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THE ROLE OF BONE MARROW DERIVED MESENCHYMAL STEM CELLS IN ATTENUATION OF RENAL FAILURE IN ADULT MALE ALBINO RATS

By

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ABSTRACT

Background: Stem cell therapy holds a great promise for the repair of injured tissues and organs, including the kidney. **Objective**: Study the possible role of bone marrow derived mesenchymal stem cells (BM MSCs) in regeneration of kidney tissue in acetaminophen-induced renal failure (RF). **Material and Methods**: Twenty four adult male albino rats of local strain were chosen as an animal model for this study. They were divided into control, RF rats, RF received culture media and RF received BM MSCs in a dose of one million cells / rat by intravenous injection. Mesenchymal stem cells were separated from rat bone marrow, being identified by their morphology and immunophenotype (CD29, CD45 and CD90) by flow cytometry. BM MSCs were labeled with PKH26 dye before injection. RF was induced by oral administration of acetaminophen. At the end of the experiment (24 days), blood samples were obtained for serum creatinine and urea. Animals were sacrificed; kidneys were obtained for histopathological examination and measurement of tissue levels of malondialdehyde (MDA) and glutathione peroxidase (GPx). **Results:** BM MSCs were successfully separated from bone marrow, being identified as mesenchymal stem cells showing plastic adherence properties and fibroblastoid shape. The cells showed +ve expression of CD29 and CD90, -ve expression of CD45 proving that it is mesenchyml cells not hematopoitic cells. Acetaminophen showed significant increase in serum creatinine, urea and tissue MDA. Also, there was significant decrease of tissue GPx. BM MSCs showed significant decrease in serum creatinine, urea and tissue MDA in addition to significant increase in tissue GPx. Histopathological examination revealed regeneration of the damaged kidney tissue and restoration of normal architecture of BM MSCs-treated group in comparison with RF group.

Conclusion: BM MSCs have a curative role in regeneration of kidney tissue in acetaminophen-induced renal failure.

Key words: Bone marrow derived mesenchymal stem cells, renal failure.

INTRODUCTION

End stage renal disease (ESRD) can result from a variety of factors, including genetic (e.g. congenital dysplasia or aplasia, reflux, polycystic kidney disease, Alport syndrome, Finnish nephropathy etc.) and environmental (IgA nephropathy, Type II diabetes, toxic or viral insult). The etiology will influence the potential strategy for maintenance and/or replacement of renal function **(Lysaght, 2002).**

ESRD constitutes worldwide health problem. There is a steady increase in numbers of patients as well as rising costs of treatment and extended therapy periods. Increased morbidity and mortality rates are associated with maintenance dialysis. Outcomes are considerably improved following transplantation, but organ supply lags far behind demand and this therapeutic option is very expensive **(McTaggart and Atkinson, 2007).**

Stem cells are undifferentiated cells that undergo both self-renewal and differentiation into one or more cell types **(Weissman, 2000).** Among stem cells, mesenchymal stem cells (MSCs) have several advantages for therapeutic use such as ability to migrate to the sites of tissue injury, strong immunosuppressive effects (**Abdi et al., 2008 and Volarevic et al., 2010)**, and better safety after infusion of allogeneic MSCs (**Hare et al., 2009 and Lee et al., 2010).**

Previous studies have shown that MSCs are able to differentiate into several cell types, including cardiomyocytes, vascular endothelial cells, neurons, hepatocytes, epithelial cells, and adipocytes, making them a potentially important source for the treatment of debilitating human diseases. Such multipotent differentiation characteristics coupled to their capacity for self-renewal and capability for the regulation of immune responses, described MSCs as potentially new therapeutic agents for treatment of chronic renal failure **(Pittenger et al., 1999).** An increasing number of data has showed that the therapeutic effects of MSCs not only rely on their differentiation ability to repair damaged tissue, but also depend on their potency to modulate local environment, activate endogenous progenitor cells, and secrete various factors **(Togel et al., 2007 and Zhang et al., 2007).**

The present work was a trial to investigate the role BM MSCs in attenuation of renal failure in adult male albino rats.

