Original Research Article

Comparative study between bone marrow- mesenchymal stem cells and adipose tissue- mesenchymal stem cells in restoration of male fertility

Running title

<mark>Bone marrow- mesenchymal</mark> stem cells and adipose tissue- mesenchymal stem cells

ABSTRACT:

Background: Male infertility is considered a major cause of couple infertility as it represents 25-50 % of infertility cases. In addition, non-obstructive azoospermia (NOA) represents 10% of all infertile men.There is no definite curable treatment for such cases.

Aim of work: The purpose of this research was to assess the efficacy of BM-MSCs and AT-MSCs in treating busulfan-induced azoospermia in wistar rats.

Materials & Methods: BM-MSCs were extracted from the femur bones of five adult Wistar rats and AT-MSCs from preperitoneal adipose tissue. They were then evaluated for morphology, MSC markers, osteogenic and adipogenic differentiation. To produce azoospermia in male Wistar rats, two doses of busulfan (15 mg/kg and 30 mg/kg) were administered intraperitoneally during a 21-day period. The seminiferous tubules of each testis were injected with 2.5106 MSCs 35 days after the second busulfan injection. 12 weeks after cell therapy, the testes were examined histopathologically and histomorphometrically.

Results: Like the control group, the seminiferous tubules treated with BM-MSCs demonstrated normal spermatogenesis in compared to the busulfan-induced azoospermic testes. In contrast to the busulfan-induced azoospermic testes, the seminiferous tubules treated with AT-MSCs exhibited a practically typical look of spermatogenesis.

Conclusion, BM-MSCs and AT-MSCs were shown to be beneficial in treating azoospermia in the wistar rat model, restoring fertility to busulfan-induced azoospermic animals after MSC transplantation. As a result, this discovery may pave the way for the future use of MSCs in the treatment of human azoospermia, although more research should be conducted to confirm the findings.

Key words: Bone marrow, Adipose tissue, Mesenchymal stem cells, Busulfan, Azoospermia**.**

Introduction:

Male factor is implicated in 50% of infertile partnerships. Impairment of spermatogenesis owing to failure of germ cell proliferation and differentiation is considered a key cause of male infertility. **[42].** Men with nonobstructive azoospermia, in instance, have little or no sperm in their sperm and are unable to conceive biological children. Nonobstructive azoospermia is often characterized by undersized testes, low testosterone levels, sperm deficiency, and increased FSH.**[1].** NOA may be caused by a variety of disorders, including genetic or congenital defects, infections, toxic exposure, drugs such as systemic chemotherapy or radiation to the testis, and idiopathic reasons **[42]**. <mark>The self-renewal and the multilineage differentiation capacities of adult stem cells (ASCs) show great promises for regenerative medicine. Despite of the greater</mark>

differentiation potential of embryonic stem cells (ESCs) compared to ASCs, ethical concerns and governmental restrictions are the main obstacles of the ESCs standing in the way of their clinical applications **[18].** On the other hand, bone marrow-derived MSCs (BM-MSCs) have been extensively examined, and their potential to cure a range of disorders, including erectile dysfunction and male infertility, has been proven. Alternatively, owing to their equivalent differentiation and therapeutic potential, adipose-tissue-derived MSCs (AT-MSCs) might be employed in future clinical applications in place of bone marrow stem cells, although AT-MSCs are simpler and safer to collect.**[41].** Stem cells were only recently adopted as prospective therapeutic agents in andrology studies on erectile dysfunction and infertility. The studies related in this area showed that ESC could participate in spermatogenesis by forming functional male germ cells *in vitro* or by supporting the maturation of primordial germ cells into haploid male gametes **[36]**. The first step forward in adult stem cell research for treating infertility was the differentiation of murine BM-MSC into male germ cells, which was followed by the same group.**[28]**. Busulfan-treated infertile mice showed that BM-MSCs could divide into germ cells, Sertoli cells, and Leydig cells **[11] & [21]**. Given the above information, the purpose of this work was to create a model of male infertility (NOA) via busulfan injection and to document the following structural changes in the testes, as well as to assess the therapeutic effect of BMMSCs and ADMSCs in the treatment of such model.

Materials and methods:

Animals. Rats: The experimental animal unit at Benha university's faculty of medicine housed 35 male Wistar rats (8-12 weeks old, an average weight of 200-220 grammes each) obtained from Holding company for biological products and vaccines (Vacsera), Helwan, Egypt. The recipient animals were housed under conventional circumstances at a temperature of 22-25o with a 12h light/dark cycle, three rats per cage in metal cages with access to tap water and commercial feed throughout the trial. The animal facilities adhered to and monitored all ethical procedures for animal care.

Busulfan: Busulfan was obtained from Sigma Chemical Company as Busilvex (Sigma, St.Louis, USA) in the form of 60mg/10ml vial thus each 1ml contains 6mg busulfan . Busulfan was solved in 250 μL DMSO (dimethyl sulfoxide; Sigma, USA) and 250 μL distilled water (1:1) freshly at room temperature.

Experimental design. Animals were placed into four groups at random. The rats in group I (control group) were evenly split into subgroups Ia and Ib; each subgroup had five rats. Subgroup Ia rats were injected intraperitoneally with 0.1 ml saline/100 g body weight and euthanized after 8 weeks. Subgroup Ib members donated bone marrow and adipose tissue stem cells. Group II rats (busulfan-treated group) (n=5) received two intra-peritoneal busulfan doses separated by three weeks: 15mg/kg body weight dissolved in 0.1 ml normal saline and 35mg/kg body weight. Rats were euthanized five weeks following the second dosage. Five weeks following the second busulfan injection, 100l of BM-MSCs combination (106 cells in 0.1 ml normal saline/testis) intra-testicularly was injected into the group III participants. After a stem cell injection, animals were killed 12 weeks later.

 AT-MSCs were injected into the testicles of rats in group IV (n=10) following the second busulfan injection, which was five weeks after the second busulfan injection for group II (n=10). 12 weeks after receiving stem cell injections, the animals were put down.

Induction of azoospermia. The experimental group (25 animals) was injected with two doses of Busulfan to induce azoospermia. The first dose was 15mg/kg body weight (2.5ml/kg) of Busilvex intraperitoneal. The second dose was 35mg/kg body weight after a three-week interval.

The azoospermic animals were divided into three subgroups according to the type of stem cells used for treatment: group II (Busulfan treated group), group III (BM-MSCs treated group) and group IV (AT-MSCs treated group).

Isolation of BM-MSCs. After inducing anesthesia in the rats (60 mg/kg ketamine and 6 mg/kg xylazine intraperitoneally) and dorsal recumbency, the knee area was thoroughly shaved and cleansed with 70% ethanol and povidone iodine. The needle was rotated into the bone lumens of the proximal tibia and distal femur. The BM was then gently sucked with a syringe containing 0.1 ml heparin sulphate. All BM samples were immediately combined with 1 ml complete culture medium [high-glucose Dulbecco's modified eagle medium (DMEM; Gibco, Paisley, UK), 15% foetal bovine serum (FBS; Gibco, Paisley, UK), 100 units/ml penicillin G, and 100 units/ml streptomycin (Gibco, Paisley, UK)]. Centrifuged at 1200 rpm for 5 minutes, the BM/medium mixture was resuspended in new warm culture media in 25 cm2 cell culture flasks and transported to an incubator (37oC and humidified 5% CO_2 atmosphere). The medium was replaced every 34 days, and the cells were subcultured prior to primary culture for 12-14 days or until big colonies formed. When big colonies formed (80%–90% confluence), cultures were twice washed with phosphate buffered saline (PBS) and cells were trypsinized for 5 minutes at 37°C with 0.25 percent trypsin in 1ml EDTA (GIBCO/BRL). Cells were resuspended in serum-supplemented media and incubated in a 25 cm2 culture flask after centrifugation (2400 rpm for 20 min). First-passage cultures are the civilizations that arose consequently. Adhesion and fusiform shape are used to identify MSCs in culture. MSCs were identified on day 14 based on their appearance, adhesion, and capacity to grow into osteocytes and chondrocytes.[42].

Isolation of AT-MSC. One rat was anaesthetized with xylazine (10 mg/kg intraperitoneal injection, Alfazyne, Woerden, Holland). & Woerden, Netherlands-based Ketamine (100 mg/kg intraperitoneally). A little amount of preperitoneal adipose tissue (1-2cm3) was excised. Multiple times using Hank's balanced salt solution mixed with 5% antibiotic-antimycotic solution, tissue samples were washed, and vascular structures were removed. The yellowish white tissue was chopped and enzymatically processed for 60 minutes at 37oC in MEM medium with 0.075 percent collagenase2. Cell suspensions were filtered with a 70 m sieve. Reconstituted the cells in MEM media supplemented with 1% penicillin/streptomycin and 15% FBS. Following 10 minutes of centrifugation at 1200rpm, cells were grown in normal culture media in 25cm2 culture flasks. After seven days, the medium was changed with new medium and then twice a week afterwards. The culture was cleared of erythrocytes and other non–adhesive cells. After attaining 70-80% confluence, cells were harvested for 3 minutes with 0.25 percent trypsin – EDTA, centrifuged, and subcultured at a 1:3–1:4 ratio. Trypan blue was used to count the cells. After mixing (1:1), the blue staining of the cells will be utilized as a sign of cell death.[7]&[23] PKH26 was used to mark stem cells; PKH26 is a red fluorochrome. Its excitation (551 nm) and emission (567 nm) wavelengths are comparable with those used in rhodamine or phycoerythrin detection systems. The present study used the PKH26 red fluorescence cell linker kit (Sigma Aldrich, USA) to label mesenchymal stem cells according to the manufacturer's procedure. After the third passage, subconfluent MSCs were removed and washed three times with serum-free media before being resuspended in 1mL of dilution buffer. The cells' suspension was treated for 5 minutes with an equivalent volume of the labelling solution containing 4x10-6 M PKH26 in

the dilution buffer. 2 ml of FBS was added to bring the response to a halt. 4 ml complete media was added to the cells, and they were rinsed three times. Then Cells were resuspended in complete medium at a concentration of 1106 cells and utilized within 30 minutes. To determine the viability and ability of PKH26-labeled MSCs to differentiate, cells were reseeded onto plates and then subjected to differentiation tests upon reaching confluence.[33].

Fluorescent microscope examination of the testes specimens of the rats that were treated with the labeled MSCs (group III&IV) was done to insure their incorporation into the testicular tissue.
Transplantation of BM-MSCs. The recipient rat's seminiferous tubules were injected with MSCs cells via the rete testis. The same method used for BM collection was used to sedate the recipient rats before to transplantation. A perpendicular incision was made in the upper portion of the scrotum, perpendicular to the midline, to remove the testis from the body. At a concentration of 8-10106 cells/ml, the cell suspension was transferred into the rete testis using a tiny, thin pipette (each testis got around 2.5105 labelled cells). The testis was returned to the body at the end of the treatment, and the incised muscle and skin were stitched back together.[35].

Histopathologic and histomorphometric assessment. For light microscopic examination, testis samples were fixed in 10% buffered formalin, dehydrated, cleared, and embedded in paraffin. Serial 5 μm sections of the testis were stained with hematoxylin and eosin (H&E) **[5]**.

Morphometric measurements: All seminiferous tubules of each testis were examined for the presence of spermatogonia, spermatocytes, and spermatids in a single cross-section. In 10 circular transverse slices of seminiferous tubules from various locations of the testis, luminal and cellular dimensions were measured **[31].** The cellular (germinal epithelium) and luminal portions were measured using diameter data. Image Analysis LS Report Software was used to quantify tubule diameters in transverse sections.

Two seminiferous tubule diameters, D1 and D2, were averaged at right angles to establish the average diameter (D).**[12].**

Scales of zero to six were used to assess the spermatogenic potential of each testis. On the basis of the number of cells in each layer of the testis, as well as their kinds, as well as their presence in seminiferous tubules, the index was created. The following were the index and criteria for the index: All cell types are present, but there are less than 100 late spermatids in each tubule; spermatogonia, spermatocytes, and round spermatids are all present; and spermatogonia, spermatocytes, and round (early) spermatids are all present with more than 100 late spermatods per tubule**. [8].**

Transmission electron microscope (TEM) examination, Testis specimens (1 mm3) were preserved in a 2.5 percent phosphate-buffered gluteraldhyde solution, dried, and embedded in epoxy resin. Semithin slices (1 m thick) were cut and stained with 1% toluidine blue for light microscopy examination. On copper grids, ultrathin slices of 50-60 nm were obtained and

stained with uranyl acetate and lead citrate.**[5].** Finally, the sections were examined and photographed by JEOL TEM at 80kV in EM unit, Faculty of medicine, Tanta University.

Immunohistochemical study of PCNA:
PCNA is an endogenous nuclear protein that is generated in the late G1 and S stages of the cell cycle and serves as a good marker for cell proliferation. Cell proliferation is required for its production and expression. Because spermatogenesis is a complicated cell cycle characterised by rapidly proliferating cells that culminates in the release of sperms, PCNA was utilised to quantitatively investigate spermatogenesis in this work. The sections were deparaffinized in xylene and hydrated in ethanol at progressively higher concentrations. Endogenous peroxidase was inhibited for 30 minutes by treatment in 3% hydrogen peroxidase. Immunohistochemical staining was performed using a primary antiserum to PCNA (DAKO A/S Denmark, Clone PC 10). The main antibody was diluted 1:50 in Trisbufferd saline, as specified on the data sheet. The slices were treated overnight at + 4°C with the primary antibody. The main antibody's binding was determined by utilising a commercial avidinbiotin-peroxidase detection method indicated by the manufacturer (DAKO, Carpenteria, USA). After three washes with PBS, sections were incubated for 1 hour with peroxidase-labeled IgG and then stained with diaminobenzidine (DAB) as the chromogen to visualise the PCNA-bound complex. Counterstaining the sections with Harris hematoxylin **[10].**

Morphometric study:
By using "Image J" software image analyzer computer system, the mean gray value in control and experimental groups were done the area percent of PCNA immunoreactivity were measured. The measured data were recorded and statistically analyzed.

Immunohistochemical study of CD105:
This antibody is intended for use in in vitro diagnostic applications. This antibody was developed to detect CD105 specifically in formalin-fixed, paraffin-embedded tissue slices. Immunohistochemistry (IHC) is a two-step method that begins with the binding of a primary antibody to the antigen of interest and concludes with the detection of the attached antibody using a chromogen. The main antibody may be employed manually or with the BioGenex Automated Staining System in IHC. Positive and negative controls should be performed concurrently on all specimens that have been treated.

Specimen Collection and Preparation
Tissues fixed in 10% (v/v) formalin, prior to paraffin embedding.

