Role of Bone Marrow Derived Mesenchymal Stem Cells and the Protective Effect of Silymarin in Cisplatin-Induced Acute Renal Failure in Rats

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ABSTRACT

Background: Cisplatin is a highly effective antitumor agent whose clinical application is limited by its nephrotoxicity, which is associated with high mortality and morbidity rates. We aimed to study the protective role of silymarin and mesenchymal stem cells as a therapeutic tool of cisplatin nephrotoxicity.

Materials and Methods: We injected rats with cisplatin in a dose of 5 mg/kg body weight for 5 days to induce acute renal failure (ARF). Silymarin was administrated 6 hours before cisplatin injection and mesenchymal stem cells were injected 24 hours after cisplatin-induced ARF.

Results: We assessed the ARF biochemically by elevation of kidney function tests and histopathologically by an alteration of the histological architecture of the renal cortex in the form of shrinkage of glomeruli, lobulated tufts and glomerular hypertrophy with narrowing capsular space. The tubules showed extensive tubular degeneration with cellular hyaline materials and debris in the lumen of the renal tubules. The renal blood vessels appeared sclerotic with marked thickened walls. When silymarin was given in different doses before cisplatin, it decreased the toxic effect of cisplatin in the kidney but sclerotic blood vessels remained. Injection of mesenchymal stem cells in rats with cisplatin-induced ARF improved the histopathological effects of cisplatin in renal tissues and kidney function tests were significantly improved.

Conclusions: There was a significant improvement in kidney function tests and renal histopathology by using silymarin as protective mechanism in cisplatin-induced ARF. Administration of mesenchymal stem cells denoted a more remarkable therapeutic effect in ARF.

Key Indexing Terms: Cisplatin; Silymarin; Mesenchymal stem cells; Acute renal failure. **[Am J Med Sci 2018;355(1):76–83.]**

INTRODUCTION

Cis-Diamminedichloroplatinum II (cisplatin), is one of the most effective chemotherapeutic agents for treatment of many solid tumors, but the use of cisplatin can cause tissue toxicity, especially in the kidneys as it induces acute renal failure (ARF).[1] The prevalence of cisplatin nephrotoxicity is high, occurring in about one-third of patients treated by cisplatin.[2]

ARF is defined as rapid and reversible loss of kidney function with or without oliguria and can be detected simply by elevated serum creatinine and blood urea.[3]

Renal tissue damage, characterized by tubular cell death, is a common histopathological feature of cisplatin nephrotoxicity. Under this condition, cell death is due to both necrosis and apoptosis.[4] Several studies have suggested that free radicals, like superoxide radical, play an important role in cisplatin-induced nephrotoxicity.[5] Cisplatin depletes glutathione (GSH) and increases lipid peroxidation in the mitochondria. It also suppresses the activities of superoxide dismustase and catalase in the renal tissues.[6]

The natural mechanism of tissue regeneration may result from proliferation of surviving dedifferentiated cells from renal stem cells that reside inside the kidney and migrate to the site of regeneration or from bone marrow cells that gain access to the injured epithelium and differentiate into mature cells.[7] Stem cells are biological cells found in all multicellular organisms. Bone marrow contains at least 2 kinds of stem cells, hematopoietic stem cells and stem cells of nonhematopoietic tissue (mesenchymal stem cells [MSCs]).[8] MSCs are interesting as they can easily isolate from a small aspirate of bone marrow and readily generate single-cell-derived colonies. MSCs also can differentiate into different types of cells, so they are currently being tested as a therapeutic option in a number of diseases.[9]

Many antioxidants agents have been investigated for cisplatin-induced nephrotoxicity prevention. Some studies advised the use of diets enriched with natural antioxidants, such as vitamin E and methionine.[10] Another study reported the use of sulfhydryl-containing

drugs, such as *N*-acetylcysteine and lipoic acid, as they could exert antioxidant activity.[11]

Silymarin is a natural antioxidant and is extracted from the seeds of milk thistle. It has been used in the treatment of chronic inflammatory liver disease and hepatic cirrhosis. The hepatoprotection of silymarin could be attributed to the antioxidant properties of its scavenging free radicals (which have a role in cisplatin nephrotoxicity) and increasing intracellular concentration of GSH.[12]

We aimed to study the protective role of silymarin and MSCs as a therapeutic tool to prevent cisplatin-induced nephrotoxicity.

