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Insulin-like growth factor (IGF)-1 and IGF binding protein-1 and -3 in the follicular fluid of infertile patients with endometriosis

J.S.L.Cunha-Filho, N.A.Lemos, F.M.Freitas, K.Kiefer, M.Faller and E.P.Passos[1]

Obstetrics and Gynecology Department, Hospital de Clínicas de Porto Alegre, Universidade Federal do Rio Grande do Sul, Brazil

[1]To whom correspondence should be addressed at: Setor de Reprodução Hospital de Clínicas, Universidade Federal do Rio Grande do Sul, Ramiro Barcelos, 2350, Sala 1125, Porto Alegre 90035–003, Brazil. E-mail: epp@via-rs.net

BACKGROUND: Endometriosis is associated with pituitary–ovarian axis dysfunction. The study of the follicular fluid in patients with endometriosis is important to elucidate the pathophysiological mechanism of this disease. The objective of this present paper was to analyse the dosages of insulin-like growth factor-1 (IGF-1) and IGF binding protein-1 and 3 (IGFBP-1 and IGFBP-3) in the follicular fluid environment of infertile patients with endometriosis. METHODS: A total of 41 infertile patients undergoing IVF between January 1999 and January 2000 participated in the cross-sectional prospective study. Patients were divided into three groups: group I, minimal/mild endometriosis (n = 12); group II, moderate/severe endometriosis (n = 10); and group III, tubal obstruction (n = 19). The ultrashort protocol was used in association with recombinant FSH for ovulation induction. Follicular fluid analysis was performed using radioimmunoassay with specific kits. RESULTS: Follicular fluid IGF-1 and IGFBP-3 levels were not significantly different among the groups; however, follicular fluid IGFBP-1 levels were lower in those patients with moderate/severe endometriosis ($P < 0.05$). Comparison of ovulation induction time, number of recombinant FSH units, number of follicles, oocytes and embryos, and fertilization and gestation/cycle rates showed non-significant differences. CONCLUSION: Infertile patients with moderate/severe endometriosis, which is associated with ovulatory dysfunction, presented lower levels of IGFBP-1 in the follicular fluid when undergoing IVF.

Key words: endometriosis/follicular fluid/infertility/insulin-like growth factor/insulin-like growth factor binding protein

Introduction

Endometriosis is associated with alterations in the hypothalamic–pituitary–ovarian axis. This association suggests a disorder in the follicular function, with altered LH and prolactin secretion, reduced ability of the pre-ovulatory follicle and of the oocyte to be fertilized, and altered luteal function (Cahill and Hull, 2000; Cunha-Filho *et al*., 2002). Alterations in the follicular fluid of patients with endometriosis suggest a cause for infertility in this group of patients, since oocyte alterations result in low-quality embryos, with reduced implantation rates (Garrido *et al*., 2000). Moreover, we demonstrated that patients with minimal/mild endometriosis present altered prolactin secretion after thyrotrophin-releasing hormone administration, with a higher prevalence of defects in the luteal phase (Cunha-Filho *et al*., 2001, 2002).

The assessment of follicular fluid is very important in the study and understanding of ovulation. Increased concentrations of estradiol/androstenedione have been observed when mature oocytes are present. In addition, the regulatory and amplificatory effect of follicular fluid growth factors has been evaluated in different groups of patients and shown to be fundamentally important for an adequate ovarian function (Erickson *et al*., 1998).

The study of the follicular fluid in patients with endometriosis is important to elucidate the pathophysiology of this disease. Some investigators demonstrated a decreased concentration of vascular endothelial growth factor (VEGF) and a higher level of progesterone in the follicular fluid of endometriotic patients (Pellicer *et al*., 1998a,b,c). In a recent study, the same authors published different results regarding follicular fluid VEGF concentration in patients with endometriosis (Garrido *et al*., 2001); however, a different methodology was used in this study (flow cytometry), and the small number of subjects (n = 6) did not allow a definite conclusion.

The most convincing data regarding oocyte quality in patients with endometriosis come from a study regarding oocyte donation carried out by Pellicer *et al*. (1994). The authors showed that the implantation rate was significantly lower in those who received oocytes from patients with endometriosis. Other investigators (Akande *et al*., 2000) studied inhibin A and B and activin A concentrations in the follicular fluid of endometriotic patients: inhibins were not

significantly altered, but the concentration of activin A was higher in patients with the disorder, which may result in a compensatory mechanism.