Reagents Provided
Mouse monoclonal antibody to CD105 diluted in PBS, pH 7.6, including 5% BSA and 0.09 percent sodium azide, from cell culture supernatant. CD105 is a mesenchymal stem cell marker. 0.1 ml primary antibody (CD105) rabbit polyclonal Ab (ab27422) and incubate for 30-60 minutes at room temperature in a wet chamber. Tonsil specimens were utilised as positive controls. The cell membrane is responsible for cellular localisation. By omitting the step of administering the main antibody, one of the testis sections was utilised as a negative control.**[5].**

Results:
This stain is cytoplasmic and cell membranous stain giving positive reagent brown colors.
Statistical analysis: The various groups' measured parameters were compared using the statistical package for the social sciences (SPSS software version 19) analysis of variance (one way ANOVA) and post-hoc least significant difference (LSD). When the p value was 0.05,

differences were judged significant. The data were summarised using the mean and standard deviation (SD).
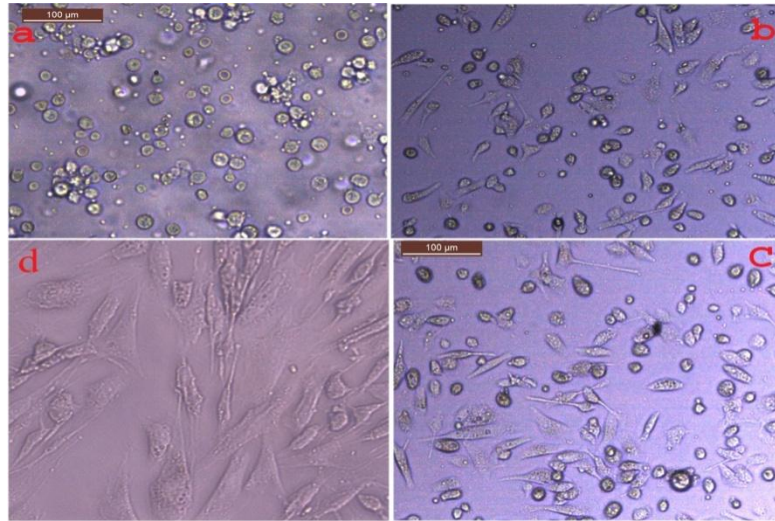
Results:

Fig. (1): The primary culture of MSCs was examined under inverted microscope showing the change of the rounded mononuclear cells into fibroblast like cells. **(a)** *Inverted microscopic pictures of a primary culture of MSCs in the first day of isolation and culture x200.* Most of the cultured cells appear rounded in first day. **(b)** *Inverted microscopic picture x200* few cells start to be spindle shape on 2nd day. **(c)** *Inverted microscopic picture x200* shows crowded cultured cells having variable size and shape (N.B: some cells showing evidence of changing their shape and start to form processes) 4th day *(d)* *Inverted microscopic pictures of a primary culture of MSCs on reaching 80% confluent x200 in second week.*

Histological examination of seminiferous tubules:

Control group:

Examination of Hx. & E-stained sections of the testes have shown normal architecture of the seminiferous tubules with average amount of interstitial tissue. The tubules have appeared regularly arranged, rounded or oval in shape and nearly of similar diameter. They have attained narrow lumina, lined by stratified germinal epithelium. The tubules are separated by interstitial spaces of average size *(Figs. 2a&2b)*. The seminiferous tubules are ensheathed by basal lamina. Each tubule is lined by epithelial cells including the Sertoli cells and the germ cells of various stages. Spermatogenic cells have included several distinguishable types; as arranged from the basal laminae towards the lumen, are spermatogonia, primary spermatocytes, secondary

spermatocytes, spermatids and sperms. The spermatogonia has appeared oval in shape and also lay directly on the basal lamina in between the sertoli cells. The spermatogonia are followed by several rows of primary spermatocytes that appeared as the largest of the spermatogenic cells having rounded outlines and large nuclei. Secondary spermatocytes are scarcely seen in these sections due to the rapid division processes. The spermatids follow the spermatocytes towards the tubular lumina and they have been recognized by their small size and their rounded outline. The spermatids finally have changed into sperms whose tails gathered within the tubular lumina. The Sertoli cells have rested on the basal lamina and fill the narrow spaces between the germ cells. They look tall, irregular with indistinguishable cell borders, pale cytoplasm and ovoid pale nuclei *(Figs. 2a&2b)*. The interstitium between the seminiferous tubules consists mainly of loose reticular connective tissues, clusters of large-sized Leydig cells and blood vessels. The Leydig cells have an extensive eosinophilic cytoplasm and rounded nuclei and are disposed either single or in small groups *(Figs. 2a& 2b)*.
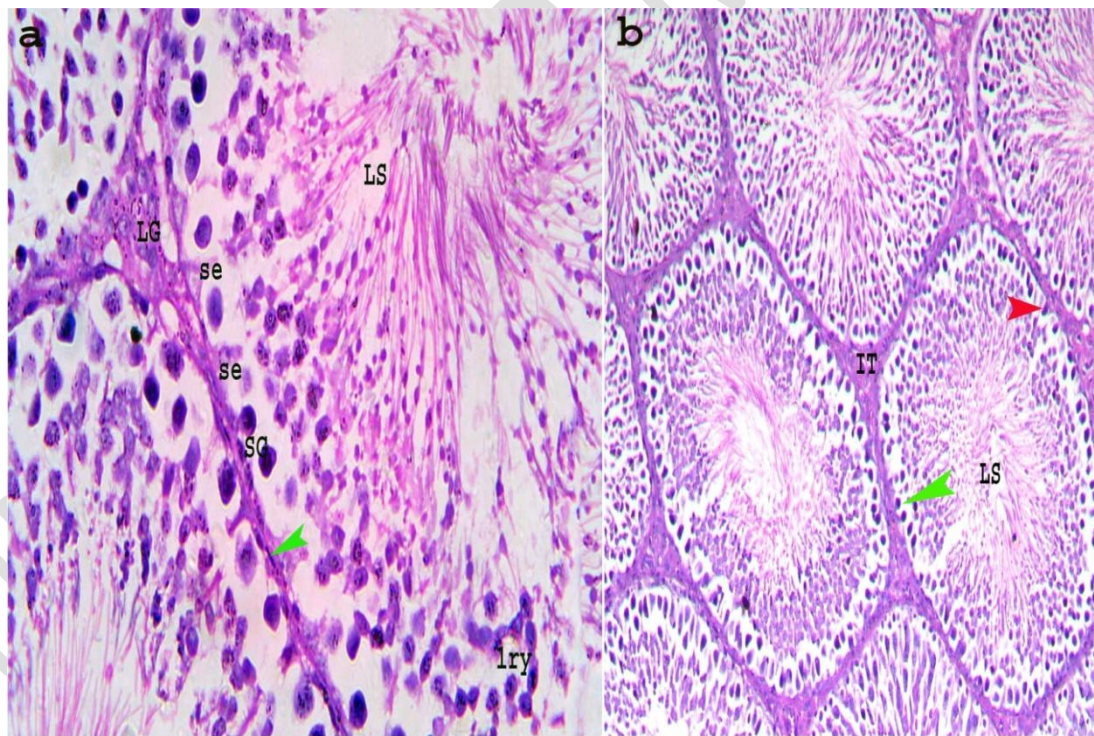

Fig. (2):a) H&E stained sectioned from control group showing seminiferous tubules with interstitial tissue inbetween contains leydig cells(LG), Basal lamina of seminiferous tubule shows Myoid cell (green arrow head), the tubule shows spermatogonia (SG), sertoli cells (se), 1ry spermatocyte (1ry) and late spermatid (LS) (groupI

H&E X400).   b) showing Regular seminiferous tubules densely packed with spermatogenic cells with thin interstitial tissue (IT) in between, the tubules show Spermatogonia (green arrow head),   Sertoli cell (Red arrow head) and late elongated spermatid (LS).   (group I H&E X100).

Ultrastructural observation:

Spermatogonia in a normal testis come in two flavours: A and B. Flattened nuclei parallel to the basal lamina and light, non-condensed chromatin granulations have been seen in spermatogonia with oval-shaped nuclei (Fig. 4c).. Spreading from the tubule's base to its lumen, the sertoli cell grows. Large, pale nucleus with nuclear membrane infolding; rather homogenous and well-defined chromatin material; and an extensive nucleolus are some of the descriptions given to it. In the cytoplasm, lipid droplets, endoplasmic reticulum, and Mitochondria of spherical or cylindrical shape fill the space (Figs. 3a&3b). There are two kinds of spermatocytes, the primary and the secondary. At one end of the cell, there are clusters of Golgi apparatus and a large nucleus with finely granular chromatin. The cytoplasm of this organism has many signet ring mitochondria (Fig.3c). In germinal cells, secondary spermatocytes are rare. There were several stages of acrosomal cap formation in the round spermatids we saw (Fig.3d). There is a large nucleus in the Leydig cell, which is surrounded by a thin ring of heterochromatin. There are many lipid droplets, medium-sized mitochondria, secretory granules, and an extensive smooth endoplasmic reticulum (SER) in the cytoplasm of Leydig cells, which contribute to their eosinophilic properties (fig. 4b). This core is surrounded by several fibrous structures, including a central axoneme, nine coarse fibres that continue from the middle piece, and numerous fibrous structures **(Fig. 4a).**
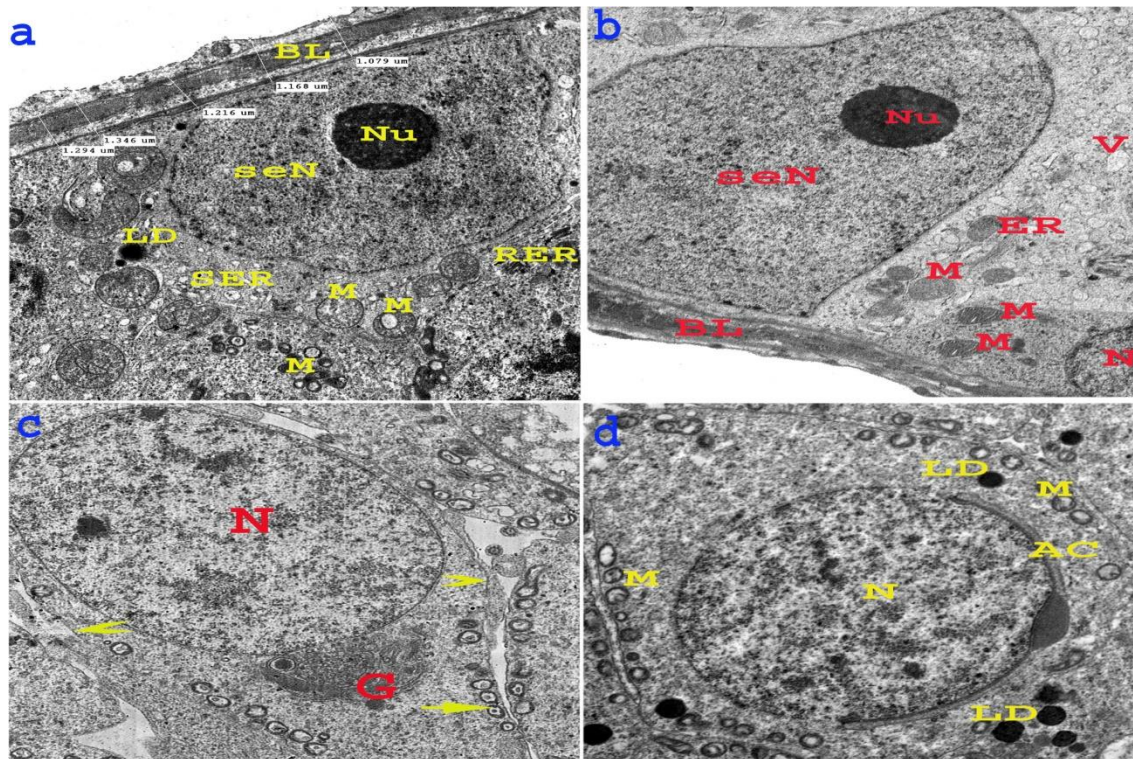
Figure (3): E/M of group I a) & b) showing sertoli cell contains elongated nucleus (N) with prominent nucleolus (Nu) resting on basal lamina (BL), the cytoplasm contains multiple mitochondria (M), dense lipid droplets (LD), rough endoplasmic reticulum (RER),and smooth endoplasmic reticulum(SER), endoplasmic reticulum (ER) and lipid vesicles(V). Notice the measurements of thickness of basal lamina varies between 1.079 to 1.346 µm in different regions. The adjacent spermatogonium cytoplasm contains mitochondria (M) and nucleus (N). c) showing 1ry spermatocyte with pale large and rounded nucleus (N), Golgi complex (G) close to nucleus, multiple signet-ring mitochondria(arrow) close to cell membrane (arrow head).d) shows Round spermatid with large rounded nucleus (N) .The acrosomal cap (AC) close to the nuclear membrane , the cytoplasm contains multiple signet-ring mitochondria (M). Notice lipid droplets (LD).   (groupI E/M   X3000)
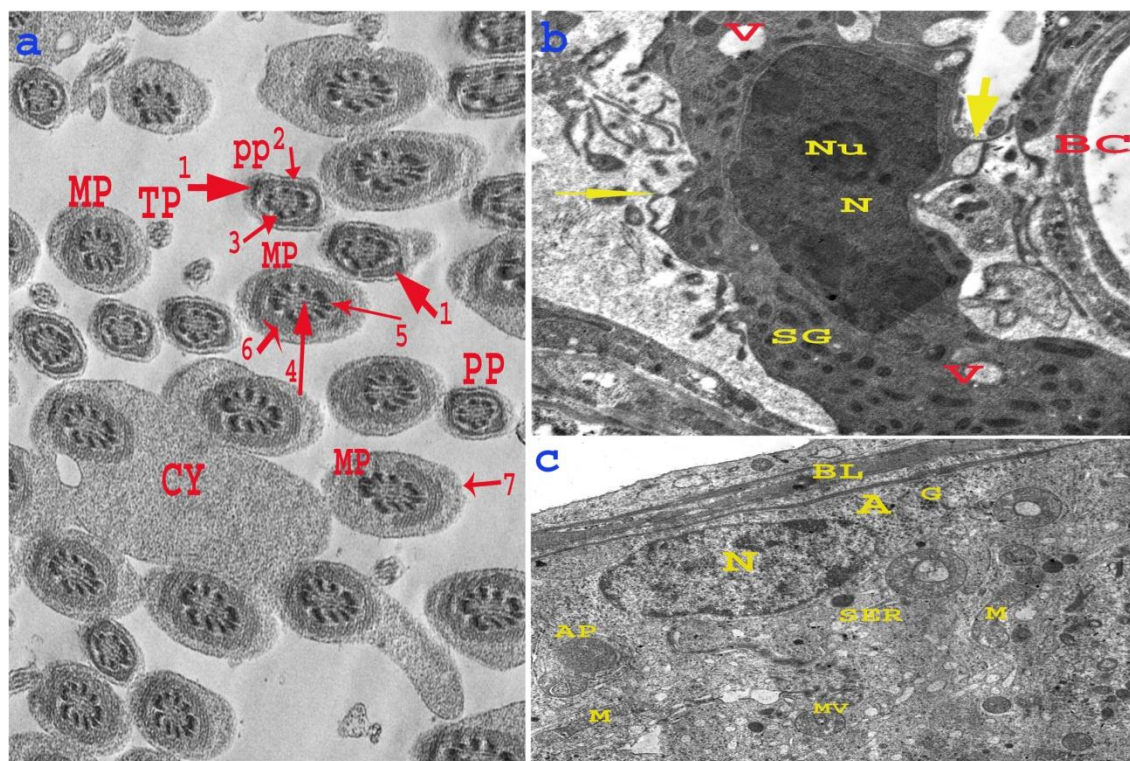
Figure (4):E/M of group I a) showing Cross sections of different parts of sperm tail. The middle piece (MP) includes Axoneme (4), nine outer dense fibers (5), flagellar sheath (6). Principal piece (pp) which includes 2 longitudinal columns (1), connecting ribs (2) , nine longitudinal fibers (3) and end piece (TP). b) showing leydig cell lies between blood capillaries (BC). This cell has many cytoplasmic processes (arrow). The nucleus (N) of this cell is dark , irregular and elongated with prominent nucleolus (Nu).The cytoplasm contains many secretory granules (SG) and electron lucent vesicles (V). c) showing Type A spermatogonium (A) with oval nucleus (N) resting on basal lamina (BL), its cytoplasm contains smooth endoplasmic reticulum (SER), rosette-shaped glycogen granules (G) and elongated mitochondria (M), also the figure shows the cytoplasm of adjacent sertoli cell, which contains autophagosome (AP) , elongated mitochondria (M) and Multivesicular body (MV)．(groupI E/M   X3000).

