Follicular fluid markers of oocyte developmental potential

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BACKGROUND: Concentrations of certain substances in follicular fluid (FF) are related to fertilization outcome and early post-fertilization development. The study aim was to identify FF markers with which to predict embryo implantation potential. METHODS: Concentrations of selected hormones, cytokines and growth factors in individual FF samples obtained during assisted reproduction treatment were related with treatment outcomes. RESULTS: Mean concentrations of LH, growth hormone (GH), prolactin (PRL), 17β-estradiol (E2) and insulin-like growth factor (IGF)-I were higher, and that of interleukin-1 (IL-1) was lower, in treatment attempts leading to a clinical pregnancy as compared with those in which no pregnancy was established. Concentrations of FSH, progesterone, tumour necrosis factor-α and IGF-II were similar in successful and unsuccessful attempts. In successful attempts, LH and GH levels were higher in those follicles from which oocytes giving rise to transferred embryos (i.e. embryos with best morphology and fastest cleavage rate) originated, as compared with other follicles from which a mature oocyte was recovered but was cryopreserved for later use. CONCLUSIONS: FF levels of LH, GH, PRL, E2, IGF-I and IL-1 may serve to analyse cases of repeated assisted reproduction failures and to assess effects of modifications of the ovarian stimulation protocol.

Key words: cytokines/follicular fluid/growth factors/pituitary and steroid hormones/pregnancy

Introduction

During gonadotrophin treatment for controlled ovarian stimulation, follicular fluid (FF) composition shows dynamic changes as individual cells of the ovarian follicle respond to the administered gonadotrophins by secreting different hormones, growth factors and cytokines. Of these substances, at least some are likely to influence, either directly or indirectly, oocyte viability and developmental potential. A number of previous studies were aimed at establishing correlations between the concentration of various substances in FF and oocyte quality (Mendoza *et al.*, 1999, review). Most of these studies, however, were burdened with the uncertainty of whether the follicular samples studied originated from follicles that actually contained a mature oocyte at the time of follicle aspiration because oocyte maturity was not assessed on the day of oocyte recovery. Recently, this problem was overcome by analysing FF accompanying oocytes to be used for ICSI and whose cumulus oophorus and corona radiata were thus removed shortly before recovery; this made it possible to evaluate the immediate oocyte maturity status (Mendoza *et al.*, 1999). It was found that FF concentrations of progesterone, growth hormone (GH), prolactin (PRL), interleukin-1 (IL-1) and

tumour necrosis factor (TNF)α are positively related with the rate of normal fertilization, and that morphologically normal and rapidly cleaving embryos develop from oocytes recovered from follicles with high concentrations of LH and IL-1 (Mendoza *et al.*, 1999).

These previous findings raised two important questions. The first question is whether the intrafollicular concentration of a given hormone, cytokine or growth factor is predominantly an intra-individual variable related to the quality of each follicle developing in the patient's ovaries during hormonal stimulation, or is it mainly an inter-individual variable potentially associated with the fecundity status of each woman? The second question is whether the abnormal FF patterns are always reflected by an easily detectable oocyte or embryo characteristic (such as fertilization failure of embryo cleavage speed and regularity), or do certain values represent independent features predicting otherwise undetectable problems of embryo pre-implantation and post-implantation development.

In the search for answers to these questions, the present study compared mean concentrations of selected hormones, cytokines and growth factors in FF from women with oocytes where ongoing clinical pregnancies were established, and in

those women who failed to become pregnant. In addition to the previously used set of hormones and cytokines, this study also examined insulin-like growth factor (IGF) -I and -II, both of which are well-characterized ovarian growth factors that have been suggested to mediate the local action of FSH and GH (Adashi, 1998; Hillier, 2001). Moreover, FF patterns were compared, within the same patient groups, between follicles from which the oocytes giving rise to transferred embryos were obtained and those corresponding to oocytes that were not transferred. This latter analysis was performed separately for treatment attempts resulting in a pregnancy and for those that failed to establish a pregnancy.

