Original Article

Effect of Catechin on Serum Levels of Inflammatory Cytokines, Antioxidant Enzymes Activity and DNA Oxidative Damage of Ovarian Tissue in Polycystic Ovarian Syndrome Rat Model

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Abstract:

Introduction:
Women with polycystic ovary syndrome (PCOS) suffer anovulation due to hormonal disorders, oxidative stress and ovarian tissue inflammation. Catechin is the most important green tea flavonoids (Camellia sinensis) with antioxidant properties. This study aimed to determine the effects of catechin on serum levels of inflammatory cytokines, antioxidant enzymes activity and DNA oxidative damage of ovarian tissue in polycystic ovarian syndrome rat model.

Materials & Methods:
In this experimental study, 24 Wistar female rats were divided into 4 equal groups (n=6) of control (saline solution, 24 days, ip), non-treated PCOS (saline solution, 24 days, ip) and PCOS treated with catechin (50 and 100 mg/kg, 24 days, ip). Polycystic ovarian syndrome was induced by single intramuscular injection of estradiol valerate (4 mg/kg). At the end of treatment period, serum levels of TNF-α, IL-1β, IL-6, SOD, CAT, GPX and levels of MDA and HOdG-8 in ovarian tissue were measured by ELISA.

Results:
Compared to non-treated PCOS group, in treated groups with 50 and 100 mg/kg of catechin, serum levels of TNF-α, IL-1β and IL-6 significantly decreased dose-dependently ($p<0.05$), levels of SOD, CAT, GPX in ovarian tissue significantly increased dose-dependently ($p<0.05$) and levels of MDA and HOdG-8 significantly decreased dose-dependently ($p<0.05$).

Conclusion:
Catechin decreases serum levels of cytokines, increases activity of antioxidant enzymes, decreases lipid peroxidation and DNA oxidative damage of ovarian tissue in polycystic ovarian syndrome rat model.

Keywords: Polycystic Ovarian Syndrome, Catechin, Cytokine, Oxidative Stress, Rat

Introduction

Polycystic ovary syndrome (PCOS) occurs as a result of disorders in the endocrine system. In PCOS, the secretion of luteinizing hormone (LH) is elevated compared to the follicle-stimulating hormone (FSH). This increase stimulates ovarian follicle cells, thereby promoting the synthesis and secretion of androgens. The level of estrogen progenitors, i.e. testosterone and androstenedione, is increased, as well. Insulin resistance, carbohydrate metabolism disorder, lipid

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disorders, hypertension, and obesity are common in patients with PCOS (1). The increase in androgens affects the formation and release of ovum during ovulation, creating a large number of follicles. The growth of follicles stops during the dominant follicle selection stage in patients with PCOS. Therefore, cystic follicles are formed below the thick ovarian capsule (2). According to studies, the elevated level of cytokines, e.g. tumor necrosis factor alpha and interleukin 1 beta, indicates systemic and local inflammation in the body. Moreover, evidence suggests a direct and close relationship of systemic and local inflammation with PCOS (3). Tumor necrosis factor alpha has a significant role in regulating the normal activity of ovaries in the follicle growth stage. However, if it increases, it can cause cystic follicles by inducing apoptosis in granulosa cells of ovarian follicles (4). Interleukins are a group of cytokines which regulate immune responses and inflammatory reactions (5). It has been proven that the level of interleukins, especially interleukin 1 alpha and interleukin 1 beta, is increased in patients with PCOS. The increased serum level of cytokines stimulates the hypothalamus, thereby enhancing the secretion of hypothalamic releasing hormones which, in turn, increase the activity of the hypothalamic–pituitary–gonadal axis (6).

According to research, oxidative stress is an imbalance in the production and inhibition of free radicals by the antioxidant defense system. Oxidative stress increases the production of androgens, causes disorders in the formation of ovarian follicles, and increases ovarian tissue injury in patients with PCOS. In addition, reports introduce the disordered synthesis of ovarian steroids as one of the causes of oxidative stress in these patients (7). According to studies, the serum level of oxidative stress indicators, including reactive oxygen species (ROS), is elevated while the blood total antioxidant capacity (TAC) is decreased in patients with PCOS (8).

Furthermore, the abnormal increase in lipid peroxidation damages cell membrane and organelles. Malondialdehyde (MDA) is the final product of fatty acid peroxidation. As a lipid peroxidation indicator, the level of MDA is of special clinical significance in determining free radical levels (9). Research has shown that guanine has a higher oxidation potential than other purines and pyrimidines. As a result of hydroxyl radical attack on position 8 of guanine, 8-hydroxy-2' -deoxyguanosine (8-OHdG) is produced. As this compound shows the dynamic balance between DNA oxidative damage and repair speed, its measurement is essential for DNA damage evaluation (10).

