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Potentiating Renal Regeneration using Mesenchymal Stem Cells

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Abstract

Background—The potential of a Mesenchymal Stem Cell (MSC) therapy to accelerate the repair of ischemically damaged human kidneys during 24 hours of warm perfusion was evaluated. The hypothesis was that by administering MSC directly to the renal tissue, there would be an improved opportunity for cellular repair mediated by intrarenal paracrine effects.

Methods—Studies were performed using the Exsanguinous Metabolic Support (EMS) tissue-engineering platform. Five pairs of human kidney allografts from donation after cardiac death (DCD) donors were studied. One human kidney was EMS perfused for 24 hours (control), while its paired kidney was EMS perfused with MSC (1×10^8). The kidneys were evaluated for DNA synthesis, cytokine/chemokine synthesis, cytoskeletal regeneration and mitosis.

Results—Treatment with MSC resulted in reduced inflammatory cytokines synthesized by the kidneys. MSC treatment led to a significant increase in the synthesis of ATP and growth factors resulting in normalization of metabolism and the cytoskeleton. Toluidine Blue staining of MSC

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treated kidneys demonstrated a significant increase in the number of renal cells undergoing mitosis (26%) compared to EMS perfusion alone.

Conclusions—To our knowledge, our work is the first to have demonstrated actual renal regeneration while ischemically damaged human kidneys are perfused ex vivo for 24 hours. The observed regeneration entails: increased synthesis of ATP, a reduced inflammatory response, increased synthesis of growth factors, normalization of the cytoskeleton and mitosis. The ability to regenerate renal tissue ex vivo sufficiently to result in immediate function could revolutionize transplantation by solving the chronic organ shortage.

Introduction

For the >650,000 patients with end-stage renal disease (ESRD) in the U.S. the pool of deceased donor kidneys has remained essentially stagnant over the past few decades [1–8]. The deceased donor kidney pool remains largely dependent upon traditional donation after brain death (DBD). The DBD donor represents a small fraction of the deaths from traumatic injuries, approximately 4% [9]. The ability to recover warm ischemically (WI) damaged kidneys from uncontrolled deceased by cardiac death (uDCD) donors represents the best near-term chance for expanding the kidney pool. The uDCD kidneys are rarely considered for organ donation because the WI has represented an obstacle [10–17]. The resulting paradigm is a large discrepancy between the growing demand for kidneys by ESRD patients, a number doubling each decade, and the pool of deceased kidney donors demonstrating little growth. This is a healthcare issue because although transplantation provides a better quality of life and is more cost-effective, the renal allograft shortage prevents it from becoming a widespread solution. Thus, the dialysis population is expected to reach >2-million patients in the next decade at an aggregate cost of >$1-trillion USD [18].

We have previously demonstrated the regeneration of severely ischemically damaged renal allografts, using a tissue-engineering platform referred to as Exsanguinous Metabolic Support (EMS). EMS is composed of an acellular medium, perfusion system, disposable organ chamber with biosensors to monitor metabolism and a control module. Rather than suppressing metabolism by >96%, as is the case with hypothermic preservation, the restored oxidative metabolism during EMS perfusion is of sufficient magnitude to support new synthesis that provides the basis for cellular reparative processes [19,20]. We believe the ability to repair ischemic damage ex vivo will provide for a significant expansion of the deceased donor kidney pool by facilitating the use of uDCD donor kidneys that are not utilized today.

The potential to further accelerate the regeneration of WI damaged human renal allografts was evaluated by introducing a mesenchymal stem cell (MSC) treatment during 24 hours of EMS perfusion. MSC were selected for this study because the cells have been shown to be immune evasive and can be transplanted without requisite immunosuppression. MSC have also been shown to secrete bioactive molecules such as cytokines/chemokines and growth factors including: granulocyte-colony stimulating factor, leukemia-inhibitory factor, macrophage-colony stimulating factor, PGE2, IL-10, TGFβ, IDO, HO-1, HGF, VEGF, FGF & IGF-1 [21–26]. The MSC do not directly replace denuded renal epithelial cells. Instead the

cells modulate renal regenerative responses that in turn have been shown to accelerate the recovery phase [27–29]. This is significant since the cells replacing lost renal epithelium are known to be derived from within the kidney itself [30]. Surviving renal cells dedifferentiate and replicate to restore the epithelium [31]. In contrast, previous work has demonstrated that resident kidney stem cells represent a small population that may be insufficient themselves to therapeutically regenerate a severely damaged human DCD kidney [28].

The study hypothesis was that by localizing the MSC to the vascular circulation within damaged human kidneys the repair processes could be potentiated resulting in accelerated recovery of the damaged renal cells. We focused on addressing 2 specific issues:

1. What was the appropriate dose of MSC that could be administrated within the human kidneys for mediation of their paracrine effects without adversely affecting the renal perfusion dynamics?
2. Would this dose of MSC demonstrate a beneficial effect on accelerating renal repair processes?

The evaluations of the repair potential mediated by the MSC included: DNA synthesis, restored cytoskeletal integrity, chemokine/cytokine & growth factor synthesis and histology. Also, several biomarkers reflecting renal damage were tested. The results of these studies demonstrate therapeutic efficacy when the paracrine effects produced by the circulating MSC are localized to the kidney.

Materials and Methods

Paired Human Renal Allograft Model –

Kidneys were procured for transplantation according to standard operating procedure and subsequently released to the National Disease Research Interchange (NDRI) once the waiting list had been exhausted. The use of discarded human kidneys exempts studies from ethics board (IRB) approval. A dosing study was first conducted that entailed 10 human kidneys to determine the requisite number of MSC to be used in the subsequent studies. The objective was to identify the highest dose of MSC that did not adversely affect the perfusion pressures, vascular flow or oxygen consumption.

