

Original Article: Oral Administration of Menthol Alleviate Adverse Effects of Polycystic Ovarian Syndrome on Blood Biochemical Parameters and Antioxidant Status in Wister Rats



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ABSTRACT

Background: Polycystic Ovarian Syndrome (PCOS) has been related to dyslipidemia and suppression of antioxidant status. However, novel agents such as menthol can be an efficient strategy for alleviation of PCOS. This study was conducted to evaluate the effects of different levels of menthol in blood biochemical parameters and antioxidant status in Wistar rats induced with PCOS.

Materials and Methods: Fifty Wistar rats were used in this study and the animals divided into five groups including; 1. Control group (Control) or healthy rats; 2. PCOS group (PCOS) that did not receive any menthol; 3, 4 & 5. Animals that received 2, 4 and 6 mg/kg of body weight (PCOS-2, PCOS-4 and PCOS-6). To induce the PCOS, 5 mg estradiol valerate was administered. At the end of the trial session, blood samples were taken to evaluate plasma concentrations of glucose, insulin, total cholesterol, Triglycerides, HDL-C, LDL-C, Ferric-Reducing Ability of Plasma (FRAP), Advanced Oxidation Protein Product (AOPP) and Total Oxidation Status (TOS).

Results: Induction of PCOS increased glucose, insulin, total cholesterol, Triglycerides, LDL-C, FRAP, AOPP and TOS, and decrease HDL-C. The use of menthol especially in higher levels improved the mentioned parameters. PCOS also increased body weight and oral treatment of menthol helped to maintain the body weight.

Conclusion: It is concluded that menthol improved antioxidant status in rats with PCOS. Thus, use of menthol is recommended for treatment of PCOS.

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Introduction

Polycystic Ovarian Syndrome (PCOS), so called Stein-Leventhal syndrome has been known to have metabolic and reproductive endocrinopathy disorders [1]. Prevalence of PCOS has been reported to be 5-20% in reproductive age [2]. Patients with PCOS are highly sensitive to some diseases and disorders including obesity, insulin resistance, type II diabetes, cardiovascular disease, infertility, malignancy, and psychological disorders [2]. The exact etiology of the PCOS is still unknown, but could be attributed to complex interactions among different factors including genetic, environmental, and behavioral factors. Anxiety, depression, and poor quality of life have been diagnosed in patients with PCOS [3].

Insulin has been known as one of the atherogenic hormones [4] which along with hyperinsulinemia could participate in the development of diabetes, hypertension, and dyslipidemia, which is often combined with elevated total cholesterol and Low-Density Lipoprotein (LDL), Triglyceride (TG), and reduced High-Density Lipoprotein (HDL) levels in patients with PCOS [5]. Hyperinsulinemia has been known to have the ability to promote ovarian androgen overproduction [6]. Dyslipidemia and sex steroids have also been known to have important effects on cardiovascular diseases [7]. Elevated oxidative stress and reduced antioxidant capacity can contribute to the increasing risk of cardiovascular disease in patients with PCOS, in addition to insulin resistance, hypertension, central obesity, and dyslipidemia [7].

The allopathic drugs have commonly been used to treat the PCOS, which include clomiphene citrate, metformin, letrozole, tamoxifene and troglitazone. These drugs have been known to have severe side effects including hot flushes, arthritis, joint or muscle pain and psychological side effects [8]. Since conventional medicine can have side effects, the alternative drugs such as herbal medicines and their derivations can play a significant role. Menthol is one natural cyclic monoterpene alcohol which is found in *Mentha* species. Menthol is also one of the most important constituents of some essential oils including eucalyptus, lemongrass, and palmarosa [9]. Rozza et al. have shown that menthol can have a gastro protective role against ethanol-induced gastric ulcers and treatment with elevated levels of the anti-inflammatory cytokine IL-10 [10].

Menthol has also been known to have antioxidant properties [11]. It seems that menthol has improved antioxi-

dant and lipid profile in animals with PCOS. This study was therefore conducted to evaluate the effects of oral administration of menthol on blood biochemical parameters and antioxidant in mice with PCOS.

