Original Article

Histological Evaluation of the Protective Effect of Stem Cell Derived Exosomes Versus Chitosan Nanoparticles on the Methotrexate Induced Testicular Injury in Albino Rat Model

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ABSTRACT

Introduction and Objectives: Methotrexate (MTX) is a chemotherapeutic agent used in the treatment of malignancies and autoimmune diseases. MTX testicular cytotoxicity has been proved in clinical and experimental studies. Therefore, this study was designed to compare the possible protective effect of adipose stem cells derived exosomes (ADSCs-EXOs) versus chitosan nanoparticles (CH-NPs) on methotrexate (MTX)-induced testicular injury in adult albino rats.

Materials and Methods: This study included 38 adult male albino rats and was divided into four groups in addition to the donor group. These groups included group I (Control) and three experimental groups; group II (MTX-treated rats); rats received IP injection of MTX (8mg/kg/week) for four weeks, group III (ADSCs-EXOs); rats were treated as group II concomitantly with IV injection of 1ml exosomes suspension (100µg/ml) once daily during the first seven days of the experiment, group IV (CH-NPs); rats were treated as group II concomitantly with chitosan nanoparticles (140 mg/kg/day) orally once daily for four weeks. Serum free testosterone level and testicular tissue malondialdehyde (MDA) and glutathione (GSH) levels were measured. Testicular sections were stained with H&E, PAS, and iNOS & N-cadherin immunostaining. Morphometric measurements were compared between groups.

Results: Group II showed disorganized seminiferous tubules with obviously degenerated spermatogenic epithelium and absence of spermatozoa. Also, this was associated with significant decrease in serum testosterone level, tissue GSH level, spermatogenic epithelium height, seminiferous tubules diameter, and N-cadherin +ve immunoreaction, as well as significant increase in tissue MDA level and iNOS +ve immunoreaction. While groups III and IV showed restoration of the normal histological tissues, biochemical and morphometric parameters. Moreover, there was no significant difference between both groups.

Conclusion: Both ADSCs-EXOs and CH-NPs have proved their potent cytoprotective effects against MTX-induced testicular injury in adult male rats and their protective effects were almost similar.

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Key Words: Chitosan nanoparticles; exosomes; methotrexate; rat; testis.

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INTRODUCTION

Methotrexate (MTX) is a chemotherapeutic drug that is utilized for the treatment of malignancies and autoimmune diseases by inhibiting the folic acid metabolism as well as the synthesis of DNA[1] which can result in cytotoxic damage to many organs such as liver[2], kidney[3], and testis[4]. Therefore, it can be provided as a relevant model of testicular injury[5]. Methotrexate destructive action occurs mainly through the imbalance between the oxidative and non-oxidative status as it leads to decreased antioxidants like catalase, glutathione, and superoxide dismutase and increased reactive oxygen species (ROS) hence damage of DNA and mitochondria[6]. Therefore, MTX can induce testicular injury causing apoptosis of viable cells[7] due to disruption of membrane integrity and damage of spermatozoa genetic material[8].

In the testis, MTX can disrupt the cell membrane integrity through affection of N-cadherin proteins. N-Cadherin is a class of membrane proteins that mediate cell adhesion. It possesses a transmembrane domain, two intracellular domains, five extracellular domains (ECDs) that bind Ca2+ ions and the neighboring cell's cadherin. Actin filaments are linked by β-catenin, which is linked by α-catenin, which is bound by intracellular domains[9].

In the adult testes, germ and Sertoli cells express N-cadherin. It is a crucial part of the blood testis barrier's cell adhesion complexes. Moreover, N-cadherin was demonstrated to be a part of the ectoplasmic specializations that allow the germ cells to adhere to the Sertoli cells as well as the adhesion of Sertoli cells to basal lamina[10].

Exosomes (EXOs) are endosomal membrane-derived nanovesicles; measuring about 20 to 200 nm in diameter;

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produced by the eukaryotic cells; released into different body fluids; carry bioactive materials like proteins, lipids, and genetic materials; and help in immune regulation and intercellular communication[11,12]. Mesenchymal stem cells (MSCs) derived exosomes have proved a therapeutic impact on many tissue injuries like liver fibrosis[13], spinal cord injuries[14], and degenerative eye diseases[15]. Also, they exhibited a cytoprotective effect on ischemia/ reperfusion injuries induced in rat testis mediated by their anti-inflammatory, antioxidant, and anti-apoptotic actions[16,17]. Moreover, exosomes ameliorated cisplatin-induced testicular injury[18]. Adipose tissue mesenchymal stem cells-derived exosomes (ADSCs-EXOs) have many bioactive molecules which can help in decreasing inflammation and enhancing the survival of injured tissues[19].

Chitosan is a polysaccharide derivative of chitin by its deacetylation[20]. Compared to the bulk form of chitosan, the nanoform has an increased surface-to-volume ratio which improves the loading capacity allowing it to act as a nanocarrier of many drugs especially anticancer drugs[21]. Also, they increase the drugs saturation solubility which increases their bioavailability[21]. They also decrease free radicals, increase antioxidant levels[22], have anti-inflammatory actions, and stimulate immune regulation[23].

Therefore, the current study purpose was to compare the possible protective effect of ADSCs-EXOs versus that of chitosan nanoparticles on testicular injury induced by MTX in adult albino rat.

MATERIALS AND METHODS

Drugs

Methotrexate (MTX)

It was purchased from Sigma Aldrich Company, in Cairo, Egypt in the form of yellowish powder. It was prepared weekly by dissolving about 32 mg of MTX powder in 20 ml phosphate buffered saline (PBS) and it was administered to rats as an intraperitoneal injection at a dose of 8 mg/kg/week[5].

Adipose tissue mesenchymal stem cells derived exosomes (ADSCs-EXOs)

The exosomes were extracted from the ADSCs and labelled with Paul Karl Horan (PKH26) (Cat, Number MIN126, Sigma Aldrich Company, Cairo, Egypt) to track homing of exosomes in the testis[24]. These steps were carried out at the Histology Department Tissue Culture Unit, Faculty of Medicine, Cairo University. Finally, exosomes were provided as suspension of 100µg exosomes/ 1ml PBS and injected through the intravenous route in the tail vein at a dose of (1 ml/rat/day)[25].

Chitosan nanoparticles (CH-NPs)

They were purchased from Nano-Tech, Emerald Dreamland, Giza, Egypt in powder form. The powder was prepared in 1% acetic acid and given orally (140 mg/kg/ day)[26]. The solution was prepared daily by dissolving 224 mg in 8 ml 1% acetic acid.

Isolation, culture and characterization of ADS Cs

A- Cell isolation[27]: Cells were separated by fragmenting adipose tissue followed by digesting tissue using a proteolytic enzyme collagenase type I followed by low-speed centrifugation.

B- Cell culture[28]: ADSCs were kept in (DMEM) supplemented with 10% fetal bovine serum and 1%penicillin/ streptomycin to approximately 80% confluency with three passages.

C- Characterization of ADSCs[27]: The inverted microscope was used for the morphological characterization of ADSCs in passage 3. Also, flow cytometry was used to detect mesenchymal stem cell surface markers (Figure 1). Stem cells (passage 3) were harvested from adipose tissue via digestion by trypsin/ Ethylenediamine tetra-acetic acid (EDTA). Subsequently, they were suspended and concentrated (2x105 cells) in 100µl PBS. The cells were cultivated in the dark with fluorescein isothiocyanate (FITC)- conjugated anti-rat monoclonal antibodies (CD59, CD73, CD44, CD14 and CD45) (Thermo-Fisher Scientific, USA) over 1h. Following centrifugation, the cells were suspended in 300µl PBS, fixed in formaldehyde 1% then analyzed by a FACS Calibur Flow cytometer (BD Biosciences). Cell Quest software was employed to analyze data. The Cells displayed positive reaction with CD 59, CD 73 and CD 44 and negative reaction with CD 45 and CD 14.

