Increased serum levels of nitric oxide metabolites among users: a possible role in progestin-induced bleeding

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BACKGROUND: Nitric oxide (NO) is a potent angiogenic and vasodilator factor that could be involved in progestin-induced bleeding. This study aimed to assess possible changes in the serum levels of NO metabolites in progestin-only contraceptive users and to identify any correlation between some of their clinical characteristics and NO metabolite levels. METHODS: This cross-sectional study included 37 contraceptive users; a single 5 ml venous blood was collected at different periods of contraceptive use. Women were divided into users with acceptable menstrual bleeding (n = 13) and those having abnormal bleeding patterns (n = 24). The controls are 13 age-matched healthy women; they were fertile, had regular menstruation and did not use any contraceptive method in the previous 3 months. NO was determined by the evaluation of its oxidation products (nitrites and nitrates) where the nitrates were reduced to nitrites with cadmium filings; total serum concentrations of nitrites were measured by using the Griess reaction. RESULTS: The mean serum levels of NO metabolites were significantly higher in the contraceptive users than in the controls (mean ± SE) 34.9 ± 11.3 versus 6.1 ± 1.5 μmol/l (P < 0.000). The mean serum levels of NO metabolites were significantly higher in the contraceptive users with abnormal bleeding patterns than in those with normal bleeding patterns (mean ± SE) 41.3 ± 7.4 versus 23.2 ± 5.8 μmol/l (P < 0.000). There was a positive correlation between NO levels and both prolonged spotting and heavy/prolonged bleeding days (P < 0.001 and P < 0.01, respectively) and negative correlation between NO levels with the duration of use and length of the menstrual cycle (P < 0.05). CONCLUSION: The significantly increased serum levels of NO metabolites among contraceptive users may primarily reflect an increase in its endometrial production, possibly secondary to its increased liberation by systemic vascular endothelium. This may result in enhanced endometrial angiogenesis and vascular dilatation which can induce and perpetuate abnormal excessive/prolonged uterine bleeding.

Key words: abnormal bleeding/progestin-only contraceptive/serum NO metabolites

Introduction

Over 20 million women use progestin-only contraceptive methods, with ~6–7 million of them using Norplant®, a levonorgestrel-releasing implant. Disturbances of vaginal bleeding are almost inevitable with the use of progestin-only contraceptive methods (30–90% depending on the particular product). Nearly 10–30% of women abandon the method because of bleeding problems (Meirik et al., 2003).

There is increasing evidence from in vivo and in vitro studies that endometrial microvascular appearance is altered by progestogen exposure. Superficial vascular dilatation (Hickey et al., 1997), neovascular formation and relative increase in endometrial vascular density with lack of stromal support (Hickey et al., 1999) were reported in women chronically exposed to low dose progestogens (McGavigan et al., 2003). Among Norplant® users, hysteroscopic studies had suggested that superficial endometrial vessels are abnormally fragile (Hickey and Fraser, 2002). Also, increased endometrial expression of vascular endothelial growth factor (VEGF) contributing to vascular permeability and dilatation has been observed (Lau et al., 1999).

It is believed that the effects of estrogen and progestin on angiogenesis are mediated indirectly, predominantly via the paracrine actions of prostaglandins (White et al., 1991), polypeptide growth factors, such as platelet-derived growth factor (PDGF) (Folkman and Klagsburn, 1987) and VEGF (Lau et al., 1999), and nitric oxide (NO) (Chwalisz and Garfield, 2000) generated by endometrial epithelial, stromal and/or endothelial cells. It appears that continuous exposure to progestogens in the presence of low to moderate levels of estrogen results in endometrium that demonstrates ‘suppressed secretory changes’, associated with disturbed angiogenesis and a tendency to release a variety of molecules capable of causing focal endometrial epithelial and endothelial damage and abnormal bleeding (Hickey and Fraser, 2002).
VEGF and NO are key players in endometrial angiogenesis and they could be involved in initiation, maintenance and control of menstrual bleeding (Lau et al., 1999; Chwalisz and Garfield, 2000). Thus, it can be extrapolated that abnormal uterine bleeding among Norplant® users may be associated with an increased endometrial production of NO, as a potent vasoactive and angiogenic factor, as well as a similar increase in its serum levels.

