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ORIGINAL ARTICLE *Early pregnancy*

Cytokine and hormonal profile in serum samples of patients undergoing controlled ovarian stimulation: interleukin-1β predicts ongoing pregnancy

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BACKGROUND: Changes in the endometrium are not regulated exclusively by ovarian hormones; the immune system has also been implicated in normal endometrial function, similar to processes taking place during inflammatory and reparative path. Many cytokines are crucially important for reproductive processes, and the role of cytokines in the female reproductive system function has been broadly investigated during controlled ovarian stimulation (COS) for IVF attempts. The aim of this study was to evaluate the levels of serum cytokines and hormones, and the clinical outcomes of women who underwent COS and ICSI procedures.

METHODS: The study prospectively included 96 patients (aged 22–43 years, unexplained or male infertility, $n = 61$; female infertility factors, $n = 35$) who underwent ICSI cycles. Serum levels of interleukin (IL-8, IL-6, IL-1β, IL-10, IL-12), tumour necrosis factor and leukaemia-inhibitory factor (LIF) and the hormones FSH, estradiol, progesterone, anti-Mullerian hormone and Inhibin-B were measured on the day of oocyte retrieval.

RESULTS: The ongoing pregnancy rate was 25.3%. The presence of serum IL-1β positively affected the implantation rate ($P = 0.004$) and increased the chance of becoming pregnant by 15 fold. Furthermore, the percentage of patients with detectable serum IL-1β levels who conceived (62.5%) was higher than those who failed to conceive (37.5%; $P = 0.019$). The LIF was undetectable in all serum samples, and no other factors influenced the clinical outcomes of patients undergoing ICSI cycles.

CONCLUSIONS: Our findings revealed that detectable serum levels of IL-1β on the day of oocyte retrieval in patients undergoing COS and ICSI are predictive of successful implantation and ongoing pregnancy.

Key words: ICSI / implantation / cytokines / interleukin-1β / oocyte retrieval

Introduction

Ovarian physiology is a complex network of regulatory mechanisms involving steroid hormones, gonadotrophins, growth factors and cytokines. This immune-endocrine interaction is triggered by the action of immune cells within the ovary and modulates ovarian function, acting through the secretion of regulatory soluble factors, especially cytokines (Adashi, 1992). The regulation of endometrial changes is not exclusively by ovarian hormones, and the immune system has been implicated in normal endometrial function, similar to processes taking place during inflammatory and reparative path. Thus, cytokines and immune cells play a major role in endometrial tissue regeneration, growth, differentiation and shedding throughout the normal menstrual cycle and in remodelling during embryonic implantation and growth (Trundley and Moffett, 2004; Salamonsen *et al.*, 2007).

Although progesterone appears to be the main factor initiating decidualization, a number of other factors, including cytokines, appear to facilitate this event. This inflammatory process is

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characterized by edema and angiogenesis of the endometrial tissue, leukocyte recruitment and the enhanced synthesis of vasoactive compounds, such as prostaglandins and cytokines, that are involved in the communication between mother and embryo (Chabbert-Buffet and Bouchard, 2002; Castro-Rendon *et al*., 2006).

It is well recognized that many cytokines are crucially important for reproductive processes, such as follicular development, ovulation, fertilization, implantation and embryonic development. The role of cytokines in the female reproductive system function has been broadly investigated during controlled ovarian stimulation (COS) for IVF attempts, and it is clear that under COS, the functions of the female reproductive system differ from those of natural cycles.

The aim of this study was to evaluate serum cytokine and hormone levels and the clinical outcomes of women who submitted to COS and ICSI procedures. For this study, pituitary blockage protocols and infertility etiologies were taken into account. The investigated cytokines included interleukin (IL)-8, IL-6, IL-1b, IL-10, IL-12, tumour necrosis factor (TNF) and leukaemia-inhibitory factor (LIF) and the hormones FSH, oestradiol (E_2), progesterone anti-Mullerian hormone (AMH) and inhibin-B.

