Hormonally mediated disturbance of angiogenesis in the human endometrium after exposure to intrauterine levonorgestrel

C.Jay McGavigan¹, Peter Dockery², Vasiliki Metaxa-Mariatou¹, Dianne Campbell¹, Colin J.R.Stewart³, Iain T.Cameron¹,4 and Steven Campbell¹,5

¹Department of Obstetrics and Gynaecology, University of Glasgow, Glasgow Royal Infirmary, Glasgow, UK, ²Department of Anatomy, University College, Cork, Ireland, ³Department of Pathology, Glasgow Royal Infirmary, Castle Street, Glasgow and ⁴Maternal, Fetal and Neonatal Physiology Group, Fetal Origins of Adult Disease Division, University of Southampton, Princess Anne Hospital, Coxford Road, Southampton, UK

To whom correspondence should be addressed at Department of Obstetrics and Gynaecology, University of Glasgow, Glasgow Royal Infirmary, 10 Alexandra Parade, Glasgow G31 2ER, UK. E-mail: gqta05@udcf.gla.ac.uk

BACKGROUND: The levonorgestrel intrauterine system (LNG-IUS) is a contraceptive device that is used for treatment of menorrhagia. The system induces inter-menstrual bleeding within the first few months after insertion. We hypothesized that this bleeding might be associated with a change in vascular development. METHODS: A randomized, controlled study was undertaken on 48 women. RESULTS: Hysterectomy specimens were obtained and immunocytochemistry was carried out with antibodies to CD31, α-smooth muscle actin and myosin. Stereological measurement of blood vessels was also undertaken. Most vessels appeared normal, including the arterioles. Large thin-walled vessels were present in the superficial endometrium of the treated group but were almost completely absent in the controls. The distribution of cytoskeletal markers revealed well-formed basal arterioles with more widespread expression in the superficial stroma than was found in untreated tissue. The volume fraction of blood vessels (P = 0.0001), the number of vessel cross-sections per unit area (P = 0.0003) and the cross-sectional diameters of the largest vascular lumens (P = 0.0001) were significantly increased following treatment with LNG-IUS. However, there was no difference in the median values of vessel diameter or the vascular surface density. CONCLUSION: These findings suggest that the LNG has a localized effect on some vessels within the superficial endometrium.

Key words: breakthrough bleeding/endometrium/levonorgestrel intrauterine system/progestogen/vasculature

Introduction

Unscheduled breakthrough bleeding is a common side-effect of progestogen-only contraceptives including Norplant® (s.c. delivery of levonorgestrel), oral progestogen preparations, slow release injectable progestogens and the levonorgestrel intrauterine system (LNG-IUS, Mirena®) (Odland, 1998; Hickey and Fraser, 2000a; Rogers et al., 2000; Thomas et al., 2000). The LNG-IUS is an intrauterine contraceptive system which is also licensed for the treatment of menorrhagia. It has been shown to reduce menstrual blood loss by up to 94% at 3 months and 97% at 12 months, and 20–30% of women are amenorrhoeic after a year of treatment (Andersson and Rybo, 1990; Irvine et al., 1998). Despite this beneficial effect on menstrual loss, compliance is often hampered because of the initial troublesome side-effect of breakthrough bleeding which usually presents as light spotting between menstrual periods. Breakthrough bleeding with the LNG-IUS is most commonly seen during the first 4–6 months after insertion of the system (Lahteenmaki et al., 1998). The presence of breakthrough bleeding and reduction in menstrual loss suggest that the progestogen may affect the endometrial vascular system directly.

Both Norplant and the LNG-IUS have profound effects on endometrial structure that could in turn affect the function and stability of the endometrial vasculature (Skinner et al., 1999; Jones and Critchley, 2000). The LNG-IUS delivers a high dose of LNG to the endometrium and induces a very rapid decidualization response in the endometrial stroma (Silverberg et al., 1986; Telfer et al., 1997; Critchley et al., 1998). Ultrastructural examination has also demonstrated changes within the surface and glandular epithelium (Pakarinen et al., 1998). Exposure to Norplant, although at a much lower effective dose to the endometrium, results in vascular changes that can be observed in tissue sections or by hysteroscopy (Rogers et al., 1993; Hickey et al., 1998). These changes include the presence of petechiae and ecchymoses in
the superficial endometrium (Hickey et al., 1996, 1998; Hickey and Fraser, 2000b). Mean superficial vascular diameter was greater in women using low dose LNG implants compared with those with menorrhagia (Hickey et al., 1998).

In view of these in-vivo observations, we postulated that a common mechanism might underlie the pathogenesis of intermenstrual bleeding irrespective of the dose or route of delivery of progestogen. We therefore sought to demonstrate the effect of short-term exposure to high-dose intrauterine LNG on endometrial vessels in a randomized, controlled study in women with menorrhagia scheduled for hysterectomy.

