

Original Article

Ozone Therapy Modulates Histopathological and Biochemical Markers in Mice with Induced Polycystic Ovary Syndrome

Reza Dadfar, Maryam Ghorbani, Morteza Izadi, Hadi Esmaeili Gouvarchin Ghaleh, Mojtaba Sepandi, Hosein Bahadoran, Javad Raouf Sarshoori

Baqiyatallah University of Medical Sciences, Tehran, Iran

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Abstract: Background — Polycystic ovary syndrome (PCOS) is an endocrine disorder characterized by polycystic ovaries and associated oxidative stress. This study assessed the potential beneficial effects of ozone therapy on ovarian tissue in mice with induced PCOS.

Methods — Forty-two female mice were distributed between six groups: a control group, a PCOS group, a PCOS group treated with ozone (45, 60, and 70 $\mu\text{g}/\text{mL}$), and a PCOS group treated with clomiphene citrate (1.5 mg/kg). PCOS was induced by injection of estradiol valerate (EV), and treatment was administered for four weeks. Data were analyzed using SPSS software, with a significance level of $p < 0.05$.

Results — Histological analysis showed that PCOS significantly disrupted the ovarian structure, decreasing the number of primary (7.80 ± 0.45 vs. 13.00 ± 0.71 ; $p < 0.05$) and preantral follicles (3.00 ± 1.58 vs. 9.80 ± 0.84 ; $p < 0.05$), while increasing the number of antral follicles (12.20 ± 0.45 vs. 6.20 ± 0.45 ; $p < 0.05$) and follicular cysts (7.60 ± 0.55 vs. 0 ; $p < 0.05$). Ozone at a dose of 45 $\mu\text{g}/\text{mL}$ significantly restored the number of follicles and improved the corpus luteum count, as well as the thickness of the follicular membrane and granulosa cell layer ($p < 0.05$) compared with other doses. Biochemically, PCOS increased the levels of MDA (25.82 ± 4.61 vs. 3.04 ± 0.45 nM/ml; $p < 0.05$) and LH (16.72 ± 1.54 vs. 8.52 ± 1.87 mIU/ml; $p < 0.05$), and decreased the level of FSH (1.80 ± 0.01 vs. 6.86 ± 0.32 mIU/ml; $p < 0.05$). Ozone at a concentration of 45 $\mu\text{g}/\text{mL}$ significantly normalized these levels ($p < 0.05$), while all doses of ozone and clomiphene citrate reduced the elevated level of DHEA. Gene expression analysis revealed PCOS-induced increase in IGF (~22-fold), Cox2 (~28-fold), and NF- κB (~3.5-fold), as well as a ~0.2-fold decrease in Nrf2 ($p < 0.05$). Ozone at a dose of 45 $\mu\text{g}/\text{mL}$ effectively reversed these pathological changes ($p < 0.05$) compared with other doses (60 and 70 $\mu\text{g}/\text{mL}$) in a PCOS mouse model.

Conclusion — Ozone therapy at a dose of 45 $\mu\text{g}/\text{mL}$ (compared with other doses in this study) reduced histopathological damage, biochemical imbalances, and inflammatory gene expression in PCOS.

Keywords: polycystic ovary syndrome, PCOS, follicular reservoir, folliculogenesis, ozone therapy, oxidative stress, mice.

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Correspondence to Javad Raouf Sarshoori. Phone: +989153151962. E-mail: raoufsar@gmail.com.

Introduction

Polycystic ovary syndrome (PCOS) is a complex endocrine disorder characterized by a combination of clinical and biochemical features affecting a noteworthy proportion of women of reproductive age [1]. This condition is often defined by the presence of polycystic ovaries, hyperandrogenism, and menstrual irregularities, with a prevalence estimated at approximately 6–12% [2]. The pathophysiology of PCOS involves a multifactorial interaction of genetic, hormonal, and environmental factors, leading to insulin resistance and hypothalamic-pituitary-ovarian axis dysregulation [3]. Although current treatment strategies primarily focus on symptom relief and restoration of reproductive function, ongoing research aims to unravel the complex mechanisms to develop more effective therapeutic interventions [4]. Ozone therapy is the medical application of ozone for the treatment of various medical conditions. It is believed that it exerts its therapeutic effects via oxidation processes, immune system modulation [5], analgesia [6], antimicrobial action [7], and

beneficial effects on the cardiovascular system [8]. However, despite clinical data supporting the effectiveness of ozone therapy, the lack of standardized protocols and a comprehensive understanding of the mechanism of action requires further research [9]. Cellular oxidation is a fundamental biochemical process involving the transfer of electrons between molecules, leading to energy conversion in the cell. This process is mainly driven by oxidative phosphorylation in the mitochondria [10]. Although cellular oxidation is necessary for energy production, it also generates reactive oxygen species (ROS) as byproducts, the excess of which can lead to oxidative stress and cellular damage, thereby contributing to the development of various pathologies including cancer, neurodegenerative diseases, and cardiovascular disorders [11]. The balance between cellular oxidation and antioxidant defense is crucial for maintaining cellular homeostasis and preventing oxidative damage. Ozone therapy has significant therapeutic properties due to the induction of oxidative stress and activation of the nuclear factor erythroid 2-related factor 2 (Nrf2) pathway [12]. This activation triggers the transcription of

antioxidant response elements, resulting in increased activity of endogenous antioxidant enzymes such as superoxide dismutase, glutathione peroxidase, catalase, heme oxygenase-1, and phase II detoxification enzymes [13]. As a result, the antioxidant capacity of cells is increased, which not only counteracts oxidative damage but also suppresses proinflammatory pathways such as nuclear factor kappa B (NF- κ B) and NLRP3 inflammasome activation, thereby reducing inflammation and providing cytoprotection [14]. According to some publications, it is likely that this mechanistic basis is a beneficial effect of ozone therapy [15].

Since PCOS may disrupt the ovarian redox balance, this experimental study investigated the effects of different doses of ozone on reducing PCOS symptoms in female NMRI mice.

Material and Methods

Ethical considerations and animal grouping

All experimental procedures were carried out in accordance with the Declaration of Helsinki [16] and under the supervision of the University Ethics Committee (ethical approval number: IR.BMSU.AEC.1402.019), including optimal physiological conditions (24 \pm 3 °C, relative humidity of 50%, 12-hour-light/12-hour-dark cycle, and free access to food/water). Forty-two adult female NMRI mice (25 \pm 5 g, 8 weeks old) were obtained from the university animal facility and distributed between 6 groups (n=7 animals per group), including control (no treatment), PCOS (disease induction without ozone therapy), PCOS+Ozone-45 μ g/ml (disease induction followed by ozone therapy at a dose of 45 μ g/ml), PCOS+Ozone-60 μ g/ml (disease induction followed by ozone therapy at a dose of 60 μ g/ml), PCOS+Ozone-70 μ g/ml (disease induction followed by ozone therapy at a dose of 70 μ g/ml) and PCOS+Clomiphene Citrate (disease induction followed by oral administration of clomiphene citrate at a dose of 1.5 mg/kg for 16 consecutive days) [17]. Ozone therapy and clomiphene citrate were administered intraperitoneally and orally, respectively.