Materials and methods

Patients

The present study involved 54 couples suffering from male infertility and who were undergoing assisted reproduction attempts using ICSI. The inclusion criteria were age of the female partner (24–35 years) and absence of any apparent female pathology. Low responders were not included in this study. Informed consent for use of the FF samples obtained during oocyte recovery was provided by all couples.

Ovarian stimulation, FF sampling and oocyte collection

Previously described techniques and protocols (Mendoza *et al.*, 1999) were used. Briefly, ovarian stimulation was performed by combined administration of purified urinary FSH (Neofertinorm®; Serono, Madrid, Spain) and human menopausal gonadotrophin (HMG; Lepori, Madrid, Spain) after pituitary desensitization with leuproreline acetate (Procrin®; Abbot, Madrid, Spain) started in the midluteal phase. When at least three follicles had reached a diameter of >18 mm, ovulation was induced with 10 000 IU HCG (Profasi®; Serono).

Transvaginal, ultrasound-guided aspiration of ovarian follicles was performed 36 h after the HCG injection. Follicles were aspirated manually with a 10 ml syringe which was changed after the aspiration of each individual follicle. Samples of FF in which an oocyte–cumulus complex was identified were centrifuged for 10 min at $500 \times g$, and the supernatants were stored at –20°C for further analysis. Samples with massive blood contamination (red colour) were excluded from further analysis.

Measurement of hormone, cytokine and growth factor concentrations

Follicular fluid concentrations of 17β-estradiol (E2), progesterone, FSH, LH, PRL, IL-1β and TNFα were determined using commercial enzyme immunoassay kits (Boehringer Mannheim, Mannheim, Germany). The intra- and inter-assay variabilities were respectively 2.7 and 5.0% for E2, 1.5 and 4.1% for progesterone, 1.8 and 5.3% for FSH, 1.8 and 5.1% for LH, 2.8 and 3.8% for PRL, 2.7 and 6.6% for IL-1, and 3.1 and 5.5% for TNFα. Direct radioimmunoassay was used for the determinations of GH (Sorin Biomedica, Vercelli, Italy), IGF-I and IGF-II (both assay kits purchased from Nichols Institute Diagnostics). The intra- and inter-assay variabilities were respectively 1.9 and 4.5% for GH, 2.9 and 9.8% for IGF-I, and 3.4 and 8.6% for IGF-II. All measurements were carried out according to the manufacturers' instructions.

Assessment of oocyte maturity, ICSI and embryo culture

Within 3 h after follicular aspiration the cumulus oophorus and the corona radiata were removed from oocytes by a brief incubation (10–20 s) in a solution of 40 IU/ml of hyaluronidase (Hyase®;

Scandinavian IVF Science, Gothenburg, Sweden) followed by repeated aspiration into a finely drawn Pasteur pipette. All these manipulations were carried out at 37°C. Denuded oocytes were assessed for maturity. Only metaphase II oocytes, identified by the presence of the first polar body, were used in this study.

ICSI was performed 3–6 h after oocyte recovery by using previously described techniques and instrumentation (Tesarik and Sousa, 1995). After ICSI, the injected oocytes were cultured at 37°C in IVF medium (Scandinavian IVF Science) equilibrated with 5% CO_2 in air. Fertilization was assessed 16–20 h after ICSI. Only normally fertilized oocytes (two pronuclei and two polar bodies) were considered further for eventual embryo transfer. These were cultured for an additional 24–30 h at 37°C in fresh CO_2-equilibrated IVF medium.

Embryo grading, selection and transfer

Embryo development was evaluated 2 days after ICSI by determining the number of blastomeres and the relative proportion of embryo volume occupied by anucleate cell fragments. Embryos with <10% fragments, with 10–20% fragments, with 20–30% fragments, and with >30% fragments were referred to as grade 1, 2, 3 and 4 respectively. Two to three embryos with the highest number of blastomeres and with the best morphological grade were selected for transfer in each treatment attempt. All the remaining embryos that had undergone at least one cleavage division and developed from normally fertilized oocytes were cryopreserved on the second day after ICSI.

