Journal of
Stem Cell Research & Therapy

Abdel Aziz et al., J Stem Cell Res Ther 2011, 1:3
DOI: 10.4172/2157-7633.1000109

Research Article Open Access

Mesenchymal Stem Cells Therapy in Acute Renal Failure: Possible Role of Hepatocyte Growth Factor

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Introduction

Acute renal failure (ARF), also known as acute kidney injury, is a rapid loss of renal functions due to damage to the kidneys, resulting in retention of the nitrogenous compounds (urea and creatinine) and non nitrogenous waste products that are normally excreted in urine [1].

Depending on the severity and duration of renal dysfunction, this accumulation is accompanied by metabolic disturbances, such as metabolic acidosis and hyperkalemia, changes in body fluid balance, and effects on many other organ systems. It can be characterized by oliguria or anuria [2]. It is a serious disease and treated as medical emergency.

In clinical practice, ischemia-reperfusion (I/R) injury is the most common cause for acute renal failure. The pathogenic events in ischemia/reperfusion injury include acute tubular necrosis, apoptosis, glomerular injury and inflammation.

Management of acute renal failure depends first on correction of the metabolic abnormalities like the correction of hyperkalemia and correction of metabolic acidosis then treatment of the cause as correction of the hypovolemic state during shock or immunosuppressive therapy for glomerulonephritis [3]. Although a number of agents and growth factors have been proven effective in the amelioration of ARF in otherwise healthy animals, no significantly effective new therapy has been introduced into clinical practice in decades. It is for these reasons that fundamentally new strategies for the treatment of ARF are needed.

Stem cell therapy holds a great promise for the repair of injured tissues and organs, including the kidney. Stem cells are undifferentiated cells that undergo both self-renewal and differentiation into one or more cell types [4], & are found in adult and embryonic tissues and have potential uses in therapies designed to repair and regenerate organs. There has been considerable focus on the ability of stem cells to differentiate into non-haematopoietic cells of various tissue lineages, including cells of the kidney [5]. This growing evidence has led to a reconsideration of the source of cells contributing to renal repair following injury.

The mechanism of action of stem cell therapy is unclear in most disease conditions. Very-low-level organ engraftment of circulating bone marrow-derived stem cells has been shown [6] but was not corroborated by others [7]. The percentage of incorporated stem cells varies widely, but it is usually below 1% in a given organ, and, in addition, its magnitude depends on the studied disease model. Other mechanistic possibilities for the therapeutic effects of stem cells include fusion with resident organ cells [8], immunomodulation [9] and paracrine mechanisms elicited through trophic mediators [10] that result in the inhibition of fibrosis and apoptosis, enhancement of angiogenesis, stimulation of mitosis and proliferation & differentiation of organ-intrinsic precursor of stem cells.

Hepatocyte growth factor (HGF), first identified by Russell et al. [11] then purified and cloned by Nakamura et al. [12] as a potent mitogen for fully differentiated hepatocytes.

Hepatocyte growth factor exerts mitogenic responses in renal epithelial cells derived from distinct regions and species, including rabbit and rat proximal tubular cells [13] and rat glomerular epithelial cells. HGF stimulates the proliferation of renal epithelial cell lines, including a rat visceral glomerular cell line [14], proximal tubular cell lines [15]. Likewise, HGF exhibits mitogenic action on renal endothelial cells [16].

Many studies demonstrated that HGF can play a role in the treatment of renal diseases such as acute renal failure caused by nephrotoxic drug administration (for example, cisplatin, cyclosporine A, tacrolimus, and antibiotics) and renal ischemia [17].

This work aims: To study the effect of mesenchymal stem cells (MSC) as a line of treatment for acute renal failure and the possible mechanism by which they act through studying their effect on the inflammatory and vascular manifestations. Also, to study the effect of hepatocyte growth factor (HGF) as another line of treatment for acute renal failure with comparison between both lines.