PCOS induction and ozone therapy

The estrus phase was determined prior to PCOS induction. For this purpose, a vaginal smear was fixed in 96% alcohol and stained using the Papanicolaou protocol, followed by microscopic confirmation of keratinized epithelial cells. Subsequently, estradiol valerate (EV, Sigma Chemical Co., St. Louis, MO, USA) was administered intraperitoneally (2 mg/0.2 ml/day) for two consecutive days. Following PCOS induction, animals were observed for 60 days to confirm successful PCOS induction, including irregular estrous cycles, followed by the appearance of persistent vaginal keratinization [18]. Subsequently, ozone therapy was administered at three different doses: 45 [19] μ g/ml, 60 μ g/ml [19], and 70 μ g/ml [20] for eight treatment sessions. Initially, animals with PCOS were injected with ozone intraperitoneally for four consecutive days (once daily at 10:00 AM), followed by four additional doses administered once a week (for four consecutive weeks).

Animal necropsy

After the final ozone dose, animals were first anesthetized with a combination of ketamine and xylazine (10 IU, intramuscular injection), and then cervical dislocation was performed [21]. Thoracotomy was performed immediately, and blood samples

were collected from the heart. Blood was centrifuged (3,000 rpm, 15 min), and serum was stored (-80 °C) for analysis of hormones (LH, FSH, DHEA) and oxidative stress markers (MDA). During laparotomy, the ovaries were isolated, and the right and left ovaries were subsequently fixed in 10% formalin for histopathological examination (hematoxylin and eosin staining) and gene expression analysis (NF- κ B, Nrf2, Cox2, IGF), respectively [22].

Histopathological assessment and follicle quantification

Ovarian samples were fixed in 10% formalin for two weeks, followed by tissue processing. The samples were then embedded in paraffin and cut into thin sections (5 μ m). Finally, the sections were stained using the hematoxylin and eosin method [23]. Regarding the microscopic evaluation, all types of follicles were detected and counted as follows: *primordial follicles* (characterized by the primary oocyte surrounded by a single layer of flattened granulosa cells), *primary follicles* (cuboidal granulosa cells in one or more layers and zona pellucida begins to form around the oocyte), *secondary follicles* (characterized by multiple layers of granulosa cells and theca layer begins to develop around the follicle along with a more prominent zona pellucida layer), *preantral (tertiary) follicles* (with increased number of granulosa cell layers and prominent theca layers), *antral follicles* (characterized by a well-defined antral space with clearly identifiable internal and external thecae), and *follicular cysts* (with excessive fluid accumulation in the antral space resulting in dilation of the follicle). We also recorded corpus luteum count, follicular sheath thickness, and granulosa layer thickness [18].

Hormonal assessment (LH, FSH, and DHEA) and oxidative stress evaluation (MDA)

For the ELISA, blood serum samples and control samples were diluted and added to wells according to the kit instructions. The plates were incubated at 37 °C for 2 hours, washed several times to remove unbound substances, and then biotinylated detection antibody was added and incubated. Avidin-HRP conjugate was then added. After incubation, the wells were washed again. Substrate solution was added, and color development was monitored. After stopping the enzymatic reaction with stop solution, absorbance was measured at 450 nm. Data analysis included comparison of absorbance values with a standard curve to calculate luteinizing hormone (LH), follicle-stimulating hormone (FSH), dehydroepiandrosterone (DHEA), and malondialdehyde (MDA) concentrations. The entire protocol required strict adherence to the kit-specific instructions (Atlas Medical, USA, 96-well plate, CAT numbers 299, 948, 294, 119 for LH, FSH, DHEA, and MDA, respectively) and the inclusion of triplicate assays for accuracy and reliability, which ultimately facilitated the reliable assessment of these hormone levels in murine blood samples [24].

Quantitative PCR protocol

Total RNA was extracted from mouse ovarian tissue using Trizol reagent (Invitrogen) according to the manufacturer's protocol. The tissue was homogenized in Trizol to facilitate cell lysis and RNA release. The quality and integrity of the extracted RNA were assessed using a NanoDrop ND-200 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE); cDNA synthesis was performed using the TAKARA cDNA kit according to the authors'

instructions. Moreover, sense and antisense primers for target and reference (GAPDH: *F: 5-TGCACCACCACTGCTTAGC-3*, *R: 5-GGCATGGACTGTGGTCATGAG-3*) genes were designed using robust primer design software such as IGF (*F: 5-GAGGAGGCTCAGAGGAAGGA-3*, *R: 5-CACCAGGCTTGTGGTGTGT-3*) [25], NF-κB (*F: 5-CCTGGAAGGAGGCAAGATG-3*, *R: 5-CCAGGAAAGGACACAGGACA-3*) [26], Nrf2 (*F: 5-TGGGCTCAGTTCAAGTGCT-3*, *R: 5-CCCTTCTTCAGCCCAATTCT-3*) [27], and Cox2 (*F: 5-TGCTGCTGCTGTCTTCTCTG-3*, *R: 5-TCTTCCAGTGTAGGCTTGCT-3*) [28]. Finally, qPCR was performed using the Ampliqon Kit with the Rotor-Gene 600 system (Corbett Research, Australia). The protocol begins with an initial denaturation of the reaction mixture at 95 °C for 15 min, followed by 40 amplification cycles (including denaturation at 95 °C for 40 s, annealing at 60 °C for 40 s, and extension at 72 °C for 40 s). For data analysis, relative quantification was performed using the Livak method ($2^{-\Delta\Delta Ct}$) and presented as a fold change value.

Data analysis

Normal data distribution was confirmed using the Kolmogorov-Smirnov test. Unpaired t-tests were used for comparisons between two groups. One-way analysis of variance (ANOVA) was performed to compare multiple groups in terms of a single factor, while two-way ANOVA was employed to analyze two independent variables (e.g., treatment group and follicle type). Following ANOVA, multiple comparisons were performed using Tukey's post-hoc analysis to identify pairwise differences. Statistical analysis was performed using SPSS software (version 16). Data are presented as mean±standard deviation, and p-values less than 0.05 were considered statistically significant.

Results

Histopathological assessment and ovarian reserve

As shown in [Figure 1](#), histological examination of the control group ([Figure 1A](#)) revealed normal ovarian architecture characterized by a balanced distribution of follicles at various stages of development, including primordial, primary, preantral, and antral follicles. The granulosa and theca cell layers were intact, and the presence of a corpus luteum was evident, indicating regular ovulatory cycles. A section from the PCOS group ([Figure 1B](#)) revealed significant histopathological changes, including an increased number of follicular cysts and a marked decrease in the number of healthy antral and preantral follicles. Histological sections stained with hematoxylin and eosin demonstrated ovarian stromal hypertrophy. The granulosa cell layer in many antral follicles appeared sparse, and there was evidence of luteinization of non-dominant follicles, implying impaired folliculogenesis. In [Figure 1C](#), histological changes in the ovaries showed slight improvement compared to the PCOS group, as evidenced by a slight increase in the number of primary and preantral follicles. However, in some cases, follicular cysts remained relatively common. Changes in the granulosa and theca layers were generally minor. In [Figure 1D](#), no visible improvement in follicular development was observed in animals with PCOS. In some cases, the corpus luteum structure also appeared more clearly defined than in the PCOS group. As shown in [Figure 1E](#), the highest ozone dose resulted in minor histopathological improvements. An increase in the number of follicular cysts was evident, indicating an adverse effect of ozone therapy at a dose of 70 µg/ml on ovarian tissue repair and regeneration. Furthermore, ovarian morphology

in the PCOS+Clomiphene Citrate group demonstrated increased formation of antral follicles and corpus luteum vs. the PCOS group, similar to observations in the ozone therapy groups. Histopathological data indicate partial restoration of normal ovarian function, although some abnormalities persisted.

