Effect of Adipose-derived stem cells versus clomiphen on treatment of experimental polycystic ovary in rats: Histological and Immunohistochemical study

Original Article

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ABSTRACT

Background: Polycystic ovarian syndrome (PCO) is a syndrome of ovarian dysfunction with polycystic ovary morphology and features of hyperandrogenism.

Aim of work: This work aimed to compare the histological effects of adipose-derived stem cells (ADSC) to clomiphen on the treatment of letrozole experimentally-induced polycystic ovary in rats.

Materials and methods: 50 adult female albino rats were divided into: Group I (control), group II (PCO group) that received Letrozole for 21 days. Then 5 rats from each group were sacrificed to prove PCO. Remaining 20 rats of PCO group were subdivided: Subgroup IIB (Recovery group), Subgroup IIC (clomiphene-treated group), Subgroup IID (ADSCs-treated group), Subgroup IIE: (clomiphene + ADSCs treated group), while the control group was subdivided into subgroups IB, IC, ID and IE, that received the solvents for 3 more weeks as in the corresponding subgroups in G II. Ovarian specimens were processed and stained with H and E, Periodic acid Schiff (PAS) reaction, immunohistochemichal stain using Ki 6. Morphometric and statistical analyses were done.

Results: PCO group showed dilated ovarian cysts, thinned granulosa layer, cell debris appeared in the follicular cavity. Clomiphen and ADSC improved the ovarian histological architecture where various stages of ovarian follicles were noticed, including corpora lutea and few cysts.

Conclusion: Clomiphen effect was found to be comparable with ADSC effect , while combining ADSC + Clomiphen gave the best results.

INTRODUCTION

Polycystic ovary syndrome (PCOS) is a complex metabolic/ endocrine disorder that affects 6% of women in reproductive age[1]. It is considered to be one of the important causes of female subfertility[2]. There is evidence that some genes may be responsible for the occurence of this syndrome, including those which codify the enzymes related to the metabolism of androgen in the ovary[3].

Treatment of this syndrome includes: changing of life style, medical and surgical treatment[4]. Clomiphene was used to treat cases of oligomenorrhea and anovulation. Women undergoing treatment with clomiphen were found to have high rates of pregnancy[5]. Clomiphen use was associated with many side effects as ovarian enlargement; ovarian hyperstimulation syndrome; hot flushes; multiple pregnancies; gastrointestinal discomfort[6].

Premature ovarian failure is a cause of female infertility. It has been found that ADSC significantly improved ovarian function after ovarian injury induced by chemotherapy. It was found that ADSC could increase oocyte and follicle number through changes in gene-expression and through production of cytokines[7].

The main experimentally-induced PCO models in rat, that have been approved, include sex steroid-induced models, constant light exposure, hypothalamic lesions[8]. Moreover, Letrozole is an aromataze enzyme inhibitor that causes androgen excess and leads to development of polycystic ovary syndrome[9].

AIM OF THE WORK

This work aimed to compare the effect of adipose-derived stem cells to clomiphen on the treatment of experimentally-induced polycystic ovary in rats.

MATERIALS AND METHODS

(I) Adipose derived stem cells (ADSC):

PKH26 labeled ADSC were purchased from the Biochemistry Department, Faculty of Medicine, Cairo University. Cells were provided as first-passage culture

cells suspended in phosphate buffered saline (PBS) solution. PBS was purshased from Gibco/ Invitrogen, Grand Island, New York, USA.

(II) Drugs:

1. Letrozol (Trade name Femara): The drug was purchased from Novartis pharmaceutical company, Switzerland, in the form of tablets containing 2.5 mg of Letrozol. Tablets were crushed and dissolved in carboxmethlycellulose (CMC). CMC was purchased from Allergan pharmaceutical company, Ireland, in the form of eye drops (Trade name Refresh plus) containing 0.5% of carboxmethlycellulose sodium.

2. Clomiphen (Trade name Clomid): The drug was purchased from Sanovi aventis pharmaceutical company, USA, in the form of tablets containing 50 mg of clomiphen citrate. Tablets were crushed and dissolved in normal saline.

(III) Animals:

The study was done at the Animal House of Kasr Al Ainy Faculty of Medicine, following the ethical guidelines for the use and care of Laboratory Animals, all the used procedures were approved with the local Ethics Committee. Fifty adult female rats, weighing 150-200g were used. All rats were selected in the estrous phase based on vaginal smears (Fig. 1A). Estrous phase was identified by the presence of large cornified cells arranged in clumps. Rats were housed in hygienic stainless steel cages and kept in clean and well ventilated room. They were allowed free access to water and were fed standard chow diet.
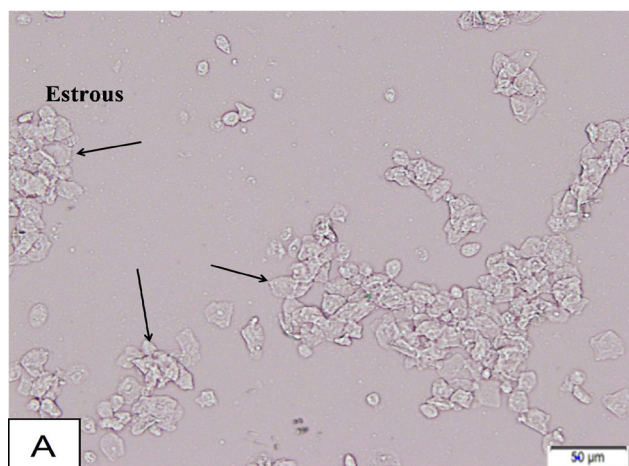
Fig. 1A: A photomicrograph of unstained vaginal smear from female rate (X 200) at the estrous phase, showing predominance of large cornified cells arranged in clumps (black arrows).

(IV) Experimental Design:

Rats were divided into two groups:

1. Control group (Group I): (n= 25rats) that received vehicle only (0.5 ml aqueous solution of carboxmethlycellulose (CMC) by intragastric intubation, once daily orally for 21 days.

2. PCO group (Group II): (n= 25rats) that received Letrozole at a concentration of 1 mg/kg (0.15mg - 0.2mg) dissolved in 0.5 ml of CMC orally once daily given by intragastric intubation for 21 days[10].

On the day subsequent to last letrozole dose administration, 5 rats from control group (Subgroup IA) and from the PCO group (Subgroup IIA) were sacrificed; their ovaries were excised. Specimens were fixed in buffered formalin solution and processed for paraffin sections. The sections were examined histologically to confirm the development of polycystic ovary in group II.

The remaining 20 rats of the control group were subdivided into four subgroups:

a. Subgroup IB: (n=5rats) that continued without treatment for another fourteen days.

b. Subgroup IC: (n=5rats) that received 0.5ml normal saline daily orally given by intragastric intubation for fourteen days.

c. Subgroup ID: (n=5rats) that received single IV injection of 0.3 ml of PBS (pH 7.4) into the rat tail vein. The injection was repeated again the following day.

d. Subgroup IE: (n=5rats) that received single IV injection of 0.3 ml of PBS (pH 7.4)) into the rat tail vein for two successive days, then rats received 0.5ml normal saline daily orally for 12 days.

The remaining 20 rats of the PCO group were subdivided into four subgroups:

a. Subgroup IIB: (Recovery group) (n=5rats) that continued without treatment for another fourteen days.

b. Subgroup IIC: (n=5rats) that received clomiphene at concentration of 1 mg/kg (0.15mg - 0.2mg) dissolved in 0.5ml normal saline orally given by intragastric intubation for fourteen days[10].

c. Subgroup IID: (n=5rats) that received a single IV injection of 1×106 autologous ADSCs in a volume of 0.3 ml of PBS) into the rat tail vein. The injection was repeated again the following day at the same dose[7].

d. Subgroup IIE: (n=5rats) that received two injections of ADSCs on two successive days, followed by clomifen as previously described for 12 days.

After five weeks from the beginning of the experiment, all the rats were sacrificed; ovaries were excised.

