Novel delivery of cellular therapy to reduce ischemia reperfusion injury in kidney transplantation

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Funding information
This study was supported by Kidney Research UK, the National Institute for Health Research (NIHR) Newcastle Biomedical Research Centre and the NIHR Blood and Transplant Research Unit in Organ Donation and Transplantation at the University of Cambridge, in collaboration with Newcastle University and in partnership with National Health Service.

Ex vivo normothermic machine perfusion (NMP) of donor kidneys prior to transplantation provides a platform for direct delivery of cellular therapeutics to optimize organ quality prior to transplantation. Multipotent Adult Progenitor Cells (MAPC®) possess potent immunomodulatory properties that could minimize ischemia reperfusion injury. We investigated the potential capability of MAPC cells in kidney NMP. Pairs (5) of human kidneys, from the same donor, were simultaneously perfused for 7 hours. Kidneys were randomly allocated to receive MAPC treatment or control. Serial samples of perfusate, urine, and tissue biopsies were taken for comparison. MAPC-treated kidneys demonstrated improved urine output ($P = .009$), decreased expression of injury biomarker NGAL ($P = .012$), improved microvascular perfusion on contrast-enhanced ultrasound (cortex $P = .019$, medulla $P = .001$), downregulation of interleukin (IL)-1β ($P = .050$), and upregulation of IL-10 ($P < .047$) and Indolamine-2,3-dioxygenase ($P = .050$). A chemotaxis model demonstrated decreased neutrophil recruitment when stimulated with perfusate from MAPC-treated kidneys ($P < .001$). Immunofluorescence revealed prelabeled MAPC cells in the perivascular space of kidneys during NMP. We report the first successful delivery of cellular therapy to a human kidney during NMP. Kidneys treated with MAPC cells demonstrate improvement in clinically relevant parameters and injury biomarkers. This novel method of cell therapy delivery provides an exciting opportunity to recondition organs prior to transplantation.
１ | INTRODUCTION

The UK kidney transplant waiting list stands at over 5000 patients. To bridge the gap between supply and demand, there has been increased use of donation after circulatory death (DCD) and extended criteria donors (ECD). １ Concerns regarding inferior outcomes from DCD and ECD organs can lead to underutilization of this valuable resource. ２, ３ DCD and ECD kidneys are more susceptible to ischemia reperfusion injury (IRI) manifesting as delayed graft function (DGF) and this can diminish long-term graft survival. ４, ５ IRI is the result of hypoxia followed by restoration of blood flow leading to microvascular dysfunction, inflammation, immune activation, and tissue injury. ６ As the transplant community becomes increasingly reliant on marginal donors, new therapeutic approaches to reduce IRI and optimize utilization of kidneys are leading to a greater focus on improving organ preservation.

Normothermic machine perfusion (NMP) is a method of organ preservation that facilitates restoration of cellular metabolism, re- viving the organ ex vivo to resume normal physiological functions. ７ Over the last few years, a number of NMP techniques and commercially available devices have been adopted into clinical practice for kidney, heart, liver, and lung transplantation. ８– １０ Kidney NMP was first described in 2008. １１ In this system, a pediatric cardiopulmonary bypass machine and membrane oxygenator perfuse an ex vivo kidney with oxygenated red blood cells suspended in crystalloid at 37°C. １２ A UK multicenter phase III randomized controlled trial (RCT) is currently underway investigating its potential to minimize DGF. １３

NMP provides a unique opportunity to deliver organ-directed, reconditioning therapies in an isolated platform to a metabolically active organ. １４ This could be transformative, facilitating direct delivery of cell therapies and reducing off-target, systemic effects in recipients. A recent study described the use of perfusion technology to preserve a liver for up to 1 week, providing a long potential therapeutic window for optimization and reconditioning. １５

Multipotent adult progenitor cells (MAPC) are adult, bone-marrow derived, mesenchymal origin, stromal cells. １６ MAPC cells represent an attractive “off-the-shelf,” nonimmunogenic cell therapy option as they lack major histocompatibility complex (MHC) Class II, or costimulatory molecules (CD80, CD86, and CD40) and have low expression of MHC Class I. １７ MAPC cells release anti-inflammatory, immunomodulatory and pro-tolerogenic cytokines thereby limiting infiltration and proliferation of pathogenic immune cells. １８, １９ In the transplant setting, a rat heterotopic heart transplant model demonstrated allogeneic MAPC cells could successfully replace standard pharmacological immunosuppression to maintain long-term graft survival. ２０

There have also been a number of successful clinical trials harnessing the immunomodulatory potential of MAPC treatment for graft vs host disease, ２１ acute respiratory distress syndrome, ２２ myocardial infarction, ２３ and an ongoing phase III clinical trial in ischemic stroke. ２４ In 2015, the first successful use of allogeneic MAPC therapy in human liver transplantation was also reported. ２５ The recipient’s leucocyte population displayed diminished immune activation and a pro-tolerogenic profile.

