

Research Article

American Ginseng Regulates Gene Expression to Protect against Premature Ovarian Failure in Rats

Lei Zhu,¹ Ji Li,² Nannan Xing,¹ Dongwei Han,² Haixue Kuang,³ and Pengling Ge^{1,4}

¹Department of Pharmacology, School of Basic Medical Sciences, Heilongjiang University of Chinese Medicine, 24 Heping Road, Harbin 150040, China

²The Key Laboratory of State Administration of Traditional Chinese Medicine of the People's Republic of China, Department of Formulas of Traditional Chinese Medicine, School of Basic Medical Sciences, Heilongjiang University of Chinese Medicine, Harbin 150040, China

³The Key Laboratory of Chinese Ministry of Education, Department of Traditional Chinese Medicine, School of Pharmacology, Heilongjiang University of Chinese Medicine, Harbin 150040, China

⁴The Key Laboratory of Myocardial Ischemia (Harbin Medical University) of Chinese Ministry of Education, Harbin 150086, China

Correspondence should be addressed to Haixue Kuang; kuanghaixue@hljucm.net and Pengling Ge; penglingge@126.com

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Premature ovarian failure (POF) is defined as lost ovarian functions before the age of 40. Three possible molecular markers (*PLA2G4A*, *miR-29a*, and *miR-144*) have been identified in our previous study by integrated analysis of mRNA and miRNA expression profiles. The present study aimed to evaluate American ginseng root's protective potential against POF by studying transcriptional and protein variations between American ginseng treatments and controls in rats. 4-Vinylcyclohexene diepoxide (VCD) was administered to rats for 14 days to induce POF. Additionally, American ginseng was administered to POF rats for one month, and *PLA2G4A*, *miR-29a*, and *miR-144* expressions were measured in rat ovaries by qRT-PCR. *PLA2G4A* protein expression was examined by Western Blot, and PGE₂, LH, FSH, and E2 serum levels were detected by ELISA. *PLA2G4A* mRNA and protein were downregulated in American ginseng-treated rats, *miR-29a* and *miR-144* levels increased, and PGE₂ serum levels decreased, while LH, FSH, and E2 increased compared to POF induction alone. Analysis of transcriptional and protein variations suggested that American ginseng protects the ovary against POF by regulating prostaglandin biosynthesis, ovulation, and preventing ovarian aging. High hormone levels (PGE₂, FSH, and LH) were reduced, and E2 secretion approached normal levels, leading to improved POF symptoms and abnormal ovulation.

1. Introduction

Premature ovarian failure (POF), also known as premature ovarian insufficiency, is characterized by amenorrhea for at least four to six months before the age of 40 with sex steroid deficiency and raised serum concentrations of follicle-stimulating hormone (FSH) of more than 40 IU/L occurring at least 1 month apart [1]. Clinical diagnosis of POF is recognized as amenorrhea (>6 months) with estrogen deficiency and high concentrations of luteinizing hormone (LH) and FSH (>20 IU/L) before the age of 40 [2]. Ovarian insufficiency often starts as secondary amenorrhea with increased FSH levels, which is also known as transitional ovarian failure [3].

Many risk factors may contribute to POF, such as physical and chemical factors, radiation and chemotherapy, ovarian failure following hysterectomy, autoimmune diseases, or hereditary factors. There is still no effective clinical treatment for POF because its etiology still remains unclear. Many women with POF are advised to undergo long-term hormone replacement therapy (HRT), which helps to relieve the symptoms of perimenopausal syndrome that can have significant impact on the woman's quality of life. In the current study, we successfully induced the occurrence of POF in rats using an ovotoxic chemical 4-vinylcyclohexene diepoxide (VCD), which can specifically accelerate the atresia of primordial and primary follicles in rodents when primordial follicles are depleted [4].

