

Title

Ex-vivo administration of mesenchymal stromal cells in kidney grafts against ischemia reperfusion injury – effective delivery without kidney function improvement posttransplant

Short title

Ex-vivo cellular therapy of kidney grafts prior to transplantation

Author names and affiliations

Stine Lohmann^{a,b,*}, MD, PhD

Marco Eijken^{b,c}, PhD

Ulla Møldrup^d, MD

Bjarne K. Møller^{a,c}, MD, Ass. Professor

James Hunter^e, MD, Bsc(hons), MBChB

Cyril Moers^f, MD, PhD

Henri Leuvenink^f, PhD, Professor

Rutger J. Ploeg^e, MD, PhD, Professor

Marian C. Clahsen-van Groningen^g, MD, PhD

Martin Hoogduijn^h, PhD

Carla C. Baan^h, PhD, Professor

Anna Krarup Keller^{a,b,d}, MD, PhD

Bente Jespersen^{a,b}, MD, DMSc, Professor

- a) Department of Clinical Medicine, Aarhus University, Aarhus, Denmark
- b) Department of Renal Medicine, Aarhus University Hospital, Aarhus, Denmark.
- c) Department of Clinical Immunology, Aarhus University Hospital, Aarhus, Denmark.
- d) Department of Urology, Aarhus University Hospital, Aarhus, Denmark.

- e) Nuffield Department of Surgical Sciences, Oxford Biomedical Research Centre, University of Oxford, Oxford, United Kingdom.
- f) Department of Surgery–Organ Donation and Transplantation, University of Medical Center Groningen, Groningen, the Netherlands.
- g) Department of Pathology, Erasmus MC, University Medical Center, Rotterdam, the Netherlands
- h) Department of Internal Medicine, Nephrology and Transplantation, Erasmus MC, University Medical Center, Rotterdam, the Netherlands.

Corresponding author

Bente Jespersen. Department of Renal Medicine, Aarhus University, Palle Juul-Jensens Boulevard 99, 8200 Aarhus N, Denmark.

Tel.: +0045 78452411/40896664. E-mail address: bente.jespersen@clin.au.dk

Author contributions

Research design: Lohmann, Eijken, Keller, Møldrup, Møller, Hunter, Moers, Leuvenink, Ploeg, Hoogduijn, Baan, Jespersen.

Performance of the research: Lohmann, Eijken, Keller, Møldrup

Analysis and interpretation of data: Lohmann, Eijken, Keller, Møldrup, Møller, Hunter, Moers, Leuvenink, Ploeg, Clahsen-van Groningen, Hoogduijn, Baan, Jespersen.

Drafting of manuscript: Lohmann, Eijken, Keller, Jespersen.

Critical revision: Lohmann, Eijken, Jespersen, Keller, Hunter, Moers, Leuvenink, Ploeg, Hoogduijn, Baan.

Funding information

This work was supported by The Lundbeck Foundation. Grant number: R198-2015-184.

The Lundbeck Foundation had no role in the study design; in the collection, analysis or interpretation of data; in the writing of the report or the decision to submit the article for publication.

Disclosure

The authors report no proprietary or commercial interest in any product mentioned, concept discussed or personal relationships with other people or organizations that could influence their work and conclusions in this article.

Data availability statement

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

Abbreviations

CD	Cluster of differentiation
DCD	Donation after circulatory death
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ENRAGE	Extracellular newly identified RAGE-binding protein
FFPE	Formalin-fixed paraffin-embedded
GADPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFR	Glomerular filtration rate
HGF	Hepatocyte growth factor
IL	Interleukin
IRI	Ischemia-reperfusion injury
MSC	Mesenchymal stromal cells
MSR	Male specific repeat
NGAL	Neutrophil gelatinase associated lipocalin
PBS	Phosphate-buffered saline
PLAU	Plasminogen activator, urokinase
qPCR	quantitative polymerase chain reaction
SD	Standard deviation
TGF- β	Transforming growth factor beta
MAG3	Mercaptoacetyltriglycine
UW	University of Wisconsin
VEGF	Vascular endothelial growth factor
WI	Warm ischemia

Abstract

Background: Mesenchymal stromal cell (MSC) therapy may improve renal function after ischemia-reperfusion injury in transplantation. Ex-vivo renal intra-arterial 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 min of warm ischemia and sixteen hrs of static cold storage were intra-arterially infused ex-vivo with ten 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 fourteen days.

Results: Postoperatively, peak p-creatinine was 1230 and 1274 $\mu\text{mol/L}$ (Tx-controls vs Tx-MSC, $p=0.69$). During follow up, no significant differences over time were detected between groups regarding p-creatinine, p-NGAL, or u-NGAL/creatinine ratio. At day fourteen 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.

In conclusion, we demonstrated intra-arterial 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 fourteen days of observation.

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 USA.^{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 non-function.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 administration with a very rapid clearance within 24 hrs.^{23,24}

Local treatment such as intra-arterial infusion in the kidney may offer improved availability of MSCs to the site of injury²⁵ and could potentially be more effective and safer than intravenous-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 two-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 intra-arterial MSC delivery is complex in the fragile postoperative phase, while an ex-vivo route of administration opens the way for cell treatment of grafts prior to transplantation. The aims of this study were to examine the feasibility of a novel delivery method of ex-

vivo intra-arterial MSC infusion prior to 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 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 intra-arterial administration of MSCs to vehicle controls in a blinded, randomized design. Following renal warm ischemia of 75 min and sixteen hrs of static cold storage (SCS) in total, grafts were randomized to controlled intra-arterial infusion of ten 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 fourteen 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 group²⁷. Briefly, after induction of anesthesia with an intravenous (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 day one to four, intramuscular buprenorphine was administrated three times a day. In sterile settings, a permanent semi-central venous catheter (5 Fr, 20cm, Careflow, BD, NJ, USA) was placed, and after midline incision, left nephrectomy was performed retroperitoneally and the kidney exposed to 75 min of warm ischemia (Figure 1A). Afterwards, the graft was flushed with cold Belzer UW

(Bridge to Life, London, UK) and put on cold storage at 4°C for the following sixteen hrs. The midline incision was closed and the pig returned to the housing facility after extubation.

