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ORIGINAL ARTICLE

Lipocalin 2 enhances mesenchymal stem cell-based cell therapy in acute kidney injury rat model

Mehryar Habibi Roudkenar · Raheleh Halabian · Hossein Abdul Tehrani · Fatemeh Amiri · Ali Jahanian-Najafabadi · Amaneh Mohammadi Roushandeh · Zahra Abbasi-Malati · Yoshikazu kuwahara

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Abstract Acute kidney injury (AKI) is one of the most common health-threatening diseases in the world. There is still no effective medical treatment for AKI. Recently, Mesenchymal stem cell (MSC)-based therapy has been proposed for treatment of AKI. However, the microenvironment of damaged kidney tissue is not favorable for survival of MSCs which would be used for therapeutic intervention. In this study, we genetically manipulated MSCs to up-regulate lipocalin-2 (Lcn2) and investigated whether the engineered MSCs (MSC-Lcn2) could improve cisplatin-induced AKI in a rat model. Our results revealed that up-regulation of Lcn2 in MSCs efficiently enhanced renal function. MSC Lcn2 up-regulates expression of HGF, IGF, FGF and VEGF growth factors. In addition, they reduced molecular biomarkers of kidney injury *such as* KIM-1 and Cystatin C, while increased the markers of proximal tubular epithelium such as AQP-1 and CK18 following cisplatin-induced AKI. Overall, here we over-expressed Lcn2, a well-known cytoprotective factor against acute ischemic renal injury, in MSCs. This not only potentiated beneficial roles of MSCs for cell therapy purposes but also suggested a new modality for treatment of AKI.

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M. H. Roudkenar (✉)
Department of Medical Biotechnology, Paramedicine Faculty, Guilan University of Medical Sciences, Rasht, Iran
e-mail: roudkenar@gums.ac.ir

M. H. Roudkenar
Neuroscience Research Center, Guilan University of Medical Sciences, Rasht, Iran

R. Halabian · H. A. Tehrani
Department of Medical Biotechnology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran

F. Amiri · Z. Abbasi-Malati
Blood Transfusion Research Center, High Institute for Research and Education in Transfusion Medicine, Tehran, Iran

A. Jahanian-Najafabadi
Department of Pharmaceutical Biotechnology, and Isfahan Pharmaceutical Sciences Research Center, School of Pharmacy, Isfahan University of Medical Sciences and Health Services, Isfahan, Iran

A. M. Roushandeh
Anatomical Sciences Department, Medicine Faculty, Hamadan University of Medical Sciences, Hamadan, Iran

Y. kuwahara
Department of Radiation Biology and Medicine, Faculty of Medicine, Tohoku Medical and Pharmaceutical University, 4-4-1, Komatsushima, Aoba-ku, Sendai 981-8558, Miyagi, Japan

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Introduction

Acute kidney injury (AKI) is a clinically disturbing disease associated with an unacceptably high mortality rate following its development to end-stage renal disease (Hoste et al. 2006; Ishani et al. 2009), and it is concurrent with increasing incidence of various complications (Chertow et al. 2006; Ftouh and Thomas 2013). AKI-associated mortality has been affected by different geographic variations including economic and regional factors, however, it has higher rates in patients under serious care and after cardiac surgery (Susantitaphong et al. 2013).

Currently, the therapeutic choices are confined to supportive cares and preventive strategies. However, none of these have shown a decrease in mortality rate caused by AKI (Masoud et al. 2012). Recent investigations have been focused on translating basic pathophysiologic understanding into clinical advances in an effort to develop selective approaches mediating effective targeted therapeutic interventions (Molitoris et al. 2009). Kidney transplantation is an alternative option; however, it has practically been limited due to some problems including the scarcity of donors, high cost, and immune rejection. It is, therefore, imperative to find other therapeutic options that could regenerate the tubular epithelium, restore kidney function and reverse the effects of AKI. In recent years, mesenchymal stem cell (MSCs)-based cell therapy has been confirmed to be another therapeutic option for AKI. MSCs secrete an array of growth factors such as vascular endothelial growth factor (VEGF), insulin-likegrowth factor-1 (IGF-1), hepatocyte growth factor (HGF) and anti-apoptotic cytokines (Khan et al. 2011; Amiri et al. 2015). After transplantation, however, the therapeutic effects of MSCs are mainly dependent on their survival in the recipient tissue (Bernhardt et al. 2006). Of note, during transplantation, cells are exposed to a hypoxic nutritionally-poor environment, oxidative stress and masses of cytotoxic factors leading to an inflammatory cytokine storm (Toma et al. 2002; Zhu et al. 2006) affecting the efficacy of MSC-based cell therapy. Therefore, several strategies have been developed in order to improve their therapeutic potential by making them strongly resistant to the environment into which they will be transplanted (Amiri et al. 2015; McGinley et al. 2011). Wilson et al. developed a system of locally introduced bone marrow–derived macrophages into inflamed glomeruli during nephrotoxic nephritis (Wilson et al. 2002). Chemical reagent and manipulation of cytoprotective and antioxidant genes of MSCs is another recent solution which has been the focal point of many investigations (Xie et al. 2012; Noiseux et al. 2012; Gao et al. 2011; Liu et al. 2011).

Lipocalin 2 (Lcn2) belongs to Lipocalin family which is expressed in several normal tissues and pathophysiological phenomena (Missiaglia et al. 2004; Nielsen et al. 1996). Also, Lcn2 appears as a protection factor against acute ischemic renal injury, inflammation, and infection (Berger et al. 2006). This mysterious protein serves to provide protection against bacterial infection and modulate oxidative stress. It acts as a cytoprotective factor against oxidative stress (Roudkenar et al. 2008). Interestingly, in response to renal tubular injury, the expression of Lcn2 rises up to 1000-fold in human and rodents, and its fast appearance into urine and serum makes it an early useful biomarker of renal failure (Schmidt-Ott et al. 2006).

Mishra et al. showed that Lcn2 can be used for a novel therapeutic intervention in ischemic acute renal failure based at least in part on its ability to tilt the balance of tubule cell fate toward survival (Mishra et al. 2004).

More recently, we showed that Lcn2-overexpressing-MSCs prevented cisplatin-induced cytotoxicity and apoptosis in kidney-derived cells i.e., HK-2 and HEK293 cells, and increased their proliferation rate and expression of growth factors (Halabian et al. 2015).

Therefore, we hypothesized that up-regulation of Lcn2 could strengthen the MSCs' capability of renal protection. In our study on AKI-affected rats, we report that Lcn2-engineered MSCs might be considered as a new modality for AKI treatment.

Materials and methods

Isolation and cultivation of MSCs

Bone marrow-derived MSCs were collected from the aspirates of femurs and tibias of 4-week-old male Sprague-Dawley rats (approximately 100 g), and were cultivated in 10 ml of Dulbecco's modified Eagle's medium (DMEM)-low glucose (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum

(FBS) (Invitrogen, Carlsbad, CA, USA) and 1% antibiotic penicillin and streptomycin solution. Cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO_2. After 48 or 72 h, non-adherent cells were discarded and the adherent cells were thoroughly washed with phosphate buffered saline (PBS). The fresh complete medium was added and replaced every 2 or 3 days for about 10 days. To further authenticate the MSCs, they were characterized by immunophenotyping (Halabian et al. 2013).

