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Use of Human Amniotic Fluid-Derived Mesenchymal Stem Cells In Treatment Of Cisplatin-Induced Renal Injury In Sprague-Dawley Rats

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Background And Objectives:*Cisplatin is a nephrotoxic chemotherapeutic agent. So, preventive measures worth to be evaluated. Human amniotic fluid stem cells (hAFSCs) in prevention or amelioration of cisplatin-induced acute kidney injury (AKI) in Sprague-Dawley rates have been tested.*

Methods:*80 Sprague-Dawley rats (250∼300g) were used and divided into 4 major groups, 20 rats on eachgroup. Group I:Cisplatin-injected group (7mg/kg I.P). Group II: Cisplatin-injected and hAFSCs-treated group (2×10^6 hAFSCs).Group III: Cisplatin-injected and DMEM culture media treated group). Group IV: Saline-injected group. One day after cisplatin administration). Each major group was further divided into 4 equal subgroups according to the timing of sacrifice; 4, 7, 11 and 30 days post-cisplatin injection. Renal function tests were done. Kidney tissue homogenate oxidative stress parameters malondialdehyde (MDA), superoxide dismutase (SOD) and glutathione (GSH) were determined.*

Results:*hAFSCs characterization was proved. Cisplatin injection resulted in a significant increase in serum creatinine and MDA and decrease in SOD, GSH and creatinine clearance. These changes were attenuated early by day 4 with the use of hAFSCs. Cisplatin injection induced tubular necrosis, atrophy, inflammatory cells infiltration and fibrosis. The use of hAFSCs was associated with significantly lowered injury score at day 4, 7, 11 and 30 with marked regenerative changes starting from day 4.*

Conclusion:*hAFSCs have both a protective and regenerative activities largely through an antioxidant activity. This activity cut short the acuteness of cisplatin nephrotoxicity.*

Keywords:*Cisplatin, nephrotoxicity, Human amniotic fluid Stem cells, Oxidative stress*

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I. Introduction

Acute kidney injury (AKI) is a serious clinical condition associated with high morbidity and mortality. It refers to a clinical syndrome characterized by a rapid decrease in renal excretory function, with the accumulation of nitrogenous end-products such as creatinine and urea (1). Drugs seem to contribute to AKI in about 20% of patients, especially in critically ill patients (2). Cisplatin is a potent chemotherapeutic agent with evident nephrotoxicity. Several mechanisms have been proposed for cisplatin-induced nephrotoxicity including direct toxicity to renal tubular epithelial cells (3), apoptosis (4), activation of the mitogen-activated protein kinase (MAPK)-signaling pathway (5), oxidative stress (6), and inflammation (7).

Based on these different mechanisms, numerous renoprotective approaches have been developed; however, the offered renoprotection is mostly partial and combination strategies may be better (8). Stem cell-based therapy is a new promising therapeutic approach for organ repair (9). Several groups successfully demonstrated the use of different stem cell types in the treatment of AKI in different experimental animal models. Most of these studies focused on amniotic fluid-derived stem cells (AFMSCs) and in vitro expanded MSCs (10).

Human amniotic fluid is a new promising source of stem cell (hAFSCs) with high plasticity and their subsequent differentiation into the three types of germ layer cells (11). In this context, the use of hAFSCs is considered ethically acceptable compared to embryonic stem cells as it is easily available without destruction of human embryo (12).

The novelty of this study originates from the long term 30 days follow up of the cisplatin-induced renal oxidative stress studying the probable antioxidant activity of hAFSCs. In addition, approve that amniotic fluid stem cell can repair kidney injury caused by toxicity of cisplatin.

II. Aim Of The Work:

The aim of this research is to isolated and in vitro characterized the amniotic fluid mesenchymal stem cells.

After that we would test in vivo, the hypothesis that the treatment with mesenchymal stem cells of amniotic fluid origin could improve renal function and attenuate tubular injury in Kidney disease induced in rat by injection of cisplatin.

III. Materials and Methods

The study design and protocol was revised and approved by Mansoura Medical research, faculty of medicine,Mansoura University.

Agents:

Cisplatin obtained from David Bull Laboratories, Dulbecco's modified Eagle's medium (DMEM) containing 20% fetal bovine serum (FBS) obtained from Invitrogen (Invitrogen, Carlsbad, CA, USA).

Harvest and Culture of human amniotic fluid stem cells (hAFSCs):

Mesenchymal stem cells were isolated from the human amniotic fluid (AF) of 15 women who underwent a cesarean delivery for breech presentation after having their consent according to the instruction of ethical committee of the faculty of medicine of Mansoura University.

The mean±SD pregnancy duration (fetal age **+** 2 weeks) was 38±1 weeks and the mean volume of the AF samples was 11.2±4.7 mL.

