Role of Mesenchymal Stem Cells Versus their Conditioned Medium on Cisplatin-Induced Acute Kidney Injury in Albino Rat. A Histological and Immunohistochemical Study

Original Article

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ABSTRACT

Introduction: Acute kidney injury (AKI) is a syndrome of rapidly declining renal function. Cisplatin nephrotoxicity is a major cause of AKI in about one third of patients under cisplatin treatment. Stem cell therapy have been suggested as a protective measure against cisplatin-induced AKI. The conditioned medium of the stem cells (serum-free culture medium) has been also found to minimize renal injury and might develop a new therapeutic strategy that avoids stem cells administration.

Aim of the Study: This study was conducted to compare the effect of intravenous injection of bone marrow-derived mesenchymal stem cells (BMSCs) versus their conditioned medium (CM) in minimizing the cisplatin-induced acute renal injury.

Material and Methods: Sixty adult female albino rats were divided into 4 main groups. Group (I) served as a control group, Group (II) (cisplatin treated group) that were subdivided into two subgroups IIa and IIb, Group (III) (BMSCs-treated group) and Group (IV) (CM-treated group). Five young male rats were additionally used for obtaining the BMSCs. Rats of all groups were sacrificed on 4th day of the experiment, except for the rats of subgroup (IIa (which were sacrificed after one day of cisplatin administration. Renal specimens were prepared for histological and immunohistochemical techniques. Morphometrical studies and statistical analysis were performed.

Results: Treatment by BMSCs resulted in obvious improvement of renal structure with significant increase in Proliferating Cell Nuclear Antigen (PCNA). However, conditioned medium (CM) was less effective in treatment of acute AKI.

Conclusion: BMSCs administration is preferable than their CM in reversing the acute structural damage of the kidney induced by cisplatin.

Key Words: Acute kidney injury, bone marrow-derived mesenchymal stem cells, cisplatin, conditioned medium.

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INTRODUCTION

Deterioration of renal function over a short period is now called "acute kidney injury" (AKI), which replaced the old term "acute renal failure"[1]. It is a syndrome of rapidly declining renal function induced by a number of different insults[2], with a mortality rate ranging from 30 to 80%. Recent dialysis techniques, continuous renal replacement therapy have no significant impact on overall mortality. Furthermore, the efforts to develop new pharmacological therapies have been largely unsuccessful[3].

Cisplatin is an anti-neoplastic drug used in the treatment of many solid-organ cancers including those of head, neck, lung, testis, ovary and breast[4]. However, nephrotoxicity is one of the main dose-limiting side effects of cisplatin[5], occurring in about one-third of patients undergoing cisplatin treatment[6]. Therefore, cisplatin was chosen to induce AKI in the present study.

Cell-based therapeutic approaches have been suggested to protect against AKI experimental models induced by cisplatin[7], glycerol[8] and ischemia-reperfusion injury[9]. Homing of mesenchymal stem cells (MSCs) to injured kidney had been demonstrated in recent researches. MSCs were documented to localize in damaged kidney promoting both morphological and functional recovery[8].

The conditioned medium (CM) of the BMSCs is the supernatant of the cultured cells containing growth factors, and suspended in serum-free complete culture medium. Interestingly the CM has been found to minimize cisplatin-

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induced renal injury, improved both renal function and structure[10]. However, the results obtained using the CM are still controversial. Hence, this study was undertaken to evaluate the capability of BMSCs versus their conditioned medium in minimizing the renal tubular structural damage using cisplatin induced acute renal injury model.

MATERIAL AND METHODS

Sixty adult female albino rats were used in the current study, each weighing between 150 – 180 gm. In addition, five young male albino rats of average weight of 80 gm each were used as a source of mesenchymal stem cells. The animals were purchased and raised in the Medical Research Center, Faculty of Medicine, Ain Shams University. The animals were housed in plastic cages with mesh wire covers and were given food and water ad libitum. The study was performed in accordance to the guidelines for care, use of laboratory animals and approved by ethical committee of Ain Shams University, Cairo, Egypt.

* *Isolation of BMSCs [11]:*

BMSCs were isolated from rat bone marrow under very restricted conditions after being anaesthetized by diethyl ether. The skin was sterilized by Betadine, The femurs and tibiae of the rats were carefully dissected from adherent soft tissues. Then they were placed into sterilized container containing phosphate buffered saline (PBS) and delivered to the laminar flow cabinet (NUAIRE, Biological Safety Cabinet, Class II Type A/B3, USA) to extract the bone marrow. The epiphyses of both ends of the bones were removed using sterile sharp scissors. A sterile syringe, filled with 2 ml of DMEM was inserted into one end of the bones to expel the marrow plugs from the opposite ends of bones to be collected into sterile tubes.

* *Culturing of BMSCs[11]:*

The sterile tubes containing the collected bone marrow specimens were flushed with DMEM and centrifuged at 1500 round per minute (rpm) for 5 minutes. The supernatant was removed using sterile pipettes (Greiner bio-one Company, Germany), and complete culture medium was added, formed of DMEM, 10% fetal bovine serum, 5 % sodium biarbonate and 1% penicillin/streptomycin (all purchased from Lonza Company, Swiss).

The mixture of bone marrow and complete culture medium was transferred to tissue culture flasks. They were then incubated at 37°C in a humidified atmosphere containing 5% CO2 and 95% air in a standard incubator.

The cultured cells were examined daily by using the inverted microscope (Axiovert 100- ZEISS) to follow up the growth of the cells and to detect any bacterial or fungal infection. The medium was first changed on the third day of culture to remove the non-adherent. After reaching about 90 % confluency, a subculture was performed on the 8th day. The old media was removed, and the cultured cells were washed twice with PBS to remove as much extracellular proteins as possible. One ml of pre-warmed trypsin /EDTA (37c◦) was added to the tissue culture flask for and left for five minutes. Complete medium was added to the trypsinized cells to stop any further effect of trypsin/ EDTA. The culture medium was then changed every third day. The third passage cells were used for injection into the rat tail vein after performing cell counting and viability using haemocytometer

* *Characterization of cultured BMSCs:*

Morphological characterization of the adherent BMSCs was performed by Giemsa staining on the 8th day of the culture. Immunohistochemical characterization was performed by using streptavidin-biotin immunoperoxidase technique for CD44, CD 105, CD 45 and CD 34[12].

* *Preparation of the conditioned medium[13]:*

BMSCs (2x106) were cultured with 2 ml of FBS-free DMEM for 48 hours. The conditioned medium was aspirated out, collected in Eppendorf tube, filtered through 0.20 um sterile syringe filter (Corning, NY14831, Germany).

* *Experimental design: Sixty animals were divided into four main groups:*

• **Group I (Control group):** Consisted of 20 rats that were further subdivided equally into two subgroups:

- Subgroup Ia: rats were given 0.5 ml of 0.9% physiological saline, once intraperitoneally.

- Subgroup Ib: rats were given single dose of 0.5 ml phosphate buffered saline (PBS) into tail vein.

- All animals of this group were sacrificed on the 4th day of the experiment.

• **Group II (cisplatin-treated group):** Consisted of 20 rats that were given single intraperitoneal injection of cisplatin (Unistin vials, 10 mg/10 ml, EIMC United Pharmaceuticals, Badr city, Cairo, Egypt), in a dose of 10 mg /kg body weight[14]. The rats that were then subdivided into two subgroups, ten animals each:

- Subgroup IIa: rats were sacrificed after one day of cisplatin injection to confirm the induction of AKI.

- Subgroup IIb: rats were sacrificed after three days from cisplatin injection (4th day of the experiment)[12].

