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Guishen-erxian decoction can improve ovarian function in rats with premature ovarian failure by inhibiting oxidative stress and granulosa cell DNA fragmentation mediated by the PI3K/Akt/FOXO3a pathway

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Summary. Background. The aim of this study was to establish a rat model of premature ovarian failure (POF) with cyclophosphamide (CTX), and explore the molecular basis of POF and the mechanism of Guishen-Erxian Decoction (GSEXD) to improve POF from the perspective of oxidative stress regulation of ovarian granulosa cell (OGC) DNA fragmentation.

Method. The study utilized SD rats to establish a POF model via CTX. Rats were divided into Control, POF group, three GSEXD dosage groups (low, medium, high), and a GSEXD+PI3K agonist group to assess GSEXD's therapeutic effects on oxidative stress, DNA fragmentation and ovarian damage.

Result. GSEXD can improve the body weight, ovarian index, pathological status, and hormone secretion of POF rats, and inhibit ovarian oxidative stress and DNA fragmentation. In addition, GSEXD inhibits the activation of the PI3K/Akt/FoxO3a pathway. PI3K agonist 740 Y-P can reverse the effects of GSEXD on ovarian function, ovarian antioxidant capacity, and granulosa cell DNA fragmentation in POF rats.

Conclusion. Inhibition of oxidative stress damage and excessive DNA fragmentation of granulosa cells is a key pathway for GSEXD to promote follicle growth and development and alleviate ovarian function decline, which may be related to the inhibition of the PI3K/AKT/FOXO3a pathway by GSEXD.

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Introduction

Premature ovarian failure (POF), also known as premature ovarian insufficiency, is a condition characterized by the cessation of menstrual periods before age 40 due to ovarian dysfunction. POF is marked by elevated gonadotropin levels and reduced estrogen, significantly affecting fertility and reproductive health in women of reproductive age. Epidemiological surveys indicate an incidence of approximately 1% to 3%, with a trend toward earlier onset and increasing prevalence (Szeliga et al., 2021). Currently, the primary treatments for POF include hormone replacement therapy (HRT) and assisted reproductive technology. However, the latter is expensive, and its success rate is limited by egg quality (Lin et al., 2021). In addition, although HRT can alleviate some symptoms, it also increases the risk of breast cancer, stroke, and gallbladder disease, significantly limiting its long-term clinical application (Marjoribanks et al., 2018; Jiang et al., 2022). Therefore, to further understand the pathological mechanism of POF and develop a safer treatment with fewer side effects is of great clinical significance for improving reproductive function and preventing long-term complications.

Premature ovarian failure (POF) is characterized by follicular depletion and hormonal imbalance, with oxidative stress and granulosa cell apoptosis recognized as central contributors to its pathogenesis (Shi et al.,

2023). Chemotherapeutic agents, such as cyclophosphamide (CTX), are known to exacerbate ovarian damage by inducing excessive reactive oxygen species (ROS) production and impairing cellular survival pathways (Bhardwaj et al., 2023). Emerging evidence suggests that herbal interventions may counteract these effects through antioxidants and anti-apoptotic mechanisms. For instance, studies on extracts of Olea europaea, ginsenosides, Xuanfei Baidu decoction, and Jinfeng pills have demonstrated improved ovarian reserve in CTX-induced POF models via ROS scavenging and apoptosis suppression (Shoorei et al., 2019; Yan et al., 2021; Zhao et al., 2022; Hu et al., 2023). Similarly, activation of the PI3K/Akt pathway has been linked to enhanced follicular survival in POF (Liu et al., 2020), highlighting its therapeutic potential. However, the role of Guishen-Erxian Decoction (GSEXD) in modulating oxidative stress, apoptosis, and PI3K/Akt/FOXO3a signaling in POF remains unexplored, warranting further investigation.

Compared with modern HRT, traditional Chinese medicine (TCM) emphasizes syndrome differentiation and holistic regulation. Numerous clinical and experimental studies have demonstrated that TCM significantly improves menstrual disorders and reduces adverse effects in POF patients, highlighting its potential as a complementary therapy (Cai et al., 2021). Among TCM prescriptions, GSEXD, which integrates the principles of Guishen Pill and Erxian Decoction, is widely used in clinical practice for POF management. Derived from classical TCM formulas for kidney deficiency syndromes, Guishen Pill contains *Rehmanniae Radix Praeparata*, *Dioscoreae Rhizoma*, *Corni Fructus*, *Lycii Fructus*, *Cuscutae Semen*, *Eucommiae Cortex*, *Angelicae Sinensis Radix*, and *Poria*, which synergistically nourish kidney essence and regulate reproductive endocrine function. Experimental studies indicate that Guishen Pill enhances ovarian reserve in mice by modulating follicle-stimulating hormone (FSH) levels and promoting granulosa cell proliferation, thereby improving ovarian follicular development (Tian et al., 2022). Erxian Decoction is a renowned formula for warming kidney yang. Erxian Decoction comprises *Curculiginis Rhizoma*, *Epimedii Folium*, *Morindae Officinalis Radix*, *Angelicae Sinensis Radix*, *Phellodendri Chinensis Cortex*, and *Anemarrhenae Rhizoma*. These herbs collectively tonify kidney yang, balance hormonal axes, and counteract hypogonadism. Erxian Decoction has been shown to restore ovarian function in POF model rats by suppressing apoptosis via the Bcl-2/Bax pathway and enhancing antioxidant enzymes (Liu et al., 2023a; Shi et al., 2023). Additionally, it regulates the hypothalamic-pituitary-ovarian (HPO) axis, improving estrogen synthesis and follicular survival (Siyu et al., 2024). By combining Guishen Pill's kidney essence-tonifying effects with Erxian Decoction's yang-warming and anti-apoptotic properties, GSEXD exerts a dual regulatory action on ovarian dysfunction. Preliminary studies confirm that GSEXD not only promotes follicular development in CTX-induced POF rats but also reduces oxidative damage and granulosa cell apoptosis. However, further research is needed to elucidate its molecular mechanisms, particularly its interactions with key signaling pathways such as PI3K/AKT.

The PI3K/Akt signaling pathway has emerged as a pivotal focus in current POF research due to its regulatory role in ovarian follicular development and cellular survival (Bai and Wang, 2022). Within this pathway, FOXO3a serves as a critical transcription factor that integrates oxidative stress responses and mediates downstream effects on apoptosis and follicle maturation (Zheng et al., 2022). Notably, FOXO3a dysfunction has been implicated in accelerated follicular depletion, a hallmark of POF pathogenesis. While prior studies, such as the observed downregulation of FOXO3a by Erxian Decoction in cisplatin-induced granulosa cell injury (Zheng et al., 2022), suggest the therapeutic potential of herbal interventions. Building on these insights, this study investigated whether GSEXD alleviates CTX-induced POF by suppressing oxidative stress and granulosa cell apoptosis via the PI3K/Akt/FOXO3a pathway. Using a CTX-induced POF rat model, we assessed GSEXD's effects on ovarian function and redox balance, combined with PI3K activation to validate pathway specificity. Our results reveal GSEXD's mechanistic basis for targeted POF intervention.

