Human mesenchymal stem cells derived from adipose tissue reduce functional and tissue damage in a rat model of chronic renal failure

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Abstract
Therapeutic approaches for CKD (chronic kidney disease) have been able to reduce proteinuria, but not diminish the disease progression. We have demonstrated beneficial effects by injection of BM (bone marrow)-derived MSCs (mesenchymal stem cells) from healthy donors in a rat model with CKD. However, it has recently been reported that BM-MSCs derived from uraemic patients failed to confer functional protection in a similar model. This suggests that autologous BM-MSCs are not suitable for the treatment of CKD. In the present study, we have explored the potential of MSCs derived from adipose tissue (AD-MSCs) as an alternative source of MSCs for the treatment of CKD. We have isolated AD-MSCs and evaluated their effect on the progression of CKD. Adult male SD (Sprague–Dawley) rats subjected to 5/6 NPX (nephrectomy) received a single intravenous infusion of 0.5 × 10⁶ AD-MSCs or MSC culture medium alone. The therapeutic effect was evaluated by plasma creatinine measurement, structural analysis and angiogenic/epitheliogenic protein expression. AD-MSCs were detected in kidney tissues from NPX animals. This group had a significant reduction in plasma creatinine levels and a lower expression of damage markers ED-1 and α-SMA (α-smooth muscle actin) (P < 0.05). In addition, treated rats exhibited a higher level of epitheliogenic [Pax-2 and BMP-7 (bone morphogenetic protein 7)] and angiogenic [VEGF (vascular endothelial growth factor)] proteins. The expression of these biomarkers of regeneration was significantly related to the improvement in renal function. Although many aspects of the cell therapy for CKD remain to be investigated, we provide evidence that AD-MSCs, a less invasive and highly available source of MSCs, exert an important therapeutic effect in this pathology.

Key words: adipose-tissue-derived mesenchymal stem cell, chronic kidney disease, nephrectomy, proteinuria, renal functional recovery

INTRODUCTION
CKD (chronic kidney disease) affects millions of people in the world and represents an important public health problem [1,2] because of the increasing incidence and prevalence and the high costs of treatment [3]. Many therapies have been developed in an attempt to reduce damage progression in CKD. A major impact has been achieved by RAAS (renin–angiotensin–aldosterone system) blockers [4,5]. Although these drugs reduce proteinuria, they have not shown significant improvement of the GFR (glomerular filtration rate) or tubular function; moreover, chronic administration of these drugs increases the risk of hyperkalaemia [5]. These facts have fuelled the search of new therapeutic strategies that can achieve better results.

Previous investigations have reported the ability of the kidney to self-repair after ischaemic ARF (acute renal failure) [6–8]. We have demonstrated in an experimental model of ARF the re-expression of epitheliogenic and angiogenic proteins involved in

Abbreviations: Ab, antibody; ARF, acute renal failure; AU, arbitrary units; BM, bone marrow; BMP-7, bone morphogenetic protein 7; BUN, blood urea nitrogen; CKD, chronic kidney disease; CM, culture medium; CMFDA, 5-chloromethylfluorescein diacotate; DAB, diaminobenzidine; EMT, epithelial–mesenchymal transition; GFR, glomerular filtration rate; H&E, haematoxylin and eosin; IHC, immunohistochemistry; mAb, monoclonal Ab; MSC, mesenchymal stem cell; AD-MSC, adipose-tissue-derived MSC; NPX, nephrectomy; PAP, peroxidase-antiperoxidase; SC, stem cell; SD, Sprague–Dawley; SR, Sirius Red; VEGF, vascular endothelial growth factor; WB, Western blotting; α-SMA, α-smooth muscle actin.

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kidney development in a similar pattern to that observed during embryogenesis [8], thus suggesting a pathway by which renal repair might be achieved.

Recent studies have reported resident kidney SCs (stem cells), supporting the idea of a possible physiological mechanism of kidney repair, based on the proliferation and differentiation of SCs [6,9–12]. MSCs (mesenchymal stem cells) have been involved in regeneration of many tissues subjected to different types of injury [13–17], including ARF [18] and recently CKD [19,20]. However, although it has been described that MSCs derived from BM (bone marrow) confer functional protection in a rat model of CKD, as evaluated by improvements in urea levels, systolic BP (blood pressure), proteinuria, GFR, glomeruloesclerosis and tubular atrophy, disease amelioration is not observed in response to uremic BM-MSCs [21].