Exosomes isolation[28]: Isolation of exosomes was done via differential and ultracentrifugation methods. Cell culture supernatant was firstly centrifuged at 300xg for 10 minutes at 4oC and 5000 xg for ten mins (at 4oC) to eliminate the cell debris and dead cells then it was centrifuged at 10000xg for thirty mins (at 4oC) to eliminate larger particles. Finally, the supernatant was exposed to centrifugation at 100000xg for 70 mins (at 4oC) so exosomes were pelleted and the pellet obtained was dissolved by PBS.

Identification of exosomes

Examination of exosomes by Transmission Electron Microscope (TEM) (Figure 2).

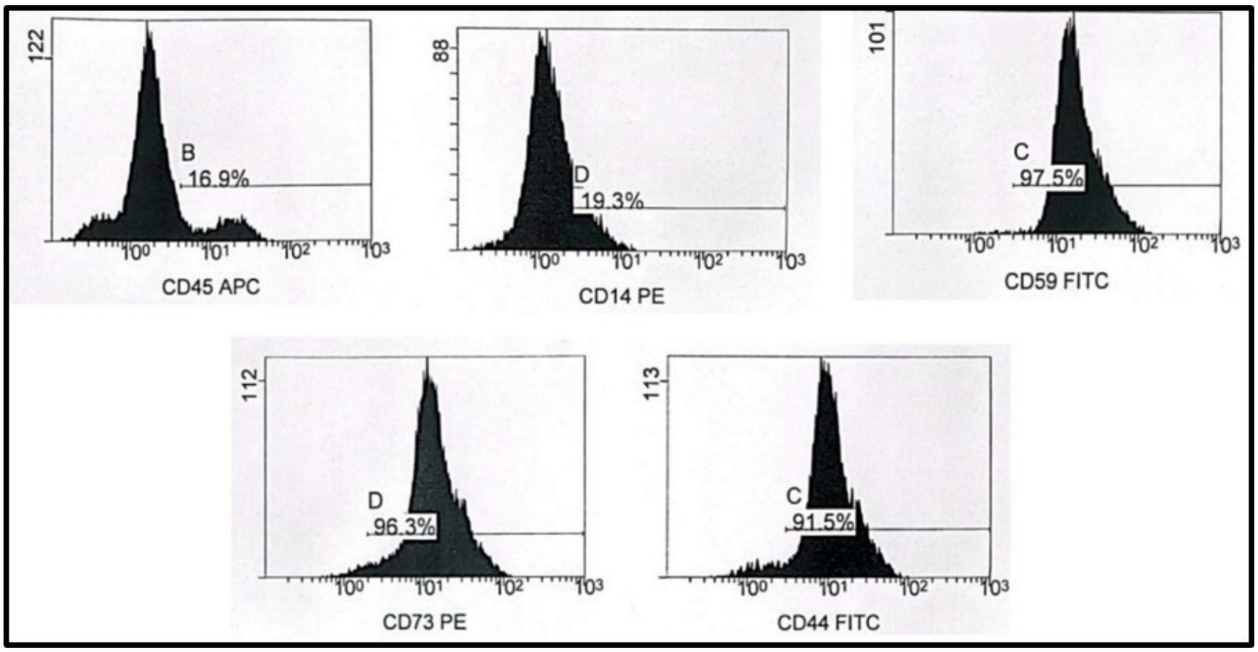

Fig. 1: Flow cytometry of stem cell surface markers showing +ve CD59, CD73, CD44.

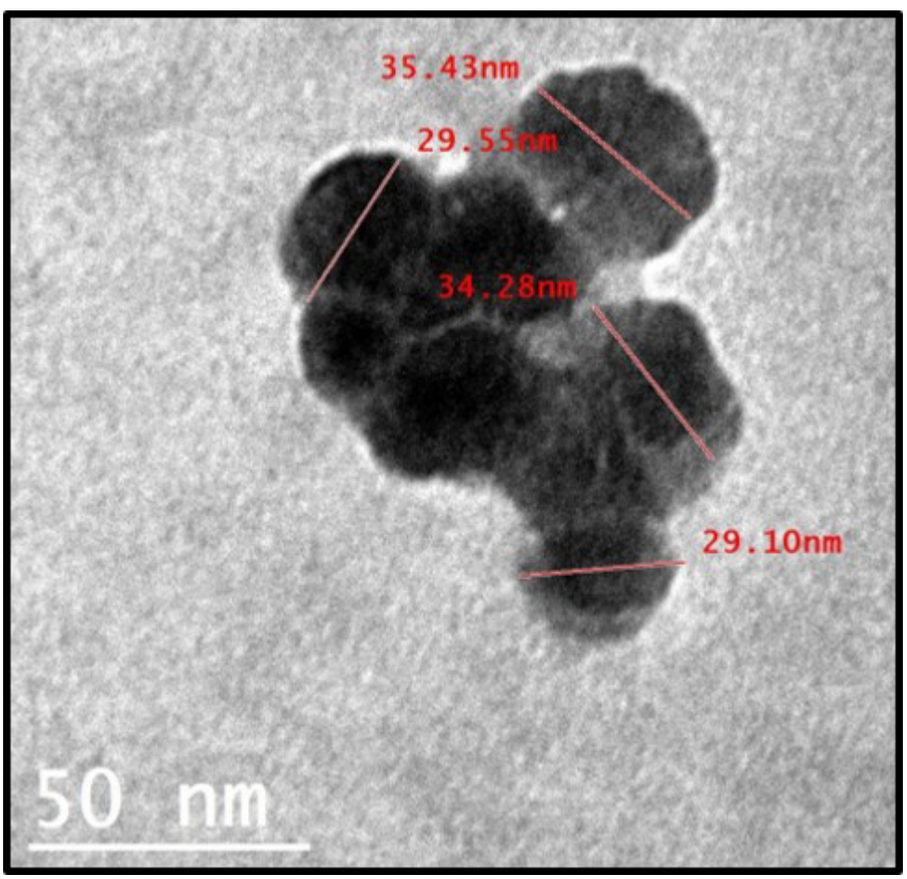

Fig. 2: Electron micrograph showing ADSCs - EXOS (X120000).

Two to five drops of exosomes were placed onto sheet of parafilm, directly on EM grids stained with osmic acid. This examination was done at the Electron Microscope Research Unit, National Research Center, Cairo University.

Exosomes have spherical morphology under TEM with a diameter of 20-200nm[11].

Labelling of exosomes[29]

For labelling of exosomes, they were dyed by PKH-26 red fluorescent cell linker kits. PKH-26 dye was diluted to a final concentration of 8μM in 100μL diluent C. (dye solution). The exosomes were then diluted with 80μL diluent C in 20μL Dulbecco's phosphate-buffered saline (DPBS), mixed with the dye solution & incubated for a period of 5 minutes with gentle pipetting. Following a 1mL DPBS dilution, the exosomes were pelleted by ultracentrifugation at 100000g for seventy mins (at 4°C). Resuspension of the pellet was done gently in 50μL DPBS.