Next day, intra-arterial 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 one hour after reperfusion. The midline incision was closed and the pig returned to the housing facility for fourteen days of observation with periodic blood and urine collection.

On day fourteen, 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-MAG3. Lastly, graftectomy was performed and the pigs were terminated under general anesthesia.

Healthy kidney tissue

This study did not include a control group without ischemic injury. In order to compare the protein and gene expression results at study end to non-injured kidneys we included six 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 (150mL/hr). After fourteen days observation, pigs underwent GFR measurement (⁵¹Chrom-EDTA clearance) and bilateral nephrectomy. The left healthy kidneys were included in this study.

Tissue Collection

On day fourteen, nephrectomy was performed and the kidney coronally sliced. For histology, tissue samples containing both medulla and cortex from upper and lower pole 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 pole 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).

Blood and urine collection

Blood collection was carried out using the central venous catheter at baseline (day minus one), 1 hour after reperfusion and on posttransplant day one to seven, nine and fourteen. Urine was collected in an ostomy

bag placed around external genitals of the pig at day two, four and seven, for which the animal did not have to be restrained²⁷. At day minus one and fourteen, urine was collected directly from the graft ureter peroperatively.

Neutrophil Gelatinase Associated Lipocalin (NGAL)

At baseline, day two, four, seven and fourteen plasma and urine NGAL were determined by the pig NGAL sandwich ELISA kit (Kit 044, BioPorto, Hellerup, Denmark). The assay was set up according to manufacturer's protocol.

MSC expansion, characterization and preparation

Porcine adipose-tissue derived MSCs from a total of three male donors were isolated, expanded (in minimum essential medium supplemented with 15% FBS) and characterized as described previously²⁸. After expansion, cells were cryopreserved at passage three. 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. Prior to MSC infusion ten million cryopreserved MSCs were thawed and washed in culture medium by centrifugation (440 g, 5 min). Cell pellets were resuspended in 50 mL cold UW solution, filtered through a 70µm 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 prior to transplantation

Approximately ten min 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 10mL/min.

Kidney tissue dissociation

In total, 0.5 g kidney tissue was dissociated using a GentleMACS dissociator (Miltenyi Biotec) as described previously²⁸. After dissociation, cells were pelleted by centrifugation and resuspended in three mL culture medium, used immediately for analysis, or cryo-preserved.

Fluorescent MSC tracing

MSCs were labelled 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 previously²⁸.

Male MSC detection

Cellular DNA, collected an hour after reperfusion and at day fourteen, was isolated from ten mg tissue or cell pellets using a NucleoSpin Tissue DNA isolation kit (Macherey-Nagel) according to the manufacturer's protocol. Cell free DNA from one mL plasma was isolated using a QIAasympphony and a QIAasympphony DSP Virus/Pathogen Kit (Quiagen) according to the manufacturer's protocol. Y-chromosome DNA was detected by qPCR using primers for the male specific repeat (MSR) located on the porcine Y-chromosome.²⁹ The porcine S100C gene was used as pig DNA control. Primer sequences are listed in Supplemental Table 1. The qPCR mix consisted of 0.5 μ L (cellular) or 5 μ L DNA (cell free), ten pmol of each primer and 1x 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

500 μ L cryo-preserved dissociated tissue from day fourteen was thawed and seeded in a T75 culture flask (Nunc) Δ surface; Thermo Scientific) with ten mL MSC medium and incubated for one day at 37°C and 5% CO₂. Next day, the culture medium was fully replaced and cells were incubated for four hrs. Before trypsinisation, cells were washed twice with PBS (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 hrs 4% formalin fixation samples, collected at study end, were stored in PBS, dehydrated, and next embedded in paraffin (FFPE). Two- μ m sections of FFPE were stained with hematoxylin and eosin, Periodic acid–Schiff–diastase (PAS), and Sirius Red according to standard diagnostic practice. Slides were analyzed by a renal pathologist (M.C-vG), 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, one 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) was used according to the manufacturer's protocol. In each kidney collected at study end, average collagen content was calculated by averaging the levels from three tissue samples.

TGFβ, IL-6 and IL-10 levels

One mL of RIPA Lysis and Extraction Buffer (ThermoFisher) supplemented with PIERCE protease inhibitor tablets (ThermoFisher) was added to ten mg of cortical kidney tissue from day fourteen. 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. IL-10 was measured using a porcine IL10 ELISA (Thermo Fisher, KSC0101) 4000x diluted, IL-6 was measured using a porcine IL6 ELISA (Thermo Fisher, ESIL6) 10x diluted, and human TGFβ was measured using a TGFβ cytoset (Thermo Fisher, CHC1683) 100x diluted. Levels for each kidney were calculated by averaging the levels from three tissue samples.