These findings suggest that BM-MSCs from uremic patients cannot be used successfully in the treatment of CKD. Therefore this scenario requires new approaches. MSCs derived from adipose tissue (AD-MSCs) could be a new therapeutic alternative that has not been evaluated for CKD. These cells have a similar differentiation potential compared with BM-MSCs [22]; additionally, they are easy to obtain and are present in significant amounts in mammals. These findings support the hypothesis that AD-MSCs could be an interesting option for CKD treatment.

The objective of the present study was to evaluate the effect of AD-MSCs in the progression of damage in CKD. We hypothesized that injection of AD-MSCs may have positive effects in delaying or even abolishing tissue injury in this disease. We propose that AD-MSCs may stimulate kidney repair by inducing the expression of repairing proteins that, in turn, would activate genes involved in the regeneration processes. To evaluate the potential action of AD-MSCs, we performed functional, morphological and molecular studies.

MATERIALS AND METHODS

Animals

Adult male SD (Sprague–Dawley) rats (220–250 g) were maintained in 12 h light/dark cycle, with food and water ad libitum at the University animal care facilities. All procedures were in accordance with institutional and international standards for the human care and use of laboratory animals (Animal Welfare Assurance Publication A5427-01, Office for Protection from Research Risks, Division of Animal Welfare, National Institutes of Health) as described previously [23].

Isolation of human AD-MSCs and in vitro expansion

SCs derived from adult human adipose tissue were collected with the informed consent from the subjects and after approval of the ethics committee of Los Andes University by flushing the abdominal wall subcutaneous tissue with sterile PBS, passed through a 70 μm Falcon cell strainer (BD Biosciences) and centrifuged at 350 g for 10 min. After centrifugation, cells were resuspended in α-MEM (α modified Eagle’s medium; Gibco), supplemented with 10% heat-inactivated MSC-qualified FBS (fetal bovine serum; Gibco) with 100 units/ml penicillin and 100 μg/ml streptomycin (Gibco), and then cultured at a density of 1×10^6 nucleated cells/cm^2 at 37°C in a 5% CO₂ atmosphere. Non-adherent cells were removed after 48 h and CM (culture medium) was changed every 3 days. At 80% confluency cells were subcultured by treatment with TrypLE® Select (Gibco), washed and cultured at 10,000 cells/cm^2. After three passages, adherent cells were detached, washed and resuspended in X-VIVO™ medium (Lonza).

Characterization of MSCs

MSCs are characterized by their adherence, fibroblast-like morphology and capacity to differentiate into specific cell lineages: adipocytes, chondrocytes and osteoblasts. To induce adipogenic differentiation, confluent cells were cultured in the medium supplemented with 1×10⁻⁶ M dexamethasone, 0.02 mg/ml indomethacin and 10 μg/ml insulin (Sigma–Aldrich). After 12 days, cell differentiation into lipid-laden adipocytes was confirmed by Oil Red O staining (Sigma–Aldrich). For chondrogenic differentiation, cells were incubated at 5×10⁵ cells/μl in 10 μl of CM for 1 h to achieve the conditions for micromass formation. Cells were cultured in the medium supplemented with 1×10⁻⁷ M dexamethasone, 50 μg/ml ascorbic acid and 10 ng/ml of TGFβ3 (transforming growth factor β3) (Sigma–Aldrich) for 7 days, assessing chondrogenic differentiation with Safranin O staining (Merck). To induce osteogenic differentiation, adherent cells were grown at 3×10⁴ cells/cm² in CM with 1×10⁻⁷ M dexamethasone, 50 g/ml ascorbic acid and 10 mM β-glycerophosphate (Sigma–Aldrich). After 21 days of culture, calcium deposits were detected by Alizarin Red staining (Sigma–Aldrich).

By phenotypic characterization of MSC, we determined the expression of CD90, CD54, CD45 and CD38 using mAbs [monoclonal Abs (antibodies)] conjugated to FITC, and CD73, CD105, CD34 and CD44 with mAbs conjugated to PE (phycoerythin) (BD Biosciences). The flow cytometry analysis was performed on a Coulter® Epics® XL™ cytometer (Beckmann Coulter).

In vivo MSC tracking

To assess the migration of MSCs to the kidney, we performed cell-tracking experiments with dye-labelled cells [24]. Long-term tracker Green CMFDA (5-chloromethylfluorescein diacetate; Molecular Probes) was used since it is retained through several generations in living cells [25,26].

