Fresh and cryopreserved, uncultured adipose tissue-derived stem and regenerative cells ameliorate ischemia–reperfusion-induced acute kidney injury

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Abstract

Background. Acute kidney injury (AKI) represents a major clinical problem with high mortality and limited causal treatments. The use of cell therapy has been suggested as a potential modality to improve the course and outcome of AKI.

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Methods. We investigated the possible renoprotection of freshly isolated, uncultured adipose tissue-derived stem and regenerative cells (ADRCs) before and after cryopreservation in a rat ischemia–reperfusion (I–R) model of AKI.

Results. We demonstrated that ADRC therapy drastically reduced mortality (survival 100% vs. 57%, ADRC vs. controls, respectively) and significantly reduced serum creatinine (sCr on Day 3: 3.03 ± 1.58 vs. 7.37 ± 2.32 mg/dL, ADRC vs. controls, respectively). Histological analysis further validated a significantly reduced intratubular cast formation, ameliorated acute tubular epithelial cell necrosis and mitigated macrophage infiltration. Furthermore, a reduced RNA expression of CXCL2 and IL-6 cast formation, ameliorated acute tubular epithelial cell necrosis (sCr on Day 3: 4.64 ± 2.43 vs. 7.24 ± 1.40 mg/dL in controls).

Conclusions. Collectively, these results suggest a potential clinical role for ADRC therapy in patients with AKI. Importantly, cryopreservation of ADRCs could offer an autologous treatment strategy for patients who are at high risk for AKI during planned interventions.

Keywords: acute renal failure; adipose tissue-derived stem and regenerative cells; adult stem cells; cell therapy; ischemia–reperfusion injury

Introduction

Kidney disease is a leading cause of morbidity and mortality in hospitalized patients and represents an annual cost of at least $32 billion for the care of end-stage renal disease alone, representing more than a quarter of annual Medicare expenditures [1]. Currently, acute kidney injury (AKI) is diagnosed in >300,000 Americans annually [1] and is defined by an abrupt and sustained impairment of renal function [2–5] that can be initiated by various insults, including ischemia, bacterial infections and nephrotoxins. Renal ischemia is often a secondary result of procedures such as cardiopulmonary bypass, nephron-sparing surgery and kidney transplantation, and is the most common initiator of AKI [6–9].

Despite advances in modern medical technology, no effective therapies for AKI beyond supportive treatment are currently available [10,11]. While still in the early stages of research, recent advancements in cell-based therapies offer new potential therapeutics for the treatment of AKI [12].

One such novel potential therapy for AKI lies within the regenerative properties of adipose tissue-derived stem and regenerative cells (ADRCs), which are an easily accessible and abundant source of regenerative cells available for real-time, autologous use. They can be isolated in large quantities by a minimally invasive liposuction, requiring no ex vivo expansion, thus making it an appealing source for immediate cell-based therapies [13,14]. Furthermore, the therapeutic benefits of freshly isolated ADRCs have been shown recently in large and small animal models of acute myocardial infarction [15,16].

It is important to note that ADRCs are composed of several cell populations, including adipose-derived stem cells (ADSC), endothelial cells, endothelial progenitor cells and vascular smooth muscle cells as well as others which have been described elsewhere [17].

While preclinical studies have shown that cultured adipose-derived cells are beneficial in cisplatin-induced AKI, the efficacy of freshly isolated and cryopreserved ADRCs in ischemic AKI has yet to be demonstrated [18]. Thus, we investigated the therapeutic efficacy of both freshly isolated and cryopreserved, uncultured, syngeneic ADRCs in a clinically relevant rat AKI model of ischemia–reperfusion (I–R).

Materials and methods

Model of I–R injury

All experimental procedures were approved by the in-house Animal Care and Use Committee. Renal I–R was performed as previously described with minor modifications (Supplementary data) [19].