MATERIALS AND METHODS

Animals

A total of 40 adult male albino rats obtained from the animal house, Moshtohor faculty of Veterinary Medicine, Benha University, Egypt, were used. Rats were an average weight (170-200 g), housed individually in clean cages in a well aerated environment, given a normal daily diet and maintained 12/12-hour light/dark cycle. All the ethical protocols for animal treatment were followed and supervised by the Animal House Facility.[13]

Drugs

Cisplatin was obtained from Merck Company (Germany) in the form of vials (10 mg/10 mL). Silymarin (Legalon) was obtained from Cid Company (Egypt) in tablet form (70 mg) and was diluted in 10 mL normal saline to get a final concentration of 7 mg/mL. Bone marrow–derived MSCs (BM-MSCs) were obtained from the department of histology, Kasr Al-Ainy Faculty of Medicine, Cairo University. They were provided as first-passage culture cells suspended in phosphate buffered saline.

The 40 adult male albino rats were divided into 6 groups as follows: group I (control group) consisted of 5 rats that did not receive any treatment. Group II (vehicle group) consisted of 5 rats that received an intraperitoneal (IP) injection of 0.1 mL of normal saline for 5 days. Group III (silymarin group) consisted of 5 rats that received an IP injection of silymarin (50 mg/kg body weight [BW]) for 5 days.[14] Group IV (cisplatin-treated group) consisted of 5 rats that received an IP injection of cisplatin (5 mg/kg BW) for 5 days.[15] Group V (silymarin protective group) consisted of 15 rats arranged in 3 subgroups according to different silymarin doses as follows: subgroup A received 25 mg/kg BW of silymarin; subgroup B received 50 mg/kg BW of silymarin[14]; and subgroup C received 75 mg/dL BW of silymarin. Silymarin was injected IP 6 hours before cisplatin injection for 5 days. Subgroup B results were used in comparing other group's results. Group VI (MSCs group) consisted of 5 rats that received MSCs in a dose of (10^6 cells) by IP infusion 24 hours after ARF was induced. Rats were killed 10 days after MSCs infusion.[15]

Preparation of BM-MSCs From Rats

Bone marrow was harvested by flushing the tibiae and femurs of 6-week-old male white albino rats with Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Nucleated cells were isolated and resuspended in complete culture medium supplemented with 1% penicillin-streptomycin. Cells were incubated at 37°C in 5% humidified CO_2 for 12-14 days as primary culture for formation of large colonies. When large colonies developed (80-90% confluence), cultures were washed twice with phosphate buffered saline and the cells were trypsinized with 0.25% trypsin in 1 mM EDTA (GIBCO/BRL) for 5 minutes at 37°C.

After centrifugation, the cells were resuspended in serum supplemented medium and incubated in culture flask. The resulting cultures were referred to as first-passage cultures.[16]

Sample Preparation

All groups were killed by CO_2 narcosis then the animals were fixed on the operating table and blood samples and kidney biopsies were taken. For blood sample collection, a craniocaudal incision of about 2 cm was made parallel and slightly to the left of the sternum through the skin and pectoral muscles to expose the ribs. Blunt curved forceps were then inserted between the fifth and sixth ribs, through the intercostals muscles. The gap was widened so that the heart became visible, then the blood samples were taken from the right ventricle for assessment of serum creatinine and blood urea in the Clinical Pathology Department, Faculty of Medicine, Benha University. Each sample of blood was centrifuged at 3,500 r.p.m. for 10 minutes and the serum was separated. Blood urea was measured in mg/dL with the use of the urease enzymatic assay[17] and serum creatinine was measured in mg/dL according to the Jaffe method.[18]

