Original Article

The Possible Protective Effect of G-CSFon Germ Cell Injury, Spermatogensis and Sperm Parameters in a Rat Model of Testicular Torsion-Detorsion. Histological and Immunohistochemical Study

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ABSTRACT

Backgroundand Objectives: Testicular torsion is a urological emergency that involves the risk of infertility as a result of germ cell ischemic/reperfusion (I/R) injury. Therefore, this study was planned to test the protective effect of hematopoietic stem cells mobilization by granulocyte-colony stimulating factor (G-CSF) on a rat model of testicular torsion- detorsion induced germ cell injury.

Material and Methods: 32 adult male albino rats were randomly divided into group I (Control group) and experimental groups which included group II (Torsion-detorsion group), group III (Spontaneous recovery group) and group IV (Torsion-detorsion with G-CSF treatment group).These experimental groups were subjected to T/D injury by rotating the left testicle 720° clockwise for 3 hours. Half an hour before detorsion in group IV, G-CSF was injected subcutaneously at a dose of 70 µg/kg/day then daily for 5 consecutive days. At the end of the study (8 weeks),hormonal assay, sperm analysis, and testicular samples were taken for histological, immunohistochemical and statistical analysis.

Results: The results revealed that the mean testosterone levels, sperm count, % of sperm viability and motility were significantly affected after torsion-detorsion with loss of the normal histological architecture of the testis and spermatogenic cells. G-CSF injection prior to detorsion showed significant improvement in testosterone levels & sperm analysis with marked improvement in histological changes.

Conclusion: G-CSF protect the testis of adult rats against ischemia/reperfusion injury induced by torsion-detorsion. Strong evidence suggested that injection of G-CSF prior to detorsion reduces testicular degenerative changes after ischemia and adverse effects of reactive oxygen species caused by reperfusion.

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Key Words: CD34, E- cadherin, G-CSF, hemopoietic stem cells (HSCs), testicular torsion-detorsion (T/D) injury.

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INTRODUCTION

Testicular torsion is caused by rotation of the spermatic cord, which first obstructs venous blood flow to the testicle, and then arterial blood flow, resulting in testicular ischemia[1]. It is a urological emergency that affects 1/4000 of the young adult male population (<25yrs) and involves the risk of infertility.Germ cell death results from testicular ischemia, which is primarily responsible for the lack of oxygen supply relative to metabolic demands, toxic metabolites accumulation and stored cellular energy depletion.The duration of testicular ischemia and the severity of cord twisting are two important prognostic factors in the survival and activity of sperms[2].

Surgical detorsion of the twisted cord within 6 hours significantly reduces the rate of permanent dysfunction on the testis.However, ahigh degree of cord twisting > 360° with symptomsfor more than 24 h results in complete or severeirreversible testicular atrophy due to cell necrosis that begins 4 h after torsion.Restoration of testicular perfusion after detorsion leads to an increase in the production of reactive oxygen species (ROS), which damage DNA, proteins and lipids of testicular cells. Also, reperfusion leads to production of pro-inflammatory cyto¬kines, including interleukin 1β and tumor necrosis factor α, which recruit macrophages and neutrophils that penetrate the testicular parenchyma. These changes cause germ cells apoptosis & disrupt Sertoli cells function, resulting inimpaired spermatogenesis and testicular atrophy[3].

Theoretically, therapy to improve ischemia/reperfusion (I/R) injury, promote spermatogenesis, or regulate the immune response could potentially prevent complications of testicular torsion-detorsion. Multipotency and tissue support enable stem cells to regenerate tissues regulated by the niche microenvironment[4]. Homing factors can also be used to obtain beneficial effects on damaged tissue, since they stimulate the migration of both hematopoietic and mesenchymal adult stem cells. Granulocyte colony stimulating factor (G-CSF) is one of the homing factors, known to mobilize hematopoietic stem cells (HSCs) from the bone marrow into the peripheral circulation. It regulates the growth, migration and facilitates the differentiation of hematopoietic cells in BM, blood and inflammatory foci[5].

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Consequently, the present study aimed at investigating the possible protective effect of G-CSF against I/R induced germ cell injury and spermatogensis following testicular torsion-detorsion in adult rat model. The effectiveness of G-CSF therapy wasmonitored by serological, histological, immunohistochemical and morphometric studies.

MATERIALS AND METHODS

Drugs

Granulocyte colony-stimulating factor (G-CSF) "Neupogen": was provided as a 0.5 ml prefilled syringe containing 300µg of filgrastim (recombinant-methionyl human G-CSF from E.coli K12,F. Hoffmann-La Roche Ltd, Basel. Kirin-Amgen Inc., Switzerland.

Animals

This study was conducted on 32 adult male albino rats with average body weight 180-250 grams. They were housed in hygienic stainless-steel cages and kept in a clean well-ventilated room in the Animal House of KasrAl-Ainy, Faculty of Medicine, Cairo University. They were allowed food and water ad libitum. All procedures were held according to the guidelines of the Animal Ethics Committee, Cairo University. Rats received surgical torsion-detorsion after a 7-day period of acclimatization.

Experimental design

The rats wererandomly divided into 3 groups

Group I(Control group), 6 rats: rats were further subdivided into 2 subgroups, 3 rats each: Subgroup Ia: each rat received sham operation (surgical inguinal skin incision on the left side without testicular torsion).

Subgroup Ib: each rat receivedsham operationfollowed by subcutaneous injection of 0.5 mL glucose 5%(solvent of G-CSF) 30 min. before closure of the wound, then daily for 5 consecutive days.

One rat from each subgroup was sacrificed after 24 hrs, 5 days and 8 weeks.

Group II(Torsion-detorsiongroup), 6 rats: each ratreceived surgery of left testicular torsion and detorsion and were sacrificed 24 hrs. after detorsion to evaluate testicular damage caused by ischemia and reperfusion[1].

Group III(Spontaneousrecovery group), 10 rats: which received surgery of left testicular torsion and detorsion as in group II and left untreated for 8 weeks.

Group IV(Torsion-detorsion with G-CSF treatment), 10 rats: which received operation of left testicular torsion followed by subcutaneous injection of G-CSF at a dose of 70 µg/kg/day diluted in 0.5 mL glucose 5% 30 min. before detorsion then daily for 5 consecutive days[6].

Four rats from each of group III & IV were sacrificed after 5 days from the start of the experiment to detect mobilization & homing of BM-derived HSCs into the testis[5],while the remaining rats were sacrificed at the end of the experiment (after 8 weeks).

