Comparative multiplex analysis of cytokines, chemokines and growth factors in follicular fluid of normoresponder women undergoing ovum donation with gonadotropin-releasing hormone agonist versus gonadotropin-releasing hormone antagonist protocols

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Submission: 21.05.2013
Review completed: 03.09.2013
Accepted: 05.10.2013

Access this article online

Quick Response Code:

Website: www.jhrsonline.org

DOI: 10.4103/0974-1208.121424

ABSTRACT

BACKGROUND: Conflicting results were yielded about the superiority of gonadotropin-releasing hormone agonist (GnRH-a) versus gonadotropin-releasing hormone antagonist (GnRH-ant) protocols used in ovarian stimulation in *in vitro* fertilization (IVF) set-up. Reports also indicate that any single specific individual marker in follicular fluid collected at the time of oocyte retrieval bears inconclusive value as a predictor of oocyte quality. **AIMS:** Simultaneous analyses of large numbers of cytokines, chemokines and growth factors in ovarian follicular fluid and perifollicular vascularity in both protocols for ovarian stimulation in IVF program to address the above mentioned lacunae. **SETTINGS AND DESIGNS:** Normoresponder women ($n = 45$) were subjected to either GnRH-a (Group 1; $n = 23$) or GnRH-ant (Group 2; $n = 22$) for ovarian stimulation in IVF clinics. **MATERIALS AND METHODS:** The fluid samples of dominant follicles collected at oocyte retrieval from women in Group 1 (GnRH-a; $n = 20$) and Group 2 (GnRH-ant; $n = 16$) were used for simultaneous quantitative assays of 48 cytokines. Perifollicular vascularity was assessed by Doppler hemodynamics to assess the ovarian vascular response in all participants in Groups 1 and 2. **RESULTS:** Despite demographic and reproductive parameters studied remained comparable, higher follicular fluid concentration of interleukins, IL-3 ($P < 0.01$), IL12p70 ($P < 0.05$) and vascular endothelial growth factor ($P < 0.01$), P4 ($P < 0.05$) and pulsatility index ($P < 0.04$) along with a lower number of oocytes in metaphase II stage ($P < 0.03$) was observed in Group 2 compared with Group 1. GnRH-a protocol appeared to be superior to GnRH-ant protocol for ovarian stimulation in normoresponder women.

KEY WORDS: Cytokines, follicular fluid, multiplex analysis, ovarian stimulation, perifollicular hemodynamics

INTRODUCTION

In a natural cycle, the close interplay of follicle stimulating hormone (FSH) and luteinizing hormone (LH) through a series of feedback mechanisms affects theca and granulosa cell functions toward the selection of the dominant follicle.[1] Gonadotropin-releasing hormone (GnRH) analogues are used to induce multiple ovulations in women undergoing *in vitro* fertilization (IVF) treatment.[2,3] The two main categories of analogs are agonists and antagonists, respectively; both are frequently used in assisted reproduction programs. There is an on-going debate concerning the efficacy of gonadotropin-releasing hormone antagonist (GnRH-ant) when compared to the most commonly used gonadotropin-releasing hormone agonist (GnRH-a) toward achieving reproductive outcomes.[4,5] In a Cochrane collaboration review, it has been reported

