Plasma concentrations of nitrate during the menstrual cycle, ovarian stimulation and ovarian hyperstimulation syndrome

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BACKGROUND: Nitric oxide (NO) is predominantly a locally acting mediator, affecting several functions in the human female reproductive tract. In vivo, it is quickly metabolized to its stable end product nitrate, which is cleared by the kidney. METHODS AND RESULTS: The aim of the present study was to evaluate possible fluctuations of plasma nitrate concentrations during the menstrual cycle, ovarian stimulation as well as ovarian hyperstimulation syndrome (OHSS). During the menstrual cycle (n = 19 women) the mean nitrate concentrations were between 26.7 and 29.5 µmol/l at all stages except for the day of ovulation, when the concentrations were significantly (P < 0.001) increased (mean 37.2 µmol/l ± 2.0). Significantly lower concentrations of plasma nitrate (P < 0.01) were measured at the end of gonadotrophin-releasing hormone (GnRH) down-regulation (24.6 µmol/l ± 1.4) compared with the concentrations found at day 8 of follicle-stimulating hormone (FSH) stimulation (34.9 µmol/l ± 2.6) and at the day of human chorionic gonadotrophin (HCG) (35.6 µmol/l ± 3.3). The concentrations of nitrate (33.4 µmol/l ± 3.4) in women with OHSS (n = 13) were similar to those seen 5 days after embryo transfer (33.2 µmol/l ± 2.3). CONCLUSIONS: The results indicate that NO synthesis is increased at the time of spontaneous ovulation. GnRH treatment inhibits NO synthesis, while NO production is not increased in women with OHSS.

Key words: in-vitro fertilization/menstrual cycle/nitrate/nitric oxide/ovarian hyperstimulation syndrome

Introduction

Nitric oxide (NO) is a free radical gas, which is known to be involved in diverse physiological and pathophysiological processes in various organ systems, including the human female reproductive tract (Ånggård, 1994; Rosselli et al., 1998). It is a highly reactive substance and its half-life in vivo is only a few seconds. A major metabolic pathway of endogenously formed NO is oxidation to nitrite (NO2-) and nitrate (NO3-) (Wennmalm et al., 1993). Under normal physiological conditions the endogenous formation of NO is low and in healthy individuals plasma concentrations of nitrate are close to zero (Lee et al., 1986; Kelm et al., 1992). On the other hand, plasma concentrations of the other NO metabolite, nitrate, are normally around 20–50 µmol/l, but may rise to 100 µmol/l in septic shock (Ochoa et al., 1991). Nitrate is eliminated via the kidney with a half-life in blood of 5–8 h (Ånggård, 1994) and with a plasma clearance of 20 ml/min in healthy humans (Wennmalm et al., 1993). Direct measurements of NO have proven to be extremely difficult and since no other endogenous source for the generation of plasma nitrate than NO exists, measurement of plasma nitrate concentrations is used as a well established index of NO production (Wennmalm et al., 1992, 1993).

Physical exercise, certain pharmacological agents and some food products lead to an increase in plasma concentrations of nitrate (Jungersten et al., 1996, 1997). These confounding factors therefore have to be eliminated when plasma nitrate is monitored as an index of endogenous NO formation.

Nitric oxide production may be regulated by oestrogens and women within reproductive age may be protected against cardiovascular disease due to oestrogen-mediated up-regulation of endothelial NO production (Rosselli et al., 1995; Cicinelli et al., 1997, 1999).

The present study was undertaken to investigate endogenous NO formation in healthy women of reproductive age by monitoring plasma concentrations of nitrate during the normal menstrual cycle, ovarian stimulation as well as in women hospitalized due to ovarian hyperstimulation syndrome (OHSS) of moderate degree. Since previous studies have demonstrated that NO production reaches its highest concentrations around mid-cycle (Rosselli et al., 1994; Cicinelli et al., 1996; Manau et al., 1999) close monitoring of plasma nitrate concentrations were performed at the time point of luteinizing hormone (LH) peak and ovulation.

