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Research Article

Assessment of VEGF and TNF-alpha levels in patients with an unexpectedly poor and suboptimal response during the treatment of infertility using ART methods

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Abstract

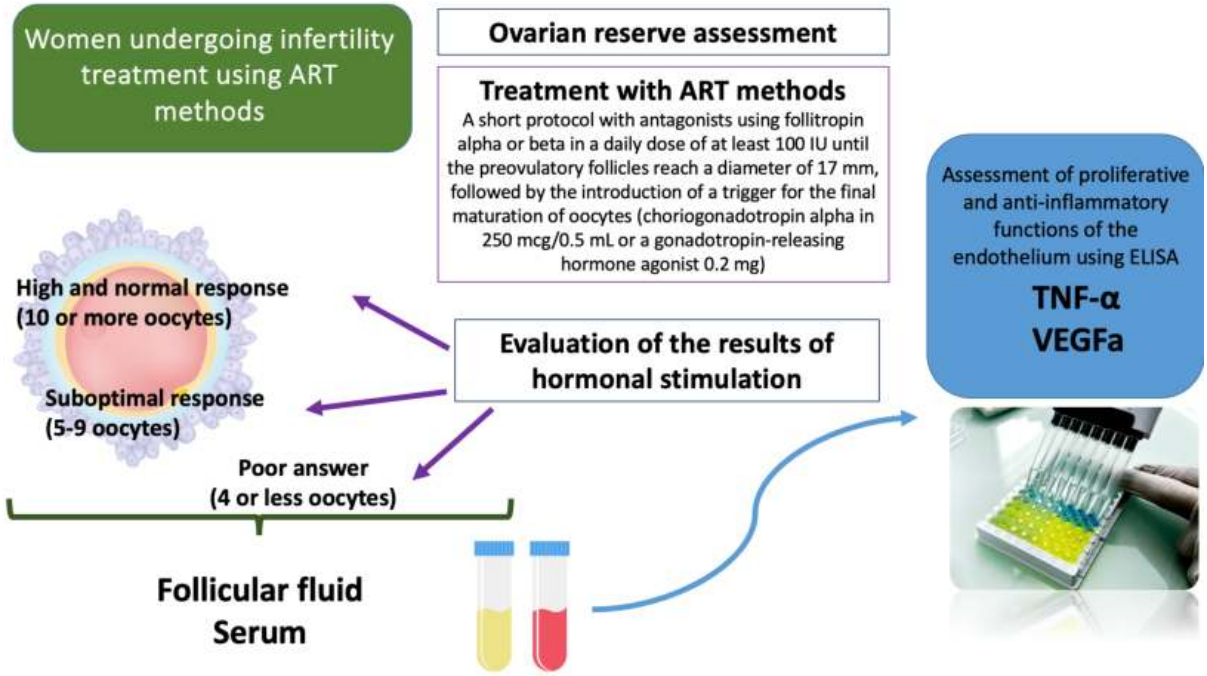
Introduction: The problem of infertility treatment currently has medical, socio-demographic and economic significance. Progress in the field of reproductive technologies has improved the situation, but the issue has not yet been completely resolved. Infertility is diagnosed in 8-12% of couples of reproductive age; in Russia this figure exceeds 15%, and, according to WHO, it is a critical level. It is known that immunological factors can disrupt the reproductive process at the stages of folliculogenesis, ovulation, and implantation. These include vascular endothelial growth factor (VEGF), tumor necrosis factor-alpha (TNF-α) and others. **The aim of this study:** to assess the levels of VEGF and TNF-α in women with different outcomes of hormonal stimulation of the ovaries during the treatment of infertility using ART methods.

Materials and methods: Based on voluntary informed consent, a simple open comparative randomized study was conducted with the participation of 71 women in the Volgograd region undergoing infertility treatment using ART methods. Inclusion criteria were the age up to 42 years inclusive and the anti-Mullerian hormone level over 1.2 ng/mL. Before ovulation stimulation with gonadotropins, data on ovarian reserve parameters were collected. Based on the results of stimulation, women were divided into 3 groups: Group 1 – with high and normal ovarian response – 10 or more oocytes were obtained (control group); Group 2 – with suboptimal ovarian response – 5-9 oocytes received; Group 3 – with a poor response – 4 or less oocytes were received. After venipuncture, which was performed in preparation for standard anesthesia, VEGF and TNF-α were quantitatively determined in the blood plasma using an enzyme-linked immunosorbent assay.

