〈Regular Article〉

Comprehensive analysis of cytokines in the follicular fluid and serum during in vitro fertilization and embryo transfer

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ABSTRACT Pregnancy rate for in vitro fertilization (IVF) remains low, for which the quality of ovum may be a contributing factor. Hence, comprehensive analysis of cytokines in follicular fluid was performed to identify potential markers of ovum quality.

Ova from women undergoing IVF for male factors or unknown etiology, excluding those undergoing treatment for female factors, were selected for cytokine analysis. Comprehensive cytokine/chemokine analysis of follicular fluid was performed. Next, enzyme-linked immunosorbent assay (ELISA) for quantifying interleukin (IL)-4, IL-17A, and IL-1ra levels in the serum and follicular fluid of 142 randomly selected patients was conducted.

The comprehensive analysis revealed significant differences in the levels of IL-4, IL-17A, and IL-1ra in the follicular fluid from all ovum grades. The cytokine levels in serum revealed a significant difference in IL-17A levels between women whose ova developed into blastocysts and women whose ova did not. However, no significant differences were found in the cytokine levels of follicular fluid.

No significant difference were found between the follicular fluid of women whose ova developed into blastocysts and those whose ova did not; however, serum IL-17A levels significantly differed. Therefore, serum IL-17A levels may be the marker of ovum quality.

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Key words：Blastocyst, Cytokines, Follicular fluid, IL-17A, In vitro fertilization

INTRODUCTION

The number of couples who require infertility treatment continues to increase because of social factors, including high age at marriage and spread of sexually transmitted diseases[1, 2)]. In vitro fertilization and embryo transfer (IVF-ET), a form

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of assisted reproductive technology (ART), was first developed in 1978, and since then, its application has rapidly spread worldwide to a point where one in 20 births in Japan was the result of ART in 2017[3]. However, the pregnancy rate per cycle of IVF-ET remains less than 30%, and difficulty in determination of ovum quality is one of the associated reasons[4].

Conventionally, the quality of ova is determined subjectively by doctors based on their shape. According to the Japan Society of Obstetrics and Gynecology, in principle, a single embryo should be returned to the uterus after IVF, and hence, the development of an objective method for selecting good-quality ova is important from the standpoint of maternal physical burden, among other aspects. Follicles consist of oocytes and the surrounding somatic cells (granulosa and theca cells), and their development progresses from primordial follicles to primary follicles, preantral follicles, antral follicles, and Graafian follicles. Follicular maturation is divided into three phases based on the dependence on gonadotropin (Gn). In the developmental phase from the primordial follicle to the preantral follicle, the follicle matures independent of Gn, whereas follicular maturation after preantral follicular stage is Gn-dependent. Local paracrine and autocrine factors are deeply involved in the Gn-independent follicular maturation from the primary to preantral follicle, and the maturation is regulated by the cross-talk between these factors. These factors mainly include various growth factors and cytokines, and a set of factors belonging to the transforming growth factor-β superfamily, such as growth differentiation factor-9 (GDF-9) and bone morphogenetic proteins (BMPs), play particularly important roles in follicular maturation. GDF-9 and BMP-9 are produced by oocytes in the primary follicular and subsequent stages. In GDF-9-knockout mice, follicular maturation has been shown to stop at the primary follicular phase[5]. In BMP-15-knockout mice, follicular maturation has been shown to proceed normally but with reduced fertility[6]. However, sheep with mutations in *BMP15* were infertile[7], and humans with mutations in *BMP15* reportedly exhibited premature ovarian insufficiency[8]. This suggests that inter-species differences may exist in reproduction. Graafian follicles initiate a series of processes leading to ovulation as a result of the luteinizing hormone (LH) surge. LH receptors in the ovaries are only present on granulosa and theca cells, and their absence on oocytes suggests that the paracrine factors from granulosa and theca cells may be implicated in oocyte nuclear and cytoplasmic maturation. After LH surge, the ovum restarts meiotic division and undergoes nuclear and cytoplasmic maturation for the preparation of future fertilization and embryonic development. Therefore, We aimed to investigate whether paracrine factors released from granulosa and theca cells could be considered as the factors contributing to ovum quality.