In the ovaries, insulin-like growth factor-1 (IGF-1) seems to have an autocrine/paracrine action. It is produced by granulosa cells after stimulation with FSH, and its activity is controlled by catalytic proteins and by insulin-like growth factor binding proteins (IGFBP), which act as inhibitors (Giordano *et al*., 1992). IGF-1 activates the production of estrogen and progesterone and the activity of aromatase, and also modulates the effect of FSH and LH (Erickson *et al*., 1989; Giudice *et al*., 1990).

IGF-1 acts as a local amplifier of the action of FSH in the follicular fluid. In addition, the ovulation control exerted by IGF-1 is evidenced by the fact that in induced assisted reproduction technology (ART) cycles, follicles with higher IGF-1 concentrations require a lower number of FSH ampoules and a shorter induction time (Oosterhuis *et al*., 1998).

On the other hand, IGFBP-1 exerts an inhibitory effect on IGF-1, acting as an anti-gonadotrophic agent (Adashi, 1995; Yoshimura, 1998) and modulating IGF-1 activity. Recently, Erickson and Shimasaki (2001) also showed the importance of IGFBP-4 and pregnancy-associated plasma protein-A (PAPP-A) in the control of ovarian function.

Several associations between response to ovarian stimulus and abnormalities in IGF-1 concentrations have been discussed, but none has been confirmed. In a study carried out by Neubourg *et al*. (1998), no differences were found in follicular fluid IGF-1, IGFPB-1, and IGFBP-3 concentrations when the response to ovarian stimulus was poor or inadequate.

With the aim of elucidating the role of some important growth factors in the pathogenesis of endometriosis-associated ovulatory dysfunction, we decided to analyse the follicular fluid environment of infertile patients with endometriosis through the analysis of IGF-1, IGFBP-1 and IGFBP-3 concentrations.

Materials and methods

Design

A cross-sectional prospective study was performed in order to analyse the follicular fluid of infertile patients with endometriosis undergoing ART.

Patients

A total of 107 patients was studied, however only 41 women met the inclusion criteria (see below) and, consequently, were selected.

IGF-1, IGFBP-1, and IGFBP-3 concentrations were assessed in the follicular fluid of 41 infertile patients with endometriosis (cases) or tubal obstruction (controls) submitted to IVF (first cycle) as the treatment for infertility at the Human Reproduction Unit, Hospital de Clínicas de Porto Alegre, Brazil, between January 1999 and January 2000. Patients were informed about the procedures and spontaneously signed an informed consent form. The research project was approved by the Ethics Committee and registered at the Graduate Research Group of the hospital.

Patients were divided into three groups: group I, patients with minimal or mild endometriosis (n = 12); group II, patients with moderate or severe endometriosis (n = 10); group III, patients with tubal obstruction (controls) (n = 19).

All patients underwent laparoscopy for investigation of infertility, the procedure was always performed by the same investigator. Endometriosis staging was performed according to the classification of The American Society for Reproductive Medicine (1985).

The following inclusion criteria were established: (i) patients with previous endocrine disorders; (ii) cases in which the cause for infertility was other than endometriosis (except for patients with tubal obstruction, in the control group); (iii) patients with serum FSH dosage >10 IU/ml in the early follicular phase (day 3 of the menstrual cycle). The criteria proposed by the World Health Organization (1992) were used for the analysis of sperm.

IVF

IVF was performed for the treatment of infertility due to the presence of endometriosis or tubal obstruction. The ultra-short protocol (MacName *et al*., 1989) was used for ovulation induction, with GnRH analogue.

On the third day of the first menstrual cycle after the beginning of the study protocol, recombinant FSH was administered at the initial dose of 150 IU, with dose adjustment according to patient response. Patients received leuprolide acetate from days 2–4 of the menstrual cycle (1 mg/day s.c.).

Ovulation induction was monitored by daily ultrasonography (Marrs *et al*., 1993). When at least one ovarian follicle had reached an 18 mm diameter, hCG at the dose of 10 000 IU was administered i.m. and oocyte collection occurred from 34 to 36 h afterwards.

Samples of follicular fluid, measuring >18 mm, in which an oocyte–cumulus complex was found, were centrifuged at 2500 rpm for 10 min and frozen at –20°C for subsequent analysis, using only clear fluid (without blood). In order to standardize the analysis, we pooled a small sample of follicular fluid of all follicles with equal volumes for retrospective comparison.