Materials and Methods

Preparation of menthol

Peppermint essential oil was purchased from Barij Essence Company (Kashan-Iran). Menthol oil was crystallized through chilling in the +14, +10 and -5°C for 8 hours each, respectively, by using sealed plastic containers in freezers. Menthol crystals were isolated from peppermint essential oil because dementholized peppermint essential oil can still contain certain amounts of menthol, racemic and isomenthols and menthone. In order to recover the menthol crystals and to remove the menthone, it had to be treated with 8 g boric acid in distillation flask for a period of 3 hours. The rest of the distillation containing borates of menthol were saponified using steam distillation on 70 g of 15% NaOH solution, and finally crystals were separated and dried in 26°C and production was investigated.

Animals

In the current study, fifty female Wistar rats, 13-15 weeks of age, weighing 170±20 g, were purchased from Pasture Institute, Tehran-Iran. To adapt, the female rats were grouped into 5 groups in the controlled temperature of 22-24°C and a lighting diet of 12 h light: 12 h darkness cycle. Food and water were ad libitum supplied.

Induction of PCOS and treatments

The Wistar rats were distributed into five groups including; 1. Control group (Control) or healthy rats that did not receive any menthol; 2. PCOS group (PCOS) that did not receive any menthol; 3, 4 & 5. Animals that received 2, 4 and 6 mg/kg of body weight (PCOS-2, PCOS-4 and PCOS-6). In the PCOS induction phase, rats were induced with PCOS by applying 5 mg estradiol valerate, as reported by previous studies [12]. Wistar rats were treated with menthol for 28 days. Body weight was recorded in days 1, 14 and 28 post-induction.

Blood sampling and biochemical analyses

At the end of the trial, all the Wistar rats were anesthetized by using ketamine/xylazine HCl (75/10 mg/kg intraperitoneally). The collected blood samples from the aorta with anticoagulant were centrifuged for 12 min

in 3, 500 rpm/min to achieve the plasma and stored in -20°C until biochemical analysis for investigation of glucose, insulin, total cholesterol, Triglycerides, HDL-C and LDL-C. The plasma concentrations of glucose, insulin, total cholesterol, Triglycerides, HDL-C and LDL-C were investigated by using Pars Azmoon commercial Kits.

Evaluation of Ferric-Reducing Ability of Plasma (FRAP)

FRAP was evaluated as previously reported [13]. In summary, FRAP reagent was produced and heated in 37°C while also a mixture of the following solutions was applied: 1. 0.3 M sodium acetate buffer solution (pH 3.6); 2. 10 mM 2,4,6-tripyridyl-1-5-triazine in 40 mM HCl solution; and 3. 20 mM FeCl_3 solution at the ratio of 10:1:1 (v/v/v). A level of plasma (10 μL) was incubated along with 90 μL of FRAP reagent in a micro plate for 30 minutes in room temperature in the dark. The level of absorbance of the mixture was assessed in the wavelength of 595 nm by a spectrophotometer. The levels of FRAP values were measured by a calibration standard curve of FeSO_4 (0-2000 μM).

Investigation of levels of Advanced Oxidation Protein Product (AOPP)

Samples were made as follows: in a tube, 20 μL of plasma from each rat was diluted into 100 μL in phosphate-buffered saline by the inclusion of 10 μL of 1.16 M KI and 20 μL of absolute acetic acid. The absorbance of the reaction mixture was rapidly read by a Spectra Max 1601 spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) in 340 nm against a blank, having 100 μL of phosphate-buffered saline, 20 μL of acetic acid, and 10 μL of KI solution. As the linear range of chloramine-T absorbance in 340 nm is between 0 and 100 μM , AOPP concentrations were

reported in μM chloramine-T equivalents. All evaluations were conducted simultaneously.

Total Oxidation Status (TOS)

The plasma concentration of TOS was evaluated by a colorimetric measurement procedure. In order to evaluate the TOS, 225 μL of Reagent 1 (xylenol orange 150 μM , NaCl 140 mM, and glycerol 1.35 M in 25 mM H_2SO_4 solution, pH 1.75) was mixed with 35 μL of plasma sample, and the absorbance of each sample was investigated spectrophotometrically in 560 nm as a sample blank. Then, 11 μL of Reagent 2 (ferrous ion [5 mM] and o-dianisidine [10 mM] in 25 mM H_2SO_4 solution) was added to the mixture within 3-4 min. The last absorbance was investigated in 560 nm. The assay was calibrated by using H_2O_2 , and the results have been reported in terms of micromolar H_2O_2 equivalent per liter ($\mu\text{mol H}_2\text{O}_2$ equiv/L). The detection limit of the procedure was investigated by evaluating the zero calibrator 10 times.