OHSS is characterized by ovarian enlargement and by fluid accumulation in the peritoneal and pleural cavities that results
in intravascular volume depletion and haemoconcentration. It has been suggested that the abnormal hormone concentrations during ovarian stimulation may cause arteriolar vasodilatation and increased permeability, resulting in changes in systemic circulation and activation of homeostatic mechanisms that lead to salt and water retention (Balasch et al., 1998). OHSS can be classified as mild, moderate and severe (Golan et al., 1989). Mild OHSS is characterized by ovarian enlargement <10 cm, abdominal tension, swelling and mild pain. Moderate OHSS includes cases in which symptoms are more pronounced, ascites and the ovaries are enlarged from 10–12 cm in diameter. Significant OHSS causes clinical evidence of ascites, ovaries are >12 cm in diameter and increased haemoconcentration, hypovolemia, hydrothorax, decreased renal perfusion and oliguria. The pathophysiological mechanisms of OHSS are mostly unknown, but several mediators with actions on the vasculature have been suggested to be of importance. Since NO is a well known mediator of vascular smooth muscle regulation and thereby vascular tone, the possibility of a linkage between plasma nitrate concentrations and OHSS was examined.

### Materials and methods

**Patient selection**

The study was approved by the Human Ethics Committee of Göteborg University, Sweden, and before being included in the study all women gave their written consent.

Nineteen women, aged 30.0 years ± 0.7 (mean ± SEM) agreed to take part in the study. Only healthy, non-smoking women of normal weight [body mass index (BMI) <23] with regular menstrual cycles (26–33 days) were asked to participate. Before being asked to participate, the women and their male partners had been through standard clinical work-up due to primary infertility. In each separate case, it had been confirmed that the underlying cause of infertility was a severe male factor. No contributing female infertility factors were found during investigation prior to IVF.

To avoid influences on plasma nitrate concentrations from food products or physical exercise, the participants were requested to follow a low-nitrate diet (Table I) and to avoid physical exercise for at least 48 h before blood sampling. Blood samples [for analysis of nitrate, oestradiol, progesterone, follicle-stimulating hormone (FSH), LH] were collected between 0700 and 0830 h after overnight fasting. After immediate centrifugation at 3000 g for 10 min, the supernatant was frozen at −70°C until assayed.

In addition, 13 women, aged 33.1 years ± 1.2 (mean ± SEM), who were admitted to hospital due to OHSS of moderate degree (Golan et al., 1989) agreed to participate. None of these women had been on any medication for the last 3 months before the start of IVF treatment.

### Study design

The study was designed as a prospective longitudinal study of two parts. In addition, blood samples were obtained from women admitted for OHSS.

**Assessment of plasma nitrate concentrations during the menstrual cycle**

Blood samples were obtained 4 days after start of menstrual shedding (CD 4), the day of LH surge (LH), the following day (LH +1), 8 days after LH surge (mid-luteal = ML) and the first day of menstrual shedding of the following cycle (CD 1; Table II). LH surge was detected by means of urinary Clearplan® (Unipath Limited, Bedford, UK). Evaluation of follicular size was performed by transvaginal sonography (TVS).

**Assessment of plasma nitrate concentrations during ovarian stimulation for IVF treatment**

The same women (except for one woman whose partner proved to be suffering from non-obstructive azoospermia, verified by testicular biopsies) were down-regulated by daily intranasal administration of 0.9–1.2 mg of buserelin acetate (Suprecu®; Hoechst Marion Roussel, Frankfurt am Main, Germany). After 3 weeks of treatment serum oestradiol concentrations were <0.20 nmol/l and down-regulation was verified by TVS. Thereafter, ovarian stimulation was performed using recombinant FSH (rFSH) 150–225 IU/day (Gonal-F®; Serono, Rome, Italy) s.c. together with 0.45–0.6 mg/day of buserelin acetate. The dosage of rFSH was adjusted according to the rise in oestradiol concentrations and TVS findings. After 12–14 days of stimulation, when at least three follicles attained a mean diameter of 18 mm, 10 000 IU of human chorionic gonadotrophin (HCG) (Profasi®; Serono, Rome, Italy) was administered s.c. to induce oocyte maturation. Oocyte retrieval was performed ~37 h later by means of TVS-guided aspiration. Routinely, as luteal support, 2500 IU of HCG was administered s.c. on the days of aspiration and embryo transfer, followed by 1250 IU four times at 3 days intervals. Embryo transfer was performed 2 days after oocyte retrieval. In four women, 50 mg of progesterone was administered i.m. daily from the day of embryo transfer due to high predicted OHSS risk determined by high concentrations of oestradiol and more than 15 follicles at TVS at the end of the stimulation period.

