Original Article

Effects of adipose derived mesenchymal stem cells and erythropoietin in testicular torsion-induced the germ cell injury in the adult albino rat

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ABSTRACT

Background: The impairment of spermatogenesis due to a failure in the germ cell proliferation and differentiation is considering one of the major factors of male infertility which is a common complication due to ischemic injury of the testis. So far, even after surgical correction and orchiopexy, there is no an effective method to restore the spermatogenesis. Erythropoietin (EPO) and different types of stem cells were used separately to rescue the testis from this complication.
Aim of the work: To compare the separate and combined effects of erythropoietin and adipose-derived stem cells (AD-MSCs) in the rat testis after the torsion de-torsion (T/D) injury.
Material and methods: A total of sixty adult male albino rats weighing (12-week-old) 200-250 grams were used in this study. They were divided randomly into five groups (10 rats for each group), in addition to 10 rats used as a source for AD-MSCs. The groups were: Group I (Control group): which subdivided into a negative control and Sham operated rats), Group II(torsion detorsion (T/D) group): Torsion of the left testis by rotating the testis 270^{O} in a clockwise direction for 2 hours, followed by detorsion in an anticlockwise direction and then fixed in position till the scarification after 6weeks, Group III (Erythropoietin-treated): The left testes of all rats were exposed to 720^{o} torsion for 2 hours de-torsion and intra testicular injection of 3X106AD-MSCs suspended in 0.5 ml of DMEM as a vehicle. Group IV (MSCs-treated group): The left testes of all rats were exposed to 720o torsion for 2 hours, de-torsion and received a single intra venous injection of erythropoietin 3000 u/Kg. Group V: (MSCs/erythropoietin-treated): The left testes of all rats were exposed to 720o torsion for 2 hours, de-torsion and received a single intra venous injection of erythropoietin 3000 u/Kg in addition to intra testicular injection of 3X106 MSCs suspended in 0.5 ml of DMEM. After the end of the study, all rats from different groups were scarified and the left testicular tissue was obtained, weighed, examined grossly and prepared for a histopathological (a light and an electron microscope) and an immunohistochemical examination. As well, a quantitative assessment of the spermatogenesis was done statistically.
Results: Histopathological examinations showed a severe destruction of seminiferous tubules of the left testes with a failure of the sperm maturation. Also; recognizable abnormalities of the ultrastructure of Sertoli, Leydig cells and all the stages of spermatogenesis in Group II (T/D) were noticed. In addition, there was a significant decrease in the testicular weight and the number of sperms. Affection was also observed but to a lesser degree in Groups III &IV (EPO and AD-MSCs).The greatest amelioration of the tissue damage and the best histological improvement of spermatogenesis, and the testicular weight with a high statistically significance were seen in Group V (EPO and AD-MSC, respectively).
Conclusion: The administration of EPO and AD-MSCs has a great protective effect on the testis and the spermatogenesis. It may be used as a promising therapy after T/D to keep the fertility.

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Key Words: Adipose, erythropoietin, rat, stem cells, testis, torsion de-torsion.

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INTRODUCTION

Tissue ischemia-reperfusion (I/R) may lead to serious pathological changes in theaffected organ. Many organs have been investigated for I/R injuries, such as brain (Lipton, 1999), heart *(Kajstura et al. 1996)*, kidney (Rabb&Postler, 1998), and testis *(Yurner et al. 1997)*. Several mechanisms are encountered in IR injuries, such as neutrophils activation *(Connolly et al. 1996)*, pro-inflammatory cytokine production *(Mitsuti et al. 1999)*, mitochondrial dysfunction *(Jassem et al. 2002)* and production of reactive oxygen species (ROS) (Li and Jacson, 2002). These changes usually result in cell apoptosis leading to organ dysfunction *(Yin et al. 1997)*.

Testicular torsion is one of the most serious urologic emergencies. It begins with a sudden onset and an intractable pain due to cutting off blood flow to the testis. The incidence of testicular torsion is around 1/4,000 of the male population, frequently observed in the newly-born, children and adolescents *(Mansbach et al. 2005)*. Testicular injury is correlated with the degree of cord twisting and duration of testicular ischemia (Visser&Heyns, 2003). Permanent testicular damage can be avoided by the early diagnosis and the immediate surgical intervention. Misdiagnosis or a delay in the intervention usually followed by male infertility due to testicular necrosis, and impaired spermatogenesis. In 36% of patients suffered from testicular torsion, sperm counts was less than 20 million/ml (*Perotti et al. 2006* and Drlik&, Kocvara, 2013).

Erythropoietin (EPO) is a glycoprotein induces the erythrocytes formation in the bone marrow and it is used in the treatment of anemia. Many studies reported the useful roles of exogenous EPO administration on I/R of lung, eye, kidney, spinal cord in animals *(Akeora et al. 2007)*. The functional EPO receptors (EPO-R) were found in the rat and human placenta, brain, and kidney cells. The expression of EPO mRNA was increased in hypoxic testis tissue *(Akeora et al. 2007)*. It has been reported that EPO stimulates testosterone production in the rat by its effect on Leydig cells steroidogenesis. Intravenous injection of EPO increases testosterone in patients complaining of renal failure *(Yamamoto et al. 1997)*.

Stem cells are non-differentiated cells that have the capacity to proliferate, regenerate, and transform into differentiated cells. When mesenchymal stem cells (MSCs) administrated systemically, it will reach to the injured organ and differentiate into new cells similar to the tissue *(Larijani et al. 2012)*. Interestingly, several experimental studies have established that MSCs has potential therapeutic effects on acute ischemic injuries such as acute myocardial infarction *(Hare et al. 2009)*, traumatic brain injury *(Wang et al. 2015)*, and acute liver failure (Volarevic et al. 2014).Rare studies have been reported about MSCs as a therapy for acute ischemic germ cell injury after testicular torsion *(Hsiao et al. 2015)*.

The use of adipose-derived (AD-MSCs) has more advantages than those of bone marrow origin with the former showing more potent anti-inflammatory and immuno-modulating functions *(Banas et al. 2008)*.

In the light of these data, the aim of this study is to evaluate the possible therapeutic effects of EPO and AD-MSCs on I/R injury in the rat model subjected to torsion detorsion of the spermatic cord.

MATERIAL AND METHODS

Animals: A total of sixty Sprague- Dawley adult male albino rats (12-week-old) weighing 200-250 grams each were housed according to the guidelines of the medical research center (Ain Shams University). Water and food were available ad libitum in plastic cages at all times in addition to the continuous veterinary care. Ten rats of them were used as a donner for the adipose tissue, while the others were randomly allocated into 5 equal groups of 10 rats each.

Experimental design:

Group I (Control): This group subdivided into two subgroups:-

Negative control: Rats only received distilled water in a dose of 0.5ml/ rat/ day.

Sham control: the left testes of all rats were manipulated gently through lower abdominal incisions. After 6 weeks they were dissected and prepared for histological sections.

Group II: (Torsion detorsion T/D): The left testes of all rats were exposed to 720 o the torsion in a clockwise direction for 2 hours, de-torsion and intra testicular injection of 0.5 ml DMEM (Dulbecco's Modified Eagle Medium)

Group III (Erythropoietin-treated): The left testes of all rats were exposed to 720o torsion for 2 hours, de-torsion and received a single intra venous injection of erythropoietin 3000 u/Kg *(Rashed et al.2013)*.

Group IV (MSCs-treated): The left testes of all rats were exposed to 720o torsion for 2 hours de-torsion and intra testicular injection of 3X106 AD-MSCs suspended in 0.5 ml of DMEM as a vehicle *(Hsiao et al. 2015)*.

Group V: (MSCs/erythropoietin-treated): The left testes of all rats were exposed to 720° torsion for 2 hours, de-torsion and received a single intra venous injection of erythropoietin 3000 u/Kg in addition to intra testicular injection of 3X106 MSCs suspended in 0.5 ml of DMEM.

After 6 weeks all the animals in the last 4 groups were sacrificed with overdose of sodium pentobarbital, and left sided orchiectomies were performed for histological examinations.

Testicular Torsion and De-torsion:

All procedures were approved by the medical research center, Ain Shams University and are in line with international standards for animal experimentation. The rats were anesthetized with an intraperitoneal injection of sodium Phenobarbital (20 mg/kg). The inguinal area was shaved well; the skin of the lower abdomen and scrotum was incised. The coverings of the left testis were incised genital and the testis was delivered outside them. 720o torsion was performed clockwise and the testis was fixed in position by one stitch to the scrotum to prevent spontaneous de-torsion (fig.1).The exposed testes were covered by wet dressing to prevent their dryness. After 2 hours de-torsion and orchiopexy were done *(Ozbal et al. 2012 and Rashed et al. 2013)*.The rats received analgesics and antibiotics during the 5 days following the operation.

Collection of the Prerenal and Inguinal Fat:

Incision of the anterior abdominal wall skin along the midline took place and exposure of the inguinal fat was performed. The inguinal fats were collected in a sterile falcon tube containing complete media (DMEM supplemented by 10% fetal bovine serum and 1%penicilline/ streptomycin, all from Sigma Aldrich). The anterior abdominal wall muscles were performed longitudinally along the mid line also then they were reflected to both sides and complete exposure to both kidneys and the pre-renal fat was achieved. Pre-renal fat was collected carefully in a sterile Falcon tubes containing complete medium (figs. 2A&2B) *(Niyaz et al. 2012)*.

Isolation, Culture and Subculture of Adipose Tissue Derived Mesenchymal Stem Cells (AD-MSCs):

The collected inguinal and pre-renal fats were washed 4 times with phosphate buffered saline(PBS) (Sigma Aldrich), dissected into fine fragments and immersed in collagenase type I 0.1% (Sigma Aldrich) (0.1 g collagenase dissolved in 100 ml PBS) then kept in a shaking water path adjusted to 80r/min and 37oC for 20-30 minutes.Several vigorous pipetting was performed during this period to assure complete dissociation of cells. Sieving of this suspension took place with 70µ stainless steel mesh (Sigma) to prevent clusters and large fragments. The complete medium was added to the suspension to prevent enzymatic digestion of the cells by collagen ase. Centrifugation of the suspension was then performed at 4°C and 1800 rpm for 10 minutes to separate the vascular-stromal fraction. Then the vascular-stromal fraction re-suspended and plated in at a density of 35 mm tissue culture flask at a density of 1×10^6 / flask in complete medium (figs.2C, 2D& 2E).By the 3rd day of the culture the medium was exchanges to get rid of non-adherent cells. The adherent cells were returned to the incubator and left to be confluent with medium changes every 3 days. When the primary cultured cell was sub-confluent the culture was trypsinised using 0. 25% trypsin/ EDTA (Gibco, South America), washed and re-plated at a density of 3.5x105 cells/75cm^2 flask for a passage 1 "P1". The same steps were done for P2- P5 *(Maumus et al. 2011)*.