Briefly, once detached, suspension of MSC in X-VIVO™ medium was incubated with 5 μM of Green CMFDA or with DMSO (vehicle) for 30 min at 37°C, according to manufacturer’s instructions. Then, the MSC suspension was centrifuged at 350 g for 5 min and resuspended with PBS. After washing twice, the MSC suspension was centrifuged again and the pellet resuspended in X-VIVO™ medium and was equilibrated at 37°C for 30 min. Finally, rats were injected with labelled cells or MSC vehicle as described above. After 24 h rats were killed and kidneys quickly dissected, embedded in Tissue-Tek embedding, frozen with liquid nitrogen and stored at −80°C. Sagital cryostat sections (5 μm thick) were cut and fixed with ethanol (70%) for 20 min at −20°C. After washing in PBS, the slides were washed

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with distilled water and counterstained with haematoxylin as described previously [27]. Slides were observed in an Olympus BX 51WI1 upright microscope with water immersion lenses. The NIH Image program ImageJ (US National Institute of Health, http://rsweb.nih.gov/ij/) was used to perform the imaging analyses.

**CKD induced by 5/6 NPX (nephrectomy) and injection of AD-MSCs**

A model that mimics the structural and functional damage of CKD was used [28]. Rats where anaesthetized with ketamine/xylazine (25:2.5 mg/kg of body weight intraperitoneally), then a retroabdominal incision in the left flank was performed and the kidney mass was reduced by clamping two renal artery subdivisions; after 1 week, rats were subjected to a contralateral NPX. This time point was considered as the initiation of kidney damage, which was prolonged for 5 weeks. Animals were randomized in four groups: (i) Sham rats; (ii) NPX rats; (iii) NPX rats injected in a tail vein with 450 μl of X-VIVO™ medium (without MSC); and (iv) NPX rats injected in the tail vein with 450μl (0.5×10⁶ cells) of AD-MSCs in X-VIVO™ medium [29], (n = 7 for all groups). All rats were injected with the corresponding solution immediately after contralateral NPX. Rats were killed 35 days after NPX; the kidney was processed for IHC (immunohistochemistry) and WB (Western blotting).

**Functional and histological damage assessment**

Functional damage was assessed through plasma creatinine levels [30]. Tissue damage was evaluated by morphological analysis using H&E (haematoxylin and eosin), SR (Sirius Red) staining and IHC of macrophages, ED-1 and α-SMA (α-smooth muscle actin).

Tubulointerstitial injury was graded (0–5+) in a blinded manner on the basis of the presence of tubular cellularity, basement membrane thickening, TD (tubular dilation), and PT (protein cast) as follows: 0, no changes present; grade 1, 10% tubulointerstitial changes present; grade 2, 10–25% tubulointerstitial involvement; grade 3, 25–50%; grade 4, 50–75%; and grade 5, 75–100%. For each biopsy, the entire cortical and outer medullary regions were evaluated (16–30 fields of 1 mm²), and a mean score per biopsy was calculated.

**Tissue processing and IHC analysis**

Immunohistochemical studies in Paraplast-embedded sections were performed as previously described [31]. Immunolocalization studies were performed using an indirect immunoperoxidase technique [31]. Briefly, tissue sections were incubated with the primary Ab, followed by incubation with the corresponding secondary Ab and with the PAP (peroxidase–antiperoxidase) complex, revealed using 3,3’-DAB (diaminobenzidine). For some Abs, immunoreactivity was revealed using a secondary Ab conjugated to ALP (alkaline phosphatase), in the presence of NBT/BCIP (Nitro Blue Tetrazolium/5-bromo-4-chloroindol-3-yl phosphate) (4.5/3.5 μl/ml) in 100 mM Tris pH 9.5. Controls for the immunostaining procedure were prepared by omission of the first Ab and by its replacement with normal or preimmune serum of the same species [32].

**Antibodies**

The primary Ab used corresponds to the same Ab used recently by us [20,23,30,31]: Pα-2, BMP-7 (bone morphogenetic protein 7) and VEGF (vascular endothelial growth factor) were obtained from Santa Cruz Biotechnology. ED-1 was from Biosource, and α-SMA was from Sigma–Aldrich. The presence of SCs was determined by Oct-4 expression.

Secondary Ab and corresponding PAP complexes were purchased from MP Biomedicals. Other chemicals were purchased from Sigma–Aldrich.