Materials and Methods

Patients

The study prospectively included 96 patients undergoing ICSI cycles in the Fertility - Assisted Fertilization Center, Brazil between January 2007 and December 2008. The patients age ranged from 22 to 43 years old (median $\pm$ SEM: 33.85 $\pm$ 0.44). All patients presented the following inclusion criteria: both ovaries present, regular menstrual cycle, BMI lower than 35 kg/m^2, no ongoing infectious disease, no uterus pathology, basal FSH $<$14 IU/ml, basal E_2 $<$70 pg/ml. All ejaculated semen used for ICSI presented motile sperm concentrations above 5×10^6 sperm/ml. Infertility was defined as unexplained infertility (32.3%), male infertility (31.2%), male- and female-associated factors (11.5%), endometriosis (12.5%), ovarian factors (7.3%) and tubal obstructions (5.2%). The serum samples were collected and sent to the Molecular Gynecology Laboratory in the Gynecology Department of the Federal University of São Paulo, Brazil, for laboratory analysis. This study was approved by the Ethics Committee of the Federal University of São Paulo, and an Informed Consent form was obtained from all participants.

COS and sample collection

The pituitary blockage was achieved with a GnRH agonist (Lupron kit™, Abbot SA Societé française des laboratories, Paris, France) (63.5% of patients) or GnRH antagonist (Cetrotide®, Serono, Geneva, Switzerland) (36.5% of patients), and the ovarian stimulation was performed using recombinant FSH (Gonal-F®, Serono, Geneva, Switzerland). When at least two follicles reached a diameter of 16 mm each, final follicular maturation was triggered with a 250 μg of recombinant hCG (Ovidrel®, Serono, Geneva, Switzerland). Oocyte retrieval was performed 35–36 h later using the vaginal route with ultrasound-guidance, and the luteal phase was supported by daily vaginal administration of 90 mg progesterone (Crinone®, Serono, Geneva, Switzerland). A peripheral blood sample was obtained from each patient on the day of oocyte retrieval. The sera were separated and stored at -80°C for further analysis.

Sample analysis

Serum samples were assessed for the cytokines IL-8, IL-6, IL-1β, IL-10, IL-12p70, TNF and LIF using a flow cytometry technique ('IL-8, IL-6, IL-1β, IL-10, IL-12p70 and TNF—Human inflammation kit' and 'Human LIF flex set', BDTM Cytometric bead array, CBA, BD Biosciences, USA). CBA kits are validated against free proteins using samples, such as stimulated PBMCs, cell lines or serum/plasma, spiked with recombinant standards. Events acquisition and analyses were performed by CellQuest and FCAP Array software (BD Bioscience), respectively, in a fluorescence-activated cell sorter Calibur 4-colour flow cytometer (BD Bioscience).The lower detection limit was 2.0 pg/ml for IL-8, IL-6, IL-1β, IL-10, IL-12p70 and TNF and 5.5 pg/ml for LIF. Owing to the high number of patients presenting undetectable levels of IL-1β, IL-10, IL-12p70 and TNF, these variables were categorized and evaluated only as present or absent.

Serum FSH, E_2 and progesterone levels were determined using an automated multi-analysis system with a chemi-luminescence technique (Advia Centaur, Siemens). The lower detection limits were 0.3 mIU/ml for FSH, 7.0 pg/ml for E_2 and 0.15 pg/ml for progesterone. An enzyme-linked immunosorbent assay (ELISA) was used to determine AMH (ELISA kit, Diagnosis System Laboratories, Inc., USA) and inhibin-B levels (Inhibin B ELISA Kit, Diagnosis System Laboratories, Inc., USA): the lower detection limits were 0.006 ng/ml and 7 pg/ml, respectively.

Statistical analysis

Data analyses were performed using Minitab 14 for Windows (Minitab, USA). Patient demographic data were evaluated by descriptive statistics, which included information on means, frequencies and cross-tabulations. Continuous variables were given as means $\pm$ SEM and compared using a Student's *t*-test, the nominal variables were tested by chi-squared or Fisher's exact tests as appropriate and a Pearson's correlation analysis was used to evaluate the relationship between variables.

