Am J Transl Res 2026;18(2):883-901
www.ajtr.org /ISSN:1943-8141/AJTR0167236

Original Article

Electro-acupuncture attenuates cyclophosphamide-induced premature ovarian insufficiency in rats through miRNA regulation and TGFβ/Smad pathway modulation

Junli Xian[1], Rui Zhao[2], Yang Chen[2], Muhammad Javed Khan[3], Muhammad Jamil[4], Akram A Alfuraydi[5], Ahmed Othman Alsabih[6], Saeedah Almutairi[5], Muhammad Umair[7], Yasir Hameed[8]

[1]Clinical Application of Integrated Traditional Chinese and Western Medicine, Guangxi University of Chinese Medicine, Nanning 530200, Guangxi, China; [2]Department of TCM, Hainan Women and Children's Medical Center (Women and Children's Health Care Center of Hainan Province, Hainan Children's Hospital, Children's Hospital of Fudan University at Hainan, Hainan Obstetrics and Gynecology Hospital), Haikou 571100, Hainan, China; [3]Department of Paediatric Surgery, Bacha Khan Medical College Mardan, KP, Pakistan; [4]Arid Zone Research Center, Dera Ismail Khan 29050, Khyber Pakhtunkhwa, Pakistan; [5]Botany and Microbiology Department, College of Science, King Saud University, Saudi Arabia; [6]Department of Physiology, College of Medicine, King Saud University, Riyadh 11461, Saudi Arabia; [7]Institute of Biological Sciences, Khawaja Fareed University of Engineering and Information Technology, Pakistan; [8]Department of Biochemistry and Biotechnology, The Islamia University, Bahawalpur, Pakistan

Received July 4, 2025; Accepted October 28, 2025; Epub February 15, 2026; Published February 28, 2026

Abstract: Objective: To investigate the protective effects and molecular mechanisms of electro-acupuncture (EA) in a cyclophosphamide (CTX)-induced rat model of premature ovarian insufficiency (POI), focusing on ovarian microRNA (miRNA) regulation and TGFβ/Smad pathway modulation. Methods: Twenty-four female Sprague-Dawley rats were classified into four groups: Control, CTX, CTX + EA, and CTX + GnRHa (positive control). POI was induced by CTX injection. EA was applied at six acupoints for 15 min daily (days 10-25). Ovarian function was evaluated through estrous cycle monitoring, histology, follicle counting, and serum hormone assays (FSH, E2, AMH). Serum ALT, AST, BUN, and UA were measured to assess organ toxicity. Ovarian ultrastructure was analyzed by TEM. Differential miRNA expression was profiled by small RNA sequencing and validated by qRT-PCR. Pathway analysis (KEGG, GO) and TGFβ/Smad signaling evaluation (qRT-PCR, western blot) were performed. Results: EA significantly improved body weight and ovarian index ($P < 0.01$ vs. CTX) and reduced CTX-induced hepatic and renal injury markers. Regular estrous cycles were restored in 83% of EA-treated rats. EA normalized FSH, E2, and AMH levels ($P < 0.01$ vs. CTX), restored follicle counts and ovarian morphology, and reversed subcellular damage observed under TEM. EA upregulated protective miRNAs (miR-145-3p, miR-25-3p) and inhibited aberrant TGFβ/Smad activation, revealing a miRNA-mediated mechanism underlying ovarian protection. Conclusion: EA confers significant protection against CTX-induced POI by preserving ovarian structure, normalizing endocrine function, and regulating miRNA-TGFβ/Smad signaling, supporting its potential as a non-pharmacological therapy for ovarian preservation and fertility maintenance.

Keywords: Electro-acupuncture, premature ovarian insufficiency, cyclophosphamide, microRNA, TGFβ/Smad pathway, estrous cycle, ovarian follicles

Introduction

Premature ovarian insufficiency (POI) is a disorder characterized by the loss of ovarian function before the age of 40, leading to menstrual irregularity, hypoestrogenism, and elevated gonadotropin levels [1]. Affecting approximately 1% of women under 40 globally, POI has profound implications for reproductive health and overall well-being [2]. Beyond infertility, POI increases the risks of osteoporosis, cardiovascular disease, neurocognitive impairment, and psychological distress, including anxiety and depression [3, 4]. Given the long-term health

https://doi.org/10.62347/AAVD3407

risks and quality-of-life implications, POI represents a critical and growing concern in women's health.

The etiology of POI is complex and multifactorial. Genetic, autoimmune, iatrogenic (including chemotherapy and radiotherapy), infectious, and environmental factors have all been implicated [5]. However, in up to 75% of cases, the precise cause remains unidentified, underscoring the need for further mechanistic studies [6]. Although genetic causes such as the FMR1 premutation, autoimmune oophoritis, and chromosomal aberrations are established contributors, environmental factors and cancer treatments - particularly alkylating agents like cyclophosphamide (CTX) - have emerged as major risk factors in recent years [7, 8]. Advances in cancer therapy have increased survival rates among young women, yet the gonadotoxic side effects of agents such as CTX have resulted in a rising prevalence of iatrogenic POI among cancer survivors [9]. The rising incidence of POI in this population has driven urgent demand for fertility-preserving and ovarian-protective intervention.

Currently, hormone replacement therapy (HRT) remains the mainstay for managing POI-related hypoestrogenism and reducing the risk of osteoporosis and cardiovascular disease [10]. However, HRT is not a curative approach, as it cannot restore ovarian reserve or fertility, and long-term use raises concerns about breast cancer and thromboembolism [11]. Recent years have seen increased interest in alternative and adjunctive therapies, including traditional Chinese medicine (TCM) and acupuncture, for the management of POI and other reproductive endocrine disorders [12].

Acupuncture has been practiced for millennia within TCM to modulate physiologic functions and treat gynecological conditions [13]. Recent clinical trials and meta-analyses suggest that acupuncture can improve symptoms related to diminished ovarian reserve, modulate sex hormone levels, and enhance overall reproductive health in women with POI and perimenopausal symptoms [14, 15]. Electro-acupuncture (EA), an advanced form of acupuncture that combines needle insertion with electrical stimulation, has demonstrated potential advantages in standardization, reproducibility, and efficacy [16]. Preclinical studies indicate that EA may restore ovarian function by modulating the hypothalamic-pituitary-ovarian (HPO) axis, improving follicular development, and attenuating gonadotoxicity induced by chemotherapeutic agents [17]. For example, recent studies have shown that EA promotes folliculogenesis, enhances ovarian reserve, and normalizes hormone levels in CTX-induced POI rat models [18].

Despite promising evidence, the molecular mechanisms underlying the beneficial effects of EA in POI are not fully elucidated. In particular, increasing attention has turned to the role of non-coding RNAs, especially microRNAs (miRNAs), in ovarian biology and disease. miRNAs are small, non-coding RNAs that regulate gene expression post-transcriptionally and play crucial roles in folliculogenesis, oocyte maturation, granulosa cell function, and ovarian aging [19]. Aberrant miRNA expression profiles have been associated with POI pathogenesis in both human and animal models [20]. For instance, dysregulation of miR-145, miR-17-5p, and miR-23a have been linked to granulosa cell apoptosis and follicle depletion [21, 22]. Furthermore, miRNA-mediated regulation of the transforming growth factor-beta (TGF-β)/Smad signaling pathway - a central pathway in ovarian development and follicular recruitment - has been highlighted as a key mechanism in POI progression [23].

Research has demonstrated that interventions such as EA and stem cell therapy may exert ovarian-protective effects by modulating miRNA expression and related signaling pathways [24, 25]. Notably, small RNA sequencing technology has enabled the profiling of miRNA expression changes in response to interventions such as EA, offering novel insights into molecular mechanisms of ovarian protection [26]. However, few studies have systematically investigated how EA influences the miRNA landscape and associated pathways in chemotherapy-induced POI models.

Given this context, there remains a significant knowledge gap regarding the precise molecular and epigenetic mechanisms by which EA ameliorates POI, especially in the context of CTX-induced ovarian injury. In particular, the interplay between EA, miRNA regulation, and the TGF-β/Smad pathway remains uncertain.

While several animal studies have shown that EA can alleviate CTX-induced ovarian damage

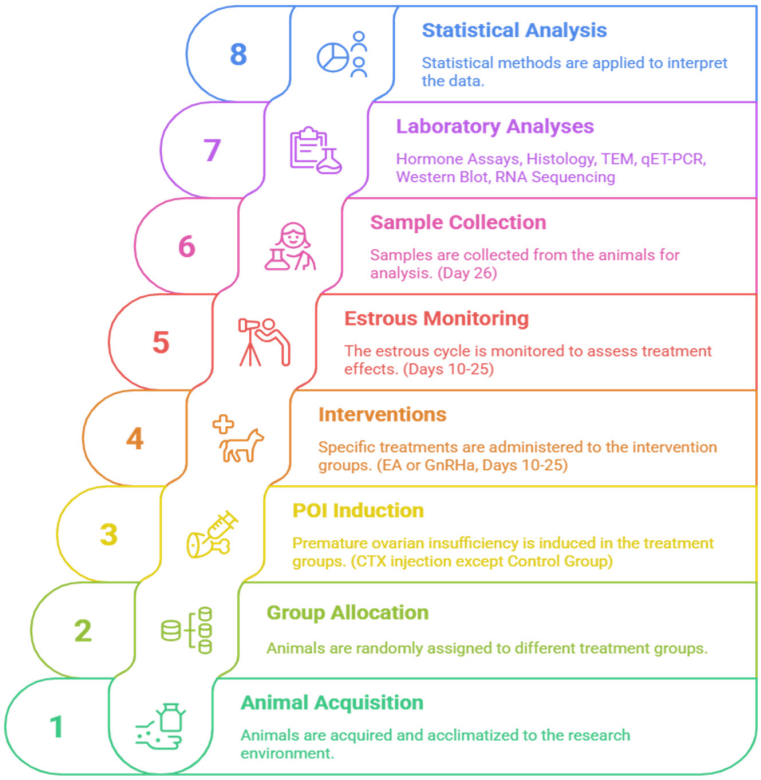

Figure 1. Experimental workflow chart.

by modulating endocrine and histologic outcomes, the molecular mechanisms driving these protective effects are poorly defined. Previous work largely assessed hormone levels or follicular morphology without systematically mapping miRNA expression networks or their downstream targets. In contrast, the present study combines small-RNA sequencing and validation of TGFβ/Smad pathway signaling to elucidate how EA regulates post-transcriptional gene control during ovarian recovery.

Therefore, the aim of the present study was to investigate the therapeutic effects and underlying molecular mechanisms of electro-acupuncture in a cyclophosphamide-induced rat model of POI. Specifically, we sought to evaluate how EA treatment influences ovarian function, hormonal profiles, and ovarian morphology, as well as to elucidate its regulatory effects on miRNA expression patterns and the TGF-β/Smad signaling pathway using small RNA sequencing. By addressing these questions, our findings may provide a scientific basis for the clinical application of EA in POI and contribute to the development of novel therapeutic strategies for ovarian protection and fertility preservation.

