ORIGINAL ARTICLE   OPEN ACCESS

Therapeutic potentials of the caffeine in polycystic ovary syndrome in a rat model: Via modulation of proinflammatory cytokines and antioxidant activity

Amir Raoofi[a,b†], Mohammad Jafar Rezaie[c†], Ali Delbari[a,b], Seyed Amir-Hossein Ghoreishi[d], Pegah Hadi Sichani[e], Shahram Maleki[f], Davood Nasiry[g], Maedeh Akhlaghi[d], Vahid Ebrahimi[h*], Amin Mousavi Khaneghah[i*]

[a]Cellular and Molecular Research Center, Sabzevar University of Medical Sciences, Sabzevar, Iran
[b]Department of Anatomy, Sabzevar University of Medical Sciences, Sabzevar, Iran
[c]Department of Anatomical Sciences, School of Medicine, Kurdistan University of Medical Sciences, Sanandaj, Iran
[d]Student Research Committee, Sabzevar University of Medical Sciences, Sabzevar, Iran
[e]Department of Nutrition Science, Isfahan Medical University, Isfahan, Iran
[f]Department of Clinical Sciences, Faculty of Veterinary Medicine, Lorestan University, Khorramabad, Iran
[g]Department of Anatomy, Faculty of Medicine, Mazandaran University of Medical Sciences, Sari, Iran
[h]Department of Anatomy, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran
[i]Department of Fruit and Vegetable Product Technology, Prof. Wacław Dąbrowski Institute of Agricultural and Food Biotechnology—State Research Institute, Warsaw, Poland

[†]These authors have contributed equally to this work.

Abstract

Recent studies have shown that polycystic ovary syndrome (PCOS) affects about 6% of women worldwide. It is associated with reproductive and metabolic dysfunction. Caffeine is naturally found in tea, cocoa, and coffee. It has been shown that caffeine can change hormonal profiles, stimulate ovulation, and enhance fertility. Therefore, in this study, the effects of caffeine on rats with PCOS were investigated. For this purpose, 40 female rats were divided into five groups: (1) control group (without any intervention), (2) sham group (administration of olive oil as a caffeine solvent), (3) PCOS group (injection of 2 mg of estradiol valerate for each rat), (4) caffeine group (administration of 37.5 mg/kg caffeine for each rat), and (5) PCOS + caffeine group. After 21 days of treatment, the ovaries of rats were removed and prepared for further evaluations, including hematoxylin and eosin staining, TUNEL assay, real-time PCR, and biochemical analysis. Administration of caffeine in PCOS mice considerably reduced both the volume of the ovary ($P < 0.05$) and follicular clusters

*Corresponding authors: Vahid Ebrahimi, Department of Anatomy, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran. *Email address:* ebrahimi.v67@gmail.com; Amin Mousavi Khaneghah, Department of Fruit and Vegetable Product Technology, Prof. Wacław Dąbrowski Institute of Agricultural and Food Biotechnology—State Research Institute, Warsaw, Poland. *Email address:* amin.mousavi@ibprs.pl

(P < 0.01). However, superoxide dismutase and glutathione peroxidase were dramatically active in the PCOS + caffeine group compared to others (P < 0.05). Besides, caffeine treatment in PCOS mice led to Bax reduction and increased Bcl-2 expression. On the other hand, the expression of IL-6 and TNF-α in PCOS + caffeine group was high compared to other groups. We found that caffeine can reduce apoptosis and inflammation in PCOS ovaries and enhance the unpleasant symptoms of PCOS.

Introduction

Polycystic ovary syndrome (PCOS) is a heterogeneous endocrine disorder that affects about 6% of women worldwide.[1-3] As a multifactorial syndrome, this is relatively complex and has unclear mechanisms. Several factors such as hyperandrogenism, chronic inflammation, and oxidative damage can cause PCOS.[4] Besides, activation of inflammatory cytokines, some nutrients, environmental causes, and some genes are involved in the instigation and progression of the disease.[4] Moreover, oxidative stress can affect ovulation, fertilization, and fetal development. In PCOS patients, oxidative stress increases significantly.[5] The main diagnostic criteria are hyperandrogenism, ovulatory dysfunction (Anovulation or oligoovulation), and polycystic ovaries.[6-9] PCOS can lead to metabolic disorders and increase the risk of type 2 diabetes, cardiovascular disease, breast cancer, hypertension, and fatty liver in the long term.[5-7,10-12] Treatment strategies include lifestyle changes, physical activity, weight loss, a low-calorie diet, and medications such as metformin and oral contraceptives containing estrogen and progestin.[4,7] Some food ingredients can be involved in the development or aggravation of the syndrome. Tea, coffee, and other compounds used in most people's daily diet contain significant amounts of caffeine.[13] Caffeine is a natural alkaloid that can affect mental alertness and mood.[14]