• **Group III** (stem cells-treated group): Consisted of ten rats which received cisplatin in a dose and route similar to group II. One day after cisplatin injection, the BMSCs suspension was injected into the rat tail vein, in a dose of 2 x106 in 0.5 ml PBS/rat[13]. Rats of this group were sacrificed three days after cisplatin injection (4th day of the experiment).

• **Group IV** (conditioned medium-treated group): Consisted of ten rats which received single intraperitoneal injection of cisplatin in a similar dose and route as group II. One day after cisplatin injection, the conditioned medium was injected in the rats' tail vein at dose of 0.5 ml/rat[13]. Rats of this group were also sacrificed three days after cisplatin injection (4th day of the experiment).

All animals were sacrificed using ether inhalation. Both kidneys were dissected out. Right kidneys were fixed in 10% formalin, and processed to obtain paraffin blocks. Five µm-thick sections were cut and stained with Hematoxylin and Eosin stain (H&E), Periodic acid Schiff's reaction. In addition, immunohistochemical staining was performed using avidine-biotin peroxidase technique for detection of Proliferating Cell Nuclear Antigen (PCNA[15]. The antibody was purchased as Mouse Monoclonal Anti-Rat PCNA Antibody, Product Number P8825 (dilution of 1:3000), Sigma- Aldrich, Saint Louis, Missouri, USA. Sections were counterstained with hematoxylin, dehydrated, cleared and mounted. The reaction appeared as brownish nuclear granules. Positive control were done on tonsil specimens. Negative control was done after omitting the primary antibody.

Homing of the male-derived MSCs, bearing the male-specific sex determining region of Y chromosome (SRY) gene, in renal tissues of recipient female rats was assessed by PCR[16]. Genomic DNA was extracted from left kidneys tissue homogenate of control and stem cell-treated groups, using Wizard Genomic DNA purification kit (Promega. Madison, WI, USA) in the Biochemistry department, Faculty of Medicine, Cairo university.

* *Morphometric study and statistical analysis:*

1- Histo-pathological scoring of acute renal tubular injury was done based on the percentage of affected tubules in the kidney sections [17]. The score reflected the grading of tubular necrosis, cast formation and tubular dilatation. The injury score percentage was calculated in each field as follow: Injury score % = (number of injured tubules/ number of whole tubules) x 100.

The score of the injured tubules was then graded as follows:

Affected tubules ≤ 10% = Grade 1, ≤ 11- 25 % = Grade 2, ≤ 2645%- = Grade 3, ≤ 46- 75% = Grade 4 and ≤ 76-100% = Grade 5.

2- Thickness of the basement membrane of renal tubules was measured in PAS stained sections.

3- Number of PCNA positive cells were measured in the immunohistochemically-stained sections of all groups.

The measurements were performed using the image analyzer Leica Q500 MC program in the Histology Department Faculty of Medicine Ain Shams University. The PC was connected to an Olympus XB microscope-Japan. The values were taken per high power fields using: 200x in scoring and 40x in PAS and PCNA-stained sections. This was done in five randomly chosen non overlapping fields in each of five stained sections obtained from five animals in each group or subgroup.

The standard error of mean (SEM) was calculated and statistical analysis was carried out using SPSS statistical program version 17; IBM Corporation, NY 10589. Data were evaluated by using the one-way analysis of variance test (ANOVA). Regarding the probability, the least significant level used was at P value less than 0.05. and distal convoluted tubules. The renal corpuscle was seen composed of a glomerulus formed of a lobulated tuft of capillaries surrounded by Bowman's capsule. The PCTs were seen more numerous having narrow lumina and lined by pyramidal cells with indistinct cell boundaries. Their cytoplasm was strongly acidophilic with rounded vesicular nuclei. The DCTs were apparently less numerous than the PCTs in the renal cortex. They had relatively wider lumen, and were lined by cubical cells with faint acidophilic cytoplasm and apical rounded vesicular nuclei (Fig. 9). The mean histopathological score was of grade (1) (Table I).

RESULTS

I- Morphology of BMSCs:

On day seven of primary culture, the cells were mostly confluent (about 90- 95%), branched and interdigitated with each-others (Fig. 1). Giemsa stain on the 8th day showed cells with bluish granular cytoplasm and vesicular nuclei (Fig. 2). In sub-culture, BMSCs appeared mostly spindle in shape, with granular cytoplasm and vesicular nuclei (Fig. 3).

Immunostaining of BMSCs showed positive cytoplasmic brownish reaction for CD105 and CD44 (Figs. 4, 5 respectively). However, negative immunoreactivity was detected for CD34 and CD45 (Figs. 6, 7 respectively).

II- PCR assessment:

A product of 224 base pairing (bp) fragment on 2% agarose gel electrophoresis was yielded in the stem cell-treated group only. No PCR products were detected in the control group (Fig. 8).

III- Histological examination of the kidney specimens:

Examination of the control sections (group I) of both subgroups IA and IB showed the same histological picture in all stains performed, hence, collectively the term control group will be used thereafter.

H&E-stained sections:

Examination of H&E-stained sections of control rat showed renal cortex containing renal corpuscles, proximal and distal convoluted tubules. The renal corpuscle was seen composed of a glomerulus formed of a lobulated tuft of capillaries surrounded by Bowman's capsule. The PCTs were seen more numerous having narrow lumina and lined by pyramidal cells with indistinct cell boundaries. Their cytoplasm was strongly acidophilic with rounded vesicular nuclei. The DCTs were apparently less numerous than the PCTs in the renal cortex. They had relatively wider lumen, and were lined by cubical cells with faint acidophilic cytoplasm and apical rounded vesicular nuclei (Fig. 9). The mean histopathological score was of grade (1) (Table I).

In subgroup IIa (one day after cisplatin administration), focal changes of renal cortical structure were noticed. Loss of tubular architecture was seen in many areas. Few dilated tubules with flattened epithelium were seen, while other tubules were seen as acidophilic masses with ill-defined nuclei. Some tubules showed sloughing of parts of their epithelium inside their lumina. Cytoplasmic vacuolization was seen in many tubular cells. (Fig. 10). Mononuclear cellular infiltration was noticed in the interstitium and infiltrating the vessels' wall. Dilatation of peritubular capillaries and congestion of glomerular capillaries were observed. (Fig. 11). Significant increase in the mean histopathological score ($P<0.05$), was detected as compared to that of the control group, and was assessed as grade (4) (Table I).

These changes were apparently aggravated in subgroup IIb (three days after cisplatin administration). Dilatation of some renal tubules with flat epithelial lining were still observed (Fig. 12). Shedding of individual cells inside the tubular lumen and separation of the epithelial lining of some renal tubules leaving denuded areas was also seen (Figs. 12, 13). Some tubular cells appeared having deeply acidophilic cytoplasm with pyknotic or karyolitic nuclei (Fig. 13). Homogenous acidophilic material (hyaline casts) were seen inside the lumen of other renal tubules (Fig. 14). Congested glomerular capillaries were still seen in some sections (Fig. 12). Extravasation of red blood corpuscles was seen in the renal interstitium (Figs. 13, 14). Grade (4) renal tubular injury was detected reflected by a significant increase ($p<0.05$) in the mean histopathological score of the affected tubules as compared with the control group and subgroup IIa (Table I).

Sections of group III (stem cells-treated group) showed the structure of renal cortical tubules nearly similar to the control group. Closely packed cortical tubules were seen with their intact epithelial lining, however, some congested glomerular capillaries were still encountered (Fig. 15). Grade (1) tubular injury was detected reflecting a significantly decreased histopathological scoring ($p<0.05$) as compared with both cisplatin subgroups and a non-significant difference ($p>0.05$) as compared with the control group (Table I).