Statistical analysis

Data were analysed for normality of distribution using the Kolmogorov–Smirnov test. Distribution was normal for E2, progesterone, FSH, LH, PRL, GH, IGF-I and IGF-II, for which differences between individual groups of oocytes and embryos were evaluated by ANOVA and paired Student's *t*-test. For IL-1 and TNFα, for which distribution was not normal, the Kruskal–Wallis test was used. An unpaired Student's *t*-test was used to compare the numbers of embryos transferred in different groups of patients.

Results

Comparison of treatment attempts leading to pregnancy and those without pregnancy

Treatment attempts that led to a pregnancy did not differ from those that failed to do so with regard to the number and morphology grade of embryos transferred (Table I). The number of blastomeres per transferred embryo (mean ± SD) was not significantly different in both groups of patients (3.8 ± 0.1 in the pregnancy group versus 3.9 ± 0.1 in the no-pregnancy group).

When mean intrafollicular concentrations of substances measured in FF samples obtained during a single treatment attempt were compared between those attempts that gave a clinical pregnancy and those that failed to do so, the successful treatment attempts showed significantly higher values for LH, GH, PRL, E2 and IGF-I (Table II). In contrast, significantly lower values were measured in the successful treatment attempts for IL-1 (Table II), whereas no differences were found in FSH, progesterone, TNFα and IGF-II.

When this comparison was made taking into account only those follicles that gave rise to the embryos transferred in the reported treatment attempts, the same differences between the successful and the unsuccessful attempts were found (Table III).

Table I. Per-patient numbers of transferred and frozen embryos with different morphological grades in women who achieved a clinical pregnancy and in those who failed to do so

Pregnancy	Embryos transferred per patient			Embryos frozen per patient		
	Grade 1	Grade 2	Grade 3	Grade 1	Grade 2	Grade 3
Yes ($n = 29$)	1.8	1.3	0.1	0.8	1.2	0.2
No ($n = 25$)	1.7	1.1	0.2	1.0	1.4	0.4

For all values within the same column, $P > 0.05$.

Table II. Mean concentrations of selected hormones, growth factors and cytokines in follicular fluid from all follicles of women who achieved a clinical pregnancy and in those who failed to do so

Pregnancy	E2 (pg/ml)	Progesterone (pg/ml)	FSH (IU/l)	LH (IU/l)	PRL (ng/ml)	GH (ng/ml)	IL-1 (pg/ml)	TNFα (pg/ml)	IGF-I (ng/ml)	IGF-II (ng/ml)
Yes ($n = 29$)	998 ± 120[a]	18.4 ± 3.2	5.1 ± 0.8	0.63 ± 0.02[b]	1100 ± 150[b]	4.1 ± 0.4[b]	21.9 ± 9.2[a]	7.6 ± 2.3	103.6 ± 15.2[a]	2.8 ± 0.9
No ($n = 25$)	764 ± 85[a]	17.5 ± 3.1	4.9 ± 0.8	0.20 ± 0.02[b]	699 ± 120[b]	2.1 ± 0.3[b]	37.3 ± 10.0[a]	7.4 ± 2.4	73.8 ± 12.6[a]	2.4 ± 0.8

Values are mean ± SD.
[a]For values within column, $P < 0.05$.
[b]For values within column, $P < 0.01$.
E2 = estradiol; GH = growth hormone; IGF = insulin-like growth factor; IL = interleukin; PRL = prolactin; TNF = tumour necrosis factor.

Table III. Mean concentrations of selected hormones, growth factors and cytokines in follicular fluid from follicles corresponding to transferred embryos in women who achieved a clinical pregnancy and in those who failed to do so

Pregnancy	E2 (pg/ml)	Progesterone (pg/ml)	FSH (IU/l)	LH (IU/l)	PRL (ng/ml)	GH (ng/ml)	IL-1 (pg/ml)	TNFα (pg/ml)	IGF-I (ng/ml)	IGF-II (ng/ml)
Yes ($n = 29$)	991 ± 118[a]	13.1 ± 2.9	5.0 ± 0.8	0.75 ± 0.02[b]	1185 ± 129[b]	4.2 ± 0.5[b]	22.5 ± 7.4[a]	7.8 ± 2.5	115.1 ± 11.5[a]	2.9 ± 0.5
No ($n = 25$)	660 ± 85[a]	14.4 ± 3.5	4.9 ± 0.9	0.36 ± 0.01[b]	721 ± 133[b]	2.9 ± 0.1[b]	35.3 ± 8.9[a]	7.5 ± 2.4	70.4 ± 8.7[a]	2.6 ± 0.5

Values are mean ± SD.
[a]For values within column, $P < 0.05$.
[b]For values within column, $P < 0.01$.
E2 = estradiol; GH = growth hormone; IGF = insulin-like growth factor; IL = interleukin; PRL = prolactin; TNF = tumour necrosis factor.

