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Ultrastructural and histological study of testicular torsion-detorsion on rat and protective effects of fibroblast and macrophage conditioned medium

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Abstract

Testicular torsion-detorsion (TD) or ischemia/reperfusion causes reactive oxygen species over-production and has extensive destructive effects on testicular tissue. Following TD, this study evaluated the therapeutic effects of conditioned medium (CM) of fibroblast, macrophage and co-culture on testicular histomorphometric and ultrastructure. Adult rats were divided into 7 groups. Healthy control, Control sham, TD, and Dulbecco's Modified Eagle Medium groups. Experimental groups: Fibroblast, macrophage and co-culture. The supernatant was obtained from the separate cultures of fibroblasts and macrophages and co-culture were injected. All injections were made through rete testis. Thirty-five days after the operation, the testis was sampled for histomorphometric and ultrastructural studies. However, the ultrastructural study of testicular tissue also showed that extensive changes occurred in Sertoli cells' nucleus, nucleolus and mitochondria. This study showed that in fibroblast and slightly less in macrophage groups, there were good improvements in all histomorphometric and ultrastructure parameters similar to the healthy control group. It was also shown that the Dulbecco's Modified Eagle Medium group had slightly better recovery conditions than the TD group, but the co-culture group showed similar conditions to the TD. Overall, it could be concluded that the CM of fibroblast was very effective. In the groups receiving fibroblastic and macrophage CM, all parameters exhibited favorable improvement but in the fibroblast group, most of the parameters were similar to those in the healthy controls group. It was also shown that the CM of fibroblast-macrophage co-culture could not be improved on TD condition but made it worsened.

Introduction

Infertility is a health problem, that its prevalence is increasing and effects on 10.00 - 15.00% of couples in the world.[1] Testicular pathology is resulted of direct testicular injury following varicocele, cryptorchidism, testicular torsion, mumps orchitis, gonadotoxic effects of drugs, genetic disorders and idiopathic.[2] The annual incidence of spermatic cord torsion is 4.50 *per* 100,000 men aged 1 - 25 years. Reperfusion is essential for the survival of testicular cells, spermatic cord detorsion is associated with biochemical and histological changes following a phenomenon called ischemia-reperfusion. Both ischemia and reperfusion cause overproduction of reactive oxygen species, pro inflammatory cytokines including tumor necrosis factor (TNF)-α and interleukin (IL)-1β, chemokines, cell adhesion molecules and resulting chemotactic movement of neutrophils and other leukocytes into the testicular tissue. Oxygen species are caused oxidative stress, through oxidation of mitochondrial membranes, cell membrane lipids, proteins and DNA, which lead to cell dysfunction and apoptosis of germ cells in the testes. Testicular reperfusion after ischemia affects spermatogenesis in both the ipsilateral and contralateral tests.[3] The number of testicular interstitial macrophages fluctuates with age in the tests. These macrophages produce IL-1 and TNF-α. The IL-1β recruits more cells to enter the cell cycle and synthesize DNA. The IL-1α and TNFα also significantly stimulate DNA synthesis in Leydig cells. Thus, IL-1α may play an important role in Leydig cell proliferation during prepubescent development in immature rats.[4] Clinical and experimental data have shown that testicular torsion causes a range of injuries depending on the duration and degree of torsion.[5]

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Ischemia and reperfusion at the site of injury leads to overproduction of reactive oxygen species and DNA chain breakdown.[5,6] Oxygen free radicals oxidize membrane lipids, proteins and DNA, and leading to cellular dysfunction and sometimes cell death.[7]

Fibroblast growth factor (FGF) were first discovered in the pituitary extract in 1973 and are widely expressed in cells and tissues. Acidic FGF (FGF1) and basic FGF (FGF2) were essentially isolated from the brain and pituitary gland as FGFs for fibroblasts. Then, at least 22 FGFs were identified and isolated. The FGFs regulate a wide range of biological functions including proliferation, survival, migration and cell differentiation.[8] Macrophages are mononuclear phagocytic cells that spread widely throughout the body. These cells help development and homeostasis and contribute to innate immune responses and adaptation.[9]

Clear populations of testicular macrophages have been identified, one of them which lies on the surface of the seminiferous tube. These macrophages express proliferation and induction-differentiation factors such as colony stimulating factor 1 and enzymes involved in retinoic acid biogenesis. Temporal depletion of these macrophages results in the termination of spermatogenesis.[10] Proteins secreted by a particular type of cells are called secretomes, that they play an important role in regulating of many physiological processes through paracrine/autocrine mechanisms. They appear to be encoded by 10.00% of the human genome.[11] Secretory factors of secretomes, microsomes or exosomes can be found in medium culture where the stem cells are cultured, so called conditioned medium (CM). Secretory factors alone without stem cells may repair various conditions in tissue/organ damage,[12] and contain various growth factors and tissue repair agents that are secreted by stem cells.[13,14]

The use of CM containing secretome has many advantages over the stem cells, because CM can be produced, freeze-dried, packaged and transport easily. In addition, it is free- cell, therefore, there is no need for matching between donor and recipient to avoid transplant rejection problems.[15] This study was conducted to evaluate CM included various FGFs and its regenerative effects on histomorphometry and ultrastructure of rat testis following torsion-detorsion.

