

Original Research

Biphasic Effect of TNF- α on the Survival and Endocrine Function of Human Ovarian Granulosa Cells

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Abstract

Background: TNF- α has a wide range of functions in processes within the ovaries, including follicular development, ovarian hormones synthesis, ovulation, granulosa cell necroptosis, and follicular atresia. Innate immune response in the ovaries is the main source of TNF- α . The purpose of this study is to explore the dose- and time-dependent interactions between TNF- α and human granulosa cells. **Methods:** The follicular fluids (FF) were obtained from infertile patients undergoing IVF with PCOS (n = 20) and healthy women (n = 19). Granulosa cells were cultured and treated with varying concentration of recombinant human TNF- α . Cell viability was detected by CCK-8 method, the levels of TNF- α , estradiol, and inhibin were measured with ELISA, and cell apoptosis was detected by flow cytometry. Q-PCR and western-blot were used to evaluate the expression of TNFR1/2, P450, Inhibin α/β A, and caspase 3. **Results:** The level of TNF- α increased gradually with the development of follicles and was remarkably higher in the FF of PCOS patients than in that of healthy women. Treatment with TNF- α as low as 0.2 ng/mL promoted the proliferation of granulosa cells, thus upregulating P450 aromatase and inhibin A. On the other hand, a high concentration of TNF- α (0.4, 0.8, 2, and 8 ng/mL) showed dose- and time-dependent inhibitory effects on cell viability and E2 and inhibin A secretion. However, no marked differences in the expression of the two types of TNFR were observed in TNF- α -treated granulosa cells. **Conclusions:** The data presented here verify a biphasic effect of TNF- α on human ovarian endocrine and follicular development.

Keywords: TNF- α ; TNF- α receptor; granulosa cell; P450 aromatase; inhibin A

1. Introduction

TNF- α is a cytokine with multiple functions, such as participating in proinflammatory responses, regulating cell differentiation, controlling tissue renewal and restructuring, and so on, which depends on the cell type, concentration, and receptor type present [1–4]. It's well known that TNF- α and its two types of receptors are expressed in the ovaries of most species [3–6]. TNF- α is mainly expressed in human oocytes and cumulus granulosa cells from aspirated follicles, and TNF- α immunoreactivity even can be observed in oocytes of human primordial follicles [1,3,4]. In rodents, TNF- α is observed not only in neonatal oocytes but also in oocytes of all follicle stages in adults [1,7]. Our previous studies have demonstrated that granulosa cells in the ovary generally express Toll-like receptor 3 (TLR3), retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5), which mediate innate activation, the main source of TNF- α in the ovaries [2,3]. Therefore, TNF- α produced locally in the ovary rather than that

from peripheral blood plays an important regulatory role in ovarian function [1,8].

Many studies have revealed that TNF- α can regulate granulosa-luteal cell growth and steroidogenesis in human and murine ovary [9–12] and the granulosa cells from small and large follicles exhibit differential response [13,14]. TNF- α consistently inhibits estrogen secretion from granulosa cells of small follicles but stimulates progesterone secretion in granulosa cells of large follicles, which may determine whether the follicle remains healthy or becomes atretic during its course of development [14]. It is further verified that TNF- α represses P450 aromatase and inhibin α -subunit expression by activating the inducible repressor isoform of cAMP-responsive element binding modulator in a MAPK dependent mechanism [15]. Further, high levels of TNF- α in follicular fluid (FF) and granulosa cells induce oocyte, granulosa, and luteal cell death, which contribute to some extent to polycystic ovary syndrome (PCOS) and premature ovarian failure (POF) [4,16]. It has been demon-



strated that the effects of TNF- α vary dramatically depending on the dose [17,18]. It has been reported that treatment of rats with relatively low (0.1 ng/mL) and high (10 ng/mL, 50 ng/mL) doses of TNF- α has no effects on either oocyte/follicle numbers or apoptosis, while an intermediate dose (1 ng/mL) significantly reduces oocyte/follicle numbers and stimulates apoptosis [17]. However, no promoting effect on granulosa cell proliferation has been observed within this concentration range.

The aim of this study was to evaluate the effect of TNF- α on human granulosa cell proliferation, P450 aromatase and inhibin A/B expression, and estradiol and inhibin synthesis. Furthermore, the expression of two distinct receptors, TNFR1 and TNFR2, was detected after TNF- α treatment.

2. Materials and Methods

2.1 Ethics Statement

Our study group consisted of 20 infertile patients with PCOS without metabolic syndrome and 19 normally ovulating women diagnosed with male factor or tubal infertility who were undergoing their first cycle of in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI) from September 2019 to August 2020 in the Reproductive Medical Center of Anhui Provincial Hospital. All patients provided written informed consent before participation, and all procedures of the study were approved by the human ethics committee of Anhui Provincial Hospital (approve ID: 20170108). The ages and body mass index (BMI) values of the two groups were comparable.

For each patient, FF samples were aspirated from follicles with a diameter ≥ 18 mm under the guidance of transvaginal ultrasound, and were all free of blood contamination. After oocytes were isolated, the FF samples were transported on ice to the laboratory immediately, and then centrifuged at $240 \times g$ at 4°C for 10 min to isolate supernatant for TNF- α detection and granulosa cells for culture.

Twelve histological specimens of normal ovaries were retrieved from storage at the Department of Pathology of China-Japan Friendship Hospital. These ovarian tissues were obtained from patients of childbearing age who underwent hysterectomy and double appendectomy for invasive cervical cancer. The project was approved by the Ethics Committee of China-Japan Friendship Hospital (approval number: 2020-28-k20).

2.2 Cell Culture and Treatment

Granulosa cells from FF samples were washed twice with 3 mL culture medium (serum-free DMEM/F12). After centrifuged at $1000 \times g$ at 4°C for 10 min, the supernatant fluid was discarded and the cells were resuspended. Cell viability and cell count were checked by 2% Trypan Blue staining. Then, the granulosa cells were diluted and cultured in DMEM/F12 (11320-033, Gibco, USA) containing 25 mM HEPES, 2 mM L-glutamine, and 10% fetal bovine

serum in 6-well plates (Nunc, Thermo Fisher, USA) at 37°C with 5% CO_2 . When the cells adhered to the bottom of the plates, the medium was refreshed with serum-free DMEM/F12. As for E2 detection, 10^{-5} M testosterone was added as the substrate. Then, the cells were treated with varying concentrations (0, 0.2, 0.4, 0.8, 2.0, 4.0 ng/mL) of recombinant human TNF- α (Cat. 300-01A, PeproTech, Rocky Hill, NJ, USA). After 48 h, the medium was collected as conditioned medium, which was frozen at -70°C until E2 and inhibin assays were performed.