Caffeine is quickly absorbed by the gastrointestinal tract and affects different body organs. Caffeine can alter the hormonal profile and affect the menstrual cycle function.[15] Some studies have indicated that these events may be due to the stimulation of ovulation.[16] The study by Chavarro et al. signified that caffeine consumption did not have much effect on ovulation disorders, but more extensive investigations are needed.[17] Caffeine has stimulating effects on the central nervous system, cardiovascular system, urinary system, adrenaline secretion, gastric acid secretion, smooth muscle relaxation, fat lipolysis, and triglyceride hydrolysis.[13] In the study by Caan et al., a significant increase in fertility was reported in women who drank more than half a cup of tea daily.[18] Caffeine consumption also reduced testosterone secretion.[19] Exposure to high doses of caffeine can lead to changes in reproductive hormones and imbalances in the endocrine system.[20] Caffeine has been reported to increase estradiol production, while moderate caffeine intake is associated with decreased estradiol concentrations.[21] It has been suggested that caffeine inhibits egg maturation, steroid production, and phosphodiesterase activity in animal models or may interfere with estrogen metabolism by inhibiting aromatase, the enzyme that converts androgen to estrogen.[21] On the other hand, some evidence suggests a link between high caffeine consumption and apoptosis. These effects seem to depend on hydrogen oxide, and caffeine consumption in low concentrations can have antioxidant possessions.[22] A study also found that high caffeine consumption reduces the length of the menstrual cycle to less than 25 days.[15] To the best of our knowledge, studies on the effects of caffeine on the ovarian cycle, especially in PCOS, have been performed sporadically, and conflicting results have been presented. Here, we attempted to investigate the therapeutic potentials of caffeine on ovarian function in a rat model of PCOS.

Materials and Methods

All animal experiments and informed consent were approved by the Institutional Ethics Committee of Sabzevar University of Medical Sciences, Sabzevar, Iran (code no IR.MEDSAB.REC.1400.033). All materials used in this study were purchased from Sigma-Aldrich (St. Louis, MO), except for the cases specified otherwise.

PCOS induction

The induction of PCOS was performed according to our previous study.[23] Briefly, the estradiol valerate was used in a single dose (2 mg/kg), dissolved in olive oil, and administered via subcutaneous injection. To validate PCOS, the vagina was examined daily for 60 days using vaginal smears and persistent vaginal cornification, one of the symptoms of follicular cysts in the ovary.

Animals and study design

Forty adult female Wistar rats (weighing 200–250 g) were purchased from the Laboratory Animal Research Center, Sabzevar University of Medical Sciences, Sabzevar, Iran. They were maintained individually in standard conditions in clean polypropylene cages. All animals were divided into five groups (n = 8 per group): The non-PCOS untreated rats (control group), non-PCOS treated with olive oil as a solvent (Sham group), non-PCOS treated with caffeine (Caffeine group), the PCOS mice not treated with caffeine (PCOS group), and the PCOS treated with caffeine (PCOS + caffeine group). In the treatment groups, caffeine was administered with drinking bottles containing 37.5 mg/kg. The caffeine solution was prepared every 2 days, and the bottles (which are dark to prevent caffeine oxidation) were refilled between 7:00 p.m. and 8:00 p.m. The duration of caffeine administration was 21 days.

Histological and stereological analyses

The tissue samples were harvested and processed at the end of the study. Then, they were embedded in paraffin, and serial sections with a thickness of 5 μm (to determine the total volumes) or 20 μm (to determine the number of the primordial,

primary, secondary, and antral follicles) were prepared. For each sample, 10 sections with equal distances were selected. The sections were stained using hematoxylin and eosin (H&E) method. All stereological measurements were done according to the method reported by Howard and Reedas.[24]

Estimation of the total volumes of the ovary

For this purpose, the Cavalieri method was used. At first, 10 sections were selected for each sample, and a grid of points was superimposed on each section. Then, to estimate the total volumes of the ovary, all the points which overlay on each tissue were counted, and the following equation was used:

$$V_{total} = \sum P \times \frac{a}{p} \times t$$

Where, "ΣP" is the total number of points counted in the 10 sections, "a/p" (mm^2) is the area related to each square formed between four points, and "t" (mm) is recognized as the distance between the selected sections.

Estimation of the number of the primordial, primary, secondary, and antral follicles

To determine the number (N_v) of the primordial, primary, secondary, and antral follicles in the ovarian tissue, the optical disector method and the following equation were used:

$$Nv = \frac{\sum Q}{\sum P \times h \times \frac{a}{f}} \times \frac{t}{BA}$$

Where "ΣQ" is the number of nuclei, "h" (μm) is the height of the disector, "Σp" is the total number of the counted frames, "a/f" (mm^2) is frame area, "BA" (μm) is the block advance microtome (set at 20 μm), and "t" (μm) is real section thickness.

Immunostaining for apoptosis

To determine the total density of apoptosis, immunohistochemistry was performed against Caspase-3 protein. Briefly, 10 sections with equal distances from each other were selected. Sections were incubated in Goat normal serum (to block nonspecific site) and then exposed in anti-Caspase-3 rabbit polyclonal antibody (1:100 in phosphate buffered saline [PBS, v/v], Abcam ab4051) overnight at 4°C. Then, the samples were washed with PBS and incubated with a secondary antibody conjugated with horseradish peroxidase (goat anti-rabbit IgG, Abcam ab205718). Finally, in order to detect the positive reactions, diaminobenzidine tetrahydrochloride was added for 5 min. The samples were then dehydrated and mounted. To calculate the average Caspase-3-positive cells in the ovarian tissue of the 10 evaluated sections, the total positive cells were counted. Finally, the sum of positive cells in the studied sections was calculated and statistically compared between the experimental groups.