Materials and Methods

Animals and experimental groups. In this study, 42 male Wistar rats were used with almost weighing 180 - 220 g. The rats were kept at a temperature of about 22.00 ˚C and a light cycle of 14 hr and dark 10 hr. Rats were kept in a clean cage and had free access to water and feed.

Veterinary Ethics Committee of Faculty of Veterinary Medicine, Urmia University, Urmia, Iran (Reference No. IR-UU-AEC 3/841/DA), and the Urmia University Research Council approved all of the experiments. 1- Healthy control group: In this group, the rats were not operated, 2- Sham control: Rats underwent laparotomy without torsion-detorsion. 3- Torsion-detorsion (TD) group: Rats underwent torsion-detorsion operation. 4- Dulbecco's Modified Eagle Medium (DMEM) group: After TD operation, 10.00 µL DMEM (Sigma-Aldrich, St. Louis, USA) was injected into the rete testis. 5, 6, and 7- Experimental groups received fibroblast, macrophage and co-culture groups, respectively: After TD operation, 10.00 µL CM from fibroblast macrophage and co-culture was injected into the rete testis.

Torsion-detorsion induction. Rats were anesthetized by intraperitoneal injection of 40.00 mg kg^{-1} ketamine (Rotexmedica, Trittau, Germany) and 5.00 mg kg^{-1} xylazine (Alfasan, Woerden, Netherlands) and after aseptic preparation of the surgical site, surgical excision was performed on the skin of the posterior middle and subsequently the white line of the abdominal wall. Spermatic cord and left testicular torsion were performed to produce testicular and ischemic curvature, and the spermatic cord was twisted counterclockwise for 1 hr with a 720-degree torsion. Thus, ischemia occurred when the bloodstream was interrupted. Reperfusion was performed after 1 hr by detorsion it and restore blood circulation. After surgery, 10.00 µL supernatant from cell culture (CM) were injected into the rete testis in the recipient groups after induction of reperfusion.

Tissue sampling for obtaining fibroblast culturing CM. First, the animal was anesthetized by intraperitoneal injection of ketamine and xylazine. After anesthesia, the animals was completely fixed on the operating table and the skin of the groin area was shaved on both sides and abdomen and the area scrubbed with iodine alcohol, Then the skin was removed under sterile conditions by fine scissors and forceps and placed in a dish containing DMEM, streptomycin/ penicillin and amphotericin B. The skin fragments were washed in three steps, then to remove the dermis from the epidermis, skin fragment was inserted into the culture medium containing 0.25% trypsin (Gibco, Grand Island, USA), and incubated for 3 - 4 min. After this time, the contents of the tube were discharged into the sterile petri dish and all sterile steps were performed under the laminar hood. Using a fine forceps and scissors, the connective tissue of the dermis was separated from the epidermis 1.00 mm in size and implanted into six-well plate. Few pieces of connective tissue were implanted within each well. A drop of fetal bovine serum (Gibco) was added for attaching of fragments to the bottom of the well and the plates were incubated at 37.00 ˚C and 5.00% CO_2 for 1 - 2 hr. After this time, DMEM 20.00% culture medium (20.00 mL of fetal bovine serum and 80.00 mL with antibiotics as above) was added to the above samples on plate and incubated for

4 - 5 days. After this time the fibroblast cells began to migrate out of the connective tissue. After the cells completely filled the plate with an 80.00% population, were passaged three times until the cells reached 80.00% confluency. The supernatant was collected and discarded, and the cells were washed twice with phosphate buffered saline and incubated for 24 hr in DMEM without fetal bovine serum. After this time, the supernatant was collected and centrifuged at 500and 3,000 *g* was then reduced to one-fourth by the Freeze dryer (Farayand Tajhiz Sahand, Tabriz, Iran) and sterilized and kept at – 20.00 ˚C until use.

Preparation of macrophages and cell culture for CM. After euthanizing the rats by overdose of ketamine (80.00 mg kg^{-1}) and xylazine (10.00 mg kg^{-1}) intra-peritoneally, macrophages were obtained by peritoneal lavage with 10.00 mL cold phosphate buffered saline. The cells from the lavage were centrifuged at 400 *g*, coated into flask and incubated. After 1 night of incubation, the cells were detached from bed by frozen and cold medium and counted and were re-cultured at a suitable density of at least 500,000 cells. Cell culture medium was removed for the preparation of supernatant after 4 days of culture and cultured for 24 hr in serum-free culture medium. After this period, the supernatant was removed and centrifuged at 400 *g* and then reduced to one-fourth volume by Freeze dryer, filtered sterile and kept at – 20.00 ˚C until use.

