

Original Article**Role of Mesenchymal Stem Cell in Identification of Hemotoxic Effect of sFas in cases of Nephrotoxicity**

Alshaimaa Mahmoud Sami Ali¹, Mahmoud Abd El Ghani Nomier¹, Hoda Rajab El Said¹, Anthony Atala²

Forensic Medicine And Clinical Toxicology Department, Faculty Of Medicine Zagazig University¹, Wake Forest Institute For regenerative medicine ,Faculty of Medicine-Wake Forest University- USA²

**ABSTRACT**

Nephrotoxic nephritis is a clinical condition with a high mortality rate. People with nephritis are susceptible to anemia. This may be due to the presence of sFas. Mesenchymal stem cells (MSCs) are multipotent stem cell population that mostly resides in the umbilical cord. MSCs hold therapeutic potential in repairing tubular injury. The aim of the present study was to investigate role of BM-derived MSCs cell therapy on mercuric chloride experimentally induced nephrotoxicity and their effect on sFas level in an in vivo albino rat. 90 adult female albino rats were divided equally in to 3 groups. Group I -ve control group, Group II received an intramuscular injection of Mercuric chloride for 2 weeks (5 days/ week) (1 mg/kg bw). Group III received an intramuscular injection of Mercuric chloride for 2 weeks (5 days/ week) (1 mg/kg bw). After establishment of kidney function tests (KFT), single dose of labeled MSCs with GFP (Green Fluorescent Protein) injected in the tail vein at a dose of labeled MSCs 1×10^6 cells. After 2 weeks from 24 hours after last mercuric chloride dose, and 3 weeks from labeled MSCs, 15 rats from each group were suspected to blood samples collection from infraorbital. Rats from each group were suspected for biochemical estimation of kidney function tests (KFT); serum creatinine, Blood Urea Nitrogen (BUN) and complete blood count (CBC) then rats were sacrificed. Kidney were extracted and subjected to histopathologic examination by H&E and immunohistochemical examination by PCNA and sFas expression. MSCs suppressed serum urea and creatinine levels in HgCl₂-treated rats with increased tissue regeneration and inhibition of tubular cell apoptosis, with decreased expression of PCNA and sFas and increased of the RBCs, Hct and Hb levels compared to the mercuric chloride group reflecting the improvement of anemia, indicating a protective therapeutic action in the kidney.

Keywords: sFas, Mesenchymel Stem Cell, Nephrotoxicity, Hemotoxicity, Anemia

INTRODUCTION

The kidney is an essential organ; it regulates the extracellular environment, like detoxification, and excretion of toxic metabolites and drugs. Nephrotoxicity is which excretion does not go smoothly due to toxic chemicals or drugs. About 20% of nephrotoxicity is caused by drugs. Moreover the elderly medication increases the incidence of nephrotoxicity up to 66%.

Drug-induced nephrotoxicity is accompanied with acute or chronic renal injury (Ferguson et al., 2008 & Rached et al., 2008).

Nephrotoxicity can be diagnosed through a simple blood test. It includes the measurements of blood urea nitrogen (BUN), concentration of serum creatinine. However, these assessments of nephrotoxicity are only possible when a

majority of kidney function is damaged (Kirtane et al., 2005).

Anemia and nephrotoxicity are major health issues due to the associated morbidity and mortality. Kidney received the highest need of cardiac out-put among the body in relation to organ weight. The anemia directly decreases the oxygen delivery. Epo is produced by peritubular cells of the kidney which is an essential growth factor for erythropoiesis (Seung et al., 2015).

The renal hypoxia is the main stimulus for erythropoietin synthesis. As kidney disease progresses, there are fewer cells producing erythropoietin within the kidneys. The hematopoietic growth factor erythropoietin controls erythropoiesis in bone marrow (Fishbane & Barry, 2008).

The main cause of anemia in nephrotoxicity is the lack of erythropoietin (Maiese et al., 2008) Moreover, there are several uremic toxins that suppress erythropoiesis in the bone marrow. Several experimental and clinical studies have associated with the cytokines effect on decreased Epo synthesis and bone marrow responsiveness. These cytokines inhibit erythropoiesis not only in vitro but also in vivo, through inhibiting erythroid colony-forming units (Bonomini & Sirolli, 2003).

Erythropoietin binds to its receptor present on the surface of erythroid progenitor cells. It has the greatest effect on colony-forming unit-erythron (CFU-E) cells. These cells contain the largest number of erythropoietin receptors (Fisher 2003).

Experimental evidence supports a role for cytokines as TNF/TNFR (TNF receptor) and Fas ligand (FasL)/Fas in renal injury. Fas is a type 1 membrane protein that belongs to the tumor necrosis factor, which activates the intracellular signaling after binding of Fas-ligand (FasL). Fas ligation leads to a series of intracellular signaling events, culminating in activation of the death-inducing signaling complexes, which

promote the activation of caspase-3-mediated apoptosis (Paunel et al., 2011).

The Successful derivation of human pluripotent stem cells is promising not only for use in the treatment of patients suffering from cell or tissue damage but also for drug discovery and the exploration of human developmental biology (Takahashi et al 2007).

The Umbilical Cord Blood is a source of hematopoietic stem cells (HSCs). MSCs play an essential role in providing the significant microenvironment for hematopoiesis. MSCs secrete many growth factors that enhance hematopoiesis. MSCs prepare a scaffold for hematopoiesis. They protect primitive progenitor cells. Also MSCs expand and maintain HSCs. Therefore, the UC can be considered as an alternative source of MSCs for clinical applications (Sara et al., 2017). **The aim of the present study was** to investigate role of BM-derived MSCs cell therapy on mercuric chloride experimentally induced nephrotoxicity and their effect on sFas level in an in vivo albino rat animal model.

MATERIALS AND METHOD

Study design:

This study was carried out on 90 adult female albino rats that were purchased from the animal

breeding house of Faculty of Medicine Zagazig university . Rats' weighed 150-200 gm (according to IRB instructions). They were housed in a temperature-Controlled and light-controlled room (12-h light/dark cycle), with free access to food and water. Before starting the experiment, all animals were left for 7 days of passive preliminaries for house acclimatization to ascertain their physical wellbeing and to exclude any diseased animal. Rats were divided equally and randomly in to 3 groups.

Group I (-ve control group): Rats were left without intervention, received only

regular diet and tap water, to measure the basic parameters.

Group II (mercuric chloride +ve control group): Rats were received an intramuscular injection of Mercuric chloride for 2 weeks (5 days/ week) (1 mg/kg body weight) (Sarkar et al., 2007). This dose is responsible for the induction of nephrotoxicity.

Group III (MSCs treated group): Rats were received an intramuscular injection of Mercuric chloride for 2 weeks (5 days/ week) (1 mg/kg body weight). After establishment of nephrotoxicity by kidney function test (KFT), single dose of labeled MSCs with GFP (Green Fluorescent Protein) injected in the tail vein at a dose of labeled MSCs 1×10^6 cells per rat suspended in 0.5 ml phosphate-buffered saline (Mohamed et al., 2014).

After 2 weeks from 24 hours after last mercuric chloride dose injection, 15 rats from each group were suspected to Blood samples collection from infraorbital. Rats from each animal group were suspected for biochemical estimation of kidney function test (KFT); serum creatinine, Blood Urea Nitrogen (BUN) and complete blood count (CBC) then rats were sacrificed. Kidney were extracted and subjected to histopathologic examination by H&E and immunohistochemical examination by PCNA. Moreover sFas expression from kidney tissue is detected by immunohistochemical study.

After 3 weeks from labeled MSCs injection. The remaining rats were subjected to blood samples collection for biochemical estimation of serum Blood Urea Nitrogen (BUN), creatinine and complete blood picture (CBC), then rats were sacrificed, kidneys were extracted and subjected to histopathological and

immunohistochemical examination for PCNA and sFas expression. Moreover, homing of labeled MSCs were performed by fluorescence microscope.