In group IV (conditioned medium-treated group), little improvement was detected when compared to groups II or III, as focal structural changes of cortical tissue were still seen. Dilated tubules with disrupted epithelial lining were noticed. Separation of epithelial lining of some tubules was detected. Cytoplasmic vacuolization was seen in many tubular cells. In addition, karyolitic nuclei were seen in some tubular cells (Fig. 16). Hyaline casts were also seen in the lumen of some tubules (Fig. 17). Grade (3) tubular injury was detected reflecting a significantly increased histopathological scoring ($p<0.05$) as compared with the control and stem cell-treated groups. However, it was significantly decreased ($p<0.05$) as compared with both cisplatin subgroups (Table I).

PAS-stained sections:

Examination of sections of the control group showed a well-defined apical brush borders of the PCTs. A PAS positive basement membranes was seen surrounding the renal tubules, glomerular capillaries and parietal layer of Bowman's capsule (Fig. 18).

In subgroup IIa (one day after cisplatin administration), focal loss of the apical brush border of some PCTs was noticed. Loss or disrupted PAS reaction was also detected in some tubular basement membranes. However, Significant thickening of other tubular basement membranes was detected ($p<0.05$) (Table I), together with apparent thickening of parts of parietal layer of Bowman′s capsule as compared to that of the

control group (Fig. 19).

Examination of PAS-stained sections of subgroup IIb (three days after cisplatin injection), revealed weak PAS reaction of the apical brush border of most of the PCTs. Disrupted basement membrane was seen in some tubules, while other tubular basement membranes were significantly thicker (P<0.05) as compared to the control group. However, this thickening was non significantly increased (p>0.05) when compared to that of subgroup IIa (Table I). In addition, apparent thickening of parts of the parietal layer of some of Bowman′s capsules was seen (Fig. 20).

In group III (stem cell-treated group), Some PCTs exhibited interrupted PAS positive apical brush borders. Regular basement membrane of many tubules, parietal layer of Bowman′s capsule and glomerular capillaries was seen. However, mild thickening of basement membrane of some tubules was noticed. This thickening was significantly decreased (p<0.05) as compared to both cisplatin subgroups, and significantly increased (p<0.05) when compared to that of the control group (Fig. 21 and Table I).

Sections of group IV (conditioned medium-treated group) showed regular apical brush border in few tubular cells. Other cells were seen with disrupted or even absent brush borders. Basement membrane of renal tubules was well seen in some parts, while other tubular basement membranes and parietal layer of Bowman's capsules were disrupted. The tubular basement membrane thickness was significantly decreased (p<0.05) when compared to both cisplatin subgroups. However, it was still significantly increased (p<0.05) as compared to control and stem cells-treated groups (Fig. 22, Table I).

The immunohistochemically-stained sections for PCNA:

Kidney sections of the control group showed positive reaction for PCNA in few nuclei of some tubular cells (Fig. 23 and Table I).

In subgroup IIa, (one day after cisplatin administration), positive reaction for PCNA was seen in few nuclei of tubular cells and in some nuclei of renal interstitium. Significant increase in PCNA immunoreactivity (p<0.05) was detected as compared to the control group (Fig. 24 and Table I).

Three days after cisplatin administration in subgroup IIb, significant increase in PCNA immunoreactivity (p<0.05) was seen in nuclei of some tubular cells as compared to the control group. Meanwhile, this immunoreactivity was non-significantly increased when compared to subgroup IIa (Fig. 25 and Table I).

After BMSCs treatment in group III, significantly increased PCNA immunoreactivity (p<0.05) was detected in nuclei of many tubular cells as compared to that of the control and cisplatin-treated groups (Fig. 26 and Table I).

However, after treatment by conditioned medium in group IV, PCNA immunoreactivity was seen in some tubular cell nuclei. It significantly decreased (p<0.05) as compared to cisplatin and stem cell-treated groups. Meanwhile, it was significantly increased (p<0.05) as compared to the control group (Fig. 27 and Table I).

Table 1: Means of morphometrical measurements in different groups:

Measurements	Control group (I)	Cisplatin group		BMSCs-treated group (III)	CM-treated group (IV)
		subgroup (IIa)	subgroup (IIb)		
Histopathological Scoring.	9.52 ±0.10	46.07 ±0.20a	60.09 ±0.16ab	10 ±0.15bc	28.88 ±0.22abcd
Mean thickness of tubular basement membrane.	0.56 ± 0.06	1.31 ± 0.10a	1.72 ± 0.17a	0.87 ± 0.04bc	1.1 ±0.10a
Mean number of PCNA positive tubular cells.	2.2 ±0.33	7.0 ±1.32a	8.3 ±1.23a	18.3 ±0.96abc	8.0 ±1.6ad

- Values are expressed as means±SEM, LSD = least significant difference
- a = Significance calculated by (LSD) at P<0.05 from the control group (I).
- b =Significance calculated by LSD at P<0.05 from subgroup (IIa).
- c= Significance calculated by LSD at P<0.05 from subgroup (IIb).
- d =Significance calculated by LSD at P<0.05 from group (III).

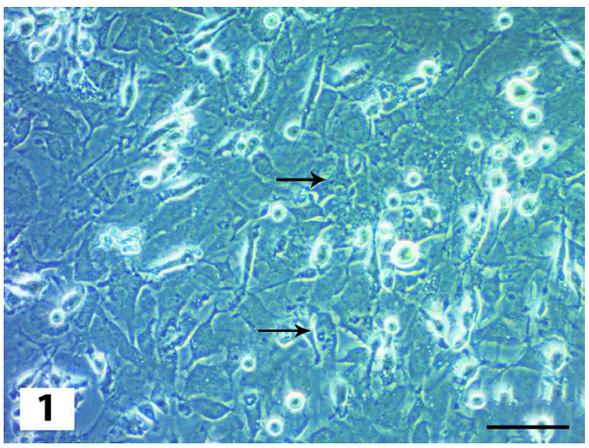

Fig. 1: Day 7 of primary culture of MSCs showing 90-95% confluent cells (↑).
(Phase contrast microscope x 200, scale bar=100μm)

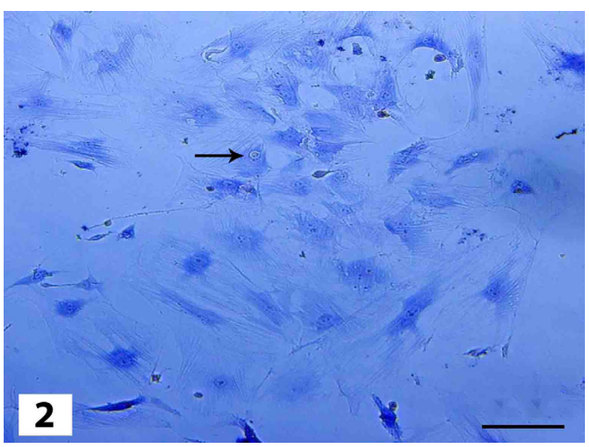

Fig. 2: Giemsa staining of MSCs (↑) showing their bluish cytoplasm and vesicular nuclei.
(Phase contrast microscope x 200, scale bar=100μm)

