Immunoreactive endothelin-1, endothelin-2 and big endothelin-1 in follicular fluids of women undergoing ovulation induction for in-vitro fertilization

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Endothelin-like immunoreactivity specific for endothelin-1 (ET-1), endothelin-2 (ET-2) or big endothelin-1 (big ET-1) was measured, using commercially available radioimmunoassay kits, in follicular fluid collected at the time of oocyte aspiration from 36 women undergoing ovulation induction by human menopausal gonadotrophin (HMG). The relationship of ET concentrations to HMG dose, peak serum oestradiol concentration, the number and size of follicles (by ultrasound), the number of retrieved oocytes and the fertilization rate per retrieved oocyte were studied. Overall, 94% of follicular fluid samples were positive for ET-1, 92% were positive for ET-2, and 100% were positive for big ET-1. Mean ET-1, ET-2 and big ET-1 concentrations were 17.23 ± 12.20, 32.42 ± 14.32 and 34.55 ± 16.34 pg/ml respectively. Endothelin-like immunoreactivity in follicular fluid samples was found in an order of ET-1 < ET-2 < big ET-1. There was a highly significant positive correlation (r = 0.8711, P = 0.0001, n = 32) between follicular ET-1 and ET-2 concentrations. No significant correlation of follicular big ET-1 was established either with ET-1 or ET-2. However, big ET-1 was found to be negatively correlated with number of oocytes (P = 0.03) and number of follicles (P = 0.04). Control plasma ET-1 and follicular ET-1 were not significantly different. There was no significant correlation between ET concentrations and any of the other studied parameters. The results demonstrated that immunoreactive ET-1, ET-2 and big ET-1 exist in human follicular fluid collected at the time of oocytes retrieval for in-vitro fertilization and may be involved in the regulation of reproductive function. The clinical significance and physiological role of follicular fluid ET deserve further studies.

Key words: big endothelin-1/endothelin-1/endothelin-2/IVF/OHSS

Introduction

Endothelin (ET), a 21-amino acid endothelium-derived peptide first described by Yanagisawa et al. (1988), induces vasoconstriction in a variety of vascular beds, possibly by directly or indirectly modulating vascular smooth muscle dihydropyridine-sensitive calcium channels or by activating other pathways of transmembrane signalling (Simonson et al., 1989). Three isoforms, denoted as endothelin-1 (ET-1), endothelin-2 (ET-2) and endothelin-3 (ET-3) with different biological activities have been identified by screening of the human genomic library (Inoue et al., 1989). Active ET-1 is derived from further cleavage of big ET-1 by a putative ET-converting enzyme (Sawamura et al., 1990). ET have been found in a variety of biological fluids and tissues, and ET receptors are widespread in uterus, endometrium, myometrium, placenta and amnion (Yanagisawa and Masaki, 1989; Cameron and Davenport, 1992; Word et al., 1992; Rubanyi and Polokoff, 1994). Economos et al. (1992) have demonstrated that ET-1 expression occurs in two endometrial cell types, i.e. glands and stroma, and that there is modulation of precursor message throughout the ovarian/menstrual cycle. From a microvascular point of view, the uniform events of cyclic menstrual shedding and regrowth of the endometrium have been generally ascribed to the actions of prostaglandins and growth factors respectively. Vasoconstriction is an important process to prevent haemorrhage at the time of menses, and vasodilation is necessary to maintain adequate blood flow to the uterus and endometrium during the establishment of pregnancy (Giudice and Saleh, 1995).