I-Biochemical Study:

A) Kidney function test (Blood Urea Nitrogen, Serum Creatinine):

1ml of blood is collected in Eppendorf for kidney function test (KFT). Then centrifuge Eppendorf at 3000 rpm for 15 minutes for serum separation. Aspirate the serum in a clean Eppendorf. The KFT studies are done using creatinine & urea diamond kit (MDSS GmbH, Schiffgraben41, 30175 Hannover, Germany) in Robonik suction machine.

B) Complete Blood Count (CBC):

1ml of blood is collected in a tube with anticoagulant EDTA for CBC for detection of RBCs and Hb levels. The CBC pictures are done using hema 21 machine.

II-MSCs isolation and subculture:

A) Isolation of MSCs:

Before MSCs injection they are prepared according to the following technique: Isolation of Nucleated cells from human bone marrow..

The inclusion criteria for MSC donors include: Age: young age as MSC efficacy was negatively affected by donor age. The gender of the cell donor had no influence on cell growth and metabolism. Informed consent is taken.

The exclusion criteria were: any risk factor for transmissible infectious diseases; the presence of age-related diseases, such as diabetes and heart failure, can also negatively affect cell functionality.

Human bone marrow sample was obtained from prof. Dr. Hala Gabr, Professor of clinical pathology, Faculty of Medicine, El kasralainy University and vice-president of the ESPCR; Egyptian Society For Progenitor Cell Research after taken the consent from an individual with average age with all the inclusion and the exclusion criteria

Cells are suspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin. Cells are incubated at 37 °C in 5% humidified CO₂ in culture upon formation of large colonies.

B) MSCs Subculture:

When large colonies developed (80–90% confluence), the cells are trypsinized with 0.25% trypsin in 1mM EDTA. Prepare culture T25 flask, add 1.5 ml warm gelatin in T25 flask and put at 37°C incubator for 5 minutes. Remove the gelatin from the flask immediately by pipet prior use. Check MSCs under microscope for cell confluence. When cell confluent is 70%-80%, remove MSCs media from flask by suction.

Put about 10 ml PBS in the flask to wash flask from media. Remove PBS by suction then add 2.5 ml trypsin 0.25% and incubate for 2 minutes. When cells are rounded up most cells are detached, add 5 ml MSCs media to dilute trypsin mix well then put all media and trypsin in 15 ml tube. Centrifuge cells at 1200rpm for 7 minutes, then aspirate the supernatant carefully.

Resuspend the cell in 5ml MSCs media, mix well and count the cell by hemocytometry. Take 10 ul of cell and mix it with 10ul of trypan blue. Calculate cell number as follow: total number of viable bright cells/ number of squares x2(diluted factor) x 10⁴ then multiply with total amount of media to calculate total cell number. Divide the total amount of cells in 2 or 3 flasks according to cell number.

Transfer flasks in to 37°C incubator then examine cell every day to check cell viability and confluency then repeat cell culture for 2 passages until receive the desired number of MSCs for injection. MSCs are labeled with green fluoresce protein (GFP) through transfection before injection in animals. MSCs cell viability were 92% after GFP transfection. MSCs are counted by hemocytometer and then used

for injection at a concentration at 1×10^6 cells in PBS.

C) Flowcytometry for MSCs: For characterization of cells, they are examined using flowcytometry. Cells are trypsinized and incubated with monoclonal antibodies for CD105, CD 34, CD45, CD271 and CD90 before injection in the MSCs treated animal group.

D) Homing of MSCs: labeled MSCs with (GFP) are detected after scarifying animal 3 weeks after injection, by staining MSCs with anti GFP then using Florescence microscope (Optika microscope Italy) at 10x and 40x.

III- Histopathological Study: Kidney organs were collected from all animals' groups. They kept in 10% formaldehyde. Paraffin blocks were sectioned at 3-4 μ thick and stained with hematoxylin and eosin (H&E) stain (**Kiernan, 2001**).

IV-Immunohistochemical Study:

Immunohistochemical reactions were carried out using streptavidin biotinimmunoperoxidase staining technique according to **Bancroft and Gamble, (2008)** Formalin-fixed, paraffin-embedded kidney tissues were cut into (3- 4-μm)thick sections and transferred to 3-aminopropyltriethoxysilane (APTS) coated glass slides. Then, sections were subjected to dewaxing, rehydration, blocking with hydrogen peroxide, and antigen retrieval that was performed by heating specimens at 100°C for 20 min in citrate buffer (pH 6.0) with microwave. One to two drops of the primary ready-to-use monoclonal ant - PCNA antibody [PC10] (ab29) (Mouse monoclonal antibody, ready to use, abcam), sFas anti body (abcam, ab65613) were then placed on the sections on separate slides. Slides were incubated at room temperature for 60 min. Incubation with secondary antibody and product visualization (Dako) was performed with DAB chromogen (3, 3-Diaminobenzidine tetrahydrochloride).

Sections were counterstained with hematoxylin, dehydrated with ethanol and xylene and mounted permanently with Di-n-butylPhthalate in Xylene (DPX).

Image Analysis and Quantitative Morphometric Measurements:

Image analysis and quantitative morphometric measurements were done by a method described by (Mustafa et al., 2015). Twelve non-overlapping fields for each specimen were selected randomly and analyzed. The optical density (OD) of sFas immunostaining was measured by using the NIH ImageJ (v1.50) program.

Optical density (OD) was calculated by the following formula:

Optical density (OD) = $\log \left(\frac{\text{max intensity}}{\text{mean intensity}} \right)$, where max intensity = 255 for 8-bit images.

The degree of immune reaction is indicated by optical density value; the darker it is, the larger the value is (Cheng and Zhang, 2014).

V. Characterization of Bone Marrow-Derived mesenchymal stem cells (BM-MSCs):

(1) Microscopical appearance:

Overtime, small round-shaped cells in culture change into larger spindle shaped cells with high proliferation and differentiation capacity. After three passages in culture, the cell population showed characteristic adhesiveness, and displaying typical fibroblast-like/ fusiform morphology detected by inverted microscope.

(2) Expression of surface markers of MSCs:

Majority of isolated cells (about 76.72%) showed CD105 positive expression. This phenotype of culture-expanded BM stem cells confirmed to the criteria for MSCs.

(3) Tracing the distribution and homing of injected MSCs:

To verify the homing of BM-MSCs labeled with GFP into the kidney tissue after being injected in the tail vein of group III (MSCs treated group) rats, the labeled cells are stained with anti GFP(abcam ab6556) and then examined by fluorescent microscope (Optika microscope Italy) at 10x and 40x.

Renal tissue damage by mercuric chloride had an essential role in attracting injected cells to the kidney, due to the high expression of chemical inflammatory attractants. Homing was confirmed by visualization of labeled BM-MSCs within the renal tissue.

VI-Statistical analysis:

The statistical analysis was done by Epi-info statistical package program version 7.2.2.16. The collected data were expressed as Mean \pm SD (SD). Multigroup comparisons of the means were carried out by one way analysis of variance (ANOVA) test. Least significant difference (LSD) test was used to compare the difference between the experimental groups and the control group.

RESULTS

Kidney Function Test (BUN, serum creatinine):

The results of the present study showed highly significant difference (P <0.001) in the mean values of BUN, serum creatinine among control , mercuric chloride group , MSCS treated group after 2 weeks of treatment by ANOVA test (p < 0.001)

By LSD test, the results of comparison revealed:- a highly significant increase in BUN, serum creatinine levels (P<0.001) in mercuric chloride , MSCs treated groups when compared with (-ve) control group , but there was a non-significant increase in BUN, serum creatinine levels (P>0.05) in mercuric chloride treated group when compared with MSCs treated group (table 1).

Table (1): Statistical comparison of BUN, serum creatinine in different studied groups after 2 weeks from induction of nephrotoxicity by (ANOVA test).