Based on the data shown in [Table 1](#), histopathological evaluation of ovarian tissue (n=7 in each group) revealed significant changes in follicular dynamics and ovarian morphology after PCOS induction. Compared with the control group, PCOS mice exhibited a significant reduction in the number of primordial follicles (7.80±0.447 vs. 13.00±0.707; p<0.05), preantral follicles (3.00±1.581 vs. 9.80±0.837; p<0.05) and the number of corpora lutea (2.00±0.707 vs. 5.80±0.137; p<0.05). In addition, there was a significant increase in the number of antral follicles (12.20±0.447 vs. 6.20±0.447; p<0.05), follicular cysts (7.60±0.548 vs. 0; p<0.05) and follicular sheath thickness (10.32±0.23 µm vs. 5.32±0.18 µm; p<0.05) accompanied by a noticeable thinning of the granulosa layer (22.43±2.89 µm vs. 36.43±3.12 µm; p<0.05). Ozone therapy at a concentration of 45 µg/ml showed probable beneficial effects in the form of increased numbers of primordial follicles (10.40±2.074 vs. 7.80±0.447 in PCOS; p<0.05) and preantral follicles (16.60±0.548 vs. 3.00±1.581 in PCOS; p<0.05), and reduced numbers of antral follicles (9.00±1.581 vs. 12.20±0.447; p<0.05) and follicular cysts (1.75±0.837 vs. 7.60±0.548; p<0.05). This treatment also improved ovulation, as evidenced by a significant increase in the number of corpora lutea (5.00±1.581 vs. 2.00±0.707 in PCOS; p<0.05), and normalized ovarian structure suggested by the reduced follicular membrane thickness (6.02±0.11 µm vs. 10.32±0.23 µm in PCOS; p<0.05) and increased granulosa layer thickness (46.54±3.81 µm vs. 22.43±2.89 µm in PCOS; p<0.05). Ozone at a dose of 60 µg/ml decreased the number of antral follicles (10.80±0.447; p<0.05 compared with PCOS) but caused statistically nonsignificant changes in primordial, primary and preantral follicles compared with PCOS. Contrariwise, ozone at a dose of 70 µg/ml aggravated the development of antral follicles (14.40±0.548; p<0.05 compared with PCOS) and only partially reduced the number of follicular cysts (5.80±0.447; p<0.05 compared with PCOS). Clomiphene citrate (1.5 mg/kg) significantly reduced the number of antral follicles (7.40±1.140; p<0.05 vs. PCOS) and follicular cysts (2.40±0.548; p<0.05 vs. PCOS), but caused weaker regeneration of granulosa layer (38.12±3.41 µm) vs. ozone in the concentration of 45 µg/ml (46.54±3.81 µm; p<0.05).

Serological assessment

According to [Table 1](#), biochemical analyses revealed a significant increase in MDA levels in the PCOS group (25.82±4.61 nM/ml) vs. the control group (3.04±0.45 nM/ml), indicating increased oxidative stress (p<0.05). Ozone therapy at a dose of 45 µg/ml significantly decreased MDA levels (8.80±1.98 nM/ml) compared to the PCOS group (p<0.05), while higher ozone doses (60 and 70 µg/ml) showed a nonsignificant decrease (24.45±3.88 and 22.70±2.34 nM/ml, respectively; p>0.05). Clomiphene citrate also significantly decreased the MDA level (13.70±3.71 nM/ml) compared with PCOS (p<0.05). Regarding the hormonal profile, the serum LH level was significantly increased in PCOS mice (16.72±1.54 mIU/mL) compared with the control group (8.52±1.87 mIU/mL; p<0.05). Ozone treatment at a dose of 45 µg/ml significantly decreased LH levels (9.01±0.98 mIU/ml) compared to PCOS (p<0.05), while doses of 60 and 70 µg/ml had no significant

effect on LH levels (15.19 ± 1.67 and 16.83 ± 2.09 mIU/ml, respectively; $p > 0.05$). Clomiphene citrate significantly decreased LH levels to values close to the control ones (8.32 ± 0.44 mIU/ml; $p < 0.05$). FSH levels were statistically significantly reduced in patients with PCOS (1.80 ± 0.01 mIU/ml) vs. the control group (6.86 ± 0.32 mIU/ml; $p < 0.05$). Ozone treatment at a dose of $45 \mu\text{g/ml}$ significantly increased the FSH level (5.79 ± 0.30 mIU/ml) vs. the PCOS group ($p < 0.05$), whereas higher ozone doses demonstrated no statistically significant effect (3.28 ± 0.03 and 2.11 ± 0.45 mIU/ml; $p > 0.05$). Clomiphene citrate maintained the FSH level at a level similar to the PCOS+Ozone45 group (6.09 ± 1.02 mIU/ml; $p > 0.05$). DHEA level was significantly increased in PCOS patients (12.98 ± 2.01 ng/ml) vs. the control group (0.89 ± 0.01 ng/ml; $p < 0.05$). All ozone doses and clomiphene citrate significantly reduced DHEA levels compared to the PCOS group ($p < 0.05$), with the greatest reduction observed in the $45 \mu\text{g/ml}$ ozone dose group (2.24 ± 0.30 ng/ml).

Gene expression assessment

As shown in [Figure 2](#), significant changes in the gene expression of IGF, Nrf2, Cox2, and NF- κ B were observed across the experimental groups ([Figure 2A-D](#)). In the PCOS group, IGF mRNA levels increased statistically significantly vs. the control group (approximately 22-fold, $p < 0.05$). Ozone therapy at a dose of $45 \mu\text{g/ml}$ significantly reduced IGF expression (approximately threefold, $p < 0.05$ compared to PCOS), while higher ozone doses (60 and $70 \mu\text{g/ml}$) resulted in a nonsignificant reduction (in both cases approximately 17-18-fold, $p > 0.05$ vs. PCOS). Treatment with

clomiphene citrate also resulted in a significant decrease in IGF expression (approximately sevenfold, $p < 0.05$ vs. PCOS), but the level remained elevated vs. the control. In case of Nrf2, a key regulator of antioxidant defense, PCOS induction caused a significant decrease in its expression (0.2-fold vs. the control, $p < 0.05$), while ozone at a dose of $45 \mu\text{g/ml}$ restored Nrf2 expression to near-normal levels (1.2-fold, $p < 0.05$ compared to PCOS). Ozone at a concentration of $60 \mu\text{g/ml}$ also significantly increased Nrf2 levels (0.8-fold, $p < 0.05$ compared to the PCOS group), while ozone at a concentration of $70 \mu\text{g/ml}$ and clomiphene citrate showed nonsignificant changes (in both cases approximately 0.3-0.4-fold, $p > 0.05$). Expression of Cox2, an indicator of inflammation, was markedly increased in PCOS mice (approximately 28-fold vs. the control group, $p < 0.05$), and ozone at a concentration of $45 \mu\text{g/ml}$ significantly decreased Cox2 levels (approximately 14-fold, $p < 0.05$ compared to the PCOS group). Ozone at concentrations of 60 and $70 \mu\text{g/ml}$ caused a nonsignificant decrease in Cox2 levels (approximately 22-24-fold, $p > 0.05$), while clomiphene citrate resulted in a moderate decrease (approximately 17-fold, $p < 0.05$ compared to PCOS). Similarly, NF- κ B mRNA level was significantly increased in the PCOS group (approximately 3.5-fold compared to the control group, $p < 0.05$), and ozone at a concentration of $45 \mu\text{g/ml}$ significantly lowered NF- κ B expression ($p < 0.05$ compared to PCOS). Ozone at concentrations of 60 and $70 \mu\text{g/ml}$ exhibited a nonsignificant decrease (approximately 2.5-3-fold, $p > 0.05$), while clomiphene citrate treatment resulted in a moderate but statistically significant decrease (approximately twofold, $p < 0.05$ vs. PCOS).