2.3 Cell Viability

Granulosa cells with a concentration of 5×10^3 cells/well were seeded in 96-well plates and treated with different concentration of TNF- α as described above. Cell viability was evaluated using a WST-8 Cell Counting Kit (CKK-8, Dojindo, Kyushu, Japan) per the manufacturer's instructions at 24, 48, 72, and 96 h.

2.4 Immunohistochemistry and Immunocytochemistry

Four-micrometer-thick tissue sections were cut from paraffin-embedded tissue blocks, mounted onto glass slide, deparaffinized in xylene and rehydrated in graded ethanol series sequentially. Then the slides were immersed in 0.01 M citrate buffer (pH 6.0) buffer and placed in microwave oven for 10 min for antigen retrieval. After cooling to $20\text{--}28^\circ\text{C}$, endogenous peroxidase was blocked with 3% H_2O_2 for 10 min. For immunocytochemistry, granulosa cells were grown on coverslips to semiconfluency, fixed with cold acetone for 10 min and rinsed with PBS. After blocking non-specific sites with 5% normal goat serum for 1 h at $20\text{--}28^\circ\text{C}$, the sections were incubated overnight at 4°C in a humidified chamber with primary antibodies as follows: anti-TNF- α at a 1:200 dilution (Rabbit monoclonal [TNF/1500R], ab270264), anti-TNF receptor 1 at a 1:500 dilution (Rabbit polyclonal, ab19139), anti-TNF receptor 2 at a 1:200 dilution (Rabbit monoclonal [EPR1653], ab109322), and Ki-67 at a 1:200 dilution (Rabbit monoclonal [SP6], ab16667). All of the primary antibodies were purchased from Abcam (USA). For the negative controls, preimmune rabbit serum were used in place of the primary antibodies. After rinsing with PBS, the sections were sequentially treated with biotinylated secondary antibodies at $20\text{--}28^\circ\text{C}$ for 1 h, and diaminobenzidine substrate chromogen system to visualize specific staining. For counterstaining, the sections were dipped in Mayer's hematoxylin for 30 s.

2.5 Enzyme-Linked Immunosorbent Assay (ELISA)

The concentrations of TNF- α in the supernatants of FF and the concentrations of estradiol and inhibin in the conditioned medium were measured with ELISA kits according to the manufacturers' instructions. The kit for TNF- α (Cat. #BMS223HS, Thermo Fisher Scientific, Waltham, MA, USA) and the kit for inhibin- α (Cat. SEA395Hu,

Table 1. Primers used for real-time quantitative RT-PCR.

Gene name	Primer pairs (5'-3')	
	Forward	Reverse
<i>TNFR1</i>	GCTGCCACTGGAACCTACTT	GGTTTTCTGAAGCGGTGAAGG
<i>TNFR2</i>	CGGGAGCTCAGATTCTTCCC	CACTGTGAGCTGTGGTCAGA
<i>P450arom</i>	TGCATGGGAATTGGACCCC	GGTTGTAGTAGTTGCAGGCAC
<i>Inhibin-α</i>	GATGTCTCCAAGCCATCCTTT	CTGGCAGCTGACTTGTCCCTCAC
<i>Inhibin-βA</i>	AGTGCCAATACCATGAAGAGG	AATTCTCTTTCTGGTCCCCACTC
<i>Inhibin-βB</i>	GCGAGAACCCTCAACTGACA	ACCGCATCCATTTGCTGGTA
<i>GAPDH</i>	GGACCTGACCTGCCGTCTAG	TAGCCCAGGATGCCCTTGAG

Cloud-Clone Corporation, Wuhan, China) were sandwich enzyme immunoassay for quantitative determination while the kits for estradiol (Cat. #KAQ0621, Thermo Fisher Scientific) was a competitive binding immunoassay. Bring the strips and reagents of the kit and samples to 20–28 °C before use. The serum-free medium and sample diluent were used as sample control and blank, respectively. Each sample, standard, sample control and blank were assayed in triplicate. At the end of reaction, all the wells were analyzed by spectrophotometry and read the absorbance of each well at 450 nm. The kit for TNF- α had a sensitivity of 0.13 pg/mL and precision (CV%) of 8.5% (intra-assay) and 9.8% (inter-assay). The sensitivity, intra-assay and inter-assay CV for the estradiol kit and inhibin- α kit were 5 pg/mL, 4.3%, 6.1% and 5.5 pg/mL, <10%, <12%, respectively. Standard curves and sample values were plotted using GraphPad Prism Software (Version 6.0, San Diego, CA, USA).

2.6 RNA Isolation and Real-Time PCR Quantification

Total RNA was isolated with Trizol reagent (15596018, Life Technologies, Carlsbad, CA, USA) and reverse-transcribed into the first strand of cDNA by using SuperScript III reverse transcriptase (2680, TaKaRa, Kyoto, Japan). Then the cDNA were used for real-time PCR with specific primers (Table 1). Real-time PCR was performed on a CFX96 Touch instrument (Bio-Rad) using the iQTM SYBR Green Supermix kit (170-8880, Bio-Rad, USA). The cycling conditions of PCR included 95 °C for 15 s, 60 °C for 30 s, repeating 40 cycles. Data were normalized to GAPDH levels [19].

2.7 Immunoblotting

Antibodies specific to TNF receptor 1 (Rabbit polyclonal, ab19139, 1:1000), TNF receptor 2 (Rabbit monoclonal [EPR1653], ab109322, 1:1000), Caspase 3 (Rabbit monoclonal [EPR18297], ab184787, 1:1000), and aromatase (Rabbit polyclonal, ab18995, 1:500) were obtained from Abcam. Anti-GAPDH antibody (Rabbit monoclonal [14C10], #2118, 1:2000) was purchased from Cell Signaling Technology.

