

Original Article

Mesenchymal stem cells versus their conditioned medium in the treatment of cisplatin-induced acute kidney injury: evaluation of efficacy and cellular side effects

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Abstract: Mesenchymal stem cells (MSCs) and their conditioned medium (CM) have been shown to ameliorate acute kidney injury (AKI). Potential risks of using stem cells are their participation in fibrosis and their differentiation to unwanted cells. The development of such adverse effects after therapy with CM is unknown. This study compares both the efficacy and safety of using MSCs or their CM in AKI model. Cisplatin was used to induce AKI in Sprague-Dawley rats. Rats were treated with either sub capsular injection of MSCs; or intraperitoneal injection of CM. Appropriate control was included and rats were sacrificed 5, 10, 30, and 60 days later. Kidney function parameters, urinary kidney injury molecule-1 (Kim-1), renal tissue damage, apoptosis, and proliferation were all determined. Special stains to detect adipocytes, osteoblasts, and fibrosis were also used. MSCs and their CM equally ameliorate kidney function deterioration, Kim-1 shedding in urine, renal tissue damage and tubular cell apoptosis. Even after 2 months, both equally reduced interstitial fibrosis. The MSC-treated group showed more enhancement of proliferation. However, adipocytes and osteoblast-like cells were detected where the stem cells were injected but not in the CM group. These results indicate that the use of CM might be an acceptable alternative to MSC therapy as it can attain comparable efficacy without the development of unwanted cells.

Keywords: Acute kidney injury, cisplatin, conditioned medium, fibrosis, maldifferentiation, mesenchymal stem cells

Introduction

Acute kidney injury (AKI) is a devastating disorder in clinical medicine [1]. AKI is associated with high mortality and morbidity despite improvements in intensive care [2]. As reviewed elsewhere [3, 4], mesenchymal stem cells (MSCs) were proven to be successful in ameliorating different types of acute or chronic renal injury. More specifically, these cells were capable of protecting the kidney against cisplatin-induced AKI in murine models [5-12].

It is now widely accepted that MSCs act mainly by secreting several growth factors, cytokines and other molecules which act collectively to

produce immunomodulatory [13, 14], trophic, and anti-apoptotic effects [7, 15, 16]. Such theory explains the success of using exosomes and the conditioned medium (CM) which were derived from cultivation of MSCs in ameliorating AKI induced by cisplatin [7, 11, 12, 17]. However, these studies did not directly compare the efficacy of using MSCs to the use of their CM. moreover, ordinary markers, as serum creatinine and blood urea nitrogen (BUN), are unreliable and poorly correlated to the degree of renal damage [18, 19]. Fortunately, the recently discovered kidney injury molecule-1 (Kim-1) was found to be a sensitive marker for AKI [20-22] and could be used for comparison.

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Although the results of experimental work using MSCs in tissue regeneration were encouraging, concerns about maldifferentiation of MSCs hindered the progress of MSCs use in human. Osteogenic differentiation of MSCs in the heart [23] and the appearance of MSC-derived adipocytes in the treated kidneys [24] are just examples. Moreover, evidence was provided that BM derived cells can give rise to myofibroblasts and may contribute to fibrosis in an experimental model of renal ischemia/reperfusion injury [25]. In addition, stem cells secrete several growth factors which were found to be antifibrotic while others appear to actively participate in fibrosis [26-28]. Whether such concerns would occur with therapy using CM is yet to be determined.

The present study was designed to compare the efficacy of MSCs or their CM using urinary Kim-1 as a sensitive marker of cisplatin-induced AKI. In addition, both strategies were compared regarding the appearance of unwanted cells and the extent of fibrosis after 2 months of follow up.

Materials and methods

The experimental protocol was approved by the Local Ethical Committee, Faculty of Medicine, Mansoura University.

Preparation, characterization and labeling of bone marrow stem cells

MSCs were prepared from the bone marrow of 8-week-old male Sprague-Dawley (SD) rats according to a standard method [29]. Bone marrow cells were plated in T-75 flasks in complete Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Lonza, Verviers, Belgium) at 37°C in a humidified atmosphere that contains 5% CO₂. Cells were used for experiments after the 3rd passage.

MSCs were characterized using fluorescence activated cell sorting (FACS) analysis. Pellets of 10⁵ to 0.5×10⁶ cells were incubated for 30 minutes at 4°C with fluorescent labeled antibodies specific for CD90, CD29, CD44, CD34 and CD45 (eBioscience, San Diego, CA, USA). Cells were then washed twice, fixed in flow buffer and the fluorescence intensity was evaluated

by EPICS-XL flow cytometry (Coulter, Miami, USA).

Fibroblast colony-forming unit assay, adipogenic and osteogenic differentiation of MSCs were performed according to standard methods [30]. Adipogenic and osteogenic differentiation of MSCs was demonstrated using fresh oil red-O solution and alizarin red S respectively. MSCs were labeled using 10 μM/L of 5-bromo-2 deoxyuridine (BrdU; Sigma-Aldrich, Taufkirchen, Germany) for 48 h at 37°C to ensure labeling of more than 90% of the incubated cells [31].

Preparation of stem cells conditioned media

As described by Bakondi et al. [32] with modification, MSCs at passage three were cultured in complete DMEM until 80% confluence. Then, the medium was switched to serum-free DMEM medium. After 48 hours, the supernatants were collected, filtered, and frozen at -20°C for no more than 2 weeks.

Study design

The study was conducted on adult female SD rats weighing 160-200 g. The 129 rats which completed the study were divided into 6 groups. Therapeutic interventions took place 24 hours after cisplatin injection. Cisplatin (Hospira UK Limited, Warwickshire, UK) was given as single dose (5 mg/kg) intraperitoneal; I.P. to all groups except group I.

Group I (Control; *n* = 6), animals were not subjected to any interventions.

Group II (Cisplatin only; N; *n* = 21), animals received only cisplatin.

Group III (Cisplatin + vehicle; V; *n* = 24), animals received 0.3 ml of serum-free DMEM under the capsule of the left kidney.

Group IV (Stem cells-treated group; Sc; *n* = 27), animals received 2×10⁶ cells suspended in 0.3 ml of serum-free DMEM under the capsule of the left kidney.

Group V (Cisplatin + medium; M; *n* = 24), animals were given serum-free DMEM (3 ml) intraperitoneal (I.P.) twice daily for 7 days except for the subgroup which were sacrificed at the 5th day (treated until the time of scarify).