Characterization of AD-MSCs:

Immunophenotypic analysis of AD-MSCs was performed with a flow cytometry. Rat AD-MSCs from P3 to P5 passages were trypsinized, fixed in a neutral 4% paraformaldehyde solution for 30 min in a cell concentration of $10X10^6$ cell / ml. The fixed cells were washed twice with PBS and incubated with FITC coupled antibodies against the rat CD34 and CD105 (AbDSerotec) in the dark at room temperature for 30 min. The rat AD MSCs were fixed with paraformaldehyde for 15 min after cells were washed with PBS. The flow cytometer was used to analyse the samples for the incidence of expression the mentioned markers *(Pawitan et al., 2013)*.

Intra-testicular Injection of AD-MSCs:

Cells from P3-P5 were trypsinized, washed and re-suspended in DMEM. 0.5 ml of the suspension containing $3X10^6$ cell was injected intra-testicular in the left testis of each rat of the MSCs- treated group and MSCs/ erythropoietin treated group half an hour after the testicular detorsion *(Hsiao et al. 2015)*.

Analysis of Testicular Weight, Length and Width:

At the time of euthanasia the left testis was compared grossly to the right one and the left testis weights were measured without removing the tunica. The length and width of testis were estimated using a Vernier caliper.

Histopathological Evaluation:

The left testicular tissue samples were fixed in 10% formalin in phosphate buffer for 5 days. Paraffin blocks were prepared using routine histological methods and sections of 5 µm thickness were obtained. After deparaffinization and rehydration, the sections were stained with hematoxylin-eosin (H&E).

Immunohistochemical Evaluation:

After deparaffinization and rehydration, sections of the testis were then treated with 10mM citrate buffer (Cat No. AP-9003-125 Lab Vision) for 5minutes. Then, the sections were washed with PBS and incubated in a solution of 3% H2O2 for 5min at room temperature to inhibit endogenous peroxidase activity. After washing with PBS sections were incubated with normal serum blocking solution at 37◦C for 30 min. Sections were again incubated in a humid chamber, +4◦C with rat monoclonal antibody against CD 105, thereafter with biotinylated IgG, and then with streptavidin conjugated to horseradish peroxidase at 37◦C for 30min each prepared according to kit instructions (Invitrogen-Plus Broad Spectrum 85-9043). The sections were finally stained with DAB (Roche Diagnostics, Mannheim, Germany) and counterstained by hematoxylin and analyzed by using a light microscope.

Transmission Electron Microscopy:

At the end of the study small fragments of the left testis were fixed for 1 h in a freshly prepared 2% paraformaldehyde, 2% glutaraldehyde in 0.05 mol/l sodium cacodylate, pH 7.2 (Sigma). Following fixation, pellets were washed in cacodylate buffer and post fixed for 1 h in 4% freshly made osmium tetroxide. After rinsing in water, pellets were stained in bloc in 1% uranyl acetate for 40 min. Samples were then dehydrated through a graded series of alcohol, transferred to propylene oxide, and infiltrated with epon. Sections of 90 nm were cut (Reichert Ultracut, Ziess, Germany), mounted, and stained with lead acetate and uranyl acetate. Sections were examined using a TEM 10 transmission electron microscope (Zeiss, Jena, Germany).

Sperm Count:

The left epididymis was dissected and macerated in 5 ml of PBS, and left at 4ºC for two hours. Thereafter, the suspension was filtered by a disposable mesh, 0.100 µm, and the cell count was performed using a hemocytometer *(Vargas et al.2011)*.

Statistical Analysis: The analysis of data was done with SPSS 16.0. P-values <0.05 were regarded as a statistically significant. One way Analysis of Variance (ANOVA) test was performed and post hoc multiple comparisons were done with least-squares differences (LSD).

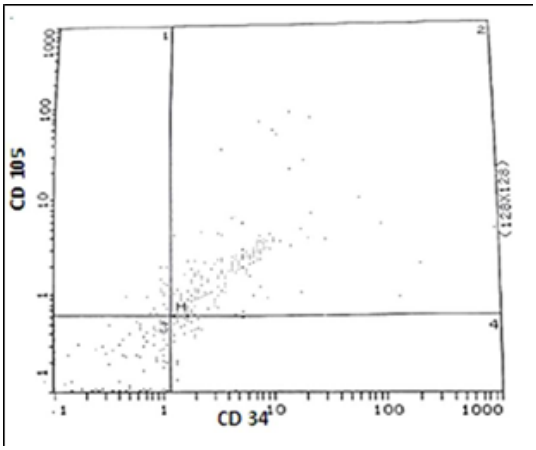
RESULTS

Culture and sub-culture of AD-MSCs: MSCs attached to the culture flasks sparsely 3 days after culture and displayed different morphological character during the initial days of incubation. Cells reached confluency within the 2nd week post seeding. The proliferation capacity and tendency of the cells to grow into small colonies were seen during the 1st week of primary cultue and onward till the end of the 5th passage. During the later passages MSCs exhibited a large, flattened fibroblast-like morphology with a well defined cytoplasmic membrane and a centerally located single nucleus when stained with Giemsa (Figs.3&4). The viability of cells was testedby Trypan blue before transplantation and found to be 95±2%.

Flow Cytometric Analysis:

For characterization of the cultured cells before their injection flow cytometric analysis of expression of CD 34 and CD 105 was performed and revealed that very few cells (<20%) expressed the hematopoietic marker, CD 34 and most of them expressed the mesenchymal stem cell marker, CD 105 (>80%) (Histogram-1).

Histogram1: Flow cytometric analysis of adipose tissue derived stem cells



Flowcytometric analysis of adipose tissue derived stem cells showed that most of the cells expressed CD 105 (>80%) and very few cells (<20%) expressed CD 34 marker.

Gross Morphology, Testis weight and Volume: Naked eye examination of the left testis was performed comparing them of the right testis of the same animals. The testis of the T/D group showed marked atrophy and change of color, consistency and outline while that of the treated groups restored their consistency, color and outline with many blood vessels seen on their surfaces (Fig.s 5; 5A-5E). The weight and volume (length and width) of the T/D group showed a significant decrease in comparison to the control group while the treated groups showed an increase in the weight and the volume of the left testis in comparison to the T/D group with a high significance in the group treated with both erythropoietin and stem cells (Table).

Histopathological Evaluation

Light microscopic examination of sections of the testes of the control adult albino rats showed that the testicular parenchyma was formed of densely packed seminiferous tubules lined by stratified germinal epithelium with all stages of spermatogenesis were evident,spermatogonia in the outer layer, the spermatocytes in the middle and the spermatozoa and spermatids protruding toward the lumen. The seminiferous tubules were separated by a narrow interstitium containing clusters of interstitial cells and blood vessels (Fig. 6-A).

The testes of T/D rats revealed that the seminiferous tubules were distorted, and separated by a very wide interstitium. Reduction in the thickness of the germinal epithelium was observed in some tubules. The germ cells decreased markedly in number and were vacuolated andseparated from each other. The widened interstitium contained homogenous acidophilic material and haemorrhage (Fig. 6-B).

The testes of erythropiotein-treated T/D adult albino rats showed that the testicular parenchyma was formed of apparently normal in some seminiferous tubules. Some tubules were lined by well-formed germinal epithelium with narrow interstitium but some showed increased and condensed germinal cells and parts were in full spermatogenesis. In another tubules, the germinal epithelium was separated from the basement membrane. Some interstitium was wide, hemorhagic and showed thickness of its blood veseles (Fig. 6-C).

The testes of stem cells-treated T/D adult albino rats showed that the testicular parenchyma looks like the normal. few seminiferous tubules showed exfoliated germ cells in the lumen and arrested spermatogenesis. In another tubules, the germinal epithelium was separated from the basement membrane. Some interstitium showed degenration of its cells, another showed increased thickness of its blood veseles (Fig. 6-D.

The testes of both stem cells and erythropiotein -treated T/D adult albino rats showed that the testicular parenchyma was nearly like the normal ones with full spermatogenesis. Some seminiferous tubules showed exfoliated germ cells in the lumen. Some interstitium was dilated and hemorhagic (Fig. 6-E).

Immunohistochemical analysis of testicular tissue:

CD-105 was used as a marker of MSCs. Seven weeks after intravenous injection of the MSCs, immunohistochemical staining of the testicular tissue using CD-105 antibody revealed a marked increase of positively stained cells in the testes of both stem cells and erythropiotein erythropoietin -treated T/D group, within the seminiferous tubules and interstitial spaces. Erythropoietin-treated group and stem cells-treated groups showed moderate expression of the same cells with preservation of normal architecture of the testis in stem cell-treated group and its loss in erythropoietin-treated group. There was no evidence of positively stained cells in the control and T/D groups (Fig.7).

Results of Transmission Electron Microscopy: The testes of the control group (Group II) revealed

that; Sertoli cells had large euchromatic nuclei and prominent nucleoli. Spermatogonia appeared with rounded nuclei resting on a regular basement membrane. Primary spermatocyte had rounded nuclei and a thin rim of cytoplasm. Spermatids had rounded euchromatic nuclei and numerous peripherally arranged mitochondria. Some of them had acrosomal caps. Small electron-dense acrosomal granules were seen at its cytoplasm. Cross sections in the mid, principal, and end pieces of the sperms showed a central axoneme formed of many microtubules with a central and a peripheral cell membranes . The axoneme of the mid pieces was surrounded by electron-dense bundles of fibrous sheath and mitochondrial sheath, whereas in the principal pieces, it was surrounded by fibrous sheath only. Leydig cells' nuclei appeared euchromatic with peripheral heterochromatin. Their cytoplasm contained mitochondria, and lipid droplets (Figs. 8A-8D).