	Group 1 (-ve control) ($\bar{X} \pm SD$)	Group 2 (Mercuric chloride) ($\bar{X} \pm SD$)	Group 3 (MSCs treated) ($\bar{X} \pm SD$)	P
BUN	32.16 \pm 8.08 ^b	64.66 \pm 6.53 ^a	77.83 \pm 5.15 ^a	0.0001
creatinine	0.81 \pm 0.10 ^b	1.76 \pm 1.13 ^a	1.78 \pm 1.13 ^a	0.0001

X = arithmetic mean SD: standard deviation. (Number of rats in each group=15 rats). p < 0.05 = Significant difference. p < 0.001 = highly Significant difference. a ; Significantly different from the control (LSD test; p < 0.001). b; Significantly different from Mercuric chloride group (LSD test; p < 0.001)

The results of the present study showed highly significant difference in the mean values of BUN, serum creatinine among -ve control, mercuric chloride group, MSCS treated group after 3 weeks of treatment by ANOVA test (p < 0.001)

By LSD test, the results of comparison revealed: - a highly significant increase in BUN, serum creatinine levels (P<0.001) in mercuric chloride treated when compared with (-ve) control group and MSCs treated group levels but there was a non-significant increase in BUN, serum creatinine levels (P>0.05) in MSCS treated group when compared with -ve control group (**table 2**). These results indicate the role of MSCs therapy in amelioration of kidney function test.

Table (2): Statistical comparison of BUN, serum creatinine in different studied groups after 3 weeks from MSCs injection by (ANOVA test).

	Group 1 (-ve control) ($\bar{X} \pm SD$)	Group 2 Mercuric chloride ($\bar{X} \pm SD$)	Group 3 MSCs treated ($\bar{X} \pm SD$)	P
BUN	29.3 \pm 5.84 ^b	76.6 \pm 5.88 ^a	32.6 \pm 7.42 ^b	0.0001
Serum creatinine	1.13 \pm 0.08 ^b	1.83 \pm 0.104 ^a	0.67 \pm 0.30 ^b	0.0001

X = arithmetic mean SD: standard deviation. (Number of rats in each group=15 rats). p < 0.05 = Significant difference. p < 0.001 = highly Significant difference. a; Significantly different from the control (LSD test; p < 0.001). b; Significantly different from Mercuric chloride group (LSD test; p < 0.001).

Complete Blood Count:

Complete blood count is done for all animal groups after 2 weeks from the occurrence of nephrotoxicity. The result of the present study demonstrates highly significant difference in the mean values of RBCs, Hb and Hct among the -ve control , mercuric chloride and the MSCs treated groups (P < 0.001).

By LSD test, the results of comparison revealed a highly significant increase in RBCs, Hb and Hct mean values (P<0.001) in mercuric chloride, MSCs treated groups when compared with (-ve) control group levels, but there was a non-significant increase in RBCs, Hb and Hct mean values (P<0.001) in MSCs treated group compared to the mercuric chloride group (**table 3**).

Table (3): Statistical comparison of RBCs, Hb, and Hct in different studied groups after 2 weeks from induction of nephrotoxicity by (ANOVA test).

CBC	Group 1 -ve control ($\bar{X} \pm SD$)	Group 2 Mercuric chloride ($\bar{X} \pm SD$)	Group 3 MSCs treated ($\bar{X} \pm SD$)	P
RBCs	7.9 ± 0.52 ^b	6.2 ± 0.50 ^a	6.6 ± 0.64 ^a	0.0001
Hb	14.2 ± 1.2 ^b	11.5 ± 0.61 ^a	11.6 ± 0.77 ^a	0.001
Hct-Hematocrit	40.1 ± 2.6 ^b	34.3 ± 3.3 ^a	35.5 ± 2.2 ^a	0.004

X = arithmetic mean SD: standard deviation. (Number of rats in each group=15 rats). p < 0.05 = Significant difference. p < 0.001 = highly Significant difference. a ; Significantly different from the control (LSD test; p < 0.001). b; Significantly different from Mercuric chloride group (LSD test; p < 0.001).

Complete blood count is done for all animal groups after 3 weeks from the MSCs therapy. The result of the present study demonstrates highly significant difference in the mean

values of RBCs, Hb and Hct among the -ve control, mercuric chloride and the MSCs treated groups (P < 0.001).

By LSD test, the results of comparison revealed a highly significant increase in RBCs, Hb and Hct mean values (P < 0.001) in mercuric chloride group, when compared with (-ve) control and MSCs treated groups levels, but there was a non-significant increase in RBCs, Hb and Hct mean values (P < 0.001) in MSCs treated group compared to the (-ve) control group (**table 4**).

Table (4): Statistical comparison of RBCs, Hb, and Hct in different studied groups after 3 weeks from MSCs injection by (ANOVA test).

CBC	Group 1 -ve control ($\bar{X} \pm SD$)	Group 2 Mercuric chloride ($\bar{X} \pm SD$)	Group 3 MSCs treated ($\bar{X} \pm SD$)	P
RBCs	7.92±0.16 ^b	6.50±0.38 ^a	7.76±0.52 ^b	0.001
Hb	14.25± 0.46 ^b	10.61±0.42 ^a	14.63±0.48 ^b	0.0001
Hct-Hematocrit	42.6±1.64 ^b	40.53±0.82 ^a	43.8±1.34 ^b	0.0008

X = arithmetic mean SD: standard deviation. (Number of rats in each group=15 rats). p < 0.05 = Significant difference .p < 0.001 = highly Significant difference. a ; Significantly different from the control (LSD test; p < 0.001). b; Significantly different from Mercuric chloride group (LSD test; p < 0.001).

Histopathologic study:

After 2 weeks from nephrotoxicity induction, kidney tissues are stained with (H&E). Examination of kidney tissues among the animal groups by light microscope indicate the pathologic changes in renal tissue in both mercuric chloride +ve control(G2) and MSCs treated groups(G3) in relation to the -ve control group(G1).

The pathological changes are in the form of distorted glomeruli with narrowing of the Bowman's space. Also marked mononuclear inflammatory infiltrate was detected. Moreover, there is marked congestion of the inter-tubular blood vessels. These changes indicate the nephrotoxicity establishment (**Fig.1**