Experimental groups

Two separate, blinded experiments were performed to evaluate the efficacy of freshly isolated ADRCs and cryopreserved ADRCs. A total of 57 rats were subjected to bilateral renal arterial and venous clamping in the fresh group. Approximately 20 min after reperfusion, animals were randomized and received an intra-arterial infusion of 200 μL of either vehicle control (phosphate-buffered saline, PBS) or 5 × 10^6 ADRCs. Twenty-nine rats (ADRC: n = 15 and Control: n = 14) had serum creatinine (sCr) monitored prior to surgery (baseline) and daily for 1 week after AKI. Survival rates were recorded daily. For mechanism evaluation, 28 rats were euthanized at 5 min (ADRC: n = 2), 2 h (ADRC: n = 6 and Control: n = 3), 24 h (ADRC: n = 6 and Control: n = 3) or 72 h post-surgery (ADRC: n = 5 and Control: n = 3). In 10 of the ADRC-treated rats (n = 3 each at the 2- and 24-h sacrifice time points, and n = 2 at the 5-min and 72-h sacrifice time points), cells were DiI-labeled for tracking ADRC engraftment within the kidneys. The remaining rats (ADRC: n = 3 and Control: n = 3 at each time point) that were sacrificed at 2 and 24 h post-AKI had one kidney snap-frozen in liquid nitrogen and stored at −80°C (for RNA isolation), while the other kidney was used for histology. All six rats (ADRC: n = 3 and Control: n = 3) sacrificed at 72 h post-AKI were evaluated histologically.

In the second experiment, 19 rats were randomly assigned to the two different groups, and received an intra-arterial infusion of 200 μL of either vehicle (Control: n = 9) or 5 × 10^6 recovered ADRCs from cryopreservation (n = 10). sCr was evaluated on Day 1–5 and 7 after AKI, with daily monitoring of animal survival.

Isolation of ADRCs

ADRCs were isolated from adult male Fisher 344 rats (100–200 g) as previously described with minor modifications [16]. Briefly, inguinal subcutaneous adipose tissue was removed and minced, then digested with 0.09% collagenase (Sigma-Aldrich, St. Louis, MO, USA) for 45 min at 37°C. The ADRC fraction was separated by centrifugation at 600 g for 5 min at 37°C. The ADRC fraction was separated by centrifugation at 600 g for 5 min and passed through 100- and 40-μm Falcon™ cell strainers (BD Biosciences, San Jose, CA, USA), sequentially. Cells were washed in PBS and incubated with Intravasc™ (Cytori Therapeutics, San Diego, CA, USA) for 10 min followed by more PBS washing. ADRCs were re-suspended at 25 × 10^6 cells/mL in PBS.

ADRC labeling for tracking

Freshly isolated ADRCs were labeled with Vybrant® DiI (Supplementary data).
ADRC cryopreservation

Briefly, ADRCs were isolated and frozen in 10% syngeneic Fisher 344 rat serum and 10% dimethyl sulphoxide in lactated Ringer’s solution using a Cryogenic Control Rate Freezer 2000 (MVE Biological Systems, Marietta, GA, USA) presenting optimized conditions for recovery and viability. Cooling was performed at −1°C/min from 4°C to −50°C, and at −10°C/min to −80°C. Then, cells were stored in liquid nitrogen for at least 48 h. Prior to infusion, cells were thawed rapidly and re-suspended in 10× volume of PBS. The cells were centrifuged at 400 g for 10 min, washed in PBS and re-suspended at 25 × 10⁶ cells/mL.

Flow cytometric analysis of ADRCs

Flow cytometric studies were performed on fresh and cryopreserved cells (Supplementary data).

Assessment of renal function

sCr concentration was measured (Supplementary data).

Histology and immunohistochemistry

Sections from post-AKI Day 3 kidneys (n = 3 for each group) were stained with hematoxylin and eosin. Tubular necrosis [20] and intratubular cast formation [21] were scored as described with modifications (Supplementary data). Other kidneys were collected at 24 h after AKI for Ki-67 staining (Supplementary data).

For the assessment of in vivo cell tracking, sections were mounted with Vectashield mounting medium with DAPI (Supplementary data).

