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Ex Vivo Administration of Mesenchymal Stromal Cells in Kidney Grafts Against Ischemia-reperfusion Injury—Effective Delivery Without Kidney Function Improvement Posttransplant

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Background. Mesenchymal stromal cell (MSC) therapy may improve renal function after ischemia-reperfusion injury in transplantation. Ex vivo renal intraarterial administration is a targeted delivery method, avoiding the lung vasculature, a known barrier for cellular therapies. In a randomized and blinded study, we tested the feasibility and effectiveness of MSC therapy in a donation after circulatory death autotransplantation model to improve posttransplant kidney function, using an ex vivo MSC delivery method similar to the clinical standard procedure of pretransplant cold graft flush. **Methods.** Kidneys exposed to 75 minutes of warm ischemia and 16 hours of static cold storage were intraarterially infused ex vivo with 10 million male porcine MSCs (Tx-MSC, n = 8) or vehicle (Tx-control, n = 8). Afterwards, the kidneys were autotransplanted after contralateral nephrectomy. Biopsies an hour after reperfusion confirmed the presence of MSCs in the renal cortex. Animals were observed for 14 days. **Results.** Postoperatively, peak plasma creatinine was 1230 and 1274 $\mu\text{mol/L}$ (Tx-controls versus Tx-MSC, $P = 0.69$). During follow-up, no significant differences over time were detected between groups regarding plasma creatinine, plasma neutrophil gelatinase-associated lipocalin, or urine neutrophil gelatinase-associated lipocalin/creatinine ratio. At day 14, measured glomerular filtration rates were 40 and 44 mL/min, $P = 0.66$. Renal collagen content and fibrosis-related mRNA expression were increased in both groups but without significant differences between the groups. **Conclusions.** We demonstrated intraarterial MSC infusion to transplant kidneys as a safe and effective method to deliver MSCs to the graft. However, we could not detect any positive effects of this cell treatment within 14 days of observation.

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

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INTRODUCTION

The use of older and higher risk kidney donors such as donation after circulatory death (DCD) donors has been increasing over the past decade in both Europe and the United States.^{1,2} DCD is associated with increased ischemia-reperfusion injury (IRI) of the transplanted organs resulting in a higher risk of delayed graft function and primary nonfunction.³⁻⁸ Thus, there is a need for effective supplemental therapies in kidney transplantation to prevent or reduce IRI as endogenous repair mechanisms in the damaged kidney may only have limited capacity.⁸

Mesenchymal stromal cells (MSCs) have immunomodulatory and regenerative properties,⁴ and their therapeutic potential has been tested in several experimental and clinical studies with promising results.^{3,5,6,9} The potentially beneficial properties of MSCs are highly desirable in kidney transplantation for several reasons: anti-inflammatory effects might decrease IRI,⁷ damaged tissue regeneration could be stimulated, and in animal models, MSC treatment has resulted in decreased fibrosis of several organs.^{7,10-14} Thus, MSCs could initiate mechanisms that would have long-term beneficial effects, possibly reducing chronic allograft nephropathy¹⁰ and therefore extending graft survival. Despite early findings, the advance of MSC therapy in human kidney transplantation has been slow due to lack of understanding key issues such as the mechanism of action of MSCs and optimal delivery of MSCs leading to studies with wide spectrum of scientific aims.¹⁵⁻¹⁹ Several studies have infused MSCs intravenously, which results in the cells mainly ending up in the lungs,^{5,20-22} and with traces of MSCs in other organs including the kidney.^{20,21} In addition, MSCs are known to have a short life span after intravenous (iv) administration with a very rapid clearance within 24 hours.^{23,24}

Local treatment such as intraarterial infusion in the kidney may offer improved availability of MSCs to the site of injury²⁵ and could potentially be more effective and safer than iv-infusion with entrapment in the lung capillaries. MSC administration into the renal artery has been carried out with success in preclinical studies.^{14,21,26} The first 2-dose study (1.0×10^5 and 2.5×10^5 cells/kg) included patients with atherosclerotic renovascular disease, and found positive efficacy with both increased renal blood flow and stable glomerular filtration rate (GFR).⁹ In vivo intraarterial MSC delivery is complex in the fragile postoperative phase, while an ex vivo route of administration opens the way for cell treatment of grafts before transplantation. The aims of this study were to examine the feasibility of a novel delivery method of ex vivo intraarterial MSC infusion before transplantation and to examine potential graft improvements posttransplant.

MATERIALS AND METHODS

Ethics and Animals

Female laboratory pigs (Danish Landrace and Yorkshire crossbreed) weighing 50 kg were used. All animal care and procedures followed guidelines from the European Union (directive 2010/63/EU) and local regulations; the study was approved by The Animal Experiments Inspectorate (reference number: 2016-15-0201-01145). All personnel involved in the animal experiments had Federation for Laboratory Animal Science Associations licenses.

