Vol 5|Issue 3| 2015 |158-169.

International Journal
of
Pharmacy Review & Research
www.ijprr.com

e-ISSN: 2248 – 9207
Print ISSN: 2248 – 9193

ROLE OF BONE MARROW MESENCHAYMAL STEM CELLS (MSCs) ON RESTORATION OF FERTILITY IN MALE RATS AFTER EXPOSURE TO ENDOCRINE DISRUPTER

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ABSTRACT

This study aimed to examine the role of bone marrow mesenchymal stem cells (MSCs) in the restoration of fertility and reproductive functions in male rats after testicular failure induced butributyltin oxide (TBTO). Rats were divided into 5 groups: Group 1 (-ve control group) with no treatment; Group 2 (TBTO vehicle control) ; Group 3(TBTO group) rats were given orally TBTO 0.062 mg/kg b.wt in 1ml distilled H_2O containing 50µ ethanol twice a week for 3 months ; Group 4 (TBTO and MSCs vehicles control) and Group 5(TBTO and MSCs treated group) rats were given (0.062) mg/kg b.wt TBTO twice a week for 3 months and then injected intravenously with a single dose of MSCs ($3x10^6$ cells in 0.5 ml phosphate buffer saline) per animal. After 5 months, male rats were allowed to mate with untreated females and then dissected. Serum samples were collected and testis sections were imune-histochemical stained for detection of CD44 positive and proliferating cell nuclear antigen (PCNA)-positive cells. Results showed that, TBTO caused significant reduction in the body weight, fertility rate and reproductive functions. The Seminiferous tubules appeared atrophied with obstructed lumen and oligospermia. However, the depletion of germ cells and decrease of PCNA-were noticed. Transplantation of MSCs markedly restored the reduction of body weights, fertility rate, serum testosterone, LH, FSH hormones, testicular enzymes, sperm counts and improved testicular DNA fragmentation. In the testicular tissue, CD44 positive cells were detected after 3months of MSCs transplantation with increase in the proliferative capacity (PCNA) stained cells. Even more, MSCs transplantation showed normal spermatogenesis process and complete recovery in germinal layers. In conclusion, MSCs transplantation can restore male fertility after disruption induced by tributyltin oxide in rats. If this protocol was proven to be functional in human, this would provide a new therapeutic concept for male infertility treatment.

Key words: Mesenchymal stem cells, Endocrine disrupters, Fertility index, Hormones, Testicular functions, Comet assay, Immunohistochemistry, Histology.

INTRODUCTION

Infertility affects 13-18% of married couple. Evidences exist from clinical and epidemiological studies suggesting an increasing incidence of male reproductive problems [1].

There is the potential for endocrine disrupting chemicals (EDCs) to act at any level of the hypothalamo-pituitary-gonadal (HPG) axis but there is general support for the view that the development and programming of the axis during fetal life could be the most sensitive window to permanently alter the homeostatic mechanisms of the endocrine system [2].

Organotinsastributyltin (TBT) are a diverse group of widely distributed environmental pollutants that have been implicated as reproductive toxicants and endocrine disruptors, and mainly used as biocides in antifouling boat paints and in plastics industry as heat and light stabilizers for poly vinyl chlorideplastics. If these endocrine disrupters mimic endogenous hormone activities, immature male rats would be more vulnerable during the

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pubertal period where sexual maturation and reproductive organs are still under development.

Stem cells are non-differentiated cells that have the ability to proliferate, regenerate, and transform into differentiated cells and it has been reported that mesenchaymal stem cells(MSCs) when transplanted systemically, it home into the damaged site and finally differentiate into cells specific to the tissue. Bone marrow represents a major source for tissue-derived MSCs. The differentiation capabilities of MSCs and their relative ease of expansion in culture clearly make MSCs potentially ideal candidates for tissue repair and gene therapy [3].

Many diseases as leukemia, Parkinson's disease, diabetes, vision impairment and heart tissue injuries are currently being successfully treated by using stem cell technology [4]. Some of the recent studies applied stem cells for the treatment of breast cancer [5], fertility and reproductive functions[6] and others applied stem cells for the treatment of liver diseases [7] and kidney injury[8].

The target of this study is to examine the role of bone marrow mesenchymal stem cells (MSCs) in the restoration of fertility and reproductive functions in young rats after testicular failure induced by tributyltin oxide (TBTO).

MATERIALS AND METHODS

Animals

A total number of 115 young male Sprague-Dawley rats (85-90 g.) and 84 female rats (140-150 g.) were obtained from Animal House of El Salam Farm, Giza-Cairo, Egypt. The animals were acclimatized to the laboratory conditions for two weeks prior to the start of the experiment. The rats were housed in metal cages at temperature of 24-27 °C at48-54% humidity,12 hours dark/ light cycle. All rats were fed on a standard diet food and water was available all time throughout the experimental period. The experimental procedures complied with the guidelines of the Committee on Care and Use of Experimental Animal Resources, AinShams University, Cairo, Egypt.

Chemical

Tributyltin oxide (TBTO) was purchased from Sigma (Sigma Aldrich, Sigma Chemical Co., St. Louis, Missouri, USA). TBTO was dissolved in distilled water and ethanol 99.5% (50µ ethanol in 1ml water for each rat).

Determination of LD50 of TBTO

LD_{50} was determined according to the method of Behreus & Karbeur [9].A total of 40 male rats were divided into 4 groups (n=10). The groups were injected 0.1, 0.2, 0.3and 0.4 mg/kg b.wt TBTO, respectively. All groups were observed for one week and the number of dead rats in each group was recoded. LD50 = The highest dose - $\sum$ a b/n(number of rats in each group) Where: a: is a constant factor between two successive doses. b: is the mean of dead rats in two successive groups. The determined LD_{50} of TBTO for male rats was 0.25 mg/kg b.wt.

