Histological Study on Effect of Mesenchymal Stem Cell Therapy on Experimental Renal Injury Induced by Ischemia/Reperfusion in Male Albino Rat

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Background and Objectives: Acute kidney injury (AKI) represents a major clinical problem with high mortality and limited treatment protocols. This study was planned to evaluate the therapeutic effectiveness of bone marrow - derived mesenchymal stem cells (BM-MSCs) in a rat model of ischemia/reperfusion (I/R) AKI.

Methods and Results: This study was carried out on thirty adult male albino rats. Animals were divided equally into three groups. Group I (control sham-operated group) (n=10), were subdivided equally into two subgroups; Ia and Ib. The experimental group (n=20) were all subjected to I/R injury by clamping both renal pedicles for 40 minutes. Half of the I/R animals did not receive MSC therapy (group II) [non-MSC treated group]. The other half of the I/R animals received single intravenous injection of PKH26 labelled BM-MSCs immediately after removal of the clamps and visual confirmation of reflow (group III) [MSC treated group]. Animals were sacrificed 24 hrs (subgroups IIa & IIIa) and 72 hrs (subgroups IIb & IIIb) after intervention. Serological measurements included serum urea and creatinine. Kidney specimens were processed for H&E, PAS and PCNA. Mean % of renal corpuscles with affected glomeruli, mean % of affected tubules, mean area % of PAS-positive reaction and mean area % of PCNA immunoreactivity were measured by histomorphometric studies and statistically compared. MSCs-treated group exhibited protection against renal injury serologically and histologically.

Conclusions: Results of the present study suggest a potential reno-protective capacity of MSCs which could be of considerable therapeutic promise for cell-based management of clinical AKI.

Keywords: Acute kidney injury, Ischaemia reperfusion, Mesenchymal stem cells, Proliferating cell nuclear antigen

Introduction

The kidney is a highly complex organ, composed of more than 30 different cell types in different compartments. These cells differ in their proliferation rate, turnover and regenerative potential. Even the different compartments of the nephron exhibit different regenerative capacity (1).

Glomerular visceral epithelial cells "podocytes", are terminally differentiated cells and their proliferation rate is virtually zero (2). If podocytes are lost due to necrosis, apoptosis or detachment, they are not replaced by proliferation of a neighboring podocyte (3). Proximal tubular cells, on the other hand, have a slow cell turnover under normal physiological conditions. After a damaging event, the tubular cells respond with diffuse proliferation (1).

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 (4).

Acute kidney injury (AKI) is a critical clinical condition

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associated with a high degree of morbidity and mortality despite the best supportive care. However, at present, no effective treatment-improving disease outcome is available (5). A great number of patients with AKI require hemodialysis. The mortality rate of patients requiring dialysis as a result of AKI is twice as high compared to patients without AKI (6).

Ischemia reperfusion injury (IRI) is one of the main causes of AKI. It occurs in a broad spectrum of clinical settings including transplantation surgery, trauma, dehydration or sepsis leading to renal hypoperfusion, acute tubular necrosis (ATN) and functional disturbances namely AKI. In renal transplantation, it is a well known risk factor for delayed graft function, which prolongs hospitalization, increases costs and needs a greater complexity of immunosuppressive drug management (7).

Clinical management of AKI has improved over the last years, but a specific therapy to improve renal function after AKI has not been developed yet. Complications arise from the inability of the kidney to regenerate lesions with functional tubular epithelial cells (8). Failure to replace damaged cells gives rise to tubulo-interstitial fibrosis and scarring, increasing the susceptibility for chronic renal injury (1).

"Stem cell-based approaches" have enormous potential for the development of future therapies. Bone marrow-derived stem cells are among the most promising candidates for clinical applications. MSCs have been of high interest for regenerative therapies because they are easy to harvest, can be readily expanded in culture and differentiate into a number of cell types *in vitro* (9).

It has been proposed that treatment with pluripotent adult stem cells offers, compared with pharmacological interventions, a broad therapeutic spectrum through which vascular, inflammatory and other manifestations of ischemic AKI can be simultaneously targeted. This reasoning is based on the fact that administered stem cells readily reach intra-renal sites of injury via the circulation. There, they can react physiologically to different local stimuli, for example, hypoxia or ischemia, in turn leading to the release of vasoactive factors, growth factors, immunomodulatory cytokines and chemokines (10).

Some studies have shown that administration of bone marrow-derived MSCs after renal ischemia/reperfusion (I/R) promoted recovery of renal function and morphological damage, indicating potential promise of healing damaged kidney after acute I/R injury with MSCs (11).