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Group VI (CM-treated group; Cm) ($n = 27$), animals were given 3 ml of the previously prepared CM I.P. twice daily for 7 days except for the subgroup which were sacrificed at the 5th day (treated until the time of scarify).

Except for the control group, each group is further divided into four subgroups where rats were sacrificed 5, 10, 30, and 60 days after cisplatin injection ($n = 5-6$ for each). For the vehicle- and the CM-treated groups, an additional subgroup ($n = 3$ in each) was designed to evaluate adipogenic differentiation at day 60 using oil red-O staining of frozen sections from the kidney. For the stem cell-treated group, three rats were sacrificed at day 60 and three rats were sacrificed at day 10 to evaluate adipogenic differentiation.

Blood samples (0.2 ml) were collected through retro-orbital venous plexus puncture. Morning and 24-hours urine was collected using metabolic cages (Nalgene; Rochester, USA). Samples were taken at 5, 10, 30, and 60 days after cisplatin injection. Rats were sacrificed using thiopental overdose. Blood samples were taken by heart puncture and the kidneys were harvested.

Measurement of biochemical parameters

Biochemical parameters were measured using an automated spectrophotometer (Slim Plus, Italy) and specific kits for creatinine (Diamond Diagnostics, Egypt) and BUN (Stanbio Lab, Texas, USA).

Measurement of urinary kidney injury molecule-1

Kim-1 in urine was measured using enzyme-linked immunosorbent assay (ELISA) kit (Oxi-Select™, Cell Biolabs, San Diego, USA). Urine samples from rats which were sacrificed at the 10th day were collected using the metabolic cage, the volume of voided urine between 8 and 12 a.m. at days 0 and 4 of the experiment was measured.

Renal morphology

Except for the subgroup that was designed to be stained with oil red-O, the kidneys were perfused, formalin-fixed, and paraffin sections (4 μm thick) were stained with hematoxylin and eosin to evaluate the degree of tubular injury in

the outer stripe of the outer medulla (OSOM). Both kidneys in the stem cell-treated group and the left kidney in all other groups were evaluated. According to Kinomura et al. [33], twenty randomly selected fields were observed (magnification $\times 400$). Tubular injury was quantified as a score between 0 and 5 as the following: 0: normal; 1: tubular cells exhibiting desquamation from the tubular basement membrane, swelling, vacuolar degeneration and necrosis involving <20% of the tubules; 2: 20-40% tubules are involved; 3: 40-60%; 4: 60-80%; and 5: 80-100%.

Detection of tubular cells proliferation and apoptosis (5th and 10th days)

The degree of tubular cells proliferation was assessed using a specific kit that detect Ki-67 antigen in rat cells (DakoCytomation, Glostrup, Denmark). The number of Ki-67-positive nuclei in the OSOM was counted in 20 randomly selected fields in the OSOM at 400x magnification [33]. Tubular cells apoptosis was evaluated using the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) method. A specific kit (Takara Bio. Inc., Japan) was used and the number of TUNEL-positive cells per field in the OSOM was counted and the results were expressed as positive cells/ 10^3 cells [9].

Detection of total collagen and detection of interstitial fibrosis

Renal tissue samples from days 10, 30, and 60 of the experiment were stained with Masson trichrome. It stains collagen blue while nuclei are stained red to blue [34]. To quantify interstitial fibrosis, the fibrotic area was measured by a color image analyzer (ImageJ 1.32) in five randomly selected fields in the OSOM and corticomedullary junction. The blood vessels were avoided. The percentage of fibrotic area per unit area was calculated [35].

Detection of adipocyte

Three rats from each of the vehicle-, stem cell-, and the CM-treated groups were sacrificed at 60 days after cisplatin injection. From the stem cell-treated group, other three rats were sacrificed at day 10 after cisplatin injection. The abdominal cavity was opened and kidneys were perfused in a retrograde fashion through the abdominal aorta using saline 0.9% till complete

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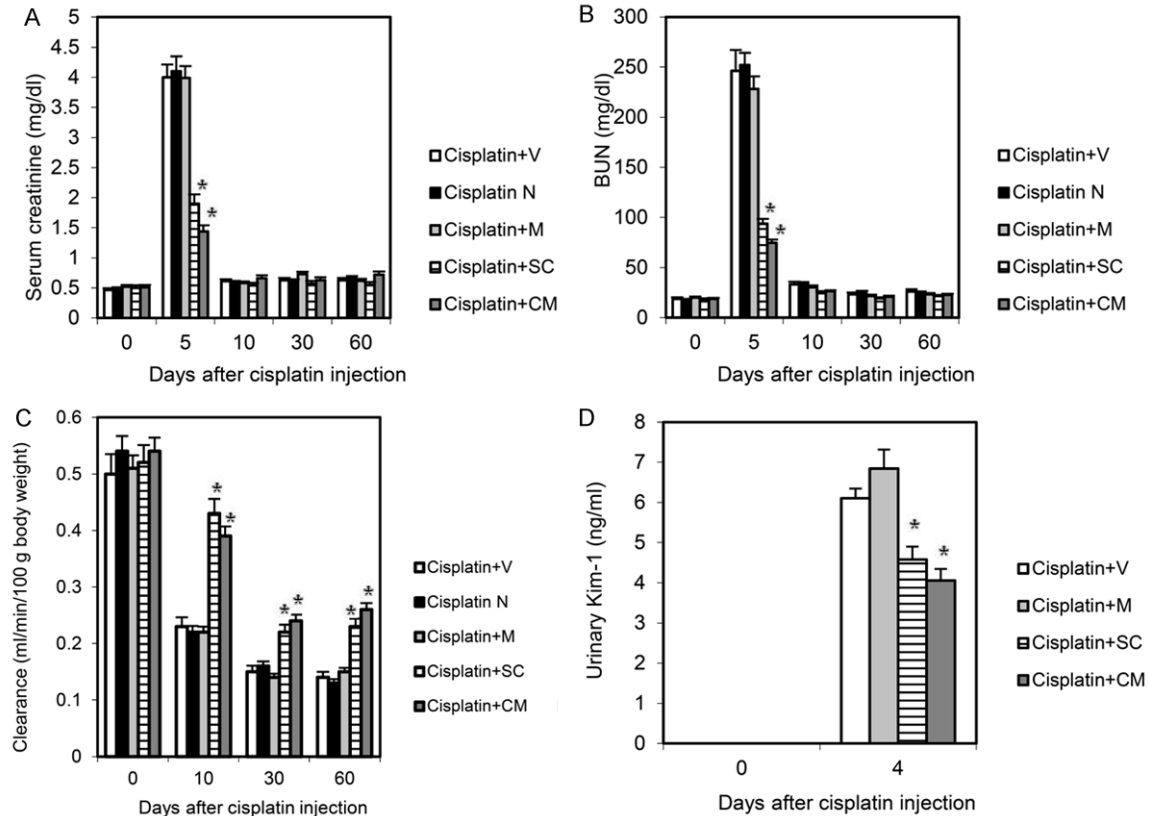