Comparison of follicles yielding oocytes that gave rise to transferred embryos and embryos that were not transferred

In treatment cycles leading to a clinical pregnancy, FF aspirated from follicles corresponding to oocytes that gave rise to the transferred embryos (embryos showing the fastest cleavage and best morphological appearance) showed significantly higher values of LH and GH, as compared with follicles corresponding to oocytes giving rise to embryos that were cryopreserved for later transfer (Table IV). Values of FSH, E2, PRL, progesterone, IGF-I, IGF-II, IL-1 and TNFα did not differ between these two groups of follicles.

In the treatment cycles that failed to establish a pregnancy, the only difference between follicles from which oocytes giving rise to the transferred embryos and the other follicles from which a mature oocyte was recovered concerned LH, which was significantly higher in the former group of follicles (Table V).

Discussion

This study used two different types of comparison to analyse the relationship between oocyte quality and the concentration of selected hormones, cytokines and growth factors in FF. Firstly, FF values were compared between treatment attempts that led to a clinical pregnancy and those that failed to do so. This comparison was expected to provide information about inter-individual differences in the ovarian response to a standard stimulation protocol. The second comparison was made between follicles from which oocytes giving rise to transferred embryos were recovered and those yielding oocytes which gave rise to embryos that were cryopreserved for later transfer. This comparison was made separately for the treatment cycles leading to a clinical pregnancy and for those that failed to do so. This comparison served to evaluate the intra-individual variability within the same groups of patients.

This study was undertaken with the same set of selected

Table IV. Concentrations of selected hormones, growth factors and cytokines in follicular fluid from follicles corresponding to transferred and non-transferred embryos in women who achieved a clinical pregnancy

Transfer	E2 (pg/ml)	Progesterone (pg/ml)	FSH (IU/l)	LH (IU/l)	PRL (ng/ml)	GH (ng/ml)	IL-1 (pg/ml)	TNFα (pg/ml)	IGF-I (ng/ml)	IGF-II (ng/ml)
Yes ($n = 98$)	991 ± 118	13.1 ± 2.9	5.0 ± 0.8	0.75 ± 0.02^a	1185 ± 129	4.2 ± 0.5^a	22.5 ± 7.4	7.8 ± 2.5	115.1 ± 11.5	2.9 ± 0.5
No ($n = 63$)	822 ± 130	13.9 ± 3.1	5.2 ± 0.5	0.32 ± 0.02^a	1114 ± 122	1.9 ± 0.3^a	25.4 ± 8.2	6.9 ± 2.7	95.5 ± 10.9	3.1 ± 0.6

Values are mean $\pm$ SD.
[a]For values within column, $P < 0.01$.
E2 = estradiol; GH = growth hormone; IGF = insulin-like growth factor; IL = interleukin; PRL = prolactin; TNF = tumour necrosis factor.

Table V. Concentrations of selected hormones, growth factors and cytokines in follicular fluid from follicles corresponding to transferred and non-transferred embryos in women who did not achieve a clinical pregnancy

Transfer	E2 (pg/ml)	Progesterone (pg/ml)	FSH (IU/l)	LH (IU/l)	PRL (ng/ml)	GH (ng/ml)	IL-1 (pg/ml)	TNFα (pg/ml)	IGF-I (ng/ml)	IGF-II (ng/ml)
Yes ($n = 74$)	660 ± 85	14.4 ± 3.3	4.9 ± 0.9	0.36 ± 0.01^a	721 ± 133	2.9 ± 0.1	35.3 ± 8.9	7.5 ± 2.4	70.4 ± 8.7	2.6 ± 0.5
No ($n = 75$)	675 ± 99	15.6 ± 3.6	5.1 ± 0.8	0.15 ± 0.02^a	688 ± 110	3.1 ± 0.4	39.5 ± 9.9	7.2 ± 3.3	81.5 ± 13.5	2.4 ± 0.4