Materials & Methods

Preparation of the animal model

Experimental animals: The study was carried on 50 female white albino rats, of an average weight 150-200 gm. Rats were bred and maintained in an air-conditioned animal house with specific pathogen-free conditions, and were subjected to a 12:12-h daylight/darkness and allowed unlimited access to chow and water. All the ethical protocols for animal treatment were followed and supervised by the animal facilities, Faculty of Medicine, Cairo University. They were divided into 5 groups as follow:

Group 1: 10 control female albino rats

Group (2) 10 female albino rats with induced acute renal failure received saline.

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Received September 06, 2011; **Accepted** October 31, 2011; **Published** November 02, 2011

Citation: Abdel Aziz MT, Wassef MA, Rashed LA, Mhfouz S, Omar N, et al. (2011) Mesenchymal Stem Cells Therapy in Acute Renal Failure: Possible Role of Hepatocyte Growth Factor. J Stem Cell Res Ther 1:109. doi:10.4172/2157-7633.1000109

Acute renal failure was induced by ischemia / reperfusion injury by anaesthetizing the rats with sodium thiopental through intramuscular injection then doing mid abdominal laparotomy then kidneys were exposed and bilateral renal pedicles were clamped with atraumatic vascular clamps for 60 minutes. Then the vascular clamp was released to allow the reperfusion of the ischemic kidneys. The mid abdominal laparotomy wound was then sealed by continuous 6/0 stitches in 2 layers. Finally, antibiotic ointment (teramycin) & powder (neomycin) were applied to the wound [18].

Group (3) 10 female albino rats with induced ARF received HGF in a dose of (o.25mg/kg body weight) by IV infusion at the rat tail vein 24 hours after the induction of ARF.

Group (4) 10 female albino rats with induced ARF received MSCs, which were processed and cultured for 14 days, in a dose of (10^7) by IV infusion at the rat tail vein 24 hours after the induction of ARF.

Group (5) 10 female albino rats with induced ARF received both MSCs, which were processed and cultured for 14 days, in a dose of (10^7) & HGF in a dose of (0.25mg/kg body weight) by IV infusion at the rat tail vein 24 hours after the induction of ARF.

Blood samples were collected from the retro-orbital vein. Sera were separated and used for measurement of Creatinine & urea.

The rats of all groups were sacrificed (by co2 narcosis) after 72 hours of induction of the acute renal failure to obtain renal tissue specimens. These tissues were examined for:

-quantitaive analysis of tumor necrosis factor-α (TNF- α), interleukin-10(IL-10), vascular endothelial growth factor (VEGF) gene expression by Real Time PCR.

-Histopathological examination of renal tissue by haematoxylin and eosine and by differential stains (periodic acid shift PAS stain and masson trichrome stain).

-Detection of the MSCs homing in kidney tissue after its labeling with PKH26 dye by fluorescent microscope to detect its red fluorescence.

Preparation of BM -derived mesenchymal stem cells 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 (DMEM, GIBCO/BRL) supplemented with 10% fetal bovine serum (GIBCO/BRL). Nucleated cells were isolated with a density gradient [Ficoll/Paque (Pharmacia)] and resuspended in complete culture medium supplemented with 1% penicillin–streptomycin (GIBCO/BRL). Cells were incubated at 37°C in 5% humidified CO_2 for 12–14 days as primary culture or upon formation of large colonies. When large colonies developed (80–90% confluence), cultures were washed twice with phosphate buffer saline(PBS) and the cells were trypsinized with 0.25% trypsin in 1mM EDTA (GIBCO/BRL) for 5 min at 37°C. After centrifugation, cells were resuspended in serum-supplemented medium and incubated in 50 cm^2 culture flask (Falcon). The resulting cultures were referred to as first-passage cultures [19]. Cells were identified as being MSCs by their morphology, adherence, and their power to differentiate into osteocytes and chondrocytes. Differentiation into osteocytes was achieved by adding 1-1000 nM dexamethasone, 0.25 mM ascorbic acid, and 1-10 mM beta-glycerophosphate to the medium. Differentiation of MSCs into osteoblasts was confirmed by morphological changes, Alzarin red staining of differentiated osteoblasts. Differentiation into chondrocyte was achieved by adding 500 ng/mL bone morphogenetic protein-2 (BMP-2;R&D Systems, USA) and 10 ng/ml transforming growth factor b3 (TGFb3) (Peprotech, London) for 3 weeks. In vitro differentiation into chondrocytes was confirmed by morphological changes, Alcian blue staining of differentiated chondrocytes. CD29 gene expression was also detected by RT-PCR as a marker of MSCs and CD45, CD44 by flow cytometry analysis.

