Possible contribution of follicular interleukin-1β to nitric oxide generation in human pre-ovulatory follicles

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The aim of this study was to investigate the relationships between follicular nitric oxide (NO) metabolite concentrations and several related variables, with special reference to follicular interleukin-1β (IL-1β). The follicular fluid from the leading and secondary follicles was collected individually from 20 women undergoing in-vitro fertilization (IVF) treatment, and the concentrations of nitrite (NO$_2^-$) and nitrate (NO$_3^-$) were determined fluorometrically using 2,3-diaminonaphthalene. Both follicular nitrite ($r = 0.42, P < 0.01$) and nitrate ($r = 0.49, P < 0.001$) were found to be significantly correlated with follicular IL-1β concentrations. There were also significant positive correlations between follicular nitrate and the number of oocytes retrieved ($P < 0.01$) and serum oestradiol concentration on the day of human chorionic gonadotrophin (HCG) administration ($P < 0.05$). When follicular cells were incubated in vitro with 10 ng/ml of IL-1β for 24 h, nitrate generation was significantly ($P < 0.01$) elevated compared with the control. In conclusion, our study demonstrates that follicular IL-1β and the number of developing follicles are significant variables that affect follicular NO concentrations, and points to the possible contribution of IL-1β to NO generation in human pre-ovulatory follicles.

**Key words:** follicular fluid/interleukin/IVF/nitric oxide/ovarian follicle

**Introduction**

Recently, the importance of nitric oxide (NO) as an intra- and intercellular mediator has become recognized in a variety of biological processes, including ovarian physiology (Moncada et al., 1991). During the process of follicular atresia in the rat ovary, interleukin-1β (IL-1β) suppresses follicular cell apoptosis, and this action appears to be mediated by NO (Chun et al., 1995). In cultured human luteal cells, the addition of a NO donor significantly inhibits production of oestradiol and progesterone from the luteal cells, suggesting that NO is a possible autocrine regulator of steroidogenesis (Van Voorhis et al., 1994). NO has been implicated in the ovulatory process, because ovulation involves changes that are common to inflammation (Espey, 1980), and NO is known to be a possible mediator of several inflammatory reactions (Moncada et al., 1991). In a primary culture of rat ovarian dispersates, IL-1β, which appears to play an intermediary role during ovulation, induces expression of the inducible isoform of NO synthase (Elluman et al., 1993). Administration of NO synthase inhibitors suppresses the human chorionic gonadotrophin (HCG)-induced ovulation of immature rat in a dose-dependent manner, whereas concomitant administration of a NO donor completely reverses this effect (Shukovsky and Tsafiriri, 1994).

The inhibitory effect of NO synthase inhibitors on ovulation is also observed in in-vitro isolated, perfused rabbit ovaries (Hesla et al., 1997).

The suggestion that NO may be involved in the mechanisms of ovulation or follicular maturation has raised interest in the dynamics and regulation of NO in pre-ovulatory follicles. So far, two studies have investigated the relationships between the concentration of NO metabolites in human pre-ovulatory follicles and other variables, including follicular fluid volume, sex steroid concentrations in the follicular fluid, ovarian artery and intra-ovarian blood flow as measured by Doppler ultrasound (Antebi et al., 1996), and follicular cell apoptosis (Sugino et al., 1996). In vivo, NO is formed from L-arginine either by a constitutive calcium-dependent, or a pro-inflammatory cytokine-inducible, NO synthase. Endothelial cells in the blood vessels possess the endothelial form of NO synthase, and transcripts of this enzyme have also been identified in human luteal cells (Van Voorhis et al., 1994). On the other hand, inducible NO synthase is present in leukocytes, which commonly reside in the ovarian follicles (Loukides et al., 1990). Human follicular fluid contains various cytokines (Khan et al., 1988; Wang and Norman, 1992), and certain cytokines appear to contribute to NO generation in ovarian follicles by activation of inducible NO synthase. However, little information is available about the relationships between follicular NO generation and cytokines in the human pre-ovulatory follicles.