Dose selection and induction of testicular failure

After determination of LD_{50}, a total of 16 immature male rats were divided into 4 groups each of 4 rats one group was kept as a control, and the other three groups were orally injected with TBTO as follow: One group was injected with 1/10 LD_{50} (0.025), the other group was injected with 1/5 LD_{50} (0.05) and the last group was injected with 1/4 LD_{50} (0.062) mg/kg b.wt. Two rats were sacrificed after 1 month and another two after 3 months for histopathological examination and determination of serum testosterone level in order to confirm testicular failure. The present work found that, testicular failure was induced by injection with 1/4 of LD_{50} for 3 months. So, 1/4 of LD_{50} (0.062) mg/kg b.wt was selected during the experiment as the dose that induced testicular failure.

Preparation of Bone Marrow-derived Mesenchymal Stem Cells MSCs

Under sterile conditions, the bone marrowsof 15 male albino rats (6-week-old) were harvested by flushing the tibiae and femurs with Dulbcco's modified Eagles medium (DMEM) (Lonza Company, Swiss). The harvested cell suspension was divided into 7easy flask 25 cm with DMEM containing 12% fetal bovine serum(Lonza Company, Swiss), 1% penicillin- streptomycin (Lonza Company, Swiss) and 0.05% Amphotericin B (Lonza Company, Swiss). Cells were incubated at 37°C in 5% humidified CO_2;the cultured cells were examined daily by using the inverted microscope to follow up the growth of the cells and to detect the appearance of any bacterial or fungal infection among the cultured cells. After 5 days the supernatant that contained the non-adherent cells was removed by aspiration using a sterile pipette. The adherent cells were then washed twice with a sterile phosphate buffer saline (PBS). Finally 10 ml of fresh complete media was added to the dish. MSCs were distinguished from other BM cells by their tendency to adhere to tissue culture plastic [10].The second exchange of media was done after 3 days [11]. On day 9 the cultured cells showed confluent appearance. Onday 12 the culture was washed with PBS and released with 0.25% trypsin in 1 mmol/l EDTA (Lonza Company, Swiss) (4 min at 37°C). After centrifugation, the cells were re-suspended and counted with trypan blue stain 0.4%(100µL of cell suspension with 100µL of stain) using Neubauer haemocytometer as described by Belsey *et al*[12]. MSCs were distinguished from other BM cells by their tendency to adhere to tissue culture plastic[10], and by negative expression of CD34 and positive expression of CD44 (Fig. 1) in immune-staining, described by Li *et al*[13]. Briefly, the cultured cells were fixed in petri dishes on day 12 of culture by Acetone: Methanol (1:1) then covered by H2O2 (10%). Dishes then immersed in a preheated citrate buffer solution (PH 6) and maintaining heat in a microwave, then left to cool, washed in distilled water and incubated in normal blocking goat serum. The primary antibody (CD34 or CD44 monoclonal mouse anti-human) was added and left overnight in the humidified chamber, then washed. The secondary biotinilated anti mouse antibody was added and incubated. Then, dishes were washed and covered by

Streptavidin horseradish peroxidase conjugate. Color was developed using Di-amino-benzidine.

Experimental design

Male rats were divided into 5 groups (n=7 each): Group 1 (-ve control group) with no treatment; Group 2 (TBTO vehicle control) : Rats were given orally 50µ ethanol in 1ml distilled H_2O twice a week for 3 months ; Group 3: rats were given orally TBTO 0.062 mg/kg b.wt in 1ml dist. H_2O containing 50µ ethanol twice a week for 3 months ; Group 4 (TBTO and MSCs vehicle control): Rats were given orally 50µ ethanol in 1ml distilled H_2O twice a week for 3 months, and then injected intravenously with a single dose of 0.5 ml phosphate buffer saline (PBS) (MSCs vehicle) per animal until 5 months; Group 5: Rats were given (0.062) mg/kg b.wt TBTO twice a week for 3 months, and then injected intravenously with a single dose of MSCs ($3x10^6$ cells in 0.5 ml PBS) per animal until 5 months.

Immune-histochemical staining

Immune-histochemical staining for the CD44 antigen using anti-CD44 antibodies was used to detect CD44-positive cells in testis tissues after 3 months and 5 months of MSCs transplantationand for proliferating cell nuclear antigen (PCNA) after 5 months of MSCs transplantation which was carried out by using the avidin–biotin peroxidase complex ABC technique [14]. Paraffin sections were deparaffinized and hydrated. After blocking the endogenous activity of peroxidase using 10% hydrogen peroxide, the sections were incubated with primary antibodies,CD44v6 (variant 6) Ab-1(Clone VFF-7) monoclonal mouse anti-human (Labvision.USA). PCNA antibody is a mouse monoclonal antibody PC 10 (Novocastra, Milton, Keynes, USA). After washing with phosphate buffer, the secondary antibody was applied (biotinylated goat antirabbit). The slides were incubated with labeled avidin–biotin peroxidase, which binds to the biotin on the secondary antibody. The site of antibody binding was visualized after adding (diaminobenzedine) chromogen, which is converted into a brown precipitate by peroxidase. CD44-positive cells showed brown cytoplasmic deposits. PCNA-positive cells showed brown nuclear deposits.

Mating trails

Mating trails were allowed 10 days before dissection. Each male of both control and treated groups was paired separately with normal untreated mature females (at proestrous stage) for 7 to 10 days. Vaginal smears were made daily to detect the presence of sperms. When +ve vaginal smears were detected this is considered as day one of pregnancy. Males from each group were separated and sacrificed; females with positive vaginal smears were isolated and kept under observation then sacrificed at the 20^{th} day of gestation. Uteri were weighed and dissected and the number of implantation sites and number of embryos were recorded. Fetuses were examined morphologically for any changes and weighed and the average body weight and body length as well as placental weights were recorded.

Male dissection

After 3 months, rats from (-ve) control, (TBTO vehicle control) and (TBTO treated group), were sacrificed. While after 8 months, rats from (-ve) control group, (TBTO plus MSCs vehicle control) group and TBTO plus MSCs treated group were sacrificed. The blood samples (6-7 ml per rat) were collected from heart (ventricles) for all experimental animals by plastic tuberculin syringes, 5 ml were put into clean centrifuge tubes, left at room temperature in an oblique position to coagulate and centrifuged at 3000 rpm for 25 minutes to separate serum samples. A clear serum with no hemolysis were divided into 4-5 parts in Eppendorf tubes and frozen at $-20C^o$ until used for the biochemical analysis. After collection of blood samples, the testes and cauda epididymis were removed immediately after dissection, washed in saline. Testes from each rat were weighed; the left testis was frozen at $-20\ C^o$ until the biochemical analysis and comet assay. The right testes were fixed in Bouin's solution for histological examinations. Two caudaepididymus from each rat were dissected; each of them was minced in 2ml 0.9% NaCl. The semen was carefully mixed and kept at $4\ C^o$ for sperm counts. Films were spread on clean slides, left to dry and stained with H&E stain for the examination of sperm morphology.

