Role of Pomegranate Molasses on Estradiol Valerate Induced Polycystic Adult Female Albino Rat Ovary: Histological and Immunohistochemical Study

Original Article

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ABSTRACT

Interoduction: One of the most well-known endocrinopathies in females in the period of reproduction is the Polycystic ovary syndrome (PCOS). PCOS is activated by the long-acting steroid, estradiol valerate (EV) that leads it to appear quickly due to metabolic and physiological abnormalities. It has been found that oxidative stress may lead to PCOS. Among the natural antioxidant products, pomegranate molasses (PM) is widely used.

Objective: The goal of this work was to explore the possible protection of PM on EV induced PCOS in female adult albino rats.

Materials and Methods: Forty normally cyclic female adult albino rats were separated into four groups (ten rats for each group); Group I: control group, Group II: pomegranate molesses treated group, Group III: estradiol valerate treated group and Group IV: estradiol valerate and pomegranate molesses treated group. After 6 weeks, cytological analysis of the vaginal smear for rats in all groups was performed to evaluate the state of the estrous cycle and then all animals were sacrificed and their ovaries were excised and handled for histological and immunohistochemical studies.

Results: Estradiol valerate treated group showed irregular cycles or constant cycles in estrous phase (Persistent Vaginal Cornification, PVC) which were considered as signs of PCO. Histologically, there was a significant decline in the number of the different healthy follicles in the cortices with the presence of multiple cystic and atretic follicles and also large congested blood vessels with hemorrhage. Immunohistochemically, the number of i-NOS positive granulosa, theca, and interstitial cells increased significantly in this group with increase of image optical density (IOD) for all these positive cells. Pomegranate molesses supplementation with estradiol valerate in group IV has ameliorating effect on the vaginal smear, histological and immunohistochemical abnormalities of the ovaries.

Conclusion: There is a potential benefit in using pomegranate molesses with EV induced PCOS.

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Key Words: Estradiol valerate; oxidative stress; PCOS; pomegranate molasses.

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INTRODUCTION

One of the most common endocrinopathies in women of reproductive age is the polycystic ovary syndrome (PCOS).[1] It is the most common etiology of infertility and its reported prevalence ranges from 2.2% to 26% worldwide.[2] It is considered as the sum of certain clinical findings rather than a disease.[3] It is an endocrine metabolic disorder with increased androgen, hirsutism, oligomenorrhea, amenorrhea, anovulation and infertility characteristics.[4] Diagnostic guidelines for PCOS are based on two of the three indicators of numerous cysts on ultrasound of ovary, irregular anovulatory cycles or hyperandrogenism.[5]

Obesity and increased body fat are commonly seen in this disorder.[6] Endometrial hyperplasia, cancer, cardiovascular disease, type 2 diabetes, anxiety and depression are all associated to long term PCOS.[7]

Rats are chosen to develop several good PCOS experimental models due to their ease of processing, shortened reproductive period, stable genotype and brief regular estrous cycles (4 or 5 days), with separate proestrus, estrus, metestrus and diestrus stages. It can be done by using different methods, such as implantation or subcutaneous injection of letrozole, antiprogesterone, androgens and estrogen.[8,9]

Estradiol valerate (EV) is a variety of estrogen that has a long half-life. It induces hypothalamic-pituitary dysfunction of gonadotrophin releasing hormone (GnRH) during its administration, leading to disturbed luteinizing hormone (LH) release and storage and causes a rapid appearance of PCOS due to metabolic and physiological disturbances.[1,8]

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PCOS may result in an imbalance between oxidant molecules and the antioxidant defensive system; therefore, antioxidants can help to reduce the infertility associated with this disease.[10]

Pomegranate (Punica granatum) has been highly valued for its beneficial properties, and it has often been planted and served as a fruit or beverage, particularly in Mediterranean area.[11] The fruit, juice, and peel of the pomegranate are high in antioxidants.[12] It contains a lot of polyphenols, notably anthocyanins, condensed tannins and ellagitannins.[13] In the Middle East, pomegranate molasses (PM), a pomegranate juice concentrate, is commonly utilized. It is possible that it is higher in effective antioxidants than the juice. It is widely used in salads and a lot of dishes in Egypt to enhance the taste and smell. It is made just by boiling the juice while adding sugar to it.[14]

Pomegranate could be used as a chemotherapeutic, chemopreventive, anti-inflammatory and anti-atherosclerotic agent.[15] So its consumption has grown tremendously.[16] This raised our interest to detect the possible protective effect of pomegranate against the oxidative damage associated with PCOS. And so, this work pointed to see if PM could protect female adult albino rats from developing PCOS caused by EV.

MATERIALS AND METHODS

Drugs

Estradiol valerate (EV): Cycloprogynova tablets were obtained from a local pharmacy, manufactured by Bayer Weimar GmbHb und Co. KG, Germany, each white tablet contained 2 mg estradiol valerate, the tablets were crushed and dissolved in distilled water to be given by intramuscular injection.

Pomegranate molasses (PM): 250 ml bottle of 10% pomegranate juice concentrate, was obtained from a local market, manufactured by Kemal Kukrer Company, Turkey.

Polyclonal Anti-inducible Nitric Oxide Synthase antibody (iNOS antibody): IgG isotype, from rabbit host, reactive to human, mouse, rat and rabbit was obtained from SNF Medical Company, Cairo

Animals

The study was carried on forty healthy cyclic adult female albino rats weighing 200±30 g obtained from Anatomy Department Animal House, Faculty of Medicine, University of Alexandria.

Methods

The animals were maintained under standard laboratory conditions of temperature and humidity and accommodated to laboratory environment for two weeks before the experiments. Guidelines for care and use of animals, officially accepted by the Animal House Center, Faculty of Medicine, Ethics Committee of Alexandria University, were followed. All animals were included in the current research after displaying at least two consecutive regular estrous cycles (4-5 days). Identification of the estrous cycle stages was carried out by daily examination of the vaginal smear of all rats before and after the study period.

Experimental protocol

The rats were divided into four equal groups at random, each included 10 rats:

Group Ia (control group): The rats received daily normal diet and water ad libitum.

Group Ib: receiving distilled water

Group II (Pomegranate molasses group): The rats received pomegranate molasses (PM) in a daily dose of 0.5ml dissolved in 0.5ml distilled water orally for 6 weeks[14,17]

Group III (Estradiol valerate group): The rats received estradiol valerate (EV) as a single intramuscular injection at a dose of 4 mg EV dissolved in distilled water for each rat to induce a well-defined PCOS.[1,2]

Group IV (EV+PM): The rats received EV as in group III in addition to PM as in group II.[1,2,14,17]

The experimental period ended after 6 weeks which was selected as a single EV injection causes anovulatory chronic PCO disorder in female adult rats after a latency period of four to six weeks. Cytological analysis of the vaginal smear for rats in all groups was performed at the end of the current study to evaluate the state of the estrous cycle. PCOS signs were irregular estrous cycles or persistent vaginal cornification (PVC).[1] Under ether inhalational anaesthesia, all rats from each study group were operated upon and dissected to obtain both ovaries which were processed for histological and immunohistochemical studies.