MATERIAL AND METHODS

Animals: Twenty four adult male albino rats of local strain were chosen as an animal model for this study. They were kept in suitable cages (20x32x20 cm for every three rats) at room temperature, with the natural light-dark cycle. They weighed 120 -140 g (average weight was 130 g). They were fed on a standard food in addition to green vegetables with free water supply. They were kept for 10 days for the adaptation to the new environments before the start of the experiment. The animals were divided into four equal groups:

Group I (control group) received distilled water 0.5 ml/rat by oral gavage for three days. **Group II (RF)** HF was induced by oral administration of acetaminophen 500mg/kg for three days ***(Gopi et al., 2010).*** **Group III (RF + vehicle)** received culture media (Dulbecco's modified Eagle's medium DMEM), by single intravenous injection in the caudal vein of 1ml/rat in the fourth day after induction of RF. **Group IV (RF + BM MSCs):** received BM MSCs by intravenous injection in the caudal vein, one million cells per rat in the fourth day after induction of RF (**Abdel Aziz et al., 2007**). After twenty day from BM MSCs injection, blood samples were taken from the retro-orbital vein for measurement of

serum creatinine and urea the animals were sacrificed to obtain kidney tissue for histopathological examination and detection of tissue levels of MDA and GPx.

Methods: RF was induced by oral administration of acetaminophen 500 mg/kg for 3 days according to ***(Gopi et al., 2010).*** Acetaminophen was dissolved in boiling water ***(USP31, 2007).***

Bone marrow was harvested by flushing the tibiae and femurs of 6-week-old male white albino rats with Dulbecco's modified Eagle's medium (DMEM, GIBCO/BRL) supplemented with 10% fetal bovine serum (GIBCO/ BRL). Nucleated cells were isolated with a density gradient [Ficoll/ Paque (Pharmacia)] and resuspended in complete culture medium supplemented with 1% penicillin–streptomycin (GIBCO/BRL). Cells were incubated at 37°C in 5% humidified CO2 for 12–14 days as primary culture or upon formation of large colonies. When large colonies developed (80– 90% confluence), cultures were washed twice with phosphate buffer saline(PBS) and the cells were trypsinized with 0.25% trypsin in 1 mM EDTA (GIBCO/BRL) for 5 min at 37°C. After centrifugation, cells were resuspended in serum supplemented medium and incubated in 50 cm2 culture flask (Falcon). The resulting cultures were referred to as first-passage cultures (**Abdel Aziz et al., 2007**) Cells were identified as being MSCs by their morphology, plastic adherence (**Dexter et al., 1981)** and immunophenotyping using flowcytometry for CD29,CD45 and CD90 **(Bieback et al., 2004).** BM MSCs were labeled with PKH26 supplied by Sigma Company (Saint Louis, Missouri USA). Cells were centrifuged and washed twice in serum free medium. Cells were pelleted and suspended in dye solution. Cells were injected intravenously into rat tail vein. After twenty days, kidney tissues were examined with a fluorescence microscope (Leica Microsystem, USA) to detect and trace the cells **(Shao-Fang et al., 2011)**. Measurements were done for serum creatinine ***(Folin and Wu, 1976),*** serum urea ***(Patton and Crouch, 1977)*** tissue levels of MDA ***(Satoh, 1978)***, and GPx tissue levels ***(Paglia and Valentine 1967).***

Kidney was excised for histopathological studies and detection of BM MSCs homing in the kidney tissue. The specimens were preserved in 10% formalin solution. Paraffin blocks were then made for the tissue samples, and different sections were obtained and slides were stained with hematoxyline and eosin (Hx and E) stains and examined using a light microscope. Other slides were kept without staining to be examined by fluorescent microscope for detection of BM MSCs homing.

All the statistical analyses were processed using Statistical Program of Social Sciences (SPSS) for windows (version 17, SPSS Inc., Chicago, IL, USA), Values of the measured parameters were expressed as mean value ± standard deviation (SD), and the differences and significance were verified by one-way ANOVA followed by the Fisher's least significant difference (LSD) *post hoc* test. *P* values less than 0.05 were considered statistically significant.