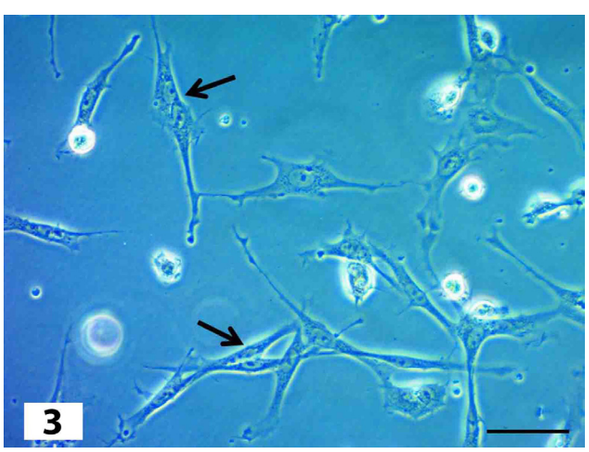

Fig. 3: Sub-culture of MSCs showing spindle-shaped cells (↑), having granular cytoplasm, vesicular nuclei and long cytoplasmic processes
(Phase contrast microscope x 200, scale bar=100μm)

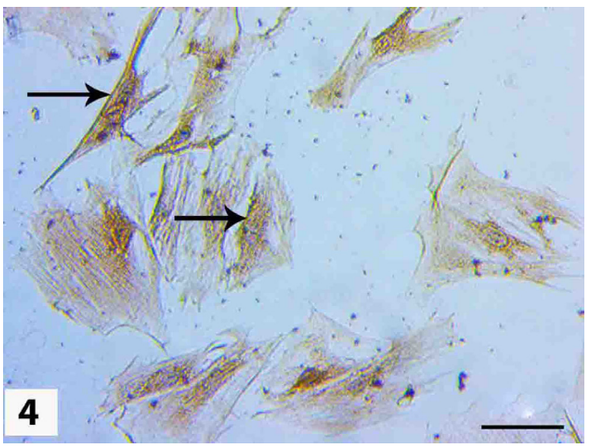

Fig. 4: Positive brownish immunoreactivity for anti CD105 antibody (↑).
(Phase contrast microscope x 200, scale bar 100μm)

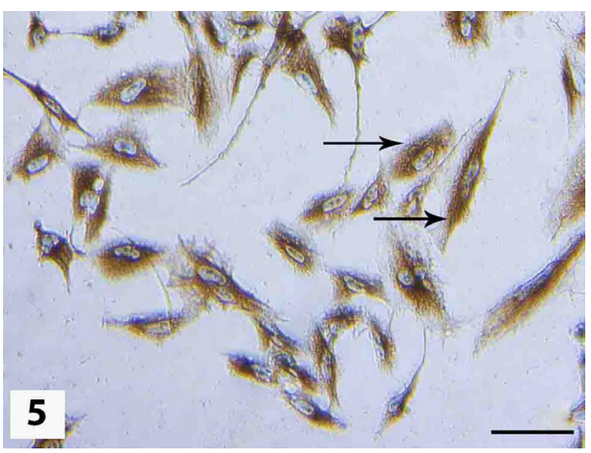

Fig. 5: Positive brownish immunoreactivity for anti CD44 antibody (↑).
(Phase contrast microscope x 200, scale bar=100μm)

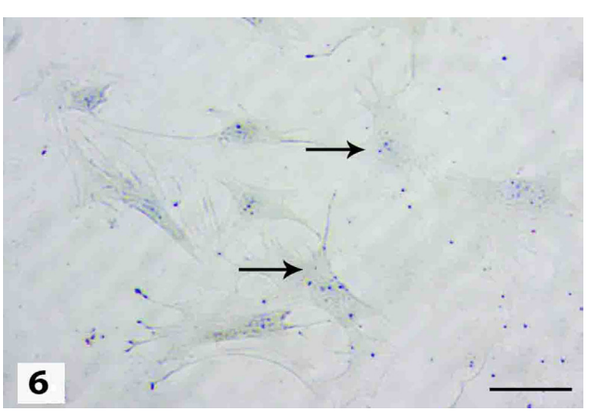

Fig. 6: Negative immunoreaction for anti CD 34 antibody (↑).
(Phase contrast microscope x 200, scale bar=100μm)

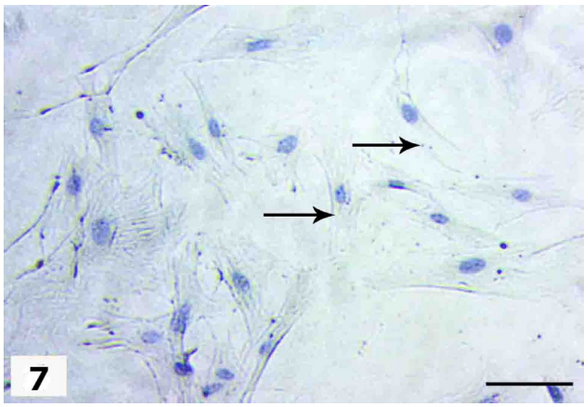

Fig. 7: Negative immunoreaction for anti CD 45 antibody (↑).
(Phase contrast microscope x 200, scale bar=100μm)

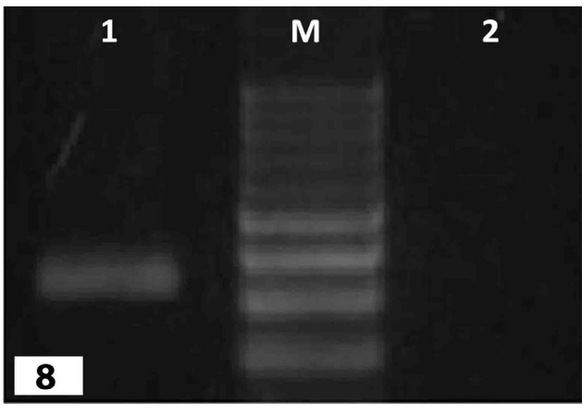

Fig. 8: An agarose gel electrophoresis showing PCR products of SRY gene expression. Lane1: PCR products in stem cells-treated rats. Lane 2: No PCR products in control group. Lane 3: DNA marker with 100 bp.

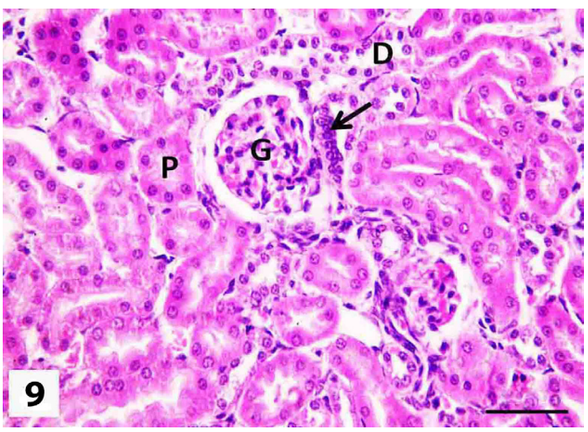

Fig. 9: Renal corpuscle formed of a glomerulus surrounded by Bowman's capsule (G). The PCTs with narrow lumen and cubical epithelial lining having dark acidophilic cytoplasm (P). The DCTs with wider lumen and pale acidophilic cubical cells (D). Notice macula densa (↑).
(Control group H&E x 400, scale bar=50μm)