Animals

Thirty-eight adult male albino rats, aged 12 weeks, and weighed 200 g were included in the current work. The animals were housed in the animal house at Kasr Al Ainy, Faculty of Medicine, Cairo University; in a hygienic environment within stainless-steel cages; and provided with standard food and water freely. Animals were maintained for 48 hours in these conditions before starting the study to enable adapting to the new environment. The experiment was done in parallel with the guidelines of Institutional Animal Care and Use Committee of Cairo University (CU – IACUC) [Number of approval: CU/III/F/12/23].

Experimental design

Donor group: two rats were used for ADMSCs isolation, culturing and phenotyping of exosomes.

Therefore, the remaining 36 animals were separated into 4 groups:

Group (I) (Control): included 18 animals, subdivided into three subgroups equally (6 rats in each subgroup):

- Subgroup Ia: corresponding to group II, rats received an intraperitoneal (IP) injection of 1 ml PBS once weekly for 4 weeks.
- Subgroup Ib: corresponding to group III, rats treated as subgroup Ia concomitantly with 1ml PBS once per day as intravenous (IV) injection in the tail vein for the first 7 experimental days only.
- Subgroup Ic: corresponding to group IV, rats treated as subgroup Ia concomitantly with 1 ml acetic acid 1% by oral gavage once daily for 4 weeks.

Experimental groups included three groups, 6 rats in each group:

Group II (MTX-treated rats): rats received IP MTX injection by 8mg/kg/week dissolved in PBS (1ml/rat/week) for four weeks[5].

Group III (ADSCs-EXOs): rats were treated as group II with IV injection of 1ml exosomes suspension (100μg/ml/rat) in the tail vein once every day during the first seven days of the experiment only[25].

Group IV (CH-NPs): rats were treated as group II with oral gavage of 140 mg/kg/day of chitosan nanoparticles dissolved in 1% acetic acid (1ml/rat/day) once daily for four days[26].

Biochemical studies

After four weeks, blood samples were taken through the tail veins of animals after24 hours of the last treatment using the butterfly needles for the analysis of free testosterone levels. Then, the animals were sacrificed by phenobarbital IP injection (120mg/kg)[30]. The testes of all rats were gently dissected free from the scrotum. Left testes were kept in micro-tubes and homogenized for biochemical analysis of tissue glutathione (GSH) levels and malondialdehyde (MDA) while the right testes were used for histological studies. The biochemical studies were done at Biochemistry department, Faculty of Medicine, Cairo University.

Histological studies

In all rats, the right testicles were cut and fixed into 10% formol saline for about 24 hours. Then, they were dehydrated through ascending alcohol concentrations (70%, 95%, and 100%) followed by clearing them in the xylene, finally, they were embedded into the paraffin wax. Serial sections were made with a thickness of 7μm which were subjected to the following:

A) Fluorescent microscopy study[5] by taking random non-stained sections from the ADSCs-EXO group and examining them with the immunofluorescent microscope for tracking of the exosomes in the testicular tissues.

B) Light microscopic study using the following stains:

- H&E stain[31].
- PAS stain[32].
- Immunohistochemical staining[32]:
    - Inducible nitric oxide synthase (iNOS) is an oxidative stress marker[1]. Anti-iNOS primary antibody is a rabbit polyclonal antibody, ready to use (Cat. No. AB5380, Sigma Aldrich chemical company, Cairo, Egypt).
    - N-cadherin is a marker of cell–cell adhesion protein complex (anchoring junction)[10].

It is present at the Sertoli cell-germ cell interface[33]. Anti-N-cadherin antibody is a rabbit polyclonal antibody, ready to use (Cat. No. SAB5700641, Sigma Aldrich chemical company, Cairo, Egypt).

Morphometric Studies: Using Lieca Qwin 500 C imaging analyzing system (Cambridge, England). From each rat 50 randomly chosen tubules were examined at

magnification x400 in the following measurement under examination of the light microscope:

1. Height of spermatogenic epithelium.
2. Diameter of seminiferous tubule.
3. The optical density of the PAS reaction in the basal lamina.
4. Area % of positive iNOS & N-cadherin immunoreactivity.

Statistical analysis

It was done for morphometric and biochemical findings using SPSS software and presented as mean ± standard deviation (SD). In order to compare between various groups, ANOVA test was employed, then a post hoc Tukey test was established. Statistical significance was determined by assessment of the p-value, and differences were deemed of statistical significance when the *p-value* was lower than 0.05[34].

RESULTS

General observations

Throughout the experiment, no mortality or morbidity was reported in all rats. Because the rats in the control subgroups (Ia, Ib, and Ic) produced similar outcomes, they were combined and presented as the control group.

Biochemical tests

The serum testosterone level and GSH level were significantly decreased in group II (MTX-treated rats) versus group I (control). Also, they were increased in group III (ADSCs-EXOs) and group IV (CH-NPs) versus group II (MTX-treated rats) without any significant differences versus each other or versus group I. On the other hand, the MDA tissue level was significantly increased in group II versus group I. Also, they were decreased in group III and group IV versus group II without any significant differences versus each other or versus group I (Table. 1).

Histological results

A) Immunofluorescent microscopic results

The testicular sections of group III (ADSCs-EXOs) demonstrated intense red immuno-fluorescence confirming the homing of PKH-26 fluorescent dye-labelled ADSCs-EXOs in the testicular tissue. Several PKH-26 labelled ADSCs-EXOs were found in interstitial tissue and seminiferous tubules (Figure 3).

B) Light microscope findings

H&E stain: In group I, the microscopic examination revealed normal testicular architecture. The seminiferous tubules contained spermatozoa and were surrounded by blood capillaries and Leydig interstitial cells-containing interstitial tissue. The spermatogenic epithelium rested on a regular basement membrane which was surrounded by flattened peritubular myoid cells. Sertoli cells exhibited pale oval nuclei and prominent nucleoli. Many spermatogonia were observed adjacent to the basement membrane, in addition to primary spermatocytes, early spermatids, and attached mature spermatids in the vicinity of the lumen (Figures 4 a,b).

Testicular sections of group II (MTX treated rats) showed that most seminiferous tubules were disorganized with the irregularly interrupted basement membrane, obviously damaged, disorganized spermatogenic epithelium with few separated layers of cells and disappearance of spermatozoa in the lumen. Some cells with dark pyknotic nuclei and others with fragmented nuclei were desquamated (detached) and shed off in the lumen. The lining epithelium illustrated Sertoli cells, some of which appeared to be normal and the others were degenerated and difficult to identify due to disturbed shape. Interstitial tissue showed marked edema forming wide interstitial tissue spaces and Leydig cells showed dark pyknotic nuclei, (Figures 4 c,d).

While the testicular sections of rats belonging to group III (ADSCs-EXOs) & group IV (CH-NPs) illustrated apparent protection of the testes. The seminiferous tubules had almost preserved normal architecture. They contained spermatozoa in their lumen and were surrounded by interstitial tissue containing Leydig's cell clusters. The spermatogenic epithelium rested on a regular basement membrane which was surrounded by flattened peritubular myoid cells. It displayed all spermatogenic epithelial layers with attached mature spermatids. Also, Sertoli cells appeared normal exhibiting pale oval nuclei with prominent nucleoli (Figures 5 a,b for group III, Figures 5 c,d for group IV).

PAS stain: In group I, group III, and group IV, the examined sections revealed mild positive PAS reaction in regular basement membranes of the seminiferous tubules (Figures 6 a,c,d respectively). In group II, the sections showed intense PAS reaction within irregular basement membranes of the seminiferous tubules (Figure 6b).