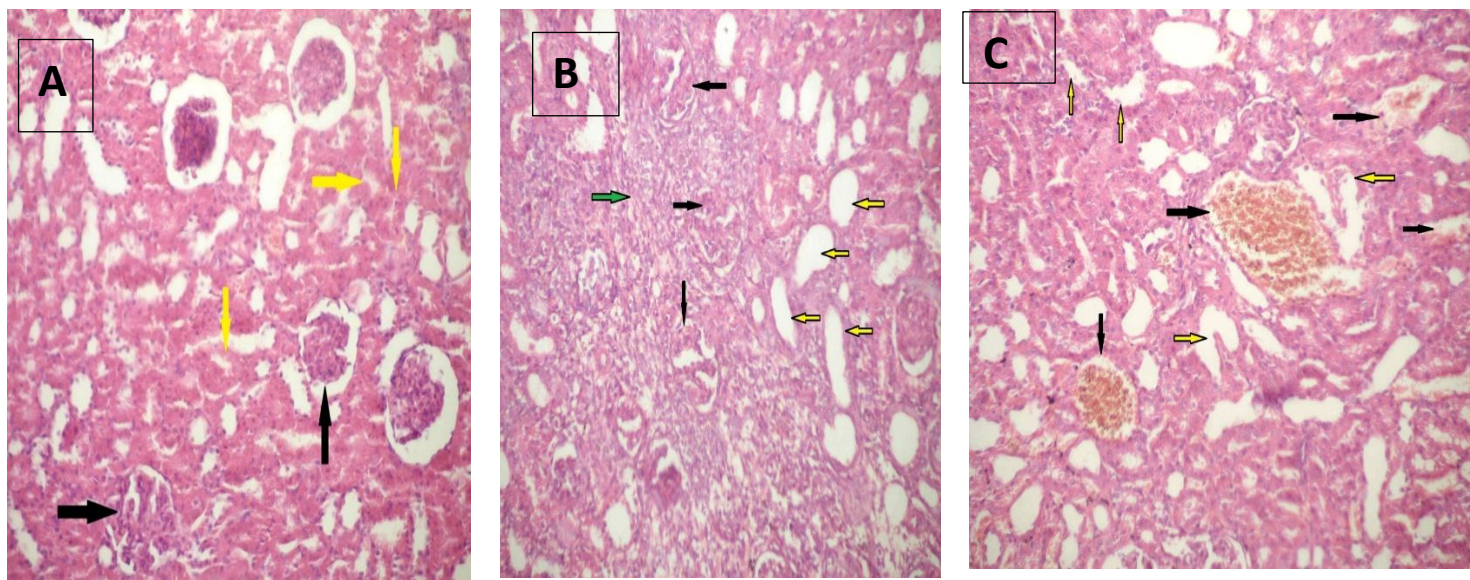


Fig. (1) A photomicrograph of a section in kidney of **A**(-ve) control rat showing normally looking glomeruli (black arrows) with well. **B**) **G2** mercuric chloride treated rats showing distorted glomeruli with narrowing of the Bowman's space (black arrows), most tubular cells were swollen and degenerated with some tubules lost their epithelial lining (yellow arrows). Marked mononuclear inflammatory infiltrate was detected (green arrow) **C**) **G3** showing marked congestion of the inter-tubular blood vessels (black arrows) and dilated tubules with exfoliation of its epithelial lining (yellow arrows) after 2weeks of treatment (H&E x200).

After 3 weeks from the MSCs therapy, the renal tissue showed improvement in the MSCs treated group (G3) in the form of well-formed glomeruli with regular Bowman's space and regenerated tubules in relation to mercuric chloride (G2) +ve control group.

The mercuric chloride group (G2) showing shrunken glomeruli associated with numerous markedly affected tubules with sloughed necrotic epithelial cells (**Fig.2**).

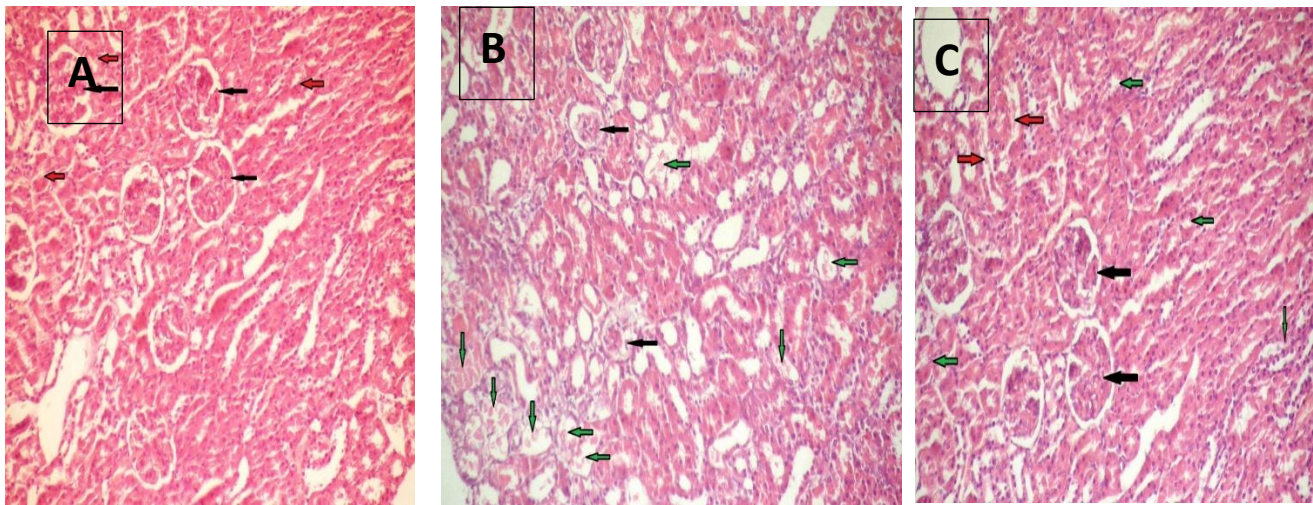


Fig. (2): showing the pathologic progress of the kidney tissue in the 3 animals groups after 3 weeks from injection of labeled MSCs. **A) G1** (-ve) control rat showing several normal glomeruli (black arrows) with well-formed tubules having central regular lumen (red arrows) **B) G2** A photomicrograph of a section in kidney of mercuric chloride treated rats showing shrunken glomeruli (black arrows) associated with numerous markedly affected tubules with sloughed necrotic epithelial cells (green arrows) **C): G3** A photomicrograph of a section in kidney of MSCs treated rats showing well-formed glomeruli with regular Bowman's space (black arrows) associated with regenerated tubules (green arrows). The red arrows demonstrate renal tubules with residual intra-luminal casts (H&E x 200).

Immunohistochemical Study:

1-PCNA:

After 2 weeks of nephrotoxicity induction, the Immuno-histochemical examination of the kidney tissues of -ve control showed few numbers of tubular and glomerular cells

with positive immune reaction to PCNA While mercuric chloride (G2) and MSCs treated groups (G3) showed many proximal and distal convoluted tubules with strong positive immune reaction to PCNA antibody in their nuclei (**Fig.3**).

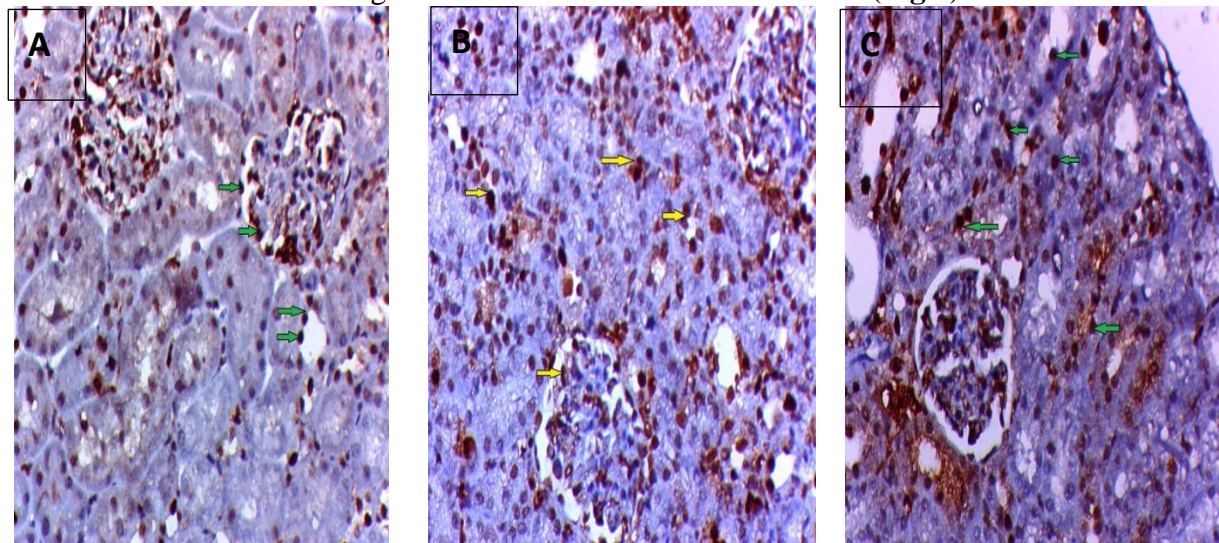


Fig. (3): showing the PCNA immunostaining of the kidney tissue in the 3 animals groups after 2 weeks of nephrotoxicity induction. **A):G1**): showing nuclear immunostaining of PCNA in some tubular and glomerular cells (green arrows) of the kidney tissue of control group **B)G2:** PCNA immunostaining of the kidney tissue in mercuric chloride group showing more labeled nuclei (yellow arrows) in proximal and distal convoluted tubule **C) G3:** showing nuclear immunostaining of PCNA in some tubular cells (green arrows) of the kidney tissue of MSCs group (PCNA immunostain X 400).