During ovarian stimulation, blood samples were drawn at the end of the down-regulation period/start of gonadotrophin stimulation (G-ST), day 8 of rFSH stimulation (G 8), the day of HCG (HCG), the following day (HCG +1), and finally 5 days after embryo transfer (embryo transfer +5).

**Assessment of plasma nitrate concentrations of nitrate during OHSS**

Blood samples were drawn from 13 women who were hospitalized due to moderate OHSS. Sampling was done immediately upon admission and before any medical therapy had been initiated. Naturally, blood samples were collected at different times of the day and none of these participants had been asked to follow neither of the specific dietary nor physical restrictions (see above).

### Assay methods

Nitrate was analysed using a gas chromatography/mass spectrometry method, previously described in detail (Ringqvist et al., 1997). Briefly,...
a known volume of plasma was added to a known volume of K\textsubscript{15}NO\textsubscript{3} (Sigma Chemical Co., St Louis, MO, USA), as internal standard. Endogenous and \textsuperscript{15}N-labelled nitrate in plasma was converted to nitrobenzene by shaking a 50 µl portion for 30 min with 750 µl of benzene and 110 µl TFMS (trifluoro-methanesulphonic acid; Sigma Chemical Co.). Before adding the plasma samples, the benzene and TFMS containing tubes were frozen at −70°C. The organic phase was separated and washed with 150 µl 0.5 M Na\textsubscript{2}CO\textsubscript{3}. Subsequently, 1 µl portion was injected into a Varian 3400 gas chromatograph (Varian, Walnut Creek, CA, USA) equipped with a 30 m XTI-5 capillary column operated with a temperature programme (60–240°C). It was connected to a Varian Saturn II mass spectrometer operated in the positive ion/chemical ionization mode, using methane as the reactant gas and selective monitoring of mass equivalent (m/e) 124 for endogenous nitrate and m/e 125 for the \textsuperscript{15}N-labelled internal standard. The detection limit for endogenous nitrate was 0.1 µmol/l and the variation coefficient was <5%.

Serum oestradiol concentrations were assayed by means of a commercial kit (Abbott IMx, Abbott Park, IL, USA) with a sensitivity limit of 0.09 nmol/l, while serum progesterone was measured by time-resolved fluoroimmunoassay (DELFIA; Pharmacia Diagnostics, Uppsala, Sweden). Serum FSH and LH were assayed using ELISA assays, AxSYM\textsuperscript{®} FSH Reagent Pack and AxSYM\textsuperscript{®} LH Reagent Pack respectively (Abbott IMx). These methods all had interassay and intra-assay coefficients of variation <10% and are used as standard procedures of measurements at our clinic.

Statistical analysis
All values were calculated as means ± SEM. Data concerning longitudinal variations of NO were compared by ANOVA followed by Fisher’s post hoc test. Student’s t-test was used to compare different groups at corresponding time points. A concentration of \( P < 0.05 \) was considered to be significant.

Results

Menstrual cycle
The lowest concentrations of plasma nitrate were found at CD 1 (26.7 µmol/l ± 1.2; Table II). Although an increase in plasma nitrate was seen during the follicular phase, this increase was not significant (Figure 1). A marked rise in nitrate concentrations (\( P < 0.001 \)) occurred at LH +1. At ML the plasma concentration of nitrate had decreased to concentrations similar to those of the follicular phase.

