Microvascular density in conditions of endometrial atrophy

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Irregular menstrual bleeding in users of hormonal contraception represents the single major reason for discontinuation of these contraceptive methods. The mechanisms which underlie these bleeding disturbances are poorly understood, but appear to be associated with changes in the endometrial microvasculature following abnormal patterns of sex steroid exposure. Endometrial microvascular density is known to be increased in users of the low-dose levonorgestrel contraceptive implant, Norplant®. This study explores microvascular density in other conditions of spontaneous (post-menopausal) and induced (danazol and goserelin) endometrial atrophy. Endometrial biopsies were fixed, paraffin-embedded and sections were immunostained using anti-CD34 antibody to identify vascular endothelial cells. The mean microvascular density (± SEM) for control samples was 186 ± 8 vessels/mm². There were no statistically significant changes in vascular density observed across the menstrual cycle. Mean microvascular density in spontaneous and induced endometrial atrophy did not differ significantly from that observed in the control population. The mean microvascular density was 230 ± 17 vessels/mm² in 31 post-menopausal women, 269 ± 67 vessels/mm² in 25 subjects treated with danazol was and 191 ± 45 vessels/mm² in nine subjects treated with goserelin. These findings suggest that the mechanisms controlling microvascular density in conditions of endometrial atrophy may vary according to the nature of the atrophic stimulus.

Key words: atrophy/endometrium/vascular density

Introduction

The disruption of normal menstrual bleeding patterns by long-acting progestogenic contraception presents a major challenge to contraceptive providers. Non-hormonal contraceptive systems may also influence menstrual bleeding. Copper-containing and inert intrauterine devices (IUD) are associated with an increased menstrual blood loss (Andrade and Orchard, 1987).

Recent attention has focused on the role of endometrial blood vessels in the initiation and control of menstrual blood loss and breakthrough bleeding (BTB) (Fraser and Peek, 1992; Rogers et al., 1993). Histological and hysteroscopic examination of the endometrium in women complaining of BTB associated with steroid hormone contraceptives suggests that the source of the bleeding may be capillaries and small veins (Fraser and Peek, 1992; Hickey et al., 1996). Dilated and disrupted small venules located close to the endometrial surface following vaginal (Hourihan et al., 1986) and intrauterine (Sheppard et al., 1987) progestogen exposure are a potential site of irregular bleeding.

Angiogenesis (the formation of new blood vessels from existing vessels) does not usually occur in the adult human apart from during pathological processes such as retinopathy, rheumatoid arthritis, tumour growth or wound repair. The female reproductive tract is an exception, where there is periodic growth and regression of blood vessels. The process of angiogenesis and vascular regression in these tissues appears to be hormonally regulated. However, the local mechanisms that control these processes have yet to be determined.

Distinct periods of angiogenesis have been identified in the endometrium during the menstrual cycle (Markee, 1940; Ferenczy et al., 1979; Kaiserman-Abramof and Padykula, 1989). The mechanisms controlling vascular proliferation in the endometrium are not fully understood and angiogenesis at this site may occur by a different process from that in other tissues (Goodger and Rogers, 1994).

Endometrial atrophy describes the loss of glandular and stromal elements of the endometrium and may arise in response to exogenous steroid hormones or following the withdrawal of endogenous ovarian steroids. The response of the endometrial vasculature to an atrophic stimulus may differ from that of the glands and stroma (Hourihan et al., 1986; Rogers et al., 1993). Two clinical treatments that may induce endometrial atrophy are danazol and gonadotrophin-releasing hormone (GnRH) agonists.

Danazol is an isoxazol derivative of 17α-ethinyl testosterone. It has numerous effects on the reproductive system; it alters the pulsatile secretion of GnRH, inhibits the lutemizing hormone (LH) and follicle stimulating hormone (FSH) surge, causes abnormal follicular maturation and thus reduces oestrogen production. Direct endometrial effects include the suppression of oestrogen and progesterone receptors, rendering the endometrium functionally hypo-oestrogenic and hypo-progestogenic (Jeppson et al., 1984). The endometrium following prolonged danazol exposure is likely to be atrophic (Greenblatt et al., 1971). Bleeding patterns following danazol exposure are determined by treatment dose. At 800 mg/day...
the majority of patients became amenorrhoeic (Fraser, 1985), whilst at lower doses (200 mg/day) they may continue to menstruate regularly, but with a significant reduction in menstrual blood loss (Chimbira et al., 1980). Between 200–800 mg/day, the clinical response to danazol is varied and bleeding patterns are generally irregular with scanty loss and a dose-related increase in amenorrhoea (Fraser, 1985).