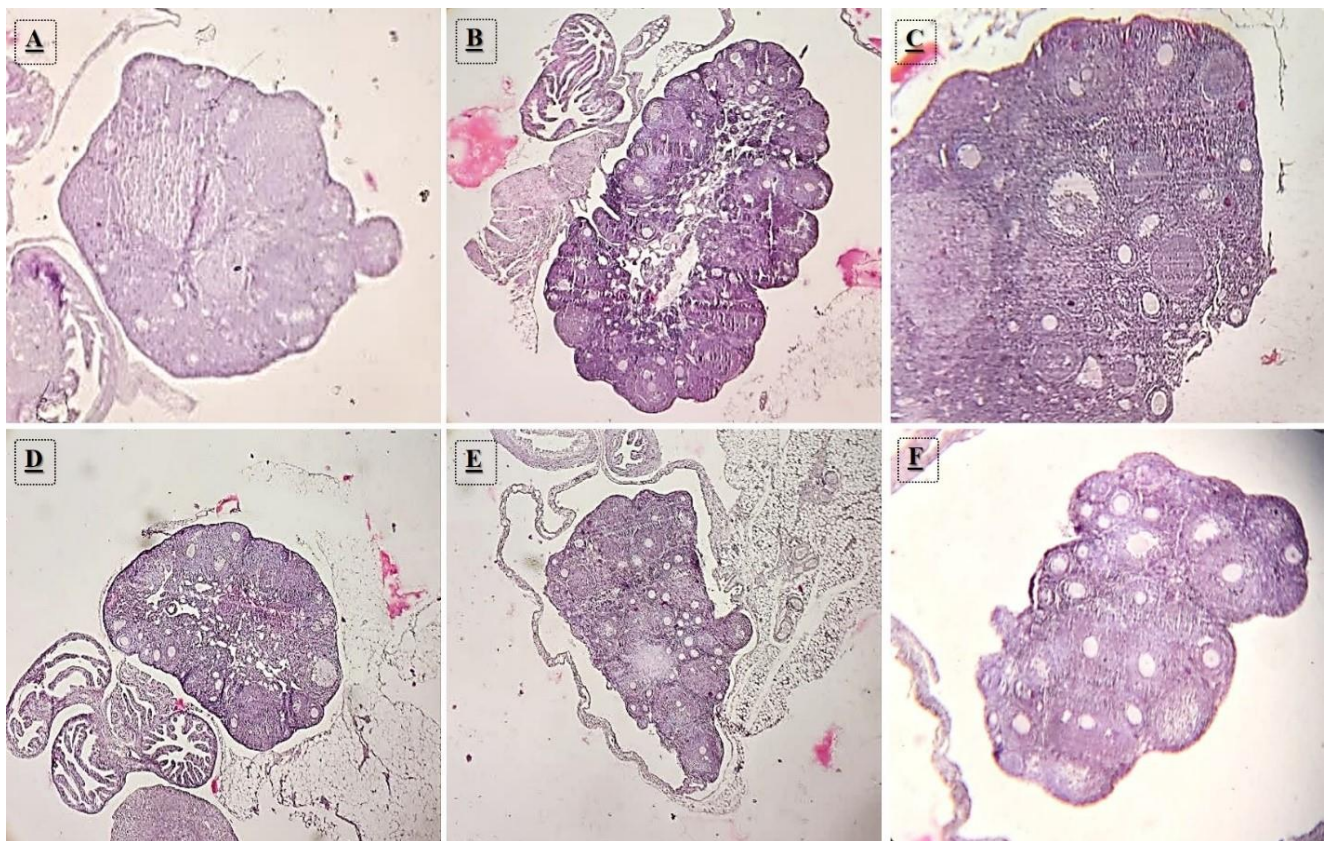


Figure 1. Histopathological (hematoxylin and eosin staining) features of the ovary in the control group (A), PCOS group (B), ozone treatment groups (C: PCOS+ozone $45 \mu\text{g/ml}$; D: PCOS + ozone $60 \mu\text{g/ml}$; E: PCOS + ozone $70 \mu\text{g/ml}$), and clomiphene citrate treatment group (F: PCOS + clomiphene citrate) ($n=7$ animals/group). Magnification $100\times$ and $400\times$.

Table 1. Data on the ovarian reserve, follicular sheath thickness, mean granulosa layer thickness, and serum levels of MDA, LH, FSH, and DHEA in control, experimental and treatment groups

Main groups	Follicle types	Control	PCOS	PCOS + Ozone45	PCOS + Ozone60	PCOS + Ozone70	PCOS + Clomiphene Citrate	
Ovarian reserve (NO)	Primordial	24.20±0.873	22.60±0.548 NS vs. Control	24.00±0.707 NS vs. PCOS	19.40±.548 NS vs. PCOS	22.40±.543 NS vs. PCOS	23.80±.447 NS vs. PCOS+Ozone45	
	Primary	13.00±0.707	7.80±0.447 ↓ vs. Control	10.40±2.074 ↑ vs. PCOS	8.00±.707 NS vs. PCOS	6.40±0.548 NS vs. PCOS	9.20±.837 NS vs. PCOS+Ozone45	
	Preantral	9.80±0.837	3.00±1.581 ↓ vs. Control	16.60±.548 ↑ vs. PCOS	4.00±0.707 NS vs. PCOS	4.00±0.707 NS vs. PCOS	8.20±1.095 ↓ vs. PCOS+Ozone45	
	Antral	6.20±0.447	12.20±0.447 ↑ vs. Control	9.00±1.581 ↓ vs. PCOS	10.80±.447 ↓ vs. PCOS	14.40±.548 ↑ vs. PCOS	7.40±1.140 ↓ vs. PCOS+Ozone45	
	Follicular cysts	0	7.60±0.548 ↑ vs. Control	1.75±.837 ↓ vs. PCOS	6.20±0.447 NS vs. PCOS	5.80±0.447 ↓ vs. PCOS	2.40±.548 NS vs. PCOS+Ozone45	
	Corpus luteum	5.80±0.137	2.00±0.707 ↓ vs. Control	5.00±1.581 ↑ vs. PCOS	3.40±1.140 ↑ vs. PCOS	3.20±0.447 ↑ vs. PCOS	4.20±1.095 NS vs. PCOS+Ozone45	
	Follicular sheath thickness (µm)	5.32±0.18	10.32±0.23 ↑ vs. Control	6.02±0.11 ↓ vs. PCOS	7.18±0.74 ↓ vs. PCOS	6.02±0.22 ↓ vs. PCOS	6.98±0.66 NS vs. PCOS+Ozone45	
	Mean granulosa layer thickness (µm)	36.43±3.12	22.43±2.89 ↓ vs. Control	46.54±3.81 ↑ vs. PCOS	20.45±2.91 NS vs. PCOS	23.5±2.03 NS vs. PCOS	38.12±3.41 ↓ vs. PCOS+Ozone45	
	Oxidative stress (nM/ml)	MDA	3.04±0.45	25.82±4.61 ↑ vs. Control	8.80±1.98 ↓ vs. PCOS	24.45±3.88 NS vs. PCOS	22.70±2.34 NS vs. PCOS	13.70±3.71 ↓ vs. PCOS+Ozone45
	Hormones	LH	8.52±1.87	16.72±1.54 ↑ vs. Control	9.01±0.98 ↓ vs. PCOS	15.19±1.67 NS vs. PCOS	16.83±2.09 NS vs. PCOS	8.32±0.44 ↓ vs. PCOS+Ozone45
FSH		6.86±0.32	1.80±0.01 ↓ vs. Control	5.79±0.30 ↑ vs. PCOS	3.28±0.03 ↑ vs. PCOS	2.11±0.45 NS vs. PCOS	6.09±1.02 NS vs. PCOS+Ozone45	
DHEA		0.89±0.01	12.98±2.01 ↓ vs. Control	2.24±0.30 ↓ vs. PCOS	8.13±0.03 ↓ vs. PCOS	9.14±1.03 ↓ vs. PCOS	3.13±0.02 ↓ vs. PCOS+Ozone45	