After 3 weeks from MSCs therapy, The Immuno-histochemical examination of the kidney tissues of -ve control showed focal PCNA nuclear staining (arrows) distributed within the glomeruli and tubules. While mercuric chloride (G2) showed marked PCNA nuclear immunostaining mainly

encountered within many proximal and distal convoluted tubules MSCs treated groups (G3) showed mild PCNA nuclear immunostaining distributed within the renal glomeruli and tubules reflecting improvement of renal tissue pathology (Fig.4).

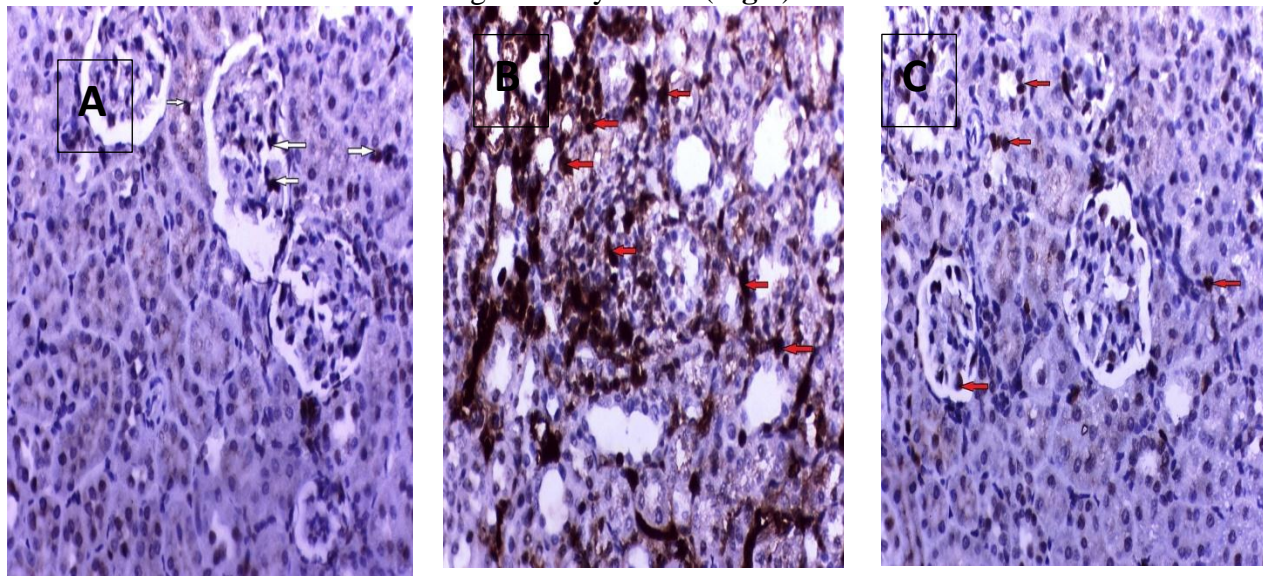


Fig. (4): define the PCNA immunostaining progression of the kidney tissue in the three groups after 3 weeks of the injection of the labeled MSCs. **A):G1** -ve control group showing focal PCNA nuclear staining (arrows) distributed within the glomeruli and tubules after 3 weeks of the injection of the labeled MSCs **B): G2** mercuric chloride group showing marked PCNA nuclear immunostaining mainly encountered within the affected tubules (red arrows) **C) G3** PCNA immunostaining of the kidney tissue of rats of MSCs treated rats showing mild PCNA nuclear immunostaining distributed within the renal glomeruli and tubules (red arrows) (PCNA immunostaining x400).

2- sFas expression :

After 2 weeks of nephrotoxicity induction, the Immuno-histochemical examination of the kidney tissue of -ve control group (G1, average cell count is 667) showed weak immunoreactivity for sFas while, the Immuno-histochemical examination of the kidney tissue of mercuric chloride (G2, cell count average is 800) and MSCs treated groups (G3 cell count average is 592,)

showed many glomeruli and tubules with strong positive immune reaction to sFas antibody in their cytoplasm (Fig.5).

The optical density (OD) of sFas immunoreaction in the renal tissue of mercuric chloride and MSCs treated groups showed a significant increase when compared to -ve control group (table 5)

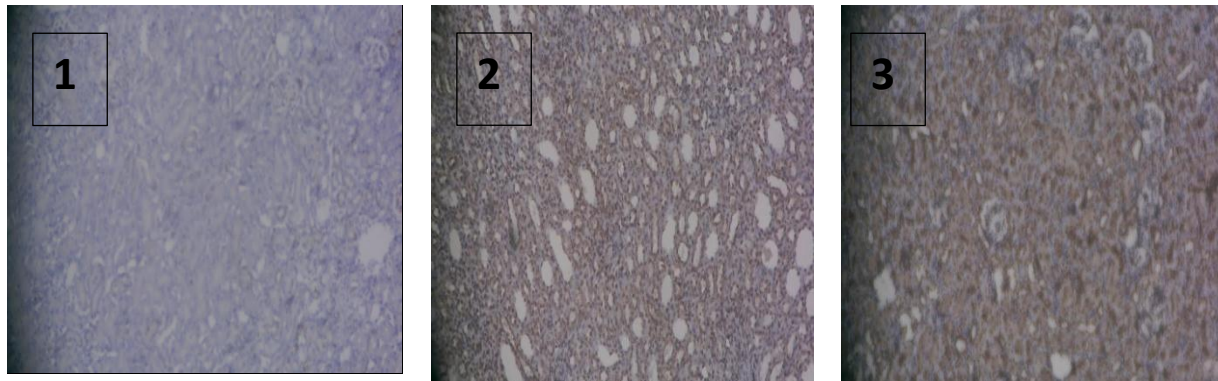


Fig (5): showing the immunostaining of sFas antibody in the 3 animal groups 2 weeks after nephrotoxicity induction. **G1:** control group showing negative secretion of sFas. **G2:** (Mercuric chloride +ve control group). Showing positive secretion of sFas **G3:** (MSCs treated group before therapy). Showing positive secretion of sFas.

Table (5): Statistical comparison of the optical density (OD) of sFas in different studied groups after 2 weeks from nephrotoxic induction by (t- test).

sFas immunostaining	Group 1 -ve control ($\bar{X} \pm SD$)	Group 2 Mercuric chloride($\bar{X} \pm SD$)	Group 3 MSCs treated($\bar{X} \pm SD$)	P
Cell intenisty	113.88± 0.872	253.15 ± 31.051*	273.42 ± 4.383*	0.002

*= significant different from group1. X = arithmetic mean. SD = Standard Deviation. P < 0.05 = Significant difference. P > 0.05 = Non-significant. P < 0.01 = Highly significant difference

3 weeks after labeled MSCs therapy, The Immuno-histochemical examination of the kidneys of -ve control (G1, cell count average is 715) and MSCs treated groups (G3, cell count average is 799) showed focal sFas immunostaining distributed within the glomeruli and tubuleswhile mercuric chloride treated group (G2, average cell

count is 616) showed strong positive immune reaction to sFas antibody in their cytoplasm (**Fig.6**).

It was noticed that optical density (OD) sFas immunoreaction level was significantly decreased in group MSCs treated group as compared to mercuric chloride group values (**table 6**).