The aim of this study was to evaluate the therapeutic potential of BM-MSCs on induced acute kidney injury in a rat model of ischemia reperfusion, monitored by sero-

logical, histological and morphometric examination.

Materials and Methods

Material

Animals: This study included 30 adult male albino rats, 180~200 (185 ± 1.25 grams) body weight. They were housed in hygienic stainless steel cages and kept in clean well-ventilated room. They were fed standard chow diet and allowed free access to water. The experiment was carried out in the Animal House of Faculty of Medicine, Cairo University, according to the Ethical Guidelines for the Care and Use of Laboratory Animals.

Animals were divided into three groups, ten rats each, as follows:

Group I (control sham-operated): 10 rats were subjected to sham operation. Rats received saline as a vehicle intravenously. They were subdivided into two subgroups, five rats each (subgroups Ia & Ib), which were sacrificed with the corresponding experimental subgroups.

Experimental group: 20 rats were subjected to renal ischemia-reperfusion (I/R) injury by clamping both renal pedicles for 40 minutes (12).

Rats were divided as follows

Group II (non-MSCs-treated group): 10 rats that received single injection of saline immediately after reflow. They were sacrificed as follows:

Subgroup IIa: 5 rats were sacrificed 24 hours (13) after removal of the clamps and visual confirmation of reflow (14).

Subgroup IIb: 5 rats were sacrificed 72 hours (12) after removal of clamps.

Group III (MSCs-treated group): 10 rats that received single injection of MSCs immediately after removal of the clamps and visual confirmation of reflow.

They were sacrificed as follows:

Subgroup IIIa: 5 rats were sacrificed 24 hours after MSCs injection.

Subgroup IIIb: 5 rats were sacrificed 72 hours after MSCs injection.

Methods

Induction of ischemia-reperfusion acute kidney injury: Animals were anesthetized with ketamine xylazine. Midline abdominal incision was performed after disinfection of the abdominal wall using povidone iodine. Kidneys were exposed and renal pedicles were bilaterally clamped for 40 minutes by atraumatic vascular clamps to induce renal ischemia (10). The clamps were then re-

moved to allow kidney reperfusion. Kidneys were observed to ensure reflow i.e swift dis- and re-coloration of the kidney (14). The incision was closed in two layers using 4-0 silk sutures, followed by topical application of garamycin cream to reduce the risk of wound infection.

Sham operation: Sham-operated animals underwent the same surgical procedure, except that the clamps were not applied (15).

Preparation of bone marrow derived-mesenchymal stem cells (BM-MSCs): Rat bone marrow derived, PKH26 labeled, mesenchymal stem cells (MSCs) were purchased from Biochemistry Department, Kasr Al-Ainy Medical School.

Bone marrow was harvested by flushing the tibiae and femurs of 6-weeks-old male albino rats with Dulbecco's modified Eagle's medium (DMEM, GIBCO/BRL) supplemented with 10% fetal bovine serum (GIBCO/BRL). Nucleated cells were isolated with a density gradient (Ficoll/Paque [Pharmacia Fine Chemicals]) and resuspended in complete culture medium supplemented with 1% penicillin-streptomycin (GIBCO/BRL). Cells were incubated at 37°C in 5% humidified CO₂ for 12~14 days, until formation of large colonies (80~90% confluence). The culture was washed with phosphate buffered saline (PBS) and released with 0.25% trypsin in 1mM EDTA (GIBCO/BRL) (5 min at 37°C). After centrifugation, the cells were resuspended with serum-supplemented medium and incubated in 50 cm² culture flask (Falcon) (16). MSCs in culture were characterized by their adhesiveness and fusiform shape (17).

Labeling of stem cells with PKH26 dye: MSCs were harvested during the 2nd passage and were labeled with PKH26 fluorescent linker dye (18). PKH26 was purchased from Sigma Company (St. Louis, MO, USA). Cells were centrifuged and washed twice in serum free medium. Cells were pelleted and suspended in dye solution. Fluorescent labeled MSCs were injected I.V. Renal tissue was examined with a fluorescent microscope (Olympus BX50F4, No. 7M03285, Tokyo, Japan) to detect and trace the cells stained with PKH26.

Injection of MSCs: Immediately after reflow, rats of group III received labeled MSCs diluted with 1 ml of saline, loaded in a 1-ml sterile syringe and injected via tail vein for each rat (12).

Laboratory investigations: Using capillary tubes, blood samples were drawn from retro orbital veins. Serum urea and creatinine were measured for all rats, before and after intervention throughout the period of the experiment (at day 0, day 1, day 2 and day 3 after induction of I/R). Measurements were estimated by conventional colorimetric

method using Quanti Chrom TM assay kits based on the improved Jung and Jaffe methods, respectively (DIUR-500 and DICT-500) (19). Measurements were done at Biochemistry Department, Kasr Al-Ainy Medical School.