Samples were collected by puncturing the membranes after the uterine muscle was opened for the cesarean delivery. Cells were isolated from the AF samples no more than 4 hours prior to use. Samples were centrifuged at 1100 rpm for 5 minutes and all the isolated cells were plated in six 35-mm Petri dishes containing low-glucose Dubelco modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 100 U/mL of penicillin, 0.1 mg/mL of streptomycin, 10 ng/mL of basic fibroblast growth factor, 10 ng/mL of epidermal growth fac- tor (all from Peprotech, Rocky Hill, NJ, USA), and 20% of fetal bovine serum (Invitrogen). The medium was re- newed after incubation of the cells at 37oC with 5% humidified carbon dioxide for 7 days and the non-adhering AF cells were removed.

The medium was replaced twice weekly until the cells reached 70% confluence, then they were treated with 0.25% trypsin and 1 mM EDTA (Invitrogen) for 3 minutes. The released MSCs were collected and replated in a split ratio of 1:3 under the same culture conditions. Cells were used for experiments after the 3rd passage.

In Vitro Assessment of Stem Cells.

Viability Test:

The viability of stem cells was checked by trypan blue exclusion according to the method of Fibroblast-Like Colony-Forming Unit Assay

Fibroblast like colony growth was evaluated on primary cells grown on tissue culture six-well dishes. Total stem cells were plated at the density of 25×10^6 cells/well. After 7 d, the capability of MSC to form fibroblast-like colonies was assessed. Images that showed MSC morphology were acquired by contrast-phase microscope.

Flow cytometry for cell surface expression assay

The AF-derived stem cells at passage 3 were released by trypsinization and analyzed by fluorescence-activated cell sorting (FACS) analysis. The cells were centrifuged at 1200 rpm for 5 minutes, and then solved in phosphate buffered saline (PBS) at the concentration of (1×106/ml).

The cells were stained with different fluorescently labeled monoclonal antibodies (mAb) according to Perin et al. The fluorescent labeled directed antibodies CD34, CD14,CD29, CD90, CD13, CD105 and Oct4 (10µl for eachsample) were added and incubated for 30 minutes at room temperature. Labeled cells were thoroughly washed with two volumes of PBS and fixed in flow buffer (1% form- aldehyde in PBS). The labeled cells were analyzed on a FACS Caliber (Becton-Dickinson, Franklin Lakes, NJ, USA) by collecting 10000 events with the Cell Quest soft- ware program (Becton-Dickinson, Franklin Lakes, NJ, USA) (13).

Animals and Experimental Design

Rats were kept on a regular 12 h dark/light cycle with free access to standard rat chow and tap water ad libitum. Eighty inbred female Sprague-Dawley rats (8 weeks old, weighing 180 to 210 g) from Medical Experimental Research Center (MERC), Mansoura Faculty of Medicine were used and divided into the following groups:

Group I (n=20): (cisplatin 'CDDP'-treated group) was injected with a single dose of cisplatin (7mg/kg I.P) in 1 ml saline.

Group II (n=20): (cisplatin and human amniotic fluid stem cells-treated group) this group was injected with 0.5 ml of culture media containing 5×106 MSCs into the tail vein.

Group III (n=20): (cisplatin and DMEM culture media-treated group) this group was injected with 0.5 ml culture media into the tail vein.

Group VI (n=20): (negative control) was injected with 1ml normal saline alone (I.P). On day one after kidney disease induction, groups II received stem cell suspended in fresh culture media; while, group III injected with fresh culture media alone. Five rats were killed at different time intervals in all groups at days 4, 7, 11, and 30 for obtaining kidney tissue and blood samples.

Biochemical Measurements

The following parameters were determined with the use of commercially available kits: serum and urinary creatinine (Diamond diagnostics, Jaffe. Colorimetric – kinetic,Hannover, Germany) and blood urea nitrogen (BUN) using urea kits, Berthelot enzymatic colorimetric method (Diamond Diagnostics Company, Hannover, Germany) according to manufacture instruction.

Lipid peroxidation (malondialdehyde 'MDA' production) and reduced glutathione (GSH) contents and superoxide dismutase activity (SOD) in the kidney tissue were determined.

Statistical analysis

All analyses were conducted using SPSS (version 16.0, SPSS, IL, USA). The biochemical data were tested for Gaussian distributions by Kolmogorov-Smirnov test. For the survival rate, the significance was computed with a log-rank test. Descriptive statistics were reported as mean±standard deviation (SD) for continuous variablesor median (min–max) for categorical variables. Differences in continuous variables were analyzed by one-way analysis of variance (ANOVA) followed by posthoc multiple comparisons (Scheffé test). Categorical variables were analyzed by Kruskal-Wallis H (K-W) followed by MannWhitney's tests. p value<0.05 was considered statistically significant at confidence interval 95%.

IV. Results

After injection of cisplatin in tail vein of rats, 5 rats will be killed at different time intervals in all groups at days 4 7 11 and 30 for obtaining kidney tissue and blood sample.