Torsion/detorsion animal model[7]

All surgical procedures were performed using aseptic technique under anesthesia with an intraperitoneal injection of 50 mg / kg of ketamine and 45 mg / kg of xylazine (Ketalar andCitanest, 2%; Eczacıbas¸ı, Turkey). After the left inguino-scrotalincision, a unilateral testicular torsion was created by rotating the left testicle 720° clockwise followed by hemi-scrotalfixation with 4/0 silk suture for 3 hours. The exposed left testis was protected with moist gauze and warm light during the operation.After 2.5 hours, G-CSFwas administrated subcutaneously. Thirty minutes after the injection of G-CSF, the twisted cord was detorsed via surgical reduction and the wound was closed. In the sham-operated control group, animals subjected to the same surgical procedure without torsion and detorsion. After sacrifice, left orchiectomy was performed and the tissue samples were divided into testes and epididymis.

Serological study

Before sacrifice tail vein blood samples were collected and centrifuged at 3000 rpm /20 min to separate plasma. Serum testosterone level was measured by solid phase radioimmunoassay (RIA) using components of a commercial kit (Coat-, Siemens Diagnostics, Los Angeles, USA)[8] in the Biochemistry department, Faculty of medicine, Cairo university.

Assessment of sperm parameters[9]

After sacrifice, epididymis' were excised, minced, and incubated in normal saline for 2 h at 37 °C to extract the sperm. The caudal epididymis was dissected out; an incision (approximately 1 mm) was made in the caudal epididymis. Semen was then squeezed onto a microscope slide. Epididymal spermatozoa were counted using a hemocytometer and expressed in millions / ml suspension. Sperm motility wasestimated by counting the number of motile sperms per unit area and expressed as percentage of motility. The sperm viability was also determined by Eosin/Nigrosin stain. Sperm parameters were assessedin the Biochemistry department, Faculty of medicine, Cairo university.

Histological study

At the end of mentioned time points, Testis specimens were dissected out, fixed in Bouin's solution and embedded in paraffin. Serial sections of 5–7 µm thickness werecut and subjected to the following:

a. Hematoxylin and Eosin (H & E) stain[10].
b. Masson's trichrome stain[11] to demonstrate collagen fibers.
c. Periodic acid schiff reaction (PAS)[11] to demonstrate basal laminae of seminiferous tubules.
d. Immunohistochemical staining using the avidin-biotin peroxidase complex technique[12] for:

1. AntiCD34 antibody for detection of BM-derived HSCs.It is a ready-to-usemouse monoclonal antibody (Lab Vision Corporation laboratories, CA 94539, USA, catalog number MS-363-R7).CD34 positive cells showed membranous brown reaction.
2. AntiE-cadherinantibody as a marker forintercellular junction. It is a ready-to-use rabbit polyclonal antibody (Lab Vision Corporation (Thermo scientific) Laboratories, USA, catalogue number RB-9036-R7). E-cadherin positive cells showed membranous and cytoplasmic brown reaction.

Application of the primary (1ry) antibodies was followed by incubation in a humid chamber at room temperature for 60 min. Tonsils were used as +ve control specimens for both CD34 and E-cadherin. On the other hand, one of the testicular sections was used as –vecontrol by passing the step of applying the 1ry antibody.

Morphometric Study

Data were obtained and analyzed using "Leica Qwin 500 C" image analyzer computer system (Cambridge, England) in the Medical Histology and Cell Biology department, Faculty of medicine, Cairo university.From each slide, 10 non-overlapping fields were examined at a magnification of x100 for all parameters (Optical density x400). The following were measured:

a. Diameter of seminiferous tubules and thickness of spermatogenic epithelium in H & E stained sections.
b. Area % of collagen in Masson's trichrome stained sections.
c. Optical density of PAS +vereaction.
d. Number of CD34 immunopositive cells.
e. Area% of E- cadherin +ve immunoreactivity.

Changes in seminiferous tubules and spermatogenetic activity were assessed using the Johnson score (Table 1). In each H&E stained slide, 10 seminiferous tubules were examined under the light microscope at a magnification of x400 and scored on a scale of 1–10 according to the degree of spermatogenesis[13].

Table 1: Johnsen tubular biopsy score

Score	Definition
10	Complete spermatogenesis with many spermatozoa
9	Disorganized spematogenesis with many spermatozoa
8	Only a few spermatozoa (<5–10/tubule)
7	No spermatozoa, many spermatids
6	Only a few spermatids (<5–10/tubule)
5	No spermatids, many spermatocytes
4	Only a few spermatocytes (<5/tubule)
3	Only spermatogonia
2	Only Sertoli cells
1	No cells detected in tubular section

Statistical Analysis

The measurements obtained were analyzed using Statistical Package for Social Science (SPSS) software version 16 (SPSS, Chicago, USA). Comparison between different groups were made by using one-way analysis-of-variance (ANOVA). The results were expressed as mean ± standard deviation (SD). The differences were considered statistically significant when probability *(p) value* is < 0.05[14].

RESULTS

Serological results

The mean values of serum testosterone levelof group III (recovery group) were significantly decreased as compared to the other groups (Table 2).

Sperm analysis

There was a significant decrease in the mean values of sperm count, mean % of sperm viability and motility of group II& group III compared to control and group IV. Additionally,there was a significant decrease in themean % of sperm motility of group IVas compared to the control value (Table 3).

Histological results

(a)Hematoxylin and Eosin Stained Sections

Sections from control rats (subgroups Ia and Ib) revealedregular seminiferous tubules with interstitium in between.Each tubule was surrounded by a basal lamina with flat myoid cells and lined by stratified epithelium of Sertoli and spermatogenic cells. Spermatogenic cells represented the different stages of spermatogenesis; beginning with

spermatogonia in the outer layer, spermatocytes in the middle, spermatids near the lumen and spermatozoa within the lumen of the seminiferous tubules.The interstitial tissue contained clusters of interstitial cells of Leydig around blood vessels (Figure 1a).

Testicular sections from group II (torsion-detorsion group)showed loss of the normal architecture of seminiferous tubules with degeneration and separation of most of germ cells from the basement membrane and their shed off in the lumen.Some of these cells showed darkly stained nucleiand others had fragmented nuclei (Figure 1b).

Sections in the rat testes ofgroup III (recovery group) revealed distorted and shrunken seminiferous tubules withreduced thickness of the germinal epitheliumand wide interstitium in between them. The germ cells markedly decreased in number and some of them showed vacuolated cytoplasm. The interstitium exhibited homogenous acidophilic material (exudate) and many inflammatory cells. The interstitial cells of leydig can't be differentiated (Figure 1c).

Testicular sections from group IV(torsion-detorsion with G-CSF treatment)recruitedapparently normal seminiferous tubules lined by almost the normal stratified epithelium (Sertoli and spermatogenic cells). Some tubules showed separation of their basal laminae at few areas. The interstitial tissue contained apparently normal leydig cells with the presence of minimal exudate (Figure 1d).