Light microscopic studies: At the end of each experimental period, right sided kidney specimens were fixed in 10% buffered formalin solution for 48 hours, dehydrated in ascending grades of ethanol and embedded in paraffin. Serial sections of 5~7 µm thickness were cut, mounted on glass slides and subjected to the following techniques:

1. H&E stain for histological assessment (20).
2. Periodic acid schiff (PAS) stain to assess brush border and basement membrane of proximal convoluted tubules (21).
3. Immunohistochemical staining for proliferating cell nuclear antigen (PCNA). Kidney sections were incubated with mouse monoclonal anti-PCNA antibody (PC 10, Novocastra, Milton, Keynes, USA), using the avidin-biotin peroxidase complex technique. Sections were counterstained with Meyer's hematoxylin (22). PCNA positive cells showed brown nuclear deposits.

Positive tissue control: Sections of human tonsil were immunostained for PCNA positive cells. PCNA immunoreactivity appeared as brown nuclear deposits.

Negative control: Additional specimens of kidney were processed in the same way but skipping the step of applying the primary antibody.

4. Examination of PKH26 labelled MSCs using Fluorescent Microscope (Japan, 7M03285) in unstained sections.

Morphometric study: Using a Leica Qwin 500 LTD Image Analysis Computer System (Cambridge, UK), the following parameters were measured:

1. Number of total and affected Malpighian renal corpuscles.
2. Number of total and affected tenal tubules.
They were counted in every fifth section of H&E-stained sections, using "counting" option on the interactive measuring menu. From each fifth section, ten non-overlapping fields were measured at a magnification of ×100. Numbers were tabulated and calculated as percentage of total number.
3. Mean area percent of PAS-positive reaction, in PAS-stained sections, measured in 10 random fields for each specimen at a magnification of ×400. The area percent represented the area of the positive reaction, which was masked by a binary colour to the area of the standard measuring frame.
4. Mean area % of positive immunoreactivity for PCNA,

measured in 10 high-power fields for each specimen at a magnification of $\times 400$.

Statistical analysis: All measurements were subjected to statistical analysis using Student T test and ANOVA test using SPSS Software ver. 9.0 Chicago, IL, USA (23).

Results

Results of laboratory investigations

Measurements of serum urea levels in the studied groups (Table 1): On day zero (before intervention), there was no significant difference in the mean serum urea values between all subgroups and the corresponding control. On day 1, mean serum urea values for all experimental subgroups showed a significant increase ($p < 0.05$) when compared to the corresponding control. On days 2 and 3, mean serum urea values for subgroups IIb and IIIb represented a significant increase when compared to the corresponding control. Mean serum urea values for subgroup IIIb showed a significant decrease when compared to subgroup IIb.

Measurements of serum creatinine levels in the studied groups (Table 2): Mean Serum creatinine values showed no significant difference between all subgroups and the corresponding control on day 0. On day 1, mean serum creatinine levels for all experimental subgroups showed a significant increase ($p < 0.05$) when compared to the corresponding control. On days 2 & 3, mean serum creatinine levels for subgroups IIb and IIIb represented a significant increase when compared to the corresponding control. Mean serum creatinine level for subgroup IIIb showed a

significant decrease when compared to subgroup IIb.

Histological results

Confirmation of homing of MSCs into the renal tissue: MSCs showed strong red autofluorescence after transplantation into rats, confirming that these cells were seeded into kidney tissue. Sections in the renal cortex of subgroup IIIa (MSC injected, 24 hrs) showed many MSCs labeled with the PKH26 detected within the corpuscles and tubules (Fig. 1).

Hematoxylin and eosin-stained sections: In the present study, examination of cortical kidney sections belonging to subgroups Ia & Ib were similar and did not show histological variations, therefore, results of both groups

Fig. 1. Photomicrograph of a section in the renal cortex of subgroup IIIa (MSC injected, 24 hrs) showing several PKH26 MSCs (red fluorescent) within the renal corpuscles and renal tubules (blue arrows) (immunofluorescence, $\times 400$).