The herb American ginseng (*Panax quinquefolius* L., Araliaceae) is one of the top ten selling natural health products in the United States [5]. American ginseng is used as an antifatigue drug and as an immunostimulant during periods of stress. In a study by Duda et al., American ginseng was shown to induce the expression of pS2, a protein that may exhibit estrogen-like effects on estrogen receptor-positive breast cancer cells [6]. In addition, American ginseng was previously studied in the mouse adipose cell line 3T3-L1 to determine its potential to inhibit proliferation, decrease the percentage of cells in S phase, and induce the expression of adiponectin, a euglycemic agent [7]. Another study showed that ginseng saponin treatment can ameliorate central nervous system (CNS) disorders and neurodegenerative diseases [8, 9]. In addition, several studies have indicated that American ginseng saponins can significantly improve cognitive abilities and emotional fluctuations [10–12].

Ginseng has been used as a nutritional supplement in East Asia for thousands of years and has recently gained popularity in the West because of its various pharmacological properties. Many experimental studies have shown that ginseng has estrogenic [6], anticancer [13], and hypoglycemic effects [14, 15] and can improve impaired memory and learning [16], which may contribute to its effect on the prevention or treatment of POF secondary diseases, such as dementia, diabetes mellitus, metabolic syndrome, osteoporosis, and certain cancers. Thus, we hypothesized that American ginseng may exert protective effects against POF and its associated complications.

In the last few years, microRNAs (miRNAs) have been found as new cell regulators for messenger RNA (mRNA) gene expression [17, 18]. miRNAs are small, noncoding RNAs in length of 20–24 nucleotide that can repress mRNA expression by binding to the 3' untranslated regions (UTR) of target mRNA, which leads to translational repression, mRNA cleavage, and deadenylation [19, 20]. Each miRNA can influence the expression of multiple target mRNAs and each mRNA can be regulated by several miRNAs [21].

In this preliminary observational study, we confirmed that *PLA2G4A* was overexpressed in POF ovarian tissues. We hypothesized that *PLA2G4A* was a candidate gene indirectly involved in POF occurrence and development by increasing prostaglandin concentration, which is important in ovulation. We also determined efficient associations between expression of both miRNAs and mRNA in target gene sets, by investigating the miRBase, MiRanda, and miRDB databases [22]. The expression of *miR-144* and *miR-29a* has been shown to suppress *PLA2G4A* transcription or translation or to cut the mRNA so that it is targeted for degradation. In our previous study, we also demonstrated that *miR-29a* and *miR-144* expression decreased in POF ovarian tissues. Therefore, the mechanism by which American ginseng is protective against POF may involve regulation of such potential targeted gene and miRNAs.

PLA2G4A expression can induce arachidonic acid (AA) release [23]. Prostaglandin (PG) biosynthesis is dependent on AA release [24] and synthetases, including PG endoperoxide synthase (PTGS) and specific PG synthase enzymes [25]. AA is converted to PGG₂ through the bifunctional

enzymes cyclooxygenase- (COX-) 1 or COX-2, resulting in the intermediate PGH₂, which is ultimately converted into prostaglandin estradiol (PGE₂) and other subtypes or thromboxanes (TX) by cell-specific synthases [26]. Matsumoto and Espey previously showed that prostaglandins regulate a series of important physiological processes, including female fertility and the reproductive lifespan [27, 28]. During the 1970s, the importance of prostaglandins and their connection to ovulation became increasingly apparent. PGE₂ is secreted by follicles and regulates ovulation [25, 29]. *PLA2G4A* expression is important in this process, as injection of rats with the specific *PLA2G4A* inhibitor arachidonyl trifluoromethyl ketone significantly decreased intraovarian bursal ovulation and total ovarian PGE₂ synthesis [30]. These data highlight a role for *PLA2G4A* activity in ovulation and PGE₂ synthesis.

Based on previous studies and the various pharmacological properties of American ginseng, our study aimed to establish the association between American ginseng and POF. Furthermore, we explored the molecular mechanism by which American ginseng may regulate gene expression and hormone secretion to protect against POF.

2. Materials and Methods

2.1. Animals and Treatment. Adult female Sprague Dawley rats (200 ± 20 g weight) were provided by the Yisi Laboratory Animal Technology Co., Ltd. (Changchun, China).