Biochemical analysis

The biochemical analysis was performed according to Nasiry et al.[25] Briefly, the obtained tissue samples were thoroughly cleaned of blood and immediately frozen at −80°C and stored for assays. Biochemical markers measured included malondialdehyde (MDA), glutathione peroxidase (GPx), and superoxide dismutase (SOD). The MDA level was determined by measuring thiobarbituric acid reactive substances, which was determined spectrophotometrically by the absorbance at 535 nm. To determine GPx level, 10% trichloroacetic acid was used, which was determined spectrophotometrically by the absorbance at 412 nm. The determination of SOD activity was based on the inhibition of superoxide radical reaction with pyrogallol, which was determined spectrophotometrically by the absorbance at 420 nm.

Gene expression analyses

The harvested tissues were homogenized using the Lyser device, and then the total RNA was extracted using TRIzol (Invitrogen®, US). The cDNA was reverse transcribed based on the Takara Bio cDNA synthesis kit protocol (Takara Bio, Inc., Kusatsu-Shi, Japan). To determine the gene expressions of Bax, Bcl-2, Caspase-3, IL1-α, IL-6, and Tumor necrosis factor-alpha (TNF-α), qRT-PCR in three biological replicates was performed using a real-time PCR system (Applied Biosystems StepOne instrument), SYBR Green Master Mix, and sets of primers (Table 1). The StepOne software (Applied Biosystems; version 2.1) was used to analyze the results. The expression levels of each target gene were normalized to its internal control, ß-actin, and the final analyses were performed using the comparative CT method ($2^{-\Delta\Delta ct}$).

Statistical analysis

The statistical analyses were performed using SPSS software (IBM SPSS Statistics, version 23), and the graphs were plotted using Prism software (GraphPad Prism 8.0.2.263). Results were presented as mean ± SD. The K-S test was used to evaluate the normality. One-way ANOVA followed by Dunnett's post-hoc test was used to compare the groups. $P < 0.05$ was considered statistically significant.

Results

Histomorphometric findings

In this experimental study, we conducted H&E staining to determine the effects of caffeine on ovarian volume and the number of different types of ovarian follicles (Figure 1).
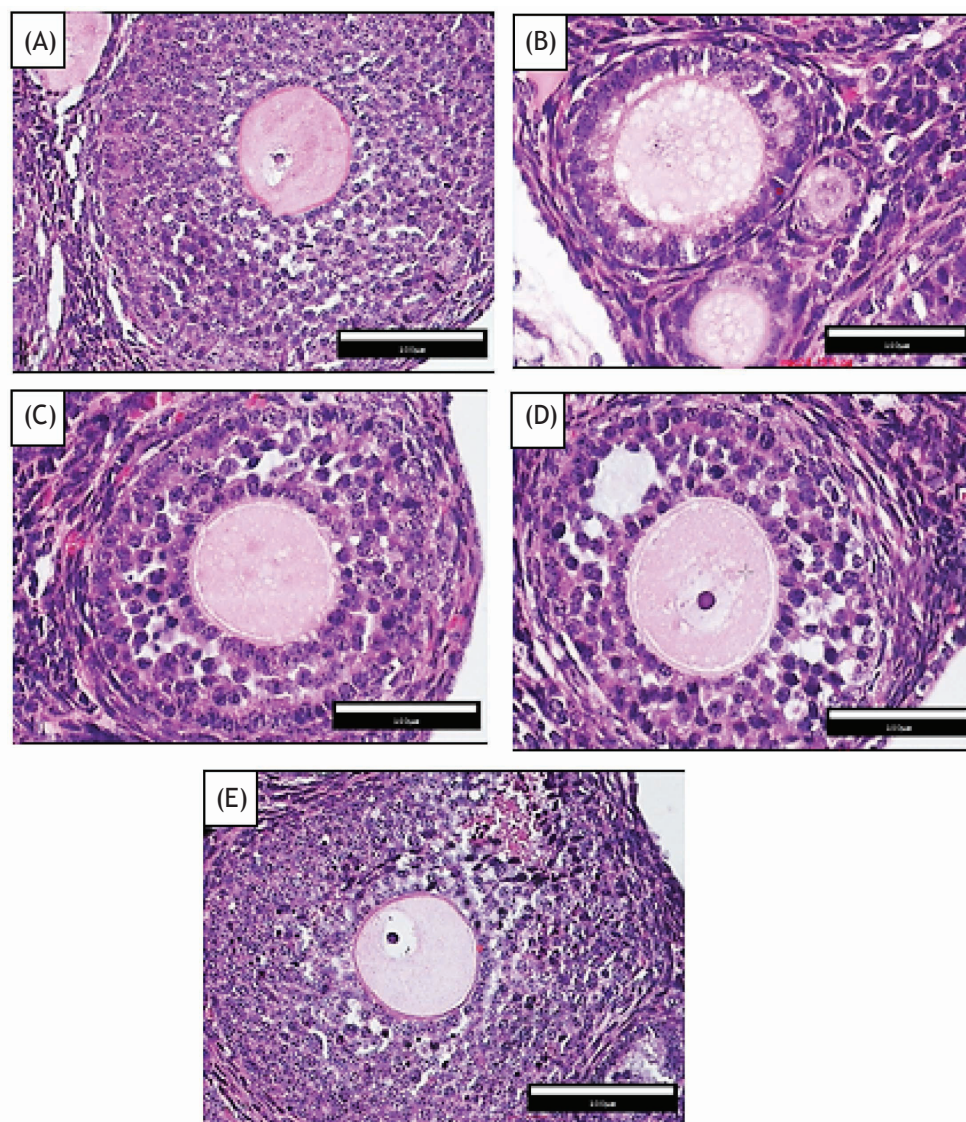
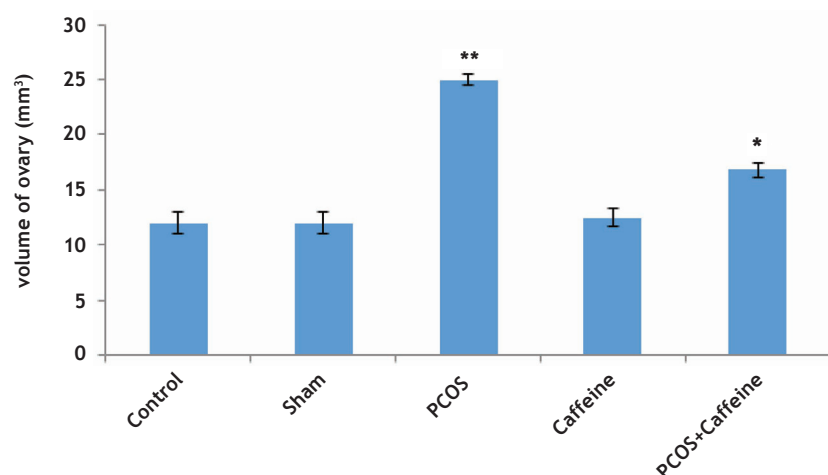*Effects of administration of caffeine on the volume of ovary*
Figure 2 represents the ovarian volume in different groups. As shown, the total volume of the ovary in the PCOS group was notably high compared to the control, sham, and

