Cytokines in Older Patients Undergoing In Vitro Fertilization: The Relationship to the Response to Controlled Ovarian Hyperstimulation

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Submitted: September 15, 1998
Accepted: November 9, 1998

Purpose: *Our purpose was to assess the endocrine, autocrine, and paracrine milieu in follicles of older women undergoing stimulated cycles, comparing normal (NR) and low (LR) responses, based on the measurement of interleukin (IL)-1β, IL-6, and vascular endothelial growth factor (VEGF) in serum and follicular fluid (FF).*
Methods: *A total of 40 women entered the study, divided into three groups: (1) older patients (>37 years) with NR (age-NR; n = 18); (2) older women with LR (age-LR; n = 11); and (3) normal controls, aged <35 years (control; n = 11). IL-1β, IL-6, and VEGF measured in serum (day of ovum pickup) and FF, employing ELISAs.*
Results: *Follicular fluid IL-6 was significantly ($P < 0.05$) higher in age-LR compared to the other two groups. IL-6 and VEGF showed a 4- to 20-fold increase in FF compared to blood, suggesting the ovary as an additional source of both cytokines. IL-1β levels remained unchanged in FF compared to blood and, also, among groups.*
Conclusions: *These data provide further evidence that the endocrine, paracrine, and/or autocrine status in vivo of older patients is different from that of younger women and suggest that cytokines, specifically IL-6, may be involved in the changes observed during senescence within the ovary.*

KEY WORDS: age; low responders; follicular fluid; serum; interleukin-1β; interleukin-6; vascular endothelial growth factor.

INTRODUCTION

One of the most difficult challenges in clinical reproductive endocrinology is the management of women who have reached the limit of their reproductive age, since we know from epidemiological studies that there is an age-related decline in reproductive performance (1). Whether the uterus, ovary, and/or hypothalamus/pituitary glands are responsible for this impairment of fertility is a matter of intensive research. The ovary may be the major site of action of senescence within the reproductive axis, and three basic aspects should be considered when this issue is analyzed: the decrease in the population of stimulatable follicles with age, the health of the follicles that can be recruited with drugs, and the quality of the enclosed oocytes that are to be fertilized.

We have long been interested in the second point and undertook a series of studies in vitro so as to determine the effects of age on granulosa-cell functions (2). We showed a decline in immunoreactive α-inhibin production by human granulosa–luteal cells in vitro in older patients, which appeared to be the consequence of a reduction in the maximum ability of the granulosa cell to produce the peptide. We also found a reduction in the steroidogenic ability of cells from women >40 years old. This decrease was evidenced by measuring progesterone accumulation in vitro under basal and human chorionic gonadotropin (hCG)-stimulated conditions. These studies have been extended recently by the development of specific assays to analyse dimeric inhibin A and B (3,4).

In addition to the importance of inhibin in ovarian folliculogenesis, other molecules acting at a paracrine/autocrine level may also be relevant. One of the key features in the regulation of the ovary is angiogenesis, which may be a clue for follicular selection and further growth. In fact, using transvaginal pulsed color Doppler, we showed reduced blood flow during natural cycles around the dominant follicle in low responders

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1058-0468/99/0500-0247$16.00/0

(LR) to ovarian stimulation compared to controls (5). A low response is characteristic of older patients.

In the human ovary, a gonadotropin-dependent preovulatory induction of interleukin (IL)-1β transcripts in the theca–interstitial cell layer has been described (6). The mediation of IL-1β as a modulator of vascular permeability throughout the production of vasoactive substances, such as vascular endothelial growth factor (VEGF) and IL-6, which are released in response to IL-1β (7–9), should be considered. Hence, IL-6 has also been shown to induce VEGF expression in several cell lines (10). In keeping with this concept, Friedman *et al.* (11) have recently shown increased VEGF follicular fluid (FF) concentrations in older women compared to controls, consistent with a hypoxic environment due to a hypothetically deficient microcirculation.