recently that the use of GnRH-ant leads to lower incidence of severe ovarian hyperstimulation syndrome, however, with similar live-birth rates when compared to GnRH-a.[6] It has been reported that higher numbers of retrieved oocytes showed less cytoplasmic abnormality and greater numbers of fertilizable oocytes, however, with a lower number of blastomeres on day 2 after fertilization in the GnRH-a treated group when compared to GnRH-ant treated group; there was however no significant difference between clinical pregnancy rates.[7] On the other hand, in a prospective randomized trial, it was observed that there were no differences in oocyte morphology between the antagonist multi-dose protocol and long-term agonist protocol.[8] The issue is further compounded by the observation that morphological criteria are not always predictive of oocyte quality.[9] Ovarian follicular fluid contains a variety of autocrine and paracrine factors responsible for the regulation of follicle competence, oocyte growth and ovulation. Thus, probing follicular fluid collected at the time of oocyte retrieval may help to provide suitable biochemical markers of oocyte quality.[10] Several previous reports have indicated the putative, but often inconclusive value of specific individual cytokines, chemokines, growth factors and hormones in follicular fluid collected at the time of oocyte retrieval as likely predictors of successful pregnancy in IVF cycles.[11-24] It has been suggested that multiplex analysis to explore the pattern of expression of a cohort of cytokines, chemokines and growth factors of follicular fluid may reveal robust indication of oocyte quality.[25,26] Further, development of either a single dominant follicle in a natural cycle or multiple follicles in gonadotropin induced cycles is highly dependent upon the development of perifollicular microvasculature networks that are reflected in the composition of follicular fluid and oocyte development.[27] To the best of our knowledge, there is no report of simultaneous analysis of expression profiles of a cohort of cytokines, chemokines and growth factors of follicular fluid retrieved from dominant follicles and perifollicular vascularity from women subjected to GnRH-a versus GnRH-ant treatment protocols. In the present study, we have performed multiplex analysis of follicular fluid obtained from women undergoing ovum donation treatment cycles in IVF program with either GnRH-a or GnRH-ant treatment schedules through the simultaneous assay of 48 cytokines and chemokines using microbead assay system and that of progesterone and beta-estradiol in follicular fluid along with the analysis of perifollicular vascularity to predict oocyte quality.

MATERIALS AND METHODS

Patients

The present prospective observational study was approved by the Institutional Ethics Review Committee and was conducted at the Assisted Reproduction Unit of the Department of Obstetrics and Gynecology and the Department of Physiology of the institute between November 2008 and December 2011. The study design and its operational execution complied with the principles in the Declaration of Helsinki, adopted by the 18th World Medical Assembly, Helsinki, Finland, June 1964 and further amended at the 59th World Medical Assembly, Seoul, Korea, October 2008. All participating women gave informed consent prior to the start of the study after understanding the objectives of the study and their rights. A group (n = 45) of normoresponder women from 65 patients who were eligible and willing to participate were finally included in the present study. All women were in the age group of 20-40 years, undergoing their first ovum donation cycles in IVF program and having plasma FSH <12 mIU/ml. The women with endometriosis, polycystic ovary syndrome, known endocrine disorders or undergoing intra-cytoplasmic sperm injection (ICSI) were excluded from the study.

Stimulation protocols

Controlled ovarian hyper-stimulation was done using standard protocols with either GnRH-a (Group 1; n = 23) or GnRH-ant (Group 2; n = 22),[28,29] based on the individual choice of the participants following discussion about both protocols. Briefly, the agonist protocol involved pituitary down regulation with daily subcutaneous administration of 1 mg leuprolide acetate (lupride; Sun Pharmaceutical, Mumbai, India), beginning from the mid luteal phase of the previous menstrual cycle until ovarian quiescence. Once down regulation was confirmed based on results from ultrasound examination and hormone assays (serum estradiol concentration: <50 pg/ml; serum LH concentration: <2 mIU/ml), the dose of leuprolide was halved and ovarian stimulation was initiated with recombinant FSH (Gonal F; Merck Serono, Mumbai, India) in doses of 150-300 IU depending on the age, body mass index and basal FSH levels. The dosage of gonadotropins was adjusted according to response seen on serial ultrasound examinations. Recombinant FSH and leuprolide were given until the day of ovulation trigger. The antagonist protocol involved ovarian stimulation with recombinant FSH beginning on the second day of the menstrual cycle. A daily dose of GnRH-ant (Cetrotide; Merck Serono, Mumbai, India) was initiated on day 6 of stimulation. Recombinant human chorionic gonadotrophin (HCG) (Ovitrelle; Merck Serono, Mumbai, India) was administered for ovulation trigger when at least two leading follicles reached a mean diameter of 18 mm. Trans-vaginal oocyte retrieval was done 36 h after ovulation trigger. The follicular fluid was collected from the dominant follicle mapped at previous scans, centrifuged at 250 × g for 15 min to separate out cellular debris and stored at −80°C until analysis. The maturity and

the quality of corresponding oocytes were done according to that described earlier.[30]