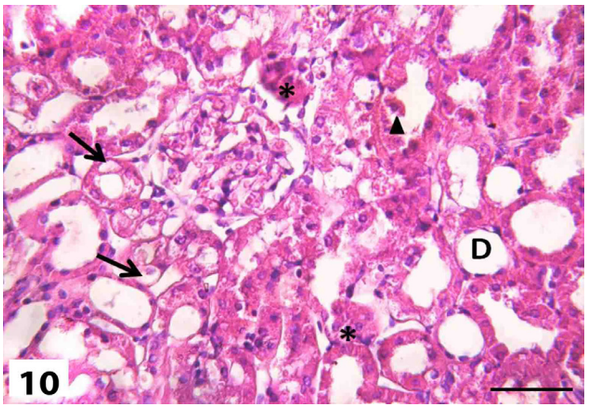

Fig. 10: Some tubules are seen as spherical acidophilic masses with ill-defined nuclei (*). Dilatation of other tubules with flattened epithelial lining can be observed (D). Notice the detachment of part of the epithelial lining in some tubules (▲). Cytoplasmic vacuolization is seen in many tubular cells (↑)
(Cisplatin-treated subgroup IIa, H&E x 400, scale bar=50μm)

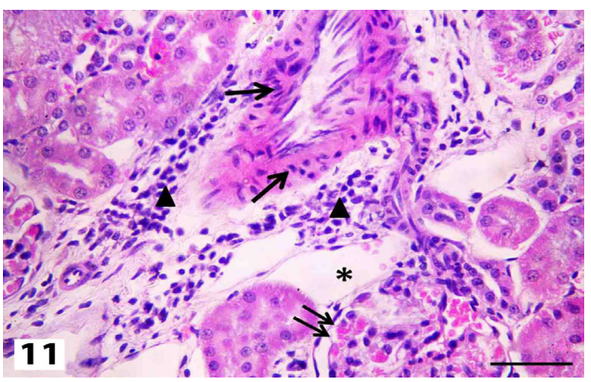

Fig. 11: Interstitial mononuclear cellular infiltrate (▲) and dilated peritubular capillaries (*) are seen. Longitudinally cut blood vessel with monnuclear cellularly infiltrated wall also is seen (↑). Notice part of the glomerulus with its congested capillaries (↑↑)
(Cisplatin-treated subgroup IIa, H&E x 400, scale bar=50μm)

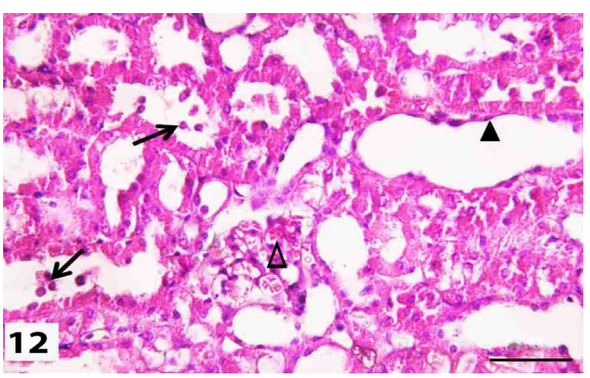

Fig. 12: Shedding of tubular epithelial cells can be seen inside the lumen (↑). Dilatation of some tubules with flattening of epithelial lining is noted (▲). Congested glomerular capillaries are noticed (Δ).
(Cisplatin-treated subgroup IIb, H&E x 400, scale bar=50μm)

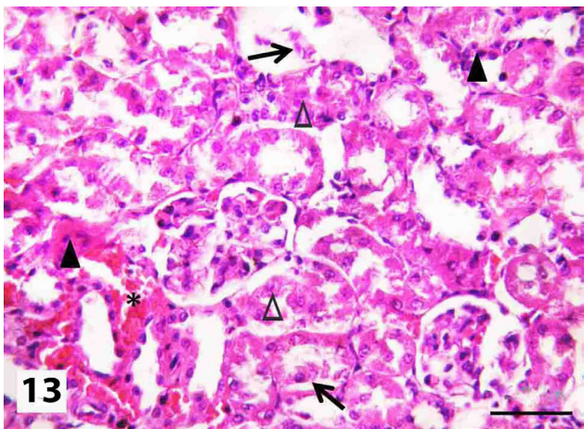

Fig. 13: Separation of epithelial lining of some tubules is noticed (↑). Some tubular cells show pyknotic nuclei and deep acidophilic cytoplasm (▲). Karyolitic nuclei are also seen (Δ). Notice extravasation of RBCs in the interstitium (*).

(Cisplatin-treated subgroup IIb, H&E x 400, scale bar=50μm)

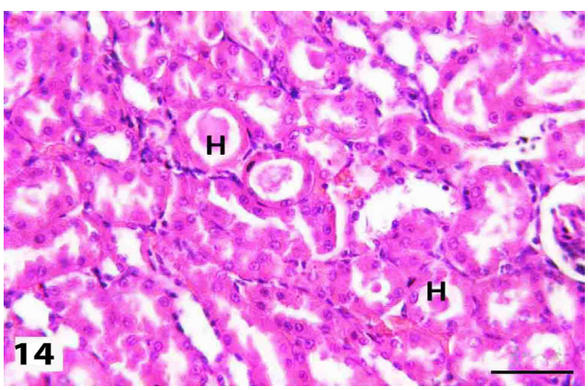

Fig. 14: Homogenous acidophilic hyaline casts are seen in the lumen of some tubules (H).

(Cisplatin-treated subgroup IIb, H&E x 400, scale bar=50μm)

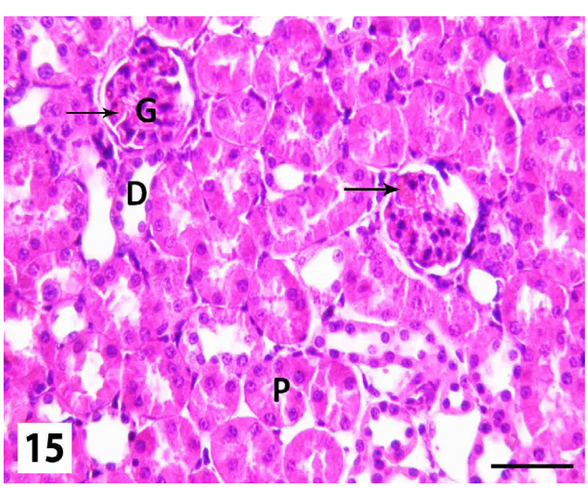

Fig. 15: Closely packed tubules are seen and regular structure of renal glomerulus (G), PCTs (P) and DCTs (D). Notice the congested glomerular capillaries (↑).

(Stem cell-treated group, H&E x 400, scale bar=50μm)

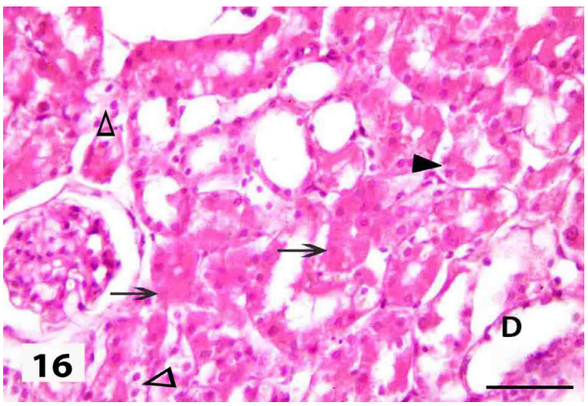

Fig. 16: Dilated tubules with disrupted epithelial lining (D) is seen. Separation of epithelial lining of some tubules (▲) can be seen. Cytoplasmic vacuolization in some tubular cells (Δ). Notice the karyolitic nuclei (↑).