The long-term outcome after ADRC administration was evaluated through histology and macroscopic pathology 3 months after cell injection in all surviving animals of the fresh and cryopreservation group (fresh ADRCs efficacy study: Control n = 6; fresh ADRCs treated: n = 9; and cryopreserved ADRCs efficacy study: Control n = 3; cryopreserved ADRCs treated: n = 9) (Supplementary data).

PCR array and TaqMan RT-PCR

Angiogenesis PCR arrays were used for preliminary screen, and candidate genes were further validated with TaqMan real-time PCR system. Specific TaqMan primers and probes for interleukin-6 (IL-6) and chemokine (C-X-C motif) ligand 2 (CXCL2) were applied (Supplementary data) [22].

Statistical analysis

All data were expressed as mean ± standard deviation (SD) and analysed using (as appropriate) Student’s t-test, ANOVA, Kaplan–Meier method and log-rank as well as Wilcoxon/Kruskal–Wallis test (JMP 7 software, SAS Institute, Cary, NC, USA) with a P-value ≤0.05 considered significant.

Results

Characteristics of ADRCs

The cell yield of freshly isolated ADRCs was 1.24 ± 0.39 × 10⁶ cells per gram of the pooled rat adipose tissue (n = 9). Flow cytometric evaluation of these cells showed that they comprised an average of ~44% CD45+ cells (blood- and tissue-derived leukocytes), 3.4% endothelial cells (CD45−/CD31+), and 51% cells that expressed neither CD45 nor CD31 (Supplemental Figure 1A, see online supplementary material for a color version of this figure). CD11b+ cells (neutrophils, monocytes and tissue macrophages) comprised the majority of CD45+ cells, while CD73 and CD90 were expressed by the majority of CD45− cells (Supplemental Figure 1B–D, see online supplementary material for a color version of this figure). Epitope expression was retained following cryopreservation (Supplemental Table 1) with a slight reduction in the frequency of CD45+/CD11b+ cells and a corresponding increase in CD45−/CD31+ cells consistent with greater sensitivity of neutrophils to freezing/thawing.

ADRC treatment abolished AKI-induced mortality

This I–R model of AKI resulted in a high mortality (43–67%) in rats that received control treatment with a peak seen between 3 and 5 days after injury (Figures 1 and 2). As shown in Figure 1, the infusion of 5 × 10⁶ freshly isolated ADRCs rescued all animals from acute mortality, resulting in a significant difference in survival (100% vs. 0% at Day 7).
ADRCs preserve post-AKI renal function

57%, \( P = 0.005, \) log-rank test). Similarly, animals treated with \( 5 \times 10^6 \) cryopreserved ADRCs also showed a significant improvement in survival. As demonstrated in Figure 2, only 33% of the control rats survived compared with 90% of the ADRC-treated group (\( P = 0.019, \) log-rank test).

**ADRC administration improved renal functional recovery**

The baseline (prior to I–R injury) sCr value of all rats was similar (Figures 3 and 4) with a significant elevation at 1 day post-AKI in all animals. However, while the sCr level in control rats continued to rise through Day 3, rats treated with ADRCs showed a significantly accelerated recovery with overall lower sCr value. It is important to consider that while the control rats also demonstrated recovery of sCr level, albeit delayed, these data are skewed as these only include surviving animals leading to fewer animals for comparison after Day 3. The largest difference in sCr level was observed on post-AKI Day 3 (sCr: 3.03 ± 1.58 vs. 7.37 ± 2.32 mg/dL, \( P < 0.0001, \) ADRC vs. Control).

![Figure 2. Kaplan–Meier survival curves demonstrating that cryopreserved ADRCs also dramatically improved survival after ischemic AKI. \( P = 0.019 \) vs. control (PBS-treated) group (log-rank test).](image)

![Figure 3. Renal function was evaluated by determining serum creatinine (milligram per decilitre) levels over 7 days after ischemic AKI. Rats infused with freshly isolated ADRCs (\( n = 15 \)) presented a significant decrease in sCr values at Day 1–7 after ischemia compared with control (PBS-treated) rats (\( n = 14 \)). ****\( P < 0.0001, **\( P < 0.01, *\) \( P < 0.05 \) vs. control (PBS-treated) group.](image)
but significant differences could be seen as early as 24 h after cell administration (Figure 3). Cryopreserved ADRCs showed a similar response in sCr values with the largest difference seen on Day 3 as well (4.64 ± 2.43 vs. 7.24 ± 1.40 mg/dL, *P* < 0.05, ADRC vs. Control, Figure 4).