I- Vaginal smear

It was used before the study period to include only the animals with normal estrus cycles and also after the study period to assess the effect of the study materials on the estrus cycle. Vaginal secretion was collected with a plastic pipette filled with 10 mL of normal saline (NaCl 0.9%). The dry fixed slides were stained by hematoxylin and eosin (H&E) stain.[18]

II-Histological study

The ovaries were fixed at 10% neutral formaldehyde. The specimens then were processed for Paraffin sections of about 5-6 µm thickness. Sections were obtained, then stained with hematoxylin and eosin for studying the histological changes of the ovarian tissues.[19]

III-Immunohistochemical study

To evaluate the oxidative damage in the ovaries of different experimental groups, paraffin sections were deparaffinized in xylene and then incubated with a rabbit anti-inducible NOS polyclonal antibody which has cross reactivity with rats, utilizing the avidin biotin peroxidase

method to assess iNOS localization in granulosa, theca and interstitial cells in the animal groups at Faculty of Medicine, University of Alexandria. Incubation of primary antibodies was followed by incubation with the biotinylated secondary antibody and reacted with the avidin-biotinylated peroxidase complex. Negative control sections were processed by replacing the primary antibody with buffer alone.[2]

IV- Image Analysis Study

This was carried out using digital image analyzer and analysis software (image J) to quantify the number of different ovarian follicles in H&E histological study, iNOS immunoreactive positive granulosa, theca and interstitial cells in immunohistochemical study and their image optical density in five non-overlapping fields taken from at least five sections / animal in Histology department, Faculty of Medicine, University of Alexandria using a 20 X objective (Bar = 100).[20]

Statistical analysis

Statistical analysis of data was fed to the computer and analyzed using IBM SPSS software package version 20.0. (Armonk, NY: IBM Corp).[21] The Kolmogorov-Smirnov test was used to verify the normality of distribution. F-test (ANOVA) was used for normally distributed quantitative variables to compare between more than two studied groups. The quantitative data were described using mean and standard deviation. Significance of the obtained results was judged at the 5% level.[22]

RESULTS

Vaginal smear results

Control rats and rats administrated PM had brief regular estrous cycles (4 or 5 days), with separate proestrus, estrus, metestrus and diestrus stages. The proestrus showed the presence of rounded nucleated epithelial cells (Figure 1-a). The estrus revealed the presence of cornified irregular non nucleated epithelial cells (Figure 1-b). The metestrus showed the existence of both rounded nucleated and irregular cornified non nucleated epithelial cells with small rounded leukocytes at the vaginal smear (Figure 1-c). The diestrus revealed a large number of small rounded leukocytes predominantly at the vaginal smear (Figure 1-d).

EV group showed irregular cycles or constant cycles in estrous phase (Persistent Vaginal Cornification, PVC) which were considered as signs of PCO. While rats administrated EV&PM showed relatively regular cycles.

Histological results

Light microscopic examination with Hematoxylin & Eosin stain:

Histological paraffin sections of ovary of the rats of the control group (group I) showed normal architecture of the ovary covered by a single layer of columnar germinal epithelium. Each ovary consisted of outer cortex and inner medulla. The cortices were occupied by multiple growing follicles at different stage of development separated by interstitial cells. The primordial follicles were very peripheral in site and consisted of central oocyte surrounded by a single layer of flattened cells. The primary follicles included central oocyte with surrounding single or multiple layers of cuboidal cells. The secondary follicles included single or multiple antral cavities between the surrounding granulosa cells encircling the central oocyte. The graafian follicles had a specific architecture including central healthy oocyte surrounded by a well-defined zona pellucida then corona radiata protruding inside a large antral cavity which was surrounded by regularly organized granulosa cells then inner cellular theca interna and outer fibrous theca externa. Multiple corpora lutea were also incorporated inside the cortex containing central vacuolated granulosa lutein cells and peripherally located smaller darker theca lutein cells. The central medulla showed connective tissue with multiple blood vessels. (Figures 2-a, 3-a)

Examination of histological paraffin sections of ovaries of PM treated rats (group II) showed better histological structure than those of the control rats (group I). There was larger number of growing healthy follicles and also a larger number of corpora lutea than those of the control rats (group I). (Figures 2-b, 3-b)

Examination of histological paraffin sections of ovaries of EV treated rats (group III) showed that the ovary had lost its characteristic architecture compared with the control. The number of growing healthy follicles in the cortices declined with the presence of multiple cystic follicles which had thin granulosa layer and thick theca layer with irregular outline, degenerated granulosa cells, vacuolated granulosa cells, intraluminal desquamated granulosa cells and large eosinophilic colloidal fluid. There were also multiple atretic follicles which had degenerated oocytes and large congested blood vessels with hemorrhage. (Figures 2-c, 3-c)

Examination of histological paraffin Sections of ovarian tissues obtained from rats treated with EV & PM (group IV) showed marked better histological structure than those of EV treated rats. The number of growing healthy follicles and also corpora lutea raised than those of EV treated rats with a marked decrease of the cystic follicles indicating restoration of ovulatory function of the ovary. (Figures 2-d, 3-d)

The immunohistochemical results

Paraffin sections stained with i-NOS antibody used avidin biotin complex and DAB chromogen in all experiment groups. The immunohistochemical staining appeared as brown granules stained in the cytoplasm of the positive granulosa, theca and interstitial cells of all experimental groups.

There were few i-NOS positive granulosa cells, small number of i-NOS positive theca cells and large

number of i-NOS positive interstitial cells with weak immunoreactivity of all these positive cells in the control group (Figure 4-a) and in the ovaries of rats administrated PM (Figure 4-b). Ovaries of rats administrated EV showed large number of i-NOS positive granulosa cells, large number of i-NOS positive theca cells and huge number of i-NOS positive interstitial cells with increase of the immunoreactivity for all these positive cells. (Figure 4-c). Ovaries of rats administrated EV&PM showed marked protective effect of the ovaries with few i-NOS positive granulosa cells, small number of i-NOS positive theca cells and large number of i-NOS positive interstitial cells with weak immunoreactivity of all these positive cells. (Figure 4-d).