In the uterus, ET-1 is produced and released from endometrium but not from myometrium (Orlando et al., 1990). It has also been reported that ET-1 or ET-3 can stimulate preovulatory follicles to release progesterone, testosterone and oestradiol, and oestradiol release increases the density of ET receptors on myometrium (Maggi et al., 1991; Usuki et al., 1991a). ET-1 has been found in human seminal fluid in relatively high amounts and correlated inversely with gonadotrophin concentrations (Casey et al., 1992; Hammami et al., 1994). Recently, ET-like immunoreactivity has been found in porcine and human follicular fluid and in cultures of granulosa cells (Iwai et al., 1991; Kamada et al., 1993; Abac et al., 1994). Human uterine smooth muscle has been shown to respond to ET-1 with forceful contractions (Garfield et al., 1990). Human placenta expresses prepro ET-1 gene and secretes big ET-1, ET-1 and ET-3 peptides and very low amounts of ET-2 (Benigni et al., 1991). Immunoreactive ET has been identified in the corpus luteum of superovulated rats (Usuki et al., 1991b). ET-1 is secreted by endometrial cells (Orlando et al., 1990), increasing intracellular calcium and inducing myometrial contraction (Word et al., 1990). Kubota et al. (1995) demonstrated ET-1 synthesis and the presence of receptors in human endometrium throughout the normal...
menstrual cycle and further suggested that ET-1 may have a potential autocrine and/or paracrine function in human stromal cells. While the effects of ET on endocrine tissues remain unclear in many cases, evidence suggests that they may play an important part in a complex network of autocrine and paracrine factors which modulates the actions and production of classical endocrine hormones (Kennedy et al., 1993).

Human menopausal gonadotrophin (HMG) has been successfully used to induce multiple follicular development in normally ovulating women for the purpose of oocyte harvesting and in vitro fertilization (IVF) programmes (Jones et al., 1982; Laufer et al., 1983). Follicular factors, including the number of follicles, follicular diameter and serum hormone concentrations as indirect measurement of follicular maturation, have been thought to influence the pregnancy rate (Lopata, 1983).

In the present study, we measured the concentrations of ET-1, ET-2 and big ET-1 in follicular fluids and their significance in relation to the number of follicles, size of follicles, number of oocytes, oestradiol concentrations and oocyte fertilization of women undergoing ovulation induction for IVF with the administration of HMG.

Figure 1. Relationship between the levels of endothelin (ET)-1 and ET-2. Each point represents the ET concentrations in one follicular fluid sample. Results are the average of duplicate measurements ($r = 0.8711, P = 0.0001, n = 32$).

Figure 2. Relationship between the concentrations of big endothelin (ET)-1 and number of oocytes. Each point represents one patient. Results are the average of duplicate measurements ($r = -0.3598, P = 0.031, n = 36$).

Materials and methods

Subjects
Follicular fluids were obtained from 36 women during oocyte retrieval as previously reported (Lee et al., 1991) as part of the IVF programme being conducted at the King Faisal Specialist Hospital and Research Centre, Riyadh, Saudi Arabia. All women were <41 (range 22–41) years of age, had spontaneous ovulatory cycles without hyperprolactinaemia and had completed routine infertility investigations, including laparoscopy. The indications for IVF were tubal damage ($n = 22$), male factor ($n = 8$) and unexplained ($n = 6$). Blood samples from 30 normal controls (males) were collected into vacutainer tubes containing ethylenediaminetetraacetic acid. These were then centrifuged at 3500 g for 5 min, after which the plasma was separated, transferred to sterile polypropylene tubes and frozen at −70°C until used for the assay.

Protocol
Cycles were monitored by measurements of serum oestriol and transvaginal ultrasonography. The follicular growth was induced with 225 IU of HMG (Humegon; Organon, Oss, The Netherlands) administered on day 3 of the cycle; the dose was increased in a stepwise manner, depending on the individual response. Ovulation was induced by i.m. injection of 10 000 IU of human chorionic gonadotrophin (HCG, Profasi; Serono, Randolph, MA, USA) when at least two follicles >15 mm in diameter and serum oestradiol concentrations from 3000 to 20 000 pmol/l were found. Oestradiol concentrations were determined according to the protocol of the manufacturer (Kodak Clinical Diagnostics Ltd., Amersham, UK). Aspiration of the leading follicles was performed using a single lumen 16-gauge needle, and oocytes were identified, removed from the follicular fluid and processed for fertilization. The follicles were not flushed with any medium. After identification and removal of the oocyte, the condition of the follicular fluid was assessed. Cleared follicular fluid was used for ET assay, while grossly blood-contaminated follicular fluid was discarded. Follicular supernatant was then separated by centrifugation at 800 g for 10 min, transferred to sterile polypropylene tubes and frozen at −70°C until assay. Prior to embryo transfer, each embryo was re-evaluated at 48 h after aspiration for cleavage and development. The number of embryos replaced in the uterus ranged from one to five.