Measurements

Serum FSH levels (day 3) were analysed using chemiluminescence kits (Immulite Ltd., USA), with the largest inter- and intra-kit variation being 8.1 and 7.7% respectively.

Follicular fluid (IGF-1, IGFBP-1 and -3) analysis was performed at the radioimmunoassay laboratory of Hospital de Clínicas the Porto Alegre, Brazil, with specific kits (DSL, Texas, USA). The result was obtained through the two-site immunoradiometric assay (IRMA) (Milles *et al*., 1974; Oosterhuis *et al*., 1998). IRMA is a highly sensitive and specific assay method that uses the competition between radiolabelled and unlabelled substances in an antigen–antibody reaction to determine the concentration of the unlabelled substance. The utilization of IRMA as the assay method permits an accurate measurement of IGFs in the follicular fluid, as demonstrated by others (Oosterhuis *et al*., 1998; Amato *et al*., 1998; Mendoza *et al*., 2002).

The minimal detection limit of IRMA was 0.8 ng/ml (corrected to 1:30 dilution) or 27 pg/ml (1.4 pg/tube) for IGF-1, 0.33 ng/ml for IGFBP-1, and ~0.5 ng/ml for IGFBP-3. The coefficients of variation were 1.5% for IGF-1, 2.7% for IGFBP-1 and 0.5% for IGFBP-3.

Statistical analysis

Since the variables showed a non-Gaussian distribution, we used the Kruskal–Wallis test and Dunn's multiple comparison for the analysis of continuous data. Otherwise, categorical data were analysed using Fisher's exact test.

The sample size was calculated (power of 80%) to compare differences regarding IGF-1, IGFBP-1 and IGFBP-3 levels in the follicular fluid.

Table I. Clinical characteristics (values are expressed as medians and 95% confidence intervals)

	Group I (n = 12)	Group II (n = 10)	Group III (n = 19)	Statistics P-value
Age (years)	34* (29.8–36.2)	26 (21.5–29.7)	30.5 (28.2–33.2)	0.001
BMI (kg/m^2)	23.5 (20–27)	23 (22–30)	24.5 (22–28)	NS
Day-3 FSH (IU/l)	4.30 (3.78–5.45)	5.15 (4.37–6.36)	5.00 (4.60–5.30)	NS
Cycle length (days)	28 (27–31)	29 (28–30)	28 (27.5–29)	NS
Reproductive history (infertility)	Primary: 58% Secondary: 42%	Primary: 70% Secondary: 30%	Primary: 63% Secondary: 37%	NS

BMI = body mass index.
*Post-hoc Dunn's procedure.

Table II. IVF outcomes (values are expressed as medians and 95% confidence intervals)

	Group I (n = 12)	Group II (n = 10)	Group III (n = 19)	Statistics P-value
rFSH (IU) administered	550 (370.78–629.22)	750 (505.88–824.11)	750 (569.23–830.77)	NS
Induction length (days)	8.5 (5.76–9.74)	9 (7.71–10.86)	9.5 (8.13–10.37)	NS
Number of follicles >16 mm	3 (1.69–4.91)	3 (1.32–5.83)	3 (2.39–6.78)	NS
Number of oocytes	3 (1.69–4.91)	5 (1.82–7.04)	4 (3.16–7.84)	NS
Fertilization rate (%)	75	85	88	NS
Number of embryos	1 (1.39–3.11)	2 (1.44–3.96)	2 (1.13–3.19)	NS
Clinical pregnancy rate/cycle (%)	25	10	21	NS

rFSH = recombinant follicle-stimulating hormone.

Table III. Follicular fluid levels of IGF-1, IGFBP-1 and IGFBP-3 (values are expressed as medians and 95% confidence intervals)

	Group I (n = 12)	Group II (n = 10)	Group III (n = 19)	Statistics P-value
IGF-1 (ng/ml)	125 (61.32–161.43)	144 (66.87–176.27)	134 (94.73–161.77)	NS
IGFBP-1 (ng/ml)	121.5 (88.99–151.75)	77* (46.26–108.31)	99.5 (84.5–124.34)	0.007
IGFBP-3 (ng/ml x100)	2352.5 (1495.70–2930.54)	2852 (2715.20–3045.38)	2621 (2212.91–2958.76)	NS

IGF-1 = insulin-like growth factor-1; IGFBP-1 = insulin-like growth factor binding protein-1; IGFBP-3 = insulin-like growth factor binding protein-3.
*Post-hoc Dunn's procedure.