Materials and methods

Preparation for prescription

The following herbs were weighed: *Rehmanniae Radix Praeparata* (15 g), *Dioscoreae Rhizoma* (15 g), *Epimedii Folium* (10 g), *Morindae Officinalis Radix* (10 g), *Corni Fructus* (10 g), *Lycii Fructus* (10 g), *Poria* (10 g), *Angelicae Sinensis Radix* (9 g), *Curculiginis Rhizoma* (6 g), *Anemarrhenae Rhizoma* (6 g), and *Phellodendri Chinensis Cortex* (6 g). These 107 g of medicinal herbs were soaked in a sevenfold amount of water for one hour, boiled and extracted for 30 min, filtered, and the extract was obtained. This was concentrated under reduced pressure, dried, and obtained as a dry paste, which was the sample required for subsequent animal experiments.

Experimental animals and the environment

The animal experimental protocol was approved by the Animal Ethics Committee (Approval No. IACUC-MIS20230056) and complied with institutional guidelines. Female SPF-grade SD rats (10 weeks old, 200±10 g, purchased from Zhuhai Baitry Tong Biotechnology Co., LTD) were housed under controlled conditions (26°C, 60% humidity, 12h light/dark cycle) with *ad libitum* access to food/water.

Animal treating

After one week of acclimatization, rats (n=35) received intraperitoneal CTX (60 mg/kg on Day 1, then 10 mg/kg/day for 14 days) to induce POF. (Li et al., 2019; Cai et al., 2024; Song et al., 2024) During modeling, vaginal secretions were scraped daily from day 7 with a sterile cotton swab dipped in normal saline, applied on a slide, dried naturally, and stained with a Gram staining kit (G1060, Solarbio, China). The smears were sealed with neutral gum (G8590, Solarbio, China), and the cell morphology was observed under an optical microscope to assess estrous cycle changes in the rats. In the POF model evaluation, vaginal smears observed from the 7th day showed a prolonged or disrupted estrous cycle, indicating successful modeling.

When the POF model was successfully established, we divided rats into two parts for different purposes in this study. In Part 1, we investigated the effects of GSEXD on oxidative stress, DNA fragmentation, and ovarian damage in POF rats. In Part 2, we examined the regulatory effects of GSEXD on the PI3K/Akt/FOXO3a signaling pathway and ovarian function. The specific administration methods for the two groups can be found in Table 1 and Figure 1. After 30 days of administration, the rats were anaesthetized with 10% chloral hydrate and euthanized in proestrus.

Observation of routine indexes in rats

From the beginning of modeling to the end of the experiment (days 0-44), changes in the general conditions of the rats in each group were recorded weekly, including changes in mental state, activity levels, diet, fur color, and body weight.

Calculation of the ovarian index

After the rat was euthanized, the ovarian tissue was excised, fat was trimmed away, and the tissue was rinsed with normal saline, dried with filter paper, weighed, and used to calculate the unilateral ovarian index. The formula is as follows: Ovarian index (g/100 g) = single ovarian weight (g)/ rat weight (g) x 100 (1) (Li et al., 2023).

Ovarian histopathology was observed by hematoxylin and eosin (HE) staining

After the rat was euthanized, ovarian tissue was promptly fixed in 4% paraformaldehyde (P0099-500 ml, Beyotime, Shanghai, China) for 24h, followed by

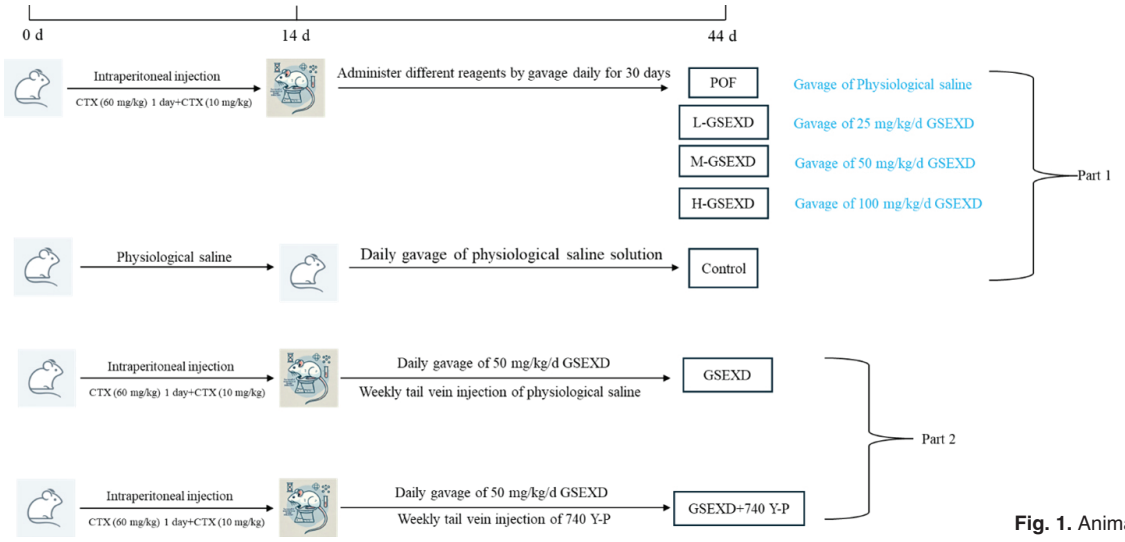

Fig. 1. Animal experiment flowchart.

Table 1. Animal Treatment Methods.

Part	Group	n	Method and dosage	Number of days
Part 1	Control	5	Administer 4 mL of 0.9% saline solution by gavage daily	30
	POF	5		30
	L-GSEXD	5	Daily gavage of GSEXD at a concentration of 25 mg/kg/d (Li et al., 2023)	30
	M-GSEXD	5	Daily gavage of GSEXD at a concentration of 50 mg/kg/d (Li et al., 2023)	30
	H-GSEXD	5	Daily gavage of GSEXD at a concentration of 100 mg/kg/d (Li et al., 2023)	30
Part 2	GSEXD	5	GSEXD at a concentration of 50 mg/kg/d was orally administered, with an equal amount of saline injected into the tail vein every week	30
	GSEXD+740 Y-P	5	50 mg/kg/d was orally administered, with an equal amount of 740-Y-P injected into the tail vein every week	30

standard procedures for clearing, paraffin embedding, and sectioning into 5-μm slices. Partial paraffin sections were taken and stained with hematoxylin and eosin (HE) (C0105M, Beyotime, Shanghai, China). Sections were dehydrated in a graded ethanol series (HB15, Guangzhou Chemical Reagent Factory, China), cleared with xylene (IC02, Guangzhou Chemical Reagent Factory, Guangzhou, China), and sealed with neutral gum (G8590, Solarbio, Beijing, China). Morphological changes in ovarian tissue, including variations in primordial, primary, secondary, mature, and atretic follicles, as well as corpus luteum number, and pathological alterations in the ovarian cortex, medulla, oocytes, granulosa cells, and interstitial cells, were observed under a light microscope.