This assumption is based on observing the results of several experimental and human studies showing that the genital organs can be a potent source of increased NO production in serum during physiological and pathological conditions (Ekerhovd et al., 2002). Moreover, NO metabolites were measurable in blood as well as in peritoneal fluid (Revel et al., 1996). Interestingly, it was found that follicular fluid NO concentration and circulating plasma concentrations did not vary significantly during ovarian stimulation (Manau et al., 2000).

With this background in mind, this study was undertaken to: (i) assess the possible changes in the serum levels of NO metabolites (nitrites and nitrates) among Norplant® users; and (ii) identify any correlation between the serum levels of NO metabolites and some clinical characteristics of these women.

**Subjects and methods**

**Subject selection**

This cross-sectional study included 37 Norplant® users recruited from the attendees of the Family Planning Clinic, Assiut University Hospitals, Egypt. They were currently using Norplant® implants (six capsules) (Leiras Pharmaceuticals, Turku, Finland) and were instructed to record all bleeding events in their Menstrual Diary Sheets with regular follow-up visits. From each woman, a single 5 ml venous blood sample was collected after overnight fasting, at different periods of Norplant® use (range 10–60 months). The users were divided into two main groups: women with menstrual bleeding similar to normal menstruation (n = 13) and those having abnormal bleeding patterns (n = 24). Abnormal bleeding patterns included prolonged spotting (n = 9) and prolonged/heavy bleeding (n = 15).

According to the WHO definitions, women with menorrhagia and polymenorrhoea were described as Norplant® users with ‘prolonged/heavy bleeding’, while those with an irregular bleeding pattern, in the form of on/off spotting, were referred as users with ‘prolonged spotting’ (World Health Organization, 1992). Daily records of vaginal bleeding were kept by each woman for at least 9 months (three reference periods) before inclusion in the study. Spotting is the amount of blood requiring no more than one sanitary towel per day, while bleeding requires more than one sanitary towel per day.

The control group included 13 age-matched ‘healthy’ women, who were fertile, had a regular menstrual pattern and did not use any method of contraception during the previous 3 months. Ethical approval for the study was obtained from the Ethical Committee Board in the Department of Obstetrics and Gynecology, Assiut University Hospital, Egypt.

**Timing of blood sample**

Among Norplant® users with irregular uterine bleeding (prolonged spotting/heavy bleeding), it was difficult to identify follicular or luteal phases due to loss of normal menstrual cyclicity. For standardization purposes, we obtained the blood samples 3 days after the start of menstruation in the controls and in the Norplant® users with regular menstruation. Samples were obtained from users with irregular bleeding patterns 3 days after the onset of the last bleeding or spotting episode.

Also, to minimize influences on plasma nitrate concentration, women were carefully selected (controls and users) to exclude associated endocrine or medical disorders, smoking or taking drugs that interfere with NO synthesis. They were sampled after a fasting period of 10–12 h and were asked about types of food eaten during the previous 24 h to rule out foodstuffs that could influence the serum levels of NO.

**Sample preparation and NO assay in serum**

All blood samples were collected in glass tubes and allowed to clot spontaneously at room temperature, then the serum was prepared by centrifugation for 15 min at 3000 r.p.m. Aliquots were stored at −70°C until NO assay (4–6 weeks). NO was determined by the evaluation of its oxidation products (nitrates and nitrites), where the nitrates were reduced to nitrites with cadmium filings, then the total serum concentrations of nitrites were measured by using the Griess reaction. Griess reagent was composed of 0.5% sulphanilamide, 0.05% naphthylethene-diamine dihydrochloride and 2.5% H₃PO₄.

The details of this technique are fully described by Van Bezooijen et al. (1998).