Gene expression analysis

RNA from approximately 20 mg tissue collected at day fourteen was isolated using a GeneJET RNA purification kit (ThermoFisher). In total, 1µg 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 µM primers and an Applied Biosystems 7300 Real-Time PCR machine. Primer sequences are listed in Supplemental Table 1. Gene expression was presented relative to the housekeeping gene Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) using the following formula $2^{-(Ct \text{ value GAPDH} - Ct \text{ value gene of interest})} \times 1000$. mRNA levels for each kidney were calculated by averaging the levels from three tissue samples.

Statistics

STATA software version 15.1 (StataCorp, Texas, USA) was used for statistical analysis and SigmaPlot 13 (Systat Software, San Jose, CA, US) used for graphical presentation. Results are presented as means with standard deviation (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 analysis of variance (ANOVA) with time and group as fixed factors and pig as random factor. If unequal standard deviations and correlations in the two groups occurred, the factors were taken into account in the analysis. Model validation was performed afterwards by comparing observed and expected within subject standard deviations and correlations. On categorical variables, Fisher's exact tests were applied. A p-value <0.05 was considered to indicate statistical significance.

Results

Ex-vivo renal intra-arterial MSC infusion

Prior to this autotransplantation study, the efficiency of the MSC administration procedure was investigated by infusing fluorescently pre-labelled male MSCs into porcine kidneys ex-vivo. Porcine kidneys were exposed to 75 min warm ischemia and sixteen hrs of static cold storage followed by slow infusion of ten million MSCs in the renal artery. Analyzing the collected renal venous outflow during MSC administration showed that of the ten 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 sub-localization in the glomeruli (see Figure 2D).

Animal characteristics and peroperative data

In total, sixteen female pigs (n=8 per group) were randomized to ex-vivo administration of ten million MSCs or vehicle. One pig (Tx-MSC) was terminated on day four due to uremic symptoms with primary non-function. Furthermore, one pig (Tx-MSC) had cardiac arrest during general anesthesia on day fourteen.

Autopsy determined that it was not related to the kidney. Data from these two pigs until their death was included. Both groups had a constant mean arterial blood pressure >60 mmHg 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 one 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 fourteen days of follow-up, MSCs could still be detected in kidney tissue; however, Y-chromosome levels were on average less than 5% compared to the levels in the one-hour biopsies, $p=0.002$ (Figure 3B). MSC clearance during the first two 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 fourteen, the plastic adherent fraction of dissociated kidney tissue was analyzed for the presence of Y-chromosome DNA. This confirmed indeed that kidneys at day fourteen still contained viable male MSCs (Figure 3D).

Renal function and injury

Due to the chosen model, all animals had impaired renal function postoperatively, resulting in anuria until day two. Figure 4A shows that peak levels of p-creatinine were reached on day four with a mean of $1230 \pm 178 \mu\text{mol/L}$ (Tx-control) and $1274 \pm 245 \mu\text{mol/L}$ (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 p-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 (Supplemental Figure S1).

NGAL, an early marker of acute renal injury, was measured in plasma and urine at baseline, postoperative day two, four, seven and fourteen. P-NGAL and u-NGAL/creatinine ratios remained significantly elevated throughout follow up in both groups vs. baseline levels (all $p\text{-values} \leq 0.009$) (Figure 5A and 5B). 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. U-protein/creatinine ratio was also without difference between groups during the follow up, $p=0.14$ (Figure 5C).

GFR, measured at day fourteen as urinary clearance of $^{51}\text{Chrom-EDTA}$, at day fourteen was 40 ± 9 mL/min (Tx-control) and 44 ± 17 mL/min (Tx-MSD), $p=0.66$ (Figure 5D). No statistically significant difference was detected in concurrent measurements of effective renal plasma flow (urinary clearance of Tc-MAG3) resulting in a mean of 133 ± 35 mL/min (Tx-control) and 146 ± 36 mL/min (Tx-MSD), $p=0.49$ (Figure 5D).

Histology

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

Collagen and fibrosis markers

To examine potential initiation of fibrosis at day fourteen, 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-MSD, $p=0.031$). The difference in total collagen content between the Tx-control and Tx-MSD was not significant, $p=0.14$ (Figure 7A). At the mRNA level, collagen1a1, collagen3a1 and fibronectin significantly increased in the Tx-control kidneys vs. healthy kidneys. However, MSD 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 (VEGF) in renal cortex tissue demonstrated significantly lower levels in Tx-control and Tx-MSD compared to healthy controls but there was no difference between the two transplanted groups (Figure 8A). Expression of hepatocyte growth factor (HGF) mRNA was equal in the three groups (Figure 8B).

Analysis of an mRNA panel of inflammation related genes in renal cortex tissue demonstrated equal mRNA expression of ENRAGE, IL-8, IL-10, and PLA2 in healthy control kidneys, Tx-controls and Tx-MSD 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 intra-arterial 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 fourteen days posttransplant.

MSCs infused prior to 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 group³¹. We proved that the remaining MSCs in the kidney after fourteen days were viable, suggesting that low amounts of MSCs can continue to modulate their microenvironment in the transplant kidney. Plasma analysis 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,³²⁻³⁶ 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 administration and the fast immunomodulatory effects of MSCs can lead to long-term therapeutic effects.^{21,37}

Warm ischemia 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 other hand, 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 intra-arterial infusion of MSC in the used dose is safe⁹.