Figure 1. Changes in serum creatinine (A), blood urea nitrogen BUN (B), creatinine clearance (C), and urinary kidney injury molecule-1 (Kim-1) (D) in rats after cisplatin injection in the vehicle (V), the cisplatin only (N), the medium (M), the stem cells (Sc), and the conditioned medium (CM) treated groups. Data represented as mean \pm SEM. *Significant difference ($P < 0.05$) versus the untreated groups.

clearance of the perfusion fluid. Neutral buffered formalin was not used. Both right and left kidneys were harvested, immediately processed for frozen section, and stained with oil red-O for demonstration of adipocytes. At least 6 sagittal sections from each kidney were examined while the cortex and area under the capsule was avoided.

Detection of osteoblasts

In the vehicle-, stem cell-, and the CM-treated groups, renal tissue samples from day 60 of the experiment were stained with Alizarin Red S to detect calcium deposition that characterizes the osteoblasts. Similarly, renal tissue samples from the stem cell-treated group were evaluated at day 10 after cisplatin injection.

Detection of stem cells in the renal tissue

The presence of BrdU-labeled MSCs in the rat right and left kidneys was evaluated at days 10, 30, and 60 using a specific monoclonal antibody against BrdU (Sigma-Aldrich).

Statistical analysis

Statistical analysis was carried out using the SPSS software (version 16.0, SPSS, IL, USA). Significance of the survival rate was computed with a log-rank test. Pathological injury scores were evaluated using Kruskal-Wallis (K-W) test followed by Mann-Whitney test for significance between individual groups. Values were expressed as median (min-max). Other parameters were evaluated by analysis of variance (ANOVA) and post hoc test for multiple comparisons (Bonferroni). Values were expressed as mean \pm SD. P -values < 0.05 were considered statistically significant.

Results

Phenotyping of stem cells

The isolated MSCs were characterized by their adherence to plastic, predominant spindle-shaped morphology, and formation of fibroblast-like colonies. By FACS analysis, MSCs were negative for the hematopoietic lineage

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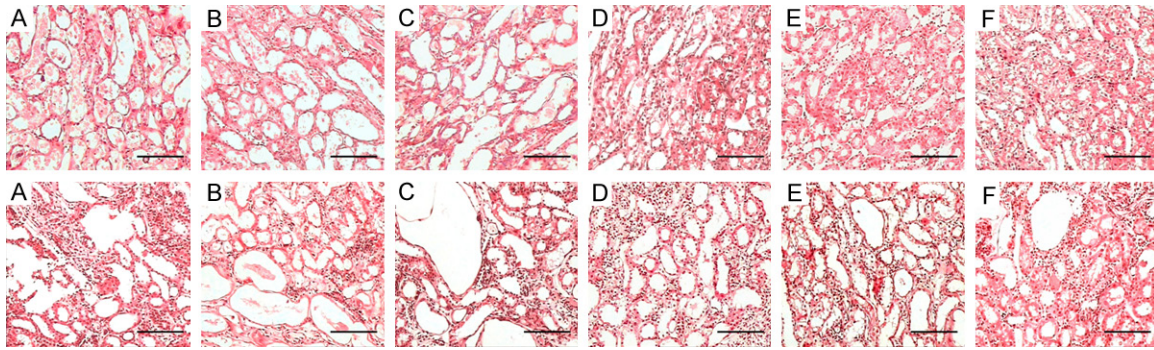


Figure 2. Histological changes of the outer stripe of the outer medulla in the rat kidney after cisplatin injection (H&E). After 5 days (upper row), necrotic changes were attenuated in the left (D) and the right (E) kidneys of the stem cell-treated group and the conditioned medium-treated group (F). Necrosis affected almost all the tubules in the cisplatin only-injected (A), the vehicle-treated (B), and the medium-treated groups (C). After 60 days (lower row), chronic changes as tubular atrophy and dilatation, microcysts formation, and mononuclear cells infiltrating the interstitium were evident in all groups but less pronounced in the treated groups (D-F). In comparison to the cisplatin only-injected (A), the vehicle-treated (B), and the medium-treated groups (C). Of note, interstitial mononuclear cells infiltration in the left kidney (D) where stem cells were injected was more pronounced than the right kidney (E) and the conditioned medium group (F). Scale bar = 100 μ m.

markers, CD45 and CD34, but positive for mesenchymal markers CD29, CD44 and CD90. In the suitable differentiation media, MSCs were able to differentiate into adipocytes and osteoblasts (Supplementary Figure 1).

Results of cell or conditioned medium therapy of cisplatin-induced AKI

Survival: Starting with 149, only 129 rats completed the study. Considering cisplatin-related mortality, no significant differences in survival was evident between the study groups ($P = 0.116$).

Changes in biochemical parameters and urinary Kim-1

Injection of cisplatin resulted in elevation of serum creatinine, and BUN to reach a peak at the 5th day. Levels of serum creatinine, but not BUN, were normalized by the 10th day. Compared to the control groups injected with cisplatin, groups injected with either MSCs or CM had significantly attenuated changes in serum creatinine and BUN at day 5 (Figure 1A, 1B). Within each group, cisplatin injection significantly reduced creatinine clearance and clearance had never recovered to its original values. Treatment with MSCs or their CM equally attenuated the changes in creatinine clearance (Figure 1C) but failed to prevent the progressive decline in clearance at days 30 and 60 in comparison to the 10th day. Considering the untreated groups, treatment with MSCs or their

CM reduced urinary Kim-1 levels at the 4th day of the experiment. Of note, no significant differences between MSCs or their CM were evident (Figure 1D).