Macrophage-fibroblasts co-culture. Macrophage-fibroblasts co-cultured in 24-well plates at a density of 2.50×10^4 each of macrophage and fibroblast cells. Cell culture medium was collected 3 days after co-culture. The cells were cultured with medium without serum for 24 hr. After this period the supernatant was removed and the centrifuged. Supernatant was reduced to one-fourth volume by the freeze dryer. Then filtrate sterilized and stored at – 20.00 ˚C until use.

Histological studies. Thirty-five days after operation, the animal was euthanized with high-dose anesthetic ketamine and xylazine as mentioned before. After opening the abdominal wall, the testes were removed. A part of left testis was transferred into the Bouin's fixation solution for histological study after being weighed. After alcohol dehydration in ascending degree of ethyl alcohols and clearing with xylene solutions and paraffin impregnation and sectioning by microtome. Then the tissue sections were stained with Hematoxylin and Eosin, and testicular specimens were examined histologically. Also, a portion of the left testis was inserted into the foil and stored in – 20.00 ˚C freezer and used for biochemical and histochemical evaluations. All the histomorpho-metrical parameters were obtained by a digital camera (Dino-Eye-AM-7023; AnMo Electronics, Hsinchu, Taiwan) and analyzed using Dino Capture Software (version 2.0; AnMo Electronics).

Electron microscope. Transmission electron microscopy (Metropolitan Vickers Co. Manchester, UK) study was performed in control and experimental groups. First, a small 1.00 mm in thickness of testis tissue from the control and experimental groups was sampled and immersed in 3.00% glutaraldehyde fixative solution. It was then washed with phosphate buffer pH: 7.30 for 30 min and three times (three substitutions) and then fixed in 1.00% osmium tetroxide for 1 - 2 hr. After dehydration of the samples with acetone ascending degrees concentration, the samples were embedded with resin impregnation and molding stages were performed. Then sections were performed with an ultramicrotome apparatus, and fine slices were transferred onto 3.05- mm grids. For staining, fine slices were placed in uranyl acetate (Sigma-Aldrich) for 7 min. After washing with distilled water, they were placed in lead citrate (Sigma-Aldrich) and then washed in distilled water and finally all grids were observed by transmission electron microscopy.

Statistical analysis. The results were analyzed by SPSS software (version 20.0; SPSS Inc., Chicago, USA), one-way ANOVA, and Tukey post hoc test. Results were evaluated as mean ± SE and significant differences between all groups were set at $p < 0.05$.

Results

Thickness of testicular capsule. Mean thickness measurements of testicular capsule in different groups showed that the lowest thickness was in healthy control group which had no significant difference with sham control group, However, TD group had the highest capsule thickness which showed no significant difference with DMEM and co-culture groups. It was also shown that the thickness of capsule in fibroblast and macrophage groups were significantly ($p < 0.05$) lower than the TD and DMEM groups (Table 1; Fig. 1).

Thickness of germinal epithelium. Mean thickness of germinal epithelium in seminiferous tubules in different groups showed that healthy control group had the highest thickness which were not significantly different with sham group However, the lowest epithelium thickness belonged to TD group, which showed no significant difference with DMEM and co-culture group. Also germinal epithelial thickness in fibroblast and macrophage groups was significantly higher than the TD and DMEM groups ($p < 0.05$), (Table 1; Fig. 1).

Diameter of seminiferous tubules. Measurement of mean diameter of seminiferous tubules showed that healthy and sham control, fibroblast and macrophage groups had the highest diameter and no significant difference with each other, however co-culture, TD and DMEM groups had the lowest diameter and these groups had significant difference with the other aforementioned groups ($p < 0.05$; Table 1; Fig. 1).