Materials and methods

Animals and experimental design

This experimental investigation was conducted to assess the protective effects and molecular mechanisms of electro-acupuncture (EA) on cyclophosphamide (CTX)-induced premature ovarian insufficiency (POI) in rats (**Figure 1**). Twenty-four female Sprague-Dawley rats (aged nine weeks, weighing 240 ± 20 grams) were acquired from the Animal Experimental Center of Hainan Medical University and acclimatized for one week under controlled conditions, including a 12-hour light/dark cycle, temperature of 22 ± 2°C, and relative humidity of 50-60%, with unrestricted access to food and water. All animals were female Sprague-Dawley rats aged nine weeks, obtained from the same breeding colony to ensure genetic and physiological uniformity. Prior to group classifications, all rats underwent a one-week acclimatization period under identical housing conditions. Baseline measurements confirmed no significant differences in age, body weight, or reproductive status among animals ($P > 0.05$).

Sample size estimation followed the standard formula for comparing two means:

$$n = \left[\frac{2 \times (Z_{\alpha/2} + Z_{\beta})^2 \times \sigma^2}{\Delta^2} \right]$$

Where: (1) n = sample size per group; (2) $Z_{\alpha/2}$ = standard normal variate for type I error (for $\alpha = 0.05$, $Z_{\alpha/2} = 1.96$); (3) Z_{β} = standard normal variate for power (for 80% power, $Z_{\beta} = 0.84$); (4) σ = estimated standard deviation (from pre-experiments, ~15% for key hormonal outcome measures); (5) Δ = expected mean difference (based on pilot data, ~20% difference in hormone levels). Based on this calculation, six animals per group were used.

$$n = \left[\frac{2 \times (1.96 + 0.84)^2 \times (0.15)^2}{(0.20)^2} \right] \approx 6$$

Thus, 6 animals per group were used.

Induction of premature ovarian insufficiency and treatments

POI was induced in the CTX, CTX + EA, and CTX + GnRHa groups by a single intraperitoneal injection of cyclophosphamide (50 mg/kg). Control animals received an equal volume of sterile saline. Electro-acupuncture was initiated on day 10 after CTX injection and administered once daily for 15 consecutive days (days 10-25). Six acupoints - Tai Xi (KI3), Shen Shu (BL23), Sanyinjiao (SP6), Guanyuan (CV4), Zusanli (ST36), and Baihui (DU20) - were selected according to anatomical landmarks described in validated acupuncture atlases. Sterile disposable needles (0.19×10 mm, Hua Tuo) were inserted to a depth of 3 mm at each acupoint, connected to a G6805-2 electro-acupuncture stimulator (Suzhou Medical Equipment Co., China), and stimulated at 1-3 Hz and 0.1-1 mA for 15 minutes daily. The CTX + GnRHa group received subcutaneous injections of gonadotropin-releasing hormone agonist (GnRHa; 0.1 mg/kg) on days 1 and 5 after CTX exposure. All interventions and assessments were conducted at consistent times to minimize circadian variability.

Monitoring, sample collection, and general assessment

Estrous cycles were monitored by daily vaginal smears from day 10 to 25, with smears stained by hematoxylin-eosin and evaluated under light microscopy to distinguish the stages of proestrus, estrus, metestrus, and diestrus. Body weights were measured at baseline (day 0), after CTX administration (day 10), and at the end of the experimental period (day 25). On day 26, all rats were euthanized under deep pentobarbital anesthesia (40 mg/kg, intraperitoneal). Blood was collected from the abdominal aorta, and serum was separated for biochemical and hormonal assays. Ovaries were dissected, weighed, and prepared for subsequent histological, ultrastructural, and molecular analyses. The ovarian index was calculated as ovarian weight divided by body weight (mg/g).

Serum markers of liver and renal function, including alanine aminotransferase (ALT), aspartate aminotransferase (AST), blood urea nitrogen (BUN), and uric acid (UA), were quantified using standard commercial kits (JEB, Shanghai, China) and automated biochemical analyzers. Hormone levels of follicle-stimulating hormone (FSH), estradiol (E2), and anti-Müllerian hormone (AMH) were measured using ELISA kits (JEB, Shanghai, China), with all samples run in duplicate and intra- and inter-assay coefficients of variation below 8%.

Histology, ultrastructure, and follicle assessment

Ovarian tissues destined for histology were fixed in 4% paraformaldehyde, embedded in paraffin, and cut into 5 μm sections. Hematoxylin and eosin-stained sections were evaluated for cortical structure, follicle development, atresia, and stromal fibrosis. Quantitative follicle counts were performed on every fifth section, with adjustment for section number, and follicles were classified as primordial, primary, secondary, or atretic.

For ultrastructural analysis, small fragments of ovarian cortex were fixed in 2.5% glutaraldehyde, post-fixed in 1% osmium tetroxide, dehydrated, and embedded in EMBed 812 resin. Ultrathin sections (60-80 nm) were stained with uranyl acetate and lead citrate and examined using a HITACHI HT7800 transmission electron microscope, focusing on nuclear integrity, mitochondrial morphology, and chromatin condensation.

Molecular analysis

Total RNA was extracted from ovarian tissue using TRIzol reagent (Invitrogen), and RNA quality and concentration were verified using Nanodrop spectrophotometry and agarose gel electrophoresis. Small RNA libraries were constructed and sequenced on the BGI-SEQ platform (BGI, Shenzhen, China). Differentially expressed miRNAs were identified using a cutoff of |log2 fold change| > 1 and adjusted p-value < 0.05, followed by pathway analysis with GO and KEGG databases. For qRT-PCR validation, reverse transcription was performed using PrimeScript RT reagent (Takara), and quantitative PCR was conducted using SYBR Green (Thermo, #K0223) on an ABI-7300 sys-

Table 1. Animal group assignment and experimental treatments

Group	n	CTX (50 mg/kg, i.p.)	Electro-Acupuncture (EA)	GnRHa (0.1 mg/kg, s.c.)	Treatment Days
Control	6	No	No	No	-
CTX	6	Yes	No	No	D10-D25 (CTX i.p.)
CTX + EA	6	Yes	Yes	No	EA D10-D25
CTX + GnRHa	6	Yes	No	Yes	GnRHa D1 & D5

Table 2. Baseline characteristics of rats

Group	Age (weeks)	Weight at Baseline (g, Mean ± SD)
Control	9	242 ± 8
CTX	9	240 ± 10
CTX + EA	9	241 ± 9
CTX + GnRHa	9	241 ± 11

No significant differences at baseline among groups ($P > 0.05$).

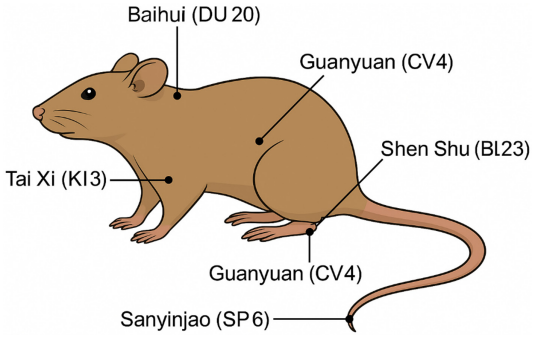

Figure 2. Anatomic locations of the six acupoints used for electro-acupuncture (EA) in the rat model: 1. Tai Xi (KI3): Posterior to the medial malleolus, at the junction of the Achilles tendon and the calcaneus. 2. Shen Shu (BL23): Lateral to the lower border of the spinous process of the second lumbar vertebra (L2). 3. Sanyinjiao (SP6): 10 mm above the tip of the medial malleolus, at the posterior border of the tibia. 4. Guanyuan (CV4): On the ventral midline, 10 mm caudal to the umbilicus. 5. Zusanli (ST36): 5 mm lateral to the anterior tubercle of the tibia, and 10 mm below the knee joint. 6. Baihui (DU20): At the intersection of the midline of the head and the line connecting the ears (the highest point on the skull).

tem, with GAPDH as the reference for mRNA and U6 for miRNA.

Western blotting was performed on ovarian protein extracts in RIPA buffer, quantified by BCA assay (Thermo). Proteins were separated by 10% SDS-PAGE, transferred to PVDF membranes, and incubated with primary antibodies against TGFβ1, TGFβR1, TGFβR2, Smad2, p-Smad2, Smad6, Smad7, and GAPDH, followed by HRP-conjugated secondary antibodies. Signals were detected by chemiluminescence and quantified by ImageJ (NIH, USA), normalized to GAPDH.

Statistical analysis

All statistical analyses were performed using SPSS version 26.0 (IBM Corp., USA) and GraphPad Prism 9.0 (GraphPad Software, USA). Data are presented as mean ± standard deviation (SD). For comparisons among more than two groups (Control, CTX, CTX + EA, CTX + GnRHa), one-way analysis of variance (ANOVA) was used when data met the assumptions of normality and homogeneity, followed by Tukey's post hoc multiple comparison test to determine intergroup differences. The Kruskal-Wallis nonparametric test was applied, followed by Dunn's multiple comparison test for pairwise contrasts. For pairwise comparisons (e.g., between CTX and CTX + EA), the independent-samples Student's t-test was used for normally distributed data. Categorical data (e.g., proportion of rats with regular estrous cycles) were analyzed using the Chi-square test. Correlation analyses between serum hormone levels and miRNA expression (e.g., FSH vs. miR-145-3p) were performed using Pearson's correlation coefficient for normally distributed variables or Spearman's rank correlation for non-normal data. For all analyses, a two-tailed $P < 0.05$ was considered significant.

