

The Effect of Wet Cupping Therapy on Anxiety and Thiol/Disulfide Homeostasis in Premature Ovarian Failure: An Experimental Study

Prematür Overyan Yetmezlikli Ratlarda Yaş Kupa Terapisinin Anksiyete ve Tiyo/Disülfid Homeostazı Üzerine Etkileri: Deneysel Bir Çalışma

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ABSTRACT Objective: The effect of wet cupping therapy on anxiety and thiol/disulfide homeostasis was evaluated in rats with premature ovarian failure (POF) induced by cyclophosphamide (CYC). **Material and Methods:** Twenty four Wistar Albino female rats were divided into control (n=6), POF (n=6), wet cupping (n=6), POF+wet cupping (n=6) groups. CYC at a dose of 200 mg/kg was injected intraperitoneally to POF and POF+wet cupping groups. One week later, wet cupping therapy was applied to wet cupping and POF+wet cupping groups for 5 minutes. The next day, Open Field Test and Elevated Plus Maze Test were applied to all experimental groups to evaluate anxiety. After sacrifice, to assess pattern formation, endometrial thickness and ovarian reserve were measured with hematoxylin-eosin staining. Thiol/disulfide homeostasis and erythrocyte oxidized-reduced glutathione levels were measured in serum to measure oxidative stress. **Results:** Although there was no statistical significance, it was observed that anxiety decreased in POF+wet cupping compared to POF group. There was no change in thiol-disulfide values in POF+wet cupping compared to POF group (p>0.05). **Conclusion:** Wet cupping therapy did not reduce anxiety in the experimental POF model and did not shift the balance to positive in thiol/disulfide homeostasis in favor of thiols. However, the obtained graphics, numerical data and high standard deviation suggest that meaningful data could be obtained with more subjects.

Keywords: Cupping therapy; cyclophosphamide; premature ovarian failure; anxiety; sulfhydryl compounds

ÖZET Amaç: Siklofosfamid [cyclophosphamide (CYC)] ile indüklenmiş prematür overyan yetmezliği (POF) olan ratlarda yaş kupa terapisinin anksiyete ve tiyo/disülfid homeostazı üzerindeki etkileri değerlendirildi. **Gereç ve Yöntemler:** Yirmi dört adet Wistar Albino dişi rat kontrol (n=6), POF (n=6), yaş kupa (n=6), POF+yaş kupa (n=6) gruplarına ayrıldı. POF ve POF+yaş kupa gruplarına 200 mg/kg CYC intraperitoneal olarak enjekte edildi. Bir hafta sonra yaş kupa ve POF+yaş kupa gruplarına 5 dk süreyle kupa terapisi uygulandı. Ertesi gün, anksiyeteyi değerlendirmek için tüm deney gruplarına Açık Alan Testi ve Yükseltilmiş Artı Labirent Testi uygulandı. Sakrifikasyonun ardından model oluşumunu değerlendirmek için endometrial kalınlık ve over rezervi hematoksilen-eozin boyası ile ölçüldü. Oksidatif stresi değerlendirmek amacıyla toplanan serumlardan tiyo/disülfid homeostazı ve eritrosit okside-redukte glutatyon düzeyleri bakıldı. **Bulgular:** İstatistiksel olarak anlamlı olmamakla birlikte POF+yaş kupa grubunda POF grubuna göre anksiyetenin azaldığı görüldü. Tiyo-disülfid değerlerinde ise POF+yaş kupa grubunda POF grubuna göre bir farklılık bulunmadı (p>0,05). **Sonuç:** Yaş kupa terapisinin deneysel POF modelinde anksiyeteyi azaltmadığı ve tiyo/disülfid homeostazında dengeyi tiyoller yönünden pozitif balansa kaydırmadığı gözlemlenmiştir. Ancak elde edilen grafikler, sayısal veriler ve standart sapmanın yüksek oluşu daha fazla denek ile anlamlı sonuçların elde edilebileceğini düşündürmektedir.

Anahtar Kelimeler: Kupa terapisi; siklofosfamid; prematür overyan yetmezlik; anksiyete; sülfidril bileşikleri

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Peer review under responsibility of Journal of Traditional Medical Complementary Therapies.

