

# Therapeutic Effects of Eugenol in Polycystic Ovarian Rats Induced by Estradiol Valerate: A Histopathological and A Biochemical Study

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## Abstract

**Background:** Polycystic ovary syndrome (PCOS) is a common type of endocrinopathy in women which is accompanied by androgens elevation, insulin resistance, and metabolic dysfunction. Eugenol is a phenolic component of clove oil that has an antioxidant, anti-inflammatory, and anti-diabetic activity. The present study aimed to evaluate the therapeutic effects of eugenol on the PCOS models of rats.

**Materials and Methods:** In this experimental study, thirty adults female Wistar rats weighing between 180 and 200 g were used. Estradiol valerate-induced PCOS rats (4 mg/rat) were treated with eugenol (12 and 24 mg/kg) for 28 days. The effects of eugenol were studied on levels of glucose, lipid profile, liver enzymes, reproductive hormones, oxidative stress, and the expression of cyclooxygenase-2 (*Cox-2*) and peroxisome proliferator-activated receptor alpha (*Ppar-α*) genes, using biochemical analysis of blood and histopathological evaluation of ovaries.

**Results:** Estradiol valerate-induced PCOS resulted in the formation of cystic follicles in the ovaries, hyperinsulinemia, hyperglycemia, hyperlipidemia, hyperandrogenism, and anovulation. It altered the *Cox-2* and *Ppar-α* gene expression and increased oxidative stress and activities of liver enzymes. Eugenol treatment improved the PCOS-associated endocrine and metabolic disorder and histopathological alterations, mostly through antioxidant, anti-diabetic, anti-hyperlipidemic, and anti-androgenic properties. It showed beneficial effects on serum glucose, serum insulin, fat profile, reproductive hormones, liver activity, oxidative stress, expression of *Cox-2* and *Ppar-α* genes, as well as restoration of normal ovulation in the PCOS animals.

**Conclusion:** Eugenol could represent a promising natural product to prevent PCOS or reduce its symptoms.

**Keywords:** *Cox-2*, Eugenol, Metabolic Dysfunction, Polycystic Ovary Syndrome, *Ppar-α*

**Citation:** Kokabiyan Z, Yaghmaei P, Jameie SB, Hajebrahimi Z. Therapeutic effects of eugenol in polycystic ovarian rats induced by estradiol valerate: a histopathological and a biochemical study. *Int J Fertil Steril.* 2022; 16(3): 184-191, doi: 10.22074/ijfs.2021.537724.1176.

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## Introduction

Polycystic ovary syndrome (PCOS) is a highly frequent hormonal disease that affects women in their childbearing ages. The symptoms of PCOS vary with each woman; however, it is marked especially by amenorrhea, hirsutism, hyper androgens (male hormone), hyperinsulinemia, obesity, ovarian enlargement, ovulatory dysfunction, and infertility. PCOS is one of the important reasons for female infertility. In women with this syndrome, the irregularity of hormones interferes with the monthly growth and release of eggs from the ovarian tissues which is called ovulation (1).

The main reason for PCOS is not fully understood, although it usually begins with an increase in levels of reproductive hormones such as luteinizing hormone (LH), androgen, or estrogen. These conditions result in an abnormal cycle of gonadotropin secretion by the pituitary gland (1). There is evidence that resistance to insulin and an increase in the amount of insulin can stimulate ovarian/adrenal androgen secretion. Studies have shown that most

women with PCOS suffer from insulin resistance and also show type 2 diabetes and cardiovascular disorder in comparison with healthy women. Obesity is a major cause of insulin resistance (2). There are also growing concerns about a link between PCOS and systemic inflammation as evidenced by a significant increase in inflammatory factors including C-reactive protein (CRP), interleukin-18, monocyte chemoattractant protein-1, white blood count, and increased oxidative stress. Elevated CRP is an independent prediction marker for the risk of cardiovascular disorder and type 2 diabetes that highlights the role of inflammation in PCOS (3). In addition, it has been proven that oxidative stress in women with PCOS increases, and the relation between oxidative stress and inflammation is undeniable (4). Therefore, this supports the association of PCOS with systemic inflammation.

Various therapeutic strategies are used for clinical management of PCOS such as lifestyle changes including weight loss, diet, and exercise for women's obesity and

Received: 29/August/2021, Accepted: 04/December/2021

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Royan Institute  
International Journal of Fertility and Sterility  
Vol 16, No 3, July-September 2022, Pages: 184-191

anovulation; ovulation-inducing oral medications; and lowering insulin levels by taking diabetes medications such as metformin (5). In recent decades, considerable attention has been paid to herbal drugs in both developing and developed countries instead of the use of chemical drugs because of their minimal side effects, easy availability, and comfortable administration (6, 7). Eugenol (4-allyl-2-methoxy phenol) is a phenolic component of clove oil extracted from a variety of other plants such as *Eugenia caryophyllus* and *Myristica fragrans*. Anti-inflammatory and antioxidant activities of eugenol have been reported (8). Eugenol administration has been shown to suppress NF- $\kappa$ B activation in a rat model of gastric carcinogenesis (9) that has an essential role in inflammation (10).