Immunohistochemical results

iNOS immunostained sections

Group I (control) testicular sections were examined, and the results showed negative immunoreaction in the cytoplasm of interstitial cells of Leydig and faint positive immunoreaction in very few solitary spermatogenic epithelial cells (Figure 7a). Testicular sections of rats of group II (MTX-treated rats) showed marked positive cytoplasmic and nuclear immunoreaction in most of the interstitial cells of Leydig, myoid cells in addition to some spermatogenic epithelial cells (Figure 7b). Sections of group III (ADSCs-EXOs) and group IV (CH-NPs) demonstrated mild to moderate positive cytoplasmic and nuclear immunoreaction in the interstitial cells of Leydig and faint immunoreaction in the spermatogenic epithelium (Figures 7 c,d).

N-cadherin immunostained sections

Examination of testicular sections in group I (control) showed strong positive membranous N-cadherin immunoreaction at Sertoli cell surface, in between Sertoli cells and spermatogonia, spermatocytes and spermatids. Also, positive immunoreaction was observed in the sites of hemidesmosomes at the Sertoli cell-basal lamina interface. Also, it revealed positive immunoreaction at the sites of the blood testicular barrier (Figure 8a). While in group II (MTX-treated rats), almost all seminiferous tubules showed negative N-cadherin immunoreaction in the Sertoli cells and spermatogenic cells in the severely disorganized spermatogenic epithelium (Figure 8b). Sections of group III (ADSCs- EXOs) and Group IV (CH-NPs) revealed strong positive membranous N-cadherin immunoreaction at the Sertoli cell surface, in between Sertoli cells and spermatogonia, spermatocytes and spermatids. Also, positive immunoreaction was observed in the sites of hemidesmosomes at the Sertoli cell-basal lamina interface (Figures 8 c,d respectively).

Morphometric and statistical results

The mean values of spermatogenic epithelial height, seminiferous tubules diameter, and area percentage of N-cadherin positive immunoreactivity were significantly decreased in group II (MTX-treated rats) versus group I while they were significantly increased in groups III (ADSCs-EXOs) and IV (CH-NPs) versus group II without any significant differences versus each other or versus group I.

On the other hand, the optical density of PAS +ve reaction in the basal lamina of seminiferous tubules and the area percentage of iNOS positive immunoreactivity were significantly increased in group II versus group I while they were significantly decreased in groups III (ADSCs-EXOs) and IV (CH-NPs) versus group II without any significant differences versus each other or versus group I (Table. 2).

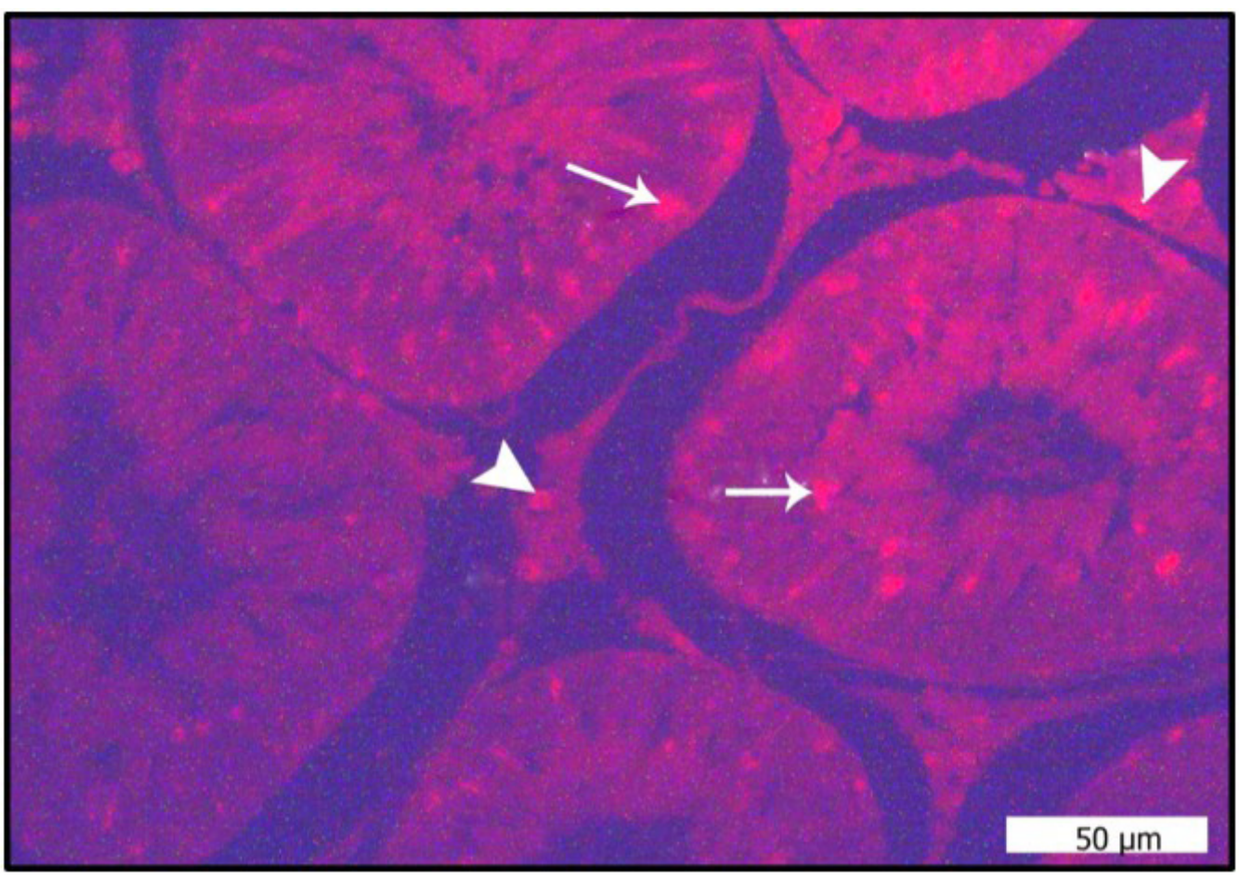

Fig. 3: Photomicrograph of a testicular section of a rat in group III (ADSCs-EXOs) showing intense red immunofluorescence of homed PKH-26 labelled ADSCs-EXOs in the seminiferous tubules (arrows) and the interstitial tissue (arrowheads) (PKH-26 fluorescent dye, x200).

