Original article

Blood TNF-α and GABRA-1 levels in individuals with poor ovarian response who underwent *in vitro* fertilization

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Abstract

Background: Poor ovarian response (POR) to ovarian stimulation during *in vitro* fertilization (IVF) procedures is a critical issue that contributes to decreased ovarian reserves and cancelation of IVF cycles. Nevertheless, the complete etiology of this condition remains poorly understood.

Objective: This study aimed to determine the levels of a blood pro-inflammatory cytokine (TNF-α) and apoptotic marker (GABRA-1) between patients exhibiting normal ovarian response (NOR) and those with POR during the process of IVF.

Methods: This was a cross-sectional study that involved the collection of whole blood from 25 participants (NOR group n = 15, POR group n = 10). The relative gene expression levels of TNF-α and GABRA-1 were assessed using reverse transcription quantitative polymerase chain reaction.

Results: Our findings indicated that the relative gene expression levels of TNF-α were markedly elevated in the POR group compared with the NOR group (POR = 6.1 ± 1.8 *vs*. NOR = 3.1 ± 1.2, $P < 0.001$). Furthermore, a positive correlation was observed between the relative gene expression levels of TNF-α and various factors, including ovarian reserve (anti-Müllerian hormone), antral follicle count, and the total number of retrieved oocytes. In contrast, the relative gene expression levels of GABRA-1 did not exhibit any significant differences between the participant groups.

Conclusion: Elevated levels of TNF-α may be a crucial factor that contributes to the pathogenesis of POR, including diminished ovarian reserve and low quantity of retrieved oocytes, after ovarian stimulation and IVF programs.

Keywords: Apoptosis, *in vitro* fertilization, ovarian response, pro-inflammatory cytokine.

A substantial proportion of women (reaching up to 35.0%) participating in the *in vitro* fertilization (IVF) process are unable to recruit an adequate number of follicles following the standard ovarian stimulation program. [1] This is referred to as poor ovarian response (POR), and individuals confronted with this challenge have a decreased number of retrieved oocytes, IVF cycle cancelation, and diminished probability of a pregnancy outcome. [1, 2] To enhance the effective treatment protocols for POR, various factors that could influence POR conditions and etiology have been explored, including age-related, immunological, and hormonal elements. [3, 4] In addition, the roles of peptides, growth factors, and pro-inflammatory cytokines present in the ovarian microenvironment have been studied. [5, 6] Nevertheless, the pathogenesis of POR is intricate and is only partially understood.

Recently, emphasis has been focused on the roles of inflammatory and cell apoptosis complexes within the follicle microenvironment, particularly their contribution to the diminished ovarian reserve following

the IVF program. [7] In an animal study on mice deficient in apoptosis-associated speck-like protein containing a C-terminal caspase recruitment domain (ASC), the reduction of the inflammatory phenotype in ASC$^{-/-}$ mice was associated with the retention of follicle reserves during reproductive aging. [8] This observation highlights that inflammation is complex and plays a crucial role in age-related follicle depletion and the ovarian milieu. [8] Regarding pro-inflammatory cytokines, tumor necrosis factor-alpha (TNF-α) is considered the principal pro-inflammatory cytokine produced by various cell types that exhibit diverse effects in multi-diseases. [9] In human ovarian physiology, TNF-α plays a substantial role in the proliferation and apoptosis cascades of the antral follicles, thereby affecting the follicle development response. [10] In an *in vitro* experiment, supplementation of exogenous TNF-α to the culture medium inhibited follicle viability and increased the number of apoptotic cells in ovarian tissues. [11] Furthermore, previous evidence has indicated that TNF-α may interact with apoptotic factors. For example, a correlation between TNF-α and the apoptotic marker gamma-aminobutyric acid type a receptor subunit alpha1 (GABRA-1), which triggers oxidative stress and activates the brain cell apoptosis pathway in Alzheimer's disease, has been reported in mice. [12] According to the ovarian physiology of avian species, GABRA-1 can inhibit granulosa cell proliferation and enhance cell apoptosis. [13]

Although TNF-α has been investigated in patients undergoing IVF, most studies have focused on the relative mRNA expression levels in the blood, and its correlation with ovarian stimulation outcomes is limited. Moreover, there is a lack of corresponding GABRA-1 data from studies conducted on human reproductive physiology. In this regard, the primary purpose of this study was to investigate the relative gene expression levels of pro-inflammatory cytokines (TNF-α) and apoptotic markers (GABRA-1) in the white blood cells retrieved from reproductive-age women with different ovarian stimulation outcomes during an IVF program, i.e., normal and poor ovarian responses.