Radioimmunoassay
Immunoreactive concentrations of ET-1, ET-2 and big ET-1 were measured using commercially available kits (Peninsula Laboratories, Belmont, CA, USA). Radioimmunoassay of ET in follicular fluids was performed essentially as reported previously (Abac et al., 1994). The assay is based on the competition of 125I-labelled peptide and unlabelled peptide (either standard or unknown) for binding to the limited quantity of antibodies specific for the standard peptide in each reaction mixture. Relative to human ET-1 (100%), the antisera had stated cross-reactivities with human ET-2 and big ET-1 of 7 and 17% respectively. The radioimmunoassay was performed according to the instructions of the manufacturer using 125I-labelled ET-1, ET-2 and big ET-1 as bound tracers. The standard curves with these ET encompassed a range of 1–128 pg per tube. All the samples were performed in duplicate and, after plotting % B/B0 for each standard directly on the y-axis and ET concentrations on the x-axis, the ‘best fit’ curve was drawn and the amounts of ET were calculated.

Statistical analysis
Data are expressed as the mean ± SD. Pearson correlation was used to calculate the relationship between various variables. Significance
fertilization after ovulation induction with HMG follicular fluids and other parameters studied in women undergoing in-vitro fertilization.

Immunoreactive ET-1, ET-2 and big ET-1 concentrations in follicular fluids and other parameters studied in women undergoing in-vitro fertilization after ovulation induction with HMG.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Observations (n)</th>
<th>Mean (±SD)</th>
<th>Range (min-max)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ET-1 (pg/ml)</td>
<td>34</td>
<td>17.23</td>
<td>12.19</td>
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<td>ET-2 (pg/ml)</td>
<td>33</td>
<td>32.42</td>
<td>14.32</td>
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<tr>
<td>Big ET-1 (pg/ml)</td>
<td>36</td>
<td>34.55</td>
<td>16.34</td>
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<td>Oestradiol (pmol/l)</td>
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<td>10.203 - 44.54</td>
<td>2936-20 892</td>
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<td>HMG (IU)</td>
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<td>93</td>
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<tr>
<td>Number of follicles</td>
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<td>5.64</td>
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<tr>
<td>Size of follicles (mm)</td>
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<tr>
<td>Number of oocytes</td>
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<td>9.22</td>
<td>6.60</td>
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<tr>
<td>Fertilized oocytes</td>
<td>36</td>
<td>4.94</td>
<td>4.81</td>
</tr>
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ET-1 = endothelin-1; ET-2 = endothelin-2; big ET-1 = big endothelin-1; HMG = human menopausal gonadotrophin.

Discussion

In this study we demonstrated the presence of immunoreactive ET-1, ET-2 and big ET-1 in the follicular fluid of human ovary. The immunoreactivity of ET-1 in follicular fluid was positively correlated with that of follicular ET-2. Follicular big ET-1 was negatively but significantly correlated with the number of follicles as well as with the number of oocytes. The concentrations of ET measured were in an order of ET-1 < ET-2 < big ET-1 in human follicular fluid. The immunocytochemical localization of ET immunoreactivity in basal endometrium suggests that these potent peptides may not only have a role in endometrial vasoconstriction, but may also be involved in the mediation of uterine contractions, endometrial proliferation and implantation (Cameron et al., 1992). ET-1 derived from endometrial stroma cells may participate in processes leading to menstruation. ET-1 secreted by these cells may gain access to the adventitial surface of the spiral arterioles and cause vasospasm of these vessels.