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 hrs of cold ischemia.³⁸ The 75 min of WI was higher than the average clinical WI in DCD donors.³⁹ To mimic the IRI in DCD other studies have varied durations of WI, often 30 min.⁴⁰⁻⁴³ As remarked by Darius et al., 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.⁴⁰ The pigs studied were younger and more healthy than most human DCD donors with better healing potential. Like in other studies,⁴⁴⁻⁴⁷ 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.⁴⁸ 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.^{48,49} Most studies report that administration of allogeneic MSCs is safe,⁵⁰⁻⁵³ however, a few studies suggest that allogeneic MSCs can cause undesired reactions, such as the induction of antibodies.⁵⁴⁻⁵⁶ Interestingly, in a study of local administrated allogeneic MSCs to Crohn's disease patients with fistulas, Avivar-Valderas et al. could not correlate the observed donor-specific antibodies with the MSC therapeutic efficacy.⁵⁷ As the impact is uncertain, additional analysis of immunological cell infiltration at the site of MSC homing, and measurements of allo-antibodies 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.⁴⁸ 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,^{58,59} through which they modulate a wide range of immune and progenitor cells.^{58,59} 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 TNF- α , which stimulates the- migration of MSC across the endothelium might result in a more efficient repair of IRI⁶⁰.

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 fourteen 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.^{6,61} Several factors could be responsible for this difference, such as MSC source and production methods, MSC activity and dose and also recipient-related factors.^{62,63} 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 one 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 fourteen 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.^{41,46,47,64-66} In addition, the compensatory perfusion and function of the remaining kidney after contralateral nephrectomy may also have affected the results in our experimental model.⁶⁷ Overcoming this phenomenon and achieving substantial fibrosis development in the experimental model might be possible with follow-up beyond fourteen days posttransplant utilizing an adult minipig model.

In conclusion, the intra-arterial 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 ten 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.

References

1. NHS. NHS Blood and Transplant Organ Donation and Transplantation website. Annual Activity Report 2016/2017. https://nhsbtdbe.blob.core.windows.net/umbraco-assets-corp/4657/activity_report_2016_17.pdf. Accessed July 1st, 2018. Accessed.
2. Hart A, Smith JM, Skeans MA, et al. OPTN/SRTR 2016 Annual Data Report: Kidney. *Am J Transplant*. 2018;18 Suppl 1:18-113 <http://dx.doi.org/doi:10.1111/ajt.14557>
3. Baraniak PR, McDevitt TC. Stem cell paracrine actions and tissue regeneration. *Regen Med*. 2010;5(1):121-143 <http://dx.doi.org/doi:10.2217/rme.09.74>
4. Erpicum P, Detry O, Weekers L, et al. Mesenchymal stromal cell therapy in conditions of renal ischaemia/reperfusion. *Nephrol Dial Transplant*. 2014;29(8):1487-1493 <http://dx.doi.org/doi:10.1093/ndt/gft538>
5. Reinders MEJ, van Kooten C, Rabelink TJ, et al. Mesenchymal Stromal Cell Therapy for Solid Organ Transplantation. *Transplantation*. 2018;102(1):35-43 <http://dx.doi.org/doi:10.1097/tp.0000000000001879>
6. Rowat P, Erpicum P, Detry O, et al. Mesenchymal Stromal Cell Therapy in Ischemia/Reperfusion Injury. *J Immunol Res*. 2015;2015:602597 <http://dx.doi.org/doi:10.1155/2015/602597>
7. Souidi N, Stolk M, Seifert M. Ischemia-reperfusion injury: beneficial effects of mesenchymal stromal cells. *Curr Opin Organ Transplant*. 2013;18(1):34-43 <http://dx.doi.org/doi:10.1097/MOT.0b013e32835c2a05>
8. Wise AF, Ricardo SD. Mesenchymal stem cells in kidney inflammation and repair. *Nephrology (Carlton)*. 2012;17(1):1-10 <http://dx.doi.org/doi:10.1111/j.1440-1797.2011.01501.x>
9. Saad A, Dietz AB, Herrmann SMS, et al. Autologous Mesenchymal Stem Cells Increase Cortical Perfusion in Renovascular Disease. *J Am Soc Nephrol*. 2017;28(9):2777-2785 <http://dx.doi.org/doi:10.1681/asn.2017020151>
10. Franquesa M, Herrero E, Torras J, et al. Mesenchymal stem cell therapy prevents interstitial fibrosis and tubular atrophy in a rat kidney allograft model. *Stem Cells Dev*. 2012;21(17):3125-3135 <http://dx.doi.org/doi:10.1089/scd.2012.0096>
11. Ohnishi S, Sumiyoshi H, Kitamura S, et al. Mesenchymal stem cells attenuate cardiac fibroblast proliferation and collagen synthesis through paracrine actions. *FEBS Lett*. 2007;581(21):3961-3966 <http://dx.doi.org/doi:10.1016/j.febslet.2007.07.028>
12. Reinders ME, de Fijter JW, Rabelink TJ. Mesenchymal stromal cells to prevent fibrosis in kidney transplantation. *Curr Opin Organ Transplant*. 2014;19(1):54-59 <http://dx.doi.org/doi:10.1097/mot.0000000000000032>
13. Baulier E, Favreau F, Le Corf A, et al. Amniotic fluid-derived mesenchymal stem cells prevent fibrosis and preserve renal function in a preclinical porcine model of kidney transplantation. *Stem Cells Transl Med*. 2014;3(7):809-820 <http://dx.doi.org/doi:10.5966/sctm.2013-0186>
14. Ebrahimi B, Eirin A, Li Z, et al. Mesenchymal stem cells improve medullary inflammation and fibrosis after revascularization of swine atherosclerotic renal artery stenosis. *PLoS One*. 2013;8(7):e67474 <http://dx.doi.org/doi:10.1371/journal.pone.0067474>
15. Perico N, Casiraghi F, Gotti E, et al. Mesenchymal stromal cells and kidney transplantation: pretransplant infusion protects from graft dysfunction while fostering immunoregulation. *Transpl Int*. 2013;26(9):867-878 <http://dx.doi.org/doi:10.1111/tri.12132>
16. Perico N, Casiraghi F, Inrona M, et al. Autologous mesenchymal stromal cells and kidney transplantation: a pilot study of safety and clinical feasibility. *Clin J Am Soc Nephrol*. 2011;6(2):412-422 <http://dx.doi.org/doi:10.2215/cjn.04950610>
17. Reinders ME, de Fijter JW, Roelofs H, et al. Autologous bone marrow-derived mesenchymal stromal cells for the treatment of allograft rejection after renal transplantation: results of a phase I study. *Stem Cells Transl Med*. 2013;2(2):107-111 <http://dx.doi.org/doi:10.5966/sctm.2012-0114>
18. Tan J, Wu W, Xu X, et al. Induction therapy with autologous mesenchymal stem cells in living-related kidney transplants: a randomized controlled trial. *Jama*. 2012;307(11):1169-1177 <http://dx.doi.org/doi:10.1001/jama.2012.316>
19. Mudrabettu C, Kumar V, Rakha A, et al. Safety and efficacy of autologous mesenchymal stromal cells transplantation in patients undergoing living donor kidney transplantation: a pilot study. *Nephrology (Carlton)*. 2015;20(1):25-33 <http://dx.doi.org/doi:10.1111/nep.12338>