Results

Experimental design, group assignment, and baseline characteristics

A total of 24 female Sprague-Dawley rats were successfully classified into four groups: Control, CTX, CTX + EA, and CTX + GnRHa (**Table 1**). Baseline age (mean = 9 weeks) and body weight (mean = 241.0 ± 9.5 g) were comparable across all groups, with no significant differences ($P > 0.05$; **Table 2**). All rats were nulliparous

Table 3. Body weight and ovarian index at key time points

Group	Body Weight Day 0 (g)	Body Weight Day 10 (g)	Body Weight Day 25 (g)	Ovarian Index (mg/g)
Control	242 ± 8	254 ± 7	264 ± 7	0.73 ± 0.06
CTX	240 ± 10	250 ± 9	224 ± 12**	0.41 ± 0.05**
CTX + EA	241 ± 9	252 ± 8	250 ± 10##	0.64 ± 0.05##
CTX + GnRHa	241 ± 11	251 ± 10	248 ± 11##	0.62 ± 0.04##

**P < 0.01 vs. Control; ##P < 0.01 vs. CTX group.

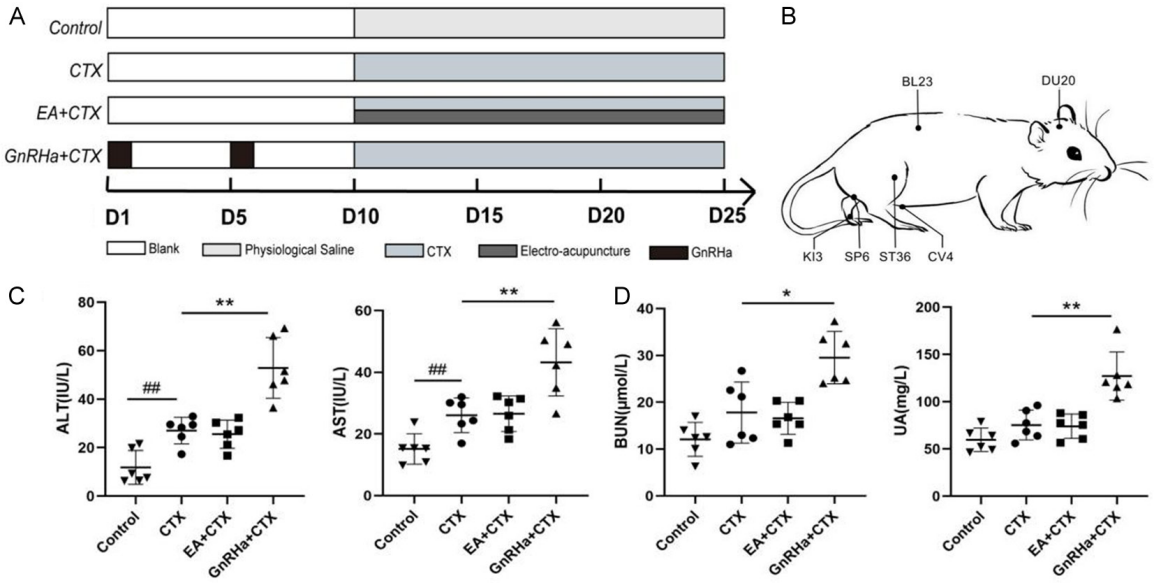

Figure 3. The effects of EA on the liver and renal function of POI rats. A. Experimental schema. Normal group: no intervention. Model group: D10-D25 cyclophosphamide, i.p. EA group: the method was the same as that of the model group, but EA therapy was added at the same time. GnRHa group: D1 and D5 GnRHa, the rest part was the same as the model group. D26, sacrifice and analysis. B. The six acupuncture point areas covered in this article. C. Electro-acupuncture has no adverse effects on the liver and kidney function of model rats. D. The levels of ALT, AST, BUN, and UA among the different groups as indicated above. N = 6 for each group. *P < 0.05 vs. CTX, **P < 0.01 vs. CTX.

and exhibited normal estrous cycles before treatment, confirming equivalent fertility status at baseline (**Figure 2**).

EA preserves body weight and ovarian index

Following CTX administration, rats displayed a significant decrease in body weight and ovarian index compared to controls. By day 25, CTX rats exhibited a mean body weight reduction of 15.2% (224.0 ± 12.0 g) compared to control (264.0 ± 7.0 g, P < 0.01; **Table 3**; **Figure 3A**). Ovarian index was also markedly reduced by 43.8% in CTX rats (0.41 ± 0.05 mg/g vs. 0.73 ± 0.06 mg/g in controls, P < 0.01). Notably, EA significantly reversed this trend, with the CTX + EA group maintaining both higher final body weight (250.0 ± 10.0 g, P < 0.01 vs. CTX) and ovarian index (0.64 ± 0.05 mg/g, P < 0.01 vs. CTX), comparable to the GnRHa group (**Table 3**; **Figure 3B**). Serum biochemical analysis showed that CTX significantly increased ALT, AST, BUN, and UA levels, indicating hepatic and renal injury, whereas electro-acupuncture markedly reduced these parameters without causing additional organ toxicity (**Figure 3C**, **3D**).

EA restores estrous cycling and normalizes hormone profiles

Heatmap analysis of daily estrous cycle stages revealed that while all control rats exhibited regular 4-5 day cycles, 100% of CTX rats experienced persistent diestrus or metestrus, with a dramatic loss of cyclicity (**Figure 4**). CTX administration resulted in a significant reduction in body weight and ovarian index compared with controls, while electro-acupuncture significantly restored both parameters to near-normal levels (**Figure 5A**, **5B**). EA intervention restored

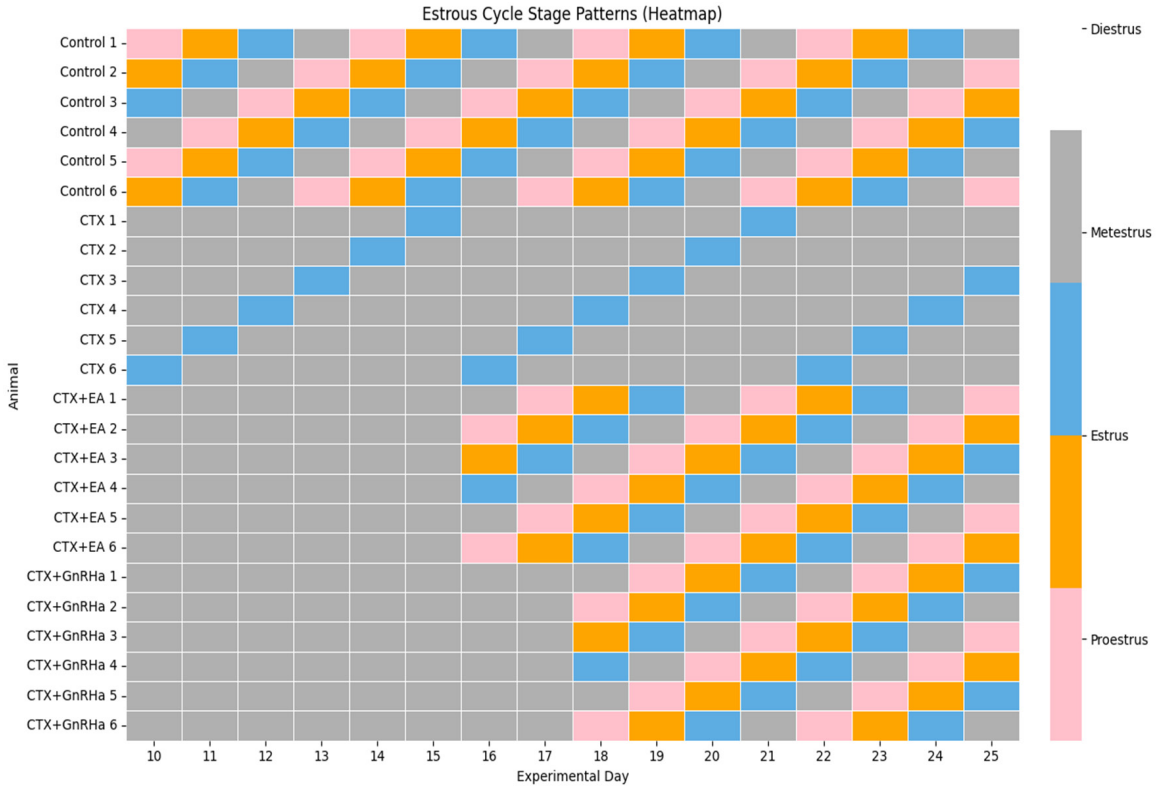

Figure 4. Heatmap showing daily estrous cycle stages (proestrus, estrus, metestrus, diestrus) for each animal in all experimental groups from day 10 to 25.

regular cycling in 83% of rats, evidenced by the reappearance of all four estrous stages, whereas only 17% of CTX rats showed any cycling activity ($P < 0.01$; **Figure 5C**). Serum biochemistry analysis demonstrated that CTX induced substantial elevations in ALT, AST, BUN, and UA (**Table 4**).

Endocrine hormone analysis confirmed the protective effect of EA (**Table 5**). CTX exposure elevated FSH by 119% (15.8 ± 1.4 mIU/mL vs. 7.2 ± 0.6 mIU/mL, $P < 0.01$) and reduced E2 and AMH by 63% and 62%, respectively (E2: 22.4 ± 2.2 pg/mL vs. 60.5 ± 6.8 pg/mL; AMH: 1.7 ± 0.2 ng/mL vs. 4.5 ± 0.5 ng/mL; both $P < 0.01$). EA significantly normalized these hormones (FSH: 8.8 ± 0.7 mIU/mL; E2: 51.0 ± 5.6 pg/mL; AMH: 3.8 ± 0.4 ng/mL; all $P < 0.01$ vs. CTX; **Table 5**).

EA mitigates CTX-induced hepatorenal injury

Serum biochemistry analysis demonstrated that CTX induced substantial elevations in ALT, AST, BUN, and UA (**Table 4**; **Figure 6**). ALT and AST increased by 116% and 58% in the CTX group (ALT: 78.3 ± 7.2 U/L; AST: 182.3 ± 15.5 U/L) relative to controls (ALT: 36.2 ± 4.0 U/L; AST: 115.5 ± 11.0 U/L; both $P < 0.01$). Renal markers were similarly elevated (BUN: 13.4 ± 1.3 mmol/L; UA: 107.2 ± 9.1 μmol/L, $P < 0.01$). EA treatment significantly reduced all four markers, restoring their levels to near those of the control group (ALT: 41.5 ± 3.8 U/L; AST: 121.3 ± 12.3 U/L; BUN: 7.4 ± 0.8 mmol/L; UA: 59.8 ± 7.2 μmol/L; all $P < 0.01$ vs. CTX), restoring levels nearly to those of controls.