Specimens were fixed in10% buffered formalin solution

and processed for paraffin sections of 5~7 μm thickness, sections mounted on canada balsam coated slides in case of ordinary and special staining and poly-L-lysine coated and charged slides in case of immunostaining. The sections were subjected to the following histological stains :

- Hematoxylene and Eosin[11].

- Periodic acid Schiff (PAS) reaction[11].

- Immunohistochemical staining using and Ki 67 antibodies to test for proliferation of granulosa cells[12].

## Staining procedures for immunohistochemistry:

Paraffin sections were deparaffinized in xylene for 30 minutes and then rehydrated in descending grades of ethanol and then brought to distilled water for 5 minutes. Sections were incubated in hydrogen peroxide for 30 minutes and then rinsed in PBS (3 times, 2 minutes each). Primary antibody {Anti Ki-67 antibody: Ki67 (Clone SP6): A rabbit monoclonal antibody (Lab Vision Corporation laboratories, USA, RM-9106-R7)} was applied to the sections , 2 drops or 100μl for each section. Then slides were rinsed well in PBS (3 times, 2min. each), incubated for 20 minutes with 2 drops of biotinylated secondary antibody for each section then rinsed well with PBS. Each section was incubated with 2 drops (100 μl) enzyme conjugate "Streptavidin-Horseradish per oxidase" for 10 minutes at room temperature then washed in PBS. Substrate-chromogen (DAB) mixture 2 drops was applied to each section and incubated at room temperature for 5~10 min. then rinsed well with distilled water. Slides were counterstained with hematoxylin, dehydrated and mounted. All steps were performed in a humidity chamber to prevent drying of the tissues. Non-specific background elimination step was omitted. Ki-67 antibody: +ve cells showed brown nuclear deposits[12].

Isolation, culture and labeling of ADSC

Adipose tissue was excised from the inguinal pad of fat in anaesthesized rat under complete aseptic condition. The adipose tissue was resected and placed into a sterile tube containing 15 ml of a PBS. Enzymatic digestion was performed using collagenase II (Serva Electrophoresis GmbH, Mannheim) in balanced Salt Solution for 60 minutes at 37°C. Digested tissue was filtered and centrifuged, and erythrocytes were removed by treatment with erythrocyte lysis buffer. The cells were transferred to tissue culture flasks with Dulbecco Modified Eagle Medium (DMEM, Gibco/ BRL, Grand Island, New York, USA) supplemented with 10% fetal bovine serum (Gibco/BRL) and, after an attachment period of 24 hours, non-adherent cells were removed by a PBS wash. Attached cells were cultured in DMEM media supplemented with 10% fetal bovine serum FBS, 1% penicillin-streptomycin (Gibco/ BRL), and 1.25 mg/L amphotericin B (Gibco/BRL), and expanded in vitro. At 80-90% confluence, cultures were washed twice with PBS and the cells were trypsinized with 0.25% trypsin in 1 mM EDTA (Gibco/BRL) for 5 min at 37°C.

After centrifugation, cells were resuspended with serum-supplemented medium and incubated in culture flask (Falcon). The resulting cultures were referred to as first-passage cultures and expanded in vitro until passage four[13].

Subgroups IID and IIE received single IV injection of 1×106 PKH26 labelled ADSC in a volume of 0.3 ml of PBS (pH 7.4). The injection was repeated again the following day[7]. Localization of PKH26 labelled ADSC was visualized by the fluorescent microscope, in the ovaries of the injected rats (Fig. 1B).
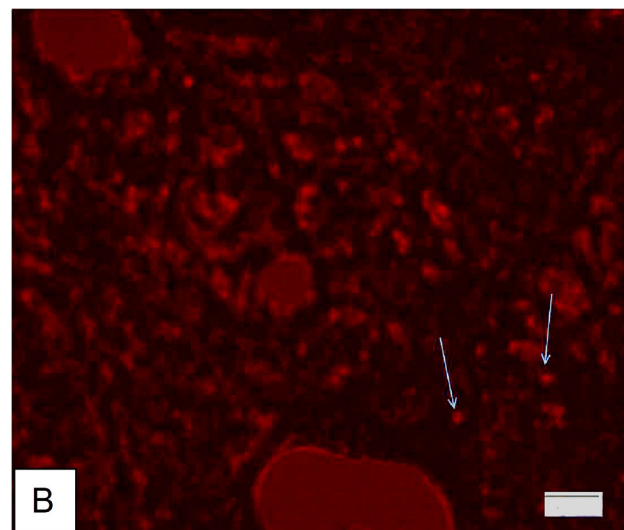
Fig. 1B: A photomicrograph of an ovarian section of adult female albino rat in subgroup IID showing positive red immunofluorescent stem cells housed inside the ovarian follicles (arrows). (PKH26 immunofluorescence x 400)

Morphometric study:

Morphometric study done using Leica Qwin 500 LTD image analyser (Cambridge UK), measuring mean area percent of PAS, optical density of PAS reaction and immunopositivety of Ki 67 antibodies.

Statistical analysis:

Data were tabulated in the form of mean ± standard deviation. Comparisons between groups were done using unpaired t test when comparing 2 groups and analysis of variance (ANOVA) with multiple comparisons post hoc test when comparing more than 2 groups. The probability (P) value obtained from statistical tables was directly supplied to the computer using SPSS software version 16. Results were considered statistically significant when *P value* was < 0.05.

RESULTS :

I- Histological study:

1. Light microscopic examination of ovarian sections of the control group stained with Hematoxylene and Eosin showed the normal histological structure of the ovary (Fig. 2A). PCO group (subgroup IIA) showed large cystic ovarian follicles,

ovarian follicles with thin granulosa layer and cell debris within the follicular cavity (Fig. 2B). Subgroup IIB (Recovery group) appeared similar to those of PCO group with almost same findings (Fig. 2C). Subgroup IIC (received clomiphen) showed improvement in the histological structure of the ovaries, where various stages of ovarian follicles were noticed, including corpora lutea (Fig. 2D). Subgroup IID (received ADSC) showed few ovarian cysts and ovarian follicles in different stages of development (Fig. 2E) Subgroup IIE (received ADSC+ clomiphen) showed histological structure similar to that seen in the control group (Fig. 2F).

2. PAS stained sections: Examination of ovarian sections of the control group showed strong positive PAS reaction in the intercellular spaces between granulosa cells, within the theca layer and in the granulsa basement membranes and in zona pellucida (Fig. 3A). PCO group (subgroup IIA) showed decreased PAS reaction in the intercellular spaces within the follicular wall and strong one in the theca layer (Fig. 3B). Subgroup IIB (recovery group) showed almost same results as subgroup IIA (Fig. 3C). Subgroup IIC (received clomiphen) strong positive PAS reaction in the intercellular spaces between granulosa cells, within the theca layer and in the granulsa basement membranes and in zona pellucida (Fig. 3D). Subgroup IID (received ADSC) showed strong positive PAS reaction in the intercellular spaces paces between granulosa cells, within the theca layer and in the granulsa basement membranes and in zona pellucida (Fig. 3E). Subgroup IIE (received ADSC + clomiphen) strong positive PAS reaction in the intercellular spaces between granulosa cells, within the theca layer and in the granulsa basement membranes and in zona pellucida. (Fig. 3F).

Morphometric results (Tables 1- 4):

Three weeks after starting the experiment, the mean area % of PAS positive reaction showed significant decrease in the granulosa of PCO group compared to the control, however the mean area % in the theca layer of the PCO group showed non-significant increase compared to the control. Five weeks after the experiment, the mean area % showed a significant decrease in the granulosa and a non-significant increase in the theca of the recovery group compared to the control subgroups.The mean area % in the granulosa of subgroup IIC (received clomiphen), subgroup IID (received ADSCs) and subgroup IIE (received ADSCs + clomiphen) were significantly higher than that in the recovery group, however, the comparison was non-significant in the theca layer. On comparing subgroup IIC and subgroup IID there was no significant difference in mean area % neither in the granulosa nor in the theca. On comparing subgroup IIE either with subgroupIIC or with subgroup IID there was also no significant difference

neither in the granulosa nor in the theca. Moreover, the optical density of PAS positive reaction showed similar statistical results except that on comparing subgroup IIE with subgroup IID, the optical density was significantly higher in granulosa of subgroup IIE, while no significant difference was found in the theca.