20. Assis AC, Carvalho JL, Jacoby BA, et al. Time-dependent migration of systemically delivered bone marrow mesenchymal stem cells to the infarcted heart. *Cell Transplant*. 2010;19(2):219-230
<http://dx.doi.org/doi:10.3727/096368909x479677>
21. Eggenhofer E, Luk F, Dahlke MH, et al. The life and fate of mesenchymal stem cells. *Front Immunol*. 2014;5:148 <http://dx.doi.org/doi:10.3389/fimmu.2014.00148>
22. Fischer UM, Harting MT, Jimenez F, et al. Pulmonary passage is a major obstacle for intravenous stem cell delivery: the pulmonary first-pass effect. *Stem Cells Dev*. 2009;18(5):683-692
<http://dx.doi.org/doi:10.1089/scd.2008.0253>
23. de Witte SFH, Luk F, Sierra Parraga JM, et al. Immunomodulation By Therapeutic Mesenchymal Stromal Cells (MSC) Is Triggered Through Phagocytosis of MSC By Monocytic Cells. *Stem Cells*. 2018;36(4):602-615
<http://dx.doi.org/doi:10.1002/stem.2779>
24. Eggenhofer E, Benseler V, Kroemer A, et al. Mesenchymal stem cells are short-lived and do not migrate beyond the lungs after intravenous infusion. *Front Immunol*. 2012;3:297
<http://dx.doi.org/doi:10.3389/fimmu.2012.00297>
25. Zonta S, De Martino M, Bedino G, et al. Which is the most suitable and effective route of administration for mesenchymal stem cell-based immunomodulation therapy in experimental kidney transplantation: endovenous or arterial? *Transplant Proc*. 2010;42(4):1336-1340
<http://dx.doi.org/doi:10.1016/j.transproceed.2010.03.081>
26. Eirin A, Zhu XY, Ferguson CM, et al. Intra-renal delivery of mesenchymal stem cells attenuates myocardial injury after reversal of hypertension in porcine renovascular disease. *Stem Cell Res Ther*. 2015;6:7
<http://dx.doi.org/doi:10.1186/scrt541>
27. Lohmann S, Eijken M, Moldrup U, et al. A Pilot Study of Postoperative Animal Welfare as a Guidance Tool in the Development of a Kidney Autotransplantation Model With Extended Warm Ischemia. *Transplant Direct*. 2019;5(11):e495 <http://dx.doi.org/doi:10.1097/txd.0000000000000941>
28. Sierra-Parraga JM, Munk A, Andersen C, et al. Mesenchymal Stromal Cells Are Retained in the Porcine Renal Cortex Independently of Their Metabolic State After Renal Intra-Arterial Infusion. *Stem Cells Dev*. 2019;28(18):1224-1235 <http://dx.doi.org/doi:10.1089/scd.2019.0105>
29. Gruessner RW, Levay-Young BK, Nakhleh RE, et al. Portal donor-specific blood transfusion and mycophenolate mofetil allow steroid avoidance and tacrolimus dose reduction with sustained levels of chimerism in a pig model of intestinal transplantation. *Transplantation*. 2004;77(10):1500-1506
<http://dx.doi.org/doi:10.1097/01.tp.0000128298.12937.b2>
30. Pool M, Eertman T, Sierra Parraga J, et al. Infusing Mesenchymal Stromal Cells into Porcine Kidneys during Normothermic Machine Perfusion: Intact MSCs Can Be Traced and Localised to Glomeruli. *Int J Mol Sci*. 2019;20(14) <http://dx.doi.org/doi:10.3390/ijms20143607>
31. Sierra-Parraga JM, Munk A, Schmidt Andersen C, et al. Mesenchymal stromal cells are retained in the renal cortex independently of their metabolic state after renal intra-arterial infusion. *Stem Cells Dev*. 2019
<http://dx.doi.org/doi:10.1089/scd.2019.0105>
32. Yao W, Mei C, Nan X, et al. Evaluation and comparison of in vitro degradation kinetics of DNA in serum, urine and saliva: A qualitative study. *Gene*. 2016;590(1):142-148 <http://dx.doi.org/doi:10.1016/j.gene.2016.06.033>
33. To EW, Chan KC, Leung SF, et al. Rapid clearance of plasma Epstein-Barr virus DNA after surgical treatment of nasopharyngeal carcinoma. *Clin Cancer Res*. 2003;9(9):3254-3259. Published 2003/09/10.
34. Khier S, Lohan L. Kinetics of circulating cell-free DNA for biomedical applications: critical appraisal of the literature. *Future Science OA*. 2018;4:FSO295 <http://dx.doi.org/doi:10.4155/fsoa-2017-0140>
35. Diehl F, Schmidt K, Choti MA, et al. Circulating mutant DNA to assess tumor dynamics. *Nat Med*. 2008;14(9):985-990 <http://dx.doi.org/doi:10.1038/nm.1789>
36. Celec P, Vlkova B, Laukova L, et al. Cell-free DNA: the role in pathophysiology and as a biomarker in kidney diseases. *Expert Rev Mol Med*. 2018;20:e1 <http://dx.doi.org/doi:10.1017/erm.2017.12>
37. Luk F, de Witte SFH, Korevaar SS, et al. Inactivated Mesenchymal Stem Cells Maintain Immunomodulatory Capacity. *Stem Cells and Development*. 2016;25(18):1342-1354 <http://dx.doi.org/doi:10.1089/scd.2016.0068>
38. Summers DM, Johnson RJ, Hudson A, et al. Effect of donor age and cold storage time on outcome in recipients of kidneys donated after circulatory death in the UK: a cohort study. *Lancet*. 2013;381(9868):727-734 [http://dx.doi.org/doi:10.1016/s0140-6736\(12\)61685-7](http://dx.doi.org/doi:10.1016/s0140-6736(12)61685-7)
39. Scalea JR, Redfield RR, Arpali E, et al. Does DCD Donor Time-to-Death Affect Recipient Outcomes? Implications of Time-to-Death at a High-Volume Center in the United States. *Am J Transplant*. 2017;17(1):191-200 <http://dx.doi.org/doi:10.1111/ajt.13948>