(Conditioned medium-treated group, H&E x 400, scale bar=50μm)

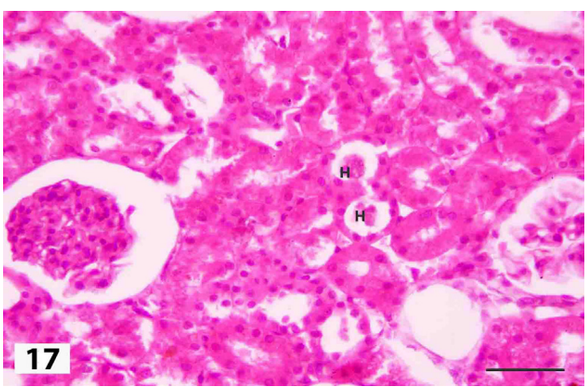

Fig. 17: Hyaline casts (H) can be seen inside the lumen of some tubules.

(Conditioned medium treated group, H&E x 400, scale bar=50μm)

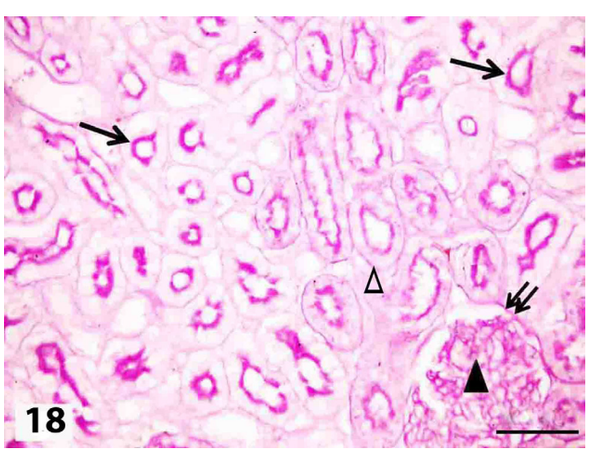

Fig. 18: PAS positive apical brush borders of the cells lining the PCTs is clearly seen (↑). The basement membrane of the renal tubules (Δ), glomerular capillaries (▲) and parietal layer of Bowman′s capsule (↑↑) are noticed.

(Control group, PAS x 400, scale bar=50μm)

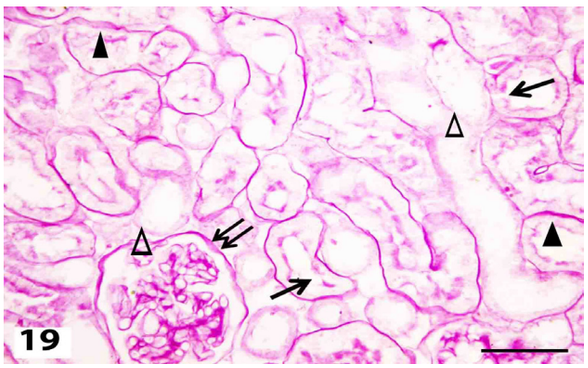

Fig. 19: Focal areas of the brush border of some proximal tubular cells are lost (↑). Loss of PAS reaction of many tubular basement membrane is obvious (Δ). Notice thickening of parietal layer of Bowman′s capsule (↑↑) and basement membrane of some tubules (▲).

(Cisplatin-treated subgroup IIa, PAS x 400, scale bar=50μm)

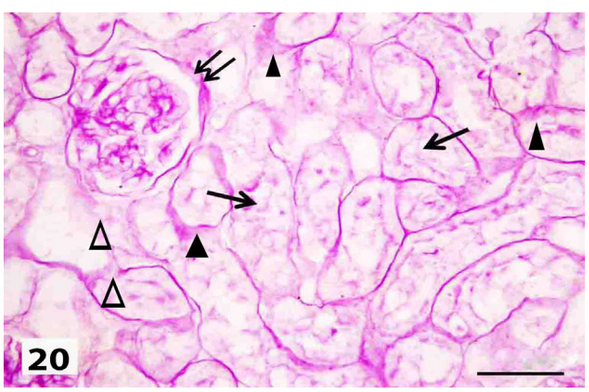

Fig. 20: Weak PAS reaction of brush border of most of proximal tubular cells (↑) is seen. Disruption of basement membrane of some tubules can be observed (Δ) and its thickening in other tubules (▲), and parietal layer of Bowman′s capsule (↑↑).

(Cisplatin-treated subgroup IIb, PAS x 400, scale bar=50μm)

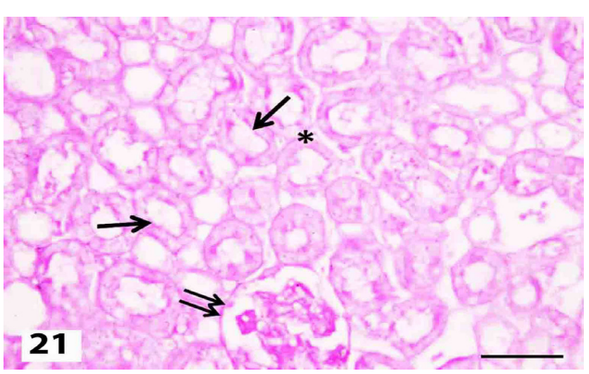

Fig. 21: Apical brush border of some PCTs is focally interrupted (↑). Mild thickening of basement membrane of some tubules (*) is seen. Notice regular basement membrane of many tubules, parietal layer of Bowman′s capsule and glomerular capillaries (↑↑).

(Stem cell-treated group, PAS x 400, scale bar=50µm)

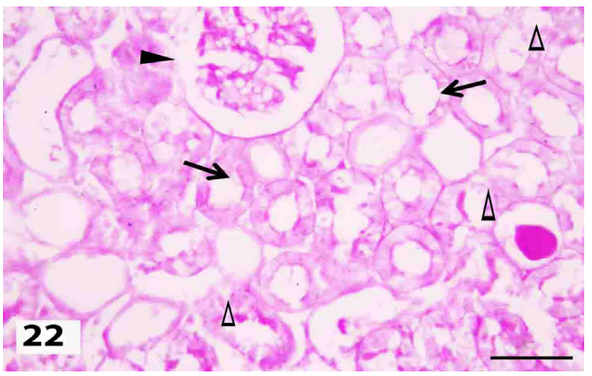

Fig. 22: Focal disruption of the apical brush border of cells of most PCTs (↑). Basement membrane of some tubules (Δ) and parietal layer of Bowman′s capsule (▲) are disrupted in some parts.

(Conditioned medium treated group, PAS x400, scale bar=50μm)

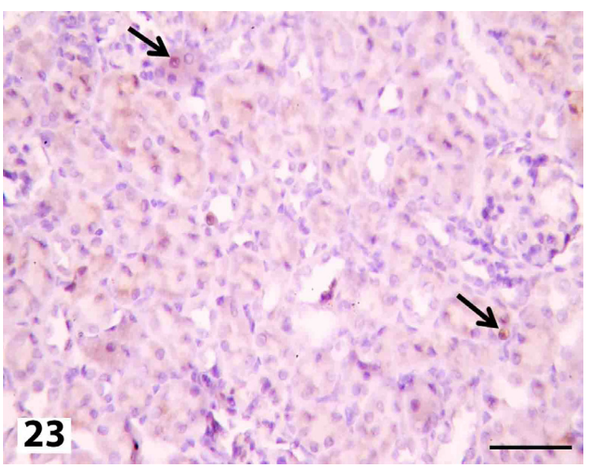

Fig. 23: Positive immunoreaction for PCNA in few tubular cell nuclei seen (↑).

(Control group, PCNA x 400, scale bar=50μm).