The implantation rate was defined as the number of gestational sacs per number of embryos transferred per patient. To evaluate the association between independent variables (serum cytokines and hormones) and the implantation rate, we used a linear regression; the results were given as the coefficient, standard error of coefficient and *P*-value. Clinical pregnancy was defined as the presence of a gestational sac with the heart beat visualized by ultrasound 4–6 weeks after embryo transfer. For the ongoing pregnancy rate (number of patients with confirmed clinical pregnancy per number of patients with embryos transferred) analysis, we used the binomial logistic regression; the results were given as the odds ratio, 95% confidence interval and *P*-value.

To evaluate the influence of factors measured in this study on ICSI outcomes according to patients' reproductive conditions, the patients were sorted on the basis of their infertility diagnosis as unexplained or male infertility ($n = 61$, 63.5%) and female infertility factors ($n = 35$, 36.5%), and outcomes were reanalysed for each group. Data were analysed using a multiadjusted approach that was adjusted for patient age, and *P*-values $\leq$0.05 were considered statistically significant.

Results

Demographic and clinical data of the studied patients, listed according to the pituitary blockage protocol and reproductive conditions, are presented in Table I. Although significant differences were noted in the number of follicles and retrieved oocytes according to patients' reproductive conditions, the clinical outcomes did not differ with respect to the reproductive condition.

Hormone and cytokine concentrations in sera

LIF was undetectable in all serum samples. IL-1β, IL-10, IL-12 and TNF were detected in a small number of serum samples; therefore, they were

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Table I Demographic and clinical data of patients undergoing controlled ovarian stimulation (COS) and ICSI.

	GnRH agonist group	GnRH antagonist group	*P*-value	Male or unexplained infertility	Female infertility factors	*P*-value
Number of patients	61	35		61	35	
Age (years)	33.7 ± 0.6	34.1 ± 0.7	0.660	33.2 ± 0.6	34.9 ± 0.7	0.071
BMI (kg/m^2)	23.2 ± 0.5	23.5 ± 0.5	0.640	23.0 ± 0.4	23.9 ± 0.6	0.169
Total FSH (IU) administered	2414.0 ± 86.0	2347.0 ± 130.0	0.659	2342.0 ± 88.0	2469.0 ± 125.0	0.398
Number of follicles	19.6 ± 1.4	17.5 ± 1.5	0.340	21.1 ± 1.3	14.8 ± 1.5	0.004
Number of oocytes recovered	14.5 ± 1.1	11.5 ± 1.0	0.077	15.7 ± 1.0	9.3 ± 1.1	<0.001
Metaphase II oocyte rate	70.8%	78.3%	0.082	71.5%	77.1%	0.194
Fertilization rate	72.7%	70.0%	0.555	70.5%	73.8%	0.492
Rate of high-quality embryo transferred	73.1%	84.7%	0.119	80.2%	73.3%	0.362
Implantation rate	19.5%	19.0%	0.949	19.3%	19.3%	0.993
Biochemical pregnancy rate	32.1%	34.3%	0.833	33.9%	31.4%	0.805
Ongoing pregnancy rate	25.0%	25.7%	0.939	26.8%	22.8%	0.673

Values are for patients receiving GnRH agonist and GnRH antagonist protocols, according to patients' reproductive condition.

interpreted as categorical variables (presence or absence). IL-1β was detected in eight samples (8.3%), IL-10 was detected in nine samples (9.4%), IL-12 was detected in five samples (5.2%) and TNF was detected in five (5.2%) serum samples. Circulating IL-8 and IL-6 levels were detected in most serum samples. Circulating hormone levels were detected in all samples, with the following values (mean ± SEM): IL-8, 19.0 ± 4.7 pg/ml; IL-6, 1.9 ± 0.2 pg/ml; FSH, 6.8 ± 0.3 mIU/ml; E_2, 1499.0 ± 102.0 pg/ml; progesterone, 22.9.0 ± 1.0 ng/ml; AMH, 1.2 ± 0.1 pg/ml and Inhibin-B, 330.5 ± 43.4 ng/ml.

