

Effects of Stem Cells and Granulocyte Colony Stimulating Factor on Reperfusion Injury

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Introduction. Bone marrow-derived stem cells have a potential capacity to differentiate and accelerate recovery in injured sites of body. Also, factors like granulocyte colony stimulating factor (GCSF) can promote their mobilization to the injured sites. We aimed to investigate the role of GCSF as an alternative therapeutic option instead of mesenchymal stem cells (MSCs) in reperfusion injury.

Materials and Methods. Twenty-nine rats with induced reperfusion injury were divided into 3 groups to receive MSC, GCSF, or nothing (control). Kidney function was assessed by blood urea nitrogen and serum creatinine levels. Histological grading was performed to evaluate the extent of tubular injury and the rate of recovery.

Results. All the rats reached recovery after 14 days. Rats in the MSC group reached early functional and histological recovery compared to the controls on the 7th day of the study ($P = .01$ and $P = .02$, respectively). Compared to the control group, the GCSF group showed a more significant histological recovery on the 7th day ($P = .04$), but kidney function was ameliorated on the 14th day ($P = .04$). Both the GCSF and control groups had a significant number of CD34+ cells, which were detected by flow cytometry on the 7th day after reperfusion injury.

Conclusions. We found therapeutic effects following administration of both MSC and GCSF which was more evident with MSC in the setting of reperfusion injury. More investigation is required to find optimal time, dose, and route of administration as well as other possible contributing factors.

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INTRODUCTION

Acute kidney injury (AKI) is a clinical term refers to a syndrome with a wide variety of manifestations.¹ Reperfusion injury is one of the main causes of AKI, which can manifest histologically as acute tubular necrosis (ATN).² Acute tubular necrosis is seen in hospitalized patients, especially in the setting of intensive care units, in about 7% of patients.³⁻⁷ This potentially reversible condition is associated with a high mortality rate, which reaches up to 80% in

hospital-acquired settings.^{2,3}

There are continuing controversies about the pathophysiologic process and compensatory mechanisms that are involved in the course of ATN, in which the pathway begins with reperfusion injury and terminates to necrosis. In this context, the potential role of 2 main mechanisms is in debate; direct tubular engraftment of bone marrow-derived stem cells, especially mesenchymal stem cells (MSC) and paracrine or endocrine effects may ensue.⁸⁻¹³

Recent studies have shown reperfusion injury induces mobilization of bone marrow-derived stem cells,^{2,9,12-15} and also increases the serum level of granulocyte colony stimulating factor (GCSF).¹⁶ Granulocyte colony stimulating factor is a potent inducer of bone marrow stem cells mobilization.¹⁷ Kidney function is affected in several ways by the GCSF, as attenuating to worsening, reported by different studies in mouse models.¹⁸⁻²²

Considering the high burden of morbidity and mortality due to AKI, investigating for an efficient, practical, and easy practice to reverse the injury and to accelerate the treatment process seems to be among the first priorities of nephrology. In this study, we aimed to assess the effects of two different treatment options, exogenous MSC versus GCSF administration, in a rat model of ATN. We also compared the effects of endogenous stem cells recruitment and GCSF induction in the setting of reperfusion injury, looking for any new lightening and side of vision in a spectrum of reperfusion injury to necrosis.

MATERIALS AND METHODS

Reperfusion Model of Rats

Thirty-one inbred male Wistar rats weighing 220 g to 250 g were used in this experimental study. Animal care was conducted in conformity with the institutional guidelines that are in compliance with the national and international regulations and policies. The rats were kept in a constant place with a 12-hour-light 12-hour-dark cycle, and they were fed with a standard diet. All of the rats were anesthetized by ketamine/acepromazine and underwent right-side surgical nephrectomy. Reperfusion injury was induced by clamping the left renal pedicle for 47 minutes. In this procedure, we used microvascular nontraumatic clamps. The ischemic time was determined based on our previous pilot study to induce reversible ATN.²³ Reperfusion was induced by releasing the clamp after 47 minutes, and it was confirmed by observation of gross macroscopic change in the color of the left kidney after releasing the clamp.

