PHYSIOLOGY

Relationships Between Concentrations of Tumor Necrosis Factor-α and Nitric Oxide in Follicular Fluid and Oocyte Quality

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Purpose: *Our objective was to explain a relationship between concentrations of tumor necrosis factor-α (TNF-α) and nitric oxide (NO) in follicular fluid, oocyte quality, and outcomes of in vitro fertilization–embryo transfer (IVF-ET).*
Methods: *The concentrations of TNF-α and NO were measured in 115 follicular fluid samples collected from 43 patients undergoing IVF-ET program, due to tubal obstruction, some with endometriosis (8 patients) or hydrosalpinx (5 patients). A correlation of these factors concentrations and the oocyte quality, the oocyte maturity, and infertility-associated diseases was analyzed.*
Results: *No correlation was found between concentrations of NO and TNF-α in follicular fluid. NO concentrations in follicular fluids were significantly higher in patients with endometriosis ($P < 0.001$) or hydrosalpinx ($P < 0.01$) compared to the patients with just tubal obstruction. Follicular NO concentration differences according to oocyte maturity and oocyte quality were not found. In contrast, TNF-α concentrations in follicular fluids were significantly higher in poor quality oocytes ($P < 0.05$) but were not associated with infertility-associated diseases, such as hydrosalphinx or endometriosis, and the oocyte maturity. No significant differences in follicular levels of NO and TNF-α as well as IVF-ET parameters of pregnant and nonpregnant groups were revealed.*
Conclusions: *There is no significant correlation between the concentrations of NO and TNF-α in follicular fluid. NO levels in follicular fluid are altered in infertility-associated diseases. However, TNF-α levels but not NO levels influence oocyte quality. These results suggest that the production of NO and TNF-α in follicular fluid may be regulated via different pathways and can be tempered with infertility-associated diseases, thereby influencing oocyte quality locally.*

KEY WORDS: follicular fluid; nitric oxide; tumor necrosis factor-α; oocyte quality.

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INTRODUCTION

Oocyte quality is one of the most important factors associated with successful pregnancy in in vitro fertilization and embryo transfer (IVF-ET). The microenvironment of the follicle is vital for normal oocyte development, folliculogenesis, and timely ovulation. Since follicular fluid is the environment where oocyte maturation occurs, it should influence fertilization and early embryo development. In this respect, alteration of cellular components within follicular fluid could play an important role in determining oocyte quality.

Numerous studies have identified tumor necrosis factor-α (TNF-α) and nitric oxide (NO) in human follicular fluid (1,2). Moreover, TNF-α increases gradually during the follicular phase, reaching the maximal level around the preovulatory period (3). Also, plasma concentration of NO rises in the follicular phase respect to the secretory phase, and peaks at midcycle (4). TNF-α in various cell systems commonly activates an

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inducible nitric oxide synthase (iNOS). Mobilization of this enzyme generates NO from L-arginine (5,6). Both TNF-α (7) and NO (8) regulate apoptosis, which is associated with follicular atresia (9), and modulate the ovarian steroidogenesis (10–12). Especially, NO is known to have a double-edged role for apoptosis (8). These data indicate that alterations in the levels of TNF-α and NO within follicular fluid can disturb oocyte maturation and folliculogenesis, thereby influencing oocyte quality.

However, it is still unclear whether the levels of NO and TNF-α in the follicular fluid is correlated with oocyte quality and, in turn, the outcomes of IVF-ET. Thus, in an attempt to understand their functional roles in oocyte quality and results of IVF-ET, we investigated the concentrations of NO and TNF-α in follicular fluid collected during oocyte retrieval after controlled ovarian hyperstimulation (COH) and IVF-ET.