To gain access to the renal system, the previous incision was continued through the animal's anterior abdominal wall, the abdominal cavity was entered by cutting the muscles, the peritoneum was reflected and the intestine displaced. The kidneys were removed and dissected, then fixed in 10% neutral buffered formalin. After fixation, specimens were processed for paraffin embedding and sectioned at a thickness of 5-7 μg then stained by hematoxylin and eosin. Renal tissue sections were examined to evaluate the histological architecture changes in the kidney tissues.

Immunohistochemical Study

CD105 immunostaining is the marker for MSCs. A 0.1 mL prediluted primary antibody (CD105) rabbit polyclonal Ab (ab27422) was incubated at room temperature in a moist chamber for 30-60 minutes. Cellular localization was the cell membrane. One of the kidney sections was used as a negative

control by bypassing the step of applying the primary antibody.[15]

Statistical Analysis

Data were expressed as mean ± standard deviation. The data were analyzed by Student's *t*-test. The differences were considered to be statistically significant when $P < 0.05$.

RESULTS

Biochemical Analysis

Serum creatinine and blood urea were measured in all study groups. The levels were normal in groups I, II and III. Cisplatin-induced ARF in group IV, as serum creatinine and blood urea levels were increased (2.8 ± 0.2 and 83 ± 3.94 mg/dL, respectively). When silymarin was given before cisplatin, there was improvement of serum creatinine and blood urea levels (1.87 ± 0.1 and 58.6 ± 2.7 mg/dL, respectively) in group V (subgroup B). After MSC transplantation, the improvement of serum creatinine and blood urea levels increased (1.72 ± 0.18 and 51.2 ± 2.39 mg/dL, respectively). All these changes were statistically significant ($P < 0.05$), as shown in Table 1.

TABLE 1. Comparison between the studied groups.

	Group I	Group II	Group III	Group IV	Group V (subgroup B)	Group VI	F test	P Value
Creatinine (mg/dL)								
Mean ± SD	0.80 ± 0.04	0.79 ± 0.03	0.82 ± 0.02	2.8 ± 0.20[abc]	1.87 ± 0.10[abcd]	1.72 ± 0.18[abcd]	233.7	0.001**
Range	0.75-0.85	0.75-0.82	0.80-0.85	2.5-3.0	1.7-1.95	1.5-2.0		
Urea (mg/dL)								
Mean ± SD	25.2 ± 1.10	25.0 ± 1.0	24.8 ± 0.84	83.0 ± 3.94[abc]	58.6 ± 2.70[abcd]	51.2 ± 2.39[abcde]	547.8	0.001**
Range	24-27	24-26	24-26	80-89	55-62	49-55		

SD, standard deviation.
[a] significant with Group I
[b] significant with Group II
[c] significant with Group III
[d] significant with Group IV
[e] significant with Group V, subgroup B

Histopathological Examination

Hematoxylin and eosin stained sections of the kidney tissue from rats in group I showed normal structure as the glomeruli consisted of a tuft of blood capillaries surrounded by capsular space and Bowman's capsule. The proximal convoluted tubule (PCT) was lined by pyramidal cells with acidophilic cytoplasm and rounded, basally located basophilic nuclei and having a free striated brush border. The distal convoluted tubule (DCT) was lined by cubical epithelium with light acidophilic cytoplasm and rounded, centrally situated basophilic nuclei and the lumina of the DCT were wider than that of the PCT (Figure 1A). The same results were found in groups II and III (Figure 1B). In group IV, 82% of renal sections of the rats had glomerular changes in form of glomeruli shrinkage with lobulated tufts or glomerular hypertrophy with narrowing capsular space. Overall, 100% of the tubules in all sections had tubular degeneration as cytoplasmic cavitations. Intratubular hyaline material and debris were found in 87% of all sections. Inflammatory cells infiltration was found in only 61% of all sections and 45% of all sections had hemorrhage. The renal blood vessels appeared sclerotic with marked thickened walls in 85% of all renal sections (Figure 1C-F; Table 2). In group V, a partial improvement in the histopathological changes in all renal sections of the 3 subgroups was found; but the improvement was less in subgroup A and almost equal in subgroups B and C (Figures 2C and D and 3; Table 3). The frequency distributions of the histopathological changes in subgroup B were 5% in glomeruli, 20% in tubules, no