**Statistical analysis**

Data were entered into a database using SPSS for Windows, version 9.00. The mean ± SEM were used to present data. The two-tailed Student’s t-test for unpaired data was used to compare equal normally distributed data and the Mann–Whitney test was used for skewedly distributed data. To assess if there is any correlation between serum levels of NO metabolites and various clinical parameters, the non-parametric Spearman’s correlation coefficient was calculated. A P-value of ≤0.05 was taken as significant.

**Results**

**Admission characteristics**

Table I presents the differences in the selected admission characteristics between Norplant® users with normal (n = 13) and abnormal (n = 24) bleeding patterns. Women with abnormal bleeding patterns had a significantly shorter duration of Norplant® use (P = 0.043), prolonged number of bleeding days (P = 0.011), prolonged number of spotting days.

<table>
<thead>
<tr>
<th>Character</th>
<th>Users with normal bleeding (n = 13) mean ± SEM</th>
<th>Users with abnormal bleeding (n = 24) mean ± SEM</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>36.1 ± 6.2</td>
<td>34.1 ± 5.6</td>
<td>0.3</td>
</tr>
<tr>
<td>Parity</td>
<td>5.6 ± 2.1</td>
<td>5.7 ± 2.2</td>
<td>0.8</td>
</tr>
<tr>
<td>Duration (month)</td>
<td>38.6 ± 13.9</td>
<td>28.4 ± 15.8</td>
<td>0.043</td>
</tr>
<tr>
<td>Bleeding days</td>
<td>3.8 ± 0.8</td>
<td>5.6 ± 1.1</td>
<td>0.011</td>
</tr>
<tr>
<td>Spotting days</td>
<td>3.3 ± 1.2</td>
<td>8.4 ± 3.1</td>
<td>0.004</td>
</tr>
<tr>
<td>Cycle length (days)</td>
<td>31.2 ± 3.6</td>
<td>25.1 ± 7.4</td>
<td>0.049</td>
</tr>
<tr>
<td>Period length (days)</td>
<td>6.6 ± 1.5</td>
<td>11.2 ± 4.1</td>
<td>0.013</td>
</tr>
</tbody>
</table>

Prolonged bleeding = prolonged spotting and prolonged/heavy bleeding days.
Table II. Serum levels of NO metabolites in Norplant® subgroups according to the type of bleeding pattern

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Mean ± SEM (μmol/l)</th>
<th>95% CI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>13</td>
<td>6.1 ± 1.5</td>
<td>5.2–6.9</td>
<td>–</td>
</tr>
<tr>
<td>All Norplant® users</td>
<td>37</td>
<td>34.9 ± 11.3</td>
<td>25.3–38.4</td>
<td>&lt;0.000</td>
</tr>
<tr>
<td>Users with normal bleeding</td>
<td>13</td>
<td>23.2 ± 5.8</td>
<td>19.6–27.6</td>
<td>&lt;0.000</td>
</tr>
<tr>
<td>Users with prolonged/</td>
<td>15</td>
<td>40.1 ± 6.9</td>
<td>35.1–43.2</td>
<td>&lt;0.000</td>
</tr>
<tr>
<td>heavy bleeding</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Users with prolonged spotting</td>
<td>9</td>
<td>44.3 ± 7.8</td>
<td>40.3–49.1</td>
<td>&lt;0.000</td>
</tr>
</tbody>
</table>

P-values calculated against the control group.
CI = confidence interval.

Table III. Correlation between serum levels of NO metabolites and some clinical characteristics of Norplant® users

<table>
<thead>
<tr>
<th>Character</th>
<th>NO serum levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duration of use</td>
<td></td>
</tr>
<tr>
<td>r-value</td>
<td>-0.414</td>
</tr>
<tr>
<td>P-value</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Bleeding days</td>
<td></td>
</tr>
<tr>
<td>r-value</td>
<td>0.531</td>
</tr>
<tr>
<td>P-value</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Spotting days</td>
<td></td>
</tr>
<tr>
<td>r-value</td>
<td>0.693</td>
</tr>
<tr>
<td>P-value</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Cycle length</td>
<td></td>
</tr>
<tr>
<td>r-value</td>
<td>-0.431</td>
</tr>
<tr>
<td>P-value</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Period length</td>
<td></td>
</tr>
<tr>
<td>r-value</td>
<td>0.571</td>
</tr>
<tr>
<td>P-value</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

r = Spearman’s correlation coefficient for the non-parametric data.