Because of the importance of infertility treatment in women and the significant role of PCOS in anovulation, here we examined the therapeutic potential of eugenol on the ovulation improvement, liver activity, and cyclooxygenase-2 (*Cox-2*) and peroxisome proliferator-activated receptor alpha (*Ppar- $\alpha$* ) genes expression in a Wistar rat model of PCOS. COX-2 has cyclooxygenase and peroxidase functions that give rise to oxidative stress (11). In addition, studies have demonstrated that Cox-2 is expressed following the activation of NF- $\kappa$ B in inflammatory reactions (10). *Ppar- $\alpha$* , also known as NR1C1 (nuclear receptor subfamily 1, group C, member 1), is a nuclear hormone receptor and a regulator of lipid and glucose metabolism and has anti-inflammatory activity. It has been shown that *Ppar- $\alpha$*  can decrease inflammation through the inhibition of the signaling pathway of NF- $\kappa$ B or reducing the level of activated NF- $\kappa$ B (12).

## Materials and Methods

### Experimental animals

In this experimental study, 30 adults female Wistar rats weighing between 180 and 200 g were purchased from Pasteur Institute of Tehran, Iran. Animals were kept in the animal house of Islamic Azad University, Science and Research Branch, Tehran, Iran, under standard laboratory conditions of constant temperature (22°C) and humidity with a 12H: 12H cycle of light and dark. They had free access to a normal diet and water. Animals were taken care of following the Guide for the Care and Use of Laboratory Animals (13). All procedures explained in this study were validated by the Animal Care and Use Committee of Islamic Azad University (IR.IAU.SRB.REC.1396.51). All animals were weighed weekly and body weight changes were recorded throughout the experiment. To familiarize the rats with the laboratory environment, animals were kept in the laboratory for 14 days without any intervention.

Intraperitoneal injection of a single dose of estradiol valerate (4 ml, 4 mg/rat, or 16 mg/kg; Aburaihan Pharmaceutical Co, Tehran, Iran) was used to induce PCOS during 28 days (14). To confirm PCOS induction, histological analysis of ovaries and hormonal and biochemical analysis of blood were performed on three rats.

Animals were randomly divided into 5 groups (each

group consists of 6 rats) as follows:

- i. The control group (C) receiving a normal diet.
- ii. The PCOS group receiving a normal diet.
- iii. Sham operation group: PCOS animals (as control of PCOS) which received intraperitoneal injection of the tween (80%) as eugenol solvent for 28 days.
- iv. Experimental group 12 (EXP-12): PCOS animals that received intraperitoneal injection of eugenol at a dose of 12 mg/kg body weight (Sigma Chemical Co, St. Louis, MO, USA) for 28 days.
- v. Experimental group 24 (EXP-24): PCOS animals that received intraperitoneal injection of eugenol at a dose of 24 mg/kg body weight for 28 days.

### Hormonal and biochemical analysis

Ovary, liver tissues, and serum samples were collected at the end of the experiment as described previously (15). Briefly, anesthesia was performed using ketamine (80 mg/kg) and xylazine (10 mg/kg), and blood samples were obtained from the heart. After 2 hours of incubation at room temperature, centrifugation was done at 2500  $\times$  g for 5 minutes for serum separation. Serum samples were kept at -20°C until further analysis. The concentration of total cholesterol (TC), high-density lipoprotein (HDL), low-density lipoprotein (LDL), triglyceride (TG), glucose, aspartate transaminase (AST/GOT) enzyme, alanine transaminase (ALT/GPT) enzyme, and alkaline phosphatase (ALP) enzyme were measured by quantitative photometric kits purchased from Parsazmun Company (Karaj, Iran) based on the manufacturer's recommendations. Also, a blood glucose meter (Accu-Chek, Germany) was used to determine the amount of whole blood sugar in the blood collected from the animal tail.

Serum levels of estradiol and progesterone were determined using rat ELISA kits (Monobind Co., USA) based on the manufacturer's instructions. Levels of insulin, testosterone, LH and follicle-stimulating hormone (FSH) were measured using rat/mouse ELISA test kits (Cosmo Bio Co. Ltd. Japan) as per the manufacturer's instructions.

To evaluate liver superoxide dismutase (SOD) and malondialdehyde (MDA), small fractions of liver tissue were removed and homogenized in ice-cold KCL (0.15 M, 3 ml/g liver tissue). The liver homogenate was centrifuged at 10000 rpm at 4°C for 20 minutes and then the supernatant was separated and stored at -70°C. Nasdox and Nalondic ELISA kits were used to evaluate the activities of SOD and MDA, respectively (Navand Salamat Co., Urmia, Iran) according to the manufacturer's instructions.