Results: The study results showed a statistically significant difference in VEGF levels in follicular fluid and serum between the groups of women with high/normal, suboptimal and poor ovarian response to gonadotropin stimulation. A higher level of this marker was observed in the serum of patients with high/normal and poor response – 48.15±4.23 and 41.29±8.26 pg/mL, respectively, while in women with a suboptimal response a lower VEGF level was determined –29.19±3.41 pg/mL. The levels of VEGF in the follicular fluid of women included in the high/normal, suboptimal and poor response groups were 35.95±3.20; 27.42±2.53 and 41.22±3.23 pg/mL, respectively (Table 1). As for TNF-α, its serum level in women with a high and normal response was lower than in the patients with a suboptimal response. In the follicular fluid of patients of group 3, there was a higher level of TNF-α, compared to groups 1 and 2, where the indicator was almost the same. However, the difference was not statistically significant.

Conclusions: Thus, VEGF is directly involved in the mechanisms of regulation of oocyte maturation and can be not only a marker of poor ovarian response, but also a predictor of unsatisfactory results of ovarian stimulation in the treatment of infertility using ART methods. TNF-alpha in follicular fluid does not have a statistically significant effect on follicle development in the treatment of infertility using assisted reproductive technologies.

Graphical abstract



Keywords

infertility, assisted reproductive technologies, immunological factors, VEGF, TNF-α, follicles

Introduction

The problem of infertility treatment currently has medical, socio-demographic and economic significance (Massarotti et al. 2019). Progress in the field of reproductive technologies has improved the situation, but the issue has not yet been completely resolved (Bahamondes and Makuch 2014; Fauque et al. 2020). Infertility is diagnosed in 8-12% of couples of reproductive age; in Russia this figure exceeds 15%, and, according to WHO, it is a critical level (Sugurova et al. 2021).

There are many causes of female infertility: polycystic ovary syndrome, fallopian tube obstruction, endometriosis, numerical and structural chromosome abnormalities, etc. It is known that immunological factors can disrupt the reproductive process at the stages of folliculogenesis, ovulation, and implantation. These may be vascular endothelial growth factor (VEGF), tumor necrosis factor α (tumor necrosis factor-alpha, TNF-α) and others (Bouet et al. 2020; Abhari et al. 2022; Guzmán et al. 2023).

It was found that the onset of pregnancy is associated with the process of angiogenesis, which is important for

the growth of the dominant follicle, initiation of the second meiotic division and implantation. Dysfunction of the formation of new vessels and the growth of the vascular wall is considered as one of the key pathophysiological factors limiting the implantation of embryos and contributing to the development of vascular complications during pregnancy. The vascular endothelial growth factor-A (VEGFA) system is a set of proteins with multiple angiogenic and antiangiogenic isoforms and receptors. Members of the VEGF system influence the proliferation, survival and migration of endothelial and non-endothelial cells and play an essential role in the regulation of follicular angiogenesis. Production of VEGF by secondary follicles stimulates their preantral development by directly acting on follicular cells to facilitate the development of follicular vasculature and formation of the underlying antrum. VEGF can provide a pro-angiogenic environment, trigger angiogenesis, stimulate follicular cells and antral follicle growth (Guzmán et al. 2023). In addition, VEGF is a necessary component of reproductive processes, embryonic development, and placenta formation. It has been shown that under the influence of follicle-stimulating hormone, the expression of VEGF in granulosa cells increases and the total number of blood vessels in the ovarian follicles of mice increases (Li et al. 2020). Inhibition of angiogenic factors impairs preantral and antral follicular growth and ovulation (Guzmán et al. 2021). It was found that a decrease in the area of the vascular network of the rat ovaries reduces the population of preantral follicles, whereas an increase increases, and the addition of VEGF to the culture medium has been shown to have a positive effect on the development of preantral follicles and allows obtaining mature oocytes (Araújo et al. 2011). Zhao et al. (2010) studied the influence of VEGF, nitric oxide (NO) and endothelin-1 in follicular fluid on the outcome of *in vitro* fertilization (IVF) procedures. It was shown that the content of VEGF and NO in the follicular fluid of antral follicles increased significantly in women who became pregnant, compared to those who did not become pregnant. At the same time, excess production of VEGF may be associated with such an unfavorable complication of infertility treatment as ovarian hyperstimulation syndrome (Naredi et al. 2014).