Discussion

Few data are available on the role played by NO in the human endometrium as a modulator of blood loss during menstruation. NO could be involved in initiation and maintenance of menstrual bleeding by induction of tissue breakdown and vascular relaxation as well by inhibition of platelet aggregation (Chwalisa and Garfied, 2000). In the human female reproductive tract, NO is synthesized in the uterus, the Fallopian tubes and the ovary (Ekerhovd et al., 2002). Endothelial nitric oxide synthase (eNOS) and its inducible form (iNOS) had been localized to the endometrial glandular epithelium in the non-pregnant uterus. Also, weak iNOS immunoreactivity was observed in endometrial stromal cells (Telfet et al., 1995).

Findings suggest that the milieu that exists in the normal secretory phase may be mimicked in women using progestin-only contraceptives and corresponds to maximal endometrial capillary proliferation. This milieu could lead to increased endometrial expression of eNOS (Lebovic et al., 2000). Thus, among Norplant® users, it has been hypothesized that there is increased endometrial expression of NOS and NO production; similarly, the serum levels of NO metabolites (nitrites and nitrates) may rise.

The concept of enhanced angiogenesis and vasorelaxation by progesterone is consistent with the well-known phenomenon of unscheduled bleeding during chronic administration of synthetic progestins. Antiprogestins not only block progesterone receptors but also inhibit estrogen-dependent proliferation and angiogenesis, and induce amenorrhea in primates (Chwalisz et al., 1999) and monkeys (Zelinskiwooten et al., 1998) by an unknown mechanism. In the human endometrium, it was found that treatment with the antiprogestin mifepristone significantly decreased eNOS expression in the endometrial glandular epithelium (Sun et al., 2003). This may point to the ability of progesterone/progestins to upregulate endometrial eNOS and in turn stimulate NO production.

Among Norplant® users, it was found that there was a significant positive correlation between the serum levels of NO metabolites and the number of both spotting and bleeding days. A number of pieces of evidences now point to the links between NO and the mechanisms that regulate endometrial angiogenesis and vascular dilatation. This in turn gives a possible explanation for the role of increased NO levels in the development of abnormal uterine bleeding in some women, e.g. perimenopausal bleeding and progestin-only contraceptive-induced bleeding.

In their study, Hurksainen et al. (1999) showed a significant inverse correlation between uterine artery pulsatility index and the amount of blood loss in women with menorrhagia. They suggested that local concentrations of

Serum levels of NO metabolites

The mean serum levels of NO metabolites were significantly higher in the Norplant® users than in the controls (mean ± SEM) 34.9 ± 11.3 versus 6.1 ± 1.5 μmol/l; P < 0.000. Also, the mean serum levels of NO metabolites were significantly higher in the Norplant® users with normal bleeding patterns than in those with a normal bleeding pattern (mean ± SEM) 41.3 ± 7.4 versus 23.2 ± 5.8 μmol/l; P < 0.000.

The 37 Norplant® users were divided into three subgroups, according to the type of bleeding pattern: users having a normal bleeding pattern (n = 13), those having heavy/prolonged bleeding (n = 9) and others having prolonged spotting (n = 15). Compared with the controls, it was found that the mean serum levels of NO metabolites were significantly higher in all Norplant® subgroups. There was a progressive increase in the following order: prolonged spotting > heavy/prolonged bleeding > normal bleeding > controls (P < 0.000 for all subgroups) (Table II).

