Original Article

Differential expression of Oct4 and VEGF in ovaries of Model of Polycystic Ovary in albino Rats after treatment with Conditioned Media and Exosomes Derived from Mesenchymal Stem Cells

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ABSTRACT

Introduction: One of the most famous endocrine diseases affecting female is Polycystic ovary syndrome (PCOS). In regenerative medicine, stem cell-free therapy as conditioned media (CM), and exosomes (EX) represents alternate strategies in treatment of such cases. Angiogenesis was one of the hypothesis in progression of PCOS. Vascular endothelial growth factor (VEGF) is cosidered an important marker for angiogenesis. Oct4 (Octamer-binding transcription factor) is one of the key transcription factors needed to produce induced pluripotent stem cells (iPS) and induce angiogenesis.

Aim of the Work: To differentiate the expression of Oct4 and VEGF in ovaries of model of PCO after treatment with conditioned media (CM)and exosomes (EX).

Materials and Methods: Seventy albino rats; (12 adult male served as breeders, 10 rats were utilized to harvest CM and EX and 48 female rats were divided into 4 groups : The control group, the PCO group , the Ex-treated group and CM-treated group. Female rats were given oral letrozole (1 mg/ kg/day) once a day for 5 weeks to induce PCO. Biochemical analysis, histological , and immunohistochemistry for VEGF and Oct4 immune-expression have all been carried out. The fertility rate was evaluated.

Results: CM showed more significant therapeutic effects than EX in restoring the normal architecture of ovaries. Furthermore, female rats treated with CM showed significant higher fertility rate than those treated with EX.

Conclusion and Recommendation: A safer and more practical way for tissue regeneration is cell free therapy. The PCO model demonstrated that stem cell generated CM restored the histological structure of ovaries and fertility more effectively than EX. Although MSCS derived exosomes are effective in treatment of many diseases, they may have disadvantages of overstimulating angiogenesis. More studies are required for comparing CM and exosomes in different diseases models.

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Key Words: Conditioned media (CM), exosomes, polycystic ovary syndrome (PCOS), VEGF Oct4 (Octamer-binding transcription factor) ovary.

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INTRODUCTION

The Polycystic ovary syndrome is a famous endocrine -gynecological condition that affect females reproductive life. One in ten females are thought to have PCOS prior to menopause. As of right now, the precise cause of PCO remains unknown[1]. However, multiple factors, including hyperandrogenism, insulin resistance, , genetics, environment with epigenetics, have been implicated as major causes[2].

The rate-limiting enzyme that changes androgens into estrogens is called aromatase. Since drug such as letrozole which is considerd a non-steroidal aromatase inhibitor . Its work in prevention of testosterone from being converted to estrogen, it can cause hyperandrogenism and ovarian polycystic alterations. For this reason, it has been extensively utilized to make a rat model of PCO[3].

Current studies on the biology of stem cells have given rise to new hopes for the treatment of complicated illnesses and conditions that have not yet been cured. The stem cells have ability for differentiation into numerous types of cells in the body. They repair damaged specialized cells[4]. Numerous studies suggest that the main therapeutic activity observed in mesenchymal stem cells (MSCs) are factors secreted by paracrine mode of secretion[5]. Molecules that MSCs release are known as the secretome; these molecules include extracellular vesicles (EVs), trophic factors , chemokines, growth factors and cytokines. The derivatives of these secretome including extracellular vesicles or conditioned medium[6].

The utilization of MSCs as cellular treatments is subject to many restrictions, not withstanding their advantageous characteristics. These include the possibility of tumor development, abnormal differentiation, poor engraftment, and a brief half-life of transplanted cells. It looks promising to employ the MSCs secretomes as a therapeutic agent since it could get around some of the drawbacks of cell-

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based methods. The capacity of the MSCs secretomes to influence the local immunological microenvironment, reduce damage, and encourage epithelial healing is becoming increasingly understood[7].

In our previous research using CM and exosomes in the treatment of PCO animal models the CM showed more beneficial effect than exosomes[8]. In this study more immunohistochemical staining were used in atrial to understand the mechanism beyond the differences in both treatment modalities.

Oct4 (Octamer-binding transcription factor) is one of the most crucial transcription factors needed for preservation pluripotency and undifferentiated condition in early embryonic cells, such as mouse and human embryonic stem cells[9]. Furthermore, Oct4 is considered recently the only transcription factor that needs to be injected externally into differentiated cells for purpose of production of iPS[10]. It is frequently expressed in embryonic cancer cells, germ cells, unfertilized eggs, embryonic germ cells and the inner cell mass of a blastocyst, and. Oct4 is also expressed in adult ovaries during the oocyte growth phase[11]. According to reports, adult ovaries with reduced Oct4 expression exhibit an increase in antral and atretic follicles and death of the primordial germ cells[12].

VEGF triggers the proliferation and migration of endothelial cells. High levels of VEGF in the serum and follicular fluid of PCOS patients in PCOS ovaries was reported. Additionally, it has been found that ovulation disorders are linked to VEGF gene overexpression in the ovarian stroma of PCOS patients[13].

MATERIALS AND METHODS

Animals and ethics

For this study, seventy albino rats were employed; twelve adult male rats served as breeders, forty-eight female rats were randomly assigned to one of four groups, and ten rats were utilized to harvest CM and exosomes from mesenchymal stem cells. In Beni Suef, Egypt, at Nahda University of Beni Suef (NUB), there is a research animal home where the animals were supplied with no contaminants. The research followed all protocols that were authorized for the use of animals in research by the Minia University Institutional Ethics Committee (permission number: 44:6/2021).

Drugs

From (Novartis Pharmaceutical Company, Egypt), Femara c tablets of 2.5 mg of letrozole were provided. The pharmacy department at Minia University pulverized the pills and combined them with 1% carboxymethylcellulose.

Conditioned media and exosomes

Preparation of bone marrow- mesenchymal stem cells

Ten albino rats of about four to six weeks old and weighed about 70-80 gm were used to obtain mesenchymal stem cells following the methodology described by Abdelwahab and colleagues[14].

Obtaining the BM-MSCs derived conditioned media

MSCs from 3rd to 5th passages were used to prepare the conditioned medium in T75 flasks. At 80–90% confluence, the BM-MSC culture media were removed and discarded. The cells underwent three PBS washes in order to remove any residual serum. The conditioned media were obtained by growing the cells for a full day in serum-free Rosswell Park Memorial Institute Medium (RPMI). To get rid of any remaining cell debris, the media were spun at 500 g for 5 minutes at 4 °C after collection. Afterwards, the media were divided and kept at a temperature of -80 °C for use in the study[15]. After that the cultivated cells were trypsinized then counted. Each flask contained about 5×10^6 cells. Each one had about an average of about 4 ml of CM.

Obtaining the BM-MSCs derived exosomes

Isolation

0.5% bovine serum albumin was added to RPMI media without fetal bovine serum for culture of the MSCs. The supernatants of the culture of third passage MSCs were collected to get the exosomes that these cells produce. The spinning the supernatant for twenty minutes at 2000 g after discarding the pellet used for removing dead cells. After removing the pellet, the centrifugation at 10,000 g for 20 minutes was used for pellet in order to further remove any remaining cell debris. ThermoFisher, Japan's SORVALL MXT 150 micro-Ultracentrifuge was used to centrifuge the cell-free supernatants for 70 minutes at 4°C at a force of 100,000 g. Following aspiration of the supernatant using a micropippetor 1000, the exosome-containing pellets were cleaned in PBS. Once more, a centrifugation was performed for seventy minutes at 100,000 g., A micropipettor 100 was used for extraction of the pellets containing exosomes, placed in a 1.5 ml eppendorf tube and stored at -80 until they were needed for the experiment[16].