Labeling of MSCs with PKH26

MSCs were harvested during the 4th passage and were labeled with PKH26, which is a red fluorochrome. It has excitation (551nm) and emission (567 nm) characteristics compatible with rhodamine or phycoerythrin detection systems. The linkers are physiologically stable and show little to no toxic side-effects on cell systems. Labeled cells retain both biological and proliferating activity, and are ideal for in vitro cell labeling, in vitro proliferation studies and long, in vivo cell tracking. In the current work, MSCs were labeled with PKH26from Sigma Company (Saint Louis, Missouri USA). Cells were centrifuged and washed twice in serum free medium. Cells were pelleted and suspended in dye solution. Cells were injected intravenously into rat tail vain. After one month, kidney tissue was examined with a fluorescence microscope to detect and trace the cells.

RT-PCR Detection of CD29 gene expression

Total RNA was extracted from cells using RNeasy Purification Reagent (Qiagen, Valencia, CA), and then a sample (1 µg) was reverse transcribed with M-MLV (Moleny – Murine Leukemia virus) reverse transcriptase (RT) for 30 minutes at 42°C in the presence of oligo-dT primer. Polymerase chain reaction (PCR) was performed using specific primers (UniGene Rn.25733) forward: 5'-AA TGTTTCAGTGCA GA GC-3' and reverse: 5'- TTGGGAT GA TGTCGGGAC-3'. PCR was performed for 35 cycles, with each cycle consisting of denaturation at 95°C for 30 seconds, annealing at 55°C to 63°C for 30 seconds, and elongation at 72°C for 1 minute, with an additional 10-minute incubation at 72°C after completion of the last cycle. To exclude the possibility of contaminating genomic DNA, PCRs were also run without RT. The PCR product was separated by electrophoresis through a 1% agarose gel, stained, and photographed under ultraviolet light.

Real-time quantitative analyses for VEGF, TNF alpha and IL10 gene expression

Total RNA was extracted from kidney tissue homogenate using RNeasy purification reagent (Qiagen, Valencia, CA). cDNA was generated from 5 µg of total RNA extracted with 1 µl (20 pmol) antisense primer and 0.8 µl superscript AMV reverse transcriptase for 60 min at 37°C.

The relative abundance of mRNA species was assessed using the SYBR® Green method on an ABI prism 7500 sequence detector system (Applied Biosystems, Foster City, CA). PCR primers were designed with Gene Runner Software (Hasting Software, Inc., Hasting, NY) from RNA sequences from GenBank (Table 1). All primer sets had a calculated annealing temperature of 60°. Quantitative RT-PCR was performed in duplicate in a 25-µl reaction volume consisting of 2X SYBR Green PCR Master Mix (Applied Biosystems), 900 nM of

each primer and 2-3 µl of cDNA. Amplification conditions were 2 min at 50°, 10 min at 95° and 40 cycles of denaturation for 15 s and annealing/extension at 60° for 10 min. Data from real-time assays were calculated using the v1·7 Sequence Detection Software from PE Biosystems (Foster City, CA). Relative expression of VEGF, TNF alpha and IL10mRNA was calculated using the comparative Ct method. All values were normalized to the beta actin genes and reported as fold change over background levels detected in ARF.

Biochemical analysis

Serum urea and creatinine levels were measured using the conventional colorimetric method using Quanti Chrom TM assay kits based on the improved Jung and Jaffe methods, respectively (DIUR-500 and DICT-500).