Stable Lcn2 expression by linearized pcDNA3.1/CT-GFP-Lcn2 in MSCs

To construct the Lcn2 expression plasmid, full-length human Lcn2 cDNA lacking termination codon were cloned into the *EcoR I* and *NotI* restriction sites of the mammalian expression vector pcDNA3.1/CT-GFP (Invitrogen, Carlsbad, CA, USA) as previously described (Halabian et al. 2013).

Fidelity of cloning was evaluated by DNA sequencing (Bioneer Corporation, Daejeon, South Korea) and the recombinant plasmid was designated as pcDNA3.1/CT-GFP-Lcn2. Next, the MSCs were transfected with a pcDNA3.1/CT-GFP-Lcn2 plasmid (MSC-Lcn2) as previously described (Halabian et al. 2013).

In addition, some MSCs were transfected with the empty pcDNA3.1/CT-GFP vector (MSC-V) and considered as control group. The expression level of Lcn2 was evaluated by RT-PCR, Real-time PCR, ELISA (R&D Systems, Minneapolis, MN, USA) (Halabian et al. 2013).

Preparation of rat AKI models

Animal experiments were conducted in accordance with the NIH Guide for care and use of laboratory animals. Female *Rattus norvegicus*, 6 weeks ages; 160–180 weights, were fed with normal chow and allowed to free excess of water. AKI was induced and set up by intraperitoneal (IP) injection of 3–16 mg/kg cisplatin (Sigma, St. Louis, MO, USA). Induction of AKI was confirmed with biochemical and histological methods during 24, 48 and 72 h.

MSCs transplantation

MSCs, MSC-V, and MSC-Lcn2 were administered to AKI-induced rats 48 h after induction of injury. MSC-V and MSC-Lcn2 were also injected to normal rats without AKI induction. The rats were divided into eight groups of 12 rats in each group including (1) control (Cont.) group in which the rats were injected intravenously with physiological saline solution; (2) MSC group which received MSCs without any manipulation; (3) MSC-V group which received MSC-V; (4) MSC-Lcn2 group which received MSC-Lcn2; (5) cisplatin (Cis) group or AKI group (in which animals were injected with 13 mg cisplatin (Sigma) per 1 kg of body weight; (6) cisplatin plus MSCs without any manipulation (AKI/MSC) group (in which animals received both cisplatin and the MSC; (7) cisplatin plus MSC-V (AKI/MSC-V) group (in which animals received both cisplatin and the MSC-V; and (8) cisplatin plus MSC-Lcn2 (AKI/MSC-Lcn2) group (in which animals received both cisplatin and the MSC-Lcn2. 1.5×10^6 MSCs, MSC-Lcn2 or MSC-V were suspended in 300 µl PBS and were intravenously injected in to the rats of any corresponding group.

Biochemical analysis of renal function

To determine the concentration of Blood Urea Nitrogen (BUN) and Serum Creatinine (SCr), blood samples were collected 24, 48 and 72 h after cisplatin injection as well as from MSC-transplanted-rats on the 4th, 10th and 21st days after cell therapy. In this regard, the rats were anesthetized using ketamine (100 mg/kg) and xylazine (10 mg/kg), and then their blood was collected by intracardiac puncture. Levels of both BUN and SCr were measured with BUN and SCr laboratory kits (Biosystem. S.A, Barcelona, Spain) using an automatic analyzer (BT 3000 PLUS, Biotecnica Instruments, Roma, Italy).

Preparation of histological samples

Kidney tissues were collected from transplanted rats on the 4th, 8th, 12th and 21st days post transplantation. The kidneys were sectioned in blocks, fixed with 4% paraformaldehyde, dehydrated in graded concentrations of alcohols and then embedded in paraffin. Kidney blocks were cut into 5-µm sections and stained with haematoxylin and eosin (H&E) (Sigma). The magnitude of tubular epithelial cell loss, necrosis, intratubular debris and tubular cast formation were scored according to six levels based on the percentage of tubule affection under a high-power field (HPF) using a light microscope. The kidney tissues were also subjected to use for PCR, Real-time PCR, and ELISA.

Table 1 The designed primer sets criteria

Genes	Primer sequence	Amplicon size (bp)
β.actin	Forward: 5′-TTCTACAATGAGCTGCGTGTGG-3′	115
	Reverse: 5′-GTGTTGAAGGTCTCAAACATGAT-3′	
HGF	Forward: 5′-GGTGCATCAGAAACAAGGGC-3′	136
	Reverse: 5′-AGGTCAAATTCATGGCCAAACC-3′	
IGF-1	Forward: 5′-CACAGACGGGCATTGTGGAT-3′	55
	Reverse: 5′-CTGAGTCTTGGGCATGTCAGT-3′	
FGF-2	Forward: 5′-ATGAAGGAAGATGGACGGCTG-3′	59
	Reverse: 5′-TTCTGTCCAGGCCCCGTTTT-3′	
VEGF	Forward: 5′-GCGAGGCAGCTTGAGTTAAA-3′	111
	Reverse: 5′-GGTGAGAGGTCTAGTTCCCG-3′	
AQP1	Forward: 5′-CTGCTGGCCATTGACTACAC-3′	181
	Reverse: 5′-GGTCTGTAAAGTCGCTGCTG-3′	
CK-18	Forward: 5′-CTGGGGCCACTACTTCAAGA-3′	188
	Reverse: 5′-CCTTGCGGAGTCCATGAATG-3′	
KIM-1	Forward: 5′-TCCTCCAACAAGACCCACAA-3′	115
	Reverse: 5′-TGGTGTTGGAGTAGAGGTGG-3′	
Cystatin C	Forward: 5′-AGTACAACAAGGGCAGCAAC-3′	188
	Reverse: 5′-TGCCTTCCTCATCAGATGGG-3′	
SRY	Forward: 5′-TTGGCTCAACAGAATCCCAG-3′	189

Detection of SRY DNA by PCR

For detection of SRY, the transplanted-female-rats were sacrificed on the 4th, 8th and 12th days after the infusion of MSCs (experimental group) or physiological saline (control group). Renal DNA was extracted according to the manufacturer's protocol (Roche, Mannheim, Germany) and subjected to the following PCR and real-time PCR conditions for amplification SRY DNA: 94 °C for 5 min, 40 cycles of PCR (94 °C for 30 s, 57 °C for 60 s, 72 °C for 120 s) and 72 °C for 5 min.

RT-PCR and real-time PCR analysis of kidney tissue

To evaluate the effects of Lcn2 on the expression of growth factors and renal markers in kidney tissue, RNA was extracted from renal tissue using Tripure (Roche, Mannheim, Germany) according to the manufacturer's protocol. Two micrograms of RNA were utilized to be converted into cDNA using the First Strand cDNA Synthesis Superscript kit (Invitrogen) according to the manufacturer's protocol. RT-PCR was performed to amplify growth factors and renal markers cDNA using specific primers. The criteria of these mentioned primers are presented in Table 1. Quantitative real-time PCR was performed using SYBR Green Mastermix (Invitrogen), 2 μl of the cDNA and 15 p mol primers for genes of interest or the β-actin gene. Reactions were performed in triplicate with Rotor-gene RG-3000 (Corbett Research, Sydney, Australia) and the specificity was monitored using melting curve analysis after cycling. Relative mRNA expression was quantified using the $\Delta\Delta$Ct method and the β-actin was used as an internal control.

Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) analysis was performed for growth factors including vascular endothelial growth factor (VEGF; R&D Systems), hepatocyte growth factor (HGF; R&D Systems), FGF-1 (Immunodiagnostic Systems, Franfurt am Main, Germany) and IGF-1 (R&D Systems) as well as for repair markers including aquaporin 1 (AQP1) (R&D Systems) and cytokeratin-18 (CK-18) (Immunodiagnostic Systems), and injury markers including kidney injury molecule-1 KIM-1 (R&D Systems), Cystatin C (Santa Cruz, Heidelberg, Germany), according to the manufacturer's instructions. Furthermore, acute kidney injury was evaluated with rat argutus AKI test assay kit (Meso Scale Discovery, Rockville, MD,

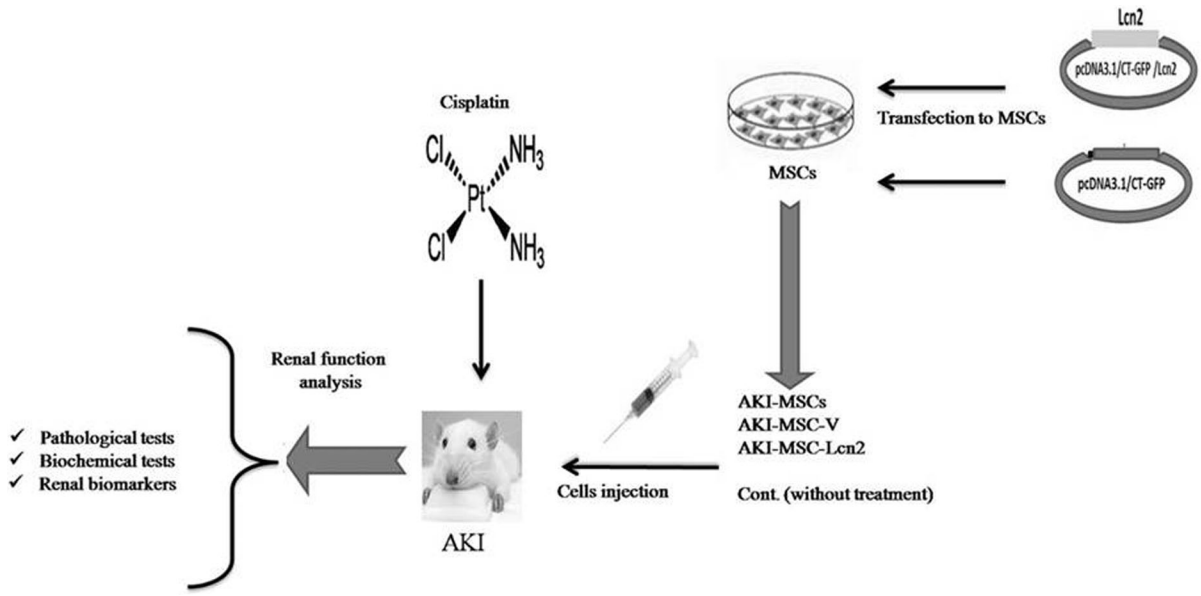


Fig. 1 Study overview. The isolated *Lcn2* was cloned into pcDNA3.1/CT-GFP vector. Then, MSCs were transfected with the recombinant vector. MSCs, MSCs-V and MSCs expressing recombinant Lcn2 (MSC-Lcn2) were infused to AKI rats. Finally, renal functions were evaluated by pathological and biochemical tests as well as renal molecular biomarkers

USA), too. This kit includes a cocktail of αGST, GSTYb1, and RPA-1, the combination of which allows researchers to stratify acute kidney injury.

Statistical analysis

Data were analyzed by standard statistical methods and analysis of variance (ANOVA) followed by Student–Newman–Keuls post hoc test for multiple comparisons or the nonparametric Kruskal–Wallis test. Group data are expressed as mean ± SD. $p \leq 0.05$ was considered as statistically significant.

Results

Study overview

Rat bone marrow MSCs were isolated and characterized as described previously (Halabian et al. 2013). Human Lcn2 cDNA was also cloned into the pcDNA3.1/CT-GFP vector. Then, the MSCs were transfected with pCDNA3.1/CT-GFP-Lcn2 or pCDNA3.1/CT-GFP constructs to generate MSC-Lcn2 and MSC-V cells, respectively, and stable clones were selected (Halabian et al. 2013). Then, the multi-differentiation potential of the MSC-Lcn2 into bone, adipogenic, and chondrogenic lineages was verified (Halabian et al. 2013). Next, these cells were transplanted into rats affected with cisplatin-induced AKI. Further elaborations on this issue are given in Fig. 1.

Incidence of AKI after cisplatin injection

AKI development following cisplatin administration (3–16 mg/kg) was evaluated by biochemical method. Elevations in both BUN (61 ± 5 vs. 16 ± 2 mg/dl, $p \leq 0.001$) and SCr levels (1.7 ± 0.2 vs. 0.4 ± 0.1 mg/dl, $p \leq 0.001$) were observed 48 h after injection of 13 mg/kg cisplatin in comparison to control (sup Fig. 1). Administration of 16 mg/kg cisplatin led to the death of the rats after 72 h; however, after administration of 13 mg/kg cisplatin, the level of BUN and SCr remained high and the rats stayed alive. Therefore, the dose of 13 mg/kg was considered as an optimized dose in our study (sup Fig. 1).

Lcn2 enhanced the re-construction potential of MSCs in AKI model

Different MSCs groups were administered to AKI-induced-rats 48 h after injury. Pathological examination of the kidney sections indicated that experimental

groups were recovering in comparison with the control group. The degree of micro-aggregate-like degeneration and necrosis of renal tubular epithelial cells were lower in MSC-treated rats (all experimental groups) compared with the AKI group (Fig. 2a). Next, the number of casts and tubular necrosis were estimated per field. In all experimental groups, the number of both cast and necrosis was lower than in the AKI group (Fig. 2b). However, in the AKI/MSC-Lcn2 group, interestingly, the number of casts was lower than in the AKI/MSC-V group on the 21st day (Fig. 2b). Altogether, these results strongly suggest that MSCs-based cell therapy in the injured kidneys not only ameliorate the severity of injuries but also reconstructed and repaired the damaged tissue. This fact is a matter of great consideration in AKI/MSC-Lcn2 group.

Higher level of SRY gene expression was detected in the AKI/MSC-Lcn2 group

For detection of the expression level of the SRY gene in the AKI model, following infusion of male rats derived MSCs into female rats, the SRY DNA was detected from different parts of the rat's kidney tissue by RT-PCR and real-time PCR. RT-PCR results revealed that there was no detectable SRY DNA in