Values are mean $\pm$ SD.
[a]For values within column, $P < 0.01$.
E2 = estradiol; GH = growth hormone; IGF = insulin-like growth factor; IL = interleukin; PRL = prolactin; TNF = tumour necrosis factor.

hormones and cytokines (E2, progesterone, FSH, LH, PRL, GH, IL-1 and TNFα) whose concentrations in FF had previously been related to fertilization results after ICSI (Mendoza *et al.*, 1999). Because no relationship between the intrafollicular concentrations of E2 and progesterone on the one hand, and embryo quality on the other hand, was detected in our previous study (Mendoza *et al*., 1999), and data published by other authors were inconsistent (Wramsby *et al*., 1981; Botero-Ruiz *et al*., 1984; Uehara *et al*., 1985; Hartshorne, 1989), it was considered whether any relationship might be found between the concentrations of these hormones in FF and the implantation potential of the corresponding embryos. To date, it appeared that this question had not yet been addressed directly in humans.

Intrafollicular concentrations of FSH and LH reflect respectively the local impact of exogenous gonadotrophin administration after previous pituitary suppression by a GnRH agonist and the degree of this suppression. Moreover, previous data have suggested a positive correlation between the residual LH concentration in FF from GnRH agonist-suppressed women and the embryo cleavage speed (Mendoza *et al*., 1999).

Higher intrafollicular concentrations of the cytokines IL-1 and TNFα have been shown to be associated with normal fertilization, and IL-1 also correlated positively with embryo cleavage speed in humans (Mendoza *et al*., 1999), in agreement with data from animal experiments (Adashi, 1990 for review).

GH concentration in FF has been shown previously to be positively correlated with both normal fertilization and pre-implantation embryo morphology and cleavage speed (Mendoza *et al*., 1999). Moreover, GH is known to enhance intrafollicular metabolic events required for oocyte maturation, such as the FSH-dependent E2 production by granulosa cells (Lanzone *et al*., 1996), the formation of FSH and LH receptors in granulosa cells (Jia *et al*., 1986) or the stimulation of androgen production by theca cells (Apa *et al*., 1996). PRL

concentrations have also been shown to be higher in follicles yielding oocytes that subsequently show normal fertilization (Mendoza *et al.*, 1999).

The rationale for the addition of two growth factors, IGF-I and IGF-II, was based on the presumable role of these factors as local mediators of FSH and GH actions in the ovary (Adashi, 1998), the previously reported strong relationship between intrafollicular GH and fertilization rates after ICSI (Mendoza *et al.*, 1999), and the existence of conflicting data about the expression of both factors in the human ovary (Hillier, 2001).

It can be expected that the concentration of substances potentially useful as markers of oocyte quality might be different in follicles corresponding to transferred and non-transferred embryos in the successful treatment attempts. The substances showing similar differences between mean follicular values in the successful and unsuccessful treatment attempts, and between follicles corresponding to the transferred and non-transferred embryos in the successful attempts, may be considered as markers with which to estimate the patient's individual fertility and/or the adequacy of the protocol used for ovarian stimulation.

Out of the ten potential markers tested in this study, three showed similar differences in the two types of comparison. Of these, LH and GH were consistently higher, and IL-1 was consistently lower, in the successful attempts and in the follicles corresponding to the transferred embryos in the successful attempts. With regard to LH, these findings are in agreement with a previous study showing that higher concentrations of LH and GH in FF are associated with faster post-fertilization development and better embryo morphology (Mendoza *et al.*, 1999). These findings complement the recent clinical experience showing that LH activity in menotrophins optimizes folliculogenesis in controlled ovarian stimulation (Filicori *et al.*, 2001).