Analysis of kidney histopathology

Kidney samples were collected into PBS and fixed overnight in 40 g/L paraformaldehyde in PBS at 4°C. Serial 5-µm sections of the cortex and the medulla of the kidney were stained with hematoxylin and eosin (H&E).

Statistical analysis

Data were expressed as mean ± SD. Significant differences were determined by using ANOVA and post-hoc tests for multiple comparisons using SPSS 9.0 computer Software. Results were considered significant at $p<0.05$.

Results

MSCs culture, identification & homing

Isolated and cultured undifferentiated MSCs reached 70-80% confluence at 14 days .In vitro osteogenic and chondrogenic differentiation of MSCs were confirmed by morphological changes and special stains (Figure 1A,B and Figure 2A,B respectively) in addition MSCs were identified by surface marker CD45 (-ve) & CD44 (+ve) detected by flow cytometry and CD29 (+) by PCR (Figure 3A,B&C) respectively. MSCs labeled with PKH26 fluorescent dye was detected in the renal tissues confirming that these cells homed into the kidney tissue (Figure 4).

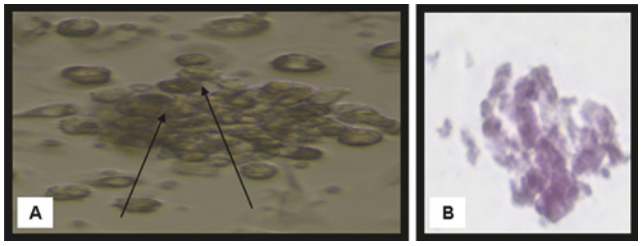

Figure 1: Morphological and histological staining of differentiated BM-MSCs into osteoblasts. (A) (×20) Arrows for differentiated MSCs osteoblasts after addition of growth factors. (B) (×200) Differentiated MSCs into osteoblasts stained with Alizarin red stain..

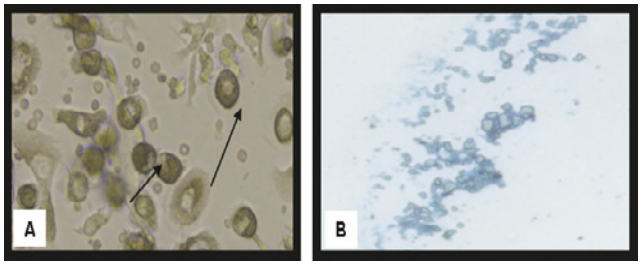

Figure 2: Morphological and histological staining of differentiated BM-MSCs into chondrocytes. (A) (×20) Arrows for differentiated MSCs chondrocytes after addition of growth factors. (B) (×200) Differentiated MSCs into chondrocytes stained with Alcian blue stain.

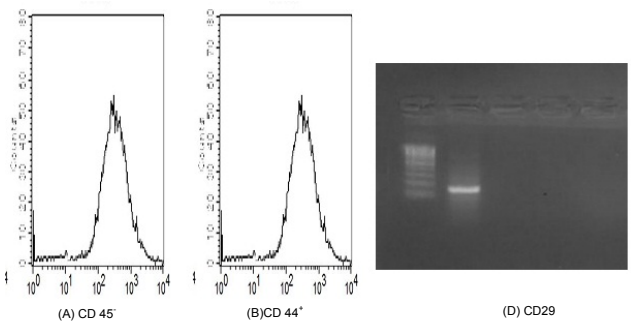

Figure 3: Flow cytometric characterization analyses of bone marrow-derived MSCs. Cells were uniformly negative for CD45(A), and positive for CD44(B). Figure 3(C) Agarose electrophoresis of CD29 PCR products of MSC in culture.

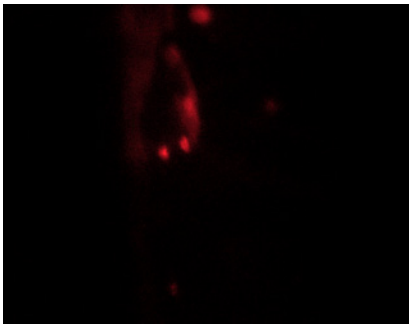

Figure 4: Detection of MSCs labeled with PKH26 fluorescent dye in kidney tissue. MSCs labeled with the PKH26 showed strong red autofluorescence after transplantation into rats, confirming that these cells were seeded into the kidney tissue.