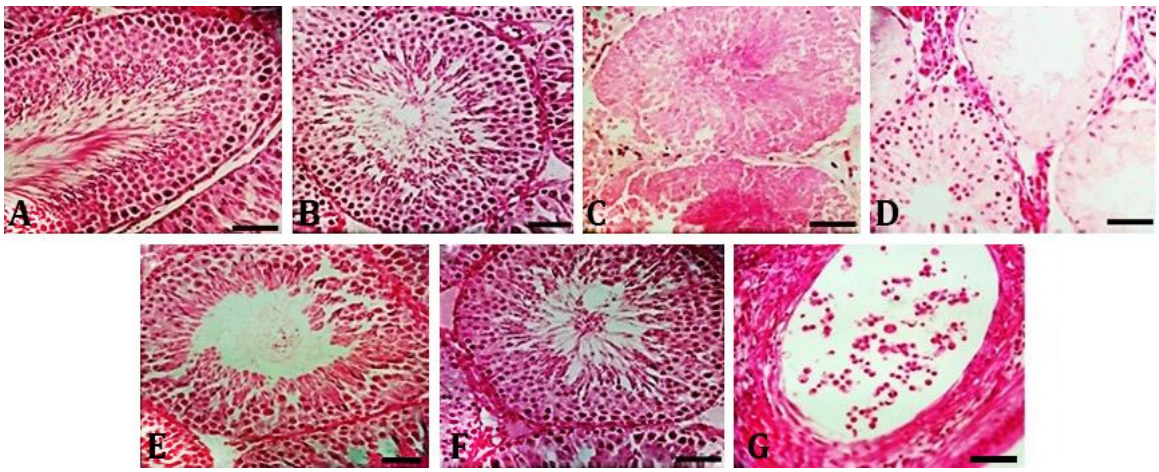

Fig. 1. Histological view of seminiferous tubules and testicular interstitial tissue using light microscopy. As shown in the figure seminiferous tubules in torsion-detorsion (TD), Dulbecco's Modified Eagle Medium (DMEM), and co-culture groups were depleted. **A)** Healthy control; **B)** Sham control; **C)** TD; **D)** DMEM; **E)** Fibroblast; **F)** Macrophage, and **G)** Co-culture. (Hematoxylin and Eosin staining; Scale bar = 50.00 µm).

Primary spermatocyte count. The mean number of primary spermatocytes in the germinal epithelium of seminiferous tubules in different experimental groups showed that the healthy control, sham control, fibroblast and macrophages groups had the highest mean number of primary spermatocytes that there was significantly different with the DMEM, co-culture and TD groups ($p < 0.05$), however, these groups were not significantly different with each other (Table 1).

Mean number of germinal cell layers. Evaluation of the average number cell layers in germinal epithelium of seminiferous tubules in different experimental groups showed that the TD, DMEM and co-culture groups had the lowest mean number that showed significant differences with the healthy control, sham control, fibroblast and macrophage groups ($p < 0.05$; Table 1).

Leydig cells. The mean number of Leydig cells in testicular interstitial tissue with spherical nucleus and euchromatic and extensive acidophilic cytoplasm were decreased in TD, DMEM and co-cultured groups and had significant differences with the fibroblast and macrophage groups ($p < 0.05$; Table 1).

Active Sertoli cells. Estimating of mean number of active Sertoli cells (Sertoli cells with a number of sperms growing at their apex) in the different groups indicated that the healthy control group had the highest number of active Sertoli cells, and had a significant difference with the other groups, except for sham control group ($p < 0.05$), while in co-culture, DMEM and TD groups the significantly lowest mean number of active Sertoli cells was observed compared to the other groups ($p < 0.05$). Also, fibroblast and macrophage groups had a high number of active Sertoli cells, which were not significantly different between themselves and with sham control group (Table 1).

Electron microscopy study. Based on the electron microscope study in healthy controls, it was found that Sertoli and spermatogonia cells were located on the basal layer, the nuclear envelop was intact and the Sertoli cell had well define nucleoli and the chromatin appeared to be clear and uniform, whereas, in the spermatogonia cell a slight heterochromatin state was observed. In both cell types the mitochondria were either spherical or oval and their cristae were elongated and the inner mitochondrial cavity was reticular. The endoplasmic reticulum was homogeneous inside the cytoplasm. The sham control group had healthy ultrastructural features as well as the healthy control group and the rough endoplasmic reticulum and nucleus membrane were uniformly observed. In TD group, in the Sertoli cells wide degenerative changes in intracellular components were observed, and nucleus had deep slit that could be due to damage of nuclear envelop, thereby, cytoplasmic organelles entered into the nucleus and mitochondria were found in the nucleoplasm, and nucleolus had a higher density than the healthy control group, however, a uniform density and also rugged edges were observed and that was fragmented to be detached and dispersed in the nucleoplasm. The mitochondria was dumbbell shaped inside the cytoplasm of Sertoli cells and fine components were isolated from mitochondria that were dispersed in the cell cytoplasm.

In DMEM group, the basal layer was strongly folded where the Sertoli cells were located on and it was damaged and broken in some parts. Large vacuole was also observed in adjacent basal layer and it seemed that these vacuoles were inside the cytoplasm of Sertoli cell. In this group, the Sertoli cell membrane appeared to be intact and nucleolus had a relatively normal shape and mitochondria inside the cytoplasm was healthier than the TD group, however, dumbbell state in some of them was still observed.

Table 1. Statistical comparison of histomorphometrical parameters of testis among different experimental groups (Mean ± SE).

