Interleukin 15 concentrations in follicular fluid and their effect on oocyte maturation in subfertile women undergoing intracytoplasmic sperm injection

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Abstract

Purpose To calculate the concentrations of interleukin 15 (IL-15) in follicular fluid (FF) and evaluate their relation with oocyte maturation, follicle size, and patients' body mass index (BMI) and age.

Methods Follicular fluid specimens were obtained from 56 subfertile women undergoing intracytoplasmic sperm injection (ICSI) during oocyte retrieval for measurement of IL-15 concentrations with ELISA. Wilcoxon's test and Pearson's correlation coefficient were used to correlate FF concentrations of IL-15 with follicular size and stage of oocyte maturation, along with patients' BMI and age.

Results IL-15 concentrations in FF of follicles with immature oocytes were significantly greater than those from follicles with mature ones (median 5.333 vs. 3.250 pg/ml, respectively, $p < 0.001$). There was a significant negative correlation between IL-15 concentrations and follicle size ($r = -0.333$, $p = 0.003$). No significant correlation was observed between IL-15 concentrations and patients' BMI and age ($p > 0.05$).

Conclusions IL-15 concentrations in FF are adversely related with the size of the follicles and the maturity of the corresponding retrieved oocytes in a cohort of expected normal responders undergoing intracytoplasmic sperm injection (ICSI). Follicular fluid concentrations of IL-15 should be investigated as a possible predictive factor for oocyte maturity.

Keywords IL-15 · Follicular fluid · Oocyte maturation · Cytokines

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Introduction

The assessment of oocyte quality plays a key role in assisted reproductive techniques (ART). The need to identify the best embryo(s) for transfer at the most competent stage has directed research to the description of oocyte morphology and efficiency.

The differentiation of mammalian oocytes and the acquisition of developmental competence require a unique microenvironment from various molecules. A cascade of cytokines, chemokines, and growth factors acts upon the oocyte before fertilization and the embryo before implantation. Follicular fluid (FF) results from the transfer of plasma components through the blood-follicular barrier and from granulosa and thecal secretions [1]. It is the microenvironment for the development of the oocyte. The complex composition of regulatory factors, such as cytokines, chemokines, and growth factors, as well as their concentrations in FF impacts oocyte viability and developmental potential [2, 3]. Studies have reported the presence of various cytokines, such as interleukins (IL), including IL-15, in FF [4–10].

It has been shown that the levels of various cytokines are significantly correlated with BMI [11–13]. There are indications that increased body weight alters the inflammation network in FF and specifically the levels of soluble IL-6 receptor (sIL-6R). Body weight perturbs intrafollicular IL-6-mediated inflammatory networks with increased levels of sIL-6R, thereby possibly affecting fertility and IVF outcomes [14].

Cytokines act as important intercellular regulators in a wide range of physiological and pathophysiological events in the human body [15]. They regulate ovarian function and, specifically, folliculogenesis and ovulation [16–22]. The FF IL-1 concentrations were found increased in unsuccessful ART attempts as compared to those in successful (leading to pregnancy) ones [3]. Interestingly, the increased FF IL-1 concentrations were found in follicles that yielded mature oocytes but unsuccessful embryo transfer. According to the authors, it is possible that FF IL-1 is associated with oocyte maturation without improvement of post-fertilization embryo viability [3].

Interleukin-15 is a pleiotropic glycoprotein that belongs to the four α -helix bundle family of cytokines [23, 24]. The FF concentrations of IL-15 are reported to be greater in women with an unsuccessful ART outcome (median 1.4 pg/ml) compared to those in women who achieved clinical pregnancy (median 0.8 pg/ml) [7]. In the same context, increased FF concentrations of IL-15 have been associated with poor prognosis on subsequent pregnancy rates and failure in oocyte retrieval, while they appear predictive of ART failure, especially when combined with poor embryo morphology [9].

The purpose of this cross-sectional study was to examine the possible relation of FF concentrations of IL-15 with oocyte maturation, as well as their relation with follicular size, and patients' BMI and age in women undergoing intracytoplasmic sperm injection (ICSI).