Received: 07 Apr 2021

Received in revised form: 14 Oct 2021

Accepted: 21 Oct 2021

Available online: 01 Nov 2021

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Premature ovarian failure (POF) is the absence of menarche with the loss of normal function of the ovaries before the age of 40, cessation of folliculogenesis, and premature reduction of ovarian follicles. The disease causes hypoestrogenism, hypogonadotropic amenorrhea, female infertility, and premenstrual syndrome.¹ POF can develop based on genetic anomalies, chemotherapy-radiotherapy, autoimmune diseases, infections, and surgical oophorectomies. POF affects 1-2% of women under the age of 40, and 0.1% of women under 30. Its incidence increases with age and the incidence for 20, 30 and 40 years old is 0.01%, 0.1%, and 1%, respectively.²

The hormone profile in POF is similar to menopause. High follicle-stimulating hormone (FSH) (FSH>40 IU/L) and low 17-beta estradiol (E2) (E2<50 pmol/L) cause vasomotor symptoms (hot flashes and night sweats), endocrine and metabolic changes (osteoporosis, infertility, Type 2 diabetes, and cardiovascular diseases) and psychological symptoms (anxiety, depression, irritability, mood changes, and sleep disorders).³ Although the cause of vasomotor symptoms in POF is shown to be estrogen deficiency, it is known that anxiety and vasomotor symptoms are related to each other.^{4,5}

Hormone replacement therapy (HRT) benefits vasomotor, endocrine, metabolic, and psychological symptoms that occur in POF, and HRT is given for cardiovascular and bone health by treatment standardization.⁶ However, the fact that HRT causes breast and endometrium cancer limits its use. In this process, additional treatments are needed for vasomotor symptoms along with anxiety.⁴ For this purpose, gabapentin, selective serotonin, and norepinephrine reuptake inhibitors are frequently prescribed to treat vasomotor symptoms and anxiety. On the other hand, due to side effects of these treatments such as dry mouth, nausea, insomnia, distraction, and interaction with other drugs, patients turn to complementary and alternative medicine methods.⁷

Traditional and complementary medicine is frequently used for the treatment of anxiety and vasomotor symptoms. Acupuncture is most commonly used in this area to benefit anxiety and vasomotor

symptoms.^{8,9} Cupping therapy is one of the essential methods of integrative medicine; the places of application are the same as acupuncture, and its effects are similar.¹⁰ Although there are many hypotheses about the mechanism of action of cupping therapy, it is said to have hematological, immunological, metabolic, and psychological effects.¹¹

Experimentally, with the administration of 200 mg/kg cyclophosphamide (CYC), both POF and anxiety model are formed in animals.^{12,13} CYC is an alkylating antineoplastic agent used to treat various cancers and autoimmune diseases, either alone or in combination. CYC is closely related to female infertility and teratogenicity, as it increases oxidative stress by causing the production of free radicals and destroying the primordial follicles.¹⁴ Since thiol-disulfide homeostasis is a good marker of oxidative stress, and it could be used to measure the toxicity caused by CYC. Chemical groups (-SH) in thiols have a protective effect on oxidative stress and reduce oxidative stress by forming reversible disulfide bonds with oxygen radicals.¹⁵ In addition, measurement of erythrocytes oxidized and reduced glutathione levels is a sensitive method that is frequently used to show total oxidant and antioxidant status.¹⁶

In this study, the therapeutic effect of wet cupping on anxiety-like behaviors and oxidant processes in POF-induced CYC will be investigated. Thus, it is aimed to contribute to the literature by experimentally investigating the controversial placebo effect of wet cupping therapy.

MATERIAL AND METHODS

ETHICAL STATEMENT

Ethics committee approval was obtained from the Bolu Abant İzzet Baysal University Animal Research Ethics Committee (date 05.02.2020, decision number: 2020/05). This study was carried out in line with the Guidelines for the Care and Use of the Laboratory Animals (<http://www.nap.edu/catalog/5140.html>) and animal rights were protected.

ANIMAL CARE

Eighty-four adult Wistar albino rats weighing 200-250 g, 2-3 months old, were obtained from Bolu

Abant İzzet Baysal University Experimental Animal Application and Research Center. The rats used in the experiments were kept in 12 hours dark and 12 hours light photoperiod, placed in plastic cages (16'31'42 cm) and fed ad-libitum with feed and water. Animals were kept at a constant 22±2 °C room temperature and 60-65% relative humidity.