MSCs and or HGF improve the kidney function

The results of the present study show a significant improvement in kidney function . Serum urea and creatinine were decreased in the ARF/MSC, ARF/HGF groups compared to the ARF group ($P<0.05$) (Table 1).

Gene expression of inflammatory and angiogenic markers

Concerning gene expression, VEGF & IL-10 genes were significantly decreased in ARF group ($P<0.05$) compared to control group. Whereas their level was significantly increased in the group that received either MSC alone or MSC and HGF but insignificant in group that received HGF alone (Figure 5), also these factors showed negative correlation with P value= $P<0.05$and R value = - 0.686 and P value= $P<0.05$and R value = - 0.744 (Figure 6B&C) respectively. On the other hand, the TNF-α gene which is one of inflammatory marker significantly decreased in the rat group that received either MSC alone

or MSC and HGF but insignificant in group that received HGF alone (Figure 5) and a significant positive correlation with serum creatinine concentration among the studied groups (Figure 6A) with P value= P<0.05 and R value = 0.868.

Histopathological changes

Histopathological examination of kidney tissue of ARF group showed Tubular atrophy of both proximal & distal tubules with marked lumen dilatation & cell debris in lumen & patchy loss of proximal tubule cells with regenerative change in tubular cells. (Figure 7 A PASX400) following MSC injection there was dense interstitial, periglomerular, perivascular and diffuse interstitial tissue infiltrates of cells between tubules at corticomedullary junction (Figure 7 B(PAS X400 X1000). In ARF/HGF there was minimal kidney damage with Patchy focal glomerular dilatation of Bowman's space. The space is partially filled with fibrin and cell debris (Figure 7C (HEX200) while in ARF/ MSCs+HGF there was cellular infilteration (Figure 7 D (HEX200).

Discussion

Bone marrow–derived stem cells contribute to cell turnover and repair in various tissue types, including the kidneys [20,21]. Mesenchymal stem cells (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 [22]. MSCs are commonly defined as bone marrow–derived fibroblast-like cells, which despite the lack of specific surface markers can be selected by their adherence characteristics in vitro and which have the ability to differentiate along the three principal mesenchymal lineages: osteoblastic, adipocytic, and chondrocytic [23,24]. In the present study, bone marrow derived mesenchymal stem cells were isolated from male rats, grown and characterized by their adhesiveness and fusiform shape and by detection of CD 29, one of surface marker of rat mesenchymal stem cells and were used to detect their possible anti-inflammatory and vascular role in amelioration of renal function in experimental model