Pathological changes in the kidney

Administration of cisplatin resulted in AKI that was restricted to the OSOM and was more evident at the 5th day (Figure 2). Treatment with MSCs or their CM resulted in significant lower levels of injury score at 5, 10, and 30 days (Table 1). An exception is the medium-treated group at day 30 as the injury score was unexpectedly low. At 30 and 60 days after cisplatin injection, chronic changes became more evident (Figure 2). No significant differences in injury score were detected between MSC- and CM-treated groups or between the right and the left kidneys of MSC-treated group (Table 1). Of note, the left kidneys of MSC-treated group showed a higher mononuclear cell infiltration (Figure 2).

Proliferation and apoptosis of renal tubular cells

Five days after administration of cisplatin, treatment with MSCs or CM resulted in enhancing proliferation in the OSOM and even the cortex. No significant differences were detected between the right and the left kidneys of MSC-treated group but both showed more proliferation than the CM. Proliferation significantly increased in the untreated groups by the 10th day

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Table 1. Injury score of the renal outer stripe of the outer medulla after cisplatin injection in vehicle (V), cisplatin only (N), medium (M), left (Sc left) and right (Sc right) kidneys of the stem cells, and conditioned medium (CM) treated groups

| Day | Control | Cisplatin + V (n = 5 ^a) | Cisplatin (N) (n = 5 ^a) | Cisplatin + M (n = 6) | Cisplatin + Sc left (n = 5 ^a) | Cisplatin + Sc right (n = 5 ^a) | Cisplatin + Cm (n = 6) | (K-W) Chi-Square | P value |
|-----|---------------|--|--|--------------------------------|--|---|----------------------------------|---------------------|---------|
| 5 | 0.0 (0.0-0.0) | 5.0 (5.0-5.0) ¹ | 5.0 (5.0-5.0) ¹ | 5.0 (4.0-5.0) ¹ | 4.0 (2.0-4.0) ^{1,2,3,4} | 4.0 (2.0-5.0) ^{1,2,3,4} | 3.0 (1.0-5.0) ^{1,2,3,4} | 29.76 | 0.001 |
| 10 | 0.0 (0.0-0.0) | 4.0 (4.0-5.0) ¹ | 4.0 (4.0-5.0) ¹ | 4.0 (4.0-5.0) ¹ | 3.0 (3.0-4.0) ^{1,2,3,4} | 3.0 (2.0-3.0) ^{1,2,3,4} | 3.0 (1.0-3.0) ^{1,2,3,4} | 34.68 | 0.000 |
| 30 | 0.0 (0.0-0.0) | 3.0 (2.0-3.0) ¹ | 3.0 (2.0-3.0) ¹ | 1.0 (1.0-2.0) ^{1,2,3} | 1.0 (1.0-1.0) ^{1,2,3} | 1.0 (1.0-1.0) ^{1,2,3} | 1.0 (1.0-1.0) ^{1,2,3} | 34.01 | 0.000 |
| 60 | 0.0 (0.0-0.0) | 1.0 (1.0-2.0) ¹ | 1.0 (1.0-2.0) ¹ | 1.0 (1.0-1.0) ¹ | (0.0-2.0) ¹ | 1.0 (0.0-1.0) ¹ | 1.0 (0.0-1.0) ¹ | 19.96 | 0.003 |

¹Significant difference ($P < 0.05$) versus control, ²versus V, ³versus N, ⁴versus M; Data are expressed as median (min-max); n = number of animals, Abbr.: K-W; Kruskal-Wallis; ^an = 6 at day 10.

Table 2. Number of proliferating cells (/HPF) and apoptotic cells (/10³ cells) and the percentage of fibrotic area in the outer stripe of the outer medulla after cisplatin injection in the vehicle (V), the cisplatin only (N), the medium (M), left (Sc left) and right (Sc right) kidneys of the stem cells, and the conditioned medium (CM) treated groups

| | Day | Control (n = 6) | Cisplatin + V (n = 5 ^a) | Cisplatin (N) (n = 5 ^a) | Cisplatin + M (n = 6) | Cisplatin + Sc left (n = 5 ^a) | Cisplatin + Sc right (n = 5 ^a) | Cisplatin + Cm (n = 6) | P value |
|------------------------|-----|--------------------|--|--|-----------------------------|--|---|-----------------------------------|---------|
| Proliferation | 5 | 8.83 ± 0.75 | 5.45 ± 1.98 | - | 0.44 ± 0.14 | 41.90 ± 12.59 ^{1,2,4} | 43.3 ± 13.16 ^{1,2,4} | 24.85 ± 8.10 ^{1,2,4,5,6} | 0.000 |
| | 10 | 8.83 ± 0.75 | 50.02 ± 9.04 ¹ | - | 42.60 ± 4.15 ¹ | 47.40 ± 6.82 ¹ | 36.90 ± 5.84 ^{1,2} | 30.35 ± 8.83 ^{1,2,4,5} | 0.000 |
| Apoptosis | 5 | 0.00 ± 0.00 | 30.60 ± 9.76 ¹ | - | 10.67 ± 2.07 ^{1,2} | 2.00 ± 0.71 ^{2,4} | 2.60 ± 1.14 ^{2,4} | 1.33 ± 0.52 ^{2,4} | 0.000 |
| | 10 | 0.00 ± 0.00 | 2.40 ± 1.14 | - | 4.67 ± 2.25 ¹ | 2.50 ± 1.04 ¹ | 2.33 ± 1.03 ^{1,4} | 1.67 ± 0.82 ⁴ | 0.000 |
| Percentage of fibrosis | 10 | 0.14 ± 0.04 | 1.96 ± 0.47 ¹ | 2.02 ± 0.75 ¹ | 1.37 ± 0.52 ¹ | 1.42 ± 0.63 ¹ | 1.55 ± 0.36 ¹ | 1.13 ± 0.19 ¹ | 0.000 |
| | 30 | 0.14 ± 0.04 | 4.83 ± 1.19 ¹ | 5.02 ± 0.86 ¹ | 4.88 ± 0.95 ¹ | 2.48 ± 0.55 ^{1,2,3,4} | 2.02 ± 0.56 ^{1,2,3,4} | 2.42 ± 0.51 ^{1,2,3,4} | 0.000 |
| | 60 | 0.14 ± 0.04 | 9.88 ± 2.33 ¹ | 10.3 ± 1.19 ¹ | 9.13 ± 0.50 ¹ | 3.96 ± 1.00 ^{1,2,3,4} | 2.74 ± 0.85 ^{1,2,3,4} | 3.00 ± 1.31 ^{1,2,3,4} | 0.000 |

¹Significant difference ($P < 0.05$) versus control, ²versus V, ³versus N, ⁴versus M, ⁵versus Sc left, ⁶versus Sc right. Data are expressed as mean ± SD; ^an = 6 at day 10.