In the follicular phase (Schiff et al., 1993), ET-1 correlates with oocyte fertilization or the hormonal ovarian response during the follicular phase. ET-1 and big ET-1 in follicular fluid may function, usually at the time of ovulation, to modulate uterine contractions that may affect the transport of spermatozoa to the upper female reproductive tract. In the reproductive system, ET appears to correlate with oocyte fertilization or the hormonal ovarian response during the follicular phase. ET-1 and big ET-1 in follicular fluid may function, usually at the time of ovulation, to modulate uterine contractions that may affect the transport of spermatozoa to the upper female reproductive tract.
act on ovarian and uterine cells as a regulator of hormone action and may be a local mediator or modulator similar to cytokines. The vasopressor effect of ET has been documented in several systems, including human placenta, uterine vasculature and myometrium (Wilkes et al., 1990; Mombouli et al., 1993; Rae et al., 1993). ET could play an important role in modulating ovarian circulation in physiological and pathological states such as ovarian hyperstimulation syndrome (OHSS). OHSS is known to be associated with elevated oestrogen concentrations and high numbers of follicles (Dhont et al., 1995). Our findings of a negative correlation (not significant) of big ET-1 with the oestradiol concentration, the number of follicles and the number of oocytes could suggest that a maintenance pressor threshold sustained by ET is lost after their concentrations decline in association with an elevation of oestradiol concentration, number of oocytes and number of follicles. This then leads to vasodilation and increased vasculature permeability, causing OHSS. Our data concerning follicular big ET-1 are also comparable to those of Polderman et al. (1993), who showed that sexual steroid hormones (17β-oestradiol and testosterone) have opposite effects on immunoreactive ET. 17β-Oestradiol increased the density of ET receptor on myometrium; however, it did not stimulate the increase in ET receptor of myometrium in the presence of progesterone (Maggi et al., 1991). Very recently it has been reported that 17β-oestradiol stimulates prostacyclin, but not ET-1, production in human vascular endothelial cells (Mikkola et al., 1995). In the present study, we were unable to see any significant correlation of oestradiol concentrations with follicular ET (ET-1, ET-2 and big ET-1). Since 17β-oestradiol did not modify directly the density of ET-1 binding sites in vitro, the increase in ET-1 binding sites in vivo may be mediated by other factors.

It appears that ET play both a central role in the secretion of gonadotrophin in the pituitary gland and a peripheral role in modulating gonadotrophins and sex steroids at the ovary or uterus (Masaki, 1993). Our data showed no correlation of follicular ET-1 and ET-2 with follicular size, number of oocytes or fertilization. These findings are inconsistent with an earlier report (Kamada et al., 1993) that ET-1 might be related to follicular development and significantly correlated with the size of follicles. Further experiments are needed to understand more of the mechanism of action of follicular ET. The concentrations of ET-1 in human follicular fluid and plasma reported by Abae et al. (1994), 37 and 22 pg/ml respectively, are comparable to our present findings. However, ET-1 concentrations in human follicular fluid reported by Kamada et al. (1993) and Kubota et al. (1994) were much higher (>500 pg/ml) than those detected by us (17.23 pg/ml) (Table I). Apparent discrepancies in circulating plasma or follicular fluid concentrations of immunoreactive ET may be due to the numerous and various assay systems used, particularly with respect to differences in sensitivity between commercially available polyclonal antibodies used in radioimmunoassay and other isopeptides (ET-2) and/or precursor big ET-1. ET-1 antiserum cross-reactivity to big ET-1 and ET-2 ranges from <10 to 100% in various assays. Another reason for such discrepancies may be the use of different extraction methods (C18/C8/C2 columns).

Potential mechanisms by which ET-1 biosynthesis and action may be regulated in endometrium include regulation of prepro ET-1 transcription, modulation of prepro ET-1 mRNA concentrations by way of ribonuclease activity and, finally, regulation of the conversion of big ET-1 to ET-1. Prepro ET-1 is synthesized as a 212-amino acid precursor, which is processed in the cell of synthesis to big ET-1 (38 amino acids) and thenceforth to ET-1 (the bio-active, 21-amino acid peptide; Economos et al., 1992). It has also been reported that big ET-1 was correlated with ET-1 from cultured endothelial cells and existed in the circulating blood in amounts larger than those of ET-1. Our present study also shows the amount of big ET-1 (34.55 pg/ml) to be double that of ET-1 (17.23 pg/ml) in human follicular fluids. The present investigation has confirmed the previous studies demonstrating that ET-1 exists in ovarian follicular fluid and has shown that ET-2 and big ET-1 are also present. Further work must be performed to elucidate the origin and metabolism of ET in human follicular fluid and their pathophysiological importance. This in turn will enable the evaluation of their biological function, including their role in the complex homeostatic mechanism of the organ, their potential involvement in autocrine, paracrine and endocrine signalling processes, and their role as modulators, especially of steroid biosynthesis in pre-ovulatory follicles. 

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References


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