Image analysis results

A. Count of ovarian follicles in H&E histological study:

Data in (Table 1) demonstrated that there was a significant increase in the number of graafian follicles and corpora lutea in group II as compared to the control group, a significant decrease in group III as compared to the group II & group I and a significant increase in group IV as compared to group III (P<0.001). (Figure 5)

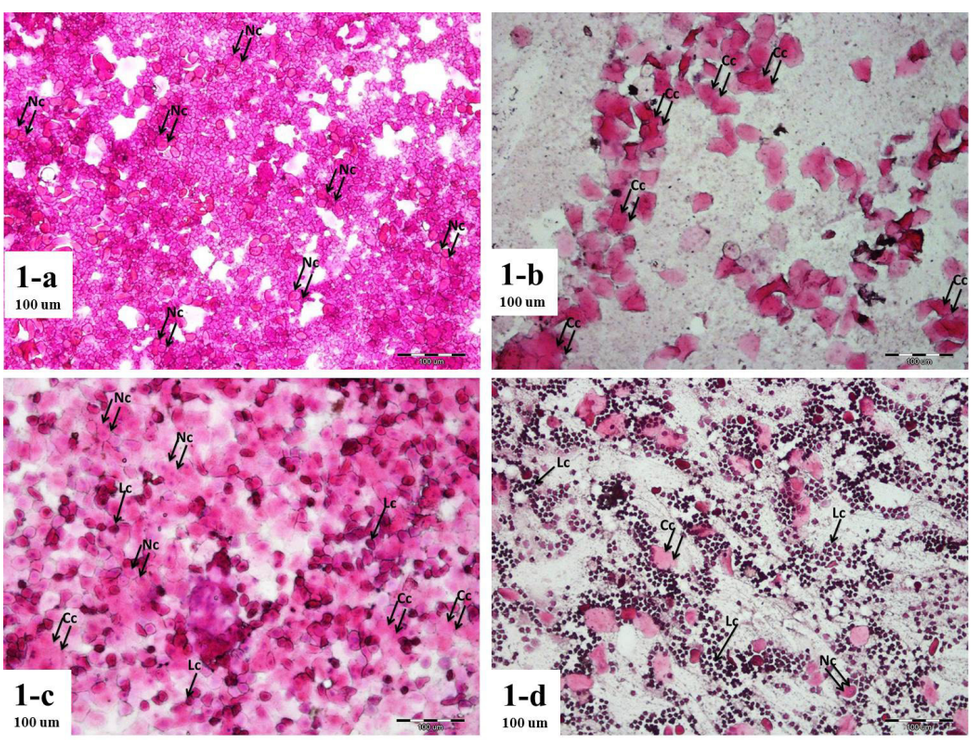
There was a significant increase in the number of cystic follicles in group III as compared to the group II & group I and a significant decrease in group IV as compared to group III (P<0.001). (Table 1, Figure 5)

B. Count of iNOS immunoreactive positive granulosa, theca and interstitial cells in immunohistochemical study:

Data in (Table 2) demonstrated that there was a significant decrease in the number of iNOS immunoreactive positive granulosa, theca and interstitial cells in group II as compared to the control group, a significant increase in group III as compared to the group II & group I and a significant decrease in group IV as compared to group III (P <0.001). (Figure 6)

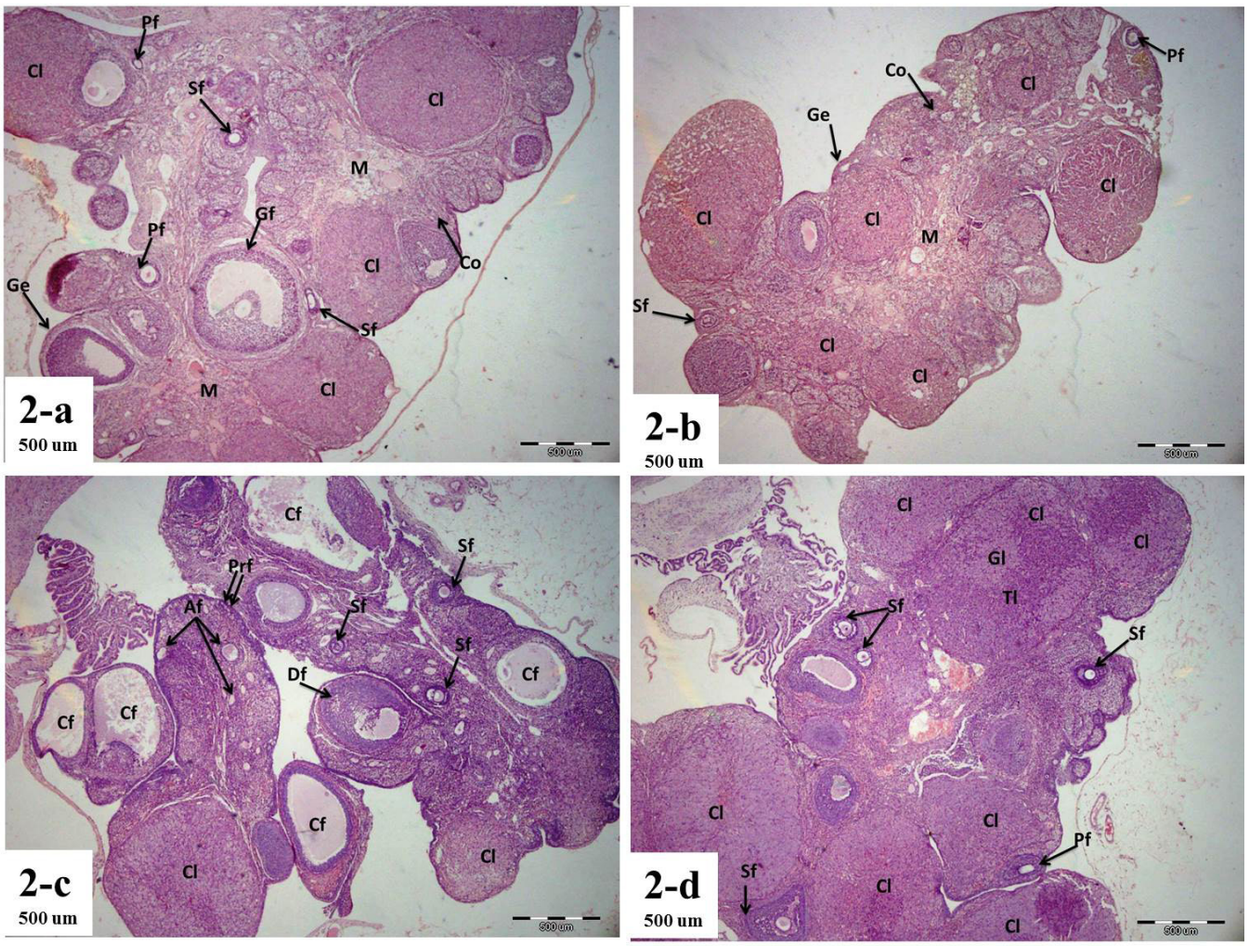
C. Image optical density (IOD) of iNOS immunostaining in the positive granulosa, theca and interstitial cells in immunohistochemical study:

There was a significant decrease in the image optical density (IOD) of iNOS immunostaining in the positive granulosa, theca and interstitial cells in group II as compared to the control group, a significant increase in group III as compared to the group II & group I and a significant decrease in group IV as compared to group III (P <0.001). (Table 3, Figure 7).