The mean levels of serum factors were compared according to the pituitary blockage protocol and patients' reproductive conditions. No differences (except for E_2 levels) were found between patients receiving the GnRH agonist protocol and those receiving the GnRH antagonist protocol. On the other hand, the levels of E_2, progesterone and AMH were lower in patients presenting female infertility factors (Table II).

Relationship between serum factors and clinical outcomes

The implantation rate (19.3%) was positively affected by the presence of serum IL-1β and negatively affected by the presence of serum IL-10 (Table III). For patients with male or unexplained infertility ($n = 61$), a similar pattern was observed. The implantation rate was positively affected by the presence of serum IL-1β ($P = 0.003$) and negatively affected by serum IL-10 ($P = 0.039$). When only patients presenting female infertility factors were investigated ($n = 35$), serum IL-1β was detected in just one patient, IL-10 was not present in any serum sample, and no other factor present in the serum was found to influence the rate of implantation. There was no association between serum hormone levels and the implantation rate of patients undergoing ICSI cycles.

The ongoing pregnancy rate was 25.3%. The presence of serum IL-1β increased the chances of ongoing pregnancy, and there was a tendency for the presence of serum IL-10 to decrease the chance of ongoing pregnancy. For patients with male or unexplained infertility ($n = 61$), the presence of serum IL-1β also increased the chance of ongoing pregnancy by almost 17-fold ($P = 0.03$), and there was a tendency of the presence of serum IL-10 to decrease the chance of pregnancy ($P = 0.076$) (Table IV).

On the other hand, when only patients presenting female infertility factors were investigated ($n = 35$), as said before, IL-1β and IL-10 were not detected in enough patients in this group to allow statistical analysis. There was no association between other serum cytokines studied or serum hormone levels and the ongoing pregnancy rate of patients undergoing ICSI cycles.

To further support these findings, the percentage of patients presenting detectable serum IL-1β levels who conceived (62.5%) was higher than those who failed to conceive (37.5%; $P = 0.019$) in the entire group of patients

Discussion

We analysed the relationship between the clinical outcomes of ICSI cycles and the concentrations of cytokines and hormones in sera that were measured on the day of oocyte collection: this approach evaluated whether there were systemic markers of ongoing pregnancy success following ICSI cycles. We also analysed the results according to the patients' reproductive conditions, which were sorted, on the basis of their infertility diagnosis, as unexplained or male infertility, and female infertility factors. This sorting made it possible to exclude female infertility factors, which interfered with hormone production and possibly cytokine production.

Since cytokines have mostly paracrine or autocrine modes of action, many studies have investigated the role of intra-follicular cytokines on IVF outcomes, believing that intra-follicular cytokine concentrations represent their function in the ovary better than their blood

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Table II Serum cytokine and hormone measurements on day of oocyte retrieval in women undergoing COS.

	GnRH agonist $n = 61$ (mean ± SEM)	GnRH antagonist $n = 35$ (mean ± SEM)	*P*-value	Male or unexplained infertility $n = 61$	Female infertility factors $n = 35$	*P*-value
IL-8 (pg/ml)	21.4 ± 7.1	14.8 ± 3.6	0.507	22.4 ± 7.3	13.0 ± 2.1	0.338
IL-6 (pg/ml)	2.1 ± 0.4	1.7 ± 0.2	0.447	2.1 ± 0.4	1.6 ± 0.3	0.375
FSH (mUI/ml)	6.9 ± 0.4	6.7 ± 0.6	0.767	6.4 ± 0.4	7.5 ± 0.5	0.087
Estradiol (pg/ml)	1692.0 ± 134.0	1207.0 ± 147.0	0.019	1714.0 ± 136.0	1157.0 ± 135.0	0.007
Progesterone (ng/ml)	22.1 ± 1.3	24.2 ± 1.6	0.329	24.7 ± 1.4	20.0 ± 1.4	0.026
Inhibin-B (ng/ml)	382.0 ± 66.0	241.0 ± 27.0	0.117	392.0 ± 64.0	224.0 ± 34.0	0.061
AMH (pg/ml)	1.3 ± 0.2	0.9 ± 0.1	0.193	1.4 ± 0.2	0.7 ± 0.1	0.008

Values are for patients receiving GnRH agonist and GnRH antagonist protocols, according to patients' reproductive condition.
IL, interleukin; AMH, anti-Mullerian hormone.