In subsequent studies an additional 5 pairs of human kidneys were used to determine the potential effect of MSC on the renal regeneration during 24 hours of warm perfusion. The data from the control and test groups was quantified as described below and the significance was analyzed using the paired Student's *t* test. The known mean WI insult was 30 minutes in 2 pairs of the human kidneys that was calculated as the time from declaration of death following withdrawal of life support until the infusion of cold preservation solution. In 3 additional pairs the WI time was unknown because the donors were found asystole. The kidney donor characteristics are listed in (Table 1).

The kidneys were received stored statically in ViaSpan™ with a mean cold ischemic (CI) time of 29.4 ± 7.4 hours. Therefore, the paired human kidneys tested experienced both warm and cold ischemic insults. While a more prolonged WI insult would have been preferred to

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evaluate renal regeneration, the present study was limited by the current donation criteria for human kidneys procured for transplantation.

EMS Perfusion –

The kidneys were weighed, cannulated and then flushed with warm EMS solution (32°C). and placed on EMS perfusion utilizing 1 liter of recirculated perfusate. Restoration of oxidative metabolism and vasodilation were determined by standard criteria of oxygen consumption of >0.1 cc/min/g of wet kidney weight, glucose consumption of >0.01mmol/hour/g, vascular flow of >100cc/min and mean arterial systolic pressures of <60 mmHg. Once oxidative metabolism and vasodilation were adequately resuscitated the infusion of MSC into the renal artery of the test kidneys was initiated. One of each of the paired kidneys was used as a control that was warm perfused for the same total time but did not receive treatment with MSC.

MSC Culture and Handling –

MSC were received from RoosterBio Inc. frozen in vials at a concentration of $1{\times}10^7$ stem cells per vial. The cells were quickly thawed, washed and placed in cell culture for population expansion. The phenotype of the MSC was $CD166^+$, $CD105^+$, $CD90^+$, $CD73^+$, $CD14^-$, $CD34^-$ and $CD45^-$. This phenotype remained stable during the cell culture. Each lot of MSC was expanded in cell culture to a final population of $1{\times}10^8$ and immediately used or alternatively stored in the vapor phase of liquid nitrogen until human kidneys became available. In the case of frozen MSC, the cells were thawed and placed in cell culture until the time of use, normally approximately 12 hours during shipment of the human kidneys. Therefore, the MSC were taken directly from cell culture when infused.

MSCs Labeling –

Prior to infusion, the MSC in cell culture were labeled with PKH26 Red Fluorescent Cell Linker Kit (Sigma Aldrich). Cells were quantified using a Nexcelom Automated T4 Cellometer and adjusted to a working concentration of $2{\times}10^6$ MSC/ml. The preselected doses of the infused cell populations were injected intra-arterially. The MSCs were slowly infused to avoid an increase in the perfusion pressures or a decrease in vascular flow. If a negative effect was observed, the infusion was halted until the pressure and flow rate were restored to preinfusion levels. Once the perfusion dynamics became normalized the infusion was continued until the desired targeted dose of MSC was administered and the EMS perfusion continued for an additional 24 hours.

Luminex Cytokine/Chemokine Analysis –

Samples of the recirculating perfusate were taken at 30 minute and 24 hour time points during the EMS perfusion of each kidney. The samples were tested using a Multiplex MAP Human Cytokine/Chemokine Magnetic Bead Panel and analyzed using the Luminex Multiplexing Platform. In addition, the synthesis of growth factors known to be associated with regeneration was also evaluated using the Luminex platform.

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Immunohistochemistry –

Human kidney samples were flash frozen and sectioned using a Leica 1860UV Cryostat. An indirect immunofluorescence assay was performed with various primary antibodies. The secondary antibodies were conjugated with an Alexa Fluor dye and counterstained with DAPI. Images were made using confocal microscopy and analyzed using ImageJ software. Ten representative sections from each of the kidneys were tested and the results analyzed as the mean from respective sections. Proliferating cell nuclear antigen (PCNA) was evaluated as a marker of up-regulation in DNA synthesis and Zonal Occludens-1 (ZO-1) was used to evaluate cytoskeletal repair. Toluidine Blue staining was used to evaluate if there was “actual” renal regeneration by determining if there was any evidence of mitotic figures in the regenerating kidneys following 24-hours of ex vivo perfusion. Mitosis is indicative of renal regeneration by surviving cells recovering synthetic functions sufficiently to undergo cell division to replace lethally injured adjacent cells.

Renal Metabolism –

Renal metabolism during the ex vivo perfusion was evaluated by measuring ATP synthesis in tissue samples taken from each kidney following 24 hours of EMS warm perfusion. Samples were taken from representative sites from the medulla and cortex and flash frozen in liquid nitrogen. Kidney samples were tested using both a colorimetric ADP/ATP ratio assay kit (Sigma-Aldrich) analyzed on a Victor2 Multiwell Plate reader and then confirmed using HPLC.

Results

Effect of Introducing MSC in the Ischemically Damaged Human Kidney –

The introduction of 25, 50, 75 or 100 $\times 10^6$ MSC did not adversely affect the renal perfusion pressures (Table 2). Likewise, at these 4 doses the vascular flow rate and oxygen consumption was also not adversely affected. Rather, the trend was a reduction in perfusion pressures, an increase in the vascular flow and a rise in the oxygen consumption. In contrast, at a dose of 2×10^8 stem cells the rise in mean arterial pressure (MAP) was associated with reduced vascular flow and a significant reduction in the oxygen consumption. At this dose of MSC the rise in perfusion pressures remained constant and never normalized (Table 2). This can be attributed to the administration of the high concentration of MSC because the EMS warm perfusion itself is acellular.

Given these results, we selected a dose of 1×10^8 MSC for this study based upon the selection of the highest dose of MSC that did not adversely affect the perfusion of the ischemically damaged human kidneys. The rationale for this decision was the hypothesis that the highest dose of MSC would provide the best opportunity to successfully determine the feasibility of MSC mediated paracrine effects that in turn could accelerate renal regeneration. Since MSC have been shown not to integrate into parenchyma but rather mediate their regenerative effects via secreted paracrine factors, the hypothesis for a high dose was further supported.