**Immunoblotting**

Kidney sections were homogenized and protein concentration was determined as previously described [31]. WB was performed as described by Harlow and Lane [33]. For SDS/PAGE, proteins were mixed with sample buffer [100 mM Tris/HCl, pH 6.8, 200 mM DTT (dithiothreitol), 4% (w/v) SDS, 0.2% (w/v) Bromophenol Blue and 20% (v/v) glycerol], transferred on to nitrocellulose membranes and blocked as previously described [31]. After blocking, membranes were probed with the Ab, washed with TBS-T (Tris-buffered saline containing 0.05% Tween 20) and incubated with HRP (horseradish peroxidase)-conjugated secondary Ab for 2 h at room temperature (25°C). Immunoreactivity was detected using ECL (enhanced chemiluminescence) obtained from Pierce. Basal controls were obtained from Sham kidneys.

Densitometry analyses were performed using NIH Image program v1.61 (US National Institutes of Health, http://rsb.info.nih.gov/nih-image). α Tubulin total protein levels were used to correct variation in sample loading.

**Statistical analysis**

A Mann–Whitney test was used with a significance level at \( P < 0.05 \). Densitometry values are presented as means ± S.D. Values are represented by AU (arbitrary units).

**RESULTS**

**Functional characterization of MSCs**

MSCs isolated from adipose tissue of SD rats showed a stable fibroblast-like phenotype in culture. Furthermore, the AD-MSCs were able to differentiate into adipocytes, chondrocytes and osteoblasts (Figure 1a) and expressed the common markers of MSCs (Figure 1b).

**Homing of AD-MSCs to the kidney**

AD-MSCs were labelled with a cell tracker (CMFDA) to facilitate their localization within the kidneys of injected animals [24] (Figure 2a). At 24 h after injection of AD-MSCs, kidney sections counterstained with haematoxylin showed the presence of green-positive cells, observed only in animals receiving Green CMFDA-treated cells (1.63 ± 0.25 positive cells per field in four fields/kidney), but not in those treated with vehicle (CM), indicating the arrival of injected AD-MSCs to the kidney (Figure 2b).
Assessment of Oct-4 expression in the different CKD treated groups

Oct-4 is a POU family transcription factor that is expressed in embryonic and adult kidney SCs and immortalized non-tumorigenic cell lines and tumour cells, but not in differentiated cells. The presence of the renal SC marker Oct-4 was evaluated 35 days after injection. The IHC stain observed in Sham animals was minimal (Figure 3A). Oct-4 levels were inhibited in NPX animals at 35 days after surgery (Figure 3B) similar to that observed in NPX animals injected with CM (NPX-CM) (Figure 3C). NPX animals injected with AD-MSCs (NPX-AD-MSCs) had a marked increase in Oct-4 staining at 35 days after injection (Figure 3D). Oct-4 expression evaluated by WB, 35 days after injection is presented in Figure 5(E): Sham animals had basal Oct-4 expression, which was reduced in NPX animals and was slightly increased in NPX + CM animals. The NPX + AD-MSC animals had a significant increase in Oct-4 expression in comparison with all the other groups ($P < 0.05$). α Tubulin total protein level was used for normalization.

Therapeutic effect of injection of AD-MSCs on renal function

Renal function was assessed by plasma creatinine and BUN (blood urea nitrogen) levels. In NPX animals, creatinine and BUN levels levels increased (Figure 4); similarly, rats submitted to NPX + CM also had increased creatinine and BUN levels, indicating renal damage. In contrast, in both the Sham and NPX + AD-MSC groups, creatinine and BUN levels close to normal levels were found, indicating a recovery in renal function (Figure 4). H&E and SR staining of renal sections from the NPX and NPX + CM groups showed alterations consistent with chronic damage, including epithelial flattening, dilated tubules and
AD-MSCs induce an improvement in CKD

Figure 2 MSCs retain Green CMFDA in vitro and dye tracking in vivo
(a) Cultures of MSC were exposed for 30 min to the cell tracker Green CMFDA at 37° C. Microscopic images of phase contrast (left) and Green CMFDA fluorescence (right) at 72 or 96 h of incubation are shown. Scale bar, 10 μm. (b) Microscopic images show kidney sections 24 h after MSC injection incubated with vehicle (upper panels) or with the cell tracker Green CMFDA (lower panels). The nuclei were counterstained with haematoxylin (dark). The far-right-hand panel (zoom) corresponds to the area enclosed with the segmented square in the Merge panel. CM, kidney injected with CM; CM + AD-MSC, kidney injected with AD-MSC. Scale bar, 10 μm.

increased fibrotic areas [Table 1, and Figures 5a(B), 5a(C), 5a(F) and 5a(G)]. NPX animals treated with a single dose of AD-MSCs had normal glomerular and tubular morphology [Figures 5a(D) and 5a(H)], comparable with that observed in Sham kidneys [Figure 5a(A) and 5a(E)]. We did not find morphological, functional or histological differences between Sham and Sham + AD-MSCs kidneys.