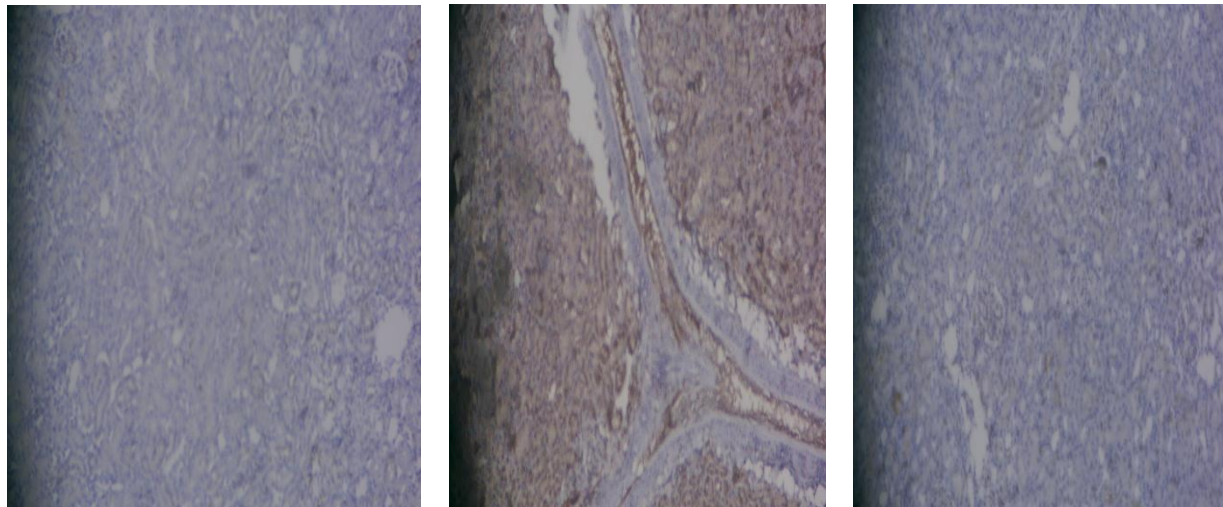


Fig (6): showing the immunostaining of sFas antibody in the 3 animal groups 3 weeks after MSCs therapy. **G1:** control group showing negative secretion of sFas **G2:** (Mercuric chloride +ve control group). Showing extremely positive secretion of sFas **G3:** (MSCs treated group): showing significant decrease of the sFas expression by immunostaining.

Table (6): Statistical comparison of the optical density (OD) of sFas in different studied groups after 3 weeks from MSCs therapy by (t- test).

sFas immunostaining	Group 1 -ve control ($\bar{X} \pm SD$)	Group 2 Mercuric chloride ($\bar{X} \pm SD$)	Group 3 MSCs treated ($\bar{X} \pm SD$)	P
Cell intensity	114.65± .0951	467.18 ± 83.28	147.66 ± 4.211*	0.004

*= significant different from group3. X = arithmetic mean. SD = Standard Deviation. P < 0.05 = Significant difference. P > 0.05 = Non-significant. P < 0.01 = Highly significant difference.

DISCUSSION

People with nephrotoxicity are susceptible to anemia because kidneys play a role in creating healthy red blood cells. They produce the erythropoietin hormone (EPO) which stimulates the bone marrow to make red blood cells (**Hoste and Kellum, 2006**). The anemia which is associated with nephrotoxicity is due to the presence of sFas. Fas (CD95) is a transmembrane glycoprotein that belongs to the family of tumor necrosis factor (TNF) (**Haasters et al., 2009**).

Soluble Fas (sFas) is a protein derived from alternative splicing of CD95. sFas is

increased in the serum of uremic patients and is associated with the presence of anemia and resistance of recombinant human EPO (rHuEPO) dosage in uremic patients. sFas levels is associated with an increased need for serum erythropoietin levels (Epo) (**Fu et al., 2006**). Mesenchymal stem cells (MSCs) are multipotent stem cell population that mostly resides in the umbilical cord (UC) and the bone marrow (BM). MSCs hold therapeutic potential in repairing tubular injury, ameliorating renal function deficits, and prolonging survival in acute renal injury (**Yener et al., 2012**).

The aim of the present study was to evaluate the effect of bone marrow Mesenchymal stem cells on the hemotoxic effect of sFas released in cases of rats with induced nephrotoxicity. In the present study Mercuric chloride caused an increase **KFT** (serum creatinine & Blood Urea Nitrogen) and **histopathologic study** changes; in the form of distorted glomeruli with narrowing of the Bowman's space. Also most tubular cells were swollen and degenerated with some tubules lost their epithelial lining. Marked mononuclear inflammatory infiltrate was detected with marked congestion of the inter-tubular blood vessels and dilated tubules with exfoliation of its epithelial lining.

These result were in line with **Yadav et al (2012)** who reported that mice administered HgCl₂ (7 mg/kg, bw) presented with high kidney function level and the pathological changes in the kidney tissue were in the form of interstitial edema, tubular dilation, and sloughing of individual epithelial cells.. Also **Sarwar et al (2007)** reported that subcutaneous administration of 4 mg/kg body weight HgCl induced renal injury evident as a marked elevation in the kidney function and the histopathological changes such as necrosis, edema and congestion of stroma and glomeruli.

The extent of injury in this study was similar to previously reported *in vivo* patterns of HgCl₂-induced nephrotoxicity, insofar as the onset of toxic effects was rapid and injury was accompanied by apoptosis. (**Langworthy et al 2009 & Wang et al 2003**)

Functional and Morphological alterations in kidney of rats following mercury intoxication were characterized by blebbing of brush border and sloughing of microvilli with desquamation into the lumens and PCT's necrosis was evident. The amount of free radicals produced by mercury actions exceeds antioxidant enzyme activities, and

thus kidney functions are disrupted and tissue damage develops (**Alam et al 2007**). The toxic effects of mercuric chloride may be due to the production of oxidative stress on kidneys as well as generation of reactive oxygen species producing a number of toxic reactions (**Prabhu et al., 2017**).

The current study demonstrated that transplanted stem cells suppressed serum urea and creatinine levels in HgCl₂-treated rats indicating a protective therapeutic action in the kidney function. Similar results were found by **Marques et al., (2013)** who demonstrated a Reno protective effect induced by stem cells through increased tissue regeneration and inhibition of tubular cell apoptosis, improved renal function.

Furthermore **Li et al (2010)** studied reported that the improvement of kidney function may be related to the prevention of the initial fall in GFR possibly through paracrine actions that contributes to proliferation of endogenous survivor cells or stimulates the proliferation and differentiation of kidney resident stem cells(**Fang et, al 2005**).

Moreover, the **CBC** results showed that, the mercuric chloride treated group had low level of Hb, Hct and RBCs. This indicates the presence of anemia in case of nephrotoxicity. In this study, there is an association between nephrotoxicity and anemia in the nephrotoxic animal models. As a result of renal injury, there is decrease in RBCs, Hb and Hct values correlated with the increase of BUN and serum creatinine.

These result coincided with **Akbar et al., 2013** who showed that RBC count, hemoglobin amount and hematocrit level were significantly lower in the nephrotoxic patient compared to the healthy one. Other study showed that, anemia is related to kidney injury because of low renal oxygen delivery. This leads to worsening oxidative stress, and impairing hemostasis (**Sickeler et al., 2014**).

In the current study MSCs treated group showed significant increase of the RBCs, Hct and Hb levels compared to the mercuric chloride group reflecting the improvement of anemia. This could be attributed to MSCs essential role in providing the significant microenvironment for hematopoiesis and their ability to secrete many growth factors that enhance hematopoiesis (**Sara et al., 2017**).

Chao et al., 2010 demonstrated that, MSC originally present in the BM. MSCs have been known as an essential component of the hematopoietic stem cell niche. They control the self-renewal, maturation, and recruitment of hematopoietic stem cells to the vascular compartment by releasing of growth factors and cytokines. Also MSCs help regeneration of damaged organs with cell-cell contact, soluble factors, and paracrine (**Han et al., 2007**).

PCNA is a marker of cell division and proliferation. PCNA found in cell nucleus and was directly involved in DNA synthesis (**Hall, et al 1990**). In this study, there was a significant increase in the number of PCNA positive nuclei in renal tubular cells of rats receiving mercuric chloride. The administration of MSCs significantly decreased the number of PCNA-positive cells, suggesting that MSCs strongly promoted tubular cell proliferation while inhibiting cell apoptosis (**Shaohua and Dongcheng 2013**).