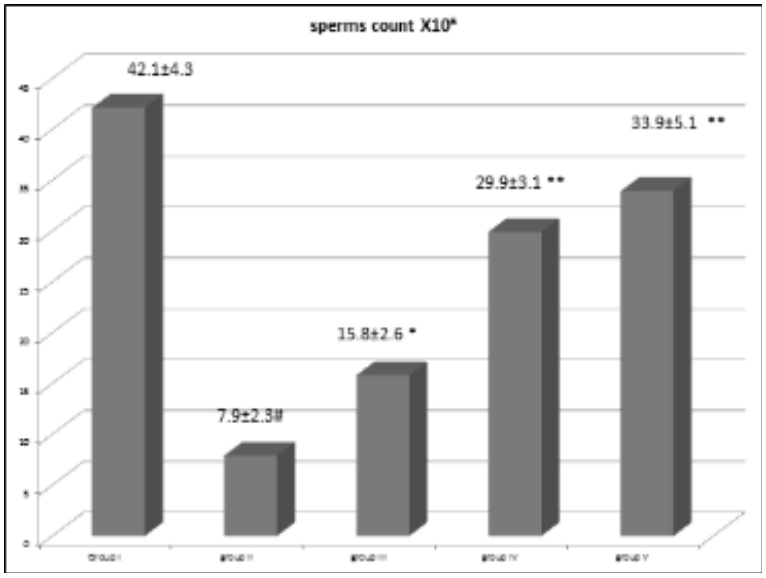
The testes of the torsion/detorsion group (Group II) showed many wide intercellular spaces between the germinal epithelial lining of the seminiferous tubules. Sertoli cells with irregular euchromatic nuclei were observed and their cytoplasm contained mitochondria with abnormal ruptured cisternae. They rested on thickened basement membrane with marked irregularity. Spermatogonia appeared with irregular nuclei. Primary spermatocytes with irregular nuclei with invagination of its nuclear membrane were observed. Some spermatids had small shrunken and distorted heterochromatic nuclei with invaginated wall and its cytoplasm contain swollen mitochondria. Other had a distorted acrosomal cap. Cross sections in the mid pieces of sperms showed marked distortion of the central axoneme, the fibrous sheath, and the mitochondrial sheath. Leydig cells had irregular euchromatic nuclei with peripheral heterochromatin, lipid droplets, and variable-size electron-dense bodies. (Figs. 9; 9A-9E).

The testes of the erythropoietin torsion / detorsion treated group (Group III) showed many small spaces between different types of germ cells. Sertoli cells had large euchromatic nuclei and its cytoplasm contains many spaces. Primary spermatocyte resembles the normal. Spermatids had irregular shape and size but euchromatic nuclei with peripherally spaces in its cytoplasm. Others showed distorted acrosomal caps. Cross sections of the mid pieces of the sperms showed normal central axoneme distorted both mitochondrial and fibrous sheathes. The nuclei of Leydig cells appeared euchromatic with peripheral heterochromatin and showed nuclear invagination. Their cytoplasm contained mitochondria, lipid droplets and variable-size electron-dense bodies (Figs.10; A-D).

The testes of the stem cells torsion / detorsion treated group (Group IV) showed fewer intercellular spaces between different types of germ cells in comparison to the previous group. Sertoli cells had large euchromatic nuclei and its cytoplasm contains few spaces and variable-size electron-dense bodies. Spermatogonia appeared with rounded nuclei resting on a well-defined regular basement membrane. Primary spermatocyte had rounded nuclei and a thin rim of cytoplasm. Primary spermatocyte resembles the normal. Spermatids had rounded euchromatic nuclei with well-defined acrosomal caps. Cross sections of the mid pieces of the sperms still showed distortion in both mitochondrial and fibrous sheathes. The nuclei of Leydig cells appeared euchromatic with condensed peripheral heterochromatin. Their cytoplasm contained lipid droplets and many variable-sized electron-dense bodies (Figs.11; 11A-11E).

The testes of combined stem cells and erythropoietin torsion / detorsion treated group (Group V) showed very few number of intercellular spaces between different types of germ cells in comparison to erythropoietin or stem cells alone groups. The histological structures of different spermatogenic cells, including spermatogonia, primary spermatocytes, and spermatids greatly resemble the normal. Sertoli cells had large euchromatic nuclei and prominent nucleoli. Spermatogonia appeared with rounded nuclei resting on a regular basement membrane. Primary spermatocytes had rounded nuclei and a thin rim of cytoplasm. Spermatids had rounded euchromatic nuclei with well-defined acrosomal caps and its cytoplasm contains mitochondria. Cross sections of the mid pieces of the sperms showed only distortion in fibrous sheathes. The nuclei of Leydig cells appeared euchromatic with peripheral heterochromatin. Their cytoplasm contained mitochondria (Figs.12; A-E).

Sperms count: The number of sperms of the torsion/de-torsion group showed a significant decrease in comparison to the negative control group ($P<0.001$) while in the treated groups the sperms count increased with various significant in comparison to the torsion/de-torsion group. The maximum increase was observed in MSCs/ erythropoietin-treated group but there was no significant difference between the stem cell treated group and the erythropoietin treated group (Histogram 2).



Histogram 2: : A bar chart showing sperms count in five groups of torsion/de-torsion injury in rats expressed in Mean ±SD shows highly significant decrease in sperm count in T/D group in comparison to the control group and a significant increase in their number in the remaining 3 groups in comparison to the control with different significance. #: p<0.001. **: P< 0.01 *: P<0.05

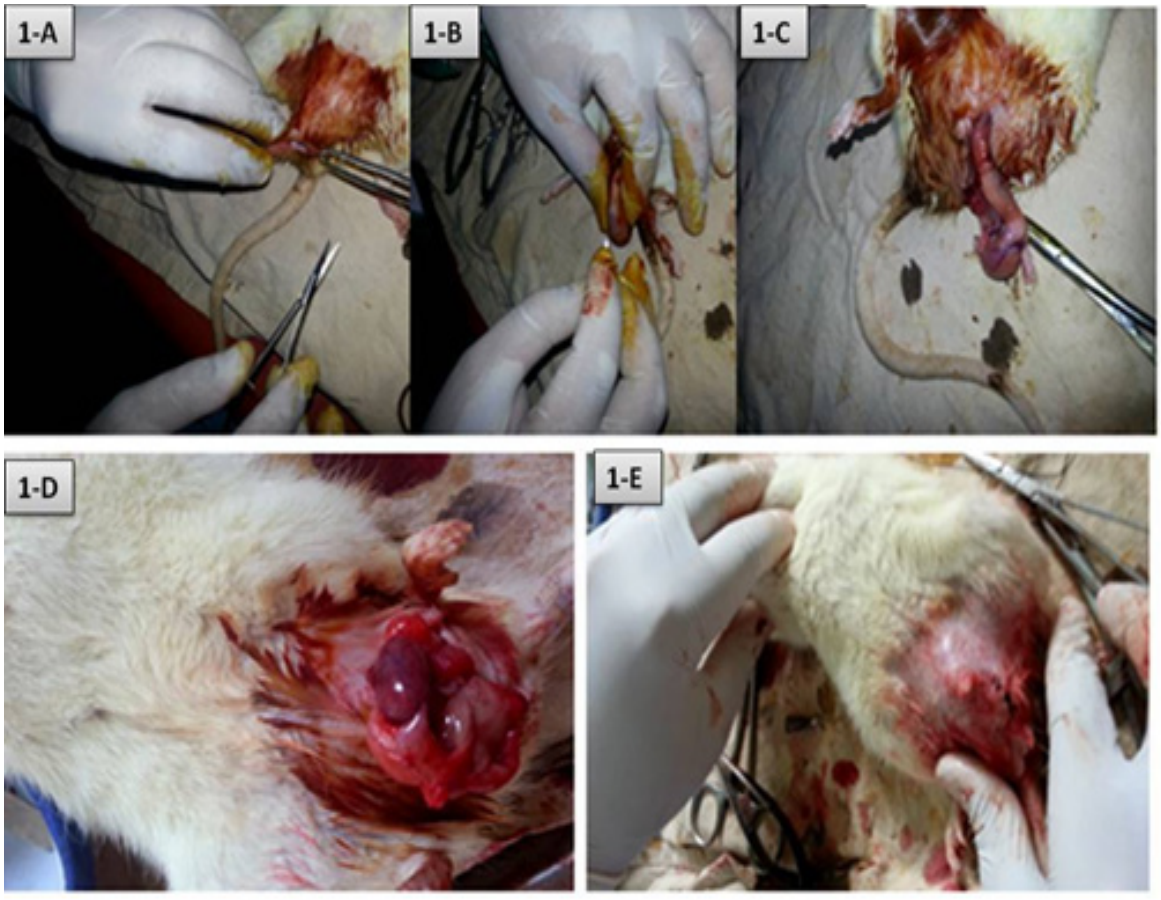


Fig.1 (1A-1E): Photographs showing the rat testicular torsion and de-torsion. 1A&1B: Incision of the covering of the testis. 1C: Delivery of the testis and the epididymis outside their coverings. 1D: Congestion of the testis after 2 hours of its torsion. 1E: Orchiopexy and sutures of the scrotum.

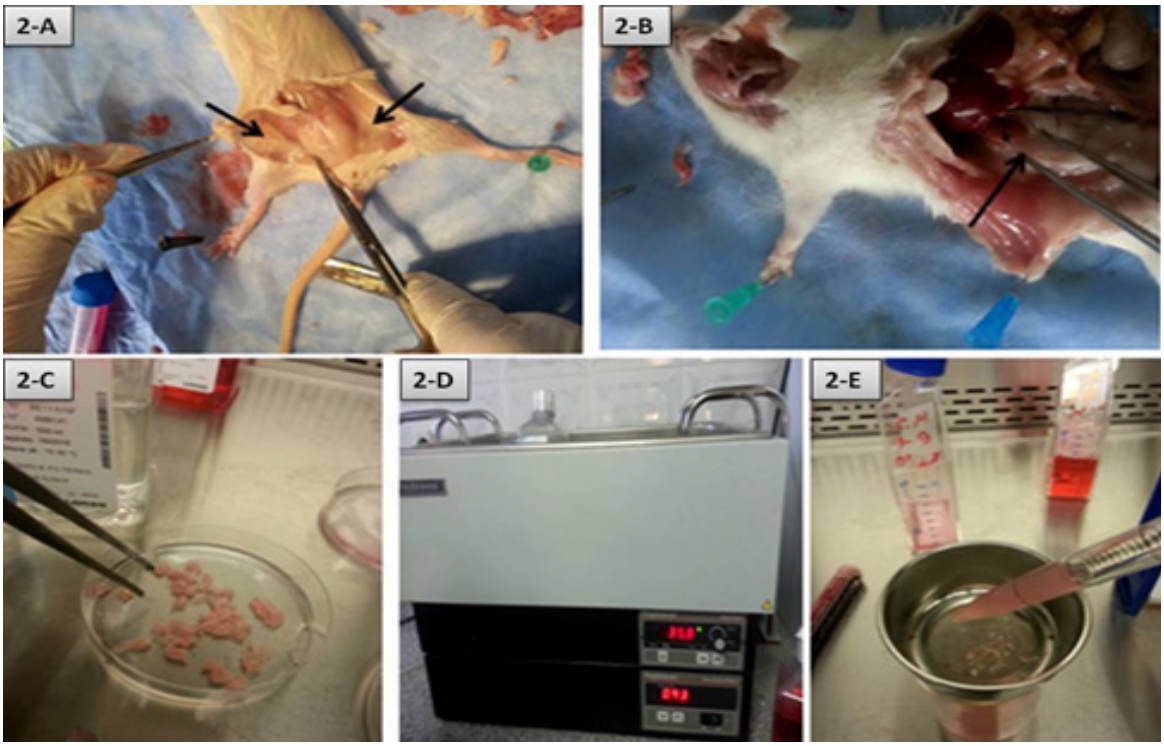


Fig.2 (2A-2E): Photograph showing isolation of the vascular-stromal fraction of adipose tissue. 2A: Excision of the inguinal fat. 2B: Collection of prerenal fat (↑)(k; kidney).2C: Fat fragments being washed and dissected before enzymatic digestion. 2D: Enzymatic digestion of the adipose tissue in a shaker water path with adjustable rotation number and temperature. 2E: Sieving of the suspension to prevent clusters.