The expression of these proteins was detected by immunoblotting as previously described [20]. The granulosa cells were lysed using RIPA lysis buffer (Cat. P0013C, Be-

yotime Biotechnology, Beijing, China). The protein concentration of the whole cell lysates was measured with a bicinchoninic acid protein assay kit (Pierce Biotechnology). Proteins (30 μ g) were mixed with 5 \times SDS-PAGE loading buffer, then loaded and separated on 10% SDS-PAGE gels and subsequently electrotransferred to polyvinylidene fluoride (PVDF) membranes (IPFL00010, Merck Millipore, Kenilworth, NJ, USA). After blocking with 5% nonfat milk for 1 h at 20–28 °C, the PVDF membranes were incubated with primary antibodies on a rocker platform at 4 °C overnight. Then, the PVDF membranes were washed with 0.1% Tween 20-containing Tris-buffered saline (TBS-T) three times, and incubated with HRP-linked Goat anti-Rabbit IgG antibody (#7074, Cell Signaling) at 20–28 °C for 1 hour, and developed with chemiluminescent substrate (34080, Thermo) after washing with TBS-T four times. Autoradiography was performed with chemiluminescence image analysis system (Tanon 5200, Beijing, China).

2.8 Apoptosis Assay

Cell apoptosis were detected with an Annexin V-FITC/PI Apoptosis Detection Kit (Cat. BMS500FI, eBioscience). After treatment with varying concentrations of recombinant human TNF- α for 96 h, 2 \times 10⁵ granulosa cells were harvested and stained with 5 μ L of Annexin V-FITC and 5 μ L of PI for 15 min in the dark at 20–28 °C. The cells were then washed, resuspended with 1 \times PBS containing 2% FBS, and analyzed by flow cytometry (FACS Calibur, BD).

2.9 Statistical Analyses

All the experiments were repeated three times. SPSS 19.0 software (IBM Corp., Chicago, IL, USA) was used for data analysis. The data are presented as the mean \pm standard deviation. As the concentration of TNF- α in the follicular fluid from PCOS patients was quite different, the data were checked the normal distribution and variance homogeneity before comparisons. The statistical significance of differences between groups was performed by Student's unpaired *t* test. One-way ANOVA was applied for multiple comparisons, further LSD test was used to inter-group comparisons. A value of *p* < 0.05 was considered as statistical significance.

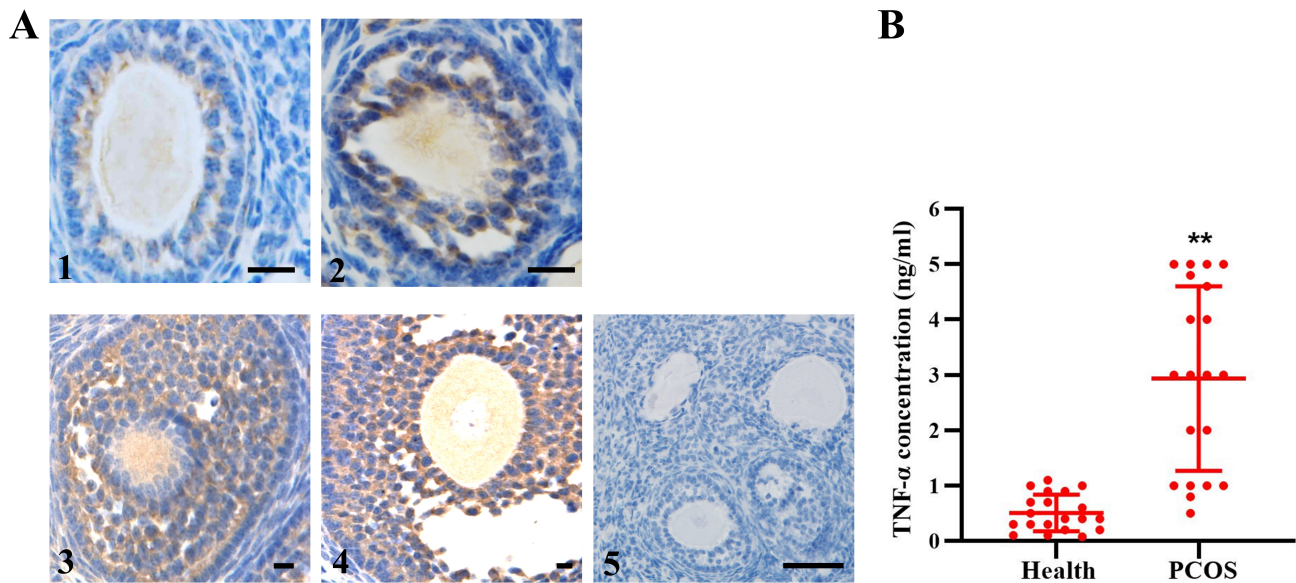


Fig. 1. TNF- α expression in human follicles. (A) Immunohistochemistry showed TNF- α expression in all stages of developmental follicles (1–4). 5, negative control. $n = 12$. (B) Quantitative detection of TNF- α in FF from healthy people ($n = 19$) and PCOS patients ($n = 20$). The data expressed as the mean \pm SD. Bar = 20 μm . **, $p < 0.01$, vs. health group.

3. Results

3.1 Expression of TNF- α in Human Follicles

TNF- α detection with immunohistochemistry staining in specimens of normal ovaries showed that the level of TNF- α increased gradually in human follicles, in accordance with the development of follicles (Fig. 1A). However, a significantly higher TNF- α level in the FF was observed for the PCOS group than for the normal group (Fig. 1B), which to some extent contributed to granulosa cell apoptosis and follicular atresia.

3.2 TNF- α does not Affect TNFR Expression

TNF- α exerts its functions in the ovary through two types of receptors, TNFR1 and TNFR2. As shown in Fig. 2A,B, the expression of TNFR1 and TNFR2 showed no significant differences in human granulosa cells. Treatment with varying concentrations of TNF- α also caused no evident effect on the expression of TNFR1 and TNFR2. However, the expression of TNFR1 appeared to increase in accordance with follicular development, while the expression of TNFR2 remained constant at all stages in follicles (Fig. 2C).

3.3 TNF- α Regulates Estradiol Synthesis in Human Ovarian Granulosa Cells

As shown in Fig. 3A,B, both mRNA and protein expression of P450 aromatase (P450arom), which catalyzes the formation of aromatic C18 estrogen from C19 androgens, was gradually inhibited by TNF- α in a dose-dependent manner (from 0.4 to 4 ng/mL) after 24 h of treatment. In addition, the concentration of E2 was decreased in the same way in the conditioned medium 48 h after TNF- α

treatment (Fig. 3C). However, surprisingly, P450arom expression was significantly upregulated meanwhile E2 concentration was also increased by treatment with 0.2 ng/mL TNF- α for 48 h (Fig. 3A–C).