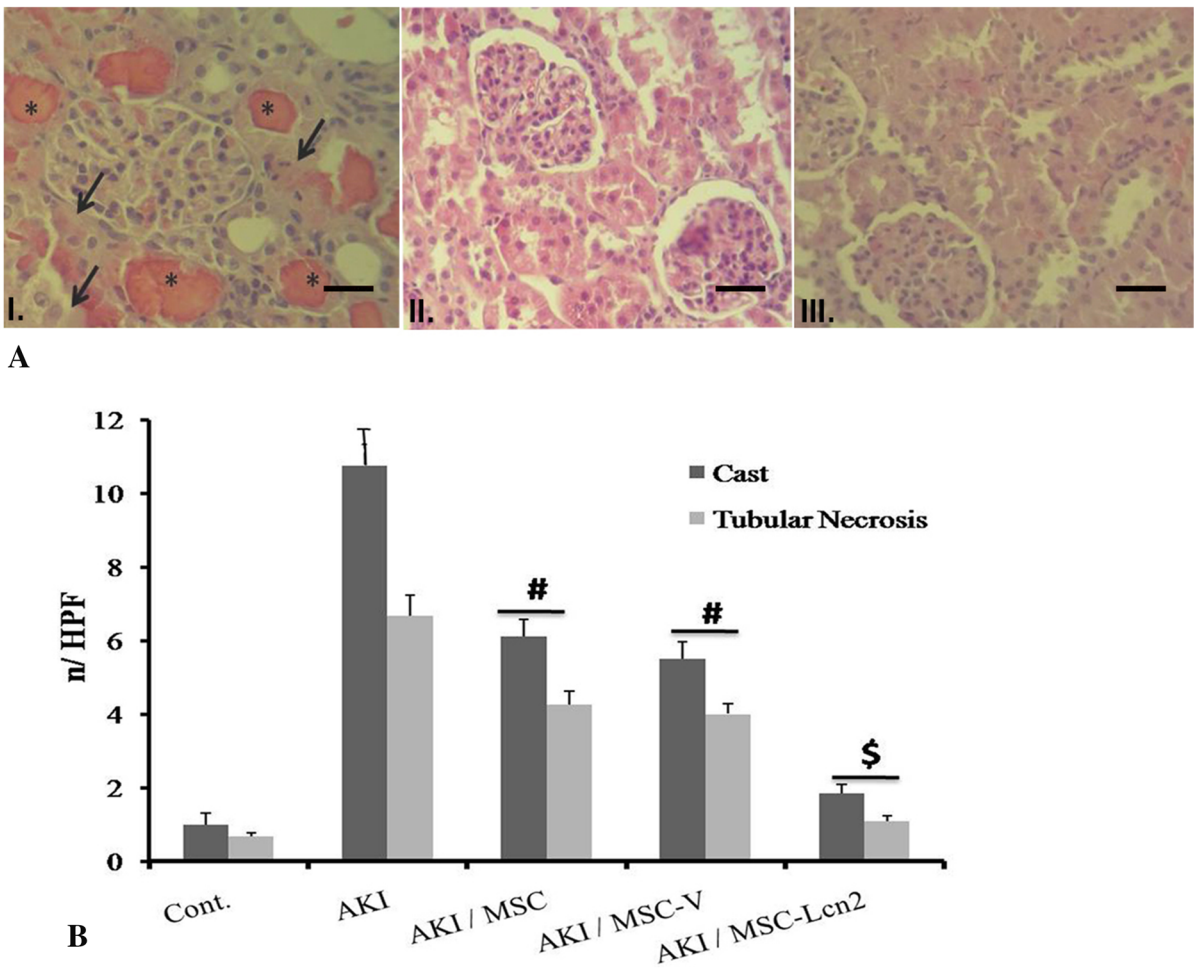


Fig. 2 Histology analysis of cisplatin-induced AKI on the 21st day. (**a**) *I* Cisplatin-induced AKI (AKI), *II* Transplantation of MSC and *III* MSC-Lcn2 following the induction of AKI. *Arrow* tubular necrosis and *Asterisk* cast. After day 21, reduction of casts in AKI/MSC-Lcn2 group was more than AKI/MSC (×400). (**b**) Number of casts and tubular necrosis. Conventional histological analysis after hematoxylin and eosin staining of kidney sections (original magnification ×400) confirmed protective effects of MSC-Lcn2 compared with control group (Mean ± SD; $ $p < 0.001$ vs. AKI, AKI/MSC-V and AKI/MSCs groups, # $p < 0.01$ vs. AKI group). ($n = 12$ rat in each groups), scale bar: 20 µm

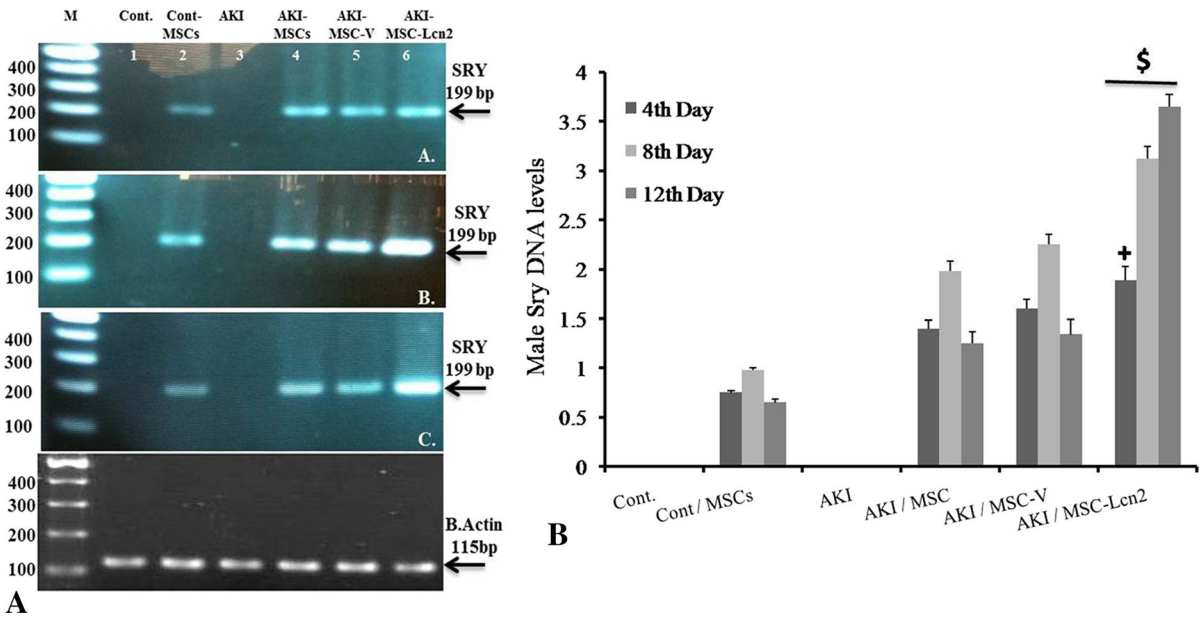


Fig. 3 Detection of SRY gene after MSCs transplantation into AKI rats. The DNA from extracted kidney sections and the distribution of MSCs in kidney tissue were quantified via SRY gene in the different groups on the 4th, 8th and 12th day after cell therapy. (**a**) PCR-based detection of donor-derived cells in the kidneys of different recipients (A. 4th day; B. 8th day; C. 12th day). (**b**) Real-time PCR for SRY. Both PCR and Real-time PCR results reveal that the expression level of the SRY gene in the AKI/MSC-Lcn2 group was higher than the other groups under cell-based therapy (AKI/MSC and AKI/MSC-V) (Mean ± SD; $ $p < 0.001$ vs. AKI, AKI/MSC-V and AKI/MSCs groups, +$p < 0.05$ vs. AKI/MSC-V and AKI/MSCs groups). ($n = 12$ rat in each groups)

rats treated with saline (control group) and the AKI group, while, it was found in AKI-MSC-Lcn2, AKI-MSCs and AKI-MSC-V groups (Fig. 3a). The level of SRY gene was also evaluated by real-time PCR in kidneys of the animals infused with male MSCs on the 4th and 8th and 12th day after infusion (Fig. 3b). Interestingly, the level of SRY gene was higher in AKI/MSC-Lcn2 than in AKI/MSCs and AKI/MSC-V treated animals (Fig. 3b). Overall, these results suggest that presence and migration of MSC-Lcn2 inside the kidney tissues of the AKI/MSC-Lcn2 group were more efficient than in the AKI/MSCs and AKI/MSC-V groups. In fact, the MSC-Lcn2 had higher survival rate than MSCs or MSC-V in injured kidney tissue.