Based on the above information, we have designed a prospective study to explore further the autocrine/paracrine environment of the follicle in stimulated cycles of older patients, including a comparison of women who were normal responders (NR) and LR. To this end, patients have been accordingly classified, and the serum and FF levels of IL-1β, IL-6, and VEGF compared.

MATERIALS AND METHODS

Patients and Ovarian Stimulation Protocols

This study was planned to analyze further the endocrine, paracrine, and autocrine status in vivo of older patients undergoing ovarian stimulation. We also wanted to learn whether or not this environment changes when an older patient also becomes a LR. Although there is no universally agreed definition of LR, it is generally accepted that women who produce fewer than four oocytes or have serum peak estradiol (E_2) levels <500 pg/ml after aggressive stimulation fit into this group of patients. It is also important for the definition of LR to have, at least, two previous unsuccessful attempts of ovarian stimulation. Thus, the study was designed considering three groups: (a) older patients (>37 years) with a NR (age-NR; n = 18); (b) older women with a LR (age-LR; n = 11); and (c) normal controls, aged <35 years (control; n = 11).

The study group included a total of 40 women with primary infertility undergoing in vitro fertilization (IVF). Their partners had normal semen analyses. The protocol for ovarian stimulation was started by pituitary desensitization with daily subcutaneous administration of 1 mg leuprolide acetate (Procrin; Abbott S.A., Madrid, Spain) and began in the luteal phase of the menstrual cycle. Serum E_2 levels <60 pg/ml (220 p*M*) and negative vaginal ultrasonographic scans were used to define ovarian quiescence. On days 1 and 2 of ovarian stimulation, 2 ampoules/day of human menopausal gonadotropin (hMG; Pergonal; Serono Laboratories, Madrid, Spain) were administered together with 2 ampoules of high-purity follicle stimulating hormone (FSH; Neo-Fertinorm; Serono). On days 3, 4, and 5 of ovarian stimulation, 1 ampoule/day hMG plus 1 ampoule/day FSH were administered to each patient. Beginning on day 6, hMG and FSH were administered on an individual basis according to serum E_2 and transvaginal ovarian ultrasound scans. The standard dose of menotropins was altered in women with advanced age, according to our previous experience. In these patients, ovarian stimulation systematically began with a higher dose of FSH/hMG, which was established on an individual basis according to what these particular patients showed in previous stimulation cycles. Thus, there was a trend toward higher doses of FSH/hMG in older than younger patients. The criteria for human chorionic gonadotropin (hCG) administration (10,000 IU; Profasi; Serono) were the presence of two or more follicles >1.9 cm in greatest diameter and serum E_2 levels >800 pg/ml (2940 p*M*). Leuprolide acetate, FSH, and hMG injections were discontinued on the day of hCG administration. Oocyte retrieval was scheduled 36–38 hr after hCG injection. The luteal phase was supported with vaginal administration of 400 mg/day micronized progesterone (Progeffik; Laboratorios Effik; Madrid, Spain).

Follicular Fluid Measurements

All visible follicles in women included in the present study were harvested by ultrasound-guided vaginal aspiration. During the process of oocyte collection, each follicle was aspirated separately and the FF collected into a sterile plastic tube by electronic suction. The FF volume was recorded and the oocyte isolated from the aspirate. When the oocyte was not initially visualized, the follicle was flushed with Ham's F-10 medium until recovery. Only follicles in which an oocyte was clearly identified were used for the purposes of the study. After isolation of the oocyte, FF aspirates were pooled and immediately centrifuged (1500*g*), and the supernatant was aliquoted and stored at −80°C until required for analysis. FF that, in the

supernatant, contained red blood cells was excluded. At the time of oocyte recovery, a blood sample was taken. The serum was isolated and properly stored as was the FF.

The isolated oocytes were placed in 20 μl IVF-medium droplets (Medicult, Copenhagen, Denmark) under mineral oil, inseminated, and cultured for 20 to 24 hr. Thereafter, the cytoplasm was checked under the inverted microscope for the presence of normal fertilization and the zygotes further incubated in new medium for an additional 24 hr. On the day of transfer (approximately 48 hr after retrieval), the number of blastomeres of each embryo was recorded.