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Fate of the MSC –

Because the MSC were fluorescently labeled it was possible to determine if the cells migrated from the vasculature into the renal parenchyma or rather simply remained in the circulation. Upon histologic examination of tissue sections, it was determined that no significant migration out of the vasculature into the renal parenchyma had occurred. Collecting the perfusate following the warm perfusion, allowed for the quantification of the MSC in the circulating perfusate because the EMS perfusate itself is acellular. The interpretation of the histologic findings was further supported by the determination that MSC remain predominately in the circulating perfusate. The perfusate was collected at the termination of the 24-hour perfusion, centrifuged and the MSC were quantified using an automated cell counter. More than 95% of the MSC initially infused into the kidneys were recovered from the recirculated perfusate. These results provide initial evidence that the MSC do not integrate into the renal tissue during 24 hours of ex vivo warm perfusion but rather remain in circulation through the vascular compartment.

ATP Synthesis –

When the ATP concentration in the renal tissue was measured (nmol per gram), a statistically significant increase in the concentration of ATP was observed in the MSC treated kidneys, in both the renal cortex ($p = .0046$) and medulla ($p = 0.013$) by 24 hours of ex vivo warm perfusion (Figure 1). The positive effect of MSC treatment on ATP synthesis was most pronounced in the medulla where the ATP synthesized was more than 3-fold the concentration over the paired control kidneys that were warm perfused without MSC. While we do not yet know the mechanism(s) mediated by the MSC treatment that resulted in such increased availability of ATP, nonetheless the increased availability of ATP is known to be associated with increased synthetic functions. Increased synthetic functions are requisite to regeneration of damaged renal tissue.

Renal Synthesis –

Using the Luminex platform we evaluated the synthesis of inflammatory cytokines and found that stem cell treatment led to a reduced proinflammatory state. The results of the known proinflammatory cytokines are listed in (Table 3). In each individual inflammatory cytokine that was tested it was observed that treatment with MSC resulted in a reduced inflammatory status in comparison to the paired control kidneys. The degree of inhibited synthesis varied from cytokine to cytokine. However, the reduced inflammatory state is clinically significant because cytokines such as TNF-α are known to induce expression of adhesion molecules such as ICAM on the surface of vascular endothelial cells lining the vasculature. The up-regulated expression of cell adhesion molecules is detrimental to outcomes following reimplantation of an allograft because such up-regulation results in enhanced margination and diapedesis of recipient immune cells within the microvasculature of the graft.

In addition, it was found that MSC treatment resulted in increased synthesis of EGF, FGF-2 and TGF-a in the test kidneys compared to the paired control kidneys (Table 3). While it was not determined whether the increased concentration of growth factors associated with MSC treatment was produced by the MSC or the renal cells, nonetheless the increased

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concentration of growth factors was statistically significant ($p<0.05$). Also, of note is the known positive effect of growth factors on regenerative pathways following ischemic injury. Growth factors mediate their positive effect by acting as trophic factors and functioning as second messengers. The growth factors are also known to mediate increases in metabolic rate, are mitogenic for damaged renal tubules, promote restoration of cytoskeletal integrity and help to restore cell surface polarity.

Cytoskeletal Repair –

ZO-1 is a tight junctional protein that was used to evaluate repair to the cytoskeleton. ZO-1 is normally expressed exclusively at the plasma membrane localized to the point of cell-to-cell contact. Following an ischemic injury that damages the cytoskeleton, ZO-1 diffuses from the cell membrane becoming dispersed within the cytoplasm. The dispersion of ZO-1 into the cytoplasm can therefore be used as a marker of cytoskeletal injury and the restoration of tight cellular junctions can be interpreted as a reparative process to the cytoskeleton. Using Image J software to objectively quantify differences in fluorescence, a 4.81% increase in restoration of cytoskeletal integrity was observed in MSC treated kidneys. The control kidneys displayed significant normalization of the cytoskeletal integrity supported by the warm perfusion alone at 24 hours. The test kidneys that were treated with MSC only marginally increased the rate of normalization following 24 hours of warm perfusion, $44.18 \pm 8.67\%$ compared to $48.99 \pm 5.95\%$ of total fluorescence where a decrease represents reduction in cytoplasmic staining. While this marginal increase was not statistically significant, the phenomenon was observed in all the test kidneys demonstrating the reproducibility of the reparative process.

PCNA –

We used PCNA as a marker of up-regulation in DNA synthesis. Using Image J, we found the mean number of PCNA positive nuclei in the control group kidneys was 194.8 ± 45.92 per 413 nuclei (47.1%). In MSC treated kidneys we found an average of 215.3 ± 24.63 per 413 nuclei (52.1%) (Figure 2). Using a paired Student's *t* test it was determined that there was a significant difference between the 2 groups ($p = 0.049$). In previous studies it was observed that PCNA up-regulation during the EMS perfusion occurred within 6 hours, far sooner than the 24 hours threshold observed in vivo following an acute kidney injury [32]. By testing for PCNA at 24 hours a more pronounced difference may have been underestimated because of earlier up-regulation.

Mitosis –

During the evaluation of the H&E stained kidney sections, cells in various stages of mitosis were observed. The tissue sections were then further studied using toluidine blue staining. The positive stained cells were counted per field with a total of 40 separate fields. While renal cells with mitotic figures were observed in all warm perfused human kidneys, the kidneys that were MSC treated demonstrated an increased number of renal cells with mitotic figures. In the control kidneys the mean number of cells positive for mitotic figures was 47.7 ± 4.4 per 168 cells (28%), while in the MSC treated kidneys the cells undergoing mitosis increased to 60.2 ± 3.84 per 168 cells (35.9%). The results demonstrated a 26% increased incidence of mitosis associated with the MSC treatment ($p<0.05$) (Figure 3). In a normal

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kidney, the number of renal cells undergoing division is quite low, with normal cell turnover occurring in terms of years [33].