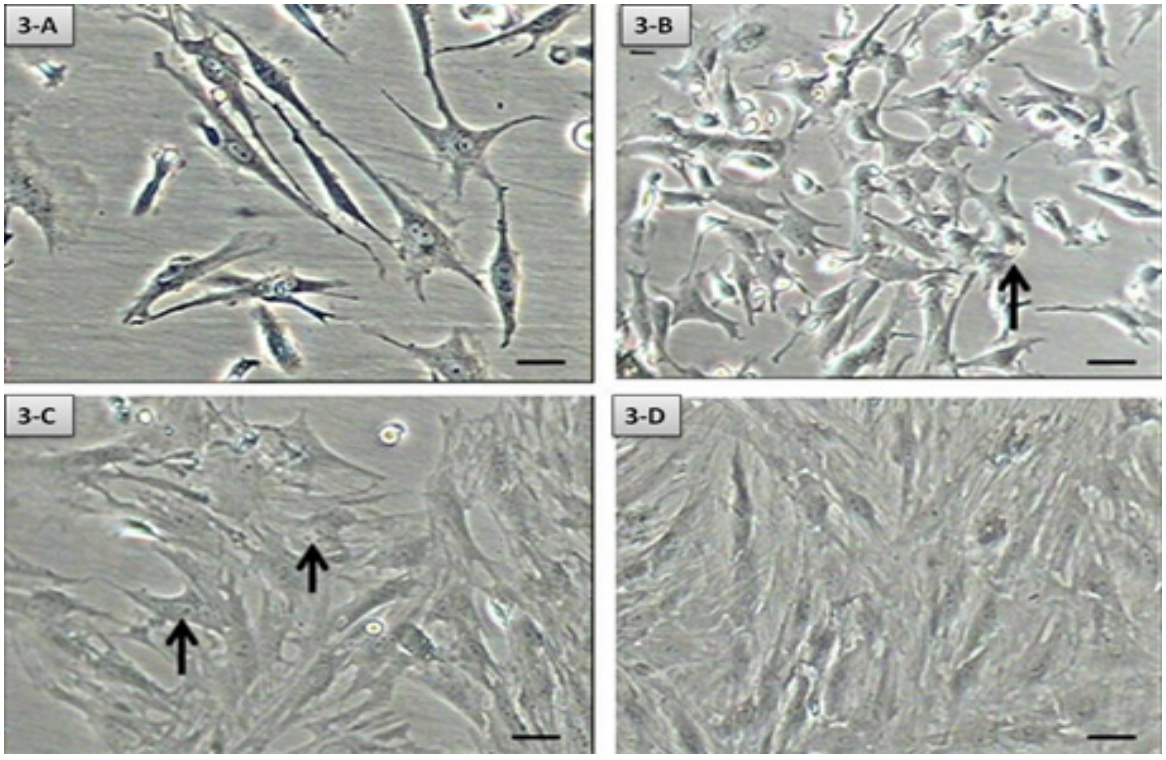


Fig.3 (3A-4D): A phase contrast of rat AD-MSCs in primary culture. 3A, 3B and 3C: showed the cells 3, 5,7 and 10 days after seeding respectively. The cells were polymorphic with centrally located nucleus (↑) and reached confluence within the 10th day after seeding (3D). Scale bar 40 µm X200.

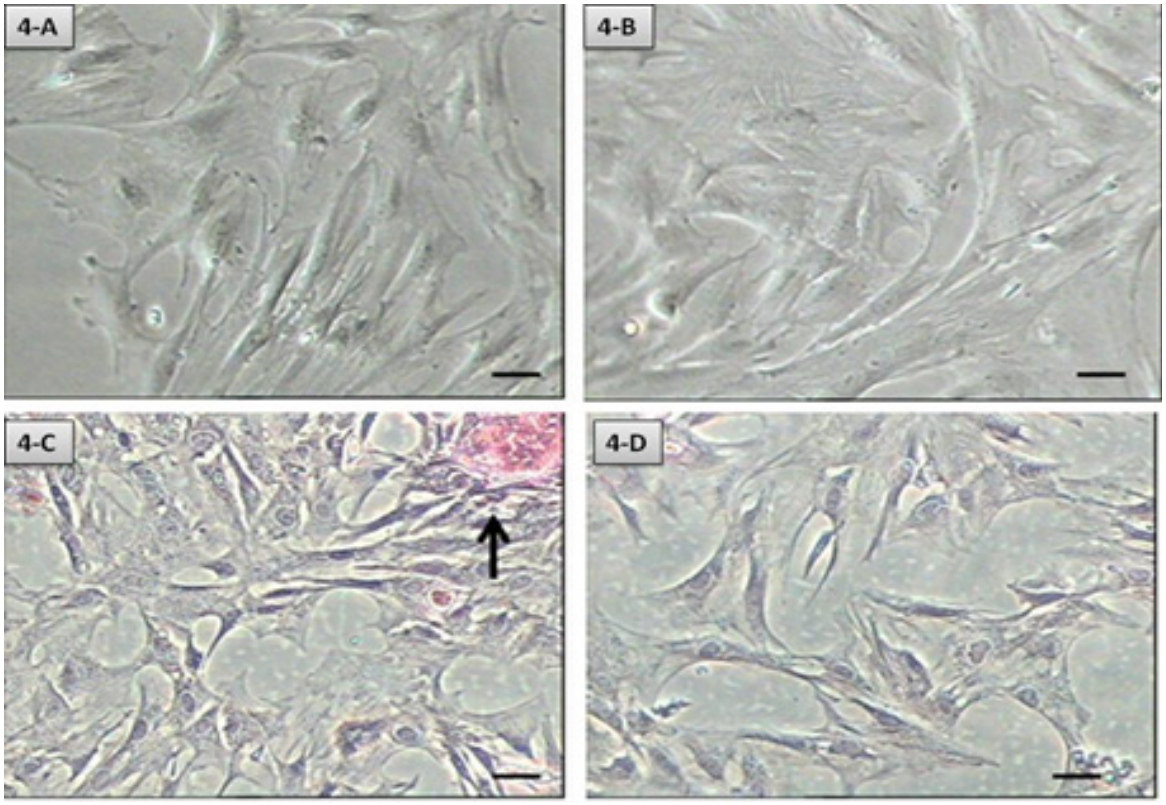


Fig.4 (4A-4D): A phase contrast of rat AD-MSCs during the 3rd passage (4A&4B) stained with geimsa (4C&4D). The cells displayed a single phenotypic morphology, fibroblast-like. Colonies were evident (↑). The cells had well defined borders and central rounded nuclei when stained with a geimsa. Scale bar 40 µm X200.

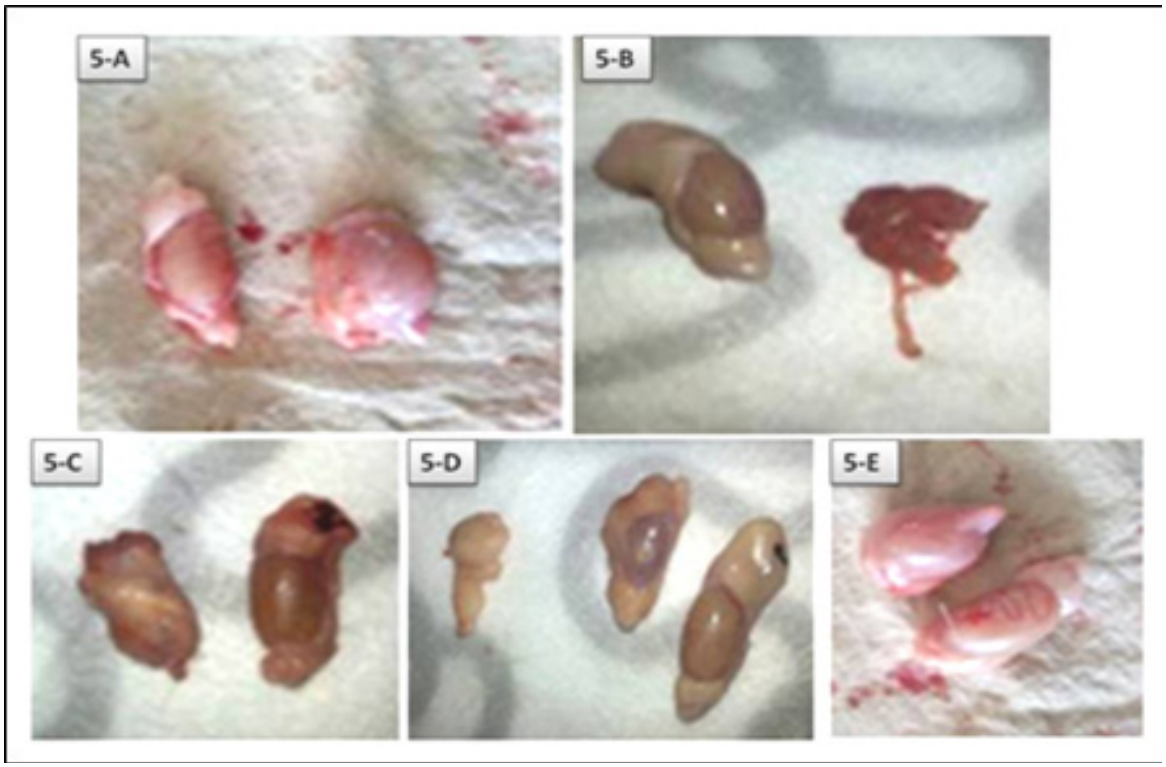
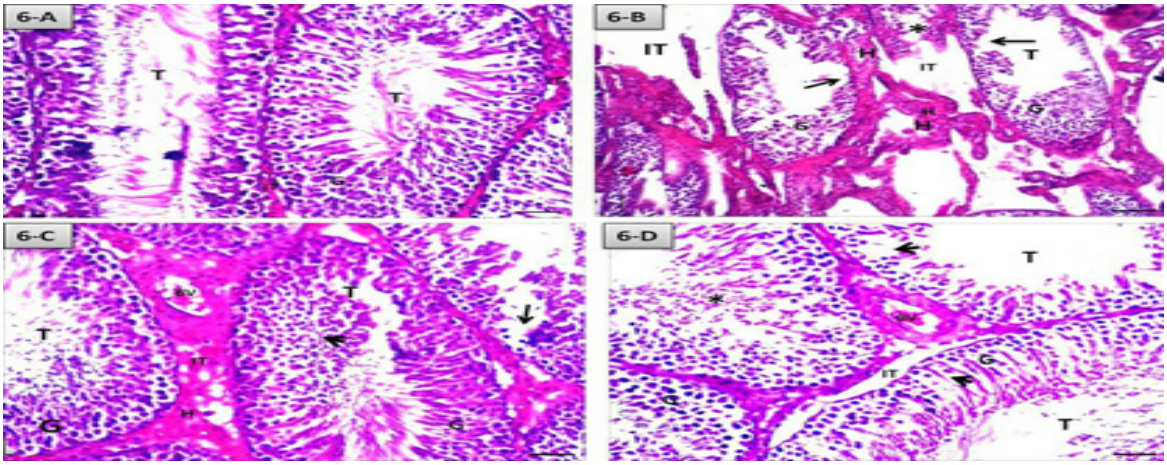