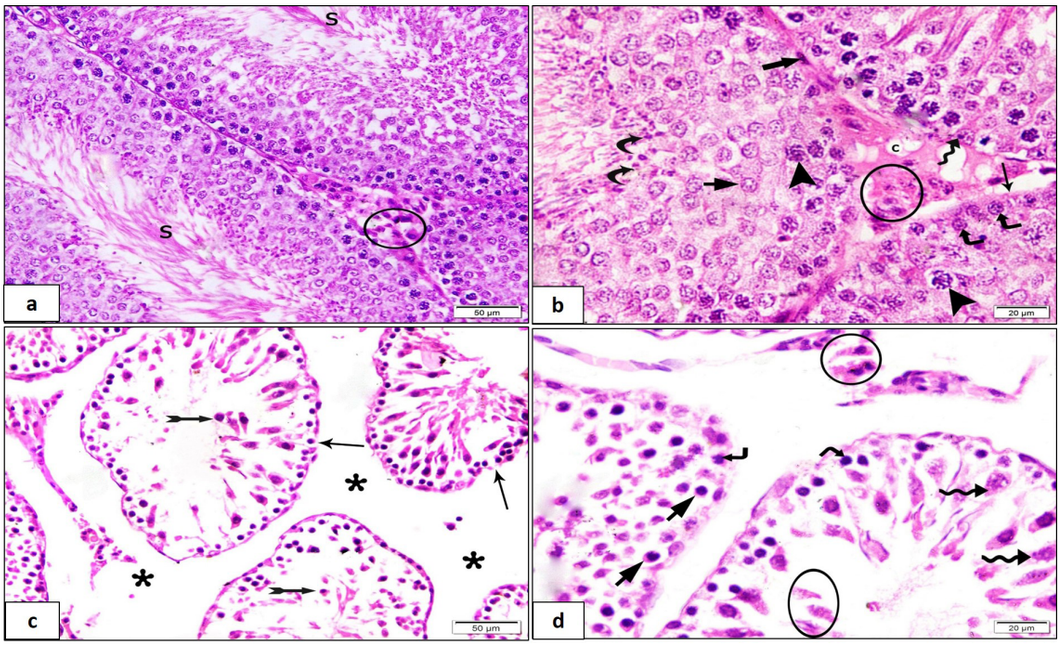

Fig. 4: Photomicrographs of H&E stained sections of group I (Control) (a,b) illustrating: a: normal structure of seminiferous tubules containing free spermatozoa (S) in the lumen and interstitial tissue containing clusters of Leydig's cells with vesicular nuclei (circle) (X200). b: Spermatogenic epithelium shows many compact layers of cells resting on a regular basement membrane (thin arrow), surrounded by myoid cells (thick arrow) and interstitial tissue containing Leydig's cells (circle) & capillaries (c). The spermatogenic epithelium includes numerous spermatogonia (angled arrows), primary spermatocytes (arrowheads), early spermatids (short arrow), attached mature spermatids (curved arrows) & Sertoli cells with vesicular nuclei and prominent nucleoli (spiral arrow) (X400). Group II (MTX-treated rats) (c,d) showing: c: disorganized seminiferous tubules with irregular interrupted basement membrane (thin arrows), obvious desquamation of the spermatogenic epithelium in the lumen (bifid arrows) with the disappearance of spermatozoa in the lumen. Note wide interstitial tissue spaces (stars) (X200). d: spermatogenic epithelium shows few separated layers of cells including spermatogonia (angled arrows) & Sertoli cells (spiral arrows). Most spermatogenic cells have pyknotic nuclei (short arrows). Some cells have fragmented nuclei (oval). Leydig's cells with dark pyknotic nuclei (circle) are also seen (X400).

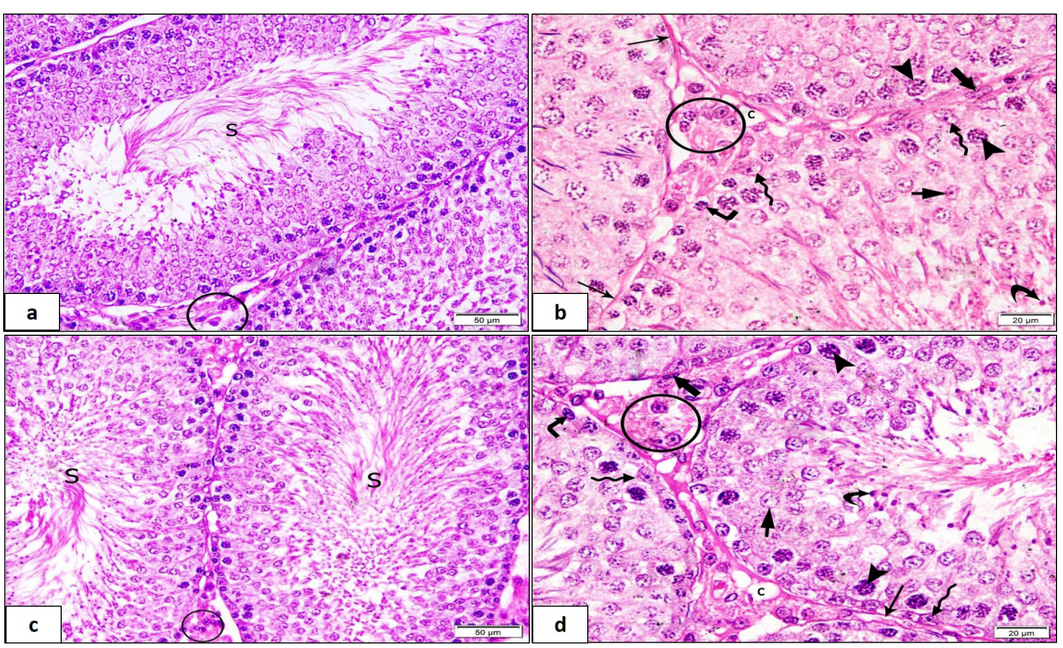

Fig. 5: Photomicrographs of H&E sections of group III (ADSCs-EXOs) (a,b) and group IV (CH-NPs) (c,d) demonstrating: a&c: preserved normal architecture of seminiferous tubules containing spermatozoa (S) in their lumen and surrounded by interstitial tissue containing clusters of Leydig's cells (circle) (X200). b&d: spermatogenic epithelium shows many compact layers of cells resting on regular basement membrane (thin arrows), surrounded by myoid cells (thick arrow) & interstitial tissue containing Leydig's cells with vesicular nuclei (circle) & capillaries (c). Spermatogenic epithelium includes numerous spermatogonia (angled arrow), primary spermatocytes (arrowheads), early spermatids (short arrow), attached mature spermatids (curved arrow) & Sertoli cells (spiral arrows) (X400).

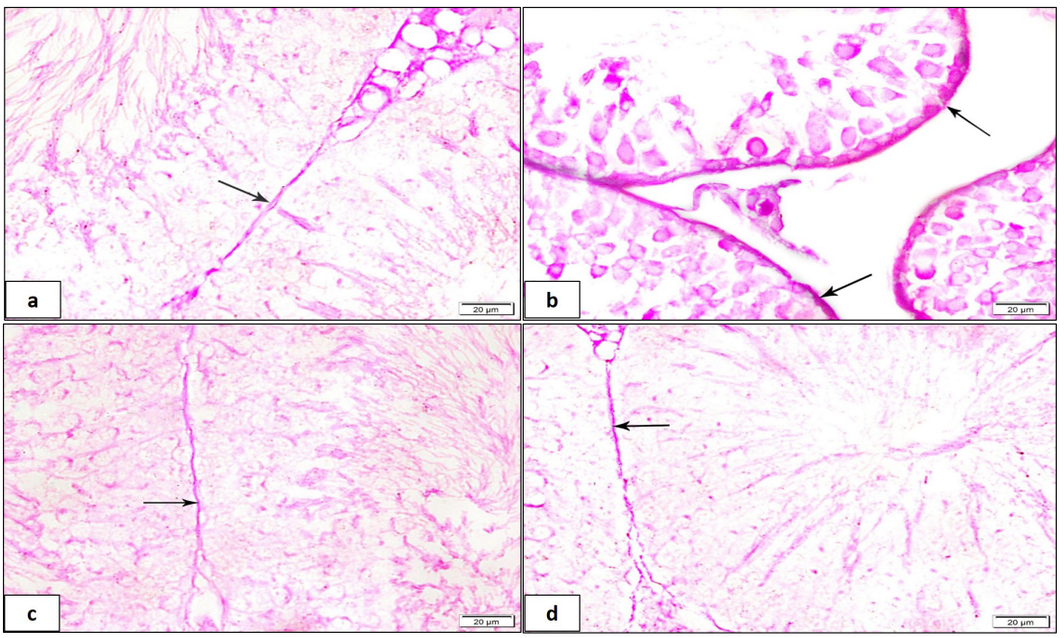

Fig. 6: Photomicrographs of PAS stained sections of all studied groups (X400): a, c and d: groups I, III and IV respectively showing mild PAS +ve reaction in the regular basement membranes of seminiferous tubules (arrows). b: group II demonstrating intense PAS-positive reaction in the irregular basement membranes of seminiferous tubules (arrows).