RESULTS

Twenty four hours from the primary culture (passage 0 = P0) of bone marrow derived mesenchymal stem cells, the cultured cells appeared crowded and suspended. They were variable in size and shape. Most of the cells appeared rounded (Figs.1a). Three days from the primary culture, the native MSCs were seen attached to the culture flasks sparsely and sporadically. Some MSCs appeared spindle-shaped, while others started to form processes. Seven days from the primary culture, the MSCs of cultured flasks were proliferated and reached 70% confluency. The cells exhibited different shapes with well-developed cytoplasmic processes, granular cytoplasm and vesicular nuclei (Figs.1). Twelve days from the primary culture, the adherent cells reached 80-90% confluency (Fig.1d) and appeared triangular, star shaped and spindle shaped (Fig.1c).

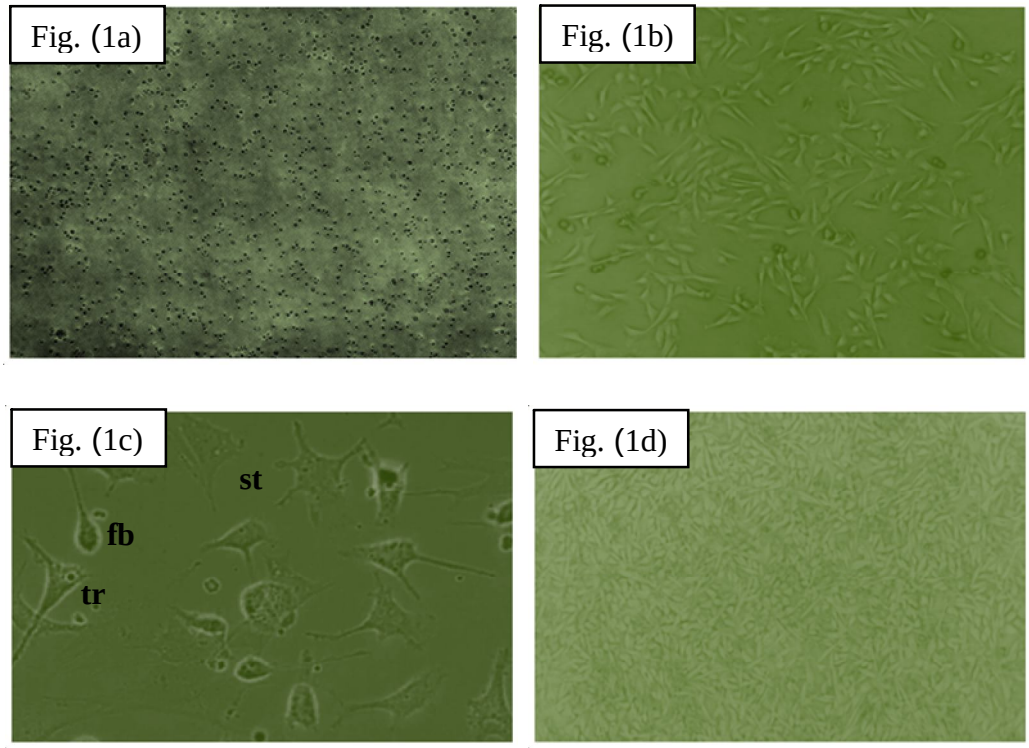

Figure (1): Phase contrast micrograph of BM MSCs by inverted microscope, showing cells appeared rounded 24 hrs after separation (1a). The cells turned to fibroblastoid (fb) shape, other cells taken star shape (st) and triangular shape (tr) with cytoplasmic processes (1c), reaching 70% confluency (1b), then reached to about 90% confluency (1d).