A key factor in the success of IVF is the quality of mature ova. If good-quality mature ova could be selected for fertilization and return to the uterus, this would increase the success rate of IVF and reduce the burden on patients. However, doctors select embryos for uterine implantation empirically based on factors such as appearance. No indicator can clearly define the quality of embryos, thereby limiting the probability of establishing successful pregnancies. A marker that measures ovum quality would reduce the physical and financial burden on patients and contribute to medical development. Therefore, We investigated whether any cytokine or chemokine in the follicular fluid or serum sampled during egg collection indicates the possibility of ovum to successfully progress towards the blastocyst stage. Moreover, we evaluated whether cytokine/chemokine levels in serum and follicular fluid correlate with each other. If such a correlation between the serum and follicular fluid could

be established, serum test might be sufficient to investigate ovum quality. In addition, the identification of cytokine or chemokine markers in the serum sample alone might be useful in clinical practice.

MATERIALS AND METHODS

Subjects

This study enrolled women for whom IVF was indicated and egg collection had undergone. They underwent IVF procedure at Kojin Hospital (Marugame City Kagawa Japan), and follicular fluid and serum samples used in this study were residual samples. Information on opting out of the study was published on the official websites of both Kawasaki Medical University Hospital and Kojin Hospital.

During egg collection, ovulation was induced with a minimal stimulation cycle using clomiphene citrate and human menopausal gonadotropin formulation to mature the follicles, whereas the status of ovarian maturation was monitored by ultrasound and blood tests [estradiol (E2) and LH levels]. When the ovaries matured to a point where the follicle diameter was ≥ 18 mm and hormone levels indicated imminent ovulation, an ovulation-stimulating drug was administered, and ova were collected 2 days later. To prevent stress from puncture, only Graafian follicles were included in the study. The follicular fluid remaining after the separation of ova was frozen for subsequent analyses.

Study 1

1. Subjects

The study involved 18 women for whom IVF was indicated (13 with unknown etiology and 5 with male factor infertility) (Table 1, Fig. 1). Fertilized ova from 14 of these women reached the blastocyst stage, whereas fertilized ova from the other 4 women did not reach the blastocyst stage. Blastocysts were graded according to the Gardner's system (grade A-C), and blastocysts from nine and five women were graded AA and grade B/C, respectively (Fig. 1).

Table 1. Clinical characteristics of 18 subjects selected for the comprehensive cytokine analysis.

Sr. No.	Age	BMI	Medium preparation method	Blastocyst feature	AMH	Ovulation induction method	Indication
1	35	21.51	Conventional	3AA	2.38	Minimal stimulation	Unknown etiology
2	43	25.32	Conventional	Not reached	0.649	Minimal stimulation	Unknown etiology
3	37	21.65	ICSI	3AB	0.528	Minimal stimulation	Male factors
4	41	20.91	Conventional	4AA	0.844	Minimal stimulation	Unknown etiology
5	42	21.79	ICSI	Not reached	0.344	Minimal stimulation	Male factors
6	39	23.85	Conventional	3CB	2.12	Minimal stimulation	Unknown etiology
7	30	20.93	Conventional	4AA	5.87	Minimal stimulation	Unknown etiology
8	40	19.9	Conventional	4BA	1.12	Minimal stimulation	Unknown etiology
9	44	21.17	ICSI	3AA	0.983	Minimal stimulation	Male factors
10	26	35.97	Conventional	3AA	4.15	Minimal stimulation	Unknown etiology
11	39	21.57	Conventional	4AA	1.67	Minimal stimulation	Unknown etiology
12	38	18.72	Conventional	Not reached	2.75	Minimal stimulation	Unknown etiology
13	44	21.17	ICSI	3AA	0.983	Minimal stimulation	Male factors
14	29	25.47	Conventional	3AA	8.47	Minimal stimulation	Unknown etiology
15	38	21.01	Conventional	4AA	0.367	Minimal stimulation	Unknown etiology
16	40	19.78	Conventional	Not reached	0.176	Minimal stimulation	Unknown etiology
17	38	26.6	ICSI	3BA	0.622	Minimal stimulation	Male factors
18	40	19.78	Conventional	3CC	0.176	Minimal stimulation	Unknown etiology

BMI, body mass index; ICSI, intracytoplasmic sperm injection; AMH, anti-Müllerian hormone (ng/mL).