Today, medications such as metformin, clomifene, and letrozole are used to treat anovulation in patients with PCOS, each with their specific mechanisms and numerous side-effects (11, 12). As oxidative stress and lipid peroxidation in patients with PCOS can cause many problems (8), it is valuable to find compounds which can decrease these side-effects.

Green tea (Camellia sinensis) belongs to division Angiosperm, class Dialypetalae, order Parital, family Teacae, and genus Camellia. There are three major groups of polyphenols in green tea: catechins, thioflavins, and thearubigins. Catechins are a type of antioxidant and among the most important flavonoids, comprising 25-35 dry weight % of green tea (13). Green tea catechins include epicatechin, epicatechin gallate, epigallocatechin, and

epigallocatechin-3-gallate, with epigallocatechin gallate being the most common and active catechin in terms of antioxidant activity (14). Research has shown that, because of its antioxidant effects, green tea accelerates burn and surgical wound healing in rats (15). According to studies, green tea essential oil can greatly enhance sperm mobility and natural morphology in addition to the diameter of seminiferous tubules, lumen, and germinal epithelium in rats consuming sodium arsenite. As a strong antioxidant, green tea essential oil inhibits the oxidative stress resulting from sodium arsenite, thereby reducing lipid peroxidation in sperms and thus preventing sperm death (16). The consumption of green tea may have a protective role in liver necrosis caused by acetaminophen and decrease serum levels of alanine transferase and aspartate transferase in rat models of acute acetaminophen-induced hepatoxicity (17). According to studies, catechin can inhibit free radicals and act as a biological antioxidant (18). It has also been shown that this substance can inhibit superoxide and hydroxyl radicals (18) and, in addition to direct antioxidant effects, indirectly increase intracellular antioxidants. The level of internal antioxidants including glutathione peroxidase, reductase, superoxide dismutase, and catalase is increased in rats receiving catechin (19). According to research, the catechin in green tea prevents the induction of apoptosis in damaged cells by inhibiting free radicals and decreasing DNA oxidative stress (20). With regard to the numerous therapeutic effects and diverse applications of green tea and its active compounds in traditional medicine as well as the antioxidant effects of catechin, the present study aimed to examine the effect of catechin on the serum levels of inflammatory cytokines, enzymatic activities of antioxidant enzymes, and ovarian tissue DNA oxidative damage in rat models of PCOS.

Methods and Materials

Adult female Wistar rats were used in the present experimental study. Twenty-four rats weighting 8±190 g and aging approximately 5±85 days were prepared from the Animal House of Payam-e Noor University, Mashhad, Iran. The animals were kept at 24±3 °C, 35±4% relative humidity, and a 12:12 light-dark cycle. They were housed in standard transparent polycarbonate cages (Razi Rad Co., Iran) with ad libitum access to water with a 500 mL plastic bottle. Moreover, during the study period, they were fed standard chow diet (Danedaran Toos, Iran). To adapt the rats to the environment, tests were performed after minimum 10 days of being housed. Rats were randomly assigned to four groups of 6: controls (treatment with 0.6 mL of saline solution as the catechin solvent); non-treated PCOS (receiving 0.5 mL of saline solution as the medicine solvent); and two groups of PCOS treated with catechin (Sigma-Aldrich, Germany) at the concentrations of 50 and 100 mg/kg (24 days of intraperitoneal injection). In the present study, all the ethical considerations related to research were taken into account and all surgical and sampling procedures were done under general anesthesia. Also, an attempt was made to use the least possible number of acceptable sample. The ethical considerations of research on laboratory animals had been approved by the Ethics Committee of Islamic Azad University, supervised by the Young Researchers and Elite Club of Mashhad, Iran, in 2016.

In the present study, rats which had 2-3 regular estrous cycles over 12-14 days of vaginal smear observation were selected. The regularity of estrous cycles was assessed using vaginal smears. To this end, 0.3 mL of physiological saline solution was slowly injected into the animals' vaginas using a sampler (Transferpette®S; Brand, Germany). Then, 1-2 drops of the noted liquid were taken and a smear was prepared. Samples were examined by a light microscope (CX21FS1; Olympus, Japan) at 400x magnification. Rats which were in the estrus phase of the reproductive cycle were selected for the following steps. In the estrus phase, vaginal smear has more cornified cells than epithelial cells and lacks leukocytes (21).