MSC-Lcn2-based cell therapy ameliorates renal dysfunction caused by cisplatin-induced acute renal injury

To determine whether Lcn2 over-expression in MSCs would increase their protective effects on AKI, biochemical analysis of BUN and SCr concentrations were performed 4, 10 and 21 days after injection of MSCs in different rat groups. Four days following cell therapy, no protective effect was observed, as reflected by a non-significant reduction in BUN and SCr levels compared with the control group (Fig. 4a, b). On the 21st day, MSCs and MSC-V groups exhibited a protective effect as was reflected by the lower BUN and SCr levels compared with the control group ($p < 0.01$). Interestingly, over-expression of Lcn2 in MSCs increased the effectiveness of MSCs on reduction of BUN and SCr levels and their levels were dropped off (BUN, $\sim 23.9 \pm 2.9$ mg/dl, $p < 0.001$ and creatinine, $\sim 0.72 \pm 0.04$ mg/dl, $p < 0.001$) (Fig. 4c, d). Taken all together, these results indicate that the over-expression of Lcn2 enhances the efficacy of MSC-based cell therapy in cisplatin-induced renal injury.

MSC-Lcn2 up-regulates some critical growth factors

To assay, the effect of Lcn2-over-expression on MSCs paracrine ability, the expression level of

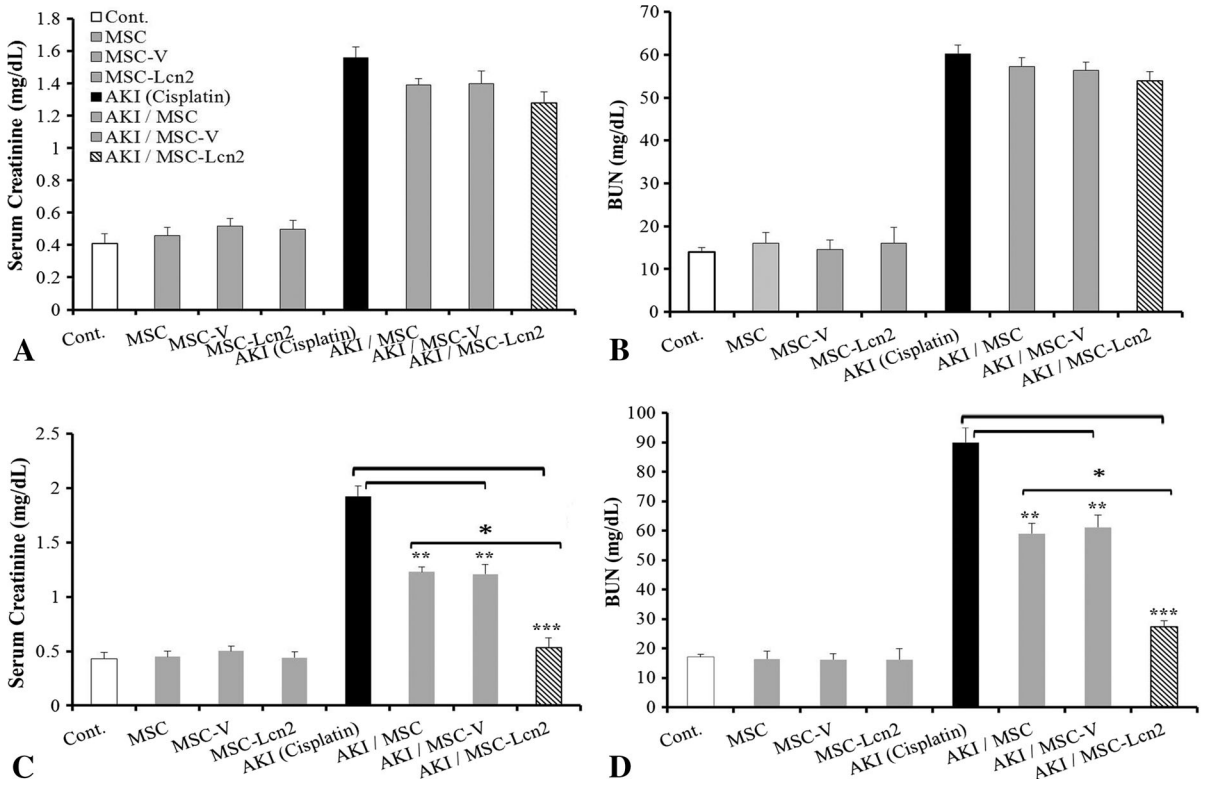


Fig. 4 Measurement of blood urea nitrogen (BUN) and serum creatinine (SCr) 4 and 21 days after cell therapy. Rats were injected intravenously with MSCs, MSC-Lcn2 or MSC-V on the 2nd day after induction of AKI, then blood was collected for determination of BUN and SCr levels on days 4 and 21. (**a**), (**b**) The SCr (**a**) and BUN (**b**) levels 4 days after cell therapy. There was no difference between the control group (AKI) and the groups under cell-based therapy. (**c**), (**d**) The SCr (**c**) and BUN (**d**) levels 21 days after cell therapy. Both BUN and SCr levels decreased in the cell-based therapy groups compared to the AKI group, but a considerable decline was observed in AKI/MSC-Lcn2 group (Mean ± SD; $^{**}p < 0.01$, and $^{***}p < 0.001$ vs. AKI group and $^{*}p < 0.05$ vs. AKI/MSC-V, AKI/MSC). ($n = 12$ rat in each groups)

some growth factors such as IGF-1, HGF, VEGF, and FGF were studied by real-time PCR and ELISA. In the AKI group with no treatment, the expression levels of IGF-I, HGF and FGF-I was down-regulated compared to the control group (without AKI). However, following MSCs, MSC-V and MSC-Lcn2 infusion to AKI rats, the mRNA levels of IGF-I, HGF, VEGF, and FGF-I was considerably up-regulated in AKI/MSC-Lcn2 group. Similar to the studies of Imberti et al. (2007) up-regulation of IGF-I was interestingly very high (Fig. 5a). These findings were further confirmed by ELISA (Fig. 5b I–IV). Altogether, the results suggest that Lcn2 plays an important role in promoting the regenerative potential of MSCs in the rat AKI model through up-regulation of some fundamental growth factors.

MSC-Lcn2-based cell therapy reduces molecular biomarkers of kidney injury following cisplatin-induced AKI

The results of RT-PCR after cell therapy revealed low expression level of AQP1 and CK-18 (markers of proximal tubule epithelium) in the AKI, AKI/MSCs, and AKI/MSC-V groups. However, rats infused with MSC Lcn2 showed high levels of AQP1 and CK-18 expression. Conversely, the expression of two injury markers i.e., Kim-1 and Cystatin C was enhanced in the AKI group (Fig. 6a). This finding was further confirmed by real-time PCR analysis (Fig. 6b). Infusion of MSCs into the rat AKI models down-regulated the expression levels of these injury markers, especially in AKI/MSC-Lcn2 groups. These findings were confirmed at the protein level by ELISA (Fig. 6c IIV).

