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Research Article

Pterostilbene attenuates VCD-induced ovarian damage through modulation of oxidative stress, inflammation, apoptosis, and SIRT1/FOXO3a gene expression

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Abstract

Background: Primary ovarian insufficiency (POI) is characterized by depletion of ovarian follicles and impaired ovarian function. Oxidative stress, inflammation, and dysregulation of intracellular signaling pathways play important roles in POI progression. Pterostilbene (PTS) is a natural stilbene compound with potent antioxidant and anti-inflammatory properties, demonstrating protective effects in several experimental models. The present study investigated the effects of PTS on oxidative stress and inflammation, as well as sirtuin 1 (SIRT1) and forkhead box O3a (FOXO3a) gene expression in a rat model of 4-vinylcyclohexene diepoxide (VCD)-induced POI.

Methods: Female Sprague-Dawley rats were randomly divided into five experimental groups (six animals per group). Intraperitoneal (i.p.) administration of VCD (160 mg/kg) was used to induce POI. The rats were then treated with 17β-estradiol, PTS (50 mg/kg) orally by gavage, or their combination according to the study design, while the control group received the appropriate vehicle only. The serum levels of total antioxidant capacity (T-AOC) and nuclear factor-kappa B (NF-κB) were determined using an enzyme-linked immunosorbent assay (ELISA). Ovarian expression of SIRT1 and FOXO3a was assessed by reverse transcription quantitative polymerase chain reaction (RT-qPCR). Immunohistochemical (IHC) staining for cleaved caspase-3 (CASP3) and 8-hydroxy-2′-deoxyguanosine (8-OHdG) was used to evaluate apoptosis and oxidative deoxyribonucleic acid (DNA) damage in ovarian tissue, respectively. The combination effect was assessed using the Bliss equation.

Results: Treatment with PTS significantly reduced NF-κB ($P = 0.0496$) and upregulated SIRT1 and FOXO3a gene expression ($P = 0.0001$), with maximum fold changes of 11.04 and 8.83, respectively. It also significantly reduced CASP3 and 8-OHdG ($P = 0.0001$), whereas the increase in T-AOC did not reach statistical significance ($P = 0.3260$). Overall, compared with 17β-estradiol, treatment with PTS produced greater effects on SIRT1 and FOXO3a gene expression. The combination treatment showed synergistic or near-additive effects for several evaluated parameters, whereas antagonistic interactions were observed for SIRT1 and FOXO3a gene expression. Combination index values for all measured parameters were as follows: T-AOC, 0.942; NF-κB, 0.690; SIRT1, 11.688; FOXO3a, 11.942; CASP3 percentage score (P-score), 1.0; CASP3 immunoreactive score (IRS), 0.997; 8-OHdG P-score, 0.943; and 8-OHdG IRS, 0.962.

Conclusion: Treatment with PTS attenuated VCD-induced ovarian injury through antioxidant and anti-inflammatory mechanisms, with modulation of SIRT1/FOXO3a gene expression and reduced NF-κB-mediated inflammation and CASP3-associated apoptosis.

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PENSOFT

Keywords

8-OHdG, CASP3, FOXO3a, NF-κB, oxidative stress, primary ovarian insufficiency (POI), pterostilbene, SIRT1, VCD

Introduction

Primary ovarian insufficiency (POI) is a condition characterized by a decline in ovarian function in women prior to the age of 40, commonly exhibited through menstrual irregularities (amenorrhea or oligomenorrhea), increased follicle-stimulating hormone (FSH) levels >25 IU/L, and variable reductions in estrogen levels. A recent meta-analysis estimated the global prevalence of POI at 3.5%, reaching up to 5.3% in developing nations (Hong et al. 2025). A variety of social, psychological, and environmental factors contribute to the increasing prevalence of POI (Li et al. 2025a, 2025b). A woman's primordial follicles decrease throughout reproductive life until menopause, and POI greatly hastens this process. The most common mechanism underlying POI is accelerated follicular atresia, which may result from genetic disorders, autoimmune diseases, and gonadotoxic drugs, leading to depletion of the ovarian reserve (Hassan et al. 2025).

Among the mechanisms contributing to follicular loss in POI, oxidative stress plays a particularly important role. Oxidative stress results from excessive production of reactive oxygen species (ROS) within the organism, thereby disrupting the balance between oxidative and antioxidative mechanisms and ultimately leading to cell damage. Antioxidants function as reactive radical scavengers, counteracting ROS generation and promoting the repair of oxidative damage to cellular morphology (Manokaran et al. 2022; Sadeghpour et al. 2025). A reduction in enzymatic antioxidants due to hazardous chemicals accelerates granulosa cell (GC) apoptosis, mitochondrial dysfunction, deoxyribonucleic acid (DNA) damage, and follicle depletion. These events have been observed in POI induced by several hazardous chemicals (Pouladvand et al. 2024).

Beyond follicular depletion, there is accumulating evidence that inflammation is an important factor in the pathogenesis of POI. Chronic ovarian inflammation may result in GC dysfunction, follicular atresia, and progressive deterioration of ovarian function (Huang et al. 2025).

In addition to oxidative stress and inflammation, apoptosis represents another key mechanism involved in the pathogenesis of POI. Follicular survival is controlled by the equilibrium between pro-apoptotic factors such as B-cell lymphoma-2 (Bcl-2)-associated X protein (BAX) and anti-apoptotic proteins such as Bcl-2. Disruption of this balance favors GC apoptosis, follicular atresia, and activation of caspase-3 (CASP3), leading to ovarian insufficiency (Shi et al. 2023; Chesnokov et al. 2024; Ju et al. 2025).

At the molecular level, sirtuin 1 (SIRT1) and forkhead box O3a (FOXO3a) are major regulators of cellular homeostasis, oxidative stress response, and follicular survival. As a highly conserved nicotinamide adenine dinucleotide (NAD+)-dependent deacetylase, SIRT1 has a multifaceted role in inflammation, oxidative stress, aging, cell proliferation, apoptosis, and other biological processes by deacetylating a number of proteins, including tumor protein 53 (p53), nuclear factor-kappa B (NF-κB), and FOXO1. Dysregulation of the SIRT1/FOXO3a axis has been implicated in ovarian aging, follicular loss, and the development of POI (Shi et al. 2023; Wu et al. 2025).

To experimentally reproduce these pathological features of POI, 4-vinylcyclohexene diepoxide (VCD) has been widely used as an ovotoxic agent. It is a toxic metabolite of 4-vinylcyclohexene (VCH) that diminishes the primordial follicle (PrF) reserve in the ovaries, which leads to POI (Zhao et al. 2025). In both *in vivo* and *in vitro* investigations, it is capable of inducing ovarian toxicity and oocyte dysfunction (Li et al. 2024a; Niu et al. 2025).

Ovarian toxicity is induced by VCD primarily through targeting primordial and primary follicles, resulting in permanent follicular loss at the earliest stages of folliculogenesis. Depletion of these follicles disturbs ovarian homeostasis and causes enhanced generation of ROS, leading to oxidative stress, mitochondrial dysfunction, and oxidative DNA damage. These changes activate NF-κB-mediated inflammatory signaling and inhibit cytoprotective pathways such as the SIRT1 and FOXO3a regulatory axis, resulting in GC apoptosis via CASP-dependent pathways. The progressive decline in GC viability favors follicular atresia and ovarian reserve depletion, leading to POI (Che et al. 2025; Huang et al. 2025). It serves as an appropriate animal model for POI because of its exclusive focus on primordial and primary follicles, mirroring the progressive decline observed in idiopathic or age-associated POI (Li et al. 2024b). However, the VCD model does not fully recapitulate the heterogeneous etiologies of human POI, despite closely mimicking several pathological features of the disease (Liu et al. 2015).

Given the involvement of oxidative stress, inflammation, and apoptosis in VCD-induced ovarian injury, compounds capable of targeting these processes may offer potential protective effects. Pterostilbene (PTS) is a natural dimethylated analog of resveratrol (RSV) present in grapes, blueberries, and several medicinal plants. Owing to its relatively high bioavailability, PTS has attracted considerable interest for its antioxidant, anti-inflammatory, and cytoprotective properties. Previous studies have shown that PTS can attenuate oxidative stress, suppress inflammatory responses and apoptosis, and regulate genes involved in cellular survival and tissue protection, suggesting potential benefits for ovarian function (Liu et al. 2024; Wang et al. 2024; Okamoto et al. 2025).