PCO was induced by a single intramuscular injection of 4 mg/kg of estradiol valerate (Aburaihan Pharmaceutical Co., Iran). The duration required for creating a PCOS model is 60 days following the injection of estradiol valerate (22). The presence of exceeding numbers of cornified cells in the vaginal smear indicates the presence of ovarian follicle cysts. From each group, one rat was randomly selected and sacrificed with a lethal dose of diethyl ether (Merck, Germany) and then the ovaries were removed. After tissue processing and staining of ovaries, ovarian cysts were viewed with a light microscope at 40x magnification and PCOS induction was confirmed.

At the end of the pharmacotherapy period, rats were anesthetized using diethyl ether. Then, the skin on the chest, sternum, and ribs was incised and blood was taken from the left ventricle using a 2 mL syringe after pushing the sternum and ribs aside. The collected blood was poured into the test tube without anticoagulant agents and placed in an incubator (INB400; Memmert, Germany) at 37 °C for 12 min. After coagulation, the tubes were centrifuged (EBA280; Hettich, Germany) at 5000 rpm for 12 min. Then, the serum on the coagulated part was removed by a sampler, transferred to another test tube, and maintained in a freezer at -80 °C (23). Using an ELISA reader (2100; Stat Fax, USA) and Finetest (China) kits, serum levels of tumor necrosis factor alpha (>46.875 pg/mL sensitivity in the range of 78.125-5000 pg/mL), interleukin 1 beta (>18.75 pg/mL sensitivity in the range of 31.25-2000 pg/mL), interleukin-6 (>37.5 pg/mL in the range of 62.5-4000 pg/mL) were measured.

Subsequently, the ovarian tissue was removed from the rats' bodies, washed with TBS (Sigma-Aldrich, Germany), and homogenized (T25 digital ULTRA-TURRAX; IKA, Germany) at 5000 rpm for 5 min. The resulting solution was then centrifuged (refrigerated centrifuge, Z366; Hermle, Germany). To prevent the enzymes and proteins from degradation, the whole procedure was conducted at 4 °C (refrigerated centrifuge) using 0.5 mM phenylmethyl sulphonyl fluoride solution (Sigma-Aldrich, Germany) as the protease inhibitor (24). After centrifugation, the clear supernatant was removed from the sedimented lower part and used for assays. Via the ELISA method and using Finetest kits, the levels of superoxide dismutase (SOD) (>9.375 pg/mL sensitivity in the range of 15.6-1000 pg/mL), catalase (CAT) (>18.75 mIU/mL sensitivity in the range of 31.2-2000 mIU/mL), glutathione peroxidase (GPX) (>18.75 pg/mL sensitivity in the range of 31.25-2000 pg/mL), 8-OHdG (>0.938 ng/mL sensitivity in the range of 1.563-100 ng/mL), and MDA (>18.75 mg/mL in the

range of 31.25-2000 ng/mL) in the ovarian tissue were measured.

The data were analyzed in SPSS 20. Due to the small sample size in each group (n=6), Kruskal-Wallis non-parametric analysis of variance and Dunn's post hoc test were used to compare the groups. Results are expressed as mean±SD, with p values<0.05 considered significant.

Results

Data analysis revealed that the activity of SOD, CAT, and GPX of the ovarian tissue was significantly reduced, while the level of MDA and 8-OHdG was significantly increased in the non-treated PCOS group compared with the controls (p<0.05). Compared with the non-treated PCOS group, the activity of SOD, CAT, and GPX of the ovarian tissue was significantly increased, while the level of MDA and 8-OHdG was significantly decreased in PCOS + 50 and 100 mg/kg catechin groups dose-dependently (p<0.05). Furthermore, the activity of antioxidant enzymes SOD, CAT, and GPX of the ovarian tissue was significantly more, while the level of MDA and 8-OHdG was significantly less in the PCOS + 100 mg/kg catechin group compared with the PCOS + 50 mg/kg catechin group (p<0.05) (Figures 1-5).

Based on the results, the serum level of cytokines, i.e. tumor necrosis factor alpha, interleukin 1 beta, and interleukin 6, was significantly elevated in the non-treated PCOS group compared with the controls (p<0.05). Compared with the non-treated PCOS group, the serum levels of tumor necrosis factor alpha, interleukin 1 beta, and interleukin 6 showed a significant decrease in PCOS + 50 and 100 mg/kg catechin groups (p<0.05). Moreover, serum levels of tumor necrosis factor alpha, interleukin 1 beta, and interleukin 6 were significantly less in the PCOS + 100 mg/kg catechin group compared with the PCOS + 50 mg/kg catechin group (p<0.05) (Figures 6-8).