Correlations

The correlation between serum levels of NO metabolites and the selected clinical characteristics was examined among the Norplant® users (Table III). There was a positive correlation between NO levels and both prolonged spotting and heavy/prolonged bleeding days (P < 0.001 and P < 0.01, respectively). On the other hand, there was a negative correlation between NO levels and the duration of use, and length of the menstrual cycle (P < 0.05).
vasoactive agents simultaneously decreased uterine blood flow resistance and promoted uterine bleeding. Similarly, Ramsay et al. (1994) indicated that NO could be a good candidate to exert both effects, since it is known that NO donors could increase uterine blood flow in humans. In addition, investigators had found an increased expression of eNOS in menorrhagic endometrium (Blumenthal et al., 2002).

Some experimental data indicate that NO may play a role in excessive/prolonged menstrual bleeding as NO can activate production of prostaglandins PGE2 and PGI2 (Zervou et al., 1999). This would promote extracellular matrix remodelling of blood vessels and vasodilatation that could initiate abnormal endometrial bleeding (Stewart and Nowak, 1996). Moreover, NO may play an integral role in the status of deficient haemostasis, which probably, in part, mediates breakthrough bleeding (Hickey and d’Arcangues, 2002).

VEGF is a potent angiogenic factor and a fundamental regulator of normal and abnormal angiogenesis in the female reproductive tract (Ferrara, 1999). NO is involved in endometrial angiogenesis as it can directly stimulate VEGF production (Fukumura et al., 2001) and, similarly, VEGF could stimulate/induce NO release from endothelial cells (Grasselli et al., 2002). It seems that such a tight and delicate paracrine loop regulating NO and VEGF production in the endometrium might be disrupted in women exposed to progestin-only methods of contraception (Papapetropoulas et al., 1997).

NO is an endogenous vasoactive substance produced in almost all human tissues. (Moncada and Higgs, 1993). Therefore, this study does not rule out the possibility that NO may be produced by other sources, rather than endometrium. Systemic vascular endothelium could be such a source for NO production, contributing, in part, to the increased serum levels of nitrates/nitrites in Norplant® users. The onset of endometrial breakdown under the effect of Norplant® could be associated with secretion of a variety of cytokines from endometrial cells, immune cells and macrophages. These cytokines can stimulate both endometrial eNOS (Ota et al., 1998) and iNOS (Tschuggue et al., 1999) to liberate NO at high concentrations. It is suggested that certain cytokines may find their way to the systemic circulation where they may stimulate both eNOS and iNOS elsewhere in the body. This assumption should be taken into consideration, since the measurement of plasma nitrites/nitrates is an indirect method for assessing the overall endogenous NO production.

Recently, it has been proposed that the mechanisms that account for the onset of abnormal uterine bleeding observed after long-term progestin treatment could arise due to reduced blood flow resulting in local hypoxia and re-perfusion injury, in turn creating an inflammatory, pro-thrombotic, pro-angiogenic environment in the endometrium. (Lockwood et al., 2004).

In the present study it was found that there was a negative correlation between the serum levels of NO and the duration of Norplant® use, i.e. the shorter the duration of use, the higher the serum levels of NO and increased endometrial angiogenesis and vasodilatation. This is supported by the results of many studies showing that the bleeding problems generally occur in the early months of progestin therapy (Faundes et al., 1978; Shoupe et al., 1991). This is not seen in the long-term Norplant® users, where fewer bleeding problems occur (Palmer et al., 1996).

Both experimental and clinical evidence is emerging pointing to links between progesterone/synthetic progestins and NO in the endometrium. Further clinical studies are definitely needed to explore the potential of antiprogestins and NO inhibitors for the treatment of abnormal uterine bleeding induced by progestin-only contraceptives.

In conclusion, our findings of the significantly increased serum levels of NO metabolites among Norplant® users may reflect primarily an increase in its endometrial production, possibly secondary to increased liberation by systemic vascular endothelium. This may result in enhanced endometrial angiogenesis and vascular dilatation which can induce and perpetuate abnormal excessive/prolonged uterine bleeding.

References


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