Busulfan-treated group:

Histological analysis of Hx. & E. stained sections from the Busulfan-treated group revealed pathological changes in the seminiferous tubules and interstitial tissues. These seminiferous tubules have shrunk significantly, become significantly twisted, and their lumina are devoid of spermatozoa **(Figs. 5a& 5b).** Histological analysis of Hx. & E. stained sections from the Busulfan-treated group revealed pathological changes in the seminiferous tubules and interstitial tissues. These seminiferous tubules have shrunk significantly, become significantly twisted, and their lumina are devoid of spermatozoa (Figs. 5a& 5b). The spermatogenic cell masses in

numerous seminiferous tubules have shrunk significantly, and the atrophic germinal epithelium has formed a narrow band around the peripheral zone of seminiferous tubules. The germ cell masses have been dissected extensively from the basal lamina (Figs. 5a& 5b). Certain tubules exhibit disarray, with germ cells exfoliating into the tubular lumen (Fig. 5a &5b). Extensive interstitial degeneration has been detected as vacuoles (Fig. 5b) or homogeneous exudate with germ cell infiltration of interstitial tissue (Fig.5a). Additionally, dilated and congested blood vessels have been detected (Fig. 5b). Sheathing of exfoliated, degraded germ cells has been seen in the tubular lumen.**(Figs. 5a& 5b). Certain tubules exhibit disarray, with germ cells exfoliating into the tubular lumen (Fig. 5a &5b). Extensive interstitial degeneration has been detected as vacuoles (Fig. 5b) or homogeneous exudate with germ cell infiltration of interstitial tissue (Fig.5a). Additionally, dilated and congested blood vessels have been detected (Fig. 5b). Sheathing of exfoliated, degraded germ cells has been seen in the tubular lumen.(Fig.5a)**
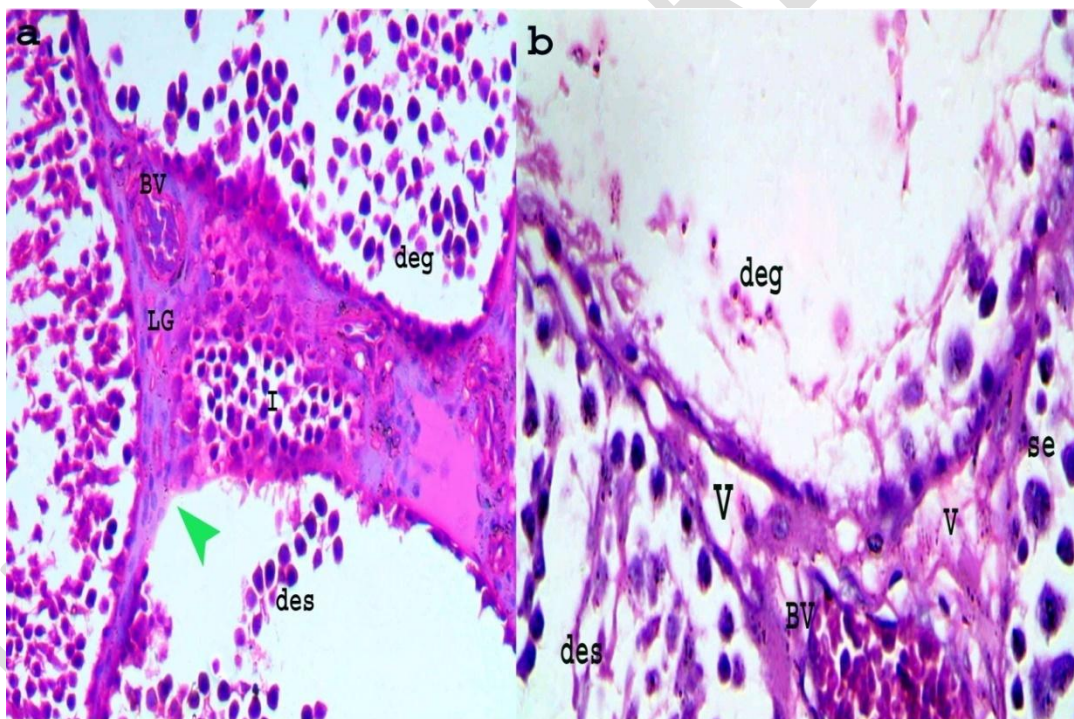

Fig. (5): a) showing interstitial tissue with cellular infiltration (I), congested bl. Vessel (B.V) and leydig cells (LG) , parts of seminiferous tubules show desquamated cells (des), degenerated spermatogonia (deg) and empty tubule (arrow head). (group II H&E X200).b) showing degenerated seminiferous tubule (deg), desquamated cells (des), sertoli cell (se) and interstitial tissue shows vacuoles (V) with dilated bl. Vessel (BV). (group II H&E X400).

Ultrastructural observations: In rats treated with busulfan, different ultrastructural changes have been seen in their testes. Basal lamina has appeared thickened with divided myoid cell **(fig. 7a),** while spermatogonia have appeared with abnormal darkly stained irregular nuclei with prominent vacuoles within and inbetween the cells **(Fig. 6a).** The cytoplasm of the germ cells and the Sertoli cells have shown large, well circumscribed vacuoles *(Figs.6a, 6b &7a).*Within the interstitial tissue, The Leydig cells have irregular nuclei and vacuoles have been observed in their cytoplasm **(Fig. 6c).** Abnormal Sertoli cell has been seen with invagination of nuclear membrane of its nucleus .Its cytoplasm contains vacuoles, swollen vacuolated mitochondria and endoplasmic reticulum **(Figs.6b&7a).** Abnormal round spermatid has been shown with degenerated acrosomal cap. Its cytoplasm has shown vacuolated mitochondria, vacuoles, vacuolated golgi apparatus and residual body (**Figs. 7b)**. Cross sections of different parts of tail of sperm have shown irregular outline and degeneration of middle piece, principal piece and degenerated end piece **(Fig.7c).**
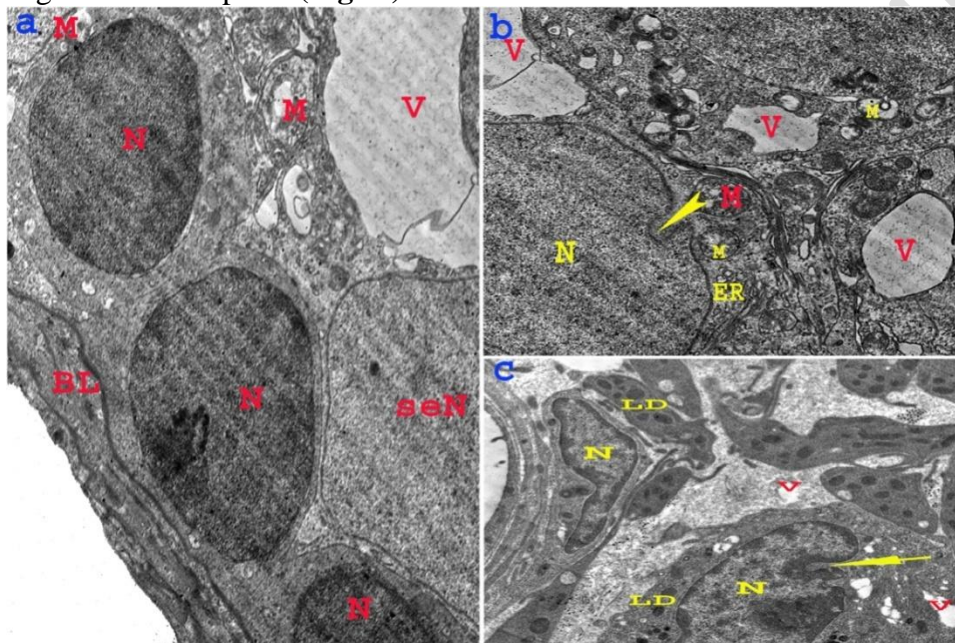

Fig. (6): a) showing spermatogonia with large dark nuclei (N) resting on basal lamina (BL) , sertoli nucleus (seN), cytoplasm contains multiple vacuoles (V) and swollen mitochondria(M). b) showing sertoli nucleus (seN) with invagination of the nuclear membrane (arrow head). Its cytoplasm contains vacuolated mitochondria (M), Endoplasmic reticulum (ER) and vacuoles (V). c) showing Multiple leydig cells of different sizes, their nuclei are elongated with indentation (head arrow) in nuclear membrane .Their cytoplasm contains vacuoles(V) and lipid droplets (LD). The interstitial tissue contains vacuoles (V). (group II E/M X3000)
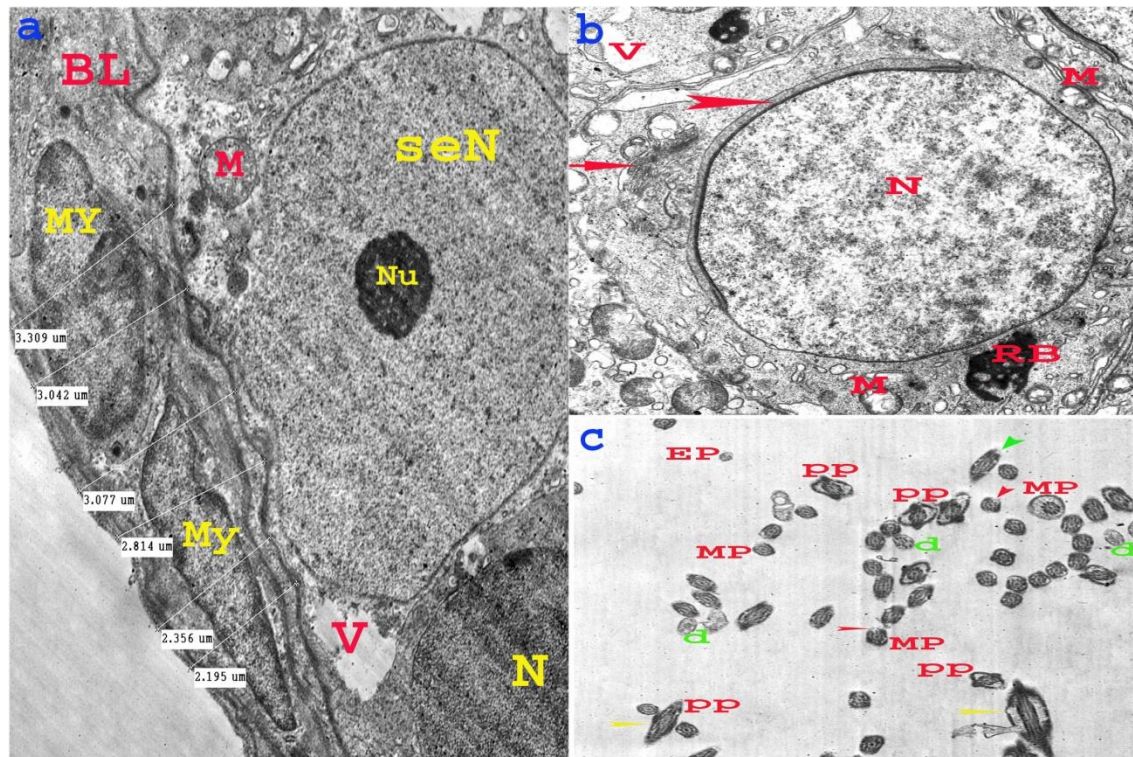
Fig. (7): a) showing sertoli cell nucleus (seN) with nucleolus(Nu). The cytoplasm contains vacuoles (V), swollen mitochondria (M), dark spermatogonium nucleus (N), both resting on thick basal lamina (BL) containing divided myoid cell (MY).Notice the measurements of thickness of basal lamina varies between 2.195 to3.309 µm in different regions. b) showing Round spermatid nucleus (N) with acrosomal cap (arrow head). The cytoplasm contains vacuolated Golgi apparatus (Red arrow), vacuolated mitochondria (M) and vacuoles (V). Notice residual body (RB). C) showing: different cross sections of tail of sperm in the form of middle piece (MP) which shows irregular outline (red arrow head) and degeneration (d) , principal piece (pp) which showing also irregular outline (green arrow head) and some are degenerated (yellow arrow head) and degenerated End piece (EP) . (group II   E/M X3000).

BM-MSCs treated group:

Histological examination of Hx. & E stained sections of rats' testes after BM-MSC implantation revealed restoration of the architecture and outline of seminiferous tubules to near-normal levels. The basal lamina's average thickness has been restored. The thickness of spermatogenic cell masses in the majority of seminiferous tubules has also been restored, as has their adhesion to the basal laminae (Figs. 8a& 8b). The seminiferous tubules' spermatogenic cell masses exhibit all phases of spermatogenesis, with a small lumen filled with spermatozoa (Figs. 8a&8b). The

interstial tissue is of ordinary thickness, and the interstitial gap has been restored to its normal size (Figs. 8a& 8b). The exudate and constriction of the blood vessels have almost disappeared. The seminiferous tubules are densely packed with stratified germinal epithelium and lined with it.**(Figs.8a&8b).**
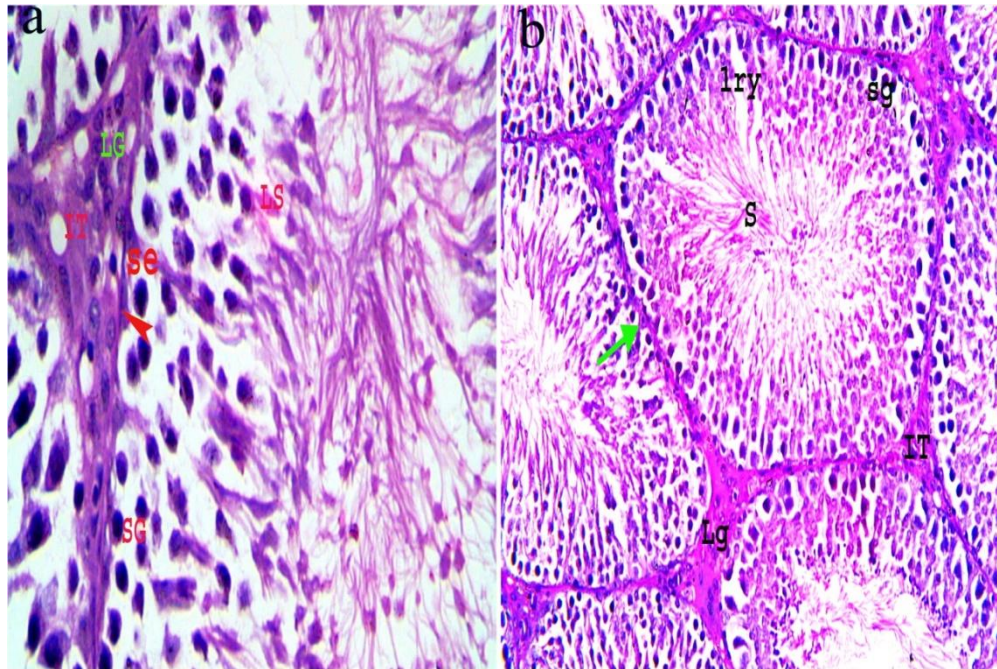