The animals were acclimated to the laboratory environment one week before starting the experiments. The animals were then randomly divided into control, POF, wet cupping and POF+wet cupping groups. Intraperitoneal (IP) saline injection was applied to the control and wet cupping groups for sham. CYC [Endoxan (Baxter, Germany) 500 mg intravenous infusion] injection, which was taken commercially at a dose of 200 mg/kg and was dissolved in 0.2 saline was administered intraperitoneally to POF (the control of POF+wet cupping) and POF+wet cupping groups to create a POF and anxiety model.¹⁷

Cupping was applied to the back area of the wet cupping and POF+wet cupping groups for therapeutic purposes. An incision without vacuum was made with a scalpel in the back region of the control and POF group for wet cupping sham to equate stress in the anxiety model.

With the wet cupping group, the cup application made to healthy people is modeled. Cupping therapy for detoxification is recommended periodically to healthy people.¹⁸ Wet cupping therapy was applied one week after CYC injection. "Open Field" and "Elevated Plus-Maze" tests were conducted 2 hours apart on the same day to measure anxiety behavior after 24 hours. A comparative balance test (counter-balance test) was used in anxiety tests.

WET CUPPING APPLICATION

Animals under ketamine (90 mg/kg) and xylazine (10 mg/kg) anesthesia, after shaving the dorsal area, 0.5 cm incisions were made on both shoulder areas and seventh cervical vertebra to reach the subcutaneous skin with the help of a lancet. Using a sterile cup with a diameter of 1 cm, vacuuming was performed at a negative pressure of 200 mmHg for 3-5 minutes, and approximately 0.3-0.5 cc of bleeding was performed.¹⁹ As a result of the procedure, the

back area of the animals was cleaned with saline, and the dressing was performed with a baticon for wound care.

APPLICATION OF BEHAVIOR TESTS

OPEN FIELD TEST

The open area test apparatus is a square-shaped apparatus made of plexiglass, 100x100x30 cm in size; the floor is divided into 16 equal squares. After the test period was initiated, all subjects were placed in the center of the Open Field apparatus, respectively, and their behaviors were recorded with a camera for 5 minutes. During this period, the parameters of "time spent in the center square", "frequency of entering the center square" and "distance traveled in the center square" were evaluated.

ELEVATED PLUS MAZE TEST

The test apparatus is 50 cm above the ground and has 4 arms, 2 of which are closed and the other 2 open. Closed arms are closed by a 40 cm high wall, while open arms are surrounded by 1 cm high plexiglass. Immediately after the test period was initiated, each subject was left in the square area at the intersection of the apparatus's arms. "The time spent in open arms" and "frequency of entering open arms" for five minutes were recorded and evaluated. In anxiety tests, the Open Field Test is always performed before the Elevated Plus Maze Test, and there is no interruption between tests.

EUTHANASIA

After conducting behavioral tests on all groups, subjects were euthanized under ketamine-xylazine (90/10 mg/kg) anesthesia by taking intracardiac blood, ovarian and uterine tissue samples for biochemical and histological examination.

HISTOLOGICAL EXAMINATION

For histomorphological examination, ovarian and uterine tissue samples from the subjects were fixed in 10% formaldehyde. Tissues were followed by tissue tracking and embedded in paraffin blocks with the broadest surface visible. 3 µm thick sections were taken from the prepared paraffin blocks and stained with hematoxylin-eosin dye. The sections were eval-

uated by the pathologist under the LEICA DM 2000 LED (Wetzlar, Germany) light microscope. Endometrial thickness was measured at the micron level. The primary follicle, secondary follicle, antral follicle, atretic follicle, and cystic follicle were counted to evaluate ovarian reserve.²⁰ Hemorrhage around the corpus luteum, vascular congestion in the stroma, mononuclear inflammatory cell infiltration, and follicular epithelial degeneration were evaluated semi-quantitatively. It was scored as 0: None, 1: Mild, 2: Moderate, 3: Severe.²¹ Hematoxylin-eosin stained sections were photographed at different magnifications with the INFINITY 3 ANALYZE Release 6.5 (Lumenera Inc., Ottawa, ON, Canada) imaging system.