Assessment of perifollicular blood flow

The perifollicular vascularity was estimated in at least two dominant follicles in each ovary on the day of HCG trigger using power Doppler blood flow analysis. All examinations were done by a single investigator with a 6.5 MHz vaginal probe (Siemens Acuson Antares, Siemens Medical Solutions, Mountain View, CA, USA). The follicles were graded according to the percentage of follicular circumference in which most flow was identified from a single cross-sectional slice as described earlier.[31] The grading was done based on per cent follicular circumference in which blood flow was identified. The blood flow velocity waveforms were then obtained by placing the Doppler gate over the color area in the perifollicular flow and activating the pulsed Doppler function. A recording was considered to be satisfactory when at least five consecutive waveforms were obtained, each demonstrating the maximum Doppler shift. The resistive index (RI), pulsatility index (PI) and peak systolic velocity (PSV) were calculated on three consecutive wave forms as described earlier.[32]

Multiplex assays of cytokines in follicular fluid

The concentrations of 48 cytokines, chemokines and growth factors in follicular fluid samples from dominant follicles in two groups as mentioned above was analyzed by quantitative cytokine assays using Bioplex Pro™ human cytokine standard 27-plex and 21-plex panels based on xMAP technology (Bio-Rad Laboratory, Hercules, CA, USA) according to the pre-optimized protocol based on the methodology provided by the manufacturer. The list of the names of the cytokines, chemokines and growth factors estimated in the Bio-Rad multiplex microbead assays and their assay characteristics is available at http://www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_5828A.pdf. Follicular fluid from three women in Group 1 and six women in Group 2 were not included in the microbead assay due to suspected poor quality. Data were collected and analyzed using a Bio-Rad BioPlex 200 instrument equipped with Bio-Plex Manager software version 6.0 (Bio-Rad Laboratory, Hercules, CA, USA). The precision based on both intra and inter-assays variations were <10% within the detection limits provided by the manufacturer. The immunoassay data were expressed in terms of mg protein estimated from Bradford protein concentration in respective follicular fluid samples using Bio-Rad microtiter microassay kit (Bio-Rad Laboratory, Hercules, CA, USA) and human serum albumin (Sigma Chemical, St. Louis, MO, USA) according to the procedure provided by the manufacturer (http://labs.fhcrc.org/fero/Protocols/BioRad_Bradford.pdf).

Enzyme immunoassays

Standard enzyme immunoassays for serum concentrations of FSH, inhibin B and LH and concentrations of estradiol 17β and progesterone in follicular fluid were done in duplicates using immunoassay kits purchased from DRG International Inc. (New Jersey, NJ, USA). The intra-assay and inter-assay coefficients of variation for enzyme immunoassays were <10%.

DATA ANALYSIS

The statistical comparison for all parameters between two groups was done by using unpaired *t*-test. Partial correlation analyses among parameters were done to estimate predicated associations between them. Analysis for partial correlation coefficient yields a measure of the strength of association between a dependent variable and one independent variable when the effect of all other independent variables is removed. This type of analysis helps to identify correlations explained by the effect of other variables, as well as, to reveal hidden correlations, i.e. that is correlations masked by the effect of other variables.[33] All statistical analyses were performed using the Statistical Package for the Social Sciences v17.0 (SPSS Inc., Chicago, IL, USA). The significance level was set at 5% ($P < 0.05$).