GnRH analogues have a higher binding affinity to GnRH receptors than naturally-occurring GnRH, with a greatly increased half-life. After an initial stimulatory effect, prolonged use of these agonists down-regulates pituitary gonadotrophic receptors, producing hypogonadotropic hypogonadism (Meldrum et al., 1982). The endometrium is generally thin and atrophic following prolonged GnRH agonist exposure (Brooks et al., 1991). In normal ovulatory women GnRH agonists will suppress ovulation and will cause amenorrhoea in up to 95% of women treated, depending on dose. The rest will have bleeding that is lighter and less regular than normal (Bergquist et al., 1981).

This aim of this study was to quantify changes in microvascular density during conditions of endometrial atrophy due to either danazol or GnRH agonist treatment, or following the menopause.

Materials and methods

Subjects

Endometrial biopsies were obtained from 73 women exposed to danazol or to goserelin, or from post-menopausal women not taking hormone therapy. The latter group had no history of post-menopausal bleeding and samples were taken from hysterectomy specimens following vaginal hysterectomy for uterovaginal prolapse. Five specimens were excluded due to lack of endometrium for analysis, and three due to inadequate patient details. This left 65 biopsies for analysis: (i) 25 following exposure to 400–600 mg of danazol/day for 6 weeks (400 mg given prior to endometrial ablation and 600 mg in the treatment of endometriosis); (ii) nine following exposure to goserelin for 12 weeks (prior to myoma resection); (iii) 31 post-menopausal women not exposed to hormone therapy.

Biopsies were obtained by outpatient curettage for 11/25 subjects from the danazol group (Pipelle; Prodimed, Neully-en-Thielle, France); by diathermy loop resection biopsy under general anaesthetic for 14/25 danazol subjects and all goserelin subjects; or by hysterectomy specimens for uterovaginal prolapse (all post-menopausal subjects). All subjects were recruited to the trial on the basis of fully informed consent. Tissue was collected in a number of research centres in Australia, and local ethical approval for these studies was obtained from ethics committees at Monash University and from Family Planning, NSW, Australia.

Controls

Control data for this study were taken from a previously published report where an identical methodology was used (Rogers et al., 1993). Normal menstrual cycle biopsies were collected as controls from 54 volunteers in Melbourne, Australia. This tissue was mainly taken from women undergoing investigation for infertility, where the cause of the infertility was shown clearly to be unrelated to uterine or endocrine factors. Tissue was not collected from women who had received exogenous sex steroid hormones or who had used an IUD in the previous 3 months. Those with known uterine pathology were also excluded. Endometrial biopsies were obtained by dilatation and curettage (D&C) or by Pipelle suction curette (Prodimed) from the uterine fundus or corpus. The isthmus was always avoided. The date of the last menstrual period was known for all subjects.

Biopsy processing, histopathology and immunohistochemistry

All biopsies were immediately placed in 10% buffered formalin at 4°C for 4–6 h, and then rinsed and stored in phosphate-buffered saline (PBS) at the same temperature. Endometrial tissue was processed by routine paraffin embedding, and 5 μm sections were cut for either immunohistochemistry or haematoxylin and eosin (H&E) staining for histopathological evaluation by an experienced gynaecological pathologist (P.R.). H&E stained sections of endometria were dated according to the general classifications of Noyes et al. (1950).

The endometrial microvasculature was visualized using the mouse monoclonal antibody against human CD34 antigen (Clone QBEND/10; Serotec, Oxford, UK), which is expressed on the endothelial cell membrane (Fina et al., 1990). Primary antibody binding was visualized using a streptavidin–biotin horseradish peroxidase immunohistochemical staining kit (Zymed Laboratories, San Francisco, CA, USA). Prior to the primary antibody incubation, endogenous peroxidase activity in the endometrial tissue was blocked by treatment with 3% hydrogen peroxide (BDH Chemicals, Port Farry, Victoria, Australia) for 10 min at room temperature. Tissue sections were incubated with the CD34 antibody for 60 min at 37°C at 1:2 dilution. Normal rabbit serum (NRS 10%; Gibco, Life Technologies Inc, Grand Island, NY, USA) was added to the CD34 antibody to prevent non-specific binding. Sections were mounted in Hydromount (National Diagnostics, New Jersey, USA) without counterstaining to avoid interference during image analysis. For negative controls, the primary antibody was replaced by mouse immunoglobulin (IgG1; Silenus Laboratories, Victoria, Australia) used at the same protein concentration, or the diluent alone was used. In each staining run, a control section of known immunohistochemical staining intensity was included as a positive control and to ensure consistency between runs. Microvascular profiles identified by CD34 staining were counted using a Zeiss Axioskop microscope (Zeiss, Göttingen, Germany) with a ×10 magnification Zeiss Achroplan Lens, without a grid. A Microcomputer Imaging Device (MCID); version 2, beta 2.4 (Imaging Research Inc., Brock University, St Catharines, Ontario, Canada) was used to accurately measure the field of view and count vessels stained with anti-CD34. Black and white images were used to enhance contrast between vessels and background staining. Image analysis assessments of microvascular density were validated by regular comparison with manual counts. Three fields of endometrial microvascular density (vessels/mm²) were calculated for each biopsy.