Study Design

The applied kidney autotransplantation animal model with extended warm ischemia (WI) was developed in a series of previous experiments to achieve sufficient renal injury with concurrent acceptable animal welfare.²⁷ In the present study, we compared intraarterial administration of MSCs to vehicle controls in a blinded, randomized design. Kidney grafts were exposed to 75 minutes of WI and a total of 16 hours of static cold storage and were randomized to controlled intraarterial infusion of 10 million MSCs in 50-mL cold Belzer UW solution, or 50-mL cold Belzer UW solution. After contralateral nephrectomy, preserved grafts were autotransplanted and animals observed for 14 days. During follow-up, renal function, biomarkers of graft injury, and inflammatory markers were measured.

Surgical Procedure

Procedures were performed as previously described by our group.²⁷ Briefly, after induction of anesthesia with an iv administration of ketamine (6 mg/kg) and midazolam (0.5 mg/kg), intubation and ventilation followed. Anesthesia was maintained by sevoflurane and iv remifentanyl for analgesic. On postoperative days 1–4, intramuscular buprenorphine was administered 3 times a day. In sterile settings, a permanent semicentral venous catheter (5 Fr, 20 cm; Careflow, BD, NJ) was placed, and after midline incision, left nephrectomy was performed retroperitoneally and the kidney exposed to 75 minutes of WI (Figure 1A). Afterwards, the graft was flushed with cold Belzer UW (Bridge to Life, London, United Kingdom) and put on cold storage at 4°C for the following 16 hours. The midline incision was closed and the pig returned to the housing facility after extubation.

Next day, intraarterial administration of MSC + UW or UW was performed, described in detail below. Following right nephrectomy, the left kidney graft was autotransplanted, with vascular and ureter anastomosis performed end-to-end. A punch biopsy was taken 1 hour after reperfusion. The midline incision was closed and the pig returned to the housing facility for 14 days of observation with periodic blood and urine collection. On day 14, the pigs were anesthetized again according to the aforementioned procedure. A feeding tube was placed in the ureter for continuous urine collection. GFR was measured as the urinary clearance of ⁵¹Chrom-EDTA and the quantity of effective renal plasma flow as clearance of Tc-mercaptoacetyl triglycine. Lastly, graftectomy was performed and the pigs were euthanized under general anesthesia.

Healthy Kidney Tissue

This study did not include a control group without ischemic injury. To compare the protein and gene expression results at study end to noninjured kidneys, we included 6 healthy kidneys from control pigs from other experiments. Pigs of similar weight and gender underwent the same anesthetic protocol, had a catheter placed in the right renal artery and administration of 50 mL saline (150 mL/h). After 14 days of observation, pigs underwent GFR measurement (⁵¹Chrom-EDTA clearance) and bilateral nephrectomy. The left healthy kidneys were included in this study.

FIGURE 1. A, Surgical procedure. Left kidney ischaemically injured by 75 min of warm ischaemia followed by static cold storage until next d. MSCs were intraarterially infused shortly before right nephrectomy and end-to-end anastomoses of artery, vein, and ureter. B, MSC administration on the back table at d 0. Suspended 10 million MSCs in 50-mL cold UW solution was digitally infused in the renal artery using a blunt cannula with a rate of approximately 10 mL/min. MSC, mesenchymal stromal cell; UW, University of Wisconsin.

Tissue Collection

On day 14, nephrectomy was performed and the kidney coronally sliced. For histology, tissue samples containing both medulla and cortex from upper and lower poles were collected and put immediately in 4% formalin. For DNA, RNA, and protein analysis, approximately 100 mg of cortical tissue was collected from the lateral side, upper and lower poles and stored in RNeasy (ThermoFisher) at -20°C . For tissue dissociation, tissue from various positions within the kidney was stored in MACS Tissue Storage Solution (Miltenyi Biotec).

Blood and Urine Collection

Blood collection was carried out using the central venous catheter at baseline (d -1), 1 hour after reperfusion and on posttransplant days 1–7, 9, and 14. Urine was collected in an ostomy bag placed around external genitals of the pig at days 2, 4, and 7, for which the animal did not have to be restrained.²⁷ At days -1 and 14, urine was collected directly from the graft ureter peroperatively.

Neutrophil Gelatinase-associated Lipocalin

At baseline, days 2, 4, 7, and 14, plasma neutrophil gelatinase-associated lipocalin (NGAL) and urine NGAL

were determined using the pig NGAL sandwich ELISA kit (Kit 044; BioPorto, Hellerup, Denmark). The assay was setup according to manufacturer's protocol.

MSC Expansion, Characterization, and Preparation

Porcine adipose-tissue-derived MSCs from a total of 3 male donors were isolated, expanded (in minimum essential medium supplemented with 15% fetal bovine serum) and characterized as described previously.²⁸ After expansion, cells were cryopreserved at passage 3. All MSC batches were $>95\%$ positive for CD29, CD44, CD90, and negative for CD31 and CD45. MSC multipotency was demonstrated by their capacity to differentiate towards adipocytes and osteoblasts. Before MSC infusion, 10 million cryopreserved MSCs were thawed and washed in culture medium by centrifugation (440g, 5 min). Cell pellets were resuspended in 50-mL cold UW solution, filtered through a 70- μm cell strainer, and transferred to a 50-mL syringe shortly before infusion. Cells were inspected by microscope to confirm a single-cell suspension and vitality by trypan blue ($>90\%$).