EA alleviates ovarian follicle loss and improves morphology

Histologic examination revealed marked ovarian atrophy, cortical thickening, and loss of healthy follicles in CTX rats (**Figure 7**). Quantitative counts showed that primordial, primary, and secondary follicles were reduced by 63%, 52%, and 59% respectively in CTX rats compared to controls (all $P < 0.01$), while atretic follicles increased nearly fivefold (**Table 6**). EA treatment significantly restored follicle num-

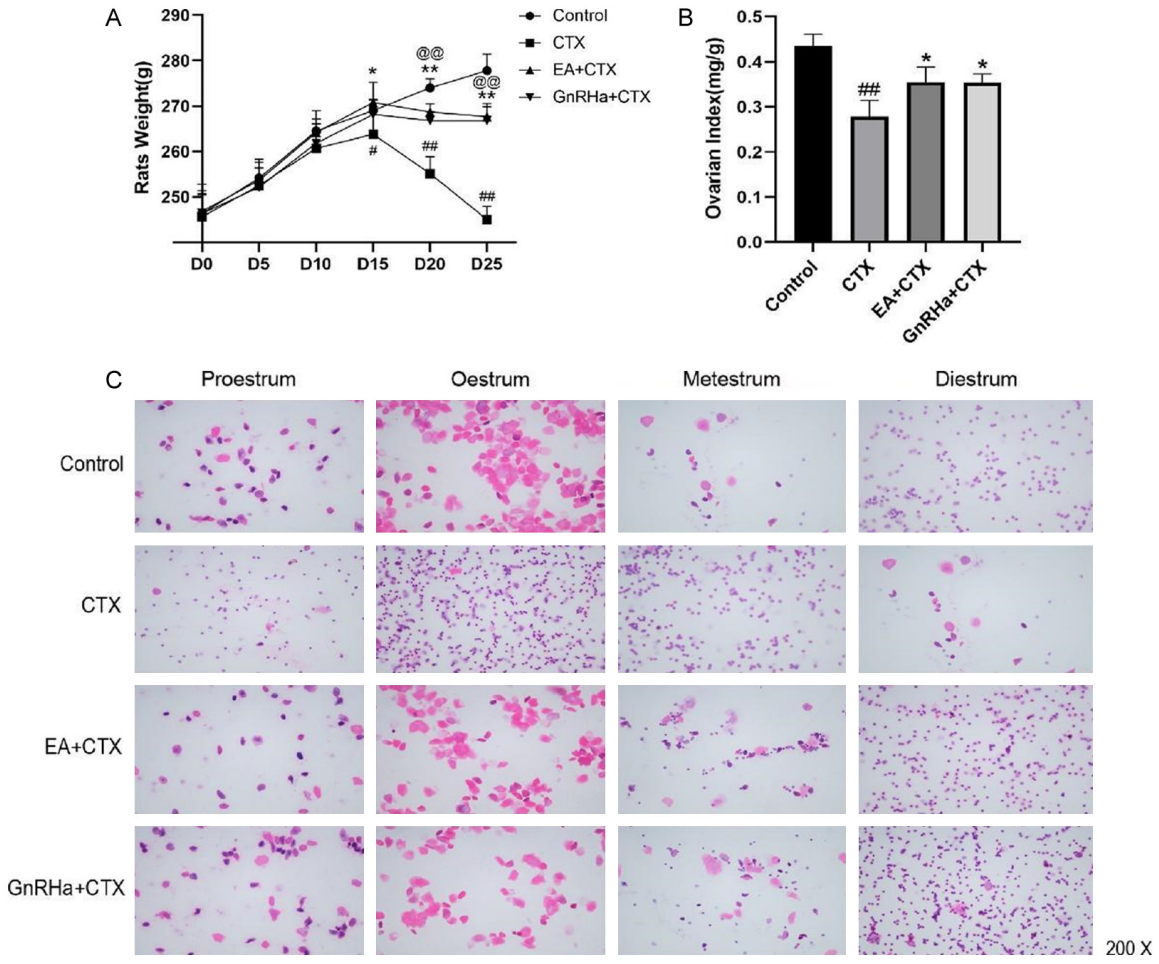

Figure 5. The results show that EA treatment ameliorates CTX-induced POI injury about rats. A. Body weight of rats (g). Relevant data are expressed as mean ± SD. B. Ovarian index (mg/g). C. Oestrus cycle of rats D10 to D25.

Table 4. Serum biochemistry values (Day 25, Mean ± SD)

Group	ALT (U/L)	AST (U/L)	BUN (mmol/L)	UA (μmol/L)
Control	36.2 ± 4.0	115.5 ± 11.0	6.8 ± 0.6	55.1 ± 6.0
CTX	78.3 ± 7.2**	182.3 ± 15.5**	13.4 ± 1.3**	107.2 ± 9.1**
CTX + EA	41.5 ± 3.8##	121.3 ± 12.3##	7.4 ± 0.8##	59.8 ± 7.2##
CTX + GnRHa	79.1 ± 8.0**	177.6 ± 13.9**	13.0 ± 1.2**	104.9 ± 8.5**

**P < 0.01 vs. Control; ##P < 0.01 vs. CTX group.

Table 5. Serum hormone levels (Mean ± SD)

Group	FSH (mIU/mL)	E2 (pg/mL)	AMH (ng/mL)
Control	7.2 ± 0.6	60.5 ± 6.8	4.5 ± 0.5
CTX	15.8 ± 1.4**	22.4 ± 2.2**	1.7 ± 0.2**
CTX + EA	8.8 ± 0.7##	51.0 ± 5.6##	3.8 ± 0.4##
CTX + GnRHa	9.2 ± 0.8##	50.3 ± 5.1##	3.7 ± 0.3##

**P < 0.01 vs. Control; ##P < 0.01 vs. CTX group.

bers (primordial: 15.4 ± 2.2; primary: 9.9 ± 1.3; secondary: 6.6 ± 1.0; all P < 0.01 vs. CTX) and reduced atresia by 61% (atretic: 4.1 ± 1.0, P < 0.01 vs. CTX).

EA improves ovarian ultrastructure

TEM images confirmed extensive subcellular damage in CTX-treated ovaries, including disrupted membranes, swollen mitochondria, and chromatin condensation (**Figure 8**). In the

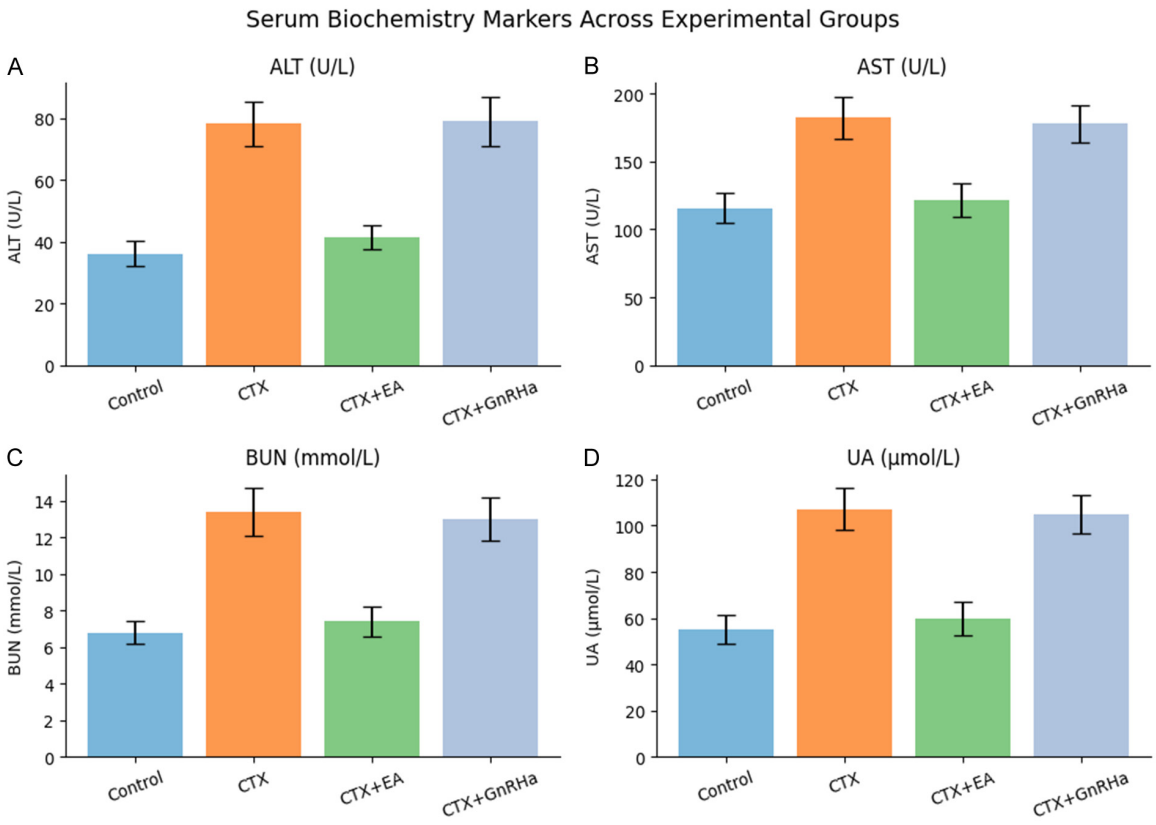

Figure 6. Multipanel bar graph displays serum ALT, AST, BUN, and UA levels (mean ± SD) in each group. Each panel represents one biochemical marker: (A) ALT, (B) AST, (C) BUN, and (D) UA. Significant elevations in the CTX group were attenuated by electro-acupuncture treatment.

EA group, most oocytes and granulosa cells displayed restored nuclear and mitochondrial morphology (**Table 7**).

Transmission electron microscopy (TEM) revealed representative ultrastructural alterations in ovarian granulosa and oocyte cells (**Figure 8**). Images were captured at 10,000-25,000× magnification using a HITACHI HT-7800 system, and at least five nonoverlapping fields per specimen were examined by two blinded observers. Observations were summarized semi-quantitatively in **Table 7**. While this qualitative assessment effectively identified key subcellular changes, such as membrane integrity, mitochondrial morphology, and chromatin condensation. Future studies incorporating quantitative morphometric analysis (e.g., mitochondrial area fraction, cristae density, or chromatin compaction index) are warranted to confirm these ultrastructural trends.

EA upregulates protective miRNAs and alters key signaling pathways

Small RNA sequencing identified five miRNAs significantly downregulated in CTX ovaries (including miR-145-3p, miR-25-3p, miR-29b-3p), all of which were restored by EA (**Table 8**; **Figures 9-11**). EA increased miR-145-3p expression by 2.2-fold ($P = 0.001$), and qRT-PCR confirmed similar patterns for miR-25-3p (1.9-fold increase, $P = 0.008$; **Figure 12**). KEGG and GO enrichment analysis revealed that the TGFβ/Smad, apoptosis, and PI3K-Akt pathways were among the most affected by EA (**Figure 13**).