### Histological analysis

Ovaries and pancreas were collected and examined for histopathological assays at the end of the procedure. Briefly, ovaries samples were removed and trimmed free of fat. Fixation was done using 10% paraformaldehyde for 24 hours. Then samples were dehydrated and cleared using alcohol and xylene solutions, placed in paraffin, sectioned at a thickness of 7  $\mu$ m by a microtome, and then mounted on

glass slides. Finally, samples were stained with Hematoxylin and Eosin (H&E) and imaged under a conventional light microscope. The number of Primary, secondary (or antral follicle), Graafian follicles, Corpus luteum, and follicular cysts were assayed in all animal ovaries according to Erickson's classification (16). Images of islets were taken in pancreas samples using a light microscope.

### Gene expression analysis by quantitative real-time polymerase chain reaction

Rats were sacrificed at the end of the experiment and ovarian tissue was removed and kept in liquid nitrogen and stored at  $-75^{\circ}\text{C}$  until the time of use. Total RNA was extracted using a High Pure RNA Isolation kit (Roche, Germany) following the manufacturer's instructions.

The integrity of RNA was assessed by agarose gel electrophoresis and also measured by a Nanodrop (Thermo Scientific, USA) at 260 and 280 nm. One microgram of total RNA was used for the cDNA synthesis, using Prime Script<sup>TM</sup> 1<sup>st</sup> strand cDNA Synthesis kit (Takara, Japan) in a final volume of 20  $\mu\text{l}$  and according to the manufacturer's recommendations. DNase I treatment was included in the cDNA synthesis process. The reaction was terminated by incubation for 5 minutes at  $70^{\circ}\text{C}$ . The cDNA product was stored at  $-20^{\circ}\text{C}$  until use.

The mRNA levels of *Cox-2* and *Ppar- $\alpha$*  were studied using quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR). Glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) was used as a reference (internal control) gene to normalize *Cox-2* and *Ppar- $\alpha$*  expression levels.

Gene-specific primers were designed using Allele ID 6.0 software and then synthesized by Macrogen (South Korea). The specificity of primer sequences for the interesting genes was checked using BLAST search. The rat primers were:

#### *Ppar- $\alpha$* -

F: 5'- GTTGAAAACAAGGAGGCAGAGG-3'

R: 5'-GAGGACAGCATGGTGAAGATGG-3'

#### *Cox-2*-

F: 5'- GGAGAGACGATCAAGATAGTGA-3'

R: 5'- GAGGATGGAGTTGTTGTAGAG-3'

#### *Gapdh*-

F: 5'- GGATAGTGAGAGCAAGAGAGAGG-3'

R: 5'- ATGGTATTGGAGAGAAGGGAGGG-3'

Real-time RT-PCR was run on a StepOnePlus Real-Time PCR (Applied Biosystems, USA) using the SYBR green PCR master mix (Takara, Japan). 10  $\mu\text{l}$  of master mix, 0.8  $\mu\text{l}$  of each primer, 0.4  $\mu\text{l}$  of ROX reference dye II, 1  $\mu\text{l}$  of cDNA template, and 7  $\mu\text{l}$  of RNase free water were added. Real-time PCR was programmed into two steps: initialization at  $94^{\circ}\text{C}$  for 30 seconds, amplification for 40 cycles with denaturation at  $95^{\circ}\text{C}$  for 5 seconds, annealing at  $60^{\circ}\text{C}$  for 30 seconds, and extension at  $72^{\circ}\text{C}$  for 30 seconds. To ensure the specificity of amplification, dissociation curve analysis was performed for all genes. Change in the fold number was calculated by using the  $2^{-\Delta\Delta\text{Ct}}$  method. RT-qPCR GraphPad Prism 6 software (GraphPad Software Inc., San Diego, USA) was used to analyze the data.

### Statistical analysis

SPSS statistical software (version 17; SPSS Inc., Chicago, USA) was used for the data analysis. All data are presented as the means  $\pm$  SEM. Shapiro-Wilk and Levine tests were performed to examine the normality of data distribution and homogeneity of variances. One-way analysis of variance (One-Way ANOVA) with t test and duncan test was used for analyzing the data and comparison of the different group means.  $P < 0.05$  was considered as significant.

### Results

#### Measurement of weight, biochemical characteristics, hormones, and enzymes

The serum levels of glucose, insulin, TG, TC, and LDL were significantly higher in PCOS and sham operation groups as compared to the control group. Administration of eugenol at a dose of 24 mg/kg body weight decreased their serum value (EXP-24 group). Administration of estradiol valerate decreased the serum amount of HDL in PCOS and sham rats in comparison to control ones. However, its level nearly increased to the normal value following eugenol administration in the Exp-24 group. In contrast to EXP-24, eugenol treatment at a concentration of 12 mg/kg (EXP-12 group) only improved the serum levels of TG and HDL and did not affect the value of glucose, insulin, TC, and LDL (Table 1).