(b) Mallory's TrichromeStained Sections

Sections from both control subgroups (Ia&Ib) revealed normal distribution of bluish stained collagen fibers in the testicular capsules (tunica albuginea), basal laminae of the seminiferous tubules and interstitial tissue in-between (Figure 2a). However, group II showed widely separated collagen fibers (by edema) in the tunica albuginea and disrupted collagen fibers in the basal laminae of seminiferous tubules and interstitial tissue (Figure 2b). The marked increase in the collagen fibers in the testicular capsules, basal laminae of seminiferous tubules and interstitium was observed in group III (Figure 2c).Group IV showed apparent normal distribution of collagen fibersas compared to the control group (Figure 2d).

(c)PAS Stained Sections

Sections in the rat testes of both control subgroups (Ia&Ib) showed thin clear basal laminae of seminiferous tubules (Figure 3a), while in group II, the basal laminae were interrupted at many sites (Figure 3b). Group III revealed increased thickness of the basal laminae in the recovery tubules (Figure 3c) and group IV demonstrated continuous thin clear basal laminae in nearly all the seminiferous tubules (Figure 3d).

(d) Immunohistochemical Results

1- CD34 immunostained sections

Sections from control testes exhibited negative immunoreactivity for CD34 (Figure 4a).On day 5, group III showed few CD34 positive HSCs in the interstitial tissue (Figure 4b). While sections from group IV displayed many HSCs immunopositive for CD34 in the interstitial tissue (Figure 4c). The immunopositive cells appeared spindle in shape with dark nuclei and brown mambranous reaction. After 8 weeks, sections from the same groups showedno immunopositive HSCs (Figures 4d,e).

2- E-cadherin immunostained sections

Sections from control rats exhibitedstrong positive and wide distribution of E-cadherin immunoreaction particularly at the basal portions of the seminiferous tubules and within Sertoli cells attaching them to spermatocytes and spermatids (Figure 5a). However, group II showed very weak cadherin immunoreactivity in the sloughed germ cells (Figure 5b) and group III revealed negative immunoreactivity in the recovery tubules (Figure 5c). Group IV showed strong positive E-cadherin immunoreactivity with lower reactivity in the central part of the seminiferous tubules (Figure 5d).

Morphometric results

In H&E stained sections, the meanJohnsen score, seminiferous tubules diameter and epithelial thickness values in torsion-detorsion group (group II) and recovery group (groupIII) showed a statistically significant decrease as compared withcontrol group (group I) and G-CSF group (group IV). In addition, a significant decrease was found in group III as compared to group II. There was no significant difference between group IV and control group (Table 4).

In group III, the area percent of collagen fibers and the optical density of PAS +ve reaction was significantly higher as compared to the other groups. However, the optical density of PAS +ve reaction in group II was significantly lower as compared to the other groups. No statistically significant difference was reported between group I and group IV as regards the both parameters (Table 5).

As regards immunoreactivity, the number of CD34 immunopositive HSCs, on day 5, showeda significant increase in group IV as compared to group I and group III. There was also a significant increase in group III as compared to group I. However, after 8 weeks, there was a significant decrease in both group III and group IV as compared to the same groups on day 5. The area percent ofE-cadherin immunoreactivity revealed a significant decrease in group III as compared to the other groups. In addition, a significant decrease was found in group II compared to both groups I & IV (Table 5).