Pro-inflammatory cytokine TNF-α is known to be involved in the development, proliferation and apoptosis of ovarian follicular cells in mammals. Its receptors are localized on oocytes, granulosa and interstitial cells, which allows for the possibility of autocrine or paracrine action. It has been shown that in bovine, TNF-α and its receptors TNFR1 and TNFR2 are expressed in preantral and antral follicles. At the same time, the addition of TNF-α to the culture medium reduces the survival of follicles and increases the number of apoptotic cells in ovarian tissue *in vitro* (Silva et al. 2020). Ma et al. (2010) assessed the number of immature porcine oocytes reaching the MII stage, and also studied the cytoskeleton and chromosomal distribution of MII oocytes when co-cultured with TNF-α. It was found that TNF-α at a concentration of 5 ng/mL reduced the rate of oocyte maturation compared to control; when exposed to 10-100 ng/mL, it led to a significant increase in the frequency of defective spindles or abnormal distribution of microfilaments, at a concentration of 200 ng/mL, which caused disruption of chromosome alignment (Ma et al. 2010).

Thus, in the literature there is sufficient data on the influence of the above-mentioned cytokines on reproductive function, but their role in the treatment of infertility using assisted reproductive technologies (ART) in women with different outcomes of hormonal stimulation of the ovaries is not sufficiently covered.

In this regard, the purpose of our study was to assess the levels of VEGF and TNF-α in women with different outcomes of hormonal stimulation of the ovaries during the treatment of infertility using ART methods.

Materials and Methods

Groups

Based on the results of ovarian stimulation, women were divided into groups:

1) women with high and normal ovarian response – 10 or more oocytes were received (control group);
2) women with a suboptimal ovarian response – 5-9 oocytes received;
3) women with a poor response – 4 or fewer oocytes were received.

Study design

A simple open comparative randomized study in parallel groups was conducted. On the basis of voluntary informed consent, 71 women of the Volgograd region were examined: they were undergoing infertility treatment using ART methods at Clinic No. 1 of Volgograd State Medical University of the Ministry of Health of Russia (Volgograd, Russia). Inclusion criteria were age up to 42 years inclusive (mean age –34.79±4.26 years) and the anti-Mullerian hormone level over 1.2 ng/mL. Exclusion criteria were age under 18 and over 42 years, "soft" ovarian stimulation protocols, and refusal to participate in a clinical trial.

The study was approved by the local ethics committee of Volgograd State Medical University: Minutes No. 049 dated February 13, 2023.

Before stimulation, an assessment of the ovarian reserve was carried out, including determination of the level of AMH and follicle-stimulating hormone (FSH), as well as the number of antral follicles. The FSH level was determined no later than 6 months before stimulation on days 2-5 of the menstrual cycle.

Hormonal stimulation of the ovaries was prescribed by doctors at Clinic No. 1 of Volgograd State Medical University of the Ministry of Health of Russia (Volgograd, Russia) in accordance with the clinical recommendations, taking into account the individual characteristics and interests of the patient.

The clinical trial included cases in which hormonal stimulation was performed using a short protocol with antagonists using gonadotropin preparations (Follitropin alfa or beta) at a daily dose of at least 100 IU (average starting dose of FSH was 125 IU, total dose – 1700 IU) until the preovulatory follicles reach a diameter of 17 mm, followed by the introduction of a trigger for the final maturation of oocytes in standard doses with a human chorionic gonadotropin drug (choriogonadotropin alpha in 250 mcg/0.5 mL or a gonadotropin-releasing hormone agonist 0.2 mg) with the exception of "soft" stimulation in the treatment of infertility using high and normal ART methods; suboptimal and poor ovarian response.

After obtaining cumulus-oocyte complexes, the results of hormonal stimulation were assessed: cumulus-oocyte complexes were counted using microscopy, the number of mature oocytes was determined after transvaginal puncture as part of the standard procedure of *in vitro* fertilization or intracytoplasmic sperm injection. The punctures were performed 36 hours after the trigger was introduced.

A single blood sample from the cubital vein and follicular fluid was taken from 8:00 to 11:00 am. After venipuncture, which was performed in preparation for standard anesthesia, blood was placed in sterile tubes without anticoagulant. Next, the samples were centrifuged at 1000 g for 20 minutes at room temperature.

Follicular fluid is not used in the fertilization procedure, but is a by-product that is usually disposed of after obtaining cumulus-oocytic complexes. The tube with follicular fluid was centrifuged at 1000 g for 20 minutes to sediment the cumulus cells. Next, follicular fluid was collected.