The aim of this study was to determine the concentrations of the NO metabolites, nitrite (NO$_2^-$) and nitrate (NO$_3^-$), in the follicular fluid in patients undergoing in-vitro fertilization (IVF) treatment, and to investigate their relationships with several variables, including the intra-follicular milieu and patient characteristics, with special reference to follicular IL-1β.

**Materials and methods**

**Follicular fluid collection**

Follicular fluid samples were obtained by follicle aspiration from 20 women participating in the IVF programme of Akita University.
Hospital, Japan. The mean age of the patients was 32.5 ± 3.3 years (range 28–35 years), and the patients included two women with polycystic ovary syndrome. In each patient, the follicular fluids of an apparently leading follicle and a secondary follicle were individually aspirated in conjunction with oocyte retrieval. At each collection time, the volume of fluid and the presence of oocyte was recorded for each follicular fluid sample. After oocyte isolation, the follicular fluids were centrifuged at 3000 g for 10 min to remove debris, blood and granulosa cells, and then frozen at −20°C until assay. Follicular fluids that were contaminated with significant quantities of blood cells were not used for analysis.

Method of ovarian stimulation for IVF

All ovarian stimulations for IVF were performed with a standard mid-luteal administration of a gonadotrophin-releasing hormone agonist (GnRHα, buserelin acetate; Hoechst Japan, Tokyo, Japan) and follicle stimulating hormone (FSH, Fertinorm; Serono Japan, Tokyo, Japan). GnRHα (300 µg) was administered intranasally every 8 h, beginning in the mid-luteal phase of the woman’s previous cycle, and ovarian stimulation with gonadotrophins was started on the second day of the cycle. The dose of FSH was adjusted to allow for individual ovarian response (usually two to three ampoules per day). When the leading follicle reached 17 mm in diameter, the administration of GnRHα and FSH was stopped, and 10 000 IU of HCG was administered. After 34 h following the HCG injection, oocytes were recovered by transvaginal echoguided follicle aspiration.

Measurement of nitrite

Concentrations of nitrite were determined by the fluorometric assay reported by Misko et al. (1993), with minor modifications. After thawing, each sample was filtered through a 10 000 Mr cut-off filter (Centricon 10; Amicon Inc., Beverly, MA, USA) by centrifugation at 10 000 g for 1 h at 4°C to remove haemoglobin resulting from cell lysis. This step is important because very small amounts of haemoglobin can interfere with the fluorescent signal of 2,3-diaminonaphthalene. After filtration, 20 µl of follicular fluid was mixed with 40 µl of deionized water and 10 µl of freshly prepared 2,3-diaminonaphthalene. Under acidic conditions, the 2,3-diaminonaphthalene reacts with nitrite to form 1-(H)-naphthoriazole, a fluorescent product. After incubation for 10 min at 20°C, 8 µl of 2.8 N NaOH was added to stop the reaction. Aliquots of 50 µl of samples were diluted in 1950 µl distilled water (total volume 2 ml), and the concentration of naphthiorazole was measured by a fluorometer at an excitation wavelength of 365 nm and emission of 450 nm. All chemicals used for the assay were purchased from Sigma Chemical Co. (St Louis, MO, USA).

A nitrite standard was routinely made fresh, dissolved in distilled water, and kept on ice prior to use. The 2,3-diaminonaphthalene agent was protected from light during the assay. Intra-assay and inter-assay variations were <15%. All samples were measured in the same assay to reduce variation.