Ex Vivo MSC Infusion Before Transplantation

Approximately 10 minutes before initiating the anastomoses, the left kidney was packed in a tissue, containing

ice, and placed on a table in the operating room. The 50-mL syringe with a blunt cannula was positioned in the artery (Figure 1B). Air was removed and the preservation fluid ($\pm$ MSCs according to randomization protocol) was infused manually at a rate of approximately 10 mL/min.

Kidney Tissue Dissociation

In total, 0.5 g kidney tissue was dissociated using a GentleMACS dissociator (Miltenyi Biotec) as described previously.²⁸ After dissociation, cells were pelleted by centrifugation and resuspended in 3-mL culture medium, used immediately for analysis, or cryopreserved.

Fluorescent MSC Tracing

MSCs were labeled with Quantum dots (Qdot) 655 (ThermoFisher) using 1.5 μ L of the reagent per million MSCs according to the manufacturer's protocol immediately before the infusion. Dissociated cortical tissue was analyzed by flow cytometry for fluorescent MSC detection as described previously.²⁸

Male MSC Detection

Cellular DNA, collected an hour after reperfusion and at day 14, was isolated from 10 mg tissue or cell pellets using a NucleoSpin Tissue DNA isolation kit (Macherey-Nagel) according to the manufacturer's protocol. Cell-free DNA from 1 mL of plasma was isolated using a QIAasympyphony and a QIAasympyphony DSP Virus/Pathogen Kit (Qiagen) according to the manufacturer's protocol. Y-chromosome DNA was detected by quantitative polymerase chain reaction (qPCR) using primers for the male-specific repeat located on the porcine Y-chromosome.²⁹ The porcine S100C gene was used as pig DNA control. Primer sequences are listed in Table S1 (SDC, <http://links.lww.com/TP/C7>). The qPCR mix consisted of 0.5 μ L (cellular) or 5 μ L DNA (cell-free), 10 pmol of each primer, and 1 $\times$ KiCStart SybrGreen qPCR ReadyMix (Sigma-Aldrich Life Science) in an end volume of 25 μ L, and run in duplicate on an Applied Biosystems 7300 Real-Time PCR machine (ThermoFisher).

Plastic Adherent Fraction of Dissociated Tissue

About 500 μ L of cryopreserved dissociated tissue from day 14 was thawed and seeded in a T75 culture flask (Nunc Delta surface; Thermo Scientific) with 10-mL MSC medium and incubated for 1 day at 37°C and 5% CO₂. Next day, the culture medium was fully replaced and cells were incubated for 4 hours. Before trypsinization, cells were washed twice with phosphate buffer saline (ThermoFisher) and detached by 2-mL 0.05% Trypsin-EDTA (ThermoFisher). Cells were pelleted by centrifugation and used for DNA isolation and PCR.

Histochemical Staining and Histomorphological Assessment

After 24 hours, 4% formalin-fixed samples, collected at study end, were stored in phosphate buffer saline, dehydrated, and next embedded in paraffin (formalin-fixed paraffin-embedded). The formalin-fixed paraffin-embedded sample sections of 2- μ m thickness were stained with hematoxylin and eosin, periodic acid-Schiff-diastrase, and Sirius Red according to standard diagnostic practice. Slides were

analyzed by a renal pathologist (M.C.C.-v.G.), blinded to the intervention. Several parameters were assessed: acute tubular necrosis (mild, moderate, or severe), fibrosis (minimal [$<10\%$], mild [10% – 25%], moderate [26% – 50%], and severe [$>50\%$]).

Collagen Content

In total, 1 mL of water was added to 50 mg of cortical kidney and tissue was homogenized using a GentleMACS dissociator and M-tubes (Miltenyi Biotec) according to the manufacturer's protocol. After homogenization, extracts were centrifuged and supernatant was used for total collagen and total protein analysis. A Pierce BCA Protein Assay Kit (ThermoFisher) (total protein) and Total Collagen Assay (perchlorate-free) Kit (Abcam) (total collagen) were used according to the manufacturer's protocol. In each kidney collected at study end, average collagen content was calculated by averaging the levels from 3 tissue samples.

Transforming Growth Factor Beta, Interleukin-6, and Interleukin-10 Levels

About 1 mL of RIPA Lysis and Extraction Buffer (ThermoFisher) supplemented with Pierce protease inhibitor tablets (ThermoFisher) were added to 10 mg of cortical kidney tissue from day 14. Tissue was homogenized as described for the collagen content assay and stored at -80°C . Total protein was measured using a Pierce BCA Protein Assay Kit (ThermoFisher) according to the manufacturer's protocol. Interleukin-10 (IL-10) was measured using a porcine IL10 ELISA (ThermoFisher, KSC0101) 4000 $\times$ diluted, interleukin-6 (IL-6) was measured using a porcine IL6 ELISA (ThermoFisher, ESIL6) 10 $\times$ diluted, and human transforming growth factor beta (TGF- β) was measured using a TGF- β cytosol (ThermoFisher, CHC1683) 100 $\times$ diluted. Levels for each kidney were calculated by averaging the levels from 3 tissue samples.