Serum levels of hormones and oxidative stress were determined by ELISA

After the end of the experiment, the rats were fasted for 12h. After inhalation anesthesia with isoflurane, blood was taken from the abdominal aorta, centrifuged at 3000 r/min at 4°C for 15 min, and the upper serum was removed for the determination of hormones and oxidative stress factors. ELISA kits measuring Estradiol (E2) (E-OSEL-R0001, Elabscience, China), FSH (E-EL-R0391, Elabscience, China), Luteinizing hormone (LH) (D731015, Sangon, China), Anti-Mullerian hormone (AMH) (E-EL-R3022, Elabscience, China), Malondialdehyde (MDA) (JL53632-96T, Jonln, China), Glutathione Peroxidase (GSH-PX) (CSB-E12146r, Cusabio, China), and Superoxide dismutase (SOD) (HB906-Ra, HB906-RA, Hnybio, China) were used to measure the serum of rats in each group. All trials were conducted in compliance with the guidelines provided by the product manuals.

The DNA fragmentation of Ovarian Granulosa Cells (OGCs) was detected by TUNEL staining

Paraffin sections were dewaxed conventionally by heating at 60°C for 60 minutes, followed by two rounds of xylene dewaxing and a graded ethanol rehydration series (100%, 95%, 80%, 75%), each for 5 minutes. The sections were permeabilized with 1% Triton X-100 at room temperature for 3-5 minutes, then washed three times in phosphate-buffered saline (PBS), each for 5 minutes. A Proteinase K working solution (ST533-0.2 ml, Beyotime, Shanghai, China) was prepared by adding 2 μl of 50X Proteinase K to 98 μl PBS per sample, applied to each sample (100 μl) for 30 minutes at 37°C, followed by three PBS washes. TdT enzyme reaction mix, consisting of 45 μl Equilibration Buffer, 1.0 μl biotin-11-dUTP, and 4.0 μl TdT Enzyme, was freshly prepared, protected from light, and added (50 μl) to each sample in a humidified, dark chamber at 37°C for 60 minutes. After three PBS washes, Streptavidin-TRITC solution (5 μl per sample in 45 μl Labeling Buffer) was freshly prepared, added (50 μl) to each sample in a humidified, dark chamber at 37°C for 30 minutes, then washed with PBS. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (C1005, Beyotime, Shanghai, China) at room temperature, protected from light for 15 minutes, followed by three PBS washes. Observations were made under a fluorescence microscope (excitation 543 nm, emission 571 nm).

Western blot (WB) assay

Cells were lysed with RIPA buffer (P0013B, Beyotime, Shanghai, China), and the protein concentration was determined with the BCA kit (WB6501, NCM Biotech, China). Protein samples were then subjected to electrophoresis and transfer. After blocking with 5% skim milk (1172, BioFroxx, China) for 2h, the membranes were incubated with primary antibodies at 4°C overnight. Finally, the second antibodies were incubated for 1h, and the developer (P2100, NCM Biotech, China) was added to the gel imaging system to collect image information and determine the gray values of the bands. The sources and dilution ratios of the antibodies used in this experiment are shown in Table 1.

Statistical analysis

Data were analyzed using GraphPad Prism 9.0 software and presented as mean ± standard deviation (SD). Multiple group comparisons were conducted using one-way ANOVA, and if there were differences between groups, SNK-q tests were used for pairwise comparisons. The difference is statistically significant when $p<0.05$.

Results

Construction of the CTX-induced POF model

As shown in Figure 2, vaginal exfoliated cells in the Control group of rats exhibited a regular pattern over 4-5 days, with numerous nuclear keratinized squamous cells observed in estrus smears. During diestrus, the vaginal mucosa thinned, allowing white blood cells to migrate from the mucosa and dominate the vaginal smear. In contrast, the POF group displayed a disrupted estrous cycle with prolonged diestrus. This indicates that POF rat modeling was successful.

Effects of GSEXD on routine indexes, ovarian morphology, and serum hormones of POF rats

As shown in Figure 3A, body weight in the Control group increased steadily throughout the experiment. In contrast, a significant decline in body weight was observed in the CTX-induced POF model group during modeling ($p<0.001$). After modeling, the high, medium, and low-dose GSEXD treatment groups showed a more pronounced weight recovery compared with the POF

group (p<0.001). Among these, the medium-dose GSEXD group exhibited the greatest weight recovery, with statistically significant differences compared with the L-GSEXD and H-GSEXD groups (p<0.001). In addition, during the modeling period, the rats consumed less food and drink, and their vitality decreased. Except for the model control group, the above conditions were improved in all groups after administration.

The ovarian index and ovarian histopathology are direct indicators of ovarian condition. Compared with the Control group, the ovarian index in the POF group was significantly reduced (Fig. 3B, p<0.001). In contrast, the high-, medium-, and low-dose GSEXD groups showed an increase in ovarian index compared with the POF group, with the most prominent increase observed in the medium-dose GSEXD group (Fig. 3B, p<0.001).

As shown in Figure 3C, HE staining revealed distinct histological alterations among groups. In the Control group, ovaries displayed normal morphology with clearly identifiable follicles at various developmental stages, including primordial follicles (PF), primary follicles (PrF), secondary follicles (SF), antral follicles (AF), and atretic follicles (AFt). PF were composed of a quiescent oocyte surrounded by a single layer of flattened granulosa cells, lacking a zona pellucida. PrF featured an enlarged oocyte encircled by cuboidal granulosa cells and an emerging zona pellucida. SF showed multiple layers of granulosa cells, a thickened zona pellucida, and a developing antral cavity. AF were characterized by a large fluid-filled antrum and an organized cumulus-oocyte complex. In contrast, AFt presented signs of degeneration, including oocyte nuclear pyknosis or fragmentation, disorganized granulosa layers, apoptotic debris, and collapsing follicular walls. Granulosa cells were well organized, and follicular structures were intact in the Control group. The POF group exhibited pronounced ovarian atrophy, with a marked reduction in follicle number, particularly SF and AF, as well as a high prevalence of AFt. The ovarian stroma appeared fibrotic, and granulosa cell layers were fragmented and disorganized, indicating severe impairment in folliculogenesis. Treatment with GSEXD resulted in varying degrees of structural improvement. The L-GSEXD group showed partial regeneration of SF and reorganization of granulosa layers, although follicle counts remained relatively low. The H-GSEXD group exhibited increased numbers of mature follicles and restoration of zona pellucida integrity. Notably, the M-GSEXD group demonstrated the most significant histological recovery, with follicle structures closely resembling those in the control group. This included an increased number of SF and AF, normal antral cavity expansion, and well-aligned granulosa cells, suggesting effective dose-dependent restoration of ovarian tissue morphology. Although full quantitative follicle counts were not performed, the representative images and visual annotations of follicle stages and atrophy provide qualitative support for the protective effects of GSEXD in CTX-induced POF.