Evaluation of the amount of protein in exosomes

The protein content within exosomes was evaluated by the Lowry Protein Assay Kit (Bio Basic Inc, Canada[17]. 150 µg of exosomal protein (extracted from about 5 ml of cultured media) suspended in PBS (100 µl) was administered intravenously to each rat[18].

Characterization of exosomes

a. Electron microscopy: MSCs produce EX which were resuspended in 30 ul of PBS. A sample of 10 ul of exosomes was loaded onto copper grids coated with carbon. Exosomes were then stained with 1% phosphotungstic acid and allowed to dry at room temperature for one minute. These pictures of the exosomes were made using transmission electron microscopy (TEM) at Al-Azhar University; at the Regional Centre for Mycology and Biotechnology (JEOL-GEM, 1010 TEM, 70 kV)[19].

b. Flow cytometry: Standard flow cytometry cannot be used for identification very tiny EX. So, they must attached to bigger beads before they can be studied using this technique[20]. Their CD 81 and CD 63 levels were measured.

c. Exosomes labelling using PKH-26 fluorescent dye: After being extracted during the 3rd passage of MSCs culture, BM-MSCs exosomes that migrated into ovarian tissue in the EX-treated group were tagged with PKH26 Red Fluorescent Cell Linker Kits; (Sigma, Aldrich, Saint Louis, USA). Next, the rats' tail veins were injected. At Kasr Elini University, researchers used fluorescence microscopy to look at ovarian tissue and verify homing while also following the exosomes that were injected[21].

Induction of polycystic ovary

To ensure that the rats' estrous cycles were regular, vaginal smear was performed for two weeks. The pipette smear method was used to do the vaginal smear. The research included all rats since they all exhibited a consistent estrous cycle. Letrozole 2.5 mg tablets were given to 36 rats. 1 mg/kg/day letrozole was taken and dissolved in 1% carboxymethyl cellulose (CMC1%) orally for 5 weeks. In order to ensure that all animals were in the polycystic ovary model. During the final two weeks of letrozole therapy, a vaginal smear was performed to confirm that they were in the persistent diestrous stage of the estrous cycle (8-44).

Experimental design

The used forty-eight female albino rats were randomly divided into four groups:

1. **Control group:** 12 rats were given CMC1% orally once daily at a dosage of two milligrams per kilogram for a duration of five weeks.
2. **Polycystic ovary group (PCO group):** Twelve rats were given letrozole for a duration of five weeks.
3. **(EX-treated group) Exosomes-treated group:** Twelve rats were administered letrozole for five weeks as described in the PCO group. After that, they were given intravenously with a single dose of about 150 µg of proteins generated from BM-MSCs, suspended in 100 µl of PBS[18].
4. **(CM-treated group) Conditioned media treated group:** Twelve rats were administered letrozole at the same dosage as the PCO group for five weeks, followed by a single tail vein injection of about 0.5 ml of CM produced from BM-MSCs[14].

Six rats were used for mating after four weeks of CM and exosomes administration in each group. The remaining female animals were sacrificed two weeks thereafter.

Breeding experiment

Six rats were selected for breeding from each group four weeks after the injection of CM or exosomes. For every 2 female rats, 1 male was utilized. For two weeks, the male and female rats were housed together in appropriate cages. The sperm plug found inside the vagina served as proof that mating had occurred. On the ninth day of the pregnancy, the diagnosis was made by manual palpation[22].

Blood and tissue collection

Rats were fasted for an entire night and given ether inhalation anesthesia. 10% buffered formalin was used to make intracardiac perfusion. A heart puncture was used to obtain blood. In heparinized tubes, a portion of the remaining blood samples were placed and the sera were stored at –20°C after centrifugation for about ten minutes at 4°C at 3000×g. For light microscopy study; Rats' ovaries were removed and fat washed and fixed in buffered formalin 10%.

Hormonal study

The serum samples were analyzed for testosterone; CUSABIO Reagent Kit, USA, Catalog Number: CSB-E05100r; follicle stimulating hormone (FSH)- (Fine Test, Catalog Number: ER0960)-, luteinizing hormone (CUSABIO Reagent Kit, USA, Catalog Number: CSB-E12654r)- and insulin (Elbascience Biotechnology Inc. Catalog Number: E-EL-R2466) using enzyme-linked immunosorbent assay kits, as directed by the manufacturing company.

Light microscopic study

The paraffin blocks were prepared by fixing ovarian samples in 10% buffered formalin. These blocks were then prepared for LM study. The hematoxylin and eosin stain were applied to serial slices that were 5-7 µm thick[23].

Additionally, Rabbit polyclonal anti Oct4 (ab18976, 1:200, abcam, USA) and sheep polyclonal against VEGF (A12303, 1:200, Abclonal, USA) antibodies were used to perform immunohistochemical labeling employing the avidin-biotin peroxidase technique. Xylene was used for deparaffination sections and rehydrated, rinsed with tape water, and then embedded in a solution of 3% hydrogen peroxide (H_2O_2) in PBS for a duration of 10 minutes in order to inhibit endogenous peroxidase. This was done in accordance with the streptavidin-biotin approach. Sections should be submerged in 0.01 µl/ml of sodium citrate buffer for 10 minutes in order to retrieve antigens, and then placed in a microwave oven. This was followed by incubation with the proper primary antibodies. The next step was to identify them by incubating the sections with goat anti-rabbit biotinylated secondary antibody (Vector Laboratories, Burlingame, CA). The tissues were visualized using the following methods: 3,3'-diaminobenzidine in H_2O_2 (DAB kit, Vector, CA) and horseradish peroxidase-avidin-biotin complex (Vectastain Elite, Vector, CA). Brownish nuclear and/or cytoplasmic granules were the reaction. Hematoxylin was then used as a counterstain, and the sections were dried and cleaned.

Image capture

Throughout the cell culture process, an inverted microscope (Labomed TCM 400, India) was used to examine the petri dishes and flasks. The exosomes that were tagged with PKH26 were examined under a fluorescence microscope. A color digital camera linked to

(Olympus BX51 microscope) (Japan) was used for capture Images of the H and E and immunohistochemical stained sections. The LC micro application software was installed on a computer and the camera was linked to it.

Morphometric measurements

All follicle types including primordial, secondary, mature Graafian and cystic, and corpora lutea had its mean count calculated from H and E stained sections (x400).

The mean number of oocytes that were positive for Oct4 was also determined. Area % of VEGF immune-expression was measured from immunostained sections. Each rat in each group had all of the aforementioned characteristics measured in 10 fields over five sections, with a magnification of 400 x.

For calculation of the area percentage of VEFG immunoreactivity, a program called Image J, developed by (the National Institutes of Health); Bethesda- Maryland, was employed.

Statistical analysis

For quantitative data analysis, Graph Pad Prism; version 7.01 for Windows, Graph Pad Software- San Diego- California- USA- www.graphpad.com) was used. For each set of parameters, we determined the mean and the standard error of the mean (SEM). Information was presented as the Mean ± SEM. A one-way analysis of variance (ANOVA) test was used to define the statistically significant differences between the groups, and for multiple comparisons, the Tukey Kramar post hoc test was chosen. Furthermore, a Chi-square test was used to evaluate the significance in fertility. Statistically significant values was considered at *p values* less than 0.05.