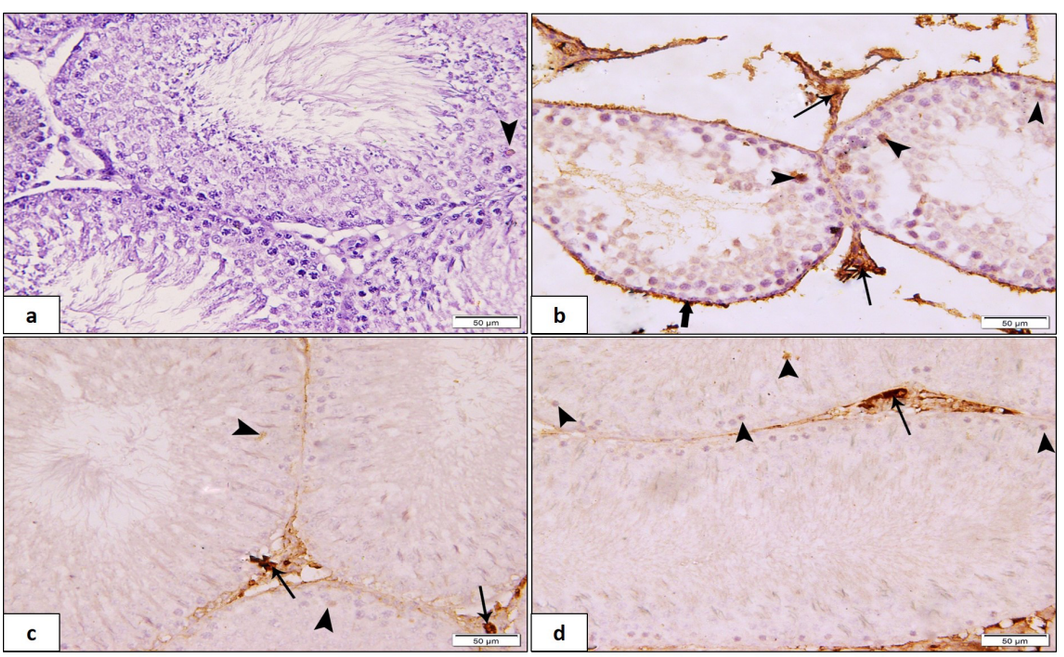

Fig. 7: Photomicrographs of iNOS immunostained sections of all studied groups (X200): a: group I showing negative immunoreaction in the cytoplasm of interstitial cell of Leydig's and faint positive immunoreaction in solitary spermatogenic epithelial cells (arrowhead). b: group II revealing marked cytoplasmic and nuclear immunoreaction in the interstitial cells of Leydig (arrows), myoid cells (thick arrow) as well as in some of the spermatogenic epithelial cells (arrowheads). c: group III & d: group IV illustrating mild to moderate cytoplasmic and nuclear immunoreaction in the interstitial cells of Leydig (arrows) and faint immunoreaction in the spermatogenic epithelium (arrowheads).

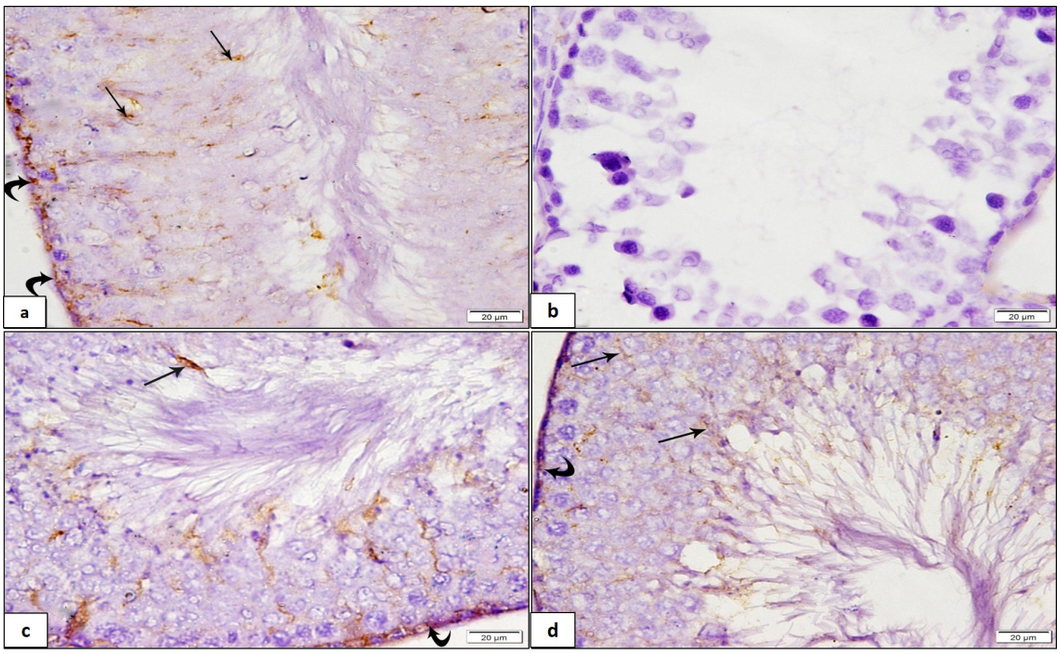

Fig. 8: Photomicrographs of N-cadherin immunostained sections of all studied groups (X400): a: group I demonstrating strong positive membranous N-cadherin immunoreaction at the surface of Sertoli cells (arrows), and positive membranous immunoreaction in the sites of hemidesmosomes (curved arrows) and blood-testis barrier (arrowheads). b: group II showing negative N-cadherin immunoreaction in Sertoli cells and spermatogenic cells of the severely disorganized spermatogenic epithelium. c, d: group III and IV respectively displaying strong positive membranous N-cadherin immunoreaction at the surface of Sertoli cells (arrows) and positive membranous immunoreaction in the sites of hemidesmosomes (curved arrows).

Table 1: The mean values (±SD) of serum free testosterone level (ng/ml), tissue MDA level (µmol/L) and tissue GSH level (nmol/ml) in the control and experimental groups.

Groups	Mean values (±SD) of serum free testosterone (ng/ml)	Mean values (±SD) of testicular tissue MDA (µmol/L)	Mean values (±SD) of testicular tissue GSH (nmol/ml)
Group I (Control)	1.09 ± 0.26	42.51 ± 17.88	92.10 ± 2.42
Group II (MTX-treated rats)	0.25 ± 0.16 *	123.28 ± 10.86 *	28.08 ± 2.61 *
Group III (ADSCs-EXOs)	0.91 ± 0.35 #	54.95 ± 21.81 #	89.86 ± 2.53 #
Group IV (CH-NPs)	0.81 ± 0.27 #	59.24 ± 23.30 #	89.36 ± 2.13 #

* Significant ($P < 0.05$) versus group I, III, IV. # Significant versus group II.

Table 2: The mean values (± SD) of spermatogenic epithelial height (µm), diameter of seminiferous tubules (µm), optical density of PAS reaction, area percent of iNOS positive immunoreactivity and area % of N-cadherin +ve immunoreactivity in the control and experimental groups.