Through the detection of the serum sex hormone content of rats in each group, serum FSH and LH levels were significantly elevated (Fig. 3D,E, p<0.001), while E2 and AMH levels were notably reduced in the POF group compared to the Control group (Fig. 3F,G, p<0.001). In contrast, the high, medium, and low-dose

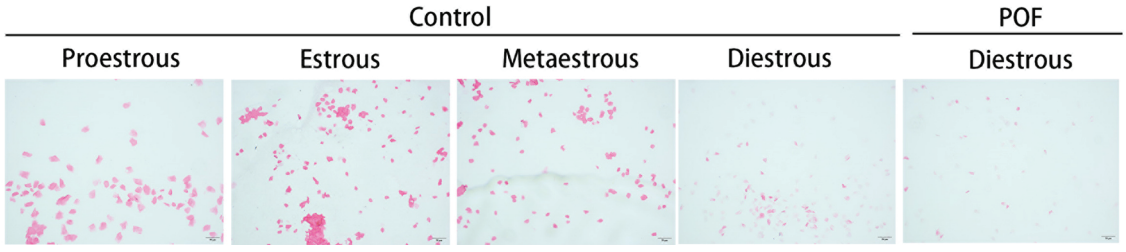

Fig. 2. Representative images of HE staining smears of exfoliated vaginal cells. x 100.

Table 2. Source and dilution ratio of antibodies used in the WB experiment.

Antibodies	Batch number	Supplier source	Dilution (application)
AKT	ab179463	Abcam, UK	1:8000 (WB)
PI3K	ab302958	Abcam, UK	1:1000 (WB)
FOXO3a	ab23683	Abcam, UK	1:1000 (WB)
p-AKT	ab38449	Abcam, UK	1:1000 (WB)
p-PI3K	ab154598	Abcam, UK	1:2000 (WB)
p-FOXO3a	ab47285	Abcam, UK	1:1000 (WB)
β-Tubulin	#2146	Cell Signaling Technology, USA	1:2000 (WB)
Goat Anti-Rabbit IgG H&L(HRP)	bs-0295G-HRP	Bioss, China	1:20000 (WB)

GSEXD groups exhibited decreased serum FSH and LH levels, alongside increased E2 and AMH levels compared with the POF group, with the medium-dose GSEXD group showing the most pronounced effects, which were statistically significant compared with both the low-dose and high-dose GSEXD groups (p<0.001). Based on these findings, it can be inferred that GSEXD has the potential to ameliorate the weight, ovarian ratio, ovarian pathological condition, and hormonal secretion in POF rats.

Effects of GSEXD on antioxidant capacity and DNA fragmentation in ovarian cells in POF rats

As depicted in Figure 4A-C, compared with the Control group, the POF group showed a significant increase in serum MDA levels (p<0.001), alongside a marked decrease in SOD activity and GSH-Px levels