**ADRC infusion dramatically attenuated acute tubular necrosis and intratubular cast formation**

Histological analysis revealed that I–R injury leads directly to acute tubular necrosis and cast formation, both of which are rarely found in healthy kidneys (Figure 5). Examination of kidneys obtained from control (PBS-treated) animals at 72 h after AKI (the time point when the majority of animals are still alive in the control group and creatinine differences are maximal between groups) demonstrated a significant degree of renal injury and exhibited degeneration of tubular structures including severe tubular necrosis, loss of brush border and tubular dilatation (Figure 5). Cast formation was found in the cortex and outer medullar region in control rats and was almost absent in healthy kidneys. There was significant reduction in cast formation in rats treated with ADRCs compared with controls on post-AKI Day 3 (0.75 ± 0.50 vs. 3.97 ± 0.17; *P* < 0.0001 ADRC vs. control animals, Figures 5D,E,F and 6).

Quantification of tubular injury/degeneration demonstrated the extent of tubular damage (3.50 ± 0.79 vs. 0.11 ± 0.32; *P* < 0.0001 Control vs. normal kidney, Supplementary Figure 3, see online supplementary material for a color version of this figure). ADRC administration markedly reduced the severity of acute tubular necrosis compared with kidneys obtained from control (PBS-treated) animals at 72 h post-AKI (0.39 ± 0.50 vs. 3.50 ± 0.79; *P* < 0.0001 ADRC vs. Control, Figure 5A,B,C andSupplementary Figure 2, see online supplementary material for a color version of this figure).

**ADRC engraftment in the injured kidney**

ADRCs were detected by the presence of DiL in the glomeruli as early as 5 min after infusion (Figure 7A). This staining was still evident at 2 h but declined thereafter with a reduced intensity at 24 h (Figure 7B and C). However, staining was still detectable within the glomeruli at 72 h after ADRC administration (Figure 7D). Faint Di staining of ADRCs could be seen in the tubular region as early as 2 h after infusion (Supplemental Figure 3, see online supplementary material for a color version of this figure) and up to 72 h (data not shown) at which time it could no longer be clearly distinguished from background fluorescence.

**ADRCs promoted tubular epithelial cell proliferation**

Abundant Ki-67-positive staining was found in the distal and proximal tubular region of kidneys treated with ADRCs at Day 1 (Supplemental Figure 4, see online supplementary material for a color version of this figure). Ki-67-positive cells were rare in control (PBS-treated) animals (0 ± 1 Ki-67-positive cells/visual field vs. 8 ± 6 Ki-67-positive cells/visual field in control and ADRC animals, respectively; *P* < 0.0001, Supplemental Figure 5, see online supplementary material for a color version of this figure).

**ADRC therapy significantly decreased CD68-positive macrophage infiltration**

Control animals exhibited prominent infiltration of CD68-positive macrophages (Figure 8C) in the tu-
lointerstitial compartment of the renal cortex and outer medulla in kidneys at 3 days post-AKI, consistent with the acute inflammatory response following I–R injury. In contrast, treatment with ADRCs resulted in a 25-fold decrease in CD68-positive cell infiltration (6 ± 7 vs. 154 ± 75 cells/visual field in ADRC and Control, respectively; P < 0.0001; Supplemental Figure 6, see online supplementary material for a color version of this figure).

ADRC treatment downregulated the inflammatory-related gene expression

The expression of CXCL2 and IL-6 was assessed at 2 and 24 h post-AKI after those genes were identified to be significantly regulated in the PCR array (Supplemental Table 2). We further validated a significant downregulation of both CXCL2 and IL-6 mRNA expression at 24 h after AKI in the ADRC-treated animals compared with control (Figure 9).