MATERIALS AND METHODS

The study was conducted on 43 patients undergoing IVF-ET program in the Pusan National University Hospital (PNUH). Written informed consent was obtained from each woman before the procedures. The consent forms and protocols used were approved by the Human Investigation Committee of PNUH. Mean age of the patients was 32.4 ± 4.9 years (mean ± SD, ranging from 25 to 43 years), and indications for IVF-ET included either one or both tubal obstruction to exclude the deviation resulting from different indications. Within the patient population, eight had endometriosis and five had hydrosalpinx.

Controlled ovarian hyperstimulation was performed by either a long or short standard protocol. In standard long protocol, 0.6 mg/day of gonadotropin-releasing hormone agonist (buserelin acetate; Frankfrut, Germany) was administrated, starting from the midluteal phase of the preceding cycle, and then buserelin acetate was reduced to half dosage on the day that gonadotropin was administered. In short protocol, 0.3 mg/day of buserelin acetate was given from the first day of the menstrual cycle. The stimulation with human menopausal gonadotropin (hMG) (Organon, Netherlands) and high purified follicle-stimulating hormone (HP-FSH) (Serono; Norwell, MA) was initiated when no sonographic evidence of ovarian follicular activity was demonstrated and serum estradiol (E_2) level was less than 50 pg/ml (conversion factor to SI: 3.671). Follicular development was assessed in all patients by daily monitoring of serum E_2 level and ovarian ultrasonography. Human chorionic gonadotropin (hCG) (pregnyl, Organon, Netherlands) was administrated when E_2 level reached the maximal peak and at least two dominant follicles were 17 mm or larger simultaneously.

Oocyte retrieval by transvaginal guidance was performed approximately 34–35 h after the hCG administration. Oocyte maturation was evaluated by the following criteria under phase-contrast microscope: (a) mature: the presence of first polar body and the radical expansion of cumulus cells; (b) intermediate I: the absence of first polar body and the radical expansion of cumulus cells; (c) intermediate II: the absence of first polar body and the compaction of cumulus cells; (d) immature: the presence of germinal vesicle; and (e) atresia: the severe abnormal morphology or ooplasm-free oocyte. Also, oocyte quality was classified into three groups: good quality oocyte with bright color and normal morphology; moderate quality oocyte with dark color and normal morphology; and poor quality oocyte with dark color and abnormal morphology.

Follicular fluid from each follicle was aspirated separately into each 15-ml cornical tube during oocyte recovery. A total of 243 follicular fluid samples were obtained, and samples that contained blood, did not have ooycte, or contained more than a single oocyte were excluded from the study. In the end, 115 samples were used for assay of the present study. All follicles were between 15 to 20 mm in diameter. As soon as the oocyte was obtained in follicular fluid, the sample was immediately centrifuged at 800 × *g* for 20 min to remove all cells. Aliquots of the cell-free supernatants were then divided into polypropylene microcentrifuge tube and were stored at −70°C until the assay.

Serum E_2 level was measured by chemiluminescence immnoassay method. Since NO is converted to nitrite (NO_2^-) and nitrate (NO_3^-), both nitrite and nitrate were measured for the best index of total NO concentration in follicular fluid, and the concentration of NO in each follicular fluid was measured as reported by Green *et al.* (14). Briefly, after nitrate was reduced to nitrite by addition of nitrate reductase (from *Aspergilus* species) for 1 hr at 37°C, nitrite was measured colorimetrically by the Griess reaction (15). The color reaction was allowed to develop for 5 min at room temperature, after which the absorbance at 540 nM was recorded. The measurement of TNF-α in each follicular fluid was performed using a commercial kit of sandwich enzyme-linked immunosorbent assay (ELISA) (Medgenix Daignostics ELISA kit; Biosource Europe S.A., Fleurus, Belgium). The intra- and

interassay coefficients of variation were 3.7% and 9.9%, respectively. The assay sensitivity was 3 pg/ml.

Statistical analysis was carried out using the unpaired Student's *t*-test and the one-way ANOVA analysis. Results are presented as mean ± SEM; $P < 0.05$ was considered statistically significant.