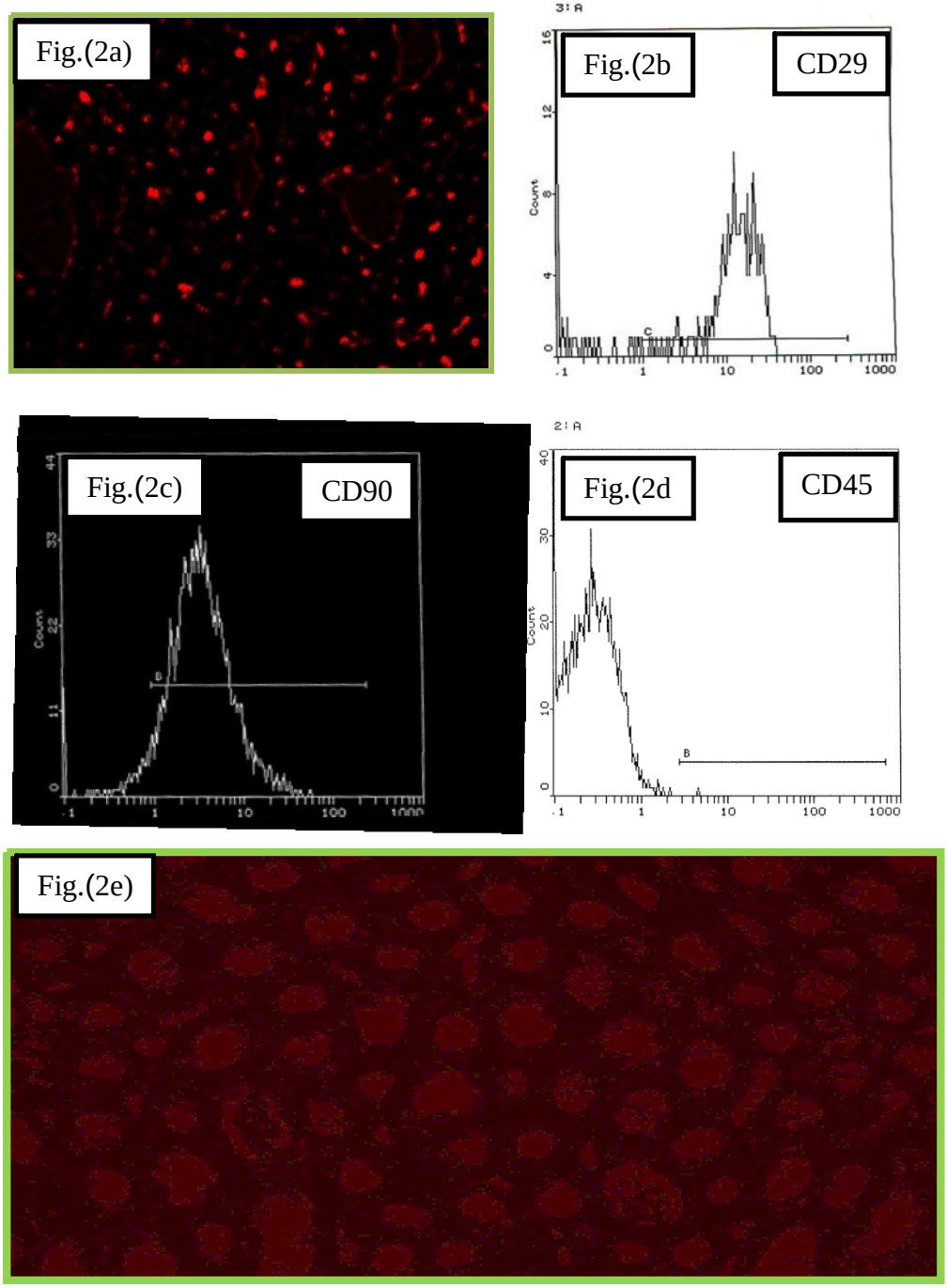

Figure (2): Labeled cells with PKH26 had taken the dye colour under flourecent microscope (2a). Histograms of flowcytometry showed +ve expression of CD29 (2b) and CD90 (2c), and –ve expression of CD45 (2d). (2e): phase contrast micrograph under fluorescent microscope showing homing of labeled cells in the kidney glomeruli.