Potential of Long Term EMS Perfusion –

To accomplish a more complete regeneration of WI damaged human renal allografts; a longer reparative process during ex vivo warm perfusion will be needed. To be assured that a longer period of EMS kidney perfusion can be successfully achieved, we conducted 2 experiments involving 2 additional human renal allografts that were perfused for 3 days. The kidneys were from DBD donors and therefore, not WI damaged. Since the kidneys were not WI damaged, it was possible to assess whether a prolonged period of ex vivo warm perfusion would mediate damage. Throughout the 3 days of ex vivo perfusion of the human kidneys the perfusion pressures remained stable (MAP <60 mmHg), vascular flow remained >170 cc/min, oxygen consumption was >0.13 cc/min/g of kidney and glucose utilization was > 0.01 mmol/hour/g. At the end of the 3-day perfusion the kidneys were flush fixed and placed in paraffin blocks after which they were sectioned. The histologic evaluations involved samples from 5 representative sites within each kidney that were H&E stained. The findings demonstrated normal vessels and glomeruli. The tubule epithelium was also normal with the occasional, mild cystic dilation of tubules. Since the parameters of the perfusion were constant throughout the 72-hours of testing and histologic evaluations did not find evidence of damage, we believe that additional days of perfusion is feasible. This observation confirms previous work performed by our group that demonstrated the feasibility of warm perfusing human renal allografts for several days [33]. A longer period of ex vivo warm perfusion would provide an increased opportunity for renal regeneration.

Discussion

To our knowledge this is the first report of active cellular regeneration in ischemically damaged human kidneys resulting in renal cells undergoing mitosis during 24 hours of ex vivo perfusion. In previously published work ex vivo warm perfusion was found to ameliorate reperfusion injury when the kidneys were reimplanted directly from EMS perfusion [20]. Cellular repair during ex vivo warm perfusion is obviously distinct from the inflammatory and coagulation responses that occur in vivo with normal physiologic responses to injury. In contrast, the repair observed during EMS perfusion is more analogous to the cellular recovery that occurs following resuscitation of oxidative metabolism in cells placed in tissue culture. We have previously reported that a period of ex vivo warm perfusion in an exsanguinous environment results in resuscitation of oxidative metabolism, reestablishment of cell volume regulation and recovery of the cytoskeletal integrity following a prelethal warm ischemic injury [19–20]. The results of this earlier work demonstrated that resuscitation of metabolism during ex vivo warm perfusion in an acellular environment was of sufficient magnitude to support de novo protein synthesis that is the basis of cellular reparative processes. However, while this work addressed cell repair following a prelethal injury, a period of DGF still occurred. The DGF represents the renal cells that were lethally injured and could not be repaired during warm perfusion but rather required that surviving renal cells regenerated sufficiently to undergo mitosis to replace the lethally injured cells. A hallmark of DGF is acute tubular necrosis (ATN) and if the ischemic

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insult is severe there is a well-recognized increased risk of primary nonfunction. Therefore, DGF represents the time requisite to regenerating the lethally injured renal cells and restoring normal renal function. Regeneration is mediated by nonlethally injured (surviving) tubular epithelial cells that dedifferentiate and proliferate to replace the lethally (irreversibly) injured tubular epithelial cells to restore renal integrity. The resulting proliferative response is rapid, involving numerous remaining tubular cells [28, 34]. The mitogenic response is driven by paracrine factors at sites of severe injury and supports the findings of MSC treatment in this study [35]. The mitogenic potential of proximal tubule cells is the reason for the therapeutic use of growth factors to accelerate recovery from acute renal failure (ARF) [36]. Similarly, an inflammatory response contributes to the injury cascade. The transmigration of immune cells into the transplant is a well-known mechanism that is associated with reperfusion injury and the development of DGF [37, 38]. The diapedesis is mediated by the chemokines released from injured renal cells such as macrophage chemoattractant protein (MCP) [36]. Injured proximal tubule cells are known to secrete proinflammatory cytokines such as TNF-α, and IL-6 [39]. In this study treatment with MSC led to a reduced inflammatory state. Our goal was to determine whether a stem cell treatment during 24 hours of ex vivo warm perfusion could be useful in accelerating the replacement of lethally injured renal cells following ischemic injury that leads to ATN. MSC treatment of ischemically damaged human kidneys resulted in a significant increase in ATP concentration compared to the paired untreated control kidneys. Of note, ATP was significantly increased in the medulla where the S3 segment tubules known to be particularly sensitive to WI are located.

With ATP depletion, disruption of the cytoskeleton occurs leading to further damage to mitochondrial function that in turn interferes with production of ATP [40]. ATP depletion, particularly in the S3 segment of the proximal tubule, leads to the disruption of cell–cell junctional complexes that maintain the cytoskeletal integrity [41, 42]. The increased availability of ATP correlated with increased synthetic functions that are dependent upon cellular energetics. The increased synthetic functions associated with MSC treatment included significantly increased synthesis of the growth factors involved with renal regeneration.

The difference in the repair of the cytoskeletal integrity was not significant between the test and control human kidneys at 24 hours of ex vivo warm perfusion. This observation was not surprising given our previous work where significant repair to the cytoskeleton of the renal cells with a prelethal insult was observed by 24 hours of warm perfusion. In lethally injured renal cells no cellular repair can be expected since the cells would need to be replaced rather than repaired. The observation of mitosis during 24 hours of ex vivo warm perfusion provides irrefutable evidence of actual renal regeneration in damaged human kidneys. A significant increase in the number of renal cells undergoing mitosis was observed in association with MSC treatment. Publications pertaining to warm perfusion of liver, lung and kidney allografts have previously used terminology such as “conditioning, reanimation, recovery, repair, etc.” to define the benefit of warm perfusion. However, actual repair on a cellular basis via mitosis has not been previously demonstrated.