2-Immuohistochemical study:

Ovarian sections of the control group (Group I) and its subgroups immunostained for Ki 67, showed strong positive immunoreactivity in nuclei of follicular cells and less one in the theca layer (Fig. 4A). PCO group (Group IIA) showed weak positive immunostaining of the thinned- out granulosa layer (Fig. 4B). Subgroup IIB showed weak positive immunostaining of the thinned- out granulosa layer (Fig. 4C).

Subgroup IIC (received clomiphen) showed strong positive immunoreaction of granulosa cells and few one in theca layer (Fig. 4D). Subgroup IID (received ADSC) immunostained for Ki 67 showed strong positive Ki 67 immunoreaction of granulosa cells and few one in theca layer (Fig. 4E). Subgroup IIE (received ADSC+clomiphen) showed strong positive Ki 67 immunoreaction of granulosa cells and few one in theca layer (Fig. 4F).

Morphometric results (Tables 5&6):

The mean area % of Ki 67 immunoreactivity, 3 weeks from the beginning of the experiment, showed a significant decrease in the granulosa of PCO group compared to the control. After 5 weeks of the experiment, this mean area % in the granulosa of the recovery group showed significant decrease when compared to the control subgroups. The mean area % in the granulosa of subgroups IIC , IID and IIE were significantly higher than that in the recovery group. On comparing subgroup IIC and subgroup IID, the mean area % was significantly higher in the granulosa of subgroup IIC. The mean area % was significantly higher in the granulosa of subgroup IIE as compared to either subgroup IIC or IID.
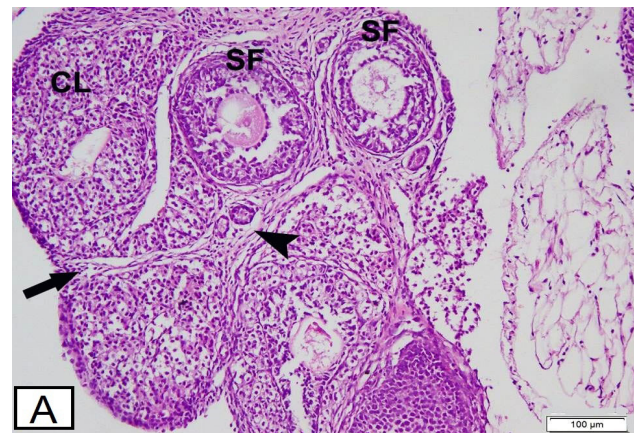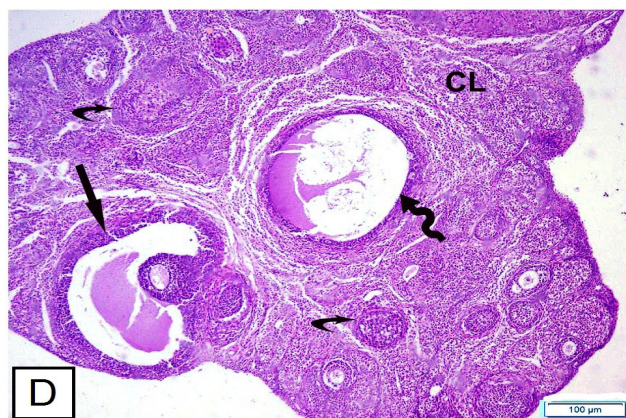

Fig. 2A: A photomicrograph of an ovarian section of adult female albino rat of the control group showing normal histological structure of the ovary, consisting of outer cortex (arrow) and inner medulla (arrow head) , secondary follicles at different stages of growth (SF), corpus luteum (CL).   (H and E X100)
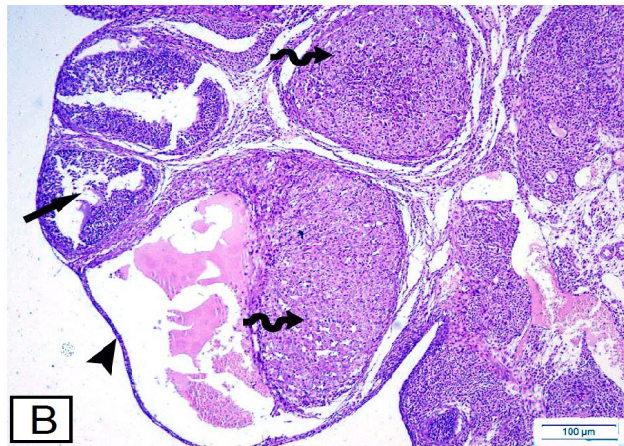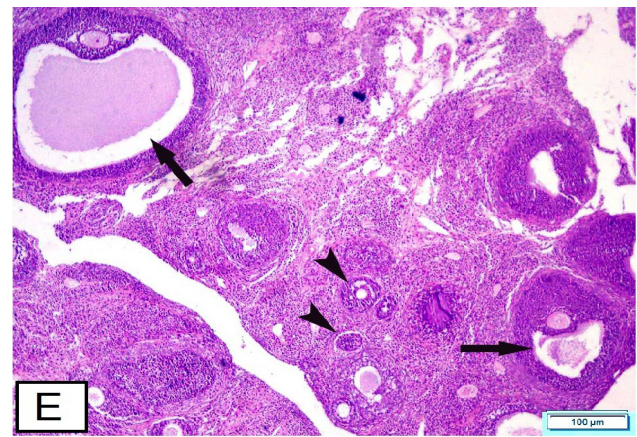
Fig. 2B: A photomicrograph of an ovarian section of adult female albino rat of the PCO group (subgroup IIA) showing a large cystic follicle having markedly thin granulosa layer (arrow head) bulging on the surface of ovary and other follicle exhibit cell debris in the lumen (arrow) and multiple degenerating follicles (spiral arrows).   (H and E X100)
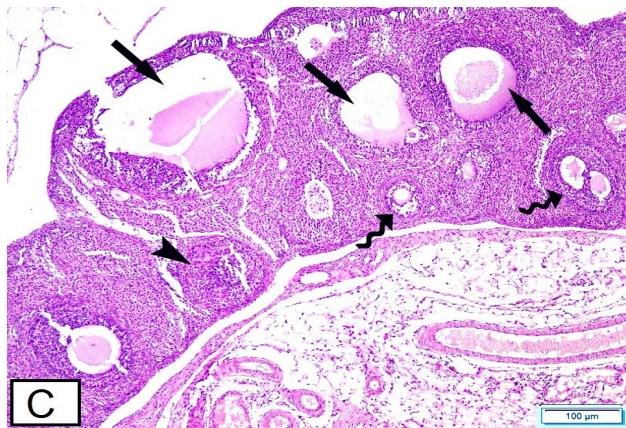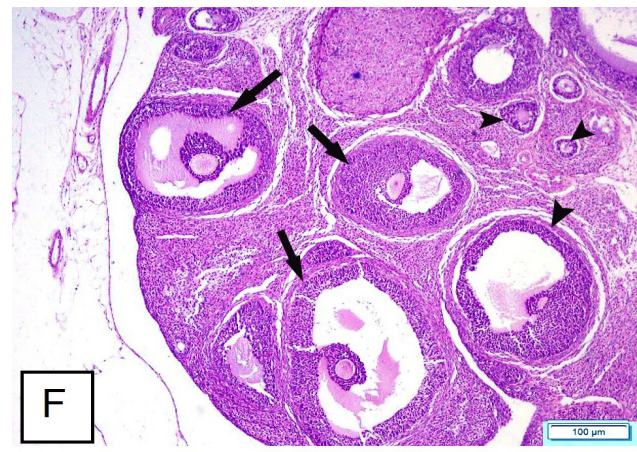