Fertility index

The fertility index was estimated according to Sadre *et al*., [15] as follow:

(%) fertility index= Total no. of pregnant females /Total no. of mated females x100.

Hormones determination

Hormonal profile of serum testosterone, lutenizing hormone (LH) and follicle stimulating hormone (FSH) were carried out using an enzyme linked immunosorbent assay ELISA kit (BioCheck, Inc., USA) according to the manufacturer's instructions**.**

Testicular functions

Alkaline phasphatase was determined colorimetrically by using a commercial kit purchased from Stanbio Chemicals (USA, Gamma Trade Co). Acid phosphatase activity was measured according to Fishman [16].GGT was estimated according to the method of Szasz [17].

Sperm count & morphology

The two caudaepididymus from each rat were be dissected, each of them was minced in 2ml 0.9% NaCl. The semen was carefully mixed; the epididymal fluid was subjected to sperms count using Neubauer haemocytometer as described by Belsey *et al.,* [12]. Films were spread on clean dray slides, left to dry and stained with HX&E stain for the examination of sperm morphology.

1

DNA analysis, Single-cell gel electrophoresis (comet assay)

Comet analysis was carried out according to the protocol described by De Boeck *et al.,* [18] and Kašuba *et al.,* [19]. All chemicals and reagents used were obtained from Sigma (Sigma Aldrich, Sigma Chemical Co., St. Louis, Missouri, USA).

Determination of anti-apoptotic serum (Bcl-2)

The Bcl-2 was determined using an enzyme linked immunosorbent assay (ELISA Kits), Glory Science Ompany, USA.

Histological examination of testicular tissues:

From each rat, right testis was carefully dissected and fixed in Bouin's, dehydrated in 70%, 90% and 100% alcohol, cleared in xylol, embedded in paraffin wax at 60C^{o}. transverse sections were cut at 5-6 microns in thickness and affixed to slides and then stained in Haematoxyline and Eosin.

Statistical Analysis

A computer program (SPSS 17.0) was used for statistical analysis. The results were expressed as means ± standard deviation (SD) using One-Sample T Test. Data were analyzed using general linear models using ANOVA one way procedures for the comparison of the groups. Differences between the groups were considered as statistically significant when $P< 0.05$, highly significant when $P< 0.01$and very highly significant when $P< 0.001$.

RESULTS

Characteristics of MSCs in culture

After approximately 5 days in culture, cell appeared as a monolayer of broad flat cells (Fig.1a), the cells differentiated into a more spindle and fibroblastic shaped cells in culture, They reached confluence appearance at 9-14 days, (Fig.1b), and attached to the culture flasks. Most of cells did not express the hematopiotic cell marker, CD34 (Fig.1c) but expressed MSC markers CD44 (Fig. 1d).

Detection of mesenchymal stem cells (MSCs) in the testis tissues

Immuno-histochemical staining of rat testis tissues with anti-CD 44 (as a marker of MSCs), after 3 months of intravenous injection of MSCs showed CD44 positive immune reactive cells, with an irregular outline (fibroblast-like) and brown cytoplasmic immune reactivity, located in the seminiferous tubule and interstitial tissues (Fig.2 a & b).

Proliferating cell nuclear antigen immunostaining PCNA

The immune-staining of testis sections from MSCs treated rats showed multiple PCNA-positive cells in the germinal layers of several seminiferous tubules comparing to control or TBTO group (Fig. 3).

Male fertility studies

Body weight

Administration with TBTO caused a reduction in the average body weights, while treatment with MSC restored the body weights near to the normal weight as compared to controls (Table 1).

FERTILITY ASSESSMENT

Fertility index and testis weights

The fertility index for male rats received TBTO for three months recorded a highly significant decrease ($p<0.01$) and recorded (44.40%)with a change (-52.15%)of +ve control which indicated that TBTO induced testicular failure. On contrary, fertility index showed significant ($p>0.05$) improvement after 5 months from treatment with MSCs, where the fertility index raised to (76.90 %), with a change (-16.60%) of+ve control (Table1).

The testes weights were decreased significantly ($p<0.001$) in the group of rats treated with TBTO with a change (-19.10%) of +ve control, while treatment with MSC restored significantly ($p > 0.05$) the testes weights nearly to the normal weight as compared to controls with the percentage of change recorded (-4.20%) of +ve control group.

Hormones

Also, TBTO administration caused decrease ($p<0.001$) in the serum levels of testosterone and LH besides a non-significant reduction ($p>0.05$) of FSH level. The treatment with MSCs restored these values to the normal values.

Testicular Enzymes

Furthermore, testicular enzymes ACP, ALP and GGT were decreased in rats induced testicular failure. Improvement of these enzymes levels were markedly confirmed after the treatment with MSC (Table 1).

Sperm Count & Morphology

Additionally, a marked reduction in sperms count (more than 90 % of +ve control), after confirmation of testicular failure, while sperms count showed marked regulation after treatment with (MSCs) with only (-11.1% of +ve control).Also, sperms abnormalities were markedly observed in the forms of abnormal head, tail and head & tail in rats administered with TBTO, while, treatment with (MSC) has great ability to recover sperm abnormalities to the normal morphology (Fig. 3c).

The results of the present work revealed that, administration of TBTO for 3 months caused significant reduction ($p<0.001$) in anti- apoptotic Bcl-2 expression, where Bcl-2 was markedly decreased to (-52.28 %) of +ve control. Treatment with mesenchymal stem cells after TBTO administration resulted in up-regulation of Bcl-2 expression to reach (46.63%) of the +ve control group (Table1).