Statistical analysis

The statistical analyses were conducted via GraphPad Prism (GraphPad Software, Inc., La Jolla, CA, USA). The Analysis of Variance (ANOVA) was used to compare among the groups, and post hoc (Tukey) was used to compare the groups. Significance was considered in $P < 0.05$.

Results

Body weight

Effects of different levels of menthol on body weight of Wistar rats are presented in Figure 1. As the results indicate, body weight was not influenced by experimental treatments in day 1 ($P > 0.05$). There was no significant difference between control and PCOS or control PCOS in day 1 ($P > 0.05$). In days 14 and 28, induction of PCOS

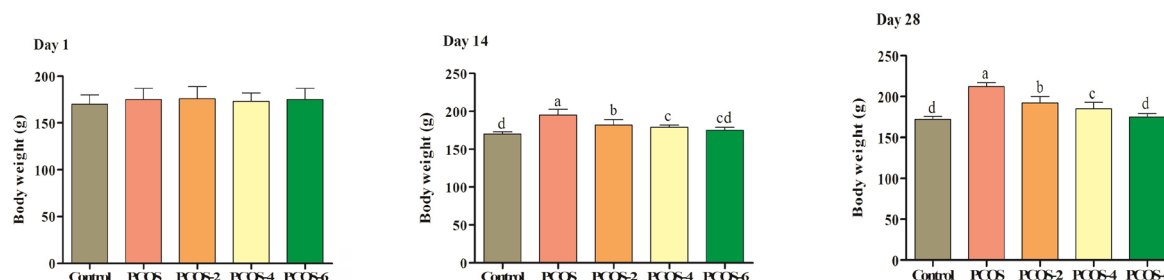


Figure 1. Effects of different levels of menthol (2, 4 & 6 mg/kg) on body weight (g) in rats with PCOS

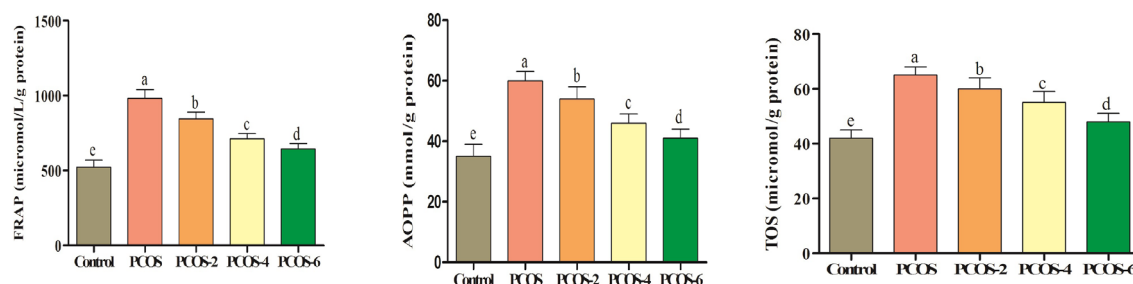
Control: Control standard without PCOS; PCOS: PCOS control without menthol; PCOS-2, 4 & 6: PCOS rats treated with 2, 4 and 6 mg/kg menthol; Superscripts (a-e) show significant differences at $P < 0.05$.

Table 1. Effects of different levels of menthol on blood biochemical parameters in rats with PCOS

Parameters	Control	PCOS	PCOS-2	PCOS-4	PCOS-6	P
HDL-C (mg/dl)	33.10±2.10 ^a	18.10±3.21 ^c	23.10±1.12 ^d	27.10±1.21 ^e	30.10±2.21 ^b	***
LDL-C (mg/dl)	26.20±3.31 ^d	45.10±3.21 ^a	39.10±2.37 ^b	32.21±3.21 ^c	28.12±3.21 ^d	***
Cholesterol (mg/dl)	76.51±3.31 ^d	135.23±6.21 ^a	115.23±6.57 ^b	98.21±6.21 ^c	82.30±7.21 ^d	***
Triglycerides (mg/dl)	82.31±5.12 ^d	126.11±11.21 ^a	99.21±3.57 ^b	92.21±4.21 ^c	81.12±3.21 ^d	***
Insulin (mIU/ml)	10.23±3.16 ^d	13.26±1.01 ^a	12.23±0.55 ^b	11.52±0.21 ^c	9.32±0.21 ^e	***
Glucose (mg/dl)	53.36±3.12 ^e	96.11±5.82 ^a	86.32±2.65 ^b	81.23±3.21 ^c	74.35±5.32 ^d	***

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Control: Control standard without PCOS; PCOS: PCOS control without menthol; PCOS-2, 4 & 6: PCOS rats treated with 2, 4 and 6 mg/kg menthol; Superscripts (a-e) show significant differences at $P<0.05$ in per row.