Despite the therapeutic benefits of hormone replacement therapy (HRT), concerns regarding its long-term adverse effects have encouraged the search for safer therapeutic alternatives (Benetti-Pinto et al. 2020; Pratiwi 2023). In this context, the protective effects of PTS demonstrated in different experimental models have highlighted its potential as a promising alternative (Wang et al. 2024; Okamoto et al. 2025). However, its role in modulating oxidative stress, inflammatory responses, apoptosis, and SIRT1/FOXO3a gene expression in VCD-induced POI remains inadequately investigated (Wu et al. 2025). Therefore, the present investigation was designed to evaluate the protective effects of PTS in a rat model of VCD-induced POI. The hypothesis was that PTS would attenuate VCD-induced ovarian injury through upregulation of the SIRT1/FOXO3a pathway, thereby reducing oxidative stress, inflammation, and apoptosis.

Materials and methods

Chemicals and reagents

VCD (Macklin Biochemical Co., Shanghai, China; Cat. No. V820469); PTS (Shanghai Bide Pharmatech Co., Ltd., Shanghai, China; Cat. No. BD149449); 17β-estradiol (HEOWNS, Tianjin, China; Cat. No. E-57600); sesame oil (Shenyang Tianfeng Biological Pharmaceutical Co., Ltd., Shenyang, China); formaldehyde solution (37%; Avonchem Ltd., United Kingdom; Cat. No. A2617-R); ketamine (10%, 25 mL; Dutch Farm International B.V., Netherlands); xylazine (2%, 20 mg/mL, 30 mL; Alfasan International B.V., Netherlands; Cat. No. 1205117-05); normal saline (0.9%, 500 mL; Pioneer Pharmaceutical Industries, Sulaymaniyah, Iraq); ethanol (70%; Al-Jabal, Iraq); enzyme-linked immunosorbent assay (ELISA) kits for total antioxidant capacity (T-AOC) and NF-κB (SunLong Biotech Co., Ltd., China; Cat. Nos. SL0776Ra and SL0537Ra, respectively); custom primers for SIRT1, FOXO3a, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (LIGO, China); anti-CASP3 monoclonal antibody (FineTest, Wuhan, China; Cat. No. FNab09938); and anti-8-hydroxy-2′-deoxyguanosine (8-OHdG) polyclonal antibody (FineTest, Wuhan, China; Cat. No. FNab11005).

Animal care and ethical approval

The study involved 30 female Sprague-Dawley rats, aged 4–6 weeks and weighing 100–130 g. The rats were sourced from the Animal House of the Iraqi Center for Cancer Research and Medical Genetics, Mustansiriyah University, Baghdad, Iraq. The animals were housed in well-ventilated cages with free access to water and standard chow pellets under optimal environmental conditions (25 °C ± 2 °C, regular light/dark cycles, and 50% humidity). Before the experiment, the animals were provided with a 10-day acclimatization period. The experimental protocol was approved by the Scientific and Ethical Committee, College of Pharmacy, Mustansiriyah University, Baghdad, Iraq (Approval No. 68; approved on 21 February 2026), and all procedures were conducted in accordance with the ethical guidelines for the care and use of laboratory animals.

Experimental design

A preliminary pilot study was performed to optimize the doses of VCD and PTS for the main experiment. Based on the pilot findings in conjunction with previously published studies, VCD (160 mg/kg, intraperitoneal [i.p.] injection) and PTS (50 mg/kg, oral gavage) were selected for the main experimental protocol (Appt et al. 2006; Surien et al. 2021; Waheed and Mshimesh 2025). Detailed findings of the pilot study were provided in Suppl. material 1.

The main experimental protocol was performed after the pilot study. Thirty female Sprague-Dawley rats were randomly assigned to five experimental groups (n = 6 in each group), as follows:

Group I (control group): Rats received sesame oil i.p. for 10 consecutive days and subsequently received oral sesame oil by gavage for 30 days.

Group II (VCD group): Rats received VCD (160 mg/kg), dissolved in sesame oil, and administered via i.p. injection for 10 consecutive days, and subsequently received oral sesame oil by gavage for 30 days.

Group III (17β-estradiol group): Rats received VCD (160 mg/kg), dissolved in sesame oil, and administered via i.p. injection for 10 consecutive days. Subsequently, the rats received 17β-estradiol (0.028 mg/kg) dissolved in sesame oil by oral gavage for 30 days.

Group IV (PTS group): Rats received VCD (160 mg/kg), dissolved in sesame oil, and administered via i.p. injection for 10 consecutive days. Subsequently, PTS (50 mg/kg), dissolved in sesame oil, was administered orally via gavage for 30 days.

Group V (combination group): Rats received VCD (160 mg/kg), dissolved in sesame oil, and administered via i.p. injection for 10 consecutive days. Thereafter, the animals received the combination of 17β-estradiol and PTS by oral gavage for 30 days.

The combined treatment effect was assessed using the Bliss independence equation. The expected Bliss effect (E expected) was calculated using the measured experimental parameters as follows:

$$\mathrm{EAB} = \mathrm{EA} + \mathrm{EB} - \mathrm{EA} \times \mathrm{EB}$$

EAB: The expected combined effect of drugs A and B (e.g., 17β-estradiol + PTS), referred to as E expected

EA: The effect of drug A (e.g., 17β-estradiol)

EB: The effect of drug B (e.g., PTS)

The expected effect calculated using the Bliss equation was compared with the observed combined effect measured experimentally using the combination index (CI):

$$\mathrm{CI} = \frac{\mathrm{Eexpected}}{\mathrm{Eobserved}}$$

The effects were represented as probabilities ($0 \leq E \leq 1$). A CI less than, greater than, or equal to 1 indicated synergistic, antagonistic, or additive effects, respectively (Foucquier and Guedj 2015). Detailed CI calculations used to assess the interaction between PTS and 17β-estradiol were provided in Suppl. material 2.

Sample collection and tissue preparation

At the completion of the experimental period (day 40), rats were euthanized with ketamine (50 mg/mL) and xylazine (20 mg/mL), for which 0.1 mL of ketamine was mixed with 0.2 mL of xylazine and administered to each rat (Sotoudeh and Namavar 2022). After opening the rib cage, a 5 mL syringe was used to collect blood directly from the heart. To isolate the serum, the samples were centrifuged at 1,000 rpm for 15 min in gel tubes. The serum was then stored in Eppendorf tubes at −20 °C until analysis of serum biomarker levels (Tuck et al. 2009). The ovaries were located in the inferior portion of the abdominal cavity. The abdominal cavity was opened, and both ovaries were carefully excised using sterile scissors and forceps. The ovaries were promptly fixed in 10% neutral-buffered formalin for immunohistochemical analysis, while the remaining ovarian tissue was processed for gene expression analysis. The ovary used for each analysis was selected randomly.

Measurements of serum biomarkers

Serum levels of T-AOC and NF-κB were quantified using commercial ELISA kits (SunLong Biotech Co., Ltd., China) following the manufacturer's instructions. Serum samples and standards were added to the microplate wells and incubated with the assay reagents. The optical density (OD) was read at 450 nm using a GloMax® Explorer Multimode Microplate Reader (Promega Corporation, USA), and biomarker concentrations were calculated from the standard curves.

Ribonucleic acid (RNA) isolation and complementary DNA (cDNA) synthesis

Total RNA was isolated from ovarian tissues using the TransZol Up Plus RNA Kit (TransGen Biotech Co., China) following the manufacturer's instructions. RNA concentration and purity were evaluated spectrophotometrically, and samples with acceptable purity were used for subsequent analysis. The synthesis of cDNA from total RNA was performed using the EasyScript® One-Step Genomic DNA (gDNA) Removal and cDNA Synthesis SuperMix (TransGen Biotech Co., China) following the manufacturer's protocol. The resulting cDNA samples were stored at −20 °C until RT-qPCR analysis.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

Gene expression analysis was performed using RT-qPCR to determine the relative messenger RNA (mRNA) expression levels of SIRT1 and FOXO3a, with GAPDH serving as the reference gene. The primer sequences used in this study are presented in Table 1. Relative mRNA expression was calculated using the 2^−ΔΔCt method. The following formulas were applied (Livak and Schmittgen 2001):

Delta cycle threshold (ΔCt) = Ct (target gene) – Ct (reference gene)

Comparative / delta-delta Ct (ΔΔCt) = ΔCt (sample) – ΔCt (control)

Relative gene expression = 2^−ΔΔCt

Immunohistochemistry (IHC)

The analysis was performed to assess the ovarian expression of cleaved CASP3 as an apoptotic marker and 8-OHdG as a marker of oxidative DNA damage in ovarian tissue sections.

Paraffin-embedded ovarian tissue sections were deparaffinized, rehydrated, and processed for antigen retrieval. Endogenous peroxidase activity was blocked using EnVision™ FLEX Peroxidase-Blocking Reagent for 5 min at room temperature. The sections were then incubated for 20 min at room temperature with a monoclonal anti-CASP3 antibody or a polyclonal anti-8-OHdG antibody. After washing, the sections were exposed to EnVision™ FLEX/horseradish peroxidase (HRP) reagent for 20 min, and the antigen–antibody complexes were visualized using EnVision™ FLEX 3,3′-diaminobenzidine (DAB+) substrate. Finally, the sections were counterstained with EnVision™ FLEX hematoxylin, dehydrated through graded ethanol, cleared in xylene, mounted with a permanent mounting medium, and examined under a Leica DM750 light microscope (Leica Microsystems, Germany). All immunohistochemical slides were independently evaluated by two blinded observers who were unaware of the experimental group allocation.