The serum and follicular fluid collected after centrifugation were placed in Eppendorf tubes and frozen at -20ºC until analysis.

Laboratory tests

To assess the proliferative and anti-inflammatory functions of the endothelium in serum and follicular fluid, the levels of vascular endothelial growth factor VEGF and tumor necrosis factor TNF-α were determined by ELISA using ELISA Kits for Vascular Endothelial Growth Factor A (VEGFA) (Cloud-Clone Corp., USA) and ELISA Kit for Tumor Necrosis Factor Alpha (TNFa) (Cloud-Clone Corp., USA). Optical density was recorded on a SPECTROstar Nano tablet spectrometer (BMG Labtech, Germany) at a wavelength of 450 nm.

Statistical analysis

Statistical data processing was carried out using the GraphPad Prism 8 software package (GraphPad Software, USA). The normality of distribution was checked using the Shapiro-Wilk test. If the studied parameters were normally distributed, the Tukey test was used. If the distribution was different from normal, the Kruskal-Wallis test with Dunn's post-hoc test was used. In addition, an assessment of equality of variances (equal SDs) was carried out – when variances differed, Brown-Forsythe and Welch tests were used. Correlation analysis was performed using Spearman's correlation coefficient. Differences were considered statistically significant at $p<0.05$. Data are presented as M±SEM, where M is the arithmetic mean, SEM is the standard error of the mean.

Results

The age of the women taking part in the tests was the same: in the first group – 32.70±4.37 years, in the second – 36.65±3.05 years, in the third – 36.15±3.98 years. Women of all groups had a normal ovarian reserve, as indicated by the number of antral follicles – on average more than 7 and the AMH level above 1.2 ng/mL (Fig. 1). However, in response to ovarian stimulation with gonadotropins, "unexpected" poor and suboptimal responses were noted. The AMH level in patients with a poor response was 2.6 times less ($p<0.0001$), and with a suboptimal response – 1.7 times less compared to the group of patients with a high and normal response (1.97±0.16 and 2.95±1.73 versus 5.07±0.49 ng/mL, respectively). The FSH value did not differ statistically significantly in any group.

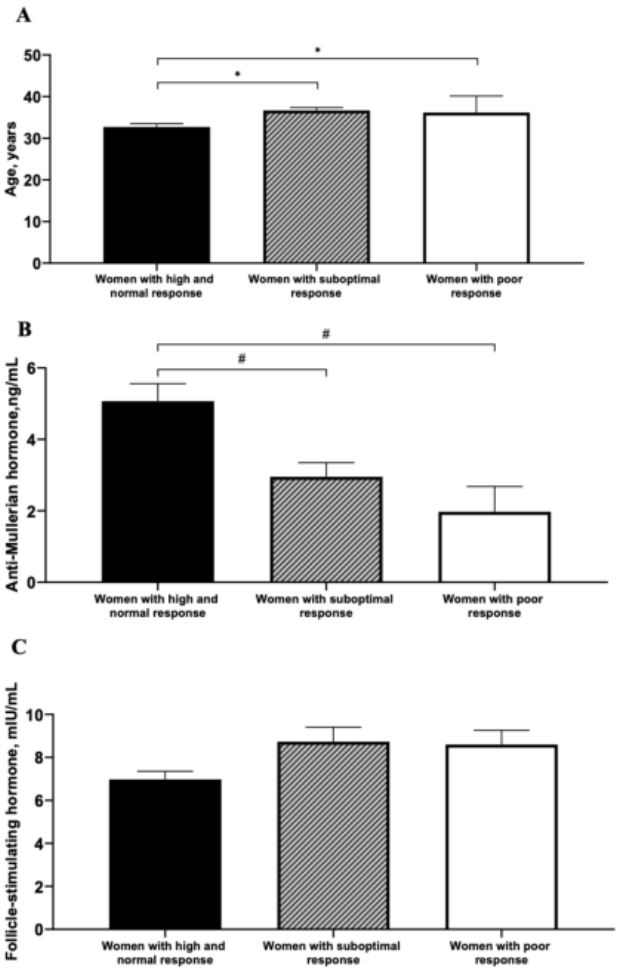


Figure 1. Age (**A**) and amount of anti-Mullerian hormone (**B**) and follicle-stimulating hormone (**C**) in women with different responses to ovarian stimulation during the treatment of infertility using ART methods. **Note:** The changes are statistically significant compared to the group of women with a high and normal response: * – according to the Tukey test, # – according to the Brown-Forsythe and Welch test, ($p<0.05$).