We tested the homogeneity and consistency of our main results (IGF-1, IGFBP-1 and -3) comparing the groups using Levene's test for variance.

In order to exclude a confounding bias (age) in association with follicular IGFBP-1 levels, we performed a regression analysis with IGFBP-1 as the dependent variable and age the independent variable.

Data analysis was considered significant when $P < 0.05$.

Results

The clinical characteristics of patients from the three groups are presented in Table I. Patients from group I were older than the others, with a median of 34 years; in group II, the median was 26 years, and in group III, 30.5 years (P = 0.001; Kruskal–Wallis test). Patients from groups II and III were similar in terms of age. Body mass index (BMI), day 3 serum FSH levels, reproductive history and cycle length were similar in the three groups.

Table II shows data related to the analysis of ovulation induction in the three groups. No differences were found regarding length of induction in days (P = 0.225, NS), number of follicles (P = 0.968, NS), number of oocytes (P = 0.773, NS), fertilization rate (P = 0.592, NS), and number of embryos (P = 0.210, NS). In addition, the number of FSH units required for ovarian induction (P = 0.064, NS) and the pregnancy rate (P = 0.575, NS) were similar among the groups.

Follicular fluid growth factor levels are shown in Table III. IGF-1 and IGFBP-3 were not significantly different among the three groups. However, group II patients (moderate/severe endometriosis) presented lower follicular fluid concentrations of IGFBP-1 than patients from the other groups (post-hoc Dunn's procedure).

Levene's test for homogeneity was not significant among the groups (inter-assay) and intra-assay ($P > 0.05$) for IGF-1, IGFBP-1 and -3.

Moreover, the regression analysis did not demonstrate a significant association between age and follicular IGFBP-1 levels among the groups [P = 0.062; 95% CI for B (–0.127–5.131)].

Discussion

The clinical characteristics of patients (age and BMI) were assessed in the three groups. Patients with minimal and mild endometriosis (group I) were older than the others, and this result was probably associated with a delay in diagnosis in this group of patients. The difference regarding age among the groups reinforces our findings, because the older group (group

I) did not present different follicular IGFBP-1 levels when compared with controls (group III).

We cannot compare results from this study with serum IGF levels previously reported (Hammerman, 1987) because our results measure follicular IGF levels. However, in the present study, younger patients (group II) have lower follicular IGFBP-1 levels and similar serum FSH (day 3) levels to the other groups. In addition, the regression analysis showed that follicular IGFBP-1 levels were not associated with age (as a confounding bias) and, consequently, did not interfere with our results.

BMI, which may influence the levels of growth factors and their binding proteins, and also the patient's response to IVF, was not different among the groups.

Fertilization and gestation rates, which have been shown by some authors to be lower in patients with endometriosis undergoing IVF (Wardle *et al*., 1985; Jansen, 1986; Pellicer *et al*., 1998b), presented similar results in patients with and without endometriosis in our study; a previous study has also reported similar findings (Olivennes *et al*., 1995). Although the group of patients with severe endometriosis (group II) presented a lower pregnancy rate (10%), this difference was not significant, probably because our sample size was too small for the evaluation of this specific outcome.

The differences found in IGFBP-1 levels reinforce the idea that these markers are associated with reproductive function (Giudice *et al*., 1990; Adashi, 1995; Jones and Clemmmons, 1995; Kawano *et al*., 1997; Yoshimura, 1998). Patients with moderate or severe endometriosis (group II) presented lower follicular fluid levels of IGFBP-1 when compared with the other groups; this decrease may be associated with the paracrine function of this marker in this group of patients.

Several studies describe the role of the IGF–IGFBP system in ovarian physiology: IGF-1 stimulates the in-vitro meiotic maturation of follicle-enclosed oocytes via receptors; IGFBP-1 inhibits gonadotrophin-induced ovulation and oocyte maturation by neutralizing IGF-1 (Adashi, 1995; Yoshimura, 1998).

Some authors also suggest an association between IGFBP-1 levels and follicular maturity (Kawano *et al*., 1997). In this study, the number of mature follicles among the three groups was not different (Veeck's criterion) (Veeck *et al*., 1983). Our findings confirm the hypothesis of other studies (Cahill and Hull, 2000), and our previous studies (Cunha-Filho *et al*., 2001, 2002) showing alterations in the hypothalamic–pituitary–ovarian axis of women with endometriosis. The decreased levels of follicular fluid IGFBP-1 shown in the present study probably constitute a compensatory mechanism to the intrinsic ovulatory dysfunction observed in infertile patients with endometriosis, increasing the active form of free IGF-1. However, we probably did not find differences in terms of free IGF-1 because of the short half-life (4.5 min) of this peptide (Pan and Kastin, 2000).