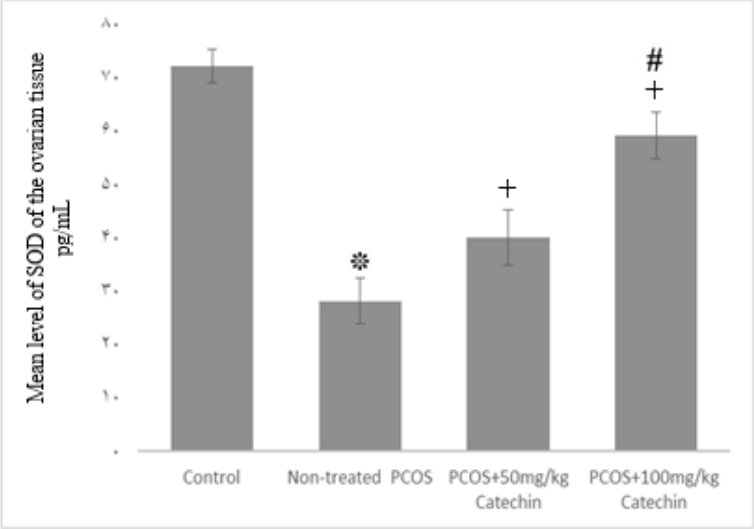

Figure 1: Mean level of SOD of the ovarian tissue in the studied groups

❄P=0.002 compared with the controls

+P=0.007 compared with the non-treated PCOS group

P=0.012 compared with the PCOS + 50 mg/kg catechin group

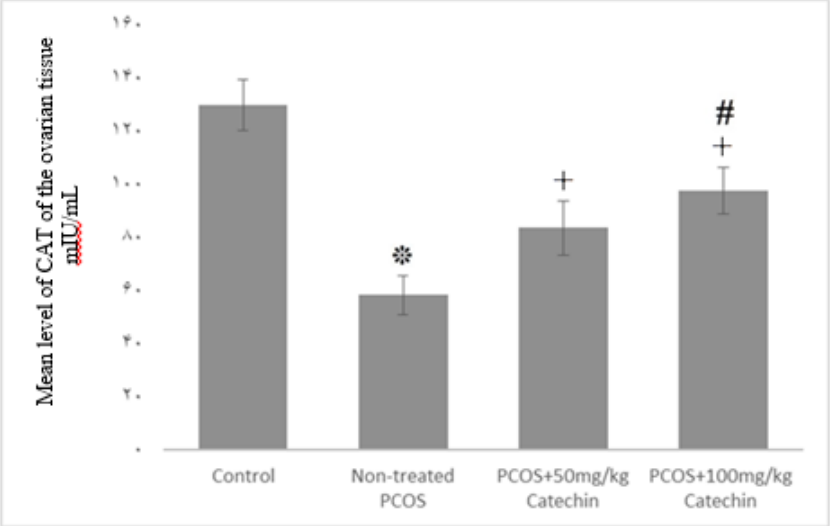

Figure 2: Mean level of CAT of the ovarian tissue in the studied groups

❊P<0.05 compared with the controls

+ P<0.05 compared with the non-treated PCOS group

# P<0.05 compared with the PCOS + 50 mg/kg catechin group

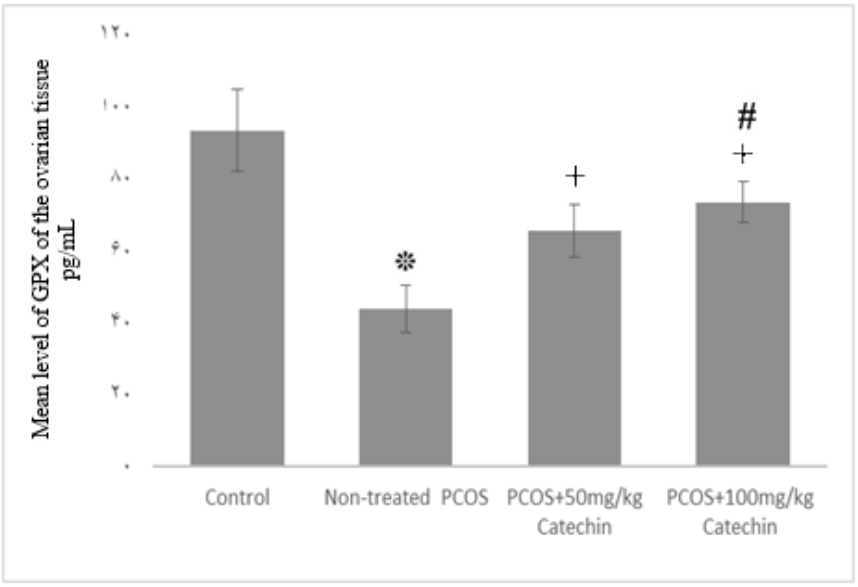

Figure 3. Mean level of GPX of the ovarian tissue in the studied groups