RESULTS

Serum endocrine profiles, ovarian hemodynamics and retrieved oocytes

Women enrolled in the IVF program of the Department of Obstetrics and Gynecology, of the institute were recruited for this study and assigned for either GnRH-agonist (Group 1: GnRH-a) or GnRH-ant (Group 2: GnRH-ant) treatment cycles. 23 women were recruited to the Group 1 (i.e., GnRH-a treatment) and 22 women recruited to the Group 2 (i.e., GnRH-ant treatment). Age of women and their body mass indices were comparable between Groups 1 and 2 [Table 1]. Both groups had similar duration of treatments. Although the mean starting recombinant follicle stimulating hormone (rFSH) dosage was apparently higher in Group 2 (GnRH-ant), it was not statistically different and the total doses of rFSH were also comparable [Table 2]. Groups 1 and 2 had similar levels of serum FSH, LH and inhibin B on day 2 of the menstrual cycle [Table 1]. The Doppler hemodynamics and ovarian response in both groups are also shown in Table 2. Although the RI, percentage of ovarian blood flow and PSV, as well as, the number of antral follicles and the number of oocytes aspirated were comparable, the PI was higher and number of oocytes in metaphase II stage was lower in Group 2 when compared to Group 1.

Table 1: Demographics and reproductive parameters

Parameter	Group 1 (*n*=23)	Group 2 (*n*=22)	*P* value
Age (years)	31.7 (4.2)	31.8 (3.8)	0.93
BMI (kg/m^2)	25.4 (4.7)	25.8 (3.7)	0.75
Mean starting rFSH dose (IU)	272.7 (78.2)	306.8 (80.8)	0.16
Total dose rFSH (IU)	2950.0 (1299.1)	3100.0 (1018.8)	0.67
Total days of stimulation	10.9 (2.3)	11.6 (4.2)	0.32
Base level, serum inhibin B (pg/ml)*	63.1 (46.1)	65.1 (49.2)	0.89
Base level, serum FSH (mIU/ml)*	6.5 (2.3)	6.6 (2.3)	0.88
Base level, serum LH (mIU/ml)*	4.9 (2.4)	6.1 (4.7)	0.28
PI	0.7 (0.3)	0.9 (0.4)	0.04
RI	0.5 (0.2)	0.5 (0.2)	1.00
Percentage ovary blood flow	62.9 (20.7)	62.4 (21.2)	0.94
PSV	6.6 (3.4)	6.4 (2.7)	0.83
Total AFC	13.5 (7.0)	12.0 (5.0)	0.42
Number of oocytes aspirated	11.5 (7.0)	9.0 (4.0)	0.15
Number of oocytes at metaphase II stage	11.0 (7.0)	6.5 (4.5)	0.03

Group 1=GnRH agonist treated; Group 2=GnRH antagonist treated; Values are shown as means±SD*on day 2 of menstrual cycle; BMI=Body mass index; rFSH=Recombinant follicle stimulating hormone; FSH=Follicle stimulating hormone; LH=Luteinizing hormone; PI=Pulsatility index; RI=Resistive index; PSV=Peak systolic velocity; AFC=Antral follicle count; SD=Standard deviation

Cytokines, chemokines, growth factors and E2 and P4 in follicular fluid

In the Bio-Rad multiplexed microbead assay system employed in the present study, 10 factors that could not be detected in any sample were: interleukin (IL)-2, IL-4, IL-5, IL-7, IL-9, IL-13, IL-15, IL-17, interferon-gamma (IFNG) and nerve growth factor β (NGFB). In addition, 10 factors, namely chemokine (C-C Motif) ligand 3 (CCL3), granulocyte colony-stimulating factor (G-CSF), IL-1a, IL-1b, IL-1RA, IL-6, IL-10, platelet-derived growth factor-BB (PDGF-BB), tumor necrosis factors (TNFa and TNFb) could be detected only in few (<10%) samples. A total of 28 factors that included CCL2, CCL4, CCL5, CCL7, CCL11, CCL27, CLEC11A, CXCL1, CXCL9, CXCL10, CXCL12, FGF2, Granulocyte-macrophage colony-stimulating factor (GM-CSF), hepatocyte growth factor (HGF), interferon, alpha 2 (IFNA2), IL-2 RA, IL-3, IL-8, IL-12p40, IL-12p70, IL-16, IL-18, LIF, M-CSF, MIF, SCF, TNFSF10 and vascular endothelial growth factor (VEGF) were detected in more than 90% of follicular fluid samples of dominant follicles in Groups 1 and 2.