Fig. (8): a) showing parts of seminiferous tubules nearly similar to control with interstitial tissue (IT) inbetween. The tubule contains sertoli cell (se), spermatogonia (SG) , late spermatid (LS) and Myoid cell (arrow head) within basal lamina.The interstitial tissue contains leydig cells (LG), (group III   H&EX400) .b) showing multiple seminiferous tubules nearly similar to control with thin interstitial tissue (IT) inbetween. The tubule contains sertoli cell (green arrow), spermatogonia (Sg) , 1ry spermatocyte (1ry) and sperms (S).The interstitial tissue contains leydig cells (LG).(group III   H&E X200)

Ultrastructural observations:

After BM-MSCs were transplanted into rats after busulfan therapy, several ultrastructural alterations in their testes were observed. They demonstrated restoration of the architecture and shape of seminiferous tubules to near-normal levels. The basal lamina's average thickness has been restored (Fig.9a). Type A spermatogonia have been described as oval in form, with a flattened nucleus parallel to the basal lamina and a thin, light, and uncondensed chromatin granulation (Fig.9a).) Sertoli cells have a big pale oval nucleus, generally uniform chromatin material, and an outwardly protruding nucleolus. The cytoplasm is densely packed with smooth

endoplasmic reticulum, thick lipid droplets, and Mitochondria that are spherical or cylindrical in form (Fig.9a). A primary spermatocyte with a spherical nucleus and fine granular chromatin has emerged. Multiple signet ring mitochondria were seen in its cytoplasm. Additionally, basal insertion of certain sperm heads between the main spermatocyte has been seen (Fig.9b). At one pole of the nucleus, round spermatids in the Golgi phase and acrosomal cap were seen, as well as many signet ring mitochondria in the cytoplasm (Fig. 9c). Multiple Leydig cells with a big nucleus and a thin ring of heterochromatin have been seen in interstitial tissue. The cytoplasm of the Leydig cell is eosinophilic and densely packed with tiny lipid droplets, moderate-sized mitochondria, golgi apparatus, and extensive smooth endoplasmic reticulum, indicating the presence of cytoplasmic processes (Fig.9d). Stages of spermiogenesis have been seen in the form of an elongated nucleus inside the future sperm head and the construction of an acrosomal cap, mitochondrial aggregation around the nine microtubule doublets, and the production of a flagellar sheath. Two centrioles and a cytoplasmic mass have been seen behind the elongated nucleus (Fig.10b). Cross sections of various segments of the sperm tail have been seen in the form of a middle piece, a major piece, and an end piece with a typical structure and regular contour that is virtually identical to that of the control. **(Fig.10a).**
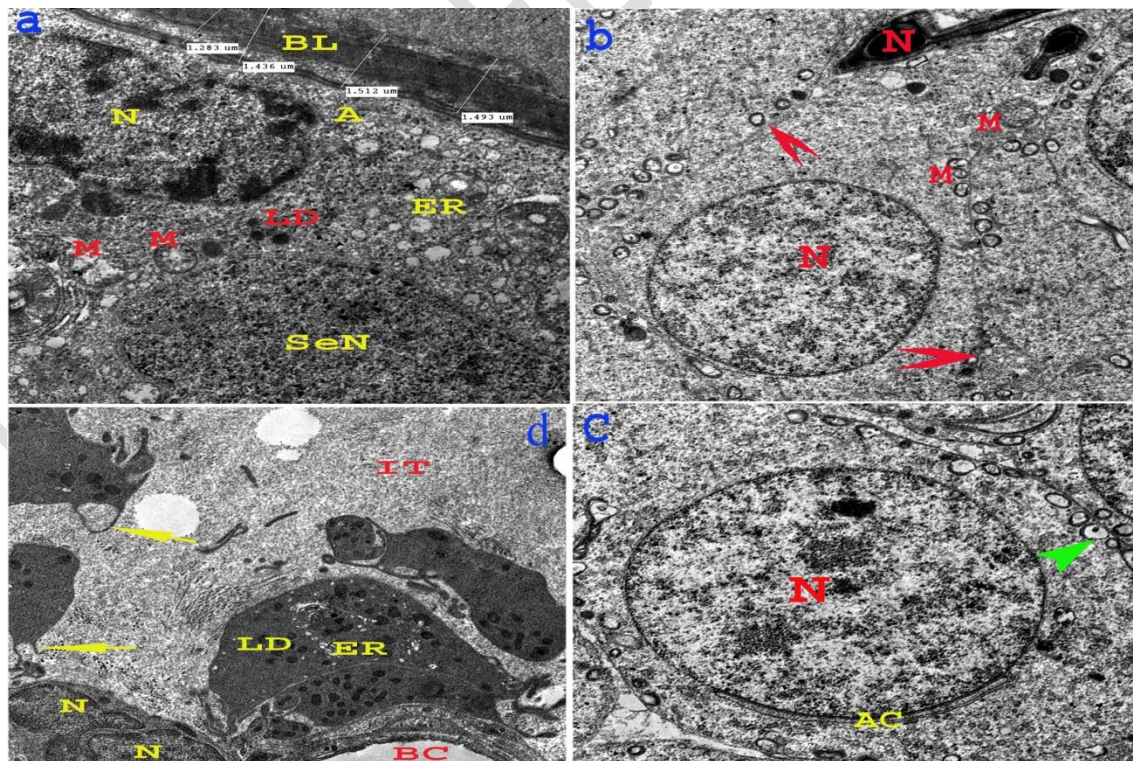
Fig. (9): a) showing Type A spermatogonium(A) with its oval nucleus(N) resting on basal lamina (BL), sertoli cell nucleus (seN). Its cytoplasm contains mitochondria(M), dense lipid droplets (LD) and endoplasmic reticulum(ER). Notice the measurements of thickness of basal lamina varies between 1.283 to 1.512 μm in different regions. b) showing Primary spermatocyte with rounded nucleus(N).Its cytoplasm contains multiple signet ring mitochondria(M) close to cell membrane (arrow head).Notice elongated nucleus of late spermatid (N). c) showing Round spermatid with its large rounded nucleus (N). The acrosomal cap (AC) is found at one pole of the nucleus. The cytoplasm contains numerous signet ring mitochondria close to cell membrane (arrow head). d) showing Interstitial tissue (IT) contains multiple leydig cells with dark nucleus (N). Its cytoplasm contains lipid droplets (LD) and endoplasmic reticulum (ER). The cell shows cytoplasmic processes (arrow). Notice bl. capillary (BC) adjacent to the cells. (group III   E/M   X3000)
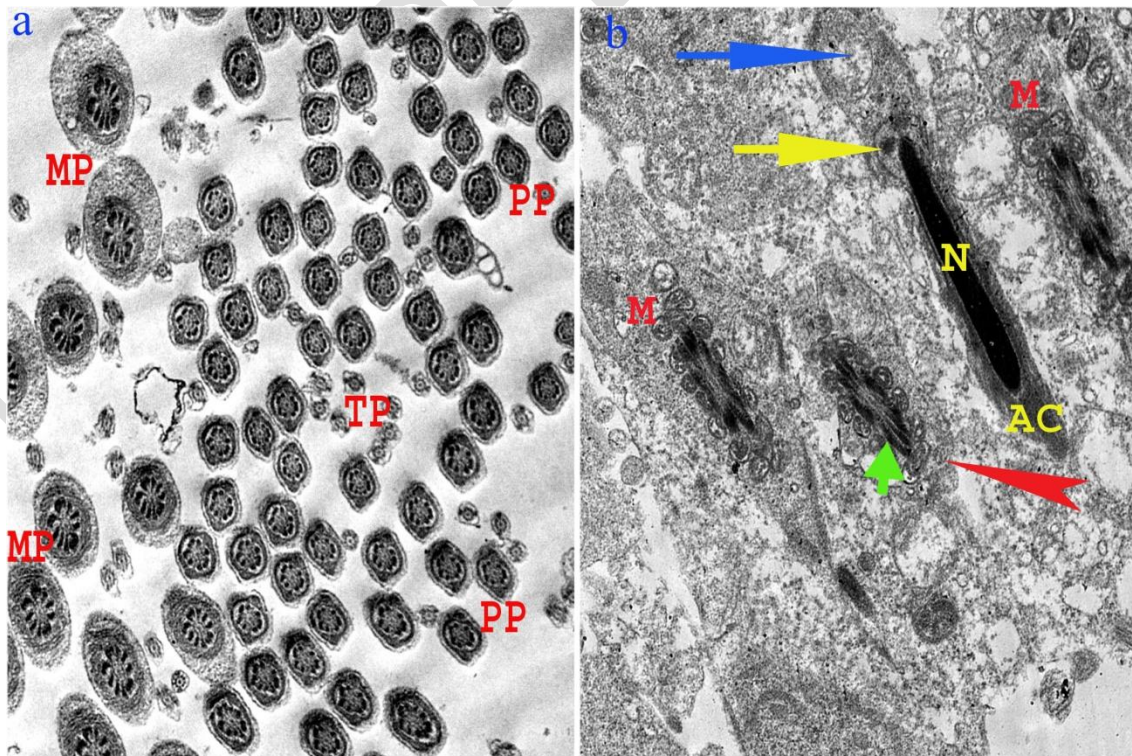
Fig. (10): a) showing Cross sections of different parts of tail of sperm in the form of Middle piece (MP), principal piece(PP) and end piece (TP). b) showing Stages of spermiogenesis in the form of ,elongated nucleus (N) within the future sperm head and formation of acrosomal cap (AC), aggregation of mitochondria (M) around the nine microtubules doublets (green arrow) and formation of flagellar sheath (Red arrow).Notice two centrioles(yellow arrow) and cytoplasmic mass (blue arrow) behind the elongated nucleus. (group III E/M X3000).

AT-MSCs treated group:

Histological examination of Hx. & E stained sections of rats' testes after AT-MSC implantation revealed restoration of the architecture and outline of seminiferous tubules to near-normal levels. The basal lamina's average thickness has been restored. The thickness of spermatogenic cell masses in the majority of seminiferous tubules has been recovered, as has their adhesion to the basal laminae (Figs. 11a& 11b). The seminiferous tubules' spermatogenic cell masses exhibit all phases of spermatogenesis, with a small lumen filled with spermatozoa (Figs.11a&11b). The interstial tissue is of typical thickness, restoring the interstitial space to its normal size but with some vacuoles.**(Fig.11a&11b).**
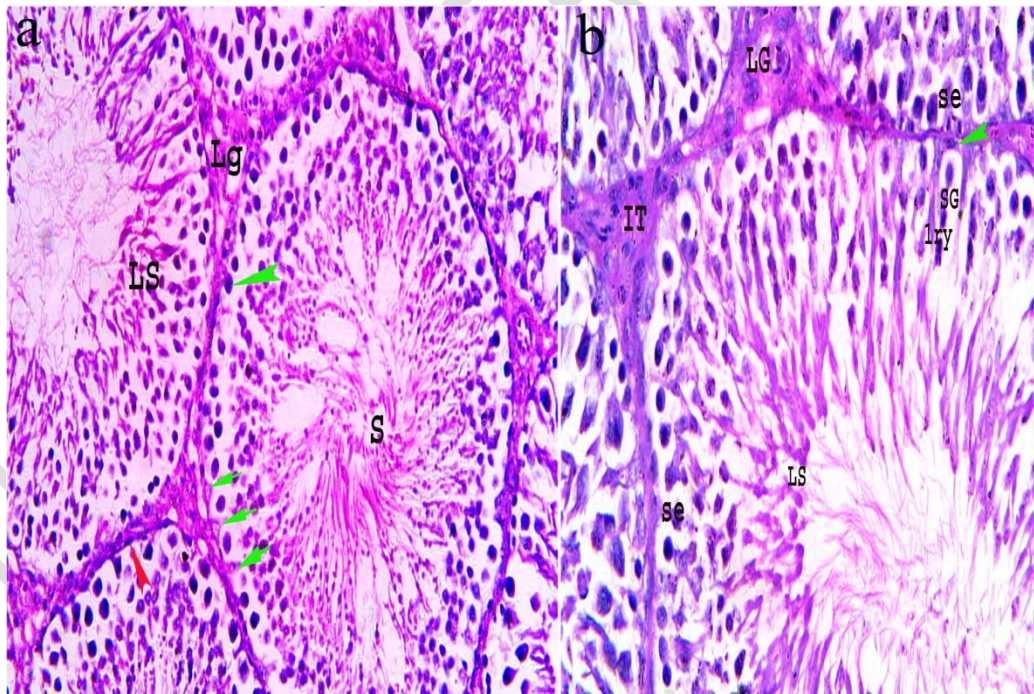

Fig. (11): a) showing spermatogonia (green arrow head), sertoli cell (green arrow), late spermatids (LS) and sperms (S) within the lumen, myoid cell (red arrow head) within basal lamina and leydig cell (Lg) in the interstitial tissue (groupIV H&EX200) .b) showing

parts of seminiferous tubules containing sertoli cell (se), spermatogonia (SG), elongated late spermatids (LS), 1ry spermatocyte (1ry), myoid cell (green arrow head) within basal lamina and leydig cell (LG) in the interstitial tissue. (group IV   H&E X400).