MAPCs and mesenchymal stromal cells (MSC) are inherently very similar. MSCs were also originally isolated from adult bone marrow. MSC culture and expansion characteristics differ from MAPCs resulting in phenotypically different populations; however, they maintain a very similar mechanism of action. ２６ There has been a successful RCT investigating autologous MSC therapy vs standard induction immunosuppression (basiliximab) in live donor kidney transplantation. Recipients who received MSC therapy were more likely to recover renal function faster, had decreased rates of rejection, less opportunistic infections, and better renal function at 1 year. ２７

However, this has not translated into widespread use of MAPC/MSC therapy in kidney transplantation. One of the main hurdles preventing widespread translation is successful delivery of the cells to the required organ. ２８ A number of animal models have explored the possibility of delivering a cell therapy during ex vivo NMP. ２９– ３１ To date, there have been no reported studies successfully administering a cell therapy to a human organ during ex vivo perfusion.

Our study aims to investigate the possible benefit of delivering MAPC cell therapy directly to marginal human kidneys in a preclinical NMP model.

２ | MATERIALS AND METHODS

2.1 | Ethics

Local research ethics committee approval for the use of human donor kidneys declined for transplantation was granted (16/NE/0230). Currently ~ 15% of kidneys retrieved from organ donors across the United Kingdom are deemed unsuitable for transplant and can be offered for research purposes. Consent for recruitment was obtained from the donor’s next of kin by specialist nurses in organ donation. Allocation of kidneys to this research project was overseen by National Health Services Blood & Transplant’s Research Innovation and Novel Technologies Advisory Group (RINTAG).
2.2 | Human kidney normothermic machine perfusion

On arrival at our center kidneys were surgically prepared on ice. The renal artery was cannulated to facilitate connection to the NMP circuit. The ureter was also cannulated so urine output could be measured and sampled. Kidney pairs were perfused simultaneously for 7 hours with an oxygenated red-cell-based perfusate at a mean temperature of 36.5°C and mean arterial pressure of 75 mm Hg, according to published protocols.32 The volume of perfusate in the circuit was kept constant by matching the urine output with a crystalloid solution via continuous infusion. All physiological parameters accessible during NMP were recorded and analyzed including: perfusate blood gas analysis, biochemical analysis, urine production, and flow rate and scored according to the validated “quality assessment tool”.33 The kidney histopathology pre- and postperfusion was assessed by a consultant histopathologist (Figure S2D).

2.3 | MAPC treatment

The MAPC cells used in this study were obtained in collaboration with Athersys Inc (Cleveland, OH). The MAPC cells were research-grade MultiStem® cultures isolated from human bone marrow with consent from a single healthy donor as previously described.34 The MAPC cells were confirmed to express phenotypic markers,35 be of >95% purity,36 and were fluorescently labeled with cytoplasmic dye CellTracker Red CMPTX (ThermoFisher, Waltham, MA).

For MAPC treatment of kidneys during NMP, following 60 minutes of perfusion, the right or left kidney was randomly allocated to a prescribed MAPC dose (50 × 10^6 cells). Immediately prior to infusion the MAPC cryovial aliquots were gently thawed in a water bath at 37°C and for delivery cells were resuspended in 10 mL of perfusate. This was infused via a 3-way tap on the arterial cannula (Figure S3). Control kidneys simply received a 10 mL bolus of crystalloid. The cell dose was extrapolated from phase II/III clinical trials of systemic MAPC therapy taking into account the average weight of a human kidney and volume of circulating perfusion fluid.29

MAPC cells viability was evaluated using a AnnexinV/Propidium iodide flow cytometry assay and revealed 90.9% viability immediately after thawing prior to infusion into the NMP circuit. To gauge potency, a T cell proliferation assay demonstrated MAPCs had immunomodulatory capability immediately upon thawing at various MAPC: peripheral blood mononuclear cells ratio (Figure S2E&F).