RESULTS

The levels of NO and TNF-α in each follicular fluid were measured to investigate the relationship between their production, and no correlation was found between the concentrations of NO and TNF-α in follicular fluids (Fig. 1). Among 43 patients studied in the research, 16 patients were clinically pregnant (the pregnancy rate of 37.2%), which was defined by the transvaginal ultrasound visualization of a gestational sac following 4–5 weeks after ET. There were no significant differences in the concentrations of NO and TNF-α in follicular fluids as well as IVF-ET parameters like age and fertilization rate of pregnant and nonpregnant groups (Table I). The follicular fluid NO and TNF-α levels were indifferent to patients' age (Table 2).

Also, follicular fluid TNF-α and NO levels of patients with endometriosis and hydrosalpinx were compared to those of women with tubal obstruction. The levels of these substances were measured separately in 19 follicular fluid samples from 8 patients with endometriosis and 13 samples from 5 patients with hydrosalpinx. Follicular fluid NO levels were significantly higher in women with endometriosis (36.25 ± 4.49) or hydrosalpinx (29.74 ± 2.12) compared to women with just tubal obstruction (17.66 ± 1.32) ($P < 0.001$ and $P < 0.01$, respectively), but follicular fluid TNF-α concentrations had no significant differences with cause of infertility (Fig. 2).

In addition, we investigated the effects of concentrations of NO and TNF-α in follicular fluid on quality and maturity of oocyte. No differences in follicular fluid NO and TNF-α concentrations were detected in relation to oocyte maturity (Table III). Although oocyte quality was not correlated with NO levels, it was significantly correlated to the TNF-α concentrations in follicular fluid. The TNF-α concentration was 10.04 ±

Table I. Comparison of Pregnant and Nonpregnant Patients

	Pregnant group	Nonpregnant group	*P* value
No. of cases	16	27	
Age (years, mean ± SD)	32.3 ± 4.5	32.6 ± 5.2	NS[a]
No. of oocytes retrieved	11.2 ± 1.0	10.8 ± 1.6	NS
Fertilization rate (%)	85.2 ± 3.1	76.3 ± 3.8	NS
No. of embryos transferred	5.3 ± 0.5	5.8 ± 0.7	NS
NO concentration (μM)	21.4 ± 2.6	24.3 ± 2.2	NS
TNF-α concentration (pM)	11.2 ± 1.3	12.5 ± 2.1	NS

[a] NS, Not significant.

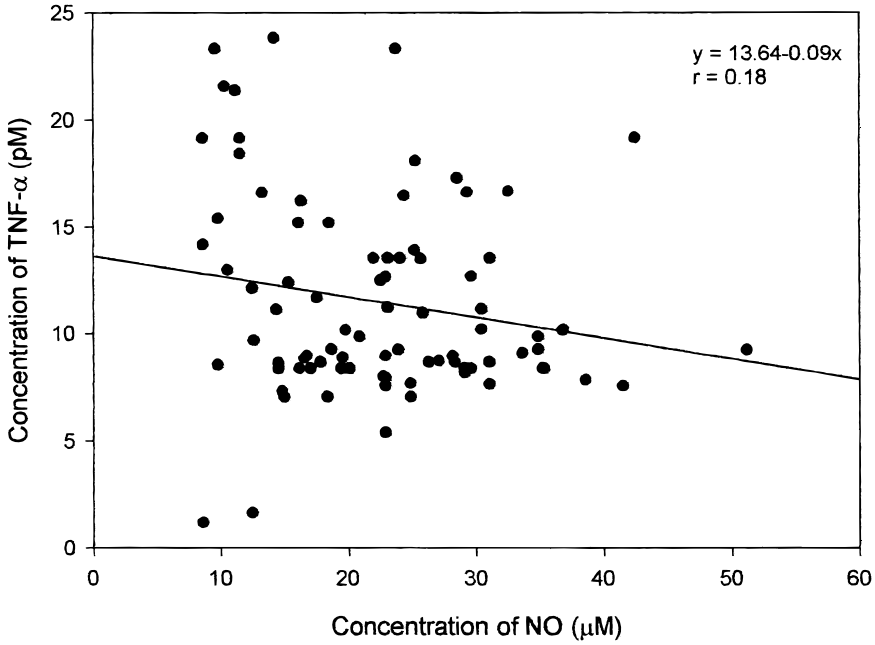

Fig. 1. Linear regression analysis showing the negative correlation between the concentrations of NO and TNF-α in follicular fluid ($r = 0.18$).