Measurement of nitrate

In order to measure nitrate concentrations, the nitrate in each sample was first converted to nitrite by the action of nitrate reductase (from Aspergillus niger). For this purpose, 20 µl of follicular fluid after filtration was incubated with 8 µl of 500 mM α-nicotinamide adenine dinucleotide phosphate (NADPH, Sigma Chemical Co.), 8 µl of 1 IU/ml nitrate reductase, and 24 µl of distilled water to a total volume of 60 µl for 10 min at 20°C. Since follicular fluid contains variable amounts of nitrate reductase inhibitors, such variations were controlled by a previously described method (Reveł et al., 1996). Briefly, three tubes, one of which contained the original sample and the other two which contained different concentrations (25 and 50 µM) of exogenous nitrate, were prepared for each sample. From the recovered nitrite concentration, determined in each tube, a graph of nitrite input versus recovery was constructed for each sample. From the graph, the value of nitrate plus nitrite was determined by extrapolation of the line to zero recovery, which gave a negative value for the initial sample concentration. The nitrate concentration of each sample was then calculated by subtracting the nitrite concentration from the total NO metabolite (nitrite plus nitrate) concentrations measured by the above method.

Follicular cell isolation and in-vitro culture

Follicular fluid samples obtained from several IVF patients were collected and subjected to centrifugation for 5 min at 200 g. Follicular aspirates, recovered from all follicles with a diameter of >10 mm from several patients, were mixed together, and used for each experiment. The cell pellets were suspended in phosphate–bicarbonate solution containing 0.1% hyaluronidase, and incubated at 37°C for 15 min. After incubation, the cells were placed on a 50% Percoll cushion, and subjected to centrifugation for 10 min at 400 g. The cell pellets were washed twice in phosphate–bicarbonate solution and resuspended in GIT medium (serum-free, Wako Chemicals, Osaka, Japan). Approximately 5×10⁶ cells in 500 ml GIT medium were placed in each well of a collagen-coated 24-well multi-well plate (Celltight C1 Plate 24F; Sumitomo Bakelite Co, Tokyo, Japan). The follicular cells were incubated with or without IL-1β (Sigma Chemical Co.) at 37°C with an atmosphere of 5% CO₂ in air. After 24 h culture, the medium was collected and, after centrifugation, the supernatant was stored frozen at −80°C for determination of nitrate according to the method described above. In each experiment, NO₃⁻, nitro-L-arginine (Sigma Chemical Co), an inhibitor of NO synthase, was added to several wells in parallel at a final concentration of 500 µM, in order to inhibit NO generation in the wells. The concentration of nitrate generated in each sample was calculated by subtracting the concentration of nitrate in the medium containing the NO synthase inhibitor from the total nitrate concentration of the medium.

Measurement of oestriadiol, progesterone and IL-1β

Progesterone and oestradiol were determined using radioimmunoassay kits purchased from Nippon DPC Corporation (Tokyo, Japan). IL-1β was determined using a commercially-available immunoenzymometric assay kit purchased from Medgenix Diagnostics SA (IL-1β ELASIA™ kit; Fleurus, Belgium). The intra- and inter-assay coefficients of variance in these assays were <15%.

Statistical analysis

Data were expressed as mean ± SE. The values between the leading and secondary follicles were compared by Student’s t-test. Correlations were evaluated both by linear regression analysis and F-test. The nitrate concentrations among groups in the in-vitro culture were compared by one-way analysis of variance, followed by Scheffe’s test for multiple comparisons. P < 0.05 was considered to be significant. Statistical analysis was performed using the StatView 4.0 (Abacus Concepts, Berkely, CA, USA) statistical package on an Apple Macintosh personal computer (Apple Computers Incorporated, Cupertino, CA, USA).

Results

The concentrations of nitrite and nitrate in follicular fluid were determined to be 1.1 ± 0.1 and 50.8 ± 7.9 µM respectively, with a significant correlation (r = 0.45, P < 0.003) between
Table I. Concentrations of nitrite, nitrate, sex steroids, and interleukin-1β in the follicular fluid of leading and secondary follicles*. Values are expressed as means ± SEM

