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The Effects of Clove Oil on The Biochemical and Histological Parameters, and Autophagy Markers in Polycystic Ovary Syndrome-Model Rats

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Abstract

Background: This study aimed to determine whether syzygium aromaticum (clove) could help polycystic ovary syndrome (PCOS) rats.

Materials and Methods: In this experimental study, forty adult female Wistar rats (weighing 250 ± 10 g) were divided randomly into five groups; G1: control, G2: PCOS group, G3: PCOS+clove (30 mg/kg/ orally/daily) group, G4: PCOS+clove (60 mg/kg/orally/daily) group, and G5: PCOS+gonadectomy group. The PCOS was induced by a single dose injection of estradiol valerate (16 mg/kg/IM). Following PCOS induction, the rats were treated for 14 days. Histological parameters, follicle apoptosis, mRNA expression of autophagy markers (*Lc3, Beclin1*), oxidative stress markers, insulin and blood glucose levels, as well as serum levels of aromatase and testosterone were evaluated in these rats. Finally, the ratio of serum luteinizing hormone (LH) to follicle-stimulating hormone (FSH) levels was also calculated.

Results: The autophagy markers (*Lc3, Beclin1*), histological parameters, oxidative stress, insulin, and hormone levels changed significantly in the PCOS rats (G2). In G3 and G5 groups, it was observed that the levels of LH/FSH and testosterone decreased significantly in comparison to the PCOS group, and inhibition of autophagy was also observed in these groups. Treatment with cloves in the G3 group significantly improved oxidative stress, histological parameters, and insulin levels.

Conclusion: These findings demonstrated that oxidative stress, apoptosis, and excessive autophagy could be improved by treatment with low doses of clove and gonadectomy. Cloves may help to improve these parameters by regulating and inhibiting excessive autophagy. However, discovering the direct role of this extract in regulating the parameters such as oxidative stress, insulin, and androgens requires further investigation. In the present study, $P<0.05$ was considered statistically significant.

Keywords: Apoptosis, Autophagy, Oxidative Stress, Polycystic Ovary Syndrome, Syzygium Aromaticum (Clove)

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Introduction

One of the prevalently complex endocrinopathy disorders is "polycystic ovary syndrome" (PCOS), affecting up to 17.8% of women of reproductive age. The etiology of this syndrome is still unclear, although environmental, genetic, and hormonal factors may be necessary for its development (1, 2). PCOS has been associated with numerous reproductive, metabolic, and biochemical abnormalities such as elevated androgens, oxidative stress, insulin resistance disruption of the hypothalamic-pituitary-gonadal axis (the ratio of luteinizing hormone (LH)/follicle-stimulating hormone (FSH) ≥ 1) (3, 4), and finally, dysregulation of ovarian steroidogenesis, folliculogenesis (5). In PCOS, the PI3K/AKT/MTORE signaling pathway plays a vital role in regulating the interactions among androgens, insulin, and growth factors. Autophagy, which is induced by oxidative stress (6), is one of the main pathways of destruction and is essential

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for cellular homeostasis, as well as maintaining energy production and synthesis of new macromolecules. This process is vital for separating natural components from inefficient and unnecessary substances. Additionally, the protective function of this process has been proven during proliferation, differentiation and regulation of reproduction (7, 8). Although the autophagy process typically occurs in the ovary and is essential for folliculogenesis, this process has a detrimental effect on the ovary through prolonged activation (5). The importance of autophagy in PCOS-related disorders is well known, including dysregulation of androgen, gonadotropins, insulin levels, and most importantly, increased follicular atresia, which leads to decreased ovarian reserve (9). Due to the role of autophagy in metabolic disorders of PCOS, the use of antioxidant inhibitors of autophagy is a reasonable treatment (10).

The *Syzygium aromaticum*, generally known as clove, is originally from the Asiatic region. Clove essential oil (eugenol, phenols, flavonoids, oleanolic acid, and tannins) has been shown to minimize damage caused by toxicity or oxidative stress in the brain, liver, heart, kidneys, and testicles (11, 12). Clove oil is a useful and valuable herb with anti-apoptotic, antioxidant, anti-diabetic, anti-hyperlipidemic properties, and is effective in treating sexual disorders (12). Furthermore, it inhibits autophagy as an antiviral therapy (12, 13). Since the typical clinical symptoms of this syndrome are associated with autophagy disorder, in this study, clove extract was used as an autophagy regulator to improve PCOS rat model parameters, including apoptosis, oxidative stress, and and the levels of androgen, gonadotropins, aromatase, and insulin. Accordingly, the present study was conducted with the aim of investigating the potential ability of clove extract in regulating parameters related to autophagy in PCOS rats.