Immunoreactivity was evaluated using a light microscope (×100). The proportion of positively stained cells was determined as (positive cells/total cells) × 100. Staining intensity was graded as follows: 0 = no staining, 1 = weak, 2 = moderate, and 3 = strong. The percentage score (P-score)

Table 1. Primers and their supplier used for RT-qPCR in this study.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Supplier
GAPDH	AGAACATCATCCCTGCATCC	CAACGGATACATTGGGGGTA	LIGO, China
FOXO3a	GGGGAGTTGGGTCAATCAGA	TTTGCATAGACTGGCTGACG	LIGO, China
SIRT1	AGGGAACCTCTGCCCTACT	CCTTTTTGTTTCGTGGAGGT	LIGO, China

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; FOXO3a, forkhead box O3a; SIRT1, sirtuin 1.

was graded as 0 = 0%, 1 = 1–25%, 2 = 26–50%, 3 = 51–75%, and 4 = 76–100%. The immunoreactive score (IRS) was calculated as P-score × intensity score (0–12) (Lee et al. 2023).

Statistical analysis

Data were statistically analyzed using the Statistical Package for the Social Sciences (SPSS) software, IBM Statistics v. 26 (2019). The obtained data were expressed as the mean ± standard error (SE). One-way analysis of variance (ANOVA) was applied to compare differences among the experimental groups, followed by the least significant difference (LSD) test for multiple comparisons between means (IBM Corp 2019). A probability (P) value >0.05 was considered non-significant, $P \leq 0.05$ was considered statistically significant, and $P \leq 0.001$ was considered highly significant.

Results

Effect of PTS treatment on oxidative stress and inflammatory markers

Serum T-AOC level

No statistically significant differences were detected in serum T-AOC levels among the experimental groups (P = 0.326). The control group showed the highest T-AOC level (14.15 ± 4.47 U/mL). A reduction was observed in the VCD-induced group (7.06 ± 1.36 U/mL). Slight increases were observed in the 17β-estradiol-treated group (7.56 ± 2.77 U/mL), PTS-treated group (11.49 ± 4.49 U/mL), and combination-treated group (11.96 ± 5.64 U/mL). The CI value was 0.942 (Fig. 1).

Serum NF-κB level

A significant overall difference was detected in serum NF-κB levels among the experimental groups (P = 0.0496). The VCD-induced group exhibited the highest NF-κB level (1292.13 ± 279.68 pg/mL), which was significantly higher than those in the 17β-estradiol-treated group (695.86 ± 98.75 pg/mL), PTS-treated group (688.11 ± 77.45 pg/mL), and combination-treated group (734.67 ± 131.60 pg/mL). Although numerical differences were observed between the control group (850.59 ± 83.94 pg/mL) and the other experimental groups, the control group did not differ significantly from the VCD-induced, 17β-estradiol-treated, PTS-treated, or combination-treated groups. The CI value was 0.690 (Fig. 2).

Effect of PTS treatment on ovarian gene expression by RT-qPCR

Quantification of SIRT1 expression by RT-qPCR

Gene expression of SIRT1 differed significantly among the experimental groups ($P < 0.001$). The VCD-induced group showed a slight increase in SIRT1 expression compared with the control group. Treatment with 17β-estradiol further increased SIRT1 expression, whereas PTS treatment resulted in the highest SIRT1 expression. The combination-treated group showed a moderate increase in SIRT1 expression and had significantly higher expression than the control group; however, it did not differ significantly from either the VCD-induced or 17β-estradiol-treated group. The calculated CI value was 11.688 (Fig. 3; Table 2).

Quantification of FOXO3a expression by RT-qPCR

Gene expression of FOXO3a differed significantly among the experimental groups ($P < 0.001$). The VCD-induced group showed a slight increase in FOXO3a expression compared with the control group. Treatment with 17β-estradiol further increased FOXO3a expression, whereas PTS treatment resulted in the highest FOXO3a expression. The combination-treated group showed an intermediate increase in FOXO3a expression and had significantly higher expression than the control group; however, it did not differ significantly from either the VCD-induced or 17β-estradiol-treated group. The calculated CI value was 11.942 (Fig. 4; Table 3).

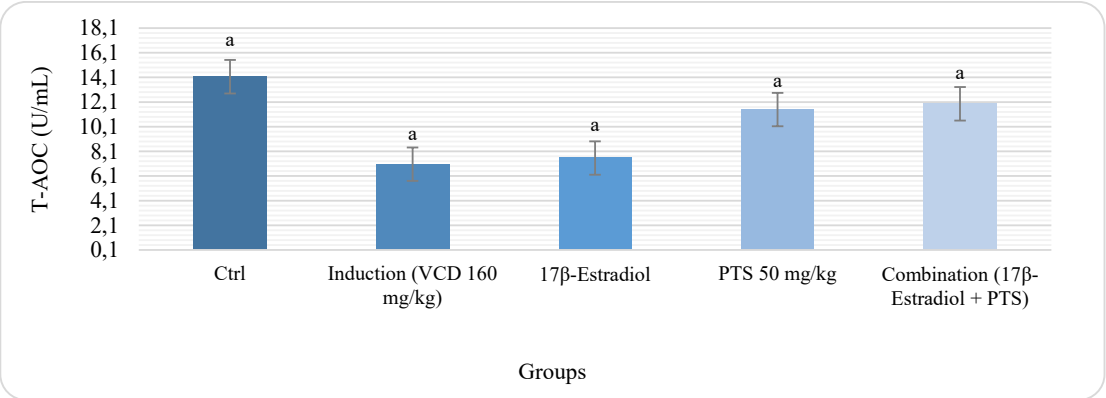

Figure 1. Effects of 17β-estradiol, PTS, and their combination on serum T-AOC levels in rats with VCD-induced POI. Results are expressed as bars representing mean ± standard error (SE). Statistical analysis was performed using one-way ANOVA followed by the LSD multiple comparisons test (n = 6). Ctrl, control; T-AOC, total antioxidant capacity; VCD, 4-vinylcyclohexene diepoxide; PTS, pterostilbene. The same lowercase letters indicate no statistically significant differences among the experimental groups ($P > 0.05$).

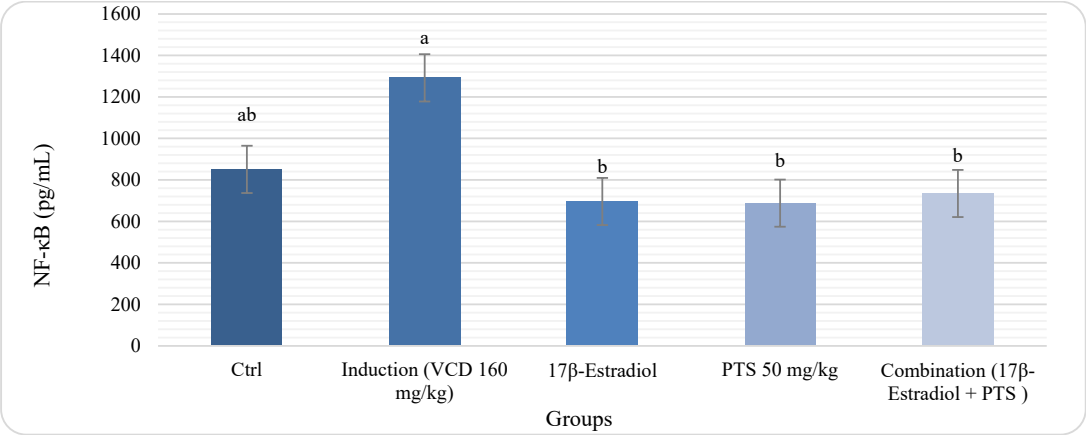

Figure 2. Effects of 17β-estradiol, PTS, and their combination on serum NF-κB levels in rats with VCD-induced POI. Results are expressed as bars representing mean ± standard error (SE). Statistical analysis was performed using one-way ANOVA followed by the LSD multiple comparisons test ($n = 6$). Ctrl, control; VCD, 4-vinylcyclohexene diepoxide; PTS, pterostilbene; NF-κB, nuclear factor kappa B. Different lowercase letters indicate statistically significant differences between groups ($P < 0.05$).