Ovarian stimulation
Significantly lower concentrations of plasma nitrate (\( P < 0.01 \)) were measured at the end of gonadotrophin-releasing hormone (GnRH) down-regulation (24.6 µmol/l ± 1.4) compared with the concentrations found at day 8 of rFSH stimulation (34.9 µmol/l ± 2.6) and at the day of HCG injection (35.6 µmol/l ± 3.3; Figure 2; Table III). The concentrations of plasma nitrate at HCG +1 (34.1 µmol/l ± 3.3) and at embryo transfer +5 (33.2 µmol/l ± 2.3) were similar to the concentrations measured during rFSH stimulation. The increase in plasma nitrate was significantly (\( P < 0.05 \)) less than the increase in serum oestradiol during ovarian stimulation (Table III).

OHSS
The plasma concentrations (33.4 ± 4.9) in patients admitted for OHSS were similar to those measured at embryo transfer +5 or at the comparable time phase of the menstrual cycle, ML.

Discussion
The instability of the NO gas makes direct measurements of concentrations in body fluids difficult to perform. In blood vessels, endothelial-derived NO diffuses into the underlying smooth muscle and thus maintains vasodilatation and a nutritious blood flow (Ånggård, 1994). In the human female reproductive tract, NO is synthesized in the uterus, the Fallopian tube as well as the ovary. In-vitro studies have demonstrated that NO is a potent mediator of smooth muscle relaxation in these organs (Buhimschi et al., 1995; Ekerhovd et al., 1998;
Ekerhovd et al., 1999; Norman et al., 1999) as well as of regulation of steroidogenesis (Vega et al., 1998).

In the present study we found evidence for a menstrual cyclicity of the nitrate concentrations. There appeared to be an increase in nitrate concentrations during the follicular phase with maximal concentrations seen at the day of ovulation. Thereafter, at the mid-luteal phase, a decrease in plasma nitrate occurred. These results are an extension of results presented in previous studies, which have shown that the highest concentrations of plasma nitrate are found around mid-cycle (Rosselli et al., 1994; Cicinelli et al., 1996; Manau et al., 1999). In the cited studies, plasma nitrate concentrations were not monitored as frequently as in the present study. Moreover, in the present study, positive urinary Clearplan® results, indicating an ongoing LH peak, were also confirmed by serum measurements of LH. The thorough monitoring in the present study enabled us to detect the dramatic increase of nitrate concentrations on the day after the LH peak. This marked elevation of NO production has not been demonstrated before. It seems reasonable to suggest that the high nitrate concentrations found to exist at this time-point of the menstrual cycle, may reflect NO-mediated mechanisms in the process of spontaneous ovulation. Several experimental studies in animals support the notion that NO plays an essential role in ovulation. Thus, the administration of nitric oxide synthase (NOS) inhibitors causes suppression of ovulation in a concentration-dependent manner in the rat as well as in the rabbit (Shukovski and Tsafriri, 1994; Bonello et al., 1996; Yamauchi et al., 1997, Mitsube et al., 1999). Likewise, studies of NOS gene-targeted mice have demonstrated a reduction of ovulations in both endothelial (eNOS) (60% reduction) and inducible (iNOS) (40% reduction) knock-out mice (Olson, 1997; Jablonka-Shariff and Olson, 1998). Thus, based on these studies it seems likely that both eNOS and iNOS are involved in the process of ovulation.

Since the nitrate concentrations increased during the follicular phase, at the same time as the oestradiol concentrations showed a continuous increase, it is tempting to speculate that a linkage exists between oestradiol and NO production in women of reproductive age. However, the possible effects of oestradiol on NO production seem to be measurable in plasma after a lag time of several hours, since we could observe this increase at LH +1. These results are in accordance with a previous study, demonstrating that oestrogen replacement therapy in post-menopausal women results in higher concentrations of NO metabolite products (nitrite and nitrate) ~24 h after administration (Cicinelli et al., 1997).

In women monitored throughout the menstrual cycle, blood flow around the developing follicle increases during the follicular phase. By means of TVS and colour Doppler imaging a marked increase in blood flow over the peri-ovulatory period has been demonstrated (Campbell et al., 1993; Balakier and Stronell, 1994). In addition, there is a redistribution of blood flow during ovulation so that blood flow increases at the base of the follicle but decreases at the apex (Brännström et al, 1998). Similarly, treatment with the NO donor glyceryl trinitrate seems to decrease pulsatility index in vessels at the rim of the dominant follicle in cycling women (Zachrisson et al, 1998). The mechanisms for these effects are not fully understood, but may be triggered by LH (Jansson, 1975) and carried out by NO (Bonello et al., 1996).

