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IL-5 in follicular fluid as a negative predictor of the intracytoplasmic sperm injection outcome

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ABSTRACT

Purpose: To determine the prognostic value of intrafollicular concentrations of some cytokines from women undergoing ovarian stimulation in the outcome of intracytoplasmic sperm injection/embryo transfer (ICSI/ET) cycles.

Methods: A total of 80 patients were included in this study following ovarian stimulation and ICSI. Follicular fluids (FF) were collected at the day of oocyte retrieval. Ten cytokines including: tumor necrosis factor- alpha (TNF-α), interleukin (IL)-1β, IL-2, IL-4, IL-5, IL-6, CXCL8/IL-8, IL-10, granulocyte-macrophage colony stimulating factor (GM-CSF), and interferon gamma (IFN-γ) were measured using magnetic multiplex immunoassays.

Results: Only the concentration of IL-5, IL-4, and GM-CSF in FF were significantly different ($p < 0.05$) between ICSI cycles that resulted in pregnancy and those that failed. Elevated FF IL-5 levels were associated with poor oocyte quality, which decreases the chance of both biochemical and clinical pregnancy. Higher FF GM-CSF associated with decrease of mature oocytes, while higher FF IL-4 concentrations were linked to good ICSI outcome through increased fertilization rate.

Conclusions: The elevated intrafollicular concentrations of IL-5 seem to be a negative predictor to the pregnancy outcome in ICSI cycles.

1. Introduction

Cytokines are key regulators of ovarian physiology, particularly in relation to folliculogenesis and ovulation, where they contribute to creating an environment supporting follicle selection and growth. Their manifold functions include regulating cellular proliferation/differentiation, follicular survival/atresia, and oocyte maturation [1–4]. Some of the redundancy and pleiotropy between certain cytokines that share an accessory signal transducing subunit result in their pro- as well as anti-inflammatory properties [4]. Several cytokines, such as transforming growth superfamily beta (TGF-β) members, are involved at all stages of folliculogenesis while the production of others including members of the glycoprotein gp130 cytokine family (IL-11 and LIF), colony-stimulating factors (CSFs), IL-1, IL-15, and IL-33 is stage-dependent [3]. Some cytokines such as IL- 1β influence oocyte fertilization and embryo quality and others correlated with successful pregnancy such as IL-8, IL-18, and MIP-1β [5].

Bidirectional somatic cell–oocyte signaling is essential to creating a changing intrafollicular microenvironment that controls primordial follicle growth into a cohort of growing follicles, from which one antral follicle is selected to ovulate a healthy oocyte [6]. The quality and viability of oocytes will be intimately linked to the intraovarian/perifollicular cytokine milieu [3]. Follicular fluid (FF), an oocyte microenvironment, is a unique biological fluid, rich in growth factors and cytokines that exert paracrine and autocrine effects on implantation. The events of oocyte, follicular maturation and somatic cell-germ cell communication occur within the FF. Due to the intimate proximity of FF to the maturing oocyte, this fluid provides a unique window into the processes occurring during follicular maturation [6–8]. In addition, FF is easily available during oocyte pick up and represents an optimal source of non-invasive biochemical predictors of oocyte quality [9].

Granulocyte-colony stimulating factor (G-CSF) is the only known

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cytokine in FF that entirely associated with assisted reproductive techniques (ART) success by three distinct experiments conducted by the European network for research, Embryo Implantation Control (EMBIC) [10]. G-CSF is a non-invasive biomarker for implantation, as it correlates with the potential of the corresponding embryo to result in a live birth in *in vitro* fertilization (IVF) [11]. It also predicts the oocyte competence and subsequent birth, combined with FF IL-15 [12]. Moreover, G-CSF provides a better choice of embryos, limits multiple pregnancies, reduces embryo cryostorage according to the oocyte morphology alone [13]. Other cytokines had a contradicted and conflicted results respective to their correlation with IVF/ICSI outcome such as IL-1β [14,15], IL-6 [16,17], and TNF-α [18,19]. In addition, some other cytokines are rarely studied or have no effect in ART treatments such as IL-3 and IL-5.