Figs. (1a-d): Vaginal smear photomicrograph of a control adult female rat (G I) showing:
Fig. (1-a): rounded nucleated epithelial cells (Nc) of proestrus phase.
Fig. (1-b): cornified irregular non nucleated epithelial cells (Cc) of estrus phase.
Fig. (1-c): both rounded nucleated (Nc) and cornified irregular non nucleated (Cc) epithelial cells with small rounded leukocytes (Lc) of metestrus phase.
Fig. (1-d): both rounded nucleated (Nc) and cornified irregular non nucleated (Cc) epithelial cells in small amount with large number of small rounded leukocytes (Lc) of diestrus phase.

(H&E stain, Bar = 100µm)



Figs. (2a-d): Paraffin section photomicrograph of an adult female rat ovary:

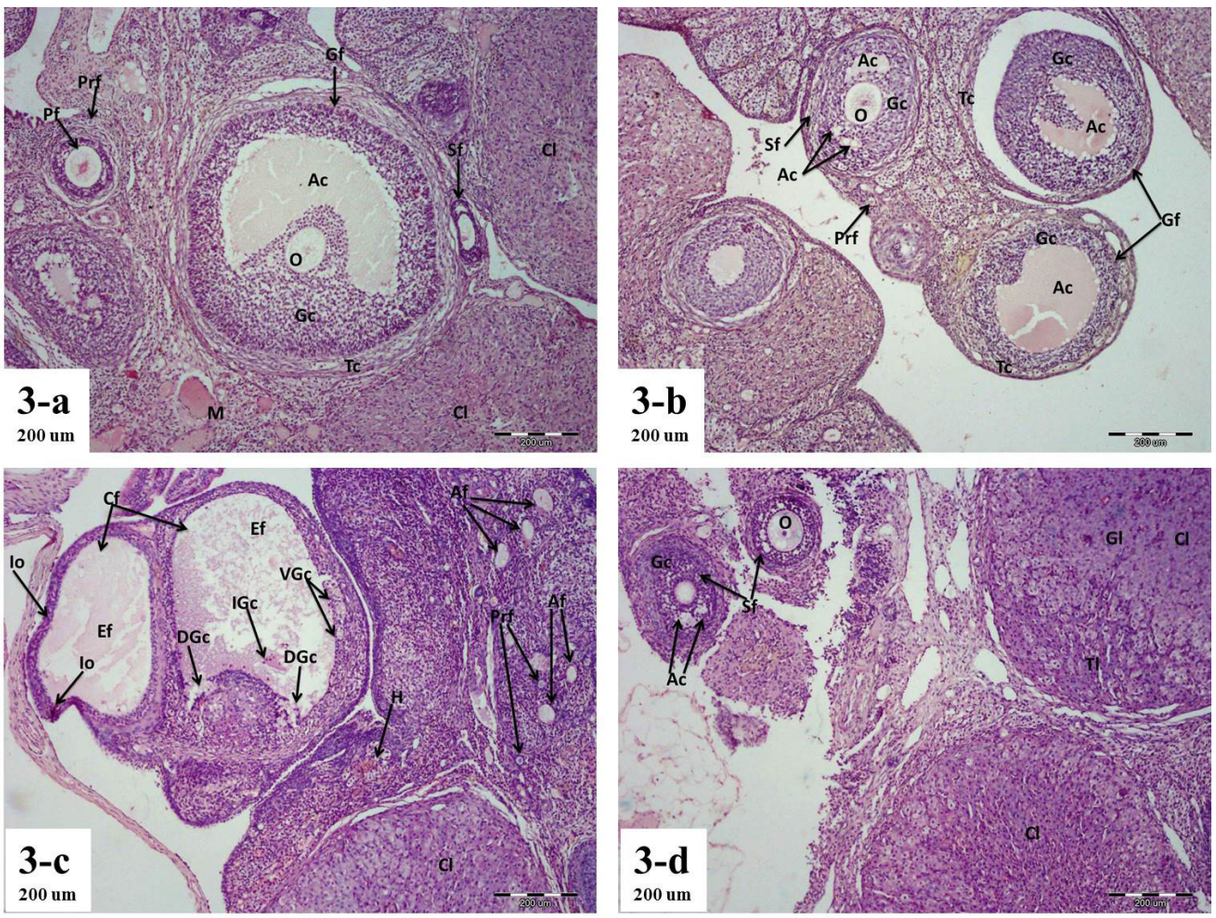
Fig. (2-a): a control adult female rat ovary (G I) showing, a single layer of columnar germinal epithelium (Ge) enclosing outer cortex (Co) and inner medulla (M). Notice the presence of primary (Pf), secondary (Sf), graafian (Gf) follicles and multiple corpora lutea (Cl) inside the cortex.

Fig. (2-b): an ovary of an adult female rat administrated PM (GII) showing, regular outline covered by single layer of columnar germinal epithelium (Ge) enclosing outer cortex (Co) and inner medulla (M). Notice the presence of primary (Pf), secondary (Sf) follicles and a large number of corpora lutea (Cl) inside the cortex.

Fig. (2-c): an ovary of an adult female rat administrated EV (GIII) showing, decrease in number of the growing follicles including primordial (Prf), secondary follicles (Sf) and only two corpora lutea (Cl). Notice the presence of multiple atretic follicles (Af) and cystic follicles (Cf) with thin irregularly organized granulosa layer and thick theca layer with large eosinophilic colloidal fluid. Notice the presence of degenerating follicle (Df) with intraluminal desquamated granulosa cells.

Fig. (2-d): an ovary of an adult female rat administrated EV&PM (GIV) showing, large number of the growing follicles including primary (Pf) and secondary follicles (Sf) and corpora lutea (Cl) with central vacuolated granulosa lutein cells (Gl) and peripherally located smaller darker theca lutein cells (Tl).

(H&E stain, Bar = 500µm)



Figs. (3a-d): Paraffin section photomicrograph of an adult female rat ovary:

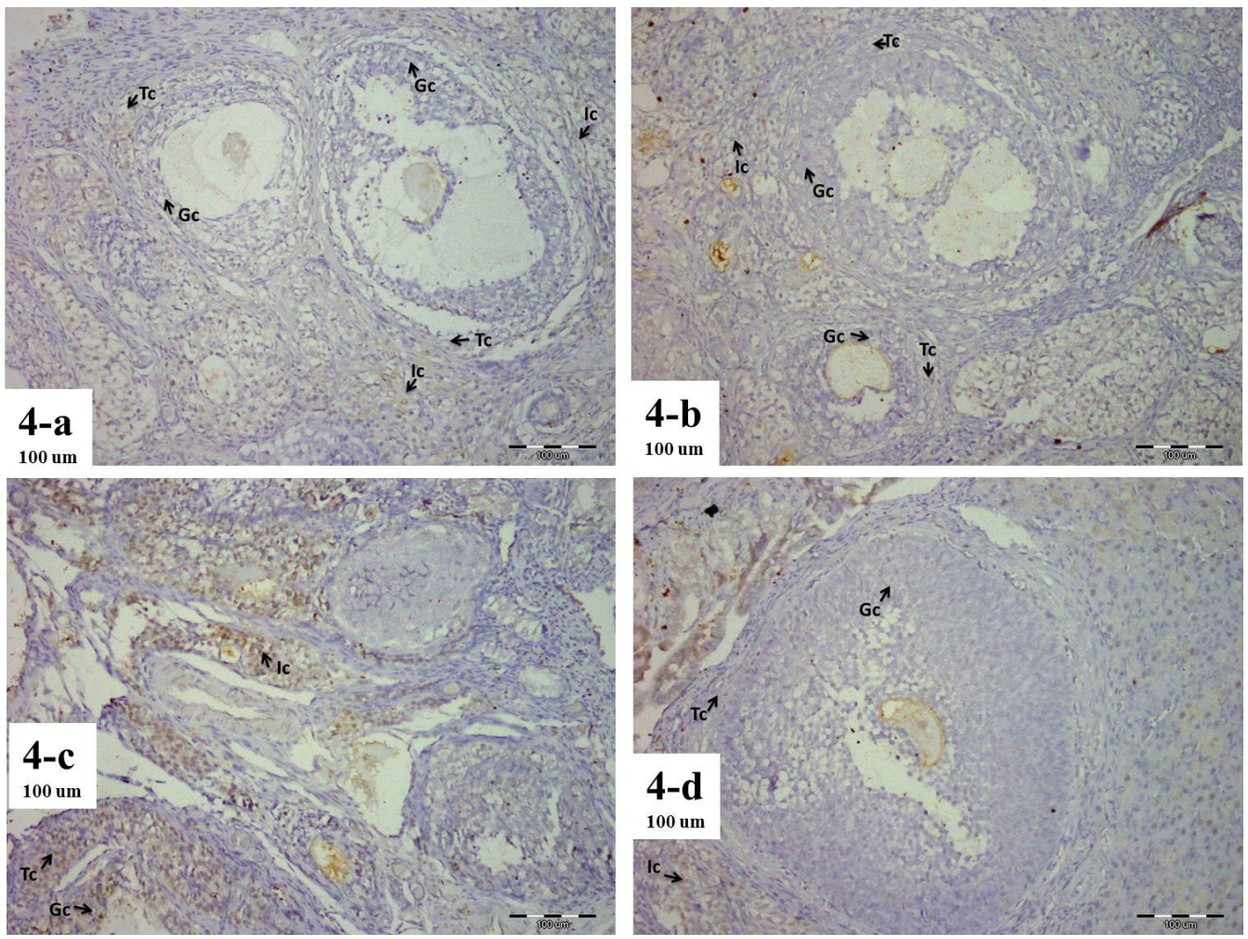
Fig. (3-a): a control adult female rat ovary (G I) showing, primordial (Prf), primary (Pf), secondary (Sf), graafian (Gf) follicles and two corpora lutea (Cl) inside the cortex with the presence of multiple blood vessels inside the medulla. Notice the components of the graafian (Gf) follicle which are oocyte (O), regularly organized granulosa cells (Gc), theca layers (TC) and a large antral cavity (Ac).

Fig. (3-b): an ovary of an adult female rat administrated PM (GII) showing primordial (Prf), secondary (Sf) and two graafian (Gf) follicles. Notice the components of the graafian (Gf) follicles with regularly organized granulosa cells (Gc), theca layers (TC) and a large antral cavity (Ac). Notice the components of the secondary (Sf) follicle with regularly organized granulosa cells (Gc) and multiple small antral cavities (Ac).

Fig. (3-c): an ovary of an adult female rat administrated EV (GIII) showing decrease in number of the growing follicles including primordial follicles (Prf) and one corpous luteum(Cl). Notice the presence of hemorrhage in between the different follicles and multiple atretic follicles (Af) with degenerated oocytes. Notice the presence of two large cystic follicles (Cf) with thin granulosa layer and thick theca layer with irregular outline (Io), degenerated granulosa cells (DGc), vacuolated granulosa cells (VGc), intraluminal desquamated granulosa cells (IGc) and large eosinophilic colloidal fluid (Ef).

Fig. (3-d): an ovary of an adult female rat administrated EV&PM (G IV) showing, two secondary follicles (Sf) with central oocyte (O), granulosa cells (Gc) and multiple small antral cavities (Ac) and two corpora lutea (Cl) with central vacuolated granulosa lutein cells (Gl) and peripherally located smaller darker theca lutein cells (Tl). Notice the absence of cystic follicles.

(H&E stain, Bar = 200µm)



Figs. (4a-d): Paraffin section photomicrograph of an adult female rat ovary:
Fig. (4-a): a control adult female rat ovary (G I) showing, growing follicles with few i-NOS positive granulosa cells (Gc↑), small number of i-NOS positive theca cells (Tc↑) and large number of i-NOS positive interstitial cells (Ic↑). Notice weak immunoreactivity for all these positive cells.
Fig. (4-b): an ovary of an adult female rat administrated PM (G II) showing, growing follicles with few i-NOS positive granulosa cells (Gc↑), small number of i-NOS positive theca cells (Tc↑) and large number of i-NOS positive interstitial cells (Ic↑). Notice weak immunoreactivity for all these positive cells.
Fig. (4-c): an ovary of an adult female rat administrated EV (G III) showing, a cyst with large number of i-NOS positive granulosa cells (Gc↑), large number of i-NOS positive theca cells (Tc↑) and huge number of i-NOS positive interstitial cells (Ic↑). Notice the increase of the immunoreactivity for these positive cells.
Fig. (-d): an ovary of an adult female rat administrated EV&PM (G IV) showing, a graafian follicle with few i-NOS positive granulosa cells (Gc↑), small number of i-NOS positive theca cells (Tc↑) and large number of i-NOS positive interstitial cells (Ic↑). Notice the weak immunoreactivity for these positive cells.

(ABC, DAB stain, Bar = 100µm)

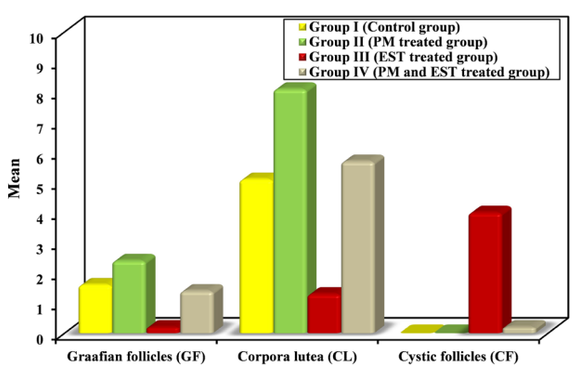


Fig. 5: Comparison between the four studied groups according to the count of ovarian follicles in H&E histological study

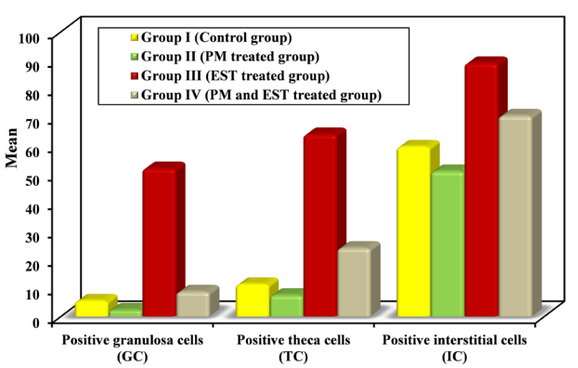


Fig. 6: Comparison between the four studied groups according to the iNOS immunoreactive positive granulosa (GC), theca (TC) and interstitial (IC) cells in immunohistochemical study