Study Groups

Two rats developed hemorrhage following the operation and were excluded from the study. The 29 remained rats were divided into 3 groups: control group (n = 10), GCSF group (n = 11), and MSC

group (n = 8). The rats in the control group did not undergo any more intervention, while those in the GCSF group received subcutaneous GCSF (PDgrastim, Pooyesh Darou, Tehran, Iran), 100 µg/kg, 6 hours after inducing reperfusion injury, for 6 days. In the MSC group, the rats received one intravenous injection of 5×10^3 CD34+ Lin- Sca-1+ c-kit+ MSCs, 6 hours after reperfusion.

One or 2 rats of each group were killed on days 2, 4, 7, and 14 after reperfusion, and the kidneys were taken for histological study and grading.

To assess kidney function, peripheral blood was collected from all rats to determine serum level of creatinine and blood urea nitrogen (BUN) before reperfusion (baseline), 24 hours after reperfusion, and before killing the animal. The upper limit reference levels of BUN and serum creatinine were 70 mg/dL and 0.8 mg/dL, respectively.

Isolation and Characterization of Mesenchymal Stem Cells

Bone marrow was obtained from the femur bones of the rats. Mesenchymal stem cells were recovered from bone marrow cells. Flow cytometry analysis was performed to obtain 5×10^3 CD34+ Lin- Sca-1+ c-kit+ MSCs to be injected to rats with reperfusion injury. To evaluate effects of GCSF and any probable role of ischemia on stem cells, flow cytometry analysis and cell count were performed in rats that received GCSF and those in the control group, by collecting peripheral blood at the 7th day of the study.

Pathologic Assessment

The removed kidneys were fixed in formalin and embedded in paraffin. Four-microgram thickness sections were stained with hematoxylin-eosin and periodic acid-Schiff staining. All of the specimens were evaluated by one pathologist. Histological changes in every section of the specimen were scored in at least 10 randomly selected nonoverlapping fields at $\times 400$ magnifications. The specimens were also examined regarding tubular cell edema, brush border loss, interstitial edema, tubular dilatation, tubular cell necrosis, and hyaline cysts, and were scored based on this grading system from zero to 4. Grade zero represents no tubular damage; grade 1, less than 25% tubular damage; grade 2, tubular damage between 25% and 50%; grade 3, tubular damage between 50% and 75%; and grade 4, more than 75% tubular damage.

Statistical Analyses

Results were presented as mean \pm standard deviation. The paired *t* test was used to analyze BUN and serum creatinine levels. Data regarding histological grading in different groups were analyzed using the nonparametric Mann-Whitney test. Statistical significance level was defined as a *P* value less than .05.

RESULTS

Kidney Function

Twenty-four hours after inducing reperfusion injury, all of the rats in the three studied groups showed a significant rise in BUN and serum creatinine levels, compared to their baseline level. On the second day after reperfusion, BUN and serum creatinine levels decreased in all rats, and the decrease was more apparent in the MSC and GCSF groups than the control group. These kidney function indicators reached their normal levels on days 5 to 6 in both the MSC and the GCSF groups, while they did not reach normal level in the control group until day 14 of the study (Table; Figures 1 and 2).

Rats in the MSC and the GCSF groups had significant decreases in BUN levels on day 14 compared to those in the control group ($P = .04$ and $P = .046$, respectively). Serum creatinine levels showed significant decreases in both MSC and GCSF groups on day 14 compared to the control group ($P = .03$ and $P = .04$, respectively). The MSC group showed a significant decrease in serum creatinine level on day 7 compared to the control group ($P = .01$), but this was not significant for the GCSF group compared to the control group ($P = .10$).