Effects of A.FMSCs on the oxidative stress caused by cisplatin:

Injection of cisplatin resulted in elevation of tissue levels of MDA and reduction of tissue levels of SOD and GSH. These changes were most evident by the 4th day. Treatment with MSCs resulted in amelioration of such changes in these parameters. The effect of stem cells on GSH outweighed that of stem cells at the 7th day. The effect of stem cells on GSH was slightly higher by the day 30.

Isolation, purification and Characterization of hAFSCs

hAFSCs were characterized on the basis of their phenotype, adherence property, and their differentiating capabilities. Cells were purified in culture media with different passages and MSCs were morphologically defined by a fibroblast-like appearance. Using appropriate differentiation media, we were able to differentiate hAFSCs in to osteocytes, adipocytes, and chondrocytes.

Flow cytometry for cell surface expression assay

The analyses revealed that the expression of surface antigens of MSCs derived from human amniotic fluid were negative for CD14 (11.3%) and CD34 (15%) while they were positive for CD29 (50%), CD90 (79.1%), CD105 (79.5%), CD13 (36.5%) and Oct4 (31.4%).

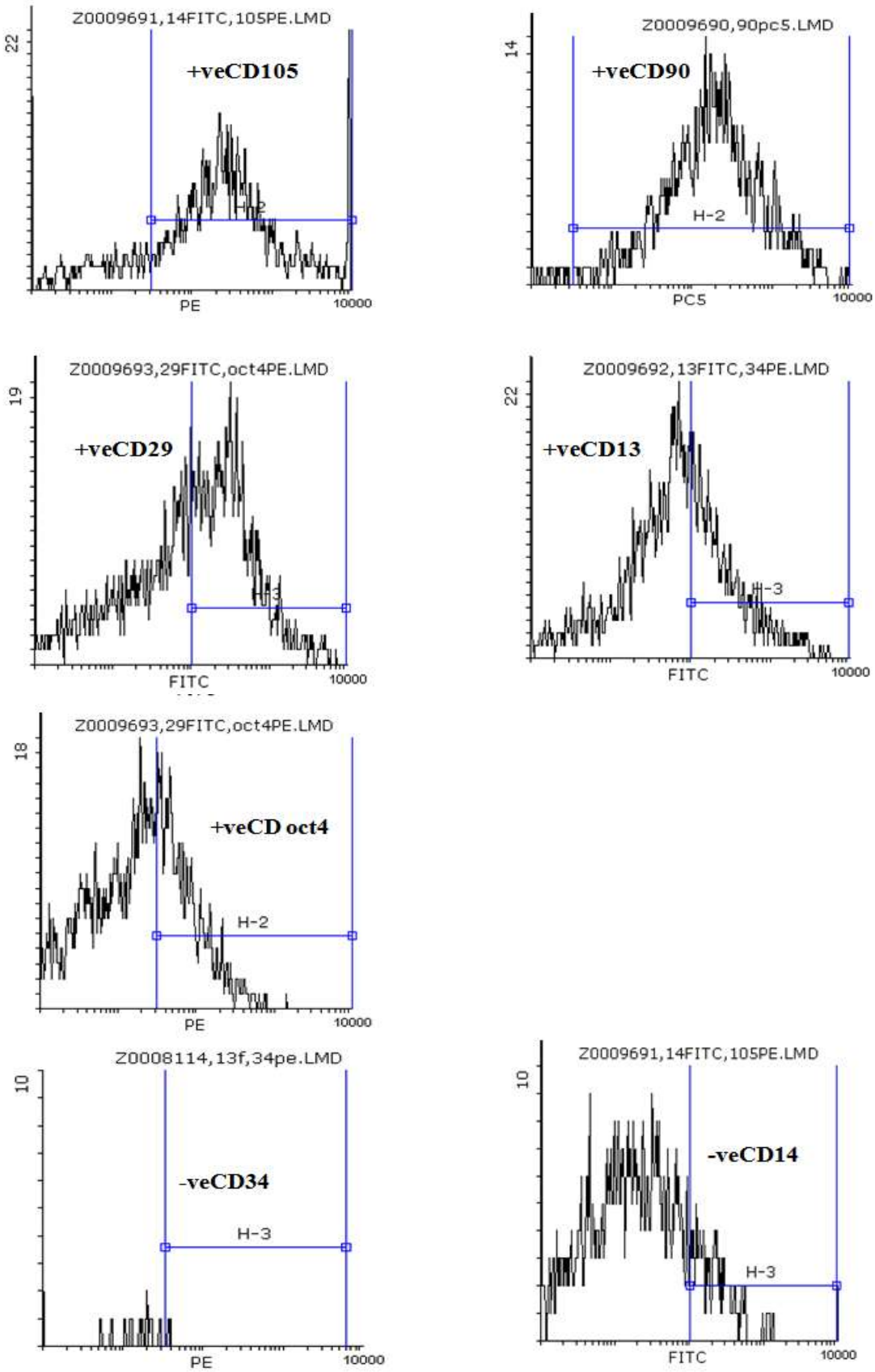


Figure1: Flow cytometry analysis of human amniotic fluid mesenchymal stem cells showingthe expression of cell surface antigens CD 105, CD 90, CD29, CD13 and Oct4. (Passage 4:8) are strongly positive; while CD34 and CD14 are negative.