2.4 | Contrast-enhanced ultrasound

Contrast-enhanced ultrasound (CEUS) was performed during NMP. This technique utilizes microbubbles of inert gas (sulfur hexafluoride in a phospholipid shell) to increase ultrasound signal return. Each bubble is approximately 2-3 μm in diameter, allowing it to pass through the capillary bed but not the interstitium. CEUS has made it possible to assess the distribution of perfusion at a micro-circulation level.37 We have previously described this technique on porcine kidneys on a cold perfusion circuit.38 To investigate tissue perfusion CEUS on kidneys pre- and post-MAPC infusion. CEUS was performed using a Philips EPIQ7 Ultrasound machine. During NMP CEUS recordings were taken at 60 minutes (before MAPC infusion) and 4 hours later. Microflow imaging designed to detect blood flow within the small vessels at high resolution with minimal artefact was also performed using the Philips EPIQ7 machine. Detailed methodology of this technique is included in the Data S1.

2.5 | ELISA

Commercially available sandwich ELISA kits (R&D Systems, Minneapolis, MN) measured interleukin (IL)-8 (DY208-05) in perfusate and kidney injury marker 1, KIM-1 (DY1750B) and neutrophil gelatinase-associated lipocalin, NGAL (DY1757) in urine according to manufacturer’s instructions.

2.6 | Mesoscale Discovery™ Multiplex

To facilitate the measurement of multiple proteins of interest in 1 assay, a custom electrochemiluminescent Mesoscale Discovery™ (MSD) Multiplex was performed on perfusate samples to quantify cytokine expression. The 10-plex panel includes IL-1β, IL-1α, IL-2, IL-6, IL-10, IL-17, tumor necrosis factor alpha (TNF-α), macrophage inflammatory protein 1 (MIP-1), interferon gamma (IFNγ), and IL-8. These assays were performed according to manufacturer’s instructions alongside technical support from an experienced MSD technician.

2.7 | High performance liquid chromatography

Protein was precipitated with 1:10 perchloric acid 60%; samples were centrifuged, filtered and, then, analyzed by high performance liquid chromatography (HPLC) to quantify kynurenine and tryptophan as described.39 Culture supernatants and perfusate were acidified with sodium acetate, pH = 4, to give a final concentration of 15 mM.

2.8 | In vitro endothelial cell line perfusate stimulation model

An immortalized endothelial cell line (human microvascular endothelial cell line [HMEC]-1 cells, ATCC) was used to investigate the impact of the perfusate secretome on endothelial function. The perfusate from the NMP series was centrifuged at 1500 G for 10 minutes at 4°C and the acellular portion reserved. HMEC-1 cells were cultured to confluence and were treated with the acellular perfusate diluted
in modified MCDB131 media. In chamber slides, cells were stimulated with 25 µL of acellular perfusate to evaluate protein expression using immunohistochemistry. In 6-well plates, HMEC-1 cells were stimulated with 250 µL of acellular perfusate to evaluate gene expression using reverse transcription polymerase chain reaction (RT-qPCR). In both situations, cells were stimulated for 4 hours. RNA extraction was performed on-column using Qiagen RNeasy Plus Mini-kit as per manufacturer’s instructions. cDNA synthesis was carried out using the Tetro cDNA synthesis kit. RNA sequence quantification was carried out using TaqMan RT-qPCR on a StepOnePlus™ Real-Time PCR System. The following primers were used: ICAM1 TaqMan Hs00164932_m1, S1PR1TaqMan Hs00173499_m1, and GAPDH TaqMan Hs02786624_g1 as a housekeeping gene.

2.9 | Mouse intraperitoneal chemotaxis assay

All animal experiments were performed in accordance with the Home Office regulations (PPL60/4521). Eight-week-old female BALB/c mice received an intraperitoneal injection of 0.5 mL of crystalloid (Ringer’s lactate), control, or MAPC perfusate (n = 5 mice per group). An additional 5 mice were not injected. Mice were euthanized after 6 hours treatment and 8 mL of phosphate buffered saline (PBS)-EDTA was injected into peritoneal cavity for lavage. A midline incision was made and lavage fluid collected. Lavage samples were centrifuged at 500 g for 5 minutes, the supernatant discarded and re-suspended in 100 µL of FACS buffer. For cell characterization the suspension was incubated  at 4°C overnight (S1PR1, ThermoFisher Scientific PA1-1040, 1:50; CD31, Abcam, ab182981, 1:2000; Aquaporin-1 Abcam ab168387, 1:100). Following wash the relevant secondary antibody (anti-rabbit Cy5 ThermoFisher Scientific 1:100; anti-rabbit FITC, Abcam, ab97050 1:200, anti-rabbit Dylight550, Immunoreagents 1:100) was added and incubated for 1 hour at room temperature. 0.1% Sudan Black B in 70% EtOH was used to quench tissue auto-fluorescence. Coverslips were mounted with Vectashield Antifade Mounting medium with DAPI (Vectorlabs). Fluorescent imaging was performed using a Zeiss Axioimager or for high resolution Leica SP8 UV AOBS within 2 weeks of staining. Images were processed and analyzed using Zen, LAS X, or Fiji software.