Rats were individually housed under constant temperature (20 ± 1°C), humidity (50 ± 5%), and light (12 h/d) conditions with standard pellet diet and water provided *ad libitum*. The animal protocol was approved by the Animal Experimental Ethical Committee of Heilongjiang University of Chinese Medicine.

Fifteen female Sprague Dawley rats (3 months old) were divided into three groups: control group ($n = 5$), 4-vinylcyclohexene diepoxide- (VCD-) induced (model) group ($n = 5$), and American ginseng-treated group ($n = 5$). VCD-induced rats and American ginseng-treated rats were both intraperitoneally (i.p.) injected with sesame oil or sesame oil plus VCD (80 mg/kg/day) for 14 consecutive days. At the same time, American ginseng-treated rats were administered American ginseng (2.25 g/kg body weight) by daily oral gavage. The control group was given equal volumes of physiological saline. After the final VCD injection, the American ginseng-treated group continued to drench for 30 days. Animals had daily access to food and water *ad libitum* for observation of toxicity and mortality.

The dosage, route of administration, and duration of treatment were based on our previous study. After 30 days, ovaries and blood serum were collected, weighed, rapidly frozen by liquid nitrogen, and stored at -80°C. The ovary weight index by the following formula: ovary weight index = ovarian weight/body weight.

2.2. RNA Extraction and qRT-PCR. Following the manufacturer's instructions, total RNA was extracted from whole ovaries by TRIzol (Invitrogen, Carlsbad, CA, USA) and an miRNA easy minikit (Qiagen, Valencia, CA, USA). RNA concentrations were determined with a NanoDrop ND-1000

TABLE 1: qRT-PCR primers for mRNA.

mRNA	Forward primer sequence [5' → 3']	Reverse primer sequence [5' → 3']
Pla2G4a	GACGCAGCGGTAGCAGAT	TCAAGG GATACGGCAGGT
β -actin	GTCAGGTCATCACTATCGGCAAT	AGAGGTCTTTACGGATGTCAACGT

TABLE 2: qRT-PCR primers for miRNA.

miRNA	DNA sequences
rno-miR-29a-3p	5'-CGTAGCACCATCTGAAATCGGTTA-3'
rno-miR-144-5p	5'-CGCGGGATATCATATATACTGTAAGT-3'
U6	5'-ACACGCAAATTCGTGAAGCGTTCC-3'

spectrophotometer (Wilmington, DE, USA). Finally, RNA integrity was assessed by denatured agarose gel electrophoresis.

For qRT-PCR experiments, total isolated RNA was reverse-transcribed into cDNA with an AccuPower RocketScript RT Premix (Bioneer, Daejeon, Korea) according to the manufacturer's instructions. The experiment was performed in duplicate using AccuPower GreenStar qPCR master mix according to the manufacturer's instructions. Tables 1 and 2 show the primer sequences used to amplify fragments. The data were normalized to expression levels of the housekeeping genes β -actin and U6, respectively, and equation $2^{-\Delta\Delta C(t)}$ was used to calculate relative expression levels. PCR products were analyzed by agarose gel electrophoresis to ensure that only a single band of the expected size was amplified.

2.3. Enzyme-Linked Immunosorbent Assay (ELISA) Analysis. PGE₂, FSH, LH, and E2 serum levels were assayed with an ELISA kit (R&D Quantikine, R&D Systems Inc., Minneapolis, MN, USA). Each test was performed in triplicate, and five independent experimental results were used for statistical analysis.

2.4. Western Blot Analysis. PLA2G4A protein expression was verified in ovarian tissue samples by Western blot, and β -actin was used as a loading control.

Protein aliquots (50 μ g) from ovaries treated with VCD, VCD and ginseng or with no treatment was loaded onto a 12% sodium dodecyl sulfate gel, and electrotransferred to a PVDF membrane. Membranes were blocked in 10% nonfat dried milk. The membrane was incubated with rat anticytosolic phospholipase A2 (PLA2G4A; Santa Cruz Biotechnology, Santa Cruz, CA, USA, 1:1000) at 4°C for 12 h. Blots were incubated with HRP-conjugated IgG (1:1000; Sigma-Aldrich, St. Louis, MO, USA) at room temperature for 1 h. The PLA2G4A protein was detected by chemiluminescence (Pierce, Rockford, IL, USA).