Comet assay

Table1 showed the mean values of DNA % tailed cells as a marker of DNA migration, which reflected DNA fragmentation and damage. In TBTO group, the damaged

testes cells (tailed cells) increased with a wide variation from +ve control (461 %) while this change markedly decreased to (103%) after MSCs treatment. With a similar comparison, the percent of DNA in tails increased (394%) while this change decreased to (89%) after MSCs treatment (Table1 and Fig 4).

Histopathological changes

Testis of control rats showed normal seminiferous tubules structure, interstitial tissue, blood capillaries and leydig cells.(Fig.5a):Examination of testis sections from the rats administered TBTO showed malformations and disorganizations of the most seminiferous tubules, some lumens of the seminiferous tubules were obstructed or filled with damaged cells and others suffered from azoospermia or oligospermia (Fig.5b). Moreover,Shrinkage, severely atrophoid, collapsed seminiferous tubules as well as vacuolation and reduction in the thickness of the germinal layers was markedly observed (Fig.5c). The nuclei of damaged cells in the forms of pyknosis, karyolysis or marked karyorrhexis along with areas of necrosis were severely detected (Fig.5d). On the other hand, testis tissues from male rats after treatment with MSCs showed marked restoration of the general structure of testicular tissues. The testicular tissues showed improvement and normal arrangement of seminiferous tubules with restoration of inter-tubular tissues (Fig. 5e). Most of the seminiferous tubules showed complete development of their germinal epithelia and the process of spermatogenesis (Fig. 5f). In spite of this recovery, some lesions such as edema and few disorganized seminiferous tubules were still observed (Fig 5e).

Mating study

After mating of treated male with TBTO for three months alone or with MSCs with normal untreated females did not show any-significant effects on the 20 day old fetuses body weights, lengths,Uteri weights, placental weights, number of implantation sites, number of live fetusesas well as fetal morphological changes compared to the fetuses of normal control groups.

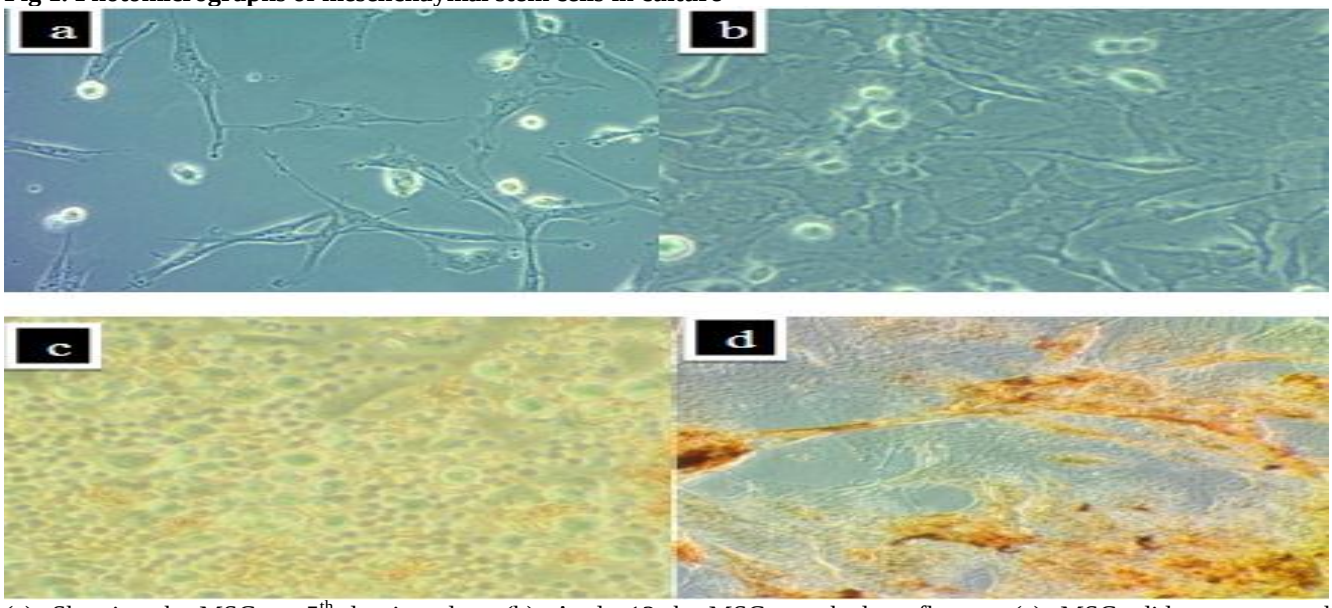
Table 1. Effect of MSCs on the body weights, fertility rate and reproduction performance after induction of testicular failure by TBTO administration for 3 months in male rats

groups / parameters	TBTO administration (for 3 months)			TBTO and MSC treatment (TBTO for 3 months then single dose of MSCs until 5 months)		
	Control (-ve)	**TBTO vehicle control**	***TBTO***	**Control (-ve)**	**TBTO and MSCs vehicle control**	***TBTO and MSC***
Body weights (g.)	253.71±6	246.85±7	236^{NS}±4.70 (- 4.40%)	407.40±20	403.33±13.65	392^{NS}±14.85 (- 2.80%)
Fertility index%	93.30	92.80	44.40^{**} (- 52.15%)	92.80	92.30	76.90^{NS} (-16.60%)
Testes weights (g.)	1.40±0.05	1.36±0.01	1.10^{***}±0.03 (-19.10%)	1.54±0.05	1.63±0.09	1.56^{NS}±0.04 (- 4.20%
Testosterone ng/ml	0.92 ± 0.03	0.87±0.05	0.27^{***}±0.008 (-68.96%)	1.99 ±0.09	1.94 ± 0.06	2.07^{NS} ± 0.11 (6.70%)
FSH ng/ml	0.46 ± 0.02	0.45±0.02	0.42^{NS} ± 0.02 (-6.66%)	0.51 ± 0.02	0.51 ±0.03	0.48^{NS}±0.02 (-5.49%)
LH ng/ml	0.51 ± 0.02	0.57±0.03	0.34^{***} ±0 .02 (- 40.35%)	0.97 ±0.01	0.96 ±0.01	1^{NS} ± 0.04 (4.16%)
Serum ACP IU/L	43.71 ±1.06	42.85±0.26	40.00^{**} ± 0.53 (- 6.65%)	45.14±0 .82	45.20 ± 1.40	47.80^{NS}±0.34 (5.75%)
Serum ALP U/ml	28.20 ±1.73	30.83 ±0 .7	39.92^{***}±1.0 (29.48%)	29 ±0 .53	29.70 ± 0.80	29.42^{NS}± 0.68
Serum GGT U/ml	17.8±0.66	18 ± 1.04	22.85^{*}±1.43 (26.94%)	20.57±0 .57	21.85 ±0 .50	21^{NS} ± 0.41 (-3.89%)
Testicular ACP µg /(g)	0.44±0.04	0.44 ±0.06	0.57^{***}±0.02 (27.9%)	0.56±0.01	0.60±0.01	0.62^{NS}±0.04 (3.33 %)
Testicular ALP µg /(g)	0.37±0.03	0.37±0.03	0.77^{***}±0.04 (105.80%)	0.43±0.02	0.43±0.02	0.48^{*}±.002 (11.10%)
Testicular GGT mg /g	0.08 ± 0.01	0.08±0.03	0.42^{***}±0.07 (412.20%)	0.09±0.04	0.10±0.03	0.11^{NS}±.0.09 (11.10%)

