Oxidative stress, vitamin E and progestin breakthrough bleeding

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Introduction

Progestin-only contraceptives were very popular in Indonesia, with more than 30% of women using depo-medroxyprogesterone acetate (DMPA; Upjohn Indonesia, Jakarta, Indonesia) injections and 8% using Norplant® (Leiras, Turku, Finland). Endometrial breakthrough bleeding (BTB) problems can be the main reason for discontinuing these methods. The local mechanisms that cause endometrial bleeding are still unclear. Following 3–12 months of Norplant use, the endometrium becomes very thin and the angiogenic response is decreased (Subakir et al., 1996). The spiral arteries are small and underdeveloped with increased dilatation of the veins and breaks in the endothelial lining (Hourihan et al., 1991).

Oxidative stress occurs when reactive oxygen species formation (e.g. $O_2^-$, $OH^-$, $H_2O_2$) and defence against reactive oxygen species is not in balance. Oxidative stress causes an oxidation of lipids, proteins, and carbohydrates and is implicated in many important pathological events.
Endometrial response to Norplant

Progestin-induced endometrial bleeding is characterized by endometrial stromal infiltration of leukocytes (Clark et al., 1996; Vincent et al., 1999) and these cells can release superoxide ions. In support of this finding, the production of epoxides in the endometrium of levonorgestrel (LNG) users with BTB were increased (White et al., 1991). Increased epoxide production increases the release of oxygen radicals (Rice-Evans and Cooke, 1991), which in turn may react with vulnerable polyunsaturated fatty acids in cell membrane. Once lipid peroxidation is initiated, a chain reaction will be propagated, which can be highly destructive if not tightly controlled by antioxidants. A possible oxidant-antioxidant imbalance in the endometrium of progestin-only contraceptive users may cause vessel fragility and disturbance in endometrial regeneration.

Normally, regeneration of the endometrium is completed 5–6 days after the initiation of menstrual shedding. The angiogenic process is important for regeneration of the endometrium. Angiogenesis involves degradation of the basement membrane of existing blood vessels, endothelial cell migration and proliferation, lumen formation, development of a functional capillary loop and maturation of the new vessel. Endometrial regeneration depends on oestrogen and polypeptide growth factors (Campbell and Cameron, 1998). Following exposure to progestin-only contraceptives, BTB tends to be prolonged or irregular, suggesting that normal endometrial repair mechanisms may be dysfunctional. In a previous study we have shown that decreased endometrial angiogenic response in Norplant users was not correlated with plasma concentrations of oestradiol, progesterone, sex hormone binding globulin (SHBG) or LNG (Subakir et al., 1996).

Vitamin E is the major physiological lipid-soluble chain-breaking antioxidant, preventing peroxidation, and also modulates the metabolism of arachidonic acid (Packer and Lanvick, 1989). We postulate that progestin-induced BTB is due in part to increased oxidative stress. One of the recommendations from the 1995 World Health Organization (WHO)-sponsored meeting on ‘Current research on progestin-only contraceptive and endometrial bleeding’ was a suggestion that the potential therapeutic benefit of a number of agents in the treatment of BTB, including vitamin E, should be investigated (Findlay, 1996). The aims of this study were to investigate the effect of progestin-only contraceptives on blood concentrations of lipid peroxides and vitamin E, and the effect of vitamin E supplementation on the in-vitro endometrial angiogenic response.

Materials and methods

Subject recruitment

In the first stage of the study, 42 Norplant users with an exposure of 3–12 months and 25 controls were recruited from the Raden Saleh Clinic in Jakarta. BTB problems occurred in 36 of Norplant users. All subjects were healthy, aged between 18 and 40 years, and were recruited to the study on the basis of fully informed consent. Ethical approval was obtained from the Medical Faculty of the University of Indonesia, Ethical Commission on Research in Humans. Control subjects were chosen from women not using a hormonal contraceptive or intra-uterine device (IUD) for at least 3 months prior to the study. Menstrual record data were analysed using the current WHO definitions and terminology. Blood levels of lipid peroxide were measured by spectrofluorometry. Endometrial biopsies from Norplant users with BTB were incubated with and without vitamin E before evaluation in an angiogenesis assay. The data from these subjects were also used as a basis for a subsequent clinical trial study (Subakir et al., 1999).

In the second study, 13 women with normal menstrual cycles were recruited from Puskesmas UI, Utan Kayu, Jakarta. Six women chose Norplant and seven women chose DMPA for contraception. All the subjects had previously never used hormonal contraceptives. The subjects were instructed not to take any additional vitamin E supplements or to vary dietary patterns during the 3 month study period. The blood concentrations of lipid peroxides and vitamin E were measured before Norplant and DMPA use, and after 3 months exposure to these contraceptives. The blood concentrations of lipid peroxide were measured by spectrophotometry and vitamin E was measured by high-performance liquid chromatography (HPLC).
Endometrial biopsy was collected from 19 women with an exposure of 3–12 months to progestin-only contraceptives (11 Norplant users, eight DMPA users), and seven control subjects. Control subjects were chosen from women not using hormonal contraceptives or IUD for at least 3 months prior to the study. All of the subjects were healthy, aged 18–40 years and recruited to the study on the basis of a fully informed consent.