Materials and methods

This study was reviewed and approved by the Institutional Review Board, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand, on January 18, 2022 (IRB no. 905/64). All participants provided written informed consent.

Participant eligibility
A total of 25 women who participated in ovarian stimulation and IVF programs at the Reproductive Biology Unit, King Chulalongkorn Memorial Hospital, and the Department of Obstetrics and Gynecology, Faculty of Medicine, Chulalongkorn University, from March 2022 to December 2022, were included in this study. The patients were categorized into two groups: those with a normal ovarian response (NOR; n = 15, aged 33 to 40 years old) and those with a POR; n = 10, aged 34 to 41 years old). For the POR group, the patients were required to exhibit at least two of the following three characteristics: advanced age (≥ 40 years old), retrieval of 1–4 oocytes on the day of oocyte pick-up, or abnormal results on the ovarian reserve tests; antral follicle count (AFC) < 5–7 follicles or anti-Müllerian hormone (AMH) levels < 0.5 - 1.1 ng/mL. [14] Patients with abnormal ovarian conditions, such as polycystic ovary syndrome, those with basal follicle-stimulating hormone (FSH) levels > 20 IU/L, or women who present any acute or chronic inflammatory or autoimmune diseases were excluded.

Blood collection and whole blood fractionation
A whole blood sample was obtained from each participant on the second day of their menstrual cycle before ovarian stimulation and the in IVF program. [15] Approximately 3 - 5 mL of whole blood was collected from each participant in a tube containing the ethylenediaminetetraacetic acid anticoagulant. The whole blood was then centrifuged at 1,500 ×g for 15 min at room temperature. This process resulted in the separation of the blood into the upper plasma layer, the lower red blood cell layer, and in between was a thin interface that contained the white blood cells, which were used for total RNA extraction.

Ovarian stimulation program
The ovarian stimulation protocol was adjusted to the specific baseline characteristics of each patient by the attending physician. Both groups of patients underwent treatment using the gonadotropin-releasing hormone (GnRH) antagonist protocol. On the third day of their menstrual cycle, an initial dose of 150–300 U/d of recombinant follicle-stimulating hormone (rFSH), either Follitropin alpha (Gonal-f®, Merck Serono SA, Switzerland) or Follitropin Beta (Puregon®, MSD, France), was administered, with subsequent adjustments made based on the ovarian response. Once the leading follicle reached 14 mm in diameter, [15] a daily dose of 250 mcg of GnRH antagonist (Orgalutran®, N.V. Organon, The Netherlands) was

introduced. A total of 250 mcg of recombinant human chorionic gonadotropin (hCG) (Ovidrel®, Serono, Rockland, MA, USA) and/or 0.2 mg triptorelin (Decapeptyl®, Ferring, Switzerland) was administered when at least three leading follicles or the majority of leading follicles in the POR group exceeded 17 mm in diameter. Finally, oocyte retrieval was performed under transvaginal ultrasound guidance from 36 to 36.5 h following hCG administration. IVF by intracytoplasmic sperm injection (ICSI) was later performed on the mature (metaphase II; MII) oocytes.

Relative gene expression level of TNF-α and GABRA-1

Total mRNA was extracted from the white blood cells utilizing an RNA Blood Mini Kit (Qiagen GmbH, Germany). The RNA concentration was subsequently assessed using a NanoDrop 1,000 Spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA), and synthesis of first-strand cDNA was performed.

All primers were obtained using NCBI BLAST®. The primer pairs (5′ to 3′) are listed below: TNF-α Forward 5′-ATCAGAGGGCCTGTACCTCAT-3′ (NM_000594.4), TNF-α Reverse 5′-AGACTCGGCAAAGTCGAGATA-3′ (NM_000594.4), GABRA-1 Forward 5′-ATGATGGAGCTCGAGGCAAA-3′ (NM_000806.5), GABRA-1 Reverse 5′-AGCTCTGAATTGTGCTGGGT-3′ (NM_000806.5), GAPDH Forward 5′-GGGGGAGCCAAAAGGGTCATCATCT-3′ (NM_001357943.2), and GAPDH Reverse 5′-GAGGGGCCATCCACAGTCTTC-3′ (NM_001357943.2).