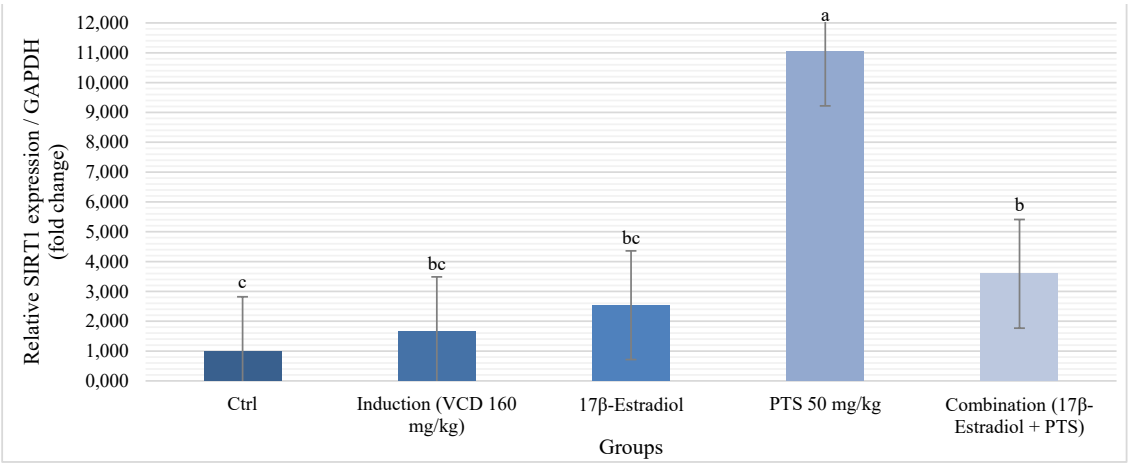

Figure 3. Quantitative analysis of ovarian SIRT1 gene expression. Results are expressed as fold change and normalized to GAPDH using the 2^−ΔΔCt method. Statistical analysis was performed using one-way ANOVA followed by the LSD multiple comparisons test ($n = 6$). Ctrl, control; VCD, 4-vinylcyclohexene diepoxide; PTS, pterostilbene; SIRT1, sirtuin 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase. Different lowercase letters indicate statistically significant differences between groups ($P \leq 0.01$).

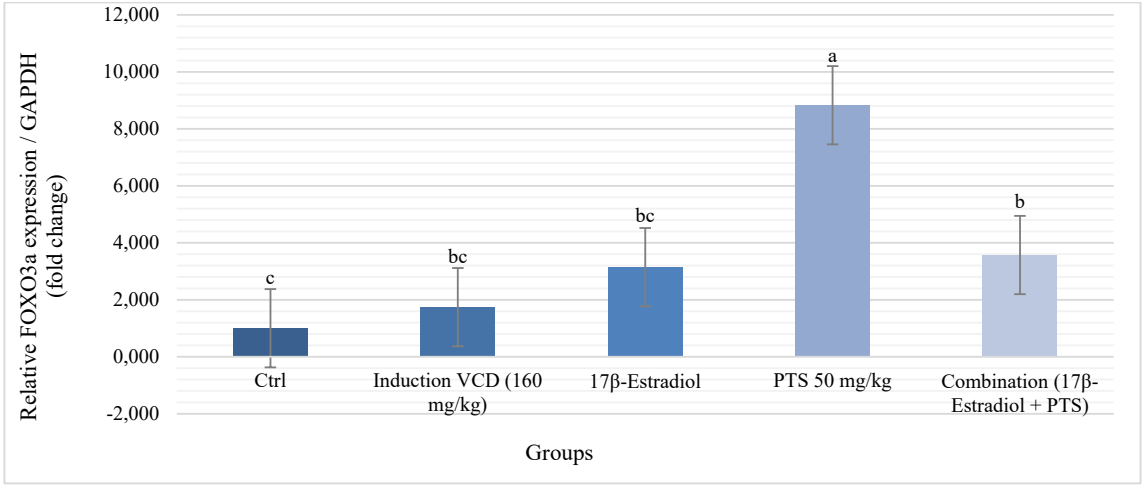

Figure 4. Quantitative analysis of ovarian FOXO3a gene expression. Results are expressed as fold change and normalized to GAPDH using the 2^−ΔΔCt method. Statistical analysis was performed using one-way ANOVA followed by the LSD multiple comparisons test ($n = 6$). Ctrl, control; VCD, 4-vinylcyclohexene diepoxide; PTS, pterostilbene; FOXO3a, forkhead box O3a; GAPDH, glyceraldehyde-3-phosphate dehydrogenase. Different lowercase letters indicate statistically significant differences between groups ($P \leq 0.01$).

Table 2. Fold change of ovarian SIRT1 gene expression among the experimental groups.

Group	Ct SIRT1	Ct GAPDH	ΔCt E	ΔCt C	ΔΔCt	Fold change
Ctrl	21.855	15.940	0.000	5.915	0.000	1.000 ± 0.00 [c]
Induction VCD (160 mg/kg)	22.148	16.143	6.005	5.915	0.090	1.667 ± 0.80 [bc]
17β-Estradiol	21.460	16.590	4.870	5.915	-1.045	2.538 ± 0.68 [bc]
PTS 50 mg/kg	23.150	20.043	3.107	5.915	-2.808	11.042 ± 4.09 [a]
Combination (17β-Estradiol + PTS)	21.621	17.388	4.233	5.915	-1.681	3.591 ± 0.81 [b]
L.S.D. (*P*-value)	–	–	–	–	–	2.407 **(0.0001)

Means with different letters in the same column differ significantly. ** ($P \leq 0.01$).
Ctrl, control; VCD, 4-vinylcyclohexene diepoxide; PTS, pterostilbene; SIRT1, sirtuin 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; *P*-value, probability value; LSD, least significant difference; Ct, cycle threshold; ΔCt, delta cycle threshold; ΔΔCt, comparative/delta-delta Ct; fold change, 2^-ΔΔCt (relative gene expression).

Table 3. Fold change of ovarian FOXO3a gene expression among the experimental groups.

Group	Ct FOXO3a	Ct GAPDH	ΔCt E	ΔCt C	ΔΔCt	Fold change
Ctrl	22.305	15.940	0.000	6.365	0.000	1.000 ± 0.00 [c]
Induction VCD (160 mg/kg)	22.378	16.143	6.235	6.365	–0.130	1.744 ± 0.79 [bc]
17β-Estradiol	21.632	16.590	5.042	6.365	–1.323	3.143 ± 0.81 [bc]
PTS 50 mg/kg	23.628	20.043	3.585	6.365	–2.780	8.829 ± 2.42 [a]
Combination (17β-Estradiol + PTS)	22.048	17.388	4.660	6.365	–1.705	3.570 ± 0.67 [b]
L.S.D. (*P*-value)	–	–	–	–	–	2.024 **(0.0001)

Means with different letters within the same column differ significantly. ** ($P \leq 0.01$).
Ctrl, control; VCD, 4-vinylcyclohexene diepoxide; PTS, pterostilbene; FOXO3a, forkhead box O3a; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; *P*-value, probability value; LSD, least significant difference; Ct, cycle threshold; ΔCt, delta cycle threshold; ΔΔCt, comparative/delta-delta Ct; fold change, 2^−ΔΔCt (relative gene expression).

Effect of PTS treatment on immunohistochemical findings of ovarian tissues

Expression of CASP3

The VCD-treated group demonstrated intense brown immunostaining for CASP3, with an increased percentage of positive cells, P-score, staining intensity, and IRS compared with the control group. Immunoreactivity and immunohistochemical scores for CASP3 were significantly higher in the VCD group than in the 17β-estradiol-treated group, while the PTS- and combination-treated groups displayed weak immunostaining and lower immunohistochemical scores, similar to those of the control group. The CI values for P-score and IRS were 1 and 0.997, respectively. The experimental groups showed statistically significant differences ($P < 0.01$) (Table 4; Figs 5, 6).

Expression of 8-OHdG

The VCD-treated group demonstrated intense brown immunostaining for 8-OHdG compared with the control group, with increased percentages of positive cells, P-score, staining intensity, and IRS. Administration of 17β-estradiol decreased 8-OHdG immunoreactivity and its corresponding immunohistochemical scores relative to the VCD group. The PTS-treated and combination-treated groups showed weak immunostaining and lower immunohistochemical scores, approaching control values. The CI values for P-score and IRS were 0.943 and 0.962, respectively. Overall, statistically significant differences were found among the experimental groups ($P < 0.01$) (Table 5; Figs 7, 8).

Discussion

The present study demonstrated that PTS exerted protective effects against VCD-induced ovarian injury. Treatment with PTS reduced serum NF-κB levels, markedly increased ovarian SIRT1 and FOXO3a gene expression, and decreased CASP3 and 8-OHdG immunoreactivity, whereas the increase in serum T-AOC did not reach statistical significance. Compared with 17β-estradiol, PTS produced greater increases in SIRT1 and FOXO3a gene expression. Bliss interaction analysis indicated additive and synergistic or near-additive effects for several evaluated parameters, whereas antagonistic interactions were observed for SIRT1 and FOXO3a gene expression. Overall, these findings support the potential antioxidant, anti-inflammatory, anti-apoptotic, and cytoprotective effects of PTS against VCD-induced ovarian injury.