Materials and Methods

The current experimental study used 40 adult female Wistar rats (weighing 250 ± 10 g). All animals were purchased from Razi Institute in Mashhad, and then they were intently maintained in an animal facility under standard conditions (12/12 hours light/dark cycles, 23 ± 2°C, and 60-70% humidity). Rats in all groups had free access to food and water. We considered ethical considerations according to the regulations of the "Gonabad University of Medical Science". The Ethical Code of the present research was specified as IR.GMU.REC.1394.32.

We randomly divided the rats into 5 experimental groups: (30 mg/kg/orally/daily)

G1) Control group, that received normal saline only (daily oral) (n=8).

G2) PCOS group, that received a single dose injection of estradiol valerate for PCOS induction only with no other treatments (n=8).

G3) PCOS+clove (30 mg/kg) group, PCOS rats that received 30 mg/kg of clove extract daily by oral gavage (n=8).

G4) PCOS+clove (60 mg/kg) group, PCOS rats that received 60 mg/kg of clove extract daily by oral gavage (n=8).

G5) PCOS+gonadectomy group, PCOS rats that underwent gonadectomy (n=8).

Experimental protocol

In the experimental process, the vaginal smear was examined daily and confirmed that all rats had regular cycles (about 8-10 days). The PCOS was induced by an intramuscular injection of a single dose (1) of estradiol valerate (16 mg/kg) dissolved in 0.2 ml of sesame oil (Riedeldehaen, Germany) (14). After confirming the induction of ovarian cysts (by evaluation with estrous cycle without ovulation), clove oil was administered orally for 14 days (30-60 mg) in G3 and G4 groups (15). Clove oil contains 13 identified compounds, among which eugenol 76.8% is considered as the main component. Other components incluse beta caryophyllene17.4%, alpha humulene 1.1%, and eugenol acetate 1.1%, which use gas chromatography-spectrum mass measurement. Finally, at the end of treatment on day 14, the rats were anesthetized with ketamine (80 mg/kg) and xylazine (10 mg/kg). Blood samples (1 cc) were taken from the cardiac apex and transferred into sterile test tubes for biochemical assessment (measure the serum levels of LH, FSH, testosterone, aromatase, insulin, and oxidative stress marker). The samples were centrifuged at 3000 rpm for 15 minutes at room temperature to separate the serum (16). Then, the separated sera without red blood cells were kept at -80°C until testing (17). In addition, all groups except for G5 were operated on for ovariectomy to examine both ovaries. Ovariectomy was performed as follows: initially, the animals were anesthetized, and a longitudinal incision was created in the midline behind the animals. Two cm incision was made below the last rib. Then, Fallopian tubes with ovaries were pulled out. The ovaries were removed, and the fallopian tubes were returned to the abdominal cavity. Finally, the incision site was sutured (3, 13). On the fourteenth day (the end of the treatment period), the rats deeply anesthetized using ketamine-xylazine and then underwent ovariectomy. The ovaries on one side were fixed in formalin for histological studies, and the other was used for molecular studies.

Vaginal smear

The PCOS model evaluation with an ovulatory estrous cycle was confirmed by examination of vaginal smears. Vaginal smears of all the rats were inspected daily at the same time to estimate the stage of the 4-day ovarian cycle. Accordingly, at first, a cotton swab was moistened and after placing it in the animal's vagina, the swab was rotated on the vaginal wall. The swab was carefully removed from the vagina and the sample on the swab was spread on a microscope slide. After fixing the sample with alcohol,

the prepared smear was stained with hematoxylin-eosin (H&E).

The observation of the slides by an optical microscope (Olympus BX51, Japan) with a magnification of 400× shows the different stages of the estrous cycle as follows:

- Proestrus: the presence of a large number of epithelial cells with round and large nuclei.
- Estrus: the presence of cornified epithelial cells without nuclei.
- Metestrus: the presence of both types of epithelial cells, nucleated and non-nucleated, along with a small number of leukocytes.
- Diestrus: the presence of leukocytes along with a small number of non-nucleated epithelial cells.

Observing a pause in the estrous cycle for two weeks (at least three periods) indicates the induction of PCOS (18, 19).

