

Relationship between tumour necrosis factor α and sex steroid concentrations in the follicular fluid of women with immunological infertility

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Experimental evidence suggests a tight relationship between cytokines and the reproductive system. Tumour necrosis factor α (TNF α), a cytokine produced by activated macrophages and mesenchymal cells, seems to participate in the control of fertility. Therefore, the present study was undertaken to evaluate the concentrations of TNF α in the follicular fluid of female patients with immunological infertility, as well as the possible role of this cytokine in follicular development. Concentrations of TNF α , 17 β -oestradiol, progesterone, androstenedione and testosterone were measured in the follicular fluid of patients with immunological infertility and patients with a tubal factor of infertility, who served as a control group. Patients with immunological infertility had significantly higher concentrations of TNF α in their follicular fluid compared to the control group. In contrast, oestradiol concentrations were significantly lower in the former group. The intrafollicular concentrations of the other steroids measured did not differ significantly between the two groups. The fertilization rate of ova from follicles included in the study was significantly lower in patients with immunological infertility compared to control subjects (19.1 and 57.1% respectively). In conclusion, this study shows that patients with infertility of immunological origin have increased follicular fluid concentrations of TNF α and lower oestradiol concentrations. We speculate that elevated TNF α concentrations in the human follicle may negatively influence both ovulation- and fertilization-related events.

Key words: immunological infertility/in-vitro fertilization/sex steroid/tumour necrosis factor α

Introduction

A number of pleiotropic factors capable of promoting cell proliferation and differentiation have been identified in the past decade. Cytokines are factors involved in the modulation of the inflammatory/immune response, as well as haemo-

poiesis (Smith, 1984). As the majority of the cytokine-related responses are local phenomena, these substances are currently regarded as paracrine and/or autocrine factors (Duggan, 1994). Cytokines are known to interact with various tissues and organs, including the endocrine system (Nathan and Sporn, 1991). Evidence for a tight relationship between cytokines and the reproductive system has accumulated (Kalra *et al.*, 1990; Petraglia *et al.*, 1990; Yamaguchi *et al.*, 1990a,b). In this connection, macrophage cytokines have been described to interfere with enzymes involved in sex steroid biosynthesis (Adashi *et al.*, 1990). Also, cytokines have been discovered to modulate the binding of gonadotrophins with their receptors in the ovary (Adashi *et al.*, 1989). In light of their effects, cytokines may influence fertility by interfering, for example, with folliculogenesis (Zolti *et al.*, 1991).

Tumour necrosis factor α (TNF α) is produced by activated macrophages and mesenchymal cells (Beutler and Cerami, 1988). Primarily characterized for its cachexia-inducing properties, TNF α is currently recognized as a factor playing a role in both physiology and illness, including fertility. In fact, a role for TNF α has been proposed in folliculogenesis and ovarian maturation (Roby and Terranova, 1988; Zolti *et al.*, 1990).

The present study was undertaken to assess possible differences in TNF α concentrations in the follicular fluid of women with infertility of immunological origin, compared to patients with non-immunological forms of infertility. Secondly, we investigated the possible role of TNF α in the pathophysiology of follicular development. To accomplish this task, we measured TNF α , 17 β -oestradiol, progesterone, androstenedione and testosterone concentrations in the follicular fluid of patients with immunological infertility and patients with a tubal factor of infertility (controls).

Materials and methods

Patients

A total of 51 follicular fluid samples were collected from 11 patients undergoing an in-vitro fertilization programme, after written informed consent had been obtained. Six patients were affected by immunological infertility, as assessed by the presence of high titres (>1:40) of antisperm antibodies in serum and/or cervical mucus (Stern *et al.*, 1992). Moreover, four of these patients were positive for antinuclear antibodies (Birkenfeld *et al.*, 1994). All patients underwent diagnostic laparoscopy. Patients with any other cause of infertility were excluded. Patient age range was 23–39 years (34.8 \pm 2.86). The remaining five patients, affected by tubal infertility, served as controls. Their age range was 29–36 years