Effect of hAFSCs on cisplatin-induced renal dysfunction

Sprague-Dawley rats injected with cisplatin developed impairment of renal function characterized by high serum levels of creatinine and BUN starting at 4th days post-injection and maintained elevated until the end of the study (Table1). Injection of hAFS cell lead to significant decrease ($p < 0.001$) of serum creatinine and BUN levels at 4th day compared with cisplatin- and DMEM culture mediatreated rats (Table 1).

Effect of hAFSCs on cisplatin-induced oxidative stress

Injection of hAFS cell lead to significant decrease ($p < 0.001$) of renal tissue MDA level with significant increase of renal tissue GSH and SOD levels starting from the 4th day till the end of the study compared with cisplatin- and DMEM culture media-treated rats (Table 2).

Table 1. Effect ofhAFSCs on biochemical measurements (n=20/group)

		GI	GII	GIII	GIV
Sr.Cr.	Day 4	1.92 ±0.04	1. ±0.09	1.89 ±0.12 *,§	0.47±0.11 $^{*,\#}$
(mg/dl)	Day 7	1.61±0.06	0.87 ±0.08	1.62 ±0.04 *,§,‡	0.48±0.13 $^{*,\#,\ddagger}$
	Day 11	0.97 ±0.14	0.72±0.06	0.98 ±0.18 *,§,‡	0.48±0.18 $^{*,\#,\ddagger,\dagger}$
	Day 30	0.76 ±0.04	0.58 ±0.08 *,‡†	0.79 ±0.05 *,§,‡	0.43±0.11 $^{*,\#,\ddagger,\dagger,\bullet}$
BUN	Day 4	266.7±8.7 4	98.2 ±4. 32 *	265.3±8.6 *,§	54.36 ±3.31 $^{*,\#}$
(mg/dl)	Day 7	228,7±18	77.3 ±4.53 *,‡	236±15 *,§,‡	53.39 ±4.51 $^{*,\#,\ddagger}$
	Day 11	137.4±14.7	69.2 ±4.65 *,‡,†	138.6±16.3 *,§,‡,†	54.43±3.91 $^{*,\#,\ddagger,\dagger}$
	Day 30	111.46±11.5.58	62.23 ±3.87 $^{*,\ddagger,\dagger,\bullet}$	112.58 ±8.4 *,§,‡	54.55±3.62 $^{*,\#,\ddagger,\dagger,\bullet}$
Cr. Cl.	Day 4	0.007±0.000	0.019±0.008 *	0.008±0.0023 *	1.6±0.86 *,‡
(ml/min/	Day 7	0.014±0.0012	0.048±0.013 *,‡	0.012±0.003 *,§,‡	1.6±0.76 $^{*,\#,\ddagger}$
100 gm)	Day 11	0.054±0.06	0.64±0.115 *,‡,†	0.056±0.02 *,‡,†	1.6±0.79 *,‡,†
	Day 30	0.34±0.24	0.89±0.232 $^{*,\ddagger,\dagger,\bullet}$	0.36±0.32 $^{*,\S,\ddagger,\dagger,\bullet}$	1.6±0.35 $^{*,\ddagger,\dagger,\bullet}$

Significant difference compared to corresponding *control, §cisplatin group and $^{\#}$cisplatin+hAFSCs group.

Significant difference compared to intragroup ‡day 4, †day 7, $^{\bullet}$day 11 by one-way analysis of variance (ANOVA) followed by *posthoc* multiple comparisons (Scheffé test) at $p \leq 0.05$.

Biochemical Study:

The data presented in fig(2) shows the statistical analysis and mean value of Serum Cr (mg/dl) biochemical measurements in cisplatin injected (GI), cisplatin injected and transplanted through tail vein with mesenchymal stem cells derived from human amniotic fluid (GII), cultured media (GIII) and normal control groups injected with saline (GIV).