The cells were examined under fluorescent microscope after labeling with PKH26 dye (Fig.2a). The kidney tissue was examined under fluorescent micro-scope for detection of homing of BM MSCs (Fig.2e).

Immunophenotyping of BM MSCs using flowcytometry showed positive expression of CD29 & CD90, and negative expression of CD45 (Fig2b,c & d).

Effects of BM MSCs on some kidney functions (Table1):

In group I, the mean ± standard deviation of serum creatinine and urea were 1.04 ± 0.05 mg/dl & 35.33±1.6 mg/dl respectively.

In group II, the mean ± standard deviation of serum creatinine and urea were 7.99±0.18 mg/dl & 102.17±5.12 mg/dl respectively. There were significant increases in serum creatinine and urea in group II when compared with group I.

In group III, the mean ± standard deviation of serum creatinine and urea were 7.87±0.25 mg/dl & 103.17±3.97 mg/dl respectively. There were no significant differences in group III when compared with group II.

In group IV, the mean ± standard deviation of serum creatinine and urea were 2.1±0.045 mg/dl & 44.16±2.93 mg/dl respectively. There were significant decreases in serum creatinine and urea in group IV when compared with group II.

Table (1): Changes in serum urea and creatinine (mean ± standard deviation).

Groups / Parameters	*Group (I)*	*Group (II)*	*Group (III)*	*Group (IV)*
Serum creatinine (mg/dl)	**1.04 ± 0.05**	**7.99±0.18 P<0.0001***	**7.87±0.25 P>0.05 ••**	**2.1±0.045 P< 0.0001***
Urea (mg/dl)	**35.33±1.6**	**102.17±5.12 P<0.0001***	**103.17±3.97 P>0.05 ••**	**44.16±2.93 P <0.0001***

Effects of BM MSCs on tissue levels of MDA and Gpx (Fig.3):

In group I, the mean ± standard deviation of tissue levels of MDA and GPx were 10.38±0.6 nmol/g tissue and 53.22±1.44 mU/g tissue respectively.

In group II, the mean ± standard deviation of tissue levels of MDA and GPx were 98.82±2.52 nmol/g tissue and 22.1±1.57 mU/g tissue respectively. There was a significant increase in MDA, and significant decrease in GPx in group II when compared with group I.

In group III, the mean ± standard deviation of tissue levels of MDA and GPx were 99.53±1.87 nmol /g tissue and 23.12±1.91 mU/g tissue respectively. There were no significant differences in group III when compared with group II.

In group IV, the mean ± standard deviation of tissue levels of MDA and GPx were 24.13±2.17 nmol /g tissue and 43.93±2.08 mU/g tissue respectively. There was significant decrease in MDA, and significant increase in GPx in group IV when compared with group II.