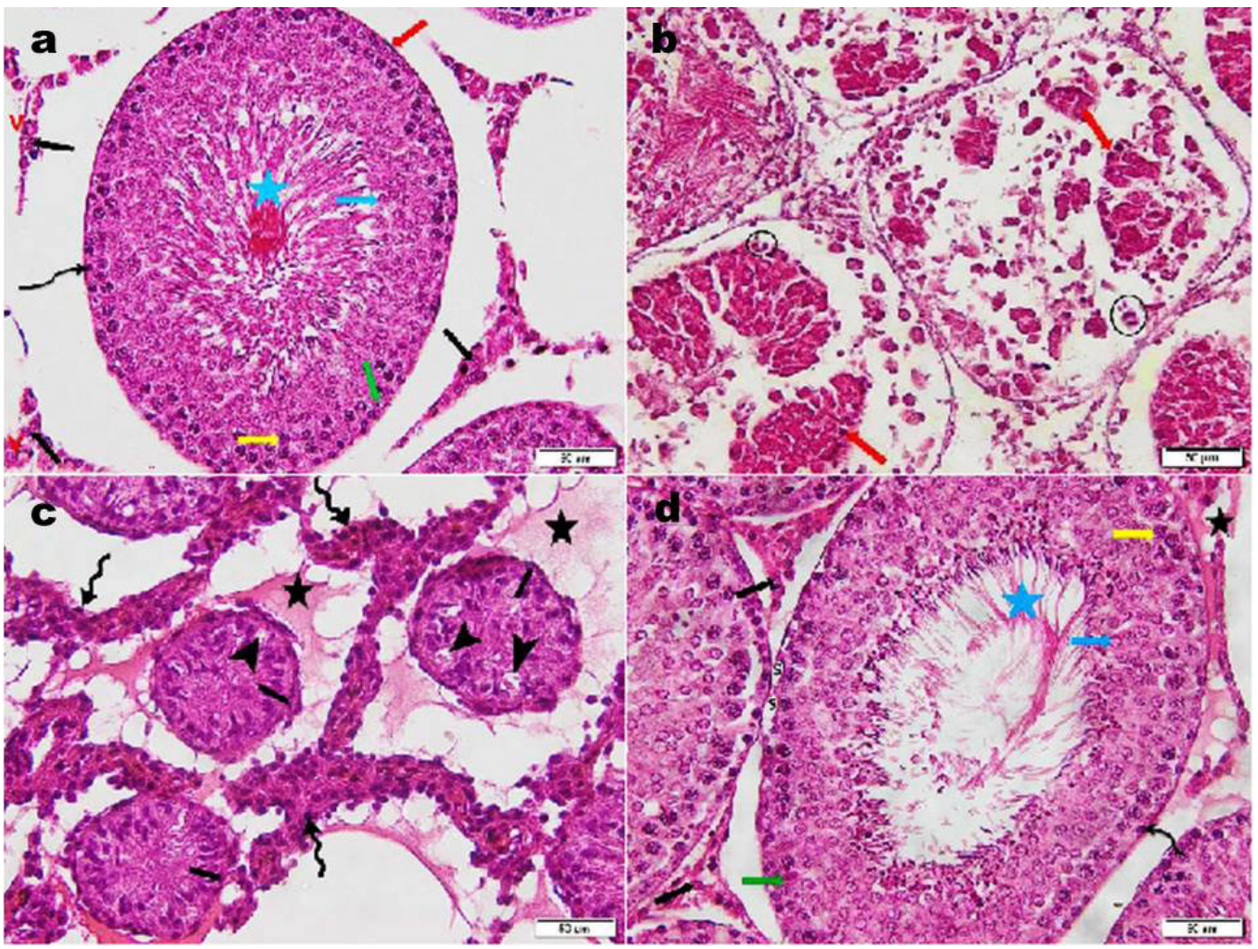

Fig. 1: Photomicrographs of testicular sections stained with H&E X200. (a) Group Ishows seminiferous tubulessurrounded by basal laminae and flat myoid cells (red arrow). The one in the center of the field displays Sertoli cells (green arrow) and different stages of spermatogenic cells including; spermatogonia (wavy arrow), primary spermatocytes (yellow arrow), spermatids (blue arrow) near the lumenand spermatozoa (bluestar) inside it. Interstitial cells of Leydig (black arrows) appear around blood vessels (V)in the interstitiun in-between tubules. (b) Group II shows distorted seminiferous tubules in which most of germ cells aredegenerated and separated from the basement membrane and shed off in the lumen. Some of these cells have darkly stained nuclei (red arrows) and others had fragmented nuclei (black circles). (c) Group III reveals distorted, shrunken and widely separated seminiferous tubules with reduced thickness of the germinal epithelium (black lines). The germ cells are markedly decreased in number and some of them show vacuolated cytoplasm (arrowheads). The interstitium contains exudate (black stars) and many inflammatory cells (wavy arrows). (d) Group IV shows apparently normal seminiferous tubules lined by Sertoli cells (green arrow) and multiple layers of spermatogenic cells; spermatogonia (wavy arrow), primary spermatocytes (yellow arrow), spermatids (blue arrow) and spermatozoa (bluestar) in the lumen. The basal lamina surrounding the tubule is separated at few areas (S). The interstitial spaces contain apparently normal Leydig cells (black arrows) and minimal exudate (black star).

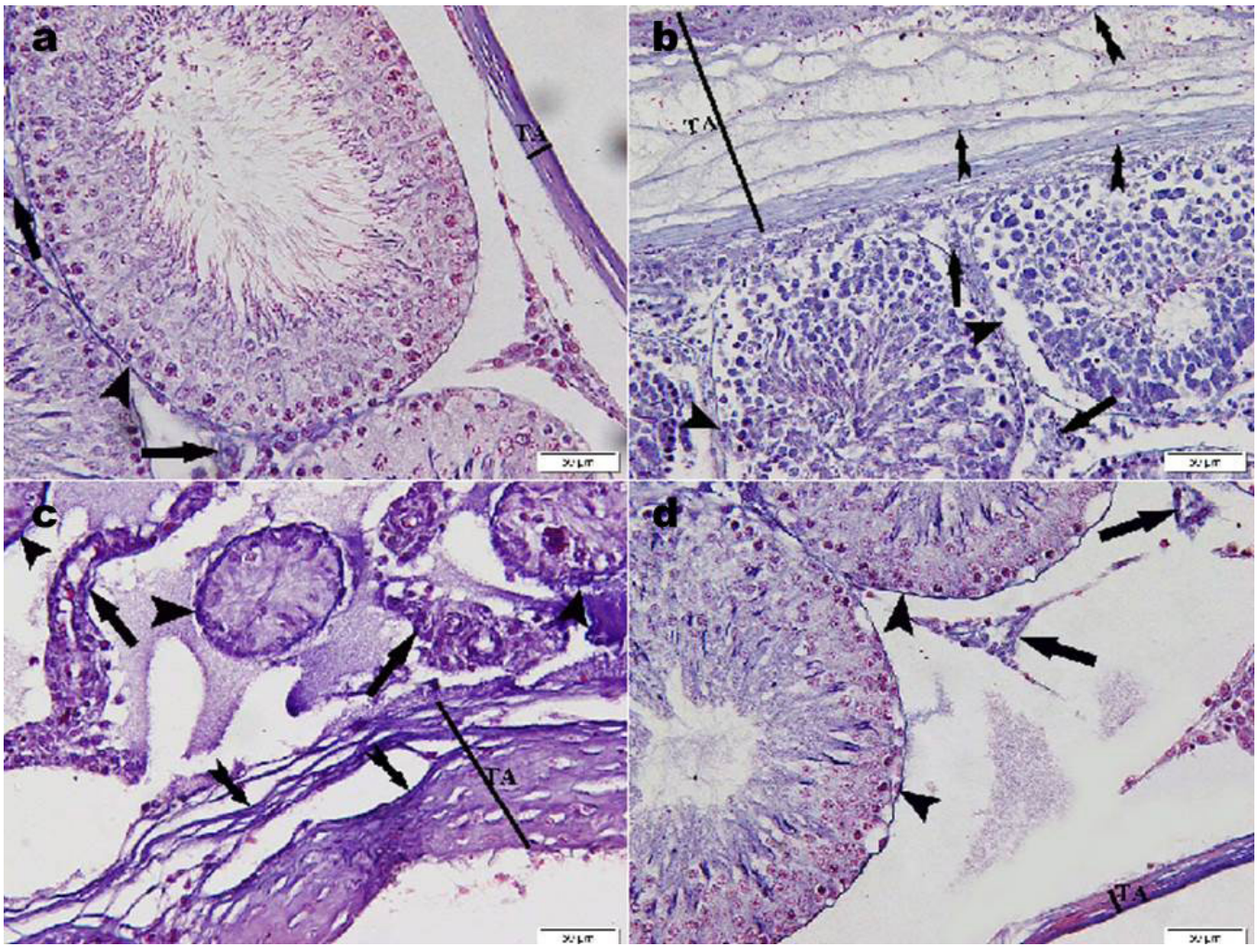

Fig. 2: Photomicrographs of testicular sections stained with Masson's trichrome X200. (a) Group I shows normal distribution of collagen fibers in tunica albuginea(TA), basallamina of seminiferous tubules (arrowhead) and interstitial tissue (arrows). (b) Group II shows widely separated collagen fibers (bifid arrows) in the tunica albuginea (TA) and disrupted collagen fibers in the basal laminae of seminiferous tubules (arrowheads) and interstitial tissue (arrows). (c) Group III reveals marked increase in the collagen fibers (bifid arrows) in the tunica albuginea (TA), basal laminae of tubules seminiferous (arrowheads) and interstitial tissue (arrows). Group IV shows apparent normal distribution of collagen fibersin tunica albuginea (TA), basal lamina of seminiferous tubules (arrowheads) and interstitial tissue (arrows).