40. Darius T, Gianello P, Vergauwen M, et al. The effect on early renal function of various dynamic preservation strategies in a preclinical pig ischemia-reperfusion autotransplant model. *Am J Transplant*. 2019;19(3):752-762 <http://dx.doi.org/doi:10.1111/ajt.15100>
41. Kathis JM, Cen JY, Chun YM, et al. Continuous Normothermic Ex Vivo Kidney Perfusion Is Superior to Brief Normothermic Perfusion Following Static Cold Storage in Donation After Circulatory Death Pig Kidney Transplantation. *Am J Transplant*. 2017;17(4):957-969 <http://dx.doi.org/doi:10.1111/ajt.14059>
42. Kerforne T, Allain G, Giraud S, et al. Defining the optimal duration for normothermic regional perfusion in the kidney donor: A porcine preclinical study. *Am J Transplant*. 2019;19(3):737-751 <http://dx.doi.org/doi:10.1111/ajt.15063>
43. Urbanellis P, Hamar M, Kathis JM, et al. Normothermic ex-vivo kidney perfusion improves early DCD graft function compared to hypothermic machine perfusion and static cold storage. *Transplantation*. 2019 <http://dx.doi.org/doi:10.1097/tp.0000000000003066>
44. Blum MF, Liu Q, Soliman B, et al. Comparison of normothermic and hypothermic perfusion in porcine kidneys donated after cardiac death. *J Surg Res*. 2017;216:35-45 <http://dx.doi.org/doi:10.1016/j.jss.2017.04.008>
45. Demos DS, Iyengar A, Bryner BS, et al. Successful Porcine Renal Transplantation After 60 Minutes of Donor Warm Ischemia: Extracorporeal Perfusion and Thrombolytics. *Asaio j*. 2015;61(4):474-479 <http://dx.doi.org/doi:10.1097/mat.0000000000000228>
46. Snoeijs MG, Matthijsen RA, Seeldrayers S, et al. Autologous transplantation of ischemically injured kidneys in pigs. *J Surg Res*. 2011;171(2):844-850 <http://dx.doi.org/doi:10.1016/j.jss.2010.05.064>
47. Tillet S, Giraud S, Delpech PO, et al. Kidney graft outcome using an anti-Xa therapeutic strategy in an experimental model of severe ischaemia-reperfusion injury. *Br J Surg*. 2015;102(1):132-142; discussion 142 <http://dx.doi.org/doi:10.1002/bjs.9662>
48. Pittenger MF, Discher DE, Péault BM, et al. Mesenchymal stem cell perspective: cell biology to clinical progress. *npj Regenerative Medicine*. 2019;4(1):22 <http://dx.doi.org/doi:10.1038/s41536-019-0083-6>
49. Stolzinger A, Jones E, McGonagle D, et al. Age-related changes in human bone marrow-derived mesenchymal stem cells: consequences for cell therapies. *Mech Ageing Dev*. 2008;129(3):163-173 <http://dx.doi.org/doi:10.1016/j.mad.2007.12.002>
50. Erpicum P, Weekers L, Detry O, et al. Infusion of third-party mesenchymal stromal cells after kidney transplantation: a phase I-II, open-label, clinical study. *Kidney Int*. 2019;95(3):693-707 <http://dx.doi.org/doi:10.1016/j.kint.2018.08.046>
51. Hoogduijn MJ, Lombardo E. Mesenchymal Stromal Cells Anno 2019: Dawn of the Therapeutic Era? Concise Review. *Stem Cells Transl Med*. 2019;8(11):1126-1134 <http://dx.doi.org/doi:10.1002/sctm.19-0073>
52. Lalu MM, McIntyre L, Pugliese C, et al. Safety of cell therapy with mesenchymal stromal cells (SafeCell): a systematic review and meta-analysis of clinical trials. *PloS one*. 2012;7(10).
53. Perico N, Casiraghi F, Remuzzi G. Clinical Translation of Mesenchymal Stromal Cell Therapies in Nephrology. *J Am Soc Nephrol*. 2018;29(2):362-375 <http://dx.doi.org/doi:10.1681/asn.2017070781>
54. Alagesan S, Griffin MD. Autologous and allogeneic mesenchymal stem cells in organ transplantation: what do we know about their safety and efficacy? *Curr Opin Organ Transplant*. 2014;19(1):65-72 <http://dx.doi.org/doi:10.1097/mot.0000000000000043>
55. Ankrum JA, Ong JF, Karp JM. Mesenchymal stem cells: immune evasive, not immune privileged. *Nature Biotechnology*. 2014;32:252 <http://dx.doi.org/doi:10.1038/nbt.2816>
56. Griffin MD, Ryan AE, Alagesan S, et al. Anti-donor immune responses elicited by allogeneic mesenchymal stem cells: what have we learned so far? *Immunol Cell Biol*. 2013;91(1):40-51 <http://dx.doi.org/doi:10.1038/icb.2012.67>
57. Avivar-Valderas A, Martin-Martin C, Ramirez C, et al. Dissecting Allo-Sensitization After Local Administration of Human Allogeneic Adipose Mesenchymal Stem Cells in Perianal Fistulas of Crohn's Disease Patients. *Front Immunol*. 2019;10:1244 <http://dx.doi.org/doi:10.3389/fimmu.2019.01244>
58. Naji A, Eitoku M, Favier B, et al. Biological functions of mesenchymal stem cells and clinical implications. *Cell Mol Life Sci*. 2019;76(17):3323-3348 <http://dx.doi.org/doi:10.1007/s00018-019-03125-1>
59. Weiss ARR, Dahlke MH. Immunomodulation by Mesenchymal Stem Cells (MSCs): Mechanisms of Action of Living, Apoptotic, and Dead MSCs. *Front Immunol*. 2019;10:1191 <http://dx.doi.org/doi:10.3389/fimmu.2019.01191>