RESULTS

Vaginal smear results

Vaginal smear to examine the four phases of the estrous cycle in rats across all groups. During the pro-estrous phase, nucleated epithelial cells were the most common type of cells, while non-nucleated cornified cells were the most common during the estrous phase. The metestrous phase showed a combination of nucleated and non-nucleated cells as well as leukocytes, and the di-estrous phase was characterized by leukocytes mainly (Figure 1).

While the PCO group displayed a persistent diestrous stage, indicating an anovulatory cycle, the control group displayed a regular estrous cycle. Meanwhile, the treated groups maintained normal cyclicity.

The characterization of exosomes

a) Flow- cytometry

CD 63 & CD 81 positivity was observed (Figure 2A).

b) The Electron microscopy

Vesicles that were spheroid in shape and have a diameter of 70-100 nm were detected that identify the exosomes (Figure 2B).

c) Flourescent microscopy

The EX-treated-group ovarian tissue sections showed positive PKH-26 labelled immunoflourescent cells (Figure 2C).

Biochemical results

When compare the PCO group with the control group, a statistically significant rise in the blood levels of insulin hormone, LH/FSH ratio, and testosterone hormone (*P value*; was < 0.0001). The EX-treated group when compared to the PCO group exhibited a significant decline as the *P value* was less than 0.0001, however a significant rise in comparison to the control group; *P value* was found less than 0.0001. Also, when compared to the PCO group and the EX-treated group, these measures in the CM-treated group decreased significantly (*P value*< 0.0001), but in comparison to the control group, no significant change was detected (*p value*> 0.05) (Tables 1,2,3).

The histological results

A) The light microscopy

1. Hematoxylin and Eosin stains results

Control group: Histological examination revealed typical ovarian structure, including the presence of an outer cortex and an inner medulla. Growing follicles and the corpus luteum were visible in the area of cortex (Figure 3A1). Mature Graafian follicles were also seen. They consisted of a single large antral cavity appeared filled with follicular fluid. An eccentric oocyte and one layer of corona radiata cells (single layer of columnar cells with oval nucleus and acidophilic cytoplasm) surrounding it was located within the cumulus oophorus. Thinner theca cell layer (theca interna cells look as polgonal cells with fibrous layer of theca externa) and thicker granulosa cell layer (multiple polygonal cells with rounded nucleus and acidophilic cytoplasm) made up the wall of mature Graafian follicles (Figure 3A2). The ovarian medulla had interstitial cells and vascularized connective tissue (Figure3A3).

PCO group: Characterized by the presence of several large cystic follicles of varying diameters throughout the cortex. Acidophilic material without oocyte in the lumen of each cyst. A highly cellular interstitial stroma were detected between the cysts. Several atretic follicles were seen (Figure 3B1). Very thin granulosa cell layer was appeared with very thick theca cell layer. Dehiscent granulosa cells with pyknotic nuclei were seen. (Figure 3B2). Ovarian medulla was observed with multiple cellular infiltrates and congested blood vessels. in addition, the interstitial medullary cells appeared as large closely packed polygonal cells with densely stained nuclei and vacuolated cytoplasm (Figure 3B3).

EX-treated group: Some developing follicles with corpora lutea reappeared in cortex (Figure 3C1). Mature Graafian follicle with many dehiscent granulosa cells, most notably those that of the cumulus oophorus (Figure 3C2). Blood vessels congestion was seen in

the medulla associated with cellular infiltrate and few interstitial medullary cells appeared as large closely packed polygonal cells with densely stained nuclei and vacuolated cytoplasm (Figure 3C3).

CM treated group: Restoration of normal follicles within the ovarian cortex observed in this group. The medulla also appeared as control group (Figure 3(D1-D3).

Morphometric results of H and E

The mean number of primordial, primary, secondary, mature Graafian follicles, and corpora lutea significantly decreased when the PCO group was compared to the control group; the *P value* was found to be less than 0.0001. When comparing the EX-treated group to the control; the mean number of these follicles decreased significantly -*P value* was calculated < 0.0002, but when comparing with the PCO group; increased significantly; *P value* was found less than 0.0001. In comparison to the control group, the CM-treated group did not vary significantly; *P value* more than 0.05. However, in comparison to the PCO group; *P value* less than 0.0001- and the EX-treated group -*P value* less than 0.0005, a significant reduction was observed. Regarding the mean number of cystic follicles; In comparison to the control group, the PCO group had a significant increase (*P value*< 0.0001). No significant difference between the the EX-treated -group and control group; *P value* observed as less than 0.05; however a significant decrease in the EX-treated group in comparison to the PCO group (*P value*<0.0001). The CM-treated group did not differ significantly from the control or EX-treated groups (*P value*>0.05). However, a significant reduction was detected if compred to the PCO group (*P value*<0.0001) (Table 4).

Immunohistochemical results

a- VEGF immunohistochemistry

In the control group, a faint positive response was seen in endothelial cells of certain blood vessels and some stromal cells (Figures 4 A1-A3). Strong positive reaction in the endothelial of the many blood vessels was observed in PCO group;, also , numerous stromal cells and all layers of granulosa cells of the cystic follicles have same observation (Figures 4 B1-B3). The EX-treated group showed a positivity in some stromal cells, superficial granulosa cells, and blood vessel endothelial cells (Figures 4 C1-C3). The expression in the group treated with CM was comparable to expression in control group (Figures 4 D1-D3).

The PCO group exhibited a significantly higher mean area fraction of VEGF expression if compared to control group; *P value* was less than 0.0001. In the group treated with EX a significant reduction in comparison to the PCO group; *P value* was calculated less than 0.0001, and a significant rise in comparison to control group as *P value* was found less than 0.0001. The CM treated group exhibited a substantial reduction when compared with the PCO group; *P value*< 0.0001 and the EX treated group; *P value*< 0.0001, but there was no significant difference with the control group (*P value*> 0.05) (Figure 4E).

b- Oct4 immunohistochemistry

The control group showed positive expression mainly in the oocyte of growing follicles (Figures 5 A1,A2), stromal cells showed mild positive expression (Figure 5A3). Positive expression in germinal epithelial cells was also observed with some cells have positive nuclear expression (Figure 5A4). PCO group showed nearly negative expression in cells of cystic follicles, stromal cells and germinal epithelium (Figures 5 B1-B4), Ex-treated group showed negative expression in the oocytes with strong positive immune-expression mainly in the stromal cells (Figures 5 C1-C3). Germinal epithelial cells also showed positive expression with some cells have positive nuclear expression (Figure 5 C4). CM-treated- group showed positive expression mainly in the oocytes (Figures 5 (D1,D2) scarcely immune-positive cells in the stroma (Figure 5D3). Positive expression of Oct4 in germinal epithelial cells was evident with some cells have positive nuclear expression (Figure5D4).

The control group if compare to the PCO group showed a significant lower mean percentage of Oct4 positive oocytes (*p-value*< 0.0001). A significant increase was seen in the Ex-treated group compared to PCO group (*p-value*< 0.0089), whereas a significant decline was observed with the control group. While there was no significant difference with control group (*p-value*=0.871), CM-treated group demonstrated a significant decline in comparison to both PCO & Ex-treated groups (both *p-values*< 0.0001) (Figure5E).

Breeding results

The PCO group had a significantly lower pregnancy rate than the control group. There was a statistically significant drop in the EX treated group compared to the control group, but insignificant difference when comparing to PCO group. CM treated group increased significantly when compared to PCO-group and EX treated group and insignificant difference when comparing to the control; (Table 5, Graph A).