❊P=0.003 compared with the controls

+ P=0.0011 compared with the non-treated PCOS group

p=0.028 compared with the PCOS + 50 mg/kg catechin group

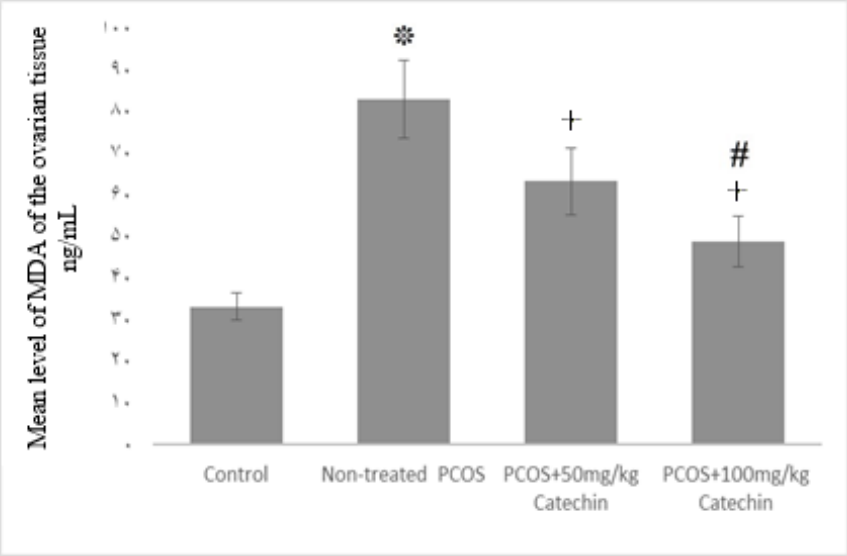

Figure 4. Mean level of MDA of the ovarian tissue in the studied groups
❊P=0.001 compared with the controls
+ P=0.019 compared with the non-treated PCOS group
# p=0.008 compared with the PCOS + 50 mg/kg catechin group

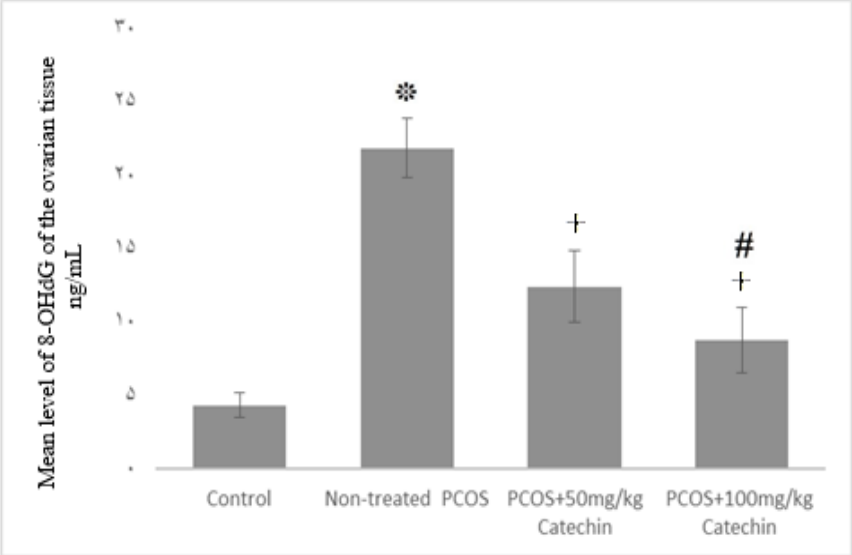

Figure 5. Mean level of 8-OHdG of the ovarian tissue in the studied groups
❊P=0.001 compared with the controls
+ P=0.007 compared with the non-treated PCOS group
# p=0.011 compared with the PCOS + 50 mg/kg catechin group