3.4 Inhibin Secretion is Regulated by TNF- α in a Dose-Dependent Manner

In this study, our data showed that TNF- α can regulate inhibin A expression and secretion in granulosa cells in a dose-dependent manner, similar to the regulation of P450arom expression (Fig. 4A,D). The transcription of inhibin α - and β A-subunit were elevated significantly in granulosa cells treated with 0.2 ng/mL TNF- α for 24 h and decreased dramatically as the dose of TNF- α increased (Fig. 4B,C). At a concentration of 4 ng/mL, inhibin A was significantly inhibited by TNF- α . The expression of β B subunit mRNA, to some extent, was also altered by TNF- α , although no significant difference was observed. Accordingly, the secretion of inhibin A was also markedly increased by 0.2 ng/mL TNF- α treatment for 48 h, but it decreased with increasing TNF- α concentrations (Fig. 4D).

3.5 Different Concentrations of TNF- α Induce Different Effects on Cell Proliferation and Apoptosis

To assess the effect of TNF- α on granulosa cell growth, a CCK-8 assay was used to evaluate cell viability at different time points. The data showed that 0.2 ng/mL TNF- α significantly promoted cell proliferation in a time-dependent manner, while a high dose of 4 ng/mL led to evident cell inhibition. Compared with the untreated group, the group treated with the medium dose (0.8 ng/mL) showed no apparent difference in cell growth (Fig. 5A). Ki-

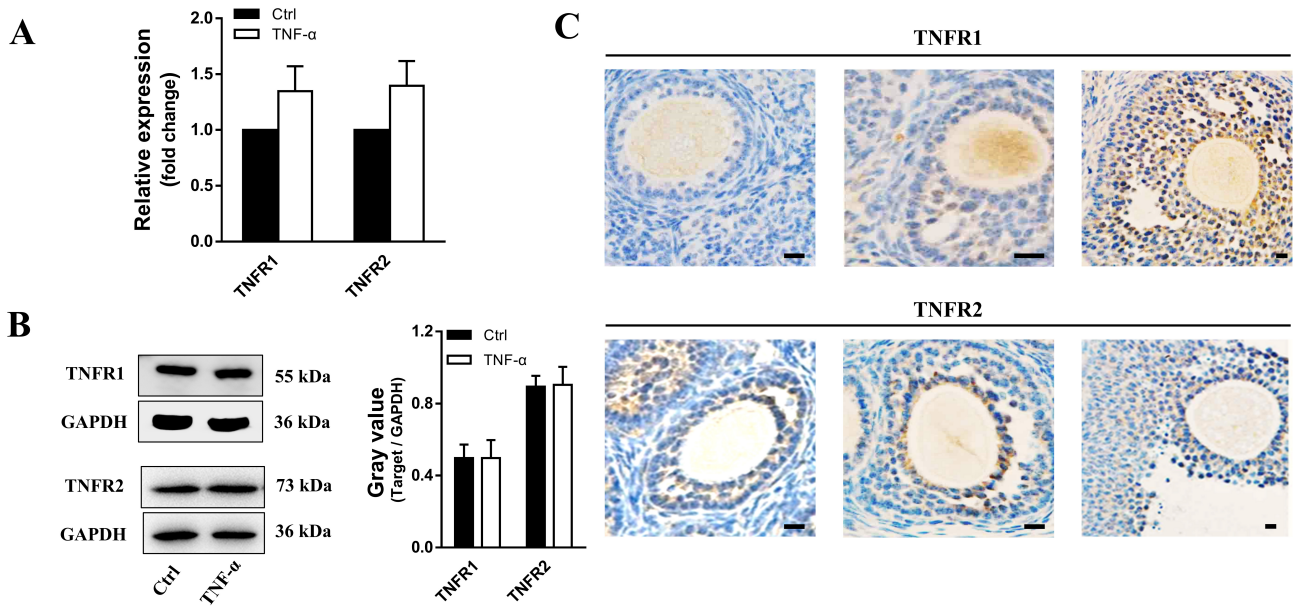


Fig. 2. TNFR expression in cultured granulosa cells and developmental follicles in ovarian tissues. (A) Transcript levels and (B) protein expression of TNFR1 and TNFR2 in granulosa cells after TNF- α treatment. $n = 19$. (C) Immunohistochemistry showing TNFR1 and TNFR2 expression in ovarian tissues. The data expressed as the mean \pm SD. Bar = 20 μ m. $n = 12$.