Regarding antioxidant status, serum T-AOC was evaluated as an indicator of systemic antioxidant capacity. The VCD-induced group exhibited the lowest serum T-AOC level among all experimental groups, suggesting impaired systemic antioxidant defenses following ovarian injury. Thus, the lower T-AOC value observed in the VCD group was consistent with the oxidative stress hypothesis of POI (Sen Halicioglu et al. 2021; Shi et al. 2023; Huang et al. 2025).

The numerical value of T-AOC in the 17β-estradiol-treated group was higher than that in the VCD-induced group; however, the difference was not statistically significant. This slight increase may indicate partial improvement of the antioxidant defense mechanisms, although less pronounced than that of specialized antioxidant compounds (Yang

Table 4. Immunohistochemical evaluation of CASP3 expression in ovarian tissue among the experimental groups using the IRS method.

Group	Mean ± SE					
	Total cells	Positive cells	Positive %	P score	Intensity score	IRS
Ctrl	28.67 ± 0.88 [a]	0.67 ± 0.33 [b]	2.45 ± 1.20 [c]	0.50 ± 0.22 [c]	0.50 ± 0.22 [c]	0.50 ± 0.22 [b]
Induction VCD (160 mg/kg)	6.83 ± 0.70 [c]	5.67 ± 0.49 [a]	83.93 ± 3.67 [a]	3.83 ± 0.16 [a]	2.67 ± 0.21 [a]	10.33 ± 1.08 [a]
17β-Estradiol	16.33 ± 0.61 [b]	5.00 ± 0.58 [a]	30.77 ± 3.63 [b]	1.67 ± 0.21 [b]	1.33 ± 0.21 [b]	2.00 ± 0.00 [b]
PTS 50 mg/kg	27.33 ± 0.80 [a]	0.50 ± 0.22 [b]	1.75 ± 0.78 [c]	0.50 ± 0.22 [c]	0.67 ± 0.33 [bc]	0.67 ± 0.33 [b]
Combination (17β-Estradiol + PTS)	29.17 ± 0.87 [a]	0.67 ± 0.33 [b]	2.30 ± 1.17 [c]	0.50 ± 0.22 [c]	0.50 ± 0.22 [c]	0.50 ± 0.22 [b]
L.S.D. (*P*-value)	2.276 ** (0.0001)	1.200 ** (0.0001)	7.150 ** (0.0001)	0.614 ** (0.0001)	0.713 ** (0.0001)	1.535 ** (0.0001)

Means with different letters within the same column differ significantly. ** (P ≤ 0.01).
Ctrl, control; VCD, 4-vinylcyclohexene diepoxide; PTS, pterostilbene; SE, standard error; *P*-value, probability value; Positive %, percentage of positive cells; P-score, percentage score; IRS, immunoreactive score; LSD, least significant difference.

Table 5. Immunohistochemical evaluation of 8-OHdG expression in ovarian tissue among the experimental groups using the IRS method.

Group	Mean ± SE					
	Total cells	Positive cells	Positive %	P score	Intensity score	IRS
Ctrl	28.67 ± 0.88 [a]	0.83 ± 0.31 [b]	2.93 ± 1.05 [c]	0.67 ± 0.21 [c]	0.83 ± 0.31 [bc]	0.83 ± 0.31 [bc]
Induction VCD (160 mg/kg)	6.83 ± 0.70 [c]	5.33 ± 0.71 [a]	77.03 ± 4.03 [a]	3.50 ± 0.22 [a]	2.33 ± 0.21 [a]	8.17 ± 0.91 [a]
17β-Estradiol	16.33 ± 0.61 [b]	5.00 ± 0.89 [a]	30.80 ± 5.87 [b]	1.50 ± 0.22 [b]	1.50 ± 0.22 [b]	2.33 ± 0.56 [b]
PTS 50 mg/kg	27.33 ± 0.80 [a]	0.83 ± 0.31 [b]	3.00 ± 1.09 [c]	0.67 ± 0.21 [c]	0.67 ± 0.21 [c]	0.67 ± 0.21 [c]
Combination (17β-Estradiol + PTS)	29.17 ± 0.87 [a]	0.50 ± 0.22 [b]	1.82 ± 0.81 [c]	0.50 ± 0.22 [c]	0.50 ± 0.22 [c]	0.50 ± 0.22 [c]
L.S.D. (*P*-value)	2.276 **(0.0001)	1.621 **(0.0001)	9.546 **(0.0001)	0.637 **(0.0001)	0.693 **(0.0001)	1.501 ** (0.0001)

Means with different letters within the same column differ significantly. ** (*P* ≤ 0.01).
Ctrl, control; VCD, 4-vinylcyclohexene diepoxide; PTS, pterostilbene; SE, standard error; *P*-value, probability value; Positive %, percentage of positive cells; P-score, percentage score; IRS, immunoreactive score; LSD, least significant difference.

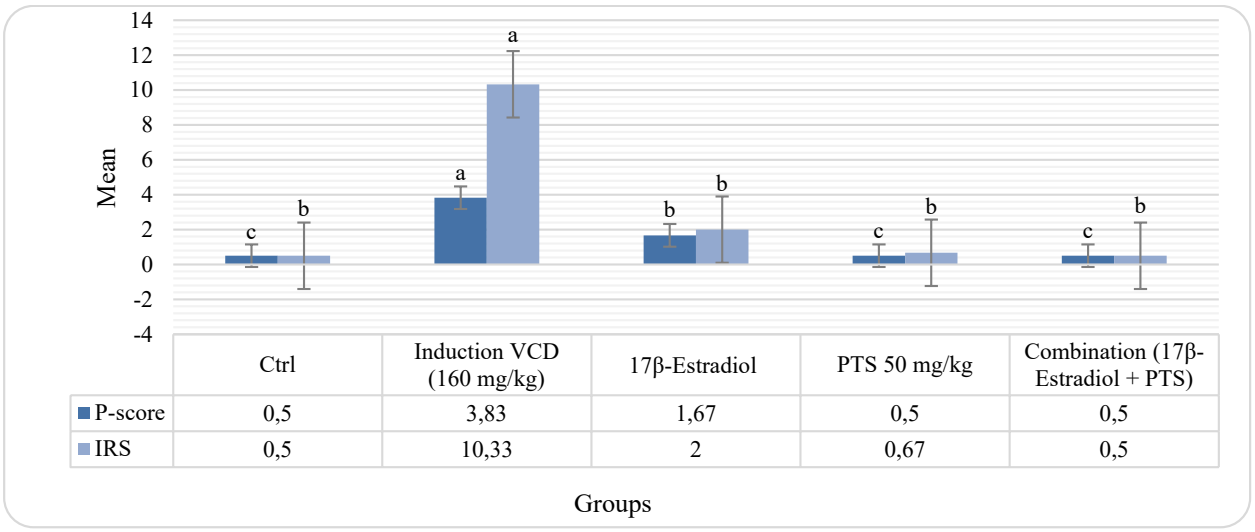

Figure 5. Effects of 17β-estradiol, PTS, and their combination on ovarian CASP3 immunohistochemical expression (P-score and IRS) in rats with VCD-induced POI. Results are expressed as bars representing mean ± standard error (SE). Statistical analysis was performed using one-way ANOVA followed by the LSD multiple comparisons test ($n = 6$). Ctrl, control; VCD, 4-vinylcyclohexene diepoxide; PTS, pterostilbene; P-score, percentage score; IRS, immunoreactive score. Different lowercase letters indicate statistically significant differences between groups ($P \leq 0.01$).