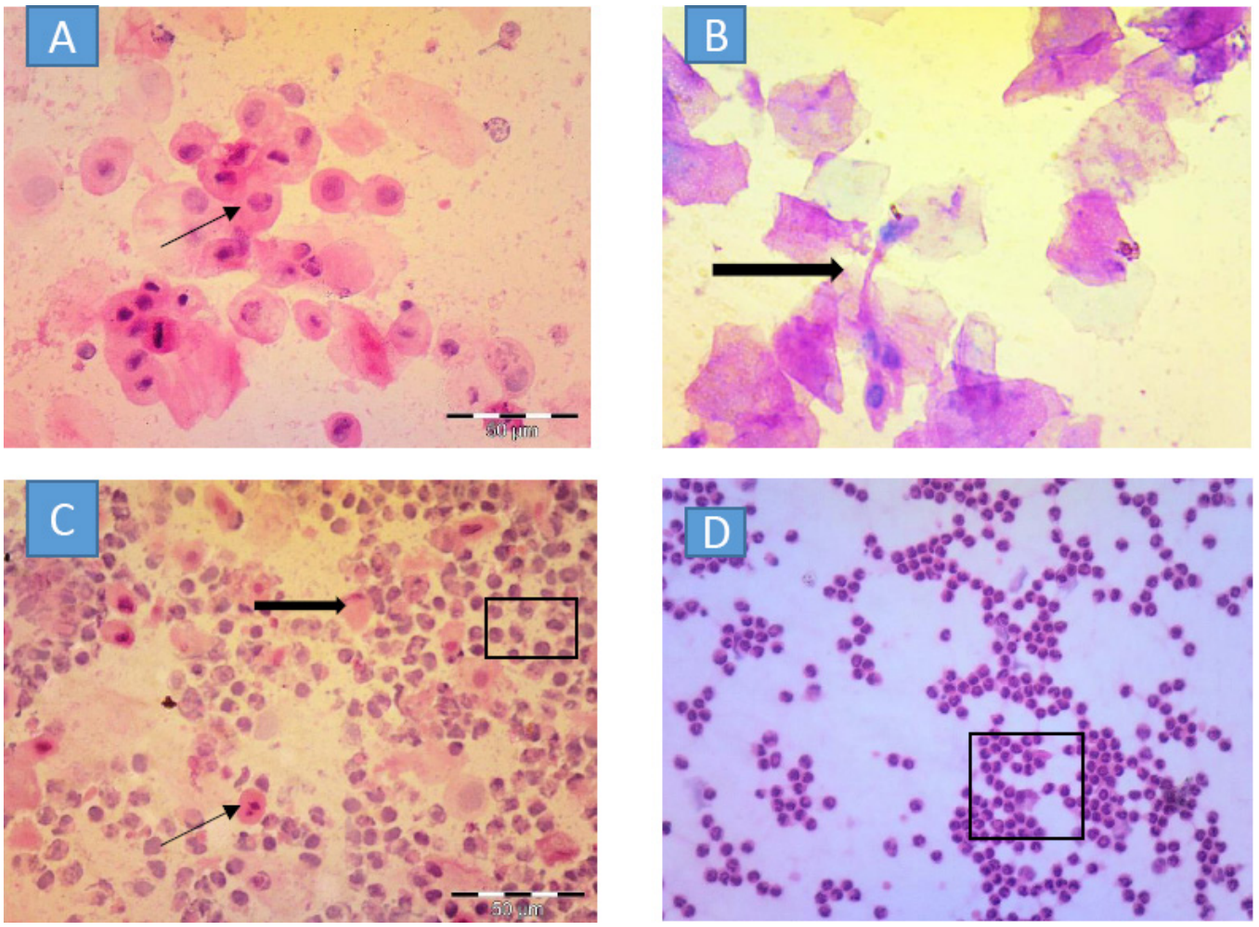


Fig. 1: photomicrographs of stained vaginal smears showing: **(A)** predominance of nucleated epithelial cells in the proestrous phase (thin arrow), **(B)** predominance of large cornified non nucleated cells in The estrous phase (thick arrow), **(C)** a mixture of nucleated epithelial cells (thin arrows), large cornified non nucleated cells (thick arrow) and leukocytes in the metestrous phase (square), while **(D)** the presence of leucocytes mainly (square) indicating the diestrous phase. (H&E X 40, scale bar = 50µm).

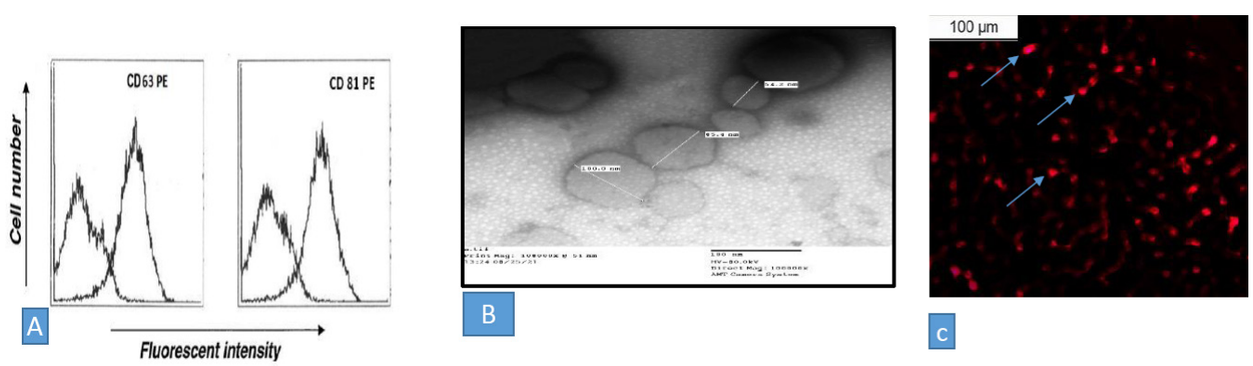


Fig. 2: Characterization of MSCs´ derived exosomes showing: **A:** analysis of exosomes by Flow cytometry showing (CD81) positive & (CD63) positive. **B:** TEM of exosomal vesicles on carbon coated copper grid showing vesicles of 70-100 nm in diameter that are spheroid in shape and having lipid bilayers , TEM: 1000000x. **C3:** Fluorescent image of PKH26 labelled exosomes in the ovarian tissues of Ex-treated group (blue arrows), scale bar =100 µm.