Ultrastructural observations: After AT-MSCs were transplanted into rats after busulfan therapy, distinct ultrastructural alterations in their testes were observed. Oval-shaped spermatogonia with a flattened nucleus parallel to the basal lamina, a thin, light, and non-condensed chromatin granulation, and a junctional complex connecting sertoli cells have been seen (Fig.12b). The basal lamina's average thickness has been restored, and a myoid cell has developed inside the basal lamina (Fig.12a). The sertoli cell has a big pale elongated nucleus with a conspicuous nucleolus and a generally uniform chromatin material. The cytoplasm is densely packed with smooth and rough endoplasmic reticulum, as well as spherical or cylinder-shaped mitochondria (Fig.12a). A primary spermatocyte with a spherical nucleus and fine granular chromatin has emerged. Its cytoplasm had many signet ring mitochondria and a black residual body with a moderately rarefied cytoplasm (Fig.13a). At one pole of the nucleus, a rounded spherical spermatid nucleus and acrosomal cap were seen, as well as several somewhat degraded signet ring mitochondria in the cytoplasm, which exhibited minor rarefaction (Fig. 13b). With basal insertion of sperm heads between primary spermatocytes, mitochondria aggregated around outer dense fibres to create a middle piece (Fig. 13d). Cross sections of various segments of the sperm tail have been seen in the form of a middle piece, a major piece, and an end piece with a normal structure and a regular contour that is almost identical to that of the control (Fig.13c). The oval nucleus of the Leydig cell has a dilated pernuclear membrane and peripheral condensation of its chromatin, while the cytoplasm includes lipid droplets, vesicles, the golgi apparatus, and an abundance of smooth endoplasmic reticulum.**(Fig.12c).**
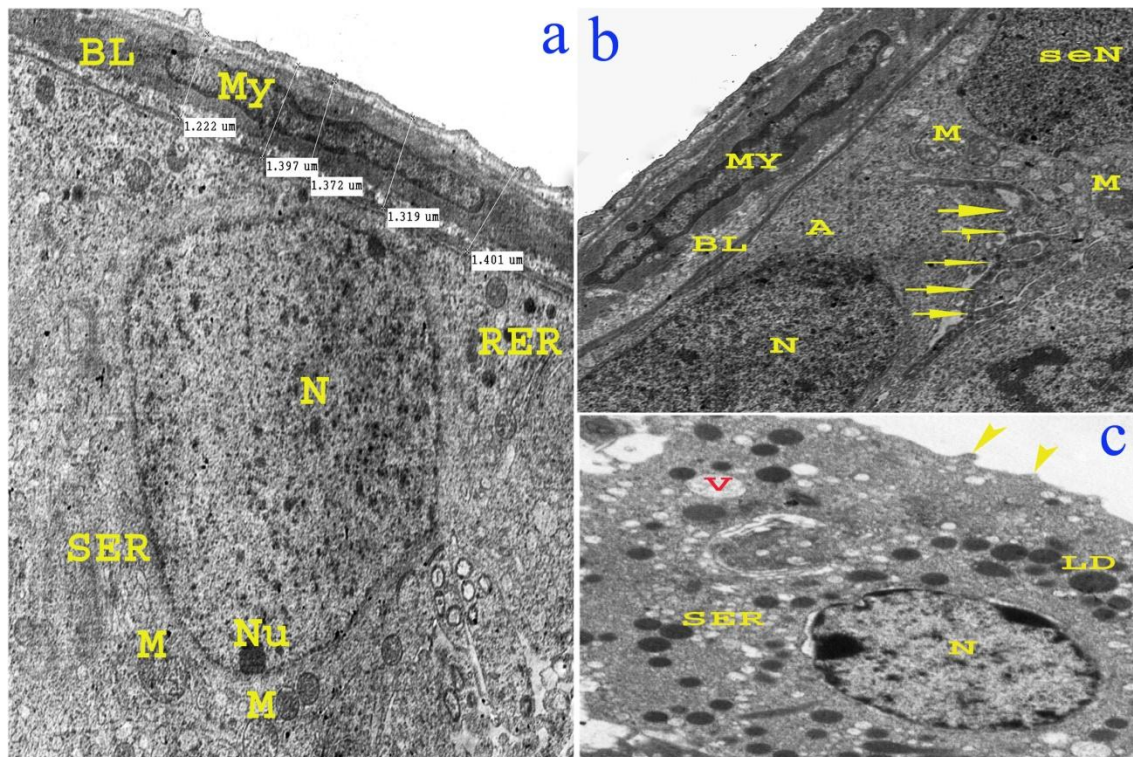
Fig. (12): a) showing sertoli cell nucleus (N) with nucleolus (Nu). Its cytoplasm contains mitochondria (M), smooth endoplasmic reticulum (SER) and rough endoplasmic reticulum (RER). Basal lamina(BL) containing myoid cell (MY). Notice the measurements of thickness of basal lamina varies between 1.222 to 1.401 μm in different regions. **b)** shows Type A spermatogonium (A) with its nucleus (N) and part of sertoli cell contains nucleus(seN). Its cytoplasm contains mitochondria (M).These two cells rest on the basal lamina (BL). Notice elongated nucleus of myoid cell (MY) within the basal lamina. Also notice Junctional complex (yellow arrows).**c)** shows leydig cell contains oval nucleus (N) with peripheral rim of chromatin. The cytoplasm contains many dark lipid droplets (LD), vesicles (V) and smooth endoplasmic reticulum (SER). Notice few cytoplasmic processes (arrow head).(group IV E/M X3000)
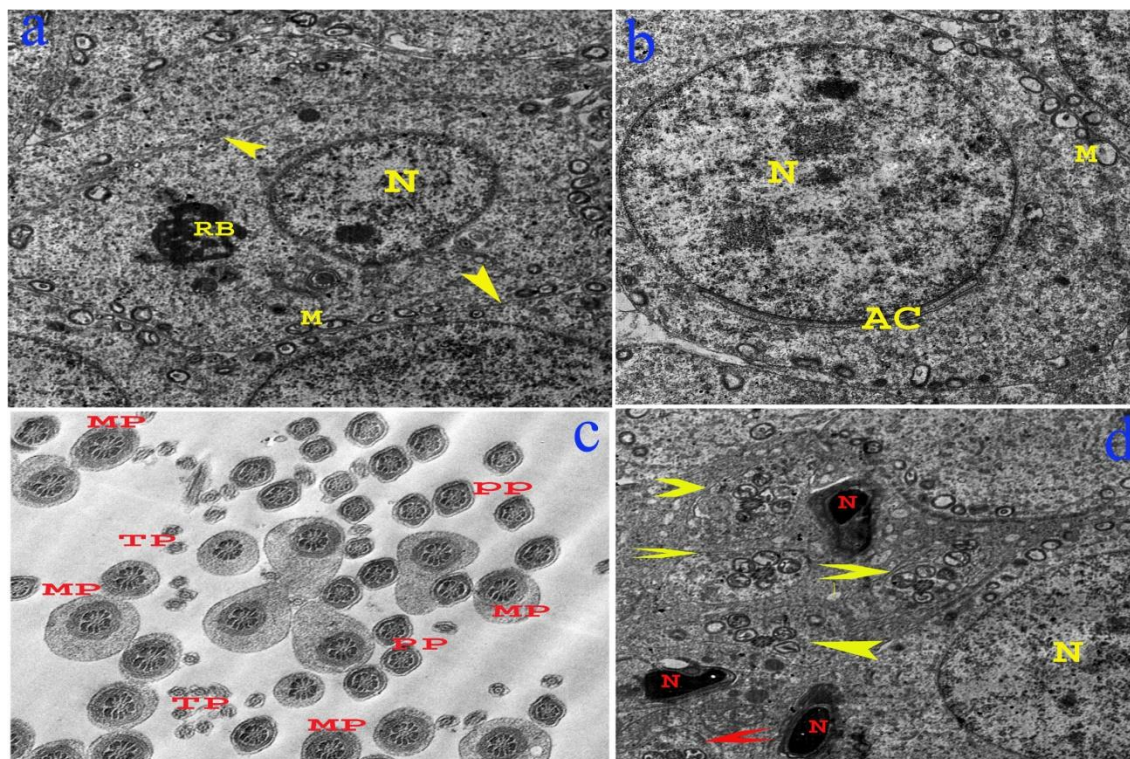
Fig. (13): a) showing1ry spermatocyte containing rounded nucleus (N) with eccentric nucleolus. Its cytoplasm contains signet-ring mitochondria (M) at periphery close to cell membrane (arrow head) .Notice a dark residual body (RB). b) showing Round spermatid nucleus (N) with acrosomal cap (AC), cytoplasm contains signet ring mitochondria(M) close to cell membrane. C) shows cross sections of different parts of tail of sperm in the form of , middle piece (MP), principal piece (pp) and end piece (TP).d) showingRound spermatid nucleus (yellow N), aggregation of mitochondria around outer dense fibers(arrow head) to form middle piece during acrosomal phase of spermiogenesis, elongation of nucleus (red N) within the head of sperm. (groupIV   E/MX3000)

Immunohistochemical examination:

PCNA staining of control testicular sections revealed a strong nuclear reaction in the majority of basal germ cells lining the seminiferous tubules in the form of dark brown pigmentation of the nucleus, indicating increased cell regeneration and proliferation, whereas other spermatogenic cells demonstrated a moderate nuclear reaction (Fig. 14a).

In testis treated with busulfan, the germinal epithelium degenerated into seminiferous tubules with gaps between the cells. The majority of germ cells have a negative response in the nuclei of their spermatogonia, i.e. (no brown pigmentation of the nucleus), indicating that they do not regenerate or proliferate. Nevertheless, just a few spermatogonia in the tubules had a positive

nuclear response. (See Figure 14b). PCNA staining of testicular sections treated with BM-MSC revealed a high positive nuclear response in the nuclei of the majority of spermatogonia, indicating increased cell regeneration and proliferation, and a moderate reaction in the nuclei of other spermatogenic cells (Fig.14c). PCNA staining of AT-MSC treated testicular sections revealed a high positive nuclear response in the nuclei of the majority of spermatogonia, indicating increased cell regeneration and proliferation, and a moderate reaction in the nuclei of other spermatogenic cells. (**Fig.14d**).
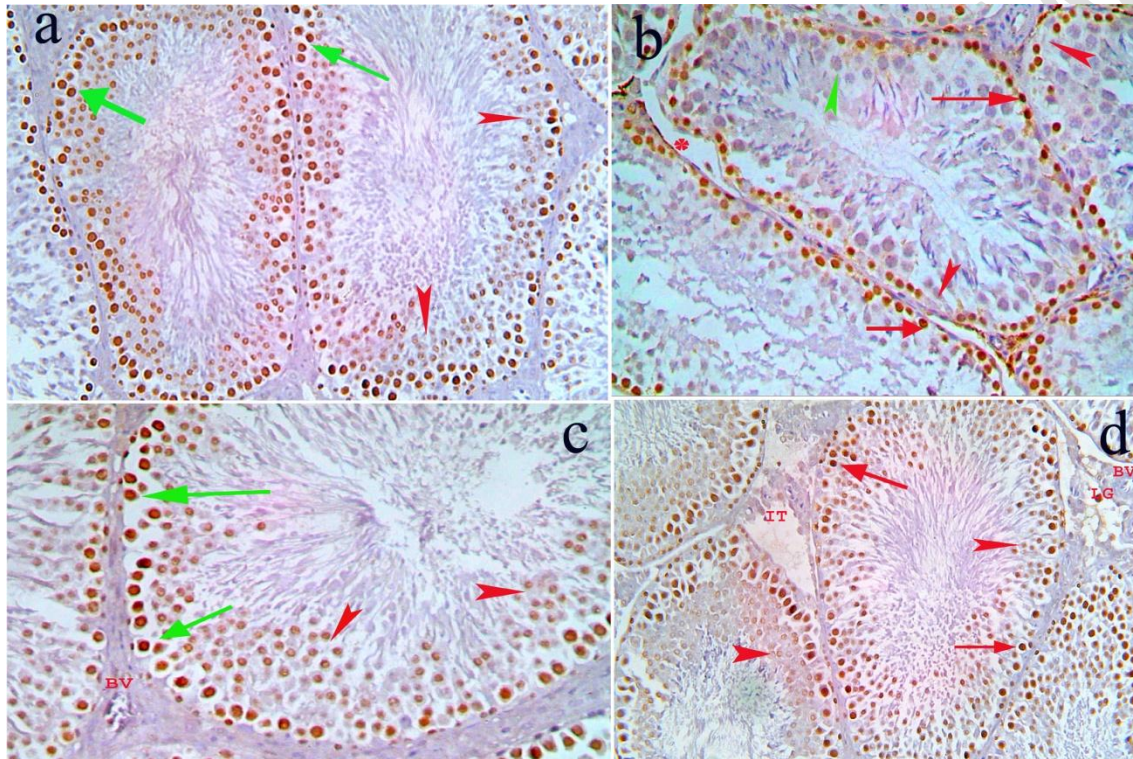

Fig. (14):a)groupI showing strong positive brown nuclear reaction in most of the basal germ cells lining the tubule(green arrow), while other spermatogenic cells show moderate nuclear reaction(red arrow head).b)groupII showing moderate positive immunostained germ cells i.e (brown nuclear reaction) (red arrow) with gaps in between (red arrow head), while most of germ cells show negative reaction (green arrow head) with partial separation from the basement membrane (star).c) groupIII shows Strong positive brown nuclear reactionin most of the basal germ cells lining the tubule(green arrow), while other spermatogenic cells show moderate nuclear reaction(red arrow head). Notice blood vessel (BV) containing blood cells in the interstitial space. d) groupIV showing strong positive brown nuclear reaction in most of the basal germ cells lining the tubule(red arrow), while other spermatogenic cells show moderate nuclear reaction(red arrow head), while interstitial tissue (IT) contains bl. Vessels(BV), leydig cell(LG) shows brown nucleus. (PCNA   X200)

There was no brown pigmentation in the spermatogenic cells' membrane or cytoplasm in slices of control rat testis, which means that CD105 was not present (Fig. 15a). Sections of testicular tissue treated with BM-MSCs showed a positive cell membrane reactivity for CD105, which shows that BM-MSCs have differentiated into these spermatogenic cells, since BM-MSCs are CD105 positive and the control testis is CD105 negative (Fig.15b). A positive cell membrane reactivity for CD105 was found in sections of AT-MSC-treated testicular tissue, showing that the transplanted AT-MSCs had developed into these spermatogenic cells, since AT-MSCs are CD105 positive, whereas control testis is CD105 negative..**(Fig.15c).**
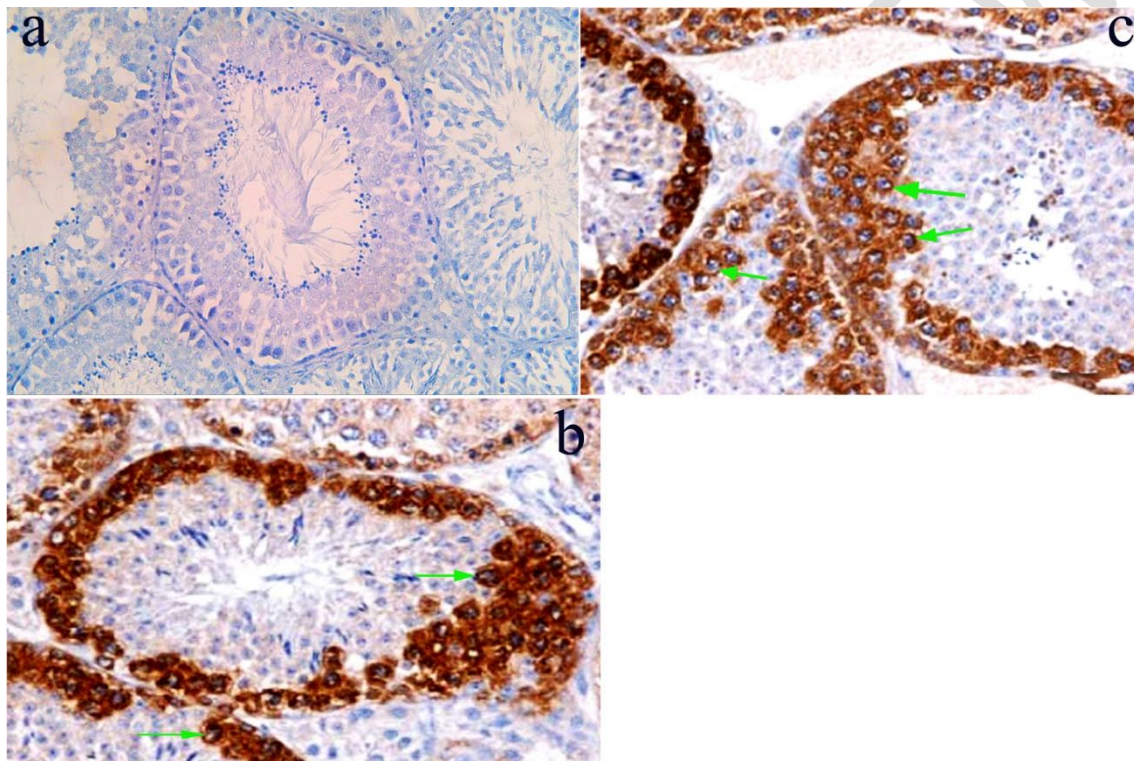
Fig. (15): a) group I showing negative reaction for CD105 antibody i.e (no brown pigmentation of cell membrane of spermatogenic cells). b)group III showing positive cell membranous reaction for CD105 antibody i.e (brown pigmentation of cell membrane and cytoplasm of spermatogenic cells)(green arrow). c) groupIV showingpositive cell membranous reaction for CD105 antibody i.e (brown pigmentation of cell membrane and cytoplasm of spermatogenic cells)(green arrow). (CD105   X200) .