Previous studies that evaluate the correlation of biomarkers and their potential in prediction of IVF/ICSI outcome used either individual FF samples [10–13,20–22] or pooled FF samples [23–27]. Pooled FF may comprehensively reflect the dynamic microenvironmental milieu in which oocytes are produced [21]. Moreover, single follicle aspiration is uncomfortable both for the patient and the physician because multiple vaginal punctures are required, with increased risk of vaginal bleeding [9]. Manifold infertility is either related to ovulatory dysfunctions, uterine abnormalities, tubal obstructions, and peritoneal or cervical causes [28]. We measured the amount of ten cytokines in pooled FF of pre-ovulatory follicles from patients undergoing ICSI due to female factor infertility to evaluate the possible role of each cytokine as a potential non-invasive biomarker of oocyte competence for subsequent pregnancy with a special attention to investigating new biomarkers for prediction of ICSI outcome.

2. Materials and methods

2.1. Study protocol

This study was done under the instructions of the Ethics Committees of Ferdowsi University of Mashhad and Mashhad University of Medical Sciences. The FF samples were collected from August 2016 to May 2017 in Milad Infertility Center, Mashhad University of Medical Sciences. Eighty patients aged 20–43 years old were included in this study. Number of previous IVF/ICSI tries, infertility period and type, superovulation protocol, and endometrial thickness were recorded. All patients were undergoing ICSI with female factor infertility (either alone or accompanied with male factor infertility) suffering from one or more of the following causes: uterine abnormalities (e.g. endometriosis, myoma, polyp, fibromas, and congenital defects), tubal obstructions, peritoneal factors (e.g. adhesions), and ovulation disorders (e.g. polycystic ovary syndrome (PCOS)). Patients with other infertility causes including male factor infertility alone, unexplained infertility, oocyte donors and patients with special diseases such as diabetes and obesity (BMI $\geq 28\ kg/m^2$) were excluded from the study.

2.2. Superovulation protocol

All patients received controlled ovarian hyperstimulation (COH) with gonadotropin releasing hormone (GnRH) protocols. Either agonist long or antagonist were selected by each physician and were individualized based on the results of patient's ovarian reserve. Thirty two patients were daily administered with agonist long recombinant FSH (Gonal-F; Merk Serono, Germany). This began at the mid-luteal phase (day 21) of the previous cycle and continued to the day of hCG injection. The size of follicles was monitored with transvaginal ultrasound every 2–3 days along with the agonist treatment. Other 48 patients were administered antagonist drugs daily (Cetrotide; Merk Serono, Germany) at the mid-follicular phase until the size of follicles reached 12 mm at least. Final oocyte maturation was triggered in both protocols by the injection of human chorionic gonadotropin (hCG) (Pregnyl; IBSA, the Netherlands). The oocyte pickup (OPU) was achieved about 36–38 h following hCG intramuscular injection.

2.3. Follicular fluid samples and multiplex cytokines determination

Under short general anesthesia, follicles were punctured along with transvaginal guided sonography to pick up the oocytes with their surrounding FF using a special needle. About half to one hour, the FF was collected as a pool respective to each patient, then centrifuged with 2000 rpm for 10 min to relieve the cellular debris. The blood contaminated samples were discarded and the clear follicular fluid samples were stored at −80 °C until they were assayed. The pooled FF samples were analyzed by magnetic beads multiplex to estimate the concentration of their cytokines at the time of oocytes retrieval. The ten measured cytokines/chemokine were included: IL-1β, IL-2, IL-4, IL-5, IL-6, CXCL8/IL-8, IL-10, GM-CSF, IFN-γ and TNF-α. FF samples were assayed by Flow Cytometry with Luminex Platform Magnetic Luminex. Human premixed multi-analyte kit (R&D System Inc. Kit code: LXSAMH-10) purchased from Bio-Rad laboratories, Italy. All the analyses were undertaken in Lapospace, Milano – Italy laboratories by Luminex instrument (Bio-Rad) (Luminex Map Technology, Milan, Italy). Samples were analyzed coupled to Bio-Plex Manger software V 6.0. The procedure of determination of multiplex cytokines was performed according to the manufacturer's instructions. Briefly, FF samples were diluted with the reagent in 1:1 and added with a quantity of 50 μl per well in duplicate. The values of the standard curve were compared with the values of the manufacturer and the prescriptions of the kit. The fluorescence intensity of each well is then converted into a concentration using a specific algorithm that is calculated automatically by the instrument.