Gene Expression Analysis

RNA from approximately 20 mg tissue collected at day 14 was isolated using a GeneJET RNA purification kit (ThermoFisher). In total, 1 μ g of total RNA was reverse transcribed using RevertAid First Strand cDNA Synthesis Kit (ThermoFisher). qPCR was performed using Power SYBR Green PCR Master Mix (ThermoFisher), 0.4 μ M primers, and an Applied Biosystems 7300 Real-Time PCR machine. Primer sequences are listed in Table S1 (SDC, <http://links.lww.com/TP/C7>). Gene expression was presented relative to the housekeeping gene GAPDH using the following formula $2^{-(\text{Ct value GAPDH} - \text{Ct value gene of interest})} \times 1000$. mRNA levels for each kidney were calculated by averaging the levels from 3 tissue samples.

Statistics

STATA software version 15.1 (StataCorp, College Station, TX) was used for statistical analysis and SigmaPlot 13 (Systat Software, San Jose, CA) used for graphical presentation. Results are presented as mean values with SD. Student's t-test and paired t-test were applied on normally distributed or log-transformed parametric variables. Repeated, continuous variables were analyzed using a mixed model ANOVA with time and group as fixed factors and pig as random factor. If unequal SDs and correlations

in the 2 groups occurred, the factors were taken into account in the analysis. Model validation was performed afterwards by comparing observed and expected within-subject SDs and correlations. On categorical variables, Fisher's exact tests were applied. $P < 0.05$ was considered to indicate statistical significance.

RESULTS

Ex Vivo Renal Intraarterial MSC Infusion

Before this autotransplantation study, the efficiency of the MSC administration procedure was investigated by infusing fluorescently prelabeled male MSCs into porcine kidneys ex vivo. Porcine kidneys were exposed to 75 minutes WI and 16 hours of static cold storage followed by slow infusion of 10 million MSCs in the renal artery. Analyzing the collected renal venous outflow during MSC administration showed that of the 10 million administered MSCs, approximately 3% passed through the kidney. MSCs were stably retained after delivery as hardly any MSCs left the kidney during additional flushing (Figure 2A).

Analyzing renal cortex tissue confirmed that MSCs were retained in the kidney. Mean MSC abundance in the tissue was approximately 3×10^4 MSCs per gram dissociated tissue. Importantly, in each kidney, MSCs were equally distributed throughout the renal cortex (Figure 2B), confirmed by Y-chromosome tracing (Figure 2C). Fluorescent microscopy demonstrated mainly cortical localization of MSCs with sublocalization in the glomeruli (Figure 2D).

Animal Characteristics and Perioperative Data

In total, 16 female pigs ($n = 8$ per group) were randomized to ex vivo administration of 10 million MSCs or vehicle. One pig (Tx-MSC) was euthanized on day 4 due to uremic symptoms with primary nonfunction. Furthermore, 1 pig (Tx-MSC) had cardiac arrest during general anesthesia on day 14. Autopsy determined that it was not related to the kidney. Data from these 2 pigs until their death were included. Both groups had a constant mean arterial blood pressure >60 mm Hg during the surgeries, they had similar duration of warm and cold ischemia, and surgery time. Selected baseline blood chemistry and fluid given intravenously during the surgery were also similar (Table 1).

MSC Retention in the Autotransplanted Kidney

To confirm MSC retention in the treated grafts, a punch biopsy was taken 1 hour after reperfusion and analyzed for the presence of Y-chromosome DNA. This showed that MSCs were successfully delivered to all treated grafts (Figure 3A). After 14 days of follow-up, MSCs could still be detected in kidney tissue; however, Y-chromosome levels were on average $<5\%$ compared to the levels in the 1-hour biopsies, $P = 0.002$ (Figure 3B). MSC clearance during the first 2 weeks was also demonstrated by the detection of cell-free Y-chromosome DNA in plasma (Figure 3C). To investigate viability of the remaining MSCs present in the kidney at day 14, the plastic adherent fraction of dissociated kidney tissue was analyzed for the presence of

FIGURE 2. MSC retention and localization after ex vivo intraarterial administration of 10 million male MSCs. Values are presented as mean and SD. A, Percentage of Qdot-labeled MSCs lost in the venous outflow during MSC administration and the following flush quantified by flow cytometry. B, Number of Qdot-labeled MSCs in the renal cortex quantified by flow cytometry. Administered MSCs were equally distributed in the upper, lateral, and lower part of the kidney. C, Detection of Y-chromosome DNA in renal cortex by qPCR. D, Qdot-labeled MSCs were mainly located in the glomeruli visualized by fluorescent microscopy. MSC, mesenchymal stromal cell; MSR, male-specific repeat; Qdot, Quantum dots; qPCR, quantitative polymerase chain reaction.