Statistical analysis

Statistical analyses were performed using the JMP program from SAS on a Macintosh 6200/75s computer. Analysis of variance was used to assess whether there was a statistically significant difference in means between two or more groups. When more than two groups were included in the analysis of variance, the Tukey–Kramer post hoc test was used to indicate which groups showed statistically significant differences in means from other groups. Statistical significance was assumed when \( P < 0.05 \). Values are means ± SEM unless otherwise stated. The power of this study to detect significant differences in sample size between the treatment groups was 52%.

Results

The mean age of the study population was 47 ± 9 years. Not surprisingly, the mean age of subjects differed between the
groups. The average age of women in the post-menopausal group was 62 ± 3 years. Average ages for the danazol and goserelin groups were 39 ± 0.9 and 41 ± 0.6 years respectively. The mean age of the control group was 34.8 ± 2 years.

**Endometrial histology**

**Controls**

Histopathological assessment of biopsies from 54 control subjects (Noyes et al., 1950) are indicated in Table I.

**Experimental group**

Histological assessment was defined as follows: (i) atrophic, with small sparse glands lined by inactive cuboidal epithelium and densely hypercellular stroma; (ii) inactive, differing from proliferative endometrium in the absence of mitoses in stromal and glandular cells; (iii) weakly proliferative with weak oestrogenic stimulation, with some irregularity of gland shape and distribution but no evidence of hyperplasia. The distributions of these different types are shown in Table II.

**CD34 immunohistochemical staining results**

CD34 antibody produced positive staining for the endothelial lining of all identifiable blood vessels in the tissue samples. The morphological appearance of the vessels did not differ from the control population. Mean microvascular densities for the different treatment groups are shown in Table III.

**Statistical analysis**

Using analysis of variance, no statistically significant differences in vascular density were identified between the post-menopausal, danazol and goserelin subjects (F ratio = 0.47, P = 0.62) or between these groups and the controls (F ratio = 1.74, P = 0.14).

No statistically significant difference in endometrial microvascular density was observed between those treated with 400 mg of danazol (mean density 310 ± 113 vessels/mm²) and treatment with 600 mg danazol (mean density 215 ± 24 vessels/mm²; F ratio = 0.52, P = 0.47).

Table IV shows the endometrial vascular density of the different treatment groups when further categorized into histological appearance.

No consistent association was observed between endometrial histology and vascular density (F ratio = 0.30, P = 0.73).

**Discussion**

The results from this study suggest that the microvascular density in these conditions of induced and spontaneous endometrial atrophy do not differ significantly from that of a control population. The large SEM observed in the danazol and goserelin treatment groups suggests that there is considerable inter-individual variation in the response to these agents.

These findings differ from that observed in Norplant users (Rogers et al., 1993) where a mean microvascular density of 294 ± 18 vessels/mm² was seen, an increase of 158% above the control population. This suggests that Norplant may affect the control of endometrial vascular regression and/or proliferation by mechanisms which differ from those of other spontaneous and induced causes of endometrial atrophy. Shaw et al. (1979, 1981) state that vascular density was increased in IUD users, but it was not possible to compare our findings with these publications since only relative changes in vascular density between control and treatment groups are given. In this study we are able to give precise values for vascular density/area of endometrium. One of the advantages of using an image analyser to measure vascular density is that it allows an exact measurement of the tissue area observed.

In the normal menstrual cycle, mean microvascular density remains static despite cyclical neovascularization and major changes in growth, oedema and regression in glandular and stromal tissues (Shaw et al., 1979, 1981; Hourihan et al., 1986; Rogers et al., 1993). This suggests that the mechanisms controlling normal endometrial vascularization are tightly regulated.

The treatment group showing the highest mean vascular density was that exposed to 400 mg danazol. This dose of danazol is associated with incomplete suppression of ovarian activity in many subjects (Fraser, 1985). Similarly, Norplant is associated with a variable ovarian response, particularly during the later months of use (Sivin, 1994). This suggests that endogenous or exogenous sex steroid may act to 'uncouple' the control of vascular and non-vascular elements in the endometrium.