2.4. Follicles and oocytes assessment

The size of follicles and their number were measured by ultrasound about 48–72 h proceeding the oocyte pickup. Follicles with a small size were smaller than 12 mm, medium sized follicles ranged between 12 and 15 mm, and the follicles larger or equal to 16 were grouped within the large sized ones. Oocyte quality were directly assessed after 20–30 min of oocyte collection; the good quality oocytes had an extended sun-flare corona radiata. The oocytes evaluated as necrotic, germinal vesicle (GV) or prophase I, and metaphase II stage (MII) according to their stage of division apparent under microscope. Only the oocytes of a class MII were inseminated, others including GV and necrotic oocytes were rejected. The proportion of each of these three stages were calculated by dividing the number of each item by the total number of retrieved oocytes per patient multiplied by 100.

2.5. ICSI outcome

Oocyte fertilization assessment was performed about 16–24 h after ICSI. Two pronuclei (2PN) were examined as an indication of fertilization and zygote formation. Fertilization rate (FR) was subsequently calculated by dividing 2PN to the number of MII oocytes and then multiplied by 100. Embryo grading and assessment was achieved at day 3 of ICSI. The produced embryos were graded as follow: Grade I, a transparent embryo, thin zona pellucida, the number of blastomeres were larger than 6 cells at day 3 with a regular blastomeres, fragmentation rate lesser or equal to 10%, equal size of blastomeres, and no multinucleated blastomeres. Other embryos were graded as grade II. Implantation rate (IR) was calculated as: the number of gestational sacs divided by the number of transferred embryos per patient. Biochemical pregnancy: positive hCG in the blood after 16 days of intrauterine transfer of the embryo(s). Clinical pregnancy: The presence of one or more gestational sacs by sonography, with positive heart sounds after 36 days of IUI in addition to amenorrhea for about 6–8 weeks.

2.6. Statistical analysis

Mean ± SD of cytokines level in pooled follicular fluid of patients were reported. Comparison of the demographics and ICSI outcome between pregnant and non-pregnant women were done using Chi-square and independent samples *t*-test for the qualitative and quantitative variables respectively. To evaluate the effects of FF cytokines on ICSI outcome, at the first step, univariate correlation of cytokines level with quantitative and binary outcome were analyzed by correlation and logistic regression test, respectively. Then cytokines which were associated with ICSI outcomes at $p < 0.2$ were selected for inclusion in the multivariate analysis. Controlling for the effects of age, type of superovulation protocol, history of previous IVF and infertility period, correlation of cytokines with quantitative outcomes including number of follicles, number of oocyte, proportion of oocyte quality, FR%, IR%, and embryo grading were evaluated by linear regression test. Also, controlling for the effects of age, type of superovulation protocol, history of previous IVF, infertility period and endometrial thickness, correlation of cytokines with binary outcomes such as results of biochemical and clinical pregnancy were evaluated by logistic regression test. Receiver operating characteristic (ROC) curve was used to evaluate the performance of each cytokine on prediction of pregnancy. Statistical analysis was performed by SPSS version 21. A value of $P < 0.05$ was considered statistically significant.

3. Results

Embryo transfer achieved in 72.5% (58/80) and canceled in 27.5% (22/80) of the patients (n = 80). The transfer was canceled due to several causes such as ovarian hyperstimulation syndrome (OHSS), uterine polyp, myoma, inappropriate endometrium, and no fertilization. Fertilization rate % was 0.70 ± 0.28. Demographics of the study population (mean ± SD with their maximum and minimum values), the mean ± SD for number and size of follicle proportion, oocytes number, the proportion of each stage (MII, germinal vesicles, and necrotic oocytes), and embryo grading as well as the results of implantation rate %, biochemical pregnancy, and the clinical pregnancy% according to the hCG blood test and sonography are presented in Table 1. From the biochemical pregnancy stage, the pregnancy data for two patients were missing (n = 78).

Result of biochemical pregnancy for 18 patients was positive, while it was negative for 60 patients. A number of quantitative variables did not show a significant difference between non-pregnant (NP) and pregnant (P) women using independent sample *t*-test including: age, number of follicles, large, medium sized follicles %, oocyte number, necrotic oocytes, grade I and II embryo, IR%. Whereas other parameters showed a significant differences ($p < 0.05$) between the two groups including: endometrial thickness, small oocytes%, MII%, and GV% (Table 2). The only cytokine which was significantly ($p = 0.033$) higher in the FF of NP women was IL-5. Although IL-6 was higher in the FF of the pregnant women and showed a trend to be linked with the biochemical pregnancy, it was insignificant ($p = 0.195$) (Table 2). None of the qualitative variables showed a significant difference between the two study groups according to Chi-square test. These variables include infertility type, superovulation protocol, and the history of previous IVF/ICSI (Table 3).