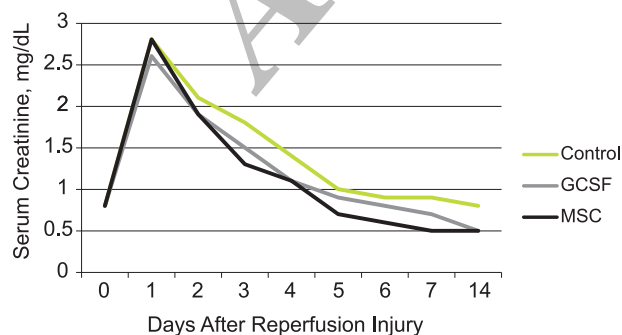


Figure 1. Serum creatinine levels trend after reperfusion injury in the three groups of rats treated with granulocyte colony stimulating factor (GCSF), mesenchymal stem cell (MSC), and nothing (control).

Kidney Function and Histology in Rats With Induced Reperfusion Injury Who Received Granulocyte Colony Stimulating Factor and Mesenchymal Stem Cell and Control Rats*

Kidney Parameters	Days After Reperfusion Injury									
	0	1	2	3	4	5	6	7	14	
Control										
BUN, mg/dL	68.0 ± 8.0	220.0 ± 10.0	173.3 ± 25.2	133.3 ± 5.8	113.3 ± 15.3	110.0 ± 10.0	100.0 ± 10.0	96.7 ± 5.8	80.0 ± 10.0	
Creatinine, mg/dL	0.8 ± 0.1	2.8 ± 0.2	2.1 ± 0.1	1.8 ± 0.1	1.4 ± 0.4	1.0 ± 0.2	0.9 ± 0.2	0.9 ± 0.1	0.8 ± 0	
Histology grade	4	...	3	2	1.5	
GCSF										
BUN, mg/dL	68.0 ± 8.0	220.0 ± 10.0	163.3 ± 20.6	155.3 ± 30.7	131.7 ± 30.1	111.3 ± 32.1	106.3 ± 30.7	86.3 ± 15.2	54.0 ± 0	
Creatinine, mg/dL	0.8 ± 0.1	2.6 ± 0.4	1.9 ± 0.3	1.5 ± 0.2	1.1 ± 0.1	0.9 ± 0.1	0.8 ± 0.1	0.7 ± 0.1	0.5 ± 0	
Histology grade	4	...	2	1	1	
MSC										
BUN, mg/dL	68.0 ± 8.0	213.0 ± 15.3	180.0 ± 20.0	133.3 ± 15.3	110.0 ± 10.0	80.0 ± 10.0	...	80.0 ± 10.0	50.0 ± 10.0	
Creatinine, mg/dL	0.8 ± 0.1	2.8 ± 0.2	1.9 ± 0.1	1.3 ± 0.1	1.1 ± 0.1	0.7 ± 0.1	0.6 ± 0.1	0.5 ± 0.1	0.5 ± 0	
Histology grade	3	...	2	0	0	

*BUN indicates blood urea nitrogen; GCSF, granulocyte colony stimulating factor; and MSC, mesenchymal stem cell.

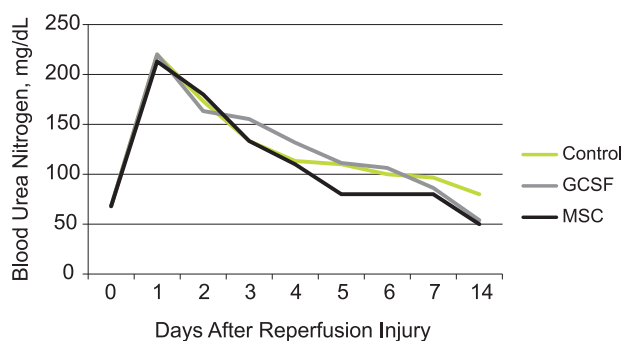


Figure 2. Blood urea nitrogen levels trend after reperfusion injury in the three groups of rats treated with granulocyte colony stimulating factor (GCSF), mesenchymal stem cell (MSC), and nothing (control).