2.10 | MAPC tracking in kidney tissue

MAPC cells were prelabeled with CellTracker CMPTX Red fluorescent dye (CT24552, ThermoFisher) facilitating cell tracking to evaluate partitioning and engraftment within the kidney. Core biopsies were taken at time 0, 1 hour, 4 hours after cell infusion and wedge biopsies at the end of perfusion. Samples were fixed in formalin and stored as paraffin embedded blocks. These were subsequently cut into 4 µM sections for fluorescence microscopy imaging using a Zeiss Axioimager.

2.11 | Immunofluorescence co-localization

Cut sections of perfused kidneys were de-waxed for 10 minutes in Xylene. The sections were rehydrated through graded ethanol (99%, 90%, and 70%) then antigen retrieval in pressure cooker with 1.5 L of Tris/EDTA for 2 minutes. Blocking was performed with 10% goat serum, 100 µL/slide for 30 minutes at room temperature. Blocking buffer was removed and incubated with 100 µL of primary antibody at 4°C overnight (S1PR1, ThermoFisher Scientific PA1-1040, 1:50; CD31, Abcam, ab182981, 1:2000; Aquaporin-1 Abcam ab168387, 1:100). Following wash the relevant secondary antibody (anti-rabbit Cy5 ThermoFisher Scientific 1:100; anti-rabbit FITC, Abcam, ab97050 1:200, anti-rabbit Dylight550, Immunoreagents 1:100) was added and incubated for 1 hour at room temperature. 0.1% Sudan Black B in 70% EtOH was used to quench tissue auto-fluorescence. Coverslips were mounted with Vectashield Antifade Mounting medium with DAPI (Vectorlabs). Fluorescent imaging was performed using a Zeiss Axioimager or for high resolution Leica SP8 UV AOBS within 2 weeks of staining. Images were processed and analyzed using Zen, LAS X, or Fiji software.

2.12 | Statistical analysis

Continuous variables are reported as mean ± standard error of mean where appropriate. Data were assessed for normality, t tests, or analysis of variance (ANOVA) were then applied as appropriate. A 2-sided paired t-test was used comparing variables between control and MAPC-treated kidney pairs from the same donor. The majority of experiments represent n = 5 pairs of kidneys; however, the CEUS experiments represent only n = 3. If another treatment group was included a 1-way ANOVA with a Dunnett’s posthoc multiple comparisons correction for continuous data was used. When recording multiple variables over time from the same kidney a repeated measures 2-way ANOVA with appropriate matching was used with Sidak’s posthoc test for multiple comparisons. P values of less than .05 were deemed statistically significant. Analyses were performed using GraphPad Prism 8.0.

3 | RESULTS

3.1 | Kidneys included in preclinical series

Five pairs of kidneys (n = 10) were included in the study (Table 1). The donors included represent a heterogeneous cohort with ages ranging from 52 to 77 years, but all were from either DCD donors or had characteristics consistent with ECD status. Cold ischemic times were significantly extended due to the delays inherent in a kidney being offered for research only purposes. All kidneys pairs were deemed untransplantable due to a suspicion regarding nonrenal malignancy identified at retrieval.

3.2 | Establishing feasibility of cell therapy delivery during kidney NMP

3.2.1 | Renal physiology

Serial measurements of physiological parameters were recorded during NMP. Perfusate samples were analyzed in real time to assess adequate oxygenation, metabolic requirements, and biochemical
parameters and compared between the kidney pairs; this demonstrated equivalent organ physiology associated with MAPC cell infusion. For physiological parameters such as, potassium, lactate, renal blood flow, and renal resistance, the kidneys were well matched throughout the 7-hour perfusion timeline (Figure 1A-D).

During NMP the ureter of the kidney was cannulated. In the kidneys treated with MAPC cells, there was significantly higher urine output compared to control kidneys, $P = .009$ (Figure 1E).

### 3.2.2 | Kidney injury biomarkers

NGAL and KIM-1 represent promising biomarkers in acute kidney injury or transplantation research. During NMP we can analyze these biomarkers to evaluate reconditioning. We assessed urinary KIM-1 and NGAL concentration over time and compared treatment groups. This demonstrated that MAPC-treated kidneys showed a significantly lower concentration of NGAL $P = .012$ (Figure 1F). Concentrations of urinary KIM-1 were matched between both treatment groups throughout perfusion (Figure S1A).