Hormonal Measurements

IL-1β, VEGF, and IL-6 were analyzed using commercially available ELISA kits (Quantikine; R&D System Inc., Minneapolis, MN). Intra- and inter-assay coefficients of variation were, respectively, 4.1% and 8.5% for IL-1β, 2.7% and 4.5% for IL-6, and 5.4% and 7.3% for VEGF.

Statistical Analysis

Data are expressed as mean ± standard error of the mean (SE). Analysis of variance (ANOVA) was employed to compare among groups. Bonferroni's and Scheffé's tests were applied when ANOVA showed statistical differences. Chi-square test was also employed. Significance was defined as $P < 0.05$. The statistical analysis was carried out using the Statistical Package for Social Sciences (SPSS Inc., Chicago, IL).

RESULTS

Table I shows some of the epidemiological and in vitro fertilization parameters of the patients included in the study. There was an obvious significant ($P < 0.05$) difference in age among groups as a result of the study design. Similarly, the number of eggs retrieved was significantly ($P < 0.05$) lower in group age-LR. Table I also shows a significant ($P < 0.05$) increase in the amount of menotrophins administered to aged women in both groups as compared to controls. In addition, the data show significantly ($P < 0.05$) better fertilization rates in the controls as compared to the age-NR patients and a significantly ($P < 0.05$) increased number of embryos transferred in the control group compared to the other two groups of aged patients. The results of embryo transfer are also shown, no miscarriage being recorded in this series.

Table II shows cytokine production. IL-1β levels in serum and FF were in the same range, and no difference among the established groups was observed. IL-6 levels had a 4- to 20-fold increase in FF compared to serum. This was also true for VEGF concentrations. IL-6 serum levels were similar among groups. In FF, however, the IL-6 concentration was significantly ($P < 0.05$) higher in the age-LR group compared to the other two groups of patients. VEGF levels did not show differences among groups in serum and FF. However, there was a clear trend toward increased serum VEGF concentration in both groups of aged women compared to controls.

DISCUSSION

The results of the present study have shown that LR of advanced reproductive age have elevated concentrations of IL-6 in FF compared to NR and younger patients and that age increases systemic levels of VEGF, whereas IL-1β levels remain basically unchanged among groups in both biological fluids tested.

IL-6 is an excellent angiogenic factor, and IL-6 mRNA is expressed during ovarian neovascularization (12). Detectable levels of IL-6 have been found in human FF (13,14). In fact, positive immunostaining for this cytokine has been shown in the thecal compartment of antral follicles and corpora lutea (14). Thus, the data presented herein may be a sign of different vascularization in the clinical situations tested. In keeping with this concept, Gaulden (15) proposed that, with aging, a deficient microvasculature developed around the dominant follicle. The resulting hypoxia predisposed to the increased incidence of aneuploid oocytes. Similarly, Van Blerkom *et al.* (16) have recently shown that oocytes from severely hypoxic follicles were associated with high frequencies of abnormalities in the organization of the chromosomes on the metaphase spindle that could lead to segregation disorders and catastrophic mosaicisms in the early embryo.

It is, therefore, assumed that women with LR have a certain degree of hypoxia. Our studies using pulsed color Doppler ultrasound also suggest this hypothesis. Indeed, when we studied blood flow around the dominant follicle in natural cycles, we found increased vascular resistance to flow in LR compared to NR (5). As a consequence, the quality of the oocytes and resulting embryos may be impaired. In fact, in this short series