Fig. 2C: A photomicrograph of an ovarian section of adult female albino rat recovery group (subgroup IIB) showing   large cystic follicles (arrow) degenerated follicle (arrow head) and few developing growing follicles (spiral arrows).   (H and E X100)
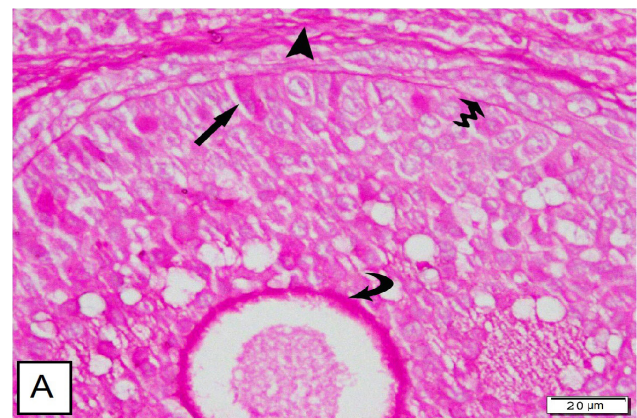

Fig. 2D: A photomicrograph of an ovarian section of adult female albino rat (subgroup IIC) receiving clomiphen showing mature Graafian follicle (arrow), secondary follicle (arrow head), dilated cystic follicle (spiral arrow) small corpora lutea (CL) and atretic follicles (rotated arrow). (H and E X100)
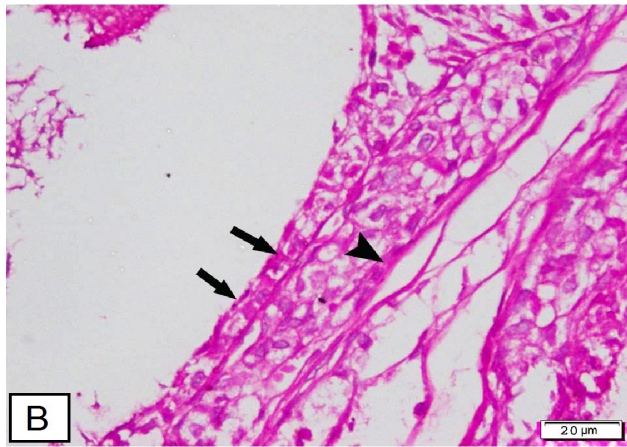

Fig. 2E: A photomicrograph of an ovarian section of adult female albino rat (subgroup IID) receiving   stem cells showing follicles in different stages of development (arrow heads) and mature Graafian follicles (arrows).   (H and E X100)

Fig. 2F: A photomicrograph of an ovarian section of adult female albino rat (subgroup IIE) receiving stem cells and clomiphen showing follicles in different stages of development (arrow heads) and numerous mature Graafian follicles (arrows).   (H and E X100)

Fig. 3A: A photomicrograph of an ovarian section of adult female albino rat of the control group showing a secondary follicle with strong positive PAS reaction in the intercellular spaces between granulosa cells (arrow), within the theca layer (arrow head),in the granulosa basement membranes (spiral arrow) and in zona pellucida (rotated arrow).   (PAS X400)

Fig. 3B: A photomicrograph of an ovarian section of adult female albino rat of subgroup IIA (PCO group) showing moderately positive PAS reaction in the intercellular (arrows) and interfibrous spaces (arrow head) within the follicular wall. (PAS X400)

Fig. 3C: A photomicrograph of an ovarian section of adult female albino rat of subgroup IIB (recovery group) showing moderately positive PAS reaction in the intercellular space (arrow) within the follicular wall and strong one in the theca layer (arrow head). (PAS X400)

Fig. 3D: A photomicrograph of an ovarian section of adult female albino rat of subgroup IIC(group receiving clomiphen) showing strong positive PAS reaction in the intercellular space (arrow) and granulosa basement membrane (spiral arrow), and also strong one in the theca layer (arrow head) and zona pellucida (rotated arrow). (PAS X400)

Fig. 3E: A photomicrograph of an ovarian section of adult female albino rat of subgroup IID (group receiving stem cells)showing strong positive PAS reaction in the intercellular (arrows) and interfibrous spaces (arrow head) within the follicular wall and theca layer. Note the strong reaction in the zona pellucida (rotated arrow) and granulosa basement membrane (spiral arrow). (PAS X400)
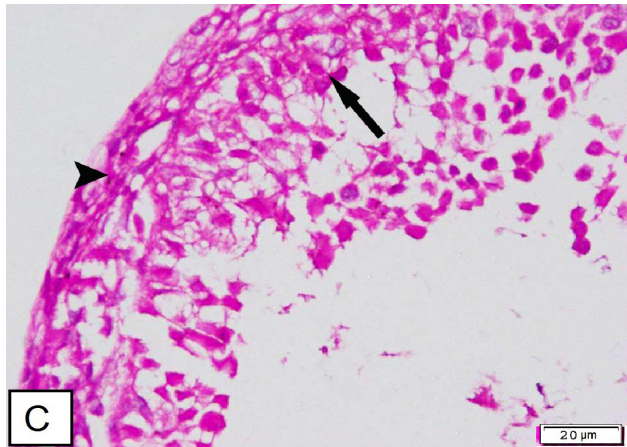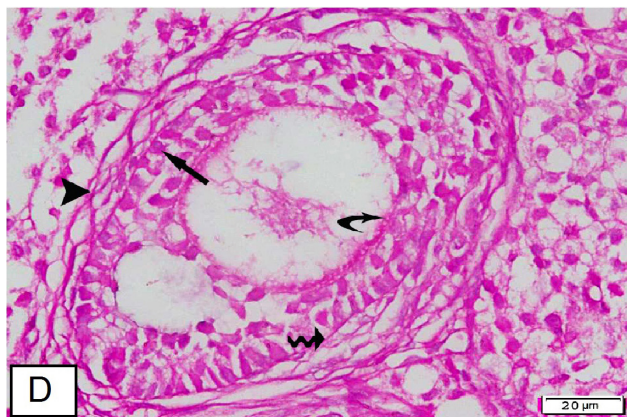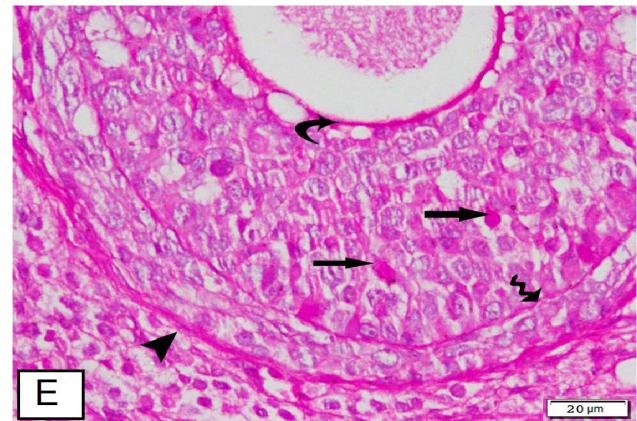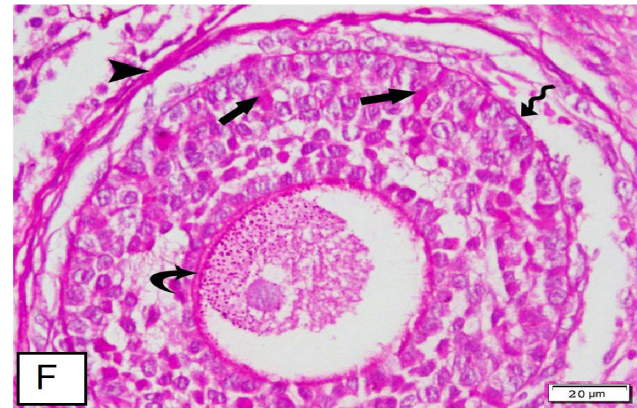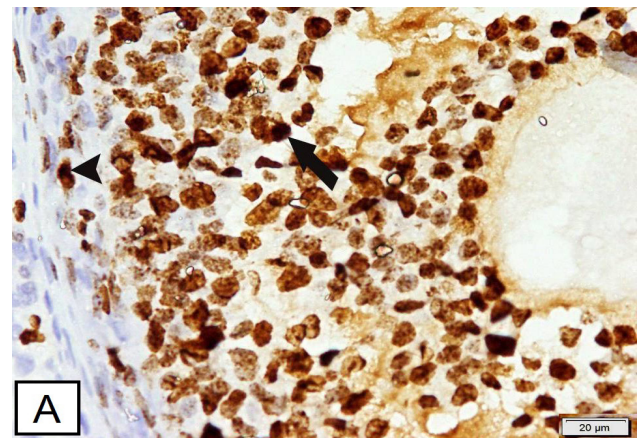