Table II. NO and TNF-α Concentrations in Follicular Fluid According to Patient's Age

	Age (years)				
	≤30	31–35	36–40	>40	*P* value
No. of cases	19	11	8	5	
NO concentration (μM)	21.49 ± 1.89	24.32 ± 5.23	24.39 ± 3.41	25.21 ± 0.98	NS[a]
TNF-α concentration (pM)	12.49 ± 2.01	18.23 ± 1.20	13.21 ± 2.60	16.78 ± 0.62	NS

[a] NS, Not significant.

1.05 in good-quality oocytes, 9.64 ± 1.02 in moderate-quality oocytes, and 17.81 ± 2.54 in poor-quality oocytes ($P < 0.05$) (Table IV).

DISCUSSION

Follicular fluid is the important microenvironment where oocyte maturation takes place with folliculogenesis. Thus, changes in cellular components of follicular fluid might influence the maturity and oocyte quality, fertilization, early embryonic development, and pregnancy outcomes. Recent studies have demonstrated the cross-communication between the immune system and the reproduction system (15). In particular, TNF-α, which is primarily characterized for its tumorcidal activity, currently is recognized as having a possible effect on folliculogenesis and ovarian maturation (10,11,16). In addition, TNF-α stimulates the production of NO by inducing iNOS on various cell systems including bovine ovarian theca cells (5,6). Also, NO plays many roles in physiological processes including reproductive events as an important intracellular and intercellular messenger (17,18). Follicular fluid or plasma TNF-α and NO levels increase gradually during the follicular phase and peak at the midcycle (3,4). These results suggest that TNF-α and NO production in follicular fluid might occur in a coordinated manner and that alterations in follicular fluid NO and TNF-α concentrations could affect quality and maturity of oocyte.

However, the present study showed that no correlation was found between follicular fluid NO and TNF-α concentrations. This negative correlation was

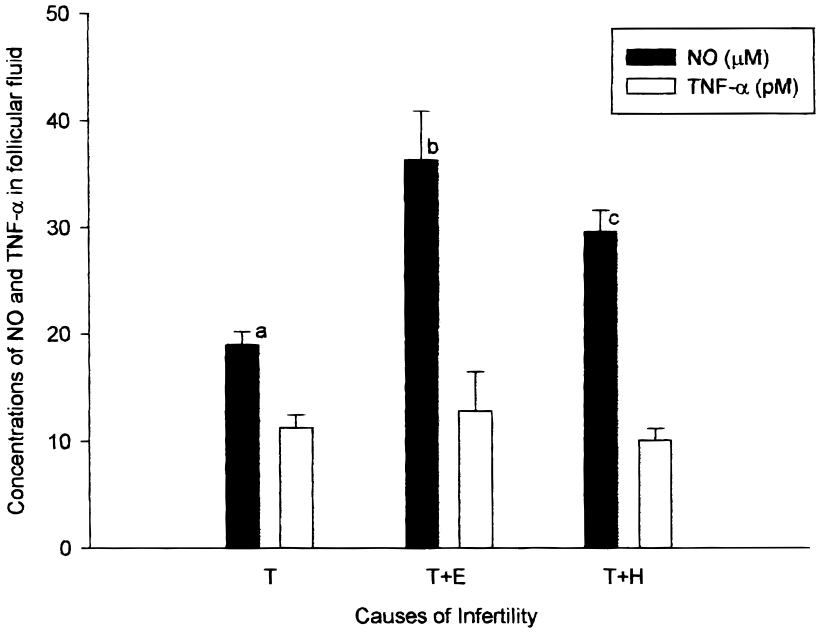

Fig. 2. Relationships between follicular fluid levels of NO and TNF-α and infertility-associated diseases. The levels of NO and TNF-α were evaluated in follicular fluid from 30 patients with just tubal obstruction (T), 8 patients with tubal obstruction and endometriosis (T + E), and 5 patients with tubal obstruction by hydrosalpinx (T + H). * $P < 0.001$ (b versus a); ** $P < 0.01$ (c versus a).