2.5. Statistical Analysis. The data were expressed as mean \pm SEM, and the means among different groups were analyzed by one-way ANOVA post hoc tests. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Differential Expression Analysis of mRNA, miRNAs, and Protein in POF. To determine whether PLA2G4A expression changed upon American ginseng treatment, we performed RT-PCR and found that PLA2G4A expression significantly decreased after American ginseng treatment. Additionally, the upstream miRNAs *miR-29a* and *miR-144* were downregulated compared to the POF model ($P < 0.05$, Figure 1(a)). Western blot analysis confirmed that PLA2G4A protein expression was downregulated compared to POF ovarian tissue (Figure 1(b)).

3.2. Ovary Weight Index. Compared to the control group, POF ovaries had clearly atrophied. Ovaries from American ginseng-treated rats had also mildly atrophied but not to the extent of POF ovaries (Figure 2).

3.3. American Ginseng Treatment Partially Rescues Serum Hormones Levels. By ELISA, we determined that FSH and LH serum levels increased after POF induction and were partially rescued by American ginseng treatment (Figure 3(a)). However, values of LH serum level were differentially expressed but showed no significant fold change ($P > 0.05$) between the treatments. Similarly, PGE₂ serum levels increased in POF animals compared to controls. Serum levels were partially rescued by American ginseng treatment. In contrast, POF induction decreased E2 levels, which were partially restored by American ginseng treatment, bringing them closer to control levels (Figure 3(b)).

4. Discussion

POF may be caused by several risk factors, including chemicals, iatrogenic agents, ovarian failure following hysterectomy, autoimmune diseases, or hereditary factors. However, the underlying molecular etiology remains complex. In our early studies, we integrated the data of several differentially expressed miRNAs and related mRNAs in ovarian tissue from POF rat models to identify potential targeted genes and estimated their efficient associations with POF [22]. Therefore, the potential targeted genes we identified may be those candidate genes regulated by American ginseng. In this study, we used the same treatment regimens and