Histological evaluation

At the end of the experimental period, the rats were anesthetized with ketamine and xylazine and then sacrificed. The sample collection method was that after opening the abdominal wall, the ovaries were carefully separated from the fallopian tube and then kept in 10% formalin for 48 hours. After ensuring the stabilization of the ovaries, the process of dehydration and clarification was done by alcohol and xylene, respectively. After embedding in paraffin, the ovaries were cut into 5 µm-thick in coronal serial sections at 50-60 µm intervals using a microtome. Ten sections were prepared from each ovary. Then, the samples are spread on the slide to be prepared for H&E staining and to identify the types of follicles in the ovary. The follicles were counted according to the following definitions:

Primordial follicle: A single layer of squamous follicular cells surrounding the oocytes.

Primary follicle: One or more layers of follicular cells that have become cubic.

Secondary follicle: This is characterized by a single, sizeable antral cavity.

Atretic follicles: The granulosa cells undergo apoptosis, and consequently, the oocyte degenerates (19).

Finally, the number of primordial, primary, antral, atretic, and cystic follicles and corpus luteum were counted by an optical microscope (Olympus BX51, Japan) with a magnification of 400×.

Apoptotic cell evaluation

In the sections prepared from the ovarian tissues, apoptotic cells were identified by the TUNEL technique. To perform this technique, first, serial sections of the sample with a thickness of 5 micrometers were obtained. The sample sections were placed on a slide, and then deparaffinization and hydration were done with xylene and decreasing concentrations of ethanol, respectively. In the next step, the slides were washed twice in PBS for ten minutes each time. After that, the slides were exposed to protein kinase for 20 minutes. After washing the slides again, the TUNEL reaction was performed according to the manufacturer's protocol (Roche, Germany). At the end of the experiment, the slides were incubated with DAB for ten minutes. Next, they were exposed to hematoxylin dye for 30 seconds to create a background color. In this staining technique, dark brown color indicates apoptotic cells (20). For calculatingthe apoptotic index (AI) in each type of follicles, we counted the number of TUNEL-positive cells, then divided that number by the total number of granulosa cells and expressed the results in percentages. Then the mean AI of each case was calculated (21).

Malondialdehyde level evaluation

The serum level of malondialdehyde was calculated by transferring 0.20 cm^3 of each serum sample into a separate microtube that contained 3.0 cm^3 of glacial acetic acid, followed by addition of 1% thiobarbituric acid (TBA) in 2% NaOH to the tube. It was then placed into the boiling water bath for 15 minutes. After cooling the vial in cold water to room temperature, the absorbance of the pink-colored product was read at 532 nm. From the standard solution obtained from malondialdehyde tetrabutylammonium salt (Sigma-Aldrich, Germany), different concentrations were prepared. A calibration curve was made using different concentrations of the standard solution (22).

Superoxide dimutase and glutathione peroxidase level evaluation

The levels of superoxide dimutase and glutathione peroxidase in serum were evaluated by an ELISA Reader (Model: ABER-2, China) - AccuBioTech) according to the manufacturer's protocols (Randox, and Ransod, UK). GPX level measurement is based on the Paglia-Valentine method. GPX catalyzes the oxidation of glutathione (GSH) by cumene hydroperoxide. The SOD level assay uses xanthine and xanthine oxidase (XOD) to generate superoxide radicals that react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazoliumchloride (I.N.T) to form a red formazan dye (23).

Evaluation of testosterone, aromatase, and the ratio of luteinizing hormone to follicle-stimulating hormone levels

Testosterone serum levels were measured according to the protocol of ELISA kits (Demeditec Diagnostics, Germany). Likewise, the serum levels of aromatase, FSH, and LH were also evaluated according to the ELISA kit protocol (ZelBio, Italy) (3).

Blood glucose and insulin level evaluation

At the end of the treatment period, the levels of serum blood sugar were measured by using a glucometer

(Bayer Corporation, USA). Serum insulin concentration was measured by enzyme-linked immunosorbent assay and it was determined using ELISA kits (Antibody and Immunoassay Services, HKU). The level of serum insulin and blood sugar were expressed as mIU/ml and mg/dl, respectively (21).