To assess the target mRNA expression levels, qPCR was performed in duplicate using a gene expression assay (KAPA SYBR® FAST qPCR Master Mix (2×) Kit, KAPABIOSYSTEMS, Sigma-Aldrich, Cape Town, South Africa) in a qPCR analyzer (Applied Biosystem™, San Francisco, CA, USA). Each reaction underwent a thermal cycling protocol of initial denaturation at 95°C for 3 min, followed by 40 cycles of 95°C for 3 s and 20 s at the specific annealing temperature for each primer. For quantification purposes, all mRNA samples were normalized against GAPDH. The relative gene expression levels were calculated by using the $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

Data analysis was performed utilizing IBM SPSS Statistics software (version 28). The independent variables comprised demographic information, hormonal profiles, and relative gene expression levels. Differences in the dependent variables between experimental groups were assessed using the *t*-test or Mann–Whitney U test. For the relation between the two designed variables, statistical analysis was conducted using Pearson's correlation. All results are presented as the mean ± standard deviation (SD). $P < 0.05$ was considered statistically significant.

Results

Demographic data

The ages of the enrolled participants were comparable across the groups, with an average of 36.5 ± 2.5 years for the NOR group and 37.6 ± 2.3 years for the POR group. Similarly, there were no significant differences in their BMI and the ages of the male partner **(Table 1).** In addition, the medical histories related to infertility causes and previous treatments, such as intrauterine insemination and IVF cycles, were found to be similar between the two groups **(Table 1)**.

Baseline hormonal profiles, ovarian stimulation, and in vitro fertilization outcomes

The baseline hormonal profiles of FSH, luteinizing hormone, and estradiol did not significantly differ between the groups **(Table 1)**. In contrast, the AMH levels were notably lower in the POR group, measuring 0.9 ± 0.4 pg/mL compared to 2.3 ± 1.1 pg/mL in the NOR group ($P = 0.004$). The average AFC was also significantly decreased in the POR group, with an average of 7.0 ± 0.7 follicles compared to the 13.7 ± 4.4 follicles in the NOR group ($P = 0.002$). For the ovarian stimulation program, the duration (days) of rFSH treatment was similar across both groups, with 10 days for the NOR group and 9 days for the POR group. On the day of hCG administration, the estradiol levels were significantly higher in the NOR group (2,036.9 ± 711.5 ng/mL) than in the POR group (1,205.3 460.5 ng/mL) ($P = 0.037$).

Table 1. Demographic data, hormonal profiles, ovarian stimulation and fertilization outcomes of participants with normal ovarian response (n = 15) and poor ovarian response to ovarian stimulation program (n = 10) (mean ± SD or %).

Baseline characteristics	Normal ovarian response	Poor ovarian response	P-value
Patient age (years)	36.5 ± 2.5	37.6 ± 2.3	0.282
BMI (kg/m^2)	21.4 ± 1.7	23.1 ± 2.8	0.051
Partner age (years)	38.5 ± 3.9	38.7 ± 3.1	0.897
Infertility (%)			
Primary	86.4	82.7	—
Secondary	13.6	17.3	—
Previous infertility treatment			
IUI (cycle)	2.0 ± 1.3	1.6 ± 2.0	0.591
IVF (cycle)	0.2 ± 0.4	0.6 ± 0.5	0.083
FSH (IU/L)	6.6 ± 1.2	6.5 ± 1.9	0.824
LH (IU/L)	5.6 ± 1.4	4.5 ± 1.2	0.781
Estradiol (ng/mL)	44.0 ± 19.2	42.0 ± 19.5	0.886
AMH (pg/mL)	2.3 ± 1.1	0.9 ± 0.4	0.004*
Antral follicle count	13.7 ± 4.4	7.0 ± 0.7	0.002*
Duration of stimulation (day)	10.0 ± 1.1	9.0 ± 0.7	0.108
Estradiol level (ng/mL) on hCG day	2,036.9 ± 711.5	1,205.3 ± 460.5	0.037*
Number of retrieved oocytes	12.3 ± 2.9	3.4 ± 0.6	<0.001*
Oocyte maturation rate (metaphase II oocyte) (%)	81.3 ± 12.2	82.8 ± 18.9	0.971
Fertilization rate (%)	93.5 ± 3.2	81.7 ± 5.2	0.043*

AMH, anti-müllerian hormone; FSH, follicle stimulating hormone; hCG, human chorionic gonadotropin; IUI, intrauterine insemination; IVF, *in vitro* fertilization; LH, luteinizing hormone.
Asterisk (*) indicates statistical difference.

Table 2. Correlation between TNF-α and selected parameters (baseline characteristics, ovarian stimulation, IVF outcomes, and relative gene expression levels GABRA-1) obtained from participants with normal ovarian response (n = 15) and poor ovarian response to ovarian stimulation (n = 10).