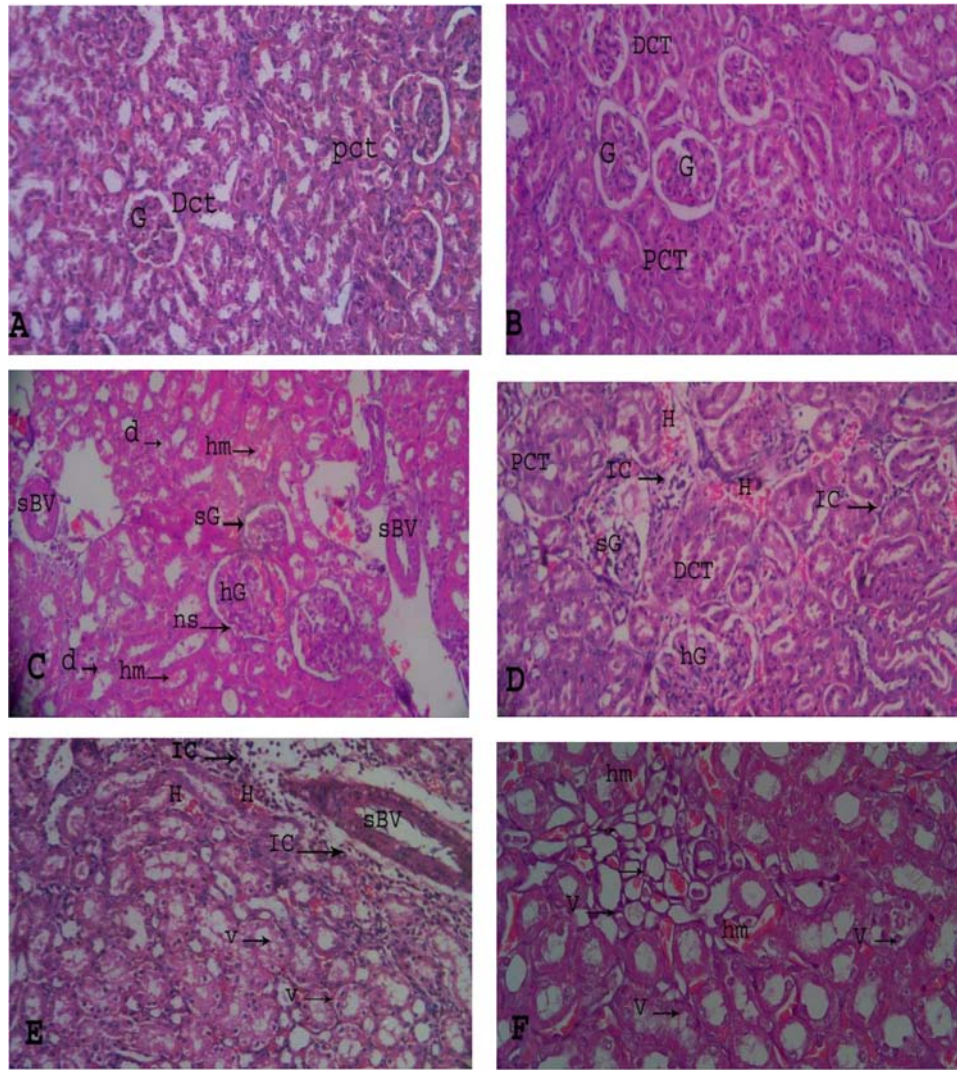

FIGURE 1. (A and B) A photomicrograph of a renal sections of groups I, II and III showing normal renal architecture of the glomeruli (G), proximal convoluted tubules (PCT) and distal convoluted tubules (DCT). (C-F) A photomicrograph of a renal section of group IV (cisplatin-treated group); sections represented shrinkage of glomeruli (sG) and hypertrophied glomeruli (hG) with narrow capsular space (ns), tubular degeneration (d). Sclerotic blood vessel (sBV) and hyaline materials filled the lumen of the tubules (hm). (D) Inflammatory cell infiltration (IC), hemorrhage (H). (E) Cytoplasmic cavitation (V) and sclerotic blood vessel (sBV). (F) Tubular degeneration with flat cell (arrow). (A-D, ×200; E and F, ×400.)

intratubular hyaline material and 9% inflammatory cells infiltration. But there was still hemorrhage (21%) and sclerotic blood vessels (35%) (Figure 2A and B). In group VI, there was a marked improvement in the histopathology as there was no glomerular affection, intratubular hyaline material or inflammatory cells infiltration in all renal sections and only 5% of tubules were affected, 4% hemorrhage and 1% sclerotic blood vessels were found after injection of MSCs (Figure 2E and F).

CD105 Immunostained Sections