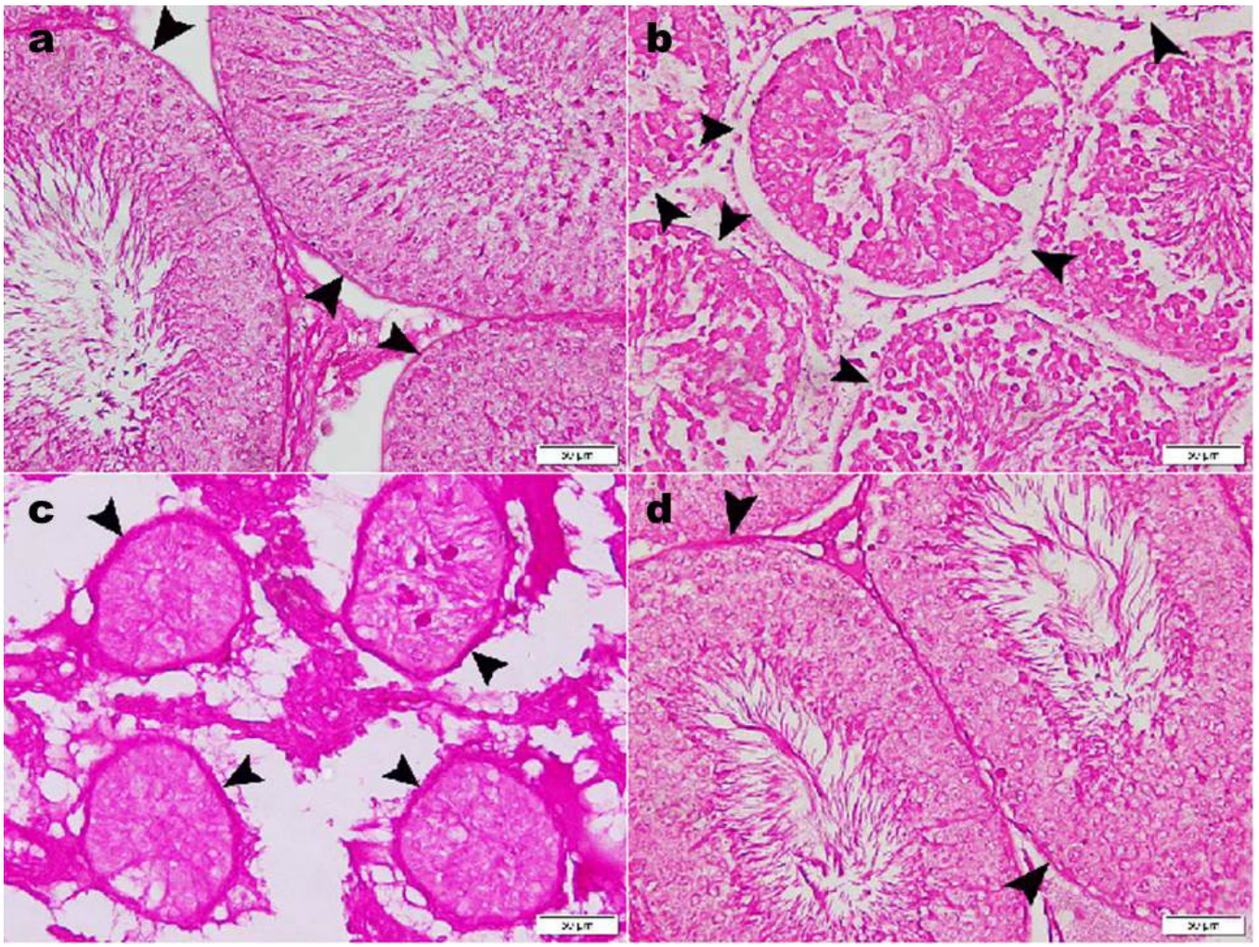

Fig. 3: Photomicrographs of testicular sections stained with PAS X200. (a) Group I shows seminiferous tubules with thin clear basal laminae (arrowheads). (b) Group II shows focal disruption of the tubular basal laminae at many sites (arrowheads). (c) Group III reveals thickened basal laminae in the recovery tubules (arrowheads). (d) Group IV shows seminiferous tubules with continuous thin clear basal laminae (arrowheads).