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We now wish to move forward with additional studies where we will extend the period of warm perfusion to several days to attempt a more complete repair of tubule damage. The irrefutable evidence of actual renal regeneration resulting in normal, life-sustaining function awaits our future planned preclinical kidney transplantation studies. The ability to regenerate damaged kidney allografts ex vivo following a severe ischemic insult sufficiently to result in immediate function upon reimplantation will be the goal of future studies. If successful, this approach could open the way to significantly expanding donor criteria by procuring kidney allografts from the uDCD.

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Abbreviations:

ESRD	End Stage Renal Disease
DGF	Delayed Graft Function
WI	Warm Ischemic
CI	Cold Ischemic
uDCD	Uncontrolled Donation after Cardiac Death
cDCD	Controlled Donation after Cardiac Death
DBD	Donation after Brain Death
EMS	Exsanguinous Metabolic Support
MSC	Mesenchymal Stem Cell
ZO-1	Zonal Occludens 1
PCNA	Proliferating Cell Nuclear Antigen
ICU	Intensive Care Unit
OPO	Organ Procurement Organization
IRB	Institutional Review Board
NDRI	National Disease Research Interchange
DNA	Deoxyribonucleic Acid
ATP	Adenosine Triphosphate
PNF	Primary Nonfunction
ATN	Acute Tubular Necrosis

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References

1. Ethics Committee, American College of Critical Care Medicine, Society of Critical Care Medicine. Recommendations for non-heartbeating organ donation. Crit Care Med. 2001;29:1826–30. [PubMed: 11546995]
2. McCall SJ, Tuttle-Newhall JE, Howell DN, et al. Prognostic significance of microvascular thrombosis in donor kidney allograft biopsies. Transplantation. 2003;75:1847. [PubMed: 12811244]
3. Oliver J, MacDowell M, Tracy A. Pathogenesis of acute renal failure associated with traumatic and toxic injury. Renal Ischemic nephrotoxic damage and the ischemic episode. J Clin Invest. 1951;30:1307–17. [PubMed: 14897900]
4. Dunnill MS. A review of the pathology and pathogenesis of acute renal failure due to acute tubular necrosis. J Clin Invest. 1974;27:2–12.
5. Solez K, Morel-Maroger L, Straer JD. The morphology of acute tubular necrosis in man. Medicine (Baltimore). 1979;58:362–70. [PubMed: 481195]
6. Jones K Ultrastructure of human acute renal failure. Lab Invest. 1982;46:254–8. [PubMed: 7199604]
7. Pagtalunan ME, Olson JL, Tilney NL, Meyer TN. Late consequences of acute ischemic Injury to a solitary kidney. J AM Soc Nephrol. 1999;10:366–73. [PubMed: 10215337]
8. Nankivell BJ, Borrows RJ, Fung CL, O'Connell PJ, Allen RD, Chapman JR. The natural history of chronic allograft nephropathy. N Eng J Med. 2003;349:2326–2333.
9. Pascual M, Theruvath T, Kawai T, Tolkoff -Rubin W, Cosimi AB. Strategies to improve long-term outcomes after renal transplantation. N Eng J Med. 2002;346:580–590.
10. Harris S, Coupes BM, Roberts SA, Roberts IS, Short CD, Brenchley PE. TGF-beta1 in chronic allograft nephropathy following renal transplant. J Nephrol. 2007;20:177–185. [PubMed: 17514622]
11. Du C, Guan Q, Yin Z, Masterson M, Zhong R, Jevnikar AM. Renal tubular epithelial cell apoptosis by Fas-FasL-dependant self-injury can augment renal allograft injury. Transpl Proc. 2003;35:2481–2482.
12. Du C, Jiang J, Guan Q, et al. Renal tubular epithelial cell self-injury through Fas/Fas ligand interaction promotes renal allograft injury. Am J Transpl. 2004;4:1583–1594.
13. Boonstra JG, vander Woude FJ, Wever PC, Laterveer JC, Daha MR, Van Kooten C. Expression and function of Fas (CD95) on human renal tubular epithelial cells. J Am Soc Nephrol. 1997;8:1517–1524. [PubMed: 9335379]
14. Adair A, Mitchell DR, Kipari T, et al. Peritubular capillary rarefaction and lymphangiogenesis in chronic allograft failure. Transplantation. 2007;83:1542–1550. [PubMed: 17589335]
15. Doucet C, Milin S, Favreau F, et al. A p38 mitogen-activated protein kinase inhibitor protects against renal damage in a non-heartbeating donor model. Am J Physiol. 2008;295:F179–91.
16. Kohmoto J, Nakao A, Sugimto R, et al. Carbon monoxide- saturated preservation solution protects lung grafts from ischemic-reperfusion injury. J Thorac Cardiovasc Surg. 2008;136:1067–75. [PubMed: 18954651]
17. McCall SJ, Tuttle-Newhall JE, Howell DN, et al. Prognostic significance of microvascular thrombosis in donor kidney allograft biopsies. Transplantation. 2003;75:1847. [PubMed: 12811244]
18. Lysaght MJ. Maintenance dialysis population dynamics: Current trends and long-term implications. J Nephrol. 2002;13:S37–40.
19. Brasile L, Stubenitsky B, Booster M, Arenada D, Haisch C, Kootstra G Hypothermia -a Limiting Factor in Using Warm Ischemically Damaged Kidneys. Am J Transplant. 2000;1:316–320.
20. Brasile L, Stubenitsky BM, Booster MH, et al. Overcoming severe renal ischemia- the role of *ex vivo* warm perfusion. Transplantation. 2002;73:897–901. [PubMed: 11923688]
21. Atoui R, Asenjo JF, Duong M, Chen G, Chiu RC, Shum-Tim D. Marrow stromal cells as universal donor cells for myocardial regenerative therapy: Their unique immune tolerance. Ann Thorac Surg. 2008;85:571–9. [PubMed: 18222266]