Sections in the renal cortex of the control rats showed negative immunostaining in the glomeruli, PCT, DCT and collecting tubule, but sections in the renal cortex of group VI showed multiple CD105 positive cells (arrows) in the glomeruli and tubules (Figure 4A and B).

DISCUSSION

Cisplatin is one of the most widely used and most potent chemotherapy agents; however, side effects in normal tissues and organs such as nephrotoxicity, neurotoxicity, ototoxicity (loss of hearing or balance or both), gastrointestinal toxicity and myelosuppression limit the use of cisplatin and related platinum-based therapeutics.[4]

In this work, rats were injected with cisplatin to induce ARF which was proven biochemically and by histopathological changes. Biochemically, measurement of serum creatinine and blood urea levels after cisplatin

TABLE 2. Frequency distribution of the histopathological changes in kidney sections of the rats in all studied groups.

	Group I (%)	Group II (%)	Group III (%)	Group IV (%)	Group V (subgroup B) (%)	Group VI (%)
Glomerular changes	0	0	0	82	5	0
Tubular degeneration	0	0	0	100	20	5
Intratubular hyaline material	0	0	0	87	0	0
Inflammatory cells infiltration	0	0	0	61	9	0
Hemorrhage	0	0	0	45	21	4
Sclerotic blood vessels	0	0	0	85	35	1