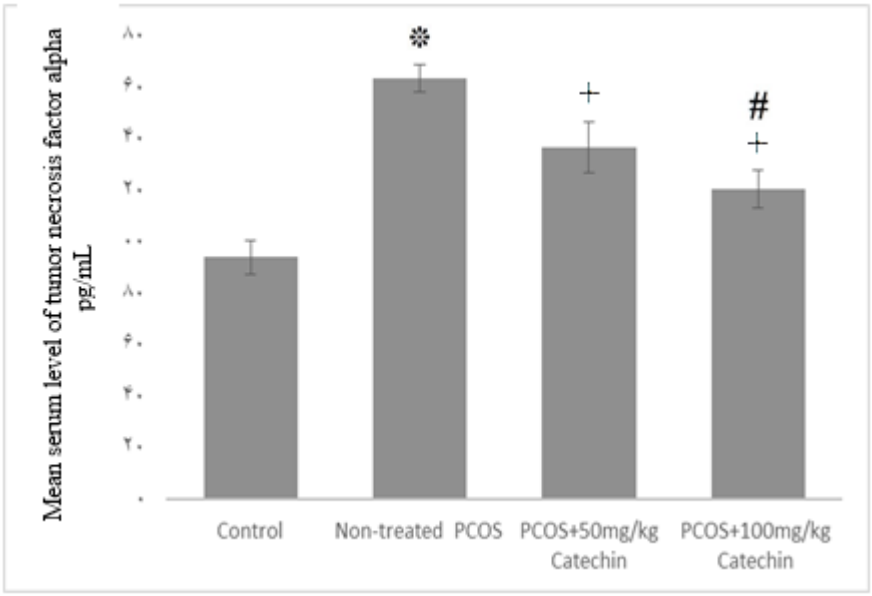

Figure 6. Mean serum level of tumor necrosis factor alpha in the studied groups
❊P=0.005 compared with the controls
+ P=0.022 compared with the non-treated PCOS group
# p=0.018 compared with the PCOS + 50 mg/kg catechin group

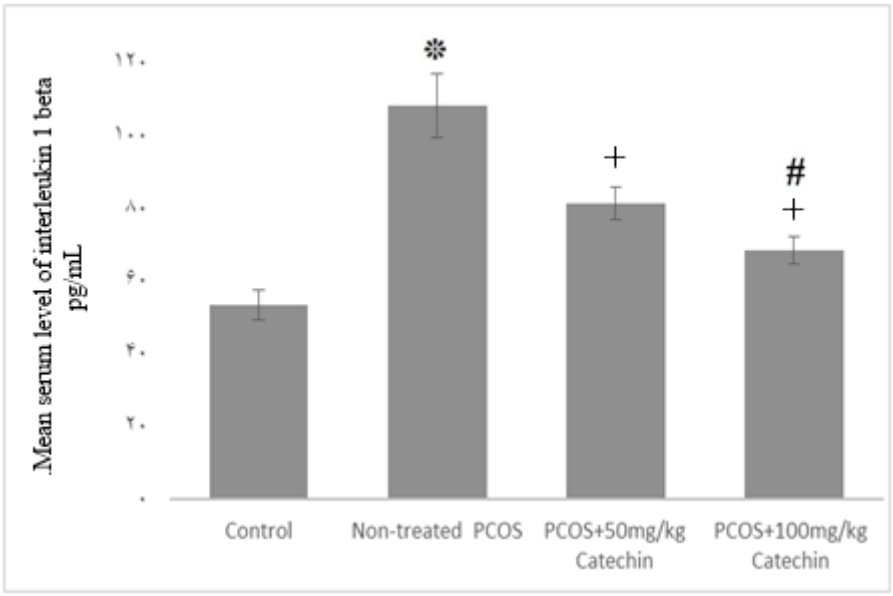

Figure 7. Mean serum level of interleukin 1 beta in the studied groups
❊P=0.002 compared with the controls
+ P=0.011 compared with the non-treated PCOS group
# p=0.021 compared with the PCOS + 50 mg/kg catechin group