Sperm counts x10^6	28.00 ±2.18	27.70 ± 1.40	2.54*** ± 0.32 (-90.83%)	45.15±1.29	45.00±0.89	40.00** ± 0.65 (-11%)
Abnormal sperm Head/1000	1.72 ±0.19	1.66 ±0.23	15.00*** ±1.71 (804%)	3.33 ±0.59	2.14 ±0.34	3.00^{NS} ±0.37 40.18
abnormal sperm Tail/1000	21.85 ±1.23	29.14±3.23	788.50***±35.53 (2606%)	20.63± 2.40	23.72 ±2.57	22.66^{NS} ±2.26 (- 4.67)
abnormal sperm Head &tail/ 1000	0.66 ±0.21	0.60 ±0.24	5.71***±1.37 852%)	0.80 ± 0.29	1.00 ± 0.29	0.80^{NS} ± .29 (20%)
% of comet tailed cells	8.71 ± 0.42	8.86 ± 0.45	49.71***± 2.31 (461%)	9.00 ± 0.43	9.14 ±0.34	18.57***± 0.68 (103%)
% of DNA in comet tail	3.78 ± 0.68	3.76 ± 0.57	18.57*** ±1.25 (394%)	4.07 ± 0.72	3.93 ± 0.46	7.43*** ± 0.29
Bcl-2µg/l	7.67±0.50	7.68±0.16	3.66***±0.33 (-52.34%)	7.80±0.35	7.71±0.38	11.45^{NS}±0.98 46.63

Values are expressed as mean ±S.D for 7 rats.
*** = very highly significant (p<0.001), compared controls.
**= highly significant (p<0.01).
*= significant (p<0.05).
a = compared to (-ve) control.
b = compared to (+ve) control.
NS= non-significant.

Fig 1. Photomicrographs of mesenchaymal stem cells in culture



(a): Showing the MSCs at 5th day in culture,(b): At day12 the MSCs reached confluence, (c): MSCs did not express the haematopioticmarker, CD34 and (d): Cells expressed MSC markers CD44. Light inverted microscope. X400.

Fig 2. (a, X100 & b, X400):Sections from testis of rats after 3 months of intravenousinjection of mesenchymal stem cells showing CD44 immune-reactive cells with an irregular outline and brown cytoplasmic immune-reactivity located in some seminiferous tubules (arrows). (Fig.cX400): after 5 months showing negative expression of CD44

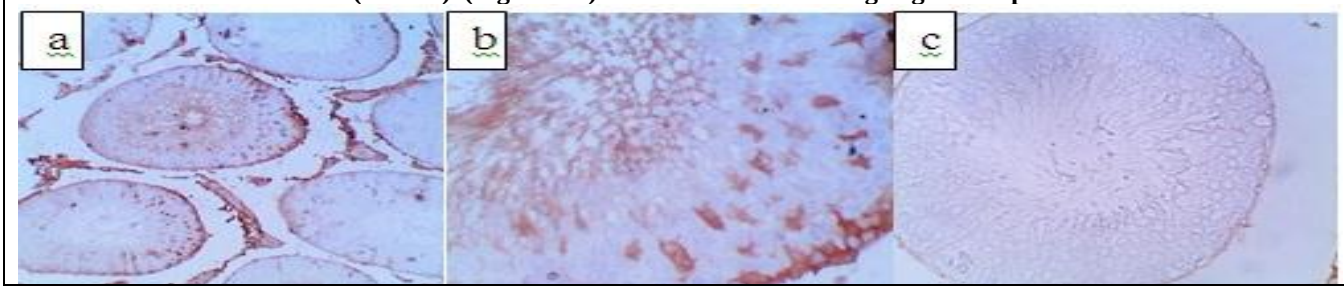
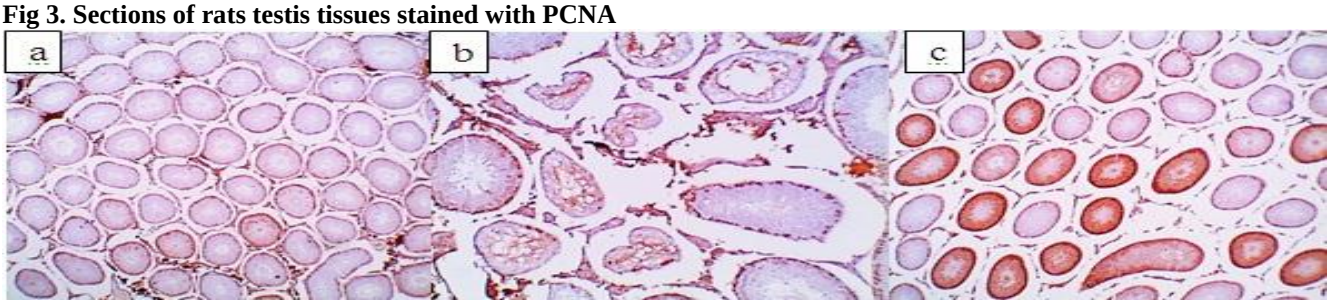


Fig 3. Sections of rats testis tissues stained with PCNA



(a): from controls showing seminiferous tubules with few PCNA- positive cells. (b): from TBTO group showing degeneration ofseminiferous tubules with few PCNA- positive cells. (c): from MSCs group showing multiple PCNA-positive cells in several seminiferous tubules.