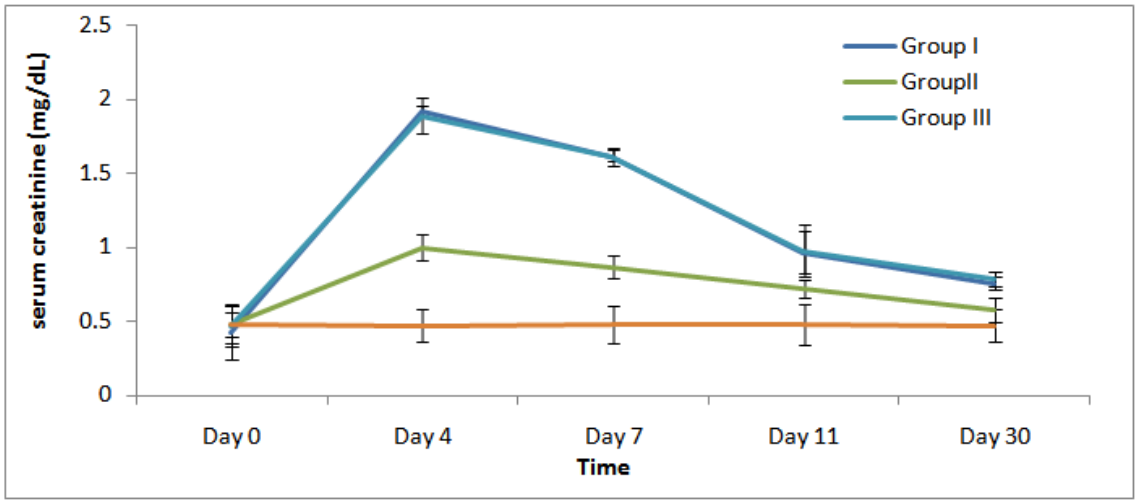
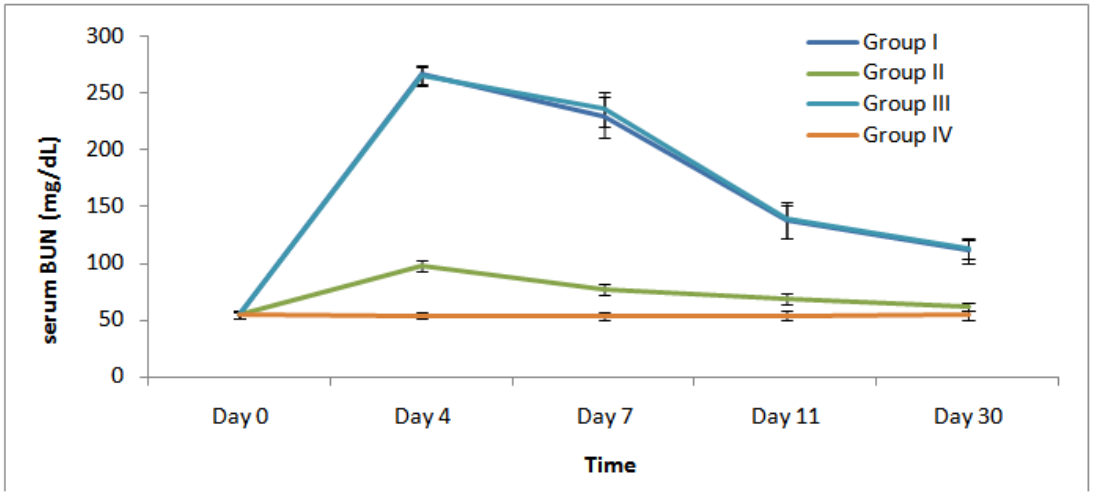


***Fig2:* Shows the biochemical measurements of serum Cr (mg/dl) for all performed experimental groups at differenttimes intervals**

From table and results showed that when injection of cisplatin in rats caused acute kidney injury of rats and then examine sample of blood and urine showed that increase in serum creatinine and when administrate of Af stem cell as adrug of repair, the kidney function were improved as showed in tables.



Fig(3) shows the statistical analysis and mean value of serum BUN (mg/dl) biochemical measurements in cisplatin injected (GI), cisplatin injected and transplanted through tail vein with mesenchymal stem cells derived from human amniotic fluid (GII), cultured media (GIII) and normal control groups injected with saline (GIV).

Table 2. Effect ofhAFSCs on renal tissue oxidative stress parameters (n=20/group)