Data are presented as mean ± standard deviation; n=7 in each group. ↑ and ↓ refer to the statistically significant (p<0.05) increase and decrease, respectively. NS, nonsignificant (p>0.05); NO, number; PCOS, polycystic ovarian syndrome; MDA, malondialdehyde; LH, luteinizing hormone; FSH, follicle-stimulating hormone; DHEA, dehydroepiandrosterone.

Discussion

This study investigated the potential role of ozone therapy in PCOS, focusing not only on ovarian histopathology but also on biochemical and molecular markers in an established NMRI mouse model. Our results demonstrate that induction of PCOS results in important disruptions to ovarian architecture, including an increase in follicular cyst numbers and a decrease in the healthy follicle population, as well as elevated oxidative stress, hormonal imbalance, and increased inflammatory gene expression. Among various experimental ozone doses, 45 µg/ml is the most effective dose for suppressing PCOS (as compared to 60 and 70 µg/ml). It restores the distribution of healthy follicles, reduces cystic changes, normalizes serum MDA, LH, FSH, and DHEA levels, and favorably modulates the expression of key genes such as IGF, Nrf2, Cox2, and NF-κB.

Histological examination of ovarian tissue from the control group demonstrates well-preserved ovarian architecture with a balanced distribution of follicles at different developmental stages, including primordial, primary, preantral, and antral follicles, along with intact granulosa and theca cell layers, and evident corpus luteum formation, indicating normal ovulatory cycles. In contrast, the PCOS model exhibits characteristic histopathological features consistent with PCOS pathology, such as an increase in follicular cysts, a decrease in the number of healthy antral and preantral follicles, and stromal hypertrophy reflecting hyperandrogenism. The observed thinning of the granulosa cell layer and luteinization of non-dominant follicles are consistent with impaired folliculogenesis and impaired follicular maturation reported in previous experimental and clinical studies [29]. Ozone therapy at a dose of 45 µg/ml can cause a slight increase in primary and preantral follicles, although follicular cysts remain common and

the granulosa and theca layers show minimal changes. After increasing the ozone dose, we observed no increase in follicular development. However, the highest dose (70 µg/ml) compared to other doses results in a reduction in the number of healthy follicles and an increase in follicular cysts, indicating cytotoxicity or oxidative stress at elevated ozone concentrations, which was also revealed in other studies [30]. These findings are consistent with previous reports where moderate ozone exposure exerts anti-inflammatory and antioxidant effects, restoring homeostasis of the follicular microenvironment, while excessive doses cause tissue damage [31]. The lack of significant improvement in primordial follicle number and follicular sheath thickness across treatment groups is consistent with the suggestion that the effects of ozone may be limited to the follicular activation and growth phases rather than the primordial follicle reserve [32]. Clomiphene citrate treatment, used as a positive control, demonstrates an increase in antral follicle and corpus luteum formation, paralleling the effects observed with low doses of ozone. This supports the potential of ozone as an adjuvant modulator of folliculogenesis [33]. From a mechanistic perspective, ozone therapy is suggested to modulate oxidative stress and inflammatory pathways in the ovarian microenvironment, which play a key role in the pathogenesis of PCOS [34]. By inducing mild oxidative preconditioning, ozone can enhance antioxidant defense systems and reduce levels of proinflammatory mediators, thereby improving granulosa cell function and follicle development [35]. This is supported by the observed histological improvements and is consistent with gene expression data regarding a reduction in inflammatory markers after ozone treatment.