TABLE 1.
Peroperative data and selected baseline values

	MSC			Control			<i>P</i>
	Obs.	Mean	95% CI	Obs.	Mean	95% CI	
Surgical time, d -1 (h:min)	8	02:26	02:12-02:40	8	02:22	02:00-02:44	0.73
Surgical time, d 0 (h:min)	8	04:23	03:57-04:48	8	04:21	03:57-04:45	0.89
WIT (min)	8	75.1	74.8-75.4	8	75.4	74.5-76.3	0.54
SCS (h:min)	8	16:04	15:54-16:14	8	16:01	15:58-16:03	0.43
IV fluid, d -1 (L)	8	3.0	2.7-3.2	8	3.0	2.6-3.4	0.83
IV fluid, d 0 (L)	8	2.9	2.7-3.2	8	3.1	2.8-3.5	0.26
Body weight, baseline (kg)	8	52.0	50.4-53.6	8	50.8	48.4-53.1	0.31
Graft weight, baseline (g)	8	164	149-180	8	137	124-150	0.01
Graft weight, d 14 (g)	6	214	182-246	8	182	150-215	0.11
Graft weight gain (g)	6	48	28-68	8	45	12-78	0.84
Potassium (mmol/L)	8	3.6	3.5-3.8	8	3.8	3.5-4.0	0.37
Leucocytes ($\times 10^9/L$)	8	19.3	17.1-21.8	8	19.6	16.8-22.9	0.85
Lactate (mmol/L)	8	1.5	1.1-1.8	8	1.8	1.2-2.4	0.25
Sodium (mmol/L)	8	141	140-142	8	141	140-142	0.85
Lactate-dehydrogenase (U/l)	8	409	341-478	8	356	242-470	0.36
Aspartate transaminase (U/l)	8	31	26-37	8	31	24-37	0.87
γ -Glutamyltransferase (U/l)	8	20	14-25	8	27	20-35	0.08
Urine-protein/creatinine ratio (mg/g)	8	6439	178-12700	7	3635	970-6299	0.33

CI, confidence interval; IV, intravenous; MSC, mesenchymal stromal cell; Obs., observed; SCS, static cold storage; WIT, warm ischemia time.

FIGURE 3. MSC retention in autotransplanted kidneys and presence of cell-free Y-chromosome DNA in plasma. Samples were analyzed for the presence of Y-chromosomes by qPCR. A, B, and D, Mean, SD, and individual levels presented. C, Mean and SD presented. A, Y-chromosome DNA levels in renal cortex 1 h after reperfusion. B, Y-chromosome DNA levels in renal cortex 14 d after transplantation, which was significantly lower vs 1-h posttransplant, $P = 0.002$. C, Detection of Y-chromosome DNA in plasma posttransplant. D, Y-chromosome DNA levels in the plastic adherent fraction of dissociated d 14 kidney tissue. MSC, mesenchymal stromal cell; MSR, male-specific repeat; qPCR, quantitative polymerase chain reaction.

FIGURE 4. Plasma creatinine of transplanted pigs from baseline (d 0) to d 14 posttransplant. A, Values are presented as mean and SD in μmol/L. All pigs had significant increases in p-creatinine from baseline to peak, $P < 0.001$. During the 14 d of follow-up, no significant difference in levels of p-creatinine was detected between groups, $P = 0.96$. B, Individual levels in μmol/L from baseline to postoperative d 14. One pig was euthanized on d 4 (Tx-MSC). MSC, mesenchymal stromal cell; p-creatinine, plasma creatinine.

Y-chromosome DNA. This confirmed indeed that kidneys at day 14 still contained viable male MSCs (Figure 3D).

Renal Function and Injury

Owing to the chosen model, all animals had impaired renal function postoperatively, resulting in anuria until day 2. Figure 4A shows that peak levels of plasma creatinine were reached on day 4 with a mean of 1230 ± 178 μmol/L (Tx-control) and 1274 ± 245 μmol/L (Tx-MSC), while Figure 4B shows individual values. The levels decreased and reached a stable plateau after one-and-a-half weeks. The MSC-treated group did not demonstrate altered levels of plasma creatinine compared to controls during the entire follow-up period, $P = 0.96$. The impaired renal function was further supported by an associated shift in electrolyte levels and fluid retention, displayed by body weight gain (Figure S1, SDC, <http://links.lww.com/TP/C7>).

NGAL, an early marker of acute renal injury, was measured in plasma and urine at baseline, postoperative days 2, 4, 7, and 14. Plasma NGAL and urine NGAL/creatinine ratios remained significantly elevated throughout follow-up in both groups versus baseline levels (all $P \leq 0.009$) (Figure 5A and B). Despite significant increase in NGAL (plasma and urine) in both groups, there was no effect of MSC treatment between the groups during the follow-up period. Urine-protein/creatinine ratio was also without difference between groups during the follow-up, $P = 0.14$ (Figure 5C).