Materials and methods

Patients

This cross-sectional study was performed at an Assisted Reproductive Unit in Athens, from September 2016 to October 2017. The trial protocol was approved by the Scientific Board and Bioethics Committee. The cohort of the study participants included 56 subfertile women undergoing ICSI. A written informed consent was obtained from all participants in the study. Following clinical evaluation along with previous medical and reproductive history, these women were categorized in terms of subfertility factor as follows: blockage or lack of fallopian tubes; presence of polyps, fibroids, or cysts; severe adhesions; male factor; and unexplained infertility.

The women included in the study were 20–45 years old (mean $\pm$ SD, 36.3 ± 6.7) with BMI between 16.5 and 37.5 (23.4 ± 4.1) and were undergoing ICSI. The exclusion criteria for the participation in the study were presence of inflammatory or infectious conditions, autoimmune and neoplastic diseases, or medication intake that might affect the immune system within 4 weeks of the oocyte retrieval. There is no robust evidence suggesting removal of polyps, fibroid, or cysts before performing ART. In two recent reviews [25, 26], this subject was reviewed. Di Spiezo Sardo et al. concluded [25] that there is no evidence about the role of hysteroscopy as a basic infertility evaluation tool or whether hysteroscopy, performed before IVF, improves pregnancy rates and live birth rates when removing submucosal fibroids or endometrial polyps. On the other hand [26], Bosteels et al. suggested that a large benefit with the hysteroscopic removal of submucous fibroids for improving the chance of clinical pregnancy in women with otherwise unexplained subfertility cannot be excluded and that hysteroscopic removal of endometrial polyps suspected on ultrasound in women prior to intrauterine insemination may increase clinical pregnancy rate. However, in that review, the authors suggest that more randomized studies are needed to substantiate the effectiveness of the hysteroscopic removal of suspected endometrial polyps, submucous fibroids, uterine septum, or intrauterine adhesions in women with unexplained subfertility or prior to ART. In the absence of data supporting an alternative clinical approach and because the present study was initiated in September 2016, we have not excluded these patients nor did we perform surgical interventions to these patients prior to the implementation of ART to avoid their impact to patients' fertility potential. Patients were enrolled during a 1-year study period and participated in the study only once. The number selected was based on previous reports [7, 9].

These women were either on a natural IVF cycle ($n = 7$) or a modified natural cycle ($n = 4$) or a short stimulation protocol ($n = 16$) or a mild stimulation protocol ($n = 29$) (Table 1). During the natural IVF cycle, no ovarian stimulation drugs were given except for the human chorionic gonadotropin (hCG) injection for oocyte maturation; in the modified natural cycle, a GnRH antagonist and a minimal dose of gonadotropins were added in the last few days of the cycle (by day 11 to the day of human chorionic gonadotropin (hCG) triggering); for the mild stimulation protocol, 150 mg clomiphene citrate or 5 mg letrozole was administered starting on day 2 or 3 for 5 days with the addition of 150–225 IU r-FSH or combination of u-FSH and r-FSH daily according to the follicular development approximately from day 5 of cycle together with 0.25 mg cetrorelix as soon as the leading follicle reached a diameter ≥ 14 mm on average and continued up to the day of hCG administration. For the short GnRH-antagonist protocol, recombinant FSH in the form of either follitropin alpha or follitropin beta was administered subcutaneously on the

Table 1 Anthropometric characteristics of patients and type of ART protocol they underwent

Number of patients	<i>n</i> = 56
Age (years, mean $\pm$ SD)	36.3 $\pm$ 6.7
BMI (kg/m ² , mean $\pm$ SD)	23.4 $\pm$ 4.1
< 20	<i>n</i> = 18
21–25	<i>n</i> = 25
> 26	<i>n</i> = 13
Protocol	
Natural	<i>n</i> = 7
Modified natural supported	<i>n</i> = 4
Mild protocol	<i>n</i> = 16
Short protocol	<i>n</i> = 29

second day of the cycle; starting doses were 200 to 450 IU, adjusted according to each patient's response to stimulation. After day 6 of cycle monitoring, cetrorelix, a gonadotropin-receptor antagonist, was initiated as soon as the leading follicle reached a diameter ≥ 14 mm on average and continued up to the day of the hCG administration. Nine women received ART for the first time, while 47 had undergone one to three rounds of ART previously.