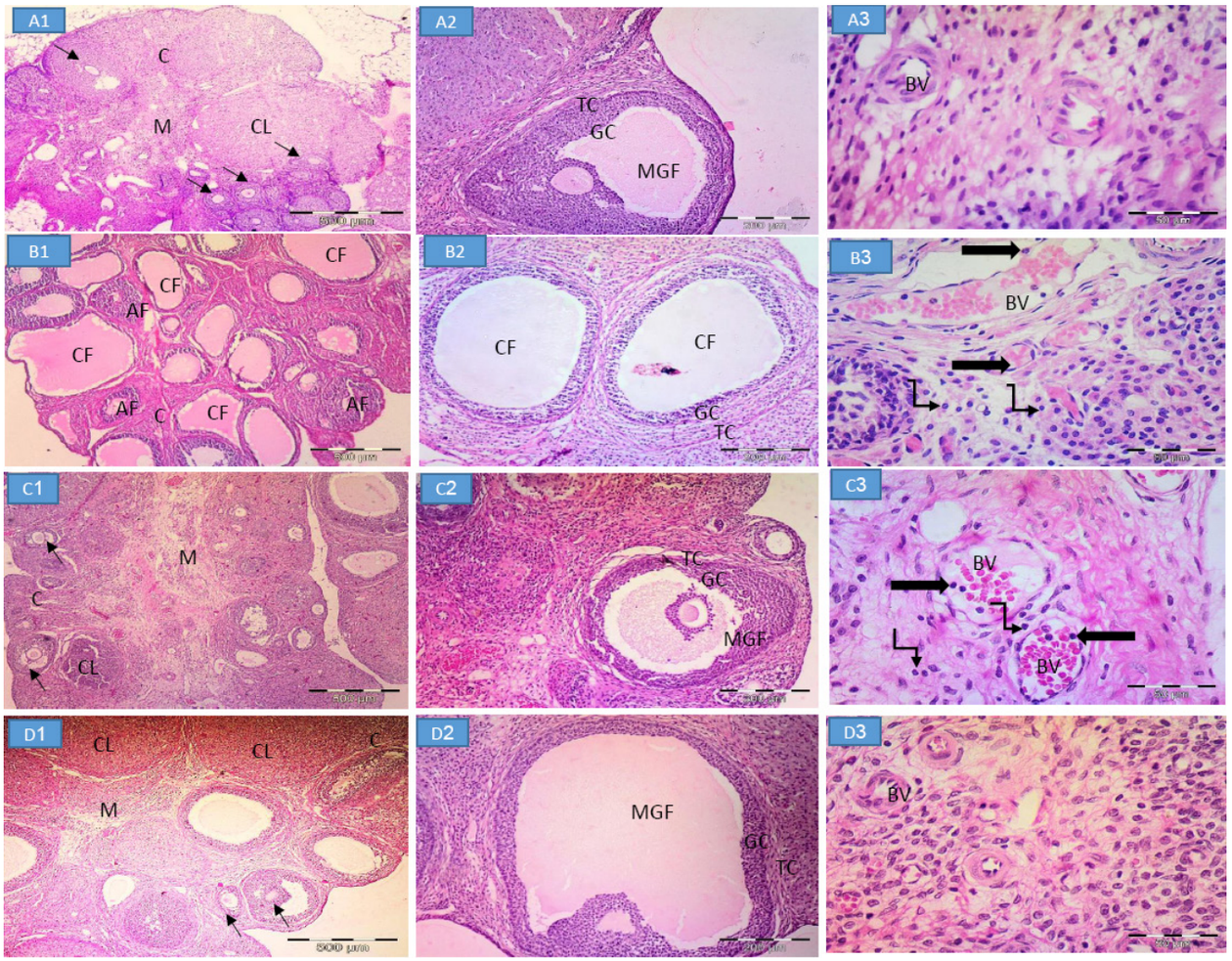


Fig. 3: Photomicrographs of rat ovarian tissues sections of the studied groups showing **(A1-A3)** Control group (A1) Normal histological ovarian appearance; the outer cortex (C) contains many growing follicles at different stages of growth (arrows) and corpus luteum (CL). (A2); Mature graafian follicle (MGF) with thick granulosa cell layers (GC) and thin theca cell layers (TC). (A3): Ovarian medulla with blood vessels (BV). **(B1-B3)** PCO group: (B1): the cortex is occupied by multiple large cystic follicles (CF) without oocyte and have acidophilic material in lumen, several atretic follicles (AF). (B3): cystic follicles (CF) has a thick theca cell layers (TC) and a thin granulosa cell layers (GC) . (B3): many congested blood vessels (BV) in the medulla with inflammatory cells (thick arrow), interstitial medullary cells appeared as large closely packed polygonal cells with densely stained nuclei and vacuolated cytoplasm (elbow arrow). **(C1-C3)** Ex- treated group : (C1): The cortex has some growing follicles (GF) and corpus luteum (CL). (C2): a mature graafian follicle (MGF) with thin theca cell layers (TC) and thick granulosa cell layers (GC) but granulosa cells of cumulus oophorus are dehiscent (CO). (C3): congested blood vessels (BV) in the medulla with inflammatory cells (thick arrow), few interstitial medullary cells appeared as large closely packed polygonal cells with densely stained nuclei and vacuolated cytoplasm (elbow arrow). **(D1-D3)** CM-treated group: (D1): Nearly normal structure of the cortex with multiple growing follicles (arrows) and corpora lutea (CL)., (D2): nearly normal mature graafian follicle that resemble that of the control group. (D3): nearly normally appearing ovarian medulla. (H&E (A1- B1- C1 -D1 x4, scale bar= 500µm), (A2- B2- C2-D2 x10, scale bar=200 µm), A3- B3- C3- D3x 40, scale bar=50).