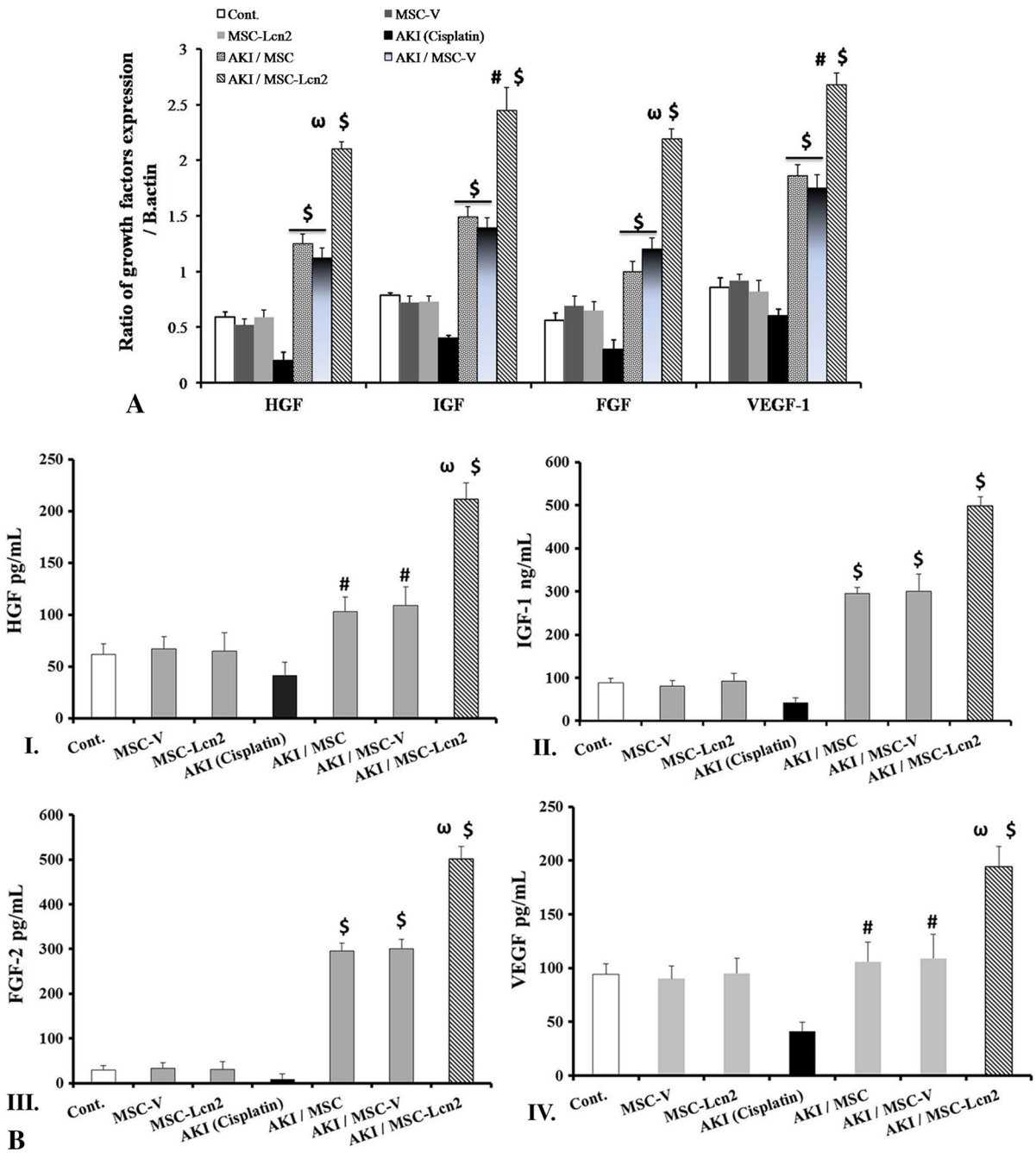


Fig. 5 Real-time PCR and ELISA for evaluation of the expression of growth factors. (**a**) Real-time PCR analysis. Results showed that Lcn2 considerably up-regulates HGF, FGF-1, IGF-1 and VEGF (Mean ± SD; **$** $p < 0.001$ vs. AKI group, **#** $p < 0.01$ vs. AKI/MSCs and AKI/MSC-V groups, **ω** $p < 0.001$ vs. AKI/MSCs and AKI/MSC-V groups). (**b**) (*I–IV*) Expression of the growth factors at protein level was also evaluated by ELISA on the 8th days. These findings were consistent with the real time PCR results (Mean ± SD; $ $p < 0.001$ vs. AKI group, # $p < 0.01$ vs. AKI group, **ω** $p < 0.001$ vs. AKI/MSCs and AKI/MSC-V groups). ($n = 12$ rat in each groups)

Taken together, the increase in repair markers and a parallel decrease in injury markers of AKI/MSC-Lcn2 groups indicate the beneficial role of Lcn2 overexpression in the regenerative process.