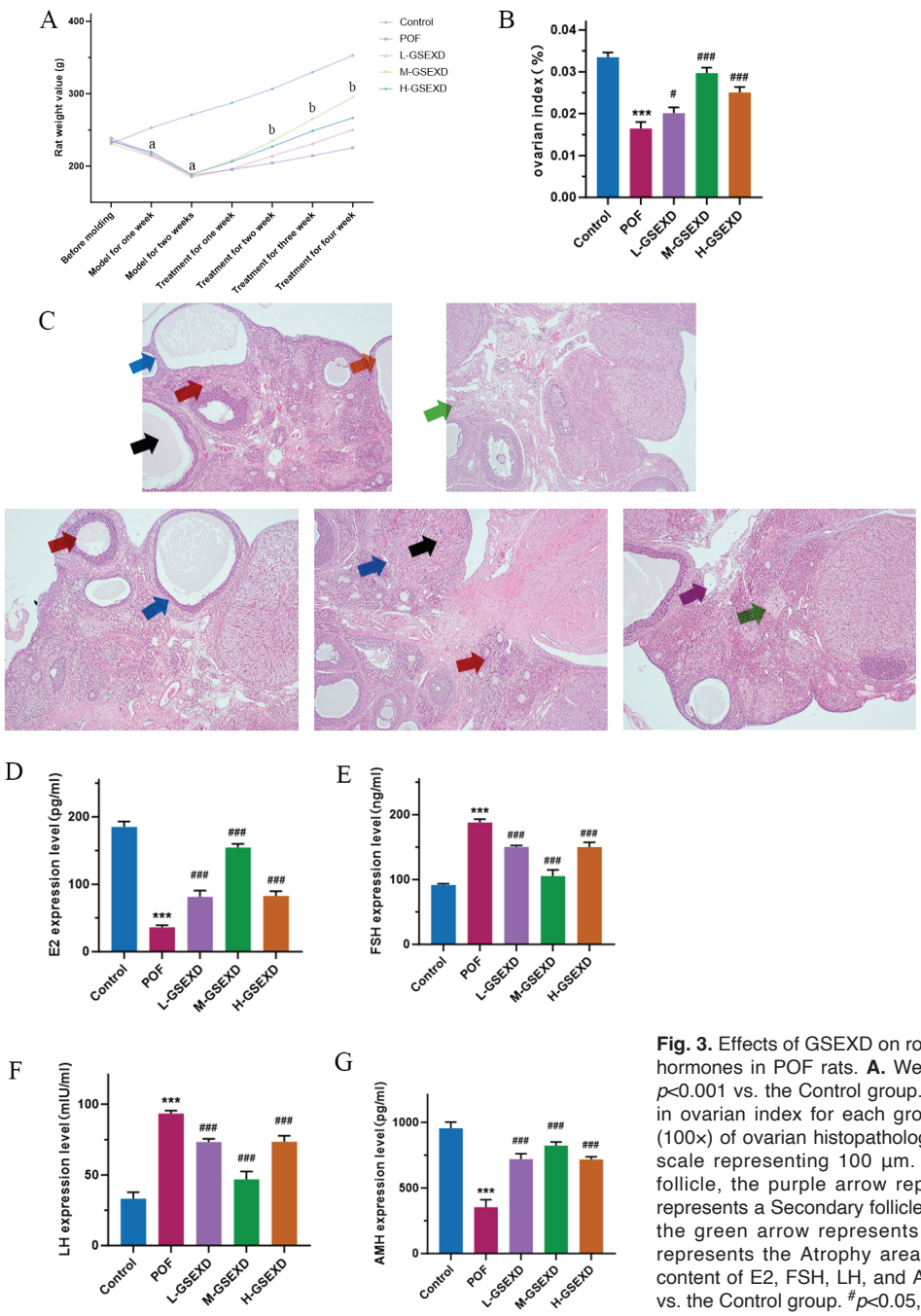

Fig. 3. Effects of GSEXD on routine indexes, ovary morphology, and serum hormones in POF rats. **A.** Weight changes in each group for 7 weeks. a: p<0.001 vs. the Control group. b: p<0.001 vs. the POF group. **B.** Changes in ovarian index for each group. **C.** HE staining: representative images (100×) of ovarian histopathological sections of rats in each group, with the scale representing 100 μm. The black arrow represents a Primordial follicle, the purple arrow represents a Primary follicle, the red arrow represents a Secondary follicle, the blue arrow represents an Antral follicle, the green arrow represents an Atretic follicle, and the yellow arrow represents the Atrophy area. **D-G.** ELISA was used to determine the content of E2, FSH, LH, and AMH in the serum of each group. ***p<0.001 vs. the Control group. $^{\#}p$<0.05, $^{\#\#\#}p$<0.001 vs. the POF group.

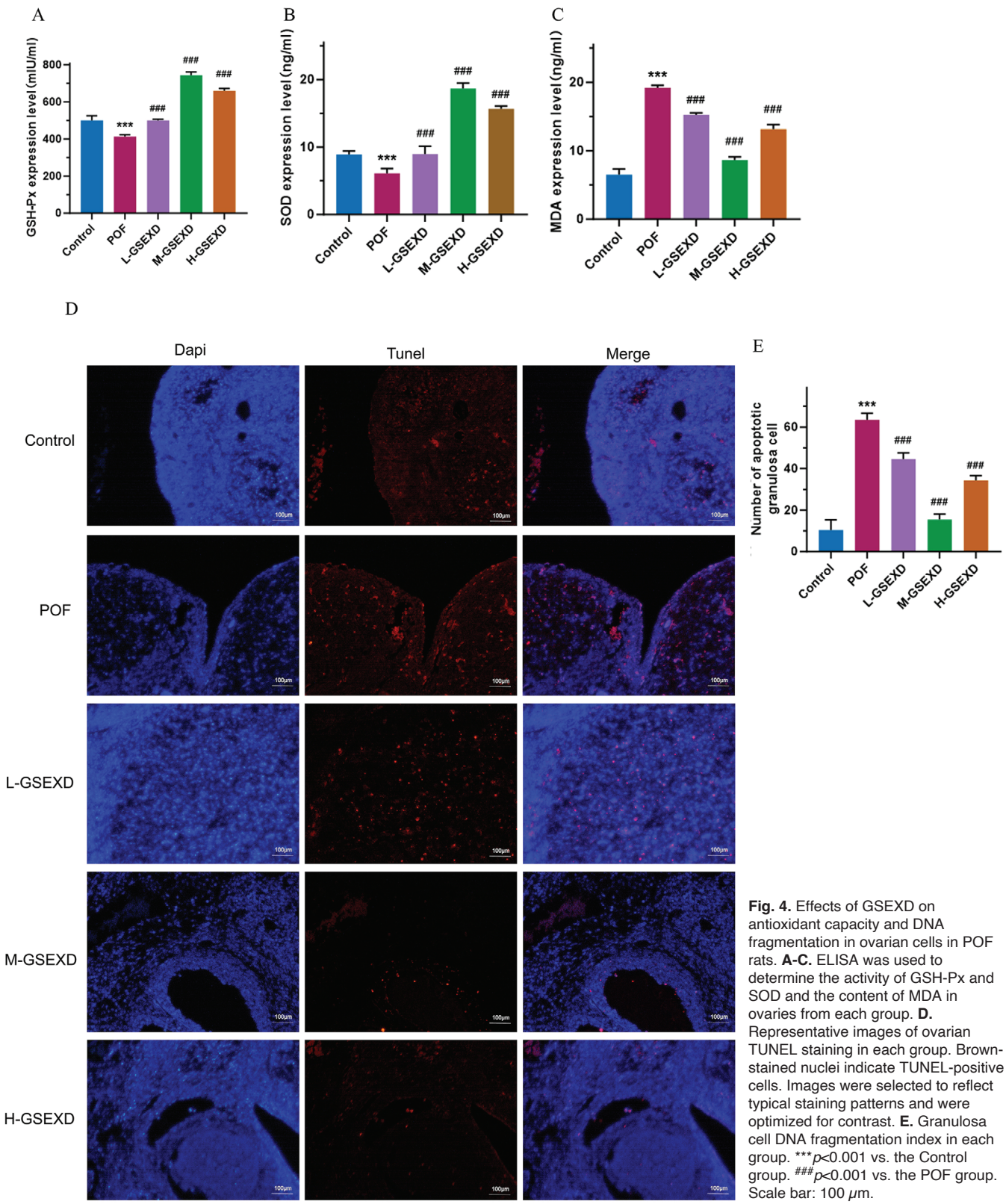

Fig. 4. Effects of GSEXD on antioxidant capacity and DNA fragmentation in ovarian cells in POF rats. **A-C.** ELISA was used to determine the activity of GSH-Px and SOD and the content of MDA in ovaries from each group. **D.** Representative images of ovarian TUNEL staining in each group. Brown-stained nuclei indicate TUNEL-positive cells. Images were selected to reflect typical staining patterns and were optimized for contrast. **E.** Granulosa cell DNA fragmentation index in each group. ***$p<0.001$ vs. the Control group. ###$p<0.001$ vs. the POF group. Scale bar: 100 μm.

(p<0.001). In contrast, the high, medium, and low-dose GSEXD groups exhibited increased GSH-Px levels and SOD activity, with reduced MDA levels relative to the POF group (p<0.001), with the medium-dose GSEXD group demonstrating the most pronounced changes (p<0.001).

The outcomes of the ovarian TUNEL examination are depicted in Figure 4D,E. The POF group showed an increase in TUNEL-positive cells compared with the Control group (p<0.001). In contrast, the high-, medium-, and low-dose GSEXD groups demonstrated a reduction in TUNEL-positive cells relative to the POF group, with the medium-dose GSEXD group showing the most significant decrease (p<0.001).