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Leading (n = 20)</th>
<th>Secondary (n = 20)</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of oocytes recovered</td>
<td>16</td>
<td>15</td>
<td>–</td>
</tr>
<tr>
<td>Follicular volume (ml)</td>
<td>4.4 ± 0.3</td>
<td>2.6 ± 0.1</td>
<td>P &lt; 0.0001</td>
</tr>
<tr>
<td>(3.0–7.2)</td>
<td>(1.5–3.4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nitrite (µM)</td>
<td>1.0 ± 0.1</td>
<td>1.2 ± 0.1</td>
<td>NS</td>
</tr>
<tr>
<td>Nitrate (µM)</td>
<td>44.8 ± 12.6</td>
<td>56.8 ± 9.7</td>
<td>NS</td>
</tr>
<tr>
<td>Oestradiol (ng/ml)</td>
<td>1890 ± 140</td>
<td>2260 ± 180</td>
<td>NS</td>
</tr>
<tr>
<td>Progesterone (ng/ml)</td>
<td>10470 ± 1030</td>
<td>7500 ± 760</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>Interleukin-1β (pg/ml)</td>
<td>121 ± 10</td>
<td>146 ± 12</td>
<td>NS</td>
</tr>
</tbody>
</table>

*Follicular fluid samples were obtained by follicle aspiration from 20 women participating in the in-vitro fertilization programme. In each patient, follicular fluids of an apparently leading follicle and a secondary follicle were individually aspirated in conjunction with oocyte retrieval. NS = not significant.

Table II. Correlations between nitric oxide metabolites (nitrite and nitrate) and follicular fluid volume, and concentrations of steroid hormones and interleukin-1β in the follicular fluid (n = 40). Values are correlation coefficients with P values given in parentheses

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Nitrite</th>
<th>Nitrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Follicular fluid volume</td>
<td>−0.27 (0.10)</td>
<td>−0.23 (0.16)</td>
</tr>
<tr>
<td>Oestradiol</td>
<td>0.24 (0.14)</td>
<td>0.15 (0.35)</td>
</tr>
<tr>
<td>Progesterone</td>
<td>−0.22 (0.17)</td>
<td>−0.19 (0.25)</td>
</tr>
<tr>
<td>Interleukin-1β</td>
<td>0.42 (0.007)</td>
<td>0.49 (0.001)</td>
</tr>
</tbody>
</table>

these two NO metabolites. The mean ± SE of follicular fluid volume, and the follicular concentrations of nitrite, nitrate, oestradiol, progesterone, and IL-1β in the leading and secondary follicles are presented in Table I. The concentrations of progesterone in the leading follicles were significantly (P < 0.05) higher than in the secondary follicles. No significant difference was found in the concentrations of nitrite or nitrate between the two follicle groups.

The correlations between the follicular NO metabolites (nitrite and nitrate) and follicular fluid volume, and the concentrations of oestradiol, progesterone or IL-1β, are presented in Table II. There were significant positive concentrations between both follicular nitrite or nitrate with IL-1β concentrations (Table II and Figure 1; r = 0.42, P < 0.01 between IL-1β and nitrite; and r = 0.49, P < 0.001 between IL-1β and nitrate).

The correlations between NO metabolite concentrations in the leading follicles and the characteristics of IVF patients, including age, the number of oocytes recovered, and the concentrations of oestradiol on the day of HCG, are shown in Table III. There were significant positive correlations between follicular nitrate concentrations and both the number of oocytes retrieved (P < 0.01) and the serum oestradiol concentrations on the day of HCG (P < 0.05).

The concentrations of nitrate generated after a 24 h culture period of follicular cells with various concentrations of IL-1β are shown in Figure 2. When follicular cells were incubated with 10 ng/ml IL-1β, there was a significant (P < 0.01) increase in the generation of nitrate above that of the control value. The concentrations of nitrite were not measured in this in-vitro experiment as the concentrations of nitrate were 50 times greater than that of nitrite and we believed that nitrate was the major NO metabolite.