GFR, measured at day 14 as urinary clearance of $^{51}\text{Chrom-EDTA}$, at day 14 was 40 ± 9 mL/min (Tx-control)

and 44 ± 17 mL/min (Tx-MSC), $P = 0.66$ (Figure 5D). No statistically significant difference was detected in concurrent measurements of effective renal plasma flow (urinary clearance of Tc-mercaptoacetyl triglycine) resulting in a mean of 133 ± 35 mL/min (Tx-control) and 146 ± 36 mL/min (Tx-MSC), $P = 0.49$ (Figure 5D).

Histology

Periodic acid-Schiff-diastase-stained sections of the upper and lower kidney poles showed no major renal abnormalities in any group (Figure 6) at day 14. Most pigs in both groups had mild tubular injury, leaving the proportions in the 2 groups similar, $P = 1.00$. Fibrosis was also scored and was absent in most kidneys while mild fibrosis was only observed in 3 Tx-control and 1 Tx-MSC pig, $P = 0.58$. The histomorphological assessments additionally revealed possible pyelonephritis with foci of neutrophilic granulocytes, which was present in 2 Tx-control and 3 Tx-MSC pigs, $P = 0.58$.

Collagen and Fibrosis Markers

To examine potential initiation of fibrosis at day 14, total collagen and a set of fibrosis-related genes were assessed. Percentage collagen of total protein significantly increased from $2\% \pm 1\%$ in healthy kidneys to $6\% \pm 3\%$ (Tx-control, $P = 0.005$) and $4\% \pm 2\%$ (Tx-MSC, $P = 0.031$). The difference in total collagen content between the Tx-control and Tx-MSC was not significant, $P = 0.14$ (Figure 7A). At the mRNA level, collagen1a1, collagen3a1, and fibronectin significantly increased in the

FIGURE 5. Renal injury and function parameters measured during the 14 d of follow-up. Results presented as mean and SD. A, The levels of p-NGAL in $\mu\text{g/L}$ were significantly higher posttransplant vs baseline in both groups, all $P \leq 0.001$. There was no evidence of treatment effect during follow-up with no significant differences in levels of p-NGAL between Tx-control vs Tx-MSC, $P = 0.29$. B, u-NGAL/creatinine in $\mu\text{g/mmol}$ were significantly increased at d 2, 4, and 7 vs baseline in both groups, $P < 0.009$. Comparing the 2 groups' mean levels throughout follow-up, there were no statistical significant differences between groups, $P = 0.31$. C, u-Protein/creatinine ratio in mg/g . There was no significant differences in the levels of u-protein/creatinine ratio between the groups throughout the follow-up, $P = 0.14$. D, GFR measured at d 14 in mL/min . Mean GFR was $40 \pm 9 \text{ mL/min}$ (Tx-control) and $44 \pm 17 \text{ mL/min}$ (Tx-MSC), $P = 0.66$. Effective renal plasma flow measured at d 14. Mean values were similar between groups, $P = 0.49$. GFR, glomerular filtration rate; MSC, mesenchymal stromal cell; p-NGAL, plasma neutrophil gelatinase-associated lipocalin; u-NGAL, neutrophil gelatinase-associated lipocalin; u-protein/creatinine, urine protein/creatinine.

Tx-control kidneys versus healthy kidneys. However, MSC treatment did not change the expression of these markers compared to the Tx-controls (Figure 7B–D).

Inflammatory and Regenerative-related Gene and Protein Expression at Study End

mRNA analysis of vascular endothelial growth factor in renal cortex tissue demonstrated significantly lower levels in Tx-control and Tx-MSC compared to healthy controls but there was no difference between the 2 transplanted groups (Figure 8A). Expression of hepatocyte growth factor mRNA was equal in the 3 groups (Figure 8B).

Analysis of an mRNA panel of inflammation-related genes in renal cortex tissue demonstrated equal mRNA expression of extracellular newly identified receptor for advanced glycation end-products binding protein, IL-8, IL-10, and plasminogen activator, urokinase type in healthy control kidneys, Tx-controls, and Tx-MSC-treated kidneys (Figure 8C–F). Finally, protein levels of TGF- β , IL-10, and IL-6 were analyzed in renal cortex tissue extracts. TGF- β and IL-10 levels in Tx-control kidneys were significantly decreased compared to healthy control kidneys, whereas IL-6 levels remained unchanged (Figure 9A–C). MSC treatment did not have an effect on TGF- β , IL-6, or IL-10 levels, $P = 0.39$ (TGF- β), $P = 0.63$ (IL-6), and $P = 0.45$ (IL-10) (Figure 9A–C).

DISCUSSION

Ex vivo intraarterial infusions of MSCs were investigated in a porcine DCD autotransplantation model using a delivery method similar to the clinical standard procedure of pretransplant cold graft flush. Targeted directly to the site of injury, the MSC delivery was efficient and safe but did not lead to beneficial effects on kidney function 14 days posttransplant. MSCs infused before anastomosis resulted in effective MSC delivery to the graft with regard to the global distribution and high retention rates of MSCs in the renal cortex after transplantation. MSCs were found mainly in the glomeruli after administration, likely as a result of size constriction of the glomerular microvasculature²⁸ in accordance with our previous study.³⁰ The relevance of glomerular localization is unclear but for optimal paracrine effects the specific localization of MSC will be of importance and a localization such as the interstitium around the tubules would be preferred. The fate of the glomeruli-localized MSCs posttransplant is yet unknown and new studies will be needed to investigate potential migration of MSC posttransplant.