Groups	Capsule thickness (µm)	Germinal epithelium thickness (µm)	Seminal tubules diameter (µm)	No. primary spermatocytes*	Germinal epithelium layers	No. Leydig cells*	No. active Sertoli cells*
Control	24.11 ± 12.78^{a}	87.34 ± 6.59^{c}	262.41 ± 18.72^{b}	49.76 ± 4.19^{b}	10.10 ± 0.44^{b}	23.20 ± 2.96^{ab}	36.50 ± 1.03^{c}
Sham	37.58 ± 22.20^{ab}	74.79 ± 6.36^{bc}	261.68 ± 11.30^{b}	44.60 ± 7.37^{b}	9.60 ± 0.53^{b}	19.53 ± 8.23^{a}	30.03 ± 1.37^{bc}
Torsion-detorsion	149.48 ± 31.04^{d}	6.40 ± 7.05^{a}	91.02 ± 20.52^{a}	7.067 ± 8 .82^{a}	1.25 ± 1.47^{a}	7.96 ± 11.96^{a}	0.33 ± 0.51^{a}
DMEM	135.20 ± 26.07^{d}	12.25 ± 9.22^{a}	99.70 ± 11.04^{a}	11.52 ± 11.009^{a}	1.96 ± 1.25^{a}	17.16 ± 12.18^{a}	5.80 ± 11.06^{a}
Fibroblast	80.96 ± 12.91^{c}	67.96 ± 10.64^{b}	242.46 ± 36.37^{b}	43.52 ± 0.28^{b}	10.44 ± 0.30^{b}	39.36 ± 4.99^{b}	27.68 ± 3.07^{b}
Macrophage	76.16 ± 11.08^{bc}	66.36 ± 6.25^{b}	241.73 ± 52.89^{b}	47.4 ± 3.56^{b}	9.60 ± 1.29^{b}	37.80 ± 2.84^{b}	23.85 ± 1.48^{b}
Co-culture	113.97 ± 8.45^{cd}	12.64 ± 1.56^{a}	90.89 ± 12.10^{a}	7.75 ± 5.80^{a}	1.72 ± 1.28^{a}	10.90 ± 7.94^{a}	0.00 ± 0.00^{a}

* Numbers were counted in 0.0625 mm^2.
$^{a\text{-}d}$ Different letters in each column indicate a significant difference between groups at the level of $p < 0.05$.

In fibroblast group, Sertoli cells were better than the DMEM and TD groups, nuclear envelop was completely intact and the chromatin appeared uniform, the nucleolus had a clear border and it looked similar to healthy control group, basal layer was complete and uniform. Mitochondria showed an oval and sometimes spherical shape, and their long cristae gave rise to a reticular view inside the mitochondria.

Ultrastructural view in Sertoli cells of macrophage group was similar to fibroblast group, it was shown to be uniform as membrane and nuclear chromatin and relatively healthy mitochondria, but overall ultrastructural conditions in fibroblast group were better than macrophage group.

In co-culture group, basal layer adjacent to Sertoli cells and spermatogonia had a highly folded state and large vacuoles were observed between the basal cell membrane and the basement membrane. The nuclei of cells had relatively uniform chromatin and clear nucleolus. Mitochondria showed a denser and darker condition than the healthy mitochondria and mitochondria was observed in a dumbbell form. The nucleus envelop seemed to be damaged, nucleoplasm and its components were observed inside the nucleus. Also, nucleolus was observed denser in damaged cells and their isolated fragments were dispersed in nucleoplasm (Fig. 2).

Discussion

Testicular torsion is an acute complication that causes severe ischemia in the testicular tissue and it causes extensive necrosis in the tissue by stress oxidative fallowing hypoxia and reperfusion of blood circulating increases of stress oxidative, and causes severe damage in the testicular tissue. Therefore, in this study, were used CM of fibroblast, macrophage and co-culture. Due to the presence of growth factors in CMs, the testis destruction was compensated. Proteins released *in vitro* have been studied to better understand the pathological conditions and mechanisms in the living organism. Secretome analysis has some challenges. The main problem is related to its collection and preparation, because often secretory proteins are in micrograms to nanogram amounts. In addition, the medium contains salts and other compounds, including phospholipids, lipids and polysaccharides which interfere with most proteomics technique.[11] The cells of the salivary glands are surrounded by a set of stromal cells such as fibroblasts that are the main cells and adjacent to the gland cells. The effect of fibroblast cell culture supernatant on biomaterials has been investigated on α-amylase expression of parotid acinar cells. This supernatant contains higher levels of neurotrophin-4 that causes an increase in the expression of secretory α-amylase from parotid gland acinar cells.[16] Fibroblasts are cells that exist throughout in human body and are essential for wound