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22. Chen L, Tredget EE, Liu C, Wu Y. Analysis of allogenicity of mesenchymal stem cells in engraftment and wound healing in mice. PLoS One. 2009;4:e7119. [PubMed: 19771171]
23. Tse WT, Pendleton JD, Beyer WM, Egalka MC, Guinan EC. Suppression of allogeneic T-cell proliferation by human marrow stromal cells: Implications in transplantation. Transplantation. 2003;75:389–97. [PubMed: 12589164]
24. Togel F, Hu Z, Weiss K, et al. Administered mesenchymal stem cells protect against ischemic acute renal failure through differentiation-independent mechanisms. Am J Renal Physiol. 2005;289:F31–42.
25. Lange C, Togel F, Ittrich H, et al. Administered mesenchymal stem cells enhance recovery form ischemia/reperfusion-induced acute renal failure in rats. Kidney Int. 2005;68:1613–17. [PubMed: 16164638]
26. Bi B, Schmitt R, Israilova M, et al. Stromal cells protect against acute tubular injury via an endocrine effect. J Am Soc Nephrol. 2007;18:2486–2496. [PubMed: 17656474]
27. Lin F, Petal V, Li L, et al. Direct evidence of renal regeneration from mature tubular epithelial cells in mice expressing tamoxifen-inducible epithelial specific cre recombinase (creERT2ksp). J Am Soc Nephrol. 2006;17:F-SA-DS412.
28. Bonventre JV. Dedifferentiation and Proliferation of Surviving Epithelial Cells in Acute Renal Failure. J Am Soc Nephrol. 2003;14:S55–S61. [PubMed: 12761240]
29. Togel F, Isaac J, Hu Z, et al. Renal SDF-1 signals mobilization and homing of CXCR4-positive cells to the kidney after ischemic injury. Kidney Int. 2005;67:1772–84. [PubMed: 15840024]
30. Gao JE, Muzic RF, Lundberg M, Caplan AI. The dynamic in vivo distribution of bone marrow-derived mesenchymal stem cells after infusion. Cells Tissues Organs. 2001;169:12–20. [PubMed: 11340257]
31. Burst VR, Gillis M, Putsch F, et al. Poor cell survival limits the beneficial impact of mesenchymal stem cell transplantation on acute kidney injury. Nephron Exp Nephrol. 2010;114:e107–e116. [PubMed: 19955830]
32. Brasile L, Stubenitsky BM, Haisch C, Kon M, Kootstra G. Repair of Damaged Organs In Vitro. Am J Transplant. 2005;5:300–306. [PubMed: 15643989]
33. Ledda-Columbano G, Columbano A, Coni P, Curto M, Faa G, Pani P. Cell Proliferation in Rat Kidney induced by 1,2-dibromoethane. Toxicol Lett. 1987;37:85–90. [PubMed: 3296322]
34. Brasile L, Stubenitsky BM, Booster MH, Haisch C, Kootstra G. NOS: the underlying mechanism preserving vascular integrity and during ex vivo warm kidney perfusion. Am J Transplant. 2003;3:674–9. [PubMed: 12780558]
35. Thadhani R, Pascual M, Bonventre JV. Acute renal failure. N Engl J Med. 1996;334:1448–1460. [PubMed: 8618585]
36. Toback FG. Regeneration after acute tubular necrosis. Kidney Int. 1992;41:226–246. [PubMed: 1593859]
37. Hammerman MR, Miller SB. Therapeutic use of growth factors in renal failure. J Am Soc Nephrol. 1994;5:1–11. [PubMed: 7948775]
38. Neto JS, Nakao A, Kimizuka K, et al. Protection of transplant-induced renal ischemia-reperfusion injury with carbon monoxide. Am J Physiol Renal Physiol. 2004;287:F979–989. [PubMed: 15292046]
39. Li L, Okusa MD. Macrophages, dendritic cells, and kidney ischemia-reperfusion injury. Semin Nephrol. 2010;30:268–277. [PubMed: 20620671]
40. Furuichi K, Wada T, Iwata Y, et al. CCR2 signaling contributes to ischemia-reperfusion injuryin kidney. J Am Soc Nephrol. 2003;14:2503–2515. [PubMed: 14514728]
41. Bonventre JV. Mechanisms of ischemic acute renal failure. Kidney Int. 1993;43:1160–1178. [PubMed: 8510397]
42. Brown D, Lee R, Bonventre JV. Redistribution of villin to proximal tubule basolateral membranes after ischemia and reperfusion. Am J Physiol. 1997;273:F1003–F1012. [PubMed: 9435690]
43. Abbate M, Bonventre JV, Brown D. The microtubule network of renal epithelial cells is disrupted by ischemia and reperfusion. Am J Physiol. 1994;267:F971–F978. [PubMed: 7810705]