The results of **sFas immunohistochemical staining** in this study showed high levels of sFas in mercuric chloride treated group. On the other hand after MSCs therapy there is obvious decrease in the sFas level in the MSCs treated group compared to the mercuric chloride treated rats. These result were in consistent with some authors reports that showed that sFas accumulates in the serum as kidney failure progresses and sFas levels correlated positively with serum creatinine (**Corina et al., 2000**). In the

kidneys, the FasL/Fas system has been detected in glomerular mesangial cells, fibroblasts, and tubular epithelial cells. Renal FasL is increased in rats and mice with proliferative glomerulonephritis. sFasL acted similarly to sFas (**Hitesh & Adebowale 2017**). Additionally **Hayden et al., (2012)** reported that sFas is elevated in AKD, CKD and also in dialysis patients. It is noticed that sFas levels were accompanied with markers of anemia. So sFas and inflammatory cytokines might play a role in anemia of critically ill patients.

CONCLUSION

MSCs therapy improved the hemotoxic effect of sFas indirectly by improving the kidney damage, as MSCs therapy in nephrotoxicity was associated with decrease sFas level in the MSCs treated animal group compared with the mercuric chloride group. This down regulation of sFas is responsible for the improvement of the CBC picture and improved the RBCs and Hb levels.

RECOMMENDATION

On light of the results of the present study, the following guidelines are recommended:

- Further studies are needed for
 - Investigation of the possible mechanisms of anemia associated with nephrotoxicity.
 - Evaluation of the role of sFas in pathogenesis of anemia and nephrotoxicity
 - Explanation of the underlying therapeutic mechanisms of MSCs and translate those into clinical practice.
- Consider experimental MSCs therapy in treatment of Anemia associated with nephrotoxicity especially in cases resistant to EPO therapy.

REFERENCES

Alam, M.S.; Kaur, G.; Jabbar, Z.; Javed, K.; Akthar, M. (2007): Eruca sativa seeds possess antioxidant activity and exert a protective effect on mercuric chloride induced renal toxicity. Food Chem. Toxicol. 45(6): 910-920.

- Akbar, D.; Mohammad, M.; Shadi, T.; Zahra, K., K.; Gholam, H., T.; Esmaeil, S., M.; Taregh, B.; Shaban, A.; Eshagh, M. (2013):** Anemia and Thrombocytopenia in Acute and Chronic Renal Failure *IJHOSCR.*, 7:(4)34-39.
- Bancroft, J.D., Gamble, M., (2008).** Connective tissue stains. In: Bancroft, J.D., Gamble, M., (Eds.), *Theory and Practice of Histological Techniques*. 6th ed. Elsevier Health Sciences, Churchill Livingstone.; London, Oxford, New York, Philadelphia, St Louis, Sydney; pp. 150.
- Bonomini, M. and Sirolli, V. (2003):** Uremic toxicity and anemia. *J Nephrol.*, 16:21–28.
- Cheng, X.L. and Zhang, J. J. (2014):** Effect of adaravone on apoptosis of hippocampus neuron in seizures rats kindled by pentylentetrazole. *Eur. Rev. Med. Pharmacol. Sci.*, 18(6).769-774.
- Chao, M.; Lee, E. J.; Nam, H.; Yang, J. H.; Cho, J.; Lim J. M. and Lee, G. (2010):** Human feeder layer system derived from umbilical cord stromal cells for human embryonic stem cells. *Fertil. Steril.*, 93(8):2525–2531.
- Corwin, H., L.; Gettinger, A. and Pearl, R., G. (2002):** Efficacy of recombinant human erythropoietin in critically ill patients: a randomized controlled trial. *JAMA.*, 288: 2827–2835.
- Fang, T.C.; Alison, M.R.; Cook, H.T. (2005):** Proliferation of bone marrow-derived cells contributes to regeneration after folic acid-induced acute tubular injury. *J Am Soc Nephrol.*; 16:1723–1732.
- Ferguson, M., A.; Vaidya, V., S. and Bonventre, J., V. (2008):** Biomarker of nephrotoxic acute kidney injury. *Toxicology* 245:182-193.
- Fishbane, S., B. and Barry, M. (2008):** Hematologic aspects of kidney disease. In: Brenner BM, ed. *Brenner & Rector's the kidney*. 8th edn. Philadelphia: Saunders Elsevier, 1733–1744
- Fisher, J., W. (2003):** Erythropoietin: physiology and pharmacology update. *Exp Biol Med.*, 228: 1–14.
- Fu, X.; Fang, L. and Li, X. (2006):** Enhanced wound-healing quality with bone marrow mesenchymal stem cells autografting after skin injury *Wound Repair Regen.*, 14: 325-335.
- Haasters, F.; Prall, W.; Anz, D.; Bourquin, C.; Pautke, C. and Endres, S. (2009):** Morphological and immunocytochemical characteristics indicate the yield of early progenitors and represent a quality control for human mesenchymal stem cell culturing. *J. Anat.*, 214:759–767.
- Hall, P.A.; et al (1990):** Proliferating cell nuclear antigen (PCNA) immunolocalization in paraffin sections: an index of cell proliferation with evidence of deregulated expression in some neoplasms. *J Pathol* 162:285- 294.
- Han, J., Y.; Goh, R., Y.; Seo, S., Y.; Hwang, T., H.; Kwon, H., C.; Kim, S., H.; et al., (2007):** Cotransplantation of cord blood hematopoietic stem cells and culture-expanded and GM-CSF-/SCF-transfected mesenchymal stem cells in SCID mice. *J Korean Med Sci.*, 22(2):242-247.
- Hayden, S., J; Albert, T., J.; Watkins, T., R. and Swenson, E., R. (2012):** Anemia in critical illness: insights into etiology, consequences, and management. *Am J Respir Crit Care Med.*, 185: 1049-1057.
- Hitesh, S. and Adebawale, A. (2017):** Early septic insult in neonatal pigs increases serum and urinary soluble Fas ligand and decreases kidney function without inducing significant renal apoptosis, *Renal Failure*, 39(1): 83-91.
- Hoste, E., A. and Kellum, J., A. (2006):** Acute kidney injury: epidemiology and diagnostic criteria. *Curr Opin Crit Care*, 12(6):531–537.
- Kirtan, A., J.; Leder, D., M.; Waikar, S., S.; Chertow, G., M.; Ray, K., K.;**

coronary syndromes and normal to mildly reduced glomerular filtration rates. *J. Am. Coll Cardiol.*, 45: 1781-1786.

Langworthy, M.; Zhou, B.; de Caestecker, M.; Moeckel, G.; Baldwin, H.S. (2009): Nfatc1 identifies a population of proximal tubule cell progenitors. *J Am Soc Nephrol.*; 20:311–321.

Li, B.; Cohen, A.; Hudson, T.E. (2010): Mobilized human hematopoietic stem/progenitor cells promote kidney repair after ischemia/reperfusion injury. *Circulation*; 121:2211–2220.

Mohamed Talaat Abdel Aziz, Mohamed Abdel Aziz Wassef, Hanan Hosni Ahmed, Laila Rashed, Soheir Mahfouz, Mayssa Ibrahim Aly, Rania Elsayed Hussein and Mai Abdelaziz (2014): The role of bone marrow derived-mesenchymal stem cells in attenuation of kidney function in rats with diabetic nephropathy *Diabetology & Metabolic Syndrome*, 6:34-44.

Maiese, K.; Chong, Z., Z. and Shang, Y., C. (2008): Raves and risks of erythropoietin. *Cytokine Growth Factor Rev.*, 19:145–155.

Marques, F.S.; Silva, J.S.; Couto, R.D.; Barreto, E.P. Jr; Ribeiro,-dos-Santos R; dos Santos W.L.; Soares, M.B. (2013): Transplantation of bone marrow mononuclear cells reduces mortality and improves renal function on mercury-induced kidney injury in mice. *Ren Fail.*; 35(5):776-81.