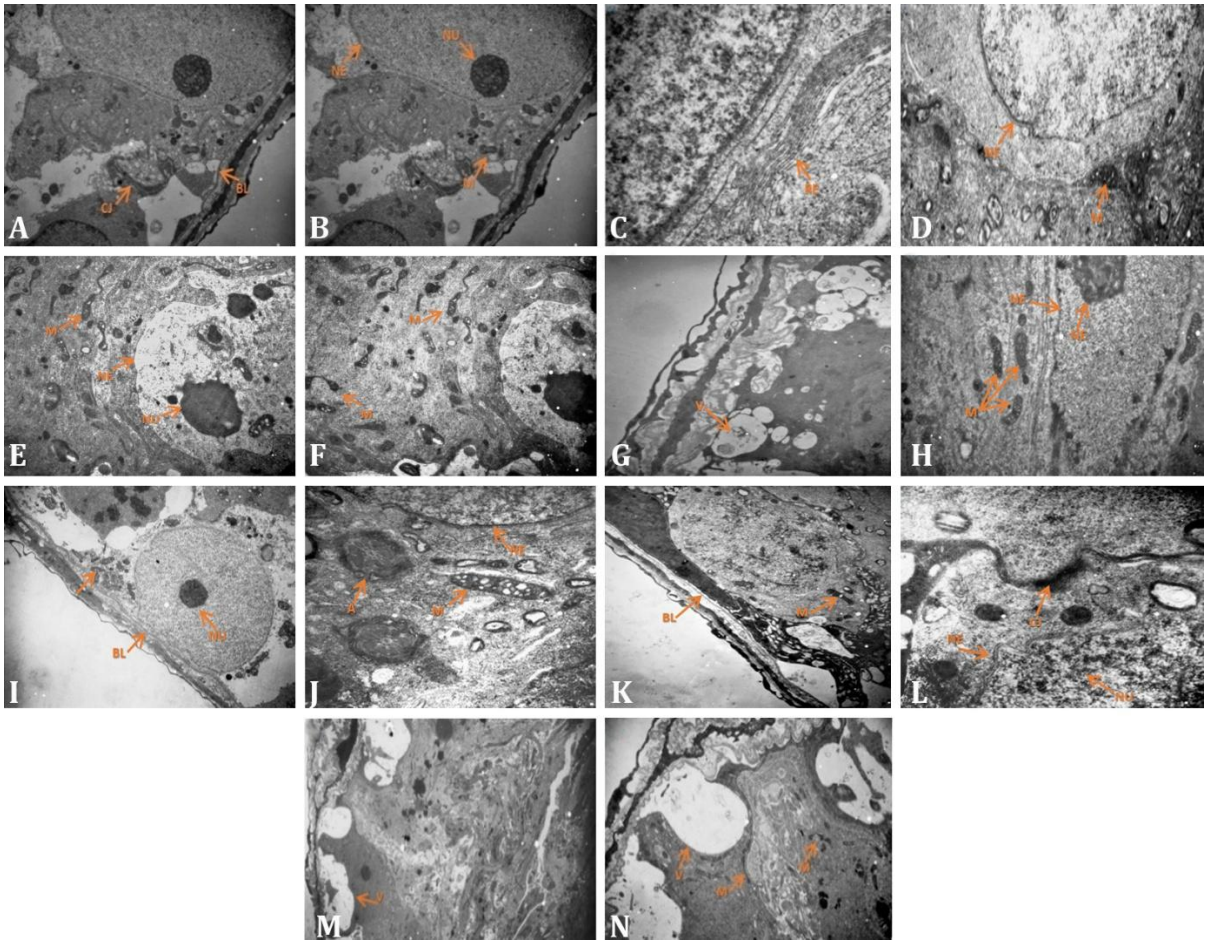

Fig. 2. Ultrastructural view of testicular tissue using electron microscope: **A** and **B)** Healthy control; **C** and **D)** Sham control; **E** and **F)** Torsion-detorsion; **G** and **H)** Dulbecco's Modified Eagle Medium; **I** and **J)** Fibroblast; **K** and **L)** Macrophage; **M** and **N)** co-culture. 30,000 (C). NE: Nuclear envelope, Nu: Nucleolus, M: Mitochondria, BL: Basal layer, RE: Endoplasmic reticula, CJ: Cell junction, and A: Axonem, V: Vacuole. Magnification = 3,000 (**I, M, N**), 4,400 (**A, B, G, K**), 7,000 (**D, E, F, H**), 12,000 (**J**), 20,000 (**L**).

healing in skin damage. Regenerating role of differentiated stem cells into fibroblast-like cells has been investigated using fibroblast cell culture CM on skin deep wound, creating a wound on animal back and injecting the cells into the wound area. It was observed that these differentiated cells had significant positive stimulatory and healing effects on the wound.[17] Limbus fibroblasts have been identified to be effective in preserving and differentiating of corneal epithelial cells, including limbus epithelial stem cells and regulating them. Supernatant from limbus fibroblasts contains factors or factors that have therapeutic benefits and that is a noninvasive therapy in the treatment of limbus stem cell defects.[18] Cell-cell interactions are important for tissue formation, homeostasis and regeneration stages.[19]