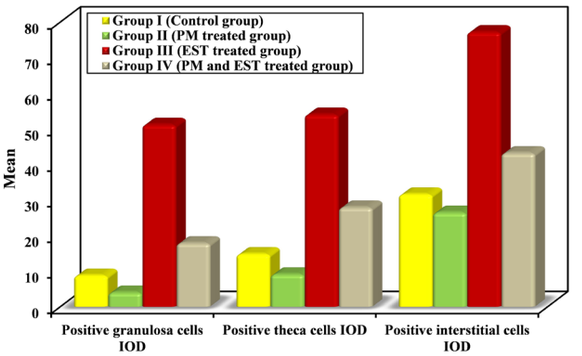


Fig. 7: Comparison between the four studied groups according to the image optical density (IOD) of iNOS immunostaining in the positive granulosa (GC), theca (TC) and interstitial (IC) cells in immunohistochemical study

Table 1: Comparison between the four studied groups according to the count of ovarian follicles in H&E histological study

Number of follicles in H&E study	Group I (n=10)	Group II (n=10)	Group III (n=10)	Group IV (n=10)	*p*
Graafian Follicles					
Min. – Max.	1.0 – 2.0	2.0 – 3.0	0.0 – 1.0	1.0 – 2.0	<0.001*
Mean ± SD.	1.60 ± 0.52	2.40 ± 0.52	0.20 ± 0.42	1.40 ± 0.52	
Median (IQR)	2.0 (1.0–2.0)	2.0 (2.0–3.0)	0.0 (0.0–0.0)	1.0 (1.0–2.0)	
pcontrol		0.053	0.001*	0.573	
Sig. bet. grps			p1<0.001*, p2=0.012*, p3=0.006*		
Corpora Lutea					
Min. – Max.	4.0 – 7.0	6.0 – 10.0	1.0 – 3.0	5.0 – 7.0	<0.001*
Mean ± SD.	5.10 ± 0.99	8.10 ± 1.45	1.30 ± 0.67	5.70 ± 0.67	
Median (IQR)	5.0 (4.0 – 6.0)	8.0 (7.0 – 9.0)	1.0 (1.0 – 1.0)	6.0 (5.0 – 6.0)	
pcontrol		0.003*	0.009*	0.415	
Sig. bet. grps			p1<0.001*, p2=0.031*, p3=0.001*		
Cystic Follicles					
Min. – Max.	0.0 – 0.0	0.0 – 0.0	2.0 – 6.0	0.0 – 1.0	<0.001*
Mean ± SD.	0.0 ± 0.0	0.0 ± 0.0	4.0 ± 1.49	0.20 ± 0.42	
Median (IQR)	0.0	0.0	4.0 (3.0–5.0)	0.0 (0.0–0.0)	
pcontrol		1.000	<0.001*	0.479	
Sig. bet. grps			p1<0.001*, p2=0.479, p3<0.001*		

Group I: Control Group II: Pomegranate Group III: Estradiol valerate Group IV: Estradiol valerate & Pomegranate IQR: Inter quartile range
SD: standard deviation.
p: *p value* for comparing between the four studied groups
pcontrol: *p value* for comparing between group I and each other group
p1: *p value* for comparing between group II and group III
p2: *p value* for comparing between group II and group IV
p3: *p value* for comparing between group III and group IV
*: Statistically significant at $p \leq 0.05$

Table 2: Comparison between the four studied groups according to the iNOS immunoreactive positive granulosa (GC), theca (TC) and interstitial (IC) cells in immunohistochemical study

Number of immunoreactive positive	Group I (n=10)	Group II (n=10)	Group III (n=10)	Group IV (n=10)	*p*
Granulosa Cells					
Min. – Max.	3.0 – 8.0	1.0 – 3.0	40.0 – 70.0	8.0 – 10.0	<0.001*
Mean ± SD.	5.50 ± 1.72	2.30 ± 0.67	52.0 ± 10.59	8.70 ± 0.82	
Median (IQR)	5.0 (4.0–7.0)	2.0 (2.0–3.0)	50.0 (40.0–60.0)	8.50 (8.0–9.0)	
pcontrol		0.552	<0.001*	0.552	
Sig. bet. grps			p1<0.001*, p2=0.055, p3<0.001*		
Theca Cells					
Min. – Max.	8.0 – 15.0	5.0 – 10.0	50.0 – 80.0	15.0 – 30.0	<0.001*
Mean ± SD.	11.30 ± 2.50	7.50 ± 1.96	64.0 ± 9.94	24.0 ± 4.90	
Median (IQR)	11.0 (10.0–14.0)	7.50 (6.0–9.0)	62.50 (60.0–70.0)	24.0 (20.0–28.0)	
pcontrol		0.463	<0.001*	<0.001*	
Sig. bet. grps			p1<0.001*, p2<0.001*, p3<0.001*		
Interstitial Cells					
Min. – Max.	50.0 – 70.0	40.0 – 60.0	70.0 – 100.0	60.0 – 80.0	<0.001*
Mean ± SD.	59.60 ± 7.34	51.0 ± 7.75	89.0 ± 10.22	70.50 ± 7.68	
Median (IQR)	60.0 (55.0–66.0)	50.0 (45.0–60.0)	90.0 (80.0–100.0)	71.50 (62.0–78.0)	
pcontrol		0.115	<0.001*	0.029*	
Sig. bet. grps			p1<0.001*, p2<0.001*, p3<0.001*		

Group I: Control Group II: Pomegranate Group III: Estradiol valerate Group IV: Estradiol valerate & Pomegranate IQR: Inter quartile range
SD: standard deviation.
p: *p value* for comparing between the four studied groups
pcontrol: *p value* for comparing between group I and each other group
p1: *p value* for comparing between group II and group III
p2: *p value* for comparing between group II and group IV
p3: *p value* for comparing between group III and group IV
*: Statistically significant at $p \leq 0.05$

Table 3: Comparison between the four studied groups according to the image optical density (IOD) of iNOS immunostaining in the positive granulosa (GC), theca (TC) and interstitial (IC) cells in immunohistochemical study