Table III The influence of serum cytokines on the implantation rate of patients undergoing COS and ICSI.

	Coefficient	SE Coefficient	*P*-value[a]
Total of patients ($n = 96$)			
Presence of serum IL-1β	35.7	11.9	0.004
Presence of serum IL-10	−23.0	11.2	0.044
Patients presenting male or unexplained infertility			
Presence of serum IL-1β	38.9	12.3	0.003
Presence of serum IL-10	−23.4	11.1	0.039

[a]Linear multiple regression.

Table IV The influence of serum cytokines on the ongoing pregnancy rate of patients undergoing COS and ICSI.

	Odds ratio	CI 95%	*P*-value[a]
Total of patients ($n = 96$)			
Presence of serum IL-1β	15.2	1.6–140.5	0.016
Presence of serum IL-10	0.09	0.01–1.52	0.094
Patients presenting male or unexplained infertility ($n = 61$)			
Presence of serum IL-1β	16.8	1.3–215.2	0.030
Presence of serum IL-10	0.08	0.0–1.36	0.076

[a]Binomial multiple logistic regression. CI, confidence interval.

concentrations (Karagouni *et al.*, 1998; Buscher *et al.*, 1999; Mendoza *et al.*, 1999, 2002; Hammadeh *et al.*, 2005; Asimakopoulos *et al.*, 2006, 2008; Nikolettos *et al.*, 2008). Few studies have evaluated blood cytokine levels and IVF outcomes (Simon *et al.*, 1996; Karagouni *et al.*, 1998; Nikolettos *et al.*, 2004, 2008; Hammadeh *et al.*, 2005; Asimakopoulos *et al.*, 2006).

The approach used in this study allowed us to evaluate the influence of cytokines from a systemic point of view, because our main outcome, ongoing pregnancy, has systemic consequences. In addition, the ability to obtain a serum sample more easily than a follicular fluid sample makes serum analysis a better option to evaluate predictive factors of IVF cycle success.

The serum concentrations of IL-1β, IL-10, IL-12 and TNF were below the assay detection limit in many samples, and to avoid bias, those variables were categorized before statistical analysis in our study. Another possible study bias could be the use of both a GnRH agonist and GnRH antagonist for pituitary blockage protocols. However, we compared the levels of evaluated serum factors, and only the levels of E_2 were slightly higher in patients who received the GnRH agonist protocol rather than the GnRH antagonist protocol. The difference in E_2 levels between the pituitary blockage protocol groups was not clinically significant, so we considered all patients equally.

We observed that the presence of circulating IL-1β could be an important predictor of ongoing pregnancy in patients undergoing ICSI cycles. This finding was mostly related to the differences observed in the group of patients presenting male or unexplained infertility. Interestingly, IL-1β was barely detectable in women presenting female infertility factors, which can be explained by infertility factors that could affect ovarian and endometrium responses to the treatment. This finding should be confirmed with a larger number of patients presenting different female infertility factors.

Karagouni *et al.* observed that ~50.0% of patients undergoing IVF cycles had detectable serum levels of IL-1β (Karagouni *et al.*, 1998), whereas we found detectable serum IL-1β levels in only 8.3% of our overall patient group and 11.5% of patients without female infertility factors. Those differences could be attributed to the techniques used for cytokine measurements or to different profiles of studied patients. Despite the small percentage of patients with detectable IL-1β levels, we observed an important association between the presence of serum IL-1β and clinical outcomes of IVF patients, a finding

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that was also reported by Karagouni *et al.*, who demonstrated that the implantation rate of patients undergoing IVF-embryo transfer who had detectable serum concentrations of IL-1α and IL-1β on the day of hCG administration was higher than the rate of IVF-embryo transfer patients who had no detectable concentrations of these cytokines (Karagouni *et al.*, 1998).