Protocol

For cycle monitoring, transvaginal ultrasonography (every 1–2 days, or as required after the sixth day of the cycle) and estradiol measurements (daily between the 8th and the 10th day of the cycle) were performed. Approximately at the 11th day of the cycle when one or more follicles reached a diameter of 18 mm and the estradiol concentrations were appropriately elevated according to the number of follicles, hCG was administered. After 36–38 h, oocytes and the corresponding FF were retrieved by transvaginal ultrasound-guided aspiration. For the research purposes of this study, the surgeon was retrieving the FF and the oocyte from the follicle with the easiest access because it was mandatory to retrieve the FF with an unused needle. Thus, the retrieved FF was certain not to have been contaminated from multiple retrievals with the same needle. The FF from the first randomly aspirated (without flushing) follicle that contained oocyte was employed for the measurement of IL-15 concentrations. The content of each follicle was collected with a single-lumen pick-up needle, the oocyte was collected in a dish with the appropriate culture media, and the total volume of FF was placed in a 15-ml sterile Falcon conical tube. The volume of the aspired FF was noted down and subsequently correlated to the diameter of the corresponding follicle as measured prior to the oocyte retrieval by ultrasound (Table 2). The FF cellular components were removed by centrifugation, at room temperature, for 5 min at 2500g, and the supernatants were frozen at -20 °C until assayed. The G-IVF™ PLUS (Vitrolife, Göteborg, Sweden) culture medium was employed. In detail, this is a bicarbonate

buffered medium containing human serum albumin and gentamicin as an antibacterial agent, for preparation and handling of gametes and for in vitro fertilization; it contains glucose and fructose and all components necessary to support both cumulus cells and sperm functionality.

Evaluation of oocyte maturity

Oocytes remained in culture media (6% CO₂, 37 °C) until insemination. Immediately prior to the ICSI procedure, COC were briefly exposed to 80 IU hyaluronidase, followed by mechanical removal of cumulus oophorus and corona radiata by repeated aspiration into a finely drawn Pasteur pipette. The maturity of the oocytes was assessed under a stereoscope. Oocytes at GV (germinal vesicle nucleus) or MI (metaphase I oocytes) stage were considered immature, while those at MII (metaphase II oocytes, 1st polar body) stage, mature.

IL-15 assay

Follicular fluid concentrations of IL-15 were determined with an enzyme-linked immunosorbent assay (sandwich ELISA) technique. Quantikine human IL-15 Immunoassay ELISA kit from R&D Systems (Minneapolis, MN, USA) was employed according to the manufacturer's guidelines. The sensitivity of the method was less than 2.0 pg/ml, and the intra- and inter-assay coefficients of variation were 4.8 and 6.6%, respectively.

Statistical analysis

The main outcome measure of this study was the comparison of the concentrations of IL-15 in FFs collected from follicles with mature and immature oocytes. Secondary outcome measures were the correlations between IL-15 concentrations and follicular size, as well as between IL-15 and patients' BMI and age.

Follicular fluid concentrations of IL-15 were correlated with the stage of oocyte maturation, patients' BMI, patients' age, and follicular size using SPSS 21.0 package (IBM Corp., released 2012, IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp.). The normality of the distributions was assessed with Kolmogorov-Smirnov's test and graphical methods. Linear regression and Mann-Whitney (Wilcoxon's test) non-parametric test was employed for the comparison of continuous variables due to their abnormal distribution, and Pearson's correlation coefficient was employed as well, as appropriate. Differences were considered as statistically significant if the null hypothesis could be rejected with > 95% confidence ($p < 0.05$). The observed power of the study using the sample size of 56 women was 90%, as shown by post hoc analysis.