ADRC administration did not cause abnormalities in kidneys or other organs

The long-term consequences of the ADRC administration was assessed in the surviving animals after 3 months by microscopically evaluating the kidneys. Furthermore, the lungs, heart, liver and spleen were assessed macroscopically. Histological analysis of the kidneys did not show any abnormal tissue growth or other abnormalities. Also, no abnormal tissue growth could be detected macroscopically in any of the other organs.
Discussion

Extensive research has been performed to investigate the therapeutic utility of cells derived from adipose tissue as well as other tissue sources. While initial theories of the regenerative potential of adult stem cells, including cultured adipose-derived cells, were focused on their ability to differentiate into multiple mature cell phenotypes [23], it is increasingly apparent that adult stem cell therapy can provide substantial benefit through mechanisms other than differentiation. This is particularly evident in ischemia or toxin-induced injury in which cell therapy appears to act primarily through the secretion of paracrine therapeutic factors. For instance, the administration of culture-expanded adipose-derived cells, as well as their conditioned medium, has proven to be beneficial in a cisplatin-induced renal injury model [18].

Here, we have used the I–R-induced renal injury model in rats, which is a well-established preclinical model pertinent to AKI [11]. Several routes of cell injection had been contemplated, and intra-arterial administration was chosen to provide the highest local cell concentration by avoiding filtering organs [24–26]. While arterial infusions of cultured cells (diameter usually >20 μm due to culture hypertrophy) have reportedly caused microvascular obstruction, it is important to note that the diameter of freshly isolated ADRCs is ~11 μm and therefore close to the capillary diameter [27,28]. The safety of intracoronary delivery of ADRCs has been shown in porcine models and is being tested in clinical trials with no reported adverse infusional events (ClinicalTrials.gov NCT00442806) [15,29]. Furthermore, ADRC injection into the left ventricle with an identical dose used in this study was tested to be safe in a rat model of myocardial infarction [16]. Overall, delivery of ADRCs into the renal artery would be desirable in a clinical setting, and we therefore used the carotid cannulation to deliver into the aorta as an approximation for delivery into the renal artery [30].

The present study is the first to demonstrate that culture expansion is not a necessary component of cell therapy, and that freshly isolated, syngeneic ADRCs have the ability to salvage renal function and prevent mortality in a preclinical rat model of AKI. The multiple end points described here suggest that ADRC therapy changes the inflammatory response and augments the regenerative response starting early in the injury process. In particular, 24 h after cell delivery, renal function was significantly improved, tubular cell proliferation was increased and a reduction in CXCL2 and IL-6 expression in the kidneys of ADRC-treated animals could be seen.

This was accompanied by a vast reduction of CD68-positive cell infiltration. The speed of this response combined with the low number of donor cells physically present within the kidney is inconsistent with a differentiation mechanism and indicates that ADRCs are, in some other fashion, reducing injury. This early response leads to increasing differences in serum creatinine, to different degrees of macrophage infiltration and tubular cast formation, and ultimately, to dramatic differences in mortality between treated and control animals.

Ischemic AKI is known to be associated with acute tubular necrosis, endothelial dysfunction and tubulointerstitial inflammation, which exacerbates the resultant tissue destruction post-injury [2,31,32]. Both tubular epithelial cell-derived cytokines/chemokines and endothelial cell-derived adhesion molecules have been shown to augment the inflammatory process and are associated with delayed tissue regeneration post-AKI [33–37]. Furthermore, the

![Fig. 6. Semi-quantitative scoring of cast formation at 3 days post-AKI. ***P < 0.0001 vs. control (PBS-treated) animals (Wilcoxon/Kruskal-Wallis tests).](image-url)
substantial inflammatory cell infiltration and activation, particularly of macrophages, are characteristic pathologic changes which are also observed clinically [38]. It has been shown that reduction of macrophages by liposomal clodronate significantly protects rats against I–R-induced AKI [39]. Thus, there could be an association between the observed reduction in macrophages in the kidneys and improvement in renal function.