These results are consistent with those obtained in endometrial secretions aspirated immediately prior to embryo transfer from 210 women undergoing IVF, where IL-1β was predictive of pregnancy in IVF cycles (Boomsma *et al.*, 2009). Some studies claim that the IL-1 system in the endometrium is regulated by steroid hormones (Simon *et al.*, 1996), however, there was no obvious relationship between IL-1 and E_2 or progesterone levels in the plasma of patients undergoing IVF (Wang and Norman, 1992; Simon *et al.*, 1996), and we also showed no correlation between serum IL-1β and the steroid hormones, or AMH and inhibin-B (data not shown). We found no correlation between the serum levels of hormones measured on the day of oocyte retrieval and clinical outcomes of ICSI cycles.

The role of IL-1 in implantation has been well documented. IL-1 was shown to increase endometrial secretions of prostaglandin and LIF, increase the expression of integrins and stimulate matrix metalloproteinase-9 activity in trophoblasts. Furthermore, animal studies demonstrated that the blockade of the IL-1 receptor type I inhibited implantation in the mouse, which further emphasizes the important role of IL-1 in implantation (Castro-Rendon *et al.*, 2006).

LIF is expressed throughout the menstrual cycle, with a striking increase in LIF mRNA levels in the middle and late secretory phases, which includes the embryo implantation period (Arici *et al.*, 1995). It was also demonstrated that IL-1β induces the LIF production by human endometrial stromal cells and decidual cells *in vitro*, indicating a synergistic effect of IL-1 and LIF on the control of blastocyst implantation (Sawai *et al.*, 1997). Overall, however, the LIF was undetectable in serum samples in our study. Since the LIF has mostly a paracrine or autocrine mode of action in the endometrium (Dimitriadis *et al.*, 2010), we believe that its levels were too low to be detected systemically.

We also showed a tendency of the presence of IL-10 to be negatively related to ongoing pregnancy. However, IL-10 is known to selectively suppress the Th1-mediated cellular immunity by inhibiting the production of inflammatory cytokines, such as interferon-γ, TNF-a and IL-1 (Mosmann and Moore, 1991). The role of IL-10 during pregnancy, as a suppressor of active maternal immunity to allow acceptance of the foetal allograft, has been a topic of study and, in the context of pregnancy, IL-10 levels increase markedly in women during early pregnancy (Thaxton and Sharma, 2010). These results do not agree with the findings of our study and should be confirmed with additional studies.

Although we have discussed mainly the potential effects of cytokines on the endometrium, it is possible that cytokines may be markers of oocyte quality, and the use of an oocyte donation model in future studies would be useful to separate these possible actions. Furthermore, the precise mechanisms involved in the positive effect of detectable serum IL-1 beta, and negative effect of serum IL-10, on implantation and pregnancy are unknown and further studies, perhaps in animal models, will be required.

In conclusion, our findings reveal that detectable serum levels of IL-1β on the day of oocyte retrieval in patients undergoing COS and ICSI are an important predictive factor of implantation and ongoing pregnancy. These results were confirmed for patients presenting male or unexplained infertility, and further studies drawing on a higher number of patients who present different female infertility factors are required to validate the positive association between IL-1β and the success of IVF cycles in those situations.

Authors' roles

The substantial contributions to conception and design, acquisition of data, analysis and interpretation of data, drafting the article and final approval of the version to be published by T C S B., R.S. and M.B. R.S. and M.B. are responsible for revising the article critically for important intellectual content; while D.P.A.F.B., substantially contributed to the acquisition of data, and revising the article critically for important intellectual content; while E.B.Jr., substantially contributed to the acquisition of data, and revising the article critically for important intellectual content and I.D.C.G.S. also substantially contributed to conception and design, and the final approval of the version to be published.

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