DETERMINATION OF SERUM E2 LEVELS-ELISA

To determine serum E2 levels, the rat E2 ELISA kit is based on the standard principle of an immunosorbent test based on the sandwich enzyme. Rat E2 antibody was coated on a well plate. Standards and test samples are added to wells, and the immobilized antibody binds E2 present in a sample. A horseradish peroxidase (HRP)-conjugate reagent was then added. HRP substrate tetramethylbenzidine (TMB) was used to visualize the HRP enzymatic reaction after washing the unbound antibody/HRP conjugates. HRP catalyzes TMB to produce a blue product that turns yellow after an acid-stopping solution is added. The intensity of the yellow color is proportional to the rat E2 caught on the plate. This ELISA kit shows no cross-reactivity of any kind.

MEASUREMENT OF SERUM THIOL-DISULFIDE HOMEOSTASIS PARAMETER LEVELS

In the first step of serum thiol-disulfide homeostasis measurement, natural (native) thiol levels in the sample were determined by 5,5'-dithiobis-(2-nitrobenzoic) acid. In the second step, dynamic and reducible disulfide bonds (-S-S) were reduced to free functional (reactive) thiol groups (-SH) in the presence of sodium borohydride (NaBH₄). The unused reductant was consumed with NaBH₄ formaldehyde and cleaned. Then, the total thiol groups, both reduced with NaBH₄ and containing native thiol, were de-

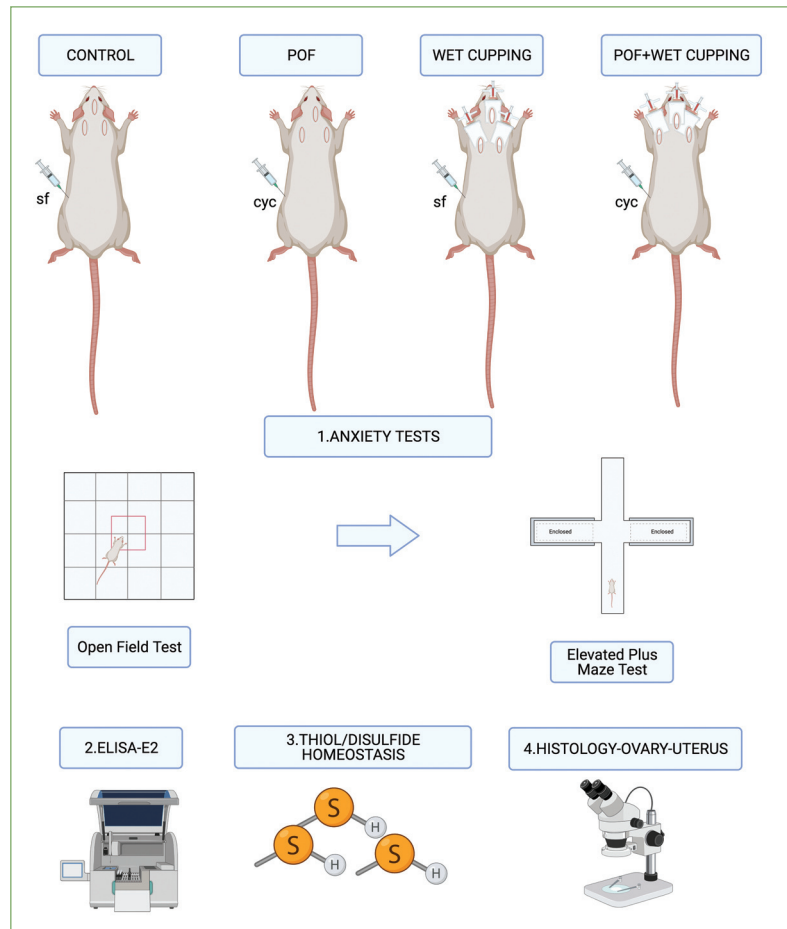
tected after the reaction with 5,5'-dithiobis-(2-nitrobenzoic) acid. After determining the native thiol (-SH) and total thiol (-SH + -S-S) concentrations and taking half of the difference between these 2 values, the dynamic disulfide amount was calculated. Disulfide(-S-S)/native thiol groups (-SH) and disulfide percentage ratios were also calculated.¹⁵