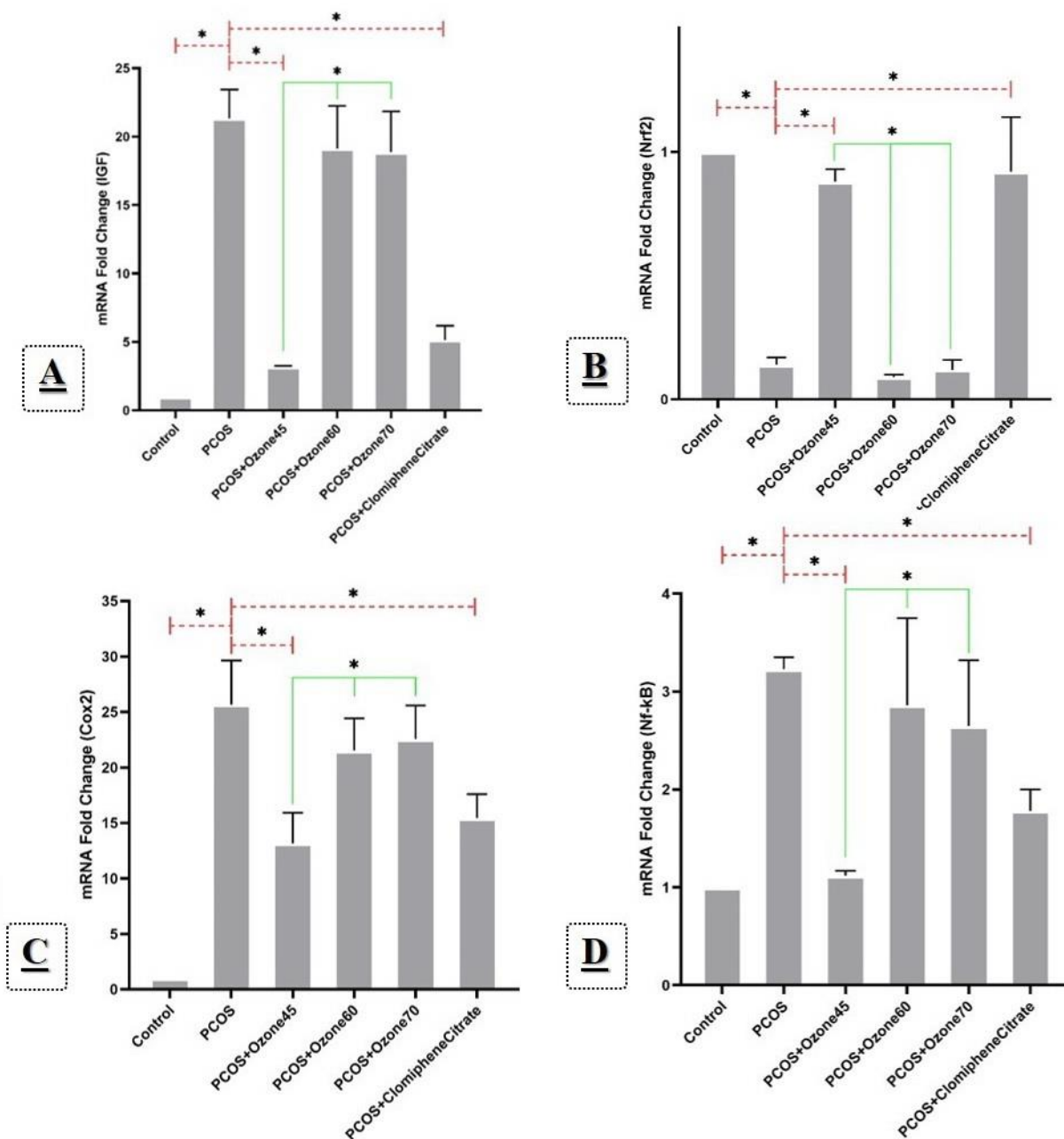


Figure 2. Fold change values of gene expression, including IGF (A), Nrf2 (B), NF-κB (C), and Cox2 (D), in different groups: control, experimental, and treatment (n=7 animals/group). Data are presented as mean ± standard deviation. * denotes significant changes (p<0.05).

In our study, consistent with the existing literature, serum DHEA and LH levels are significantly elevated in the PCOS group vs. the control group, while FSH levels are significantly reduced. Elevated levels of androgens such as DHEA are characteristic features of PCOS, reflecting hyperandrogenism caused by impaired steroidogenesis in ovarian theca cells [36]. Similarly, elevated LH levels (compared to FSH) contribute to the disruption of normal folliculogenesis by stimulating androgen production and impairing granulosa cell function [37]. These hormonal imbalances support previous data demonstrating an endocrine profile characteristic of PCOS in both clinical and experimental models [38]. Despite the promising effects of ozone therapy on ovarian histology and inflammatory gene expression, our results indicate that ozone administration at a dose of 45 µg/mL can likely normalize the

hormonal disbalances of LH, FSH, and DHEA. This is consistent with previous studies reporting limited effects of antioxidant or anti-inflammatory interventions on systemic gonadotropin and androgen levels in PCOS models [39]. The mechanism underlying the failure of ozone to modulate circulating hormones may be related to its local action, primarily in ovarian tissue, affecting oxidative stress and inflammation, rather than directly altering the regulation of the hypothalamic-pituitary-ovarian axis [40]. In contrast, clomiphene citrate, a selective estrogen receptor modulator, shows a weak and statistically nonsignificant improvement in hormonal parameters, consistent with the known mechanism of ovulation induction through hypothalamic feedback modulation [41]. These data suggest that although ozone therapy

may exert local beneficial effects on the ovaries, systemic endocrine correction may require further investigation.

PCOS is characterized by a disrupted ovarian microenvironment marked by increased inflammatory signaling and oxidative stress, which contribute to impaired folliculogenesis and ovarian dysfunction [39]. Consistent with these reports, our results demonstrate a significant increase in the expression of proinflammatory genes, including IGF, NF- κ B, and Cox2, after PCOS induction vs. the control group. Concomitantly, the expression of Nrf2, a critical transcription factor regulating antioxidant responses, is markedly suppressed. These changes are consistent with previous studies that have documented increased NF- κ B and Cox2 expression in PCOS ovaries, while decreased Nrf2 activity exacerbates oxidative damage [42]. Importantly, ozone therapy administered at a dose of 45 μ g/ml attenuates the expression of IGF, NF- κ B, and Cox2 genes compared to untreated PCOS animals. This reduction is more pronounced than that observed with clomiphene citrate treatment, suggesting a possible modulating capacity of ozone on inflammatory gene networks. These results support previous experimental data that ozone exerts immunomodulatory effects by inhibiting NF- κ B signaling, thereby reducing the production of proinflammatory cytokines [43]. Mechanistically, ozone induces mild oxidative preconditioning that activates Nrf2 pathways enhancing endogenous antioxidant defenses and mitigating tissue damage caused by oxidative stress [44].

Limitations and suggestions for future research

Despite the valuable insights obtained from the ozone therapy study, several limitations were identified. The current study focused on histopathological, hormonal, and gene expression parameters, without assessing long-term reproductive outcomes such as fertility, oocyte quality, or offspring health, which are crucial for evaluating clinical efficacy. Although key inflammatory and antioxidant genes were modulated, the precise cellular mechanisms remain unclear. Hence, future studies using proteomic and metabolomic analysis could elucidate the underlying pathways and markers of oxidative stress. Given the multifactorial nature of PCOS, combining ozone therapy with established pharmacological or behavioral interventions merits exploration to maximize therapeutic benefit. Although these results highlight the potential role of ozone therapy in restoring the balance of inflammatory and oxidative gene expression in PCOS, further protein-level studies (e.g., using western blotting technique) and functional assays are needed to definitively prove a causal relationship. It should be noted that the ozone doses used in this study were selected based on previously validated concentrations used to treat other inflammatory diseases in women. Furthermore, these selected doses were derived from studies of other inflammatory pathologies in women; consequently, doses of 45, 60, and 70 μ g/mL were also proposed for the treatment of PCOS.

Conclusion

In this study, the 45 μ g/ml dose proved to be the most effective ozone concentration among other doses (60 and 70 μ g/ml), likely exerting a modulating effect on ovarian reserve dynamics in the experimental PCOS model. Despite the persistent hormonal imbalance characteristic of PCOS, ozone therapy can improve ovarian tissue integrity and reduce inflammation, thereby

supporting its potential as an adjunctive treatment for PCOS. Further experimental and clinical studies are recommended to accurately determine the role of ozone therapy and its therapeutic effects.

Abbreviations

PCOS, polycystic ovary syndrome; ROS, reactive oxygen species; Nrf2, erythroid 2-related factor 2; NF- κ B, nuclear factor kappa B; LH, luteinizing hormone; FSH, follicle-stimulating hormone; DHEA, dehydroepiandrosterone; Cox2, Cyclooxygenase-2; IGF, insulin-like growth factor; MDA, malondialdehyde; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Author contributions

The basic study design was proposed by HB and JRSSH. RD was responsible for animal handling and data collection. All statistical analyses were performed by RD and MGH. Treatment was performed under the supervision of MI and MS. HEGGH was responsible for laboratory analyses. The initial manuscript was prepared by RD and approved by all authors.

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Data availability

The datasets used and analyzed for this study are available from the corresponding author upon reasonable request.

Conflict of interest

The authors declare that they have no conflicts of interest.

Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed by the authors of this study (ethical approval No: IR.BMSU.AEC.1402.019).