Fig. 5(5A-5E): Photographs showing the gross morphology of the left testis in comparison to the right one in the same animal in the T/D and treated groups. 5A: Group I. 5B: Group II. 5C: Group III. 5D: Group IV. 5E: Group V. Marked atrophy and a change of the color, consistency and outline were observed in the T/D group.



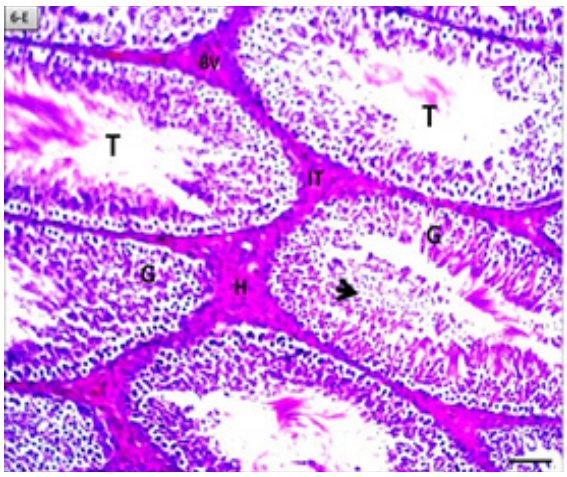


Fig.6 (6A-6E): Photomicrographs of control (Group I) rat testis shows; (6A): The testicular parenchyma contains semineferous tubules (T) lined by stratified germinal epithelium (G) and are separated by a narrow interstitium (IT) containing clusters of interstitial cells. (6B): Testis of Group II shows distorted semineferous tubules (T), with reduction of thickness of the germinal epithelium (G) containing vacuoles and separated from each other (←). The interstitium is very wide contained homogenous acidophilic material and hemorrhage (H). (6C): A testis of Group II rat showing apparently normal semineferous tubules (T) lined by well-formed germinal epithelium (G) some showed increased and condensed germinal cells and parts were in full spermatogenesis (↓). In other tubules, the germinal epithelium was separated from the basement membrane (↓). Some interstitium was dilated (IT), haemorrhagic (H) and showed thickness of its blood vessels (BV). (6D): Testis of Group IV showing exfoliated germ cells in the lumen of tubules (*). Some interstitium (IT) showed degeneration of its cells (→), thickness of its blood vessels (BV).(6E): Testis of Group V, seminiferous tubules(T) showed exfoliated germ cells in the lumen(↓), wide and haemorrhagic (H) interstitium (IT) (H&E. x 200).Scale bar 100µm

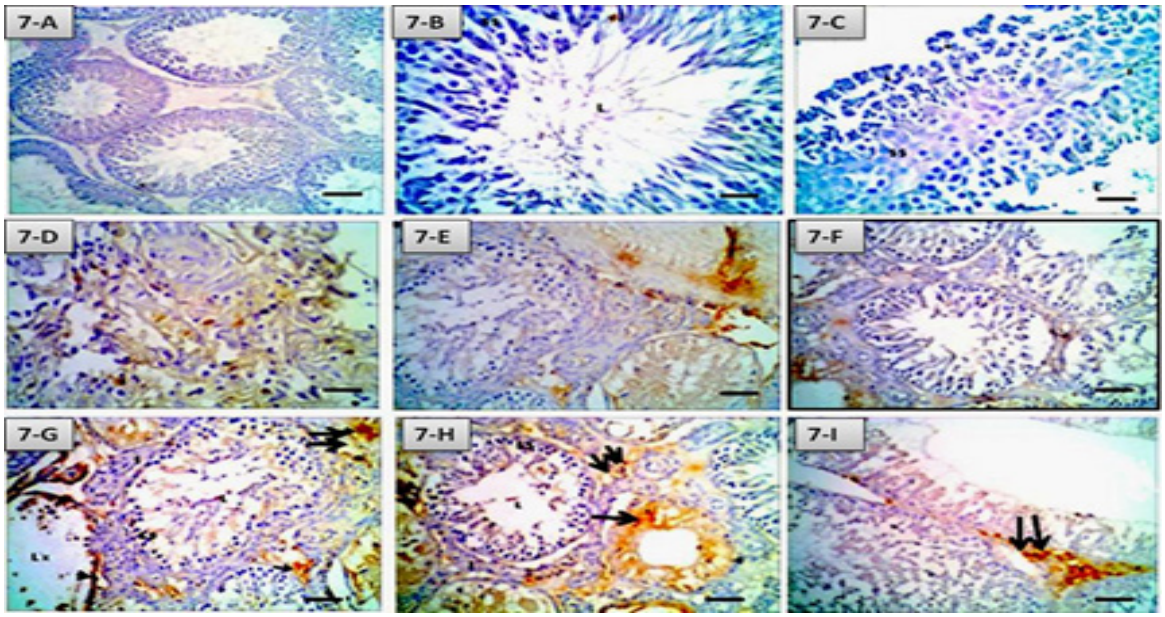


Fig. 7(7A-7I): Photographs of the rat testicular tissue 7 weeks after T/D injury, immuno-histochemically stained with anti CD-105. Group I (7A&7B) and Group II (7C) showed no evidence of positively stained cells. Group III & IV showed a moderate expression of CD-105 positively stained cells with loss of normal testicular architecture in the former (D) and slight restoration of it in the latter group (7E& 7F) respectively. Group V (G, H&I) showed a marked expression of CD-105 positively stained cells both in the seminiferous tubules (↑) and in the interstitial spaces (↑↑). Counter stains Hematoxylin X 200, scale bar 100µm. B, C&I; scale bar 20 µm X400.

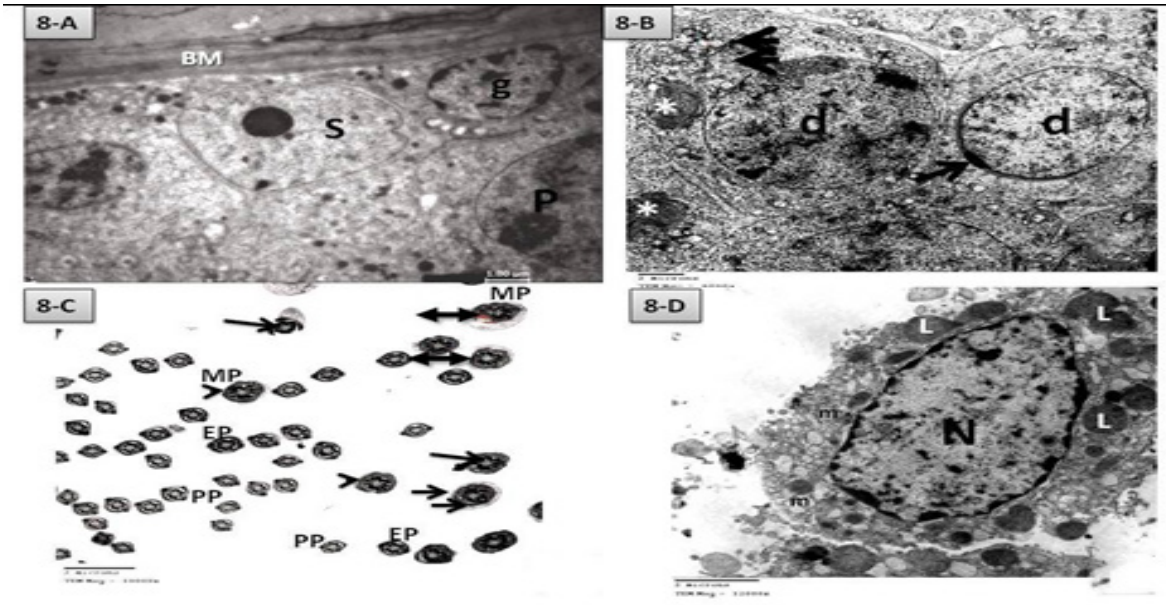


Fig. 8(8A-8D): Group I rat's testis showing; (A); Sertoli cell has a pale euchromatic nucleus with prominent nucleolus (S). Spermatogonia (g) contains rounded nucleus with peripheral heterochromatin clumps resting on a regular basement membrane (BM). Part of Primary spermatocyte (P) is appeared. (B); Spermatids (d) that contain rounded euchromatic nuclei and peripherally arranged mitochondria and acrosomic granules are also seen (white strikes). One of the Spermatids has an acrosomal cap (↑). (C); Cross sections in the mid pieces (MP), principal pieces (PP), and end pieces (EP) pieces of the sperms. All pieces have a central axoneme (►) and peripheral cell membrane (→). In the mid pieces, the axoneme is surrounded by a fibrous sheath (↔) and mitochondrial sheath (2→). The axoneme of the principle pieces is surrounded by the fibrous sheath only. In the end pieces, the central axoneme is surrounded by cell membrane. (D); Leydig cells of control adult albino rat's testis showing euchromatic nuclei (N) with peripheral heterochromatin. The cytoplasm contains mitochondria (m) and lipid droplets (L).