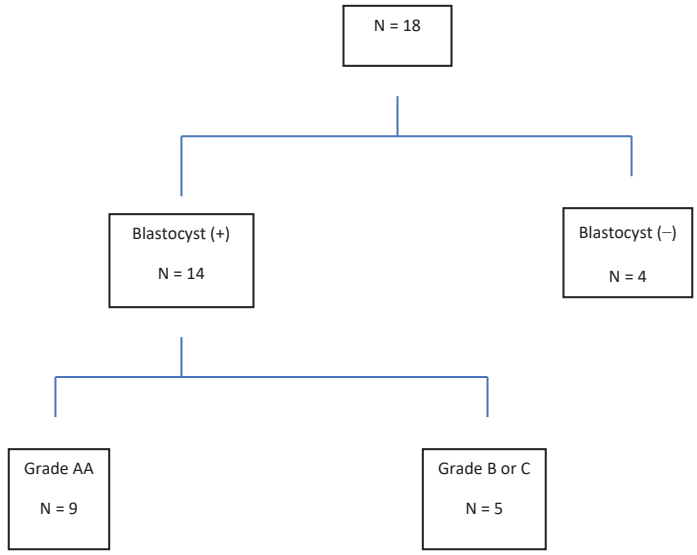

Fig. 1. Subject selection for the comprehensive cytokine analysis of the follicular fluid.

2. Correlation analysis

The E2 level, E2 level/number of follicles with diameter ≥14 mm at the collection ratio, and E2 level/number of collected eggs ratio were measured. Correlations between these parameters and the possibility of reaching the blastocyst stage and blastocyst grade were determined (grade AA blastocyst, grade B or C blastocyst, or no blastocyst stage reached). Follicle measurements were taken by transvaginal ultrasound to determine the maximum diameter. The number of oocytes retrieved ranged from 1 to 7, with an average of 2.8.

3. Comprehensive analysis of cytokines in the follicular fluid

The follicular fluid was homogenized using a homogenizer. Sonicated samples were centrifuged, and the follicular fluid was collected. The protein concentration was adjusted to 500 μg/mL in each sample. The protein samples were subject to Proteome Profile Array (Human Cytokine Array, R&D Systems, Minneapolis, USA) of CCL1, CXCL1, ICAM-1/CD54, IL-4, IL-13, IL-27, CCL2/MCP1, CXCL10, IFN-γ, IL-5, IL-16, IL-32α, MIP-1α/MIP-1β, CXCL11, IL-1α, IL-6, IL-17A, MIF, CCL5/RANTES, CXCL12, IL-1β, IL-8, IL-17E, serpinE1/PAI-1, CD40 ligand/TNFSF5, G-CSF, IL-1ra, IL-10, IL-18, TNF-α, complement component C5/C5a, GM-CSF, IL-2, IL-12, IL-21, and TREM-1 for comprehensive cytokine/chemokine quantitation. Pixel density was calculated using the Image Quant LAS4000mini® biomedical imager (GE Healthcare Japan, Tokyo, Japan) and Image Quant TL® image-analysis software (GE Healthcare Japan).

Study 2

1. Quantification of IL-1ra, IL-4, and IL-17A levels by ELISA

Following the comprehensive cytokine analysis, follicular fluid and serum samples from 142 patients were collected (Table 2, Fig. 2). Among these, each of the serum and follicular fluid were randomly selected, and cytokine levels were measured using Quantikine® ELISA Human IL-1ra/IL-4/IL-17A (R&D Systems, Bio-Techne) according to the manufacturer's instructions. The absorbance was measured at 495 nm using Varioskan Flash® (Thermo

Table 2. Clinical characteristics of patients selected for ELISA.