Table 1 Real-time PCR primers list.

Gene	Sequence	Primer sequence	TM (°C)	Accession
IL-1α	F	TCCCCAACTGGTACATCAGC	59.35	NM_031512.2
	R	GGTGAAGTCAACTATGTCCCG		
TNF-α	F	CCTCACACTCACAAACCACCA	55	NM_012675.3
	R	ACAAGGTACAACCCATCGGC		
IL-6	F	GCCTTCTTGGGACTGATGCT	55	NM_012589.2
	R	TGCCATTGCACAACTCTTTTCT		
Bcl-2	F	CAAGCCTTCTTCCTCCTGGTGA	55	NM_012963.2
	R	CGCTCTCGTACACTTCAGAGA		
Caspase-3	F	GATGAGGCAGACTTTCTGCT	60	NM_173837.2
	R	CATAGTTCACGCCAGTCAGGAT		
Bax	F	TTCCGAGTGGCAGCTGAGATGTTT	60.28	NM_171988.2
	R	TGCTGGCAAAGTAGAAGAGGGCAA		
GAPDH	F	ATCTGACATGCTGCCTGGAG	60.25	NR_046237.1
	R	AAGGTTGGAAGATGGGAGTTGC		

Figure 1 Photomicrograph of the ovary stained with H&E, 40X. (A) Control group; (B) Sham group; (C) PCOS group; (D) Caffeine group; (E) PCOS + caffeine group.

Figure 2 Comparing the mean total volume of ovary (mm^3). Effects of administration of caffeine on volume of ovary (mm^3) of rats with PCOS. **$P < 0.01$ implies the significant differences, in comparison to control, sham, and caffeine groups. *$P < 0.05$ represents the significant difference in comparison with the PCOS group.
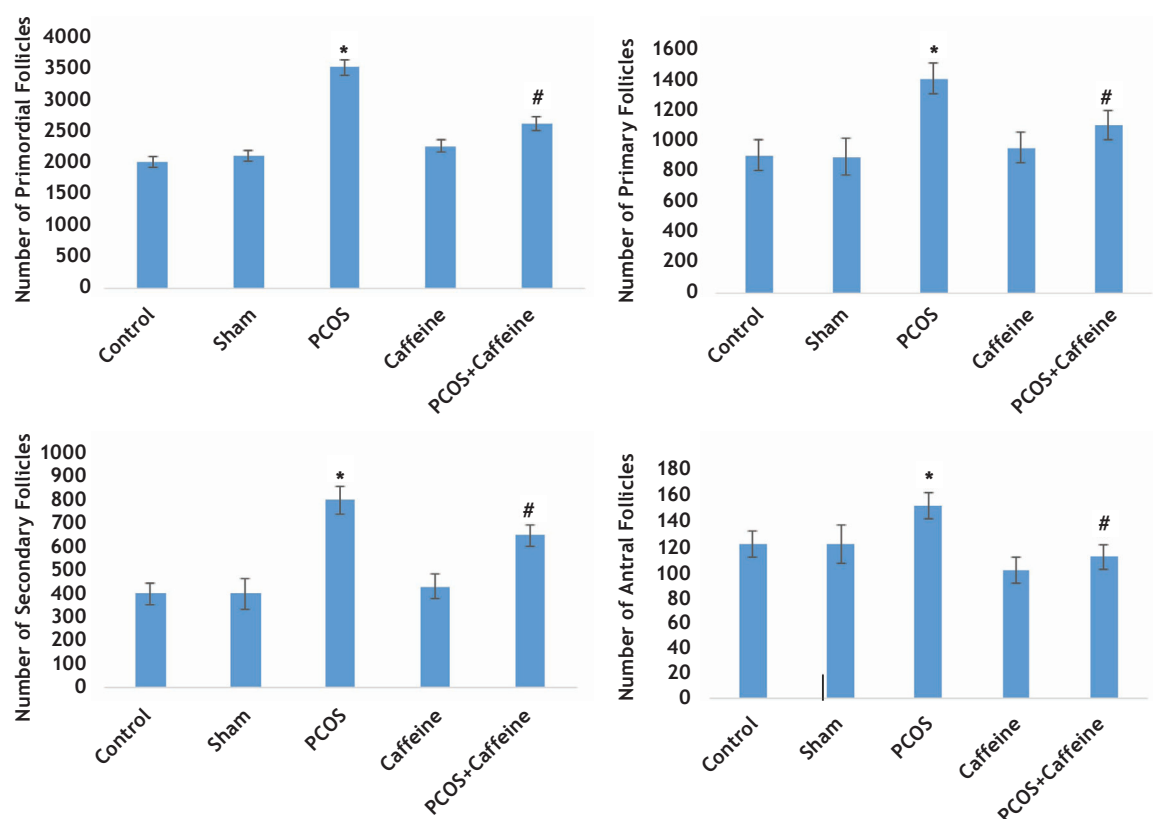