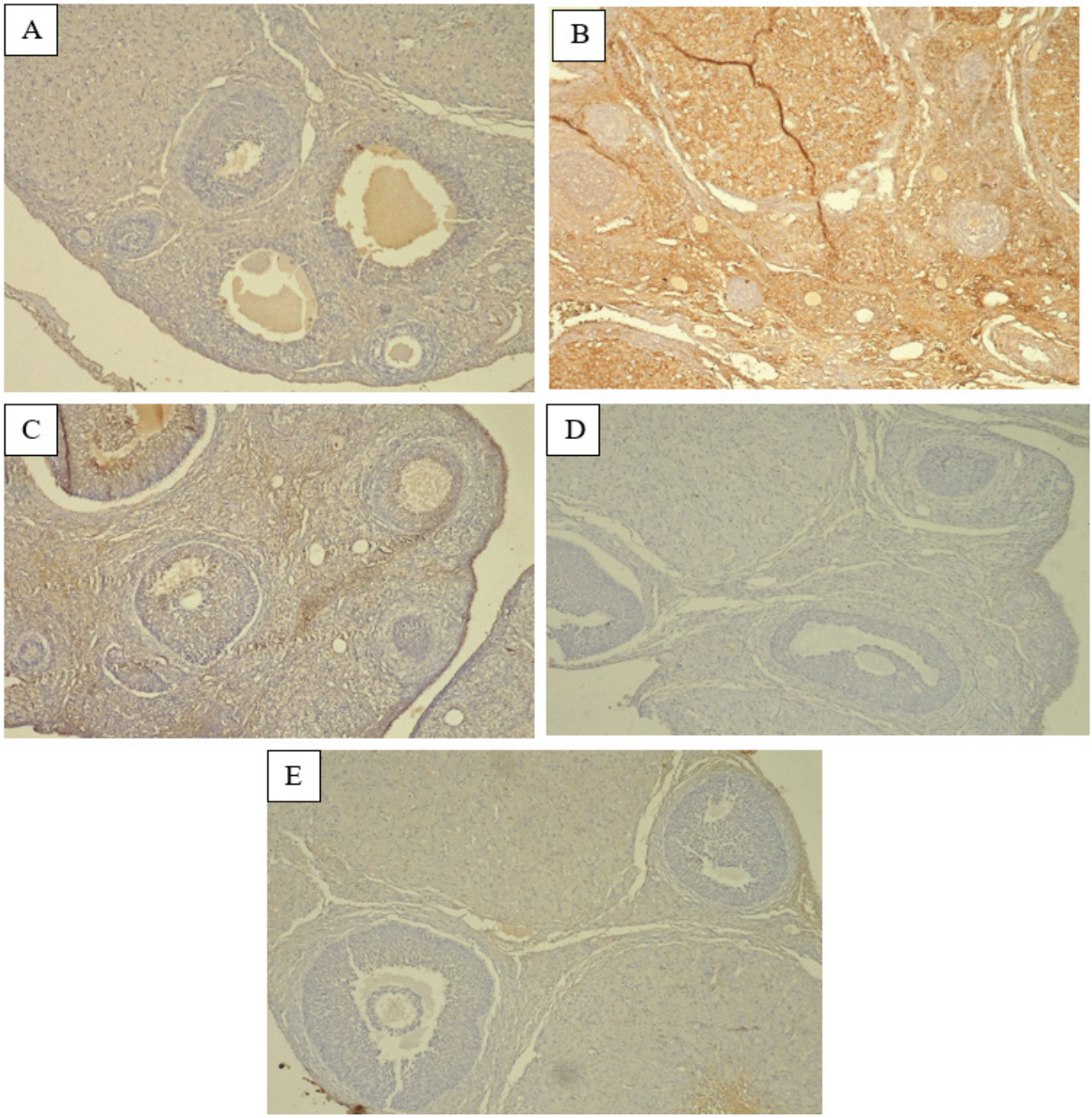

Figure 6. Photomicrographs of rat ovarian sections showing CASP3 immunohistochemical expression among the experimental groups (immunohistochemical staining; magnification ×100). **A.** Control group, staining intensity (+1); **B.** VCD (160 mg/kg) group, staining intensity (+3); **C.** 17β-estradiol group, staining intensity (+2); **D.** PTS (50 mg/kg) group, staining intensity (+1); **E.** combination (PTS + 17β-estradiol) group, staining intensity (+1).

et al. 2021). The average T-AOC level of the PTS-treated group was also higher relative to the VCD group, suggesting a possible enhancement of antioxidant defense systems and a decrease in oxidative damage (Wang et al. 2024). The combination-treated group exhibited the highest T-AOC level among the treated groups and approached that of the control group. The calculated CI value suggested a potential synergistic interaction between the two treatments. However, the observed increase in serum T-AOC level should be considered a supportive trend rather than a statistically significant improvement (Yang et al. 2021).

The lack of statistical significance in serum T-AOC does not necessarily imply the absence of oxidative stress modulation. The serum T-AOC level is an indicator of the entire antioxidant defense system. In several studies, the T-AOC difference was non-significant, although oxidative imbalance was observed through other oxidative stress markers. This may reflect the complexity of antioxidant defense systems and the limited sensitivity of T-AOC as a single biomarker for detecting subtle changes in oxidative status (Marrocco et al. 2017; Silvestrini et al. 2023; Tsao et al. 2024).

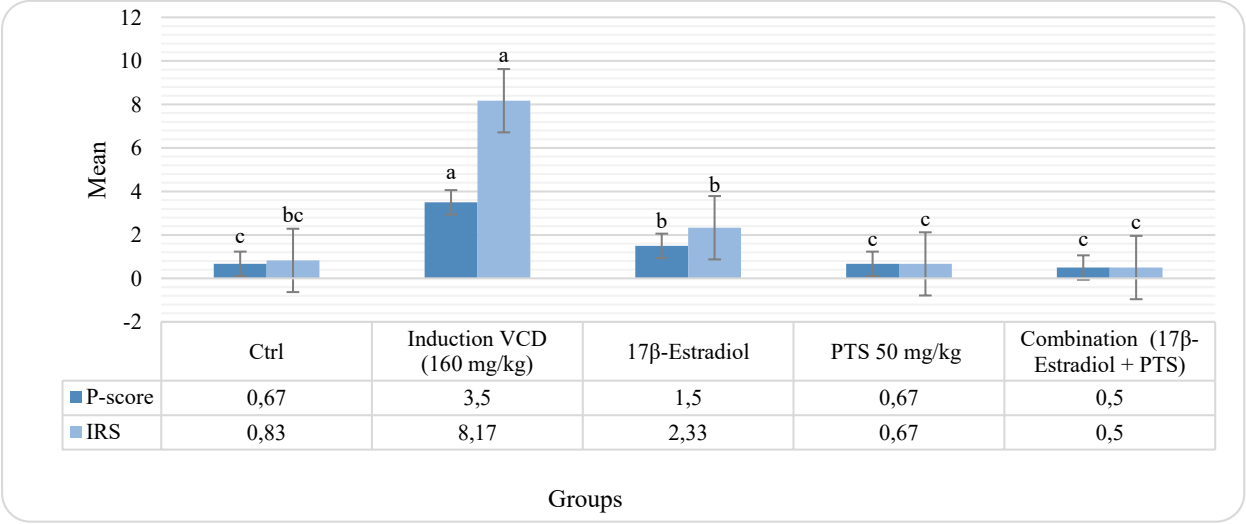

Figure 7. Effects of 17β-estradiol, PTS, and their combination on ovarian 8-OHdG immunohistochemical expression (P-score and IRS) in rats with VCD-induced POI. Results are expressed as bars representing mean ± standard error (SE). Statistical analysis was performed using one-way ANOVA followed by the LSD multiple comparisons test ($n = 6$). Ctrl, control; VCD, 4-vinylcyclohexene diepoxide; PTS, pterostilbene; P-score, percentage score; IRS, immunoreactive score. Different lowercase letters indicate statistically significant differences between groups ($P \leq 0.01$).

These findings are consistent with the reduced antioxidant capacity observed in the present study. The highest level of NF-κB was observed in the VCD group, indicating activation of inflammatory pathways due to increased oxidative stress in ovarian tissue, leading to GC injury, follicular atresia, and ovarian dysfunction and highlighting the role of inflammation in the pathogenesis of VCD-induced POI (Huang et al. 2025; Chen et al. 2026).

The level of NF-κB in the 17β-estradiol-treated group was significantly lower than that in the VCD-induced group, which may be explained by the anti-inflammatory activity of 17β-estradiol. This activity can suppress inflammatory signaling pathways and decrease oxidative stress-induced cellular injury, indicating a partial alleviation of inflammatory injury in the ovary induced by VCD (Murphy et al. 2010; Li et al. 2025a, 2025b). The PTS group also exhibited a marked reduction in NF-κB level relative to the VCD group. The observation that PTS reduced NF-κB levels aligns with Liu et al. (2024), who reported anti-inflammatory effects of PTS in various disease models, suggesting its protective role against ovarian inflammation in relation to VCD-induced injury (Zhou et al. 2025). The combination-treated group showed a greater reduction in the level of NF-κB than the VCD group. Bliss interaction analysis also showed a synergistic interaction. This synergism may be due to the complementary mechanisms of the two agents; however, this synergistic effect was marker-specific and was not consistently observed across all evaluated parameters (Devanaboyina et al. 2022; Chen et al. 2023).

To further explore the molecular mechanisms underlying the observed biochemical alterations, ovarian SIRT1 and FOXO3a gene expression was analyzed by RT-qPCR. A slight increase in SIRT1 expression was observed in the VCD-induced group compared with the control group. This increase may represent a compensatory cellular response to the oxidative stress and inflammatory injury caused by VCD (Zhang et al. 2020; Liu et al. 2023; Niu et al. 2025).