Table 2 shows the profiles of 28 cytokines, chemokines and growth factors in follicular fluid samples obtained from two groups. It is evident from Table 2 that the mean values against dispersion values are acceptable for downstream statistical analysis only for 10 cytokines (GM-CSF, HGF, IFNA2, IL-3, IL-12p70, IL-16, LIF, M-CSF, TNFSF10 and VEGF), of which three cytokines (IL-3, IL-12p70 and VEGF) displayed differential concentration profiles between the

Table 2: Concentrations of secreted cytokines, chemokines, growth factors, and sex steroid hormones in follicular fluid of women undergoing ovum donation treatments with either GnRH agonist (GnRH-a, Group 1) or GnRH antagonist (GnRH-ant, Group 2) in IVF program

Name (alias)	Concentration (mean±SD)	
	Groups	
	1 (GnRH-a) (*n*=20)	2 (GnRH-ant) (*n*=16)
Cytokines, chemokines and growth factors (pg/mg protein)		
CCL2 (MCP1)	41.7±33.1	47.1±29.2
CCL4 (MIP1b)	20.3±17.2	32.6±30.4
CCL5 (RANTES)	10.5±16.3	9.3±17.6
CCL7 (MCP3)	2.4±2.0	2.4±1.5
CCL11 (Eotaxin)	27.1±26.8	39.3±38.1
CCL27 (CTACK)	47.3±29.0	39.5±28.8
CLEC11A (SCGF)	11918.6±4035.2	9665.3±4845.2
CXCL1 (GROa)	153.2±65.6	122.4±85.6
CXCL9 (MIG)	260.1±114.4	263.0±85.2
CXCL10 (IP10)	339.9±271.0	380.1±182.4
CXCL12 (SDF1A)	62.4±24.64	57.3±28.0
FGF2 (FGFB)	3.9±7.0	4.0±6.4
GM-CSF (CSF-2)	21.0±14.9	21.7±12.8
HGF (F-TCF)	2930.4±827.2	2848.5±1105.6
INFA2	64.3±6.5	60.9±11.2
IL-2RA (CD25)	66.4±49.7	69.0±41.2
IL-3 (Multi-CSF)	11.1±4.1	15.2±4.8**
IL-8 (CXCL8)	36.8±25.1	36.3±30.4
IL-12p40 (CLMF2)	170.8±110.1	160.8±108.8
IL-12p70 (NKSF1)	12.0±4.8	17.7±8.4*
IL-16 (LCF)	184.4±54.6	157.1±65.2
IL-18 (IGIF)	38.0±11.1	42.9±32.0
LIF (DIA)	12.7±7.9	12.5±6.4
M-CSF (CSF-1)	62.5±17.2	58.5±19.6
MIF (MMIF)	4756.6±1778.5	4412.8±2306.8
SCF (KL)	54.3±24.8	50.3±23.2
TNFSF10 (TRAIL)	22.1±7.1	21.1±8.4
VEGF (VPF)	254.1±95.5	389.8±101.6**
Sex steroid hormones (nmol/mg protein)		
Estradiol-17 β (E2)	1.0±0.5	1.0±0.8
Progesterone (P4)	41.1±12.3	61.7±20.8*