Figure 3 Effects of caffeine on the number of follicles in different groups. A significant increase in all follicular clusters is detected in the PCOS group compared to the caffeine-treated group. *$P < 0.01$ implies the significant differences, in comparison to control, sham, and caffeine groups. #$P < 0.05$ represents the significant difference in comparison with the PCOS group.

caffeine groups ($P < 0.01$), while it was considerably low in the PCOS + caffeine group compared to the PCOS group ($P < 0.05$).

### Assessment of the numbers of primordial, primary, secondary, and antral follicles

The number of follicles in various stages was counted (Figure 3). According to the data, there was a significant increase in all follicular clusters in the PCOS group compared to the other groups ($P < 0.01$). The administration of caffeine in PCOS rats reduced different types of ovarian follicles. However, there was a significant difference in the numbers of follicular clusters in the PCOS + caffeine group compared to the control, sham, and caffeine groups ($P < 0.05$).
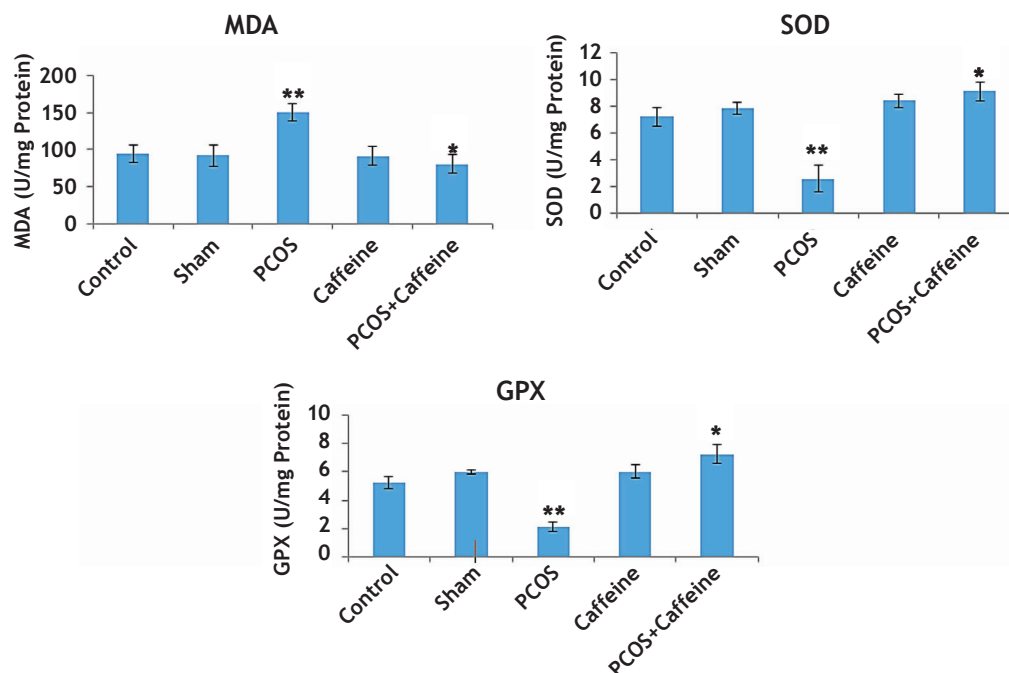
Figure 4 The effect of caffeine on changes in MDA and antioxidant enzymes in rats. Activities of SOD and GPX in ovary study groups. Values are illustrated in mean ± S.D; n = 6. **P < 0.01 indicates significant differences compared to the control, sham, and caffeine groups. *P < 0.05 indicates significant differences compared to the PCOS group.
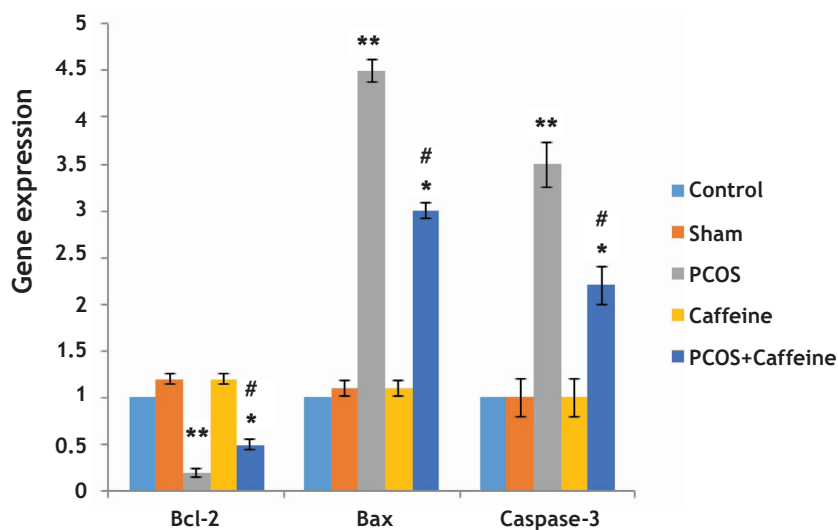
Figure 5 PCOS noticeably induced several genes related to apoptosis. Fold change of apoptosis gene expression were detected by qPCR after 3 weeks. Caffeine administration induced a significant increase in Bcl-2, while the expression levels of Bax and Caspase-3 declined. The values are expressed as mean ± SD (n = 6); **P < 0.01 indicates significant differences compared to the control, sham, and caffeine groups. *P < 0.05 indicates significant differences compared to the PCOS group. #P < 0.05 refers to the considerable differences in comparison with the control, sham, and caffeine groups