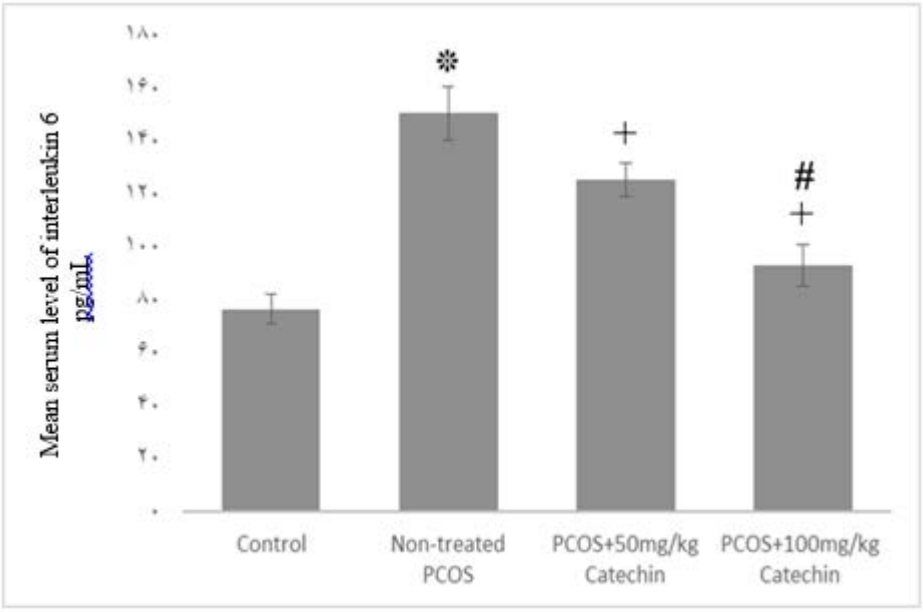

Figure 8. Mean serum level of interleukin 6 in the studied groups
❊P=0.003 compared with the controls
+ P=0.009 compared with the non-treated PCOS group
# p=0.013 compared with the PCOS + 50 mg/kg catechin group

Discussion

The present study examined the effect of catechin on the serum level of cytokines, i.e. tumor necrosis factor alpha, interleukin 1 beta, and interleukin 6; level of antioxidant enzymes, i.e. SOD, CAT, and GPX of the ovarian tissue; level of DNA oxidative damage; and level of lipid peroxidation of the ovarian tissue of rat models of PCOS. The tissue examination to confirm the rat model of PCOS revealed that the use of this dose in approximately 60 days can lead to ovarian cysts of different dimensions. In line with the present study, it has been shown than estradiol valerate can create a rat model of PCOS, followed by the formation of large cystic follicles in ovaries (25). According to another study, 8 weeks after the injection of 40 mg/kg of estradiol valerate, the number of ovarian cysts and apoptotic granulosa cells significantly increases (22).

Based on the results of the present study, the serum level of cytokines, i.e. tumor necrosis factor alpha, interleukin 1 beta, and interleukin-6, was significantly increased in non-treated PCOS rats compared with the controls. Another study examined tumor necrosis factor alpha, interleukin 1 alpha, and interleukin 1 beta in the blood sera of patients with PCOS and showed that the serum level of interleukin 1 alpha and beta is increased in these patients, while tumor necrosis factor alpha has no significant change. Inflammatory cytokines and especially interleukins remarkably increase in these patients. Thus, PCOS can be considered as a mild chronic inflammatory disease (26). Moreover, various studies have demonstrated the high prevalence of cardiovascular diseases, diabetes, insulin resistance, and metabolic syndrome in these patients. Therefore, PCOS can be regarded as a pre-

inflammatory stage. Based on studies, tumor necrosis factor alpha, which has a role in systemic inflammation and stimulates the reaction of the acute phase of inflammation, is increased in the sera of patients with PCOS (27). Another study has shown that, as inflammation indicators, the serum level of inflammatory cytokines, C-reactive protein, and erythrocyte sedimentation rate (ESR) are elevated in women with PCOS (28).

The present study revealed that the activity of antioxidant enzymes of the ovarian tissue is decreased, while the level of lipid peroxidation and DNA oxidative damage is increased. Similarly, a study on oxidative stress conditions in the ovarian tissue of rat models of PCOS reported that the induction of PCOS increases oxidative stress in the noted tissue. In addition, the induction of PCOS by injecting estradiol valerate increases ROS, reduces the activity of ovarian tissue antioxidant enzyme, and thus leads to ovarian cysts (22). It was also revealed that, in patients with PCOS, serum levels of oxidative indicators, e.g. TAC, ROS, and hydroxyl radicals, are abnormal, and excessive oxidative stress can lead to ovarian mesenchymal hyperplasia, disrupting the function of the ovaries and, finally, decreasing ovulation (29). Scientific evidence suggests that the activity of ovarian tissue antioxidant enzymes is reduced in patients with PCOS. As a result, in oxidative stress conditions, ovarian androgens are increased, disrupting the creation of follicles (30). According to another study, the levels of blood lipids, including triglyceride, and oxidized lipids are higher in patients with PCOS. Furthermore, it is reported that, in these patients, lipids are not protected against oxidations as a result of the weak antioxidant defense system. Consequently, the chance of oxidative damage to lipids is increased, as manifested by elevated serum level of MDA (30). In addition, it has been shown that increased oxidative stress in the ovaries of rat models of PCOS, together with increased oxidative stress caused by ROS, damages the ovarian epithelial cell DNA and induces apoptosis in granulosa cells (31), consistent with the present results.