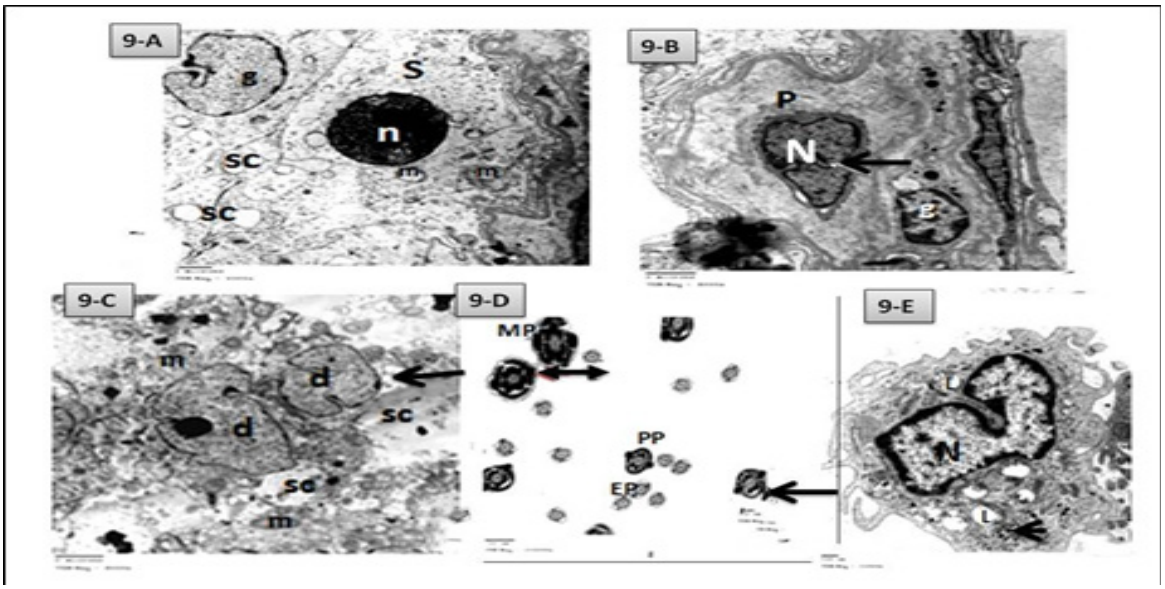


Fig. 9(9A-9E): Photomicrographs of a testis of Group II rat showing many intercellular spaces (SC) between the germinal epithelial linings of the seminiferous tubule. (9A); Sertoli cell (S) with an irregular euchromatic nucleus and a dark rounded nucleolus (n). The cytoplasm contains abnormal-shaped mitochondria (m). Spermatogonia (g) appeared with irregular nuclei. (9B); Primary spermatocyte (P) with irregular nucleus (N ←) and irregular cytoplasm are also seen. (9C); one Spermatid (d ←) with small shrunken irregular invaginated nucleus and another adjacent Spermatid containing distorted acrosomal cap. Its cytoplasm contains mitochondria (m) with wide intercellular spaces (Sc) between the Spermatids. (9D); Cross-sections in the mid pieces of sperms (MP) with marked distortion of the central axoneme (←), fibrous sheath (↔). Principal pieces (PP), and end pieces (EP) pieces of the sperms are seen (E); Leydig cell appeared with irregular euchromatic nucleus (N) containing peripheral heterochromatin. The cytoplasm contains lipid droplets (L), and variable-size electron-dense bodies (←).

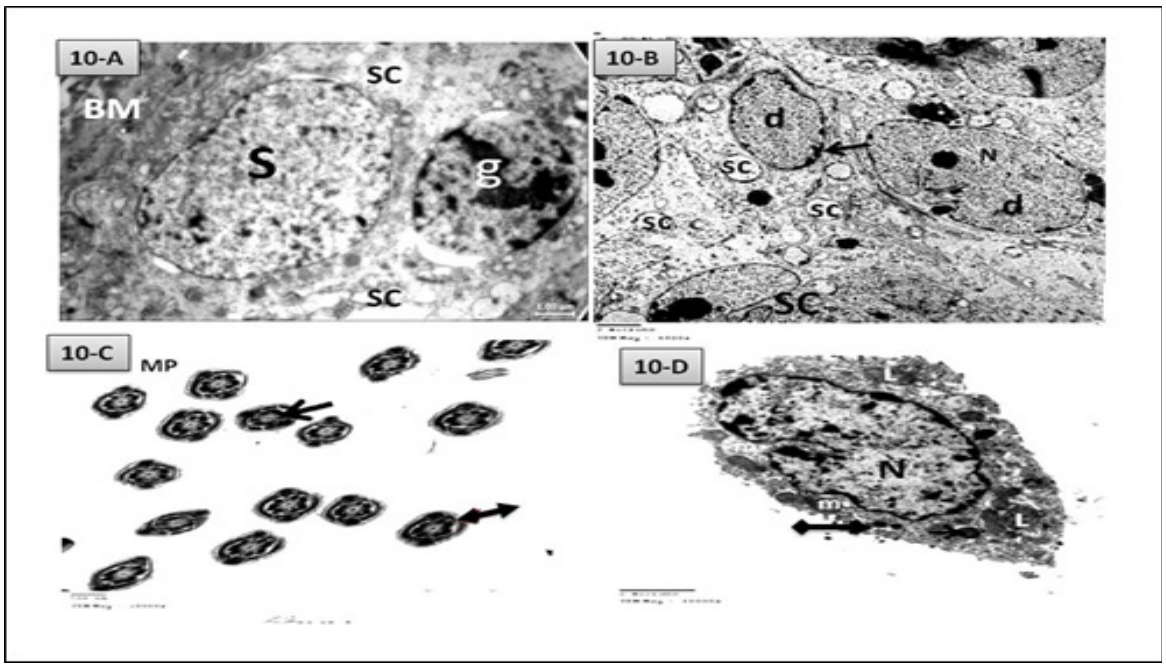


Fig.10 (10A-10D): Photomicrographs of a testis of Group III rats showing many but small intercellular spaces (Sc) between different types of germ cells. (A); Sertoli cell (S) resting on basement membrane (BM), primary spermatocyte (P), and Spermatids (d) are also observed. (B); Spermatids (d) had irregular shape and size but euchromatic nuclei with peripherally spaces in its cytoplasm and distorted acrosomal caps (←). (10C). Cross sections of mid pieces (MP) of the sperms with distorted mitochondrial sheaths (←) and fibrous sheath (↔).(10D); The Leydig cells nuclei appeared euchromatic with peripheral heterochromatin and showed nuclear invagination. Their cytoplasm contained mitochondria (m), lipid droplets (L) and variable-size electron-dense bodies (►).

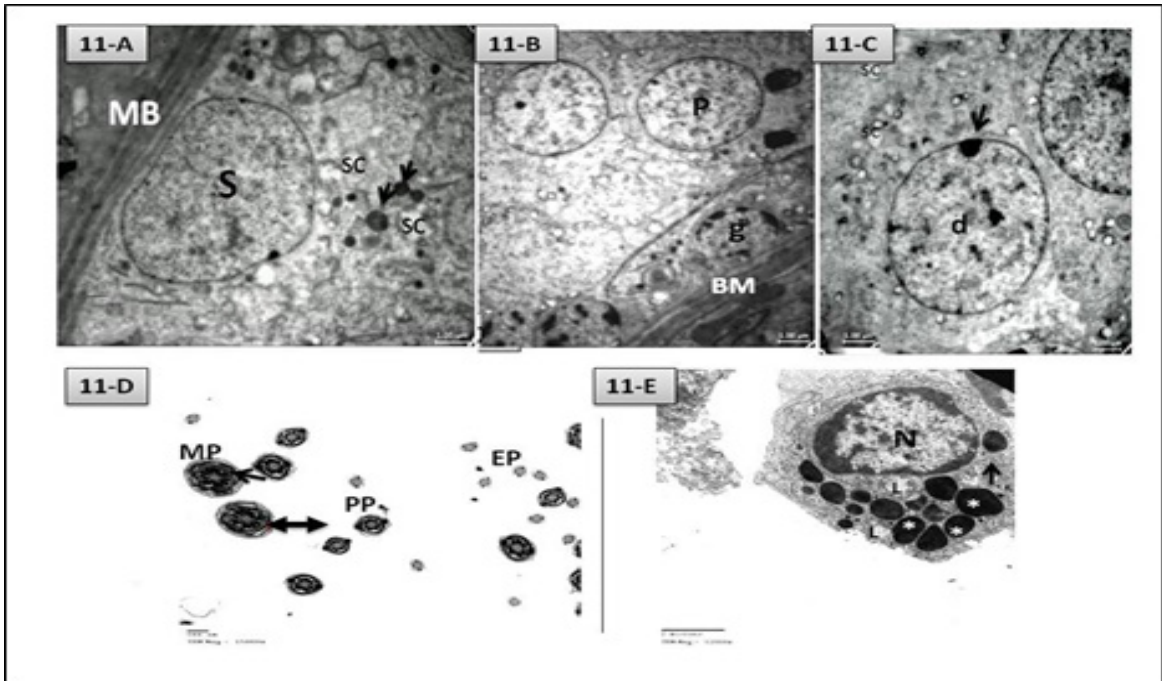


Fig.11 (11A-11E): A testis of Group IV rat showing fewer intercellular spaces (Sc) between germ cells. (11A); Sertoli cell (S) had large euchromatic nuclei and its cytoplasm contains few spaces and variable-size electron-dense bodies. (11B). Spermatogonium (g) contains rounded nucleus with peripheral heterochromatin clumps resting on the basement membrane (BM). Primary spermatocyte (P) has rounded nuclei and a thin rim of cytoplasm. (11C); Spermatids (d) had rounded euchromatic nuclei with well-defined acrosomal cap (*). (11D); Cross sections of mid pieces (MP) of the sperms with distorted mitochondrial sheaths (←) and fibrous sheath (↔) with normal principal pieces (PP), and end pieces (EP) pieces (11E). The Leydig cells nuclei (N) appeared euchromatic with condensed peripheral heterochromatin. Their cytoplasm contains lipid droplets (L) and many variable-sized electron-dense bodies (white asterisks).