Damage markers, ED-1 (macrophages) and α-SMA (myofibroblasts), were widely distributed in the renal parenchyma of NPX and NPX + CM animals (n = 7 per group) [Figures 5b(B), 5b(C), 5b(F) and 5b(G)]. However, levels of both markers were decreased in NPX + AD-MSC animals. [Figures 5b(D) and 5b(H)]. Sham rats had a normal IHC distribution of these markers [Figures 5b(A) and 5b(E)].

Localization of the angiogenic marker VEGF
At 35 days after NPX, the presence of VEGF, a transcriptional factor involved in angiogenesis, was analysed. The IHC of VEGF in Sham kidneys revealed that basal levels of this factor were observed as a weak signal (Figure 6A); however, in NPX and NPX + CM animals, VEGF was abolished (Figures 6B and 6C). The addition of AD-MSCs to NPX rats increased the level of this protein (Figure 6D). As shown in Figures 6(A)–6(D), VEGF was localized in proximal tubule cells in the inner and outer medulla.

The expression of angiogenic markers is shown in Figure 6(E). VEGF was at a basal level in Sham rats and was reduced in NPX and NPX + CM animals when measured at 35 days after damage. However, the level of this angiogenic marker was elevated in NPX + AD-MSC animals. The differences between NPX + AD-MSC and Sham, and NPX and NPX + CM groups were significant (P < 0.05). The α tubulin total protein level was used to correct for variation in sample loading.

Expression of the epithelial markers Pax-2 and BMP-7
A significant difference was detected in epithelial markers among the control and AD-MSC-treated kidneys. As shown in Figure 7(a), rats treated with AD-MSCs had a characteristic pattern of distribution of the epithelial markers Pax-2 and BMP-7. The IHC of these proteins showed minimal staining in Sham rats [Figures 7a(A) and 7a(E)] and an even weaker staining in NPX and NPX + CM kidneys [Figures 7a(B), 7a(C), 7a(F) and 7a(G)]. Injection with AD-MSCs in NPX animals (NPX + AD-MSCs) induced an increase in these markers [Figures 7a(D) and...
Figure 3  Expression and localization of renal SCs

The presence of renal MSCs was evaluated by Oct-4 marker. Oct-4 localization was evaluated by IHC in Sham (A), NPX (B) NPX + CM (C) and NPX + AD-MSC (D) kidney samples at 35 days after injection. The staining was higher in NPX + AD-MSC kidneys 35 days after injection (D). The stain was minimally observed in Sham, NPX and NPX + CM animals (A–C). Scale bar, 100 μm. Arrows show the marker localization. The immunoblot (E) shows an increased expression of Oct-4 in animals injected with AD-MSC compared with respective controls (Sham and NPX). *P < 0.05.

Figure 4  Functional renal damage

Renal function was assessed by plasma and BUN creatinine levels in Sham, NPX + CM and NPX + AD-MSC animals 35 days after injection. Sham rats showed normal plasma creatinine and BUN levels. In NPX and NPX + CM animals, the creatinine and BUN levels increased with both values being significantly higher than Sham group (*P < 0.05). In NPX + AD-MSC animals, the creatinine and BUN levels were reduced, although only the BUN levels were significantly different from those in Sham animals.

As reported previously [30] in adult kidneys of Sham animals, Pax-2 and BMP-7 are detected in the nucleus of collecting duct cells, but not in proximal tubular cells. A similar pattern was observed in the present study for Sham rats [Figures 7a(A) and 7a(E)]; whereas the expression pattern was changed to proximal tubular cells in NPX + AD-MSC animals [Figure 7a(D) and (H)].

The basal levels of Pax-2 were present in Sham kidneys, whereas, in NPX and NPX + CM animals, the expression level was even lower and was increased in NPX + AD-MSC animals (Figure 7b, left-hand panels). BMP-7 was also expressed at basal levels in Sham and was increased in NPX and NPX + CM animals (Figure 7b, right-hand panels). These differences were not significant. However, in NPX + AD-MSC animals, an increase in expression of BMP-7 was observed (Figure 7b, right-hand panels). The findings in the NPX + AD-MSC group were significantly different from the other three groups (P < 0.05). The expression of α-tubulin was used to correct for variation in sample loading.