Pathologic Findings

To assess whether the course of recovery from reperfusion injury was compatible with histological changes, we evaluated pathologic grading scores

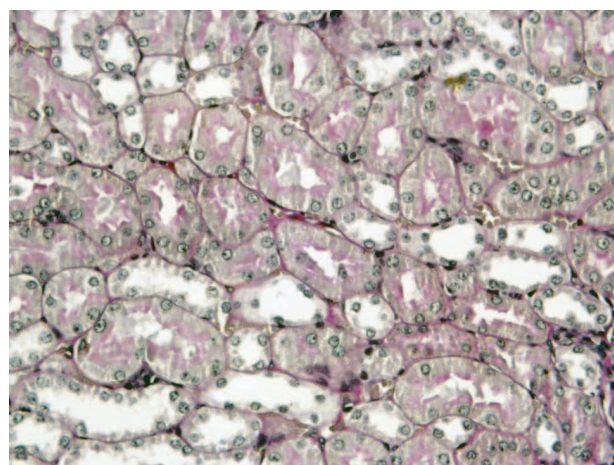


Figure 3. Normal rat kidney, corresponding to grade 0 pathology (periodic acid-Schiff, ×200).

on days 2, 4, 7, and 14 after reperfusion injury in all the three studied groups (Figures 3 and 4). Consistent with the decrease in BUN and serum