Co-culture models have been widely used to investigate the role of cell-cell physical contact, interactions (autocrine/paracrine) on cell function, as well as cell differentiation. The mixed co-culture model is often suitable for describing cell-cell contact on cell behavior *in vitro*, whereas indirect co-culture can be used to study the effects of paracrine signaling on cellular responses.[20] Co-culture techniques are available with numerous applications in biology to study the natural or synthetic interactions between cell populations. These techniques are very important in synthetic biology. Cell-cell interactions in co-culture have been strongly influenced by the cellular environment, therefore, it must be carefully selected. In this study it was identified that oxidative stress induced by TD had a proliferative effect on testicular connective tissues, thus it increased capsule thickness in TD, DMEM and co-culture groups compared to healthy controls group. Fibroblast and macrophage CM decreased oxidative stress in testicular tissue by effects of regeneration on seminiferous tube and blood vessels and testicular capsule thickness was significantly decreased in fibroblast and macrophage groups compared aforementioned groups. Mast cells number is indirectly related to the formation of fibrosis tissue in testicular cryptorchidism. When mast cells are activated, inflammatory processes are stimulated and indirectly stimulate macrophages and that is caused to produce FGFs

from activated fibroblasts that stimulate collagen and fibrosis synthesis.[21]

In the present study, the thickness of germinal epithelium and diameter of seminiferous tubules in the TD group showed the lowest rate than the other groups, which was not significantly different with DMEM and co-culture groups, however, was significantly different with other groups. This finding showed that these parameters were repaired in fibroblast and macrophage groups and that was on level of healthy groups. A histologic study showed atrophy of seminiferous tubules and extensive interstitial spaces in the torsion group. Testicular torsion is an ischemia-reperfusion injury that causes the release of free radicals following oxidative stress.[22]

In this study, it was shown that the number of cellular layers in the germinal epithelium and the mean number of primary spermatocytes were significantly decreased in TD, DMEM and co-culture groups compared to the other groups, all these findings indicated the regenerative effects of CM obtained from separate fibroblast and macrophage. Macrophage populations present in the interstitial space are directly related to spermatogonia. It seems these macrophages affect the microenvironment of spermatogonia and represent an important role on differentiation of these cells. Macrophages not only affect the differentiation and resistance of spermatogonia against native antigens but also have reversal induction to starting of spermatogenesis.[23] Macrophage secretory products such as IL-1 and TNF-α have been shown to influence Leydig cell function.[24,25] In another study, it was found that the production of inflammatory cytokines in separate cultures of primary and secondary macrophages in the presence of fibroblast CM was reduced, while the production of inflammatory cytokines in single cultures of fibroblasts in the presence of macrophage CM was increased. Compared to separate cultures, co-culture of secondary macrophages with fibroblasts significantly increased the production of monocyte chemotactic protein-1 (MCP-1) and IL-6. Co-culture of primary macrophages with fibroblasts significantly increased the production of MCP-1 alone. The TNF-α, as a fibroblast mitogenic factor, may contribute to the high levels of MCP-1 in co-cultures. Increased MCP-1 production leads to a shift of fibroblasts to a fibrotic phenotype, as fibrotic fibroblasts increase the production of TNF induced by MCP-1.[26] The FGF-1,2 stimulate proliferation of spermatogonia A and B through direct effect on DNA synthesis, as FGF-1 plays a role in communication with spermatid and Sertoli cells during spermatogenesis. The FGF-2 plays a role in proliferation and differentiation of germ cells in the rodent testis before and after birth.[27] The FGF signal has been proven to be involved in the formation of sexual organs. Four FGF receptors have been identified in the human genome that play a role in regulating gonadal function. Fibroblast growth factor receptor 1 plays role in maintaining undifferentiation spermatogonia. Fibroblast growth factor receptor 2 is associated with sex determination and testicular cord formation. It has been proven that the FGF receptor 3 is a marker of pre-spermatogonia that is associated with spermatogonia survival. Fibroblast growth factor receptor 4 plays a role in myogenesis and muscle regeneration, which is expressed on the surface on epithelium of seminiferous tubule. It is known that FGF 9 contributes in development of neurons, bone formation, lens fiber differentiation, gap junction formation, sex determination and steroidogenesis. Fibroblast growth factor 9 maintains overexpression of the *SOX9* gene. Thus, FGF 9 is associated with transient expression of FGF receptor 2, 3 and testicular development.[28]