	Total	Blastocyst grade		Blastocyst stage not reached
	N = 142	Grade AA N = 18	Grade B or C N = 28	N = 96
Age	38 ± 5.3	37 ± 4.7	38.0 ± 4.1	39 ± 5.6
BMI	22.6 ± 3.9	22.3 ± 4.2	21.9 ± 2.4	22.8 ± 4.1
Indication (N)	Male factors: 39 Female factors: 19 Unknown etiology: 77 Other (age): 7	Male factors: 4 Female factors: 2 Unknown etiology: 12 Other (age): 0	Male factors: 5 Female factors: 5 Unknown etiology: 17 Other (age): 1	Male factors: 30 Female factors: 12 Unknown etiology: 48 Other (age): 6
AMH	1.8 ± 2.33	2.3 ± 2.72	1.7 ± 1.62	1.7 ± 2.43
Ovulation induction method	Minimal stimulation cycle	Minimal stimulation cycle	Minimal stimulation cycle	Minimal stimulation cycle

BMI, body mass index; AMH, anti-Müllerian hormone (ng/mL).

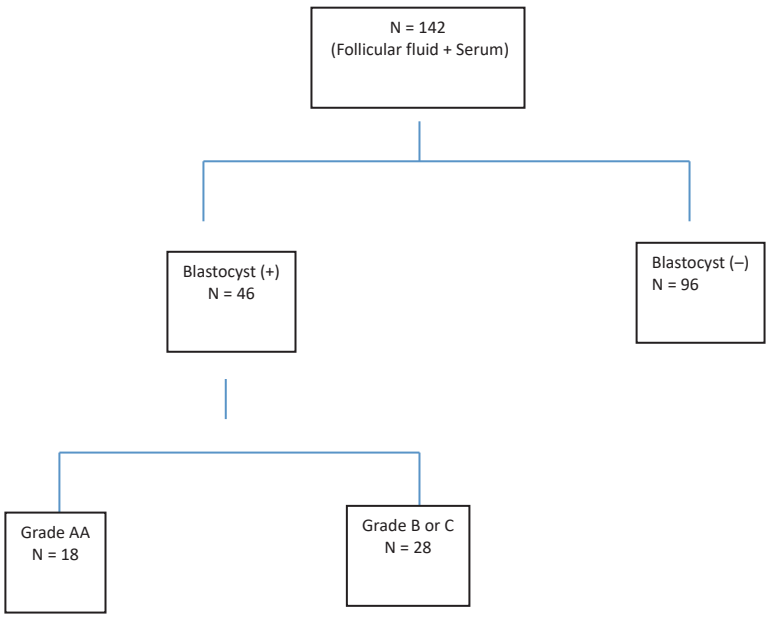

Fig. 2. Patient selection for the quantification of interleukin (IL) -4, IL-17A, and IL-1ra levels by ELISA.

Fisher Scientific, Waltham, MA, USA).

Statistical analysis

Data are presented as X. Statistical analysis was performed using the SAS software (SAS Institute Inc. Version 13.2). A stepwise method was used to select confounding factors from a number of factors (age, body mass index, smoking status, stimulation method, indications for IVF, and anti-Müllerian hormone levels). The χ^2 test and Wilcoxon test were performed for the statistical analysis. Statistical data are presented as median and range. A *P*-value of < 0.05 was considered statistically significant.

RESULTS

Study 1: Correlation analysis

The E2 level, E2 level/number of follicles with a diameter of ≥ 14 mm at collection ratio, and E2 level/number of collected eggs ratio between two sample groups (ova reaching or not reaching the blastocyst stage) or three blastocyst grade groups (grade AA blastocyst, grade B or C blastocyst, or no

blastocyst stage reached) were compared.

No significant difference was found in the E2 level, E2 level/number of follicles with diameter ≥ 14 mm at collection ratio, and E2 level/number of collected eggs ratio, between two sample groups or between three blastocyst grade groups (Table 3).

Study 1: Comprehensive cytokine analysis of the follicular fluid

Fourteen fertilized ova that reached the blastocyst stage were graded according to the Gardner's system (grade A-C), and those with grade AA were compared with blastocysts with grade B or C (Fig. 1). All 36 cytokines in the follicular fluid were examined (Table 4). The Gardner's system is a classification based on the size of the blastocoel cavity, and blastocysts of grade 3 or higher are further subdivided into A to C according to the state of the endocytotic mass and nutrient ectoderm. The Gardner's system is an ocular classification and is a subjective assessment. When confounding factors were selected using the stepwise method, age was the only confounding factor identified because of the small number of subjects. Statistical analysis by means of the logistic regression analysis with IL-4 and age as the input variables and grades AA or B or C as the target variables revealed a significant difference in IL-4 levels among different blastocyst grade groups ($p < 0.05$). The investigation of other factors using the χ^2 test revealed evident trends in IL-1ra and IL-17A levels.