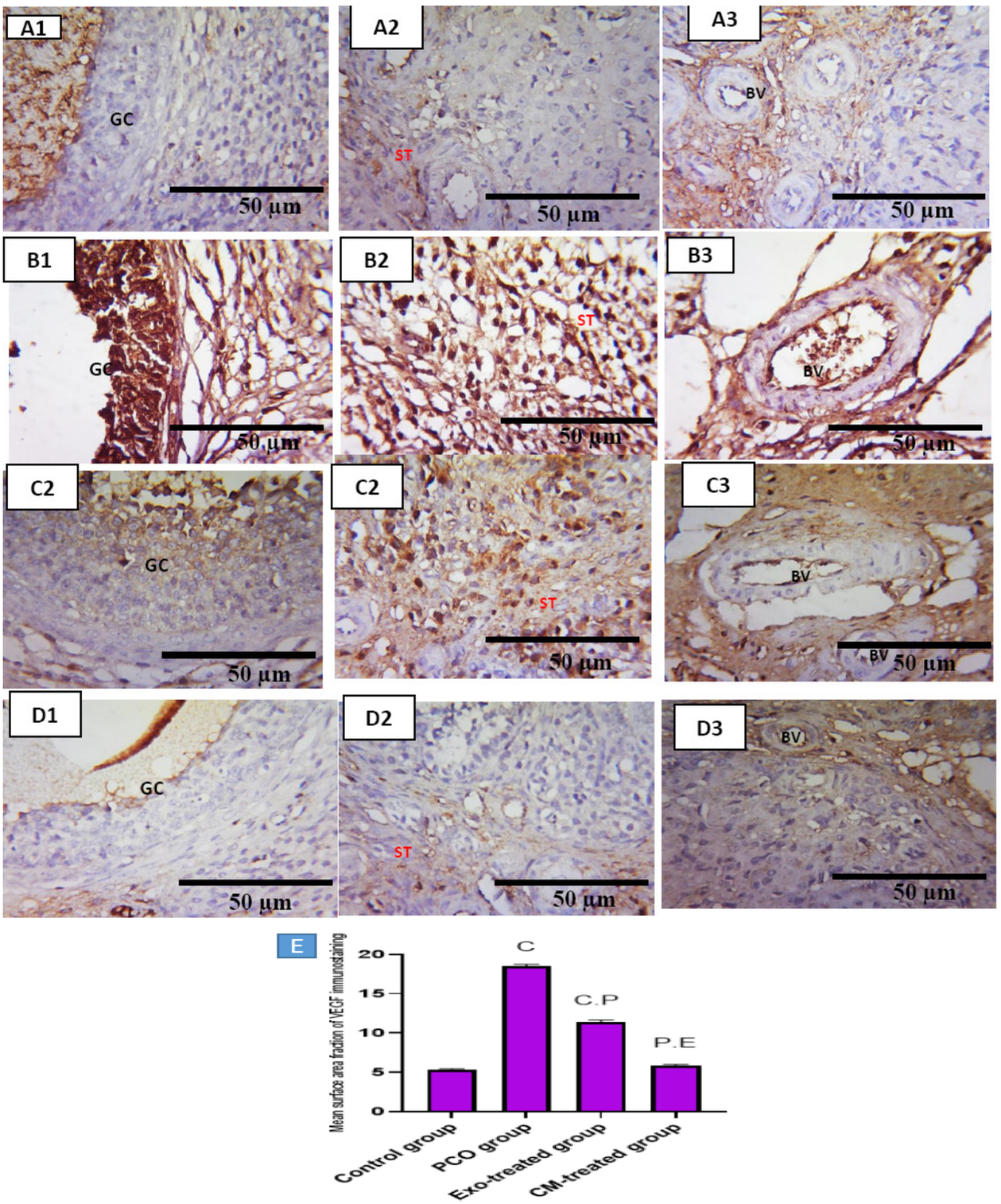


Fig. 4: Photomicrographs of rat ovarian tissues immunostained for VEGF from the control -group (A1-A3) showing negative staining except for mild positive reaction in endothelial cells of some blood vessels (BV) and some stromal cells (ST). , PCO- group (B1-B3) showing extensive positive immune-expression in granulosa cells (GC) , in endothelial cells of blood vessels (BV) and stromal cells (ST), Ex-treated -group (C1-C3) showing positive immune-reaction in only superficial granulosa cells, endothelial cells of blood vessels and many stromal cell and CM- treated -group (D1-D3) showing mild positive immune reaction in endothelial cells of blood vessels and few stromal cells. Inset showing higher magnification of blood vessels. VEGF immunohistochemistry, counterstained with H x10. (E): Histogram of the mean surface area fraction of VEGF expression in various groups. C vs control group, P vs PCO group, E vs EX- treated group.

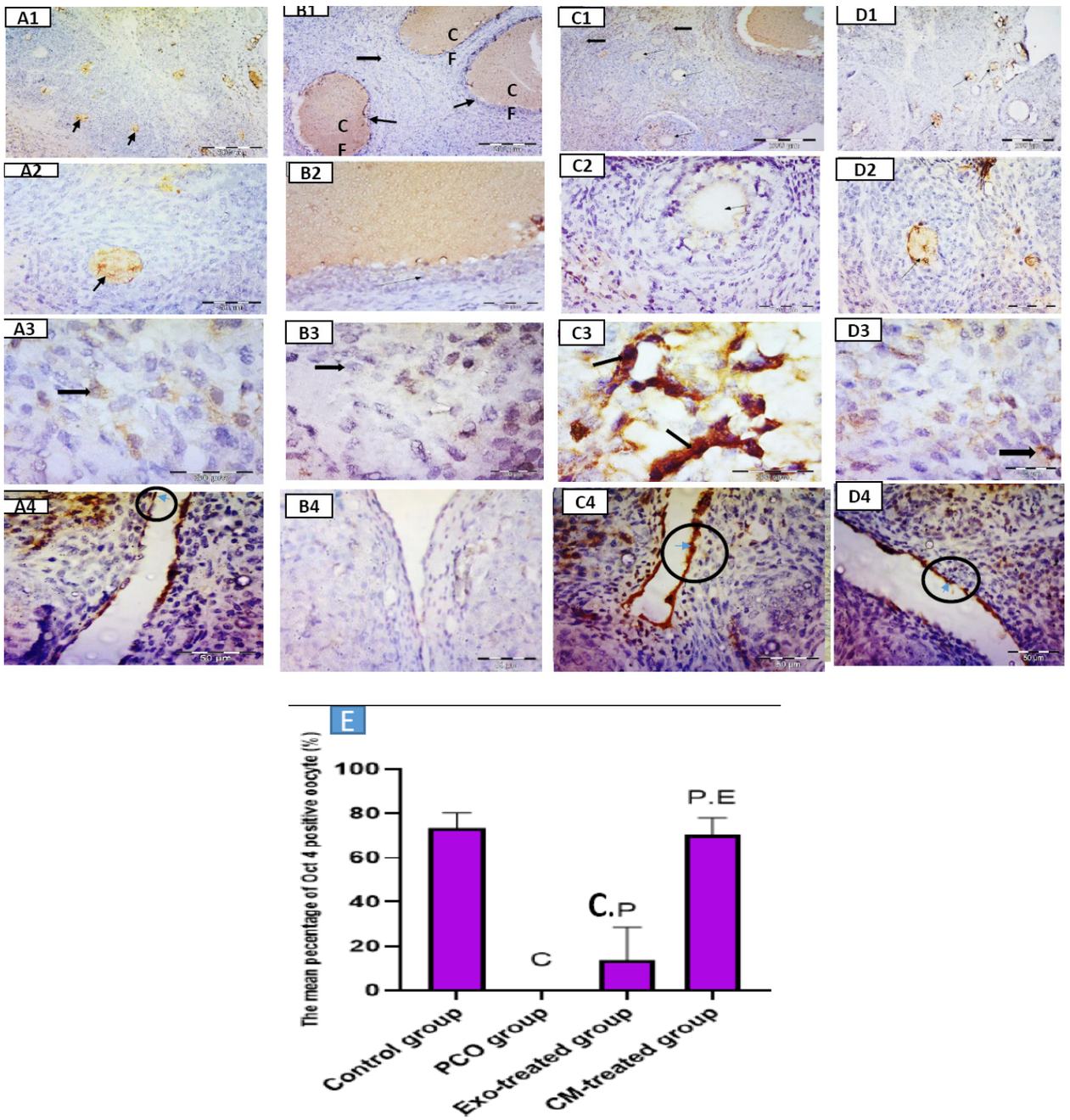


Fig. 5: Photomicrographs of rat ovarian tissues immunostained for Oct 4 from the control group (A1-A2) showing positive expression mainly in the oocyte of growing follicles (thin arrows), (A3) stromal cells showing mild positive expression (elbow arrow) and (A4) showing positive expression of Oct4 in the germinal epithelial cells with some cells with positive nuclear expression (circle). PCO group (B1-B4) showing nearly negative expression in cells of cystic follicles (arrows), stromal cells (elbow arrow) and germinal epithelium (thick arrow) , Ex- treated group (C1-C3) showing negative expression in the oocytes (C1-C2), positive expression mainly in the stromal cells (C3) and in germinal epithelial cells with some cells with positive nuclear expression (circle). (C4). CM-treated group (D1-D2) showing positive expression in the oocytes (arrows) scarcely immune-positive cells in the stroma (elbow arrow)(D3) and (D4) showing positive expression in germinal epithelial cells with some cells with positive nuclear expression (circle). OCT 4 immunohistochemistry, counterstained with H x10 (A1-A4-B1-B4,C1-C4-D1-D4)& X 40 (A2- B2- C2-D2,)&X100 (A3-B3-C3-D3) (E): Histogram showing the mean percentage of Oct 4 positive oocytes in the studied groups (n=6). C vs control group, P vs PCO group, E vs Ex- treated group; significant at $p< 0.05$.

Table 1: Serum testosterone hormone level in different groups. *P value* is significant < 0.05.

Group	Mean ±SEM	*p-value*
Control group	1. 047 ± 0.02	
PCO group	2.67 ± 0.05	0.0001^{C}
Ex-treated group	1.83 ± 0.04	0.0001^{C} 0.0001^{P}
CM-treated group	1.055 ± 0.02	0.0001^{P} 0.0001^{E}

c vs control group, P vs PCO group, E vs Ex-treated group.