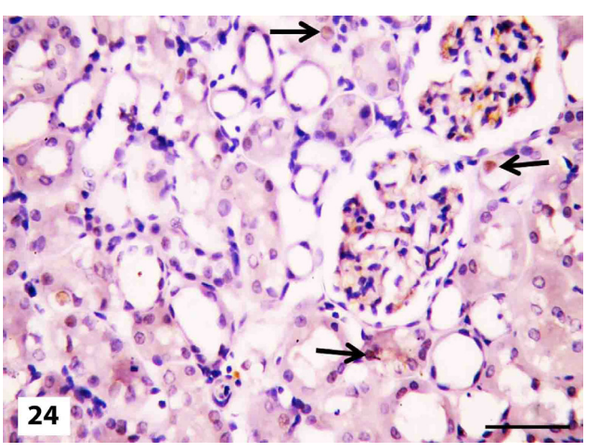

Fig. 24: Positive immuno-reaction for PCNA in few tubular cell nuclei (↑), and some interstitial cell nuclei (Δ).

(Cisplatin-treated subgroup IIa, PCNA x 400, scale bar=50μm)

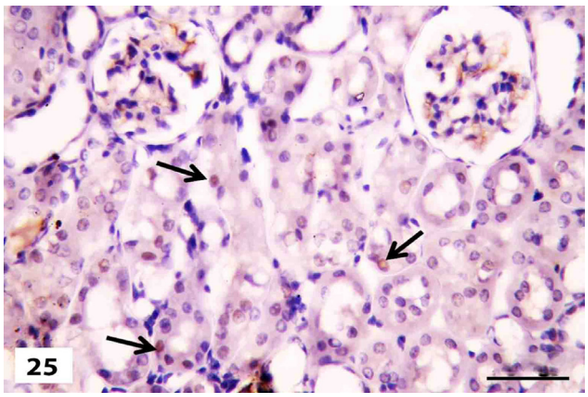

Fig. 25: Positive immuno-reaction for PCNA in some tubular cell nuclei (↑).
(Cisplatin-treated subgroup IIb, PCNA x 400, scale bar=50μm)

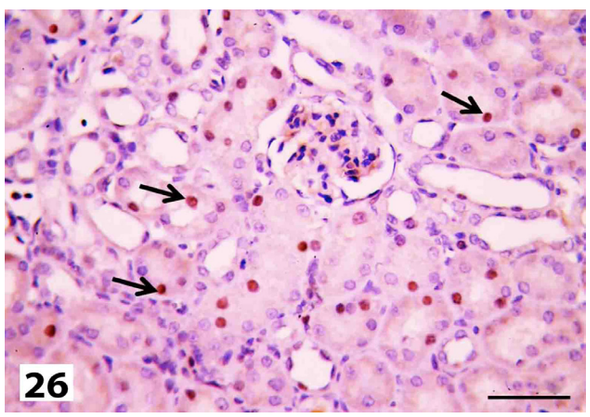

Fig. 26: Many tubular cell nuclei show positive PCNA reaction (↑).
(Stem cell-treated group, PCNA x 400, scale bar=50μm)**.**

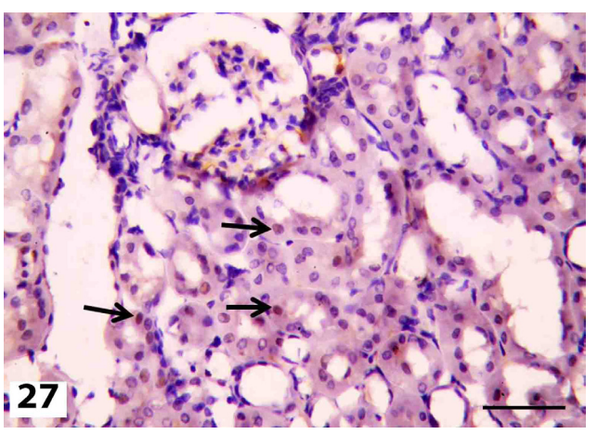

Fig. 26: Positive immunoreaction for PCNA in the nuclei (↑) of some tubular cells.
Conditioned medium treated group, PCNA x 400, scale bar=50μm)

DISCUSSION

In the current study injection of cisplatin resulted in nephrotoxicity mainly affecting the kidney tubules and renal interstitium.

Tubular cells were reported to be a target to toxins. Elevated intracellular concentrations of various reabsorbed or secreted molecules by tubular cells rendered them liable to toxic injury. In addition, presence of high concentrations of solutes inside the lumen of tubules -due to water reabsorption- could lead to tubular injury[18]. It was also reported that the concentration of cisplatin in proximal tubules was about five times the plasma concentration, thus contributing to its induced nephrotoxicity. Cisplatin was found to be accumulated by peritubular uptake primarily in proximal followed by distal tubules[19].

In cisplatin-treated group of the present study, separation of the epithelial lining of tubular cells from their basement membrane occurred. This might be attributed to redistribution or alteration of integrin which anchor the tubular cells causing detachment of cells from their basement membrane[18].

Dilatation of some tubules and flattening of their epithelial lining was also detected after cisplatin administration in the present study. Tubular debris caused by epithelial sloughing and cast formation could block out flow of urine resulting in increased intra-tubular pressure and dilatation of some tubules. Additionally, the intra-renal vasoconstriction associated with AKI result in reduced delivery of oxygen and nutrients to tubular epithelial cells leading to their flattening and atrophy[18].

The current study revealed that cisplatin caused degenerative changes in the tubular cells in the form of cytoplasmic vacuolization in some cells or deeply acidophilic cytoplasm in other cells. This was accompanied by the presence of pyknotic or karyolitic nuclei. This was in accordance with some previous researches[17, 20]. It was demonstrated that cisplatin caused direct tubular injury through multiple interacting mechanisms. One of the reported mechanisms was binding to DNA leading to formation of inter and intra strands cross-links resulting in arrest of DNA synthesis and replication. The cells that were unable to repair cisplatin-induced DNA damage underwent apoptosis. These researchers added that cisplatin-mediated anti-tumor action was through DNA damage. DNA damaged fragments and oxidative stress resulted from cisplatin initiated mitochondrial pathway of apoptosis[21]. Other investigators revealed that mitogen activated protein kinases like P38 and tumor suppressor gene P53 were activated in cisplatin

induced renal injury. Both P38 and P53 played a role in apoptosis in response to DNA damage[17, 22].

Multiple apoptotic pathways had been suggested in cisplatin-induced renal injury including mitochondrial and endoplasmic stress pathways[23]. It was reported that as cisplatin affected DNA of the cell, accumulation of misfolded proteins in the endoplasmic reticulum (ER) occur, thus activating signaling pathways that increase the production of chaperones leading to ER stress. This led to caspase activation and apoptosis[18]. Activation of caspase in renal epithelial cells might occur as early as 12 hours after cisplatin exposure *in vitro*[24]. Moreover, other investigators stated that cisplatin caused release of reactive oxygen species (ROS) that resulted in activation of apoptosis and necrosis. In addition, cisplatin caused damage of mitochondrial and nuclear DNA which might be hydrolyzed into potentially charged metabolites that accumulate into the mitochondria[25].

Significant thickening of basement membrane of some tubules was detected in the present study after cisplatin administration in both subgroups IIa & IIb. It was reported that accumulation of glycoproteins due to renal damage and up-regulation of integrin, laminin and fibronectin were responsible for basement membrane thickening[20].

The renal interstitium of cisplatin-treated subgroup IIa of the current study revealed mild mononuclear cellular infiltration. It was reported that death of tubular cells by necrosis could recruit inflammatory cells including macrophage and leukocytes[26]. Moreover, loss of renal endothelial cells and renal dysfunction in cisplatin nephrotoxicity have been reported[17], and might be the cause of the extravasation of RBCs, congestion of glomerular and dilatation of peritubular capillaries seen in group II.