Table 2 FF volumes and the corresponding follicle sizes

Follicular fluid volume (ml)	Follicle size (mm)
< 0.5	< 10
0.6–1.0	10–12
1.0–2.0	13–15
2.0–3.0	16–18
3.0–4.0	18–20
4.0–5.0	21–22
> 5.0	> 22

Results

Overall, 56 women participated in the study. Forty-three mature and 13 immature oocytes (2 GV; 11 MI) were retrieved from these women. The FF volumes and corresponding follicle sizes are presented in Table 2. Twenty follicles measured less than 15 mm, 23 measured between 15 and 20 mm, and 13 measured more than 20 mm. All immature oocytes ($n = 13$) were retrieved from follicles that measured less than 15 mm (13 out of 20; 65%). The mean concentration of IL-15 in the FFs with mature and immature oocytes was 3.450 ± 1.489 pg/ml (0.9–6.4 pg/ml) and 5.342 ± 1.630 pg/ml (3.1–7.5 pg/ml), respectively. The median of the latter was significantly greater than the median of the former (5.333 and 3.250 pg/ml, respectively; $p < 0.001$) (Fig. 1).

Mean $\pm$ SD and median values of FF IL-15 concentrations in follicles with mature oocytes from natural cycles (only mature oocytes were retrieved from these cycles), in follicles with mature oocytes from medicated cycles and in

follicles with immature oocytes from medicated cycles, are presented in Table 3. When the FF IL-15 concentrations in follicles from these three groups were compared among them, there was no significant difference between the median FF IL-15 concentrations in follicles with mature oocytes from natural cycles as compared to those from follicles with mature oocytes from medicated cycles (median 4.200 vs. 3.150 pg/ml, respectively, $p > 0.05$; Wilcoxon test), neither between follicles with mature oocytes from natural cycles and follicles with immature oocytes from medicated cycles (median 4.200 pg/ml vs. 5.000 pg/ml, respectively, $p > 0.05$; Wilcoxon test), but they were significantly lower in follicles with mature oocytes from medicated cycles as compared to those from follicles with immature oocytes from medicated cycles (median 3.150 vs. 5.000 pg/ml, respectively, $p < 0.05$; Wilcoxon test). When linear regression was employed, with FF IL-15 concentration taken as dependent variable, the model was statistically significant ($p = 0.05$) only for maturity ($p = 0.029$; $R^2 = 0.194$) among all variables included in the model as independent variables (maturity, BMI, age, size of follicle). Unfortunately, no data were obtained from the embryos derived from the oocytes observed in this study because a one-by-one oocyte-embryo cultured ART procedure was not employed. A statistically significant negative correlation was found between IL-15 concentrations measured in FFs from all follicles retrieved for this study and the sizes of the corresponding follicles ($r = -0.333$, p value < 0.05) (Fig. 2).

No correlation was found between FF IL-15 concentrations and the corresponding patients' BMIs or ages (all p values > 0.05).

Fig. 1 Whisker box-plots of IL-15 concentrations (pg/ml) in follicular fluid (FF) of mature and immature oocytes. The asterisk indicates statistically significant difference ($p < 0.05$)

Table 3 Presentation (mean $\pm$ SD; median) and statistical comparison (Wilcoxon test) of FF IL-15 concentrations (pg/ml) from follicles with mature oocytes from natural cycles (only mature oocytes were retrieved from these cycles), from follicles with mature oocytes from medicated

	<i>N</i>	Mean $\pm$ SD (pg/ml)	Median (pg/ml)
Follicles from natural cycles	7	3.800 $\pm$ 1.204	4.200
With mature oocytes	7	3.800 $\pm$ 1.204	4.200
Follicles from medicated cycles	49	3.817 $\pm$ 1.825	3.450
With mature oocytes	36	3.447 $\pm$ 1.661	3.150*
With immature oocytes	13	5.155 $\pm$ 1.825	5.000

Discussion

In this cross-sectional study, we found that FF concentrations of IL-15 were greater in follicles with immature oocytes than in those with mature ones in women undergoing ART with ICSI. This is not the first study on IL-15 measurement in FF [7, 9], but it is the first one to present significant correlations between FF IL-15 concentrations and the maturity of oocyte. The sample size is too small to assume any difference in IL-15 concentrations between follicles with the two types of immature oocytes (GV, MI). In addition, the sample size of natural cycles was small so that the comparison of FF IL-15 concentrations from follicles with mature oocytes from these cycles with the FF IL-15 concentrations from follicles with either mature or immature oocytes from medicated cycles should be interpreted with caution. However, in medicated cycles, the FF IL-15 concentrations were significantly greater in follicles with immature than in follicles with mature oocytes. The FF volumes that corresponded to the size of each follicle (Table 2) were similar to those described previously by Wittmaack et al. [27].