Effects of GSEXD on the PI3K/Akt/FOXO3a pathway in POF rats

By detecting the expression of AKT, p-AKT, mTOR, p-mTOR, FOXO3a, and p-FOXO3a in the ovarian tissue of rats in each group, the results revealed that compared to the Control group, the POF group exhibited increased protein expression of AKT/p-AKT, mTOR/p-mTOR, and FOXO3a/p-FOXO3a in ovarian tissue (Fig. 5A-D, p<0.001). In contrast, the high-, medium-, and low-dose GSEXD groups, along with the positive control group, showed reduced expression of these proteins relative to the POF group, with the medium-dose GSEXD group demonstrating the most pronounced reduction (p<0.001).

740 Y-P reversed the improvement effect of GSEXD on ovarian function in POF rats

To gain further insights into the potential involvement of the Akt/mTOR/FOXO3a pathway in the therapeutic effects of GSEXD on oxidative stress, DNA fragmentation, and ovarian damage in POF rats, we administered the PI3K agonist 740 Y-P to POF rats as an additional treatment alongside GSEXD, aiming to assess its functional rescue capabilities. As exhibited in Figure 6A-C, compared with the GSEXD group, the rats in the GSEXD+740 Y-P group had lower body weight, lower ovarian index, and poorer ovarian status (p<0.001). The findings from serum hormone analysis revealed that, in contrast to the GSEXD group, the levels of FSH and LH in the serum of rats in the GSEXD+740 Y-P group

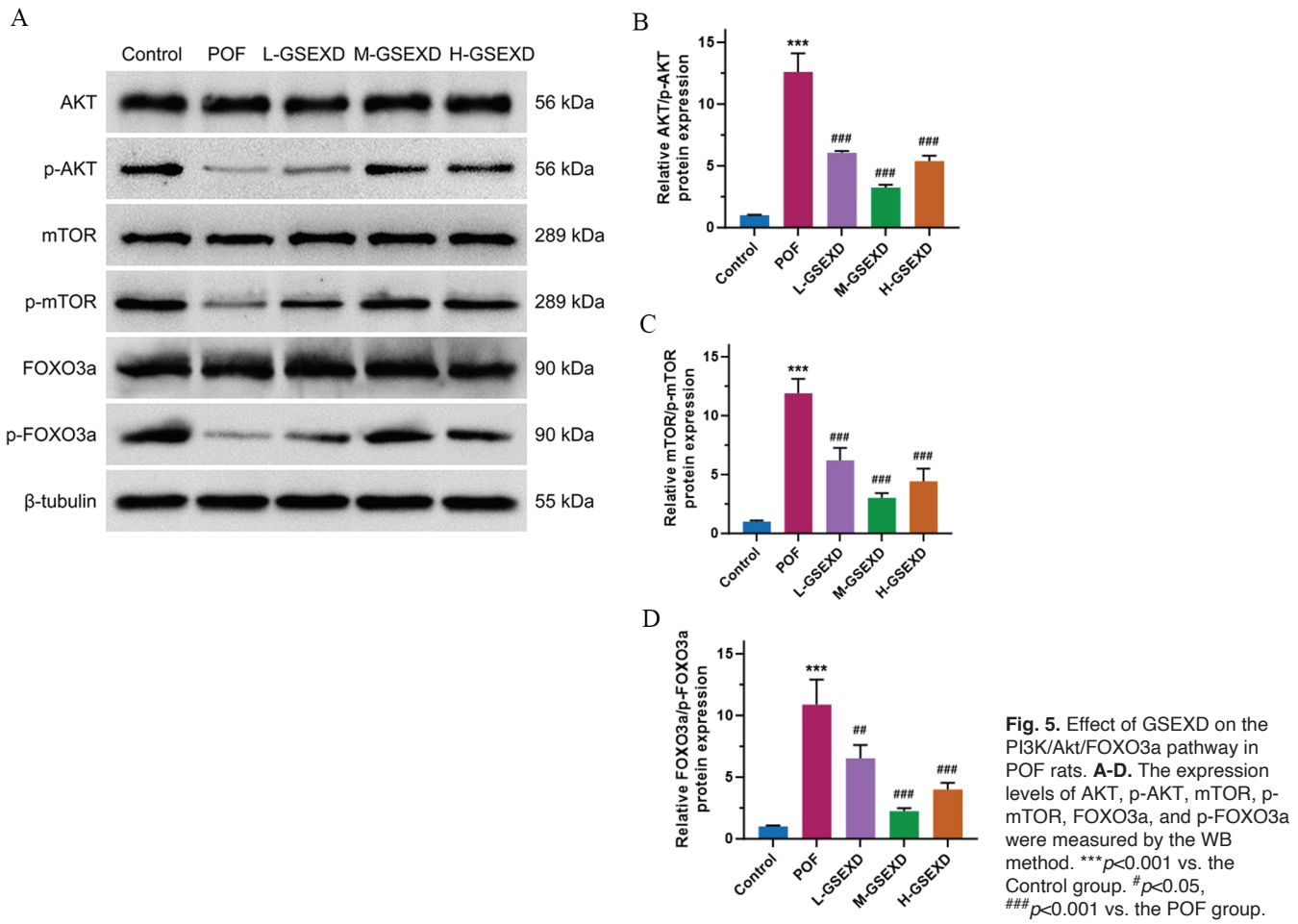

Fig. 5. Effect of GSEXD on the PI3K/Akt/FOXO3a pathway in POF rats. **A-D.** The expression levels of AKT, p-AKT, mTOR, p-mTOR, FOXO3a, and p-FOXO3a were measured by the WB method. ***p<0.001 vs. the Control group. $^{\#}p$<0.05, $^{\#\#\#}p$<0.001 vs. the POF group.