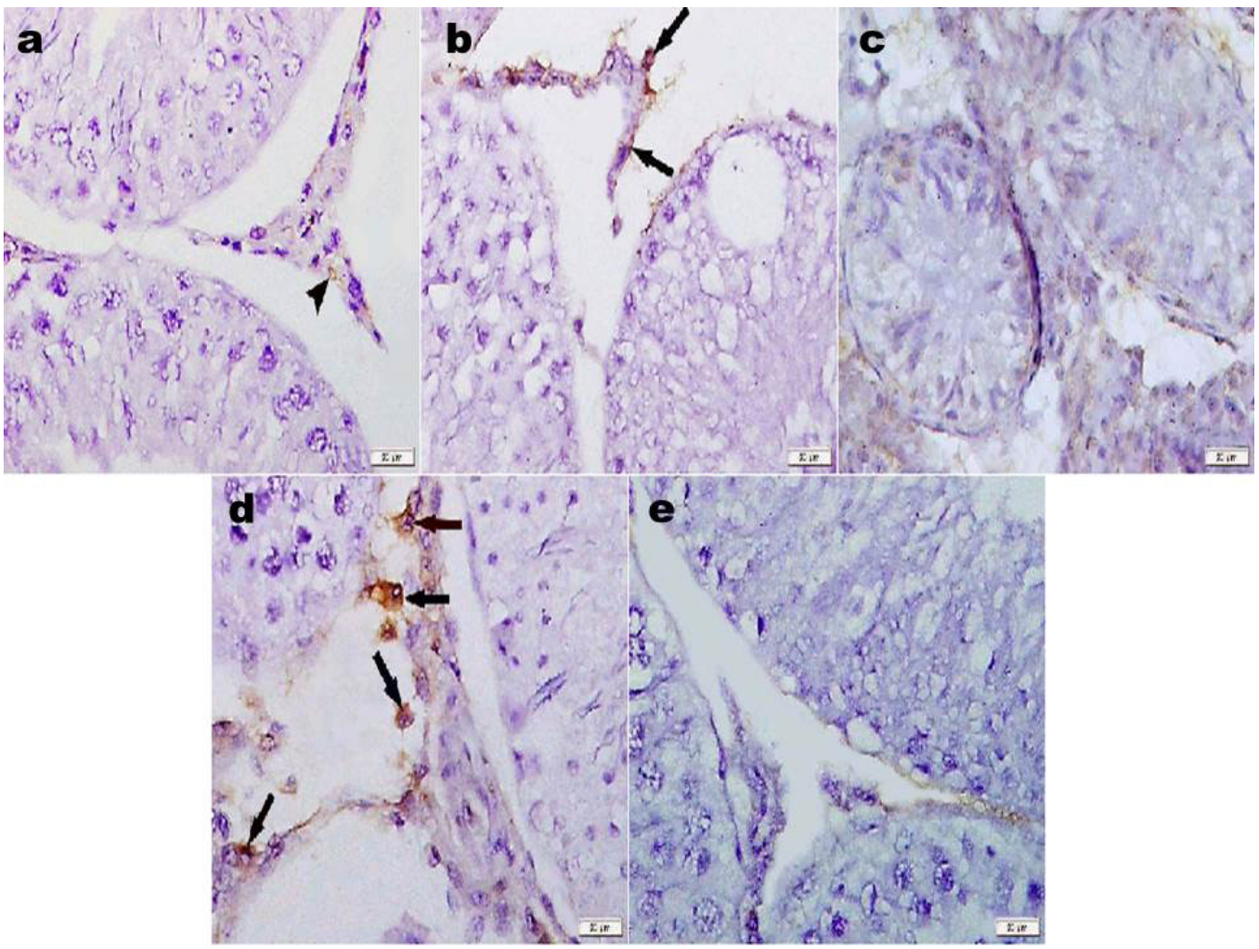

Fig. 4: Photomicrographs of testicular sections stained with CD34 immunostaining X400. (a) Group I shows positive immunoreactivity for CD34 in the wall of small blood vessel among interstitial tissue (arrowhead). No CD34 immunopositive HSCs can be detected. (b) Group III on day 5 shows few CD34 immunopositive HSCs in the interstitial tissue (arrows). (c) Group IV on day 5 reveals many CD34 immunopositive HSCs within the interstitial tissue (arrows). (d) & (e)Group III&Group IV respectively after 8 weeksshow no CD34 immunopositive HSCs.

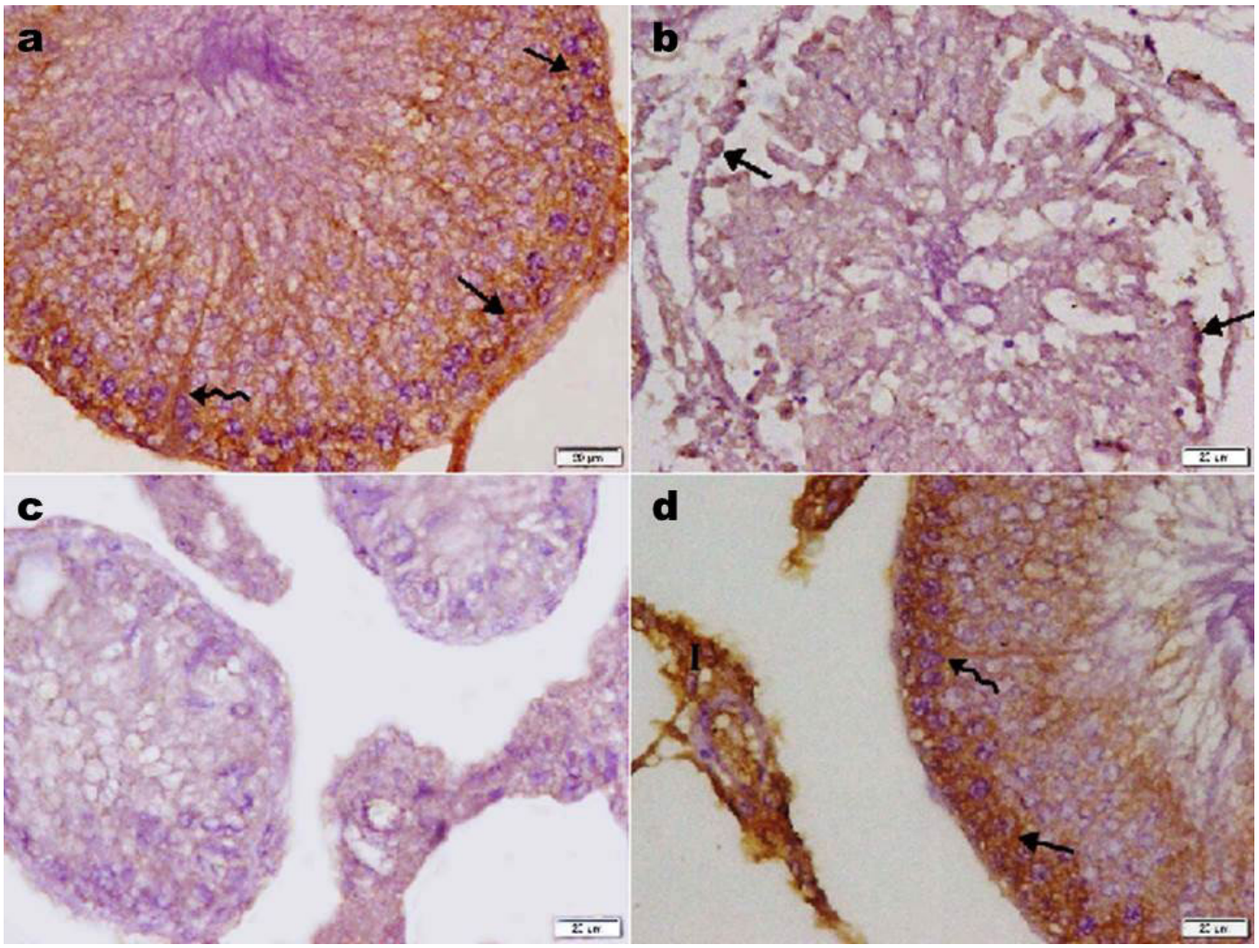

Fig. 5: Photomicrographs of testicular sections stained with E-cadherin immunostaining X400. (a) Group I showsstrong positive and wide distribution of E-cadherin immunoreactivity at the basal part of the seminiferous tubule (arrows) and in a Sertoli cell (wavy arrow) attaching its lateral walls to spermatocytes and spermatids. (b) Group II exhibits very weak cadherin immunoreaction (arrows). (c) Group III reveals negative immunoreactivity. (d) Group IV shows strong positive immunoreactivity at the basal part of the seminiferous tubule (arrow) and in a Sertoli cell (wavy arrow) and its lateral walls between it and other spermatogenic cellswith low reactivity in the central part of the tubule. Note the positive reaction in the interstitial tissue (I).

Table 2: Mean values ± standard deviation (SD)of serum testosterone level in control and experimental groups

Groups	Testosterone (ng/ml)
Group I	8.1±0.58
Group II	7.6±0.44
Group III	3.3±0.42[a]
Group IV	7.8±0.29

a significant decrease compared to the other groups

Table 3: Mean values ± SD of sperm count, mean % of sperm viability and motility in control and experimental groups

Groups	Sperm Counts (million /ml.)	Viability (%)	Motility (%)
Group I	95.10±4.75	93.00±4.05	92.00±3.33
Group II	20.00± 2.94[a]	4.70±0.34[a]	4.70±0.34[a]
Group III	22.17±6.49[a]	2.50± 0.29[a]	2.50 ± 0.29[a]
Group IV	84.60± 4.03	86.00±4.97	73.50±6.36[b]

a significant decrease compared to both groups I& IV.
b significant decrease as compared to group I.