In the present study, we evaluated the presence of BMSCs in renal tissue after their intravenous administration by using the chromosome Y localization strategy. It was detected only in renal specimens of group III (stem cell-treated group), indicating that BMSCs migrated to the site of injury.

Administration of BMSCs after cisplatin-induced AKI in group III of the present study revealed an improvement of structure of renal tubules and interstitium. In addition, significant increase in PCNA immunoreactivity was detected. These results were also recorded by previous investigators[27]. It was reported that MSCs administration to mice with AKI could reduce apoptosis and inhibit the production of pro-inflammatory cytokines. The MSCs were also found to secrete trophic growth factors promoting angiogenesis and cellular proliferation[7]. The MSCs have been also documented to trans-differentiate into tubular epithelium in mice at early stage of ischemic reperfusion renal injury. The differentiated donor cells replaced the denuded surface, therefore contributed to the maintenance of the structural integrity of renal tubules[28]. Interestingly, the surviving renal epithelial cells following renal injury might undergo dedifferentiation, acquiring mesenchymal features. These cells were reported to have the ability to proliferate to restore the denuded basement membrane[29]. Moreover, direct contact of MSCs with the cellular microenvironment might be able to increase the number of asymmetric divisions of stem cells and to recruit more stem cells from different renal stem cell niches into the cycle[30]. It was previously reported that multiple stem cell niches were found in the kidney. These niches existed in cortical tubular pool[31], Bowman's capsules[32], and renal papillae[33]. It can be suggested that the presence of multiple stem cell niches in the kidney and their activation by injected BMSCs could play a major role in rapid repair seen in the present study.

Some researchers suggested that proliferation and differentiation were at least partially attributed to interactions between MSCs and their neighboring cells. These interactions might occur through secretion of a broad array of factors involving; cell fusion[34], or micro RNA (miR) carried by the microvesicles[35]. These paracrine factors had trophic, angiogenic, immuno-modulating, anti-inflammatory and anti-fibrotic effects. So, as a result of this interaction, MSCs could modulate multiple processes such as migration and proliferation in many types of cells[34]. Microvesicles convey paracrine factors such as lipids, proteins and nucleic acids to recipient cells. These factors might be responsible for recovery of AKI after BMSCs administration by activation of the proliferative program of the tubular cells that survived the injury. Microvesicles were thought to shuttle specific subsets of mRNA associated with a mesenchymal phenotype responsible for controlling proliferation, transcription and immunoregulation. Microvesicles were also thought to contribute to the regenerative effect of BMSCs by inducing up-regulation of the anti-apoptotic gene (BCL2) in tubular epithelial cells, and downregulation of caspases. Thus, by these ways, they prevent the development of chronic renal disease[36].

In the current study, administration of conditioned medium (CM) in group IV, could not improve the tubular structure as effectively as BMSCs. There was degeneration of tubular cells and cast formation. In addition, significant increase in thickness of tubular basement membrane as compared with control group and significant decrease in PCNA immuno-

reactivity as compared to stem cell-treated group was detected. Similar results were recorded by previous investigators[13], who stated that even after administration of high consecutive doses of CM, no improvement had been observed in animal models of ischemia reperfusion kidney injury. On the contrary, other researchers reported that CM improved renal function and increased survival in AKI experimental models[37].

The MSC-conditioned medium was reported to promote macrophage to adopt a regulatory-like M2 phenotype. This phenotype was characterized by a significantly reduced production of pro-inflammatory cytokines and high production of interleukin-10 (IL-10). Thus, exerting anti-inflammatory and immuno-suppressive actions. However, it could not improve the renal structure as the stem cells did[38].

The protective paracrine effect of the CM was suggested to be also mediated at least in part by the exosome-like micro-vesicles released by BMSCs. Treatment of animals with these exosomes significantly induced cellular proliferation and inhibited the tubular necrosis[37].

From all the aforementioned data, and from the results of the present study, it is concluded that BMSCs intravenous administration successfully reversed the renal structural damage after induction of AKI, and was more effective than their conditioned medium. The CM could prevent progress of renal injury by its anti-inflammatory and anti-apoptotic effect, however it cannot replace the necrotic tubular epithelial cells. On the other hand, BMSCs can trans-differentiate into functionally active tubular epithelial cells. However, they are not useful as a protective measurement as they migrate only to the site of injury. Hence, it is recommended to use BMSCs in the treatment of AKI.

CONFLICT OF INTEREST

There are no conflicts of interest.

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الملخص العربى

دور خلايا النسيج الاوسط الجذعية مقارنة بالوسط المكيف لها على إصابة الكلى الحادة المستحثه بالسيسبلاتين فى الجرذ الأبيض. دراسة هستولوچية و هستوكيميائية مناعية

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المقدمة: تعتبرإصابة الكلي الحادة من الأمراض المسببة للهبوط السريع في وظائف الكلى. وتعتبر السمية الكلوية للسيسبلاتين سبب رئيسي لإصابة الكلي الحادة في ما يقرب من ثلث المرضي تحت العلاج بالسيسبلاتين. وقد اقترحت الخلايا الجذعية كوسيلة للوقاية ضد إصابة الكلى الحادة المستحثه بالسيسبلاتين. ووجد أيضا أن الوسط المكيف للخلايا الجذعية يقلل من اصابة الكلى، ومن الممكن ان يكون طريقة جديدة في العلاج متجنبا إعطاء الخلايا الجذعية.

الهدف من البحث: أجريت هذه الدراسة لمقارنة تأثير الحقن الوريدي لخلايا النسيج الأوسط الجذعية المشتقة من نخاع العظام مقارنة بالوسط المكيف لها فى تقليل اصابة الكلى الحادة المستحثة بالسيسبلاتين.

مواد وطرق البحث: تم تقسيم ستين جرذا من الاناث البيضاء البالغة الى أربعة مجموعات رئيسية. المجموعة الأولى تم استخدامها كمجموعة ضابطة، المجموعة الثانية (المجموعة المعالجة بالسيسبلاتين) والتي تم تقسيمها الى مجموعتين فرعيتين، المجموعة الثالثة (المجموعة المعالجة بالخلايا الجذعية) والمجموعة الرابعة (المجموعة المعالجة بالوسط المكيف). كما تم استخدام خمسة جرذان صغيرة السن لأخذ خلايا النسيج الأوسط الجذعية المشتقة من نخاع العظام. تم التضحية بكل الجرذان بكل المجموعات في اليوم الرابع من التجربة، ما عدا جرذان المجموعة الفرعية الثانية (ب) والتي تم التضحية بهم بعد يوم واحد من إعطاء السيسبلاتين. وقد تم تحضير عينات الكلى للصبغات الهستولوجية والهستوكيميائية المناعية. كما تم عمل المقاييس الشكلية والتحليل الإحصائي.

النتائج: أحدث العلاج بخلايا النسيج الأوسط الجذعية المشتقة من نخاع العظام تحسنا واضحا في تركيب الكلى مع زيادة كبيره في المستضد الخلوي النووي المتكاثر. ولكن الوسط المكيف كان أقل تأثيرا في علاج إصابة الكلى الحادة.

الخلاصة: يفضل استخدام خلايا النسيج الأوسط الجذعية المشتقة من نخاع العظام عن استخدام الوسط المكيف لها في عكس إصابة الكلى التركيبية الحادة المستحثه بالسيسبلاتين.