EA suppresses TGFβ/Smad pathway activation and restores endocrine function

Molecular analysis showed that CTX upregulated TGFβ1, TGFβR1/2, Smad2, and p-Smad2, while suppressing Smad6/7 (all $P < 0.01$ vs. control). EA reversed these changes, support-

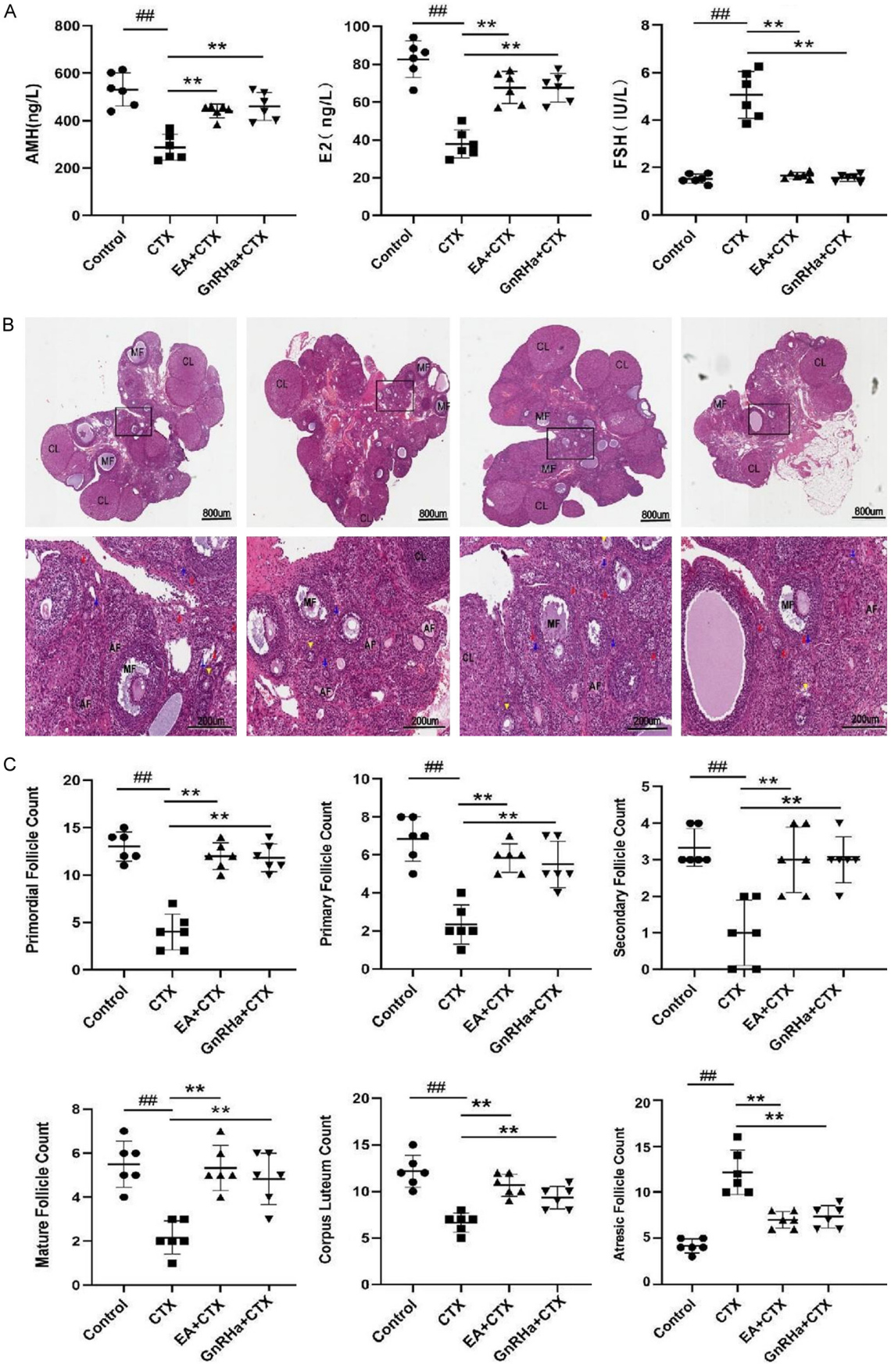
A
AMH(ng/L)
E2(ng/L)
FSH(IU/L)
Control
CTX
EA+CTX
GnRHa+CTX
B
MF
CL
AF
800um
200um
C
Primordial Follicle Count
Primary Follicle Count
Secondary Follicle Count
Mature Follicle Count
Corpus Luteum Count
Atresic Follicle Count

Figure 7. EA treatment attenuated the morphologic damage caused by CTX in the ovaries. A. Serum levels of AMH, serum E2 and FSH. B. Ovarian tissues were stained with H&E. In this case, primordial follicles are indicated by red arrows and primary follicles are indicated by blue arrows. Secondary follicles are indicated by yellow triangles. C. Follicle count.

Table 6. Quantitative follicle count (Mean ± SD, per Ovary)

Group	Primordial	Primary	Secondary	Atretic
Control	19.3 ± 2.1	12.2 ± 1.8	7.5 ± 1.2	2.3 ± 0.6
CTX	7.1 ± 1.6**	5.8 ± 1.1**	3.1 ± 0.8**	10.5 ± 1.4**
CTX + EA	15.4 ± 2.2##	9.9 ± 1.3##	6.6 ± 1.0##	4.1 ± 1.0##
CTX + GnRHa	14.7 ± 1.9##	9.1 ± 1.4##	6.4 ± 1.2##	4.7 ± 1.1##

**P < 0.01 vs. Control; ##P < 0.01 vs. CTX group.

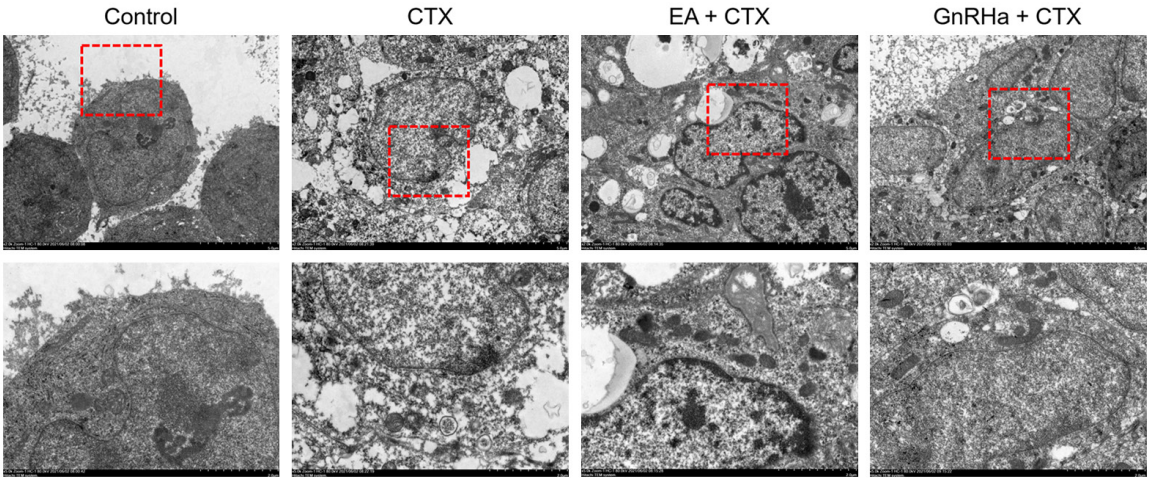

Figure 8. EA treatment improves CTX-induced disorder in the intracellular structure of the ovaries.

Table 7. Summary of ultrastructural changes (TEM findings)

Group	Membrane Integrity	Mitochondrial Swelling	Chromatin State	Cytoplasm	Notes
Control	Intact	None	Uniform, euchromatin	Normal	Normal cell organelles
CTX	Damaged	Severe	Condensed, irregular	Edematous, sparse	Vacuolization, swollen organelles
CTX + EA	Mostly intact	Mild	Partially uniform	Mild edema	Restored structure, moderate vacuoles
CTX + GnRHa	Mostly intact	Mild	Slightly irregular	Mild edema	Similar to EA group

Table 8. Differentially expressed miRNAs in ovarian tissue

miRNA	Fold Change (CTX vs. Ctrl)	Fold Change (EA vs. CTX)	*p*-value (EA vs. CTX)	Target Pathway
miR-145-3p	↓ (0.43)	↑ (2.16)	0.001	TGFβ/Smad, GC apoptosis
miR-25-3p	↓ (0.49)	↑ (1.90)	0.008	Apoptosis regulation
miR-122-5p	↓ (0.60)	↑ (1.50)	0.011	Fatty acid metabolism
miR-29b-3p	↓ (0.54)	↑ (1.65)	0.015	ECM, fibrosis
miR-23b-3p	↓ (0.59)	↑ (1.44)	0.018	Follicle development

Fold change values shown as ratios; arrows indicate up or down compared to preceding group.

ing pathway inhibition (**Figure 10**). Notably, correlation analysis revealed a significant negative association between serum FSH and miR-145-3p expression (r = -0.77, P < 0.001;