Table III. Concentrations of NO and TNF-α in Follicular Fluid According to Oocyte Maturity

	Oocyte maturity				
	Mature	Inter I[a]	Inter II	Immature	Atresia
No. of oocytes	65	22	10	8	10
NO Conc. (μM)	22.43 ± 1.32[b]	27.25 ± 4.15[b]	22.36 ± 2.15[b]	24.87 ± 2.41[b]	28.12 ± 1.71[b]
TNF-α Conc. (pM)	9.96 ± 1.59[b]	11.99 ± 1.45[b]	8.87 ± 1.66[b]	13.82 ± 5.23[b]	12.36 ± 1.23[b]

[a] Inter, Intermediate oocyte.
[b] Not significant (versus each group).

derived by the finding that NO concentrations were significantly higher in patients with tubal obstruction and endometriosis or hydrosalpinx compared to patients with just tubal obstruction, whereas TNF-α concentration were indifferent to the presence of endometriosis or hydrosalpinx. This is the first study to prove the existence of negative correlation between NO and TNF-α levels in follicular fluid. The finding implies that NO production in follicular fluid may not be regulated by TNF-α but by other factors.

NO is synthesized during the conversion from L-arginine to L-citrulline by mostly inducible and/or constitutive NOS. iNOS is activated only by cytokines, such as lipopolysaccharide, interleukin-1 (IL-1), and TNF-α. In contrast, the activity of constitutive NOS (cNOS), isolated from endothelium and brain, is calcium-dependent and also is regulated by sex hormones (19). Endothelial NOS (eNOS) and iNOS, but not neuronal NOS (nNOS), are localized in various ovarian cells and participate in ovulatory process (20,21). Particularly, eNOS is expressed in theca cells, granulosa cells, and the surface of oocyte during the follicular development. Thus, follicular fluid NO seems to be produced by either eNOS or iNOS. However, under the normal physiological conditions, follicular fluid NO seems to be synthesized from granulosa cells by eNOS, since in isolated of human follicular cells at least 90% of cells are granulosa cells even though macrophages and lymphocytes are present as well (22). In certain pathological conditions, iNOS might play a major role in NO production. In most organs, iNOS is expressed only in response to immunological stimuli. This fact is supported by our observation that follicular fluid NO levels in patients with endometriosis or hydrosalpinx were significantly higher compared to the levels found in patients with only tubal obstruction.

Geva *et al.* (23) reported that follicular fluid TNF-α concentration was not different in the (OHSS) group and the control group, whereas IL-6 levels were significantly higher in the OHSS group. In contrast, Cianci *et al.* (24) reported that follicular fluid TNF-α concentration was higher in patients with immunologic infertility than in the control (patients with tubal infertility). Also, they reported that the fertilization rate was significantly lower in patients with immunologic infertility compared to the control group. They defined immunologic infertility as the presence of high titer (>1:40) of antisperm antibodies in serum or cervical mucus and used the tubal infertility group as control. Although these facts suggest that follicular fluid TNF-α level can be changed easily with patient's characteristics associated with specific diseases or syndromes, the present study showed no difference in follicular fluid TNF-α levels between patients with endometriosis or hydrosalpinx and patients with only tubal obstruction. However, a further study is required to confirm these results, since the sample size is small in this research.