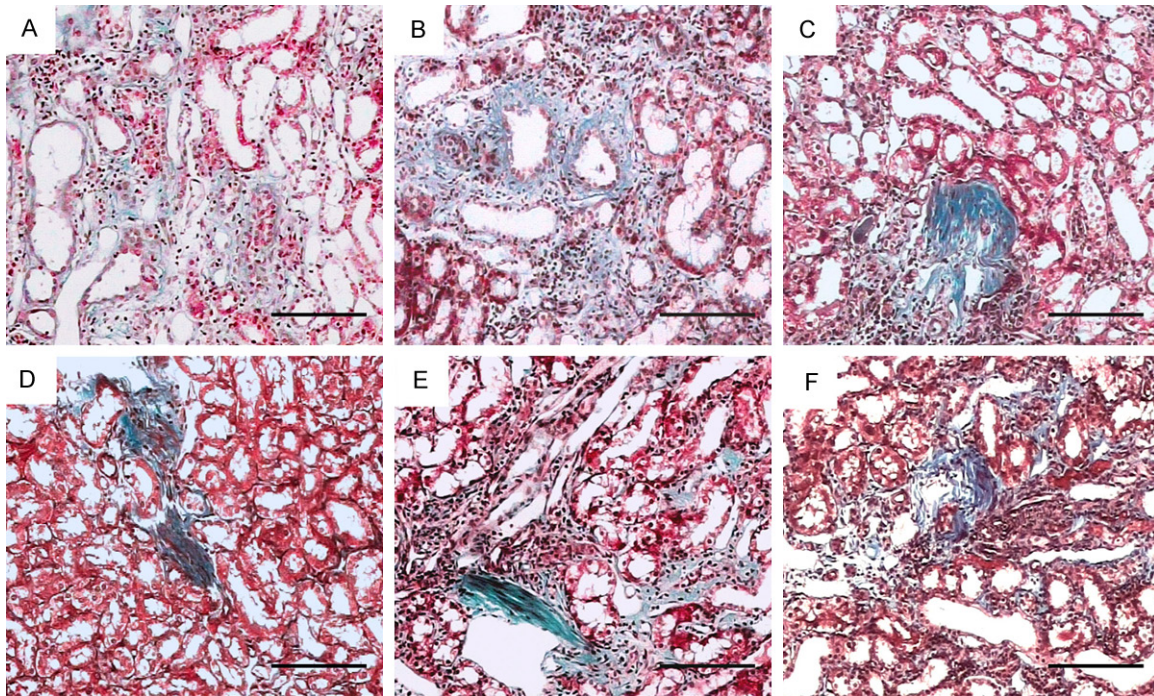


Figure 3. Masson trichrome was used to demonstrate fibrosis (blue color) in the outer stripe of the outer medulla after 60 days of cisplatin injection in rat. Fibrosis was less evident the left (D) and right (E) kidneys of the stem cell-treated, and the conditioned medium-treated (F) groups than that of the cisplatin only-injected (A), vehicle-treated (B), and medium-treated (C) groups. Scale bar = 100 μ m.

and the effect of treatment was not sustained (Table 2; Supplementary Figure 2).

Regarding apoptosis, treatment with MSCs or CM resulted in attenuating apoptosis in the OSOM in comparison to the untreated groups only in the 5th day. No significant differences were detected between the right and the left kidneys of MSC-treated group (Table 2; Supplementary Figure 2).

Changes in the fibrosis scoring index

Starting from day 10, Masson trichrome stain revealed fibrotic areas around the dilated or atrophic tubules in the cortico-medullary junction. These changes appeared to be progressive in days 30 and 60 (Figure 3) of the experiment. The groups which were treated with the CM and both kidneys of the group that was treated with stem cells showed lower levels of interstitial fibrosis in comparison to the untreated groups at days 30 and 60 of the experiment. No significant differences in the fibrosis scores were evident between the CM- and both kidneys of the stem cell-treated groups (Table 2).

Oil red-O staining for detection of adipocytes

After 60 days of cisplatin injection, adipocytes were demonstrated only in the OSOM of the left kidney of the MSC-treated group. Clusters of adipocytes were found to be distributed in the outer medulla in almost all the 18 kidney sections (Figure 4A, 4B). In 18 sections from the right kidneys, no adipocytes were detected in the OSOM. However, only three fields were positive for oil red-O and were exclusively limited to the cortex (Figure 4C). No adipocytes were evident in the CM-treated or the vehicle-treated groups after 60 days of cisplatin injection. Similarly, no adipocytes were detected in the left or the right kidney of MSC-treated group after 10 days of therapy.

Alizarin red S staining for detection of osteoblasts

Osteoblasts were demonstrated only in the outer medulla of the left kidney of the MSC-treated group at day 60 (Figure 4D, 4E). We did not find similar picture in the right kidneys of MSC-treated group, the CM-treated or the vehi-

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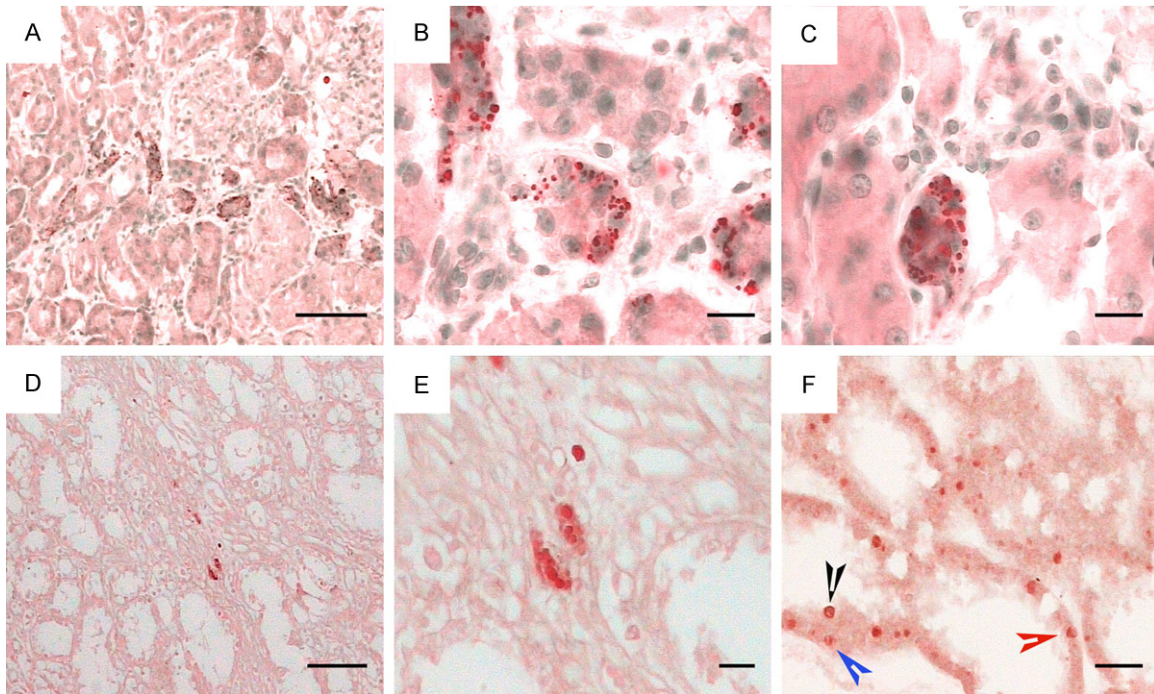