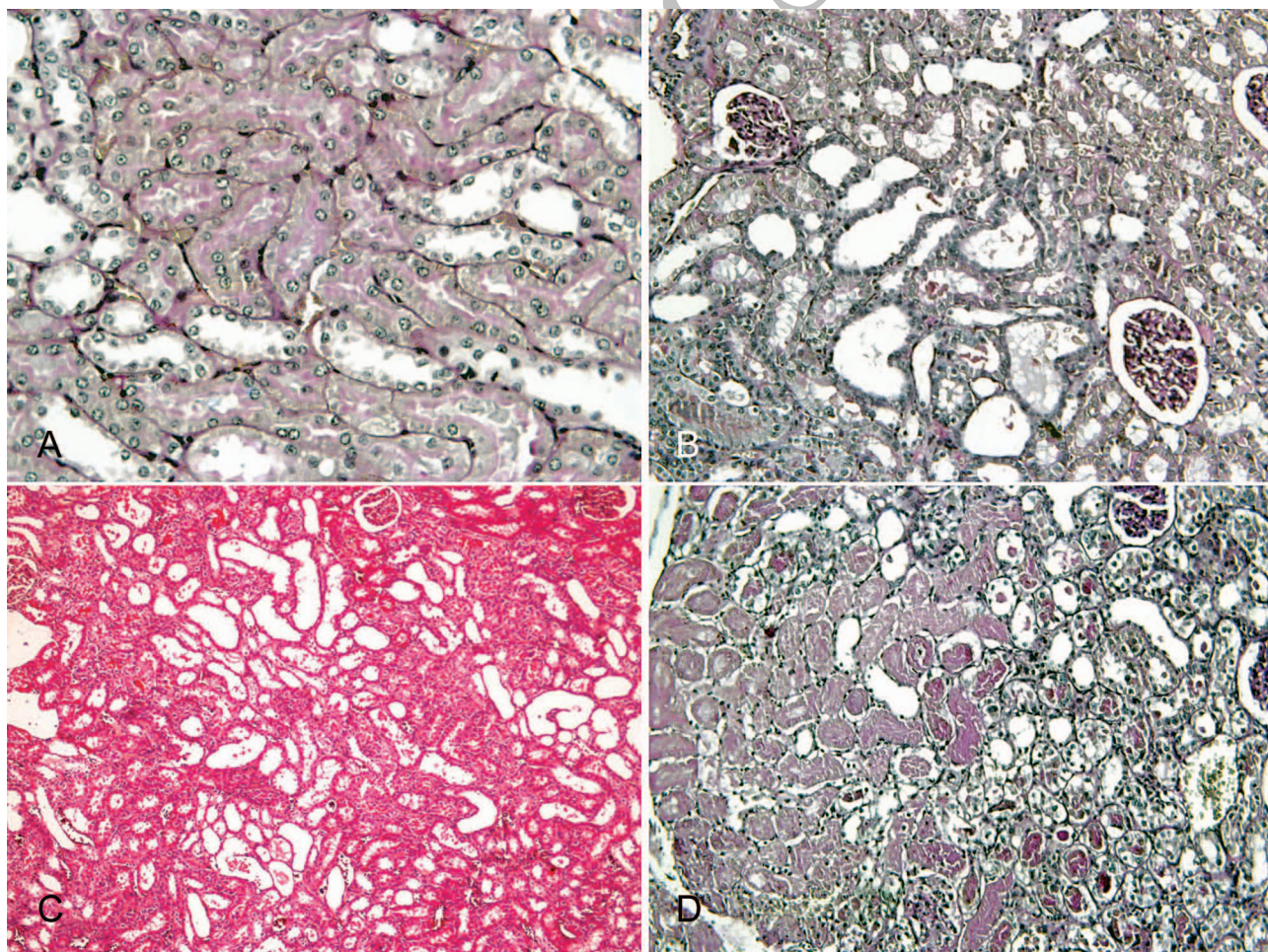


Figure 4. Pathological grading of the rat kidneys after reperfusion injury. **A**, Grade 1 ischemic injury with brush border loss (periodic acid-Schiff, ×200). **B**, Grade 2 injury showing brush border loss and tubular dilatation (periodic acid-Schiff, ×100). **C**, Grade 3 injury with tubular dilatation and necrosis (hematoxylin-eosin, ×100). **D**, Grade 4 injury with widespread necrosis (periodic acid-Schiff, ×100).

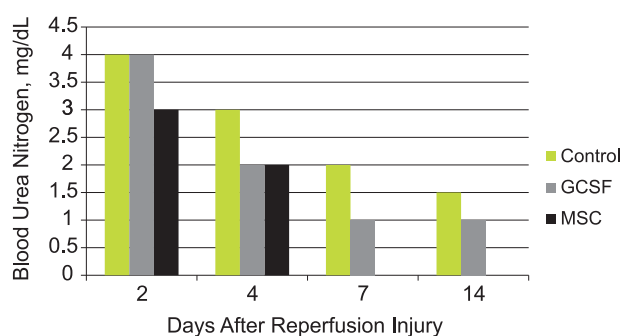


Figure 5. Histological grade of kidney injury after reperfusion injury in the three groups of rats treated with granulocyte colony stimulating factor (GCSF), mesenchymal stem cell (MSC), and nothing (control).

creatinine levels, histological grading scores in all the three studied groups improved by day 14. Rats in the MSC group had normal renal histology on the 7th day after reperfusion injury (Table; Figure 5). There was a significant difference in the histological improvement rate between the MSC and the control groups ($P = .02$) and between the GCSF and the control groups ($P = .04$), which were in favor of their potential therapeutic effects (Figure 5). There was no significant difference in the repair process according to the histological changes between the MSC and GCSF groups ($P = .06$; Figure 5).

CD34+ Cells Count in Peripheral Blood Smear

To evaluate the effect of GCSF in mobilizing bone marrow derived stem cells and to assess any inducing effect of ischemia on bone marrow, we performed CD34+ cells count in peripheral blood specimens on day 7 in the GCSF and control groups by flow cytometry. The number of CD34+ cells in peripheral blood of rats in the GCSF and control groups were 12433.3 ± 1167.6 cells/mL and 14100.0 ± 3214.0 cells/mL, respectively. Both groups showed remarkable peripheral blood count of CD34+ cells, confirmatory to the mobilizing effect of GCSF and inducing effect of ischemia on bone marrow-derived stem cells.

DISCUSSION

In our experimental study, we found administration of MSCs and GCSF can attenuate recovery course of perfusion injury and ameliorate tubular necrosis, as it was determined by evaluating kidney function, and it was evident by improvements in histological grading status. The significant differences between the MSC and control

groups and between the GCSF and control groups represent an augmented potential recovery effect of bone marrow-derived stem cells and related cytokines on repair process. We did not find any significant difference between therapeutic effects of the MSC and GCSF in our study.