Fig 4. (a): sperms from controls, showing normal sperm cells formed of curved hooked head and normal tail, (b):Sperms from rats injected with TBTO showing amorphous head and coiled tail, (c): Sperms from rats after treatment with MSC showing improvement in sperm deformations (HX &E).DNA fragment migration patterns by comet assay evaluated with a fluorescence microscope for testes cells,(d):from control rat showingintact cells; most of DNA is located in the head of the comet.(e): from TBTO group showingtailed cells, DNA fragmented and migrated from the comet head and formed a tail.(f): showingrestoration to the normal intact cells

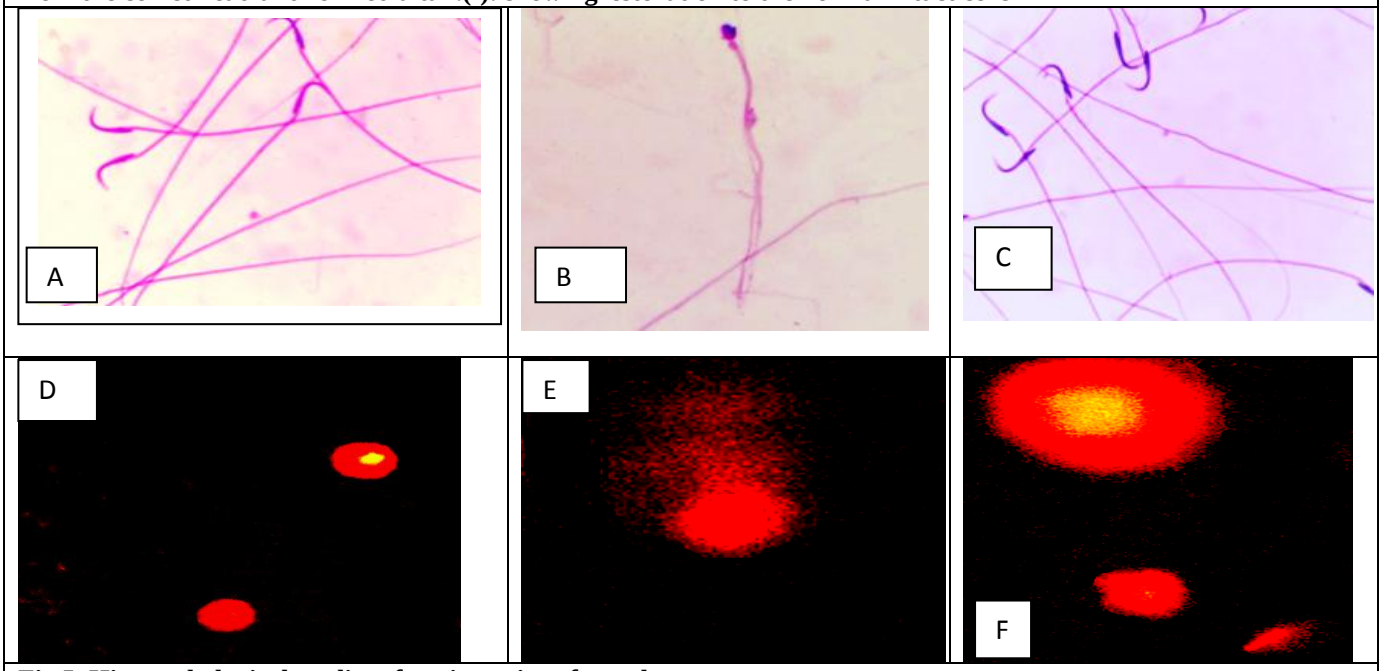
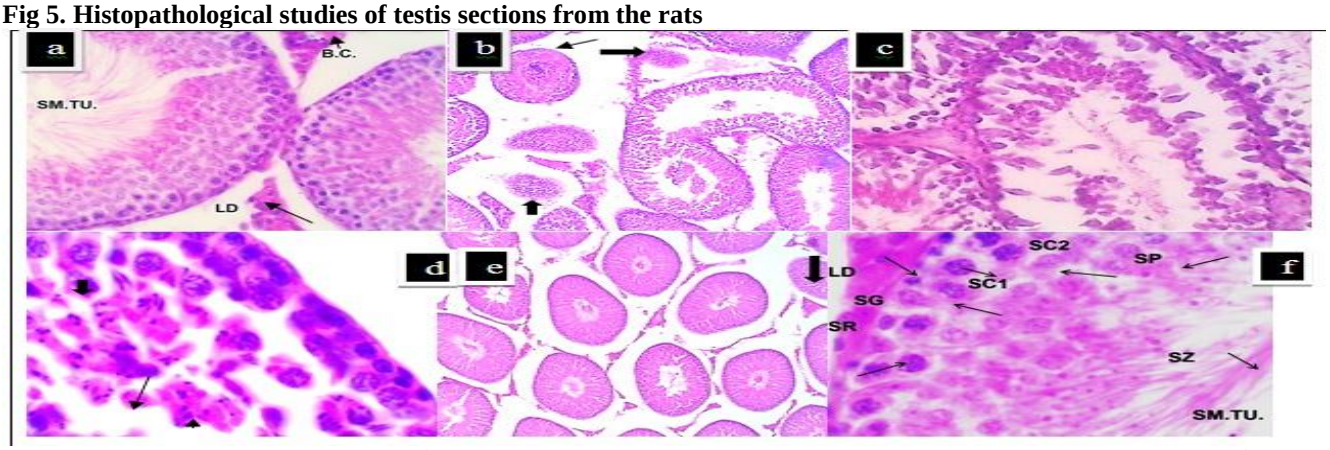