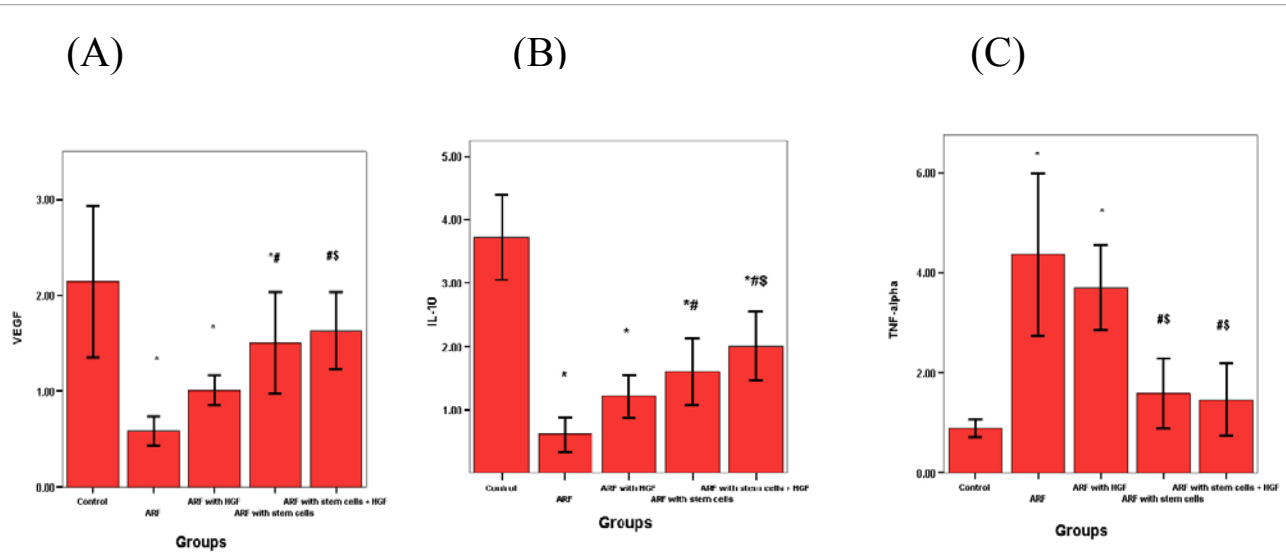

Figure 5: Quantitave analysis of VEGF(A) , IL-10 (B) &TNF-α(C) gene expression by real time PCR in different groups(# significant difference to ARF group).

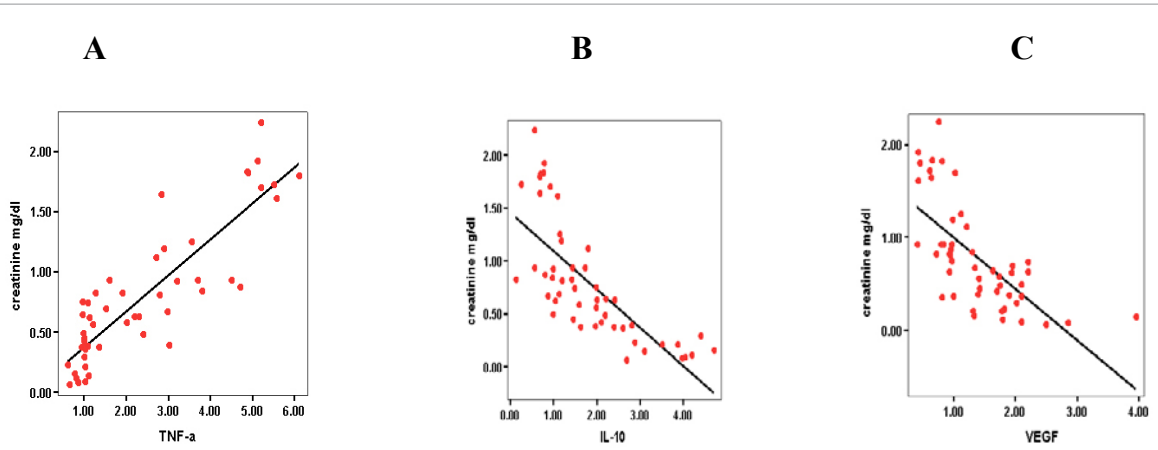

Figure 6: Correlation between TNF-α (A), IL-10 (B) & VEGF(C) with creatinine(B&C significant positive correlation (A) significant negative correlation).