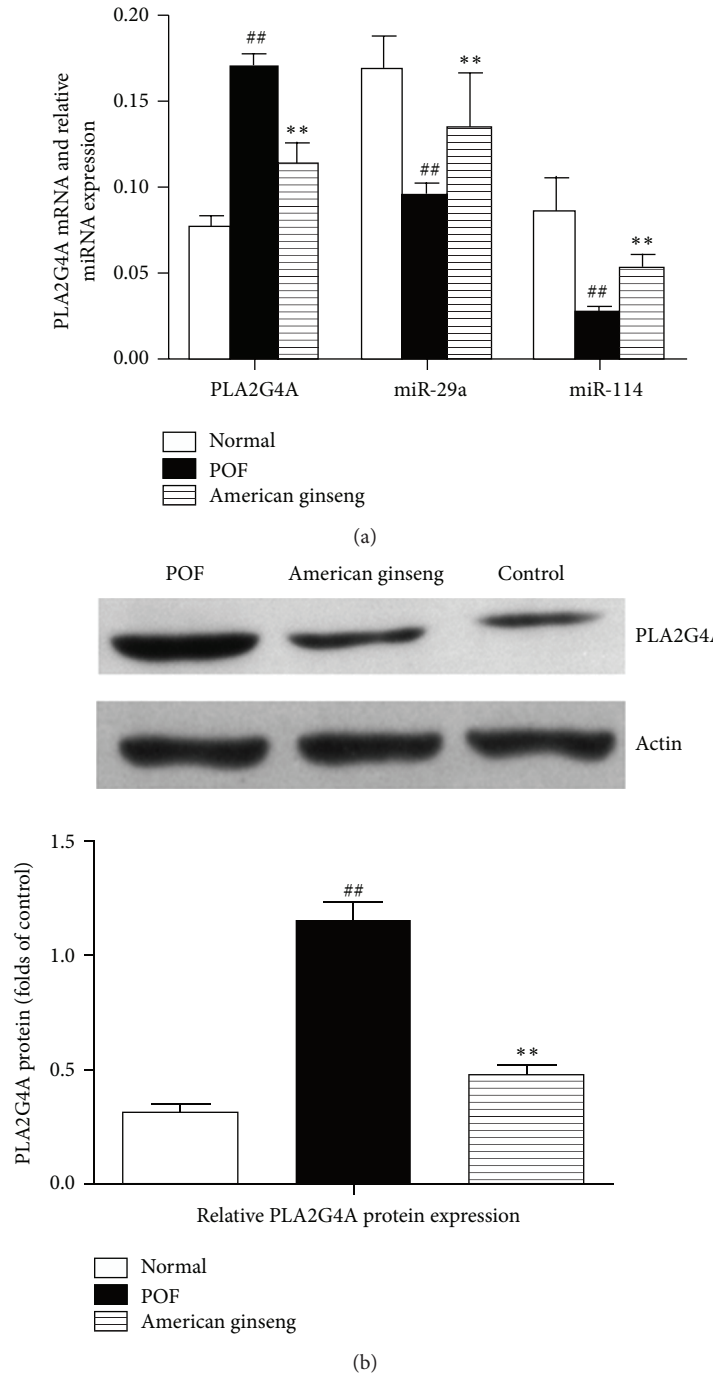


FIGURE 1: Validation of differential *miR-29a*, *miR-144*, *PLA2G4A mRNA*, and *PLA2G4A* protein expression in American ginseng-treated POF tissues. (a) *miR-29a* and *miR-144* and *PLA2G4A* levels in ginseng-treated ovaries were determined by qRT-PCR. (b) Western blotting for *PLA2G4A* protein in the absence or presence of American ginseng. The expression level trended toward control levels. Data were expressed as mean \pm SEM from five independent experiments performed in duplicate. ^{**} $P < 0.01$ versus VCD-treated groups and ^{##} $P < 0.01$ versus control.

found that American ginseng significantly reduced *PLA2G4A* expression and increased *miR-144* and *miR-29a* expression in POF ovarian tissues compared to POF induction alone. We found that the differentially expressed genes were involved in prostaglandin biosynthesis, which plays an important role in ovulation. As expected, PGE_2 concentrations decreased after

American ginseng treatment compared to POF induction alone. Furthermore, the increased ovary weight index and E2 concentrations in American ginseng-treated rats compared to POF induction alone suggest that American ginseng partially reduces ovarian atrophy and prevents abnormal ovulation and subsequent occurrence and development of

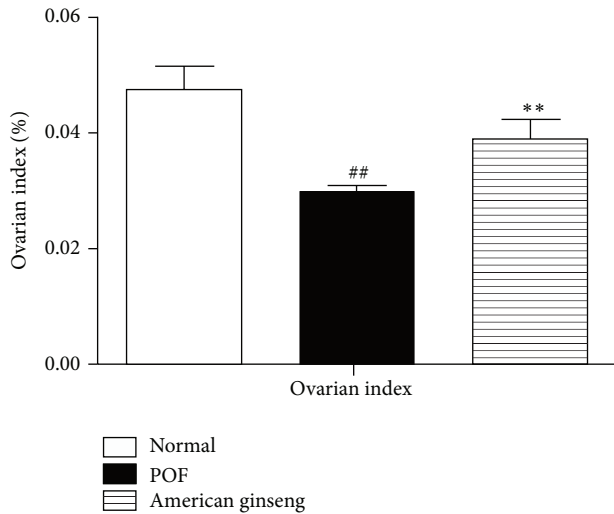


FIGURE 2: Ovary weight. Ovarian index = ovarian weight/body weight; data were expressed as mean \pm SEM. ** $P < 0.01$ versus VCD-treated groups and ## $P < 0.01$ versus control.

POF. These data further suggest that American ginseng has potent protective effects against VCD-induced POF. In the current study, we detected mRNA and miRNAs that are regulated by American ginseng and are involved in important biological events likely contributing to POF development, such as prostaglandin biosynthesis, ovulation, hormone secretion, and ovarian aging.