It has been debated whether stem cells are playing a direct structural role or inserting paracrine/endocrine effects to augment the recovery rate process. Granulocyte colony stimulating factor, as a cytokine which is induced by stem cells or has an effect to mobilize bone marrow-derived stem cells, is another presumptive involved factor. In a recent study by Semedo and coworkers,²⁴ infusion of bone marrow mononuclear cells 24 hours after reperfusion in mice induced significant decrease in BUN and serum creatinine levels compared to the control group that was without any further intervention after reperfusion injury. In this study, they showed early modulation of inflammation and a better outcome that was induced by bone marrow mononuclear cells following reperfusion injury. It was also found that protective molecules such as interleukin-10, heme oxygenase 1, and bone morphogenetic 7 increased, while some inflammatory marker such as interleukin-6, collagen I, connective tissue growth factor, transforming growth factor- β , and vimentin decreased in mice treated with bone marrow mononuclear cells.²⁴ In a previous study of this research team, they observed administration of MSCs 6 hours after reperfusion attenuated kidney injury, and 24 hours after reperfusion, mice showed decreased serum creatinine and BUN levels compared to nontreated group. They showed an anti-inflammatory state in mice treated with MSCs and indicated an early recovery period of damaged renal tubular cells after the therapy.² In another study by Kipatovskii and coworkers,²⁵ administration of MSCs compared to kidney cells in rats with reperfusion injury showed significant improvement in kidney function parameters and survival rate. Results of these studies are along with our study's results which indicate early recovery period and amelioration in kidney function after therapeutic administration of stem cells.

In a study by Zerbini and colleagues, injection of stem cells could not increase life expectancy in rats. They highlighted the probable effects of concentration of stem cells and route of

administration as variables that might have effects on the course of therapy.²⁶ In this regard, administration of subcutaneous and probably low-dose GCSF may be one of the limiting factors to reach brisk recovery response in our study.

In a study by Fang and associates bone marrow-derived cells induced augmented repair of renal tubular cells after AKI. Furthermore, evidence provided by this study supported the theory that treatment with GCSF as a stem cell mobilizing cytokine might facilitate renal tubular cell regeneration.²¹ Zhang and colleagues showed increased serum and kidney GCSF concentrations following reperfusion injury. Also, they showed that reactive oxygen species can increase GCSF mRNA in vitro and induce production of GCSF protein by outer medullary thick ascending limb cells in the kidney.¹⁶ Kale and coworkers showed the role of reperfusion in mobilization of bone marrow stem cells in mice model. They detected stem cells in peripheral blood by flow cytometry after inducing ischemia, while these cells were undetectable in control groups without ischemic injury, and they showed an induced response by ischemia to compensate the resultant injury.¹²

Considering the above studies along with our study's results, MSCs and GCSF can play a major role in attenuating recovery process after renal ischemic injury. We showed more prominent prognostic results by MSC therapy. Presence of CD34+ cells in peripheral blood of GCSF and control groups suggests the additional therapeutic role of GCSF, meanwhile reminds the probable existence of other mediators which were induced by reperfusion injury and may have potential therapeutic effects.

CONCLUSIONS

Owing to the high morbidity and mortality rates in the context of AKI and disappointing results of available therapeutic approaches, a better understanding of pathophysiologic processes of reperfusion to combat irreversible injury is highly warranted. It is likely that ischemia per se has an important role not only in modifying the extent of injury by mobilizing potential differentiating stem cells, but also by exerting compensatory responses by inducing regulatory, anti-inflammatory, anti-apoptotic, mitogenic, and angiogenic factors. In these days of molecular biology and proteomics,

further research to elucidate these pathways and to identify major responsible mediators will lead us to novel therapeutic approaches and to overcome the challenge.

CONFLICT OF INTEREST

None declared.

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