The present study demonstrated that the serum level of inflammatory cytokines, lipid peroxidation, and ovarian tissue DNA oxidative damage are reduced, while the activity of ovarian tissue antioxidant enzymes is increased in rats with PCOS treated with catechin. PCOS is a chronic inflammatory disease in which the level of inflammatory cytokines in elevated (32). According to research, because of its anti-inflammatory effects, green tea reduces serum levels of C-reactive protein, tumor necrosis factor alpha, and interleukin 6. It applies its anti-inflammatory effects in inflammatory conditions by decreasing the release of inflammatory cytokines and inhibiting caspase 3 (33). Based on another study, the oral consumption of epigallocatechin-3-gallate by old rats on high-fat diets significantly reduces C-reactive protein and tumor necrosis factor alpha compared with the controls. It can decrease the level of interleukin 6 by increasing its inflammatory effects on mild systemic inflammation (34). Various studies have indicated that the consumption of green tea improves inflammatory factors in women with type II diabetes and prevents systemic inflammation in these patients by preventing the increase of inflammation cytokines (35).

Research also shows that, as a strong antioxidant, green tea catechins inhibit oxidative stress and lipid peroxidation,

thereby preventing tissue damage. They also induce tissue protection by strengthening the antioxidant defense system and increase endogenous antioxidants and the activity of antioxidant enzymes (36). It has been reported that, as a natural antioxidant, catechin enhances the antioxidant capacity of plasma, thereby decreasing the aggregation of cellular free radicals. It also prevents the effect of mutagens on chromosomes by decreasing oxidative stress conditions (37). Another study attributed the antioxidant effects of green tea compounds in inhibiting free radicals to their catechol structure and hydroxyl groups in their structure. These compounds deactivate free radicals by connecting to them. It was also shown that, by increasing antioxidant enzyme activities, catechins can deactivate superoxide, hydroxyl, nitric oxide, and hydrogen peroxide radicals (38). Based on another study, the consumption of green tea increases the activity of catalase and GPX in groups receiving thioacetamide. The increased activity of antioxidant enzymes in rats treated with green tea has been regarded as an indicator for antioxidant effects which are attributed to green tea (39). Another study evaluated the antioxidant activity of green tea essential oil by measuring the level of MDA and the activity of hepatic tissue antioxidant enzymes. This study concluded that administration of green tea for hypertriglyceridemic and hypercholesterolemic rats decreases the serum level of hepatic enzymes, enhances the activity of antioxidant enzymes, and reduces the level of hepatic tissue lipid peroxidation (40). Research has revealed that, by inhibiting the aggregation of free radicals, catechin prevents lipid peroxidation. It was also shown that, by decreasing superoxides, peroxynitrite and hydroxyl radicals, and lipid peroxidation, epicatechin reduces the serum level of MDA (41). According to one study, epigallocatechin gallate reduces tissue DNA oxidative damage. Moreover, because of its antioxidant effects, it protects hepatic cells from 2-nitropropane (42). Catechin also reduces cellular oxidative stress and DNA damage by inhibiting ROS and gives time to DNA to repair itself due to its protective effect (43). One study explored the effect of green tea polyphenols on the level of lipid peroxidation products and DNA structural damage in pulmonary cells. Results showed that green tea compounds decrease DNA oxidative damage, thereby reducing mutation and tumorigenesis in pulmonary cells (44). An important point about patients with PCOS is insulin resistance and increased weight. The limitations of the present study are the lack of periodic examinations of body weight or checking glucose and insulin serum levels, together with failing to check insulin resistance level.

Conclusion

Administration of catechin can dose-dependently decrease serum levels of inflammatory cytokines in rat models of PCOS. Moreover, due to its antioxidant effects, catechin increases the activity of antioxidant enzymes, decreases DNA oxidative damage, and reduces lipid peroxidation in the ovarian tissue of rats with PCOS. Based on the results, green tea catechin is effective in decreasing certain complications caused by PCOS.

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Conflict of Interest

None to declare.

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