**Vitamin E assay**
The plasma vitamin E (alpha tocopherol) concentration was measured by HPLC (Shimadzu, model 420). The analytical column was an ODS (C18) RF column (inner diameter 46 mm × 30 cm), and was eluted with methanol at a flow rate of 2.0 ml/min. Tocopherol was determined by UV absorption at 284 nm at a retention time of 15.3 min.

**Lipid peroxide assay**
Lipid peroxide was evaluated by malonaldehyde (MDA) reaction with thiobarbituric acid (TBA). Decomposition of lipid peroxide to MDA was accelerated with TBA. Malonaldehyde, with two carbonyl groups, can cross-link two amino compounds to produce fluorescent molecules. The products are called aminoiminopropene Schiff base and they form readily at acidic pH values. The products were measured spectrofluorometrically at an excitation wavelength of 515 nm and emission at 532 nm.

In the second study, spectrophotometry was used to measure blood lipid peroxide with the TBA test. MDA can react in the TBA test to generate a coloured product (pink) which is detected at 548 nm.

**Endothelial cell culture**
Endothelial cells from human umbilical cord vein were isolated and cultured using a modification of a published method (Jaffe et al., 1973). Endothelial cells were harvested using collagenase type Ia (Sigma Chemical Co., St Louis, MO, USA). The culture medium was M199 (Gibco Laboratories, Grand Island, NY, USA) containing 20% fetal bovine serum (FBS; Sigma), 0.20 mg/ml garamycin (Schering, Jakarta, Indonesia), and 1.75 mg/ml fungizone (Flow Laboratories, Maclean, VA, USA). After confluence, the primary endothelial cell culture was used in the angiogenic assay. Cells were confirmed as endothelial based on morphological criteria and the formation of a contact-inhibited confluent monolayer in culture (Jaffe et al., 1973).

**Endometrial angiogenic assay**
Endometrial biopsies were taken using either a micro-hysteroscope or a pipelle (Prodimed 60530, Neuilly-en-Thelle, France) suction curette. Endometrial tissue was placed into Roswell Park Memorial Medium (RPMI) 1640 (Flow Laboratories) at room temperature and transported to the laboratory within 60 min of collection. Endometrial samples were divided: half was incubated with RPMI 1640 as controls and half was incubated with vitamin E in 20 mg/l RPMI 1640 for 60 min prior to angiogenic measurement. Endometrial angiogenic response was assayed using an endothelial cell migration assay. Explants were cultured utilizing a three-dimensional collagen matrix containing suspended endothelial cells (Folkman et al., 1989; modified by Rogers et al., 1992). Endothelial cells were suspended in NCTC (Flow Laboratories) with 20% FBS, garamycin and fungizone. The medium was adjusted with 0.1 mol/l NaOH to reach a pH of 7.4 after the addition of chilled vitrogen 100 (Collagen Corporation, Palo Alto, CA, USA). Three or four endometrial explants (~500 μm in diameter) were placed in each of the two wells of the culture plate and cultured for 96 h and observed at 24 h intervals using an inverted phase-contrast microscope. The angiogenic score ranged from 0 to 4: 0 = no endothelial cell response; 1 = elongation and some orientation toward the explant; 2 = minimal spokewheel response; 3 = medium spokewheel response; 4 = extreme spokewheel formation. Six to eight readings were obtained for each endometrial biopsy and the median values compared between treatments.

**Statistical analysis**
The endothelial cell migration score for each biopsy was calculated as the median response for each endometrial explant. Differences in endometrial score between different groups were analysed using the Mann–Whitney test. Mean lipid peroxide and
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- **Figure 1.** Blood concentrations of lipid peroxide in control (A), Norplant with bleeding (B) and Norplant without bleeding (C).
- **Figure 2.** Effect of in-vitro supplementation of vitamin E on endometrial angiogenic activity in Norplant users with bleeding problems (placebo; vitamin E).
- **Figure 3.** Blood lipid peroxide concentrations in women before (white) and after 3 months (shaded) exposure to Norplant or depo-medroxyprogesterone acetate (DMPA).

Vitamin E were calculated. Differences in blood lipid peroxide and vitamin E between groups were analysed using Student’s t-test.

**Results**

The blood concentrations of lipid peroxide from Norplant users with bleeding problems were significantly higher than controls (mean ± SE: 8.06 ± 0.46 versus 5.64 ± 0.38 μmol/l, P < 0.05), unlike those from Norplant users without bleeding problems (6.96 ± 0.786 μmol/l) (Figure 1).