The expression of SIRT1 and FOXO3a in the 17β-estradiol-treated group was higher than that in the VCD-induced group. This finding agrees with previous studies reporting that 17β-estradiol modulates cell survival pathways and inflammatory signaling, which may contribute to the enhancement of SIRT1 and FOXO3a gene expression and the maintenance of ovarian cellular homeostasis (Cagnacci et al. 2023; Tian et al. 2023).

The highest levels of SIRT1 and FOXO3a expression were observed in the PTS-treated group. This marked increase may be related to the strong antioxidant activity of PTS and its potential to activate protective pathways related to SIRT1. The finding that PTS upregulates SIRT1 expression is consistent with Wang et al. (2024), who reported that PTS increased SIRT1/Nrf2 expression in aging chickens. However, the degree of upregulation (11-fold) was greater in the present study, possibly due to differences in species, dose, or duration of treatment (Hori et al. 2013; Grzeczka et al. 2025; Camerano et al. 2026).

The expression of SIRT1 and FOXO3a was also higher in the combination-treated group than in the control and VCD-induced groups. This improvement could be due to protection against oxidative stress and inflammatory responses. Nevertheless, the expression level was still lower than that observed in the PTS-treated group, suggesting that PTS may have contributed substantially to gene activation in the combined treatment (Liu et al. 2024; Gong et al. 2025). 17β-estradiol may have modified the transcriptional response induced by PTS through feedback regulation, receptor-mediated cross talk, or competition at shared downstream signaling pathways. Accordingly, the combined treatment produced substantially lower

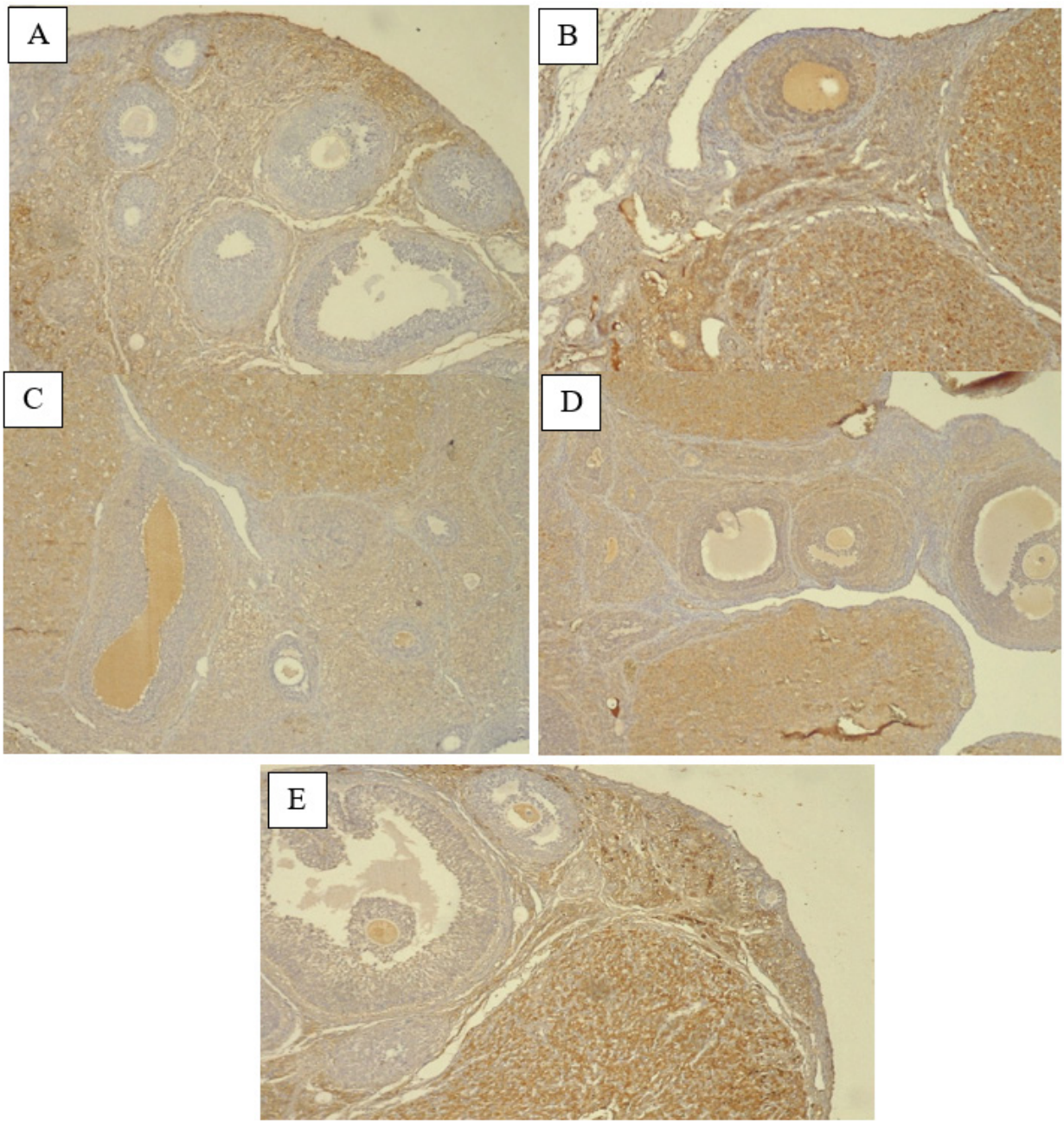

Figure 8. Photomicrographs of rat ovarian sections showing 8-OHdG immunohistochemical expression among the experimental groups under a light microscope (immunohistochemical staining; magnification ×100). **A.** Control group, staining intensity (+1); **B.** VCD (160 mg/kg) group, staining intensity (+3); **C.** 17β-estradiol group, staining intensity (+2); **D.** PTS (50 mg/kg) group, staining intensity (+1); **E.** combination (PTS + 17β-estradiol) group, staining intensity (+1).

gene expression than expected from the individual effects, which explains the high antagonistic CI (Moore and Faller 2013; Tao and Cheng 2023).

Dose of PTS used in this study (50 mg/kg/day in rats) corresponds to approximately 8 mg/kg/day in humans using allometric scaling (Reagan-Shaw et al. 2008), or approximately 480 mg/day for a 60 kg person. This dose can be approximately achieved using commercially available PTS supplements (50–250 mg/day), with a 250 mg dose taken twice daily providing 500 mg/day, suggesting that clinical translation may be feasible. However, the antagonism observed with 17β-estradiol raises concerns about combining PTS with HRT, and further studies are needed to determine optimal dosing and combination strategies.

The results suggest an association between PTS treatment and upregulation of SIRT1/FOXO3a gene expression, supporting the possibility that this pathway may mediate the antioxidant and anti-inflammatory effects of PTS. However, future studies using pathway inhibitors (e.g., EX-527 for SIRT1) or SIRT1/FOXO3a knockdown models are needed to establish causation (Fig. 9) (Hori et al. 2013; Gong et al. 2025).

Accordingly, the effects of oxidative stress and inflammation were further evaluated by assessing CASP3