A series of clinical findings have suggested that *PLA2G4A* is indirectly involved in ovulation [25]. When *PLA2G4A* was knocked out in mouse ovaries, ovulation and fertilization rates were significantly reduced compared to control littermates [31]. Previous studies have shown that ovulation is preceded by the induction of *PLA2G4A* expression in the granulosa cells of bovine ovulatory follicles [25]. In our previous studies, we confirmed that *PLA2G4A* was overexpressed in POF ovaries and hypothesized that upregulation of *PLA2G4A* may indirectly result in the occurrence and development of POF by increasing prostaglandin concentration.

PLA2G4A is a catalytic enzyme in AA biosynthesis [29]. Prostaglandin biosynthesis requires AA release and *PLA2G4A* is essential for proper induction of PTGS2, an isoform of the PTGS synthetase in prostaglandin biosynthesis [32]. PTGS2 is necessary for the response to proinflammatory stimuli [33]. Kurusu et al. have shown that *PLA2G4A* cellular and temporal expression coincides with PTGS2 expression in follicles [31]. The LH surge induces ovarian PTGS2 expression and then induces an increase in PGE₂ (a subtype of prostaglandin) production in preovulatory follicles, which are two critical factors in oocyte maturation [34] and ovulation [27]. Previous studies have also demonstrated that *PLA2G4A* is required for prostaglandin biosynthesis. In the current study, our data suggest that American ginseng can suppress *PLA2G4A* mRNA and *PLA2G4A* protein expression in ovarian tissues. Furthermore, American ginseng treatment reduced LH secretion, though not significantly, in serum of POF rats, which may lead to the downregulation of PGE₂ and the rescue of abnormal ovulation.

It has previously been reported that prostaglandin is important for ovulation. *PLA2G4A* contributes to AA release, a substrate necessary for the release of proinflammatory mediators, such as PGs and leukotrienes [35]. Our data revealed that PGE₂ serum levels decreased in POF rats treated with American ginseng. At present, ovulation is primarily considered an acute and self-steering inflammatory reaction, including local edema, white blood cell release from the blood vessel, and activation of proteolytic enzymes and collagenolytic enzymes [36]. These changes result in follicle wall rupture and mature egg release. Recently, *PLA2G4A* mRNA levels were found to increase during the proresolving phase of the acute inflammatory process [37]. PGE₂ can induce inflammation, fever, and pain and induce ovulation. Animal studies have shown that the number of CD68+ cells, macrophages, and neutrophils significantly increased in the follicular fluid after treatment with PGE₂, which can activate the expression of ovarian proteolytic enzymes and further promote follicular rupture and egg release [38]. Ristimäki et al. injected PGE₂ or its derivatives into monkeys treated with indomethacin, an inflammation inhibitor, prior to ovulation to restore ovarian function [39]. Therefore, increasing PGE₂ levels may indirectly lead to POF. Regulation of PGE₂ levels may be American ginseng's primary mechanism of action to protect against POF.

In addition, PGE₂ affects neuroendocrine cells in the hypothalamus, which can induce gonadotrophic releasing hormone (GnRH) release. GnRH stimulates the anterior pituitary gland to secrete LH and FSH [2]. LH can regulate hormone production, and FSH is responsible for follicle development. In this study, we found that ovary weight ratios increased after American ginseng treatment compared to POF induction alone, and their ratios tended to decrease towards the control rats. The restoration of ovarian weight may contribute to restoring ovarian function. Additionally, we observed increased serum levels of E2 after treatment with American ginseng and decreased FSH and LH secretion by the hypothalamic-pituitary-ovarian axis. Therefore, the decrease in FSH and LH levels may be due to the reduction of PGE₂ and increase in E2 induced by American ginseng in POF rats. The recovery of hormone production further prevented the primordial follicle pool from being abnormally depleted.