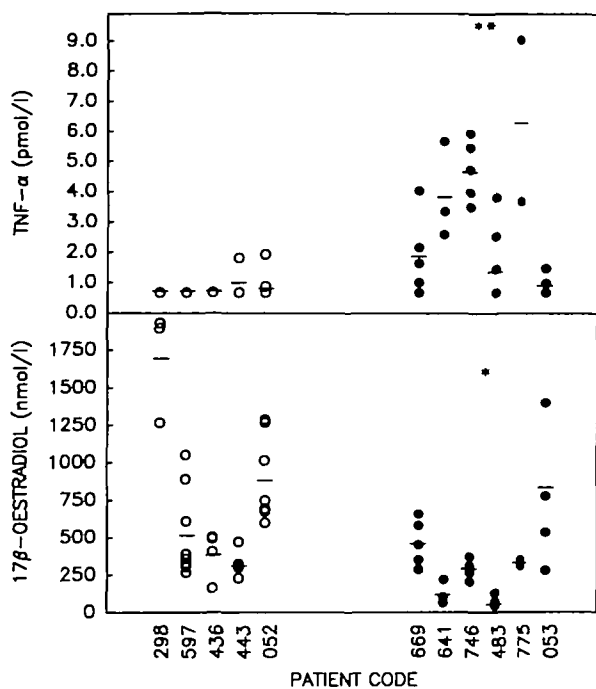


Figure 1. Upper panel: Concentrations of tumour necrosis factor α (TNF α) in the follicular fluid of women affected by immunological infertility (\bullet ; $n = 6$, no. of samples = 25) and women affected by a tubal factor of infertility (\circ ; $n = 5$, no. of samples = 26). Horizontal lines indicate the mean of each individual patient. $**P < 0.001$ (Student's t -test). Lower panel: Concentrations of 17β -oestradiol in the follicular fluid of the same groups of women indicated above. $*P < 0.05$ (Student's t -test).

(32.6 ± 1.12). The male partners had normal sperm parameters, according to WHO criteria (1992).

Antisperm antibody measurements

Serum, cervical mucus and seminal plasma antisperm antibodies were evaluated by the sperm antibody slide test (Biotec, Hannover, Germany), as previously described (Csuppan, 1988). Antinuclear antibodies were detected by an indirect immunofluorescence method, according to Rothfield (1976).

Ovarian stimulation

All patients were treated by a long protocol with GnRH agonist (GnRHa, Dtrp-6, Decapeptyl 3.75; Ipsen, Milan, Italy). GnRHa was administered on day 21 of the menstrual cycle preceding oocyte retrieval. After attainment of pituitary desensitization, indicated by serum oestradiol concentrations <127 pmol/l, and follicle diameter <10 mm, ovarian stimulation was routinely initiated with menotrophin (HMG), corresponding to 150 IU follicle stimulating hormone (FSH) + 70 IU luteinizing hormone (Pergogreen; Serono, Milan, Italy). The daily dose of exogenous gonadotrophin was continued on an individual basis, depending on follicular growth. The mean (\pm SEM) number of gonadotrophin ampoules administered to each patient was similar in the immunological infertility group and the control group (18.2 ± 1.9 and 18.9 ± 4.7 respectively). When the two largest follicles had reached at least a diameter of 16 mm and concentrations of oestradiol were consistent, 10 000 IU of human chorionic gonadotrophin (HCG, Profasi HP; Serono) was administered. Mean (\pm SEM) serum oestradiol concentrations at the time of HCG administration were 6.2 ± 0.71 and 7.3 ± 2.2 nmol/l in the immunological infertility and control groups respectively. Oocytes were retrieved 36 h later.

Follicular fluid aspiration

Follicular fluid aspiration was performed transvaginally, under vaginal ultrasonographic guidance (Aloka, SSD500, 5 MHz). Each follicle was aspirated separately and then collected in a 15 ml conical tube, avoiding flushing with culture medium. Follicular aspirates contaminated with blood or medium were discarded.