After transplantation, the MSCs were largely cleared over a period of 14 days, which is in agreement with an earlier study of our group.²⁸ We proved that the remaining MSCs in the kidney after 14 days were viable, suggesting that low amounts of MSCs can continue to modulate their microenvironment in the transplant kidney. Plasma analysis

FIGURE 6. Histomorphological assessment of kidney sections containing both cortex and medulla from upper and lower poles in transplanted kidneys. A, Histological findings presented with number of events in each group. The proportions of events were not significantly different between the groups in regards of acute tubular necrosis, fibrosis, or foci of infection (all $P > 0.05$). B, PAS-stained kidney sections from controls and MSC-treated pigs. (1) Mild acute tubular necrosis characterized by mild loss of tubular epithelium nuclei and mild dilatation of the tubule. (2) Moderate acute tubular necrosis characterized by moderate loss of tubular epithelium nuclei and moderate dilatation of the tubules with intraluminal debris. (3) Examples of urinary tract infection characterized by foci of intraluminal debris with neutrophilic granulocytes in tubuli. (1) and (2) magnification, $\times 20$. (3) Magnification, $\times 10$. MSC, mesenchymal stromal cell; PAS, periodic acid-Schiff-diastase.

FIGURE 7. Renal collagen content and fibrosis-related genes. A, Collagen mass per total protein mass, presented with individual values, mean, and SD. Collagen significantly increased in both transplanted groups vs healthy controls, $*P = 0.005$, $**P = 0.031$. (B)–(D) Fibrosis-related mRNA expression obtained by qPCR relative to GAPDH. Results are expressed with individual values, mean, and SD. Col1A1, Col3A1, and fibronectin significantly increased in the transplanted Tx-control kidneys vs nonischemic healthy kidneys, $P < 0.001$ (Col1A1), $P < 0.001$ (Col3A1), $P = 0.007$ (fibronectin). The differences in fibrosis gene expressions were insignificant between the 2 transplanted kidney groups Tx-control vs Tx-MSC, all $P > 0.05$. HGF, hepatocyte growth factor; MSC, mesenchymal stromal cell; qPCR, quantitative polymerase chain reaction.

demonstrated circulating cell-free donor Y-chromosome DNA posttransplant. As the half-life of circulating cell-free DNA is considered to be in the range of minutes to hours,^{31–35} the level of Y-chromosome DNA in plasma is a good representation of the clearance rate of male donor MSCs at a specific time point. The demonstrated clearance of MSCs is not necessarily problematic, as MSCs’ immunomodulatory effects are detectable within hours after

FIGURE 8. Regenerative gene expression and inflammatory process. mRNA expression in renal d 14 tissue obtained by qPCR relative to GAPDH. Values presented individually, as well as mean and SD. A, VEGF mRNA expression was significantly lower in the transplanted groups vs healthy control kidneys, $*P < 0.001$ (Tx-control vs healthy control); $**P = 0.002$ (Tx-MSC vs healthy control). MSC treatment did not have effect compared to the Tx-controls, $P = 0.520$. (B)–(F) HGF, ENRAGE, IL-8, IL-10, and PLAUG were without significant differences between Tx-control vs Tx-MSC, all $P > 0.05$, and healthy control vs Tx-control, all $P > 0.05$. ENRAGE, extracellular newly identified receptor for advanced glycation end-products binding protein; HGF, hepatocyte growth factor; IL, interleukin; MSC, mesenchymal stromal cell; PLAUG, plasminogen activator, urokinase type; qPCR, quantitative polymerase chain reaction; TGF, transforming growth factor; VEGF, vascular endothelial growth factor.

FIGURE 9. Protein level detections of TGF- β , IL-10, and IL-6 in d 14 renal tissue. The levels are corrected for total protein of the kidney protein extract and presented by individual values, mean, and SD in pg/mg protein. A, The levels of TGF- β were significantly lower in the Tx-control group compared to healthy controls, $*P = 0.002$. No difference was found between Tx-control vs Tx-MSC, $P = 0.388$. B, IL-10 levels were significantly lower in the 2 transplanted groups vs healthy controls, $*P = 0.002$, $**P = 0.031$. IL-10 was without statistical difference between Tx-control vs Tx-MSC, $P = 0.454$. C, IL-6 levels were without statistical differences between groups, all $P > 0.05$. IL, interleukin; MSC, mesenchymal stromal cell; TGF, transforming growth factor.

administration and the fast immunomodulatory effects of MSCs can lead to long-term therapeutic effects.^{21,36}