The number of antral follicles was 1.89 ($p<0.0001$) and 1.40 times ($p=0.0101$) times lower, respectively, in women with a poor and suboptimal response and amounted to 7.50±0.60 and 10.15±0.86 compared to the control group, in which the indicator was 14.23±0.82 (Fig. 2). The number of punctured follicles in groups 2 and 3 was less by 1.87 ($p<0.0001$) and 3.82 times ($p<0.0001$), respectively, compared to the indicators of women in group 1 (13.90±0.78 and 6. 8±0.66 versus 26.03±1.81). The number of cumulus-oocytic complexes in women with a suboptimal response was 9.05±0.58, in women with a poor response – 3.2±0.29. Compared to patients in group 1, where the indicator corresponded to 17.16±0.88, their number was 1.89 times ($p<0.0001$) and 5.51 times ($p<0.0001$) less. The number of mature oocytes obtained in groups 2 and 3 was less by 2.10 ($p<0.0001$) and 5.41 times ($p<0.0001$) (7.10±0.35 and 2.75±0.26 versus 14.90±0.73), respectively, compared to the control group (Fig. 2).

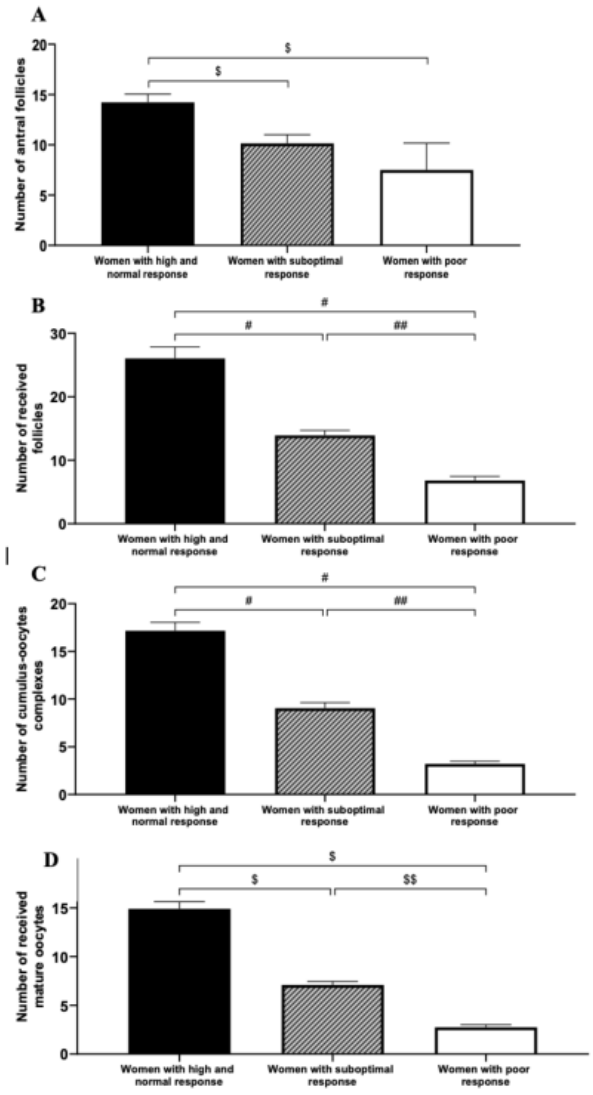


Figure 2. Number of antral (**A**) and punctate follicles (**B**), cumulus-oocyte complexes (**C**) and obtained mature oocytes (**D**) in women with different responses to ovarian stimulation during the treatment of infertility using ART methods. **Note:** The changes are statistically significant compared to the group of women with a high and normal response: $ – according to the Kruskal-Wallis test with Dunn's post-hoc test, # – according to the Brown-Forsythe and Welch test; compared to a group of women with a suboptimal response: $$ – according to the Kruskal-Wallis test with Dunn's post-hoc test, ## – according to Brown-Forsythe and Welch test ($p<0.05$).

The folliculo-oocyte index in women in the second group was 0.99±0.10, in the third - 0.47±0.061, which is 1.29 and 2.72 times less than in the first group, where the indicator was 1. 28±0.07 (Fig. 3). The follicular index of the obtained oocytes and the oocyte index (oocyte maturity index) in women with different responses to ovarian stimulation were not statistically significantly different.