*P<0.05; **P<0.01. The factors which were subjected to statistical analysis for comparisons are shown in bold. SD=Standard deviation; GnRH=Gonadotropin-releasing hormone; GM=Granulocyte-macrophage; IL=Interleukin; CSF=Colony-stimulating factor; VEGF=Vascular endothelial growth factor; MCP=Monocyte chemoattractant protein; MIP1b=Macrophage inflammatory protein 1-beta; SCGF=Stem cell growth factor; GROa=Growth-regulated alpha protein; MIG=Monokine induced by interferon-gamma; SDF1A=Stromal cell-derived factor 1A; FGF=Fibroblast growth factor; FGFB=Basic fibroblast growth factor; HGF=Hepatocyte growth factor; F-TCF=Tumor cytotoxic factor; CLMF2=Cytotoxic lymphocyte maturation factor 2; LCF=Lymphocyte chemoattractant factor; IGIF=Interferon gamma-inducing factor; DIA=Differentiation inhibitory activity; MIF=Macrophage migration inhibitory factor: MMIF=Macrophage migration inhibitory factor; SCF=Stem cell factor; KL=c-Kit ligand; TNFSF10=Tumor necrosis factor (ligand) superfamily, member 10; TRAIL=TNF-related apoptosis inducing ligand; VPF=Vascular permeability factor; IVF=*In vitro* fertilization

two groups; those were higher in Group 2 (GnRH-ant treated group) when compared to Group 1 (GnRH-a treated group). The follicular fluid concentrations of E2 and P4 detected by the enzyme-linked immunosorbent assay revealed comparable values for E2 while that of P4 was found to be significantly higher ($P < 0.05$) in follicles of Group 2.

Table 3 shows corrected partial correlation between any two cytokines in follicular fluid irrespective of any treatment groups. No significant association was observed between any vascular parameter and any follicular factor studied, except between PI and IL-12p70 ($P < 0.02$). Two interesting corollaries could be derived from correlated expression of markers:

1. There were negative correlation between CCL11 and GM-CSF, between CXCL1 and IL2RA and CXCL1 and progesterone
2. Two regulatory hubs, STATs and NF-kB, appear critical in positive co-regulation of at least five correlated markers in this process [Figure 1].

DISCUSSION

To the best of our knowledge, the present report documents for the first time the results of a study conducted for a large number of cytokines, chemokines and growth factors, estradiol-17β and progesterone in the follicular fluid of the dominant follicle following treatments with GnRH-agonist and antagonist protocols in ovum donation set up of IVF program. In the present study, IL-2, IL-4, IL-9, IL-13, IL-15 and IFNG could not be detected and IL-1RA, IL-6, IL-10, G-CSF and platelet-derived growth factor β could be detected in <10% of samples. Many of these factors (IL-1RA, IL-2, IL-6, IL-9, IL-10, IL-15, IFNG and platelet-derived growth factor) are reportedly not suitable markers of oocyte competence and pregnancy outcome.[14,25] Furthermore, we observed that 28 factors could be detected in the follicular fluid samples obtained from patients of both treatment groups. However, the dispersion values as compared to central values of concentrations of 18 factors detected in more than 90% of follicular samples in both groups of the present study were too high to undertake any further analysis for arriving at statistically meaningful difference in between-groups analysis. Of 10 factors that could be subjected to further statistical comparison, only three cytokines (IL-3, IL-12p70 and VEGF) in the follicular fluid showed meaningful results. Although observed changes in the levels of these three follicular fluid cytokines along with perifollicular PI and circulatory progesterone could be influenced by a host of external (e.g., stress, exercise and nutritional habits) and internal (aging, autonomic, endocrine and immunological) physiological factors,[34,35] the possible physiological significance of these observed changes in the two treatment protocols will be discussed in the following section.

Table 3: Profiles of partial correlations among concentrations of secreted factors in follicular fluid of women undergoing ovum donation treatments with either GnRH agonist (GnRH-a, Group 1) or GnRH antagonist (GnRH-ant, Group 2)

Name (alias)	Factors correlated to ($P<0.05$)
CCL2 (MCP1)	CXCL10, VEGF
CCL5 (RANTES)	CLEC11A, IL-12p70
CCL11 (Eotaxin)	GM-CSF (−)
CXCL1 (GROa)	IL-2RA (−), P4 (−)
IL-12p70 (NKSF1)	CCL5, CXCL9
HGF (F-TCF)	CCL7, IFNA2, IL12p40, TNFSF10
LIF (DIA)	CCL7, IL-2RA, IL-3, IL-16, M-CSF, TNFSF10