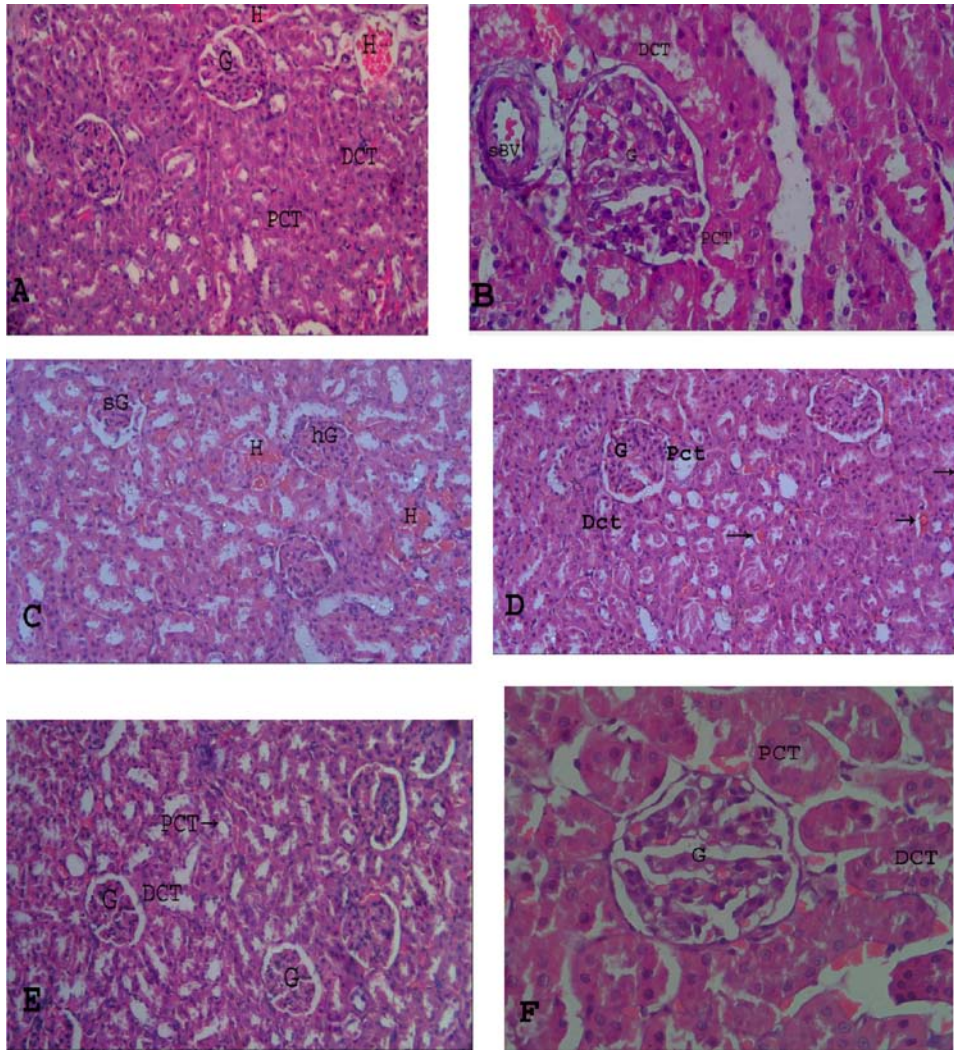

FIGURE 2. (A and B) A photomicrograph of a renal sections of group V (silymarin protective subgroup B) section represented decrease in the degenerative changes in the glomeruli (G), proximal convoluted tubules (PCT) and distal convoluted tubules (DCT) but still there are hemorrhage (H) and sclerotic blood vessel (sBV). (C) (subgroup A) sections represented decrease in the degenerative changes in the tubules but there is shrinkage of glomeruli (sG) and hypertrophied glomeruli (hG) and hemorrhage (H). (D) (subgroup C) sections represented decrease in the degenerative changes but still there is some hemorrhage (arrow). (E and F) A photomicrograph of a renal section of group VI (MSCs group): section represented marked improvement in renal tissues which reflect the potential role of MSCs in tissue regeneration showing normal glomeruli (G), proximal convoluted tubules (PCT) distal convoluted tubules (DCT). (A and C–E, ×200; B and F, ×400.)

TABLE 3. Frequency distribution of the histopathological changes in kidney sections of the rats in the subgroups of group V.

	Subgroup A (%)	Subgroup B (%)	Subgroup C (%)
Glomerular changes	35	5	5
Tubular degeneration	43	20	18
Intratubular hyaline material	17	0	0
Inflammatory cells infiltration	31	9	8
Hemorrhage	29	21	21
Sclerotic blood vessels	50	35	34

injection revealed a significant high elevation of their levels as compared with control. This result was supported in previous studies,[2,19] where it was also noted that cisplatin accumulates in the kidney, especially in the PCTs at the corticomedullary region, causing a decrease in the glomerular filtration rate and extensive cellular necrosis. Ghaly et al[20] added that serum electrolytes disturbance was recorded in their study as indicated by high significant elevation of serum sodium and potassium levels.