		GI	GII	GIII	GIV
MDA	Day 4	67.4±3.4	35.3 ±4.25 *	69.5 ±4.8 *,§	14.5±3.43 $^{*,\#}$
(nmol/g tissue)	Day 7	65.1±4.54	25.9±4.34 *,‡	63.2 ±3.87 *,§,‡	14.5 ±3.89 $^{*,\#}$
	Day 11	34.1±4.7	17.6 ±3.54 *,‡,†	37.2 ±4.6 §,‡,†	14.6 ±2.56 $^{*,\#,\ddagger,\dagger}$
	Day 30	32.8±5.1	16.7±4.45 *,‡,†	33.2±5.4 §,‡,†	14.5±2.46 $^{*,\#,\ddagger,\dagger,\bullet}$
GSH	Day 4	0.264±0.028	0.78 ±0.19	0.269±0.045	5.36± 0.17
(mmol/g tissue)	Day 7	0.53±0.041	2.732 ±0.22	0.56±0.052	5.38±1.15
	Day 11	1.38±0.42	3.63±0.24 *,‡,†	1.35±0.35 *,§,‡,†	5.35±0.18 $^{*,\#,\ddagger,\dagger}$
	Day 30	1.92±0.04	4.32±0.21 *,‡,†	1.95±0.08 *,§,‡,†	5.39 ±0.35 $^{*,\#,\ddagger,\dagger,\bullet}$
SOD	Day 4	3.25±0.64	6.74±0.54 *	3.70±0.65 *,§	20.86±1.24 $^{*,\#}$
(U/g tissue)	Day 7	6.24±0.57	10.24±0.43 *,‡	6.46±0.62 *,§,‡	20.82±1.64 $^{*,\#,\ddagger}$
	Day 11	9.96±0.30	15.45±0.53 *,‡,†	9.94±0.52 *,§,‡,†	20.78±1.28 $^{*,\#,\ddagger,\dagger}$
	Day 30	15.24±0.46	18.54±0.54 $^{*,\ddagger,\dagger,\bullet}$	15.43±0.52 $^{\S,\ddagger,\dagger,\bullet}$	20.73±0.84 $^{*,\#,\ddagger,\dagger,\bullet}$

Significant difference compared to corresponding *control, §cisplatin group and $^{\#}$cisplatin + hAFSCs group.

Significant difference compared to intragroup ‡day 4, †day 7, $^{\bullet}$day 11 by one-way analysis of variance (ANOVA) followed by *posthoc*
multiple comparisons (Scheffé test) at $p \leq 0.05$

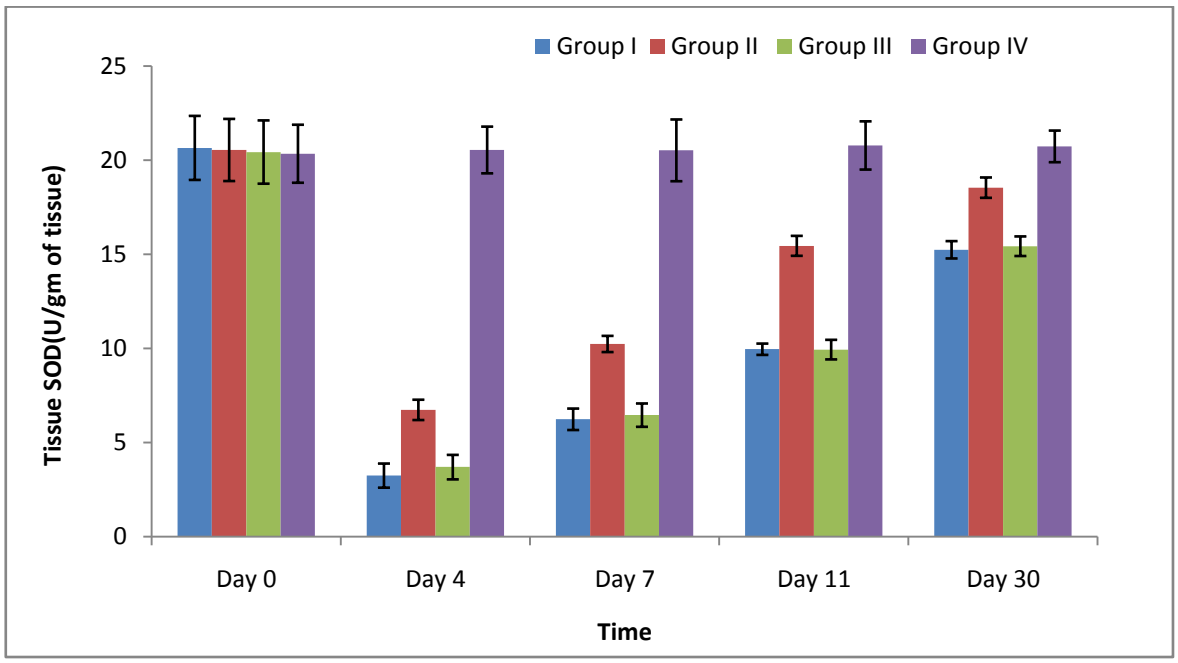


Figure (4): Renal superoxide dismutase activity (U/g of tissue) in different groups at different time intervals. A highly significant ($p<0.001$) increase in the mean value of renal GSH content was demonstrated in groups II & IV at days 4, 7, 11 and 30 when compared to groups I & V (Figure 4)