Table 4: Mean values ± SD of the Johnsen score, diameter of seminiferous tubules and thickness of spermatogenic epithelium in control and experimental groups

Groups	Johnsen score	Diameter of seminiferous tubules (µm)	Thickness of spermatogenic epithelium (µm)
Group I	9.85±0.22	373.81±3.44	105.41±2.25
Group II	5.33±0.13[a]	322.42±2.73[a]	81.61±0.66[a]
Group III	3.91±0.36[b]	209.63±5.62[b]	49.50± 0.39[b]
Group IV	9.28±0.43[c]	361.44±2.71[c]	98.62±0.03[c]

a significant decrease compared to both groups I & IV.
b significant decrease compared to the other groups.
c significant increase compared to both groups II and III but non-significant compared to group I.

Table 5: Mean values ± SD of area% of collagen fibers, optical density of PAS +ve reaction, number of CD34 +ve cells and area % of E-cadherin +ve reaction in control and experimental groups

Groups	Area % of collagen fibers	Optical density of PAS +ve reaction	Number of CD34 +ve cells		Area % of E- cadherin +ve reaction
			On day 5	After 8 wks	
Group I	10.25±2.06	1.90 ± 0.30	0.00±0.00	0.00±0.00	13.12 ± 0.74
Group II	9.44 ± 3.08	0.53±0.22[b]	-		6.56 ± 0.44[f]
Group III	22.21 ± 6.68a	3.51±0.92[a]	2.29± 0.64[c]	0.00±0.00[e]	0.01 ± 0.02[b]
Group IV	12.85 ± 2.81	1.78±0.42	15.55±1.73[d]	0.00±0.00[e]	11.99 ± 0.87

a significant increase compared to the other groups.
b significant decrease compared tothe other groups.
c significant increase compared to group I.
d significant increase compared to both groups I & III.
e significant decrease compared to the same groups on day 5.
f significant decrease compared to both groups I & IV.

DISCUSSION

Testicular torsion is the twisting of the spermatic cord, which cause a decrease or complete loss of blood flow to the affected testis. It is a common surgical emergency affecting mainly adults leading to male subfertility and infertility[15]. Oxidative stress, inflammation and immunological responses were involved in testicular torsion/detorsion pathogenesis[16].

In the current study we explored the protective effect of G-CSF on testicular injury after torsion-detorsion (T/D). The light microscopic examination of T/D rats in group II showed distorted seminiferous tubules with interrupted basal laminae at many sites. The germ cells were separated from the basement membrane and from each other with signs of degeneration. Tunica albuginea showed widely separated collagen fibers (by edema) and disrupted collagen fibers in the basal laminae of seminiferous tubules and interstitial tissue were also detected. These observations were further confirmed by quantitative morphometric measurements which revealeda significant decrease in the mean Johnsen score, seminiferous tubules diameter and epithelial thickness valuesas compared to control and G-CSF treated groups.In addition, a significant decrease in the optical density of PAS +ve reaction and area % of E-cadherin immunoreactivity was detected when compared to the other groups.

The same findings were observed by Kostakis *et al*[1] who describeddestructed seminiferous tubules with coagulative necrosis of the germ cells, along with smaller areas characterized by apoptotic germ cells and poorly defined borders of seminiferous tubules in rats subjected to torsion/detorsion of testis. The previous changes were explained by Galhom *et al*[17] who reported thattesticular T/D can cause an ischemia/reperfusion injury (IRI) to testes; a state in which the blood supply to tissue is decreased, causing hypoxia and increase in lipid peroxidation products. However, after reperfusion, when the blood supply increases, large amounts of oxygen and nitrogen-derived free radicals are formed resulting in more damage to the ischemic tissue. In addition, signs of cellular degeneration were attributed to DNA damage, protein synthesis inhibition and spermatogenesis arrest caused byT/D injury, resulting in inhibition of sperm production[18].

Intercellular communication is of great importance in male reproductive biology. E-cadherin is a structural protein in adherent junctions of Sertoli cells and plays a vital role in development of blood testis barrier[19]. Mohamed *et al*[20] mentioned that, exposure of spermatogenic cells to ROS downregulating the expression of E-cadherin and causing disruption of the blood–testis barrier, allowing passage of toxic agents between the cells and widening of intercellular spaces. Edematous tunica albuginea might be due to capillary dilatation and postcapillary venules slowing blood flow which became lodged with RBCs, as observed by[15].

In this study,There was a significant decrease in the mean values of sperm count, mean % of sperm viability and motility in groups II & III, in addition to, a significant decrease of serum testosterone of group III as compared to the control.It was stated that mammaliantestes are highly sensitive to oxidative stress due to high content of

polyunsaturated fatty acids in its plasma membranes[21]. These fatty acids are essential for male germinal cells to maintain sperm viability and mobility[22]. Thus, oxidative stress might disrupt the steroidogenesis of Leydig cellsresulting indecrease in testosterone level in addition to affecting the germinal epithelium differentiation into normal spermatozoa[15].

Group III (recovery group) revealed shrunken seminiferous tubules with reduced thickness of the germinal epithelium and thickened basemement membranes. The germ cells markedly decreased with some cytoplasmic vaculations. The interstitium exhibited exudate, many inflammatory cells and marked fibrosis. These changes were further confirmed by the quantitative morphometric analysis which proved a statistically significant decrease in the mean Johnsen score, seminiferous tubules diameter, epithelial thickness values and area % of E-cadherin immunoreactivity, however, a significant increase in the area % of collagen fibers and the optical density of PAS +ve reaction was detected as compared to the other groups.

These findings could be as a result of ischemic reperfusion injury for a longer duration in the recovery group (8 weeks) as compared to group II (24 hrs). This agreed with Wei *et al*[23] who reported that testicular torsion-detorsion produced pronounced injury in testis 3 months after detorsion. In addition, Abo Gazia[24] also observed severe histological changes in the recovery group (4 weeks after detorsion) due to ischemic reperfusion injury in the form of extensive widening of interstitial spaces, shedding of some layers of the seminiferous tubules, tubular atrophy with fibrosis, and complete absence of sperms. Shrinkage in germ and Sertoli cells could also explain the negative immunoreactivity for E-cadherin.

The thickened basement membrane could be a result of collagen deposition into the basement membrane and interstitium due to increase in its production by myoid cells or reduction of its proteolysis in the extracellular matrix[25]. This tendency towards fibrosis may be one of the possible explanations for the shrunken seminiferous tubules. In addition,AbouElnaga *et al*[26] reported that increased thickness of the basement membrane leads to impairment of the testicular metabolism causing germinal cell hypoplasia and tubular atrophy.