60. Teo GS, Ankrum JA, Martinelli R, et al. Mesenchymal stem cells transmigrate between and directly through tumor necrosis factor-alpha-activated endothelial cells via both leukocyte-like and novel mechanisms. *Stem Cells*. 2012;30(11):2472-2486 <http://dx.doi.org/doi:10.1002/stem.1198>
61. de Vries DK, Schaapherder AF, Reinders ME. Mesenchymal stromal cells in renal ischemia/reperfusion injury. *Front Immunol*. 2012;3:162 <http://dx.doi.org/doi:10.3389/fimmu.2012.00162>
62. Caplan AI. Cell-Based Therapies: The Nonresponder. *Stem Cells Transl Med*. 2018;7(11):762-766 <http://dx.doi.org/doi:10.1002/sctm.18-0074>
63. Moll G, Ankrum JA, Kamhieh-Milz J, et al. Intravascular Mesenchymal Stromal/Stem Cell Therapy Product Diversification: Time for New Clinical Guidelines. *Trends Mol Med*. 2019;25(2):149-163 <http://dx.doi.org/doi:10.1016/j.molmed.2018.12.006>
64. Bon D, Billault C, Thuillier R, et al. Analysis of perfusates during hypothermic machine perfusion by NMR spectroscopy: a potential tool for predicting kidney graft outcome. *Transplantation*. 2014;97(8):810-816 <http://dx.doi.org/doi:10.1097/tp.0000000000000046>
65. Rossard L, Favreau F, Giraud S, et al. Role of warm ischemia on innate and adaptive responses in a preclinical renal auto-transplanted porcine model. *J Transl Med*. 2013;11:129 <http://dx.doi.org/doi:10.1186/1479-5876-11-129>
66. Schreinemachers MC, Doorschodt BM, Florquin S, et al. Pulsatile perfusion preservation of warm ischaemia-damaged experimental kidney grafts. *Br J Surg*. 2010;97(3):349-358 <http://dx.doi.org/doi:10.1002/bjs.6879>
67. Kierulf-Lassen C, Nielsen PM, Qi H, et al. Unilateral nephrectomy diminishes ischemic acute kidney injury through enhanced perfusion and reduced pro-inflammatory and pro-fibrotic responses. *PLoS One*. 2017;12(12):e0190009 <http://dx.doi.org/doi:10.1371/journal.pone.0190009>

Table 1

	MSC			Control			p-value
	Obs.	Mean	95% CI	Obs.	Mean	95% CI	
Surgical time, day -1 (hh:mm)	8	02:26	02:12-02:40	8	02:22	02:00-02:44	0.73
Surgical time, day 0 (hh:mm)	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 (hh:mm)	8	16:04	15:54-16:14	8	16:01	15:58-16:03	0.43
Iv fluid, day-1 (L)	8	3.0	2.7-3.2	8	3.0	2.6-3.4	0.83
Iv fluid, day 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, day 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 (x 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
Gamma-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

Table 1: Perioperative data and selected baseline values.