The results of multivariate analysis showed that the number of follicles was significantly affected by age and infertility period; as a whole, the number of follicles increased with the prolonged infertility period, and decreased with aged patients. While other predictors, including: FF cytokines, superovulation protocol, and the history of previous IVF/ICSI attempts, had no significant effect on the follicle number. In this study, the results showed that the patients with a history of previous IVF/ICSI attempts significantly correlated with the large follicles proportion (58.6%) in comparison to their proportion in the patients with first IVF/ICSI attempt (39.5%). While other predictors including cytokines, age, and infertility period have no effect on size proportion of large follicles. The age was significantly and inversely correlated with the number of oocytes. The ten measured cytokines and other predictors had no significant effect on the number of oocytes. IL-10 was linked to an increase in oocyte number, but did not reach to the significant level ($p = 0.083$).

In all patients, the infertility period was significantly and directly correlated with GV proportion. IL-5 had a significant correlation with the GV% ($p = 0.036$), and this cytokine was positively proportioned to this proportion; for each unit increase in the FF IL-5 concentration, there was a 2.3% increase in GV%. GM-CSF was correlated with increased GV%, but not reached to the significancy level ($p = 0.071$). Other predictors including the other nine measured cytokines, in addition to the previously mentioned predictors had no significant effect on the GV%. GM-CSF had a significant correlation with the MII%, on average, with a unit increase in FF GM-CSF concentration, oocyte MII proportion decreased by 1% ($p = 0.012$). Also, IL-5 had a significant correlation with the MII% ($p = 0.046$), with one unit increase in FF IL-5 concentration, the proportion of metaphase II oocyte was 5.6% decreased. Other eight measured cytokines and predictors had no significant effect on the MII%. None of the ten estimated cytokines, or other predictors had any significant correlation with the embryo grading; both grade I and grade II embryos. In general, patients with previous IVF/ICSI attempts had a fertilization rate of (FR% = 74.4%),

Table 1
Description of study population and characteristics of ICSI cycles. Quantitative and qualitative variables were presented as mean ± standard deviation and frequency (%), respectively.

	Mean ± SD	Minimum	Maximum	Frequency (%)
Age (year)	31.35 ± 5.23	20	43	–
Infertility type				
Female	–	–	–	29/80 (36.2%)
Mixed	–	–	–	51/80 (63.8%)
Infertility period (year)	5.98 ± 3.98	5	17	–
Superovulation protocol				
Agonist	–	–	–	32/80 (40%)
Antagonist	–	–	–	48/80 (60%)
Previous IVF/ICSI attempts				
Yes	–	–	–	23/80 (28.8%)
No	–	–	–	57/80 (71.2%)
Endometrial thickness (mm)	9.22 ± 2.14	3.5	15	–
Number of follicles	16.25 ± 11.56	1	50	–
Size of follicles				
Large (%)	0.53 ± 0.32	0.00	1.00	–
Medium (%)	0.38 ± 0.24	0.00	1.00	–
Small (%)	0.09 ± 0.17	0.00	0.80	–
Number of oocytes	10.34 ± 7.29	1	45	–
Oocyte quality				
MII (%)	0.91 ± 0.19	0.00	1.00	–
GV (%)	0.02 ± 0.07	0.00	0.50	–
Necrotic (%)	0.07 ± 0.15	0.00	0.70	–
FR %	0.70 ± 0.28	0.00	1.00	–
Embryo grading				
Grade I	0.61 ± 0.27	0.00	1.00	–
Grade II	0.34 ± 0.24	0.00	1.00	–
IR %	0.25 ± 0.23	0.33	1.00	–
Biochemical pregnancy				
Positive	–	–	–	18/78 (23.1%)
Negative	–	–	–	60/78 (76.9%)
Clinical pregnancy				
Positive	–	–	–	17/78 (21.8%)
Negative	–	–	–	61/78 (78.2%)

MII: metaphase II; GV: germinal vesicle; FR: fertilization rate; IR: implantation rate.