We also investigated if MAPC therapy had an effect on flavin mononucleotide (FMN) concentration. FMN has emerged as a promising biomarker of organ viability during liver ex vivo perfusion. Increased FMN measured the perfusate is a marker of mitochondrial complex 1 injury. There was a nonsignificant reduction in FMN production in MAPC-treated kidneys (Figure S1B).

### 3.2.3 | Ex vivo ultrasound

To determine if the administration of a MAPC cell bolus was associated with any effects on the renal microvasculature we performed a number of imaging studies using ultrasound. Microflow imaging (MFI) Doppler ultrasound provided high resolution detail on blood flow within small vessels. This revealed there was restored blood flow within the renal medulla 4 hours after MAPC cell infusion (Figure 1G, indicated by white arrow). The same effect was not seen in control kidneys.

Alongside MFI, CEUS was performed before MAPC cell infusion and 4 hours later. CEUS provides a quantifiable assessment of microvascular perfusion and demonstrated a significant improvement in both cortical and medullary perfusion after 4 hours of MAPC treatment during NMP, compared to the control kidneys, $P = .019$ and $P = .001$, respectively (Figure 1H & I).

### 3.3 | Evaluating the immunomodulatory potential of MAPC therapy during NMP

#### 3.3.1 | Cytokine profiling

MAPC cells are reported to mediate their immunomodulatory capacity through increasing anti-inflammatory cytokines and down regulating pro-inflammatory cytokines. A panel of cytokines were measured at serial time points during perfusion (time 0, 1 hour, 2 hours, 4 hours, and 7 hours) to evaluate this phenomenon during NMP. This panel revealed MAPC-treated kidneys had a significant reduction in IL-1β, $P = .050$ (Figure 2A). There was also a significant increase in anti-inflammatory cytokine IL-10, $P = .047$ (Figure 2B). Alongside this was a nonsignificant reduction in other pro-inflammatory cytokines; IL-6, IL-1α, IL-17, and IL-8 but his was not the case for all cytokines; TNFα, MIP-1β, IL-2, and IFNγ. (Figure S1 C).

#### 3.3.2 | Indolamine-2, 3-dioxygenase activity

MAPC cells are reported to regulate T cells via indolamine-2,3-dioxygenase (IDO) (19). Cells expressing IDO catabolize tryptophan (Trp) to suppress effector T cells and activate Foxp3-lineage regulatory
CD4 T cells (Tregs).\(^2\) IDO activity was assessed by measuring Trp and the catabolite kynurenine (Kyn) in the perfusate using HPLC. This revealed that MAPC-treated kidneys had significantly higher IDO activity (elevated Kyn:Trp ratio) following 7 hours of perfusion when compared to IDO activity in paired control kidneys, \(P = .050\) (Figure 2C).

### 3.3.3 | MAPC perfusate secretome effect on HMEC-1 endothelial cell line model

An in-vitro endothelial cell line model was established to better understand the impact of MAPC perfusate secretome on the endothelium and microvascular integrity following reperfusion. Samples of perfusate taken after 7 hours of NMP were added to HMEC-1 cells in culture. ICAM-1 (activation status) and S1PR1 (microvascular barrier integrity) expression was analyzed in the HMEC-1 cells in response to perfusate from pairs of kidneys (control perfusate vs MAPC perfusate) (Figure 2D-G). ICAM-1 protein expression was significantly increased in the control group, \(P < .001\). However, in the MAPC-treated group this upregulation was not as marked, \(P = .022\). In contrast, S1PR1 gene and protein expression in HMEC-1 cells was downregulated by control perfusate; however, MAPC perfusate maintained S1PR1 gene expression at unstimulated levels, \(P = .032\); and increased protein expression when compared with control perfusate, \(P < .001\). This preservation of S1PR1 protein was also seen on immunofluorescence in the tissue of NMP perfused kidneys, \(P = .030\) (Figure 2H-J).

### 3.3.4 | MAPC secretome effect on mouse intraperitoneal chemotaxis assay

To evaluate if the MAPC secretome in the perfusate during NMP had an impact on leucocyte chemotaxis we used a small animal model. Samples of acellular perfusate were injected intraperitoneally to mice. There were 4 treatment groups, each containing 5 mice, as described in the methods section. Six hours after injection the intraperitoneal space was lavaged to harvest the immune cell infiltrate, which was analyzed using flow cytometry (Figure 2D-F). Control perfusate led to a significant increase in peritoneal Ly6G+ neutrophils compared to crystalloid injections alone, \(P < .001\). This increase was not seen in perfusate from MAPC-treated kidneys, (Figure 2K-L) indicating reduced inflammatory chemotaxis.