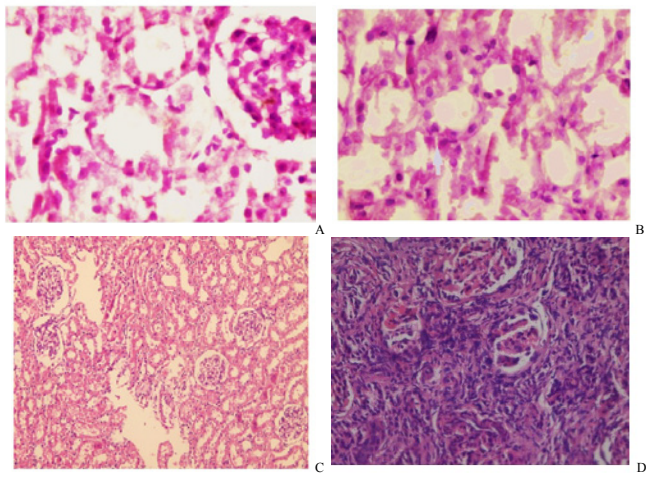

Figure 7: Histopathological examination of renal tissues in different groups: (A)ARF showed atrophy and patchy necrosis of proximal and distal renal tubules & cell debris in the lumen. (B)ARF+ MSC(B) & ARF+MSC+HGF(D) showing dense interstitial tissue infiltrate between tubules at corticomedullary junction.(D)ARF+HGF (C), showed fibrin and cell debris in cortical tubules.

	Primer sequence
VEGF	Forward:5'GCCTGAAATCTACCAGATCATGTTG 3´ Reverse:3'TTCCACAAGCTCCACGAATCTT 5´
TNF-α	Forward :5' GACCCTCACACTCAG ATC ATC TTC T -3' Reverse :3' TTGTCTTTGAGATCCATGCCA TT 5'
IL-10	Forward :5´ GAA GCT GAA GAC CCT CTG GAT ACA 3´ Reverse : 3´ TTG TCT TTG AGA TCC ATG CCA TT 5´
Beta actin	forward 5'-TGTTGTCCCTGTATGCCTCT-3' reverse 3'-TAATGTCACGCACGATTTCC-5'

Table 1: Sequence of the primers used for real-time PCR.

groups	urea (mg/dl) mean± SD	creatinine (mg/dl) mean± SD
control	33.39 ± 7.95	0.16 ± 0.08
ARF	82.73 ± 10.28*	1.63 ± 0.44*
ARF with HGF	69.79 ± 7.52*#	1.06 ± 0.27*#
ARF with stem cells	49.28 ± 6.31*#$	0.6 ± 0.16*#$$
ARF with stem cells & HGF	43.73 ± 9.20*#$	0.5 ± 0.11*#$

Table 2: Serum urea (mg/dl) & creatinine (mg/dl) in different studied groups:

*Significant p as compared to control group (P<0.05)
#Significant p as compared to ARF group (P<0.05)
$Significant p as compared to ARF with HGF group (P<0.05)

of acute renal fai decrease in serum urea and creatinine concentrations than those of the ARF group. Our results were similar to those of Dai [27], who proved that a Single Injection of naked plasmid encoding hepatocyte growth factor prevented cell death and ameliorated acute renal functions in mice. The possible mechanism is that hepatocyte growth factor exerts mitogenic responses in renal epithelial cells derived from distinct regions and species, including rabbit and rat proximal tubular cells .HGF also stimulates the proliferation of renal epithelial cell lines, including a rat visceral glomerular cell line [14], proximal tubular cell lines [15].

Following stem cell injection, those donors cells could be detected in recipient failing kidneys by autofluorescence that appeared in kidney tissue after MSCs injection .The result of the present work showed strong red auto fluorescence after transplantation in rats, confirming that these cells were actually insinuating themselves into the renal tissue as detected by fluorescent microscope. This result was in accordance to that reported by Morigi et al. [28], who labeled a human bone marrow MSCs with PKH 26 dye and administered it into mice with induced acute renal failure and found the red fluorescence of the MSC.

Fusion or transdifferentiation, this could not be answered in this study, However, both techniques definitely proved that those cells were able to maintain high population all through the study, in other words, for 3 days following MSC injection. These results agree with those of Li et al. [29]; who showed 50% replacement of proximal tubular cells with donor cells. These results also agree with Rookmaaker et al. [30]; who declared that bone-marrow-derived cells may home to injured glomerular endothelium, differentiate into endothelial cells, and participate in regeneration of the highly specialized glomerular microvasculature. In addition, they confirmed previous observations that bone-marrow-derived cells can replace injured mesangial cells [31]. Tögel et al. [18]; stated that infused MSC were detected in the kidney only early after administration and were predominantly in glomeruli