Real time analysis evaluation

Total RNA was extracted from the ovarian tissue of each rat with a RNX-plus (ParsTous, Iran) kit according to the manufacturer's protocol. The purity of RNA was defined by electrophoresis on an agarose gel. The total RNA (1 µg) was reverse-transcribed with a cDNA synthesis kit (ParsTous, Iran). Reverse transcription polymerase chain reaction (RT-PCR) was performed with ABI PRISM® 48-well optical reaction plate (Applied Biosystems StepOne, FosterCity, USA). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as an endogenous gene control. The relative gene expression levels were compared with those in control, and all real-time quantitative PCR were performed in triplicates (24). The primers were as follows:

Beclin1-F: CGAAAGGTGGTGGCAGAAAAC
R: ACTATATTCTCGCTGGTACTGAGC
Lc3β-F: TCAGTGAGAGCTGCCTCTGTC
R: AGCAGTGGGGATTTACACAGTG
Gapdh-F: GGTCTACATGTTCCAGTATGACTC
R: CATTTGATGTTAGCGGGATCTCG

Statistical analysis

Statistical analysis was performed using SPSS 20 software (IBM, USA). Kolmogorov-Smirnov test was used to measure the normal distribution of data. All data were presented in mean ± SE. The one-way ANOVA test followed by the Tukey posthoc test were used to compare histopathological values. The independent t test was used to compare and analyze the data from the real-time PCR. A P≤0.05 was considered statistically significant.

Results

Superoxide dimutase, glutathione peroxidase and malondialdehyde level analysis

The serum levels of GPX and SOD decreased significantly in the PCOS group as compared to the control group (P=0.001). In the PCOS+clove (30 and 60 mg/kg/orally/daily) and PCOS+gonadectomy groups, these levels significantly increased when compared with the PCOS group (P=0.001, Fig.1). The level of malondialdehyde (MDA) in the PCOS group was significantly higher than the control group; on the other hand, it was significantly lower in PCOS+clove (30 and 60 mg/kg/ orally/daily) and PCOS+gonadectomy groups compared to the PCOS group (P=0.001, Fig.1). No significant differences were observed in the serum levels of GPX, SOD, or MDA between the clove treatment and gonadectomy groups compared with the control group.

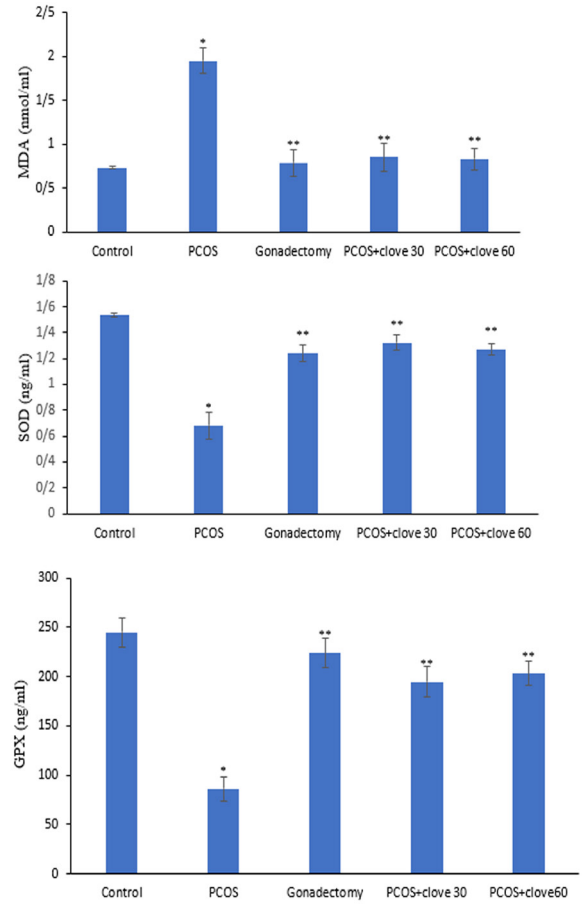

Fig.1: A comparison of the oxidative stress markers MDA, SOD, GPX in control, PCOS, PCOS+gonadectomy; PCOS+clove 30, and PCOS+clove 60 groups. Data values are mean ± SD and P<0.05 is significant. Analyzed by ANOVA test. MDA; Malondialdehyde, SOD; Superoxide dismutase, GPX; Glutathione peroxidase, PCOS; Polycystic ovary syndrome, *; P<0.05 vs. the control group, and **; P<0.05 vs. the PCOS group.