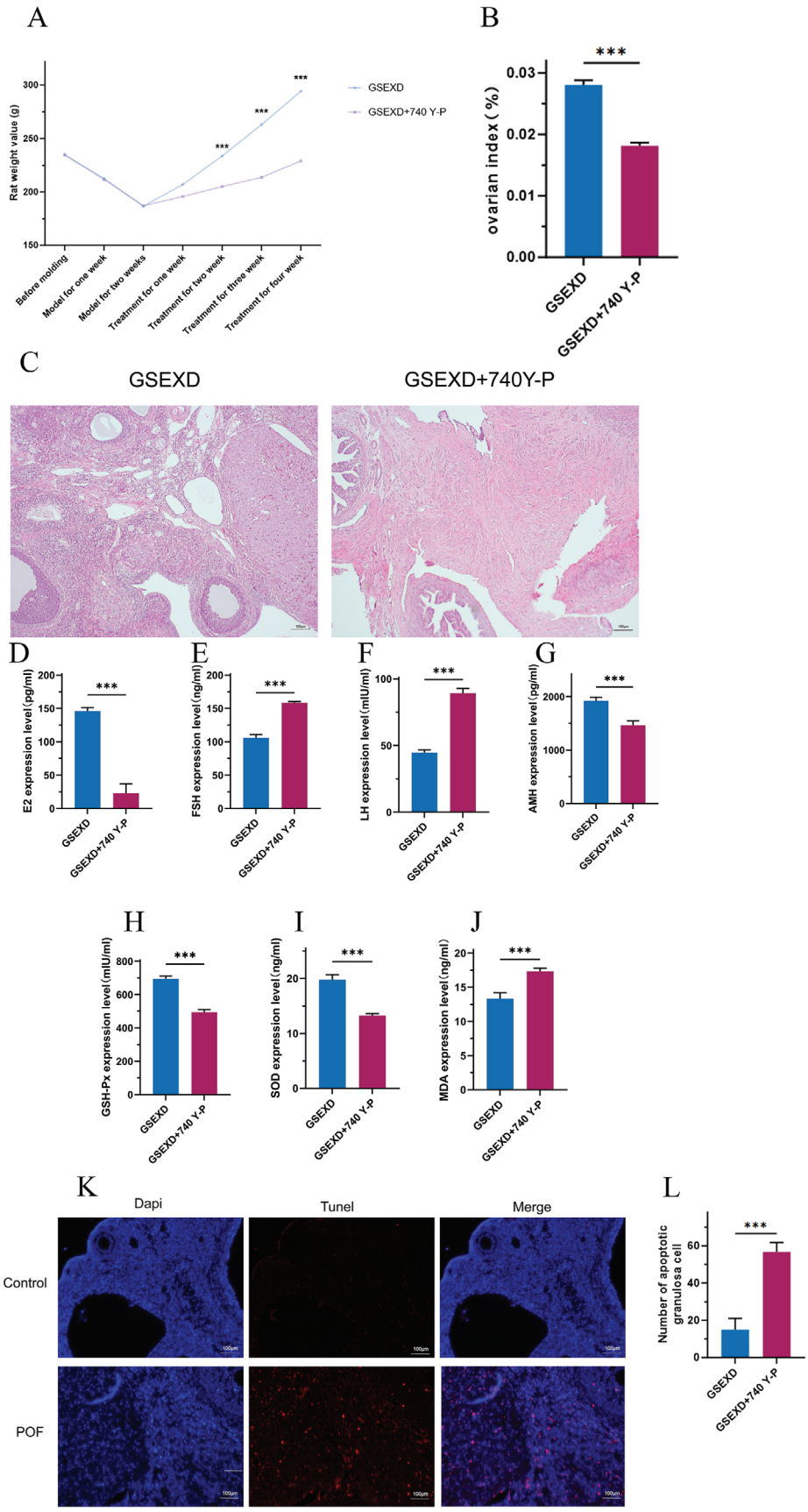

Fig. 6. Effect of GSEXD on the PI3K/Akt/FOXO3a pathway in POF rats. **A.** Weight changes in each group at 7 weeks. **B.** Ovarian index of each group. **C.** HE staining: representative images of ovarian histopathological sections of rats in each group, with the scale representing 100 μm. **D-G.** ELISA was used to determine the content of E2, FSH, LH, and AMH in the serum of each group. **H-J.** ELISA was used to determine the activity of GSH-Px and SOD and the content of MDA in ovaries from each group. **K, L.** Representative images of ovarian TUNEL staining and granulosa cell DNA fragmentation index in each group. ***p<0.001 vs. the GSEXD group. Sclae bar: 100 μm.

exhibited notable increases, while the levels of E2 and AMH showed significant decreases ($p<0.001$). Additionally, the ELISA and ovarian TUNEL staining results demonstrated that, compared with the GSEXD group, the antioxidant capacity within rat ovaries in the GSEXD+740 Y-P group experienced a decline ($p<0.001$). This was accompanied by an elevation in the levels of the oxidative stress marker MDA and an increase in the DNA fragmentation of ovarian tissue cells.

Discussion

POF significantly impacts fertility and overall health due to its complex pathogenesis involving genetic, immunological, and environmental factors. TCM, particularly formulations like Erxian Decoction and Guishen Pill, has emerged as a promising complementary approach due to its multi-target mechanisms, including estrogen regulation, inflammation reduction, and apoptosis inhibition (Li et al., 2022a,b). GSEXD is a novel clinical formula that combines Erxian Decoction and Guishen Pill through clinical prescription, for clinically treating POF. Its formulation is based on the compatibility of Guishen Pill and Erxian Decoction, which are known for their effects in tonifying the kidney and promoting blood circulation. The present study demonstrated that GSEXD effectively ameliorates POF symptoms by significantly increasing serum estrogen concentrations, promoting follicular development, and restoring ovarian reserve function. The therapeutic efficacy of GSEXD was mediated via modulation of the PI3K/Akt/mTOR signaling pathway, enhancement of antioxidant enzyme activities (SOD and GSH-Px), reduction of an oxidative damage marker (MDA), and suppression of OGC apoptosis. In addition, this study validated the critical role of the PI3K/Akt pathway through intervention experiments with the PI3K agonist 740 Y-P. Overall, this study systematically elucidated the potential molecular mechanisms underlying the beneficial effects of GSEXD on POF ovarian function, providing innovative and effective treatment options for managing premature ovarian failure.