Table I. Several IVF Parameters of the Patients Included in the Present Study

Variable	Patient group		
	Age-NR (n = 18)	Age-LR (n = 11)	Control (n = 11)
Age (yr)	40.8 ± 0.4	39.8 ± 1.1	32.5 ± 0.6*
Infertility (yr)	3.3 ± 0.5	6.4 ± 1.4	4.1 ± 0.9
Basal FSH (mIU/ml)	6.4 ± 1.1	10.5 ± 3.3	4.7 ± 0.3
Basal E_2 (pg/ml)	65.7 ± 19.8	128.9 ± 98.6	67.0 ± 12.6
Days of stimulation	10.2 ± 0.4	9.4 ± 0.5	10.0 ± 0.6
FSH/hMG ampoules (No.)	50.2 ± 4.6	51.8 ± 3.9	30.8 ± 3.7*
E_2, day of hCG (pg/ml)	1907 ± 282	856 ± 159	1381 ± 72
Oocytes	9.0 ± 0.9	2.7 ± 0.3*	11.2 ± 1.2
Fertilization(%)	44.0 ± 7.3**	57.0 ± 11.0	78.6 ± 4.6**
Blastomeres (No.)	2.7 ± 0.3	2.3 ± 0.3**	3.4 ± 0.3**
Transferred embryos	2.2 ± 0.4	1.4 ± 0.3	3.7 ± 0.4*
Pregnancies (%)	5 (27.8)	1 (9.0)	4 (36.3)
Implantation (%)	6/42 (14.3)	1/18 (5.5)	8/41 (19.5)

* Significantly ($P < 0.05$) different from the remaining groups by Bonferroni and Scheffé tests.
** Significantly ($P < 0.05$) different between groups by Bonferroni and Scheffé tests.

we observed reduced fertilization in older patients compared to controls. Implantation was also affected, but the numbers do not allow valid conclusions. The literature is, however, full of evidence pointing in this direction. Studies performed in unfertilized human oocytes showed a significant increase in chromosome abnormalities in women >35 years old (17). Similarly, Battaglia *et al.* (18) studied the influence of maternal age on the meiotic apparatus. Using high-resolution confocal microscopy, they observed that the meiotic spindle in older women is frequently abnormal, showing up to 79% aneuploid conditions, compared to 17% in younger patients. In addition, recent studies employing fluorescence in situ hybridization in human preimplantation embryos have shown that aneuploidy is more frequent in women >40 years old than in younger patients (19), suggesting that the quality of the oocyte and the resulting embryo in women >40 years old may be one of the mechanisms involved in the decline of fecundity with age. Similarly, Janny and Menezo (20) performed embryo cultures up to the blastocyst stage and showed that embryos derived from older women arrest at the morula stage at a higher degree than those coming from younger patients, again suggesting that the quality of the oocyte plays a crucial role in embryo development. In fact, these authors also showed a negative linear correlation between blastocyst expansion and patient's age (20).

Table II. Serum and FF Concentrations of Cytokines IL-1β, IL-6, and VEGF in the Three Groups Established

Variable (pg/ml)	Patient group		
	Age-NR (n = 18)	Age-LR (n = 11)	Control (n = 11)
Serum			
IL-1β	1.0 ± 0.3	1.6 ± 0.4	1.4 ± 0.3
IL-6	3.8 ± 0.6	3.0 ± 1.1	2.0 ± 1.1
VEGF	903 ± 515	1161 ± 618	238 ± 54
FF			
IL-1β	2.8 ± 0.4	1.9 ± 0.5	1.5 ± 0.3
IL-6	16.6 ± 3.4	60.4 ± 18.9*	14.9 ± 4.6
VEGF	3457 ± 658	4495 ± 1546	3762 ± 752

* Significantly ($P < 0.05$) different from the remaining groups by Bonferroni and Scheffé tests.

It is more difficult to explain how a deficient vasculature results in increased ovarian levels of an angiogenic factor. Moreover, situations of known enhanced angiogenesis, such as the ovarian hyperstimulation syndrome, are associated with increased serum and PF (peritoneal fluid) IL-6 levels (14,21,22). One plausible explanation for this discrepancy has been reported recently by Friedman *et al.* (11), who studied VEGF levels in the same population of women as in our study. Contrary to our findings, they found elevated VEGF in FF of LR of advanced reproductive age. Interestingly, they performed in vitro studies with granulosa cells, inducing hypoxia by modification of the concentration of O_2 in the culture atmosphere, and showed that hypoxia stimulates the release of VEGF to the culture medium, suggesting that this is the mechanism by which angiogenic factors are elevated in LR. Thus, IL-6 concentrations may also be regulated by this fine phenomenon.