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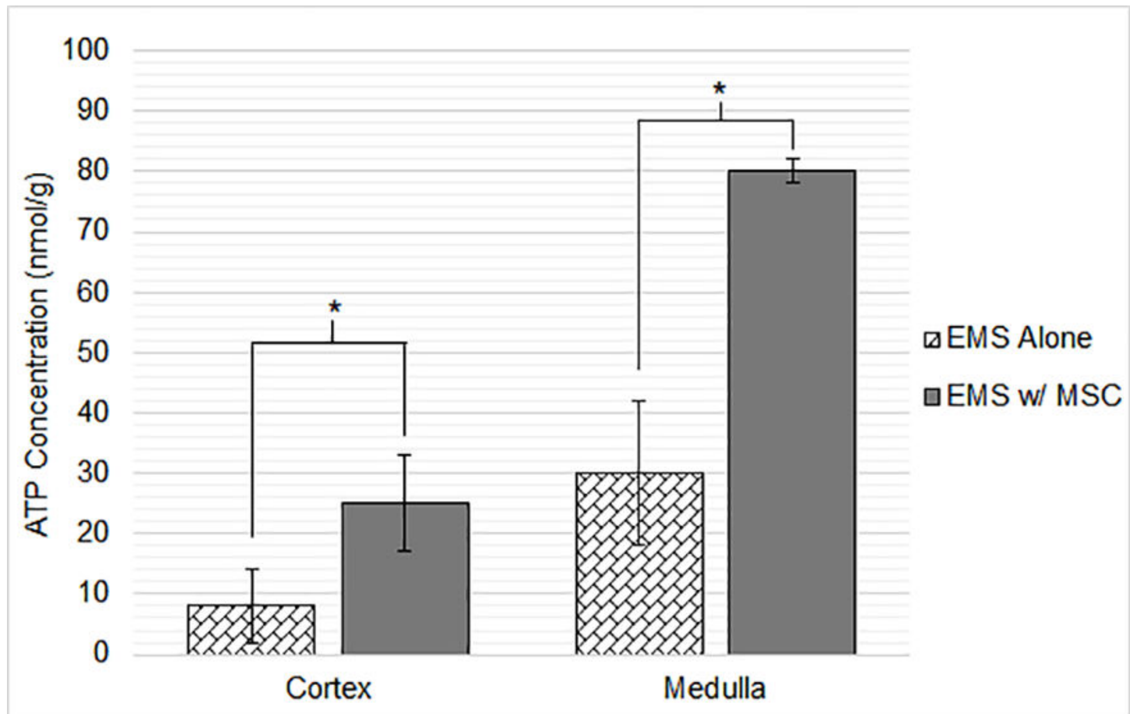

Figure 1: Pathways of Regeneration (ATP Synthesis)

ATP concentration was tested in human renal biopsies following 24 hours of Exsanguinous Metabolic Support. In test kidneys, mesenchymal stem cells were administered intra-arterially and compared to EMS treatment alone in the paired kidney. MSC treated human kidneys showed a significant increase in ATP concentration compared to controls in samples taken from the cortex and medulla (*denotes a ($p<0.05$)). ($n = 5$ pairs).

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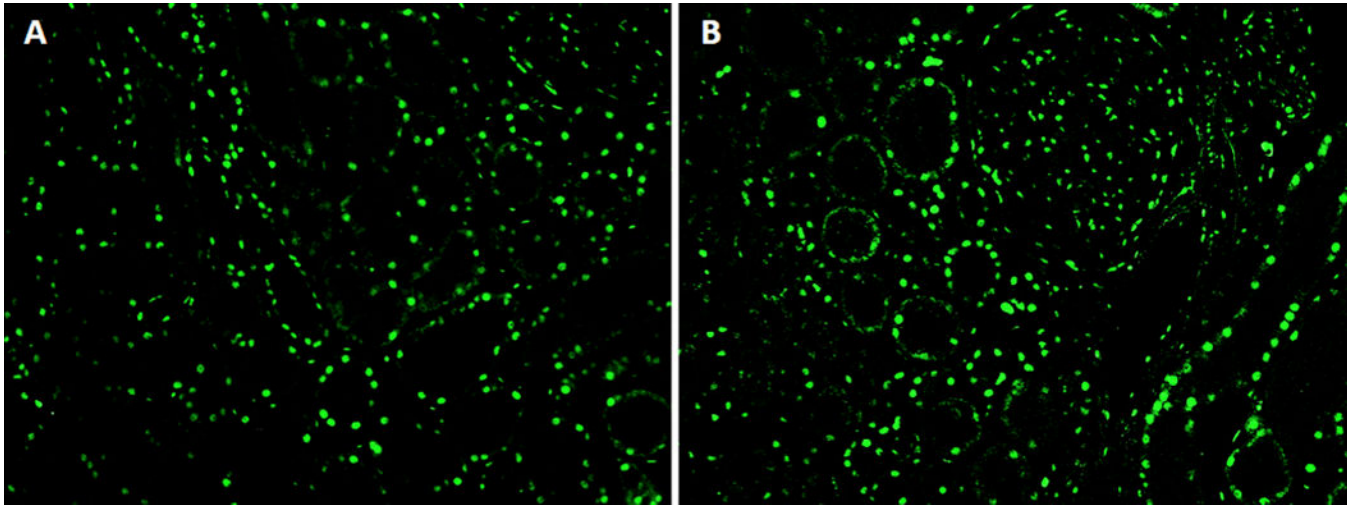

Figure 2: Pathways of Regeneration (DNA Synthesis)

Human renal biopsies were taken following 24 hours of Exsanguinous Metabolic Support with or without the addition of 1×10^8 mesenchymal stem cells. Tissue samples were fixed in 4% formaldehyde, processed and embedded in paraffin wax for sectioning. An indirect immunofluorescent assay was performed using a Mouse Anti-PCNA antibody (ABCAM) and Goat Anti-Mouse Alexa Fluor 488 secondary. A.) Represents PCNA expression after 24H of EMS perfusion B.) MSC treated human kidneys resulted in a significant increase in PCNA positive nuclei in the paired test kidneys ($p<0.049$) (n=5 pairs).