Histopathologically, cisplatin injection altered the histological architecture of the renal cortex and lead to shrinkage of glomeruli, lobulated tufts and glomerular hypertrophy with narrowing capsular space with inflammatory cell infiltration and hemorrhage. These findings were analogous to the findings of Ghaly et al,[20] as they found that the ultrastructure of the basal lamina of glomerular podocytes lacked its trilaminar appearance and were suggested to be the sequel of glomerular sclerosing process after cisplatin treatment. On the contrary, others found that the glomeruli had normal structure and the histopathological changes were mainly in the corticomedullary region.[14]

In this study, we found an extensive tubular degeneration with flat cells and cytoplasmic cavitation with cellular hyaline materials and debris in the lumen of the renal tubules. The renal blood vessels appeared sclerotic with marked thickened wall. These histological findings were in accordance with Ghaly et al,[20] who explained that the presence of hyaline materials as necrotic epithelial cells provides the matrix for tubular cast formation with subsequent tubular obstruction.

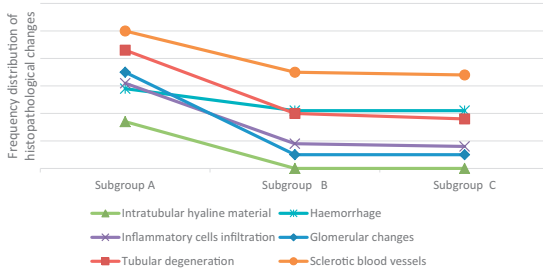

FIGURE 3. A dose response curve showed the frequency distribution of the histopathological changes in renal section according to different doses of silymarin in group V. improvement in renal tissue was less in subgroup A, and was almost equal in subgroup B and C.

Oxidative damage in cisplatin nephrotoxicity is induced by reactive oxygen species and free radicals, which directly act on cell components and destroy their structure.[21,22] Others have described the tubular damage induced by cisplatin as if cisplatin was in the interior of the cells; the hydrolysis product (chloride atoms inside it were replaced by water molecules) was believed to be the active species, reacting with GSH in the cytoplasm and DNA in the nucleus. The produced cisplatin-DNA intrastrand crosslinks resulted in cytotoxicity and were thought to be responsible for cellular death.[23]

In this study, we gave silymarin as an antioxidant 6 hours before every cisplatin injection and we found that there was a decrease in the toxic effect of cisplatin in the kidney. In histopathology, the degenerative changes in the renal glomeruli and tubules were reduced, but some blood vessels were still sclerotic. This effect of silymarin was founded with different doses used in this study. The histopathological improvement was less in subgroup A and almost equal in subgroup B and C, the silymarin protective groups, which indicates that silymarin has an actual protective effect. Biochemically, there was also a significant reduction in blood urea and serum creatinen levels comparing with the cisplatine-treated group. This has been proven in other studies, which explain the role of silymarin protective effect in cisplatin-induced nephrotoxicity as its activity against lipid peroxidation (as a result of free radical scavenging) and the ability to increase the cellular content of GSH.[14] In another study it was found that giving silymarin after cisplatin-induced ARF failed in complete protection against the pathological alteration caused by cisplatin but giving silymarin before cisplatin significantly decreased the histological and ultrastructural changes induced by cisplatin and appear highly protective.[24]

Stem cells have potential uses in therapies designed to repair and regenerate organs. The beneficial effects of stem cell occur through differentiation-therapeutic independent pathways that include increased cell survival and proliferation, decreased inflammation and suppression of immune function.[25] MSCs are attractive candidates for renal repair, because nephrons are of mesenchymal origin and because stromal cells are of crucial importance for signaling, leading to differentiation of both nephrons and collecting ducts.[26]