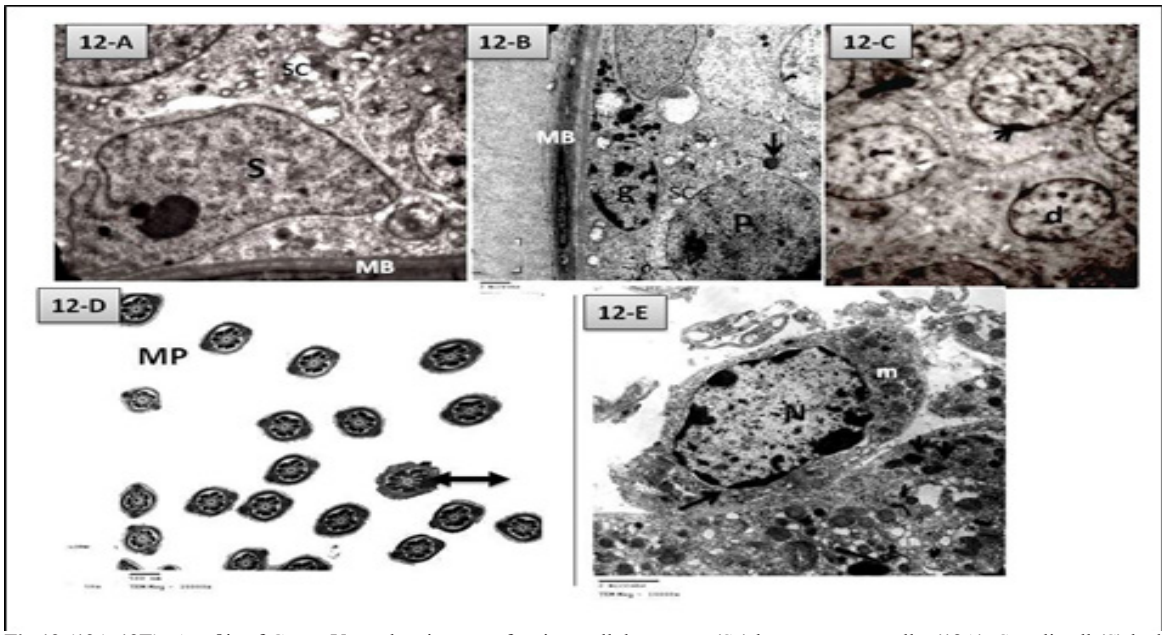


Fig.12 (12A-12E): A testis of Group V rat showing very few intercellular spaces (Sc) between germ cells. (12A); Sertoli cell (S) had large euchromatic nuclei and prominent nucleoli (12B): Spermatogonia (g) with rounded nuclei resting on the basement membrane (BM). Primary spermatocyte (P) had rounded nuclei and a thin rim of cytoplasm and electron-dense body. (C); Spermatids (d) had rounded euchromatic nuclei with well-defined acrosomal caps (→).(12D); Cross sections of the mid pieces(MP) of the sperms showed only distortion in fibrous sheath(↔) .(E); The nuclei of Leydig cells (N) appeared euchromatic with a peripheral heterochromatin. Their cytoplasm contained mitochondria (m).

Table: Changes in the left testis weight, length and width of adult male rat model of T/D injury treated with erythropoietin (or/and) AD-MSCs

Erythropoietin and stem cell-treated T/D group	Stem cell-treated T/D group	Erythropoietin-treated T/D group	T/D group	Control	Left testicle dimensions and weight
1.02±0.01**	0.93±0.02	0.91±0.04	0.05±0.01**	1.08±0.06	Weight
1.52±0.02*	1.5±0.01*	1.42±0.03	1.34±0.04*	1.63±0.03	Length
0.75±0.01	0.76±0.01	0.71±0.02	0.61±0.02*	0.83±0.011	Width

Values are represented as Mean ± SE, * = significant (P<0.05) and **=highly significant (P<0.01)

DISCUSSION

The testicular torsion is a common urological emergency among newborns, children and adolescents. Torsion usually occurs accidently without any predisposing factors. Trauma, testicular tumor, testicles with horizontal lie in the scrotum, history of cryptorchidism, an increase in the testicular volume during puberty may be considered as predisposing factors increasing the incidence of testicular torsion (Turner& Brown, 1993). The severity of testicular damage is related to the time and the degree of torsion. Therefore, late presentation or failure to diagnose and correctly manage this condition leads to massive testicular injury and subfertility (Anderson and Williamson, 1990) and the severe germ cell injury longer than 24 hours leads to a persistent infertility *(Mansbach et al. 2005)*. In this study we tested the role of erythropoietin and AD-MSCs separately and their combined effect to decrease testicular injury after T/D.

In this study, the light microscopic examination of T/D rats in comparing to the control group showed distorted seminiferous tubules which separated by a very wide interstitium contained homogenous acidophilic material and a hemorrhage which caused by T/D. This will led to ischemia which eliminates testicular blood flow followed by the reperfusion state in which the interstitial spaces were infiltrated by different cells such as leukocytes and macrophages. Reduction in the thickness of the germinal epithelium was observed and the germ cells were vacuolated and separated from each other; due to the low oxygen tension caused by decreased testicular blood flow and so, oxygen free radicals. These findings were reported by previous studies and correlated to the degree and duration of torsion

(Janetscheket al. 1991 and Beheshtian et al. 2008).

Ultrastructurally, many wide intercellular spaces were observed between the germinal cells lining the semineferous tubules. Previous researchers *(Yurtc et al. 2009)* observed a loosening of cell–cell contacts in the testicular I/R and attributed these separations to shrinkage of both germ and Sertoli cells. Former studies *(Karakaya et al. 2010)* reported that there was disorganization of germ cell structure and affection of their function. They explained disorganization by elevated level of reactive oxygen species (ROS). In addition, it was suggested that most of the semineferous tubules showed maturation arrest with only primary and secondary spermatocytes, and few tubules were completely devoid of any germinal cells *(Dokmeci et al. 2007)*. Moreover; Sertoli cells, Spermatogonia and primary spermatocytes were irregular in shape with irregular nuclei. Spermatids had small shrunken and distorted heterochromatic nuclei with invagination of its wall and their cytoplasm contained swollen mitochondria with abnormal-shaped cisternae. Others had distorted acrosomal cap.

In our study; the mid pieces of sperms showed marked distortion of the central axoneme, the fibrous sheath, and the mitochondrial sheath. Leydig cells had irregular euchromatic nuclei with peripheral heterochromatin, lipid droplets, and variable-size electron-dense bodies. It was suggested that I/R injury can induce an apoptotic reaction *(Turner et al. 1997)* and was attributed to a disturbed function of antioxidant enzymes, including superoxide dismutase, catalase, glutathione reductase, and glutathione peroxidase, which are normally present in the testis. The disturbance in these enzymes results in an accumulation of ROS within the cytoplasm resulting in a disorganization of all germ cells especially Spermatids. In addition, the release of acrosomal enzymes of distorted acrosomal caps may be involved in damage of testicular tissues. *Power et al. 2003* stated that testicular I/R injury usually lead to extraordinary apoptosis of haploid cells that might be due to the adhesion of neutrophils to the testicular venous endothelium.

Histological examination of the testes of erythropoietin-treated T/D group revealed that the testicular parenchyma was formed of apparently normal in some seminiferous tubules. Some tubules were lined by well-formed germinal epithelium with narrow interstitium but some showed increased and condensed germinal cells and parts were in full spermatogenesis. Sometimes, the germinal epithelium was separated from the basement membrane. Parts of interstitium were wide, hemorrhagic and showed thickness of their blood vessels. Electron microscopic examination of the same group revealed many small intercellular spaces between different types of germ cells. Sertoli cells had large euchromatic nuclei and their cytoplasm contains many spaces. Primary spermatocyte resembles the control. Spermatids had irregular shape and size but euchromatic nuclei with peripherally spaces in their cytoplasm. Others showed distorted acrosomal caps. The mid pieces of the sperms showed normal central axoneme distorted both mitochondrial and fibrous sheathes. The nuclei of Leydig cells appeared euchromatic with peripheral heterochromatin and showed nuclear invagination. These findings indicated that erythropoietin produced a significant improvement because of its antioxidant action that scavenges ROS and also improves the microvasculature *(Hamed et al. 2011)*. Furthermore, erythropoietin inhibits the release of the proinflammatory cytokines, resulting in decreased neutrophils infiltration and accumulation *(Buemi et al. 2002 and Ergur et al. 2008)*. In contrast, sluggishness of blood flow following torsion may limit the vascular capacity to deliver appropriate drug doses to the testes *(Köseoğlu et al. 2009)*. These could explain the still preserved lesions in some tubules.

Erythropoietin has been known to reduce cellular infiltration and has anti-inflammatory, anti-oxidant and anti-apoptotic effect during ischemic injury *(Michels et al. 2006)*.

In our study when T/D rats treated with stem cells, the light microscopic examination of testicular parenchyma was nearly as the control group. Some seminiferous tubules showed exfoliated germ cells in the lumen and sometimes showed arrested spermatogenesis. Fewer of germinal epithelium was separated from the basement membrane. Some interstitium showed degeneration of its cells, another showed thickness of its blood vessels. Ultrastructurally; fewer intercellular spaces between different types of germ cells were noticed. Sertoli cells had large euchromatic nuclei and their cytoplasm contain few spaces and variable-size electron-dense bodies. Spermatogonia and Primary spermatocytes appeared as the control. Spermatids

had rounded euchromatic nuclei with well-defined acrosomal caps, but the mid pieces of the sperms still showed distortion in both mitochondrial and fibrous sheathes. The nuclei of Leydig cells appeared euchromatic with condensed peripheral heterochromatin.

The ameliorative effect of MSCs may be through prevention of testicular apoptosis, reduction of intra testicular oxidative stress and promotion of testosterone production which stimulates spermatogenesis against torsion induced germ cell injury *(Clevers et al. 2014)* also; through its potential differentiation into another gem cell *(He et al. 2009)*. This indicated by previous studies reported the effect of MSC against ischemic injury in kidney *(Sadek et al. 2013)*, heart *(hare et al. 2009)* and lung models *(Chien et al. 2012)*.