(−)=Negatively associated; GnRH=Gonadotropin-releasing hormone; IL=Interleukin; MCP=Monocyte chemoattractant protein; GROa=Growth-regulated alpha protein alpha; CCL=Chemokine (C-C Motif) ligand; HGF=Hepatocyte growth factor; F-TCF=Tumor cytotoxic factor; LIF=Leukemia inhibitory factor; DIA=Differentiation inhibitory activity; VEGF=Vascular endothelial growth factor; CSF=Colony-stimulating factor; GM=Granulocyte-macrophage; GMCSF=Granulocyte-macrophage colony-stimulatingfactor

ILs

Based on early reports indicating that IL-3 has no specific action on ovarian function,[36] and is not secreted by ovarian surface epithelium,[37] not much attention was given to the potential role of IL-3 in ovarian follicular development. However, there are reports indicating the presence of immunopositive IL-3 in the basal lamina of avian ovarian follicle[38] and the potential role of IL-3 in embryonic development.[39] To the best of our knowledge, this is the first report showing detectable level of IL-3 in the human

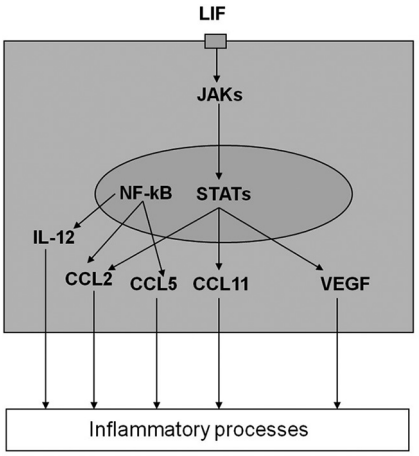

Figure 1: *Post hoc* ontological analysis of co-related markers using Thomson Reuters MetaCore portal revealed that involvement of two major regulatory hubs in STATs and nuclear factor kappa-light-chain-enhancer of activated B cells may have mediated the expression of five correlated markers (chemokine (C-C Motif) ligand 2, chemokine (C-C Motif) ligand 5, chemokine (C-C Motif) ligand 11, interleukin-12 [IL-12] and vascular endothelial growth factor [VEGF]), all which could be potentially linked to inflammatory processes. It is notable that three (CCL11, IL-12 and VEGF) of them were observed to be higher in follicular fluid from antagonist treated group

follicular fluid and its significant difference between agonist and antagonist treatment groups, the latter group showing higher concentration. Although there is no clear knowledge of any functional significance of IL-3 in follicular fluid, there is evidence that IL-3 may be involved in survival, commitment and differentiation in a variety of cell types and regulation of tissue vasculature.[40,41]

IL-12p40 and IL-12p70 are known to be present in follicles and high IL-12 concentration in follicular fluid has seen to be negatively associated with fertilizability of aspired oocytes and pregnancy outcome.[11,15,16] Further, high concentration of IL-12 in follicular fluid was seen to be associated with poor quality embryos, while high concentration of CCL5 in follicular fluid was associated with top quality embryos in ICSI program.[25] Thus, the observation from the present study indicating high concentrations of follicular IL-12p70 associated with a lower number of oocytes at metaphase II stage in the antagonist treatment group with no change in concentrations of CCL5 between agonist and antagonist treatment groups needs to be further studied to decipher the physiological link to explain the observed lesser yield in the oocyte maturation in antagonist treatment protocol.

IL-12p70 is a member of small family of heterodimeric cytokines and comprised of independently regulated disulfide-linked 40 kDa (p40) and 35 kDa (p35) subunits. The IL-12p40 either as a monomer or dimer can antagonize the action of holopeptide IL-12p70 and thus their ratios rather their respective concentrations are important in physiological regulation.[42] In the present study, we observed the ratio of IL-12p40 to p70 was 14 and 9 in agonist and antagonist treatment groups, respectively. Collectively, it indicates that a relatively higher ratio of p40 to p70 of IL-12 might provide a better protection against cytotoxic effect of IL-12p70 in the agonist treatment group yielding a higher order of oocyte maturation as observed in the present study.