It has been shown that the therapeutic effect of bone marrow-derived mesenchymal stem cells in sepsis is due to their ability to reprogram host monocytes/macrophages leading to a reduction in circulating IL-6. It was also reported that the circulating level of IL-6 is clinically associated with mortality in renal injury [38]. Immunosuppressive [40] or immunomodulatory properties of adipose-derived cells have been described, partially explained through inhibition or modulation of the T-cell response. They have been applied clinically in graft-versus-host disease (GVHD) associated with allogeneic hematopoietic stem cell transplantation in patients [41,42]. Based on this, the underlying mechanism of ADRCs upon infusion may also be related to systemic immunomodulatory characteristics.

The observation of a significant reduction in tubular epithelial cell necrosis may be directly correlated with the reduction in inflammation. However, it may also be plausible that ADRCs evoke a benefit by increasing cell survival under hypoxia [43]. More importantly, additional in vitro evidence indicated that freshly isolated ADRCs abundantly expressed vascular endothelial growth factor A (VEGF-A) and IGF-1 [44]. Also, a significant reduction in apoptotic/necrotic cardiomyocytes after acute myocardial infarction in rats treated with fresh ADRCs was demonstrated [16]. Overall, the relationship between inflammation and necrosis/apoptosis in this model remains unclear; however, it is likely that attenuation of both contributes to the benefit observed. Important-ly, this reduction in tubular cell necrosis/apoptosis also led to a reduction in tubular cast formation, thus explaining the maintenance of some level of renal function.

The contribution of renal resident stem/progenitor cells in the repair of renal injury has been recently postulated [45,46]. It was suggested that such beneficial effects might rely on the administration of cell-based therapies to generate a favorable environment for resident stem/progenitor cell expansion and proliferation [47]. Interestingly, we...
have also demonstrated that ADRCs enhance tubular cell proliferation as detected by an increase in Ki-67-positive cells as early as 24 h after cell infusion, while ADRCs at that time were found primarily in the glomeruli. Another finding in this study is the preserved ability of ADRCs to reduce mortality and improve renal function following AKI after cryopreservation. Long-term storage of autologous ADRCs could provide several benefits since...
it has been shown that cells have a reduced therapeutic potential as an organism ages [48,49]. Walter and colleagues have also shown that the number of endothelial progenitor cells inversely correlates with age [49]. These findings could support the rationale of cryogenic storage of ADRCs in preparation for future therapeutic use. ADRCs could then be utilized in patients who are undergoing scheduled procedures with an inherent risk of renal ischemic damage such as cardiopulmonary bypass surgery, kidney transplantation, and partial nephrectomy or when the administration of nephrotoxic agents, such as chemotherapeutics or contrast dyes, is planned.

Isolation of autologous ADRCs represents a crucial step that could affect its therapeutic effectiveness. It is therefore essential to establish a reliable and standardized ADRC isolation procedure, taking different depot and age factors into account in order to reduce variability in outcomes as much as feasible [50,51]. Furthermore, reports on clinical trials to date using ADRCs have not shown an unfavorable safety profile, but longer follow-up data are needed to draw final conclusions. In general, any cell therapy approach and clinical investigation should follow careful consideration of the risk/benefit ratio for the patient population to be studied, and i.e. intravascular cell administration needs to be considered and titrated carefully.

Collectively, the present study demonstrates that fresh and cryopreserved ADRCs significantly decrease mortality and increase renal function in a preclinical model of ischemia/reperfusion-induced AKI, offering a potential therapeutic approach. Administration of ADRCs modified the expression of pro-inflammatory cytokines CXCL2 and IL-6 as well as the infiltration of macrophages. The result was a significant reduction in tubular necrosis/apoptosis and an increase in the proliferation of tubular cells, thus improving functional and structural recovery, and overall survival.

Supplementary data

Supplementary data is available online at http://ndt.oxfordjournals.org.

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Conflict of interest statement. All authors are or have been employees of Cytori Therapeutics, Inc., and some are holders of stock and/or options.

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A novel method for isolating podocytes using magnetic-activated cell sorting

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Abstract

Background. A large body of accumulated data has now revealed that podocytes play a major role in the development of proteinuria. However, the mechanisms of podocyte injury, leading to foot process effacement and proteinuria, are still unclear partly due to the current lack of an appro-