Table 2
Demographics, cycle characteristics, and cytokines in non-pregnant (NP) and pregnant (P) women. Comparisons of these quantitative variables between P and NP were performed using univariate analysis independent sample *t*-test.

	Non pregnant Mean ± SD (n = 60)	Pregnant Mean ± SD (n = 18)	*P* value
Age (year)	31.67 ± 5.57	31.06 ± 3.64	0.663
Endometrial thickness (mm)	8.93 ± 2.02	10.14 ± 2.40	0.036*
Number of follicles	17.17 ± 12.88	13.77 ± 4.93	0.272
Large follicles (%)	0.52 ± 0.34	0.55 ± 0.27	0.684
Medium follicles (%)	0.37 ± 0.23	0.41 ± 0.25	0.625
Small follicles (%)	0.112 ± 0.18	0.04 ± 0.09	0.035*
Number of oocytes	10.57 ± 7.98	9.61 ± 4.49	0.520
MII (%)	0.89 ± 0.19	0.97 ± 0.089	0.020*
GV (%)	0.026 ± 0.084	0.003 ± 0.014	0.042*
Necrotic (%)	0.08 ± 0.016	0.24 ± 0.089	0.071
FR %	0.67 ± 0.29	0.79 ± 0.23	0.140
Grade I embryo (%)	0.60 ± 0.29	0.65 ± 0.25	0.102
Grade II embryo (%)	0.33 ± 0.25	0.35 ± 0.20	0.422
IL-1β	13.17 ± 0.75	13.06 ± 0.60	0.592
IL-2	30.14 ± 4.68	29.98 ± 3.55	0.894
IL-4	15.70 ± 1.19	15.88 ± 0.91	0.559
IL-5	10.37 ± 0.81	9.92 ± 0.51	0.033*
IL-6	51.09 ± 65.83	81.00 ± 131.64	0.195
CXCL8/IL8	789.50 ± 395.83	664.97 ± 365.78	0.238
IL-10	46.42 ± 9.74	44.43 ± 10.52	0.457
IFN-γ	17.80 ± 1.13	17.63 ± 0.63	0.574
GM-CSF	12.41 ± 6.32	11.63 ± 0.74	0.603
TNF-α	11.60 ± 0.90	11.47 ± 0.83	0.602

MII: metaphase II; GV: germinal vesicle; FR: fertilization rate; IL: interleukin; INF-γ: interferon gamma; GM-CSF: Granulocyte macrophage-colony stimulating factor; TNF-α: tumor necrosis factor-alpha.

* Differences were statistically significant ($p < 0.05$).

Table 3
Demographics and cycle characteristics in non-pregnant (NP) and pregnant (P) women. Comparisons of these qualitative variables between P and NP were performed using univariate analysis Chi-square test.

	Non pregnant N (%) (n = 60)	Pregnant N (%) (n = 18)	*P* value
Infertility type			
Female factor infertility n (%)	24/28 (86%)	4/28 (14%)	0.168
Mixed infertility n (%)	36/50 (72%)	14/50 (28%)	
Previous IVF/ICSI			
No n (%)	16/23(69.6%)	7/23(30.4%)	0.319
Yes n (%)	44/ 55 (80%)	11/55(20%)	
Superovulation protocol			
Agonist n (%)	20/28(71.4%)	8/28(28.6%)	0.389
Antagonist n (%)	40/50(80%)	10/50(20%)	

which was significantly greater than of those patients undergoing first ICSI attempt (FR% = 59.7%). The only significantly correlated measured cytokine was IL-4; an increase in each unit of FF IL-4, correlated with 7.5% increase in FR% (P = 0.007). IL-5 was inversely and significantly correlated with the chance of biochemical pregnancy (p = 0.026; OR = 0.223). Other nine measured cytokines and predictors had no significant effect on the chance of biochemical pregnancy. In all patients, the endometrial thickness was significantly correlated with the IR%; in general, the thicker the endometrial thickness, the greater the implantation rate. None of the ten measured cytokines had a significant correlation with IR%. IR% proportioned directly with (IFN-γ, IL-4, IL-6, TNF-α) and inversely with (IL-1β, IL-2, IL-5, IL-8, IL-10, GM-CSF), but it was insignificant.