Vascular endothelial growth factor

We have observed a relatively higher concentration of follicular fluid VEGF in the antagonist treatment group when compared to agonist treatment group in the present study. VEGF has been shown to be one of the prime movers of ovarian vascular physiology in a large number of systems.[43] In the present study, we observed a higher follicular VEGF levels along with the evidence of higher degree of leutinization in terms of PI and progesterone secretion, however, associated with lesser degree of oocyte maturation in antagonist treated group as compared to the agonist treatment group. The potential role of VEGF in the follicular fluid with reference to oocyte quality and pregnancy outcome is not very clear,[22,44] despite the general consensus that VEGF levels might locally influence follicular vascularity and thereby the process of luteinization.[45] Indeed, elevated level of VEGF in follicular fluid was seen to be associated with low pregnancy rate.[46,47] Further studies are indeed necessary to examine the possible physiological links involving concentrations of follicular VEGF, PI and progesterone production with the incidence of oocyte maturation.[27]

Finally, a few major points emerged from the results of the present study. Firstly, the observation in the present study that the concentration of CCL11, also known as Eotaxin-1 – which was found to be negatively correlated with GM-CSF – was higher in the follicular fluid from the antagonist treated group appears intriguing. Eotaxin-1 has been associated with eosinophilia and tissue aging[48,49] and negatively correlated with growth factor action of GM-CSF.[37,50] Further, it appeared that follicular concentration of CXCL1 showed an overall negative correlation with soluble IL2RA and progesterone and that there were higher progesterone and IL2RA along with relatively lower CXCL1 in GnRH-ant group. The observed correlations along with the relative levels of these cytokines are possibly indicative of insufficient tissue homeostasis and heightened immune activation[51,52] in the antagonist treated group. Furthermore, higher levels of CCL11, IL-12 and VEGF in follicular fluid of antagonist treated group were suggestive of higher inflammatory bias in dominant follicels [Figure 1]. Collectively, it appears from the results of the present study that follicular fluid from antagonist treated group bears higher potential to stimulate inflammatory process. Thus, GnRH-a protocol appears to be a superior ovarian stimulation protocol when compared to GnRH-ant protocol. Interestingly, Orvieto and Patrizio made similar conclusion based on analysis of available clinical data.[53] In the present study, we have essentially explored the predictor values of follicular fluid cytokines for oocyte quality from donor oocyte cycles. On the other hand, pregnancy outcome on embryo transfer, which although provides direct proof of concept, depends on multifactorial processes acting in a non-linear combinatorial manner, which involve embryonic growth and endometrial ecology of the recipients as well.[54,55] Furthermore, the concept of identifying molecular predictors of oocyte evaluation is an important one because substantial evidence suggests that oocyte quality significantly affects fertilization and subsequent embryo development and that morphological predictors are often misleading.[56] The present study provides the proof of this concept and it appears that molecular examination of follicular fluid along with morphological and cellular characteristics may eventually identify significant biomarkers in follicular fluid having more precise and objective predictor value in near future.[25,46]

ACKNOWLEDGEMENTS

The authors would like to thank all volunteers who provided their biological samples to conduct this study as a part of the *in vitro*

fertilization program of the All India Institute of Medical Sciences, New Delhi.

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How to cite this article: Malhotra N, Srivastava A, Rana H, Bahadur A, Sengupta J, Ghosh D. Comparative multiplex analysis of cytokines, chemokines and growth factors in follicular fluid of normoresponder women undergoing ovum donation with gonadotropin-releasing hormone agonist versus gonadotropin-releasing hormone antagonist protocols. J Hum Reprod Sci 2013;6:205-12.
Source of Support: All India Institute of Medical Sciences, New Delhi for the Bioplex Pro™ human cytokine standard 27-plex and 21-plex kits, **Conflict of Interest:** None declared.