Fig 5. Histopathological studies of testis sections from the rats



(a X400):Control testis showed normal seminiferous tubules (SM.TU.), blood capillary (B.C.) and leydig cells (LD). Figs (b-d): Testis from TBTO administered rats showed,Fig (bX200) severely atrophied seminiferous tubules (thick arrows) with obstructed lumen (thin arrows), some of the seminiferous tubules appeared suffered fromazospermia or oligospermia. Fig (c X200): markeddegeneration and disorganization of the spermatogenic layers. Fig (dX1000):The nuclei of damaged cells in the forms of pyknosis (thick arrow), karyolysis (thin arrow) or marked karyorrhexis (arrowhead). Fig (e X 200):Testis of MSCs treated rats showed regeneration of the most seminiferous tubules and intertubular tissues, Fig (f X1000): most of the seminiferous tubules (SM.TU.) showed complete development of their germinal epithelia and the process of spermatogenesis (Spermatogonia (SG), primary spermatocytes (S1), secondary spermatocytes (S2), spermatids (SP) ,spermatozoa (SZ) and Leydig cell (LD).HX&E.

DISCUSSION

The testicular failure model rat-which induced by TBTO- exhibited a decrease in the pregnancies (fertility index) and testes weights. Reduction of fertility rate may be mainly due to several reasons as sexual hormones reduction (testosterone, LH and FSH), disruption in testicular enzymes and functions, decreases of sperm count and/or quality and apoptosis of germinal epithelia. In this concern, Sofikitis *et al.,* [20] mentioned that hormones such as testosterone, FSH and LH are known to influence the germ cell fate and their removal induces germ cell apoptosis. In addition, testicular enzymes may serve as specific markers in spermatogenesis [21].Also, TBT could cause a spermatotoxic effects and the decline of sperm count and quality suggested that this chemical could impair fertility in animals[22].Decrease in testicular weight may be due to decrease in testosterone level or the atrophy of testicular tubules and spermatogenic arrest along with increase in the percentages of DNA fragmentations in testicular tissues [23], these suggestions in accordance with the results of the present study which were proven by the histological examination and comet assay.

The present study illustrated decreases of testosterone, LH as well as FSH. These results agree with many previous authors [24-27]. This reduction of these hormones may be due to the androgen-like properties of TBT; TBT may interact with the androgen receptor along the hypothalamic-pituitary-gonadal axis thus affecting steroid hormone synthesis or metabolism. This means that TBT may act as mimic androgen and activated negative feedback action. Thus the down regulation of testosterone production in the Leydig cells is due to the inhibition of FSH release in the pituitary as a result of negative feedback mechanism. This mechanism results in a decreased production of endogenous testosterone in Leydig cells and is accompanied by reduced release of LH [26]. Also, some of these chemicals bind to intracellular receptor proteins for steroid hormones [28] and evoke hormonal effects in animals [29].

The present data investigated testicular dysfunctions which were represented by increasing levels of testicular enzymes ACP, ALP and GGT. These observations are consistent with previous findings by some authors [30-31]. The increase in such enzymes may be resulted from direct interaction of TBT with membrane bound enzymes or cell membrane through the lipophilicity of lipid membrane layers and this leading to leakage of such enzymes[32].Necrosis which was confirmed in this study may contribute in the elevation of the testicular enzymes [33].

Moreover, a remarkable reduction in sperm count (to above 90 % of control) and increasing in sperms abnormality in the forms of abnormal head, tail and head & tail were noticed. On the same line, similar results were being found in some studies [34, 27]. The alteration in sperm count and their morphology in this study may be due to apoptotic effect on germ cells which was illustrated by histological examination, comet and Bcl-2 analysis in this study. Damage to DNA is one of the markers and typical characteristic of apoptosis [35]. The role of the Bcl-2 signaling pathway in regulating the mitochondria-dependent apoptotic pathway in proteins of the Bcl-2 family appears to be essential for male germ cell homeostasis [36]. Moreover, this study revealed damage of testicular DNA, confirmed by increasing of tailed cells and tail DNA% of comet analysis, down-regulation of the serum Bcl-2 level as well as testicular histological changes (malformations and disorganizations of seminiferous tubules , apoptosis and necrosis).The apoptosis may be caused by androgenic decline [20] or by cytotoxic effect of TBT, organotin interact with DNA and cause DNA damage, which is a clear symptom of cytotoxicity and also related to apoptosis, in addition, mitochondria and membrane functions seem to be a preferred target of these organotin which are lipophilic pollutants. TBT may disrupt the role of Bcl-2 during increasing the permeability transition pore in the mitochondrial membrane through the interaction of TBT with double protein layers as lipophilic character [32].

The previous result were supported by the decrease of the proliferative capacity of testis cells which confirmed by low expression of proliferating cell nuclear antigen immune-staining (PCNA) in testicular tissue of TBTO rats as comparing to control and MSCs groups. Some investigators have reported that the increase in PCNA in testicular germ cells indicated high proliferative activity and stimulation of spermatogenesis [37]. It has been shown that PCNA is also involved in DNA repair. Thus, it is possible that DNA polymerase delta might be activated to repair possible damage to the genetic material [38]. Thus TBTO may suppress the proliferation during its direct toxic effect on nuclear DNA

After mating of treated male with TBTO for three months alone or plus MSC with normal untreated females did not show any -significant changes on the 20 day old fetuses' body weights and lengths. Also, Uteri weights, placental weights, number of implantation sites and number of live fetuses were not affected. Effects of TBT on reproduction and development occur only at exposures near those causing maternal toxicity [39]. Also, TBT transferred from administrated mothers via placenta to fetuses [40, 27].

It has been reported that mesenchymal stem cells, MSCs when transplanted systemically, were able to recognize and migrate to sites of injury, suggesting that they had migratory capacity. Thus, MSCs home into the damaged site and finally differentiate into cells specific to the tissue and serve as an integrated member of the tissue, thereby contributing toward tissue repair [41].