The results of the study showed a statistically significant difference in the levels of VEGF in follicular fluid and in blood serum between groups of women with high/normal, suboptimal and poor ovarian response to stimulation with gonadotropins. A higher level of this marker was observed in the serum of patients with a high and poor response – 48.15±4.23 and 41.29±8.26 pg/mL, respectively, while in patients with a suboptimal response a lower level was determined VEGF-29.19±3.41 pg/mL. The levels of VEGF in the follicular fluid of women included in the high/normal, suboptimal and poor response groups were 35.95±3.20; 27.42±2.53 and 41.22±3.23 pg/mL, respectively (Table 1). A statistically significant difference was found between the second and third groups ($p=0.0052$).

As for TNF-α, its serum level in women with a high and normal response was lower than in patients with a suboptimal response. In the follicular fluid, patients of group 3 had a higher level of TNF-α, compared to groups 1 and 2, where the level was almost the same. The difference between groups was not statistically significant (Table 1).

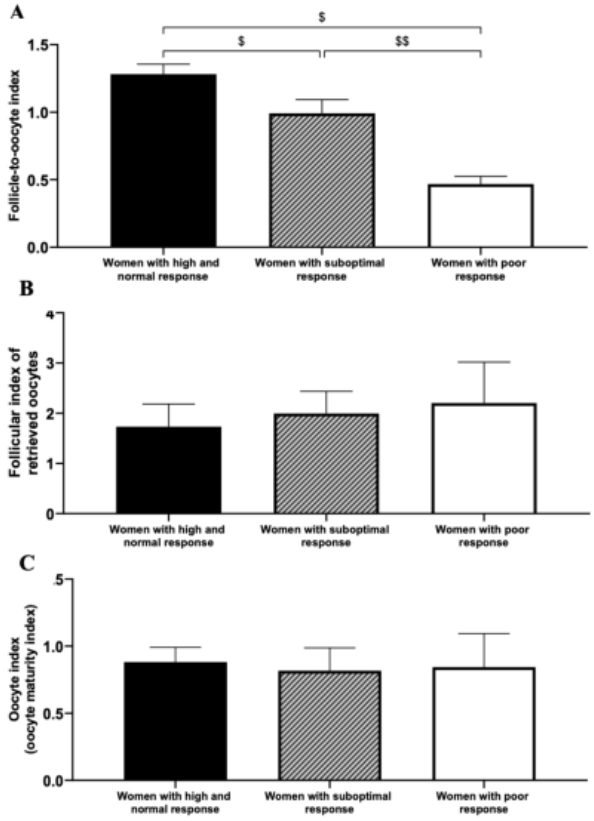


Figure 3. Follicular-oocyte index (A), follicular index of obtained oocytes (B) and oocyte index (oocyte maturity index) (C) in women with different responses to ovarian stimulation during the treatment of infertility using ART methods. **Note:** The changes are statistically significant compared to the group of women with a high and normal response: $ – according to the Kruskal-Wallis test with Dunn's post-hoc test; compared to the group of women with a suboptimal answer: $$ – according to the Kruskal-Wallis test with Dunn's post-hoc test ($p<0.05$).

Correlation analysis showed a moderate negative relationship ($r=-0.33$, $p=0.005$) between the age of patients and the number of AMH, a similar relationship was found between the age of women and the number of antral follicles ($r=-0.26$, $p=0,03$). The FSH level negatively correlated with the number of AMH ($r=-0.29$, $p=0.015$) and antral follicles ($r=-0.36$, $p=0.002$). There is a strong positive relationship between the AMH level and the number of antral follicles ($r=0.68$, $p<0.0001$), a moderate positive relationship between the AMH concentration and the follicle-oocyte index ($r=0.28$, $p=0.02$), as well as a negative relationship between the amount of AMH and the concentration of TNF-α in serum ($r=-0.33$, $p=0.006$) and follicular fluid ($r=-0.35$, $p=0.003$). The number of antral follicles negatively correlated with the level of serum TNF-α ($r=-0.28$, $p=0.02$) (Fig. 4).