Cytoplasmic vacuolation of the germ cells were attributed to the hydropic degeneration resulting from mitochondrial dysfunction and disruption of sodium pump with increased sodium influx and attraction of water[27]. The inflammatory cells infiltration could be referred to the reperfusion state which causes infiltration of the interstitial spaces by leukocytes and macrophages and this is agreed withKumar *et al*[28] who mentioned thatinflammatory cells infiltration typically begins within 1–2 weeks of tissue damage.The exudate was attributed to an increase in vascular permeability[24].

As regards CD34+ immunopositiveHSCs, on day 5, group III showed a significant increase in CD34 +ve cells as compared to the control group. This is consistent with Maeda[29] who mentioned that tissue damage stimulates SCs to move from the bone marrow into the blood circulation, then accumulate in the damaged tissue. Thus, SCs can home the injured tissue. HCSs have the ability for self-renewal and differentiation for multiple cells. They can be identified by specific cell surface markers as CD34+[30].

Our study showed marked improvement in testicular structure and function in group IV(G-CSF treated group) confirmed by all parameters measured by morphometry. Also, there was a significant increase in Johnsen score, serum testosterone, sperm count, % of sperm viability and motility in comparison to that of non-treated groups (groups II&III) and a significant decrease in the mean % of sperm motility as compared to the control.

In support,Ntemoue *et al*[31] reported that G-CSF treated mice exhibited better recovery of spermatogenesis after busalfan treatment.G-CSF, a hematopoietic growth factor, is known by its ability to enhance spermatogenic recovery and potentially preserve fertility as proved by Kotzuret *et al*[32] who reported that the mode of G-CSF action is by promotion of spermatogonia proliferation, leading to enhanced spermatogenic regeneration from the surviving spermatogenic stem cells.In addition,It can bind to the undifferentiated spermatogonia receptor and stimulate spermatogenesis.

Morever, the protective effect of G-CSF is mediated by endogenous mobilization of BM stem cells and homing into the injured tissues, where the stem cells enhance tissue recovery according to Khanlarkhani *et al*[33] who reported that endogenous mobilization of HSCs was enhanced by G-CSF after busulfan exposure. The use of G-CSF to mobilize HSCs has several advantages in comparison to collection of natural bone marrow. G-CSF can mobilize stem cells in a higher yield. It can also activate the phagocytic functions of neutrophils[34]. This was confirmed by the results of CD34 immunohistochemical staining which showed on day 5, a significant increase in group IV as compared to groups I and III. However, after 8 weeks, there was a significant decrease as compared to the same group on day 5. This could be explained by Carvalho *et al*[35] who mentioned that, the CD34 antigen expression is reduced gradually as the level of maturation of hematopoietic cell lineages increases, to the point of becoming completely absent in fully mature cells.

AboulFotouh *et al*[5] added that G-CSF can minimize inflammation by downregulation of pro-inflammatory cytokines' production and thus ameliorates the destructive inflammatory response, in addition to its ability to increase glucose and vitamin C uptake in testes, resulting in improvement of sperm viability and motility and enhancement of the antioxidant action in germ cells as spermatozoa.

CONCLUSION

Our results revealed that G-CSF has a protective action on spermatogenesis and sperm parameters after

induced testicular torsion/detorsion injury bystimulating proliferation of surviving spermatogonia, endogenous mobilization of HSCs andattenuating inflammation. Thus, it enhances spermatogenic recovery and potentially preserves fertility.

CONFLICT OF INTERESTS

There are no conflicts of interest.

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الملخص العربى

التأثير الوقائي المحتمل لعامل تحفيز مستعمرة الخلايا المحببة علي إصابة الخلايا الجنسية ، تكوين الحيوانات المنوية ومعلمات الحيوان المنوي في نموذج الجرذ لالتواء-اصلاح التواء الخصية. دراسة نسيجية وكيميائية مناعية

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الخلفية والأهداف: التواء الخصية هو حالة طارئة في المسالك البولية تنطوي على خطر العقم نتيجة لإصابة نقص تروية ثم إعادة التروية للخلايا الجنسية. لذلك ، تم التخطيط لهذه الدراسة لاختبار التأثير الوقائي لتحريك الخلايا الجذعية المكونة للدم بواسطة عامل تحفيز مستعمرة الخلايا المحببة على نموذج الجرذان لإصابة الخلايا الجنسية التي يسببها التواء-اصلاح التواء الخصية.

المواد والاساليب: تم تقسيم ٣٢ جرذًا من الذكور البالغين بشكل عشوائي إلى المجموعة الأولى (المجموعة الضابطة) والمجموعات التجريبية التي تضمنت المجموعة الثانية (مجموعة االتواء-اصلاح االتواء الخصية) ، والمجموعة الثالثة (مجموعة التعافي التلقائي) والمجموعة الرابعة (االتواء-اصلاح االتواء الخصية المعالجة بعامل تحفيز مستعمرة الخلايا المحببة, تعرضت هذه المجموعات التجريبية لإصابة الالتواء-اصلاح الالتواء عن طريق تدوير الخصية اليسرى ٧٢٠ درجة في اتجاه عقارب الساعة لمدة ٣ ساعات. قبل نصف ساعة من اصلاح الالتواء في المجموعة الرابعة ، تم حقن عامل تحفيز مستعمرة الخلايا المحببة تحت الجلد بجرعة ٧٠ ميكروجرام / كجم / يوم ثم يوميًا لمدة ٥ أيام متتالية ، وفي نهاية الدراسة (٨ أسابيع) ، تم إجراء الفحص الهرموني وتحليل الحيوانات المنوية و تم أخذ عينات من الخصية للتحليل النسيجي والكيميائي المناعي والإحصائي.

النتائج: أظهرت النتائج أن متوسط مستويات هرمون التستوستيرون وعدد الحيوانات المنوية والنسبة المئوية من حيوية الحيوانات المنوية وحركتها قد تأثرت بشكل ملحوظ بعد االتواء-اصلاح االتواء الخصية مع فقدان البنية النسيجية الطبيعية للخلايا المولدة للحيوانات المنوية والخصية. أظهر حقن عامل تحفيز مستعمرة الخلايا المحببة قبل اصلاح الالتواء تحسنًا ملحوظًا في مستويات هرمون التستوستيرون وتحليل الحيوانات المنوية مع تحسن ملحوظ في التغيرات النسيجية.

الاستنتاج: يحمي عامل تحفيز مستعمرة الخلايا المحببة خصية الجرذان البالغة من الإصابة بنقص التروية / إعادة التروية الناتجة عن االتواء-اصلاح التواء الخصية. تشير الدلائل القوية إلى أن حقن عامل تحفيز مستعمرة الخلايا المحببة قبل اصلاح الالتواء يقلل من التغيرات التنكسية في الخصية بعد نقص التروية والآثار الضارة لأنواع الأكسجين التفاعلية الناتجة عن اعادة التروية.