Study 2: Quantification of IL-4, IL-17A, and IL-1ra levels in the serum and follicular fluid

ELISA was used to quantify IL-4, IL-17A, and IL-1ra levels in the serum and follicular fluid of randomly selected patients (Table 2, Fig. 2). The protein levels in the serum and follicular fluid from these 142 patients were measured, and quantitative measurements of each cytokine were corrected (IL-4, IL-17A, and IL-1ra protein levels/protein levels in each sample).

The Wilcoxon test was performed to compare the follicular fluid from ova that developed into blastocysts with the fluid from ova that did not show any significant differences in IL-4, IL-17A, and IL-1ra levels. However, the comparison of IL-4, IL-17A, and IL-1ra levels between the serum samples of women whose ova developed into blastocysts and those whose ova did not progress towards the blastocyst stage showed a significant difference in IL-17A levels ($p < 0.05$) (Table 5). Furthermore, a

Table 3. Correlations between the E2 level, E2 level/number of follicles with diameter ≥ 14 mm at the collection ratio, and E2 level/number of collected eggs ratio and the possibility of reaching the blastocyst stage and blastocyst grade were determined (grade AA blastocyst, grade B or C blastocyst, or no blastocyst stage reached).

	Blastocyst (+)	Blastocyst (–)	*p* value
E2	1045 ± 827	919 ± 596	NS
E2 level/number of follicles with a diameter of ≥ 14 mm at collection ratio	355 ± 154	357 ± 203	NS
E2 level/number of collected eggs ratio	339 ± 143	384 ± 227	NS

	GradeAA	GradeB or C	Blastocyst (–)	*p* value
E2	803 ± 378	1158 ± 961	919 ± 596	NS
E2 level/number of follicles with a diameter of ≥ 14 mm at collection ratio	353 ± 106	355 ± 176	357 ± 203	NS
E2 level/number of collected eggs ratio	250 ± 97	380 ± 145	384 ± 227	NS

(E2: pg/mL)

Table 4. Comprehensive cytokine and chemokine analysis of follicular fluid.

Cytekine	Blastcyst	Blastcyst stage not reached	P value
CCL1/I-309	1.39 (0-2.97)	0.91 (0.02-1.97)	NS
CCL-2/MCP-1	0.51 (0.01-1.43)	0.30 (0.005-1.21)	NS
MIP-1 α/MIP-1 β	0.37 (0-1.63)	0.38 (0.02-1.16)	NS
CCL5/RANTES	0.35 (0-13.07)	0.56 (0-1.59)	NS
CD40 Ligand/TNFSF5	0.92 (0.04-2.86)	0.76 (0.36-1.95)	NS
Complement Component C5/C5a	0.49 (0-1.61)	0.46 (0.08-1.31)	NS
CXCL1/GRO α	0.64 (0.08-4.12)	1.29 (0.8-3.49)	NS
CXCL10/IP-10	1.8 7(0.07-4.90)	0.97 (0.62-2.91)	NS
CXCL11/I-TAC	1.19 (0-2.55)	0.91 (0-1.83)	NS
CXCL12/SDF-1	4.90 (0.04-21.63)	7.25 (1.52-13.50)	NS
G-CSF	0.9 3(0-2.00)	0.97 (0.01-1.51)	NS
GM-CSF	0.88 (0-2.04)	0.9 (0.06-2.05)	NS
ICAM-1/CD54	29.5 (0.04-94.46)	23.5 (18.4-41.6)	NS
IFN- γ	0.77 (0.01-2.77)	1.44 (0.3-1.86)	NS
IL-1 α/IL-1F1	1.20 (0.20-3.15)	2.19 (0.33-5.74)	NS
IL-1 β/IL-1F2	0.91 (0.31-2.63)	1.24 (0.25-1.63)	NS
IL-1ra/IL-1F3	1.19 (0-2.83)	1.40 (0.61-1.71)	P = 0.0053
IL-2	0.79 (0-1.98)	0.41 (0.01-1.58)	NS
IL-17A	0.28 (0-1.22)	0.13 (0-0.96)	P = 0.0053
IL-17E	0.70 (0.03-2.48)	1.15 (0.03-1.37)	NS
IL-18/IL-1F4	2.07 (0.37-7.56)	3.35 (0.28-10.90)	NS
IL-21	0.63 (0.01-2.24)	1.03 (0.32-3.64)	NS
IL-27	0.51 (0.15-1.94)	0.39 (0.23-2.21)	NS
IL-32 α	0.67 (0.11-6.21)	0.35 (0.13-13.19)	NS
MIF	5.50 (0.08-12.12)	8.74 (7.08-14.31)	NS
SerpinE1/PAI-1	53.25 (8.26-93.01)	45.80 (36.22-75.01)	NS
TNF- α	0.27 (0-1.64)	0.13 (0-0.57)	NS
TREM-1	0.22 (0-3.82)	0.10 (0-0.75)	NS
IL-13	1.14 (0-2.43)	1.45 (0-2.54)	NS
IL-16	1.47 (0-51.1)	1.54 (0.03-1.99)	NS
IL-4	0.57 (0-1.62)	0.28 (0.01-1.19)	P = 0.0311
IL-5	0.64 (0-1.38)	0.29 (0.01-1.22)	NS
IL-6	0.95 (0.04-2.87)	0.99 (0.42-1.81)	NS
IL-8	0.53 (0.01-3.23)	1.13 (0.61-1.62)	NS
IL-10	0.48 (0.05-2.24)	0.70 (0.30-2.57)	NS
IL-12 p70	0.68 (0.16-1.70)	0.66 (0.18-2.53)	NS