The effect of caffeine on MDA and antioxidant enzymes

As shown in Figure 4, PCOS rats had high levels of MDA activity compared to the control, sham, caffeine (P < 0.01), and PCOS + caffeine groups (P < 0.05). The activity of antioxidant enzymes, including SOD and GP_X, was evaluated, and the data showed that these enzymes were dramatically active in the ovaries of the PCOS + caffeine group (P < 0.05), while the levels of SOD and GP_X were notably low in the PCOS group (P < 0.01). According to these results, caffeine can significantly increase antioxidant activity in PCOS rats.

The effects of caffeine on the expressions of apoptotic genes and Caspase-3 protein

According to the qPCR analysis, the level of apoptosis genes such as Bax was low in the control, sham, and caffeine groups, while Bcl-2 was significantly high (P < 0.001). On the other hand, a high level of Bax and a low level of Bcl-2 were seen in the ovaries of PCOS rats compared to the control, sham, and caffeine groups (P < 0.001) (Figure 5). Three weeks of caffeine treatment in PCOS rats led to a severe reduction in the expression of Bax (P < 0.01), while Bcl-2 expression in their ovarian tissues had been considerably increased (P < 0.05) (Figure 5).
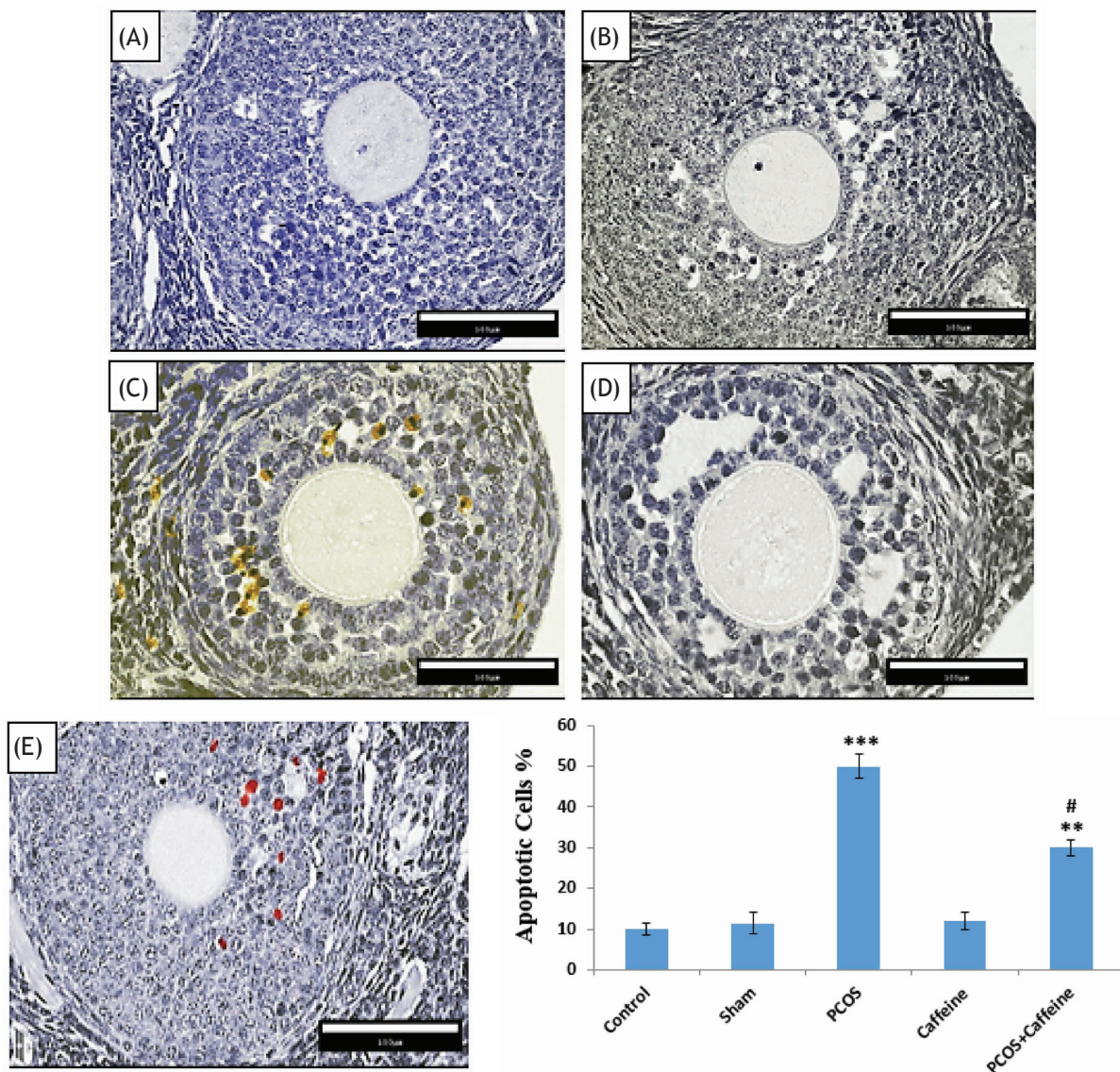
Figure 6 Caspase-3 detection of apoptotic cells in the ovary of (A) Control group; (B) Sham group; (C) PCOS group; (D) Caffeine group; (E) PCOS + caffeine group. The effect of caffeine on percent of apoptotic cells in ovary of PCOS-induced rats. Mean ± SD of the percent of apoptotic cells of ovary in different groups. ***P < 0.001 demonstrates significant differences compared to the control, sham, and caffeine groups. **P < 0.01 implies the significant differences in comparison to control, sham, and caffeine groups, and #P < 0.05 represents the significant difference in comparison with the PCOS group (n = 6 rat per group).