Groups	Mean (±SD) of height of spermatogenic epithelium (µm)	Mean (±SD) of diameter of seminiferous tubules (µm)	Mean (±SD) of optical density of PAS reaction	Mean (±SD) of area % of iNOS positive immunoreactivity	Mean (±SD) of area % of N-cadherin +ve immunoreactivity
Group 1 Control)	115.16 ± 29.33	276.82 ± 40.87	0.18 ± 0,007	1.86 ± 0.39	54.4 ± 12.14
Group II (MTX-treated rats)	19.82 ± 5.8 *	75.16 ± 26.78 *	0.27 ± 0.03 *	55.22 ± 5.36 *	5.8 ± 2.9 *
Group III (ADSCs-EXOs)	101.78 ± 25.49 #	268.3 ± 42.69 #	0.19 ± 0.01 #	4.48 ± 2.15#	50.2 ± 12.5 #
Group IV (CH-NPs)	94.62 ± 27.2#	267.96 ± 48.72#	0.2 ± .009 #	4.9 ± 2.36 #	43.77 ± 11.07#

* Significant ($P < 0.05$) versus group I, III, IV. # Significant versus group II.

DISCUSSION

Methotrexate (MTX) is a widely used and efficacious anti-cancer medication[35]; however, it is associated with profound cytotoxicity that can affect healthy tissues especially the testis which can lead to male infertility making it a critical subject for research to find protective agents to prevent the induced MTX cytotoxicity[36,37].

In this work, adult albino rats were chosen as from a histological perspective, their testicles resemble those of humans quite a little[38]. Methotrexate was reported to induce testicular tissue damage either by a single injection (20 mg/kg)[39] or by weekly administration of 8mg/kg for four weeks[5]. We chose the weekly injection method based on a previous study that concluded that more oxidative stress damage was produced by the prolonged MTX treatment than by the single dose[40].

In the present study, H&E sections of group II (MTX-treated rats) revealed marked histological injury with a significant decrease in the height of spermatogenic epithelium, diameter of seminiferous tubules and serum free testosterone level which could be explained by the oxidative stress induced by MTX.

In harmony with our findings, similar findings were reported by other studies that explained such injurious effects of MTX by the increased lipid peroxidation in the testicular tissues[41,42]. In addition, another study found that MTX was associated with increased degeneration of spermatogenic epithelial cells with decreased height and suppression of spermatogenesis and the latter explained these findings by DNA damage of the immature germinal cells with their sloughing into the tubule lumen[43].

This could support our findings of nuclear pyknosis in spermatogenic cells and Leydig cells in group II indicating apoptosis; it is explained by elevated pro-inflammatory mediators, such as IL-1β and IL-6, which started the apoptotic cascade[44,45].

In the current study, the decreased serum free testosterone level in group II could be explained by oxidative stress-induced injury of Leydig cells with a subsequent drop in testosterone secretion. In accordance with our results, another study found similar outcomes and they explained them by oxidative stress inducing either injury of the Leydig cells directly or affecting the anterior pituitary such that Leydig cells are not stimulated to generate adequate testosterone by luteinizing hormones[27].

Moreover, the observed decrease in the diameter of seminiferous tubules might be explained by edema in the interstitial tissues induced by oxidative stress hence compressing the seminiferous tubules. Previous authors reported similar findings and an explanation, adding that MTX activates Nuclear Factor Kappa B (NF-κB), allowing it to translocate from cytoplasm to the nucleus and activate genes that produce proinflammatory mediators like IL-6 and IL-1β, which cause inflammation and edema[46].

An attractive finding of MTX treated rats in this study was the appearance of some normal Sertoli cells adjacent to the basement membrane. This finding was hand in hand with another study which recorded partial affection of Sertoli cells associated with severe damage of spermatocytes[39]. Such findings emphasized the fact that the Sertoli cells are more resistant to MTX. Also, the researchers attributed resistance of Sertoli cells to oxidative stress to their increased expression of BCL2 antiapoptotic family[47].

MTX treated rats in the current study showed intense PAS +ve reaction in irregular basement membranes of the seminiferous tubules. The same finding was reported by previous researchers who induced oxidative stress testicular damage by monosodium glutamate. Additionally, they suggested that a decreased consumption of glycogen by the few sperm that are available or the suppression of glycogen phosphorylase activity were the causes of the elevated PAS reaction associated with oxidative stress[48].

The increased oxidative stress in the same group was confirmed by elevated tissue MDA, and lowered tissue GSH levels relative to the control group. This was consistent with a previous research in which testicular damage was induced by cisplatin and the researchers explained that superoxide radicals result in excessive consumption and reduction of antioxidant-affecting thiols such as GSH[49].

Additional confirmation of increased oxidative stress in group II was obtained from marked iNOS cytoplasmic as well as nuclear immunoreactivity because NF-κB was transferred from the cytoplasm to the nucleus in the majority of Leydig interstitial cells, myoid cells in addition to some spermatogenic epithelial cells that were also confirmed morphometrically by the increased area percent of iNOS positive immunoreactivity.

In the current study, MTX treated rats showed negative N-cadherin immunoreaction in Sertoli cells and spermatogenic cells damaged by oxidative stress. This finding was supported by previous studies which clarified that oxidative stress causes a direct decrease in N-cadherin expression, which breaks down the blood-testis barrier[10,50,51].

The present work investigated the protective role of ADSCs-EXOs (Group III) and chitosan nanoparticles (Group IV) on testicular damage induced by MTX. Mostly, both treatments showed similar apparent protective effects on the testicular tissue. H&E sections of both groups showed preservation of the seminiferous tubules' normal architecture. That was supported morphometrically by significantly increased spermatogenic epithelium height and seminiferous tubule diameter versus MTX treated rats. Also, these findings were supported biochemically by significantly increased serum free testosterone level and tissue GSH level and significantly decreased tissue MDA level. Both groups also showed increased N-cadherin expression and decreased iNOS expression denoting marked success of both protective therapies. All of these

findings were related to the antioxidant effect of both exosomes and chitosan nanoparticles.

Exosomes obtained from adipose tissue mesenchymal stem cells were used in this research since it has been documented that they have therapeutic properties and regulate receptor cells via intercellular communication[52]. Additionally, it has been demonstrated that they are functioning similarly to stem cells[53]. However, the clinical use of exosomes could avoid the main drawbacks of stem cells clinical use including tumorigenesis, rejection and the difficulties associated with stem cell preparation and storage[28].

In the current study, a dose of 100µg/ml/rat of exosomes was used according to previous research in which the authors compared the protective effect of different doses of exosomes (50µg/ml,100µg/ml,200µg/ml) against cyclophosphamide-induced testicular damage and found that the protective effect of 100µg/ml and 200µg/ml of exosomes was superior to that of 50µg/ml[25].

Exosomes regulate the intercellular communication via regulating the recipient cell's uptake of lipids, mRNA, proteins, and miRNA thus they could ameliorate the MTX reproductive toxicity by regulating the transmission of these molecules to the germ cells especially miRNA which increases the cellular proliferation and angiogenesis besides enhancing the immune response[54].

Additionally, they upregulate the phosphatidylinositol 3 kinase/protein kinase B (PI3K/AKT) signaling pathway mediating multiple factors like kinases, transcription factors, and phosphatases besides promoting the polarization of macrophages to M2 phenotype. This in turn reduces the inflammation which explains our findings regarding reduced signs of inflammation in the interstitial tissue and the absence of edema with preserved normal Leydig's cells[55,56]. Moreover, exosomes may contribute to Leydig cell survival by passing through the blood-testis barrier which going in parallel with our findings[57].