TNF α and ovarian steroid concentration measurements

The concentration of each hormone in the follicular fluid was measured in a single assay run. TNF α concentrations were measured by an immunoenzymatic method specific for human TNF α (BioSource International, Camarillo, CA, USA). The minimal detectable amount of TNF α in the follicular fluid was 0.68 pmol/l. This concentration increased the adsorbance value obtained without TNF α by 10%. The intra-assay coefficient of variation (CV) was $4 \pm 0.6\%$. TNF α kit cross-reactivity was $<0.01\%$ for the following substances: IL-2, IL-4, IL-6, IL-10, IFN α , IFN γ , IL-2R, IL-6R and stem cell factor.

Oestradiol concentration in the follicular fluid was measured directly, without extraction, by radioimmunoassay using an antiserum (RD/13/01; Analytical Antibodies, Segrate, Italy). This antiserum showed the following cross-reactivities: oestradiol = 100%, 17α -oestradiol = 1.8%, oestrone = 1.6%, oestriol = 0.6%, testosterone $<0.01\%$, progesterone $<0.01\%$, dehydroepiandrosterone $<0.01\%$ and cortisol $<0.01\%$. Aliquots (200 μ l) of either follicular fluid, diluted 1:2500, or standard solutions were incubated with 100 μ l of antiserum (1:80 000, final dilution), and 100 μ l of [3 H]oestradiol (~ 8000 cpm) at 4°C for 18–20 h. Ice-cold charcoal–dextran was then added to achieve separation of bound from free labelled hormone. Tubes were centrifuged at 1500 g at 4°C for 11 min, and the supernatants were collected and counted in a β -counter after the addition of scintillation fluid. Total and non-specific binding were 28.7 and 2.7% respectively. The detection limit of the assay (ED_{90}) was 10.5 pmol/l. The intra-assay CV was $11.3 \pm 1.5\%$. Serial dilutions (from 1:160 to 1:2560) of randomly selected follicular fluid samples, assayed for oestradiol, generated a curve parallel to the standard curve.

The concentration of progesterone in the follicular fluid was measured directly, without extraction, by radioimmunoassay using an antiserum (CU 413/1; Analytical Antibodies). Aliquots (100 μ l) of either follicular fluid, diluted 1:8000, or standards were incubated with 100 μ l of antiserum (final dilution 1:6000) and 100 μ l of [3 H]progesterone (~ 6000 cpm) at 4°C for 18–20 h. Separation of bound from free labelled hormone was achieved by the addition of charcoal–dextran. Samples were then centrifuged at 1500 g at 4°C for 11 min, and the supernatants were collected and counted in a β -counter. Total and non-specific binding were respectively 43 and 8.3%. The ED_{90} was 0.79 nmol/l and the intra-assay CV was $10.8 \pm 1.9\%$. Serial dilutions (1:400 to 1:12 800) of randomly selected follicular fluid samples assayed for progesterone resulted in a curve parallel to the standard curve.

Total testosterone was measured directly, without extraction, by radioimmunoassay using a kit purchased from Immunotech International (Marseille, France). Samples were assayed after a 1:5 dilution. The ED_{90} was 0.11 nmol/l. The intra-assay CV was $4.2 \pm 1.03\%$.

Androstenedione was measured by radioimmunoassay using a kit purchased from Immunotech International. Samples were extracted with ethyl ether and assayed after 1:10 dilution. The ED_{90} was 0.19 nmol/l. The intra-assay CV was $2.8 \pm 0.91\%$.

Analysis of data

Results were presented as mean \pm SEM. Statistical analysis was performed by unpaired Student's t -test, or χ^2 test, as appropriate.

Table 1. Follicular fluid concentrations (nmol/l) of progesterone, total testosterone and androstenedione in patients with immunological and tubal factor (control) infertility

	Control (n = 5, m = 26)	Immunological (n = 6, m = 25)
Progesterone	16 133.6 \pm 1.668.4	21 819.8 \pm 3175.3
Total testosterone	3.02 \pm 0.36	3.42 \pm 0.37
Androstenedione	43.6 \pm 4.21	41.2 \pm 2.6

n = number of patients in the group; m = number of follicular fluid samples.

Correlation between concentrations of various hormones in the follicular fluid were performed by Pearson's correlation test.