Analysis of hormones in serum

The serum levels of testosterone showed a significant enhancement in the PCOS group compared to the control group. In the PCOS+clove (30 mg/kg/orally/daily) and PCOS+gonadectomy groups, the testosterone levels decreased significantly when compared with the PCOS group (P=0.001, Fig.2). In comparison with the control group, the LH/FSH levels illustrated a considerable increase in the PCOS group (LH/FSH ≥1). The ratio of serum levels of LH to FSH in the PCOS+clove (30 mg/kg/orally/daily) and PCOS+gonadectomy groups were significantly decreased compared to the PCOS group (P=0.01, LH/FSH <1). Interestingly, there was no significant difference in the serum levels of testosterone and the ratio of the serum levels of LH to FSH in when comparing PCOS+clove (60 mg/kg/orally/daily) and PCOS groups (Fig.2).

Insulin level analysis

The insulin level improved remarkably in the PCOS group compared to the control group. In the PCOS+clove (30 mg/kg/orally/daily) and PCOS+gonadectomy groups, it significantly decreased in comparison with the PCOS group (P=0.001, Fig.3).

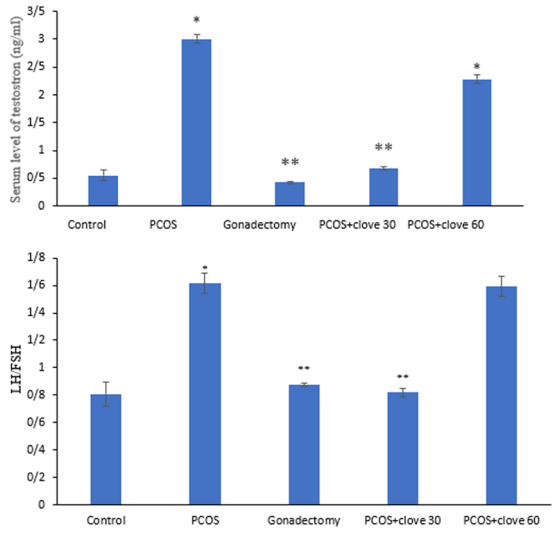

Fig.2: A comparison of the LH/FSH and testosterone in control, PCOS, PCOS+gonadectomy; PCOS+clove 30, and PCOS+clove 60 groups. Data values are mean ± SD and P<0.05 is significant. Analyzed by ANOVA test. LH; Luteinizing hormone, FSH; Follicle-stimulating hormone, PCOS; Polycystic ovary syndrome, *; P<0.05 vs. the control group, and **; P<0.05 vs. the PCOS group.

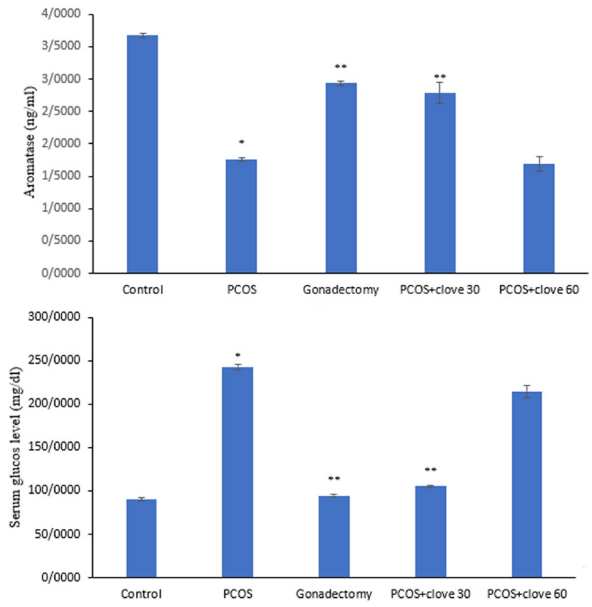

Fig.3: A comparison of the levels of aromatase, insulin, and glucose in control, PCOS, PCOS+gonadectomy; PCOS+clove 30, and PCOS+clove 60 groups. Data values are mean ± SD and P<0.05 is significant. Data were analyzed by ANOVA test. PCOS; Polycystic ovary syndrome, *; P<0.05 vs. the control group, and **; P<0.05 vs. the PCOS group.

Blood glucose level analysis

The PCOS group showed a significant increase in blood glucose levels compared to the control group. There was a significant decrease in the blood glucose of the PCOS+clove (30 mg/kg/orally/daily) and PCOS+gonadectomy groups as compared to the PCOS group (P=0.001, Fig.3).