Stem cell engraftment

Under fluorescent microscopic (Leica) examination, PKH26-stained testis tissue emitted pink fluorescence that indicated homing of stem cell and their integration into seminiferous tubule **(Figs. 16a &16b)**

Fig. (16): a) photomicrograph of a section in the rat testis 3 months after transplantation of PKH26 labelled BM-MSCs showing: strong red auto-fluorescence (green 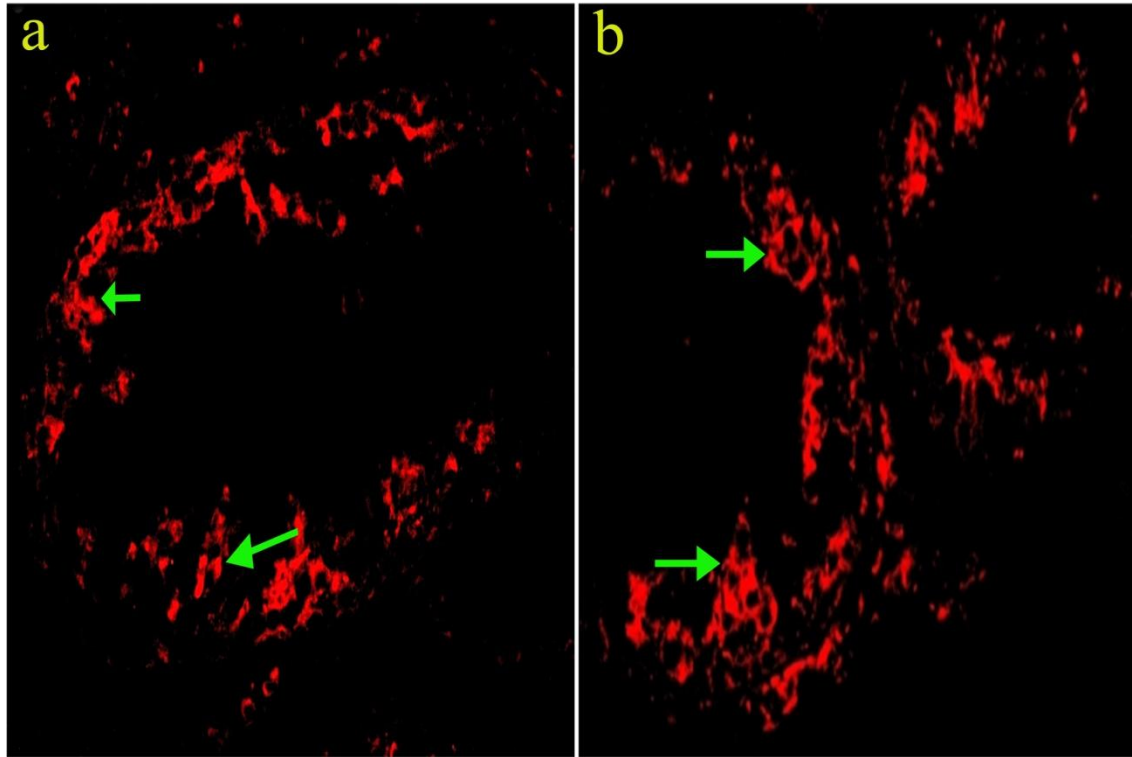arrow) ,confirming homing of these cells into the seminiferous tubule. b) photomicrograph of a section in the rat testis 3 months after transplantation of PKH26 labelled AT-MSCs showing: strong red auto-fluorescence (green arrow) ,confirming homing of these cells into the seminiferous tubule. (Fluorescent Microscope X 200).

Mean values of seminiferous tubules luminal diameter in pixel (Table 1& Graph 1):

Group	Mean±SD (Pixel)	Comparison		sig
Control	170.89±40.27	Control	Busulfan treated	**0.000****
			BM stem cells treated	**0.068**
			AT stem cells treated	**0.002****
Busulfan treated	273.78±88.92	Busulfan treated	Control	**0.000****
			BM stem cells treated	**0.000****
			AT stem cells treated	**0.000****
BM mesenchymal stem cells treated	122.61±43	BM stem cells treated	Control	**0.068**
			Busulfan treated	**0.000****
			AT stem cells treated	**0.165**
AT mesenchymal stem cells treated	86.22±20.12	AT stem cells treated	Control	**0.002****
			Busulfan treated	**0.000****
			BM stem cells treated	**0.165**
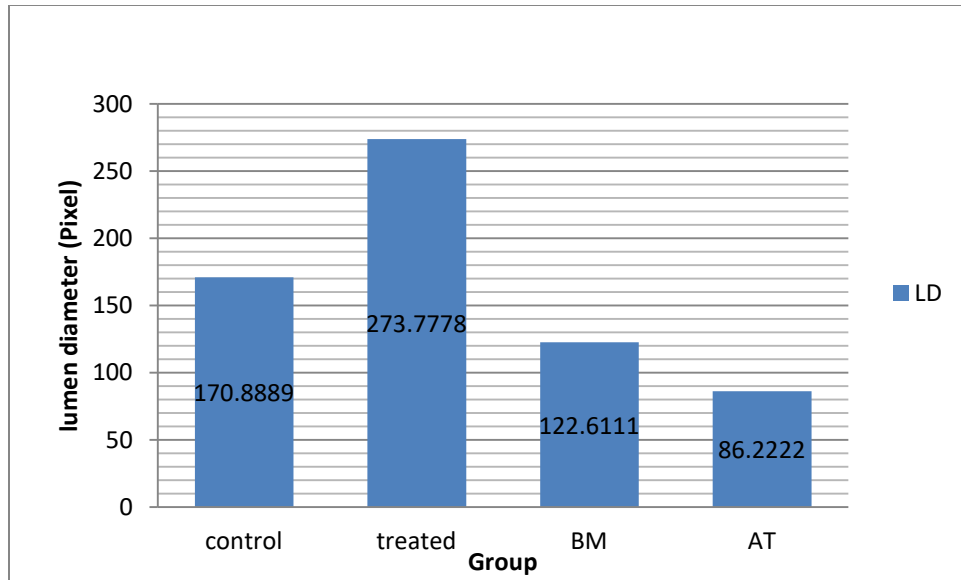
Graph 1 . Mean values of seminiferous tubules luminal diameter in pixel

Mean values of seminiferous tubules cellular diameter in pixel (Table 2& Graph 2):

Group	Mean±SD pixel	Comparison		sig
Control	326.28±52.45	Control	Busulfan treated	0.000**
			BM stem cells treated	0. 456
			AT stem cells treated	0. 051**
Busulfan treated	135.81±38.90	Busulfan treated	Control	0.000**
			BM stem cells treated	0.002**
			AT stem cells treated	0. 037**
BM mesenchymal stem cells treated	292.08±165.45	BM stem cells treated	Control	0.456
			Busulfan treated	0.002**
			AT stem cells treated	0.213
AT mesenchymal stem cells treated	234.53±72.48	AT stem cells treated	Control	0.051**

			Busulfan treated	0.037**
			BM stem cells treated	0.213
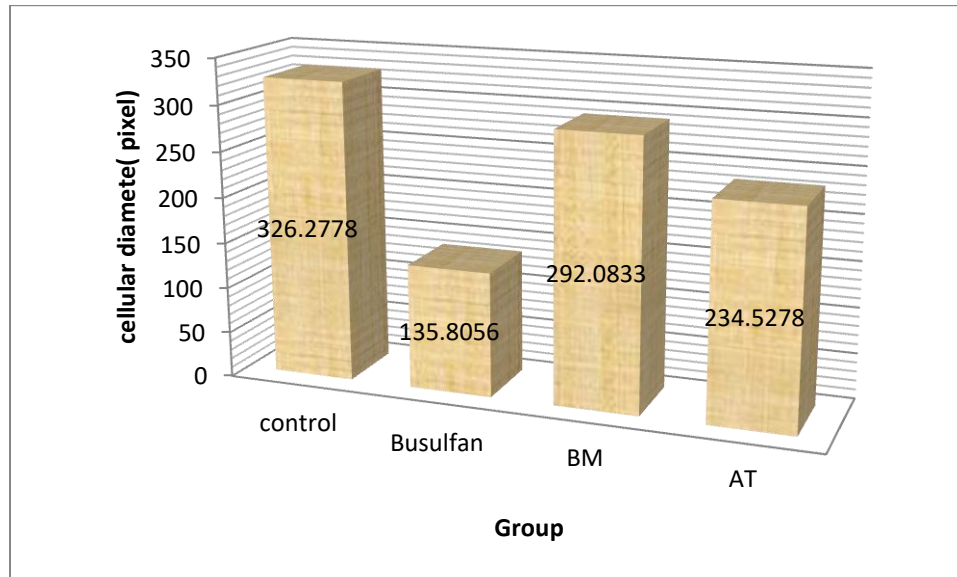
Graph 2 . Mean values of seminiferous tubules cellular diameter in pixel

Mean values of spermatogenesis index (Table 3& Graph 3):

Group	Mean±SD	Comparison		sig
Control	6.0000±1.15	Control	Busulfan treated	0.000**
			BM stem cells treated	0. 614
			AT stem cells treated	0. 086
Busulfan treated	1.14±0.69	Busulfan treated	Control	0.000**
			BM stem cells treated	0.000**
			AT stem cells treated	0. 000**
BM mesenchymal stem cells treated	6.29±0.76	BM stem cells treated	Control	0.614
			Busulfan treated	0.000**

			AT stem cells treated	0.031**
AT mesenchymal stem cells treated	5.0000±1.41	AT stem cells treated	Control	0.086
			Busulfan treated	0.000**
			BM stem cells treated	0.031**
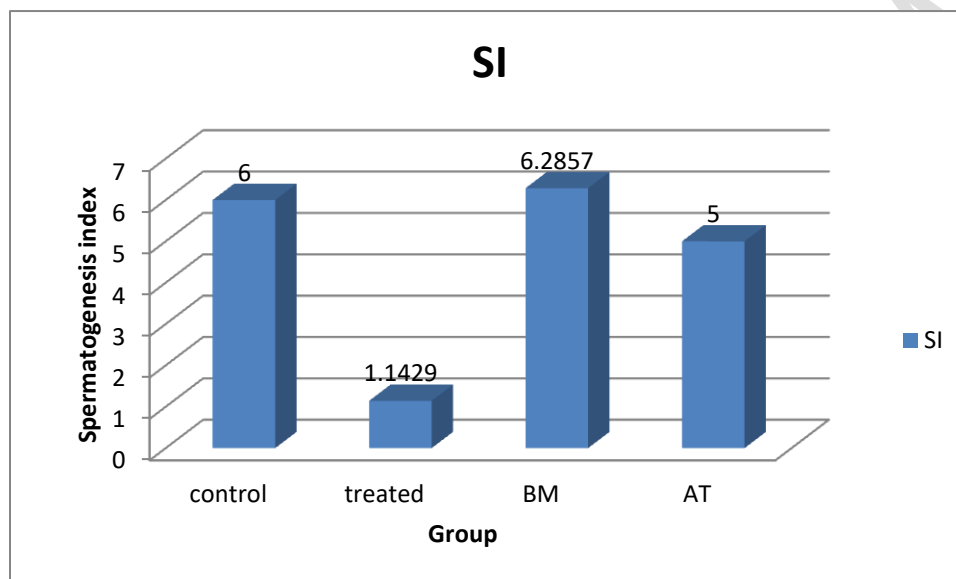
Graph 3. Mean values of spermatogenesis index

Table (4) & Graph (4): Comparison between all groups as regarding area % PCNA immunoreactivity.

Group	Mean±SD %	Comparison		sig
Control	22.18±6.03	Control	Busulfan treated	0.000**
			BM stem cells treated	0. 120
			AT stem cells treated	0. 002**
Busulfan treated	6.94±1.88	Busulfan treated	Control	0.000**
			BM stem cells treated	0.000**
			AT stem cells treated	0. 031**

			Control	0.120
BM mesenchymal stem cells treated	18.01±4.97	BM stem cells treated	Busulfan treated	0.000**
			AT stem cells treated	0.060
			Control	0.002**
AT mesenchymal stem cells treated	12.90±3.77	AT stem cells treated	Busulfan treated	0.031**
			BM stem cells treated	0.060
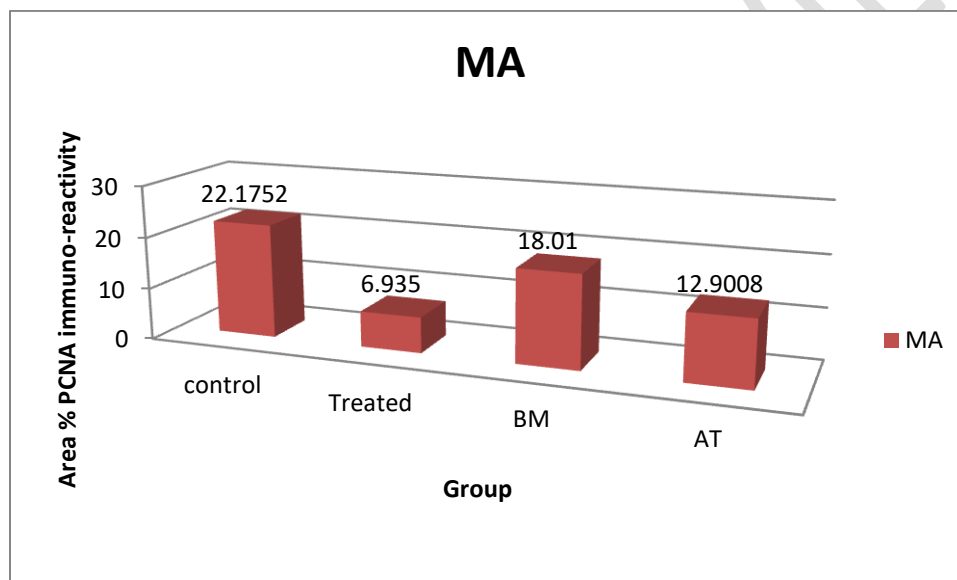
Graph 4.Comparison between all groups as regarding area % PCNA immunoreactivity.