Thirty-two out of 36 biopsies from Norplant users with bleeding problems had a median EC migratory score of 0. Supplementation of vitamin E (in vitro) resulted in a significantly higher angiogenic score than placebo (z = -2.24, two-tailed P < 0.05) (Figure 2).

The blood concentrations of lipid peroxide were significantly increased after 3 months exposure to Norplant (n = 6, mean ± SE: 1.36 ± 0.30 versus 2.92 ± 0.77 μmol/l, P < 0.05) and DMPA (n = 7, mean ± SE: 2.5 ± 0.16 versus 3.53 ± 0.46 μmol/l, P < 0.05) (Figure 3).

The blood concentrations of vitamin E were significantly decreased after 3 months exposure to Norplant (n = 6, mean ± SE: 0.92 ± 0.18 versus 0.61 ± 0.23 mg/dl, P < 0.05) and DMPA (n = 7, mean ± SE: 2.26 ± 0.76 versus 0.79 ± 0.26 mg/dl, P < 0.05) (Figure 4).

The endometrial angiogenic activity scores in Norplant and DMPA users were significantly lower than controls, and the angiogenic activity in DMPA users was higher than Norplant users (Table I).

**Discussion**

The blood concentrations of lipid peroxide in Norplant and DMPA users were significantly higher than in controls. The increased concentration of lipid peroxide may be caused by an elevated radical chain reaction in lipid membranes. This corroborates another investigation (White et al., 1991) in which there was an increase of epoxide synthesis, which correlated with vasodilatation and rupture of blood vessels in the endometrium. It has been reported that the use of DMPA is associ-
Figure 4. Blood vitamin E concentrations in women before (white) and after 3 months (shaded) exposure to Norplant or depo-medroxyprogesterone acetate (DMPA).

Table 1. EC migration score in Norplant and depo-medroxyprogesterone acetate (DMPA) users

<table>
<thead>
<tr>
<th>EC score (median)</th>
<th>Norplant</th>
<th>DMPA</th>
<th>Control</th>
<th>Total</th>
</tr>
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<tbody>
<tr>
<td>0.0</td>
<td>7</td>
<td>4</td>
<td>1</td>
<td>12</td>
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<tr>
<td>0.5</td>
<td>3</td>
<td>3</td>
<td>2</td>
<td>8</td>
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<tr>
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<td>3.0</td>
<td>1</td>
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</tr>
<tr>
<td>Total</td>
<td>11</td>
<td>8</td>
<td>7</td>
<td>26</td>
</tr>
</tbody>
</table>

Norplant (A: 2; PB: 3; IF: 2; N: 4); DMPA (A: 4; PB: 2; IF: 1; N: 1). A = amenorrhoea; PB = prolonged bleeding; IF = infrequent bleeding; N = normal cycle.

Control (proliferative: n = 4; secretory: n = 4).

EC score in Norplant < DMPA < control (P < 0.02).

associated with lymphocyte infiltration in the endometrium (Song et al., 1996). The lymphocytes release superoxide ions, which may influence endometrial integrity and lead to BTB.

Thirty-two out of 36 biopsies from Norplant users with bleeding problems had a median EC migratory score of 0. Addition of vitamin E to the endometrium explants increased the EC migratory score. Vitamin E is a fat-soluble antioxidant, which can protect the membrane cell from the damaging effects of lipid peroxidation. Support for the present results can be drawn from other investigators who have shown that vitamin E can inhibit free radical formation and can protect EC cultures from cell damage (Matsuo and Kaneko, 1990; Niki, 1990; Faruqi et al., 1994). It is interesting to note that the blood concentrations of lipid peroxide were increased and those of vitamin E were decreased after 3 months exposure to Norplant and DMPA. Although the subjects were instructed not take any additional vitamin E or to change their dietary pattern during the study, some confounding factors can influence the blood concentrations of vitamin E. However, it is suggested that the concept of oxidant–antioxidant imbalance may be at least partially responsible for cell damage in endometrium.

We have shown that endometrial angiogenic response was decreased in progestin-only contraceptive users. Seven out of 11 Norplant biopsies (63.6%) and four out of eight DMPA biopsies (50%) had a median endothelial cell (EC) score of 0 compared with one out of five for controls (20%). Increased reactive oxygen species production can induce apoptosis in several cell types. Although there was not a clear correlation between absolute lipid peroxide concentration and endometrial angiogenic activity, it is still possible that the oxidant–antioxidant imbalance is causing cell damage and reduced angiogenic activity, thus leading to BTB in the progestin contraceptive users.

It is concluded that in progestin-only contraceptive users, higher lipid peroxide and lower vitamin E blood concentration may contribute endometrial cell damage and decrease the endometrial angiogenic response. It is suggested that vitamin E supplementation may be effective in increasing angiogenic response.

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