Aromatase level analysis

The aromatase levels decreased significantly in the PCOS group compared to the control group (P=0.001). But, in the PCOS+clove (30 mg/kg/orally/daily) and PCOS+gonadectomy groups, it significantly increased compared with the PCOS group (P=0.001). In this regard, there was no significant difference between the PCOS+clove (60 mg/kg /orally/daily) and the PCOS group (Fig.3).

Apoptosis index analysis

In this study we evaluated apoptosis indicators in the primary and antral follicles. The rats displayed a standard apoptosis index in the control group. Compared with the controls, PCOS rats exhibited an increased apoptosis index in both the primary and antral follicles. Clove (30 mg/kg/ orally/daily) treatment reduced the apoptosis index as compared with the PCOS group (P<0.001). In this regard, we did not observe a significant difference between the PCOS+clove (60 mg/kg /orally/daily) and PCOS groups (Fig.4).

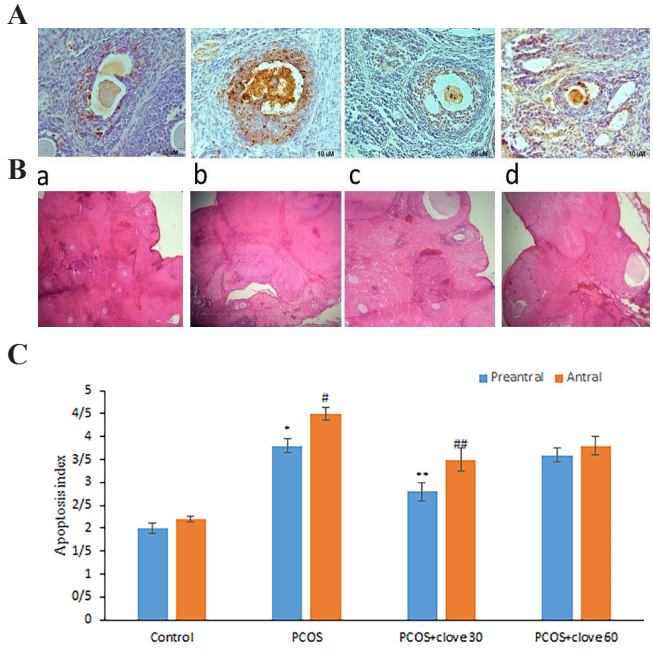

Fig.4: Effect of clove oil on PCOS symptoms in control, PCOS, PCOS+clove 30, and PCOS+clove 60 groups. **A.** Ovarian follicle apoptosis findings (scale bar: 10 µM), **B.** Ovarian histological findings, a; Control, b; PCOS group, c; PCOS+clove (30 mg/kg/ orally/daily) group, d; PCOS+clove (60 mg/kg /orally/daily) group (scale bar: 40 µM). **C.** Comparison of the apoptosis index in control, PCOS, PCOS+clove 30, and PCOS+clove 60 groups. Data values are mean ± SD and P<0.05 is significant. Analyzed by ANOVA test. PCOS; Polycystic ovary syndrome, *; P<0.05 vs. the control group, **; P<0.05 vs. the PCOS group in prenatal follicles, #; P<0.05 vs. the control group, and ##; P<0.05 vs. the PCOS group in antral follicles.

Histological parameters analysis

Rats in the control group displayed normal ovary histology. In the PCOS group the number of corpus luteum and all the follicles significantly decreased. However, the number of follicular cysts increased, and cell debris in the antrum was detected in this group. Treatment with clove (30 mg/kg/ orally/daily) exhibited well-developed antral follicles, a standard granulosa cell layer, and minimal cell debris. Also, the number of follicular cysts decreased following clove treatment. In this regard, there was no significant difference between the PCOS+clove (60 mg/kg/ orally/daily) and PCOS groups (P=0.001, Fig.4B, Table 1).