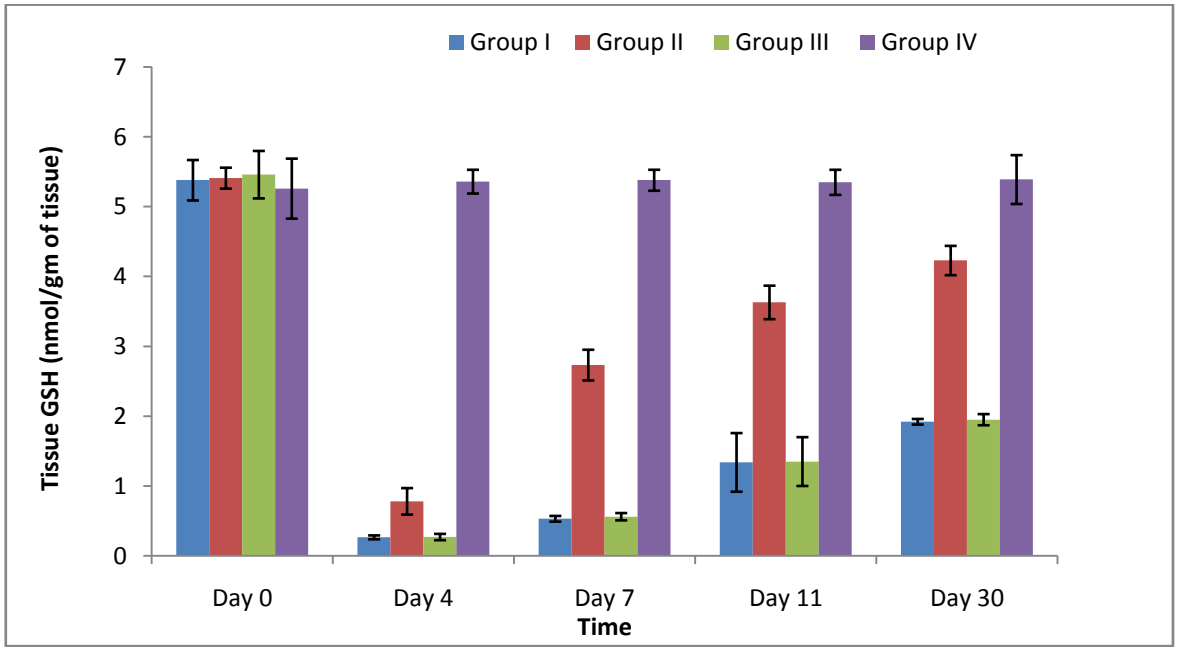


Figure (5): Show the content of renal reduced glutathione (nmol/g of tissue) in different groups at different time intervals

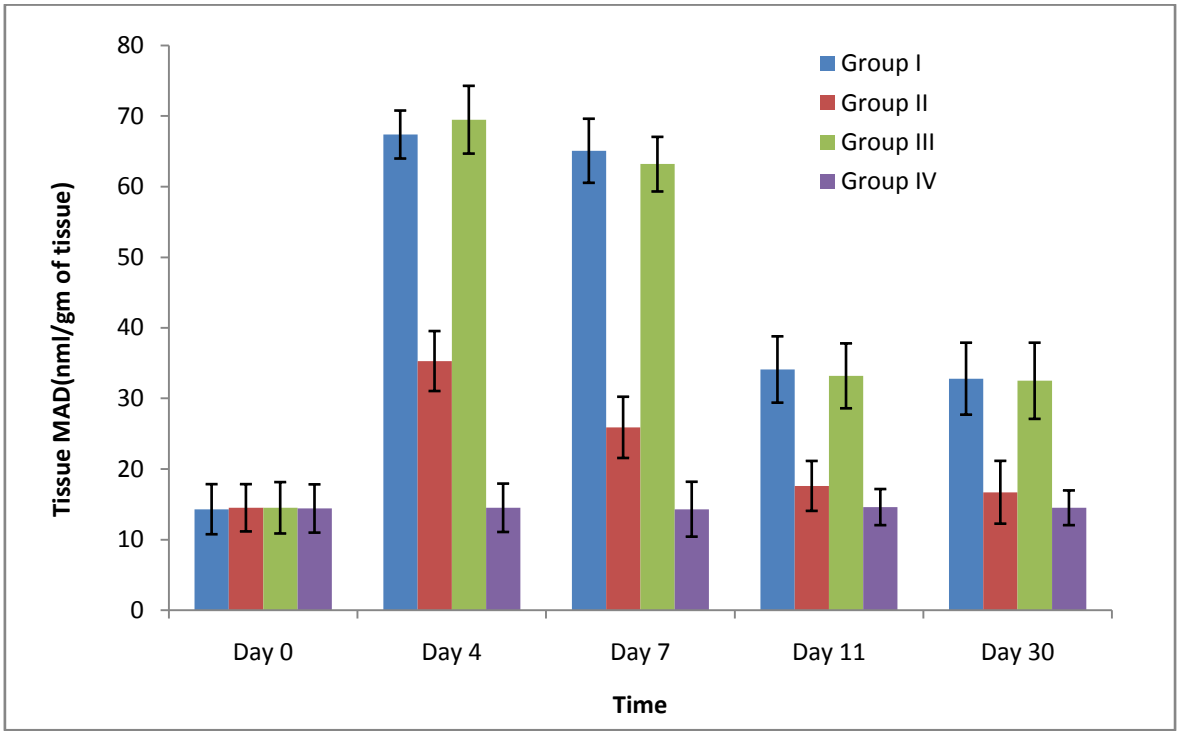


Figure (6): Show the content of MDA (nmol/g of tissue) in different groups at different time intervals