To further confirm whether the differential expression of *PLA2G4A* was directly induced by American ginseng, we performed qRT-PCR analysis to determine whether American ginseng affected the expression of *miR-144* and *miR-29a*, both of which can suppress *PLA2G4A* expression. Our results suggested that *PLA2G4A* downregulation might be secondary to the upregulation of *miR-144* and *miR-29a* induced by American ginseng treatment. However, it is also possible that American ginseng may regulate *miR-144* and *miR-29a* expression to specifically restore *PLA2G4A* levels. Indeed, Yao et al. previously showed that *miR-29a* expression was significantly suppressed in FSH-treated cultured rat granulosa cells [40]. Moreover, a recent study showed that follicular thyroid carcinoma tissues had decreased *miR-144* expression, resulting in activated mTOR signaling [41], which can accelerate ovarian aging and induce POF [42]. In our

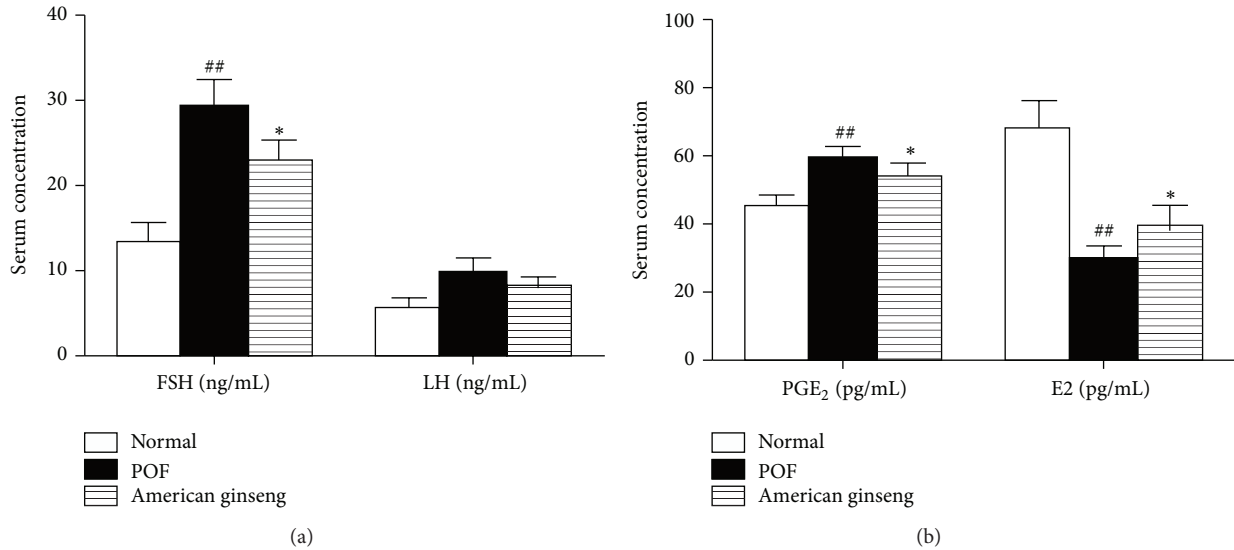


FIGURE 3: PGE₂, E₂, FSH, and LH serum levels. PGE₂, FSH, LH, and E₂ serum levels were detected by ELISA. Data are summarized and presented as mean \pm SEM from four independent experiments. * $P < 0.05$ versus VCD-treated groups and ^{##} $P < 0.01$ versus control.

previous study, we demonstrated that *miR-29a* and *miR-144* expression decreased in POF ovarian tissues. This inverse correlation might verify our current results, indicating that upregulated *miR-29a* and *miR-144* induced by American ginseng treatment may play a significant role in protecting ovarian function by regulating the response to hormone stimulation and preventing ovarian aging.

From our analysis, we found that American ginseng protects against POF by altering mRNA and miRNAs expression and hormone levels. The changes in the *PLA2G4A* and *miR-29a* and *miR-144* genes suggest that American ginseng exerts its effect by regulating prostaglandin biosynthesis, ovulation, and preventing ovarian aging. The high hormone levels (PGE₂, FSH, and LH) were reduced and E₂ secretion approached normal levels upon American ginseng treatment, which led to improved symptoms of POF and abnormal ovulation. Although further research is necessary to elucidate the active constituents of American ginseng and to validate the function of ginseng in POF women and rats, our study expands our understanding of American ginseng's pharmacological activities as an anti-POF agent.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Lei Zhu and Ji Li contributed equally to this work.

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