ERYTHROCYTE OXIDIZED-REDUCED GLUTATHIONE LEVELS MEASUREMENT

Whole blood samples were separated from plasma by washing process for determination of erythrocyte oxidized-reduced glutathione levels. Later, after the erythrocytes were exploded and the erythrocyte content was revealed, the erythrocyte protein content was precipitated with tricarboxylic acid. Before the supernatant was obtained, the native reduced glutathione levels in the sample were determined, and then oxidized glutathione was reduced with a reagent containing sodium hydroxide and sodium borohydride. Excess sodium hydroxide and sodium borohydride were removed with the help of hydrochloric acid. Subsequently, with a second reduced glutathione assay, total glutathione amounts were determined by spectrophotometry with a modified Ellman reagent. Half of the difference between total reduced glutathione levels and native reduced glutathione levels was calculated as oxidized glutathione.¹⁶

STATISTICAL ANALYSIS

Data were evaluated in the statistical package program IBM SPSS Statistics 25.0 (IBM Corp., Armonk, New York, USA). Descriptive statistics were given as number of units (n), percent (%), mean±standard deviation ($\bar{X}\pm SD$), median (Q1-Q3) values. The normal distribution of the data of numerical variables was evaluated with the Shapiro-Wilk test of normality and Q-Q graphs. In the data with normal distribution, evaluating the groups within themselves was made by using one-way analysis of variance. In the data that were not distributed normally, the Kruskal-Wallis H test was used to evaluate the groups within themselves, and the Mann-Whitney U test was used to determine the difference between the pairs (Appendix 1).



APPENDIX 1: Experimental design chart made by Biorender.

RESULTS

BEHAVIORAL RESULTS

OPEN FIELD TEST

Although there was no significant difference, it was observed that POF group subjects spent less time in the central square and traveled less than those in control ($p>0.05$). On the other hand, it was observed that the subjects in POF+wet cupping group spent more time in the central square than those in POF group. It was noted that the group that spent the last time in the central square was POF, and most time was wet cupping (Figure 1).

ELEVATED PLUS MAZE TEST

Although there was no significant difference, it was observed that the subjects in POF group spent the least amount of time in the open arms, the least fre-

quent in the open arms, and the least traveled in the open arms. On the other hand, subjects in wet cupping group are better than control group in these three parameters. It was also noted that the subjects in POF+wet cupping group spent more time in the open arms, entered the open arms more frequently, and traveled more compared to those in POF group (Figure 2).

HISTOLOGICAL RESULTS

Endometrial thickness was thinner in POF group compared to control ($p<0.05$). In evaluating ovarian reserve, the numbers of primordial follicles, primary follicles, secondary follicles, and antral follicles were found to be decreased, and the number of atretic follicles and cystic follicles were found to be increased in POF group compared to controls ($p<0.05$). Hemorrhage around the corpus luteum, vascular conges-

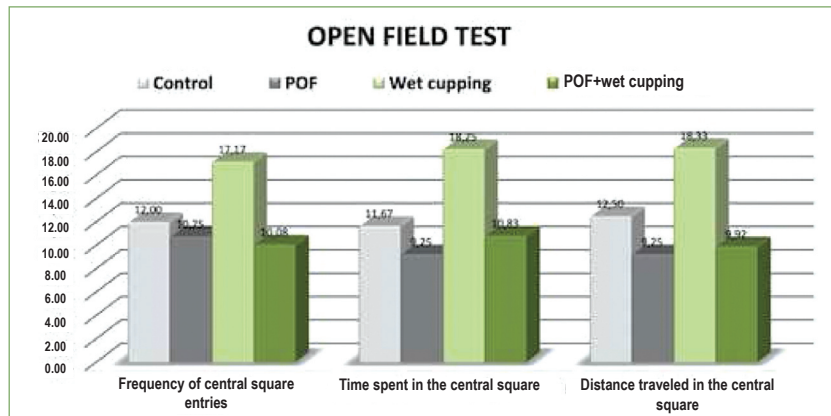


FIGURE 1: Open Field Test, frequency of central square entries, time spent in the central square, distance traveled in the central square parameters between groups. POF: Premature ovarian failure.