Table 1. The mean values of serum urea level ($\pm$ SD) in the studied groups

Day	Group I	Subgroup IIa	Subgroup IIb	Subgroup IIIa	Subgroup IIIb
Day 0	30.9 $\pm$ 3.50	30.9 $\pm$ 3.50	30.5 $\pm$ 2.79	30.7 $\pm$ 4.03	30.9 $\pm$ 3.50
Day 1	30.9 $\pm$ 4.32	92.81 $\pm$ 16.28 ^a	94.22 $\pm$ 14.29 ^a	91.11 $\pm$ 13.33 ^a	92.1 $\pm$ 15.37 ^a
Day 2	30.32 $\pm$ 4.32		105.11 $\pm$ 19.98 ^a		71.22 $\pm$ 11.55 ^{a,b}
Day 3	30.8 $\pm$ 4.31		98.6 $\pm$ 15.50 ^a		52.5 $\pm$ 10.46 ^{a,b}

^aSignificantly different from the corresponding value of the control group at $p < 0.05$. ^bSignificantly different from the corresponding value of subgroup IIb at $p < 0.05$ on the same day.

Table 2. The mean values of serum creatinine level ($\pm$ SD) in the studied groups

Day	Group I	Subgroup IIa	Subgroup IIb	Subgroup IIIa	Subgroup IIIb
Day 0	0.83 $\pm$ 0.23	0.78 $\pm$ 0.19	0.81 $\pm$ 0.20	0.81 $\pm$ 0.20	0.82 $\pm$ 0.22
Day 1	0.82 $\pm$ 0.20	2.23 $\pm$ 0.54 ^a	2.25 $\pm$ 0.49 ^a	2.21 $\pm$ 0.63 ^a	2.24 $\pm$ 0.52 ^a
Day 2	0.82 $\pm$ 0.22		3.28 $\pm$ 0.89 ^a		2.21 $\pm$ 0.53 ^{a,b}
Day 3	0.81 $\pm$ 0.21		2.46 $\pm$ 0.77 ^a		1.1 $\pm$ 0.33 ^{a,b}

^aSignificantly different from the corresponding value of the control group at $p < 0.05$. ^bSignificantly different from the corresponding value of subgroup IIb at $p < 0.05$ on the same day.

were represented as the control group (I). Sections in the renal cortex of the control group exhibited normal histological architecture demonstrating Malpighian renal corpuscles (MRCs) formed of tuft of glomeruli and Bowman's capsule enclosing Bowman's space, proximal (PCT) and distal convoluted tubules (DCT). PCTs showed narrow lumina and were lined with high cuboidal (pyramidal) cells with rounded basal pale nuclei. The lining cells exhibited apical brush border and basal striations. DCTs showed wider lumina and were lined with cubical cells with rounded central nuclei and less prominent basal striations (Fig. 2).

Examination of cortical sections of subgroup IIa showed many forms of affection and damage of renal tissue; marked destruction of renal corpuscles was noted in some fields with loss of almost all glomerular tufts. Glomerular as well as peritubular congestion was observed in some cortical sections in addition to the presence of exudation in the interstitial tissue (Fig. 3). Tubular changes included cytoplasmic vacuolation and pyknotic nuclei of most of the lining tubular cells. Marked rarefaction of tubular architecture was noted where some tubules exhibited shedding and dislodgment of the lining epithelium in the lumina (Figs. 3, 4). Acidophilic hyaline casts were detected in the lumina of some cortical tubules (Fig. 4).

Similar morphological alterations were observed in cortical structures (glomerular and tubular) on examination of kidney sections of subgroup IIb with widening of Bowman's space (Fig. 5).

On examination of renal cortical sections of subgroup

IIIa, distortion of renal corpuscles was noted, in the form of shrunken glomeruli. Some areas of tubular cytoplasm exhibited vacuolation. Cellular debris in tubular lumina was noted (Fig. 6).

Sections in the renal cortex of subgroup IIIb showed apparently normal histological architecture of renal cor-

Fig. 3. Photomicrograph of a section in the renal cortex of subgroup IIa showing complete destruction of a renal corpuscle with loss of nearly all glomerular tufts (G). The remaining glomerular capillaries are congested (arrowheads). Tubular lining cells show rarefied cytoplasm exhibiting vacuolations (V) and pyknotic nuclei (arrows). Tubular lumina contain debris (black stars). Note the presence of peritubular congestion (C) and exudation (E) in the interstitial tissue (H&E, ×400).

Fig. 2. Photomicrograph of a section in the renal cortex of group I (control sham-operated) showing Malpighian renal corpuscle containing glomerulus (G) surrounded by Bowman's space (arrowhead). PCT (P) is lined with high cuboidal cells having rounded basal pale nuclei. DCT (D) is lined with cubical cells having rounded central nuclei (H&E, ×400).

Fig. 4. Photomicrograph of a section in the renal cortex of subgroup IIa showing marked rarefaction and vacuolation (V) of the cytoplasm of the lining tubular cells. Some tubules show areas of lost nuclei of the lining cells (green arrows). Many tubules show dense hyaline casts (short arrows). Peritubular congestion is noted (C) (H&E, ×400).