With regard to GH, it is now believed that its presence in FF is due not only to the transport of systemic GH of pituitary origin (Gosden *et al.*, 1988), but that local production of GH in the ovarian follicles also contributes (Izadyar *et al.*, 1999). Within the follicle, GH is known to act as a co-gonadotrophin by stimulating the proliferation and differentiation of granulosa cells and increasing their production of E2 (Mason *et al.*, 1990; Langhout *et al.*, 1991). In-vitro experiments showed this GH effect to be direct (Mason *et al.*, 1990) and to be mediated by functional GH receptors on human granulosa cells (Carlsson *et al.*, 1992). The results of the present study show that oocytes originating from follicles with higher concentrations of GH have a better chance of giving rise to a viable embryo. Moreover, high intrafollicular GH concentrations were associated with presumably more fertile individuals because the mean GH values were higher in patients who became pregnant than in those who did not achieve pregnancy. GH concentrations were also higher in follicles corresponding to transferred embryos as compared with non-transferred embryos in treatment cycles leading to pregnancy, but not in those that did not lead to pregnancy. This suggests that a subpopulation of high-quality follicles that eventually yield oocytes with high developmental potential and that are characterized by an elevated level of GH production develop in some, but not all, women during ovarian stimulation.

Interestingly, the mean intrafollicular concentration of IGF-I was also higher in patients who became pregnant as compared with those who failed to establish pregnancy. Although no difference in the concentration of this growth factor was detected between follicles corresponding to transferred and non-transferred embryos, this finding suggests that in humans (Apa *et al.*, 1994; as in the rat), the intrafollicular action of GH may be at least partly mediated by IGF-I. In support of this hypothesis, IGF-I concentrations were found to be reduced in the FF of low responders (Oosterhuis *et al.*, 1998). In the present study, however, low responders were not included. We have shown previously that oocytes which achieved normal fertilization after ICSI and underwent regular cleavage divisions were harvested from follicles with higher concentrations of GH as compared with oocytes that failed to fertilize, and showed an abnormal fertilization pattern or were arrested or morphologically abnormal during cleavage (Mendoza *et al.*, 1999).

In contrast to LH and GH, intrafollicular levels of IL-1 were lower in treatment attempts leading to pregnancy as compared with unsuccessful attempts and in those follicles corresponding to transferred embryos in successful attempts as compared with other follicles yielding mature oocytes. This observation was surprising in view of the previously reported positive correlation of intrafollicular IL-1 with normal fertilization (Mendoza *et al.*, 1999). It is possible that IL-1 stimulates those events in oocyte cytoplasmic maturation that are necessary for normal fertilization, but without improving post-fertilization embryo viability.

The higher intrafollicular concentrations of E2 in the successful treatment attempts may be a direct consequence of the higher concentrations of LH and GH, both of which are known to promote steroidogenesis in the theca interna and granulosa cells (Hsu and Hammond, 1987; Hutchinson *et al.*, 1988; Mason *et al.*, 1990; Karnitis *et al.*, 1994; Nahum *et al.*, 1995; Lanzone *et al.*,

1996). However, they may also reflect a higher number of dominant follicles resulting in a higher number of mature oocytes, allowing a better selection of subsequent embryos. The cause of the higher values of intrafollicular PRL in this group of patients is not clear, and its explanation will require further study.

In conclusion, these data suggest that intrafollicular concentrations of LH, GH and IL-1 are related to oocyte post-fertilization developmental potential. The predictive value of these determinations is not paralleled either by embryo cleavage speed or by embryo morphology, since the treatment attempts leading to a pregnancy did not differ from the unsuccessful attempts as to the number of embryos transferred, the mean number of blastomeres per transferred embryo, and the relative representation of different embryo morphology grades in the transferred embryo cohort. Even though the cellular and molecular mechanisms underlying the association between the intrafollicular concentration of LH, GH and IL-1 on the one hand, and the oocyte quality on the other hand, are understood only partly, these findings may have practical applications in the management of ovarian stimulation protocols in current assisted reproduction programmes. Analysis of bulk FF samples representing a mixture of fluid from the totality of follicles aspirated during an IVF attempt is easy to organize, and it may contribute to a better understanding of the causes of repeated IVF failures. This approach may also be useful to monitor the effects of modifications of ovarian stimulation protocols in this category of women.

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