Other authors demonstrated that peritoneal IGF-1 levels are higher in patients with endometriosis who present low peritoneal IGFBP-2 and IGFBP-3 concentrations (Kim *et al*., 2000). Moreover, serum IGF-1 levels are higher in these patients when compared with those undergoing tubal sterilization (Gourgon *et al*., 1999), these findings may suggest the development of ectopic endometrium and infertility in patients with endometriosis.

Our finding is in accordance with other studies that show increased progesterone and activin A levels in the follicular fluid of endometriotic patients (Pellicer *et al*., 1998c; Akande *et al*., 2000), abnormalities in the granulosa cell cycle — affecting IVF results (Toya *et al*., 2000), and reduced embryo implantation rates associated with oocyte donation (Pellicer *et al*., 1994). These investigators agree with Harlow *et al*. (1996), who identified altered steroidogenesis as an important finding associated with endometriosis.

We decided not to measure steroids in follicular fluid because we selected only those patients with mature oocytes and, as already demonstrated by Pellicer *et al*. (1998c), levels of progesterone in follicular fluid increase with the severity of this disease, but testosterone acumulation decreases. These findings were not associated with embryo quality in patients with endometriosis. We can explain those findings with our results, if the availability of IGF-1 increases when follicular level of IGFBP-1 decreases and, consequently, the action of FSH is amplified during the folliculogenesis. However, in our small patient groups the decrease in IGFBP-1 was not accompanied by a significant rise in IGF-1.

The main difficulty in studying a specific clinical outcome in human reproduction is that a large number of patients are necessary to reach a conclusion. The minimum sample size required to demonstrate a 5% difference between two groups, with an outcome presenting 20–25% of prevalence, is about 1100 participants in each study arm. So, conclusions about implantation, fertilization and pregnancy rates are very difficult to obtain.

Recently, some investigators, performing a meta-analysis, suggested that patients with endometriosis undergoing IVF demonstrated a decrease in fertilization and implantation rates, with a decrease also in the number of oocytes retrieved. Patients with severe endometriosis have lower pregnancy rates than those with mild disease (Barnhart *et al*., 2002). The results of the present study are consistent with this and suggest that the effects of endometriosis are not only upon the receptivity or the endometrium, but also the embryo and oocyte development.

The study of oocyte dysfunction in endometriosis can benefit from a better understanding of follicular regulation and oocyte maturation. Several peptides and substances are currently under investigation (IGFPB-4, PAPP-A), and their importance in endometriosis-associated infertility is unknown. Moreover, the assessment of some FSH-independent growth factors (growth differentiation factor-9 and bone morphogenetic protein-15) in the follicular pre-antral stage could elucidate the ovulatory dysfunction observed in endometriotic patients (Erickson and Shimasaki, 2001).

Even using a stimulated model, our findings cannot be applied generally, because as demonstrated by Amato *et al*. (1998), serum and follicular IGF-1 did not change during physiological and FSH-stimulated cycles. More studies are necessary regarding the role of the growth hormone (GH) axis in folliculogenesis and the utilization of GH during ovarian stimulation protocols, particularly in endometriotic patients, to elucidate and confirm our results.

We decided to include only those patients with stimulated cycles undergoing IVF because: (i) our study population was more homogeneous and comparable; (ii) we controlled and excluded an intrinsic hormonal factor; (iii) follicular fluid samples were obtained during the same cycle-day; and (iv) we were able to study the ovarian response after the ultra-short protocol administration. However, our results cannot be generalized to unstimulated cycles. Interesting directions for future studies may be to obtain follicular fluid from small antral follicles during laparoscopy or to include only natural IVF cycles.

In conclusion, patients with severe endometriosis presented a decreased level of IGFBP-1 in follicular fluid**,** which may be associated with a compensatory mechanism provoked by a subtle disorder in the ovulatory paracrine/autocrine action. Endometriosis should be seen as a syndrome that may result in anatomical, peritoneal, immunological, and endocrine dysfunction, with effects on folliculogenesis, oocyte quality and embryo development and, consequently, on fertility.

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