Figure legends

Figure 1: (A) Surgical procedure. Left kidney ischemically injured by 75 min of warm ischemia followed by static cold storage until next day. MSCs were intra-arterially infused shortly before right nephrectomy and end-to-end anastomoses of artery, vein and ureter. (B) MSC administration on the back table at day zero. Suspended ten million MSCs in 50 mL cold UW solution was digitally infused in the renal artery using a blunt cannula with a rate of approximately ten mL/min. MSC, mesenchymal stromal cells; UW, University of Wisconsin.

Figure 2: MSC retention and localization after ex vivo intra-arterial administration of ten million male MSCs. Values presented as mean and standard deviation. (A) Percentage of Qdot-labeled MSCs lost in the venous outflow during MSC administration and the following flush quantified by flowcytometry. (B) Number of Qdot-labeled MSCs in the renal cortex quantified by flowcytometry. 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-labelled MSCs were mainly located in the glomeruli visualised by fluorescent microscopy. MSC, mesenchymal stromal cells; MSR, male specific repeat; qPCR, quantitative polymerase chain reaction.

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, D: Mean, standard deviation and individual levels presented. C: Mean and standard deviation presented. (A) Y-chromosome DNA levels in renal cortex one hour after reperfusion. (B) Y-chromosome DNA levels in renal cortex fourteen days after transplantation, which was significantly lower vs. one hour 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 day fourteen kidney tissue. MSC, mesenchymal stromal cells; MSR, male specific repeat.

Figure 4: Plasma creatinine of transplanted pigs from baseline (day 0) to day fourteen posttransplant. (A) Values presented as mean and standard deviation in $\mu\text{mol/L}$. All pigs had significant increases in p-creatinine from baseline to peak, $p\text{-values} < 0.001$. During the fourteen days of follow-up no significant difference in levels of p-creatinine were detected between groups, $p=0.96$. (B) Individual levels in $\mu\text{mol/L}$ from baseline to postoperative day fourteen. One pig was terminated on day four (Tx-MSC). MSC, mesenchymal stromal cells.

Figure 5: Renal injury and function parameters measured during the fourteen days of follow-up. Results presented as mean and standard deviation. (A) The levels of p-NGAL in $\mu\text{g/L}$ were significantly higher posttransplant vs. baseline in both groups, all $p\text{-values} \leq 0.001$. 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) Urine NGAL/creatinine in $\mu\text{g}/\text{mmol}$ were significantly increased at day two, four and seven vs. baseline in both groups, $p\text{-values} < 0.009$. Comparing the two groups' mean levels throughout follow up, there was no statistical significant differences between groups, $p=0.31$. (C) Urine 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 day fourteen in mL/min . Mean GFR was $40 \pm 9 \text{ mL}/\text{min}$ (Tx-control) and $44 \pm 17 \text{ mL}/\text{min}$ (Tx-MSC), $p=0.66$. Effective renal plasma flow measured at day fourteen. Mean values were similar between groups, $p=0.49$. GFR, glomerular filtration rate; MSC, mesenchymal stromal cells; NGAL, neutrophil gelatinase-associated lipocalin.

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 $p\text{-values} > 0.05$). (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 x20. (3): Magnification x10. MSC, mesenchymal stromal cells.

Figure 7. Renal collagen content and fibrosis related genes. (A) Collagen mass per total protein mass, presented with individual values, mean and standard deviation. 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 standard deviation. Col1A1, Col3A1, and fibronectin all significantly increased in the transplanted Tx-Control kidneys vs. non-ischemic healthy kidneys, $p<0.001$ (Col1A1), $p<0.001$ (Col3A1), $p=0.007$ (Fibronectin). The differences in fibrosis gene expressions were insignificant between the two transplanted kidney groups Tx-control vs. Tx-MS, all p -values >0.05 . GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HGF, hepatocyte growth factor; MSC, mesenchymal stem cells; qPCR, quantitative polymerase chain reaction.

Figure 8. Regenerative genes expression and inflammatory process. mRNA expression in renal day fourteen tissue obtained by qPCR relative to GAPDH. Values presented individually, as well as mean and standard deviation. (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-MS vs. Healthy control). MS treatment did not have effect compared to the Tx-controls, $p=0.520$. (B-F) HGF, ENRAGE, IL-8, IL-10, and PLAU were without significant differences between Tx-control vs. Tx-MS, all p -values >0.05 , and healthy control vs. Tx-control, all p -values >0.05 . GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HGF, hepatocyte growth factor; ILs, interleukin; MS, mesenchymal stem cells; 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 day fourteen renal tissue. The levels are corrected for total protein of the kidney protein extract and presented by individual values, mean and standard deviation in pg/ μ g protein. (A) 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-MS, $p=0.388$. (B) IL-10 levels were significantly lower in the two transplanted groups vs. healthy controls, $*p=0.002$, $**p=0.031$. IL-10 was without statistical difference between Tx-control vs. Tx-MS, $p=0.454$. (C) IL-6 levels were without statistical differences between groups, all p -values >0.05 . ILs, interleukin; MS, mesenchymal stem cells; TGF, transforming growth factor.

Figure S1: The impaired renal function's effect on fluid and electrolyte balance. Values presented as mean and standard deviation. (A) Weight gain postoperatively days with peak gains of 6.9 kg (Tx-control) and 7.1 kg (Tx-MS) ($p=0.81$). (B-F) Hemoglobin and electrolytes from baseline to day fourteen. There was no evidence of different levels between the groups during follow-up, $p=0.67$ (hemoglobin), $p=0.68$ (p-potassium), $p=0.86$ (p-sodium), $p=0.49$ (p-chloride), and $p=0.74$ (p-bicarbonate).