**Table 1:** Serum levels of glucose, TG, TC, LDL, HDL, and insulin in PCOS and non-PCOS rats

Group	Glucose (mg/dl)	TG (mg/dl)	TC (mg/dl)	LDL (mg/dl)	HDL (mg/dl)	Insulin ( $\mu\text{IU/ml}$ )
Control	93.75 $\pm$ 2.75	53.67 $\pm$ 4.10	68.00 $\pm$ 2.74	34.40 $\pm$ 1.55	31.00 $\pm$ 0.58	0.146 $\pm$ 0.008
PCOS	129.60 $\pm$ 6.36**	76.00 $\pm$ 2.31**	82.75 $\pm$ 2.43**	42.50 $\pm$ 0.69**	22.68 $\pm$ 1.03**	0.204 $\pm$ 0.013**
Sham	125.00 $\pm$ 6.45**	73.75 $\pm$ 2.25**	86.33 $\pm$ 3.18**	42.63 $\pm$ 0.71**	22.50 $\pm$ 1.44**	0.199 $\pm$ 0.018*
EXP-12	115.67 $\pm$ 3.18	57.67 $\pm$ 1.20 <sup>#</sup>	78.00 $\pm$ 1.00	40.40 $\pm$ 0.92*	28.00 $\pm$ 1.41 <sup>#</sup>	0.183 $\pm$ 0.014
EXP-24	100.67 $\pm$ 4.78 <sup>#</sup>	54.00 $\pm$ 2.89 <sup>#</sup>	69.00 $\pm$ 2.35 <sup>#</sup>	35.60 $\pm$ 0.60 <sup>#</sup>	29.67 $\pm$ 1.20 <sup>#</sup>	0.144 $\pm$ 0.004 <sup>#</sup>

Values are presented as mean  $\pm$  SEM from 5 rats in each group. TG; Triglyceride, TC; Total cholesterol, LDL; Low-density lipoprotein, HDL; High-density lipoprotein, PCOS; Polycystic ovary syndrome, EXP-12; Experimental group 12, EXP-24; Experimental group 24, \*; Statistically different from the control rats, #; Statistically different from the PCOS rats, ;\*, #;  $P \leq 0.05$ , and \*\*, ##;  $P \leq 0.01$ .

The serum amounts of LH, FSH, testosterone, progesterone and estradiol hormones are presented in Table 2. We observed that injection of estradiol valerate for 28 days decreased the serum level of FSH and progesterone and increased the levels of LH, testosterone, and estradiol in PCOS and sham rats in comparison to control rats. However, their levels nearly reached the normal value following eugenol administration in EXP-12 and Exp-24 groups (except for the LH hormone in the EXP-12 group).

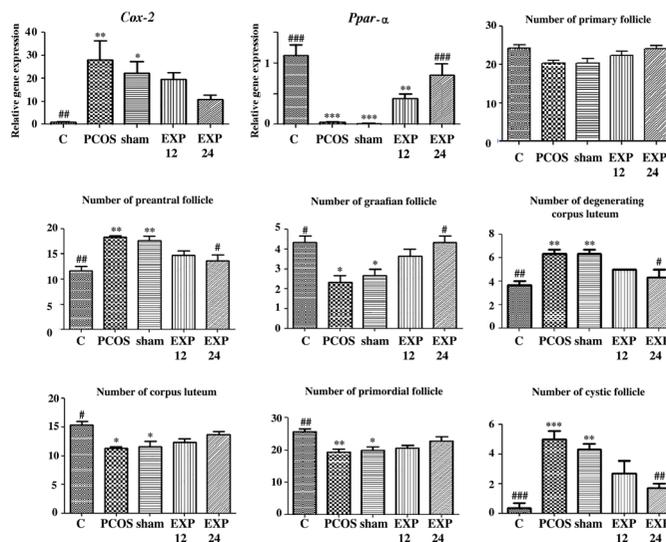
The content of SOD decreased, while the MDA, AST, ALT, and ALP significantly increased in the PCOS and sham rats in comparison to the control rats (Table 3). However, their amount improved to near normal levels following eugenol administration in EXP-12 and EXP-24 groups (except for the AST and ALT enzyme in EXP-12 rats).

**Gene expression analysis by real-time polymerase chain reaction**

The relative expression of *Cox-2* and *Ppar-α* genes was studied using quantitative real-time RT-PCR.

The expression of *Cox-2* increased, while the expression of the *Ppar-α* gene significantly decreased in the PCOS and sham rats in comparison to the control rats (Fig.1). Eugenol treatment at a concentration of 24 mg/kg improved the expression of *Cox-2* and *Ppar-α* genes in the EXP-24 group. The expression of *Cox-2* decreased, while the expression of *Ppar-α* significantly increased in the

EXP-24 group after eugenol administration. In contrast to EXP-24, eugenol treatment at a concentration of 12 mg/kg (EXP-12 group) had no effect on the expression levels of *Cox-2* and *Ppar-α* gene in PCOS rats.



**Fig.1:** The expression of *Cox-2* and *Ppar-α* genes, and the number of primary follicles, preantral follicles, Graafian follicles, degenerating corpus luteum, corpus luteum, primordial follicles, and follicular cysts in all animal groups. C; Control, PCOS; Estradiol valerate-induced polycystic ovarian rat with a normal diet, Sham; Control PCOS rat with IP injection of tween as eugenol solvent, EXP-12; PCOS rats with IP injection of eugenol (12 mg/kg body weight), EXP-24; PCOS rats with IP injection of eugenol (24 mg/kg body weight), \*; Statistically different from the control rats, #; Statistically different from the PCOS rats, \*; #; P<0.05, \*\*; ###; P<0.01, and \*\*\*\*; ####; P<0.001.