Figure 4. After 60 days of MSCs injection under the capsule of the left kidney, staining frozen sections from the kidney with oil red-O demonstrated clusters of adipocytes in the outer medulla of the left kidney (A, B) while only few adipocytes were detected in the cortex of the right kidney (C). Using Alizarin red S, osteoblast-like cells were detected only in the left kidney where stem cells were injected (D, E). Follow up of the MSCs which were injected under the capsule of the left kidney (F). By the 10th day, BrdU-labeled MSCs were detected under the capsule, in the interstitium, and integrated in the tubules of the cortex. Cells showed the filled pattern (upper left arrow), the segmented (right arrow), and punctate (bottom left arrow) signals. Scale bar = 100 μ m (A, D); 20 μ m (B, C, E, F).

cle-treated groups at day 60 or in the left or right kidneys of MSC-treated group at day 10.

Detection of stem cells in the kidney

In rats which were sacrificed at the 10th day, BrdU-positive cells were detected in the left kidney only. Cells were distributed under the capsule, in the interstitium, and even were integrated in the tubules of the cortex. BrdU-signals varied from the filled pattern to the segmented and punctuated signals indicating a gradual loss of the stain (**Figure 4F**). We were unable to detect any BrdU-positive cells at days 30 and 60 of the experiment.

Discussion

In the present study, we demonstrated that cellular therapy using MSCs have little advantage over the use of CM in cisplatin-induced AKI. However, MSCs therapy was associated with the appearance of adipocytes and osteoblast-like cells. Several studies reported the ability of MSCs to ameliorate cisplatin-induced AKI [5,

12]. In a parallel way, it was found that the growth factors-rich CM derived from MSCs was also capable of enhancing regeneration following certain models of AKI [7, 12, 36, 37]. However, such finding was not consistent in all studies. Except for one [38], most of the studies agreed that CM diminished cisplatin-nephrotoxicity [7, 10, 12]. Although different results could be attributed to differences in the dose and duration of therapy with CM, none of these studies directly compared the outcome of using MSCs to the use of their CM.

In the present study, MSCs and their CM equally blunted cisplatin-induced changes in serum creatinine and BUN. However, it is difficult to rely on these markers to compare the efficacy of therapy. For several reasons, serum creatinine and BUN are considered suboptimal markers of AKI [19, 39]. Creatinine clearance provides a more reliable assessment of renal function and appears to be affected earlier in cisplatin-induced nephrotoxicity [40]. Again, clearance could underestimate renal function for several reasons [18]. Thus, Kim-1 was included

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in the present study to provide a sensitive method to compare both strategies. Kim-1 is sensitive marker for AKI secondary to cisplatin or other causes [20-22]. We found that the estimated results from urinary Kim-1 did not reveal differences between the MSC- and the CM-treated groups. These results, in addition to the results of renal injury score and apoptosis indicated that MSCs and the CM are equally effective in the treatment of AKI. Although MSCs was superior in enhancing proliferation, such difference did not affect the final outcome after 60 days regarding creatinine clearance, chronic changes, or the extent of fibrosis. Of note, a large number of MSCs was used (2 million) in the present study while the dose of the CM used was about one third of that used by Bi et al. [7].

Another important point to consider when comparing the use of MSCs to the use of their CM is safety. Safety considerations have hindered the extrapolation of successful experiments on animals to clinical trials. Maldifferentiation of MSCs [23, 24], cyst formation in the lung [41], and possible participation in fibrosis [25] are just examples. Although one study using ischemia/reperfusion-induced AKI model did not reveal any adverse effects over 3 months of follow up [42], the authors did not specifically evaluated the maldifferentiation possibility.

After 60 days of therapy, we were able to demonstrate the presence of clusters of adipocytes and osteoblasts-like cells in the left kidneys of the stem cell treated group. In the right kidney of the same animals, few adipocytes but no osteoblasts were detected and were limited to the cortex only. In one study, it was reported that after 2 months of intra-arterial injection of MSCs in a rat model of AKI induced by Anti-Thy1.1, adipocytes and pronounced surrounding fibrosis were detected in about 20% of the glomeruli [24]. The authors suggested that this unexpected differentiation might be related to the model rather than MSCs therapy. Of note, we used a different model of kidney injury and injected MSCs via the sub capsular instead of intra-arterial route that might result in obliteration of some of the glomeruli. Even though, we were still able to detect unusual cells. Rather than being unrequired, adipocytes may be harmful. Adipocytes may enhance the fibrotic response via the proinflammatory activity of these cells [43]. As reviewed elsewhere [44],

several, but not all, studies demonstrated that differentiation resulted in loss of the immune privilege of MSCs with subsequent activation of the host immune response and loss of the benefits gained by MSCs therapy. Indeed, we noticed that mononuclear cell infiltration was more prominent in the left kidney where MSCs were injected. We could not detect the BrdU-labeled MSCs after 30 or 60 days. Thus, we could not confirm that the maldifferentiated cells originated from the injected MSCs. However, absence of maldifferentiated cells in the vehicle-treated or the CM-treated groups could be used as indirect evidence that the appearance of these cells is not a normal finding in the kidney, a finding related to the model of cisplatin, or even related to sub capsular injection of stem cells. Similarly, absence of adipocytes or osteoblasts in the left kidney of the MSC-treated group after 10 days of therapy confirm that the later detected cells were newly developed and not merely cells that contaminated the injected MSCs preparation. Still, it was reported that MSCs secret transforming growth factor- β [45] that acts as an enhancer of adipogenesis [46]. So, it is possible that MSCs did not differentiate by itself but enhanced the differentiation of other cells. Although no adipocytes were detected in the CM-treated group, we could not rely on this as MSCs secretory pattern vary with variable surrounding conditions [32].