Multiplex magnetic bead-based flow cytometry measured cytokines in the follicular fluid samples are shown in Table 4. IL-5 is the only measured cytokine which significantly affects the chance of biochemical pregnancy using multivariate logistic regression test. Using the Receiver Operating Characteristic (ROC) curve in the prediction of biochemical pregnancy, the area under curve (AUC) for IL-5 was 0.654, i.e. this cytokine has a medium ability in predicting the chance of biochemical pregnancy. Whereas other nine measured cytokines had a poor ability to predict the biochemical pregnancy (Fig. 1 and Table 4).

Table 4
Mean ± Standard deviation of multiplex bead-based measured cytokines concentrations in follicular fluid. Receiver operating characteristics (ROC) curve was done for estimation of performance of each cytokine on prediction of biochemical pregnancy status. Area under curve (AUC) is presented for each cytokine.

	Mean ± SD (pg/ml)	AUC
IL-1β	13.13 ± 0.72	0.534
IL-2	30.07 ± 4.45	0.531
IL-4	15.74 ± 1.13	0.583
IL-5	10.23 ± 0.77	0.654*
IL-6	57.19 ± 84.55	0.571
CXCL8/IL8	757.39 ± 385.97	0.592
IL-10	45.95 ± 9.95	0.576
IFN-γ	17.75 ± 1.03	0.519
GM-CSF	12.21 ± 5.48	0.518
TNF-α	11.57 ± 0.87	0.542

IL: interleukin; INF-γ: interferon gamma; GM-CSF: Granulocyte macrophage-colony stimulating factor; TNF-α: tumor necrosis factor-alpha.

* Multivariate logistic regression test showed that only IL-5 significantly affect the chance of biochemical pregnancy. AUC for IL-5 shows that this cytokine has a medium ability in prediction of biochemical pregnancy.

4. Discussion

The main finding of this study is that some measured FF cytokines such as IL-5, IL-4, and GM-CSF from preovulatory follicles were significantly associated with ICSI outcome. Elevated FF IL-5 levels were associated with poor oocyte quality, which decreases the chance of pregnancy. Higher FF GM-CSF was associated with a decrease in mature oocytes, while higher FF IL-4 concentrations linked to good ICSI outcome through increased fertilization rate.

We observed that the elevated FF IL-5 concentrations were

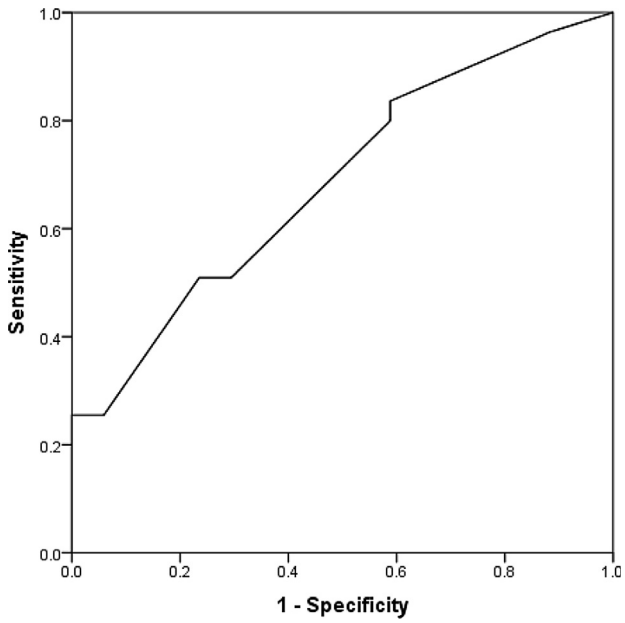

Fig. 1. Receiver operating characteristics (ROC) curve showing the performance of IL-5 in prediction of biochemical pregnancy.

associated significantly and negatively with ICSI outcome and resulted in poor oocyte quality through increasing GV% and decreasing MII%. It has been reported that IL-5 is a well-known T helper cell type 1 (Th1) cytokine produced by both hematopoietic and non-hematopoietic cells with a pleiotropic activities [29]. It affects the differentiation of myeloid cells, increment of chemotactic activity and adhesion capacity of eosinophils [30,31]. It also stimulates B-cells and act as a differentiation factor for eosinophils [25,32]. In reproductive organs, IL-5 contributes to tissue remodeling in mice ovaries [33] and endometrial tissue remodeling associated with estrus cycle [31]. Recently, Terenina et al. [34] suggested that IL-5 acts as one of the upstream key regulators of porcine ovarian follicular atresia.