By immunohistochemistry, the expression of the Caspase-3 protein was assessed. According to the results, Caspase-3 considerably increased in the PCOS group compared to the control, sham, and caffeine groups, while it remarkably declined in the caffeine treated group (Figure 6).
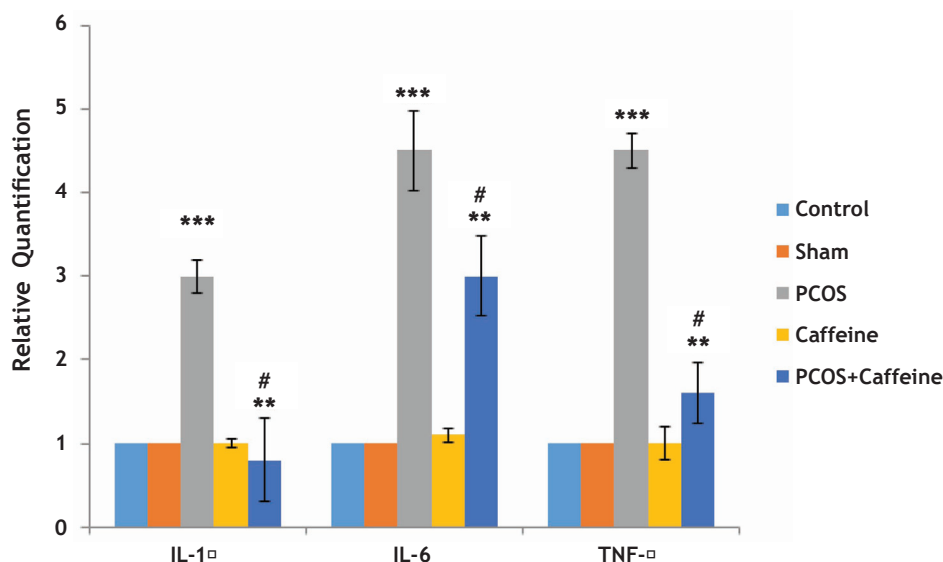
The effect of caffeine on mRNA expression of IL-1a, IL-6, and TNF-a in PCOS-induced rats

The relative expression level of mRNA in IL-1α, TNF-α, and IL-6 was quantified in control, sham, caffeine, PCOS, and PCOS + caffeine groups. According to the results, IL-1α, TNF-α, and IL-6 expression levels were remarkably high in the PCOS group compared to the control, sham, and caffeine groups (P < 0.05). The expression of IL-6 and TNF-α

in the PCOS + caffeine group was higher than in the control, sham, and caffeine groups. However, the differences between the levels of IL-1α in PCOS + caffeine compared to the control, sham, and caffeine groups were insignificant (Figure 7).