In users of high dose progestogens, Song et al. (1995) observed a decrease in microvascular density, from 169 ± 9.3
vessels/mm² in the control population, to 103 ± 9.6 vessels/mm². High-dose progestogens are associated with marked endometrial oedema, and this may account for the relative reduction in microvascular density observed by these authors.

The results from this study should be interpreted with caution, since other factors may have acted to influence vascular density in these subjects. In the danazol group, 11 women were known to have endometriosis and were treated with 600 mg of danazol for 4–6 weeks. The remainder (14) had confirmed menorrhagia and were treated with 400 mg of danazol for the same period. All those in the goserelin group were known to have uterine myomata and were treated for 12 weeks. The post-menopausal group were between 2–20 years since spontaneous or surgical menopause. Timing since induction of endometrial atrophy may influence vascular density. Longitudinal studies examining changes in microvascular density following the induction of endometrial atrophy are in progress.

A wide range of histological appearances were observed in all the groups studied, demonstrating individual endometrial variation in response to similar ‘atrophic’ stimuli. It is possible that these conditions may have acted independently to alter endometrial microvascular density. Endometrial vascular density did not differ significantly within histological groupings, although there was a tendency for atrophic endometrium to be associated with a higher vascular density. The mean vascular density for each histological group was similar to that reported by Rogers et al. (1993) for Norplant users, and these workers also failed to identify an association between microvascular density and endometrial histology. The clinical significance of these changes in vascular density and morphology are unclear. There is no evidence at present to suggest that changes in endometrial microvascular density correlate directly with bleeding patterns (Rogers et al., 1993). It is possible that new vessels or changes in the ratio of endometrial vascular and non-vascular elements associated with endometrial atrophy may result in small blood vessels that are more ‘fragile’ than normal endometrial vessels and that these may be associated with BTB. This may explain the characteristic pattern of light, prolonged and irregular bleeding in this population. Endometria exposed to Norplant, danazol and goserelin have shown the highest vascular density values, and these sex steroid hormones are more frequently associated with irregular bleeding than the post-menopausal and high-dose progestogen groups.

All biopsies in this study were obtained by blind techniques from danazol and goserelin-exposed endometrium. Normal endometrial development (Dallenbach-Hellweg, 1987) and vascular density (Shaw et al., 1979) are thought to be uniform throughout the cavity. There is hysteroscopic evidence that in women exposed to exogenous sex steroid hormones the endometrial vascular distribution is irregular and patchy (Brooks et al., 1991; Hickey et al., 1996). Directed endometrial biopsies should help to clarify the relationship between density and bleeding. Unfortunately, hysteroscopic equipment is not available to provide directed biopsy specimens of adequate size under out-patient conditions.

When endometrium cannot be obtained at endometrial biopsy, there is evidence that these subjects may differ in terms of ovarian steroids and bleeding patterns (Hadiisaputra, 1996). This may undermine the value of studies where conclusions are drawn from a selected group of subjects with sufficient tissue at biopsy.

The local mechanisms controlling these changes in endometrial vascular density are unclear, but may differ between induced and spontaneous atrophy. Receptors for GnRH have been identified in the endometrium (Bourgain et al., 1994), and danazol is known to exert direct endometrial effects via androgenic and progestogenic receptors (Barbieri, 1991). The effects of sex steroid hormones on the endometrial vasculature may depend upon the dose, duration and type of hormone, and may also show individual variation.

The role of oestrogens in the regulation of microvascular density is unclear, but Rogers et al. (1993) observed that Norplant users with higher endogenous oestradiol concentrations had a tendency towards lower microvascular densities. Oestrogen has been used to treat prolonged bleeding episodes in Norplant (Diaz et al., 1990) and depot medroxyprogesterone acetate (DMPA) (Fraser, 1983) users. Longitudinal studies of changes in microvascular density following successful management of bleeding problems with sex steroid hormones are required. If oestrogen appears to reduce bleeding by ‘normalizing’ microvascular density in the endometrium, there may be a role for other substances which oppose vascular proliferation or promote vascular regression in the control of BTB. Serum oestradiol values in this study might have provided further useful information.

In conclusion, this study suggests that endometrial microvascular density following danazol and goserelin treatment and in the post-menopause, does not differ significantly from that of normal endometrium. Variations in endometrial histological appearance were observed following exposure to danazol, goserelin and in the post-menopause. Endometrial vascular density did not differ significantly between histological groups.
These observations differ from those seen following Norplant insertion, suggesting that the mechanisms of endometrial vascular control may differ in response to this low dose levonorgestrel implant.

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