Table 2: Mean LH/FSH ratio. *P-value* is significant < 0.05.

Group	Mean ±SEM	*p-value*
Control group	0.57 ± 0.007	
PCO group	1.34 ± 0.007	0.0001^{C}
Ex-treated group	0.88 ± 0.29	0.0021^{C} 0.0001^{P}
CM-treated group	0.59 ± 0.014	0.0001^{P} 0.0037^{E}

c vs control group, P vs PCO group, E vs Ex-treated group.

Table 3: Mean Insulin hormone level in different groups. *P-value* is significant < 0.05.

Group	Mean ±SEM	*p-value*
Control group	0.57 ± 0.007	
PCO group	1.34 ± 0.007	0.0001^{C}
Ex-treated group	0.88 ± 0.29	0.0021^{C} 0.0001^{P}
CM-treated group	0.59 ± 0.014	0.0001^{P} 0.0037^{E}

c vs control group, P vs PCO group, E vs Ex-treated group.

Table 4: Mean number of various follicles in groups

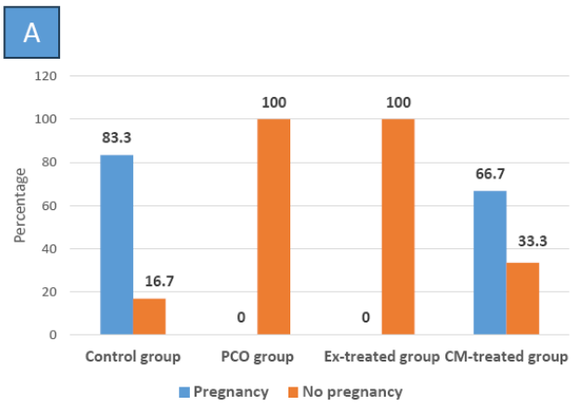
Group/follicles	Primordial follicles	Primary follicles	Secondary follicles	Mature graffian follicles	Corpus luteum	Cystic follicles
Control group	19.83 ± 0.94	10.17 ± 0.47	8.5 ± 0.42	4.66 ± 0.33	6.16 ± 0.4	0.66 ±.0.33
PCO group	$3.83 \pm 0.47^{*C}$	$4.16 \pm 0.30^{*C}$	$0.33 \pm 0.2^{*C}$	$0.16 \pm 0.16^{*C}$	$0.16 \pm 0.16^{*C}$	$4.66 \pm 0.33^{*C}$
Exo-treated group	$13 \pm 0.73^{*C.P}$	$6.83 \pm 0.47^{*C.P}$	$4.66 \pm 0.33^{*C.P}$	$3 \pm 0.25^{*C.P}$	$4.3 \pm 0.21^{*C.P}$	$1.5 \pm 0.42^{*C.P}$
CM-treated group	$13 \pm 0.73^{*P.E}$	$9.33 \pm 0.42.^{*P.E}$	$8 \pm 0.5^{*P.E}$	$4.33 \pm 0.42^{*P.E}$	$5.8 \pm 0.4^{*P.E}$	$0.83 \pm 0.3^{*P.E}$

*: significant, C: significant versus control group, *P*: significant versus PCO group, E: significant versus EX- treated group.

Table 5: Rate of pregnancy in studied group (chi square test)

Group	Total animals (n)	Pregnancy		*p value*	*P value* between all groups
		Yes	No		
Control group	6	5 (83.3%)	1 (16.7%)		
PCO-group	6	0 (0%)	6 (100%)	0.003^{*C}	
EX-treated group	6	0 (0%)	6 (100%)	0.003^{*C}	0.002
CM-treated group	6	4 (66.7 %)	2 (33.3 %)	0.505^{C} 0.014^{*P} 0.014^{*E}	

*: significant, C mean significant versus the control group, *P* mean significant versus PCO group, E mean significant versus EX-treated group. *P value* is significant if (< 0.05)



Graph A: The rate of pregnancy in studied group

DISCUSSION

The Polycystic ovary syndrome is a metabolic, reproductive and psychological problem with no definite cause[24].

Many types of stem cells, including MSCs mesenchymal stem cells have been widely used in treatment of the reproductive problems . However, because of the limitation of living cells injection, the use of their paracrine secretions seems to be a more promising option[25]. It has been reported that the therapeutic effect of these stem cells are mainly through production of their paracrine secretion such as cytokines, chemokines, growth factors, and soluble factors known as extracellular vesicles[26].

The drug called letrozole was used to induce model of PCO[44]. This work used the same drug to compare the effect of MSCS derived CM and exosomes in alleviating biochemical and morphological characters of PCO model.

The PCO model in this study was characterized by increase serum level of testosterone and insulin with high FSH/ LH ratio, the same results were obtained by[27].

In this research, both the CM-treated and EX-treated groups had improved biochemical findings; however, the CM-treated group shown a more significant improvement. This lined up with the findings of Faruk *et al*.[21], who treated a rat model of PCO using exosomes produced from stem cells. In another model of PCO established by letrozole, CM obtained from MSCs enhanced the hormonal profile[15].

H and E staining revealed several enlarged cystic follicles of varying sizes occupying the cortex and numerous congested blood vessels in the medulla. The granulosa cell layers of the cystic follicles wall were noticeably thinner, whereas the theca cell layers were noticeably thicker. A large number of atretic follicles with the lack of the corpus luteum was obvious in this study. The same results appeared lined with our previous study[8-45].

Compared to the control group, the ovarian architecture of the EX-treated group was moderately improved, with a considerable reduction in the number of developing and mature follicles. On the other hand, the CM-treated group exhibited almost normal ovarian architecture. Morphological alterations in a PCO model were shown to be ameliorated by CM produced from MSCs[15]. Zhao *et al*.[28], reported that exosomal miR-323-3p in cumulus cells of PCOS inhibits apoptosis and promotes cell proliferation through acting on programmed cell death 4 (PDCD4).

Although moderate morphological improvement occurred in Ex-treated -group regarding the number of follicles and appearance of some mature follicles and corpora lutea, the ovarian medulla still had congested blood vessels and animals of this group failed to get pregnant after breeding with males.

In a trial to understand the possible mechanisms behind these results, immunohistochemical study was performed.

The vascular endothelial growth factor (VEGF) is considered a key angiogenic growth factor that promote angiogenesis through receptor tyrosine kinase VEGF receptors[29]. VEGF expression was significantly high in the PCO group of the this study when compared with the control group. These results were also observed by study of Attia et al.,[30] who reported the high expression of VEGF with PCO problem .

A number of researches have shown that hyperandrogenism and hormone deficiency cause angiogenic abnormalities in the ovaries of PCO patients[31,32]. Angiogenesis abnormalities, particularly in the VEGF-involving pathways, have emerged as the most significant result in PCOS research during the last several years[33].

Hyper-androgenism is the hallmark of PCOS, and among the many variables that influence ,VEGF, expression, the most well-established explanation for the elevated levels of ,VEGF, in PCOS patients is hyper-androgen in the ovaries[34].

Both treated groups showed significant lower expression than PCO group, however, CM treated group showed a significant lower expression than EX-treated group. Therefore, the possible mechanism behind low fertility in the EX-treated group may be due to high level of VEGF that resulted in neoangiogenesis. The lower level of VEGF expression detected in Ex-treated groups of this study compared with PCO group was in accordance with study of Pakravan *et al*.,[35] who told that MSCs derived exosomes have anti angiogenic effect by suppression of VEGF in breast cell cancer. In contrast, other studies reported that MSCs derived exosomes have angiogenic effect and stimulate VEGF[36].