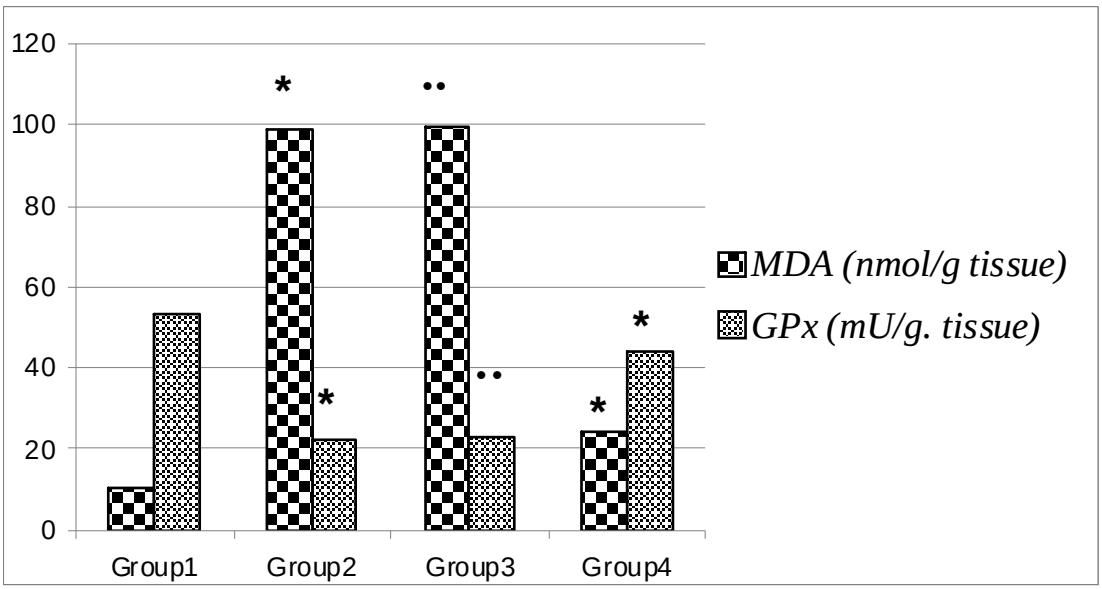

Figure (3): Effects of BM MSCs on tissue levels of MDA and Gpx (Mean±SD).

Histopathological examination:

In group I, kidney tissue showed normal histological structure of the glomeruli and tubules in the cortical portion Fig.(4a). Group II and group III showed distortion of renal architecture in the form of hyperemia with swelling in the lining epithelium of the glomerulus and partial loss of brush border of proximal convoluted tubules as well as necrotic lesions in epithelial lining of urinary tubules (Fig.4b and c), Group IV showed restoration of renal architecture and normal dilatation of renal lumens lined by regenerative cells Fig. (4d).