Fig. 3F: A photomicrograph of an ovarian section of adult female albino rat of subgroup IIE (group receiving stem cells and clomiphen) showing strong positive PAS reaction in the intercellular (arrow) and interfibrous spaces (arrow head) within the follicular wall , zona pellucida (rotated arrow) and granulosa basement membrane (spiral arrow). (PAS X400)

Fig. 4A: A photomicrograph of an ovarian section of adult female albino rat of the control group showing strong positive Ki 67 immunoreactivity in nuclei of granulosa (arrow) cells and theca layer (arrow head) in the wall of a secondary follicle. (Ki 67 immunostaining X400)

Fig. 4B: A photomicrograph of an ovarian section of adult female albino rat of subgroup IIA (PCO group)showing positive Ki 67 immunostaining of the thinned- out granulosa layer (arrows) while theca layer shows negative reaction (arrow head).　　　　　(Ki 67 immunostaining X400)

Fig. 4C: A photomicrograph of an ovarian section of adult female albino rat of subgroup IIB (recovery group) showing positive Ki 67 immunostaining of the thinned- out granulosa layer (arrows) while theca layer shows negative reaction (arrow head). (Ki 67 immunostaining X400)
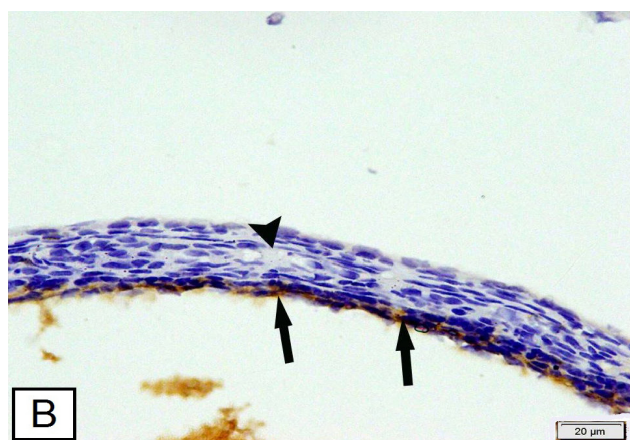

Fig. 4D: A photomicrograph of an ovarian section of adult female albino rat of subgroup IIC (group receiving clomiphen) showing strong positive Ki 67 immunoreaction of granulosa cells (arrows) and few ones in theca layer (arrow head).　　　　　(Ki 67 immunostaining X400)

Fig. 4E: A photomicrograph of an ovarian section of adult female albino rat of subgroup IID (group receiving stem cells) showing a secondary follicle with strong positive Ki 67 immunoreactivity of many granulosa cells (arrows) and few ones in theca layer (arrow head).　　　(Ki 67 immunostaining X400)
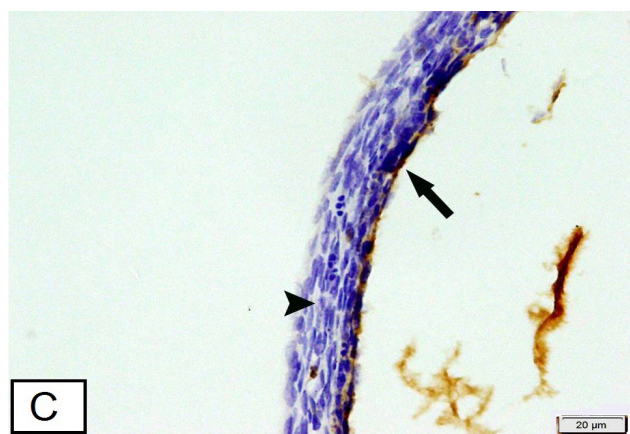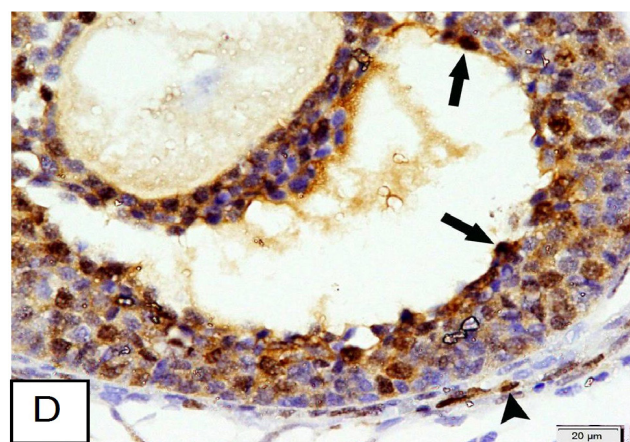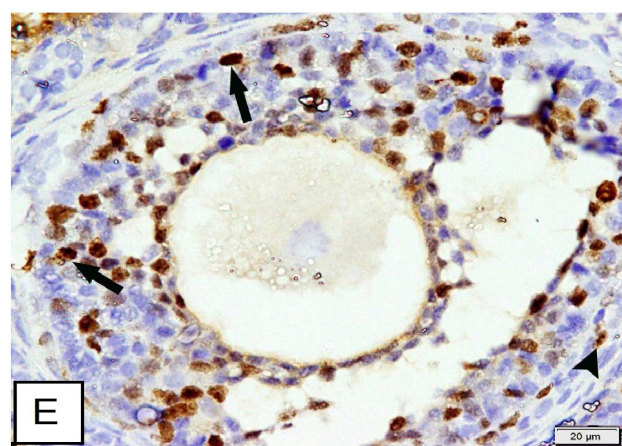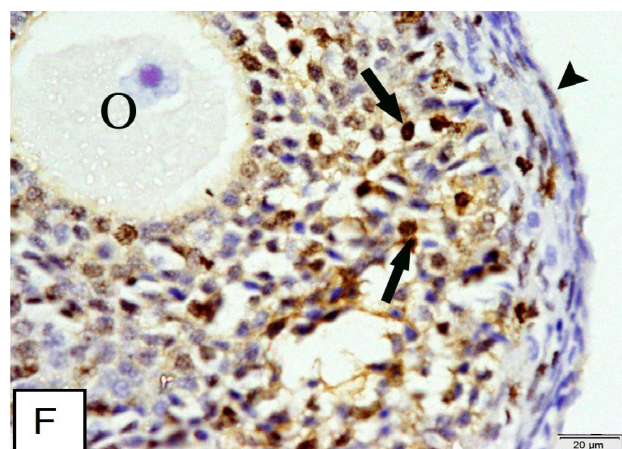

Fig. 4F: A photomicrograph of an ovarian section of adult female albino rat of subgroup IIE (group receiving stem cells and clomiphen) showing strong positive Ki 67 immunoreactivity of many granulosa cells (black arrows) and few in theca layer (arrow head) in the wall of a secondary follicle with aprominent oocyte (o).　　　(Ki 67 immunostaining X400)

Table 1: The mean area percent of glycogen and other periodate reactive carbohydrates in the control and PCO groups after 3 weeks of the experiment

	control	PCO
area % of CHO of granulosa	11.20 ±1.18	7.72±1.78*
area % of CHO of theca	6.80 ±2.03	7.33±1.97

Values are presented as mean ±SD

*: statistically significant compared to corresponding value in control group *(P<0.05)*

Table 2: Mean area percent of CHO in all the studied groups after 5 weeks of the experiment

Parameters	control	recovery	clomiphen	stem cells	Stem cells + clomiphen
area % of CHO in granulosa	11.74±0.88	7.17±1.33*	9.70±1.20*#	9.29±1.16*#	10.32±1.45#
area % of CHO in theca	6.46±0.57	7.11±1.35	7.02±0.82	7.05±1.67	6.92±1.07

Values are presented as mean ±SD

*: statistically significant compared to corresponding value in control group *(P<0.05)*

#: statistically significant compared to corresponding value in recovery group *(P<0.05)*

Table 3: Optical density of PAS positive reaction in the control and PCO groups after 3 weeks of the experiment

Parameters	control	PCO
granulosa optical density	1.64±0.16	0.63±0.15*
theca optical density	0.34±0.16	0.55±0.27

*: statistically significant compared to corresponding value in control group *(P<0.05)*

Values are presented as mean ±SD

Table 4: Optical density of PAS positive reaction in all the studied groups after 5 weeks of the experiment.