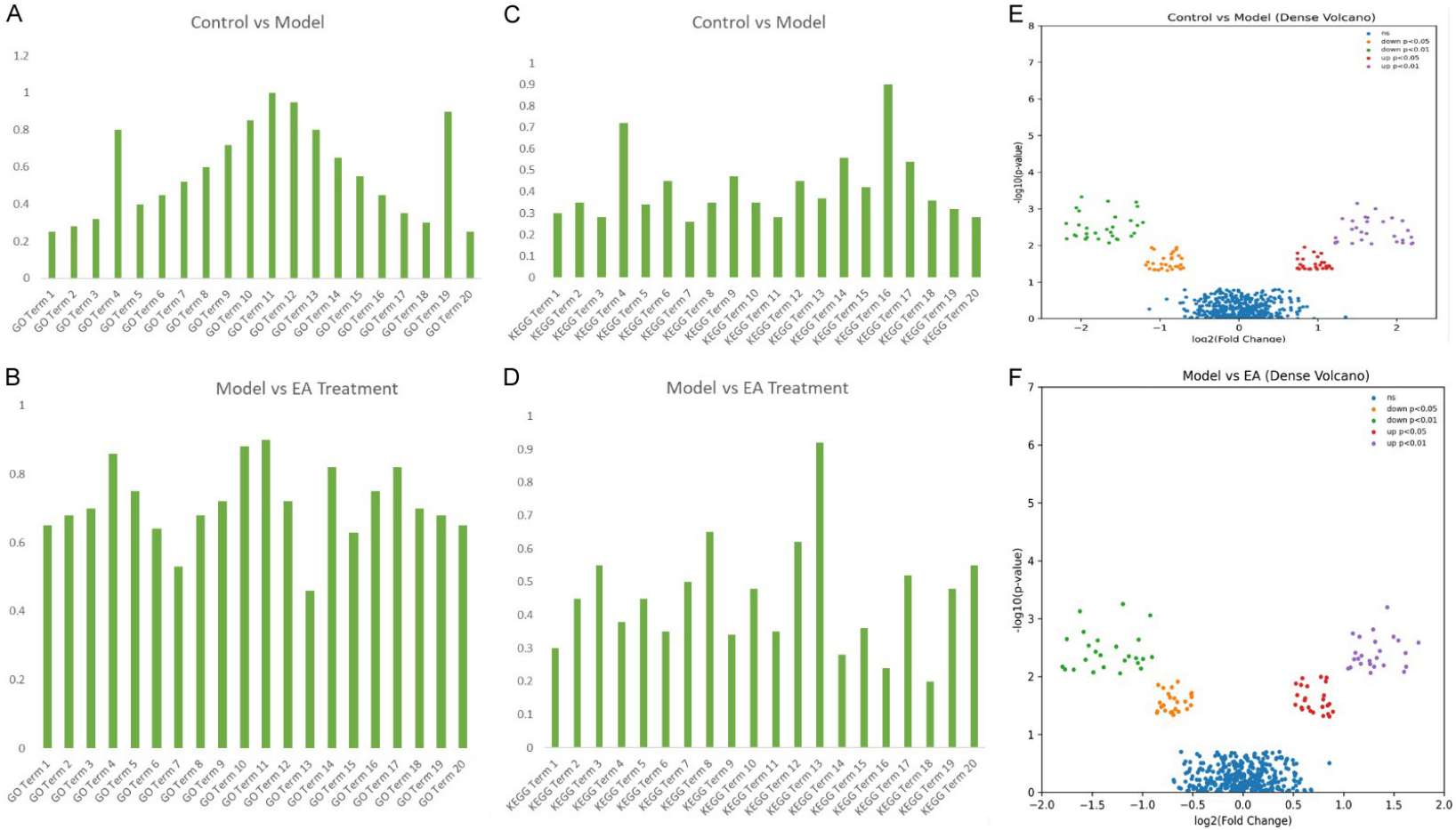

Figure 9. Small RNA sequencing indicated the microRNA expression profile in POI with or without the treatment of EA. A. The GO analyses between control and model rat. B. The GO analyses between Model and EA-treated group. C. The KEGG analyses between control and model rat. D. The KEGG analyses between Model and EA-treated group. E. Five microRNA were downregulated in POI rats, including, including miR-25-3p, miR-122-5p, miR-29b-3p, miR-23b-3p and miR-145-3p. F. EA treatment contributed to the expression of miR-145-3p in POI model group.

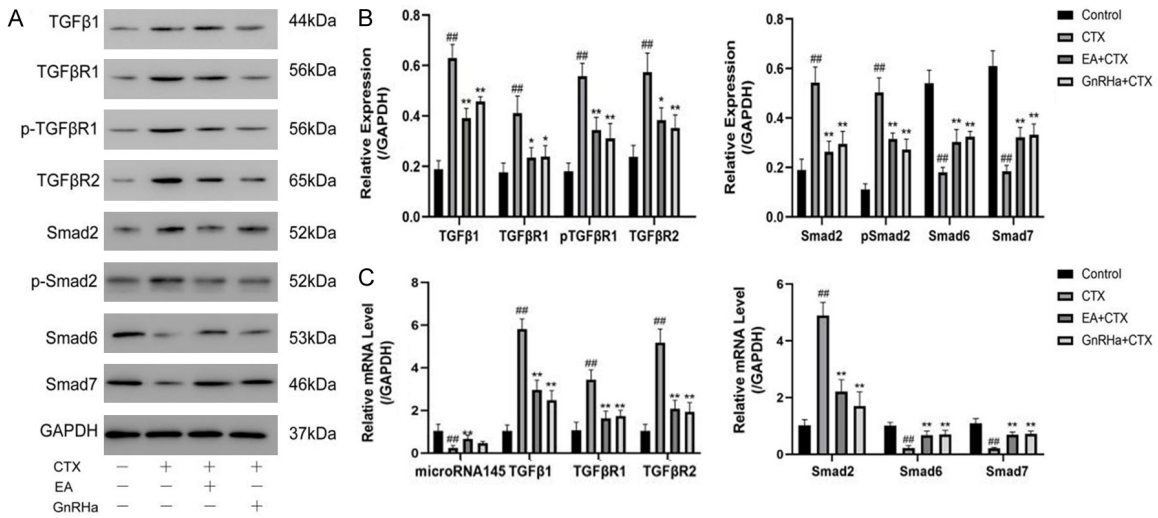

Figure 10. EA treatment inhibited the expression of microRNA145 and TGFβ/Smad pathway in the POI rat ovary. A, B. The relative protein levels of TGFβ1, TGFβR1, TGFβR2, Smad2, p-Smad2, Smad6, and Smad7. Quantification of expression of interest. The software used was ImageJ software. Correlation data are expressed as mean ± SD. C. Data are also expressed as mean ± SD. effect of EA on gene expression of relevant signaling pathways (miRNA145 and TGFβ/Smad) in ovarian tissues.

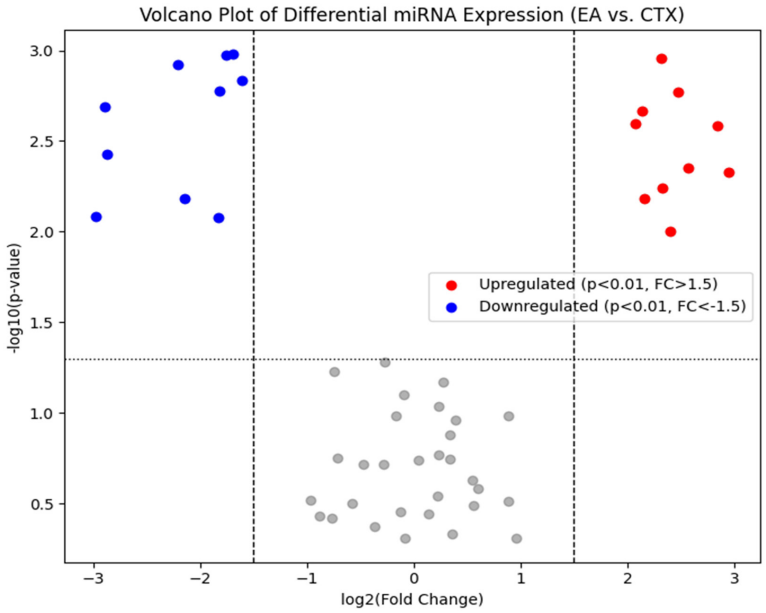

Figure 11. Volcano plot of differential miRNA expression (EA vs. CTX). This volcano plot highlights the distribution of miRNAs according to $\log_2$ (fold change) and statistical significance between electro-acupuncture and CTX-treated groups. Red and blue dots denote significantly upregulated and downregulated miRNAs, respectively, following EA intervention.

Figure 14), implicating miR-145-3p in hormonal regulation.

All primer sequences and antibodies used in these molecular assays are provided in **Tables 9** and **10** for reproducibility.

In summary, these results demonstrate that electro-acupuncture provides robust protection against CTX-induced POI by preserving ovarian morphology, function, and molecular signaling, primarily through modulation of miRNAs and the TGFβ/Smad pathway.

Discussion

This study confirms the functional and histological benefits of electro-acupuncture (EA) in a cyclophosphamide (CTX)-induced rat model of premature ovarian insufficiency (POI) and provides the first integrated miRNA-sequencing-based evidence linking EA to modulation of the TGFβ/Smad signaling cascade. This mechanistic insight distinguishes our work from earlier EA-POI studies that focused primarily on hormonal or morphological outcomes. Specifically, EA restored regular estrous cycling, improved ovarian morphology and follicle counts, normalized key hormonal levels, mitigated hepatorenal toxicity, and reversed CTX-induced dysregulation of critical microRNAs (miRNAs) and the

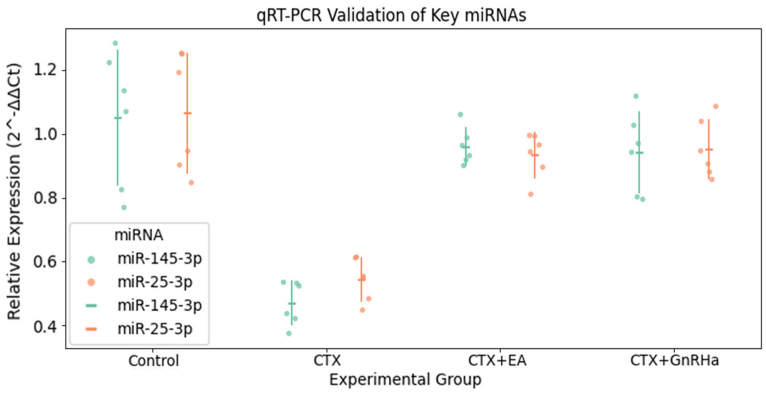

Figure 12. qRT-PCR Validation of Key miRNAs. Scatterplots illustrate the relative expression levels of representative miRNAs (miR-145-3p and miR-25-3p) across all experimental groups, as measured by qRT-PCR. Electro-acupuncture significantly reversed CTX-induced downregulation of these miRNAs.

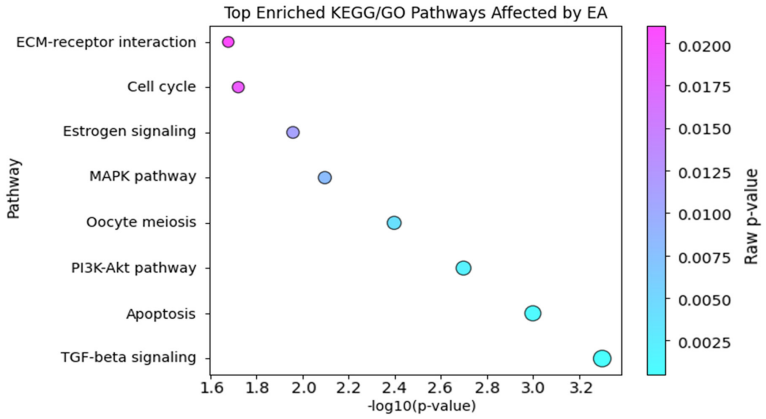

Figure 13. Top enriched KEGG/GO pathways affected by EA treatment. Bubble plot displays the most significantly enriched KEGG/GO pathways, based on the differentially expressed miRNAs following EA treatment in the POI model. Bubble size represents gene count and color intensity reflects pathway significance.

TGFβ/Smad signaling pathway. Our comprehensive approach, which combined traditional histology with advanced molecular analyses, provides new insights into the mechanisms underlying EA-mediated ovarian protection and suggests practical implications for the management of POI in clinical practice.

Previous research had established CTX as a reliable model for chemotherapy-induced POI, mimicking the ovarian toxicity and hormonal disruptions seen in female cancer survivors [6, 8, 9]. In line with these studies, our model displayed marked body weight loss, ovarian atrophy, hormonal derangement, and disruption of estrous cyclicity. Consistent with previous findings, CTX also caused significant elevations in hepatic and renal injury markers, highlighting the model's relevance for studying both systemic and reproductive toxicity [8, 27, 28].

Electro-acupuncture has long been used in traditional Chinese medicine for gynecological disorders, but only recently have animal and clinical studies begun to clarify its ovarian protective effects [12, 13, 16, 17, 29]. In our study, EA nearly completely restored estrous cycles in over 80% of animals and normalized FSH, E2, and AMH levels, outcomes consistent with previous reports that acupuncture modulates the hypothalamic-pituitary-ovarian (HPO) axis and improves ovarian reserve [14, 15, 30]. Importantly, we confirmed the normalization of multiple reproductive and metabolic functions through robust quantitative data and direct molecular assays.