DISCUSSION

During kidney development, a proliferation of undifferentiated cells and a later differentiation into specific cell types occurs, achieved by the sequential expression of a large number of renal
**Table 1 Renal damage indices**
Comparison of renal morphology in the NPX model treated with MSC (NPX + AD-MSCs) or vehicle (NPX) and Sham animals, 35 days after damage was induced. Macrophages abundance (ED-1), interstitial α-SMA, dilated tubules (DT), protein cast (PC) and interstitial fibrosis (IF). ∗ or †P < 0.05 compared with Sham; ‡P < 0.05 compared with NPX using one-way ANOVA.

<table>
<thead>
<tr>
<th>Group</th>
<th>ED-1 (score 0–5)</th>
<th>α-SMA (score 0–5)</th>
<th>DT (score 0–5)</th>
<th>PC (score 0–5)</th>
<th>IF (score 0–5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham (n = 7)</td>
<td>0.7 ± 0.5</td>
<td>1.0 ± 0.0</td>
<td>1.1 ± 0.7</td>
<td>0.3 ± 0.05</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td>NPX (n = 7)</td>
<td>4.8 ± 0.7∗</td>
<td>4.5 ± 0.5∗</td>
<td>4.9 ± 0.5∗</td>
<td>3.9 ± 0.7∗</td>
<td>4.2 ± 0.4∗</td>
</tr>
<tr>
<td>NPX + AD-MSCs (n = 7)</td>
<td>2.1 ± 0.2†‡</td>
<td>1.7 ± 0.4†‡</td>
<td>1.5 ± 0.5†</td>
<td>0.9 ± 0.3†‡</td>
<td>1.8 ± 0.4†</td>
</tr>
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**Figure 5** Histological NPX-induced damage evidence in CKD kidneys and renal damage markers induced by NPX in CKD kidneys

(a) The H&E and SR staining in kidney samples at 35 day post NPX was observed in Sham, NPX rats or NPX injected with CM (NPX + CM) or AD-MSCs (NPX + AD-MSCs). NPX rats showed disappearance of the brush border, epithelium, tubular and glomerular alterations (B, F), similar to that observed in NPX + CM rats (C, G). NPX + AD-MSCs showed morphological characteristics (D, H) similar to those observed in Sham rats (A, E). Scale bar, 100 μm. Arrows show the renal tissue damage. (b) The tissue damage was assessed by positive staining for macrophages (ED-1) and miofibroblasts (α-SMA) 35 days post-NPX observed in Sham, NPX or NPX injected with CM (NPX + CM) or AD-MSCs (NPX + AD-MSCs) groups. Both markers were observed in the interstitium of internal and external medulla in NPX and NPX + CM kidneys (B, C, F, G). A decreased staining of these markers was observed in NPX + AD-MSCs kidneys (D, H), comparable with kidneys of Sham rats (A, E). ED-1 and α-SMA markers were performed using peroxidase and developed with DAB (brown reaction). Scale bar, 100 μm. Arrows show the marker localization.
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Figure 6 Immunolocalization and expression of endothelial cell markers during AD-MSCs induced recovery in CKD

Sham, NPX, NPX + CM and NPX + AD-MSCs samples were stained for angiogenic factor VEGF (A-D). A maximum expression was observed in kidneys treated with AD-MSCs (D). The expression of this marker was observed in thick ascending limb of Henle and proximal tubule cells localized primarily in the internal and external medulla. The staining was performed using peroxidase and developed with DAB (brown reaction). 100 μm scale bar. Arrows show the marker localization. (E) Immunoblot studies were performed to evaluate the expression of VEGF. In animals treated with AD-MSCs (NPX + AD-MSCs), an increased expression of these markers was observed compared with the respective control. *P < 0.05.

Figures (A-D) show the immunolocalization of VEGF in different conditions. The results indicate a significant increase in VEGF expression in the kidneys treated with AD-MSCs compared to the control groups (NPX and NPX + CM). The staining was performed using peroxidase and visualized with DAB (brown reaction). The scale bar represents 100 μm.

E) Immunoblot studies were performed to further evaluate the expression of VEGF. The blots show a higher expression of VEGF in the AD-MSC-treated group compared to the control groups (NPX and NPX + CM). The increase in VEGF expression in the AD-MSC-treated group was statistically significant (P < 0.05).

Genes [8]. We have shown that in the repairing phases of kidney damage, a recapitulation of the genetic programme expressed in the organogenesis is activated [8].