Parameters	control subgroups	Subgroup IIB (recovery)	Subgroup IIC (clomiphen)	Subgroup IID (stem cells)	Subgroup IIE (stem cells + clomiphen)
granulosa optical density	1.64±0.16	0.62±0.13*	1.23±0.15*#	0.98±0.20*#	1.44±0.16#@
theca optical density	0.35±0.18	0.52±0.27	0.45±0.11	0.51±0.08	0.42±0.16

Values are presented as mean ±SD

*: statistically significant compared to corresponding value in control group *(P<0.05)*

#: statistically significant compared to corresponding value in recovery group *(P<0.05)*

@: statistically significant compared to corresponding value in stem cells group *(P<0.05)*

Table 5: Mean area percent of Ki 67in the control and PCO groups after 3 weeks of the experiment.

Parameters	control	PCO
area % of KI67 in granulosa	83.45±1.57	30.42±1.18*

*: statistically significant compared to corresponding value in control group *(P<0.05)*

Table 6: Mean area percent of Ki 67 in all the studied groups after 5 weeks of the experiment.

Parameters	control subgroups	Subgroup IIB (recovery)	Subgroup IIC (clomiphen)	Subgroup IID (stem cells)	Subgroup IIE (stem cells + clomiphen)
area % of KI67 of granulosa	82.81±1.79	29.94±1.03*	65.56±1.46*#	60.40±1.61*#	68.25±2.09*#$@

Values are presented as mean ±SD

*: statistically significant compared to control group *(P<0.05)*

#: statistically significant compared to recovery group *(P<0.05)*

$: statistically significant compared to clomiphen group *(P<0.05)*

@: statistically significant compared to in stem cells group *(P<0.05)*

DISCUSSION

Polycystic ovary syndrome (PCOS) has been considered as one of the most controversial syndromes in gynecology and endocrinology. Numerous discussions and consensuses for diagnosis had been created due to the heterogeneity of this syndrome[1].

Despite clomiphen was considered the first line of treatment of infertility associated with PCOS[4], it was associated with numerous side effects[6]. On the other hand, ADSC was reported to improve significantly the ovarian functions after chemotherapy-induced ovarian injury. It was suggested that it could increase oocytes and follicles number[7].

Accordingly, the present study was designed to compare the effect of adipose-derived stem cells versus clomiphen on the treatment of experimentally-induced polycystic ovary in rats.

Many rat models had been used to investigate this syndrome[14]. In the present study letrozole was used to produce an animal model of PCOS. Letrozole is a non-steroidal aromatase inhibitor, it produces complete inhibition of aromatase enyme thus blocking the conversion of androgen to estrogen leading to suppression of estrogen and increase of androgen. Many authors suggested that letrozole administration in female adult rats could induce PCO features that resemble the disease features in human beings[10].

In order to begin the experiment in the same phase for all the groups, all rats were selected in their estrous phase of estrous cycle. This selection was achieved using microscopic examination of vaginal smears. Under microscope, estrous phase was characterized by predominance of large cornified cells arranged in clumps, this agrees with what was suggested by some reporters, who identified this phase during cytological examination of vaginal cells by showing almost large cornified cells arranged in clumps[15]. Microscopic examination of ovarian

specimens from the control group, three weeks from the beginning of the experiment, showed normal histological structure of the ovary with follicles in different stages of development, in addition to corpora lutea. On the other hand, specimens from the PCO group showed dilated ovarian cysts, dilated ovarian follicles that exhibited thin granulosa layer and debris within the follicular cavity, in addition to multiple atretic follicles. Similar findings were detected by some authors who also observed multiple cysts in letrozole-induced PCO rats.. They also reported that letrozole induced-PCOS rats showed significant number of atretic follicles randomly spread between other follicles, disruption of the granulosa layer, with cell debris in the follicular cavity[15].

Five weeks from the beginning of the experiment, specimens from control subgroups appeared similar to those of control subgroups after 3 weeks. On the other hand, the specimens from the recovery group appeared nearly similar to those of the PCO group. Meanwhile, the other subgroups receiving clomiphen, ADSC and combined clomiphen with ADSC, showed improvement in their histological structure. Various stages of ovarian follicles were noticed, including corpora lutea but few cysts were still observed. Similar results were reported by some authors, who reported marked recovery in the specimens from PCO rats treated with clomiphen[10]. Similarly, other authors reported improvement in the histological structure of the ovarian tissue, in the female rats suffering from ovarian failure induced by chemotherapy, after injection of ADSC[7]. In accordance with the current work, they detected numerous follicles at all stages of development in the ovarian specimens of the ADSC-treated female mice. Concomitant observations were also mentioned by some reporters where the total number of follicles and corpora lutea was increased following ADSC therapy of ovarian failure[17].

An important character of stem cells is their ability to selectively home to sites of tissue inflammation or damage[18]. In the present study, intravenously injected ADSC have migrated to the damaged ovary. This was confirmed by localization of PKH26 labelled ADSC visualized by the fluorescent microscope in the ovaries of the injected rats.Stem Cells have the ability to recognize the damaged site, then it can home and integrate into this site. Homing of ADSC to sites of injury might be caused by certain substances released from sites of tissue damage or inflammation. Increased chemokine concentration at the inflamed or damaged sites likely directs ADSC migration to these sites[19]. ADSC express receptors for several chemokines such as Tumor Necrosis Factor-alpha, Fibroblast growth factor-2 and Endothelial growth factor[20].

In the present study, follicles at different stages of development were detected in the ovaries of the ADSC-treated group. The mechanism of recovery of ovarian function and folliculogenesis following after ADSC therapy has not been fully explained, but many studies suggested some mechanisms such as increasing number of follicles and reducing apoptosis of granulosa cells, and subsequent recovery of ovarian sex hormone function[7].

It was suggested that ADSC migrating to damaged or inflammed tissues may act by two mechanisms: differentiation into cells specific to the tissue and/or paracrine effects[21]. Moreover, analysis of the factors released from ADSC revealed that cultured ADSC, at relatively early passages, secrete hematopoietic, angiogenic and anti-apoptotic factors in addition to cytokines and growth factors[22]. After 3 weeks of the experiment, ovarian specimens from the control group stained with PAS showed strong positive PAS staining in the intercellular spaces between granulosa cells, within the theca layer, in the granulosa basement membranes and in zona pellucida. On the other hand, examination of ovarian sections of PCO group (Group II) showed significant decrease in the mean area % of positive PAS reaction in the granulosa layer compared to the control ovarian sections. However, the mean area % in the theca of this group was non-significantly increased as compared to the control. Also, comparable results were detected when measuring the optical density on the specimens of these two groups. These results appeared close to those of some reporters who observed that the theca layer showed significantly higher PAS staining in PCO ovaries, while, in the granulosa layer, the PAS staining was significantly lower in PCO ovaries. They also observed that, in the control, the granulosa layer was more significantly stained than that in PCO ovaries, while, in the theca layer the intensity of PAS staining was significantly higher in cystic ovaries than that in the control[23].