Duffield et al. [32]; state that BDMC contribute a regenerative cytokine environment that may be important in the resulting functional repair. Similarly, it was found that bone marrow–derived stem cells seemed to contribute relatively small numbers of cells (3 to 22%) to regenerating renal tubular [33] and glomerular cell populations [21]; that is, the majority of reparative cells were derived from intrinsic kidney cells. Regardless the cause, whether it's MSC differentiation, fusion or merely cytokine induced renal improvement; following MSC injection, the results of the present work showed increase in IL10 and VEGF and decrease in TNF gene expression in renal tissues. Several studies stated that after 24 h of MSCs infusion, only exceptionally scarce numbers of MSCs were found in the kidney, a pattern that essentially rules out the possibility that significant numbers of infused MSCs are retained in the kidney where they could physically replace lost kidney cells by transdifferentiation. This conclusion is furthermore supported by the fact that there were no intrarenal transdifferentiation events of MSC within 3 days of administration, whereas occasional MSC-derived capillary endothelial cells were identified only after 5–7 days. From this, it could be deduced that the mechanisms that mediate the protective effects of MSC must be primarily paracrine. This is proved by their expression of several growth factors such as HGF, VEGF, and IGF-I, all known to improve renal function in CRF, mediated by their antiapoptotic, mitogenic and other cytokine actions. Collectively, these as yet incompletely defined paracrine actions of MSC result in the renal downregulation of proinflammatory cytokines IL-1β, TNF-α, and IFN-γ, as well as iNOS, and upregulation of anti-inflammatory and organ-protective IL-10 [34], as well as bFGF, TGF-α, and Bcl-2. The lack of renoprotection obtained by infused fibroblasts may be due, at least in part, to the fact that MSC exhibit a comparatively higher expression of VEGF, HGF, and IGF-I, therefore suggesting that the combined delivery, by MSC, of these factors appears to result in superior renoprotection than that obtained with the growth factors that are more highly expressed by fibroblasts (EGF, HB-EGF, BMP-7, bFGF).

Histopathological examination of renal tissue samples of ARF group showed increased congestion & increased cellularity of the glomeruli & fibrin deposition. There was also patchy tubular atrophy & necrosis. On

the other hand normal medullary tubules with vacuolar degeneration as a late event were seen. Interstitial edema & mild inflammation also occurred. MT showed dense fibrosis invaded by a dense collection of MSCs which were indicated by autofluorescence in kidney tissue after MSCs injection. Those were examined with H&E stain, that is a more sensitive detector regarding cellular infiltration, & finally with Mason Dichromate, which is a better detector of collagen fibers hence, fibrosis & scaring. The present findings agreed with those of [29]; who recorded similar perivascular & periglomerular infiltration. In addition, they reported cell fusion, with occurrence of binucleated cells.

In the current study when HGF was administered after 24 hours, there were patchy focal glomerular dilatation of Bowman's space and the space was partially filled with fibrin and cell debris. Rest of glomeruli appeared normal. Cortical tubules showed patchy areas of minimal necrosis & degeneration mostly hyaline & vacuolar as well as atrophy & tubular dilatation. This finding agreed with Kawaida K et al. [35], who stated that HGF prevents ARF and accelerates renal regeneration in mice and in accordance with Miller et al. [36], who stated that Hepatocyte growth factor accelerates recovery from acute ischemic renal injury in rats.

Using stem cell–enriching and/or cytokine-enriching strategies after ARF, we found the effect of the injected cytokines HGF to be more important to improve kidney function than the transplanted MSCs alone. Our data therefore support the emerging findings that stem cell therapy may enhance kidney function primarily via paracrine mechanisms as opposed to a regeneration of new renal tissue. Although MSCs are capable of producing a great variety of cytokines, including HGF. HGF -enhanced MSCs showed increased cytokine expression in vivo and maximized the beneficial paracrine effects of MSC transplantation.

In conclusion, MSC & HGF can exert their effect by paracrine mechanisms through down regulation of proinflammatory cytokine TNF-α and up regulation of anti-inflammatory IL-10 and VEGF.

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