(median (range))

three-group comparison of IL-4, IL-17A, and IL-1ra levels between three blastocyst grade groups (grade AA, grade B or C, or no blastocyst stage reached) also revealed a significant difference in serum IL-17A levels ($p < 0.05$), and an intergroup comparison identified a significant difference in IL-17A levels between the serum samples of women whose fertilized ova developed into Grade B or C blastocysts and whose fertilized ova did not reach the blastocyst stage ($p < 0.05$) (Table 6).

Next, as in the comprehensive cytokine analysis, we compared serum samples obtained only from women who underwent IVF for male factors or unknown etiology, excluding those who underwent treatment for female factors. A comparison between three groups (grade AA, grade B or C, or no blastocyst stage reached) also showed a significant difference in serum IL-17A levels ($p < 0.05$). However, intergroup comparisons among these three groups did not reveal any significant differences (Table 7).

Table 5. Comparison of interleukin (IL)-4, IL-17A, and IL-1ra levels in the follicular fluid/serum samples of all subjects divided into two groups (fertilized ova reaching or not reaching the blastocyst stage) using the Wilcoxon test.

All cases, 2 groups comparison			
IL-4 Serum	Blastocyst	Blastocyst stage not reached	P value
N = 78	25	53	NS
median	0.0028	0.0031	
range	0-0.13	0-0.12	
IL-1ra Serum	Blastocyst	Blastocyst stage not reached	P value
N = 77	23	54	NS
median	8.58	7.95	
range	3.57-46.53	3.14-38.79	
IL-17A Serum	Blastocyst	Blastocyst stage not reached	P value
N = 78	23	55	< 0.05
median	0.0043	0	
range	0-0.21	0-0.14	
IL-4 Follicular Fluid	Blastocyst	Blastocyst stage not reached	P value
N = 80	21	59	NS
median	0	0.00016	
range	0-0.019	0-0.088	
IL-1ra Follicular Fluid	Blastocyst	Blastocyst stage not reached	P value
N = 69	20	49	NS
median	10.19	6.74	
range	2.80-22.7	1.61-21.92	
IL-17A Follicular Fluid	Blastocyst	Blastocyst stage not reached	P value
N = 85	26	59	NS
median	0	0	
range	0-3.72	0-0.22	

(ng/g protein)

Table 6. Comparison of interleukin (IL)-4, IL-17A, and IL-1ra levels in the follicular fluid/serum samples of all subjects divided into three groups (grade AA, grade B or C, or no blastocyst stage reached) using the Kruskal-Wallis test.