In our study, the rats were injected with BM-MSCs 24 hours after the induction of ARF, and after 10 days

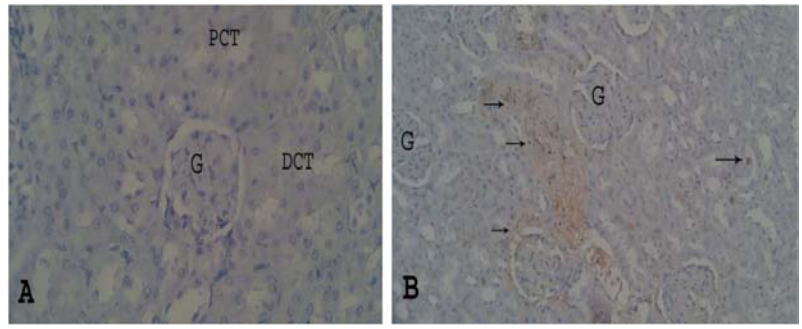

FIGURE 4. (A) A photomicrograph of a renal sections of group I of a control group showing negative immunostaining in the glomeruli (G), proximal convoluted tubules (PCT), distal convoluted tubules (DCT). (B) A photomicrograph of a renal section of group VI (MSCs group): section showing multiple CD105 positive cells (arrows) in the glomeruli (G) and tubules. (CD105 immunostaining, ×200.)

there was marked improvement of the histopathological effects of cisplatin, and both serum creatinine and blood urea levels were significantly decreased. These results were also found in another study where they injected rats with labeled BM-MSCs to detect their possible anti-inflammatory, angiogenesis and homing potential in amelioration of renal tissues and functions in an experimental model. They concluded that the MSCs could be used in the treatment of ARF, as the results showed a significant improvement in renal function tests as well as renal histopathology.[15] Ling et al[27] stated that BM-MSCs fused with renal cells and that 50% of proximal tubular cells were replaced with donor cells. Rookmaaker et al[28] added that BM-MSCs may be homed to injured glomerular endothelium and differentiate into endothelial cells in addition to participation in regeneration of the glomerular microvasculature.

One study used fetal kidney stem cells (fKSC) and found that fKSC express mesenchymal and renal progenitor markers, exhibiting an *in vitro* angiogenic potential and the ability to differentiate into cells of renal epithelial lineage. The administration of fKSC in cisplatin-induced ARF results in rapid recovery of renal function and histology, and promotes renal angiogenesis.[29] Another study used human adipose-derived MSCs (AD-MSCs) and concluded that a possible mechanism of kidney recovery with AD-MSC treatment may be that certain signals are released from the injured tubular cells that trigger AD-MSCs to produce specific cytokines.[30]

Many studies have explained the role of MSCs as a therapeutic tool in ARF as MSCs express several growth and antiapoptotic factors, such as vascular endothelial growth factor, insulin like growth factor 1 and hepatocyte growth factor; all these factors are known to improve renal function in renal failure models. One of the main beneficial effects of MSCs is the paracrine action; via this action MSCs mediate their antiapoptotic, mitogenic and other cytokine actions,[31,32] which results in the renal down regulation of proinflammatory cytokines as interleukin 1 beta, tumor necrosis factor alpha and interferon gamma as well as fibrogenic growth factors such as transforming growth factor beta.[33] On the other hand, it upregulates the anti-inflammatory and organ-protective interleukin-10 factors, such as transforming growth factor alpha.[34]

CONCLUSIONS

Silymarin, a natural antioxidant, can be used as a protective measurement of cisplatin-induced ARF and could be used in combination with cisplatin to limit its renal injury. In addition, the administration of MSCs in cisplatin-induced ARF results in improvement in both kidney function tests and renal histology indicating the promising role of the stem cells in the treatment of ARF.

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