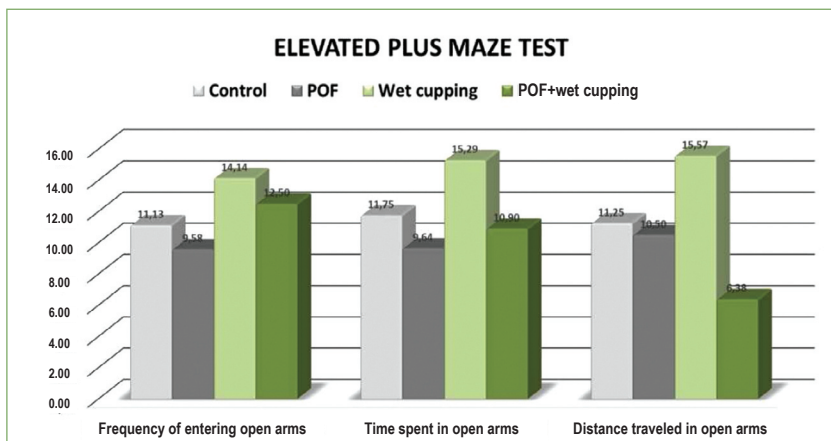


FIGURE 2: Elevated Plus Maze Test, frequency of entering open arms, time spent in open arms, distance traveled in open arms parameters between groups. POF: Premature ovarian failure.

tion in the stroma, mononuclear cell infiltration, and follicle epithelial degeneration were evaluated to be increased in POF group compared to control ($p < 0.05$). Within these results, it is observed that POF model is formed histologically with CYC injection (Figure 3).

SERUM E2 LEVEL

There was no significant difference between the groups in serum E2 values measured by ELISA (Figure 4).

THIOL/DISULFIDE HOMEOSTASIS

When the native thiol and total thiol levels were evaluated, the control group was higher than POF

($p = 0.001$) and POF+wet cupping groups ($p = 0.001$), and the wet cupping group was higher than POF ($p = 0.0001$) and POF+wet cupping groups ($p = 0.001$). There was no significant difference between POF and POF+wet cupping groups ($p > 0.05$). Wet cupping application increased native thiol and total thiol values compared to controls, but a statistically significant difference was not found ($p > 0.05$). Disulfide values of POF+wet cupping group were higher than the control ($p = 0.016$) and wet cupping group ($p = 0.002$), and also, POF group values were higher than wet cupping group ($p = 0.01$). The lowest disulfide values were seen in wet cupping group. When the ratio of disulfide/native thiol was evaluated, control group was higher than POF ($p = 0.009$) and POF+wet cupping

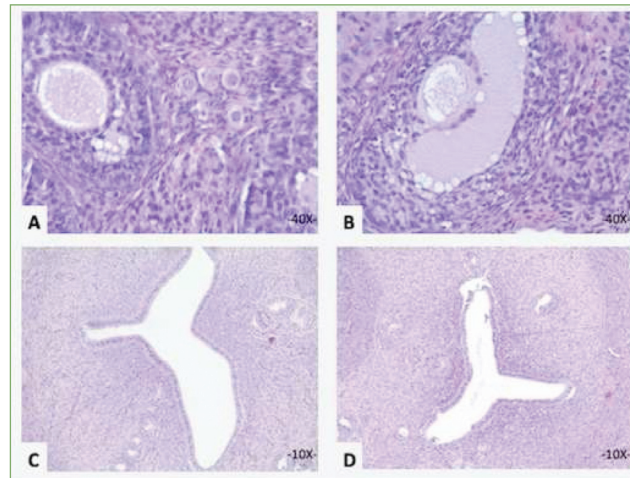


FIGURE 3: A. Control ovarian section (H&E, X40, $p < 0.05$), B. POF ovarian section (H&E, X40, $p < 0.05$); C. Control endometrial thickness (H&E, X10, $p < 0.05$) D. POF endometrial thickness (H&E, X10, $p < 0.05$).