Parameter I	Parameter II	r^2	P-value
TNF-α	AMH	0.669	0.004*
TNF-α	Antral follicle count	0.745	<0.001*
TNF-α	Number of retrieved oocytes	0.738	<0.001*
TNF-α	Maturation rate(metaphase II oocyte)	0.241	0.188
TNF-α	Fertilization rate	0.254	0.178
TNF-α	GABRA-1	0.032	0.452

The difference in ovarian stimulation outcomes was reflected in the number of retrieved oocytes, with the NOR group obtaining a significantly greater number of oocytes (12.3 ± 2.9) compared to the POR group (3.4 ± 0.6) ($P < 0.001$). Following the oocyte retrieval process, maturation rates of the oocytes (MII oocytes) did not differ between the groups ($P = 0.971$). However, the fertilization rates post-IVF/ICSI were significantly lower in the POR group ($P = 0.043$) **(Table 1).**

Relative gene expression levels of TNF-α and GABRA-1

Analysis of the TNF-α expression levels revealed significant differences between the patient groups. Specifically, TNF-α levels were markedly higher in the POR group, with relative gene expression levels of 6.1 ± 1.8 compared to the 3.1 ± 1.2 of the NOR group ($P < 0.001$). In contrast, the relative gene expression levels of GABRA-1 did not exhibit a statistically significant difference between the groups (3.2 ± 2.8 and 2.3 ± 2.2; NOR and POR, respectively) ($P = 0.217$).

Correlation analysis of the relative gene expression levels of TNF-α and other parameters

As shown in **Table 2**, a positive correlation was observed between TNF-α levels and the baseline characteristics, as well as the ovarian stimulation outcomes: AMH ($r^2 = 0.699$, $P = 0.004$), AFC ($r^2 = 0.745$, $P < 0.001$), and the number of retrieved oocytes ($r^2 = 0.738$, $P < 0.001$). Conversely, a correlation was not observed between TNF-α levels and the rate of oocyte maturation, fertilization, or the relative expression level of GABRA-1 ($r^2 = 0.241$, 0.254, and 0.032, respectively; $P > 0.05$).

Discussion

This study primarily assessed the relative gene expression levels of TNF-α, a pro-inflammatory cytokine, and GABRA-1, a cell apoptosis marker, in two distinct populations characterized by their responses to ovarian stimulation during infertility treatment with the IVF program. The primary finding indicated that the relative gene expression levels of TNF-α in the patients with POR were significantly higher than those in the patients with NOR. Furthermore, the relative gene expression levels of TNF-α exhibited a positive correlation with ovarian reserve, including AMH level, AFC, and number of oocytes retrieved following the IVF procedure. In contrast, the apoptotic marker GABRA-1 did not exhibit any significant variation in ovarian response to the stimulation protocol.

TNF-α is a potent, multifunctional pro-inflammatory cytokine that has diverse effects on multiple cell types and the development of chronic inflammatory diseases. [16] TNF-α was initially identified in 1975, [17] and it is one of more than 20 members of the tumor necrosis factor family. [18] Various cell types have been reported to possess the ability to produce TNF-α, including activated monocytes, fibroblasts, and endothelial cells. [18] In the context of human reproductive physiology, TNF-α and its receptor have been identified in the ovaries, follicles, and oocytes. [19] The preliminary research, focusing on the TNF-α serum levels and their impact on pregnancy outcomes in patients undergoing IVF, was studied in 2007. The findings did not identify a significant correlation between TNF-α levels, interferon (IFN)-γ levels, and implantation rate. [18] However, other parameters, such as AFC, number of mature oocytes, fertilization

success rate, and quality of the embryo, were not reported. [18] A subsequent investigation involving the patient diagnosed with endometriosis, a chronic inflammatory disorder, revealed that the increased levels of TNF-α in the follicular fluid negatively affected the fertilization outcomes after IVF treatment. [20] This suggests that TNF-α may play a crucial role in influencing the ovarian microenvironment. [20] In an animal model, follicle atresia was modulated by the TNF-α/receptor complex through the involvement of TNFR1-associated death domain protein and/or Fas-associated death domain along with pro-caspase protein, which resulted in the apoptosis of granulosa cells and oocytes. [21] The loss of granulosa cell function might result in the reduction of serum AMH levels, which serves as the marker of ovarian reserve, as AMH is synthesized by the granulosa cells of developing follicles. [22] This hypothesis might be supported by the study of *in vitro* cultured bovine granulosa cells. [23] The supplementation of the exogenous TNF-α diminished the endocrine activity of granulosa cells, as indicated by the reduction of the inhibin A, inhibin B, and AMH gene expression levels. [23] In addition, an *in vitro* investigation utilizing mural granulosa cells obtained from rats also indicated that the direct supplementation of TNF-α or the exogenous induction through the stress factors negatively affected oocyte development by initiating apoptosis in the ovarian cells, [24] resulting in a decreased number of retrieved follicles and oocytes.