cycles and from follicles with immature oocytes from medicated cycles. The asterisk denotes statistically significant difference from follicles with immature oocytes from medicated cycles (at the level of $p < 0.05$)

A number of previous studies aimed at establishing correlations between the concentration of various cytokines in FF and oocyte quality [2, 9]. Resident and infiltrating leukocytes are the principal sources of cytokines in FF and surrounding tissue. Ovarian somatic cells, including luteal, stromal, thecal, and granulosa cells, are an important cellular source of cytokines. Cytokines play an important role in the regulation of ovarian function, gonadal steroid secretion, corpus luteum function, embryo development, and implantation [7, 28]. There is growing evidence that the interactions between the immune and endocrine system can influence ovarian function. Consequently, the presence of regulatory factors in FF is indirectly linked to fertilization and early embryonic development [3, 29, 30]. Of these factors, at least some are likely to influence, either directly or indirectly, oocyte viability and developmental potential. Certain cytokines contribute to creating an environment for the selection of the follicles and their development [17]. Thus, they regulate cell proliferation and differentiation, follicular survival and atresia, and maturation of oocytes [16–22]. Furthermore, increased concentrations of IL-15 in FF have been associated with reduced pregnancy and take-home baby rates [7, 9]. Thus,

Fig. 2 Scatter chart representing the negative correlation ($r = -0.333$, p value < 0.05) between follicle sizes (in mm) and IL-15 concentrations (in pg/ml)

it seems that increased concentrations of IL-15 in FF might indicate improper maturing process. It has been shown that IL-15 reduces the effect of Ca^{2+} on some kinases, such as ERK1/2 and Akt, which are crucial in controlling the proliferation and differentiation of keratinocytes [31]. During the differentiation of keratinocytes, Ca^{2+} inhibits the activation of ERK1/2 and induces Akt phosphorylation, but in the presence of IL-15, these effects are attenuated and the differentiation process is disturbed. Akt is known to have a role in the cell cycle at G1 and G2 phases, while in general, various kinases play a key role in the oocyte maturation [32, 33]. As Ca^{2+} increase is crucial in triggering the complex machinery involved in gamete maturation and fertilization, and IL-15 inhibits its action on kinases, this interleukin may be important in the oocyte maturation process [34, 35].

In addition, in this study, a significant negative correlation between FF IL-15 concentrations and the sizes of the corresponding follicles was found. Of note, it is established that the size of the follicle is associated positively with the maturity of the corresponding oocyte [36]. Finally, the FF amounts of IL-15 were not associated to BMI or age of the studied patients.

From the foregoing, it is possible that the increased concentration of IL-15 in FF is directly and/or indirectly associated with growth inhibition of the follicle and eventually the oocyte maturity. Thus, it might be related negatively with the outcome of the IVF process. Alternatively, IL-15 could be a mere confounding marker of the oocyte immaturity.

A definite limitation of the current study is the lack of randomization, the reduced cohort size, the wide discrepancy of stimulation protocols employed, and the small number of FFs retrieved from natural cycles which could stand for controls. To that aim, a randomized control study should evaluate whether FF concentrations of IL-15 are effective in identifying oocytes with the best competence in patients using a conventional ART scheme. We fully acknowledge the difficulty of performing well-powered randomized control trials to answer this particular clinical question. Still, evidence remains poor in this field. Furthermore, the effect of IL-15 in the Ca^{2+} -induced oocyte maturation in vitro should be further investigated.

In conclusion, it is suggested that FF concentrations of IL-15 are related negatively with the maturity of the retrieved oocytes after ART. It is proposed that IL-15 concentrations, being related negatively to the oocyte maturation, might be employed as a marker of its potential to develop, following fertilization, into a healthy viable embryo that can be implanted and give rise to a successful pregnancy together with the classic morphological assessment. Further studies are required to determine whether the FF concentration of IL-15 can be used as a marker for the selection of the most competitive oocyte in ART.

Compliance with ethical standards

Conflict of interest All authors declare no conflict of interest.

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