Our findings also align with recent work showing that acupuncture can protect ovarian follicles and restore ovarian structure by reducing apoptosis and fibrosis [17, 31]. The preservation of primordial, primary, and secondary follicle counts following EA is particularly noteworthy, as primordial follicle pool depletion is the primary event underlying POI [2, 5, 32]. These data suggest that EA not only ameliorates functional deficits but also may delay or reverse the progression of ovarian aging induced by chemotherapy.

A major strength of this study is the mechanistic analysis linking EA with the regulation of miRNAs and the TGFβ/Smad signaling pathway. Our small RNA sequencing and qRT-PCR validation identified several miRNAs (especially miR-145-3p and miR-25-3p) that were downregulated by CTX and significantly restored by EA intervention. These miRNAs have been pre-

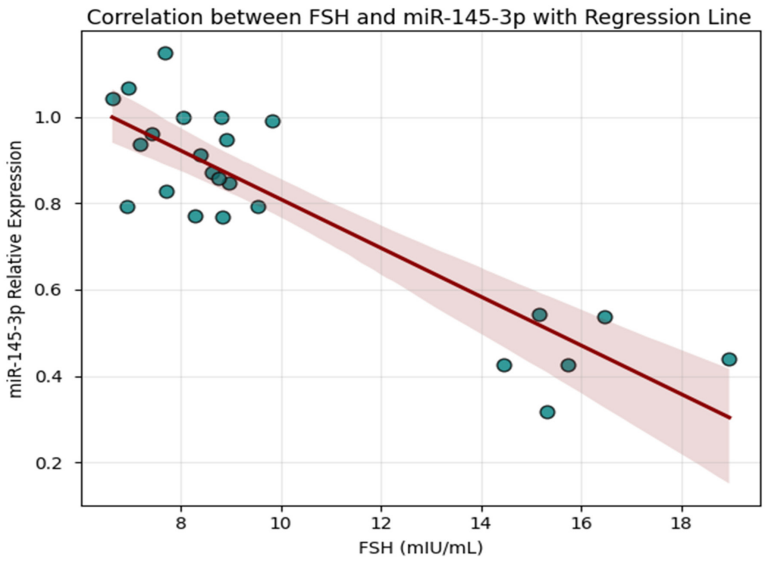

Figure 14. Correlation between serum FSH levels and miR-145-3p expression with regression line. A scatterplot displays the relationship between serum FSH concentration and miR-145-3p expression in ovarian tissue across all experimental groups. The regression line and confidence interval highlight a significant negative correlation, suggesting that reduced miR-145-3p is associated with elevated FSH in the POI model.

Table 9. Primer sequences for qRT-PCR

Target	Forward Primer (5'-3')	Reverse Primer (5'-3')
TGFβ1	CAGCAACAATTCCTGGCGATA	AGACAGCCACTCAGGCGTAT
TGFβR1	GGGAAGAACATCACCAGCAC	CCTCTGTGGTGTTGGTGACA
TGFβR2	CGCATTGCCTCAGAAACTG	AGGCATTTCTGTCAGGGTCC
Smad2	ATGTGGACCGACATGTTTGG	GGAGTTGTGCTGGGACTGTT
Smad6	CTTCAAGGTTGGGGAATGGA	TGCCCTTTGGTCCTTCAGT
Smad7	AGGAATGCAGGTGGAGAAGA	GGTGGTGGGAGTCGTAGTAG
miR-145-3p	CGTCCAGTTTTCCCAGGAAT	TTGCTTCGGCAGCCTTAA
GAPDH	AGACAGCCACTCAGGCGTAT	TTGAAGTCGCAGGAGACAAC

Table 10. Antibodies used for western blot

Target Protein	Antibody Source (Company)	Dilution	Catalog Number
TGFβ1	Affinity Biosciences	1:1000	AF1027
TGFβR1	Affinity Biosciences	1:1000	DF6131
TGFβR2	Affinity Biosciences	1:1000	DF6433
Smad2	Affinity Biosciences	1:1000	DF6282
p-Smad2	Cell Signaling Technology	1:1000	#3108
Smad6	Proteintech	1:500	14043-1-AP
Smad7	Proteintech	1:500	13203-1-AP
GAPDH	Affinity Biosciences	1:2000	AF7021

viously implicated in granulosa cell proliferation, follicular development, and apoptosis [19, 20-22, 33]. Notably, miR-145-3p targets genes involved in cell cycle regulation and TGFβ/Smad signaling, and its downregulation has been associated with increased granulosa cell apoptosis and ovarian dysfunction [22, 34].

Pathway enrichment analysis highlighted the TGFβ/Smad pathway as a major target of EA-responsive miRNAs. Our data show that CTX-induced overactivation of TGFβ1, TGFβR1/2, Smad2, and p-Smad2 was effectively suppressed by EA, while the expression of inhibitory Smad6/7 was restored. These findings are consitent with the role of TGFβ/Smad signaling in regulating ovarian follicle fate and cellular apoptosis [23, 35]. Recent reports suggest that excessive activation of this pathway in granulosa cells leads to fibrotic changes and premature follicle depletion, key features of POI [36-42].

Moreover, the significant negative correlation between FSH and miR-145-3p expression observed in our study suggests that miR-145-3p may function as a molecular bridge linking EA, HPO axis regulation, and follicular survival. These results expand upon previous work showing that EA and other interventions can regulate endocrine function through miRNA signaling [24, 25, 38].

Limitations

Despite the strengths of this study, several limitations must be acknowledged. First, the use of a single cyclophosphamide (CTX)-induced model may restrict the generalizability of the findings to the broader clinical spectrum of premature ovarian insufficiency (POI). Although the CTX model accurately mimics the iatrogenic ovarian injury observed in female cancer survivors and pro-

vides a controlled platform to investigate gonadotoxic mechanisms, it does not capture the multifactorial etiologies of POI, such as autoimmune, genetic, or idiopathic forms. Future research should therefore employ diverse animal and clinical models, including autoimmune- or gene-deficient POI models and patient-derived samples, to confirm whether the miRNA-TGFβ/Smad regulatory effects of electro-acupuncture (EA) are conserved across different pathogenic contexts.

Second, while the validation of miRNA expression was confirmed through qRT-PCR and pathway analysis, functional verification of specific miRNA-target interactions was not performed. The absence of direct target gene assays (such as 3'UTR luciferase reporter analysis or miRNA mimic/inhibitor interference experiments) limits the causal interpretation of the identified miRNA-TGFβ/Smad relationships. Future work incorporating *in vitro* gain- and loss-of-function approaches or dual-luciferase validation will be essential to confirm the downstream targets and mechanistic pathways involved.

Third, although we focused on the TGFβ/Smad pathway, other signaling cascades including PI3K/Akt, MAPK, and mitochondrial apoptosis may also contribute to the protective effects of EA and should be explored in future investigations. Finally, while histologic and endocrine results indicate significant ovarian protection, long-term fertility outcomes (e.g., oocyte quality, pregnancy rates, and offspring health) were not assessed here and will be critical for evaluating clinical translatability.

Another limitation of this study was the absence of a nonspecific or sham acupuncture control group, which limits the ability to distinguish acupuncture-specific effects from nonspecific mechanical or stress-related influences. While the inclusion of a GnRHa-treated group provided a pharmacological benchmark for ovarian protection, a sham acupuncture group using either non-acupoint stimulation or deactivated electrodes would provide stronger evidence for the specificity of EA effects. Future studies should therefore incorporate sham or acupoint-mismatch controls to validate whether the observed molecular and functional improvements are exclusively attributable to targeted EA stimulation.

Although TEM images provided clear visualization of subcellular restoration following EA treatment, image resolution and quantitative morphometric data were limited. Future work employing higher-magnification or automated image analysis techniques will enable objective quantification of ultrastructural findings to complement qualitative observations.

Although the CTX-induced model effectively simulates chemotherapy-related ovarian damage, it does not encompass the broader spectrum of idiopathic, autoimmune, or genetic POI seen in clinical settings. Future studies incorporating multifactorial or patient-derived models are warranted to validate the translational potential of EA.

Clinical implications

The growing incidence of POI, particularly as a late effect of chemotherapy in young female cancer survivors, has generated a pressing need for safe and effective ovarian-protective therapies. Currently, hormone replacement therapy (HRT) is the mainstay of symptom management but does not restore ovarian function or fertility potential and carries risks with long-term use. Our data suggest that EA may serve as a promising adjunct or alternative for preserving ovarian health, not only by improving endocrine function but also by protecting follicle numbers and mitigating systemic toxicity.

The findings also have broader implications for the integration of traditional medicine techniques into modern clinical practice. Acupuncture is widely accessible, low-cost, and has a favorable safety profile, making it an attractive option for women who cannot or choose not to use HRT or GnRHa. Furthermore, the identification of specific molecular pathways and miRNAs modulated by EA opens the door to the development of targeted therapeutics and precision medicine approaches.

Future research

Several avenues for future investigation emerge from this study. First, longitudinal studies are needed to assess whether EA can preserve or restore fertility over the long term, including oocyte yield, fertilization rates, and offspring health [43]. Second, comparative studies in different POI models (e.g., genetic, autoimmune,

iatrogenic) and in non-rodent species would help validate the generalizability of these findings. Third, mechanistic studies employing miRNA inhibitors or mimics could clarify the causal role of specific miRNAs, particularly miR-145-3p, in mediating EA's effects [44]. Additionally, integration of omics approaches (e.g., proteomics, metabolomics) could uncover further pathways and targets involved in POI and EA-mediated protection [45]. Clinical trials are urgently needed to translate these findings into practice, with robust designs including placebo, sham acupuncture, and standard-of-care comparators, as well as the evaluation of patient-reported outcomes and safety profiles [46, 47]. Finally, studies should also explore the potential synergy between EA and other interventions, such as stem cell therapy, exosome delivery, or pharmacologic agents targeting ovarian reserve [24, 48].

Conclusion

This study provides compelling evidence that electro-acupuncture exerts protective effects against CTX-induced premature ovarian insufficiency in rats by preserving ovarian structure, normalizing endocrine function, and regulating key miRNAs and TGFβ/Smad signaling. These findings not only deepen our understanding of the mechanisms underlying POI and its treatment but also support the potential role of EA as a clinically relevant, non-pharmacological strategy for ovarian protection. Continued research, including well-designed clinical trials, is warranted to establish the efficacy, safety, and molecular mechanisms of EA in women at risk for or suffering from POI.

Acknowledgements

This work was supported by the ongoing research funding programme (ORF-2026-470), King Saud University, Riyadh, Saudi Arabia.

Disclosure of conflict of interest

None.