Considering the elevated TNF-α levels in patients with POR, the direct mechanism underlying this phenomenon has not yet been clarified. However, TNF-α is regarded as a notable factor that can impede follicle development, [25] as evidenced by the reduced number of follicles observed in patients with POR. Furthermore, TNF-α could inhibit gonadotropin-stimulated steroidogenesis in the ovary. [25] The proposed mechanisms for this effect include the activation of protein kinase A (PKA). [26] This indicates that TNF-α could inhibit FSH-stimulated estradiol secretion at the post-cAMP site, with PKA identified as the subsequent key component in the signaling pathway, [26] which may result in poor follicle development competency. In addition, TNF-α cytokines are theoretically produced by monocytes or macrophages during the acute inflammatory phase, while a recent study indicated that the pro-inflammatory cytokines interleukins, such as IL-10,

were related to POR occurrence. [27] These mechanisms might indicate the underlying cause of elevated TNF-α levels in the patients with POR recruited by our study. To date, the comprehensive regulatory pathway of TNF-α related to poor ovarian function remains largely unexplored. Therefore, future research should focus on investigating the influence of various inflammatory cytokines on the pathogenesis of POR.

For GABRA-1, the GABRA-1 gene encodes the highly conserved and abundantly expressed subunit of the GABRA receptor family. [28] Pathogenic variants in GABRA-1 have been linked to the increased risk of neurodevelopmental syndromes. [28] However, recent findings have revealed that GABRA-1 serves as the functional downstream target of the non-coding RNA miR-139-5p. [29] The downregulation of this complex inhibits malignant cell proliferation via the apoptotic pathway. [29] In general, follicle atresia plays an important role in maintaining ovarian homeostasis, and any dysregulation in this process can lead to other disorders such as premature ovarian insufficiency or polycystic ovary syndrome. In contrast, research data has well established that granulosa cell death, or apoptosis, is a major factor that contributes to the atresia of developing follicles, which can notably affect the quantity of mature follicles. [30] Regarding the present data, this is the first study to evaluate the relative gene expression levels of GABRA-1 in patients with diminished ovarian reserve during an IVF program. A previous study in the avian species indicated that the apoptotic rates of granulosa cells transfected with GABRA-1 shRNA lentivirus increased via caspase-3 activity. [13] However, in our study, no differences in GABRA-1 levels between the participants with POR and those with NOR were observed. Furthermore, there is a notable lack of research addressing the role of GABRA-1 in human ovarian biology. The contradictory findings with that of avian species suggest that GABRA-1 may not be implicated in the cellular pathways associated with the proliferation and apoptosis of human follicles, oocytes, and ovarian cells, particularly in relation to inadequate follicle development in patients with POR. To validate this hypothesis, it is essential to perform studies examining the GABRA-1 gene or protein expression in human ovarian cells derived from the different categories of ovarian pathology. The current study had limitations because of its small sample size and small number of candidate genes. The gene regulation factors that influence the ovarian response could vary among individuals, thus complicating the establishment of a definitive correlation between the two candidate genes and ovarian stimulation outcomes within a limited population.

Conclusion

The current research highlights the variations in the blood pro-inflammatory cytokine TNF-α and apoptosis marker GABRA-1 in the NOR and POR cohorts. The increased relative gene expression levels of TNF-α were observed in patients with POR and revealed a positive correlation with the diminished ovarian reserve and reduced number of retrieved oocytes. Our findings primarily concluded that TNF-α might contribute to the development of POR. For clinical applications, the monitoring of blood TNF-α levels before undergoing ovarian stimulation and the IVF program may identify individuals at risk of POR. This early detection may facilitate timely interventions and contribute to the development of new biomarkers for evaluating ovarian function and predicting ovarian stimulation outcomes.

In addition, this study is the first to investigate the relative gene expression levels of GABRA-1 in blood samples from patients with infertility. Although the specific data could not be obtained, further research on GABRA-1 and other apoptosis markers associated with follicle development and ovarian response in patients with POR is warranted.

Conflict of interest statement
The authors declare the absence of any conflict of interest.

Data sharing statement

All analyzed data in the present study are included in this article. Further details are available for non-commercial purposes from the corresponding author upon reasonable request.

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