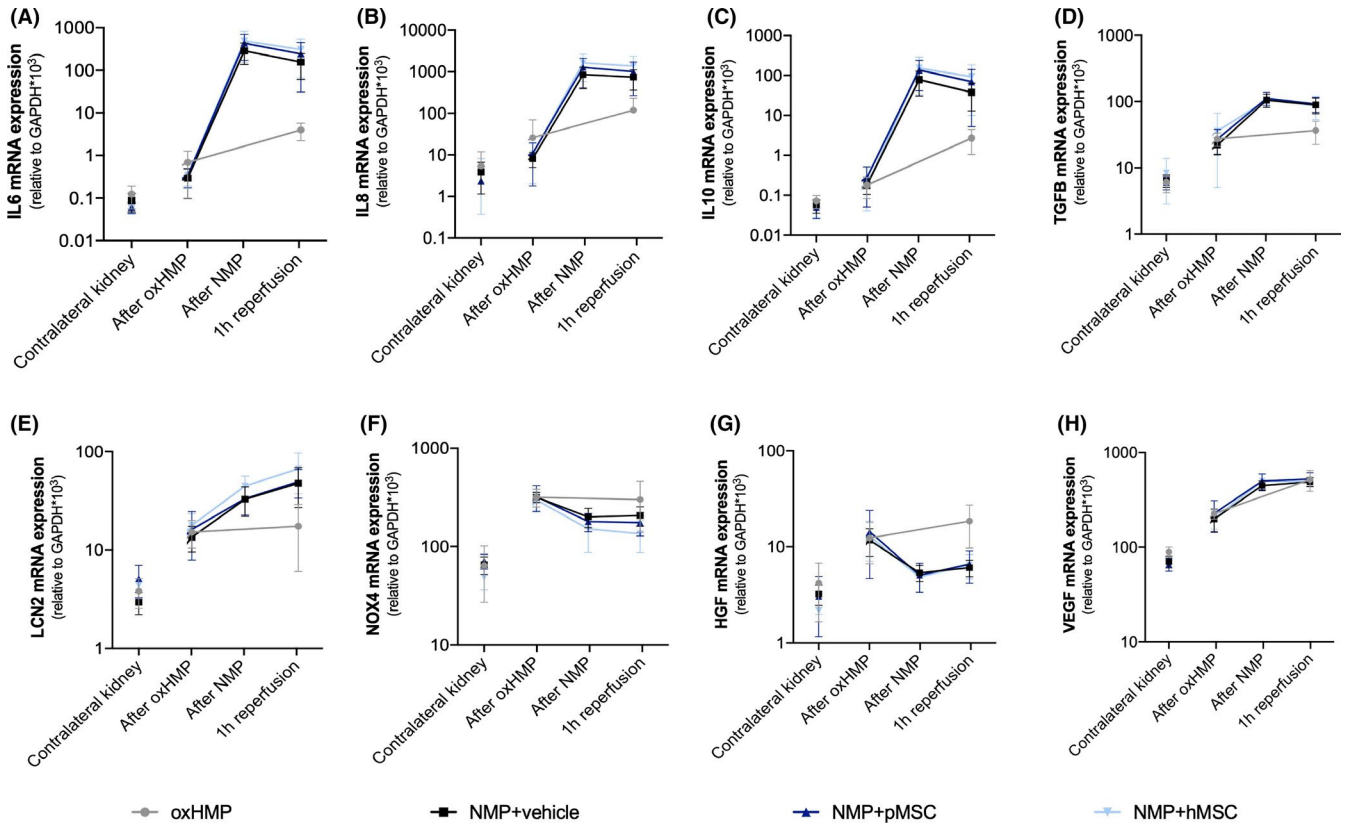

FIGURE 8 Inflammatory, injury, and repair-related gene expression relative to GAPDH in tissue of healthy kidneys and kidney tissue after ischemia and oxHMP, after NMP, after 1 h of reperfusion and 14 days after transplantation. (A) IL-6 mRNA expression; (B) IL-8 mRNA expression; (C) IL-10 mRNA expression; (D) TGFβ mRNA expression (E) LCN2 mRNA expression; (F) NOX4 mRNA expression; (G) HGF mRNA expression; and (H) VEGF mRNA expression. Data are presented as mean ± SD. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HGF, hepatocyte growth factor; IL-6, interleukin 6; IL-8, interleukin 8; IL-10, interleukin 10; LCN2, lipocalin-2; MSCs, mesenchymal stromal cells; NMP, normothermic machine perfusion; NOX4, nicotinamide adenine dinucleotide phosphate oxidase 4; oxHMP, oxygenated hypothermic machine perfusion; TGFβ, transforming growth factor beta; Tx, transplantation; VEGF, vascular endothelial growth factor [Color figure can be viewed at wileyonlinelibrary.com]

outcome. The dosing and methodology of MSC therapy prior to transplantation in this study did not improve early function or condition of ischemically damaged kidneys. Our methods allowed us to identify MSCs in treated kidneys up to 14 days posttransplant and the study provided a proof of concept for ex vivo MSC therapy during NMP conveying a number of valuable first insights with regard to targeted cell therapy in donor kidneys that have been subsequently successfully transplanted.

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DISCLOSURE

The authors have no conflicts of interest to disclose as described by the *American Journal of Transplantation*.

AUTHOR CONTRIBUTIONS

Lohmann, Pool, Rozenberg, Keller, Møldrup, Lignell, and Eijken performed the experiments. Lohmann, Pool, Rozenberg, Keller, Møldrup, Møller, Lignell, Sierra-Parraga, Hunter, Moers, Hoogduijn, Baan, Leuvenink, Ploeg, Eijken, and Jespersen involved in analysis and interpretation of data. Lohmann, Pool, and Rozenberg drafted the manuscript. Lohmann, Pool, Rozenberg, Keller, Møldrup, Møller, Lignell, Sierra-Parraga, Lo Faro, Hunter, Moers, Hoogduijn, Baan, Leuvenink, Ploeg, Eijken, and Jespersen designed the research and involved in critical revision of the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Stine Lohmann https://orcid.org/0000-0002-3774-4471
Rutger J. Ploeg https://orcid.org/0000-0001-7801-665X

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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