Mustafa, H. (2015): prophylactic role of co-enzyme Q10 and *Cynara Scolymus L* on toxicity in rat: Biochemical and immunohistochemical study. *Indian J. pharmacol.*, 47(6): 649-656.

Paunel, A.; Flohé, S.; Scholz, M.; Windolf, J. and Lögters, T. (2011): Increased serum soluble Fas after major trauma is associated with delayed neutrophil apoptosis and development of sepsis. *Crit Care.* 15:R20. doi: 10.1186/cc9965

Prabhu, N.S.; Brijender, B.; Renu, S.(2017):Evaluation of Protective Effect of *Tagetes erecta* Against Mercuric Chloride Induced Nephrotoxicity *International Journal of Pharmaceutical and Clinical Research*; 9(8): 593-598.

Rached, E.; Hoffmann, D.; Blumbach, K.; Weber, K.; Dekant, W. and Mally, A. (2008): Evaluation of putative biomarkers of nephrotoxicity after exposure to ochratoxin A in vivo and in vitro. *Toxicol. Sci.*, 103:371-381.

Sara, A.; Karim, S.; Parvin, A.; Davod, P., S. and Hamze, T. (2017): Effects of Mesenchymal Stem Cell Derivatives on Hematopoiesis and Hematopoietic Stem Cells *Adv Pharm Bull*, 7(2): 165-177.

Sarkar, P., K.; Prajapati, P., K.; Choudhary, A., K.; Shukla, V., J. and Ravishankar, B.(2007) :Haematinic evaluation of *Lauha bhasma* and *Mandura bhasma* on HgCl₂ -induced anemia in rats. *Indian J. Pharm. Sci.*, 69 (6): 791-79.

Sarwar, A. M.; Kaur, G.; Jabbar, Z.; Javed, K. (2007): *Eruca sativa* seeds possess antioxidant activity and exert a protective effect on mercuric chloride induced renal toxicity. *Food Chem Toxicol.* Jun;45(6):910-20.

Seung, S., H.; Seon, H., B.; Shin, Y., A.; Ho, J., C.; Ki, Y.,N.; Dong-Wan, C. and Sejoong, K.(2015):Anemia Is a Risk Factor for Acute Kidney Injury and Long-Term Mortality in Critically Ill Patients *Tohoku J. Exp. Med.*, 237: 287-295.

Shaohua, Q. I.; Dongcheng, W.U. (2013):Bone marrow-derived mesenchymal stem cells protect against cisplatin-induced acute kidney injury in rats by inhibiting cell apoptosis. *Int J Mol Med.* Dec; 32(6): 1262–1272.

Sickeler, R.; Phillips-Bute, B.; Kertai, M., D.; Schroder, J.; Mathew, J., P.; Swaminathan, M. and Stafford-Smith, M. (2014): The risk of acute kidney injury with co-occurrence of anemia and hypotension

during cardiopulmonary bypass relative to anemia alone. *Ann. Thorac. Surg.*, 97: 865-871.

Takahashi, K.; Tanabe, K.; Ohnuki, M. (2007): Induction of pluripotent stem cells from adult human fibroblasts by defined factors," *Cell*, vol. 131, no. 5, pp. 861–872.

Wang, Z.; Chen, J.K.; Wang, S.W.; Moeckel, G.; Harris, R.C. (2003): Importance of functional egf receptors in recovery from acute nephrotoxic injury. *J Am Soc Nephrol.* 14:3147–3154.

Yadav, N.;Rao, S. and Mukhopadhyay, A. (2012): Bone marrow cells contribute to tubular epithelium regeneration following acute kidney injury induced by mercuric chloride. *Indian J. Med. Res.*, 136(2): 211–220.

Yener, K.;Sokmen, M.; Unsal, A.; Cigerli, S. and ozagari, A. (2012): Effects of human Umbilical Cord Stem Cells and Granulocyte Colony- Stimulating Factor (G-CSF) on Carbon Tetrachloride-Induced Nephrotoxicity. *Nephro-Urol.*, 4(3): 545-550.

دور الخلايا الجذعية الميزنشيمية في تحديد التسمم الدموي لماده الفاس في حالات التسمم الكلوي
 د/الشيما محمد سامى على و أ.د/ محمود عبد الغنى نمير وأ.د/ هدى رجب السيد وأ.د/ أنطونى عطالله*
 قسم الطب الشرعى والسموم الإكلينيكية كلية الطب -جامعة الزقازيق و مركز الخلايا الجذعية بجامعة ويك فورست_الولايات المتحدة الأمريكية*

الملخص العربى

يعد التسمم الكلوي من أكثر الأمراض إنتشارا وأخطرهم نظرا لإرتفاع نسبة الوفيات في مرضى التسمم الكلوي. و يعد فقر الدم او الانيميا مشكلة شائعة في الأشخاص الذين يعانون من أمراض الكلى وذلك لان الكلى تنتج هرمون الإيثروروبوتين . وهو الهرمون المسؤل عن تنشيط النخاع العظمي لإنتاج المزيد من خلايا الدم الحمراء. ومن أسباب الإنيميا المصاحبه للتسمم الكلوي ايضا وجود بروتين الفاس وهو احد البروتينات المسؤله عن موت الخلايا المبرمج. تعد الخلايا الجذعية الميزنشيمية بابا جديدا واعداء للعلاج الفعال لأمراض التسمم الكلوي. وقد يسهم فى إعطاء فرصة جديدة لحياة آمنة. يهدف البحث الي تقييم دورالخلايا الجذعية كعلاج للتسمم الكلوي والانيميا المصاحبه له فى الجرذان البيضاء أجريت هذه الدراسة على تسعين (٩٠) من إناث الجرذان البيضاء وتم تقسيم الجرذان عشوائيا الى ثلاث مجموعات متساوية.المجموعة الضابطة و قد تم متابعة هذه الجرذان وذلك لمقارنتها بباقي المجموعات، المجموعة الثانية: (مجموعه كلوريد الزئبق) تم حقنها بماده كلوريد الزئبق ١ مجم /كجم لمدة اسبوعين وذلك لاحداث التسمم الكلوي المجموعة الثالثة (مجموعة العلاج بالخلايا الجذعية الميزنشيميه)تم حقنها بماده كلوريد الزئبق ١ مجم /كجم لمدة اسبوعين ثم تم حقنها بالخلايا الجذعية السابق تعليمها بالبروتين. وتم متابعة الجرذان لمدة ٣ اسابيع بعد الحقن.بعد اسبوعين من دراسته تم اخذ عينات الدم لقياس وظائف الكلى وصوره الدم ثم تم ذبح الجرذان (PCNA) واستخراج الكلى لفحصها هستولوجيا بالمجهر الضوئى وبأستخدام الدلائل الهستوكيميائية المناعية لتقدير عامل لتحديد نسبة تدمير الخلايا وايضا نسبة الفاس بالخلايا وتمت أعاده هذه التقييمات بعد ٣ أسابيع من العلاج بالخلايا الجذعية و تم ملاحظه تدهور وظائف الكلى مصاحب لوجود الانيميا وزيادة نسبة الفاس فى الخلايا الكلويه المدمره وكلما زادت نسبة تدمير الخلايا الكلويه كلما إرتفعت نسبة الفاس ونسبه الأنيميا ولوحظ تحسن واضح فى وظائف الكلى والأنيميا ونقص فى نسبة الفاس بعد العلاج بالخلايا الجذعية الميزنشيميه بالمقارنه بالمجموعه الضابطة والمجموعه المريضة أدي حقن الجرذان البيضاء بالخلايا الجذعية الي تحسن ملحوظ في الكلى وظيفيا وهستولوجيا مما ادي الي تحسن حاله الأنيميا المصاحبة للتسمم الكلوي . هذا وقد ساهم تحسن الكي فى تقليل نسبة الفاس وبالتالي يعتبر تأثير الخلايا الجذعية الميزنشيمية على الفاس تأثير غير مباشر.