In women during ovarian stimulation for IVF treatment, positive correlation existed between follicular fluid nitrite/nitrate concentrations and follicular volume as well as serum oestradiol concentrations (Anteby et al., 1996). In line with these results, it was suggested that oral supplementation of L-arginine, the substrate for NO biosynthesis, may improve ovarian response in poor responders during ovarian stimulation for IVF treatment (Battaglia et al., 1999).

Table III. Values (mean ± SEM) of hormones and nitrate during ovarian stimulation (n = 18)

<table>
<thead>
<tr>
<th></th>
<th>G-ST</th>
<th>G8</th>
<th>HCG</th>
<th>HCG + 1</th>
<th>Embryo transfer + 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oestradiol (nmol/l)</td>
<td>0.1 ± 0</td>
<td>2.0 ± 0.3</td>
<td>9.5 ± 1.3</td>
<td>10.5 ± 1.2</td>
<td>5.9 ± 0.6</td>
</tr>
<tr>
<td>Progesterone (nmol/l)</td>
<td>0.7 ± 0.2</td>
<td>1.1 ± 0.4</td>
<td>10.2 ± 7.3</td>
<td>35.3 ± 9.1</td>
<td></td>
</tr>
<tr>
<td>Nitrate (µmol/l)</td>
<td>24.6 ± 1.4</td>
<td>34.9 ± 2.6</td>
<td>35.6 ± 3.3</td>
<td>34.1 ± 3.3</td>
<td>33.2 ± 2.3</td>
</tr>
</tbody>
</table>

G-ST = start of gonadotrophin stimulation; G8 = 8 days after start of rFSH stimulation; HCG = day of HCG injection; HCG +1 = 1 day after HCG injection; embryo transfer +5 = 5 days after embryo transfer.

Progesterone concentrations at embryo transfer +5 are not shown since four of the women were given progesterone as luteal support.

Figure 2. Plasma nitrate concentrations (mean ± SEM) during controlled ovarian hyperstimulation (n = 18). G-ST = start of gonadotrophin stimulation; G8 = 8 days after start of rFSH stimulation; HCG = day of HCG injection; HCG +1 = 1 day after HCG injection; ET + 5 = 5 days after embryo transfer. A significant increase (P < 0.05) in plasma nitrate concentration was measured during gonadotrophin stimulation compared with G-ST.
Interestingly, in a recently published study it was found that follicular fluid concentrations and circulating concentrations of nitrite/nitrate did not differ significantly during ovarian stimulation (Manau *et al.*, 2000). No relationship between nitrite/nitrate follicular fluid concentrations and parameters of ovarian response was registered. In another study, the same authors found that circulating concentrations of serum nitrite/nitrate were significantly elevated in the late follicular phase compared with cycle day 3 of the spontaneous menstrual cycle, thus paralleling plasma oestradiol (Manau *et al.*, 1999).

In the present study plasma concentrations of nitrate after medication with GnRH were low. An increase in the concentration of plasma nitrate was seen after rFSH treatment. Thus, it seems likely that there is a linkage between FSH, or more likely oestradiol and endogenous production of NO. In fact, it is well known that oestrogens induce vasodilation, an effect that may partially be mediated by NO (Magnarson and Rosernfield, 1989). It has also been shown that 2 mg daily of oestradiol raises plasma nitrate concentrations significantly, indicating a link between NO synthesis and oestrogen concentrations (Ramsey *et al.*, 1995). However, in the present study the relative increase in plasma nitrate was significantly less than the increase in serum oestradiol during ovarian stimulation (Table III). This may be explained by mechanisms observed in other studies, indicating a complexity by which oestradiol affects vascular tone by controlling eNOS activity via a receptor-mediated system (Hayashi *et al.*, 1995). In the latter study, using cultured endothelial cells from the human umbilical vein and the bovine thoracal aorta, it was shown that preincubation with oestradiol at 10^{-12} to 10^{-8} mol/l enhanced eNOS activity, while higher concentrations of oestradiol tended to reduce eNOS activity to control concentrations. It thus seems likely that very high concentrations of oestradiol inhibit eNOS activity via a system that is not receptor-dependent. A similar negative feedback system may explain why the plasma concentrations of nitrate in the present study did not increase to extremely high concentrations during IVF treatment, when concentrations of oestradiol were supraphysiological. Likewise, women with high concentrations of oestradiol (≥10.0 nmol/l) at ovarian stimulation did not expose higher concentrations of nitrate than women with serum oestradiol concentrations <10.0 nmol/l at the end of rFSH induced ovarian stimulation. Thus, when serum oestradiol were extremely high plasma nitrate concentrations were similar, or in some cases even lower than those measured in women with a moderate increase in serum oestradiol concentrations.