Results

TNF α concentrations in the follicular fluid of patients with immunological infertility were significantly higher than those measured in control patients ($P < 0.05$, *t*-test; Figure 1, upper panel). On the other hand, the concentrations of oestradiol in the follicular fluid of women affected by immunological infertility were lower than those measured in the control group ($P < 0.05$, *t*-test; Figure 1, lower panel). TNF α concentrations ranged from undetectable values (24% of the follicles) to 9.05 pmol/l in one patient in the test group. In contrast, TNF α concentrations fell below the detection limit of our assay (0.68 pmol/l) in the majority (89%) of follicular fluid samples collected from control subjects.

Follicular fluid concentrations of progesterone, testosterone and androstenedione were not significantly different in patients affected by immunological infertility compared to women bearing a tubal factor of infertility (Table I). In addition, TNF α values were not significantly correlated with concentrations of either progesterone, testosterone, or androstenedione in both groups of infertile women (data not shown). Interestingly, the positive correlation observed between concentrations of oestradiol and testosterone in the follicular fluid of control patients ($r = 0.83$; $P < 0.001$) was not present in patients affected by immunological infertility ($r = -0.01$; not significant). The ratios between testosterone/oestradiol and androstenedione/oestradiol (0.03 ± 0.006 and 0.249 ± 0.05 respectively) in patients with immunological infertility were significantly higher ($P < 0.005$) than those found in control patients (0.0048 ± 0.0005 and 0.0725 ± 0.0071 respectively).

The rate of fertilization of ova from the follicles included in this study was 19.1% in patients with immunological infertility and 57.1% in patients affected by tubal infertility ($P = 0.03$, χ^2 test). The mean number of oocytes aspirated was similar in both groups of patients (7.17 ± 0.31 and 6.6 ± 0.98 respectively).

Discussion

TNF α has been regarded as one of the most relevant factors in the pathophysiology of ovarian function (Adashi, 1990). Previous reports have shown the presence of immunoreactive TNF α in the follicular fluids of stimulated human ovaries (Barak *et al.*, 1992; Jasper and Norman, 1995). This is the

first study showing the presence of elevated concentrations of TNF α in the follicular fluid of patients with immunological infertility. In contrast, TNF α was undetectable in the follicular fluids of patients with tubal infertility (Barak *et al.*, 1992). Elevated follicular fluid concentrations of TNF α were associated with reduced concentrations of oestradiol and increased androgens/oestradiol ratios. Interestingly, the positive correlation observed in the control group between follicular fluid oestradiol concentrations and testosterone was lost in patients with immunological infertility, suggesting that the observed high concentrations of TNF α may influence aromatase activity. Indeed, TNF α has been shown to be capable of inhibiting the activity of this enzyme induced by FSH in the rat (Emoto and Baird, 1988), and the production of oestrogens by human granulosa cells in cultures (Best *et al.*, 1994). In addition, Zolti *et al.* (1992) showed that follicles of patients affected by polycystic ovarian syndrome, with high concentrations of TNF α , contained minimal amounts of oestradiol. Whether the suppression of oestradiol production induced by TNF α involves a direct inhibition of the aromatase enzyme or is mediated by an inhibition of FSH binding to its receptor (Adashi *et al.*, 1989) is a matter for debate.

The fertilization rate of patients with immunological infertility was lower than that found in patients with tubal infertility. Whether this effect is due to the elevated concentrations of TNF α in the follicular fluid or is mediated by the low intrafollicular oestradiol concentrations (Tesarik and Mendoza, 1995) is not clear at the present time. It should also be taken into account that the lower fertilization rate may result from the tissue damage brought about by the immune response (Munck *et al.*, 1984). Indeed, patients with antisperm antibodies, in serum and/or in the cervical mucus, or with antibodies against zona pellucida in their follicular fluid have a lower fertilization rate and reduced early embryonic development (Vazquez-Levin *et al.*, 1991; Papale *et al.*, 1994).

In conclusion, although the mechanisms of interaction between cytokines and ovarian function are still unclear, we found that patients with immunological infertility have increased concentrations of TNF α and low concentrations of oestradiol in their follicular fluids. This is in line with the role proposed for cytokines in the pathophysiology of ovulatory and fertilization phenomena (Adashi, 1990).

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