Table 1. Levels of VEGF and TNF-α in serum and follicular fluid in women with different responses to ovarian stimulation during the treatment of infertility using assisted reproductive technologies (M±SEM)

Indicators studied	Group 1 – Women with high and normal response (n=31)	Group 2 – Women with suboptimal response (n=20)	Group 3 – Women with poor response (n=20)	p	Statistical test
VEGF in blood serum, pg/mL	48.15±4.23	29.19±3.41	41.29±8.26	p_{1-2}= 0.0101 p_{1-3}= 0.0443 p_{2-3}>0.9999	Kruskal-Wallis test (with Dunn)
VEGF in follicular fluid, pg/mL	35.95±3.2	27.42±2.53	41.22±3.23	p_{1-2}= 0.1368 p_{1-3}= 0.4534 p_{2-3}=0.0052	Kruskal-Wallis test (with Dunn)
TNF- α in blood serum, pg/mL	8.36±7.62	14.56±2.70	10.74±1.56	p_{1-2}= 0.0030 p_{1-3}= 0.1467 p_{2-3}=0.6409	Kruskal-Wallis test (with Dunn)
TNF- α in follicular fluid, pg/mL	11.59±1.00	11.28±1.32	13.66±1.36	p_{1-2}>0.9999 p_{1-3}= 0.6828 p_{2-3}=0.5898	Kruskal-Wallis test (with Dunn)

Note: Data are presented as M±SEM, where M is the arithmetic mean, SEM is the standard error of the mean.

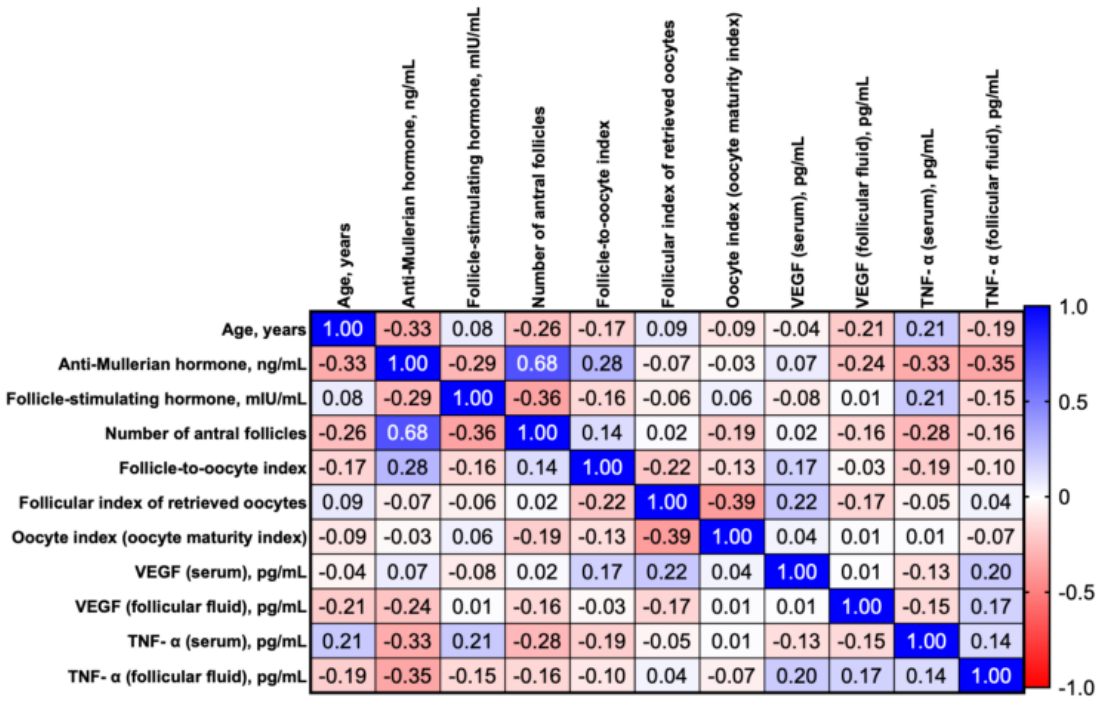


Figure 4. Correlation analysis of the relationship between VEGF and TNF-α in serum and follicular fluid in women with different responses to ovarian stimulation during the treatment of infertility using assisted reproductive technologies and ovarian reserve. **Note:** The data is presented as a heat map (the sample is not divided).

Discussion

Oocyte competence and embryo development are determined by many factors in the follicular environment (Warzych and Lipinska 2020; Babayev and Duncan 2022). Analysis of its components provides information about changes in the microenvironment of the oocyte. The possibility of studying the factors produced in the human ovary has become especially real with the introduction of IVF methods into practice.

Interactions between developing oocytes and surrounding cells are facilitated by cytokines; they also regulate all stages of folliculogenesis. Thus, their level in follicular fluid may be a prognostic marker for obtaining high-quality embryos and pregnancy. In addition, the determination of cytokines in follicular fluid in women with an unfavorable outcome of IVF may serve as a basis for the development of immunotherapy for such cases.