Discussion

In this study, the mRNA expression levels of several genes related to apoptosis, including Bax, Bcl-2, and Caspase-3, and several genes associated with inflammation, including IL-1α, IL-6, and TNF-α, in the ovary of all study groups were investigated. Based on our results, we reported that the gene expression of Bax and Caspase-3 along with IL-1α, IL-6, and TNF-α in the ovaries of rats with PCOS increased

Figure 7 The effect of caffeine on mRNA expression levels of IL-1α, IL-6, and TNF-α in PCOS-induced rats. Mean ± SD of the mRNA expression levels of IL-1α, IL-6, and TNF-α of ovary in different groups. ***P < 0.001 indicates significant differences compared to the control, sham, and caffeine groups. **P < 0.01 indicates significant differences compared to the PCOS group. #P < 0.05 refers to the considerable differences in comparison with the control, sham, and caffeine groups (n = 6 rat per group).

dramatically. On the other hand, the histological examination of our study confirmed the significant positive apoptotic cells in the ovary of PCOS animals compared to the healthy groups. Additionally, the stereological assessments showed a substantial decrease in the mean total volume of the ovary and the mean number of primordial, primary, secondary, and antral follicles in the ovaries of rats with PCOS. Based on the statistics obtained from the biochemical analysis, a significant increase in oxidative stress and a decrease in antioxidant status in the PCOS group were identified. Eventually, it turned out that caffeine was able to recover histopathological damages, cell death, inflammation, and antioxidant status in rats with PCOS.

While the etiology of PCOS rests a secret, some confirmations support the manifestation of chronic low-grade inflammation in females.[26] The appearance of this chronic condition is demonstrated by upsurges of several factors, including pro-inflammatory cytokines, oxidative stress, and endothelial inflammation. Chronic inflammatory progressions are usually related to a rise in cytokines and chemokines such as interleukins (ILs), and PCOS is also associated with an increase in these agents.[27,28]

On the other hand, oxidative stress and inflammation processes are strictly related, and there is ample evidence that inflammation persuades the production of reactive oxygen species (ROS), and oxidative stress also exacerbates inflammation.[29] It has been reported that lipid peroxidation and the amount of MDA have increased significantly in PCOS.[30] These findings complemented succeeding probes for assessing different oxidative stress markers containing lipid peroxidation and protein carbonyl content.[31,32] In contrast to the elevation of oxidative stress, females with PCOS have a declined total antioxidant capacity,[32] reduced glutathione,[33] and decreased content of haptoglobin as an antioxidant protein.[34] In addition to the declared biomarkers, additional pro-inflammatory cytokines are not raised in the peripheral circulation in patients with PCOS. For

example, a recent investigation by Escobar-Morreale et al. revealed no alteration in the serum level of IL-6 in women with PCOS compared to the controls.[35] Tumor necrosis factor-alpha (TNF-α) is an important inflammatory cytokine, mainly secreted by the visceral adipocytes. According to the above study, it was found that there was no significant change in TNF-α levels in women with PCOS compared to the controls.[35]

Besides, it was found that the Bax/Bcl-2 ratio was significantly greater in the rats with PCOS than in the control ones.[36] On the other side, Tilly et al. (1995) and Tilly (1996) have publicized that follicular atresia is closely linked to an imbalance among members of the Bcl-2 family.[37,38] As a result, perhaps it can be said that amplification in ovarian apoptosis through disparity among the Bcl-2 family members may be associated with the alterations of developing cystic follicles in PCOS.

In the study of Shah et al., the histological evaluation showed ovaries with large antral follicles and thickened theca internal and shrunken granulosa cells in PCOS animals,[39] which was similar to the previous findings.[40] This study also displayed the lack of corpus luteum formation in female rats with PCOS. The number of healthy follicles in different growing stages diminutioned, and the absence of oocyte or corona radiate cells was observed in the PCOS group. Besides, reduction in granulosa cells and atrophy of interstitial cells were detected, but quercetin usage improved normal follicles and declined cystic follicles in ovaries.[39]

Caffeine is a relatively high-consumption psychostimulant in different nations, with antioxidant, antiapoptotic, and anti-inflammatory properties that endorses our present results.[41] It has also been presented that caffeine has promising effects on some neuroimmune disorders.[42] Caffeine is able to impede lipid peroxidation, ROS production, and oxidative stress pathways,[43,44] which are consistent with the results of our study. Additionally, several experiments in

line with our outcomes indicated that caffeine is able to reduce inflammatory and apoptosis symptoms in humans and animals by improving antioxidant capacity.[45,46]

Conclusion

The results of the present study regarding the relative improvement of unpleasant symptoms caused by PCOS showed that caffeine consumption could be helpful as a complementary strategy and core therapeutic approaches. However, caffeine's exact mechanism of action in reducing inflammatory symptoms and amending ovarian histological aspects during PCOS still needs further research.

Authors' Contributions

AR and MJR helped in the conception and design of the study. SAHG, DN, PHS and SHM were involved in supervision and data analyses. AR and VE contributed to the drafting the article. AD, VE, and AMK were involved in the final approval of the version to be submitted.

Conflict of Interest

No potential conflict of interest was reported by the authors.

Ethics Disclosures

The protocol of this study was reviewed and confirmed by the Institutional Ethics Committee of Sabzevar University of Medical Sciences, Sabzevar, Iran (code no IR.MEDSAB. REC.1400.033).

Consent for Publication

Consent for publishing the resulting draft of the present research has been confirmed by all coauthors.

Acknowledgment

This work was performed at the School of Medicine, Sabzevar University of Medical Science.

Funding

This work was supported by a grant from the Vice Chancellor for Research, Sabzevar University of Medical Sciences, Sabzevar, Iran.

Availability of Supporting Data

The authors have stated that supporting data will be made available to others upon request.

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