All cases, 3 groups comparison				
IL-4 Serum	AA	BorC	Blastocyst stage not reached	P value
N = 78	10	15	53	NS
median	0.0026	0.0036	0.0031	
range	0.0018-0.0049	0-0.012	0-0.12	
IL-1ra Serum	AA	BorC	Blastocyst stage not reached	P value
N = 77	9	14	54	NS
median	9.48	8.19	7.95	
range	4.97-20.13	3.57-46.53	3.14-38.79	
IL-17A Serum	AA	BorC	Blastocyst stage not reached	P value
N = 78	9	14	55	< 0.05
median	0	0.0069	0	
range	0-0.0068	0-0.21	0-0.14	
IL-4 Follicular fluid	AA	BorC	Blastocyst stage not reached	P value
N = 80	8	13	59	NS
median	0	0.00012	0.00016	
range	0-0.00056	0-0.019	0-0.088	
IL-1ra Follicular fluid	AA	BorC	Blastocyst stage not reached	P value
N = 69	8	12	49	NS
median	9.9	10.62	6.74	
range	3.08-22.7	2.8-21.87	1.61-21.92	
IL-17A Follicular fluid	AA	BorC	Blastocyst stage not reached	P value
N = 85	9	17	59	NS
median	0	0	0	
range	0-0.26	0-3.72	0-0.22	

(ng/g protein)

Table 7. Comparison of interleukin (IL)-4, IL-17A, and IL-1ra levels in the follicular fluid/serum samples of subjects with male factor infertility or unknown etiology alone, excluding those with female factor infertility, divided into three groups (grade AA, grade B or C, or no blastocyst stage reached) using the Kruskal-Wallis test.

Male Factor・Unknown etiology, 3 groups comparison				
IL-4 Serum	AA	BorC	Blastocyst stage not reached	P value
N = 60	7	10	43	NS
median	0.0132	0.015	0.013	
range	0.007-0.034	0.003-0.039	0-0.036	
IL-1ra Serum	AA	BorC	Blastocyst stage not reached	P value
N = 60	7	10	43	NS
median	4.55	4.85	4.508	
range	3.407-7.137	3.376-8.876	2.432-14.801	
IL-17A Serum	AA	BorC	Blastocyst stage not reached	P value
N = 62	8	11	43	< 0.05
median	0	0	0	
range	0	0-0.850	0-0.483	
IL-4 Follicular fluid	AA	BorC	Blastocyst stage not reached	P value
N = 60	7	10	43	NS
median	0	0	0	
range	0-0.0015	0-0.00405	0-0.235	
IL-1ra Follicular fluid	AA	BorC	Blastocyst stage not reached	P value
N = 59	7	10	42	NS
median	7.496	10.072	5.578	
range	2.539-16.592	0-23.482	0-21.919	
IL-17A Follicular fluid	AA	BorC	Blastocyst stage not reached	P value
N = 80	8	15	57	NS
median	0	0	0	
range	0-0.218	0-0.269	0-0.451	

(ng/g protein)

DISCUSSION

As ovulation is a phenomenon that is extremely similar to an acute inflammatory reaction localized to the ovary, the involvement of various cytokines and chemokines in this process has come under scrutiny. Studies that have investigated the characteristics of cytokines in women undergoing IVF showed that MIP-1α and CD44 are associated with polycystic ovary syndrome, and IL-23, IFN-γ, and TNF-α are associated with endometriosis. In addition, increased IL-18, IL-8, and MIP-1β levels are associated with pregnancy rates in IVF[9, 10].

Various cytokines are thus believed to be present in the follicular fluid of women at ovulation, as reported in the aforementioned studies[11–16]. In this study, We selected women undergoing IVF for male factors or unknown etiology, and performed comprehensive cytokine analysis. Three cytokines that were found to be significantly different in a comprehensive analysis of cytokines in follicular fluid were targeted and correlated with serum.