**Table 2:** Serum levels of LH, FSH, testosterone, progesterone, and estradiol in PCOS and non-PCOS rats

Group	LH (mIU/ml)	FSH (mIU/ml)	Testosterone (ng/ml)	Progesterone (ng/ml)	Estradiol (pg/ml)
Control	0.36 ± 0.005	0.49 ± 0.002	0.17 ± 0.035	3.23 ± 0.273	2026.0 ± 16.74
PCOS	0.47 ± 0.003***	0.34 ± 0.003***	3.24 ± 0.074***	2.16 ± 0.130**	2413.5 ± 40.74***
Sham	0.45 ± 0.006***	0.34 ± 0.005***	3.33 ± 0.118***	2.17 ± 0.189**	2469.7 ± 28.58***
EXP-12	0.42 ± 0.015**,#	0.40 ± 0.007***,###	0.31 ± 0.045###	2.98 ± 0.107#	2142.7 ± 44.05##
EXP-24	0.38 ± 0.005###,+	0.49 ± 0.002###,+++	0.58 ± 0.086*,###	3.18 ± 0.028##	2033.0 ± 11.00###

Values are presented as mean ± SEM from 5 rats in each group. LH; Luteinizing hormone, FSH; Follicle-stimulating hormone, PCOS; Polycystic ovary syndrome, EXP-12; Experimental group 12, EXP-24; Experimental group 24, #; Statistically different from the control rats, #; Statistically different from the PCOS rats, \*; Statistically different from the EXP\_12 rats. \*, #; P<0.05, \*\*, ##; P<0.01, and \*\*\*, ###, \*\*\*\*; P<0.001.

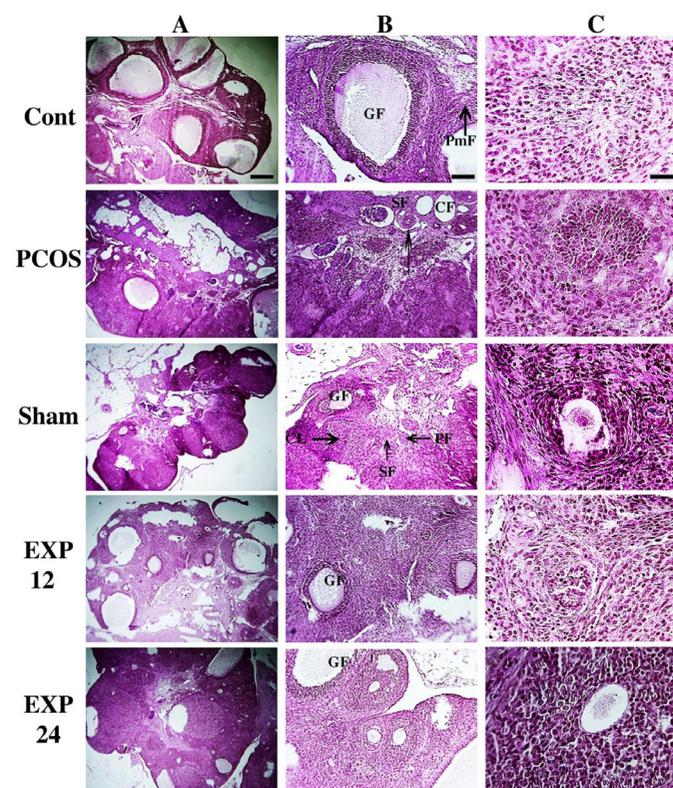
**Table 3:** SOD and MDA contents in liver tissue and serum levels of AST, ALT, and ALP in PCOS and non-PCOS rats

Group	SOD (U/ml)	MDA (μL)	AST (U/L)	ALT (U/L)	ALP (U/L)
Control	0.49 ± 0.034	3.46 ± 0.075	149.50 ± 5.14	143.00 ± 5.80	971.7 ± 40.45
PCOS	0.27 ± 0.006***	5.02 ± 0.277***	232.40 ± 8.72**	215.20 ± 10.91***	1229.0 ± 28.29**
Sham	0.28 ± 0.021***	4.94 ± 0.113***	223.33 ± 14.53*	210.00 ± 10.00**	1212.5 ± 31.46**
EXP-12	0.42 ± 0.003##	3.53 ± 0.084###	189.00 ± 14.74	186.00 ± 8.88*	1022.7 ± 15.84#
EXP-24	0.49 ± 0.008###	3.74 ± 0.174##	159.33 ± 18.55##	149.50 ± 7.41##	975.7 ± 55.64##

Values are presented as mean ± SEM from 5 rats in each group. SOD; Superoxide dismutase, MDA; Malondialdehyde, AST; Aspartate transaminase, ALT; Alanine transaminase, ALP; Alkaline phosphatase, PCOS; Polycystic ovary syndrome, EXP-12; Experimental group 12, EXP-24; Experimental group 24, #; Statistically different from the control rats, #; Statistically different from the PCOS rats, \*, #; P<0.05, \*\*, ##; P<0.01, and \*\*\*, ###, \*\*\*\*; P<0.001.