### 3.4 | Determining the physical distribution of NMP administered MAPC cells

MAPC cells were prelabeled with a red fluorescent dye in order to understand cell fate following intra-arterial delivery during NMP. To achieve this, following 7 hours of perfusion, anatomical regions of interest sampled from the cortex, medulla, artery, and collecting system of the kidney and visualized using fluorescent immunohistochemistry and confocal microscopy. Nuclear counter-staining was with DAPI (blue). Additional staining for endothelial cell marker (CD31 - green) and a proximal tubular epithelial cell marker (Aquaporin-1) was performed for morphological co-localization. This revealed the majority of MAPC cells were in the glomeruli in sections of cortex and around the peritubular space in the sections of medulla (Figure 3A-E).

The tissues sections only account for the fate of MAPC cells within the kidney; there was also a proportion of MAPC cells that remained circulating within the perfusate. A cell filter was added to the perfusion circuit after 7 hours of NMP to evaluate this. These cells were subsequently analyzed on flow cytometry, (Figure S2A). This revealed a small population of red positive cells alongside a separate population of unlabeled cells, assumed to be passenger leukocytes. A viability assay revealed the mean proportion of circulating live MAPC cells to be 21%. In contrast, the passenger leukocyte population had 44% viability. The proportion of passenger leukocytes resident in the kidney tissue was assessed and revealed no significant difference between MAPC and control kidneys (Figure S2B,C).
4 | DISCUSSION

MAPC therapy has demonstrated significant promise in treating a number of clinical conditions associated with inflammation and ischemia.\textsuperscript{22–24,43} Clinical trials have demonstrated MAPC cells have a robust safety profile. Therefore, the translation of MAPC therapy to minimize ischemia reperfusion injury in kidney transplantation represents a promising avenue for further exploration. Here we have described the first reported series of human kidneys infused with a cellular therapy using normothermic machine perfusion.

Kidneys treated with MAPC therapy demonstrated an improvement in physiological parameters and biomarkers of kidney injury.
FIGURE 2 Evaluating the immunomodulatory capability of MAPC therapy during kidney NMP. Panels A and B depict significant results from custom Mesoscale Discovery™ panel analyzing perfusate cytokine concentrations comparing control (blue) with MAPC-treated (red) NMP kidneys. Results are expressed as the mean fold change calculated relative to time zero. Panel A demonstrates a significant decreased expression of cytokine IL-1β, paired t test, n = 5 pairs, \( P < .05 \) (MAPC-treated mean fold change 6.9 ± 2.1 vs control 26.8 ± 7.7). Panel B demonstrates a significant increase IL-10 in MAPC group, paired t test, n = 5 pairs, \( P < .05 \) (MAPC-treated mean fold change 949.4 ± 288.6 vs control 252.9 ± 71.3). Panel C compares IDO activity in paired kidneys after 7 hours of NMP, paired t test, n = 5 pairs, \( P < .05 \) (MAPC treated K/T ratio 0.34 ± 0.02 vs control 0.29 ± 0.03). Panels D and E depict qPCR results from perfusate stimulated HMEC-1 cells (control kidneys vs MAPC treated). Panel D demonstrates ICAM-1 gene expression, paired t test, n = 5 pairs, \( P = ns \) (MAPC treated mean fold change on qPCR 9.6 ± 3.1 vs 6.5 ± 0.9). Panel E S1PR1 gene expression, paired t test, \( P < .05 \) (MAPC treated mean fold change on qPCR −4.9 ± 1.7 vs control 0.6 ± 0.6). Panels F and G depict perfusate stimulated HMEC-1 cells immunohistochemistry staining for ICAM-1 and S1PR1 protein expression (control vs MAPC treated). Graph F demonstrates comparison of ICAM-1 protein expression between treatment groups (MAPC treated 23.9 ± 3.3 vs control perfusate 37.3 ± 9.2). Graph G demonstrates comparison of S1PR1 protein expression between treatment groups (MAPC treated 76.5 ± 4.7 vs control perfusate 57.6 ± 4.9). Analyzed with 1-way ANOVA and Tukey’s test posthoc analysis, \( P < .05 \). Panels H and I depict S1PR1 staining visualized using fluorescence microscopy on kidney biopsies taken during NMP, blue stain is DAPI, red is S1PR1. Panel J demonstrates a significant upregulation of S1PR1 protein expression as quantified by mean fluorescence intensity, paired t test, n = 5 pairs, \( P < .05 \) (MAPC treated 0.11 ± 0.01 vs control 0.18 ± 0.02). Panels K and L demonstrate results from mouse intraperitoneal chemotaxis experiments. Panel K depicts typical flow cytometry scatter plots examining staining for Ly6G + neutrophils isolated from mouse peritoneal lavage for different treatment groups. Panel L depicts the mean number of Ly6G + cells isolated from lavage samples demonstrating neutrophil recruitment with MAPC perfusate compared to control perfusate, paired t test, n = 5 pairs, \( P < .01 \) (MAPC treated 2990 ± 506 neutrophils vs control mice 8849 ± 1675 neutrophils). ANOVA, analysis of variance; HMEC-1, human microvascular endothelial cell line 1; IDO, indolamine 2, 3 dioxygenase; IL, interleukin; MAPC, Multipotent Adult Progenitor Cells; NMP, normothermic machine perfusion; qPCR, quantitative polymerase chain reaction