The results showed a significant difference in IL-4, and evident trends in IL-17A and IL-1ra. Based on these results, we then used ELISA to measure their levels in the follicular fluid and serum sampled at ovulation, and this analysis revealed a significant difference in serum IL-17A levels between women whose fertilized ova reached the blastocyst stage and those whose ova did not. IL-17A is an inflammatory cytokine that belongs to the IL-17 family of cytokines consisting of six molecules, but fewer analyses of the other molecules in this family have been conducted compared with IL-17A. IL-17A and IL-1ra knockout mice, IL-17F and IL-1ra knockout mice, and IL-17A/F and IL-1ra knockout mice have been produced previously, and the roles of these cytokines in the incidence of autoimmune disease and prevention of infection

have been compared[3]. In the present study, we found that high IL-17A levels were associated with the progression of fertilized ova towards the blastocyst stage. In patients with polycystic ovary syndrome one report demonstrated the significant increase of IL-17A in the follicle fluid, but the other report demonstrated no difference of IL-17A of the follicle fluid[13, 15]. IL-17A levels of the follicle fluid in women complicated with endometriosis is lower than those without endometriosis[12]. These studies were examined the patients with female infertility. In the present study we examined IL-17 A levels of the follicle of the patients without ovulational disorder. These results suggest that IL-17A is possible factor associated with ovulation, but not with embryo maturation. Ova may have been more likely to reach the blastocyst stage because IL-17A is induced in the acute inflammatory reaction. Therefore, we investigated the existence of any treatment options for low IL-17A levels. A study demonstrated that both IL-17A and IL-17F are responsible for host defense mechanisms via the induction of β-defensin expression. β-Defensins are peptides having antimicrobial action that prevents the invasion and survival of pathogenic microorganisms; the α form is found in neutrophils, and the β form is found in epithelial cells. β-defensins are also widely distributed in the mucosal epithelium and lining of the respiratory organs, mouth, large intestine, kidneys, eyes, and reproductive organs, among others, and are produced in response to an infectious stimulus. This suggests that if the level of β-defensins in the ovarian epithelium is decreased because of age or other factors, the production of IL-17A might be declined. The administration of β-defensins during ovulation may therefore encourage IL-17A production. Logistic regression analysis (receiver operating characteristic curve) of serum IL-17A levels showed that the cutoff value was 0.4674 pg/g protein, and it may be possible to improve outcomes for patients with levels less than the cutoff value by β-defensin treatment. We further investigated the serum levels of IL-4, IL-1RA, and IL-17A. There is significant difference of IL-17A of the sera between the blastocyst reached group and the blastocyst not reached group.

If there was a correlation between cytokine concentrations in follicular fluid and serum, we thought it would be clinically applicable. We also further classified the patients into three groups according to the Gardner's classification (grade AA, grade B or C, or no blastocyst stage reached). we found a significant difference in serum IL-17A levels. Detailed intergroup comparisons identified a significant difference in serum IL-17A levels between women whose fertilized ova developed into grade B or C blastocysts and those whose fertilized ova did not reach the blastocyst stage. This may be because the Gardner's classification is a visual classification that provides an objective assessment. Nonetheless, further investigation of women with grade AA blastocysts could have led to better results. These results show that IL-17A levels of the sera might be the marker to indicate the embryo growth in IVF-ET. Elevated IL-17A in sera and follicular fluid were reported in women with positive antithyroid antibodies[11]. Some immunological aspect might affect the IL-17A levels in women at the ovulation phase. The mechanism of the change of IL-17A levels would be examined in the further investigation.

Conventionally, E2 has been used as an index of ovum maturity during egg collection; however, in this study, we found no significant difference in E2 levels irrespective of the blastocyst stage, and the involvement of E2 was therefore ruled out.

In conclusion, the present study findings suggest that serum IL-17A levels differed significantly between women whose fertilized ova reached the blastocyst stage and those whose fertilized ova did not reach the blastocyst stage, indicating that IL-

17A may be a marker for determining progression towards the blastocyst stage. As mentioned earlier, whether patients with low IL-17A levels could be clinically treated with β-defensin is a topic for future studies. Delayed egg collection is beneficial for patients with low serum IL-17A levels (less than the cutoff value).

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DISCLOSURES

・HUMAN RIGHTS STATEMENTS AND INFORMED CONSENT

All procedures followed were in accordance with the ethical guidelines of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1964 and its later amendments. Since residual samples were used, written informed consent was not obtained.

・CONFLICT OF INTEREST

Authors declare no conflict of interest.

・APPROVAL BY ETHICS COMMITTEE

This study was approved by the Institutional Review Board of Kawasaki Medical University (3384) for the five-year period from January 31, 2019, to March 31, 2023.

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