In the current study, the mean area % of PAS positive reaction of the granulosa showed significant decrease in the recovery group (subgroup IIB) compared to the control, while, that in the theca showed a non-significant increase. Similar results were detected on assessment of the optical density in these subgroups. Meanwhile, the mean area % of PAS reactivity in the granulosa of subgroup IIC (received clomiphen), subgroup IID (received ADSCs) and subgroup IIE (received ADSCs + clomiphen) were significantly higher than that in the recovery group. However, the comparison was non-significant in the mean area% in all three subgroups in the theca layer.These results were comparable to what was found on measuring the optical density between these subgroups. On the other hand, on comparing subgroups IIC, IID and IIE, there was no significant difference in mean area % of PAS reaction neither in the granulosa nor in the theca and comparable results were found on measuring the optical density.

It is worth mentioning that folliculogenesis is characterized by granulosa cells proliferation, differentiation of the theca cells from the ovarian stroma, and basement membrane deposition thus separating the theca layer from avascular granulosa cells. Unlike the granulosa cells, the theca layer is well vascularized and contains circumferential collagen bundles. Changes in the

extracellular matrix (ECM) can influence gene expression as well as cell migration, proliferation, apoptosis and differentiation.It was suggested that most of the ECM components in the ovarian follicles are synthesized by the granulosa cells. It was also suggested that granulosa cells secrete a mucopolysaccharide-rich fluid that coalesces forming the antrum of the secondary follicle[24].

In the present study, the strong PAS reaction seen in the theca layer, could be attributed to the increased amount of carbohydrates and GAGs associated with the increased amount of collagen present. This assumption can be based on some studies which reported increased collagen deposition in the ovaries of rats with PCOS[25].

The above suggestions point to the important role of carbohydrates and GAGs secreted by granulosa cells in the differentiation and development of ovarian follicles. This might explain the significant decrease of carbohydrates and GAGs found in the granulosa of cystic ovaries in comparison to the control and to the treated subgroups, in the present study. In contrast, the strong staining seen in the theca of cystic ovaries could be attributed to the increased amount of collagen deposition[25].

After 3 weeks of the experiment, the mean area % of Ki 67 immunoreactivity, showed significant decrease in the granulosa of the PCO group compared to the control ovarian sections. This coincided well with the results of a study that evaluated proliferation in the ovary of rats with PCOS. It reported that proliferation was significantly higher in the granulosa cells of the control specimen[12].

After 5 weeks of the experiment, the mean area % of Ki 67 immunoreactivity of the recovery group (subgroup IIB) failed to return to the normal levels as it showed significant decrease in the immunoreactivity of thinned out granulosa when compared to the control subgroups. Meanwhile, the mean area % of Ki 67 immunoreactivity in the granulosa of the treated subgroups IIC, IID and IIE were significantly higher than that in the recovery group. This substantiates more the known effective role of clomiphen being the standard first choice for treating PCOS, in addition to presenting a hopeful effective role of ADSC as an alternative or complementary therapy. This effect of ADSC could be explained by the fact that these cells probably migrated towards the ovary, to be a source of stem cells capable of regenerating the population of primordial follicles and greatly preventing their apoptosis.

On comparing subgroup IIE with subgroup IIC, the mean area % of Ki67 immunoreactivity was significantly higher in the granulosa of subgroup IIE. This again supports the possible effective role of combining ADSC and clomiphen, which may be beneficial in the future therapy of PCOS.

Moreover, in the present study, the significant increase in Ki67 immuno-expression, detected in the granulosa of the groups treated by clomiphen and/or stem cells, reflects the increased number of granulosa cells after treatment.

This assumption could be supported by some reporters, who studied the effect of clomiphen on rat ovarian histology. They found that clomiphen could increase the number of granulosa cells and they raised the possibility that clomiphen may increase the risk of granulosa cell tumours especially if used in high doses[26]. In another study done, the ability of the human embryonic stem cells to differentiate into functioning granulosa cells was tested. The authors found that stem cells could differentiate to granulosa cells that were able to transfer testosterone into estrogen indicating that they are biologically active[27].

In the present study, leaving the PCO affected rats for natural recovery was non-sufficient to restore the histological findings compared to the treated groups and control. Similarly, in another study a group of letrozole-induced PCOS rats was left for natural recovery, the authors found that the recovery period was non-sufficient to restore the histological findings as compared to Clomiphene citrate treatment, as many cysts and atretic follicles were still found[10].

Taken all together, the results of the current study might draw attention to an effective role for ADSC in treatment of PCOS as its effect was comparable to that of clomiphen, which is currently the best drug recommended for treating infertility induced by PCOS, but in some cases it is not recommended or causes various side-effects. It is to be noted that ADSC and clompihen act via different pathways of modes of action, where clomiphene acts as a selective estrogen receptor modulator, inducing a change in gonadotropin releasing hormone (GnRH) pulse frequency leading to increased release of follicle stimulating hormone (FSH) from the pituitary gland[28]. On the other hand, ADSC could repair and regenerate the damaged ovarian tissue[29]. It could be proposed that both drugs act together in synergism, where the added effect of their combination was higher than the individual result of each of them separately.This synergistic drug combination allows lower doses of each constituent drug and consequently less adverse effects[30].

CONCLUSION:

In the present study, both clomiphen and ADSC could improve ovarian structure in this rat model of PCOS. Clomiphen effect was found to be comparable to that of ADSC, while combining ADSC + Clomiphen gave the best results.

CONFLICT OF INTEREST:

There are no conflicts of interest.

REFERENCES

1. Batista J G, Soares-Jr J M, Maganhin C C, Simões R S, Tomaz G and Baracat E C. (2012): Assessing the benefits of rosiglitazone in women with polycystic ovary syndrome through its effects on insulin-like growth factor 1, insulin-like growth factor-binding protein-3 and insulin resistance: a pilot study. Clinics (São Paulo).; 67(3):283-7.

2. Al-Shaikh S, Al-Mukhatar E, Al-Zubaidy A A, Al-Rubaie B J U and Al-Khuzaee L. (2017): Use of clomiphene or letrozole for treating women with polycystic ovary syndrome related subfertility in Hilla city. Middle East Fertility Society Journal .; 22(2):105-110.

3. Miller W L. (2013): Steroid hormone synthesis in mitochondria. Mol Cell Endocrinol. 379(1-2):62-73.

4. Melo A S, Ferriani R A and Navarro P A. (2015): Treatment of infertility in women with polycystic ovary syndrome: approach to clinical practice Clinics (Sao Paulo).; 70(11): 765–769.

5. Sirmans S M and Pate KA. (2014): Epidemiology, diagnosis, and management of polycystic ovary syndrome Clin Epidemiol.; 6: 1–13.

6. Ndefo U A, Eaton A and Green M R. (2013): Polycystic Ovary Syndrome. A Review of Treatment Options With a Focus on Pharmacological Approaches. 38(6): 336-338.

7. Sun M, Wang S, Li Y, Yu L, Gu F, Wang C and Yao Y. (2013): Adipose-derived stem cells improved mouse ovary function after chemotherapy-induced ovary failure. Stem Cell Res Ther. 9;4(4):80.

8. Paixão L, Ramos R B, Lavarda A , Morsh D M and Spritzer P M. (2017): Animal models of hyperandrogenism and ovarian morphology changes as features of polycystic ovary syndrome: a systematic review Reprod Biol Endocrinol.; 15: 12.

9. Shi D and Vine D F. (2012): Animal models of polycystic ovary syndrome: a focused review of rodent models in relationship to clinical phenotypes and cardiometabolic risk .fertility and sterility . 98 (1) : 185–193.

10. Jadhav M, Menon S and Shailajan S. (2013): In vivo evaluation of mimosa pudicalinn. In the management of polycystic ovary using rat model. Int. J. App. biol. Pharmaceut. Technol., 4(1): 285-292.

11. Suvarna S K, Layton C and Bancroft J D. (2013): Bancroft's Theory and Practice of Histological Techniques , Churchill Livingstone, UK, 7th edition pp 173-225.