Differentiation of stem cells to unwanted lineage has been described in several other occasions. Ectopic osteogenic differentiation of MSCs has been observed in a model of cardiac injury [23]. Similarly, fibroblast-like differentiation of MSCs has been reported after chronic heart allograft rejection [47]. Final judgment of MSCs safety requires the consideration of the possibility of differentiation of these cells to unwanted cell types. It seems that using the paracrine function of stem cells, through their CM, might be safer than using the cells by themselves.

It might be difficult to accept that the adipocytes and osteoblasts found in the kidney of the MSC-treated group originated from MSCs owing to the idea that MSCs transplanted to tissue have short survival time. In fact, the major influence on survival of injected stem cells is the recipient environment. Most of the reports on short survival of stem cells were related to

vascular entrapment or failure to survive in ischemic tissue. Even though, a proportion of these cells (18%) could survive for weeks in the infarcted heart [48].

Regardless the primary disease, progressive interstitial fibrosis is considered a key determinant of progression to end-stage renal disease [49]. Cisplatin-induced kidney injury is usually followed by post-tubular injury-renal interstitial fibrosis [35]. In the present study, we have demonstrated that the severity of fibrosis was attenuated with MSCs or CM therapy although either strategy did not prevent other chronic changes as tubular atrophy and formation of microcysts. As the precise mechanism of fibrosis is not yet fully understood, we could not explain the mechanism by which MSC therapy reduced fibrosis in this model. Reviewing different studies on the role of MSCs in fibrosis revealed their effectiveness only before massive fibrosis is well established [50]. Reduction of fibrosis may be secondary to reducing tissue damage. In addition, MSCs can directly prevent fibrosis by secreting different growth factors [27, 28]. On the contrary, MSCs may actively participate in fibrosis by different mechanisms [51]. Although the net result of stem cell therapy in the present study was reduction of fibrosis, the experimental design considered the possibility that stem cells may participate in fibrosis. It was reported that the number of muscle actin-positive cells increased significantly starting from the 7th day after cisplatin injection [35]. For this reason and others, we planned to inject the CM for 7 days only to rule out the possibility that the CM would enhance fibrosis. No significant differences in fibrosis score were detected between the CM-treated group and the stem cell treated group. Similarly, no significant differences in fibrosis score were detected between the left and the right kidneys of the stem cells-treated group although MSCs were injected only in the left kidney. One possible explanation is that MSCs might already differentiate to alpha-smooth muscle actin positive cells which participate in fibrosis but their percentage was too small to be statistically significant. Quantification of the contribution of bone marrow cells to fibrosis in murine models of renal fibrosis revealed that most of the fibroblasts arise by local epithelial-mesenchymal transition and between 8.6 to 30% of α -smooth muscle actin-positive interstitial cells were bone marrow-derived [52-54].

By the 10 day, BrdU-labeled MSCs were found to be integrated in the tubular epithelium in the cortex. We did not evaluate whether MSCs have differentiated into functioning tubular epithelium as the overall contribution of BMSCs to the regenerating renal cells is low [55-57]. More importantly, one limitation in the present study was the use of BrdU to label MSCs. We relied on a study that detected BrdU-labeled MSCs in the retina of the rat eye 5 and 12 weeks after MSC injection [58]. We could not detect BrdU-positive cells at day 60; so that we did not confirm that the detected adipocytes and osteoblasts originated from MSCs. A gradual loss of BrdU signals from MSCs limit the value of BrdU as a follow up tool to 2 weeks after injection [59].

Finally, the present study adopted local injection of MSCs in one kidney and the evaluation of both kidneys. Except for the presence of osteoblasts and adipocytes, no differences were evident between the right and the left kidneys. Herrera et al. [60] provided evidence that MSC homing is required for the beneficial effects of MSCs. In another study, it was suggested that homing is not an absolute need but only provides a mechanism for increasing the local concentration of growth factors released by MSCs [7]. The results of the present study suggested that homing of MSCs is not a prerequisite to gain their benefits although migration of MSCs from the left kidney to the right side cannot be ruled out. In fact, we assumed that migration through blood vessels could explain the presence of few adipocytes in the cortex of the right kidney.

Conclusion

Considering efficacy, MSCs and their CM are apparently equal. However, the use of MSCs could be associated with the development of unwanted cells even after AKI. Although no apparent hazard was detected secondary to this maldifferentiation, more efforts are required to evaluate the safety of using MSCs before proceeding to clinical application.

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Disclosure of conflict of interest

None.