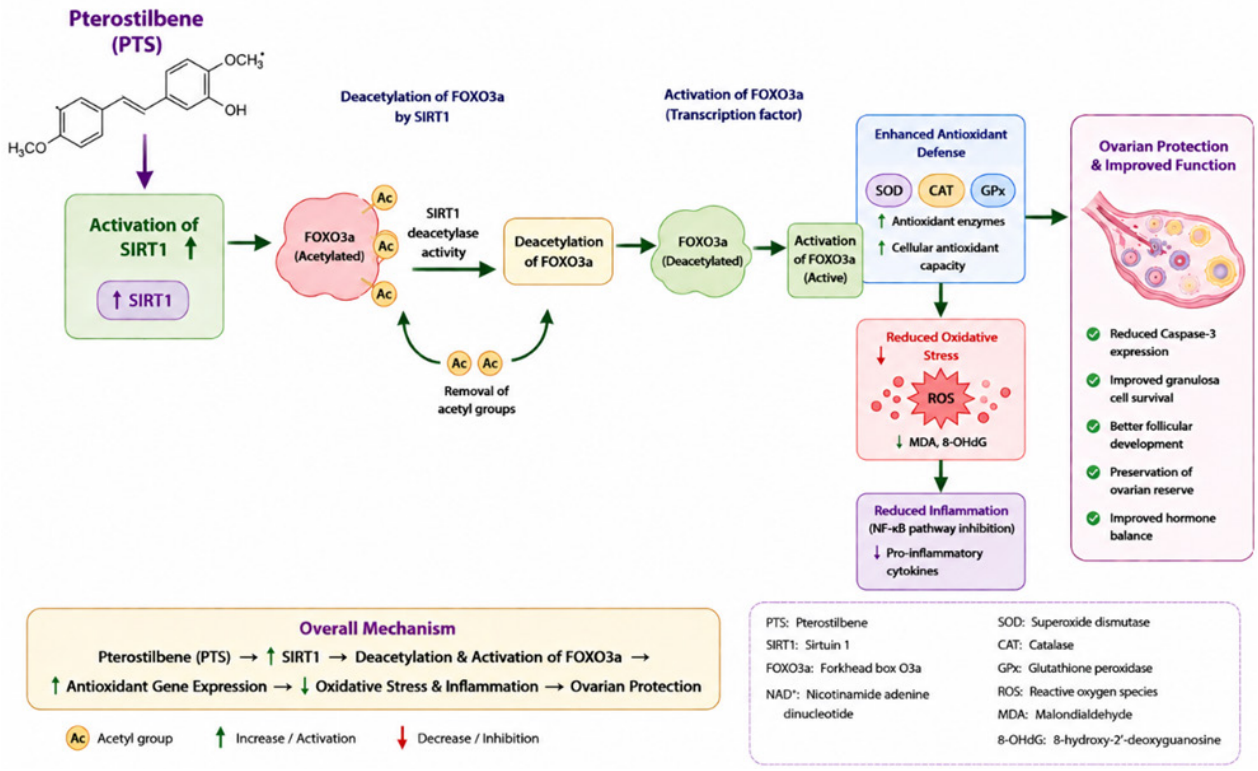

Figure 9. Schematic illustration of the proposed molecular mechanism of PTS through the SIRT1/FOXO3a signaling pathway based on published evidence (Hori et al. 2013; Iside et al. 2020; Gong et al. 2025).

and 8-OHdG immunoexpression. A marked increase in ovarian CASP3 immunohistochemical expression in the VCD-treated group might be related to oxidative stress-mediated mitochondrial damage. Excessive oxidative stress damages mitochondrial membrane integrity and increases the release of cytochrome c (Cyt c), which then activates the intrinsic apoptotic cascade via caspase-9 (CASP9) and CASP3. Thus, GC apoptosis increased, resulting in rapid follicular depletion and ovarian dysfunction (Sen Halicioglu et al. 2021; Liu et al. 2023; Chesnokov et al. 2024).

The administration of 17β-estradiol significantly reduced CASP3 immunoreactivity. However, it offered only partial protection against VCD-induced apoptosis. Experimental evidence suggests that 17β-estradiol is capable of promoting GC survival and delaying the process of follicular atresia by modulating apoptotic regulators and maintaining follicular homeostasis, although full suppression of apoptosis is not always achieved under persistent ovarian injury (Regan et al. 2018; Wang et al. 2023; Lu et al. 2026).

Interestingly, treatment with PTS almost restored CASP3 immunoreactivity to the level of the control group due to its ability to attenuate oxidative injury, preserve mitochondrial function, and suppress apoptotic signaling, thereby improving GC survival (Chen et al. 2023; Okamoto et al. 2025).

Likewise, the combination-treated group showed better preservation of GC viability and ovarian follicular architecture than the monotherapy groups. The better protection provided by the combination might be related to complementary mechanisms that simultaneously reduce oxidative damage, maintain cellular homeostasis, and inhibit apoptotic signaling, thus limiting follicular loss and preserving ovarian function. These findings were supported by additive and synergistic or near-additive effects based on CI values for both P-score and IRS (Regan et al. 2018; Tsao et al. 2024; Lu et al. 2026).

The accumulation of 8-OHdG, a recognized biomarker of oxidative DNA damage, indicates that VCD exposure caused significant oxidative injury in ovarian cells. Excess ROS can oxidize guanine residues in nuclear and mitochondrial DNA, impair mitochondrial function, and compromise GC and oocyte viability. Accordingly, the high expression of 8-OHdG in the VCD group confirms extensive oxidative damage, which may be a contributing factor to accelerated follicular depletion and ovarian dysfunction. The high expression of 8-OHdG in the VCD group was also accompanied by increased CASP3. Oxidative damage to DNA and mitochondria may activate cellular stress responses and promote the intrinsic apoptotic pathway. The concurrent elevation in 8-OHdG and CASP3 suggests that oxidative injury contributed to the increased GC apoptosis observed in the VCD group (Sen Halicioglu et al. 2021; Huang et al. 2023; Voros et al. 2025).

17β-Estradiol significantly decreased ovarian 8-OHdG immunoreactivity. This protective effect may be attributed to the ability of 17β-estradiol to maintain the homeostasis of follicular cells and reduce damage to ovarian DNA and mitochondria. Experimental evidence has demonstrated the ability of 17β-estradiol to decrease experimentally

induced oxidative damage to ovarian nuclear DNA and membrane lipids, supporting an antioxidant and cytoprotective role of estrogen within ovarian tissue (Chesnokov et al. 2024; Xu et al. 2024).

Treatment with PTS reduced 8-OHdG immunoreactivity, suggesting effective limitation of oxidative DNA damage and preservation of ovarian cellular integrity. This result is consistent with the antioxidant properties of PTS. Previous experimental studies have demonstrated that PTS enhances ovarian antioxidant defenses through activation of the SIRT1/nuclear factor erythroid 2-related factor 2 (Nrf2) pathway in an animal ovarian model and improves oocyte quality and reproductive outcomes (Chen et al. 2023; Okamoto et al. 2025).

The combination treatment provided greater protection against VCD-induced oxidative DNA injury than 17β-estradiol alone. The greater effect could be due to complementary actions, since PTS can upregulate antioxidant defenses and mitochondrial redox control, whereas 17β-estradiol can promote GC survival and follicular homeostasis. Based on the CI values, these results were further supported by additive and synergistic or near-additive effects for P-score and IRS, respectively (Regan et al. 2018; Tsao et al. 2024).

The study has several limitations. First, the sample size (n = 6 per group) was modest, and the T-AOC results were underpowered to detect significant differences. Second, the study measured gene expression but not protein levels or enzymatic activity, limiting mechanistic conclusions. Third, the study did not assess functional ovarian reserve (e.g., follicle counts, estrous cycle) or fertility outcomes. Fourth, the study used a single time point (day 40), and dynamic changes over time may have been missed. Fifth, the combination of PTS and 17β-estradiol showed antagonism for SIRT1/FOXO3a expression, suggesting that these agents may interact negatively at the molecular level, which has clinical implications for combination therapy.

Conclusion

The present study shows that PTS at 50 mg/kg/day alleviates VCD-induced ovarian damage in rats, as indicated by reduced serum NF-κB levels, decreased CASP3 and 8-OHdG immunoreactivity, and increased SIRT1 and FOXO3a gene expression. These results suggest a possible protective effect of PTS via antioxidant and anti-inflammatory mechanisms, which may include the SIRT1 and FOXO3a pathway. However, the combination of PTS and 17β-estradiol antagonized SIRT1/FOXO3a expression, suggesting that these agents may have a negative interaction at the molecular level. Future studies are needed to validate the mechanistic role of SIRT1/FOXO3a using pathway inhibitors, assess functional ovarian reserve and fertility outcomes, and determine the optimal dosing and combination strategies for clinical translation. Given the limitations of this preclinical study, PTS should be considered a promising candidate for further exploration rather than an established therapy for POI.

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Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statements

The authors declared that no clinical trials were used in the present study.

The authors declared that no experiments on humans or human tissues were performed for the present study.

The authors declared that no informed consent was obtained from the humans, donors or donors' representatives participating in the study.

Experiments on animals: The experimental protocol was granted by the Scientific and Ethical Committee, College of Pharmacy, Mustansiriyah University, Baghdad, Iraq (Approval No. 68, approved on 21 February 2026), and all procedures were conducted with accordance with the ethical guidelines for the care and use of laboratory animals.

The authors declared that no commercially available immortalised human and animal cell lines were used in the present study.

Artificial Intelligence (AI) use

The authors accept full responsibility for the content of the manuscript, including the disclosure of any use of AI.
No AI tools were used in the preparation of this manuscript.

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Author contributions

Kawthar Fadhil Abd Al-Hussein conducted the study and prepared the manuscript. The co-authors provided scientific guidance, reviewed the manuscript, and approved the final version.

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Data availability

All of the data that support the findings of this study are available in the main text or Supplementary Information.

Supplementary material 1

Results of the pilot study

Authors: Kawthar Fadhil, Suzan Jasim, Ahmed Mahmood
Data type: docx
Explanation note: Supplementary file containing the pilot study methodology and data used to determine the optimal doses of 4-vinylcyclohexene diepoxide (VCD) and pterostilbene (PTS) for the main study.
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Link: https://doi.org/10.3897/pharmacia.73.e209499.suppl1

Supplementary material 2

Combination index calculation

Authors: Kawthar Fadhil, Suzan Jasim, Ahmed Mahmood
Data type: docx
Explanation note: Bliss combination index calculations were used to evaluate the interaction between 17β-estradiol and pterostilbene, including expected effects, observed effects, and calculated combination index values.
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