A higher volume of urine output and lower urinary NGAL during NMP has previously been correlated with improved clinical outcomes following transplantation.\(^{33,44}\) Improving these key parameters with MAPC treatment is suggestive of less tissue damage and improved cellular metabolism. We have also demonstrated a significant improvement in microvascular perfusion during NMP using CEUS. The improved blood flow seen in MAPC-treated kidneys could be mediated by PGE\(_2\) or IDO, both potent vasodilators, which are reported to be part of the MAPC cell secretome in pro-inflammatory environments.\(^{45}\) Interestingly, the global renal blood flow was unchanged but vasodilation of the microcirculation seems to result in improved tissue perfusion and reestablishment of homeostasis as evidenced by increased urine output and reduction in NGAL.

Cytokine profiling revealed that MAPC therapy during kidney NMP was associated with an anti-inflammatory, pro-tolerogenic cytokine profile. This included decreased expression of IL-1β, a pro-inflammatory cytokine associated with endothelial activation. Decreased IL-1β expression during ex vivo lung perfusion correlates with improved survival following lung transplantation.\(^{56}\) There was also upregulation of pro-tolerogenic, anti-inflammatory IL-10 and this was accompanied by increased IDO activity. MAPC cells modulating differential cytokine expression in perfusate may be key in their potential role of minimizing IRI.

Tracking MAPC cells after NMP delivery revealed cells could be found throughout the kidney. The distribution was similar to that described when MSCs were delivered to pig kidneys.\(^{21}\) Previous studies demonstrated MAPC cells have a powerful ability to home to hypoxic endothelium where they migrate into tissue, taking up residence within the perivascular space.\(^{47-49}\) Indeed, in a preclinical study of acute myocardial infarction the cells actively migrated into the ischemic myocardium following transarterial delivery.\(^{50}\) This may be mirrored in our NMP model – MAPC cells are homing to hypoxic areas within the kidney where they take temporary residence. In this micro-environment, the cells release an anti-inflammatory secretome, which is having beneficial effects; increased blood flow, improved urine output, and reduced tubular injury. The proportion of circulating nonviable MAPC cells may also have a mechanistic role. Previous studies have demonstrated that apoptotic MSCs induce a significant immunosuppressive response mediated by recipient phagocytes.\(^{51}\)

NMP provides a platform for direct delivery of MAPC therapy, overcoming the limitations of systemic delivery where cell therapies can get trapped in the lungs or liver of recipients, reducing delivery to target sites.\(^{28,52}\) During NMP, the pro-inflammatory ischemic kidney provides a microenvironment to prime MAPC cells, licensing them to take on the desired immunomodulatory phenotype. This direct delivery platform that effectively primes cells could provide the solution to a previous significant barrier in MSC/MAPC therapy translation.\(^{53}\)

The timing of MAPC/MSC delivery has been demonstrated as a key factor affecting therapy efficacy and NMP facilitates early delivery of cells prior to implantation. Timing of MSC infusion relative to the organ transplant plays an important role in the potency of the cell’s immunomodulatory effect.\(^{54}\) One study demonstrated, in an animal model of semi-allogeneic heart transplantation, that the systemic infusion of MSCs into the recipient prior to transplantation conferred a stronger pro-tolerogenic therapeutic effect than if the infusion was given postoperatively.\(^{55}\) This has since been replicated in a number of settings, including kidney, and confirmed that delivery of cells posttransplant can result in graft inflammation, whereas pretransplant infusion resulted in interaction with myeloid populations and localization to lymphoid organs leading to expansion of pre-tolerogenic Tregs and diminished inflammation.\(^{56-58}\)