## Histological observation

Morphological studies of the ovaries were done after 28-day administration of eugenol. A comparison of ovarian samples from different groups showed distinct morphological alterations in the ovaries. Injection of estradiol valerate during 28 days induced the formation of cystic follicles in PCOS and sham operation groups (Figs.1, 2). In comparison with the control group, counting the number of follicles in different stages in PCOS and sham operation animals specified that the injection of estradiol valerate decreased the number of primordial follicles, Graafian follicles, and corpus luteum; however, it increased the number of preantral follicles and degenerating corpus luteum. The administration of eugenol improved the histological features of the ovaries. Treatment with eugenol (12 or 24 mg/kg), markedly decreased the number of follicular cysts in EXP-12 and EX-24 groups. Furthermore, the administration of eugenol significantly increased the number of primordial follicles, Graafian follicles, and corpus luteum; however, it decreased the number of preantral follicles and the number of degenerating corpus luteum in the treated animals. Administration of estradiol valerate or eugenol had no effect on the number of primary follicles.



**Fig.2:** Photomicrograph of a section in ovarian tissues in all animal groups using hematoxylin and eosin staining. **A.** 200  $\mu$ m scale bar, **B.** 100  $\mu$ m scale bar, and **C.** 50  $\mu$ m scale bar. Cont; Control, PCOS; Estradiol valerate-induced polycystic ovarian rat with a normal diet, Sham; Control PCOS rat with IP injection of tween as eugenol solvent, EXP-12; PCOS rats with IP injection of eugenol (12 mg/kg body weight), EXP-24; PCOS rats with IP injection of eugenol (24 mg/kg body weight), PmF; Primordial follicle, PF; Primary follicle, SF; Secondary follicle, GF; Graafian follicle, CF; Cystic follicle, and CL; Corpus luteum.

## Discussion

This study reported the potential therapeutic effects of eugenol on the improvement of the physiological changes of PCOS rats against ovarian toxicity induced by estradiol valerate. In the present work, treating rats with estradiol valerate resulted in significant histological and biochemical changes such as the formation of cystic follicles in the ovaries, hyperinsulinemia, hyperglycemia, hyperlipidemia, hyperandrogenism, anovulation, and increased oxidative stress and activities of liver enzymes indicating the development of PCOS. Previous studies of rodent models of PCOS have also shown hormonal changes, histological alterations of ovaries, and metabolic disorders (15, 17-19).

Many studies have used estradiol valerate to model PCOS in rodents (15, 17-19). Estradiol valerate is a long-acting estrogen that disrupts the secretion of hypothalamic gonadotrophin-releasing hormone (GnRH). This hormone is responsible for the secretion of FSH and LH from the anterior pituitary. Studies have reported impaired secretion of GnRH disrupts the release and storage of LH (17). In this study, administration of estradiol valerate caused a markedly increase in LH, estradiol, and testosterone and a significantly decrease in FSH and progesterone hormones similar to the characteristic of hormonal alterations in women with PCOS disorder (1). Decreased progesterone production indicates anovulation that similar to the human PCOS (1) and other rat models of estradiol valerate-induced PCOS (17-19). Hyperandrogenism (high level of testosterone) is the most typical hormonal change of PCOS that has an important role in the pathology of PCOS. Many studies of rats with estradiol valerate-induced PCOS model also have shown changes in sex hormonal secretion (18, 19).

The histological alterations in ovaries may be the result of the hormonal dysregulation caused by estradiol valerate. It is well demonstrated that pituitary gonadotropins (FSH and LH) are the main regulators of ovarian follicle evolution, oocyte maturation, and ovulation (20, 21). FSH stimulates the proliferation of granulosa cells and induces the production of estrogen hormone. It is also involved in follicle evolution from primary follicle to preantral follicle (20). LH is required for androgen synthesis and induces the onset of meiosis in oocytes, as well as the development of the corpus luteum and ovulation (21). In the present study, it has been observed that estradiol valerate-induced hormonal changes led to abnormal follicular development and an increase in cystic follicles. The number of primordial follicles, Graafian follicles, and corpus luteum, decreased markedly in PCOS animals, suggesting anovulation. This was while the number of preantral follicles increased significantly in PCOS rats, as compared to the control ones. Therefore, based on the analysis of ovaries, injection of estradiol valerate resulted in the formation of cystic follicles and a decrease in ovulation and the number of corpus luteum, probably due to hormonal alterations. In line with this data, previous

reports have revealed that continuous treatment of rats with steroid hormones causes anovulation (22).