Hand in hand with our results, researchers in a previous work observed that exosomes induced marked improvement of the testicular histology with restoration of spermatogenesis after administration of ADSCs-EXOs in a model of testicular torsion[58]. Further, another study added that exosomes can restore the disturbance in the oxidation and antioxidation balance through direct delivery of antioxidative enzymes mRNA and proteins. This means that ADSCs-EXOs not only prevent inflammation and encourage the migration and proliferation of spermatogenic cells, but they also prevent apoptosis[59].

Chitosan is a natural polysaccharide that has proven its protective effect on many organs through its antioxidant activity[60]. In the present study, chitosan was used in the form of nanoparticles to enhance its specificity, bioavailability, and solubility and decrease the pharmacological toxicity[22,61]. We used a low dose of CH-NPs (140 mg/kg/day) based on a previous study that compared the protective effect of a low dose (140 mg/kg/day) and high dose (280 mg/kg/day) on testicular damage and stress induced by D galactose and they found no significant difference between both doses as a protective therapy for oxidative testicular damage[62].

In this study, chitosan nanoparticles showed nearly normal testicular architecture going in line with the results obtained by other researchers[63]. Also, similar results were obtained by another study that explained the role of chitosan in activating protein kinases and cytokines that are responsible for alleviating oxidative stress[64]. In the same concern, another study added that the OH and amino acid groups in the chitosan nanoparticles react with unstable free radicals to produce a stable macromolecule hence explaining the scavenging activity of CH-NPs and suppression of the cellular damage[65].

Both ADSCs-EXOs and CH-NPs treated groups in this study revealed decreased positive iNOS immunoreaction. The same finding was reached by other studies which attributed their results to the ability of exosomes and CH-NPs to suppress oxidative stress and inflammation[66,67].

Also, both groups III and IV displayed strong positive membranous N-cadherin immunoreaction in the Sertoli cell surface and spermatogenic cells at sites of anchoring junctions that were significantly increased versus MTX treated rats. In agreement with our results, a previous study used exosomes in the treatment of skin wounds and found that they increased the gene expression of N-cadherin[68]. Similarily, chitosan improved the paracrine ability of testicular cells to generate enough cytokines to sustain the blood-testicular barrier and restored the disruption of cell adhesion gene expression profiles during oxidative stress[10].

An obvious observation in the current study was that the statistical analysis of all measured parameters of both ADSCs-EXOs and CH-NPs groups showed nonsignificant difference versus each other denoting similar protective efficacy.

CONCLUSION AND RECOMMENDATIONS

Both ADSCs-EXOs and chitosan nanoparticles have potent cytoprotective effects against MTX-induced testicular injury in adult male albino rats and their protective effects were almost similar to each other. They protected the testis from MTX-induced damage through their potent antioxidant effect. They also up-regulated N-cadherin protein expression, so preserving the blood-testis barrier's integrity.

Clinical studies are recommended to be directed to explore whether ADSCs-EXOs and chitosan nanoparticles have the same protective effect in patients under MTX treatment. Further studies are needed to clarify if there are any complications from the use of ADSCs-EXOs and chitosan nanoparticles. Also, further research is required to evaluate the efficacy of the used therapies in the current study if applied after MTX intake as a treatment, not as a protective measure for testicular injury.

CONFLICT OF INTERESTS

There are no conflicts of interest.

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الملخص العربى

تقييم هستولوجى للتأثير الوقائي للإكسوزومات المستخلصة من الخلايا الجذعية فى مقابل جسيمات كيتوزان النانوية على إصابة الخصية المحدثة بعقارميثوتريكسات في نموذج الجرذ الأبيض

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الخلفية والهدف: يستخدم عقار ميثوتريكسات المضاد للسرطان فى علاج السرطانات والامراض المناعية وقد اثبت تأثيره السام على خلايا الخصية بالدراسات الأكلينيكية والتجريبية. ولذلك صممت هذه الدراسه لمقارنة التأثير الوقائي المحتمل للإكسوزومات المستخلصة من الخلايا الجذعية الدهنية فى مقابل جسيمات كيتوزان النانوية على إصابة الخصية المحدثة بمادة ميثوتريكسات في نموذج الجرذ الأبيض البالغ.

المواد وطرق البحث: وقد اشتملت هذه الدراسة على ٣٨ ذكرا بالغا من الجرذان البيضاء وتم تقسيمهم الى اربع مجموعات بالأضافة الى المجموعة المانحة.

وقد شملت هذه المجموعات المجموعة الضابطة (١) وثلاث مجموعات تجريبية: مجموعة ٢ (الحيوانات المعاملة بمادة ميثوتريكسات): تم حقن الجرذان بمادة ميثوتريكسات في الغشاء البريتوني بجرعة ٨ مللي /كجم /أسبوع لمدة ٤ أسابيع. مجموعة ٣ (الأكسوزومات المستخلصة من الجذعية الدهنية): تم معالجه الجرذان مثل المجموعة الثانية بالتزامن مع حقنها ب ١ مللي من معلق الأكسوزومات في الوريد الذيلي (١٠٠ ميكروجرام /ملل (مره واحدة يوميا خلال السبع أيام الأولى من التجربة فقط. مجموعة ٤ (جسيمات كيتوزان النانوية): تم معالجة الجرذان مثل المجموعة الثانية بالتزامن مع تلقيها جسيمات كيتوزان النانوية بجرعة (١٤٠ مجم/كجم/ يوم) عن طريق الفم مرة واحدة يوميا لمده ٤ أسابيع.

وقد تم قياس مستوى هرمون التيستوستيرون الحر في المصل ومستوى المالون داي الدهايد في النسيج ومستوى الجلوتاثايون في النسيج. وقد تم صبغ قطاعات الخصية بصبغة الهيماتوكسيلين والإيوسين وصبغة شيف للحمض البيريودي وصبغة هستوكيميائية مناعيه للكشف عن الإنزيم المحفز لإنتاج أكسيد النيتروجين وعن روابط ال ن – كادهيرين. وقد تم مقارنة القياسات المورفومترية بين المجموعات قيد الدراسة.

النتائج: أظهرت المجموعة الثانية عدم انتظام في شكل الأنيبيبات المنوية وانحلال واضح في النسيج الطلائي المنوي مع اختفاء الحيوانات المنوية. وكان ذلك مصحوبا بانخفاضا ذو دلالة إحصائية فى مستوى هرمون التيستوستيرون الحر في المصل ومستوى الجلوتاثايون في النسيج وقيمة ارتفاع النسيج الطلائي المنوي وقطر الأنيبيبات المنوية والتفاعل المناعي الإيجابي لروابط ال ن- كادهيرين. بالأضافة الى ارتفاعا ذو دلالة احصائية في مستوى المالون داي الدهايد في النسيج والتفاعل المناعي الايجابي للانزيم المحفز لإنتاج أكسيد النيتروجين. بينما المجموعتان الثالثة والرابعة فقد أظهرتا احتفاظا بالنسيج الهستولوجي الطبيعى والنتائج البيوكيميائية والقياسات المورفومترية الطبيعية. كما أنه لم يكن هناك فرقا ذو دلالة احصائية بين كلا المجموعتين.

الخلاصة: اثبتت كلا من الأكسوزومات المستخلصة من الخلايا الجذعية الدهنية وجسيمات كيتوزان النانوية تأثيرها الوقائي الفعال على الخلايا ضد إصابة الخصية المحدثة بالميثوتريكسات، كما كان تأثيرهما الوقائي تقريبا متشابها.