WI induced significant renal injury, but no therapeutic effects of MSCs were detected regarding the read-outs of renal function, injury, inflammation, or fibrosis. On the contrary, we did not observe any reduced survival or worse outcomes with this targeted MSC treatment. We did not detect any systemic adverse effects such as anaphylactic shock or lung embolisms or side effects, which suggests that intraarterial infusion of MSC in the used dose is safe.⁹

The developed animal model mimicked the clinical transplantation situation in regard to main features of the surgical procedure, preservation fluid, and storage duration of 16 hours of cold ischemia.³⁷ The 75 minutes of WI was higher than the average clinical WI in DCD donors.³⁸ To mimic the IRI in DCD, other studies have varied durations of WI, often 30 minutes.^{39–42} As remarked by Darius et al,³⁹ their studied kidneys (of 30 min WI) had superior quality compared to clinical extended deceased donor kidneys, and a given intervention effect might be even more beneficial in kidneys with greater injury. The pigs studied were younger and more healthy than most human DCD donors with better healing potential. Like in other

studies,^{43–46} we intended to cure serious IRI with a maximum of translationality.²⁷

Similar to the present study, several preclinical and clinical studies make use of allogeneic MSCs.⁴⁷ Our choice was based on the easier application of off-the-shelf allogeneic MSC compared to autologous MSC in the situation of deceased organ transplantation and the fact that kidney recipients' age and illness make their MSCs more difficult to isolate in sufficient numbers and quality.^{47,48} Most studies report that administration of allogeneic MSCs is safe;^{49–52} however, a few studies suggest that allogeneic MSCs can cause undesired reactions, such as the induction of antibodies.^{53–55} Interestingly, in a study of local administered allogeneic MSCs to Crohn's disease patients with fistulas, Avivar-Valderas et al⁵⁶ could not correlate the observed donor-specific antibodies with the MSC therapeutic efficacy. As the impact is uncertain, additional analysis of immunological cell infiltration at the site of MSC homing, and measurements of alloantibodies in MSC recipients is of future relevance in transplantation.

In recent years, novel data on the mechanism of MSC action revealed that in contrast to previous belief administered MSCs do not engraft long term.⁴⁷ Instead, MSCs have a brief presence during which they mediate

immunomodulation and regeneration effects by mechanisms dependent on direct cell-cell contact and via soluble factors produced by MSC,^{57,58} through which they modulate a wide range of immune and progenitor cells.^{57,58} On the basis of these mechanisms, we expected differences in the inflammatory and regenerative responses as well as fibrosis, after kidney transplantation upon MSC treatment, between Tx-MSC and Tx-controls, which we did not find. The fact that MSC delivery was successful indicates that the functionality of the administered cells was not sufficient to induce therapeutic effects. Improvement of the properties of the cells, such as by priming by for instance by tumor necrosis factor- α , which stimulates the migration of MSC across the endothelium might result in a more efficient repair of IRI.⁵⁹

Renal fibrosis is a crucial chronic and time-dependent process in transplantation, caused by the excessive accumulation of connective tissue, mostly collagen. Neither fibrosis nor the biomarker cascades towards fibrosis were different between the transplanted groups. Since follow-up of 14 days could be too short to generate detectable differences in fibrosis development, we examined mRNA expression of a set of fibrosis-related genes. Collagen content and profibrotic gene expression increased significantly after IRI, suggesting that longer follow-up could give perceptive information on chronic treatment effects.

The lack of functional or histological improvements as well as immunomodulatory effects as a result of MSC treatment in our study of homogenous large animals with renal grafts exposed to severe ischemia before transplantation is in contrast to some published studies reporting positive MSC effects on IRI.^{6,60} Several factors could be responsible for this difference, such as MSC source and production methods, MSC activity and dose and also recipient-related factors.^{61,62} Our choice of MSC dose was based on our in vivo safety study conducted, which showed adverse effects of a higher dose of MSCs (unpublished data) and a human study, which had established safety of similar dose with potential therapeutic effect.⁹ Still, testing only 1 dose a limitation of our study that can be seen as a phase I preclinical trial showing feasibility and short-term safety.

Our biomarkers returned to nearly normal levels during follow-up and there were no substantial pathological tissue alterations after 14 days. The fairly young experimental animals used in this study might have had great endogenous renal regeneration capacity, an issue also raised in other studies.^{40,45,46,63-65} In addition, the compensatory perfusion and function of the remaining kidney after contralateral nephrectomy may also have affected the results in our experimental model.⁶⁶ Overcoming this phenomenon and achieving substantial fibrosis development in the experimental model might be possible with follow-up beyond 14 days posttransplant utilizing an adult minipig model.

In conclusion, the intraarterial administration of allogeneic MSCs to donor kidneys before transplantation is safe and feasible, enabling its use in kidney transplantation with deceased donor organs. In our experimental setting, the administration of 10 million allogeneic adipose tissue-derived MSCs did not result in beneficial effects on the damaged kidneys. Yet, our findings do not eliminate the possibility that similar therapy with enhanced MSC or different cell types or numbers may initiate regeneration after IRI in kidney transplantation.

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