Due to symptoms such as hyperinsulinemia, dysglycemia, and dyslipidemia, PCOS has been suggested to be a metabolic disorder (2). Data from the present study suggest that levels of blood glucose and insulin are markedly higher in PCOS than in the control animals, which is the property of insulin resistance. The cause of decreased insulin sensitivity in PCOS is not yet well understood, but factors that compensate for insulin resistance often have a therapeutic effect on the outcomes of the disease. A study by Holmång et al. (23) demonstrated that continuous exposure to androgens leads to insulin resistance due to effects on the transportation of glucose. In line with the present study, elevated testosterone levels may be partly involved in insulin resistance.

Insulin resistance may be the result of oxidative stress, too. There is supportive evidence that oxidative stress plays a critical role in the pathology of diabetes and the development of insulin resistance. Reactive oxygen species (ROS) can decrease insulin sensitivity by affecting insulin signal transduction and decreasing the expression of GLUT4 transporter (glucose transporter type 4) on the cell surface (24). On the other hand, oxidative stress could play a role in the development of PCOS, too. There are many reports on the role of oxidative stress in the pathology of PCOS (25). In this study, we showed that the induction of PCOS using estradiol valerate generated oxidative stress. Our data revealed that the serum levels of MDA significantly increased in the PCOS rats, while the levels of SOD decreased. MDA is a widely known biomarker for oxidative stress. It is the final product of the peroxidation of polyunsaturated fatty acids in the membrane of cells when the free radicals and ROS generate. Therefore, its increase in PCOS rats indicates the creation of oxidative stress. SOD is the mainline defense antioxidant with a primary function of dismutation of the superoxide anions into molecular oxygen and hydrogen peroxide (26). The reduction of this enzyme in the present study again supports the development of oxidative stress in PCOS rats. Therefore, insulin resistance in PCOS rats may be due to oxidative stress.

Androgens also affect the metabolism of lipoproteins in different ways. Hyperandrogenemia can affect the profile of lipids. In fact, one of the features of PCOS is a disturbance in the profile of lipids that can be caused by hyperandrogenemia (27). In the present work, estradiol valerate had adverse effects on serum lipid profile and markedly increased TG, TC, and LDL in PCOS animals. In addition, it markedly decreased HDL in PCOS animals in comparison to controls. In line with this study, Karateke et al. (28) have also shown a decrease in HDL and an increase in TG, TC, and LDL in PCOS rats.

The liver is the largest organ inside the body, responsible for almost all metabolic processes including fat metabolism, maintaining of blood glucose levels, and insulin metabolism that is all associated with PCOS (29).

Because of the central role of the liver in the homeostasis of glucose and metabolism of lipids, dysglycemia and dyslipidemia may affect the liver and leads to hepatic injury. In fact, Zhang et al. (30) have shown that an increase of androgen levels and resistance to insulin contribute to hepatic steatosis and can alter the metabolism of lipids in the liver, liver response to inflammation, and cellular function. In the present study, our data demonstrated that the level of AST, ALT, and ALP was significantly high in estradiol valerate-induced PCOS rats suggesting the dysregulation of liver function in PCOS. AST, ALT, and ALP are enzymes that are mostly present in the liver. High levels of them in the serum can indicate a liver injury, due to the release from damaged hepatocytes into the extracellular space (31). Results of this study confirmed the previous finding by Osman and co-workers. They showed an increase in AST, ALT, and ALP along with dyslipidemia and insulin resistance in estradiol valerate-induced PCOS rats. They also found that injection of estradiol valerate to female rats provokes oxidative stress, as also shown in the present study (32). Liver injury in PCOS subjects may be the result of increased oxidative stress. Parenchymal cells, hepatic stellate cells, Kupffer cells, and endothelial cells are the most sensitive cells to free radicals and oxidative stress and damage in the liver (33). Therefore, an increased level of AST, ALT, and ALP in the present study can confirm oxidative stress and above data in PCOS animals. These data led us to evaluate the expression of the *Cox-2* and *Ppar- $\alpha$*  genes in PCOS animals. *Cox-2* has cyclooxygenase and peroxidase functions that can be induced by ROS and give rise to oxidative stress (11). *Ppar- $\alpha$* , also known as NR1C1 (nuclear receptor subfamily 1, group C, member 1), is a nuclear hormone receptor and a hepatic regulator of lipid and glucose metabolism. It is expressed in organs with more oxidative activity and has a central role in fatty acid oxidation, inflammatory responses, and oxidative stress (34). As mentioned above, the expression of *Cox-2* increased, while the expression of the *Ppar- $\alpha$*  gene significantly decreased in the estradiol valerate model of PCOS rats that can indicate oxidative stress and dyslipidemia. Decrease of *Ppar- $\alpha$*  suggests that the dysregulation of lipids profile in PCOS subjects may be the result of *PPAR- $\alpha$*  inhibition. Karimzadeh et al. (35) reported similar results of increased *Cox-2* expression in PCOS rats. Wang et al. (36) induced the PCOS model in rats by DHEA and evaluated the *Ppar- $\gamma$* 's expression in adipose tissue. They found that both protein and mRNA levels of *Ppar- $\gamma$*  are reduced in the DHEA model of PCOS rats.