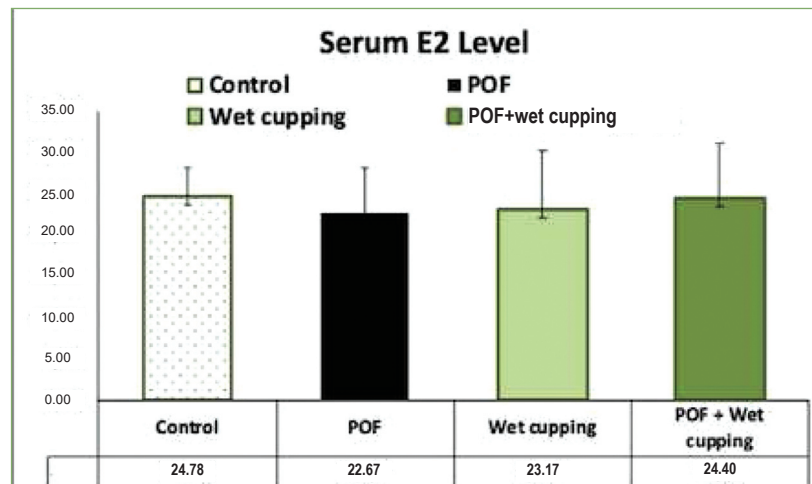


FIGURE 4: Serum E2 level. POF: Premature ovarian failure.

($p = 0.004$), and wet cupping group compared to the POF ($p = 0.002$) and POF+wet cupping groups ($p = 0.001$) was found to be below. The lowest level was seen in the wet cupping group.

ERYTHROCYTE OXIDIZED-REDUCED GLUTATHIONE LEVELS

There was no statistically significant difference between total glutathione, oxidized, and reduced glutathione values ($p > 0.05$). Serum thiol/disulfide homeostasis levels of all groups are shown in [Table 1](#).

DISCUSSION

POF, which is mainly due to premenopausal chemotherapeutics, brings along many vasomotor symptoms that reduce the patient’s quality of life and ovarian suppression in young women. Although social causes are effective, exposure to chemicals and estrogen withdrawal triggers other symptoms based on anxiety.²² At this point, patients who already suffer from chemical exposure avoid antidepressants and turn to alternative medicine methods.²³ Wet cupping, which is one of the frequently used alternative med-

TABLE 1: Serum thiol/disulfide homeostasis.

	Control	Wet cupping	POF	POF+wet cupping	p value
	Mean±Std	Mean±Std	Mean±Std	Mean±Std	
Native thiol (µmol/L)	316.66±28.2	338.9±19.3	240.7±24.0**	251.3±36.7**	0.0001
Total thiol (µmol/L)	305.83±26.3	316.3±20.9	236.7±16.4**	250.5±24.3**	0.0001
Disulphide (µmol/L)	19.03±4.3	16.21±2.8	25.53±4.1*	27.11±6.4**	0.001
SS/SH (%)	6.12±1.7	4.78±0.8	10.83±2.8**	11.27±4.1**	0.0001
Total glutathione (%)	1364.2±111.6	1060.41±123.2	1142.57±189.39	1211.08±335.3	>0.05
Reduced glutathione (%)	1246.28±128.0	936.61±131.6	1039.07±164.5	1083.78±335.0	>0.05
Oxidized glutathione (%)	59.00±15.1	61.91±12.8	51.75±15.2	63.65±10.0	>0.05
OG/RG (%)	4.84±1.6	6.81±2.1	4.94±0.9	6.38±2.2	>0.05

*Means significantly different from the only wet cupping group; Std: standart deviation; **Means significantly different from both the control and wet cupping group; SS/SH (%): Disulfide/native thiol %; OG/RG (%): Oxidized glutathione/reduced glutathione %.

icine methods due to its easy applicability and cheapness, has acupuncture-like effects such as increasing the level of endorphin and enkephalin at the level of the cerebral cortex, analgesia, increase in the level of neurotransmitters in the relevant region, the release of opiate-like substances and activating c-Fos protein in the central nervous system.²⁴ Considering the existence of a placebo effect, mainly due to culture and belief factors, limits scientific clinical studies on wet cupping, and experimental studies are needed at this stage.²⁵ Our study is the first to experimentally evaluate the effects of wet cupping therapy on anxiety-like behaviors caused by POF.

Complementary medicine is frequently used in treating depression and anxiety, and it is known that acupuncture is good for anxiety.²⁶ Although the effects of and wet cupping therapy applied to POF-induced CYC and anxiety model were not statistically significant, it was observed that wet cupping therapy reduced anxiety numerically. In the Open Field Test, anxiety-like behaviors were observed less in POF+wet cupping group compared to POF. Since the standard deviation is found to be high in behavioral experiments, it is thought that statistical significance could be shown if the number of subjects is increased. At this stage, it should be discussed again whether the anxiolytic effect of wet cupping is a placebo or not. In design of the study, the effects of CYC, which causes anxiety by increasing oxidative stress, and the hypothesis that wet cupping therapy improves anxiety-like behaviors by reducing oxidative stress measured by evaluating thiol/disulfide homeostasis and