Table IV. Concentrations of NO and TNF-α in Follicular Fluid According to Oocyte Quality

	Oocyte quality		
	Good	Moderate	Poor
No. of oocytes	62	35	18
NO concentration (μM)	22.41 ± 1.43[a]	22.54 ± 1.41[a]	26.55 ± 1.68[a]
TNF-α concentration (pM)	10.0 ± 41.05[b]	9.64 ± 1.02[b]	17.81 ± 2.54[b]

[a] Not significant (versus each group).
[b] $P < 0.05$.

Recent studies have shown that follicular atresia is associated with apoptosis (9). NO and TNF-α have been known to induce or prevent apoptosis in a dose-dependent manner (7,8), and they influence folliculogenesis and oocyte maturation by inhibiting aromatase activity and estradiol secretion in granulosa cells (12,25). In this respect, different levels of NO and TNF-α in follicular fluid seem to affect maturity and quality of oocytes. However, the present result showed that follicular fluid NO level has no correlation with maturity and quality of oocyte. This finding agrees partially with the findings of Sugino *et al.* (2), who found no significant differences between follicular fluid NO concentration and follicle size. Therefore, NO may not be involved in apoptosis, since the NO concentrations (<30 μM) demonstrated in our study are lower than the concentrations (30–40 μM) that are reported to induce DNA fragmentation (26).

In contrast, the present results showed that follicular fluid TNF-α level is not correlated with the oocyte maturity but with the oocyte quality, although there is no linear relationship between follicular fluid TNF-α levels and oocyte quality in that TNF-α levels are in the middle for good-quality oocytes, low for moderate-quality oocytes, and high for poor-quality oocytes. Because poor-quality oocytes had the significantly higher TNF-α levels compared to the other quality oocytes and most of the moderate-quality oocytes also were fertilized and developed normally like as good quality oocytes. Therefore, we think that this is not a question of linear relationship between TNF-α level and oocyte quality. Rather, it seems that there is an upper limit of TNF-α which results in deterioration of oocyte quality.

The finding that follicular fluid TNF-α level is not correlated with oocyte maturity also was observed previously by Bili *et al.* (27) and Mendoza *et al.* (28). However, they reported that follicular fluid TNF-α level did not correspond to the fertilization (27), nor was it significantly lower in unfertilized oocytes compared to the fertilized oocytes, which implies that TNF-α is associated with the normal-quality oocytes (28). When our result is compared, the reason for this discrepancy could be explained by the fact that they used the presence of fertilization as the standard for the oocyte quality and particularly, that Mendosa *et al.* (28) experimented only with the mature oocytes to determine fertilization.

Follicle size also might influence levels of NO and TNF-α in follicular fluids, since it increases with oocyte maturation during follicular development: NO metabolites and TNF-α levels also increase with follicular development (3,4). However, as mentioned previously, Sugino *et al.* (2) reported no significant differences in concentrations of NO of follicular fluid among large, medium, or small follicle size, and few data concerning TNF-α level exist in the literature. Previously, Bili *et al.* (27) and Mendoza *et al.* (28) reported that follicular fluid TNF-α level is indifferent to oocyte maturity. Therefore, if we assume oocyte maturity closely correlates to follicle size, then the findings imply indirectly that TNF-α level also is indifferent to follicle size.

Despite the fact that TNF-α did correlate with poor-quality oocytes, it did not affect the pregnancy rate after IVF-ET in this study, because of two reasons: (a) the low number of poor quality oocytes collected, and (b) the transfer of good-quality embryos into uterus. Bili *et al.* (27) previously insisted that no correlation exists between TNF-α level in follicular fluid and the achievement of pregnancy.

CONCLUSIONS

A correlation between follicular fluid NO and TNF-α concentrations is not found, which implies that their productions may be regulated via different pathways. The follicular fluid NO level can be altered by infertility-associated diseases and TNF-α level can influence oocyte quality. However, more research is needed to verify the above results, since the present study uses a small number of cases, thus further study is necessary to explain the mechanisms and sources of follicular fluid NO and TNF-α productions.

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