Table 1: A comparison of the primordial, primary, antral, cystic follicles, and corpus luteum in control, PCOS, PCOS+clove 30, and PCOS+clove 60 groups

Group	Primordial follicles	Primary follicles	Antral follicles	Cystic follicles	Corpus Leuteum
Control	49.6 ± 2.07	18.4 ± 1.14	15.6 ± 1.14	0	10.2 ± 0.83
PCOS	32.2 ± 1.87*	7.6 ± 1.14*	3.8 ± 0.83*	6.6 ± 0.54*	1.6 ± 0.89*
PCOS+clove 30	40 ± 1.78	15.4 ± 1.14†	13 ± 1.58†	1.1 ± 0.05	6.8 ± 0.83†
PCOS+clove 60	35.2 ± 1.78*	7.8 ± 0.83*	4.6 ± 1.14*	5.1 ± 0.83*	1.4 ± 0.05

Data values are mean ± SD and P<0.05 is significant. Control group; Injection of normal saline only; PCOS group; PCOS was induced by injection of estradiol valerate, PCOS+clove 30; PCOS induction along with clove extract (30 mg/ kg) treatment, PCOS+clove 60; PCOS induction along with clove extract (60 mg/kg) treatment of clove, PCOS; Polycystic ovary syndrome, *; P<0.05 vs. the control group, and †; P<0.05 vs. the PCOS group. Analyzed by ANOVA test.

Real-time analysis

Real-time PCR results showed that the Beclin1 and Lc3β mRNA levels increased significantly in the PCOS groups compared with the control group (P=0.001). However, there was a significant decrease in Beclin1 and Lc3β mRNA levels in the PCOS+clove (30 mg/kg/ orally/daily) group compared to the PCOS group (P≤0.001). However, the comparison of mRNA of the mentioned genes showed no significant difference between the PCOS+clove group (60 mg/kg/orally/day) and PCOS (Fig.5).

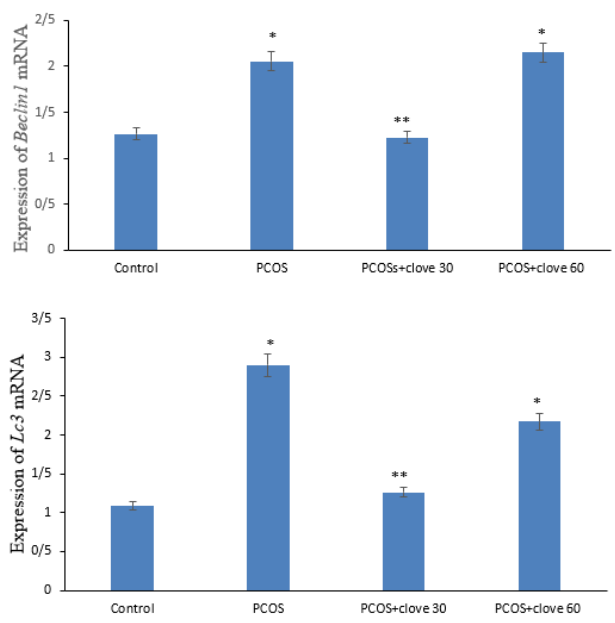

Fig.5: mRNA expression on *Beclin1* and *Lc3* in control, PCOS, PCOS+clove 30, and PCOS+clove 60 groups, as measured by the real-time PCR. Data values are mean ± SD and P<0.05 is significant. PCOS; Polycystic ovary syndrome, PCR; Polymerase chain reaction, *; P<0.05 vs. the control group, and **; P<0.05 vs. the PCOS group.

Discussion

In the present study, autophagy markers (Lc3β, Beclin1), histological parameters, oxidative stress, insulin levels, and hormone levels all changed significantly in PCOS rats following PCOS induction when compared to the control rats receiving only saline. Testosterone levels and the ratio of LH to FSH in G3 and G5 rats were significantly lower than in the rats with PCOS only (G2 group). Autophagy inhibition was also observed in these two groups. Clove treatment in the G3 group improved oxidative stress, histological parameters, and insulin levels significantly. PCOS is an endocrinological disorder. Internationally, the Rotterdam criterion describes PCOS as follows: i. Low or no ovulation, ii. Hyper-androgenic, iii. Polycystic ovaries (25). This syndrome is associated with impaired insulin levels, obesity, and diabetes in most women. In this syndrome, some morphological ovarian markers such as a few preantral follicles, antral follicles, atretic follicles, and degenerative ovarian cysts may be shown. Although this study showed all the above markers, some studies have suggested that these markers are not exclusive to PCOS (3-5).