**Figure 2.** Effects of different levels of menthol (2, 4 & 6 mg/kg) on antioxidant status in rats with PCOSGMJ
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Control: Control standard without PCOS; PCOS: PCOS control without menthol; PCOS-2, 4 & 6: PCOS rats treated with 2, 4 and 6 mg/kg menthol; Superscripts (a-e) show significant differences at $P<0.05$.

significantly increased body weight, so control PCOS rats showed higher body weight in comparison to control group ($P<0.05$). Oral gavage of menthol could significantly decrease body weight. There was positive correlation between body weight and level of PCOS ($P<0.05$), so higher levels of menthol could significantly decrease body weight ($P<0.05$).

Blood biochemical parameters

Effects of different levels of menthol on blood biochemical parameters are presented in Table 1. Induction of PCOS could significantly increase LDL-C, cholesterol, Triglycerides, insulin and glucose but also decrease HDL-C (control versus PCOS group ($P<0.05$)). However, oral administration of menthol could significantly increase HDL-C and decrease LDL-C, cholesterol, Triglycerides, insulin and glucose ($P<0.05$). The best responses were observed in highest levels of menthol (6 mg/kg).

Antioxidant status

Our findings for antioxidant status are shown in Figure 2. As can be seen in the results, the induction of PCOS increased oxidation status in terms of FRAP, AOPP and TOS ($P<0.05$) (control vs. PCOS). Oral treatment with menthol significantly improved antioxidant levels in comparison to PCOS group. As levels of menthol were increased, oxidation status was decreased respectively.

Discussion

Body weight significantly increased in PCOS rats compared to the control group. Kim et al. showed in their study that increased body weight in PCOS rats induced with dehydroepiandrosterone [14]. The difference between control group and PCOS group could be attributed to inferring obesity [15], and diets can have a major role in weight gain in animals with PCOS. It has been accepted that high-fiber diet and low-fat diets could decrease body weight [16]. In the current study, menthol comprises very small portion of the diet and thus cannot

have a role due to low fat levels. It seems that menthol decreases oxidation and subsequent dyslipidemia, and dyslipidemia could have a major role in increased body weight. Maintained body weight could be attributed to antioxidant status.

Hyperglycemia was observed in rats with PCOS. PCOS has been reported as one metabolic disorder related with type 2 diabetes mellitus [17] and it is caused by hyperglycemia in initial phases which leads to insulin resistance. Similarly, other studies have reported hyperglycemia in Letrozole induced PCOS rats [18]. Hyperinsulinemia was also observed in our study and it is known to have the ability to promote ovarian androgen overproduction [6]. Menthol decreased levels of insulin and glucose, and it seemed that menthol increases sensitivity to insulin and decreases glucose.

Dyslipidemia was also observed in rats with PCOS (control vs. PCOS). Imbalanced lipid profile has been related to hyper-androgenemia [19, 20]. Lipid peroxidation has been known as one of the markers for oxidative tissue damage. It also induces free radical damage to the components of cell membrane that cause cell necrosis and inflammation [21]. Some studies have reported oxidative stress as one of the pathological factors for PCOS [22, 23]. Elevated oxidant levels could change the stereo diagnosis in ovaries which could be attributed to raised androgen production and polycystic ovaries [22]. In the present study, menthol improves lipid profile by antioxidant system, and our findings for oxidation system supported our hypotheses. In sum, it could be stated that antioxidant activity of menthol prevents lipid peroxidation and blood parameters could be considered as index for such action.

Conclusion

In conclusion, PCOS had negative effects on almost all blood parameters as well as the antioxidant status. Oral administration of menthol improved blood parameters through antioxidant system. Future studies are needed to evaluate the effects of menthol. Therefore, we recommend the use of menthol for treatment of PCOS as a novel agent.

Ethical Considerations

Compliance with ethical guidelines

There was no ethical considerations to be considered in this research.

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Authors' contributions

All authors contributed toward data analysis, drafting and revising the paper and agreed to be responsible for all the aspects of this work.

Conflict of interest

The authors declared no conflict of interest.

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