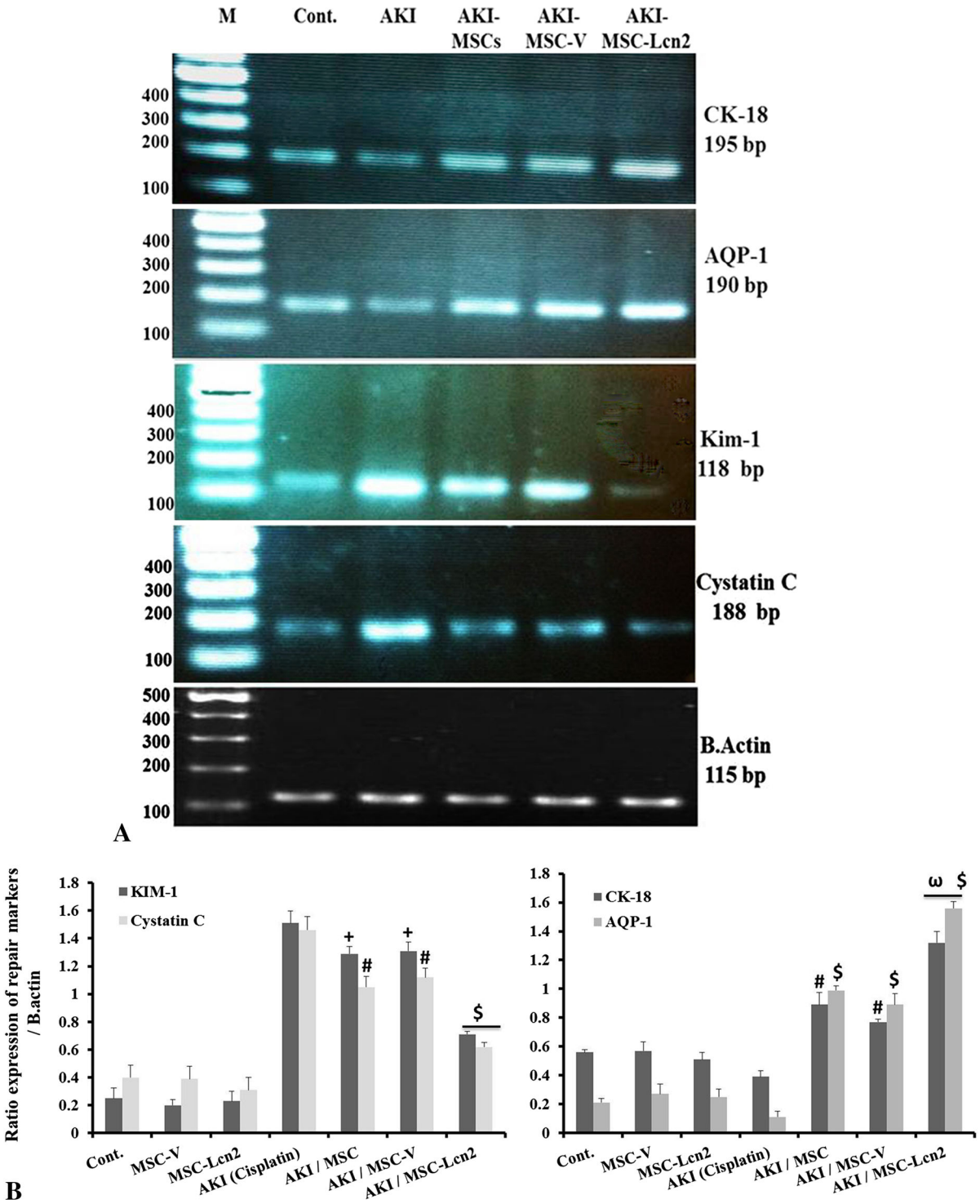
After cell therapy with MSCs, MSC-V, and MSC-Lcn2 in the AKI model, repair processing was also further assessed by rat AKI test. In this assay expression of αGST, GSTYb1, and RPA-1 was evaluated. Of note, these proteins are not naturally expressed in a healthy kidney. In the AKI group without MSCs-based cell therapy, expression of these markers was induced. However, 21 days after MSCs infusion, the levels of αGST, GSTYb1, and RPA-1 were significantly decreased in the AKI/MSCs, AKI/MSC-V and AKI/MSC-Lcn2 groups compared to the control group (AKI). Interestingly, in the AKI/MSC-Lcn2 group, there was a marked drop in the expression of this biomarker in comparison with the AKI/MSC-V and AKI/MSC groups (Fig. 6d). In other words, the therapeutic effect of MSC-Lcn2 was the most obvious finding in AKI/MSC-Lcn2 group. Overall, consistent with previous findings, these results indicated that the over-expression of Lcn2 in MSCs improve their efficacy in the treatment of the cisplatin-induced renal injury.

Fig. 6 Expression of some injury and repair molecular biomarkers of kidney in AKI rats following MSC-based therapy. (**a**) RT-PCR. Up-regulation of CK-18 and **AQP-1** (repair markers) and down-regulation of KIM-1 and Cystatin C (injury markers) were considerable in AKI/MSC-Lcn2. (**b**) These results were further confirmed by Real-time PCR (Mean ± SD; **$** $p < 0.001$ vs. AKI group, **#** $p < 0.01$ vs. AKI group, **ω** $p < 0.001$ vs. AKI/MSCs and AKI/MSC-V groups, **+**$p < 0.05$ vs. AKI group) and (**c**) ELISA (Mean ± SD; **$** $p < 0.001$ vs. AKI group, **ω** $p < 0.001$ vs. AKI/MSCs and AKI/MSC-V groups, **#** $p < 0.01$ vs. AKI/MSCs and AKI/MSC-V groups). (**d**) Argutus AKI Test on day 21. This test simultaneously evaluates expression of αGST, GSTYb1, and RPA-1 as acute kidney injury markers. These biomarkers are down-regulated in the AKI/MSC-Lcn2 group compared with the AKI/MSC-V and/or AKI/MSC groups (Mean ± SD; **$** $p < 0.001$ vs. AKI group, **#** $p < 0.01$ vs. AKI group, **ω** $p < 0.01$ vs. AKI/MSCs and AKI/MSC-V groups). ($n = 12$ rat in each groups)

Discussion

Ischemic and/or toxic injuries caused by chemotherapy, antibiotics, and shocks occurring after major surgery or infections might result in serious and common illnesses like acute kidney injury, in which renal tubular cells are damaged, and malfunction (Kelly and Molitoris 2000; Humes and Szczypka 2004; Hartmann et al. 2000). Apoptosis, necrosis, inflammation and oxidative stress are known cellular and molecular phenomena that play an important role in the severity of AKI (Chertow et al. 2006; Xie et al. 2012; Yong et al. 2011; Havasi and Borkan 2011). The current therapeutic option for AKI are based on supportive cures, in fact, there is not a real treatment for AKI yet. Therefore, it is essential to develop new and effective strategies in order to improve AKI treatment.

In this study, we suggested a new treatment strategy i.e., cell-based gene therapy in which MSCs were genetically modified to over-express lipocalin 2. We believe that Lcn2 selection for the purpose of MSCs-based gene therapy is very reasonable and intellectual. Lcn2 not only acts as an antioxidant, anti-apoptosis and anti-inflammatory protein (Halabian et al. 2013; Berger et al. 2006) but it also physiologically plays an important role in regeneration and proliferation of the tubular and epithelial cells of kidney tissue (Mishra et al. 2004). It is also well known that when the kidney is injured, the expression of Lcn2 is induced dramatically and immediately (Mishra et al. 2005; Peacock et al. 2013). It seems that this induction is a compensatory reaction to re-establish homeostasis. Interestingly, Mishra et al. showed that administration of recombinant Lcn2 ameliorates renal damage following the incidence of AKI in mice models (2004). This beneficial role of Lcn2 following AKI occurrence was further reported by Jung et al. (2012). They showed that Lcn2 not only plays an important role in the regeneration of the injured cells directly, but it highly contributes to cell proliferation by providing iron as an essential factor for growth (Jung et al. 2012). In a previous in vitro study, we reported that Lcn2-modified MSCs were protected against unfavorable microenvironments (Halabian et al. 2013). In the present study, we not only further expand our previous study in vivo but also our results clearly revealed that MSCs-Lcn2 enhances the protection against cisplatin-induced AKI. When kidney tissues are damaged, some growth factors are secreted into blood circulation, and in turn, cause MSCs to migrate toward the site of injury (Bussolati et al. 2009; Xinaris et al. 2013). However, it is controversial whether MSCs are directly involved in the regeneration process of injured kidney cells or they indirectly play a role in the process by the secretion of growth factors (Togel et al 2007; Morigi et al. 2004).