An alternative explanation for the increased production of IL-6 in aged LR may be the existence of different populations of cells in these patients. We know (Garrido and Pellicer, unpublished) that the removal of macrophages from granulosa-cell pellets impairs

IL-6 secretion in vitro, suggesting that white blood cells are the major source of IL-6 in the ovary. Thus, the increased IL-6 accumulation in vitro may be an indirect sign of increased presence of white blood cells in the ovaries of aged-LR compared to the other two groups tested. In fact, monocytes and endothelial cells release IL-6 (7,8,12).

The difference between our results on VEGF blood levels, especially in FF, and the observations of Friedman *et al.* (11) should also be discussed. We have learned that there is a large range of values concerning cytokine measurements among patients. Looking carefully at Friedman and co-workers' report, they also had a strong variability in their concentrations of VEGF in FF, which, together with the statistical methods employed, may explain the contradictory results. In any case, it is worth noting that, like Friedman *et al.* (11), the present report confirms elevated FF levels of an angiogenic substance in LR of advanced reproductive age and differences between younger and older women.

IL-1β was also tested in our study. The hypothesis was to evaluate IL-1β as a modulator of VEGF and IL-6 release (7–9). Our data did not show differences in IL-1β concentrations in serum or FF between older (NR or LR) patients and controls. The results, however, have to be viewed with caution, because the entire IL-1 system was not tested. In other words, we did not analyze the concentrations of the receptor antagonist (IL-ra) in any of the biological fluids tested. Therefore, a role for IL-1β in the systemic and local (ovarian) changes observed in older patients cannot be totally ruled out until further studies analyze the entire system.

In summary, we have shown different IL-6 levels in FF, comparing younger and older patients and NR and LR of advanced reproductive age. The data may reflect a hypoxic milieu due to a deficient microvasculature in LR or, alternatively, an increased presence of macrophages in follicles from age-LR patients. The 4- to 20-fold increase in FF IL-6 and VEGF levels further suggests an ovarian origin of these cytokines in humans.