In order to understand the underlying cause of high vascularity in EX-treated group in comparison with CM-treated group, Oct4 immunohistochemistry was performed.

Oct4, octamer-binding transcription factor 4 is a marker of stem cells that is linked to the development of many cell types. According to Heng *et al*.[37], Oct4 controls the growth of granulosa and membrane cells, and it may enhance the potency of oogenesis by promoting the proliferation of ovarian mesenchymal cells. Oct4, which is involved in oocyte development and meiosis, is found in the granulosa cells of growing follicles and has been linked to several key functions in these cell types. Gonadotropins control the expression and function of Oct4, which is abundantly expressed in the ovary[38]. According to Heng *et al*.[37], granulosa cells express much higher levels of Oct4 through FSH. The PI3K/Akt signaling system is the pathway mediating FSH-induced cellular viability via Oct4.

In the current study, the immunoexpression of Oct 4 was nearly negative in the PCO group which indicated absence of this stem cell factor from apoptotic granulosa cells of cystic follicles that did not contain oocytes. Faruk *et al*.,[21] in their study found a minimal faint expression of Oct4 in their PCO model.

Regarding the EX treated group and CM-treated group expression of Oct4 was very variant. The CM treated group expression was mainly in the oocyte, some cells of germinal epithelium and some of the interstitial stromal cells but in the EX-treated group; the expression of Oct4 was mainly in interstitial stromal cells and some cells of germinal epithelium.

Stimulatory effect of MSCs-derived exosomes on Oct4 immune-expression in the treatment of cisplatin induced ovarian damage was previously reported[39].

It was postulated that the ovary may include stem cells that play role in the development of new follicles at various points in reproductive life, which could explain why Oct4 is expressed in the germinal epithelium of this research. The stem cells home surface epithelium of the ovary, which may be found in both the adult and the menopause ovaries, as well as in ovaries of premature failure. VSELs are small population of embryonic-like stem cells expresses nuclear other markers and Oct4 specific to pluripotent and primordial germ cells. Then there are slightly larger stem cells called OSCs with cytoplasmic Oct4, which are similar to spermatogonial stem cells in the testes. These cells divide actively and are part of the ovarian stem cell pool[40]. While embedded in the ovary surface epithelium (OSE), asymmetric cell division used for renew VSELs by giving birth to OSCs. The OSCs undergo symmetric cell division in the form of meiosis, clonal expansion and ultimately develop into an oocyte, which is then assembled as a primordial follicle. Under normal conditions, the epithelial cells that line surface of the ovary provide stem cells niche. The epithelial-mesenchymal transition then undergo to become the granulosa cells, which are necessary for the construction of primordial follicle. Menopause may result from stem cells losing their capacity to develop follicles as a result of age-related decline in epithelial cell activity[41]. Therefore, the expression of, oct4, in the germinal epithelium of CM-treated-group most probably were VSELs and OSCs with nuclear and cytoplasmic expression respectively. These stem cells differentiated and finally formed ovarian follicles that were more obvious in the CM-treated- group.

A previous study by Hess *et al*.,[42] reported that knock out of Oct4 gene resulted in inhibition of angiogenesis. Another study by Ji *et al*.,[43] found that Oct4 overexpression resulted in stimulation of MSCs angiogenesis and its level is parallel with the level of VEGF.

Therefore, the high level of expression of, Oct4, in the cells of stroma of EX-treated group might result in stimulation of angiogenesis and high level of VEGF that most probably resulted in disturbed folliculogenesis and eventually low fertility. Although the, CM, contain exosomes but different pattern of expression was observed in this group. This differential expression might be due to low concentration of exosomes in the conditioned media in comparison with exosomes in the EX-treated group.

CONCLUSION AND RECOMMENDATION

The PCO model demonstrated that stem cell generated CM restored the histological structure of ovaries and fertility more effectively than Exosomes. Although MSCS derived exosomes are effective in treatment of many diseases, they may have disadvantages of overstimulating angiogenesis. More studies are required for comparing CM and exosomes in different diseases models.

CONFLICT OF INTERESTS

There are no conflicts of interest.

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الملخص العربى

التعبير التفاضلي لعامل الاوكتا فور (Oct4) وعامل النمو البطاني الوعائي في مبايض نموذج الجرذ المتعدد تكيس المبايض بعد العلاج باستخدام الوسائط المكيفة والإكسوسومات المشتقة من الخلايا الجذعية الوسيطة

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مقدمة: متلازمة تعدد تكيس المبايض هي أكثر أمراض الغدد الصماء شيوعًا التي تؤثر سلبا على النساء. في الطب التجديدي، يمثل الان العلاج الخالي من الخلايا كوسائط مكيفه، والأكسوزومات استراتيجيات بديلة لعلاج مثل هذه الحالات يعد عامل النمو البطاني الوعائي علامه وعائيه لها دور في تكيسات المبايض .كما يعد أيضا الاوكتافور Oct4 (عامل النسخ المرتبط) أحد عوامل النسخ اللازمة لإنتاج الخلايا الجذعية المحفزة المتعددة القدرات و الاوعيه الدمويه .

الهدف من الدراسه : مقارنه التمييز التفاضلي بين تعبير لعامل الاوكتا فور و عامل النمو البطاني الوعائي في مبيض نموذج الجرذان المتعدد التكيسات بعد العلاج بالوسائط المكيفة والإكسوسومات.

المواد وطريقه البحث: تم استخدام ٧٠ جرذ ابيض منها ١٢ ذكر بالغ للتزاوج و ١٠ لاستخراج الوسط المكيف والاكسوسومات و تقسيم عدد ٤٨ من إناث الجرذان عشوائياً إلى ٤ مجموعات:

المجموعة الضابطة، مجموعة تكيسات المبيض ، المجموعة المعالجة بالوسائط المكيفة ، والمجموعة المعالجة الإكسوسومات المشتقة من الخلايا الجذعية الوسيطة. تم إعطاء إناث الجرذان ليتروزول عن طريق الفم (١ مجم/كجم/ يوم) مرة واحدة يوميًا لمدة خمسة أسابيع من أجل تحفيز تكيسات المبايض تم إجراء التحليل الكيميائي الحيوي والأنسجة والكيمياء المناعية للتعبير المناعي لـ VEGF و Oct4. تم تقييم معدل الخصوبة.

النتائج: أظهرت النتائج ان الوسائط المكيفة لها تأثيرات علاجية أكثر أهمية من الإكسوسومات في استعادة النتائج الطبيعية للمبيض قد كان تعبير اوكتا فور متغيرًا. علاوة على ذلك، أظهرت إناث الجرذان المعالجة بالوسائط المكيفة معدل خصوبة أعلى بكثير من تلك المعالجة بالإكسوسومات.

الخلاصة والتوصيات : العلاج الخالي من الخلايا أصبحت الان من الطرق الأكثر أمانًا و أفضل عمليا لتجديد الأنسجة. جيث أظهر نموذج المبيض المتعدد التكيسات أن الخلايا الجذعية المولدة للوسائط المكيفة أعادت البنية النسيجية الطبيعية للمبيضين والخصوبة بشكل أكثر فعالية من الإكسوسومات من خلال تنظيم التعبيرعن عامل الخلايا الجذعية المسمي (Oct4) اوكتا فور ولكن بالرغم من ذلك كان هناك زياده في معدل تكوين الاوعيه الدمويه في موذل تكيس المبيض لذلك يجب مقارنه النوعين في نماذج الأخرى لباقي الامراض .