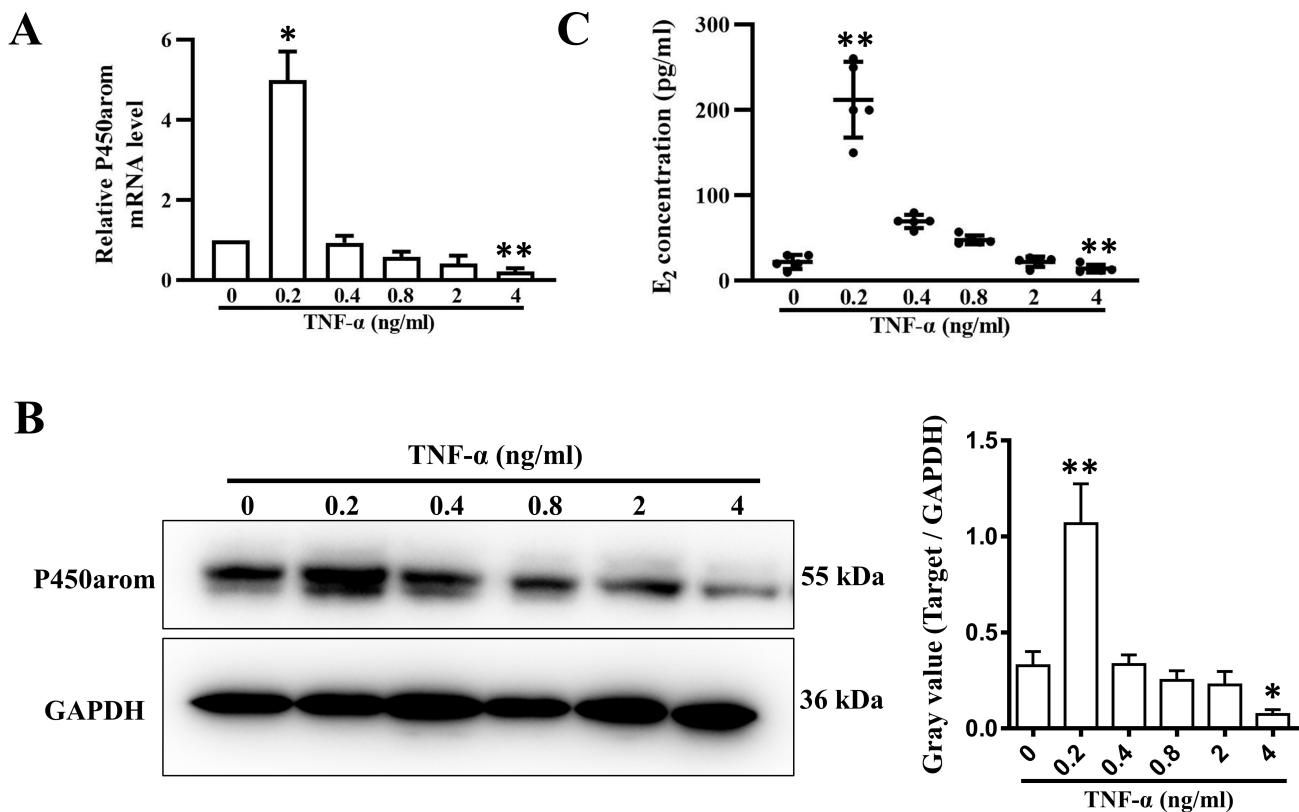


Fig. 3. P450arom expression and E₂ secretion in granulosa cells treated with varying concentrations of TNF- α . (A) Relative mRNA levels and (B) relative protein levels of P450arom in granulosa cells treated with 0.2, 0.4, 0.8, 2, and 4 ng/mL TNF- α for 24 h. (C) E₂ concentrations in the supernatants of cultured granulosa cells treated with 0.2, 0.4, 0.8, 2, and 4 ng/mL TNF- α for 48 h. The data expressed as the mean \pm SD. $n = 5$. *, $p < 0.05$; **, $p < 0.01$, vs. untreated group.

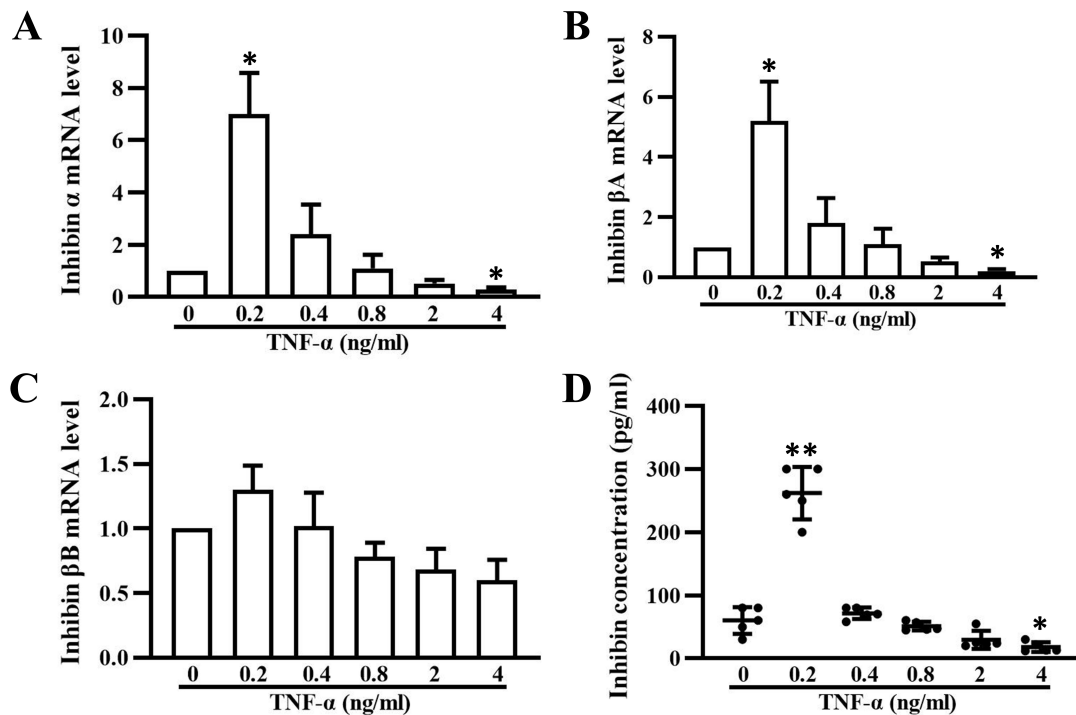


Fig. 4. Inhibin A expression and secretion in granulosa cells treated with varying concentrations of TNF- α . Relative mRNA levels of subunits α (A), βA (B), and βB (C) of inhibin in granulosa cells treated with 0.2, 0.4, 0.8, 2, and 4 ng/mL TNF- α for 24 h. (D) Inhibin A concentrations in the supernatants of cultured granulosa cells treated with 0.2, 0.4, 0.8, 2, and 4 ng/mL TNF- α for 48 h. The data expressed as the mean \pm SD. $n = 5$. *, $p < 0.05$; **, $p < 0.01$, vs. untreated group.

67 in granulosa cells was expressed in a similar way after exposure to varying concentrations of TNF- α for 96 h (Fig. 5B). Furthermore, the apoptosis assay results showed that 4 ng/mL TNF- α induced significant apoptosis and up-regulation of cleaved-caspase3 (Fig. 5C,D,E), which may have contributed to some extent to the notable decreases in E2 and inhibin A secretion.

4. Discussion

It has been reported that TNF- α in the ovaries is released from macrophages, granulosa cells, and oocytes [2,3,21] and acts as an important intraovarian regulator of steroidogenesis, follicular development, and atresia [1,8,11,12,22,23]. The ability of TNF- α to promote follicular development or atresia is dependent on the stage of follicular maturation. TNF- α is deemed to have a selective effect on progesterone secretion by granulosa cells in all types of follicles before ovulation in chickens [24]. It exerts a stimulatory effect at low doses but is inhibitory at high doses, especially in the cells of preantral and antral follicles [17,18]. In this study, our results demonstrated that 0.2 ng/mL TNF- α significantly promoted granulosa cell proliferation, while 4 ng/mL TNF- α notably inhibited cell growth, which was possibly attributable to high apoptosis. Whether TNF- α exerts this biphasic effect on granulosa cells before the development of antral follicle and luteal cells needs further investigation.