Fig. 5. Photomicrograph of a section in the renal cortex of subgroup IIb showing two MRCs with shrunken glomeruli (G) and widened bowman's space (arrowheads). The cytoplasm of the lining tubular cells exhibits vacuolation (V). Cellular debris is dislodged in the tubular lumina (black stars). Peritubular congestion (C) is noted (H&E, x400).

Fig. 6. Photomicrograph of a section in the renal cortex of subgroup IIIa (stem, 24 hrs) showing Malpighian renal corpuscle with shrunken glomerulus (G) and widened Bowman's space (arrowhead). Some of the lining tubular epithelial cells show cytoplasmic vacuolations (V). Cellular debris is observed in lumina of some tubules (black stars) (H&E, x400).

puscles with well formed capillary tufts and normal appearance of Bowman's space. Most of the tubules; PCT and DCT, exhibited apparently normal histological architecture; except for few areas of cytoplasmic vacuolation. No peritubular congestion was noted (Fig. 7).

PAS stained sections: Sections in the renal cortex of the control group showed multiple cortical tubules with preserved brush border and basement membrane (Fig. 8). Sections in the renal cortex of subgroup IIa (IRI, 24 hrs)

Fig. 7. Photomicrograph of a section in the renal cortex of subgroup IIb (stem, 72 hrs) showing apparently normal histological architecture of a renal corpuscle with well formed glomerular tuft (G) surrounded by Bowman's space. Renal tubules, PCT (P) and DCT (D), show apparently normal histological architecture, except for few areas of cytoplasmic vacuolation (H&E, x400).

Fig. 8. Photomicrograph of a section in the renal cortex of group I (control sham-operated) showing multiple cortical tubules with preserved brush border (arrows) and basement membrane (arrowheads) (PAS, x400).

(Fig. 9), subgroup IIb (IRI, 72 hrs) as well as subgroup IIIa (MSC injected, 24 hrs) showed partial loss or complete loss of the brush border in most of the tubules. Basement membrane was also interrupted at some sites. Sections in the renal cortex of subgroup IIIb (MSC injected, 72 hrs) showed many cortical tubules with preserved brush border. A continuous basement membrane was detected in nearly all the tubules (Fig. 10).

Immunohistochemical results

PCNA immuno-stained sections: Sections in the renal

Fig. 9. Photomicrograph of a section in the renal cortex of subgroup IIa (IRI, 24 hrs) showing partial loss (blue arrows) and complete loss (yellow arrows) of brush border in most of the tubules. basement membrane are also interrupted at some sites of the tubules (green arrowheads) (PAS, $\times 400$).

Fig. 10. Photomicrograph of a section in the renal cortex of subgroup IIIb (MSC injected, 72 hrs) showing many cortical tubules with preserved brush border (arrows). Continuous basement membrane (arrowheads) are detected in nearly all the tubules (PAS, $\times 400$).

cortex of the control group showed some positive PCNA immuno-reactive nuclei among the lining tubular epithelial cells (Fig. 11). An apparent increase in positive PCNA immuno-reactive nuclei in the lining tubular epithelial cells was observed in subgroups (IIb) (Fig. 12), as well as IIIb, when compared to other groups (Fig. 13).

Quantitative morphometric results

Mean percentage of renal corpuscles with affected glomeruli ($\pm$ SD) in the studied groups: The mean % of renal corpuscles with affected glomeruli in all the ex-

Fig. 11. Photomicrograph of a section in the renal cortex of group I (control sham-operated) showing some positive PCNA immuno-reactive nuclei (arrowheads) among the tubular lining cells (PCNA, $\times 1,000$).

Fig. 12. Photomicrograph of a section in the renal cortex of subgroup IIb (IRI, 72 hrs) showing many positive PCNA immuno-reactive nuclei (arrowheads) among the tubular lining cells (PCNA, $\times 1,000$).

perimental subgroups expressed a significant increase ($p < 0.05$) when compared to control. Such measurement started to decline in subgroup IIIb, representing a significant decrease when compared to subgroups IIa, IIb and IIIa. No statistically significant difference was reported between subgroups IIa, IIb and IIIa (Table 3, Fig. 14).

Mean percentage of affected tubules ($\pm$ SD) in the studied groups: The mean percentage of affected tubules in all the experimental subgroups showed a significant increase ($p < 0.05$) when compared to control. A statistically significant decrease was reported in subgroup IIb as com-

Table 3. The mean percentage of renal corpuscles with affected glomeruli ($\pm$ SD) in the studied groups

Groups and subgroups	Mean% $\pm$ SD of renal corpuscles with affected glomeruli
Group I	6.43 $\pm$ 1.43
Subgroup IIa	63.54 $\pm$ 10.14 ^a
Subgroup IIb	55.54 $\pm$ 8.98 ^a
Subgroup IIIa	59.15 $\pm$ 8.26 ^a
Subgroup IIIb	23.94 $\pm$ 5.12 ^{a,b}

^aSignificantly different from the value of the control group at $p < 0.05$. ^bSignificantly different from the values of subgroups IIa, IIb and IIIa at $p < 0.05$.