Previous studies, which investigated the possible role of IL-5 in IVF/ICSI outcome, could not detect IL-5 in the FF of women undergoing ICSI using a bead-based multiplex sandwich immunoassay [11]. Another study detected IL-5 in 25% of FF samples using the R&D systems. In the latter study, the implantation rate, embryo morphology, fragmentation or early cleavage were not significantly different between oocytes which lead to a successful birth and those that do not [10]. In a recent study, Niu et al. [35] did not find a significant correlation between FF IL-5 from the largest follicle with top-quality embryo and the embryo developmental potential in a cohort of patients with or without metabolic syndrome undergoing IVF.

It was suggested that Th1 cytokines promote allograft rejection and may compromise pregnancy, whereas the Th2 cytokines promote allograft tolerance and therefore may improve fetal survival [1,36]. Recent animal model study documented that Th2 biased response in peripheral blood mononuclear cells (PMNC) during early pregnancy is essential for successful pregnancy in cattle [37]. Interestingly, IL-5, known with its Th2 anti-inflammatory properties, decreased significantly in FF of pregnant women and was inversely correlated with both biochemical and clinical pregnancy. In addition, it has a medium ability in prediction of biochemical pregnancy based on ROC curve in our study. Pregnancy is not only as a simplistic Th1/Th2 paradigm, where the 'bad' Th1-type cytokines induce abortion and 'good' Th2-type cytokines are responsible for successful pregnancy [1]. Many cytokines, not only Th1 and Th2 cytokines, are produced by other immune and non-immune cells present at the fetomaternal interface. Those cells are pluripotent in that their functions could depend on their relative concentrations. Their activity could be mitigated by receptors and antagonists, and they could have stage-dependent functions [1,38].

The other cytokine in our study with significant difference between successful and unsuccessful ICSI cycles, was IL-4. This interleukin increased significantly in FF of pregnant women and resulted in increased fertilization rates. IL-4 is extensively expressed from the cumulus oophorus that surrounds the oocyte during ovulation and the embryo during the first 72 h before the implantation, and its effect is favored by progesterone produced by cumulus luteal cells [39]. The resolution of inflammation plays a critical role throughout pregnancy and is largely mediated by immune cells that produce IL-4 and IL-10 [40]. The study of Marzi et al. [41] demonstrated that the normal pregnancy was correlated with an increase in serum IL-4 and IL-10. However, our study does not reveal any significant correlations between FF concentrations of IL-10 and the implantation rate.

Also, GM-CSF was significantly correlated with poor oocyte quality, as an increase in FF concentrations of this cytokine was associated with a decrease MII oocytes. GM-CSF is expressed from granulosa cell (GC) and theca cell (TC) and participates in ovulation and luteinization by enhancing recruitment of macrophages [3]. It is a pro- inflammatory cytokine which regulates myeloid cell proliferation and development. It is also critical for the functions of monocytes, macrophages and dendritic cells, and is produced in large amounts under inflammatory conditions by activated cells of immune system [42]. The study of Lèdèe et al. [13] suggested that follicular GM-CSF concentrations are not related to corresponding ongoing pregnancy rates. The use of COH to stimulate a multi-follicular response using exogenous gonadotropins for ART treatment can be detrimental to oogenesis, embryo quality, and endometrial receptivity, and consequent perturbation to the intrafollicular cytokines networks, in terms of both cytokine levels and their interrelationships. This may impact oocyte maturation/fertilization and embryo developmental competence by comparing to the intrafollicular milieu [22]. This forces the production of oocytes from follicles that do not reach optimal maturation and possibly yield oocytes that are not fully competent. The similar sized pre-ovulatory follicles of unstimulated cycles have a different hormonal milieu when compared with the growing follicles as COH cycles affect not only immune processes but also meiosis and ovulation pathways. Nevertheless, these differences do not seem to be related to early stage embryonic morphology [20]. GM–CSF was higher in the FF from the antagonist superovulation protocol [43], while in our study we did not find any significant differences in FF concentrations of GM-CSF between the two protocols.