References

- Izadi M, Javanbakht M, Sarafzadeh A, Einollahi B, Futuhi F, Vahedi Z, et al. Efficacy of ozone therapy on visual evoked potentials in diabetic patients. *Diabetol Metab Syndr* 2023; 15(1): 140. <https://doi.org/10.1186/s13098-023-01114-w>.
- Chiaffarino F, Cipriani S, Dalmartello M, Ricci E, Esposito G, Fedele F, et al. Prevalence of polycystic ovary syndrome in European countries and USA: A systematic review and meta-analysis. *Eur J Obstet Gynecol Reprod Biol* 2022; 279: 159-170. <https://doi.org/10.1016/j.ejogrb.2022.10.020>.
- Di Lorenzo M, Cacciapuoti N, Lonardo MS, Nasti G, Gautiero C, Belfiore A, et al. Pathophysiology and nutritional approaches in polycystic ovary syndrome (PCOS): A comprehensive review. *Curr Nutr Rep* 2023; 12(3): 527-544. <https://doi.org/10.1007/s13668-023-00479-8>.
- Izadi M, Bagheri M, Far AB, Sureada A, Soodmand M. Effect of ozonated water and chlorhexidine mouthwash on oral health in critically ill patients on mechanical ventilation: A double-blind randomised clinical

- trial. *Intensive Crit Care Nurs* 2021; 66: 103083. <https://doi.org/10.1016/j.iccn.2021.103083>.
5. Cenci A, Macchia I, La Sorsa V, Sbarigia C, Di Donna V, Pietraforte D. Mechanisms of action of ozone therapy in emerging viral diseases: Immunomodulatory effects and therapeutic advantages with reference to SARS-CoV-2. *Front Microbiol* 2022; 13: 871645. <https://doi.org/10.3389/fmicb.2022.871645>.
 6. Hidalgo-Tallón FJ, Torres-Morera LM, Baeza-Noci J, Carrillo-Izquierdo MD, Pinto-Bonilla R. Updated review on ozone therapy in pain medicine. *Front Physiol* 2022; 13: 840623. <https://doi.org/10.3389/fphys.2022.840623>.
 7. Esmaili Gouvarchinghaleh H, Farzanehpour M, Izadi M, Aminian F. Exploring the antiviral properties of ozone therapy: A narrative review. *Ozone: Science & Engineering* 2025; 47(3): 278-292. <https://doi.org/10.1080/01919512.2025.2474453>.
 8. Ullah R, Khan M, Shah SA, Saeed K, Kim MO. Natural antioxidant anthocyanins – A hidden therapeutic candidate in metabolic disorders with major focus in neurodegeneration. *Nutrients* 2019; 11(6): 1195. <https://doi.org/10.3390/nu11061195>.
 9. Re L, Mawsouf MN, Menéndez S, León OS, Sánchez GM, Hernández F. Ozone therapy: Clinical and basic evidence of its therapeutic potential. *Arch Med Res* 2008; 39(1): 17-26. <https://doi.org/10.1016/j.arcmed.2007.07.005>.
 10. Arfin S, Jha NK, Jha SK, Kesari KK, Ruokolainen J, Roychoudhury S, et al. Oxidative stress in cancer cell metabolism. *Antioxidants (Basel)* 2021; 10(5): 642. <https://doi.org/10.3390/antiox10050642>.
 11. Juan CA, Pérez de la Lastra JM, Plou FJ, Pérez-Lebeña E. The chemistry of reactive oxygen species (ROS) revisited: outlining their role in biological macromolecules (DNA, lipids and proteins) and induced pathologies. *Int J Mol Sci* 2021; 22(9): 4642. <https://doi.org/10.3390/ijms22094642>.
 12. Franzini M, Valdenassi L, Pandolfi S, Tirelli U, Ricevuti G, Chirumbolo S. The role of ozone as an Nrf2-keap1-Are activator in the anti-microbial activity and immunity modulation of infected wounds. *Antioxidants* 2023; 12(11): 1985. <https://doi.org/10.3390/antiox12111985>.
 13. Masan J, Sramka M, Rabarova D. The possibilities of using the effects of ozone therapy in neurology. *Neuro Endocrinol Lett* 2021; 42(1): 13-21. <https://pubmed.ncbi.nlm.nih.gov/33932964>.
 14. Pires JR, Karam AM, Garcia VG, Ribeiro FS, Pontes AEF, Andrade CR, et al. Effect of systemic ozone therapy as a biomodulator of tissue regeneration and inflammatory response in rats. *Rev Odontol UNESP* 2021; 50: e20210046. <https://doi.org/10.1590/1807-2577.04621>.
 15. Rouhi M, Mohebi N, Yazdi N. Neurological adverse effects associated with ozone therapy. *Clin Neuroradiol* 2025; 35(3): 595-599. <https://doi.org/10.1007/s00062-025-01510-x>.
 16. Petkov CI, Flecknell P, Murphy K, Basso MA, Mitchell AS, Hartig R, et al. Unified ethical principles and an animal research 'Helsinki' declaration as foundations for international collaboration. *Curr Res Neurobiol* 2022; 3: 100060. <https://doi.org/10.1016/j.crneur.2022.100060>.
 17. Pahlevani P, Mosavi S, Rastgoo Haghi A, Lahotian H, Esna Ashari F, Alizadeh Z. Study of the effects of Stachys lavandulifolia alcoholic extract on histomorphometry of endometrium in polycystic ovarian syndrome rat model. *Avicenna J Clin Med* 2016; 23(1): 40-48. <http://sih.umsha.ac.ir/article-1-855-en.html>.
 18. Asghari R, Shokri-Asl V, Rezaei H, Tavallaie M, Khafaei M, Abdolmaleki A, et al. Alteration of TGFβ1, GDF9, and BMP2 gene expression in preantral follicles of an estradiol valerate-induced polycystic ovary mouse model can lead to anovulation, polycystic morphology, obesity, and absence of hyperandrogenism. *Clin Exp Reprod Med* 2021; 48(3): 245-254. <https://doi.org/10.5653/cerm.2020.04112>.
 19. Wei A, Feng H, Jia XM, Tang H, Liao YY, Li BR. Ozone therapy ameliorates inflammation and endometrial injury in rats with pelvic inflammatory disease. *Biomed Pharmacother* 2018; 107: 1418-1425. <https://doi.org/10.1016/j.biopha.2018.07.137>.
 20. Bocci V. Does ozone therapy normalize the cellular redox balance? Implications for the therapy of human immunodeficiency virus infection and several other diseases. *Med Hypotheses* 1996; 46(2): 150-154. [https://doi.org/10.1016/S0306-9877\(96\)90016-X](https://doi.org/10.1016/S0306-9877(96)90016-X).
 21. Salahshoor MR, Abdolmaleki A, Shabanizadeh A, Jalali A, Roshankhah S. *Ipomoea aquatica* extract reduces hepatotoxicity by antioxidative properties following dichlorvos administration in rats. *Chin J Physiol* 2020; 63(2): 77-84. https://doi.org/10.4103/CJP.CJP_89_19.
 22. Abdolmaleki A, Jalili C, Mansouri K, Bakhtiari M. New rat to mouse xenograft transplantation of endometrium as a model of human endometriosis. *Animal Model Exp Med* 2021; 4(3): 268-277. <https://doi.org/10.1002/ame2.12181>.
 23. Jalili C, Roshankhah S, Jalali A, Salahshoor MR. Hepatoprotective activity of royal jelly on mercuric chloride-induced damage model in rats. *J Rep Pharm Sci* 2019; 8(2): e147377. https://doi.org/10.4103/jrptps.JRPTPS_27_19.
 24. Roshankhah S, Abdolmaleki A, Salahshoor MR. Anti-inflammatory, anti-apoptotic, and antioxidant actions of Middle Eastern Phoenix dactylifera extract on mercury-induced hepatotoxicity in vivo. *Mol Biol Rep* 2020; 47(8): 6053-6065. <https://doi.org/10.1007/s11033-020-05680-4>.
 25. Gusmao DO, de Sousa ME, Tavares MR, Donato Jr J. Increased GH secretion and body growth in mice carrying ablation of IGF-1 receptor in GH-releasing hormone cells. *Endocrinology* 2022; 163(11): bqac151. <https://doi.org/10.1210/endoqr/bqac151>.
 26. Mojtabavi S, Saed A, Aboulfazli S, Kheirandish A, Najafi M, Jafari-Sabet M, et al. Evaluation of curcumin effect on IL6, Sirt1, TNFα and NFκB expression of liver tissues in diabetic mice with STZ. *J Diabetes Metab Disord* 2023; 22(1): 205-215. <https://doi.org/10.1007/s40200-022-01090-4>.
 27. Sun HJ, Ding S, Guan DX, Ma LQ. Nrf2/Keap1 pathway in countering arsenic-induced oxidative stress in mice after chronic exposure at environmentally-relevant concentrations. *Chemosphere* 2022; 303(Pt 3): 135256. <https://doi.org/10.1016/j.chemosphere.2022.135256>.
 28. Grace VB, Wilson DD, Anushya R. Regulation of inflammation and COX-2 gene expression in benzo (a) pyrene induced lung carcinogenesis in mice by all trans retinoic acid (ATRA). *Life Sci* 2021; 285: 119967. <https://doi.org/10.1016/j.lfs.2021.119967>.
 29. Papalou O, M. Victor V, Diamanti-Kandarakis E. Oxidative stress in polycystic ovary syndrome. *Curr Pharm Des* 2016; 22(18): 2709-2722. <https://doi.org/10.2174/1381612822666160216151852>.
 30. Bocci VA, Zanardi I, Travagli V. Ozone acting on human blood yields a hormetic dose-response relationship. *J Translat Med* 2011; 9: 66. <https://doi.org/10.1186/1479-5876-9-66>.
 31. Malatesta M, Tabaracci G, Pellicciari C. Low-dose ozone as a eustress inducer: Experimental evidence of the molecular mechanisms accounting for its therapeutic action. *Int J Mol Sci* 2024; 25(23): 12657. <https://doi.org/10.3390/ijms252312657>.
 32. Franks S, Stark J, Hardy K. Follicle dynamics and anovulation in polycystic ovary syndrome. *Hum Reprod Update* 2008; 14(4): 367-378. <https://doi.org/10.1093/humupd/dmn015>.
 33. Balen AH, Morley LC, Misso M, Franks S, Legro RS, Wijayarathne CN, et al. The management of anovulatory infertility in women with polycystic ovary syndrome: An analysis of the evidence to support the development of global WHO guidance. *Hum Reprod Update* 2016; 22(6): 687-708. <https://doi.org/10.1093/humupd/dmw025>.
 34. de Sire A, Agostini F, Lippi L, Mangone M, Marchese S, Cisari C, et al. Oxygen-ozone therapy in the rehabilitation field: State of the art on mechanisms of action, safety and effectiveness in patients with musculoskeletal disorders. *Biomolecules* 2021; 11(3): 356. <https://doi.org/10.3390/biom11030356>.
 35. Dias AR, Bitsaksis C, Emdin D, Bosman L, Smith AH, Merhi Z. Ozone sauna therapy and pulsed electromagnetic field therapy could potentially improve outcome in women with diminished ovarian