Oxidative stress induced by ischemia-reperfusion caused by testicular torsion-detorsion significantly decreased the mean number of Leydig cells, whereas, growth factors in CM of fibroblasts and macrophages had a notable effect on proliferation of these cells. There was a significant difference between aforementioned groups with all other groups. Progressive histopathologic damage in testicular tissue is associated with increased duration of reperfusion. Progressive oxidative stress results in lipid peroxidation in the cell membrane and the starting of apoptotic events, therefore, torsion-detorsion accelerates oxidative stress and deteriorates spermatozoa production.[5,29] Testicular macrophages are closely related to Leydig cells and play an important role in development, regeneration and production of testosterone.[23] Fibroblast growth factors are a family of heparin-binding proteins that play key roles in different stages of development. The FGF-2 plays a role in proliferation and differentiation of Leydig cells, peritubular and Sertoli cells in rodent pre- and postnatal tests.[27] However, spermatogenesis activity is very close and directly related to Sertoli cells and ultimately results in the production of spermatozoids. Therefore, to evaluate the amount of spermatogenesis activity in the germinal epithelium, estimating mean number of active Sertoli cells in different groups will be important. This study showed that the Sertoli cells had at least activity in the TD and DMEM groups, whereas, no Sertoli cells were active in the co-culture group, therefore, TD, DMEM and co-culture groups had a significant difference with the other groups. It was shown that CM from the fibroblasts and macrophages significantly increased the activity of Sertoli cells compared to TD, DMEM and co-culture groups, however, that was not the same in healthy control group. This was probably the factors in CM have been compensatory roles in the fibroblasts and macrophages groups. It has been shown that, FGF-9 deletion in the fetal gonads lead to a reversal of the sex of the fetus from male to female in healthy groups. The FGF-2 plays clear roles on proliferation and differentiation in Sertoli cells during testicular development.[30] Generally, study of Sertoli cells ultra-

structure in different groups showed that, nucleus membrane were severe damaged and degenerative changes were shown in nucleolus in TD group, but the mitochondria showed a dumbbell form or fragmented destructive structure. All of these observations, indicated that destruction of the normal activities in Sertoli cells was associated with these cellular organelles, whereas, these injuries were highly compensated in the fibroblast and macrophage groups, especially fibroblast group was very much similar to the healthy and sham control groups as nuclear membrane was healthy and the nucleoplasm looked uniform and healthy. This finding showed that the fibroblast and macrophage groups were able to undergo critical conditions of post-ischemic and reperfusion and to restored their ultrastructure by receiving growth factors. Also, these findings showed that in DMEM group, the Sertoli cell organelles were regenerated and were partly affected by receiving CM, but in co-culture group despite the presence of growth factors in the CM, ultrastructure compensatory remodeling was not complete, especially in Sertoli cells mitochondria, similar to appearance to TD group, due to active inflammatory factors. In TD group of a study, seminiferous tubules lost their natural structure. Spermatogenic cells were disrupted, degenerated and isolated from the basal layer. Numerous multi nucleus giant cells were found in some seminiferous tubules. Some tubules contained necrotic acidophilic bodies, germ cells were detachment and many tubules did not contain sperm. Widely edema was in the interstitial tissue. Epididymis tubules were filled with necrotic debris. Images from scanning electron microscopy showed the spermatogenic cells were irregular, abnormal and malformed, and their cytoplasm contained numerous vacuoles. Therefore, orchiectomy followed by testicular torsion preserve contralateral testis and epididymis structure better than detorsion only.[31] Ischemia resulting from torsion and vascular obstruction of the spermatic cord is responsible for the ultrastructural changes in contralateral testis. Lamina propria containing seminiferous tubules was thickened due to increased collagen fibrils, basal layer was continuous and had thickness and numerous folds. Distance between the basal layer and the germ cells were increased due to collagen fibers. Leydig cells had degenerated mitochondria with low crystals. Leydig cells lost their contact with adjacent cells in some areas, and these spaces were filled with collagen filaments. Germ cells had endoplasmic reticulum with large cisterns and dense bodies in cytoplasm. The beneficial effects of FGF21 are due to the preservation of mitochondrial membrane potential.[32] Overall, TD or ischemia/reperfusion on testis had a wide destruction effect which impaired testicular morphometric parameters such as, capsule thickness**,** germinal epithelial thickness, seminiferous tubules diameter, the number of Leydig cells, primary spermatocytes, cellular layers of germinal epithelium and active Sertoli cells. Ultra-structural study of testicular tissue also showed that extensive changes occurred in nucleus, nucleolus, and mitochondria of Sertoli cells. In this study, it was shown that in fibroblast group, there was good improvement in all parameters, as most parameters were similar to healthy control group. It was also shown that macrophage group had significant improvement effects but these effects were less effective than fibroblast group. It was also shown that the DMEM group had slightly better recovery conditions than the TD group, but the co-culture group showed similar conditions to the TD group due to the presence of inflammatory factors. Then, it could be concluded that CM derived from fibroblast had most beneficial effects and that macrophage CM had significant effects on improving TD-induced conditions. Hence, discovering new therapeutic targets are required to be used for treatment of TD-induced complications.

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Conflict of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this article.

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