Increased level of MDA was observed in cisplatin injected rats group I when compared to non-injected group IV at days 4, 7, 11 and 30. A significant decrease in tissue MDA was noted after intravenous injection of 5×105 MSC obtained from human amniotic fluid (group II) into syngeneic S.D rats on day 1 after receivingcisplatin at days 4, 7, 11 and 30 when compared with cisplatin injected rats treated with culture media via tail vein group III (Figur6).

V. Discussion

The use of stem cells has emerged in the past few years as an effective strategy to reduce AKI in small animal models (27). Recovery of the kidneys from AKI after treatment with MSCs was attributed to the ability of MSCs to secret growth factors and cytokines that enhance recovery and reduce tissue damage (26, 28).

As in many kidney diseases, the pathogenesis of cisplatin-induced AKI is complex and involves both local events in the kidney as well as recruitment of the circulating inflammatory cells (29). The complexity of cisplatin-induced AKI may explain why a single pharmacological agent can- not provide complete protection against cisplatin nephrotoxicity (29).

The present study demonstrated that stem cells isolated from human amniotic fluid accelerate the recovery of experimental cisplatin-induced AKI. Treatment with hAFSCs significantly improved renal function, reduced oxidative stress, and increased the regenerative capacity of the kidney.

Several studies investigated the potential role of stem cells in repair of AKI. Most of these used amniotic fluid-derived stem cells (A.FMSCs) and/or in vitro expanded MSCs that were applied to different experimental models (10). De Coppi and associates (20) isolated and characterized amniotic fluid stem cells with potential to differentiate into various cell lineages. Since then, several studies provided evidence concerning their multipotency and therapeutic potential in animal models.

In the present work, hAFSCs injected through tail vein one day after cisplatin lead to biochemical and structural improvement of AKI. These results are in accordance to Perin et al. (21) who demonstrated the efficacy of injected hAFS cells in preventing renal damage in nu/nu mice with cisplatin-induced AKI; however, in such study stem cells were given before the onset of renal damage. In addition, it was documented that in NOD/SCID mice with established cisplatin-induced AKI, hAFS cell treatment ameliorated tubular damage, limited renal function impairment, and prolonged animal lifespan (22).

In the present study, cisplatin and culture-media injected groups showed significant increase of renal tissue MDA level with significant decrease of GSH and SOD levels. These results are in agreement with previous studies that showed renal toxicity of cisplatin manifests as increased renal oxidative damage (23, 24). Injection of hAFSCs limited cisplatin-induced renal oxidative damage. Studies about the antioxidant action of stem cells are few; however, it was reported that adipose-derived stem cells exerted antioxidant action and

protected human dermal fibroblasts from oxidant damage (25).

The novelty of this study originates through combined study of the acute injury oxidative stress parameters. In addition; long term 30 days follow up permitted evaluation of the activity of hAFSCs in limiting the consequent fibrosis on top of cutting short the acuteness of cisplatin-induced acute nephrotoxicity.

The use of stem cells has emerged in the past few years as an effective strategy to reduce AKI in small animal models (2). Recovery of the kidneys from AKI after treatment with MSCs was attributed to the ability of MSCs to secret growth factors and cytokines that enhance recovery and reduce tissue damage (1, 6).

As in many kidney diseases, the pathogenesis of cisplatin-induced AKI is complex and involves both local events in the kidney as well as recruitment of the circulating inflammatory cells (21). The complexity of cisplatin-induced AKI may explain why a single pharmacological agent can not provide complete protection against cisplatin nephrotoxicity (21).

Actually hAFSCs are promising target in regenerative medicine as they carry specific advantages in comparison to other stem cell types. First; hAFSCs are easy to be obtained from the amniotic fluid without ethical concerns. Second; hAFSCs cells are less liable to form tumors in vivo as they are genomically stable. Third; hAFSCs obtained during the first trimester can be reprogrammed efficiently with maintenance of pluripotency, only with the employment of a valproic acid (11).

In conclusion, hAFSCs may provide an alternative promising source of stem cells for the prevention and treatment of cisplatin-induced AKI. hAFSCs single dose early in cisplatin induced acute nephrotoxicity cut short the acuteness of renal injury with consequent reduction of the developing renal fibrosis. Boaster dose of hAFSCs may have synergistic ant fibrotic activity.

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Potential conflict of interest

The authors have no conflicting financial interest.

Supplementary Materials

Supplementary data including six figure can be found with this article.

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