Discussion

As NO is a highly reactive radical oxygen species, its direct measurement in-vivo is practically impossible. Instead,
Nitric oxide in follicular fluid

Figure 2. Amounts of nitrate generated after a 24 h culture period of follicular cells with various concentrations of interleukin-1β (IL-1β). Values are expressed as the mean ± SE of four successive experiments. In each experiment, the concentrations of nitrate in the supernatant were determined in triplicate in each group. The levels of nitrate in the study groups are expressed relative to the mean (100%) nitrate level of the control group. When follicular cells were incubated with 10 ng/ml IL-1β, nitrate was increased by 60% above that of the control level (*P < 0.01; Scheffe’s multiple comparison test).

measurement of the stable major NO metabolites, nitrite and nitrate, has been used to investigate the dynamics of NO in biological fluid, such as ascites or blood (Rosselli et al., 1994; Revel et al., 1996). The concentrations of nitrite and nitrate in human follicular fluid in our present study, which were determined by a highly sensitive fluorometric assay (Misko et al., 1993), were in close agreement with those of the previous reports which were measured using a colorimetric assay (Anteby et al., 1996) or capillary zone electrophoresis (Sugino et al., 1996).

However, there were several differences in the correlation results among our report and the previous reports. Anteby et al. (1996) collected the content of two to four follicles from each IVF patient, and Pearson’s correlation coefficient values between NO metabolites and several follicular parameters were measured individually. They found significant correlations between NO metabolites and follicular fluid volume, oestradiol concentration and ultrasound ovarian flow parameters. Sugino et al. (1996) pooled all follicular fluid samples from 20 IVF patients and divided them into three groups by diameter. The large follicles contained higher amounts of oestradiol and progesterone, whereas small follicles contained higher amounts of testosterone and an increased number of luteal cells containing apoptotic bodies, an indication of atresia-related apoptosis. However, there were no significant differences in the concentrations of NO metabolites among three groups. In the present study, we collected follicular fluid samples of the largest growing follicle and a second largest follicle from each IVF patient, so that each group consisted of the same number of samples from the same patients and individual differences of the concentrations of NO metabolites could be neglected. The leading follicles contained higher amounts of follicular fluid volume and progesterone, indicating a state of advanced maturation, but the mean concentration of NO metabolites in the leading follicles was almost same as that of the secondary follicles. Therefore, our data support the result of Sugino et al. (1996), indicating that the concentrations of NO metabolites were not influenced by follicular size. In addition, we were unable to find positive correlations between follicular NO metabolites and follicular oestradiol concentrations which Anteby et al. (1996) had found, although the difference of the two studies may result from the difference of an analytical method of the correlation data.

The NO metabolic pathway in the follicular fluid remains unclear. In a similar manner to many substances present in the follicular fluid, the NO metabolites may be derived from blood plasma through the blood-follicle barrier. However, as there are several potential sources of NO in or around the ovarian follicles, it has been suggested that a part of these metabolites in the follicular fluid may be derived from NO generated in the ovary. It is well established that follicular fluid contains considerable amounts of IL-1β (Khan et al., 1988; Wang and Norman, 1992), and that 5–15% of the follicular cells, obtained from follicular fluid, are resident macrophages and monocytes (Loukides et al., 1990) containing an inducible NO synthase. Indeed, our present study indicates a significant positive correlation between the NO metabolites and IL-1β concentrations in the follicular fluid, and points to the possible contribution of IL-1β to NO generation in human pre-ovulatory follicles. When follicular aspirates were incubated with IL-1β in vitro, a significant stimulatory effect on nitrate generation was detected at a concentration of 10 ng/ml of IL-1β. The concentration of IL-1β that stimulated NO generation from follicular cells was two orders higher than the follicular fluid concentration of IL-1β. However, when cytokines stimulate inducible NO synthase, through autocrine or paracrine mechanisms in activated macrophages or other cells, the local concentration of cytokines around or within these cells may increase to that order. Recently, Zackrission et al. (1996) found elevation of immunoreactive inducible NO synthase in the granulosa cells at 6 h after the HCG injection in the rat ovaries, and the authors suggested that invasion of the leukocytes into the granulosa layer during that period may be an explanation of that result. Conversely, Van Voorhis et al. (1996) demonstrated a reduction of inducible NO synthase messenger RNA values after HCG injection. The negative regulation mechanism at the transcriptional levels may explain this discrepancy (Zackrission et al., 1996).