Sufficient E2 and inhibin are necessary for follicle development, ovulation, and cyclic secretion of pituitary hormones at different phases of the ovarian cycle while the granulosa cells in the preovulatory follicle just have the function of synthesizing E2 and inhibin [25,26]. It has been reported that TNF- α inhibits P450 aromatase catalytic activity and inhibin secretion in a dose-dependent manner in granulosa cells [15,27–29]. Our results showed biphasic effects of TNF- α on the expression of P450 aromatase and inhibin and the secretion of E2 and inhibin A in granulosa cells, and the inhibitory effect of the high dose may be attributable to apoptosis, as granulosa cell apoptosis is recognized to play a key role in follicular atresia [30–32]. However, other inducible repressors, such as cAMP-responsive element binding modulator (CREM), which participates in the LH-triggered downregulation of aromatase and α -inhibin genes [15], probably also contribute to endocrine inhibition, as transcriptional downregulation of P450 aromatase and α - and β -subunits of inhibin were observed in our study.

The multiple biological functions of TNF- α are mainly mediated by two types of receptors, TNFR1 (p55/p60) and TNFR2 (p75/p80), which have homologous extracellular domains but notably different intracellular domains for signaling. TNFR1 contains an intracellular death domain essential for cytotoxicity and apopto-

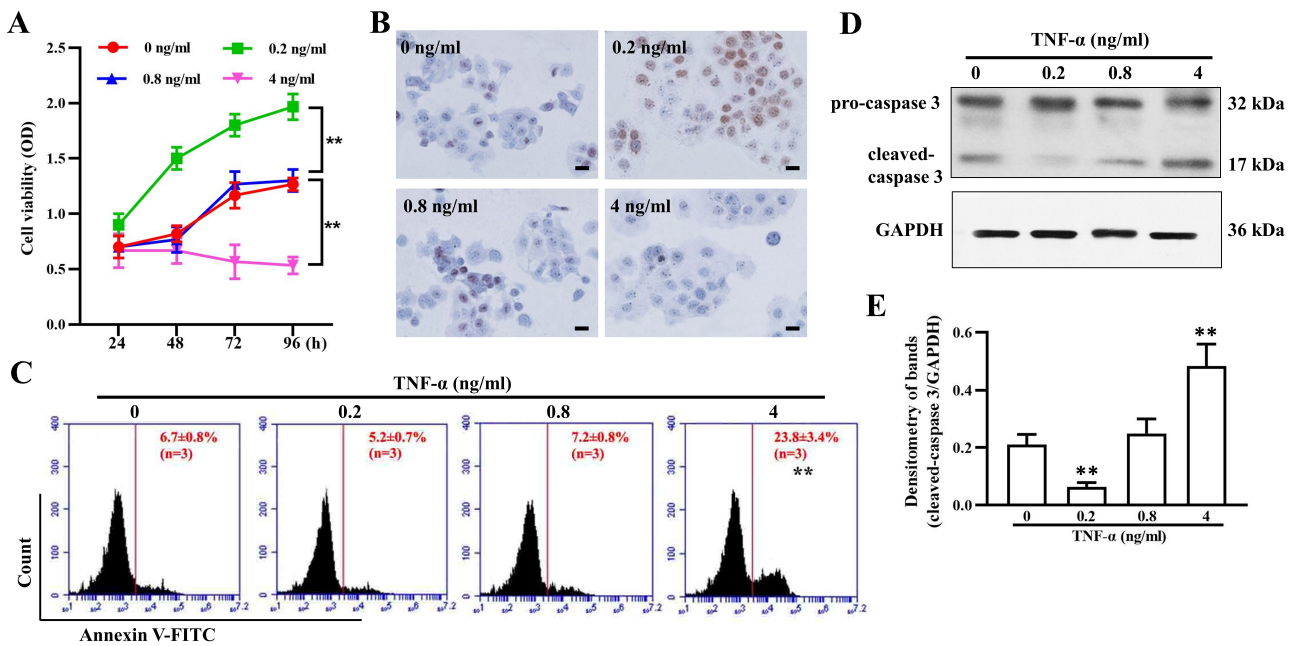


Fig. 5. TNF- α regulates granulosa cell proliferation and apoptosis. (A) Cell viability of granulosa cells treated with 0, 0.2, 0.8, and 4 ng/mL TNF- α for 24, 48, 72, and 96 h. Ki67 expression (B), cell apoptosis (C), and cleaved-caspase 3 expression (D,E) in granulosa cells after treatment with 0, 0.2, 0.8, and 4 ng/mL TNF- α for 96 h. The data expressed as the mean \pm SD. Bar = 20 μ m. n = 9. **, p < 0.01, vs. untreated group.

sis, while TNFR2 activation leads to cell survival, growth and differentiation [33–35]. It has been verified that TNF- α and its receptors are expressed in follicles at different stages from primordial to preovulatory follicles. Furthermore, the binding rate of TNF- α to TNFR2 is 20 times rapider than TNFR1 when the receptors are isolated and purified, but TNFR1 binds soluble TNF- α with a much higher affinity than TNFR2 [34]. These findings may, to some extent, help to explain the biphasic effect of TNF- α on human granulosa cells. At a low dose, TNF- α preferentially binds to TNFR2, thus promoting cell growth, while at a high dose, TNF- α may bind to both TNFR1 and TNFR2 simultaneously, leading to apoptosis.

Women with PCOS exhibit some metabolic abnormalities, such as obesity, insulin resistance, type 2 diabetes mellitus [36,37], which, to some extent, may lead to systemically higher inflammatory cells and cytokines including TNF- α in peripheral blood and greater number of macrophages and lymphocytes immersed in ovary as compared with healthy women [38]. Interestingly, high serum TNF- α level was also observed in lean PCOS patients [39]. Therefore, chronic inflammation is common in PCOS patients even though the exact pathogenesis of PCOS is not completely understood. Some studies have reported that monoclonal anti-TNF- α antibodies via intraovarian injection improves follicular development and oocyte meiotic maturation, minimizes local inflammatory response, decreases granulosa cell apoptosis, and improves viability in mouse [17,40,41], which indicates that TNF- α and