In the present study, following the induction of PCOS in adult rats, parameters such as hyper-androgenemia, hyperinsulinemia, hyperglycemia, increased LH/FSH ratio, increased aromatase level, oxidative stress, and ovarian tissue apoptosis were observed. Autophagy is considered as an intracellular homeostasis process. During this process, autophagy-related proteins, including Lc3 and Beclin, form a structure called the autophagosome, which destroys the dysfunctional and stressful components of the cell by attaching to the lysosome (26). Autophagy has been shown to regulate primary follicle growth and atresia. It is a key part of each follicle's defense mechanism in response to various intracellular and extracellular stimuli (9). As mentioned above, autophagy dysregulation has been reported in PCOS. Molecular findings of a recent study showed an increase in autophagy markers (LC3 and Beclin1) following induction of PCOS (6). Other features of PCOS include increased oxidative stress, such as intracellular ROS, which is one of the main pathways preoccupied in PCOS pathogenesis (26-28). This study showed that the MDA level significantly increased while the SOD and GPX levels significantly decreased as a result of induction of PCOS in rats. Other reports have shown that oxidative stress may lead to induction of autophagy and eventually follicular atresia through the PI3K/AKT/mTOR pathway (6, 29). The biochemical findings of this paper show an increase in androgens in rats with PCOS, which makes it possible to induce autophagy by hyperandrogenism. Studies have shown that androgens induce autophagy by regulating Beclin expression (30). On the other hand, autophagy in the PCOS ovarian theca layer has been shown to lead to hyperandrogenism (31). Autophagy and hyperandrogenism seem to have a synergistic effect.

Other biochemical results of the present study include an increased LH/FSH ratio, which provides a significant value in diagnosing PCOS (32). Studies have shown that the formation of autophagosomes have an effect on

FSH levels (33, 34). The role of autophagy in regulating the insulin associated with this syndrome is well known (35, 36). In this study, impaired insulin levels may have resulted from disruption of normal autophagic function. Due to the impairment of autophagy-related parameters, by considering the above data, *S. aromaticum* (Clove) has been suggested as a potent antioxidant and an autophagy inhibitor (37) for managing PCOS. This plant enhances antioxidant enzymes by increasing the levels of MDA, SOD, and GPX in the treatment groups. We found the highest reduction in oxidative stress in a dose of 60mg clove, but this dose could not increase aromatase and did not reduce the levels of androgen and gonadotropins. In contrast, 30mg of cloves improved insulin, androgen, and gonadotropin levels. In this regard, other studies have suggested the opposite effects of high doses over low doses of clove oil on the reproductive system (15). The findings of our study also indicated an increase in apoptosis index in antral and preantral follicles in PCOS rats. In the study, treatment with 30 mg, unlike 60 mg clove extract, improved the number of follicles and reduced the apoptosis index. The results of the treatment with 30 mg may be due to an improvement in the hypothalamic-pituitary-ovarian axis and an approximate return to normal levels of gonadotropins. Since clove has an inhibitory effect on the expression of autophagic genes, including Beclin and Lc3, it has been reported to be effective as an anti-autophagic drug in the treatment of influenza, a viral infection (38).

In this study, there was a decreased autophagy activity, with special significance in the clove-treated group. This decrease in autophagy activity was associated with normalized insulin and blood glucose levels. The present results further suggest that clove may indicate a good therapeutic strategy in the treatment of autophagy-related PCOS parameters. In addition, we found that an orally administered clove reduces LH/FSH levels as well as the abnormal secretion of androgen levels. Since autophagy plays an important role in PCOS-dependent metabolism, this study was designed to determine whether the improvement of PCOS parameters was the result of inhibition of autophagy by cloves. As one control group, we used a gonadectomy group (to eliminate the source of autophagy). We found that improvement in PCOS parameters (oxidative stress, insulin, blood glucose, gonadotropins, and testosterone levels) occurred after the removal of the source of autophagy (ovarian tissue). Therefore, clove may help to improve the parameters by regulating autophagy and inhibiting excessive autophagy. However, the direct role of this extract in regulating the parameters such as oxidative stress, insulin, and androgens cannot be ignored (39, 40).

Conclusion

This study suggests that gonadectomy may be an appropriate method for women who are not prone to fertility; however, pharmacological properties of low-dose clove as a metabolic regulator may be beneficial for women who are prone to fertility.

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Authors' Contributions

M.S., M.M., M.Sh.; Planned and designed the experiments. S.-H.A.-E., M.Sh.; Performed the experiments. S.-A.E., M.Sh., M.S.; Analyzed the data. M.Sh.; Wrote the manuscript. M.S., M.Sh., S.-H.A.-E., M.M., R.M.; Reviewed and discussed the data. All authors read and approved the finan manuscript.

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