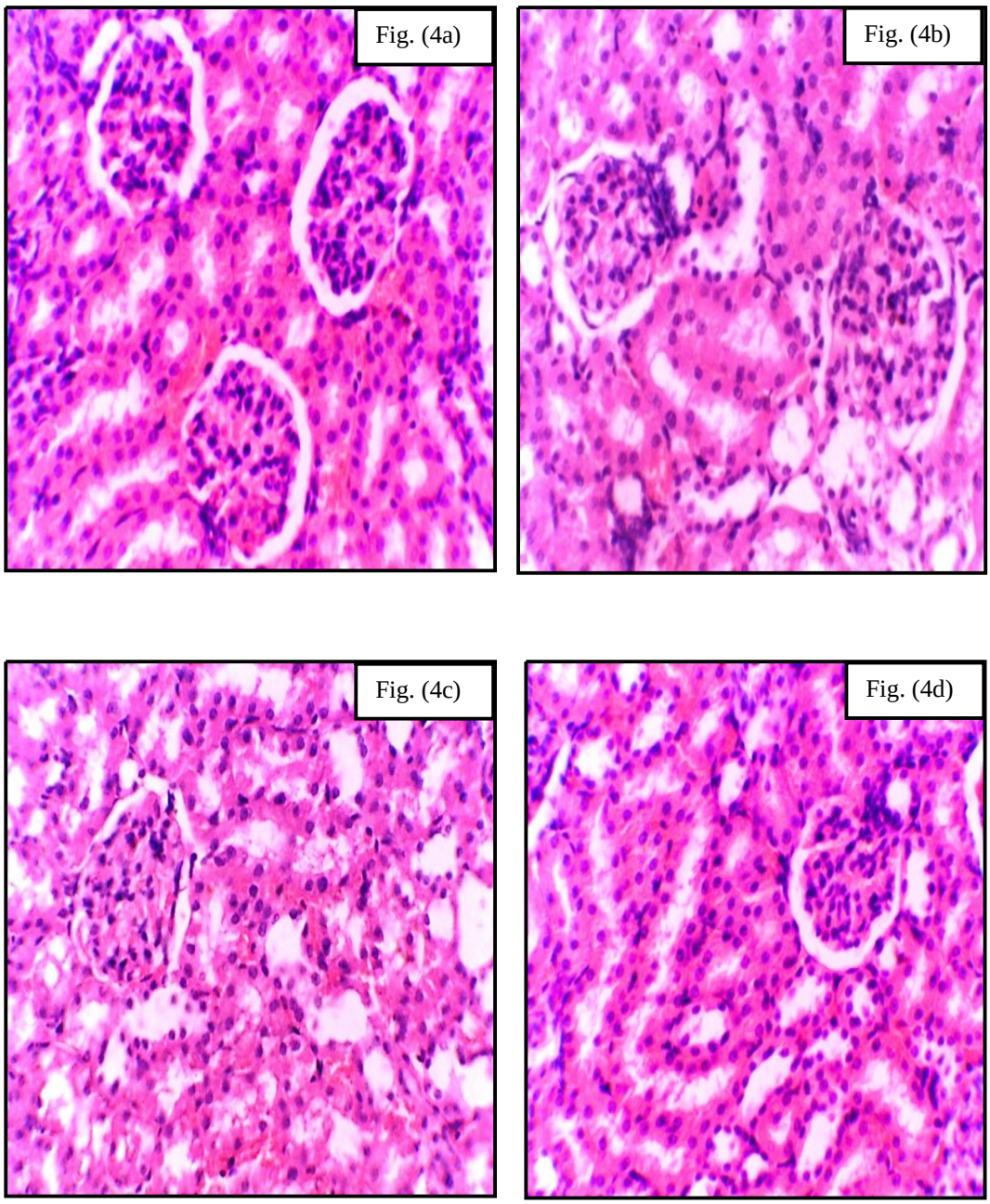

Figure (4): (4a) Kidney tissue in group I Showed normal architecture of glomeruli and kidney tubules. (4b) kidney tissue in group II showed conestion and hypremia of glomeruli and loss of brush border of kidney tubules. (4c) Kidney tissue group III showed Conestion and hypremia of glomeruli and loss of brush border of kidney with lymphocytic infiltration tubules. (4d) Kidney tissue in group IV showed restoration of renal architecture and normal dilatation of kidney Tubules (H& E, X400).

DISCUSSION

The isolated stem cells were identified as MSCs as they exhibited fibroblastic shape, plastic-adherent properties. This was in concomitant with the results of ***Dexter et al. (1981), Tan et al. (2009); Bianco et al. (2013) and Mohapatra et al. (2015)***. Immunophenotyping of the separated cells using flowcytometry showed positive expression of CD29 and CD90 which are characteristically expressed on MSCs, in addition to negative expression of CD45 which were characteristics of hematopoitic stem cells, which prove that the separated cells are MSCs not hematopoietic stem cells. This agreed with the results of ***Bieback et al. (2004) and Song et al. (2014).*** The cells were labeled with PKH26 dye to detect homing of MSCs in the kidney tissues by fluorescent microscope ***(Shah & Mao, 2011 and Abdel Aziz et al., 2014).***

In the 2006, International Society for Cellular Therapy conveyed a set of simple criteria for defining a culture of cells as a culture of MSCs. The criteria were adherence to plastic, expression of CD29, CD44, CD105, CD90 and CD73 receptors; lack of hematopoietic (such as CD14, CD31,CD33, CD34, and CD45) and endothelial markers; in vitro differentiation to cartilage, bone, and fat; chemical induction in nonclonal cultures ***(Mohapatra et al., 2015)***. Based on these criteria, isolation of MSC-like cells has been reported from different tissues other than bone marrow including adipose tissue, umbilical cord, dental pulp, skeletal muscle, periodontal ligament and even brain **(Orbay et al., 2012)**. MSCs also has been isolated from peripheral blood, cord Wharton's jelly, amniotic fluid, compact bone, periosteum, synovial membrane, synovial fluid, articular cartilage, and foetal tissues ***(Augello et al., 2010)***. However, these MSC-like populations are not identical and exhibit differences in their molecular phenotype and differentiation responses ***(Al-Nbaheen et al., 2013)***. Only bone marrow-derived MSCs have documented evidence of stemness including the ability to form bone and bone marrow organ upon serial transplantation in vivo ***(Sacchetti et al., 2007)***. In the current work, mesenchymal stem cells were separated from bone marrow and labeled with PKH26 dye for detection of homing cells in the kidney tissue. ***Shao-Fang et al. (2011***) found that labeling with PKH26 does not yield any differences in morphology, proliferation ability, apoptosis, and cell cycle of human umbilical mesenchymal stem cells, which indicates that PKH26 does not change the physiological activity of cells.