- reserve undergoing assisted reproductive technology. *Med Gas Res* 2023; 13(4): 202-207. <https://doi.org/10.4103/2045-9912.350862>.
36. Azziz R, Carmina E, Chen Z, Dunaif A, Laven JS, Legro RS, et al. Polycystic ovary syndrome. *Nat Rev Dis Primers* 2016; 2(1): 16057. <https://doi.org/10.1038/nrdp.2016.57>.
 37. Goodarzi MO, Dumesic DA, Chazenbalk G, Azziz R. Polycystic ovary syndrome: Etiology, pathogenesis and diagnosis. *Nat Rev Endocrinol* 2011; 7(4): 219-231. <https://doi.org/10.1038/nrendo.2010.217>.
 38. Teede H, Deeks A, Moran L. Polycystic ovary syndrome: a complex condition with psychological, reproductive and metabolic manifestations that impacts on health across the lifespan. *BMC Med* 2010; 8: 41. <https://doi.org/10.1186/1741-7015-8-41>.
 39. González F. Inflammation in polycystic ovary syndrome: underpinning of insulin resistance and ovarian dysfunction. *Steroids* 2012; 77(4): 300-305. <https://doi.org/10.1016/j.steroids.2011.12.003>.
 40. Bocci V, Borrelli E, Travagli V, Zanardi I. The ozone paradox: Ozone is a strong oxidant as well as a medical drug. *Med Res Rev* 2009; 29(4): 646-682. <https://doi.org/10.1002/med.20150>.
 41. Legro RS, Arslanian SA, Ehrmann DA, Hoeger KM, Murad MH, Pasquali R, et al. Diagnosis and treatment of polycystic ovary syndrome: An Endocrine Society clinical practice guideline. *J Clin Endocrinol Metab* 2013; 98(12): 4565-4592. <https://doi.org/10.1210/jc.2013-2350>.
 42. Zhao Y, Zhang C, Huang Y, Yu Y, Li R, Li M, et al. Up-regulated expression of WNT5a increases inflammation and oxidative stress via PI3K/AKT/NF-κB signaling in the granulosa cells of PCOS patients. *J Clin Endocrinol Metab* 2015; 100(1): 201-211. <https://doi.org/10.1210/jc.2014-2419>.
 43. Re L, Martínez-Sánchez G, Bordicchia M, Malcangi G, Pocognoli A, Morales-Segura MA, et al. Is ozone pre-conditioning effect linked to Nrf2/EpRE activation pathway in vivo? A preliminary result. *Eur J Pharmacol* 2014; 742: 158-162. <https://doi.org/10.1016/j.ejphar.2014.08.029>.
 44. Martínez-Sánchez G, Al-Dalain SM, Menéndez S, Re L, Giuliani A, Candelario-Jalil E, et al. Therapeutic efficacy of ozone in patients with diabetic foot. *Eur J Pharmacol* 2005; 523(1-3): 151-161. <https://doi.org/10.1016/j.ejphar.2005.08.020>.

Authors:

Reza Dadfar – Department of Anatomical Sciences, Faculty of Medicine, Baqiyatallah University of Medical Sciences, Tehran, Iran. <https://orcid.org/0000-0002-0088-9837>.

Maryam Ghorbani – Department of Pharmacology and Toxicology, Faculty of Pharmacy, Baqiyatallah University of Medical Sciences, Tehran, Iran. <https://orcid.org/0000-0001-7958-2880>.

Morteza Izadi – Health Research Center, Lifestyle Institute, Baqiyatallah University of Medical Sciences, Tehran, Iran. <https://orcid.org/0000-0002-2046-6321>.

Hadi Esmaeili Gouvarchin Ghaleh – Applied Virology Research Center, Biomedicine Technologies Institute, Baqiyatallah University of Medical Sciences, Tehran, Iran. <https://orcid.org/0000-0001-8562-2295>.

Mojtaba Sepandi – Health Research Center, Lifestyle Institute, Baqiyatallah University of Medical Sciences, Tehran, Iran. <https://orcid.org/0000-0001-6441-5887>.

Hosein Bahadoran – PhD, Department of Anatomical Sciences, Faculty of Medicine, Baqiyatallah University of Medical Sciences, Tehran, Iran. <https://orcid.org/0009-0004-6478-0453>.

Javad Raouf Sarshoori – PhD, Department of Anatomical Sciences, Faculty of Medicine, Baqiyatallah University of Medical Sciences, Tehran, Iran. <https://orcid.org/0000-0001-8155-1573>.