MSCs have been the subject of great interest in the last few years because of their potential role in regeneration and tissue repair [34]. Their experimental and/or clinical use in acute myocardial infarction, skin and skeletal muscle regeneration and ARF, has shown encouraging results [13–17]. Furthermore, positive effects of MSC in different CKD animal models have been reported recently [35,36], as evaluated by reduction in plasma creatinine levels [34], improvements in proteinuria [37,38], renal fibrosis [39,40], glomerulosclerosis, macrophage infiltration [41], and renal function [42], and in the reduction of pro-inflammatory cytokines [43]. It has recently been proposed that BM-MSCs from healthy individuals can help to maintain the angiogenesis process in CKD being also involved in secondary repairing of CVD (cardiovascular disease) induced by CKD [44]. In addition, we have reported the beneficial use of BM-MSCs in a 5/6 NPX model, evaluated by functional parameters and the induction of epitheliogenic and angiogenic proteins [20].

However, in patients who have been longitudinally followed, CKD has been associated with a lower yield and dysfunction of their BM-MSCs [45,46]. The reduced number of BM-MSCs could be an effect of defective proliferation, shortened survival or defective mobilization from the BM. Another possibility to explain BM-MSCs dysfunction is an inflammatory mechanism, since it has been shown that inflammatory markers, such as IFN (interferon), IL-6 (interleukin 6) or CRP (C-reactive protein) can induce senescence and apoptosis [47,48]. In addition, the uraemia or some of the uraemic toxins produced in these patients, such as homocysteine [49] or p-cresol [50] may inhibit differentiation and function of BM-MSCs. Lastly, complications of CKD, such as secondary hyperparathyroidism or lack of erythropoietin, may also contribute to dysfunction of BM-MSCs [41]. In addition, the functional protection induced by healthy BM-MSCs in a rat model of 5/6 NPX is not observed with uraemic BM-MSCs [21]. These data suggest that the BM-MSCs from uraemic patients cannot be used successfully in CKD treatment.

In this context, AD-MSCs are a new alternative that have recently been evaluated in a chronic renal damage model with promising results [51]. The uses of AD-MSCs in therapeutic applications depend on the availability of tissue and the ease of in vitro expansion, and AD-MSCs have been shown to have a similar differentiation potential to that of BM-MSCs [22]. In addition, recent data have shown that AD-MSCs are not affected by renal disease [52]. These data support our hypothesis that AD-MSCs can be an interesting option for CKD treatment. In the present study, we investigated the renal effect of injection of human AD-MSCs in rats submitted to 5/6 NPX by measurements of functional, histological and molecular parameters. It was demonstrated that a single intravenous infusion of AD-MSCs was able to enhance renal reparative processes and to normalize renal function.

As shown in the present study, CKD rats injected with AD-MSCs have an increased expression of VEGF; these results are in accordance with in vitro studies [53], suggesting there is a pathway related to vascular protection induced by MSCs [54]. VEGF is an essential factor in endothelial and vascular system...
AD-MSCs induce an improvement in CKD

**Figure 7 Immunolocalization and expression of epithelial markers in MSC-mediated recovery of CKD**

(a) The samples were stained for Pax-2 (A–D) and BMP-7 (E–H) in Sham, NPX, NPX + CM and NPX + AD-MSC kidneys. Pax-2 was localized in proximal tubular cells and BMP-7 in the collector tubular cells of internal and external medulla. The staining for these markers was increased in NPX + AD-MSCs (D, H) when compared with Sham (A, E), NPX (B, F) and NPX + CM (C, G) kidneys. The staining was done using peroxidase and developed with DAB (brown reaction). Scale bar, 100 μm. Arrows show the marker localization. (b) Immunoblot studies were performed to evaluate the expression of Pax-2 (A) and BMP-7 (B). In Sham and NPX rats, the expression of these markers was minimal; however, the injection with AD-MSCs in NPX kidneys induced an increase in the expression of both of the markers. *P < 0.05.