Guishen Pill is a classical prescription from Jingyue Quanshu, which exerts the effects of tonifying yin and yang, nourishing blood, and replenishing essence. Clinical studies have shown that Guishen Pill can alleviate symptoms of POF, such as hot flashes, night sweats, and scanty menstruation. Animal experiments further confirmed its ability to improve follicular function and regulate endocrine balance and apoptosis in OGCs (Li et al., 2022a,b; Zhong et al., 2022). Erxian Decoction integrates both yang-tonifying and yin-nourishing herbs to warm the kidney, replenish essence, and harmonize the reproductive axis, showing clinical efficacy in improving ovarian function (Liu et al., 2023a.b; Mai et al., 2024). Network pharmacology analysis has identified PI3K/Akt signaling as a core pathway involved in the action of both formulas, in addition to their effects on ovarian steroidogenesis (Ding et al., 2017). The PI3K/Akt pathway regulates cell proliferation and survival and is essential for PF activation, granulosa cell proliferation, and oocyte development. Granulosa cell apoptosis plays a central role in follicular atresia and ovarian reserve depletion, with the Fas/FasL–Caspase-3 pathway being a key mechanism that leads to DNA fragmentation and follicular closure (Cao et al., 2024). Our previous study demonstrated that in POF rat ovaries, Fas expression was significantly decreased, and FasL was upregulated. Treatment with GSEXD reversed these alterations, indicating that its therapeutic effect is linked to the regulation of the apoptotic pathway in ovarian tissue. Quercetin, identified through network pharmacology as one of the major active ingredients in the Erxian Decoction, is a dietary flavonoid with broad biological activity. It has been shown to regulate PI3K/Akt/FoxO3a signaling and protect against follicular depletion by preventing excessive activation of this pathway (Wang et al., 2023; Yang et al., 2024). Dysregulation of downstream molecules, such as FoxO3a and mTOR, can cause premature depletion of PF and accelerate POF progression (Kim et al., 2019). Quercetin has also been reported to inhibit CTX-induced follicle loss by modulating the PI3K/Akt/FoxO3a pathway, thereby promoting follicle development and maturation (Li et al., 2021). Collectively, these findings suggest that the combination of Guishen Pill and Erxian Decoction, now formulated as GSEXD, offers synergistic efficacy in improving ovarian function in POF, particularly in cases associated with kidney deficiency. While previous studies have not explored this specific herbal combination, our earlier research confirmed that GSEXD reduces oxidative stress and granulosa cell apoptosis in POF rat ovaries. Nevertheless, the precise mechanisms remained unclear, prompting the present investigation.

OGCs play a pivotal role in follicular development by supporting oocyte maturation, ovulation, and fertilization (Cai et al., 2021). Extensive apoptosis of granulosa cells, often triggered by oxidative stress (ROS), is a key factor in initiating follicular atresia, leading to premature depletion of the ovarian reserve and contributing to the development of POF (Liu et al., 2023a,b; Shi et al., 2023). In this study, following the successful establishment of the POF model, a significant increase in granulosa cell DNA fragmentation was observed in rats. However, with the administration of GSEXD, granulosa cell DNA fragmentation was markedly reduced. Previous studies have shown that Erxian Decoction can enhance the antioxidant and anti-apoptotic abilities of the ovary to alleviate POF (Liu et al., 2023a,b). In addition, Guishen pills have also been proven to reduce the level of apoptosis and oxidative stress in rats with Polycystic ovary syndrome (PCOS) (Tian et al., 2022). We thus hypothesize that GSEXD may alleviate ROS-induced oxidative stress, as evidenced by the increase in GSH-Px and SOD levels and the decrease in MDA levels.

In ovarian tissue damage, ROS can lead to cellular injury through both ROS-dependent and ROS-independent mechanisms (Mohammadi et al., 2022; Soltani et al., 2023). In this study, GSEXD enhanced the antioxidant capacity of the body and mitigated ROS-mediated oxidative damage. This indicates that GSEXD plays a crucial role in the ROS-dependent pathway, inhibiting cell injury and apoptosis induced by excessive ROS. ROS-induced oxidative stress is considered a potential cause of POF (Jiang et al., 2018), and several TCMs have been found to protect against ROS-induced ovarian failure by inhibiting apoptosis and oxidative stress through related pathways (Zhou et al., 2021; Chen et al., 2024; Li et al., 2022a,b). Both Guishen Pill and Erxian Decoction have also demonstrated remarkable antioxidant effects, consistent with the findings of this study.

Further research indicated that the levels of AKT/p-AKT, mTOR/p-mTOR, and FOXO3a/p-FOXO3a were significantly elevated in the ovarian tissues of the POF group, suggesting that this signaling pathway may be involved in the development of POF. Following GSEXD treatment, the protein expression levels were significantly reduced, and DNA fragmentation in ovarian cells was notably alleviated, indicating that GSEXD may protect ovarian tissue and exert therapeutic effects by inhibiting the overactivation of the PI3K/Akt/FOXO3a signaling pathway. The PI3K/Akt signaling pathway is a crucial regulator of cell proliferation, differentiation, and survival. PI3K activates the phosphorylation of Akt, which subsequently activates a series of downstream target proteins, including antiproliferative and anti-apoptotic factors such as FOXO3a, members of the Bcl-2 family, mTOR (regulates protein biosynthesis and cell growth), and p27 (maintains the PF reserve) (Wang et al., 2019; Bai and Wang, 2022). FoxO3a is a key downstream transcription factor of Akt. In its non-phosphorylated form, FoxO3a can translocate into the nucleus and induce the expression of pro-apoptotic genes (Li et al., 2019). mTOR, as a master regulator of cell growth and metabolism, works in conjunction with Akt to regulate cell survival and proliferation processes (Liu et al., 2018). Furthermore, the application of the PI3K agonist 740 Y-P reversed the beneficial effects of GSEXD on POF, suggesting that GSEXD may reduce FoxO3a phosphorylation by inhibiting PI3K/Akt signaling, preventing its nuclear translocation and the induction of apoptotic genes. Additionally, GSEXD may inhibit excessive mTOR activation, thereby restoring normal ovarian cell function.

Interestingly, GSEXD did not exhibit a dose-dependent effect in our study; instead, the medium dose demonstrated better therapeutic outcomes. This may be related to the complex active constituents within GSEXD. Furthermore, it is possible that at a medium dosage, the drug molecules of GSEXD bind with sufficient protein receptors, resulting in the maximum therapeutic effect. Moreover, GSEXD could offer POF patients a safer and more effective adjunct therapy in clinical settings, helping to delay ovarian functional decline and improve reproductive prognosis, particularly given the current limited efficacy and notable side effects of clinical treatments for POF. As a TCM formula, GSEXD shows potential in alleviating POF symptoms and enhancing fertility.

In conclusion, this study demonstrates that GSEXD can exert antioxidant effects through the regulation of the PI3K/Akt/FOXO3a pathway, improve the ovarian environment, and reduce granulosa cell DNA fragmentation, thereby offering therapeutic potential for POF. However, reproductive outcomes were not further observed in this study, and the precise mechanisms of GSEXD's multi-target intervention in POF remain unclear. Future studies should explore GSEXD's protective effects on ovarian function under various pathological conditions, focusing on additional molecular mechanisms beyond ROS. In-depth *in vivo*/*in vitro* research and larger clinical trials are needed to clarify that OGCs are the supportive cells of oocytes, playing a crucial role in regulating follicle maturation, ovulation, and fertilization through their interactions with oocytes. Currently, it is widely accepted that granulosa cell DNA fragmentation is a central factor in initiating follicular atresia; extensive DNA fragmentation can lead to follicular atresia of numerous immature oocytes, resulting in premature depletion of the ovarian reserve and ultimately causing POF. Future studies will be done to further investigate the mechanisms underlying GSEXD and verify its safety and efficacy in treating POF.

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Data availability. The data that support the findings of this study are available from the corresponding author upon reasonable request.

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