Little evidence is available that non-invasive selection at the oocyte stage (first polar body, granular cytoplasm) may be of prognostic value. Certain patterns of pronuclei such as the number and distribution of pronuclei at the zygote stage was found to correlate to treatment outcome in IVF and ICSI cycles [44], while our results revealed neither significant correlation between embryo grading at the day three after ICSI and its outcome, nor with FF fluid concentration of cytokines.

At ovulation, the ovulatory follicle undergoes maturational changes that are associated with increasing follicular size and alterations accompanied with increasing its ability to produce estradiol. The use of COH in ART treatments affects the maturation of follicles and oocytes at the induced ovulation including the interval from induced luteolysis to induced ovulation, ovulatory follicles growth rate, and ovulatory follicles size in animal models [45]. Günther et al. [46] demonstrated a significant correlation between increased FF IL-18 concentration and successful pregnancy after IVF/ICSI. The author attributed this success to increased number of oocyte retrieved due to the increase in response to ovarian stimulation. While none of the measured cytokines significantly affected the number of oocytes retrieved among our ICSI patients. Previous comparative study between GnRH agonist long and antagonist protocol and their effect on implantation rate and serum cytokines concentrations reported that FF IL-1β, IL-8, IL-12, and TNF-α had no significant differences between the two protocols [47]. We confirmed this findings regarding most of these cytokines, in addition to other cytokines including IFN-γ, GM-CSF, IL-2, IL-5, and IL-10.

An intriguing result in this study was regarding the infertility period. We found that longer infertility period was associated with a higher number of follicles, but none of the ten measured cytokines was associated. The overall likelihood of successful treatment for infertility is nearly 50%, but this varies by reason, age of female partner, history of previous fertility, and duration of infertility. A shorter duration of infertility and previous fertility increases the chances of achieving pregnancy [48]. Kalu et al. [49] concluded that young women who had a live birth and those who experienced an early miscarriage after IVF had a greater chance of achieving pregnancy and a live birth in a second cycle. The outcome of the first IVF cycle, however, does not predict subsequent IVF success in older women. Rehman et al. [14] demonstrated that higher FF IL-1β concentrations was associated with an increase in the number of retrieved, mature and fertilized oocytes in ICSI patients.

In this study, we found that the patients with previous IVF/ICSI attempts have a higher number of large follicles proportion compared with patients undergoing their first ICSI trial. Likewise, FR% was higher (74.4% versus 59.7%) in patients with previous IVF/ICSI attempts and first attempt respectively. This may be attributed to the previous ovary stimulation with a controlled ovarian hyperstimulation cycles in the previous IVF/ICSI trials that may override the ovarian response in these patients. Our results are consistent with those of Hendriks et al. [50] in that patients within a certain age frame produce a poor ovarian response to gonadotropin stimulation in their first IVF cycle.

Our results revealed that the age was inversely proportioned with the number of follicles and the number of oocytes. Age is the most prognostic factor for IVF/ICSI success. Age associated infertility appears to be primarily related to ovarian aging and the diminishing ovarian follicle count [51]. During the reproductive life, once ovary has exhausted its reserve of follicles throughout many of functions are lost [52]. Previous studies reported that the deleterious effects of advanced patient age associated with IL-6 and its negative effects on the uterine receptivity to the embryo, hence, poor IVF/ICSI outcome [25]. In our female factor infertility patients, results showed no significant correlation between age and FF concentrations of IL-6 from successful ICSI cycles and unsuccessful ones. We also revealed that the endometrial thickness was linked to increase implantation rate and the chance of clinical pregnancy, but none of the ten measured cytokines had been significantly associated to increase endometrial thickness. While the study of Rehman et al. [14] demonstrate that the IL-1β increase endometrial thickness and reproductive outcome rates in ICSI patients.

In summary, our data suggests several detrimental effects of elevated FF IL-5 on ICSI outcome. One is associated with poor oocyte quality through a significant increase in oocytes with a germinal vesicle stage accompanied with a decrease in MII oocytes proportion. The second one was associated with decreased chance of both biochemical and clinical pregnancy. Moreover, IL-5 had medium prediction ability in predicting the chance of biochemical pregnancy. So, we can conclude that the elevated intrafollicular concentrations of IL-5 seem to be a negative predictor to the pregnancy outcome in ICSI cycles. In addition, elevated FF GM-CSF was associated with poor oocyte quality, while higher FF IL-4 concentrations were linked to good ICSI outcome.

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