In this study, we searched the therapeutic potential of eugenol on ovulation improvement, liver activity, and *Cox-2* and *Ppar- $\alpha$*  genes expression in an estradiol valerate model of PCOS rats. Based on our data, administration of eugenol could attenuate symptoms of PCOS and return normal ovulation in PCOS animals. Eugenol is a phenolic component of clove oil with some potential therapeutic effects including anti-inflammatory, antioxidant, and neuroprotective activity (8). Our data revealed that after

using eugenol, the levels of FSH, LH, progesterone, testosterone, and estradiol improved to near the control value. These results are in line with some data reported previously by others. Moghimian et al. (37) have shown that clove extract could improve reproductive hormonal changes induced by morphine in rats. They observed a considerable increase in FSH levels following treatment with clove extract. Another study by Poli and Challa (38) demonstrated that eugenol could enhance the serum level of progesterone in female albino rats. A study by Barghi et al. (39) has shown that eugenol could decrease the serum level of testosterone in adult female rats after ovarian torsion/detorsion. Therefore, it seems that eugenol has beneficial effects on reproductive hormones and can improve hormonal changes in PCOS rats. We also observed that eugenol improved the histological features of the ovaries. Treatment with eugenol decreased the number of cysts in treated animals. Furthermore, we observed that eugenol can improve the number of primordial follicles, Graafian follicles, corpus luteum, preantral follicles, and decrease the number of degenerating corpus luteum in treated animals. These results may be due to the improvement of hormonal disorders following treatment with eugenol. Therefore, it may suggest that eugenol has a direct effect on the hypothalamic-pituitary-gonadal axis.

The improvement of hormonal levels and ovaries histological parameters in the EXP-1 and EXP-2 groups may be due to a decrease in oxidative stress, too. Some previous studies also have demonstrated the antioxidant activity of eugenol (8, 37, 38). As mentioned before injection of estradiol valerate produced oxidative stress in PCOS rats. Treatment with eugenol improved the levels of oxidative stress markers. The amount of SOD and MDA improved to near normal levels following eugenol administration in EXP-12 and EXP-24 groups. It increased the level of SOD and decreased the level of MDA in subjected animals. These data suggest that eugenol is beneficial as a protective agent against oxidative stress in PCOS individuals.

Benefit effects of eugenol were observed on liver function and metabolic characteristics of PCOS, too. Eugenol at a dose of 24 mg/kg body weight decreased the levels of glucose, insulin, TG, TC, and LDL; and increased the serum level of HDL in PCOS and sham rats in comparison to control ones, suggesting anti-diabetics and anti-hyperlipidemic effects of eugenol. Therefore, it seems that eugenol can prevent insulin resistance and dyslipidemia in the PCOS model of estradiol valerate. Some previous studies have demonstrated anti-hyperlipidemic and anti-diabetic effects of eugenol in the diabetic animal model (40).

As mentioned above, androgens and oxidative stress can affect the metabolism of lipoproteins and develop insulin resistance and dysglycemia. Therefore, it may be concluded that the anti-diabetic and anti-hyperlipidemic properties of eugenol are the result of a decrease in testosterone.

AST, ALT, and ALP act as biomarkers of liver function and their increase indicates hepatocyte injury

observed in PCOS rats. Eugenol (at a dose of 24 mg/kg) decreased serum levels of AST, ALT, and ALP in EXP-1 and EXP-2 groups. Because of the central role of the liver in the homeostasis of glucose and metabolism of lipids, dysglycemia and dyslipidemia may affect the liver and leads to hepatic injury. Therefore, these results may confirm the anti-diabetic and anti-hyperlipidemic properties of eugenol.

Eugenol treatment at a concentration of 24 mg/kg improved the expression of *Cox-2* and *Ppar- $\alpha$*  gene in the EXP-24 group, too. The expression of *Cox-2* decreased, while the expression of *Ppar- $\alpha$*  significantly increased in the EXP-24 group after eugenol administration, which can confirm again a decrease of oxidative stress and prevention of dyslipidemia.

## Conclusion

The present study focused on the effects of eugenol on some adverse features of estradiol valerate-induced PCOS in Wistar rats. The study provided supportive evidence that eugenol possesses potent antioxidant, anti-diabetic, anti-hyperlipidemic, and anti-androgenic properties. It showed beneficial effects against serum glucose, serum insulin, fat profile, reproductive hormones, liver activity, and oxidative stress, as well as restored normal ovulation in the PCOS animals. Eugenol could represent a promising natural product to reduce PCOS symptoms. A challenge for future studies will be to elucidate the mechanisms.

## Acknowledgments

This study was supported in part by Islamic Azad University, Science and Research Branch, Tehran, Iran. The authors declare no conflict of interest.

## Authors' Contribution

Z.K.; Contributed to all experimental work, data and statistical analysis, and interpretation of data. Z.H.; Drafted the manuscript, and contributed to data and statistical analysis, and interpretation of data. P.Y.; Contributed to conception and supervised, directed, designed, and managed the study. S.B.J.; Contributed to conception and designed the experiments. All authors read and approved the final manuscript.

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