development, and glomerulogenesis. Its expression in adult kidneys is restricted to glomerular and some tubular cells, where it has been implicated in the maintenance of the permeability and integrity of the capillaries that constitute the glomerulus. Previous results from our laboratory and others have shown a protective role of VEGF against glomerular injury [8,55] and its expression has been inversely related to the generation of glomerulosclerosis in progressive kidney diseases [56]. Furthermore, in the NPX + AD-MSCs group we observed an increased expression of BMP-7 and Pax-2 that are related to tubular development. We have also shown increased expression of these factors in the repairing processes after acute kidney damage [8,57,58]. Therefore, if these proteins are being overexpressed, the EMT (epithelial–mesenchymal transition) processes might be activated, supporting a reparation and regeneration hypothesis underlying the effects of MSC therapy [57]. In vitro studies have shown that MSCs can induce the expression of important trophic factors such as IGF-1 (insulin-like growth factor 1), EGF (epidermal growth factor), VEGF and bFGF (basic fibroblast growth factor) [53]. It is known that BMP-7 and Pax-2 participate in kidney
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development, protecting against cellular apoptosis [59] and promoting the epithelization processes [8,31,60]. The considerable increase in BMP-7 and Pax-2 in the kidneys from the NPX + AD-MSC group can be interpreted as an indicator of a cellular repair response to kidney damage.

One of the biggest challenges with respect to cell therapy, and specifically, kidney cell therapy, is the understanding of the mechanisms involved in the therapeutic effect described, which may include a paracrine effect given by the secretion of cytokines, chemokines and several mediators that would activate genes involved in the regeneration processes. Moreover, recent studies with human MSC-CM (MSC culture medium) have shown an improvement in cardiac function in animals subjected to myocardial infarction, suggesting that the therapeutic effects of SC transplantation can be mediated by paracrine factors, including microvesicles [61]. In CKD treated with MSC-CM we did not observe any significant effect on disease progression. This could be due to the fact that a higher concentration of the secreted factor is required, or that these factors alone do not have any effect on triggering the regeneration process in the kidney.

Further studies should be directed towards modifying MSCs, either genetically or by pre-incubation with specific Abs directed against factors such as VEGF or BMP-7. Additionally, comparing the effect of the MSC with direct administration of one or a combination of these factors could provide information about the importance of the cellular effect, i.e. mediated by homing, replication and differentiation of SC in kidney cells.

A small, but significant, increase in the renal SC marker Oct-4 [10] was observed after the injection of AD-MSCs in uraemic rats, suggesting an induction of tubular and epithelial cells to undergo EMT processes, to proliferate and differentiate with a consequent renal reparative phenomenon, or may indicate an induction of resident kidney SCs to proliferate and mediate kidney repair. Comparative data have shown that the Oct-4 levels induced by AD-MSCs, are higher than the levels induced by BM-MSCs, as reported previously by our group [20]. In this regard, we must emphasize that these two pathways are not mutually exclusive and that both probably play a role in tissue regeneration.

It has been proposed that the proliferation and differentiation of MSCs and resident kidney SCs into mature and functional epithelial cells capable of promoting repair is achieved by the secretion of the anti-inflammatory protein TSG-6 due to the embolization of the injected MSCs in the lung [62]. In this study, we have evidence of the arrival of MSCs into kidney, although we cannot rule out the possible embolization of injected MSCs in the lungs and the subsequent secretion of paracrine factors. In fact, after 35 days we found a minimal presence of MSCs in the kidney, which favours previous data on a paracrine mechanism of action [63,64].

In summary, our present results have shown the positive effects of AD-MSCs in an animal model of CKD. We propose that the use of AD-MSCs in CKD patients could be possible, owing to the facility to obtain and culture AD-MSCs. Considering the high prevalence and incidence of this pathology in the world, the present study opens a new perspective in SC therapy and regenerative medicine for CKD, in which AD-MSCs could play a therapeutically beneficial role.

CLINICAL PERSPECTIVES

- CKD is an important pathology worldwide. The use of BM-MSCs from healthy donors have been shown to have beneficial effects; however BM-MSCs derived from uraemic patients fail to confer functional protection. In the present study, we have investigated the potential of AD-MSCs as an alternative source of MSCs for the treatment of CKD.
- Our results have shown a positive effect of AD-MSCs in an animal model as shown by a significant reduction in plasma creatinine levels, a lower expression level of damage markers and the presence of epitheliogenic and angiogenic proteins.
- Although many aspects of cell therapy for CKD remain to be investigated, we provide evidence that AD-MSCs, a less invasive and highly available source of MSCs, exert a beneficial therapeutic role.

AUTHOR CONTRIBUTION

Sandra Villanueva, Juan Carreño, Carlos Vio conceived the experiments and co-wrote the paper; César Vergara, Rocio Strodthoff, Francisca Fajre, Carlos Céspedes, Pablo Sáez and Lorena Salazar carried out the study; and Carlos Irrázabal, Fernando Figueroa and Jorge Bartolucci analysed the data and co-wrote the paper.

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REFERENCES


S. Villanueva and others
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