12. Lombardi L A, Simões R S, Maganhin C C, Baracat M C, Silva-Sasso G R, Florencio-Silva R, Soares J M and Baracat E C. (2014): Immunohistochemical evaluation of proliferation, apoptosis and steroidogenic enzymes in the ovary of rats with polycystic ovary. Rev. Assoc. Med. Bras., 60(4): 349-356.

13. Huang S P, Hsu C C, Chang S C, Wang C H, Deng S C, Dai N T, Chen T M, Chan J Y, Chen S G and Huang S M. (2012): Adipose-derived stem cells seeded on a cellular dermal matrix grafts enhance wound healing in a murine model of a full-thickness defect. Ann Plast Surg; 69: 656–62.

14. Walters K A, Allan C M and Handelsman D J. (2012): Rodent Models for human polycystic ovary syndrome. Biol. of reprod. ;86(5):149, 1–12.

15. Byers S L, Wiles M V, Dunn S L and Taft R A. (2012): Mouse Estrous Cycle Identification Tool and ImagesPLoS One.; 7(4): e35538.

16. Rezvanfar M A, Ahmadi A, Shojaei-Saadi H A, Baeeri M and Abdollahi M (2012): Molecular mechanism of a novel Selenium based complementary medicine which confers protection against hyperandrogenism induced polycystic ovary, Theriogenology, 78: 620-631.

17. Takehara Y, Yabuuchi A, Ezoe K, Kuroda T, Yamadera R, Sano C, Murata N, Aida T, Nakama K, Aono F, Aoyama N, Kato K and Kato O (2013): The restorative effects of adipose-derived mesenchymal stem cells on damaged ovarian function. Laboratory Investigation; 93: 181-93.

18. Cuiffo B G and Karnoub A E (2012): Mesenchymal stem cells in tumor development emerging roles and concepts Cell Adh Migr.; 6(3): 220–230.

19. DiMarino A M, Caplan A I, and Bonfield T L. (2013): Mesenchymal Stem Cells in Tissue Repair Front Immunol.; 4: 201.

20. Dash B C, Xu Z , Lin L , Koo A, Ndon S, Berthiaume F, Dardik A and Hsia H. (2018): Stem Cells and Engineered Scaffolds for Regenerative Wound Healing Bioengineering,. 5 (23): 1-12

21. Li H, Xu Y, Fu Q and Li C. (2012): Effects of multiple agents on epithelial differentiation of rabbit adipose-derived stem cells in 3D culture. Tissue Engineering; 18(17): 1760–70.

22. Lee C , Burnsed O A , Raghuram V, Kalisvaart J, Boyan B D and Schwartz Z. (2012): Adipose stem cells can secrete angiogenic factors that inhibit hyaline cartilage regeneration Stem Cell Research and Therapy, 3:35.

23. Salvetti N, Gimeno E and Canal A. (2003) : Histochemical study of the extracellular matrix components in the follicular wall of induced polycystic ovaries Braz. J. morphol. Sci. 20(2), 93-100.

24. Martelli A, Russo V, Mauro A, Di Giacinto O, Nardinocchi D, Mattioli M, Barboni B and Berardinelli P. (2017): Insights into Ovarian Follicle Angiogenesis: Morphological and Chronological Vascular Remodeling from Primordial to Ovulating Follicles. SM Vasc Med.; 2(1): 1009.

25. Treesh S A and Khair N S. (2014): Effect of Thyroid Disorders on the Adult Female Albino Rats (Histological and Histochemical Study) . J Cytol Histol 5: 245.

26. Ozdemir I , Ustundag N, Guven A, Duran B and Demirci F. (2005): Effect of clomiphene citrate on ovarian, endometrial, and cervical histologies in a rat model. Gynecol Obstet Invest. ;60(4):181-5.

27. Lan C W, Chen M J, Jan P S, Chen H F and Ho H N. (2013): Differentiation of human embryonic stem cells into functional ovarian granulosa-like cells. J Clin Endocrinol Metab.; 98(9): 3713-23.

28. Balen A H. (2013): Ovulation induction in the management of anovulatory polycystic ovary syndrome. Mol Cell Endocrinol.; 373(1–2):77–82.

29. Kolaparthy L K, Sanivarapu S, Moogla S and Kutcham R S. (2015): Adipose Tissue - Adequate, Accessible Regenerative Material Int J Stem Cells; 8(2): 121–127.

30. Huang H, Zhang P, Qu X, Sanseau P and Yang L. (2014): Systematic prediction of drug combinations based on clinical side-effects Scientific Reports volume 4, Article number: 7160.

الملخص العربى

دراسة على تأثير الخلايا الجزعية المستخلصة من النسيج الدهنى في مقابل الكلوميفين في علاج تكيس المبايض التجريبي في الفئران : دراسة هستولوجية وهستوكيميائية مناعية

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المقدمة: تنتشر متلازمة تكيس المبايض بين قطاع كبير من النساء في سن الانجاب وتؤثر سلبا على الخصوبة .

الهدف من البحث: تهدف هذه الدراسة الى المقارنة بين تاثير الخلايا الجذعية المستخلصة من النسيج الدهني في مقابل عقار الكلوميفين في علاج تكيس المبايض التجريبي في الفئران.

مواد وطرق البحث: أجريت هذه الدراسة على خمسين من إناث الفئران البيضاء البالغة تراوح اوزانها بين 150 200- جرام تم تقسيمهم بالتساوى إلى مجموعتين :
المجموعة الأولي (الضابطة):
تم إعطاؤها نصف ميلليلتر من محلول الكاربوكسي ميثايل سليولوز بالفم يوميا لمدة 21 يوما.
المجموعة الثانية (مجموعة تكيس المبايض):
تم إعطاؤها ليتروزول واحد ميلليجرام لكل كيلوجرام مذابة في نصف ميلليلتر من محلول الكاربوكسي ميثايل سليولوزبالفم يوميا لمدة 21 يوما ثم تم التضحية بخمسة فئران من المجموعة الضابطة وتسميتها (المجموعة الفرعية 1أ) و خمسة فئران من مجموعة تكيس المبايض وتسميتها (المجموعة الفرعية 2أ). ثم تم تجهيز قطاعات المبيض و فحصها للتأكد من حدوث تكيس المبايض في المجموعة الثانية.
الفئران العشرون الباقية من مجموعة تكيس المبايض تم تقسيمها بالتساوى إلى أربعة مجموعات فرعية (العدد = خمسة فئران):
المجموعة الفرعية 2ب (مجموعه التعافي) استمرت دون علاج - المجموعة الفرعية 2ج تم اعطاؤها كلوميفين - المجموعة الفرعية 2د تم حقنها بالخلايا الجزعية المستخلصة من الدهون - المجموعة الفرعية 2ه تم علاجها بالخلايا الجزعية والكلوميفين.
بعد مرور خمسة أسابيع من بداية التجربة تم التضحية بجميع الفئران واستخلاص المبايض وتجهيز وصبغ القطاعات بالصبغات التالية : صبغة الهيماتوكسلين و الإيوسين. - تفاعل شيف للحمض البيريودى - الصبغات الكيميائية المناعية ضد أنوية الخلايا المتكاثرة.
الفئران العشرون الباقية من المجموعة الضابطة تم تقسيمها بالتساوى إلى أربعة مجموعات فرعية (العدد = خمسة فئران) اخذت فقط المذيبات لمدة اسبوعين .

النتائج: اظهرت مجموعة تكيس المبايض تدهور في تركيب المبيض حيث ظهر توسع كيسي لحويصلات المبيض مع رقة الطبقة المحببة ,و نجح كل من عقار الكلوميفين والخلايا الجزعيه في تحسين التركيب الهستولوجي للمبيض .

الاستنتاج: تاثير الخلايا الجزعيه المستخلصة من النسيج الدهني كان مقاربا لتاثير عقار الكلوميفين , بينما دمجهما معا اعطى افضل النتائج.