Fig. 13. Photomicrograph of a section in the renal cortex of subgroup IIa (MSC injected, 72 hrs) showing apparent increase in positive PCNA immuno-reactive nuclei (arrows) among the lining tubular epithelial cells (PCNA, $\times 1,000$). **Fig. 14.** Histogram comparing the mean percentage of renal corpuscles with affected glomeruli in the studied groups. ^aSignificantly different from the value of the control group at $p < 0.05$. ^bSignificantly different from the values of subgroups IIa, IIb and IIIa at $p < 0.05$.**Table 4.** The mean percentage of affected tubules ($\pm$ SD) in the studied groups

Groups and subgroups	Mean% $\pm$ SD of affected tubules
Group I	4.1 $\pm$ 1.33
Subgroup IIa	79.68 $\pm$ 1.96 ^a
Subgroup IIb	65.92 $\pm$ 1.27 ^{a,c}
Subgroup IIIa	78.2 $\pm$ 0.44 ^a
Subgroup IIIb	39.62 $\pm$ 1.02 ^{a,b}

^aSignificantly different from the value of the control group at $p < 0.05$. ^bSignificantly different from the values of subgroups IIa, IIb and IIIa at $p < 0.05$. ^cSignificantly different from the value of subgroup IIa at $p < 0.05$.

Fig. 15. Histogram comparison the mean percentage of affected tubules in the studied groups. ^aSignificantly different from the value of the control group at $p < 0.05$. ^bSignificantly different from the values of subgroups IIa, IIb and IIIa at $p < 0.05$. ^cSignificantly different from the value of subgroup IIa at $p < 0.05$.

pared to subgroup IIa. Such measurement represented a significant decrease in subgroup IIIb, when compared to subgroups IIa, IIb and IIIa (Table 4, Fig. 15).

Mean area percentage of PAS positive reaction ($\pm$ SD) in the studied groups: The mean area % of PAS positive reaction in all the experimental subgroups showed a significant decrease ($p < 0.05$) when compared to control. Such measurement showed a significant increase in subgroup IIIb when compared to subgroups IIa, IIb and IIIa. No statistically significant difference was recorded between subgroups IIa, IIb and IIIa (Table 5, Fig. 16).

Mean area percentage of PCNA positive nuclei ($\pm$ SD) in the studied groups: Mean area % of PCNA immunoreactivity in the tubular epithelial cells of subgroups IIa, IIb, IIIa and IIIb showed a significant increase ($p < 0.05$) when compared to control. The highest value for such measurement was recorded in subgroup IIIb, representing

Table 5. Mean area percentage of PAS positive reaction ($\pm$ SD) in the studied groups

Groups and subgroups	Mean% $\pm$ SD of affected tubules
Group I	39.55 $\pm$ 4.88
Subgroup IIa	14.32 $\pm$ 2.33 ^a
Subgroup IIb	14.32 $\pm$ 2.33 ^a
Subgroup IIIa	14.8 $\pm$ 2.52 ^a
Subgroup IIIb	29.09 $\pm$ 3.52 ^{a,b}

^aSignificantly different from the value of the control group at $p < 0.05$. ^bSignificantly different from the values of subgroups IIa, IIb and IIIa at $p < 0.05$.

Fig. 16. Histogram comparing the mean area percentage of PAS positive reaction in the studied groups. ^aSignificantly different from the value of the control group at $p < 0.05$. ^bSignificantly different from the values of subgroups IIa, IIb and IIIa at $p < 0.05$.

a significant increase when compared to all subgroups (Table 6, Fig. 17).

Discussion

Renal ischemia/reperfusion (I/R) injury, whether caused by shock or during surgery or transplantation, is the most common cause for acute kidney injury (AKI) which is associated with high morbidity and mortality rates (24).

In the present study, serological analysis went parallel with morphometric examination for all groups. A statistically significant elevation of serum levels of urea and creatinine was reported in groups II & III, 24 hrs after intervention. Such measurements confirmed the occurrence of renal dysfunction. These results are consistent with the findings of researchers who reported that animals that underwent renal I/R exhibited significant increase in the serum concentrations of urea and creatinine, compared with sham-operated animals (25).