Discussion:

   The present research examined Hx. and E stained sections of the testes of control wistar rats and found that the seminiferous tubules had a normal architecture with an average quantity of interstitial tissue. Tubules looked to be regular in form, rounded or oval in shape, and virtually identical in diameter. They have developed small lumina, are bordered by stratified germinal epithelium, and are separated by medium-sized interstitial gaps, and ultrastructural examinations have shown two distinct forms of spermatogonia: A and B. Oval-shaped spermatogonia with a flattened nucleus parallel to the basal lamina and a thin, light, and uncondensed chromatin granulation have been seen in type A spermatogonia. Spermatogonia of type B are spherical in

form and have highly stained chromatin regions in their nuclei. Sertoli cells run the length of the seminiferous tubule, from the basal lamina to the lumen. It features a big pale elongated nucleus with an infolded nuclear membrane, rather uniform chromatin material, and a conspicuous nucleolus. The cytoplasm is densely packed with smooth endoplasmic reticulum, lipid droplets, and Mitochondria that are either spherical or cylindrical in form. Primary and secondary spermatocytes are two distinct kinds of spermatocytes. There is now a large spherical nucleus with fine granular chromatin and clusters of Golgi apparatus at one end of the cell. The cytoplasm had several mitochondria with signet rings. Various stages of acrosomal cap formation have been seen in round spermatids in the Golgi phase. Leydig cells have a large nucleus that is bordered by a thin heterochromatin border. All of these factors lead to Leydig cells' distinctive appearance, which is marked by an abundance of smooth endoplasmic reticulum and many lipid droplets.. Cross sections of various parts of the sperm tail have been observed in the form of a central piece that consists of a vaflagellar axoneme surrounded by nine coarse (outer dense fibres) fibres arranged longitudinally external to this core.,Elongated mitochondria have been arranged in a tightly packed helix. The major component was composed of a central axoneme, nine coarse fibres extending from the centre piece, and a core surrounded by many fibrous ribs organised in a circular pattern. Two of the core's longitudinal fibrils fused with the surrounding ribs to produce ventral and dorsal columns.. This was consistent with **Kerr. (2010), Young et al. (2013), Ovalle and Nahirney. (2008), and Mohammad Ghasemi et al. (2008),** who said that Spermatogonia depended on the basement membrane and possessed rounded to elliptical nuclei with minimal heterochromatin and a central nucleolus. Primary spermatocytes were bigger than secondary spermatocytes. It is possible to witness many phases of miosis. They feature a big spherical nucleus and a nucleoplasm that is homogeneous in size. Basement membrane is bilayer in nature, consisting of dark and light layers. Various phases of spermiogenesis were seen, with spermatids extending with an extensively elongated and dense nucleus on which the acrosomal cap developed.

In our investigation, immunohistochemical staining of control testis with PCNA revealed a significant nuclear response for PCNA in the majority of basal germ cells lining the seminiferous tubules, but only a mild nuclear reaction in other spermatogenic cells.

These results corroborated **Kerr's.(2010).**

Recent research has shown that chemotherapy medicines such as busulfan may induce testicular injury, as seen by decreased testicular volume, oligozoospermia, and apoptotic cell death in the testicular germinal epithelium. **Mohammad-Ghasemi and colleagues (2008).** In contrast to other compounds, busulfan principally kills spermatogonial stem cells. However, other compounds, with the exception of busulfan, destroy differentiated spermatogonia **(Kanatsu-Shinohara et al., 2003; Anjamrooz et al., 2006 and Kawashima et al., 2009).** In numerous species of animals, a single intraperitoneal injection of 10, 20, 30, 40, or 50 mg/kg busulfan results in the deletion of spermatogenic cells and lifelong sterility **(Anjamrooz et al., 2006 and Kawashima et al., 2009).** Additionally, the length of sterility caused by busulfan is depending on the level of stem cell depletion **(Kanatsu-Shinohara et al., 2003).** Busulfan is capable of destroying spermatogonial stem cells from a variety of species. However, it has no impact on the production of DNA. Busulfan, on the other hand, suppresses the following mitosis when it intoxicates cells in the G1 phase. **Panahi et al. (2015)**.

Histological analysis of Hx. & E. stained sections from the Busulfan-treated group revealed that the tubules are asymmetrically structured and lack the control cellular architecture with irregular outlines. Seminiferous tubules have been significantly reduced and deformed, with their lumina devoid of spermatozoa**.** This is backed by morphometric and statistical data demonstrating that the mean diameter of seminiferous tubules reduced considerably (P 0.01) in the busulfan treated group compared to the control group, The luminal diameter of the seminiferous tubule was significantly increased in the busulfan treated group compared to the control group (P 0.01), the cellular diameter of the seminiferous tubule was significantly decreased (P 0.01) in the busulfan treated group compared to the control group, and the spermatogenesis index was significantly decreased (P 0.01) in the busulfan treated group compared to the control group. These statistical findings are **consistent with those of Hajihoseini et al. (2017), Tamadon et al. (2015), Payehdar et al. (2017), and Mehrabani et al. (2015).** Several tubules exhibited disarray, with germ cells exfoliating into the tubular lumen. While the remaining tubules seemed normal due to the progression of spermatogenic cells. Significant interstitial degeneration has been detected in the form of vacuoles or homogeneous exudate, as well as germ cell infiltration of interstitial tissue. **These findings corroborate those of Panahi et al. (2015),** who discovered that two doses of busulfan injection separated by 21 days generated azoospermia 35 days following the

final injection in rats. Additionally, Anjamrooz et al. (2006) reported in 2006 that intraperitoneal injection of busulfan at dosages of 20, 30, 40, and 50 mg/kg might produce infertility in rats after four weeks. Thus, a large dosage of busulfan greatly reduced the quantity of sperms in the epididymal lumen and rendered the animals permanently infertile, but a modest dose resulted in a decrease in the number of germ cells. A dosage of 30 mg/kg of busulfan was shown to be the best amount for depleting host germ cells and causing the least death in mice. Also These findings corroborate those of Ogawa et al. (1999), who demonstrated that the system of divided injections is the most accurate way to design an azoospermic model for studying cell transplantation into a recipient animal, as Busulfan at a dose of 25 mg/kg is lethal to rats due to their myelotoxicity. Payehdar et al. (2017) also noticed a reduction in the thickness of the germinal epithelium, range, and vacuolar space on the basement membrane of the seminiferous tubules, as well as a total loss of spermatogenesis. Monsefi et al. (2013) also reported that after two months of busulfan therapy, histological investigations of testes in the busulfan treatment group indicated certain degenerative alterations such as seminiferous tubular atrophy and germinal epithelium degradation in the majority of tubules. In certain tubules, the quantity of sperm clusters and spermatids was reduced. Pyknotic nuclei were seen in a few germinal cells. Seminiferous tubules were filled by the vast vacuolated lumen, and the atrophic germinal epithelium formed a narrow band around the peripheral zone of seminiferous tubules..

In our research, Different ultrastructural alterations in the testes of rats treated with busulfan have been observed. The basal lamina has enlarged with the appearance of split myoid cells with rarefaction of their cytoplasm, whilst spermatogonia have developed aberrant darkly pigmented irregular nuclei with conspicuous vacuoles inside and between the cells. The cytoplasm of germ cells and Sertoli cells had several big, well-defined vacuoles. Degenerated Leydig cells with irregular nuclei and vacuoles have been discovered inside the interstitial tissue. We saw an abnormal Sertoli cell with vacuoles inside the cytoplasm, large vacuolated mitochondria, and nuclear membrane invagination. Spermatocytes with deteriorated cytoplasmic organelles and vacuoles have been seen, as well as an aberrant spherical spermatid with a degraded acrosomal cap. Vacuolated mitochondria, vacuoles, and vacuolated golgi apparatus have been seen in its cytoplasm. Additionally, leftover bodies have been seen. Cross sections of several regions of the sperm tail revealed an uneven shape and degeneration of the intermediate piece, the major component, and the deteriorated end piece. Mohammad Ghasemi et al. (2008) concurred, stating

that a single dose of busulfan induced ultrastructural features of apoptosis in all experimental groups, including chromatin marginalisation of germ cell nuclei, particularly in spermatogonia, nuclei deformation, germ cell separation and the presence of large spaces between adjacent cells, cell shrinkage, the presence of vacuoles in germ cells, and apoptotic bodies in sertoli Vacuoles in the cytoplasm of germ cells and spermatogonial cells' more electron dense dark nuclei. These symptoms may be indicative of an early stage of spermatogonial apoptosis..

Immunohistochemical analysis of PCNA stain in busulfan-treated testis revealed degeneration of the germinal epithelium in seminiferous tubules with gaps between the cells in the present investigation. The majority of germ cells have a negative response in their spermatogonia's nucleus. Nevertheless, just a few spermatogonia in the tubules had a positive nuclear response. This is confirmed by morphometric and statistical data demonstrating a substantial reduction in the area percent of PCNA immunoreactivity in the busulfan treated group compared to the control group (P 0.01). These findings corroborate those of Choi et al. (2004), who reported that the proportion of tubules undergoing apoptosis rose after one week of busulfan administration and that proliferating cell nuclear antigen expression was suppressed after four weeks.

The blood-testis barrier may aid in the protection of MSCs from immunologic reactions. et al. Lue et al (2007). Not only are BM-MSCs hypoimmunogenic, but they also induce immunosurveillance or immunosuppression upon transplantation, making them an excellent candidate for allogeneic transplantation. Bibber et al. Additionally, sertoli cells are immunological tolerant cells that may promote the survival of donor BM-MSCs and shield transplanted allogeneic cells from immune or inflammatory responses after transplantation. et al. Mital et al (2010)**.**

In this study, histological examination of Hx. & E stained sections of rats' testes after BM-MSC transplantation revealed restoration of the architecture and outline of seminiferous tubules to near-normal levels. The basal lamina's average thickness has been restored. The thickness of spermatogenic cell masses in the majority of seminiferous tubules was also restored, as was their adhesion to the basal laminae. Exudate and blood vessel congestion have nearly fully resolved. Densely packed seminiferous tubules are bordered with stratified germinal epithelium. Vacuoles are all but vanished. The interstial tissue is of ordinary thickness, restoring the interstitial space to its normal size. This is confirmed by morphometric and statistical data demonstrating that the seminiferous tubule luminal diameter reduced insignificantly in the BM stem cell treatment

group compared to the control group (P> 0.05). There was a statistically significant increase in the diameter of the seminiferous tubule when compared to the busulfan-treated group (P 0.05), but there was no significant change when compared to the control group (P0.01). A considerable rise in the spermatogenesis index was seen in the BM stem cell-treated group, but there was no significant difference between the control group and the busulfan-treated group (P>0.05) (P0.01). These results corroborate those of Hajihoseini et al. (2017), Vahdati et al. (2017), who demonstrated that injected BM-SCs may stimulate spermatogenesis, and Monsefi et al. (2013), who demonstrated that transplanted BM-SCs can develop into germinal cells in wistar rats' seminiferous tubules. MSCs implanted into sterile male rats' atrophic testicular seminiferous tubules survived and established themselves in the seminiferous tubules and interestitium. SSCs derived from MSCs proliferated and generated additional germ cells including primary spermatocytes, spermatids, and spermatozoa in certain recipient rats' seminiferous tubules. Additionally, MSCs were found in the interstitial connective tissue between the seminiferous tubules, with a morphology similar to that of leydig cells. Additionally, Lue et al. (2007) demonstrated that BM-MSCs transplanted into the testis of an infertile mouse model treated with busulfan seemed to develop into germ cells, sertoli cells, and leydig cells. However, Van Saen et al. (2009) and Lassalle et al. (2008) demonstrated that in mice, BM-MSCs were incapable of differentiating into sperm.,

Busulfan treatment resulted in a variety of ultrastructural changes in the testicles of rats transplanted with BM-MSCs. They showed that seminiferous tubules were restored to near-normal levels in terms of their architecture and form. The average thickness of the basal lamina has been restored. the nucleus was flattened to lie parallel to the basal lamina and had a thin, thin, and uncondensed layer of DNA attached to it; this was characteristic of type A spermatogonia. Myoid cells may be detected resting on sertoli cells in the basal lamina. Large pale oval nucleus with infolded nuclear membrane, homogenous chromatin composition, and prominent nucleolus are some of the hallmarks of this cell. Smooth endoplasmic reticulum, thick lipid droplets, and cylindrical or spherical mitochondria fill the cytoplasm to the brim. Spermatocytes have formed in the laboratory, and this one is the first to show signs of development. There were several signet ring mitochondria in its cytoplasm, according to the findings. Additionally, some sperm heads have been shown to be inserted near the base of the spermatocyte. Several signet ring mitochondria and spherical spermatids in the Golgi phase and

acrosomal cap have been seen at one end of the nucleus.. Multiple Leydig cells with a big nucleus and a thin ring of heterochromatin have been seen in interstitial tissue. The cytoplasm of the Leydig cell is eosinophilic, with many tiny lipid droplets, moderately sized mitochondria, a golgi apparatus, and an abundance of smooth endoplasmic reticulum, and has been demonstrated to have cytoplasmic processes. Stages of spermiogenesis have been seen in the form of an elongated nucleus inside the future sperm head and the construction of an acrosomal cap, mitochondrial aggregation around the nine microtubule doublets, and the production of a flagellar sheath. Two centrioles and a cytoplasmic mass have been seen behind the elongated nucleus. Cross sections of various segments of the sperm tail have been seen in the form of a middle piece, a major piece, and an end piece with a typical structure and regular contour that is virtually identical to that of the control. There have been observations of elongated spermatids with black nuclei (condensed chromatins) and normal tail development. Metwally et al. (2017) reported similar results, stating that ultrathin sections of the seminiferous tubules of rats treated with BM-MSCs demonstrated a significant restoration of the overall structure of testicular tissues after Lambda-cyhalothrin therapy. Sertoli cells have essentially undamaged nuclei but an increased number of lysosomes in their cytoplasm. Primary spermatocytes had a big nucleus and normal cytoplasmic organelles, while round spermatids possessed well-formed acrosomes..

Proliferation and cell regeneration were evident in the majority of spermatogonia, with dark brown nuclear pigmentation, whereas other spermatogenic cells had a more modest reactivity to PCNA immunohistochemical staining of the testicles. Morphometric and statistical results show that the area percent of PCNA immunoreactivity in the BM stem cell-treated group increased significantly without a significant difference when compared to the control group (P>0.05), but increased significantly when compared to the busulfan-treated group (P0.05) (P0.01). The brown pigmentation on the cell membrane and the cytoplasm of spermatogenic cells in sections of testicular tissue treated with BM-MSCs showed that the transplanted BM-MSCs differentiated into these spermatogenic cells, whereas the control testis is CD105 negative. This indicated that the transplanted BM-MSCs differentiated into these spermatogenic cells, which are CD105 positive..

Adipose tissue-derived mesenchymal stem cells (ADMSCs) were identified as an attractive source of multipotent adult stem cells due to their relative abundance and ease of isolation, as well as their high proliferative capacity, ability to generate a large number of cells, and ability to

be expanded for a longer period of time than bone marrow-derived stem cells Umesh et al (2016).

Histological investigation of Hx. & E stained sections of rats' testes after AT-MSC implantation revealed restoration of the architecture and outline of seminiferous tubules to near-normal levels. The basal lamina's average thickness has been restored. The thickness of spermatogenic cell masses in the majority of seminiferous tubules has been restored, as has their adhesion to the basal laminae. Some seminiferous tubules have been shown to be devoid of sperm, while others have been observed to be densely packed with spermatogenic cells. The seminiferous tubules' spermatogenic cell masses demonstrated all phases of spermatogenesis, with a small lumen filled with spermatozoa. This is supported by morphometric and statistical findings that the mean luminal diameter in the AT stem cell treated group was significantly decreased when compared to the control group (P 0.01) and significantly decreased when compared to the busulfan treated group (P 0.01), the seminiferous tubule cellular diameter was significantly increased in the AT stem cell treated group when compared to the busulfan treated group (P 0.05), and the sperma diameter was significantly increased in the AT stem cell treated group when The interstial tissue is of medium thickness, restoring the interstitial space to its normal size but containing some vacuoles. Mehrabani et al., (2015) observed the existence of spermatogonia in the seminiferous tubules after ATMSC implantation. Additionally, in sections of ATMSCs-treated testis, the tubules looked to be packed with germinal cells (spermatogonia, primary spermatocytes, spermatids, and sperms). They showed that in their new niche, injected ATMSCs developed into testicular germinal cells. Additionally, Cakici et al., (2013) showed that after 12 weeks, the testes of infertile rats treated with busulfan appeared morphologically normal with spermatogenesis in certain tubules of cell treated..