One hypothesis behind the present study was that women with OHSS may have increased NO production, resulting in an increase in vascular permeability and thus leakage of fluid from blood vessels into extravascular space. Several vasoactive substances, of which some have been linked to OHSS, such as acetylcholine, histamine, thrombin, bradykinin and substance P are known to stimulate endothelial NO biosynthesis (Ånggård, 1994). In the present study eight of the 13 women with moderate OHSS later proved to be pregnant. It is well known that in IVF cycles where conception has occurred, the risk of OHSS is four-fold (Golan *et al.*, 1989). All 13 women suffering from moderate OHSS had plasma concentrations of nitrate similar to those observed during the menstrual cycle. Thus, it seems unlikely that NO is of pathophysiological significance in the fully developed OHSS. However, the present results do not rule out the possibility that NO may be a mediator of early events in OHSS development. New evidence of endogenous NO production indicates that negative feedback mechanisms evolve when high concentrations of NO are synthesized (Rosselli *et al.*, 1998). Thus, the results of the present study are in accordance with previous studies where NO metabolites were measured in blood as well as in peritoneal fluid of women with severe OHSS (Revel *et al.*, 1996; Manau *et al.*, 1998).

Since measurement of plasma nitrate is an indirect method of assessing endogenous NO production, the results obtained always have to be interpreted with some caution. Plasma nitrate is eliminated via the kidneys and fluctuations in plasma nitrate may therefore reflect changes in renal function (Davison and Noble, 1981; Mackenzie *et al.*, 1996). Plasma clearance of nitrate has been calculated to be ~20 ml/ml in healthy individuals (Wennmalm *et al.*, 1993). To our knowledge, possible fluctuations of renal clearance of nitrate during the menstrual cycle, ovarian stimulation or OHSS have not been examined. It seems likely that clearance of nitrate may be reduced in women with OHSS since glomerular filtration rate in these women often is reduced. In the present study, plasma nitrate concentrations were not increased in women with OHSS. Thus, a possible decrease in renal clearance leading to an increase in plasma nitrate concentrations was not registered.

A nitrite-restricted diet for at least 48 h before blood sampling seems to be mandatory to eliminate effects of certain food products (Jungersten *et al.*, 1996). Physical exercise tends to cause a dramatic increase in plasma nitrate concentrations since physical activity induces vascular endothelial NO synthesis, a beneficial effect of regular physical exercise (Jungersten *et al.*, 1996, 1997). Likewise, factors such as hypoxia, flow, mechanical stretching or shear stress also give rise to an enhanced endothelial NO release (Ånggård, 1994).

To summarize, the present study demonstrates that there is a significant increase in the concentration of plasma nitrate the day after LH peak. This finding gives support for the involvement of NO in spontaneous ovulation. Treatment with GnRH inhibits NO production. During ovarian stimulation an increase in plasma nitrate occurs, but when a high concentration of oestradiol is found, plasma nitrate concentration does not rise to a similar extent. On the contrary, plasma nitrate concentrations may be relatively low when oestradiol concentrations are high during ovarian stimulation. Thus, when serum oestradiol are high, negative feedback mechanisms on NO production seem to be present. Plasma nitrate concentrations are not increased in the fully developed OHSS.

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References

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