erythrocyte oxidized-reduced glutathione levels. However, it was observed that wet cupping did not increase total and native thiols in POF. A clinical study reported that wet cupping reduced oxidative stress, but the reducing effect of wet cupping in disulfide levels in POF was not detected in this study.²⁷ In this case, we could explain how cupping therapy reduces anxiety in our study numerically: Cupping and acupuncture reduce anxiety by increasing opiate-like substances and endorphin.^{24,28,29} In addition, some studies have shown that the hyperemia and hemostasis caused by cupping on acupuncture points have a therapeutic effect, but it is not known through which mechanisms this improves anxiety.³⁰ The limited number of experimental studies on wet cupping limits the discussion.

Wet cupping therapy is also widely used among healthy individuals for detoxification.¹⁹ The fact that antioxidants are higher and oxidants are lower in wet cupping group compared to the controls explains the lower level of anxiety-like behaviour in wet cupping group compared to controls in behavioral tests. Our study supports periodic wet cupping recommendation to healthy individuals.

Cyclophosphamide ideally models ovarian damage and subsequent anxiety in POF with its alkylating chemotherapeutic effect.^{31,32} The mechanisms of CYC to induce anxiety are explained by E2 withdrawal due to ovarian injury and increased oxidative stress.^{12,33} In our study, the dose of CYC determined to create a POF model caused damage

to the ovaries, created anxiety-like behaviors, and decreased serum E2 levels, but this decrease was not significant.^{12,17} This shows that E2 withdrawal time is not sufficient. Since there are contradictory values for the ideal E2 withdrawal time in POF model created with chemical and surgical applications in the literature, POF model could be created in by waiting longer.^{34,35}

CONCLUSION

In the study, the numerical reduction of anxiety in rats by the experimental wet cupping therapy without any chemicals suggested that wet cupping might be a supportive method to antidepressants used in vasomotor symptoms induced-anxiety. More experimental and clinical studies are needed to reveal the mechanisms of action of wet cupping.

Acknowledgment

The authors thank Esra FİDAN for her support in wet cupping method.

Source of Finance

During this study, no financial or spiritual support was received neither from any pharmaceutical company that has a direct connection with the research subject, nor from a company that pro-

vides or produces medical instruments and materials which may negatively affect the evaluation process of this study.

Conflict of Interest

No conflicts of interest between the authors and / or family members of the scientific and medical committee members or members of the potential conflicts of interest, counseling, expertise, working conditions, share holding and similar situations in any firm.

Authorship Contributions

Idea/Concept: Hümeyra Çelik, Ayhan Çetinkaya, Özgür Mehmet Yis, Selma Erdoğan Düzcü, Bihter Gökçe Bozat, Murat Alışık; **Design:** Hümeyra Çelik, Ayhan Çetinkaya, Özgür Mehmet Yis, Selma Erdoğan Düzcü, Bihter Gökçe Bozat, Murat Alışık; **Control/Supervision:** Hümeyra Çelik, Bihter Gökçe Bozat, Murat Alışık, Selma Erdoğan Düzcü, Özgür Mehmet Yis, Ayhan Çetinkaya; **Data Collection and/or Processing:** Hümeyra Çelik, Bihter Gökçe Bozat, Ayhan Çetinkaya, Özgür Mehmet Yis, Selma Erdoğan Düzcü, Murat Alışık; **Analysis and/or Interpretation:** Selma Erdoğan Düzcü, Murat Alışık, Özgür Mehmet Yis; **Literature Review:** Hümeyra Çelik, Bihter Gökçe Bozat, Selma Erdoğan Düzcü; **Writing the Article:** Hümeyra Çelik, Bihter Gökçe Bozat, Selma Erdoğan Düzcü, Murat Alışık, Ayhan Çetinkaya, Özgür Mehmet Yis; **Critical Review:** Ayhan Çetinkaya, Hümeyra Çelik, Özgür Mehmet Yis; **References and Fundings:** Hümeyra Çelik; **Materials:** Hümeyra Çelik, Bihter Gökçe Bozat, Ayhan Çetinkaya, Özgür Mehmet Yis, Selma Erdoğan Düzcü, Murat Alışık.

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