Image optical density of INOS immunostaining in positive	Group I (n=10)	Group II (n=10)	Group III (n=10)	Group IV (n=10)	*p*
Granulosa Cells					
Min. – Max.	7.11 – 10.52	2.11 – 5.85	43.18 – 65.06	15.23 – 20.60	<0.001*
Mean ± SD.	8.79 ± 1.21	4.05 ± 1.12	51.21 ± 6.80	17.89 ± 1.93	
Median (IQR)	8.83(7.61–9.69)	4.13(3.52–4.65)	49.93(46.14–55.12)	17.77(16.48–19.54)	
pcontrol		0.029*	<0.001*	<0.001*	
Sig. bet. grps			p1<0.001*, p2<0.001*, p3<0.001*		
Theca Cells					
Min. – Max.	11.65 – 18.98	7.78 – 10.60	42.38 – 67.19	24.25 – 33.52	<0.001*
Mean ± SD.	14.81 ± 2.54	9.14 ± 0.96	54.06 ± 8.43	27.94 ± 2.97	
Median (IQR)	14.33(12.74–16.45)	8.99(8.43–9.78)	51.67(47.91–60.56)	27.67(25.57–29.36)	
pcontrol		0.047*	<0.001*	<0.001*	
Sig. bet. grps			p1<0.001*, p2<0.001*, p3<0.001*		
Interstitial Cells					
Min. – Max.	24.07 – 39.53	22.20 – 28.82	70.56 – 83.42	40.23 – 45.30	<0.001*
Mean ± SD.	31.59 ± 6.04	26.50 ± 2.09	77.25 ± 4.37	43.22 ± 1.92	
Median (IQR)	31.08(26.17–37.57)	26.95(25.21–28.33)	77.28(74.13–80.84)	43.30(41.21–45.21)	
pcontrol		0.034*	<0.001*	<0.001*	
Sig. bet. grps			p1<0.001*, p2<0.001*, p3<0.001*		

Group I: Control Group II: Pomegranate Group III: Estradiol valerate Group IV: Estradiol valerate & Pomegranate IQR: Inter quartile range
SD: standard deviation.
p: *p value* for comparing between the four studied groups
pcontrol: *p value* for comparing between group I and each other group
p1: *p value* for comparing between group II and group III
p2: *p value* for comparing between group II and group IV
p3: *p value* for comparing between group III and group IV
*: Statistically significant at $p \leq 0.05$

DISCUSSION

Polycystic ovary syndrome (PCOS) is one of the most common endocrinological disorders, affecting women in reproductive age.[23]

Rodent models are low-cost, easy-to-handle animals with brief estrous cycles and generation times.[24] The female rat has normal ovarian cycles of 4 or 5 days, with separate proestrous, estrous, metestrous, and diestrous phases; hence, this animal is an excellent model to study the PCOS.(25) Androgens, estrogens, anti-progesterone, and letrozole are used to induce reproductive and metabolic characteristics of PCOS in rats.[25]

EV rat model was used because it resembles human PCOS in many aspects, including anovulation, changes in the ovarian histology and systemic inflammation.[26] This model is also easier, faster, and more accessible to perform than other models of this disease.[27]

In the current study, the histological examination showed that the ovaries in group control group (group I) and in the pomegranate molasses group (group II) were of good histological structure and this explained why the ovarian cycles of these two groups were regular cycles of 4 or 5 days, with separate proestrous, estrous, metestrous, and diestrous phases at the vaginal smear.

While estradiol valerate group (group III) showed decline in the number of growing healthy follicles with the presence of multiple cystic follicles and atretic follicles and this explained the induction of polycystic ovary with defective ovulation which caused irregular ovarian cycles or constant cycles in estrous phase (Persistent Vaginal Cornification, PVC) at the vaginal smears at the end of the study period.

Nofal *et al*.[2] reported that by the effect of estradiol valerate, irregular ovarian cycles or constant cycles in estrous phase (Persistent Vaginal Cornification, PVC) at the vaginal smears were considered as signs of PCO and this was similar to the findings of the present study.

According to Mirabolghasemi *et al*.[18], vaginal smears of EV treated rats showed abnormal estrus cycles and persistent vaginal cornification (PVC) as a sign of the pres-ence of ovarian cysts and early confirmation of PCOS induction and these findings coincided with the findings of the present study.

In the current study, EV treated rats (group III) showed that the ovary had lost its characteristic architecture in comparison to the control rats (group I). There was a significant decline in the number of the healthy follicles in the cortices with the presence of multiple atretic follicles, large congested blood vessels with hemorrhage and multiple cystic follicles with thin granulosa layer, degenerated granulosa cells, vacuolated granulosa cells, intraluminal desquamated granulosa cells, large eosinophilic colloidal fluid and thick theca layer. These histological findings demonstrated the defective ovulatory function with defective ovulation in PCO in the form of multiple cystic follicles with thickened theca cell layer that mainly prevented the ova release and this may be developed from presence of oxidative damage.

According to Badawi *et al*.[19], the EV-treated group showed massive different follicular loss with large number of follicular cysts and many atretic degenerative follicles and these findings coincided with the findings of the present study. These histological findings were also in accordance with Ghafurniyan *et al*.[28], Rezvanfar *et al*.[29], Wu *et al*.[30] and Nofal *et al*.[2], who showed same results.

Nofal *et al*.[2] reported that follicular rupture and release of ova can be mechanically prevented by a thickened theca cell layer and an excess proportion of collagen deposited around follicles and this explained the defective ovulation in PCOS and this was similar to the present study. Chronic inflammation could contribute to fibrosis and so it has been proven to play a role in the PCOS pathophysiology[31] Many studies reported oxidative stress as one of the pathological factors for PCOS.[32]

In the present work, to evaluate the presence of oxidative damage, iNOS expression in the ovaries of different experimental groups was assessed using an immunohistochemical technique.

Oxidative stress develops whenever the body antioxidant defenses are overwhelmed by the reactive oxygen species (ROS) increased production and therefore causes protein modification, lipid peroxidation, damage to DNA and other effects.[33] NO is produced by the inducible form of nitric oxide synthase (iNOS) in inflammatory and autoimmune diseases. The enzyme primarily responsible for the roles of NO in inflammatory processes is the iNOS which is not typically expressed in resting cells and must first be induced by certain cytokines or inflammatory products.[23] In the PCOS pathophysiology, low-grade chronic inflammation played a crucial role.[34]

In EV treated rats (group III), there was a significant increase in the number of the iNOS immunoreactive positive granulosa, theca and interstitial cells with a significant increase of the optical density of iNOS immunostaining for all these positive cells compared with the control group (group I), confirming the presence of oxidative damage in PCOS and this explained the previous histological changes that found in EV treated rats (group III).

Nofal *et al*.[2] reported that the expression of iNOS was raised in EV group in the granulosa and theca cells confirming the presence of an imbalance of oxidative/ antioxidative status in PCOS with no significant change in the interstitial cells and this was similar to the present study but there was also increase in the interstitial cells in the present study.