On the other hand, there is a tendency for MSCs to be attracted toward injured tissues (Togel et al. 2009). These observations prompted us to design the present study. However, the major challenge is the low survival of MSCs after transplantation (Chertow et al. 2006; Toma et al. 2002). However, our results

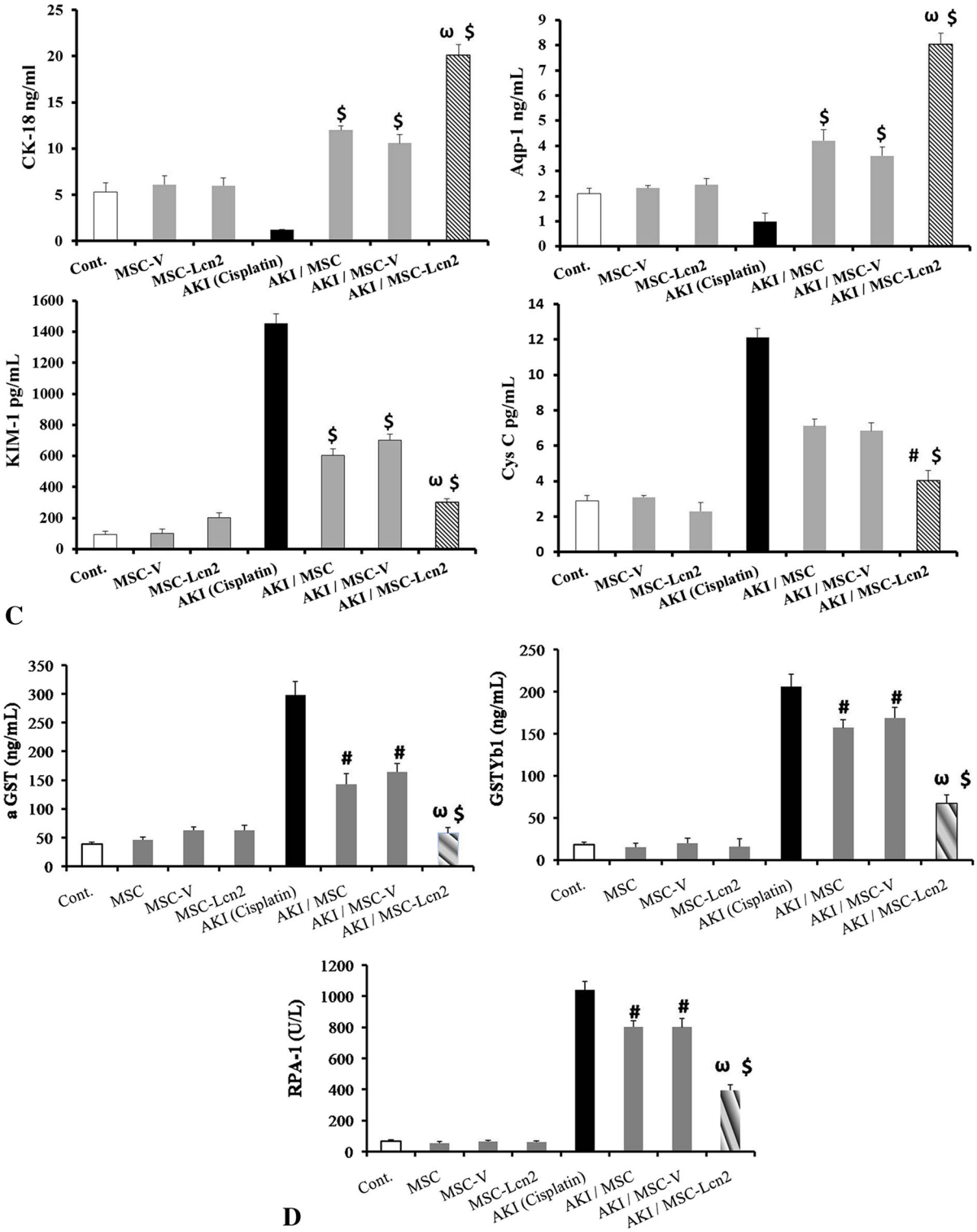


Fig. 6 continued

strongly suggested that over-expression of Lcn2 in MSCs can effectively address this concern. Our results revealed that expression of SRY gene was higher in MSC-Lcn2 group in comparison to MSC-V and MSC which suggests that Lcn2 expression enhances survival of MSCs either under ischemic or toxic microenvironment of AKI.

There are a few studies indicating that direct implication of MSCs in the regeneration of injured kidney is due to their ability to differentiate into renal tubular cells and endothelial cells. However, it has been shown in many studies that the renoprotective potential of MSCs is due to the secretion of beneficial growth factors. In fact, MSCs exert their regeneration potential through paracrine effects (Togel et al. 2009; Bianchi et al. 2014; Rodrigues et al. 2010). The growth factors secreted by MSCs can provoke anti-apoptotic, anti-inflammatory, mitogenic, and angiogenic actions in injured tissues (Asanuma et al. 2010). Different strategies could be used for the improvement of local MSCs' paracrine properties such as pre-treatment with cytokine and growth factors, or genetic manipulation (Bianchi et al. 2014). Interestingly, our results showed that over-expression of Lcn2 not only enhanced regenerative potential of MSCs in rat AKI models but also stimulated the secretion of several critical growth factors including HGF, IGF-1, FGF, and VEGF. These results were consistent with our previous in vitro study in which we reported that Lcn2 up-regulates these growth factors in MSCs especially under hypoxia, serum deprivation and oxidative stress conditions (Halabian et al. 2013). These findings again highlight that the selection of Lcn2 as a candidate protein for MSCs-based gene therapy is very reasonable. In support of our study, there is some evidence that the genetically engineered MSCs which over-express IGF-1 are able to enhance the efficiency of cell-based gene therapy in a mouse model of chronic kidney disease (Imberti et al. 2007). Furthermore, Yuan et al. (2011) reported that up-regulation of VEGF in human embryonic MSCs strengthens the renal protective effects of these cells in a nude mouse model of cisplatin-induced AKI.

BUN and serum creatinine are two biochemical markers routinely employed in laboratories to evaluate kidney injury (Edelstein 2008). However, recently it has been highly controversial whether these markers can really indicate the recovery rate of damage. Hence, in this study, we also evaluated the expression of other molecular biomarkers to examine the recovery of the damage. Our results revealed that MSC-Lcn2 reduced expression of damage markers, KIM-1 and Cystatin C, and simultaneously increased the markers of proximal tubular epithelium such as **AQP-1** and CK-18 (Sohn et al. 2013; Bonventre 2009; Dharnidharka et al. 2002). We also evaluated improvement of the damaged kidney using other molecular biomarkers e.g. αGST, GSTYb1, and RPA-1 which are all provided in the AKI test kit. These biomarkers have been recently approved by Food and Drug Administration (FDA) and European Medicines Agency (EMA) to evaluate renal damage (Hoffmann et al. 2010).

It is noteworthy that administration of MSC-Lcn2 led to enhanced survival rates without any identified tumorigenesis potential in the kidneys as well as other body organs two months after cell therapy (Supplementary Fig. 2).

Conclusion

In summary, the results of our in vivo study showed that the MSC-Lcn2 ameliorates renal dysfunction caused by cisplatin-induced renal injury. However, further and comprehensive studies are required to clarify the molecular mechanisms underlying the beneficial roles of MSC-Lcn2 on the improvement of cisplatin-induced renal injury including homing, engraftment, and survival of MSCs proliferation in the injured organ as well as differentiation potential of MSCs infused into renal tissue. On the other hand, in order to better explain the action of cells on renal tissue, experiments on renal sections to evaluate the proliferation rate of tubular cells and apoptosis in renal tissue following infusion of MSCs would be other subjects of future studies.

In addition, it should be noted that there is a major safety concern in the clinical application of genetically manipulated MSCs, therefore, further studies in this regard will be of use. One of these studies could be an assessment of beneficial effects of MSCs cultivation in the presence of MCS-Lcn2 secretome, which might alleviate the need for Lcn2 over-expression in MSCs transplantation.

Our findings might be beneficial to design a strategy for the prevention of graft cell death in MSC-based cell therapy especially for AKI in a clinical trial.

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Compliance with ethical standards

Conflict of interest The authors declare that there is no any conflict of interest.

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