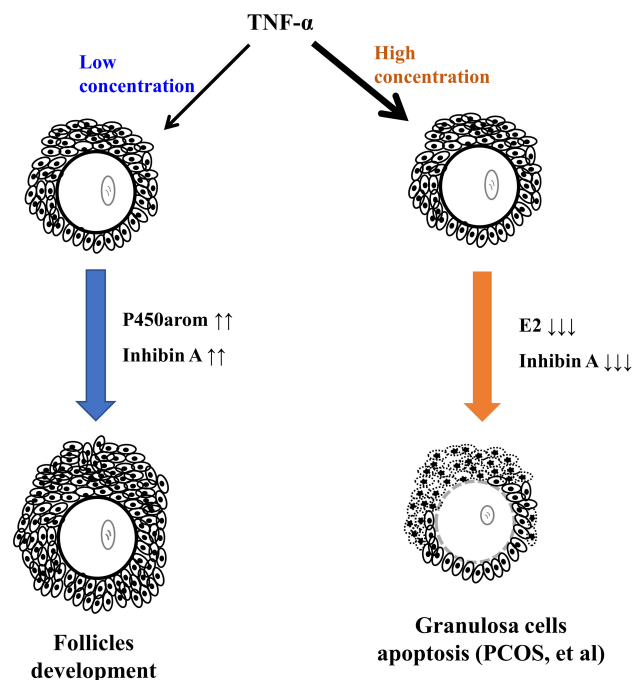


Fig. 6. The proposed model of TNF- α exerting a biphasic effect on ovarian endocrine and follicular development.

TNFR1 play essential roles in abnormal reproductive endocrine function in the ovaries. In this study, consistent with previous reports, we observed higher levels of TNF- α in FF in the group of PCOS patients than in the control

group. Thus, it is possible that high levels of TNF- α in FF and TNFR1 in follicular cells partly contribute to the pathogenesis of PCOS (Fig. 6). Furthermore, TNF- α and TNFR1 may be promising therapeutic targets for PCOS.

5. Conclusions

In summary, the results demonstrate that TNF- α exerts a biphasic effect on ovarian endocrine and follicular development.

Author Contributions

DQF and JL conceived and designed the study; HYL, DKX and WHW performed the experiments; DQF, XHT and BL analyzed the data and wrote the manuscript.

Ethics Approval and Consent to Participate

The project was approved by the Ethics Committee of China-Japan Friendship Hospital (approval number: 2020-28-k20).

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Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

References

- [1] Yamamoto Y, Kuwahara A, Taniguchi Y, Yamasaki M, Tanaka Y, Mukai Y, *et al.* Tumor necrosis factor alpha inhibits ovulation and induces granulosa cell death in rat ovaries. *Reproductive Medicine and Biology*. 2015; 14: 107–115.
- [2] Yan K, Cheng L, Liu P, Liu Z, Zhao S, Zhu W, *et al.* Polyinosinic-Polycytidylic Acid Perturbs Ovarian Functions Through Toll-Like Receptor 3-Mediated Tumor Necrosis Factor α Production in Female Mice. *Biology of Reproduction*. 2015; 93: 11.
- [3] Yan K, Liang J, Zhang X, Deng L, Feng D, Ling B. Polyinosinic-polycytidylic acid induces innate immune responses via Toll-like receptor 3 in human ovarian granulosa cells. *Immunology and Cell Biology*. 2019; 97: 753–765.
- [4] Salmassi A, Fattahi A, Simon N, Latifi Z, Ghasemnejad T, Nouri M, *et al.* Messenger RNA and protein expression of tumor necrosis factor alpha and its receptors in human follicular granulosa cells. *Journal of Cellular Physiology*. 2019; 234: 20240–20248.
- [5] Urban RJ, Nagamani M, Bodenburt Y. Tumor necrosis factor alpha inhibits transcriptional activity of the porcine P-45011a insulin-like growth factor response element. *The Journal of Biological Chemistry*. 1996; 271: 31699–31703.
- [6] Sakumoto R, Okuda K. Possible Actions of Tumor Necrosis Factor-ALPHA in Ovarian Function. *Journal of Reproduction and Development*. 2004; 50: 39–46.
- [7] Marcinkiewicz JL, Krishna A, Cheung CM, Terranova PF. Oocytic tumor necrosis factor alpha: localization in the neonatal ovary and throughout follicular development in the adult rat. *Biology of Reproduction*. 1994; 50: 1251–1260.
- [8] Terranova PF, Rice VM. Review: cytokine involvement in ovarian processes. *American Journal of Reproductive Immunology*. 1997; 37: 50–63.
- [9] Yan Z, Hunter V, Weed J, Hutchison S, Lyles R, Terranova P. Tumor necrosis factor-alpha alters steroidogenesis and stimulates proliferation of human ovarian granulosa cells in vitro. *Fertility and Sterility*. 1993; 59: 332–338.
- [10] Roby KF, Terranova PF. Effects of tumor necrosis factor-alpha in vitro on steroidogenesis of healthy and atretic follicles of the rat: theca as a target. *Endocrinology*. 1990; 126: 2711–2718.
- [11] Fukuoka M, Yasuda K, Fujiwara H, Kanzaki H, Mori T. Interactions between interferon gamma, tumour necrosis factor alpha, and interleukin-1 in modulating progesterone and oestradiol production by human luteinized granulosa cells in culture. *Human Reproduction*. 1992; 7: 1361–1364.
- [12] Fukuoka M, Yasuda K, Emi N, Fujiwara H, Iwai M, Takakura K, *et al.* Cytokine modulation of progesterone and estradiol secretion in cultures of luteinized human granulosa cells. *The Journal of Clinical Endocrinology and Metabolism*. 1992; 75: 254–258.
- [13] Rice VM, Williams VR, Limback SD, Terranova PF. Tumour necrosis factor-alpha inhibits follicle-stimulating hormone-induced granulosa cell oestradiol secretion in the human: dependence on size of follicle. *Human Reproduction*. 1996; 11: 1256–1261.
- [14] Montgomery Rice V, Limback SD, Roby KF, Terranova PF. Differential responses of granulosa cells from small and large follicles to follicle stimulating hormone (FSH) during the menstrual cycle and acyclicity: effects of tumour necrosis factor-alpha. *Human Reproduction*. 1998; 13: 1285–1291.
- [15] Morales V, Gonzalez-Robayna I, Santana MP, Hernandez I, Fanjul LF. Tumor necrosis factor-alpha activates transcription of inducible repressor form of 3',5'-cyclic Adenosine 5'-monophosphate-responsive element binding modulator and represses P450 aromatase and inhibin alpha-subunit expression in rat ovarian granulosa cells by a p44/42 mitogen-activated protein kinase-dependent mechanism. *Endocrinology*. 2006; 147: 5932–5939.
- [16] Wang LJ, Brännström M, Robertson SA, Norman RJ. Tumor necrosis factor alpha in the human ovary: presence in follicular fluid and effects on cell proliferation and prostaglandin production. *Fertility and Sterility*. 1992; 58: 934–940.
- [17] Morrison LJ, Marcinkiewicz JL. Tumor necrosis factor alpha enhances oocyte/follicle apoptosis in the neonatal rat ovary. *Biology of Reproduction*. 2002; 66: 450–457.
- [18] Marcinkiewicz JL, Balchak SK, Morrison LJ. The involvement of tumor necrosis factor-alpha (TNF) as an intraovarian regulator of oocyte apoptosis in the neonatal rat. *Frontiers in Bioscience*. 2002; 7: d1997–d2005.
- [19] Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, *et al.* The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments. *Clinical Chemistry*. 2009; 55: 611–622.
- [20] Yan K, Feng D, Liang J, Wang Q, Deng L, Zhang X, *et al.* Cytosolic DNA sensor-initiated innate immune responses in mouse ovarian granulosa cells. *Reproduction*. 2017; 153: 821–834.
- [21] Figueroa F, Mendoza G, Cardozo D, Mohamed F, Oliveros L, Fomeris M. Sympathetic innervation regulates macrophage activity in rats with polycystic ovary. *The Journal of Endocrinology*. 2018; 238: 33–45.
- [22] Cui Z, Liu L, Zhu Q, Wang Y, Yin H, Li D, *et al.* Inhibin α regulates follicular development via hormone secretion and granulosa cell behaviors in laying hens. *Cell and Tissue Research*. 2020; 381: 337–350.
- [23] Spicer LJ. Tumor necrosis factor-alpha (TNF-alpha) inhibits steroidogenesis of bovine ovarian granulosa and thecal cells in