In this study immunohistochemistry examination of testis tissues in rats injected MSCs after induction of testicular failure showed CD44 positive cells with an irregular outline (fibroblast-like or spindle shaped cells) with brown cytoplasmic immune reactivity, located in some seminiferous tubules. In similar study, spindle-shaped and branched cells in seminiferous tubules of testes sections in male rats injected intravenously with mesenchymal stem cells were confirmed [42]. The authors added that this result indicating the migration of injected

stem cells to the injured testes. More additions, in a diabetic treatment study with MSCs, pancreatic sections were subjected to the H&E and MSCs marker CD44 and the result showed increasing in the number of CD44 cells after MSCs therapy. Homing of MSCs into the damaged ovarian tissue was evident by detection of endogenous stem cells showing CD44 immune-reactivity in the chemotherapy-exposed rats [43-44].

In the current work MSCs were transplanted for 5 months and the testicular tissues showed immune-reactivity to CD44 marker 3 months after transplantation. Lue *et al.,* [45] investigated that MSCs can survive in recipient testes for at least 12 weeks after transplantation. Also, Dobrinski *et al.,* [46] reported that donor germ cells could be documented in recipient mouse testes up to 6 months after transplantation of testis. In a different study, Cakici *et al.,* [47] studied the effect of intravenous injection of bone marrow cells on mouse kidney injury and found that the powerful improvement was at 12 week from transplantation and the major homing of stem cells was at 17 weeks from transplantation. On the other hand, Abd El Aziz and Metwally [42] detected MSCs in testis tissue after 15 days of stem cells injection and they expected that the longer duration might be responsible for better improvement.

Moreover, the proliferating cell nuclear antigen PCNA immune-staining (as a standard marker in proliferating cells) showed large proliferative capacity of testis cells in MSCs rats as compared to TBTO and controls. Abd El Aziz and Metwally [42] detected moderate PCNA-positive cells in the testis cells after 15 days of stem cells injection and they expected that the longer duration might be responsible for better improvement.

The current results showed that injection of MSC, 3x 10^6 cells in male rats induced testicular failure by TBTO, caused marked restoration of fertility from only 44.4% to 76.9% in relation to positive controls after mating trails with normal females. Most of the seminiferous tubules showed complete development of the germinal epithelia and the process of spermatogenesis with more restoration of inter-tubular tissues. These histological features supported by restoration of correlated biochemical tests of testosterone, LH, FSH, testicular enzymes, apoptotic analysis

From the previous data it may be expected that, MSC may migrated and homed to the injured testis and make repairing through MSC mechanism. These suggestions may be more or less accordance with the results of several studies.Cakici*et al*[47]studied the injection of mesenchymal stem cells (MSCs) (1.4 × $10^5/cm^2$) into rete testis for 12 weeks in male rats induced azoospermia. The author detected spermatogenesis, in some seminiferous tubules and successful pregnancy was obtained when rats with stem cell treatment were mated. Similar study illustrated by Zahkook*et al*[48]who found restoration of spermatogenesis in male rats induced azoospermia by busulfunafter transplantation of bone marrow MSC into testes for 12 weeks. Additionally, restoration of fertility in infertile mice was confirmed by transplantation of male germ-line stem cells[49, 50].

There are many investigators who discussed the mechanism of MSCs in tissue repairing: Several mechanisms, included chemokine-chemokine receptor interactions and possibly several adhesion receptor-ligand pairs participate in MSCs homing [51]. Previous studies suggest that triggering of the chemokine receptor CXCR4 by its ligand stromal derived factor may play an important role in the migration of transplanted MSC to sites of injury in the brain [52] even though CXCR4 appears to be expressed at a low level on the surface of MSC [53].

Several studies demonstrated that the migration of MSC to the injured tissues is dependent on CD44 expression on MSC [54-56]. The previous studies suggested that the potential role of CD44 in MSCs migration is represented in CD44 and hyaluronic acid interaction. Eggenhofer *et al.,* [51] suggested that CD44 and hyaluronic acid interactions recruit exogenous MSC to injured renal tissue and enhance renal regeneration which recruit exogenous MSC to injured tissue and enhance renal regeneration. Some researchers have reported that stem cells can regenerate various cell lineages by trans-differentiation [57-58] or by a recovery through a mechanism of protection [59].

Other possible explanations for target organ regeneration and improvement in function include facilitating the release of vascular endothelial growth factor (VEGF) by stem cells, thus, increasing the blood supply to cells and helping to repair damaged tissue [60]. Stem cells may also act by up-regulating the Bcl-2 gene and suppressing apoptosis [61]or by suppressing inflammation in the diseased organ via the interleukin-6 (IL-6) pathway [62]. Both of these processes are thought to contribute to the regeneration of normal cells in the damaged organ [63]. In more details, some investigators [58] discussed the paracrine effects and trophic action of MSCs during tissue repair. (A): when lesion lead to the death of tissue-specific cells, and part of a blood vessel. (B): Endothelial cells become activated, immune system cells are attracted to the necrotic area, and pericytes/MSCs become activated. (C): Activated pericytes/MSCs migrate into the lesion site and proliferate. The proliferating MSCs secrete bioactive molecules that will exert (a) anti-apoptotic effects on tissue-specific cells, (b) immune-modulatory effects on immune system cells, (c) angiogenic effects (d) anti-scarring effects near the wound site and (e) chemo-attractant effects on other cells. (D): MSC paracrine effects led to stimulation of tissue-intrinsic progenitors to regenerate the damaged tissue area, modulation of immune response and consequent maintenance of self-tolerance, and re-establishment of blood supply.It is important to mention that the potential rapid disappearance of infused MSC does not rule out a functional effect of the cells [58]. It has for instance been demonstrated that the phagocytosis of dead MSC induces the generation of macrophages with a regulatory phenotype [64].

CONCLUSION AND RECOMMENDATIONS

The most important issue of this study was that intravenous transplantation of bone marrow mesenchymal stem cells (BMSCs) is a successful method for the improvement of male fertility and reproductive functions in rats after the endocrine disrupter (tributyltin oxide ,TBTO) effects. If this protocol was proven to be functional in human, this would provide a new therapeutic concept for the treatment, and the possibility to treatment of male infertility. The duration time of transplantation is represented an important role in the tissue repairing with mesenchymal stem cells.

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