Currently, there are conflicting reports regarding the effect of VEGF and TNF-α on embryogenesis. Increased levels of VEGF in follicular fluid have been associated with the development of ovarian hyperstimulation syndrome, poor fertilization and premature luteinization (Kudsy et al. 2016; Wu et al. 2021). In other studies (Monteleone et al. 2008; Chen et al. 2023), on the contrary, high levels of VEGF contributed to a higher fertilization rate, the production of better quality embryos and more frequent pregnancy.

Elevated levels of TNF-α can negatively affect embryogenesis, inhibit oocyte maturation and cause an increase in the frequency of chromosomal abnormalities. A connection between TNF-α and a decrease in the number of fertilized oocytes in an IVF cycle has been revealed (Gaafar et al. 2014). However, TNF-α has previously been shown to enhance folliculogenesis and ovulation, promote vascularization, and suppress granulosa cell proliferation and spontaneous apoptosis. TNF-α is one of the vital cytokines for antral follicle growth and selection; local dose of TNF-α can determine the follicular response (Alhilali et al. 2020).

Our results are consistent with the data of Battaglia et al. (2000) and Nouri et al. (2014), which also showed that a high ovarian response is associated with an increased level of VEGF, which may be one of the markers and predictors of the development of ovarian hyperstimulation syndrome. At the same time, Battaglia et al. (2000) showed that low ovarian reserve and poor response are associated with high levels of VEGF in comparison with a group of patients with a normal response. According to the researchers, higher levels of VEGF were associated with lower preovulatory serum estradiol levels.

Expression of the VEGF gene is controlled by several mechanisms, including the influence of the partial pressure of oxygen in the blood. Low partial pressure of oxygen induces VEGF mRNA expression. In turn, adenosine, which accumulates intensively under hypoxic conditions, activates adenosine receptors, which also leads to an increase in the concentration of cAMP and VEGF mRNA.

In patients with a poor response, there is increased resistance in the perifollicular arteries, which leads to decreased oxygen delivery to developing oocytes. Impaired perifollicular microcirculation and subsequent hypoxia can lead to an increase in the number of immature oocytes, a low follicular-oocyte and follicular index, as well as a high proportion of aneuploid eggs obtained in women with normal and high levels of ovarian reserve.

TNF-α is one of the significant factors at the stage of selection of the dominant follicle and preovulatory development; however, its role in folliculogenesis has not been fully studied. TNF-α is a hormone-like polypeptide that performs a wide range of biological functions. Its main source in the ovary are macrophages, as well as cells of the corpus luteum, theca and granulosa. This cytokine acts by binding to two types of receptors: TNFR1 is responsible for transmitting the death signal, while TNFR2 is involved in cell proliferation. Thus, in the ovary, TNF-α can initiate both apoptosis and proliferation depending on the stage of follicle development. Apparently, the decrease in the number of oocytes and the death of granulosa cells of unruptured follicles increase follicular atresia. These effects are mediated by TNF-α through autophagy and apoptosis. In a study by Alhilali et al. (2020), the level of the cytokine significantly and inversely correlated with the severity of ovarian hyperstimulation syndrome and, accordingly, with the number of oocytes obtained. The concentration of TNF-α is lower in the follicular fluid in the group of patients with severe OHSS, in which the number of eggs was higher than in normoreactive patients.

Our study found elevated levels of TNF-α in the follicular fluid of poor responder patients, which may indicate an inflammatory micro-environment during follicle development and is consistent with the literature (Alhilali et al. 2020; Huang et al. 2023). At the same time, the differences were not statistically significant and can only indicate a tendency towards an increase in the indicator.

Conclusion

Thus, VEGF is directly involved in the mechanisms of regulation of oocyte maturation and can be not only a marker of a poor ovarian response, but also a predictor of unsatisfactory results of ovarian stimulation in the treatment of infertility using ART methods.

TNF-alpha in follicular fluid does not have a statistically significant effect on follicle development in the treatment of infertility using assisted reproductive technologies.

Conflict of interest

The authors declare the absence of a conflict of interests.

Aknowledgement

The study was carried out with financial support from the Russian Science Foundation within project No. 23-25-00067 “The role of the endothelium in the regulation of oocyte maturation in the treatment of infertility using assisted reproductive technologies.”

Data availability

All of the data that support the findings of this study are available in the main text.

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