REFERENCES

1. Menken J, Trussell J, Larsen U: Age and Infertility. Science 1986;233:1389–1394
2. Pellicer A, Marí M, De los Santos, Simon C, Remohi J, Tarin JJ: Effects of aging on the human ovary: The secretion of immunoreactive α-inhibin and progesterone. Fertil. Steril 1994;61:663–668
3. Klein NA, Illingwort PJ, Groome NP, McNeilly AS, Battaglia DE, Soules MR: Decreased inhibin B secretion is associated with the monotropic FSH rise in older, ovulatory women: A study of serum and follicular fluid levels of dimeric inhibin A and B in spontaneous menstrual cycles. J Clin Endocrinol Metab 1996;81:2742–2745
4. Seifer DB, Gardiner AC, Lambert-Messerlian G, Schneyer AL: Differential secretion of dimeric inhibin in cultured luteinized granulosa cells as a function of ovarian reserve. J Clin Endocrinol Metab 1996;81:736–739
5. Pellicer A, Ballester MJ, Serrano MD, Mir A, Serra-Serra V, Remohi J, Bonilla-Musoles FM: Aetiologic factors involved in the low response to gonadotrophins in infertile women with normal basal serum follicle stimulating hormone. Hum Reprod 1994;9:806–811
6. Simón C, Tsafriri A, Pellicer A, Polan ML: The role of interleukins in the ovary. Reprod Med Rev 1996;5:51–63
7. Schindler R, Mancilla J, Endres S, Ghorbani R, Clark SC, Dinarello CA: Correlations and interactions in the production of interleukin-6 (IL-6), IL-1, and tumor necrosis factor (TNF) in human mononuclear cells: IL-6 suppresses IL-1 and TNF. Blood 1990;75:40–47
8. Li J, Perrella MA, Tsai JC, Yet SF, Hsieh CM, Yoshizumi M, Patterson C, Endege WO, Zhou F, Lee ME: Induction of vascular endothelial growth factor gene expression by interleukin-1β in rat aortic smooth muscle cells. J Biol Chem 1995;270:308–312
9. Levitas E, Chamoun D, Udoff LC, *et al.*: Periovulatory and interleukin (IL)-1 dependent upregulation of intraovarian vascular endothelial growth factor (VEGF): Potential role for VEGF in the promotion of periovulatory angiogenesis and vascular permeability. J Soc Gynecol Invest 1997;4 (Suppl): 87 (abstract)
10. Cohen T, Nahari D, Cerem LW, Neufeld G, Levi BZ: Interleukin 6 induces the expression of vascular endothelial growth factor. J Biol Chem 1996;271:736–741
11. Friedman ChI, Danforth DR, Herbosa-Encarnación C, Arbogast L, Alak BM, Seifer DB: Follicular fluid vascular endothelial growth factor concentrations are elevated in women of advanced reproductive age undergoing ovulation induction. Fertil Steril 1997;68:607–612
12. Motro B, Itin A, Sachs L, Keshet E: Pattern of interleukin-6 gene expression in vivo suggests a role for this cytokine in angiogenesis. Proc Natl Acad Sci USA 1990;87:3092–3096
13. Buyalos RP, Watson JM, Martinez-Maza O: Detection of interleukin-6 in human follicular fluid. Fertil Steril 1992;57: 1230–1234
14. Loret de Mola JR, Flores JP, Baumgardner GP, Goldfarb JM, Gindlesperger V, Friedlander MA: Elevated interleukin-6 levels in the ovarian hyperstimulation syndrome: Ovarian immunohistochemical localization of interleukin-6 signal. Obstet Gynecol 1996;87:581–586
15. Gaulden ME: Maternal age effect: The enigma of Down's syndrome and other trisomic conditions. Mutat Res 1992;296: 69–88
16. Van Blerkom J, Antczak M, Schrader R: The developmental potential of the human oocyte is related to the dissolved oxygen content of follicular fluid: Association with vascular endothelial growth factor levels and perifollicular blood flow characteristics. Hum Reprod 1997;12:1047–1055
17. Plachot M, Veiga A, Montagut J, de Grouchy J, Calderon G, Lepretre S, Junca AM, Santalo J, Carles E, Mandelbaum J, *et al.*: Are clinical and biological IVF parameters correlated with

chromosomal disorders in early life: A multicentric study. Hum Reprod 1988;3:627–635
18. Battaglia DE, Goodwin P, Klein NA, Soules MR: Influence of maternal age on meiotic spindle assembly in oocytes from naturally cycling women. Hum Reprod 1996;11:2217–2222
19. Munné S, Alikani M, Tomkin G, Grifo J, Cohen J: Embryo morphology, developmental rates, and maternal age are correlated with chromosomal abnormalities. Fertil. Steril 1995;64:382–391
20. Janny L, Menezo YJR: Maternal age effect on early human embryonic development and blastocyst formation. Mol Reprod Dev 1996;45:31–37
21. Friedlander MA, Loret de Mola JR, Goldfarb JM: Elevated levels of interleukin-6 in ascites and serum from women with ovarian hyperstimulation syndrome. Fertil Steril 1993;60:826–833
22. Revel A, Barak V, Lavy Y, Anteby E, Abramov Y, Schenker JJ, Amit A, Finci-Yeheskel Z, Mayer M, Simon A, Laufer N, Hurwitz A: Characterization of intraperitoneal cytokines and nitrites in women with severe ovarian hyperstimulation syndrome. Fertil Steril 1996;66:66–71