However, there are a number of limitations in this study. NMP as an experimental system is limited in its ability to measure the posttransplantation effectiveness of a therapy. These issues
would need to be addressed in further studies. Additionally, we cannot determine the fate and persistence of the MAPC population after transplantation of the kidney. Other animal studies investigating systemic MSC therapy have demonstrated cells are no longer traceable in the recipient at 21 days, indeed the majority are lost from the system by 72 hours.\textsuperscript{43} There is also debate about the necessity of MSC or MAPC cell engraftment to support their therapeutic actions as this may be modulated through phagocytosis of the cells by recipient monocyte/macrophage populations which contribute to subsequent immunomodulation.\textsuperscript{59} Also, the immunogenicity of MAPC cells in the NMP setting needs investigated. It has been previously demonstrated that the in vitro immunosuppressive activity of MAPC cells was independent of MHC matching.\textsuperscript{17} Reassuringly, in other clinical circumstances where allogeneic MAPC cells have been used there was no evidence of development of new anti-HLA antibodies in unsensitized patients treated after myocardial infarction\textsuperscript{23} or in 18 immunosuppressed bone marrow transplantation patients.\textsuperscript{21}

4.1 Conclusion

This is the first reported series of cell therapy successfully delivered directly to human donor kidneys in an isolated ex vivo perfusion platform. Kidneys treated with MAPC cells during NMP demonstrate improvement in clinically relevant parameters and a reduction in injury

**FIGURE 3** Determining MAPC cell fate and physical distribution following delivery during kidney NMP. MAPC cells were prelabeled with a red cytoplasmic fluorescent dye (CellTracker Red CMPTX, ThermoFisher) to facilitate tracking of the cells through the kidney following 7 hours of perfusion. The red labeled cells are the MAPC cells as noted by white arrows. Counter nuclear staining is in blue with DAPI. The green staining is endothelial marker CD31 and highlights the kidneys vasculature. The pink staining is Aquaporin 1 a marker for proximal tubular cells. Panels A and B are sections taken from the kidney cortex after 6 hours of MAPC treatment during NMP. These represent typical confocal microscopy images from kidneys treated with MAPC cells. The white letter (G) identifies the glomerulus. Panel C is a typical section taken from the kidney’s medulla; vessels identified by the CD31 green stain are identified by the green (V). Panel D is a high magnification image taken of three MAPC cells that are resident in the interstitium next to a peritubular capillary. Panel E depicts the confocal images of a blood vessel from the renal cortex with a MAPC cell in the lumen and cells that have mobilized out of the vessel into the nearby tissue. MAPC, Multipotent Adult Progenitor Cells; NMP, normothermic machine perfusion.
and pro-inflammatory biomarkers. This effect may be mediated by changes to circulating cytokines or through secreted soluble anti-inflammatory mediators. NMP represents a novel cell therapy delivery system. This represents a paradigm shift, providing an exciting opportunity to directly treat organs prior to transplantation to minimize IRI. A future clinical trial evaluating this modality of delivery could result in the transplantation of otherwise discarded organs, thereby reducing the transplant waiting list and offering hope to patients with renal failure.

**ACKNOWLEDGMENTS**

We thank all the member of the perfusion and theatre team at the Freeman Hospital for technical support. We are very grateful to staff at NHSBT Barrack Road Histocompatibility and Immunogenetics for help with tissue typing.

**DISCLOSURE**

Some authors of this manuscript have conflicts of interest to disclose as described by the American Journal of Transplantation. VDR and AT are employees of ReGenesys and Athersys, Inc, respectively and are both shareholders of Athersys stock. The other authors declare that they have no competing interests.

**AUTHOR CONTRIBUTIONS**

ET, AF, NS, SA, and CW conceived the study. ET, LB, IKI, TG, GW, LW, SJT, RF, BS, AM, KC, and HDL performed the experiments and data analysis. AS, SA, and MLN provided technical expertise and training in perfusion. VDR and AT provided the MAPC cells and scientific advice during the project. ET, NS, SA, and CW prepared and revised the manuscript. All authors read and approved the final manuscript.

**DATA AVAILABILITY STATEMENT**

All data related to this study are present in the paper or the Data S1 found at the end of this article. The data that support the findings of this study are available from the corresponding author upon request. All reagents and perfusion consumables were commercially available. MAPC cells were obtained through the material transfer agreement between Newcastle University and Athersys Inc.

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

How to cite this article: Thompson ER, Bates L, Ibrahim IK, et al. Novel delivery of cellular therapy to reduce ischemia reperfusion injury in kidney transplantation. Am J Transplant. 2021;21:1402-1414. https://doi.org/10.1111/ajt.16100