Table 6. Mean area percentage of PCNA positive nuclei ($\pm$ SD) in the studied groups

Groups and subgroups	Mean% $\pm$ SD of affected tubules
Group I	2.12 $\pm$ 0.42
Subgroup IIa	4.81 $\pm$ 1.34 ^a
Subgroup IIb	6.56 $\pm$ 2.09 ^{a,c}
Subgroup IIIa	7.78 $\pm$ 1.76 ^{a,c}
Subgroup IIIb	14.90 $\pm$ 2.13 ^{a,b}

^aSignificantly different from the value of the control group at $p < 0.05$. ^bSignificantly different from the values of subgroups IIa, IIb and IIIa at $p < 0.05$. ^cSignificantly different from the value of subgroup IIa at $p < 0.05$.

Fig. 17. Histogram comparison the mean area % of PCNA positive nuclei in the studied groups. ^aSignificantly different from the value of the control group at $p < 0.05$. ^bSignificantly different from the values of subgroups IIa, IIb and IIIa at $p < 0.05$. ^cSignificantly different from the value of subgroup IIa at $p < 0.05$.

In the present study, morphological findings detected in the Malpighian corpuscles after I/R ranged from distortion of corpuscles exhibiting marked destruction of glomerular capillary tufts with shrunken glomeruli to complete destruction and complete loss of glomerular capillaries. Marked glomerular capillary distortion or complete loss is described as a picture of "end stage kidney" resulting from acute and severe renal injury (26).

As regards the renal tubules, severe tubular damage was detected where the lining tubular epithelial cells vacuolated cytoplasm and pyknotic nuclei. Dislodgment and shedding of epithelial cells was evident with accumulation of cellular debris in tubular lumina as well as acidophilic hyaline casts. Similar changes were previously reported by other investigators who demonstrated severe acute tubular damage in kidney sections of the I/R group (27). The influx of neutrophils, macrophages and lymphocytes during reperfusion initiates a cascade of chemokines liberation

with the subsequent production of reactive oxygen species and nitric oxide resulting in further tubular damage (28). Histomorphometric analysis in the current work revealed a significant increase in the mean area % of corpuscles with affected glomeruli and the tubules when compared to control sham-operated group.

Loss of renal tubular epithelium associated with I/R is attributed to both apoptotic and necrotic cell death. I/R results in overwhelming cellular ATP depletion leading to cellular dysfunction and ultimately cell death (29). In I/R injury, shedding of tubular epithelial cells occurs secondary to ischemia and anoxia which could lead to loss of cellular integrity. This could be clarified on the basis of assumption of investigators who stated that ischemia results in rapid loss of brush border and cytoskeletal integrity which leads to loss of cell junctions and loss of polarity with mislocalization of adhesion molecules and other membrane proteins such as the Na⁺K⁺-ATPase and β -integrins leading to desquamation of epithelial tubular cells (30).

Disruption of brush border in I/R group was evident in PAS-Stained sections of the I/R group which revealed partial and complete loss of brush border in most of the tubules. This was further confirmed by morphometric analysis which proved a statistically significant decrease in the PAS mean area% in I/R sections.

Acidophilic hyaline casts demonstrated in some tubular lumina of the I/R group might represent cellular debris that underwent molecular changes. Cells and their debris that detach from the tubular basement membrane combine with proteins present in the tubular lumina resulting in cast formation (31).

Cortical sections of I/R group revealed peritubular congestion and the presence of hyaline exudates in the interstitium. This might represent an inflammatory response occurring as a sequelae of ischaemia and shortage of oxygen supply. Similar observations were previously reported by researchers who demonstrated the presence of hyaline exudation in the interstitial spaces as well as peritubular congestion (32).

Although reperfusion is essential for the survival of ischaemic tissue, there is good evidence that reperfusion itself causes additional cellular injury "reperfusion injury", which has been attributed to the generation of reactive oxygen species (ROS), depletion of ATP, neutrophil infiltration, and membrane lipid alterations, cytoskeletal dysfunction and intracellular Ca²⁺ accumulation (27).

Meanwhile, statistical analysis proved a significant decrease in the mean area % of affected tubules 72 hrs after induction of injury (IIb) as compared to 24 hrs after in-

duction of injury (IIa). Thus, the extent of injury was progressively reduced with longer periods of reperfusion. This might be due to the process of repair which occurs in response to tubular injury. It was previously reported that the greatest tubular injury was detected after 18 h of reperfusion and the extent was progressively reduced with longer periods of reperfusion due to the concomitant tubular recovery observed after 72 hrs (30).