After busulfan treatment, rats were transplanted with AT-MSCs, and numerous ultrastructural changes were identified in their testes. Protoplasmic cells have been identified as oval-shaped spermatogonia with a flattened nucleus that is parallel to the basal lamina, as well as an uncondensed, light, and chromatin-free granulation. Restoring basal lamina's average thickness, and forming a myoid cell inside it, is the goal of this treatment. It has a large pale elongated nucleus with a prominent nucleolus and chromatin material that is quite homogeneous. Smooth and rough endoplasmic reticulums, as well as spherical or cylinder-shaped mitochondria, are all found in abundance in the cytoplasm. Spermatocytes have formed in the laboratory, and this one

is the first to show signs of development. Multiple signet ring mitochondria and a black residual body with slightly rarefied cytoplasm were seen in its cytoplasm. At one pole of the nucleus, a rounded spherical spermatid nucleus and acrosomal cap were seen, as well as several somewhat degraded signet ring mitochondria in the cytoplasm, which exhibited minor rarefaction. With basal insertion of sperm heads between primary spermatocytes, mitochondria aggregated around outer dense fibres to create a middle piece. Cross sections of various segments of the sperm tail have been seen in the form of a middle piece, a major piece, and an end piece with a normal structure and a regular contour virtually identical to that of the control. The oval nucleus of the Leydig cell has a dilated pernuclear membrane and peripheral condensation of its chromatin, while the cytoplasm includes lipid droplets, vesicles, the golgi apparatus, and an abundance of smooth endoplasmic reticulum. Atalla et al. (2017) reported similar results, stating that ultrastructural analysis of the testes of the group treated with AT-MSCs showed characteristics that were almost identical to those of the control group. Spermtogonia were spotted plainly lying on a trilaminar foundation membrane. The primary spermatocytes were identified by their big rounded nuclei, whilst the spermatids were identified by their rounded euchromatic nuclei. Sertoli cells had huge irregular nuclei with conspicuous nucleoli and a significant number of mitochondria. The Leydig cells were oval in shape with big eccentric nuclei and peripherally located chromatin, with folded nuclei visible in some cells. Their cytoplasms were densely packed with mitochondria and lipid droplets..

There was a significant positive nuclear response in the nuclei of the majority of the spermatogonia labelled with PCNA, suggesting an increase in cell regeneration and proliferation, and a mild positive nuclear reaction in the nuclei of other spermatogenic cells stained with the same antibody. An increase in PCNA immunoreactivity in the AT stem cell therapy group over the busulfan treated group is shown by morphometric and statistical studies, which support these results (P 0.05). A brown pigmentation of the cell membrane and cytoplasm of spermatogenic cells was seen in slices of testicular tissue treated with AT-MSCs, showing that the transplanted AT-MSCs had grown into these spermatogenic cells...

Our findings, in conjunction with those of previous studies, may help to clarify the processes by which spermatogenesis recovers after MSC transplantation. The first idea is that MSCs can be differentiated into target cells under the right induction circumstances Gnecchi and Melo (2009).

The second idea is that MSCs secrete growth factors that promote resident spermatogonial stem cells SSCs to restore host cell function. Leatherworker (2013). The last step is the fusion of MSCs with indigenous seminiferous tubule cells in order to restore the function of wounded tissue. Mansour and co-authors (2012). Interestingly, regardless of the route or source of the MSCs, all these animal models demonstrated that MSC treatment may be effective in reducing the adverse effects of chemotherapy on spermatogenesis.

In summary, we found that transplanted MSCs were accepted by the testis of host infertile rats. MSCs injected into testicular seminiferous tubules could differentiate into all kinds of germinal cells.

Conclusion

In conclusion the BM-MSCs and AT-MSCs were shown to be beneficial in treating azoospermia in the wistar rat model, restoring fertility to busulfan-induced azoospermic animals after MSC transplantation. As a result, this discovery may pave the way for the future use of MSCs in the treatment of human azoospermia, although more research should be conducted to confirm the findings.

Ethical Approval

All animal studies were approved by the Benha university's institutional animal care committee.

Declaration of competing interest: The authors report no declaration of competing interest.

References:

1- **Agarwal A, Makker K, Sharma R (2008):** Clinical relevance of oxidative stress in male factor infertility: an update. Am J Reprod Immunol; 59:2–11.

2-**Anand S, Patel H and Bhartiya D,(2015).** Chemoblated mouse seminiferous tubular cells enriched for very small embryonic-like stem cells undergo spontaneous spermatogenesis in vitro: Reproductive Biology and Endocrinology; 015: 31-33.

3-**Anjamrooz S H, Mansoureh Movahedin, Sayed Javad Mowla and Shahram Pour Bairanvand** (2006) Assessment of Morphological and Functional Changes in theMouse Testis and Epididymal Sperms Following Busulfan Treatment Iranian Biomedical Journal 11 (1): 15-22.

4-**Atalla S, Saleh H, Abdel Gawad S, and Mohamed H (2017)** Histological study on the effect of adipose tissue -derived mesenchymal stem cells on the testis of chemically induced castration model by cacium chloride in adult albino rats: EJH Vol 40,No(4): 486-498.

5-Bancroft J, Layton C & Suvarna SK(2018): The Hematoxylin and eosin. In: Suvarna SK, Layton C and Bancroft JD (ed). Theory and Practice of histological techniques. 8th ed. Churchill Livingstone of El Sevier, Philadelphia Ch. 10 and 11 pp; 172–214.

6-Bibber, B.; Sinha, G.; Lobba, A.R.; Greco, S.J.; Rameshwar, P. A review of stem cell translation and potential confounds by cancer stem cells. *Stem Cells Int.* **2013**, *2013*, 241048.

7-Cakici, C., B. Buyrukcu, G. Duruksu, A.H. Haliloglu, A. Aksoy, A. Isik, O. Uludag, H. Ustun, C. Subasi and E. Karaoz,(2013). Recovery of fertility in azoospermia rats after injection of adipose-tissue derived mesenchymal stem cells: the sperm generation. Biomed Res. Int., 529-589.

8-Chang CLT, Fung HP, Lin YF, et al. Indenopyridine hydrochloride induced testicular spermatogenesis failure with high seminal alkaline phosphatase levels in male dog. Biol Pharm Bull 2002; 25(8): 1097-1100.

9-Choi YJ, Ok DW, Kwon DN, Chung J, Kim HC, Yeo SM, *et al*. Murine male germ cell apoptosis induced by busulfan treatment correlates with loss of c-kit-expression in a Fas/FasL- and p53- independent manner. FEBS Lett. 2004; 575: 41-51.

10-D'Andrea MR, Lawrence D, Nagele RG, Wang CY, Damiano BP (2008): PCNA indexing as a preclinical immunohistochemical biomarker for testicular toxicity. Biotech Histochem; 83:211–220

11-Drusenheimer N, Wulf G, Nolte J, Lee JH, Dev A, Dressel R, Gromoll J, Schmidtke J, Engel W, Nayernia K(2007). Putative human male germ cells from bone marrow stem cells. Soc Reprod Fertil Suppl, ;63:69-76.

12-Gilliland KO, Freel CD, Lane CW, et al. Multilamellar bodies as potential scattering particles in human agerelated nuclear cataracts. Mol Vis 2001; 7: 120-130.

13-Gnecchi, M.; Melo, L.G. Bone marrow-derived mesenchymal stem cells: Isolation, expansion, characterization, viral transduction, and production of conditioned medium. *Methods Mol. Biol.* **2009**, *482*, 281–294.

14-Hajihoseini, M., A. Vahdati , S. E. Hosseini, D. Mehrabani, A. Tamadon: Induction of spermatogenesis after stem cell therapy of azoospermic guinea pigs . Vet. arhiv 87, 333-350, 2017.

15- Kanatsu-Shinohara M, Ogonuki N, Inoue K et al., "Long-term proliferation in culture and germline transmission of mouse

male germline stem cells," *Biology of Reproduction*, vol. 69, no.
2, pp. 612–616, 2003.

16-Kawashima A, Osman BAH, Takashima M, Kikuchi A, Kohchi S, Satoh E, *et al.* CABS1
Is a novel calcium-binding protein specifically expressed in elongate spermatids of mice. Biol
Reprod. 2009; 80: 1293-304.
17-Kerr J.(2010). Functional histology.second edition, Male reproductive system Mosby
Elsevier, 441-460.

18-Kim Y, Selvaraj V, Dobrinski I, Lee H, McEntee MC, Travis AJ.(2006). Recipient
preparation and mixed germ cell isolation for spermatogonial stem cell transplantation in
domestic cats. J Androl.;27:248-256.

19-Lassalle B, Mouthon MA, Riou L, Barroca V, Coureuil M, Boussin F, *et al.* Bone
marrow-derived stem cells do not reconstitute spermatogenesis *in vivo*. Stem Cells
2008;26:1385-6.

20-Leatherman, J. Stem cells supporting other stem cells. *Front. Genet.* **2013**, *4*, 257.

**21- Lue Y, Erkkila K, Liu PY, Ma K, Wang C, Hikim AS, Swerdloff
RS (2007).** Fate of bone marrow stem cells transplanted into the testis:
potential implication for men with testicular failure. Am J Pathol.
;170:899-908.

22-Mansour A, Abou-Ezzi G, Sitnicka E, Jacobsen SE, Wakkach A, Blin-Wakkach C.
Osteoclasts promote the formation of hematopoietic stem cell niches in the bone marrow. J Exp
Med 2012;209:537-549.

**23- Mehrabani D, Mohammad A, Hassan sh, Tamadon A, Shahrokh Z, Keshavarz S,
Farhad Rahmanifar, Dianatpour M, Khodabandeh Z, Razeghian Jahromi I, Tanideh N,
Ramzi M, Aqababa H and Kuhi-Hosein abadi O (2015).** Adipose Tissue-Derived
Mesenchymal Stem Cells Repair Germinal cells Of Seminiferous Tubules Of Busulfan- Induced
Azoospermic Rats. Journal Of Human Reproductive Sciences; Vol.8:103-110.

24-Metwally H, Abd-Ellah H, Shaheen N, Afifi M and Al-Zail N (2017) Role of
Mesenchymal Stem Cells in the Treatment of Testicular Toxicity Induced by Lambda-
Cyhalothrin in Rats: Wulfenia journal, Vol 24, No. 10; 108-138.

25-Mital, P.; Kaur, G.; Dufour, J.M. Immunoprotective sertoli cells: Making allogeneic
andxenogeneic transplantation feasible. *Reproduction* **2010**, *139*, 495–504.

26-**Mohammad-Ghasemi F, Soleimanirad J, Ghanbari AA.** An
Ultrastructural Study on the Apoptotic Features of Spermatogenic Cells following Busulfan
Treatment in AdultMice. J Reprod Infertil 2008; 4: 319-29.

27-Monsefi M, Fereydouni B, Rohani L, Talaei T. Mesenchymal stem cells repair germinal cells of seminiferous tubules of sterile rats. Iran J Reprod Med 2013;11:537-44.

28-Nayernia K, Lee JH, Drusenheimer N (2006), "Derivation of male germ cells from bone marrow stem cells,*Laboratory Investigation***, vol. 86, no. 7, pp. 654–663.**

29-Ogawa, T.; Dobrinski, I.; Brinster, R.L. Recipient preparation is critical for spermatogonial transplantation in the rat. *Tissue Cell* **1999**, *31*, 461–472.

30-Ovalle, William K, Nahirney, Patrick C. Netter's essential histology 2013 second edition Philadelphia, PA 19103-2899 **by Saunders, an imprint of Elsevier Inc.**
31-Panahi M, Keshavarz S, Rahmanifar F, Tamadon A, Mehrabani D, Karimaghai N, *et al*. Busulfan induced azoospermia: Stereological evaluation of testes in rat. Veterinary Research Forum. 2015; 6 (4) 273 - 278 [In press].

32- Payehdar A, Hosseini E, Mehrabani D, Forouzanfar M Busulfan Treatment Effects on Testicular Tissue and Serum Levels of Anti- Mullerian Hormone and Testosterone in Adult Mice The Indonesian Biomedical Journal, Vol.9, No.2, August 2017, p.106-112

33-Peng L, Run Z, Haitao S, Lei C, Fang L, Chen Y, Mouxuan D, Xiaodan J.PKH26 Can Transfer to Host Cells in Vitro and Vivo stem cells and development.2013; 22(2):340-344.

34-Short, B. and R. Wagey (2013). Isolation and culture of mesenchymal stem cells from mouse compact bone.. Methods Mol. Biol., 946: 335-347.

35-Tamadon, A., D. Mehrabani, F. Rahmanifar, A. Raayat Jahromi, M. Panahi, S. Zare, Z. KHODABANDEH, I. RAZEGHIAN JAHROMI, N. TA NIDEH, M. DIANAT POUR, M. RAMZI, O. KOOHI-HOSEINABADI (2015): Induction of spermatogenesis by bone marrow-derived mesenchymal stem cells in busulfan-induced azoospermia in hamster. Int. J. Stem Cells 8, 134-145.

36-Toyooka Y et al (2003). Embryonic stem cells can form germ cells in vitro. Proc Natl Acad Sci U S A;100(20):11457–11462.

37-Umesh D, Michael S, Ravindra K, Sadanand F. Advances in Adipose-Derived Stem Cells Isolation, Characterization and Application in Regenerative Tissue Engineering. Stem Cells International. 2016; dx.doi.org/10.1155/2016/3206807.

38-Vahdati A,Fathi AR, Nasimi P,and Saki GH (2015).Busulfan induces apoptosis and cytotoxic effects on testis and epididymal sperm of adult male mouse following low dose treatment. International Journal of Biosciences (IJB), vol.6, No.5,p.70-78.

39-Van Saen D, Goossens E, De Block G, Tournaye H. Bone marrow stem cells transplanted to the testis of sterile micedo not differentiate into spermatogonial stem cells and have no protective effect on fertility. Fertil Steril 2009;91(4 Suppl):1549-1552.

40-Young Barbara, O'Dowd Geraldine & Woodford Phillip (2014) WHEATER'S FUNCTIONAL HISTOLOGY: A TEXT AND COLOUR ATLAS, sixth edition Philadelphia, PA 19103-2899

41-Yoshimura K, Sato K, Aoi N, Kurita M, Hirohi T, and Harii K,(2008)."Cell-assisted lipo-transfer for cosmetic breast augmentation: supportive use of adipose-derived stem/ stromal cells, *Aesthetic Plastic Surgery*, vol. 32, no. 1, pp. 48–55.

42-Zhang D, Liu X, Peng J, He D, Lin T, Zhu J, *et al (2014).* Potential spermatogenesis recovery with bone marrow mesenchymal stem cells in an azoospermic rat model. Int J Mol Sci;15: 13151-13165.

43- Zhao Xin-Hong, Jian-Hong Qiu, Wen-Qing Cai, Song Li and Wei Li (2013). Preparation of infertile male rabbits by local electron beam irradiation for intra-testicular transplantation of autologous bone marrow stem cells. Acta Cirurgica Brasileira; Vol 28(2):148-152.