Great improvement was noticed in combined erythropoietin and MCS treated T/D group. Light microscopic examination of testes that the testicular parenchyma was as the control group with full spermatogenesis. Few seminiferous tubules showed exfoliated germ cells in the lumen and very few interstitium was wide with few hemorrhages. Ultra-structurally, this group showed very few numbers of intercellular spaces between the germ cells in comparison to separate erythropoietin or MSCs treated groups. The histological structures of Sertoli cells Spermatogonia, primary spermatocytes, and spermatids greatly resemble the control. Spermatids had rounded euchromatic nuclei with well-defined acrosomal caps. Cross sections of the mid pieces of the sperms showed only distortion in fibrous sheath. Leydig cells appeared normal as the control group. These findings attributed to the combined effects of erythropoietin and MCS ischemic injury on testicular tissues.

In the current work most of the injected AD-MSCs expressed the CD-105 marker of mesenchymal stem cells, confirmed by flow cytometric analysis of these cells as reported in previous studies on characterization of MSCs from various sources for therapeutic applications in regenerative medicine *(Hosseinzadeh et al. 2013)*. The staining of the testicular tissue using CD-105 antibody searching for the presence of these cells revealed marked increase in number of positively stained cell in the group that received both erythropoietin and AD-MCS which present in the seminiferous tubules and interstitial spaces in comparison to group of erythropoietin T/D or MCS treated T/D group which showed moderate expression of the same cells

The presence of these cells in the interstitial spaces mainly and within the seminiferous tubules to a lesser extent was explained by *Hsiao et al.(2015)* who stated the role of AD-MSCs in supporting Leydig cells and Sertoli cells to achieve the process of full spermatogenesis. Leydig cells are interstitial cells located at interstitial spaces adjacent to the seminiferous tubules and they are testosterone-producing cells. Testosterone and FSH are essential for spermatogenesis and promote differentiation of Spermatogonia via activating Sertoli cells *(O'Shaughnessy et al. 2009)*. Despite the differentiation capacity of AD-MSCs to germ cell is limited in comparison to bone marrow-derived MSCs *(Hosseinzadeh et al. 2013)*, their differentiation to Leydig cells and their paracrine support of them were proved *(Hsiao et al. 2015)*. So the stem germ cell support and the differentiation of AD-MSCs differentiation to Leydig cells is the most accepted mechanism to guard against the testicular damage after T/D.

In our study; the number of sperms of the T/D group showed a significant decrease in comparison to the negative control group *($P<0.001$)* while in the treated groups the sperms count increased with significant increase in comparison to the T/D group. The maximum increase was observed in erythropoietin and MSC cells T/D treated group. This indicates the effect of injected mesenchymal cells in increasing spermatogenesis after ischemic injury of testicular tissues as reported by *Hsiao et al.(2015)* who used Johnsen's score (a score used to evaluate pathological infertility) and proved that orbital fat derived mesenchymal stem cells increase the score from less than 6 to more than 8 in a rat model of T/D.

The best results obtained in this study concerning the histopathological picture, ultrastructure and sperms count were obtained in the group treated with both erythropoietin and AD-MSCs. Erythropoietin has the ability of stimulation and mobilization of self-stem cells from marrow to the affected testis and provides a supporting short-term efficacy on rat testicular injury after ischemia/reperfusion *(Rashed et al. 2013)*, so we use the intravenous method to inject it, while the rout of administration of AD-MSCs was local injection as the physiological blood–testis barrier stops stem cells from entering testis tissue via the circulation *(Hsiao et al. 2015)* the augmentation of the two methods may be the key of the best improvement.

CONCLUSION

The testicular torsion induced a marked harmful effect on the testis however, combination of erythropoietin and MSC cells supplementation before the reperfusion period improved the testicular micro-structure and ultrastructure and increase the sperm count in the albino rat. Hence, injection of erythropoietin and MSC cells before the surgical intervention can be recommended to avoid the marked structural affection in the ipsilateral testis and provides a new therapeutic strategy to rescue the infertility; a sequel of the testicular torsion induced the germ cell injury.

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تأثير الخلايا الجذعية الوسيطة المستمدة من النسيج الدهنى وعقار الارثروبيوتين فى التغييرات الناتجة عن الالتواء وفك الالتواء التجريبى فى خصية الفأر الأبيض البالغ

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ملخص البحث

الخلفية: يعتبر التواء الخصية من الحالات المرضية الحرجة التى اذا لم يتم فيها التدخل الجراحى السريع قد تؤدى الى تدمير الخصية والعقم. ويعد العلاج بالخلايا الجذعية من الطرق الحديثة لعلاج معظم الأمراض ومنها التواء الخصية، كما وجد أن لعقار الارثروبيوتين دور فعال وحيوفى فى علاج الالتواء وكذلك فى تحريك الخلايا الجذعية من نخاع العظام الى الى الخصية المصابة.

الهدف من البحث: دراسة دور الخلايا الجذعية الوسيطة المستمدة من النسيج الدهنى وكذلك عقار الارثريوبيوتين كل منهما على حدة أو كخليط فى الحد من التغيرات الهستولوجية والهستوكيميائية مناعية اللناتجة عن الالتواء وفك الالتواء التجريبى فى خصية الجرذان البيضاء

الطرق البحث: أجريت هذه الدراسة على ستون من ذكورالجرذان البيضاءالبالغةخلال الدراسة. تم توزيعهاعشوائيا إلى خمس مجموعات متساوية (10لكل منهما)، بالإضافة إلى10جرذان استخدمت للحصول على الخلاياالجذعية الوسيطة المستمدة من النسيج الدهنى. هذه المجموعات الخمس تم تقسيها على النحو الأتى:
المجموعةالأولى (المجموعة الضابطة)، المجموعة الثانية (مجموعةالالتواء وفك الالتواء) حيث تم التواء الخصية اليسرى 720 درجة فى اتجاه عقارب الساعة لمدة ساعتين ثم تم فك الالتواء فى عكس عقارب الساعة، (المجموعة الثالثة): وفيها تم الحقن الوريدى مرة واحدة لعقار الارثريوبيوتين بجرعة 3000 ميكرو/ كج بعد عملية الالتواء وفك الالتواء ،(المجموعة الرابعة): وفيها تم حقن الخصية بالخلايا الجذعية الوسيطة المستمدة من النسيج الدهنى بجرعة 3X106 بعد عملية الالتواء وفك الالتواء، (المجموعة الخامسة):وتم فيها حقن خليط من الخلايا الجذعية والارثريوبيوتين بنفس الجرعات بعد عملية الالتواء وفك الالتواء.فى نهاية التجربة تم التضحية بجميع الجرذان باستخدام جرعة زائدة من بنتوباربيتال الصوديوم ثم استخرجت الخصية اليسار لكل الجرذان وتم تحضير عينات للدراسة الهستولوجية والهستوكيميائية المناعية وعينات أخرى للفحص الالكترونى. هذا وقد تمت دراسة احصائية لعدد الحيوانات المنوية فى البربخ الأيسر بجهاز الهيموسيتوميتر، وحللت جميع النتائج احصائيا

النتائج:وجد بالعين المجردة ضمور ملحوظ فى خصى الفئران التى تعرضت للالتواء وتغير لونها الى اللون الأحمر الداكن مقارنة بالمجموعة الضابطة ومجموعة الالتواء المعالجة بالخلايا الجذعية وعقار الارثريوبيوتين. كما أظهرتا لخصية اليسرى للجرذان التى تعرضت للالتواء تغييرات نسيجية على شكل تدمير شديد لبعض الأنيبيبات المنوية وبعضها وجدت مشوهة وبدت مفصولة عن بعضها بمسافات بينية واسعة احتوت على مادة متجانسة صبغت بمواد حمضية وبعض النزيفكما شوهد انخفاض ملحوظ فى سمك الجدار فى سمك الجدار الطلائى الجرثومى. بالفحص الميكروسكوبى لوحظ وجود مسافات بينية واسعة بين الخلايا الجرثومية، ونواة خلايا سيرتولى غير منتظمة وداكنة وترتكز الخلايا على غشاء قاعدى سميك ومتعرج، كما لوحظت تغيرات فى خلايا ليدج على شكل نواة غير منتظمة الشكل بها كروماتين طرفى وقطرات دهنية، وعلاوة على ذلك لوحظ عدم وجود تفاعل مناعى للأجسام المضادة الخاصة ب CD-105 فى الأنيبيبات المنوية والفراغات بين الخلوية. وبجهاز الهيموسيتوميتر وجد انخفاض ملحوظ فى عدد الحيوانات المنوية مقارنة بالمجموعة الضابطة. مع حقن الخلايا الجذعية المتوسطة المستمدة من النسيج الدهنى أو عقار الارثريوبيوتين وجد تحسن طفيف فى جميع النتائج السابقة. أما فى الجرذان المعالجة بمزيج من الخلايا الجذعية المتوسطة المستمدة من النسيج الدهنى وعقار الارثريوبيوتين فالأنيبيبات المنوية بدت طبيعية ومبطنة بخلايا جرثومية جيدة الشكل وبينها مسافات بينية ضيقة بها كميات محدودة من المواد الحمضية ويوجد خلايا جرثومية محدودة مفصولة عن الغشاء القاعدى. كما وجود تحسن ملحوظ فى شكل خلايا سيرتولى وخلايا ليدج،وعلاوة على ذلك لوحظ كثافة فى ايجابية التفاعل المناعى البنى للأجسام المضادة الخاصة ب CD-105 فى الأنيبيبات المنوية والفراغات بين الخلوية. كما زاد عدد الخلايا المنوية بشكل ملحوظ.

الأستنتاج: ان للالتواء التجريبى فى الفئران البيضاء تأثيرا ضارا على التركيب النسيجى للخصية قلة عدد الحيوانات المنوية وأن حقن الخلايا الجذعية المتوسطة المتستمدة من النسيج الدهنى أو عقار الارثريوبيوتين له قدرة متوسطة على تخفيف الأضرار النسيجية وزيادة متوسطة فى عدد الحيوانات المنوية، ولكن عند حقن مزيج من الخلية الجذعية وعقار الارثريوبيوتين فان له القدرة على تحسين الأضرار النسيجية بشكل واضح وجلى وزيادة عدد الحيوانات المنوية بشكل ملحوظ. ان حقن الحالات المصابة بالالتواء بخليط من الخلايا الجذعية المتوسطة المستمدة من النسيج الدهنى وعقار الارثريوبيوتينيمكن أخذه فى الاعتبارلتحسين الشكل التركيبى للخصية وأمل جديد فى علاج العقم.