Endothelial, constitutive NO synthase also appears to influence follicular NO concentrations. The transcripts encoding an endothelial NO synthase were identified in human luteal cells (Van Voorhis et al., 1994). In the rat ovary, concentrations of endothelial NO synthase messenger RNA increases after gonadotrophin stimulation, and peaks at 12 h after the HCG injection (Van Voorhis et al., 1996). Immunoreactive endothelial NO synthase in the granulosa cells does not increase
after HCG injection, whereas there is a significant increase in the residual ovarian tissues (Zackrisson et al., 1996). By immunohistochemical analysis of the rat ovary, although there are signals of the endothelial NO synthase in the stroma and theca of peri-ovulatory ovaries, particularly strong signals are localized in blood vessels (Zackrisson et al., 1996). Thus, generation of NO from endothelium in the ovary is suggested to influence follicular NO concentrations during the ovulatory phase. Our present study demonstrates significant positive correlations between follicular nitrate and the number of recovered oocytes, or serum oestradiol concentrations on the day of HCG. Since these parameters are well correlated with the total number of developing follicles, the ovarian response to exogenous gonadotrophins is thought to be a significant variable of follicular NO concentrations. This correlation could be explained in two ways: (i) hyperstimulated ovaries, with a great number of growing follicles, also contain numerous blood vessels around these follicles, and therefore total NO generation in such ovaries may increase due to a quantitative increase of ovarian endothelial cells. Thus, each follicle is exposed to a relatively high amount of NO; (ii) oestrogen is reported to enhance endothelial NO generation (Hayashi et al., 1995), and concentrations of circulating NO metabolites are found to increase during the late follicular phase (Rosselli et al., 1994; Cicinelli et al., 1996). High concentrations of serum oestrogen during the late follicular phase may directly stimulate generation of NO from blood vessels within the ovary, resulting in an increased follicular NO concentration.

The role of NO in pre-ovulatory ovarian follicles remains a matter of speculation. A reduction in the ovulation rate of rats by in-vivo administration of NO synthase inhibitors indicates the relative importance of NO in the ovulatory process (Shukovsky and Tsafriri, 1994). Bonello et al. (1996) observe a marked reduction of IL-1β induced ovulation rate of the in-vitro perfused rat ovary, suggesting an involvement of NO in the pre-ovulatory pathway of IL-1β. However, although IL-1β activates cyclooxygenase enzymes, which produce prostaglandins, and induces cellular cytotoxicity in the rat ovary, these IL-1β-mediated effects are not mediated by NO (Ben-Shlomo et al., 1994). A growing body of evidence indicates that NO modulates the movement of fluids and proteins out of the vasculature (Kubes, 1995), suggesting that NO may regulate follicular fluid accumulation of the pre-ovulatory follicles towards ovulation by controlling capillary vessel permeability (Anteby et al., 1996). However our data did not support this hypothesis, because there was no difference in the NO metabolite concentrations between leading and secondary follicles, although follicular fluid in the leading follicles was greater than that in the secondary follicles. NO may participate in the periovulatory modulation of ovarian blood flow by virtue of its potent vasodilatory activity (Shukovsky and Tsafriri, 1994). It is reported that a reduction of flow rate in the in-vitro perfused rat ovary, probably as a consequence of vasoconstriction, occurs after administration of NO synthase inhibitors (Bonello et al., 1996). In addition, NO may be involved in the process of leukocyte infiltration into the pre-ovulatory follicles, which are reported to be facilitatory to the ovulatory process (Hellberg et al., 1991). In the rat ovary, s.c. administration of NO synthase inhibitor is associated with a significant reduction in the number of leukocytes in the theca cell layer at the time of ovulation (Bonello et al., 1996).

In conclusion, our current study demonstrates that follicular IL-1β and the number of developing follicles are significant variables affecting follicular NO concentrations, and point to the possible contribution of IL-1β to NO generation in human pre-ovulatory follicles. These results will certainly be important in establishing a physiological role for NO in the ovary during the ovulatory process. Investigations into the role of NO in ovarian physiology are still in their initial stages, and warrant further exploration.

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