There are many methods to isolate stromal cells from bone marrow including plastic adherence ***(Dexter et al., 1981)***, gradient density centrifugation ***(Chen et al., 1991)*** and immunomagnetic selection which each have their own limitations and advantages ***(Jia et al., 2002 and Dezawa et al., 2004).*** In the current work BM MSCs were separated by plastic adherence method which is an easy method**.**

The renal failure non treated group which was subjected to oral administration of 500 mg/kg of acetaminophen showed significant increase in serum creatinine and urea compared to control group, These results agreed with ***Zaher et al.(2008) and Gopi et al. (2010).*** A reactive metabolite of acetaminophen that

caused oxidative damage to tissues might be the reason for its reno-toxic effects ***(Richie et al., 1992 and Gopi et al., 2010)***. Increased concentration of serum urea and creatinine levels in the serum was taken as the index of nephrotoxicity ***(Bennit et al., 1982 and Anwar et al., 1999).*** Acetaminophen overdose is often linked to many metabolic disorders including serum electrolyte, urea and creatinine derangements. Increased concentration of serum urea and creatinine is considered for investigating drug induced nephrotoxicity in animals and man ***(Ohkawa et al., 1979).***

The significant increase in kidney tissue levels of MDA and decrease in GPx in group II indicated occurrence of oxidative stress in the kidney tissue. Acetaminophen- induced oxidative stress results in lipoperoxidation, protein thiol oxidation, mitochondrial endoplasmic reticulum injury, altered homeotaxis, and irreversible DNA damage characterized by protein adduct formation ***(Sies, 1993; Packer et al., 1995 and Shay et al., 2008).***

MSCs contribute directly to tissue repair and have been shown to be incorporated into organ structures ***(T?gel and Westenfelder, 2011).*** MSCs were demonstrated to contribute to tubular epithelial cell regeneration in mice in a glycerol model of acute kidney injury ***(Herrera et al., 2004).*** Several groups hypothesized that paracrine mechanisms are more likely to explain observed improvements and started identifying factors that might confer renoprotection and regeneration. A number of key factors have been identified so far. Endothelial growth factor has been shown to be directly reno-protective and also has important effects on renal vasculature, which is a key component in the patho-physiological cascade of acute kidney injury **(Togel et al., 2007).** Insulin growth factor -1, a potent proliferative and survival factor that has been shown to be renoprotective when infused as a protein in rats, has been demonstrated to be highly expressed in MSCs, and knockdown of IGF-1 expression limited the protective action of MSCs (**Imberti et al., 2007**).

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خلفية البحث: العلاج بالخلايا الجذعية يعطي أملاً كبيراً في إصلاح الأنسجة والأعضاء التالفة بما فيها الكليتين.

الهدف من البحث: تقييم دور العلاج بالخلايا الجذعية المفصولة من نخاع العظم في تحسين وظائف الكلي في الفشل الكلوي المحدث تجريبيا في ذكور الجرذان البيضاء.

مواد وطرق البحث: استخدم في هذا البحث أربعة وعشرون فأرا أبيضا بالغا من فصيلة محلية و قد تم إحداث الفشل الكلوي بإستخدام مادة الأسيتامينوفين. وفي نهاية التجربة (أربعة وعشرين يوما) تم أخذ عينات من الدم لقياس بعض وظائف الكلي وكذلك تم أخذ عينات من نسيج الكلي لفحص النسيج مجهريا ولعمل بعض القياسات الخاصة بحدوث عملية الأكسدة داخل نسيج الكلي.

النتائج: أحدث الأسيتامينوفين فشلا في وظائف الكلي، بينما أحدثت الخلايا الجذعية رجوعا في المقاييس التي تم دراستها إلي المستويات الطبيعية.

الإستنتاج: العلاج بالخلايا الجذعية المفصولة من نخاع العظم له دور كبير في إلتئام نسيج الكلي في الفشل الكلوي المحدث تجريبياً.