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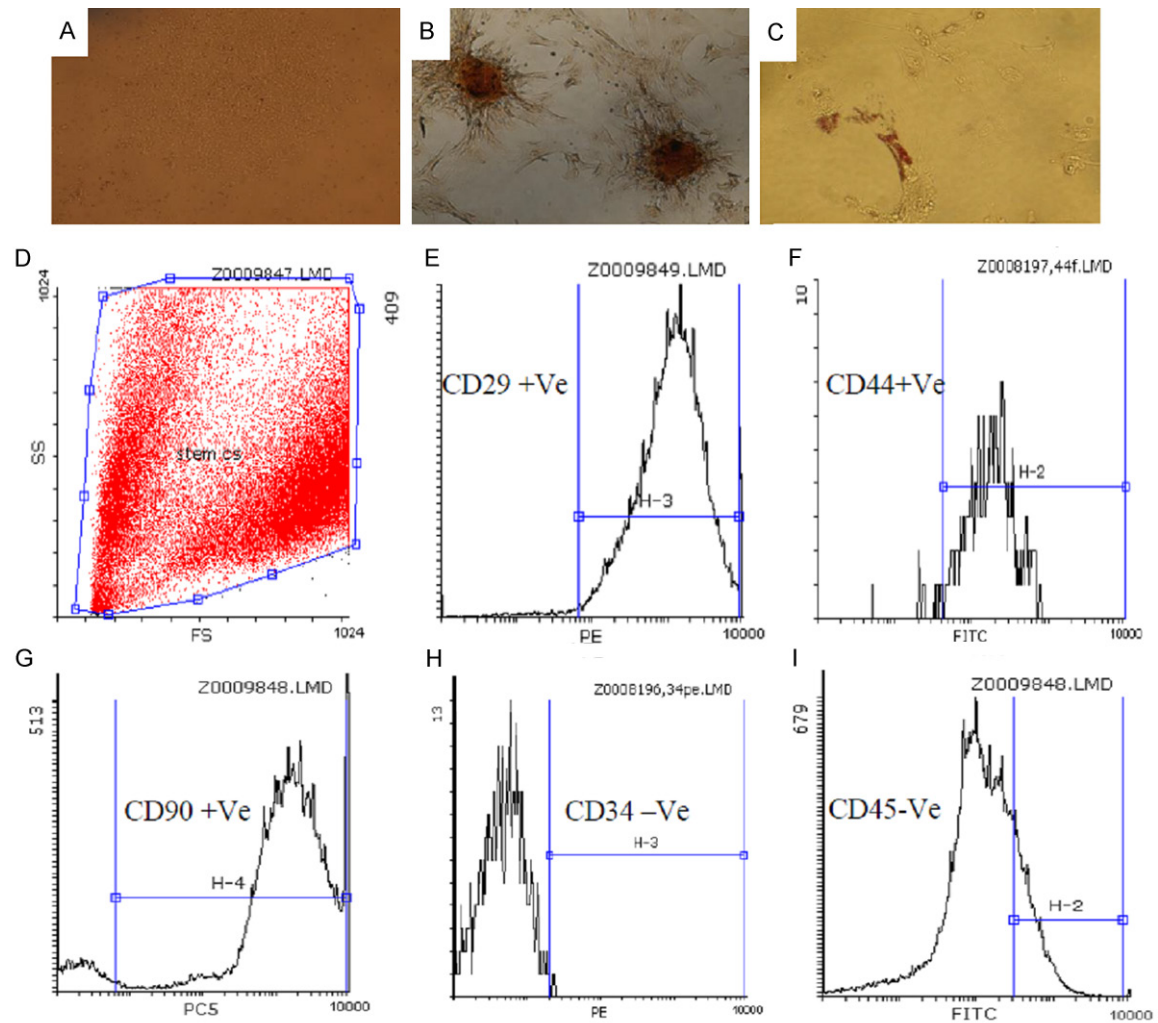
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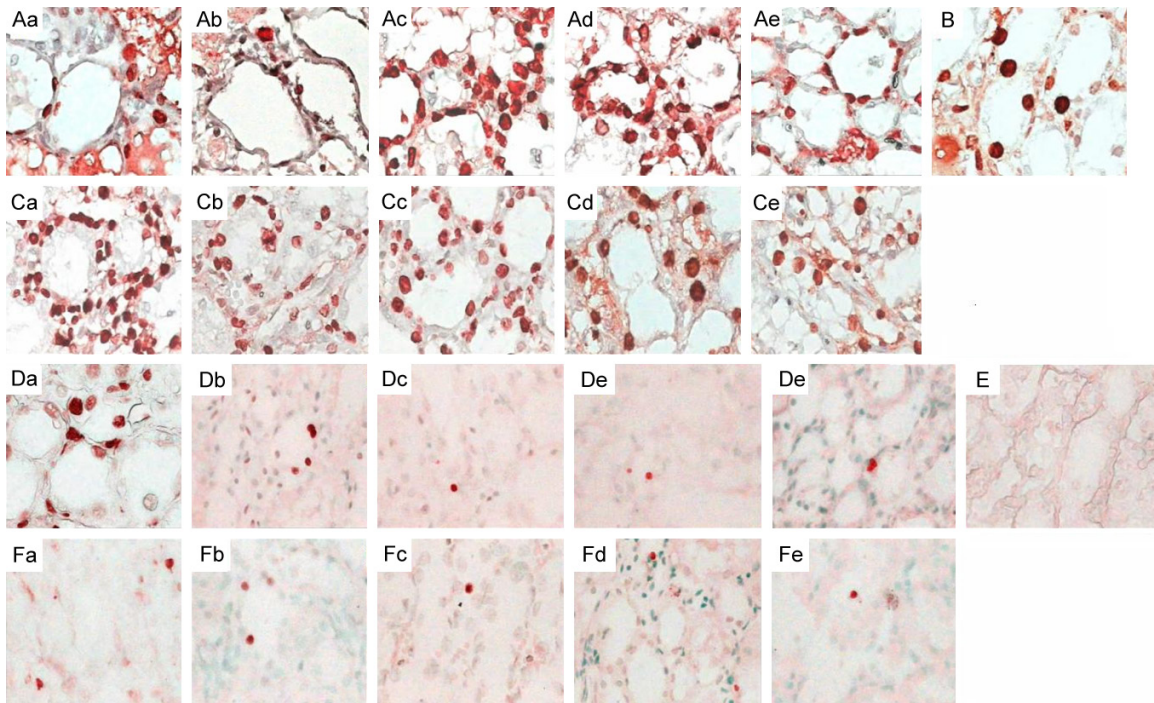
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Supplementary Figure 1. After one week in culture, mesenchymal stem cells (MSCs) were able to form colonies from spindle-shaped fibroblastoid cells (A). MSCs were able to differentiate to osteoblasts, as calcium-rich hydroxyapatite was detected with Alizarin red (B); and adipocyte showing intracellular lipid vacuoles revealed by Oil Red O staining (C). By flow cytometric analysis (D), MSCs at passage 3 expressed surface antigens CD29 (96.5%) (E), CD44 (93.7%) (F) and CD90 (56.2%) (G) but were negative for CD34 (0.2%) (H) and CD45 (15.2%) (I). Magnification $\times 100$ (A, C); $\times 200$ (B).

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Supplementary Figure 2. Assessment of proliferation by Ki-67 antibodies (A-C) and apoptosis by TUNEL (D-F) in the outer stripe of the outer medulla at 5 (A, D) and 10 (C, F) days of cisplatin injection in comparison to control (B, E). By the 5th day, few proliferating cells were detected in the vehicle (a)-, and medium (b)-treated groups in comparison to the left (c) and right (d) kidneys of the stem cell-treated group. Proliferation was intermediate in the conditioned medium-treated (e) group. By the 10th day, proliferation became high in all groups except the conditioned medium-treated (e) group. Apoptosis was significantly reduced in the treated groups (c-e) in comparison to the untreated (a, b) ones. Differences were prominent at day 5 but not at day 10. Magnification: $\times 400$.