In the present study, the experimental rats which received MSC therapy after I/R revealed that on day 2 (48 hrs after intervention) and day 3 (72 hrs after intervention), subgroup IIIb showed a significant decrease for both serum urea and creatinine levels, when compared to subgroup IIb. These results proved that administration of BM-MSCs resulted in improvement of renal functions starting on day 2.

Going parallel with serological measurements, histological examination of renal sections 24 hrs after MSC therapy revealed persistence of features of I/R damage in the form of glomerular distortion and tubular affection. However, 72 hrs after MSC therapy, apparently normal histological architecture was demonstrated for cortical renal corpuscles and tubules. Similar findings were reported by other researchers who demonstrated reversibility of cortical tubular renal damage after treatment of rats with MSCs (12). Quantitative histomorphometric analysis in the current work proved that the least percentage of corpuscles with affected glomeruli and as well as tubules was detected in rats sacrificed 72 hrs after receiving MSC therapy.

By referral to PAS-stained sections 72 hrs after MSC therapy, it was found that many cortical tubules exhibited preserved PAS positive brush border and continuous basement membrane was detected in nearly all the tubules. Morphometric analysis proved a statistically significant increase in mean area % of PAS 72 hrs after MSC when compared to other experimental groups.

In the present study, PCNA immunostaining was used to evaluate the regenerative capacity of injured tubular epithelial. The highest value was recorded in subgroup IIIb, representing a significant increase when compared to all subgroups. Such result goes hand in hand with the finding of other investigators who reported that PCNA expression in post-ischemic kidney increases from 9.4% on day 1 after I/R injury to 34% on day 2, thus it was time dependent (33).

This might be explained by the fact that under normal circumstances, the tubular epithelial cells have a slow rate of proliferation. Such low rate of turnover changes dramatically after an ischemic or toxic insult, when there is a marked increase in cell death by necrosis and apoptosis

and a vigorous response to replace these cells (30).

From the previous findings demonstrated in the present study, it could be assumed that administration of BM-MSCs resulted in improvement of renal injury, both morphologically and functionally. In the present study, intravenously injected MSCs have migrated to renal glomeruli and tubules. This was confirmed by localization of PKH26 labelled MSC which exhibited a red fluorescence when visualized by the fluorescent microscopy.

Homing of MSCs to sites of injury might be attributed to certain substances released at sites of tissue damage. Increased chemokine concentration at the inflammation site likely directs MSCs migration to these sites. Chemokines are released after tissue damage and MSCs express the receptors for several chemokines. Stromal-derived factor-1, platelet-derived growth factor and CD44 are likely candidates in the regulation of MSCs homing since their receptors are up-regulated after renal injury (34).

MSCs migrated to injured tissue might act by paracrine effects and/or differentiation. MSCs have been proved to integrate into damaged tubules and differentiate into renal epithelial cells in cases of cisplatin and glycerol induced acute kidney injury (35). However, other studies showed protection from injury by exogenous MSCs with little or no tubular incorporation. This discrepancy may be explained in part by different analytical methods and injury models (36).

MSCs beneficial effects are consistently noted within 1 to 2 days of injection, well before there is sufficient time for cellular growth, division and differentiation. Thus, direct tubule repopulation by exogenous MSCs is probably a less significant mechanism of MSCs induced renal repair (34). Given the importance of inflammation in the pathophysiology of acute kidney injury, it is very important to consider the immunomodulatory properties of MSCs and the role these may play in reno-protection (37).

The role of exogenous BM-MSCs might be also attributed to production of substances that stimulate renal stem cells. This was consistent with other researchers who suggested that exogenous MSCs paracrine activity may stimulate the endogenous renal stem cell population, leading to cellular recovery and renal injury repair (38).

Conclusion

In this rat model of ischemia reperfusion, BM-MSCs administration proved to have a potential reno-protective capacity which could be of considerable therapeutic promise in the management of AKI.

Reno-protective potential of MSCs in the current study was proved at both morphological and functional levels.

MSCs were capable of homing to injured kidney when

injected intravenously soon after injury.

Endogenous stem cells need to be augmented by exogenous administration of stem cells for more rapid improvement to attain normal function as early as possible.

Recommendations

Future studies should be directed to explore whether BM-MSCs have the same reno-protective effect in humans and to clarify the exact mechanism by which these cells provide improvement of degenerative changes.

If proved effective, it is advisable to use BM-MSCs as a therapeutic tool in case of AKI. Meanwhile, the proper dosage needed for therapeutic purposes should be determined.

Investigations of the possible role of MSCs administration on degenerative changes of other organs is also recommended.

Long-term risks, such as transformation or maldifferentiation, associated with MSCs administration, should be thoroughly investigated.

Potential conflict of interest

The authors have no conflicting financial interest.

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