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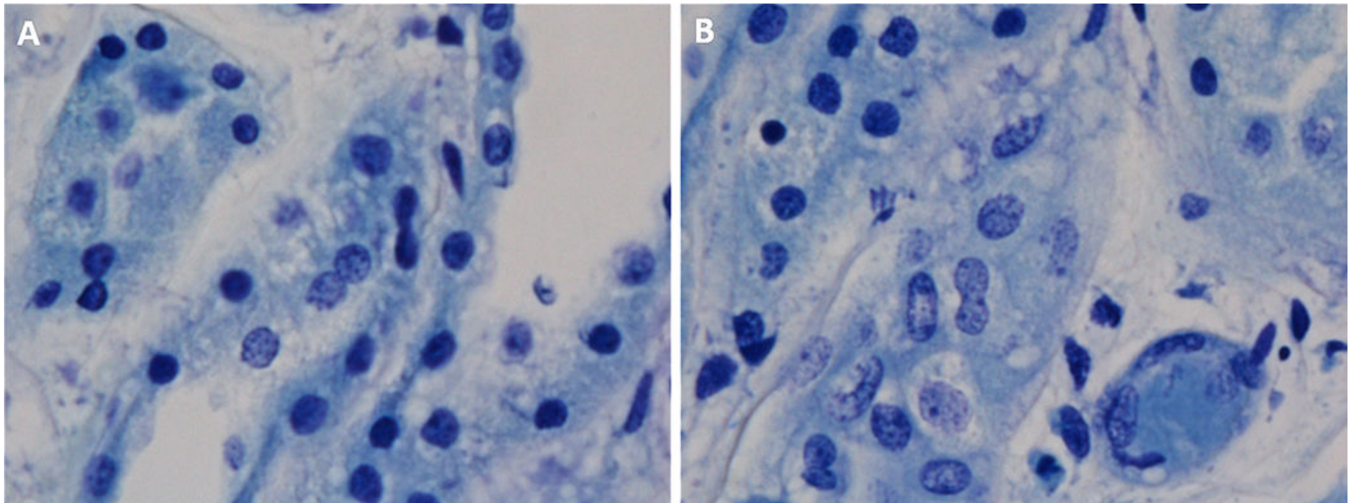

Figure 3: Pathways of Regeneration (Mitosis)

Human kidney samples were taken following 24 hours of warm perfusion with or without the addition of 1×10^8 mesenchymal stem cells. Tissue samples were fixed in 4% formaldehyde, processed and embedded in paraffin wax for sectioning. Toluidine Blue staining was performed to help visualize the presence of mitotic figures. A.) Mitotic figures after EMS perfusion. B. Mitotic figures after EMS with the addition of MSC had a 26% increase in prevalence in comparison to the paired controlled kidneys that were warm perfused alone ($p<0.05$) (n=5 pairs).

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Table 1:

Donor Characteristics of Paired Human Kidneys

Donor Pairs	Age	Sex	Cause of Death	Reason for Discard	Donor Type	WIT (Minutes)	CI (Hours)	Hypertension	HCV	Diabetes
1	25	Male	Drug Overdose	IV Drug Use	DCD	Unknown *	26	No	Yes	No
2	54	Female	Head Trauma	List Exhausted	DCD	31 +	27	Yes	No	Yes
3	33	Male	Drug Overdose	IV Drug Use	DCD	Unknown *	21	No	Yes	No
4	59	Female	Cardiac Arrest	List Exhausted	DCD	29 +	30	No	No	No
5	48	Male	Drug Overdose	IV Drug Use	DCD	Unknown*	43	Yes	Yes	No

* Donors found asystole

+ WIT is defined as the period between declaration of death following removal of life support until kidneys are cold flushed with hypothermic solution.

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Table 2:

Effect of MSC Administration in Ischemically Damaged Human Kidneys

	Pretreatment			*Posttreatment* * *(% Change)*		
MSC Infusion	**MAP (mmHg)**	**Flow (cc/min)**	**O_2 Consumption (cc/min/g)**	**MAP (mmHg)**	**Flow (cc/min)**	**O_2 Consumption (cc/min/g)**
.25 × 10^8^	27.7	113	0.15	−7.8 ↓	23.6 ↑	42.3 ↑
.5 × 10^8^	27.7	153	0.11	−6.4 ↓	25.0 ↑	38.9 ↑
.75 × 10^8^	34.0	179	0.09	− 4.1↓	3.24 ↑	55.0 ↑
1.0 × 10^8^	34.0	150	0.11	−14.6↓	28.6 ↑	21.4 ↑
2.0 × 10^8^	27.7	95	0.15	34.1↑	21.8 ↓	−66.7 ↓

N=2 for each dose of MSC

MAP = Mean Arterial Pressure

* at 24 hours of warm perfusion

$$^{+}\text{Oxygen consumption} = \frac{\left\{(\text{PaO}_2\ \text{artery}) - \left(\text{PaO}_2\ \text{vein}\right)\ \text{X Flow Rate (liter per minute)}\right\}}{\text{Weight (g)}}$$

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Table 3:

Effect of MSC Administration on Renal Synthesis

Cytokines	**Control**	**MSC Treated**	**% Change**
Eotaxin	3590.8 ± 2666.0	2088.4 ± 1945.0	−41.8
G-CSF	8416.4 ± 1583.6	5583.5 ± 4416.5	−33.7
IL-6	3247 ± 339.5	2379.8 ± 685.2	−26.7
IP-10	2839.5 ± 1821.4	603.8 ± 532.3	−78.7
MIP-1a	4441.8 ± 139.5	2611.3 ± 1835.7	−41.2
MIP-1B	4236.2 ± 503.4	2609.1 ± 2138.7	−38.4
RANTES	1788.2 ± 1361.6	477.1 ± 449.1	−73.3
TNF-a	2700.4 ± 1933.6	767.1 ± 85.1	−71.6
MCP-3	722.4 ± 417.9	384.1 ± 364.4	−46.8
Flt-3L	50.1 ± 20.3	40.1 ± 8.8	−20.0
GM-CSF	25.1 ± 4.4	5.2 ± 3.3	−79.3
Fractalkine	169.4 ± 28.7	100.5 ± 24.7	−40.7
MDC	11.4 ± 5.7	6.1 ± 1.1	−46.5
IL-1B	53 ± 41.0	4.9 ± 0.7	−90.8
Growth Factors			
EGF	10.1 ± 4.6	15.6 ± 4.13	54.5
FGF-2	19.6 ± 11.8	29.8 ± 15.8	52.0
TGF-α	10.5 ± 2.3	22.2 ± 10.6	111.4