- vitro. Involvement of TNF-alpha receptors. *Endocrine*. 1998; 8: 109–115.
- [24] Bryan MA, Grizzle JM, Saxton AM, Siegel PB, Dunnington EA. Effect of tumor necrosis factor-alpha on progesterone production by granulosa cells in laying hens of different genetic lines. *Domestic Animal Endocrinology*. 1997; 14: 161–169.
- [25] Messinis IE, Messini CI, Dafopoulos K. Novel aspects of the endocrinology of the menstrual cycle. *Reproductive Biomedicine Online*. 2014; 28: 714–722.
- [26] Noguchi M, Yoshioka K, Itoh S, Suzuki C, Arai S, Wada Y, *et al*. Peripheral concentrations of inhibin a, ovarian steroids, and gonadotropins associated with follicular development throughout the estrous cycle of the sow. *Reproduction*. 2010; 139: 153–161.
- [27] Vashisht M, Rani P, Sunita, Onteru SK, Singh D. Curcumin primed exosomes reverses LPS-induced pro-inflammatory gene expression in buffalo granulosa cells. *Journal of Cellular Biochemistry*. 2018; 119: 1488–1500.
- [28] Onnureddy K, Ravinder, Onteru SK, Singh D. IGF-1 attenuates LPS induced pro-inflammatory cytokines expression in buffalo (*Bubalus bubalis*) granulosa cells. *Molecular Immunology*. 2015; 64: 136–143.
- [29] Imai M, Yamoto M, Otani H, Nakano R. Cytokine modulation of inhibin secretion in cultured rat granulosa cells. *The Journal of Endocrinology*. 1996; 151: 449–457.
- [30] Park JH, Park SA, Lee YJ, Joo NR, Shin J, Oh SM. TOPK inhibition accelerates oxidative stress-induced granulosa cell apoptosis via the p53/SIRT1 axis. *International Journal of Molecular Medicine*. 2020; 46: 1923–1937.
- [31] Zhang J, Xu Y, Liu H, Pan Z. MicroRNAs in ovarian follicular atresia and granulosa cell apoptosis. *Reproductive Biology and Endocrinology*. 2019; 17: 9.
- [32] Matsuda F, Inoue N, Manabe N, Ohkura S. Follicular growth and atresia in mammalian ovaries: regulation by survival and death of granulosa cells. *The Journal of Reproduction and Development*. 2012; 58: 44–50.
- [33] McDermott MF. TNF and TNFR biology in health and disease. *Cellular and Molecular Biology*. 2001; 47: 619–635.
- [34] Greenfeld CR, Roby KF, Pepling ME, Babus JK, Terranova PF, Flaws JA. Tumor necrosis factor (TNF) receptor type 2 is an important mediator of TNF alpha function in the mouse ovary. *Biology of Reproduction*. 2007; 76: 224–231.
- [35] So T, Ishii N. The TNF-TNFR Family of Co-Signal Molecules. *Advances in Experimental Medicine and Biology*. 2019; 1189: 53–84.
- [36] Kim CH, Ahn JW, You RM, Kim SH, Chae HD, Kang BM. Pioglitazone treatment decreases follicular fluid levels of tumor necrosis factor-alpha and interleukin-6 in patients with polycystic ovary syndrome. *Clinical and Experimental Reproductive Medicine*. 2011; 38: 98–102.
- [37] Abraham Gnanadass S, Divakar Prabhu Y, Valsala Gopalakrishnan A. Association of metabolic and inflammatory markers with polycystic ovarian syndrome (PCOS): an update. *Archives of Gynecology and Obstetrics*. 2021; 303: 631–643.
- [38] Xiong YL, Liang XY, Yang X, Li Y, Wei LN. Low-grade chronic inflammation in the peripheral blood and ovaries of women with polycystic ovarian syndrome. *European Journal of Obstetrics & Gynecology and Reproductive Biology*. 2011; 159: 148–150.
- [39] Uzdogan A, Kuru Pekcan M, Cil AP, Kisa U, Akbiyik F. Progesterone and tumor necrosis factor-alpha in lean polycystic ovary syndrome patients. *Gynecological Endocrinology*. 2021; 37: 925–929.
- [40] Johnson ML, Murdoch J, Van Kirk EA, Kaltenbach JE, Murdoch WJ. Tumor necrosis factor alpha regulates collagenolytic activity in preovulatory ovine follicles: relationship to cytokine secretion by the oocyte-cumulus cell complex. *Biology of Reproduction*. 1999; 61: 1581–1585.
- [41] Lang Q, Yidong X, Xueguang Z, Sixian W, Wenming X, Tao Z. ETA-mediated anti-TNF-alpha therapy ameliorates the phenotype of PCOS model induced by letrozole. *PLoS ONE*. 2019; 14: e0217495.