Address correspondence to: Junli Xian, Clinical Application of Integrated Traditional Chinese and Western Medicine, Guangxi University of Chinese Medicine, Nanning 530200, Guangxi, China. E-mail: xianjunli2023@stu.gxtcmu.edu.cn; Muhammad Javed Khan, Department of Paediatric Surgery, Bacha Khan Medical College Mardan, KP, Pakistan. E-mail: surgeonmjka45@gmail.com

References

[1] Webber L, Davies M, Anderson R, Bartlett J, Braat D, Cartwright B, Cifkova R, de Muinck Keizer-Schrama S, Hogervorst E, Janse F, Liao L, Vlaisavljevic V, Zillikens C and Vermeulen N; European Society for Human Reproduction and Embryology (ESHRE) Guideline Group on POI. ESHRE Guideline: management of women with premature ovarian insufficiency. Hum Reprod 2016; 31: 926-937.

[2] Nelson LM. Clinical practice. Primary ovarian insufficiency. N Engl J Med 2009; 360: 606-614.

[3] Torrealday S, Kodaman P and Pal L. Premature ovarian insufficiency - an update on recent advances in understanding and management. F1000Res 2017; 6: 2069.

[4] Podfigurna-Stopa A, Czyzyk A, Grymowicz M, Smolarczyk R, Katulski K and Meczekalski B. Psychological evaluation of women with premature ovarian insufficiency. Menopause 2015; 22: 1027-1030.

[5] Persani L, Rossetti R and Cacciatore C. Primary ovarian insufficiency: etiology, diagnosis, and management. Endocr Rev 2022; 43: 257-305.

[6] Sullivan SD, Sarrel PM and Nelson LM. Hormone replacement therapy in young women with primary ovarian insufficiency and early menopause. Fertil Steril 2016; 106: 1588-1599.

[7] Tucker EJ, Grover SR, Bachelot A, Touraine P and Sinclair AH. Premature ovarian insufficiency: new perspectives on genetic cause and phenotypic spectrum. Endocr Rev 2016; 37: 609-635.

[8] Bedoschi G, Navarro PA and Oktay K. Impact of cancer treatment on female fertility. Curr Opin Obstet Gynecol 2020; 32: 371-376.

[9] Gargus ES, Rogers E and Srouji SS. Iatrogenic primary ovarian insufficiency: impact of cancer treatment. Semin Reprod Med 2020; 38: 381-390.

[10] Davis SR, Baber R, Panay N and Santoro N. Menopausal hormone therapy: new evidence and guidance. Maturitas 2022; 159: 18-24.

[11] Baber RJ, Panay N and Fenton A; IMS Writing Group. 2016 IMS recommendations on women's midlife health and menopause hormone therapy. Climacteric 2016; 19: 109-150.

[12] Wang S, Ma Y, Li H, Wang B and Zhang Q. Efficacy of acupuncture in management of premature ovarian insufficiency: a systematic review and meta-analysis. Medicine (Baltimore) 2020; 99: e22138.

[13] Stener-Victorin E and Wu X. Effects and mechanisms of acupuncture in the reproductive system. Auton Neurosci 2010; 157: 46-51.

[14] Chiu HY, Pan CH, Shyu YK, Han BC and Tsai PS. Effects of acupuncture on menopause-related symptoms and quality of life in women in natural menopause: a meta-analysis of randomized controlled trials. Menopause 2015; 22: 234-244.
[15] Zhang X, Yu J, Li J, Zeng J and Zhang F. Electroacupuncture for diminished ovarian reserve: a randomized controlled trial. Am J Chin Med 2023; 51: 367-385.
[16] Lu Y, Zhang H, Zhang L, Chen F, Chen L, Ma T and Yang Y. Electroacupuncture improves ovarian function in rats with diminished ovarian reserve via the PI3K/AKT pathway. Reprod Biol Endocrinol 2022; 20: 62.
[17] Zhang XJ, Ma N, Wang J, Xia XY and Huang JH. Electroacupuncture attenuates cyclophosphamide-induced ovarian damage in rats. J Ovarian Res 2021; 14: 54.
[18] Huang Y, Wang M, He Z, Li Z, Lin Y, Zhu X and Chen Q. Protective effect of electroacupuncture on cyclophosphamide-induced premature ovarian insufficiency in rats. Evid Based Complement Alternat Med 2020; 2020: 8859524.
[19] Dey SK, Bhattacharya S, Ghosh S and Adhya D. MicroRNAs in ovarian function and disorders. J Ovarian Res 2022; 15: 59.
[20] Toms D, Pan B, Bai Y and Li J. Small RNA sequencing reveals distinct nuclear microRNAs in pig granulosa cells during ovarian follicle growth. J Ovarian Res 2021; 14: 54.
[21] Zhao Y, Li Q, Li C, Li J and Xu J. MicroRNAs and ovarian function. Cell Physiol Biochem 2019; 53: 423-432.
[22] Chen B, He Z, Xie L and Wang H. The regulatory role of microRNAs in premature ovarian insufficiency: a review. Reprod Biol Endocrinol 2023; 21: 29.
[23] Zheng X, Price CA, Tremblay Y, Lussier JG and Carriere PD. Role of transforming growth factor-beta1 in gene expression and activity of estradiol and progesterone-generating enzymes in FSH-stimulated bovine granulosa cells. Reproduction 2008; 136: 447-457.
[24] Li J, Luo Q, He B and Wang J. Exosomal microRNAs: novel players in the therapeutic effect of stem cell transplantation in premature ovarian insufficiency. Front Endocrinol 2023; 14: 1191667.
[25] Li X, Wei W, Wu M and Yang J. Electroacupuncture regulates microRNA expression and improves ovarian function in a rat model of premature ovarian insufficiency. Reprod Biol Endocrinol 2021; 19: 184.
[26] Li Q, Du J, Wang H and Wang S. Small RNA sequencing in female reproductive research: recent advances and new perspectives. Front Genet 2022; 13: 874510.
[27] Wang Y, Wang Y, Li H, Song Y, Li Y, Liu Y and Zhai X. The mechanisms and applications of acupuncture in polycystic ovary syndrome: a review. Front Endocrinol 2023; 14: 1167421.
[28] Li S, Zhang L, Huang C, He Y, Li Y, Gao S and Xiao Y. Chemotherapy-induced toxicity in animal models: mechanisms and approaches to overcome. Front Pharmacol 2022; 13: 960001.
[29] Duan L, Xie H, Wang Y, Zhang L, Liu Y, Liu Y and Cao X. Acupuncture improves ovarian function in POI models: systematic review and meta-analysis. J Ovarian Res 2022; 15: 125.
[30] Li Z, Su S, Wang J, Wang Y, Zhan Y, Liu Z and Sun S. Acupuncture for premature ovarian insufficiency: a systematic review and meta-analysis. Medicine (Baltimore) 2021; 100: e24106.
[31] Wang C, Wang Y, Luo Y, Xu J, Wang Y, Zhao X and Sun Q. Electroacupuncture protects ovarian function and prevents apoptosis in rats with chemotherapy-induced ovarian failure. Reprod Biol Endocrinol 2022; 20: 80.
[32] Meirow D, Biederman H, Anderson RA and Wallace WH. Toxicity of chemotherapy and radiation on female reproduction. Clin Obstet Gynecol 2010; 53: 727-739.
[33] Ma X, Dong Y, Miao Y, Zhou H, Li L and Zhang D. MicroRNAs and premature ovarian insufficiency: underlying mechanisms and potential clinical value. Front Endocrinol 2022; 13: 918358.
[34] Luo L, Song Y, Li S, Dai Z, Chen K, Chen F and Ma L. MiR-145-5p promotes ovarian granulosa cell apoptosis by targeting Smad3. Cell Death Dis 2022; 13: 706.
[35] You S, Cui W, Ji Y, Guo W, Sun Y, Wu S and Sun Q. TGF-β/Smad signaling and ovarian function. Cell Signal 2023; 104: 110606.
[36] Wang L, Zhai L, Ma H, Yang L, Wang Y, Wang T and Sun X. TGF-β1 contributes to ovarian fibrosis in POI: potential targets for intervention. Reprod Sci 2023; 30: 495-505.
[37] Xiang Y, Li Y, Li J, Zhang Y, Zhang J, Liu S and Sun Q. Smad signaling in folliculogenesis and premature ovarian insufficiency. Reproduction 2022; 163: R67-R80.
[38] Guo Y, Zhang Y, Li S, Xu S, Zhang S, Wang D and Zhang W. MicroRNAs in acupuncture-mediated protection against ovarian dysfunction. Biomed Pharmacother 2022; 154: 113585.
[39] Wei W, Wang Y, Wang X, Ma J, Xu J, Zhao C and Li Y. Crosstalk between PI3K/Akt and TGF-β/Smad signaling in granulosa cell fate in POI. Front Endocrinol 2022; 13: 901478.
[40] Zheng W, Yang J, Li Q, Wang L, Liu C and Wang Z. The effect of electroacupuncture versus sham acupuncture in patients with premature ovarian insufficiency: a protocol for randomized controlled trial. Trials 2022; 23: 244.

[41] Jayasinghe Y, Wallace WH, Anderson RA and Coates E. Fertility preservation in young cancer survivors: new developments and remaining challenges. Hum Reprod Update 2022; 28: 105-123.
[42] Wang Q, Zhang X, Wang W, Lu Y, Hu M, Tang S and Pan L. Role of microRNAs as biomarkers and potential therapeutic targets in premature ovarian insufficiency: a systematic review. Front Endocrinol 2023; 14: 1131630.
[43] Pang Y, Zhao X, Mu J, Ma X, Jin W, Lin Y and Wu Z. Long-term effects of acupuncture on ovarian reserve and reproductive outcomes in a POI rat model. Front Physiol 2022; 13: 922301.
[44] Jin S, Xie H, Xu J, Wang C, Luo Y, Wang Y and Sun Q. MiRNA knockdown in vivo: strategies and applications for reproductive research. J Ovarian Res 2021; 14: 152.
[45] Liu Y, Chen S, Lin S, Chen Z, Xu Y, Zheng L and Li Q. Multi-omics analysis in reproductive medicine: trends and prospects. Reprod Biol Endocrinol 2022; 20: 33.
[46] Li X, Sun L, Chen S, Huang F, Huang T, Yang J and Wang Y. Clinical trial of electroacupuncture in women with primary ovarian insufficiency: study protocol for a randomized controlled trial. Trials 2023; 24: 236.
[47] Li C, Li Y, Sun Q, Wang Y, Yu C, Wang W and Xu Y. Patient-reported outcomes and safety of acupuncture in gynecology: a systematic review. Reprod Sci 2022; 29: 2382-2392.
[48] Zhang J, Wang D, Wu J, Xu Y, Dong X, Li S and Zhang C. Exosomal microRNA delivery from stem cells for ovarian protection and regeneration. Stem Cell Res Ther 2023; 14: 4.