Oxidative stress is known to be a major pathological characteristic of PCOS and the antioxidant status has been reduced in females with PCOS.[35] Natural products can often provide more safety than synthetic medications

with fewer side effects. The high polyphenol content of pomegranate fruit, which makes it a strong astringent and anti-inflammatory product, has drawn the pharmaceutical and cosmetic industries, leading to a rise in recent popularity of the fruit.[36] Oxidative stress could be protected by medicine plant product rich in polyphenol compound such as PM.[37]

In the present study, rats administrated PM (group II) showed better histological structure, a significant larger number of growing healthy follicles and also a significant larger number of corpora lutea than those of the control rats (group I). There were fewer i-NOS immunoreactive positive granulosa, theca and interstitial cells with weaker optical density of iNOS immunostaining for all these positive cells significantly compared with those of the control rats (group I) confirming the anti-oxidative effect of the pomegranate molasses and this explained the better histological structure that found in rats administrated PM (group II). This means that PM protected the state of ovulation in comparison to the control rats (group I) via its anti-oxidative effect.

Rats administrated EV&PM (group IV) showed marked better histological structure than those of EV treated rats (group III). The number of the growing healthy follicles and also corpora lutea was significantly increased than those of EV treated rats (group III) with a significant marked decrease of the cystic and atretic follicles indicating protection of ovulatory function of the ovary as simultaneous drug administration was used and also this explained the protective effect of pomegranate molasses on the ovarian cycles through displaying relatively regular estrous cycles.

There were significantly fewer iNOS immunoreactive positive granulosa, theca and interstitial cells with significantly weaker optical density of iNOS immunostaining for all these positive cells compared with those of the EV treated rats (group III) confirming the anti-oxidative effect of the pomegranate molasses and this explained the better histological structure that found in rats administrated EV&PM (group IV) than those of EV treated rats (group III). This means that PM led to protection of the ovulatory function of the ovary via its anti-oxidative effect.

The findings of the present study coincides with explanation of Mihanfar *et al*.[38] who stated that since oxidative stress played an important role in the development of PCOS, polyphenols are recommended to be an effective treatment for this disorder, but the present study was a protective study rather than a recovery study.

The present study coincides with Chitra *et al*.[39] who suggested that the use of pomegranate extract declined the complications of the polycystic ovary syndrome.

Yayla *et al*.[40] reported that pomegranate had shown protective effect on the ovarian ischemia by decreasing oxidative stress and increasing antioxidant defense system and this explains that pomegranate can be used as antioxidant agent as it used in the present study.

Based on the present study, EV induced PCO causes a serious oxidative stressful effect on ovarian follicles but this effect could be markedly protected by using natural antioxidant products (PM).

CONCLUSION

Pomegranate molasses protected the ovaries and limited the vaginal smear, histological and immunohistochemical changes induced by estradiol valerate, and therefore, there is a potential benefit in using pomegranate molasses with EV induced PCOS. Moreover, the immunohistochemical detection of iNOS may be provided as a sensitive marker for oxidative damage in PCOS.

ABBREVIATIONS

PCOS: Polycystic ovary syndrome, **EV:** Estradiol valerate, **PM:** Pomegranate molasses, **PVC:** Persistent Vaginal Cornification, **i-NOS:** inducible Nitric Oxide Synthase, **IOD:** Image optical density, **GnRH:** Gonadotrophin releasing hormone, **LH:** Luteinizing hormone, **mg:** milligram, **ml:** milliliter, **Ig:** Immunoglobulin, **NaCl:** Sodium chloride, **H&E:** Hematoxylin and Eosin stain, **µm:** Micrometer, Image **J:** Java-based image, **SPSS:** Statistical Package for the Social Sciences, **ANOVA:** Analysis of variance, **Fig.:** Figure, **DAB stain:** Diaminobenzidine stain, **ABC:** Avidin-biotin complex, **P value:** Probability value, **N:** number, **M:** mean, **SD:** Standard deviation, **IQR:** Inter quartile range, **ROS:** Reactive oxygen species, **DNA:** Deoxy-ribonucleic acid, **NO:** Nitric Oxide.

CONFLICT OF INTERESTS

There are no conflicts of interest.

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الملخص العربى

دور دبس الرمان على تكيس مبايض اناث الجرذان البيضاء البالغة الناجمة عن مادة استراديول فاليرات: دراسة نسيجية و نسيجية كيميائية مناعية

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المقدمة: أحد أكثر امثلة اعتلال الغدد الصماء المعروفة في الإناث في فترة التكاثر هو متلازمة تكيس المبايض. يتم تنشيط متلازمة تكيس المبايض من قبل مادة استراديول فاليرات و هي احدى مركبات الستيرويد طويل المفعول, و الذي يؤدي إلى ظهور المتلازمة بسرعة بسبب الاضطرابات الأيضية والفسيولوجية. و لقد وجد ان الإجهاد التأكسدي قد يؤدي إلى متلازمة تكيس المبايض. يستخدم دبس الرمان على نطاق واسع كأحد المنتجات الطبيعية المضادة للأكسدة.

الهدف: هدف هذا العمل الى اكتشاف الحماية المحتملة لدبس الرمان على متلازمة تكيس المبايض الناجمة عن مادة استراديول فاليرات في اناث الجرذان البيضاء البالغة.

مواد و طرق البحث: تم فصل اربعين من اناث الجرذان البيضاء البالغة ذات الدورات الطبيعية الى اربعة مجموعات (عشرة جرذان لكل مجموعة); المجموعة الاولى: المجموعة الضابطة, المجموعة الثانية: المجموعة المعالجة بدبس الرمان, المجموعة الثالثة: المجموعة المعالجة بمادة استراديول فاليرات, المجموعة الرابعة: المجموعة المعالجة بمادة استراديول فاليرات ودبس الرمان معا. وبعد ستة اسابيع من التجربة تم إجراء التحليل الخلوي للمسحة المهبلية لجرذان جميع المجموعات لتقييم حالة الدورة الشبقية ثم بعد ذلك تم ذبح جميع الجرذان وتم استئصال مبايضها وتحضيرها للدراسة النسيجية و النسيجية الكيميائية المناعية.

النتائج: أظهرت المجموعة المعالجة بمادة استراديول فاليرات دورات شبقية غير منتظمة أو دورات مستمرة في مرحلة الشبق (التقرن المهبلي المستمر) التي كانت تعتبر علامات لتكيس المبايض. بناءا على الدراسة النسيجية، انخفض عدد البصيلات المختلفة الصحية في قشره المبايض انخفاضا بارزا مع وجود عدة بصيلات كيسية ورتقية وكذلك اوعية دموية كبيرة محتقنة مع وجود نزيف. بناءا على الدراسة النسيجية الكيميائية المناعية, ازدادت عدد الخلايا الحبيبية و القرابية والخلالية الإيجابية للإنزيم المخلق لأكسيد النيتريك بشكل بارز في هذه المجموعة مع زيادة الكثافة البصرية لجميع هذه الخلايا الإيجابية. واثبتت ايضا النتائج ان الحاق دبس الرمان مع مادة استراديول فاليرات في المجموعة الرابعة كان له تأثير حسن على اضطرابات المسحة المهبلية والاضطرابات النسيجية و النسيجية الكيميائية المناعية في المبايض على حد سواء.

الاستنتاج: ان هناك فائدة محتملة في استخدام دبس الرمان مع متلازمة تكيس المبايض الناجمة عن مادة الاستراديول فاليرات.