Materials and methods

Study design and patient characteristics
Women attending the general gynaecology clinics at three hospitals in Glasgow, UK were recruited to the study. These women had been referred for secondary management of menorrhagia. At the first consultation, a thorough history was taken and examination was performed as well as a pelvic ultrasound scan and an endometrial consultation, a thorough history was taken and examination was referred for secondary management of menorrhagia. At the first appointment, a detailed questionnaire and height and weight were assessed independently by local pathologists and reports were corrected for the proportion of the field occupied by tissue (Howard and Reid, 1998). Grid length was measured using cycloid grids oriented perpendicular to the vertical axis of the endometrium (Howard and Reid, 1998). Grid length was measured using digital images by superimposing orthogonal grids using the image analysis program. A grid of 13 × 13 points was superimposed on the centre of each non-overlapping field. The outer surface of the endothelium was measured using cycloid grids oriented perpendicular to the vertical axis of the endometrium (Howard and Reid, 1998). Grid length was corrected for the proportion of the field occupied by tissue (Howard and Reid, 1998). Vessel diameter was measured manually with a camera lucida attachment (Olympus, London, UK). It was necessary to examine the specimen using ×20 and ×40 objective lenses in order to measure the diameters of both large and small vessels accurately. The images were spatially calibrated using a 10 μm linear graticule (Gricules Ltd, Tonbridge, UK). The number of vessel lumen cross-sections per unit area was counted using the camera lucida and a ×20 objective. A 13 × 13 orthogonal grid with an external guard zone was used to define the counting area and allow systematic accumulation of data. Vessels overlapping the basal and left lateral margins of the grid were excluded. Measurements were carried out on one section from each patient. Eleven to 20 fields were analysed per patient depending on the size of the tissue section. A total of 5548 point counts were made for the calculation of volume fraction. The total number of vessel lumen diameters measured was 2049. Preliminary analysis of the pooled data sets demonstrated that
the data were not normally distributed. The median value of each data parameter was therefore obtained as an estimator for each patient. In addition the largest single vessel diameter was used as a non-arbitrary index for the presence of large vessels (Hourihan et al., 1986). Mann–Whitney U-tests (Minitab v 13) were used to compare these sets of median or maximum values.

Results

The patients in the control and treated groups were matched for age, parity, body mass index, previous treatment of menorrhagia and family history of hysterectomy. There was, however, a greater proportion of smokers in the control group (Table I).

The morphology observed in the control specimens was characteristic of normal endometrium (Figure 1A). The response to the LNG-IUS was not consistent between patients. After short-term exposure (range 28–156 days, mean 56.7 days, median 50 days) some specimens showed a marked thinning of the endometrium in comparison with the control group. No consistent delineation between basal and superficial endometrium was observed. Other treated endometrium had a highly irregular surface. In the dense basal stroma, gland epithelial height was greater than that in the more superficial functionalis (Figure 1B). In treated specimens, less densely packed superficial tissue, characteristic of normal functionalis, was almost absent in places. In all specimens there were areas of endometrial surface irregularity or micropolypoid appearance, decidualization and reduced epithelial height in the superficial region (Figure 1C, D). Nevertheless there was considerable variation in tissue morphology. The highly irregular surface was characterized by the presence of micropolypoidal structures that in some cross-sections appeared detached from the tissue. In cross-section this had a classical micropolypoidal appearance with the presence of circular or ovoid cross-sections not directly connected with the surface within the plane of section. Such structures were not

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<sup>a</sup>Six women decided to retain the levonorgestrel intrauterine system (LNG-IUS) and not proceed to hysterectomy, as they were satisfied with the effect of the system on their menstrual loss

<sup>b</sup>Three women in the control group chose to withdraw from the study after randomization.

Disturbance of endometrial angiogenesis by LNG

Figure 1. Tissue morphology after exposure to the levonorgestrel intrauterine system (LMG-IUS). (A) Control tissue had a smooth surface with normal glandular morphology and no large thin-walled vessels within the superficial part of the tissue. (B) Endometrial glands after exposure to LNG-IUS for 50 days with basal tissue present on the right side of the figure. Within the basal part of the tissue the epithelium was of greater height in contrast to that seen in the more superficial areas (arrows). (C) After short-term exposure to LNG-IUS (50 days), the tissue surface was highly irregular and micropolypoid, making it difficult to distinguish the boundary between surface and glandular epithelium. These micropolyps sometimes appeared detached from the tissue in cross-section (arrow). (D) Higher magnification of a micropolypoidal structure showing dilated vessels (arrows), decidualized areas of stroma and irregularly thinned surface epithelium and a constricted base following 70 days exposure to the LNG-IUS. Scale bars: A = 115 μm, B = 50 μm, C = 90 μm, D = 45 μm.
observed in the control tissue (Figure 1A, C) of the present study or in any hysterectomy material from untreated women which we have examined in recent years.

Qualitative assessment of vascular morphology

Most micro-vessels were of normal appearance within the superficial endometrium. However, CD31 localization of the endothelium demonstrated the presence of large vessels in the superficial part of the tissue (Figure 2). Following treatment with LNG-IUS, CD31 immunolocalization also identified individual cells within the vessel lumen and the superficial stroma. These CD31-labelled stromal cells were less evident in the densely cellular basal parts of the stroma.

The large superficial vessels lacked an obvious muscularized wall with the distinctive layering characteristic of well-formed basal arterioles (Figure 3A, B). Despite very similar distributions of cytoskeletal markers within the myometrium, expression of α-actin and myosin was much more variable within treated (Figure 3C–E) and control tissue than previously described (Kohnen et al., 2000). Variability of expression occurred within the stroma both within the functionalis and basalis. There were no consistent patterns of expression or gradients within the stroma. In some specimens, the distribution corresponded to the previously reported pattern of basally restricted myofibroblast-like cells, but in others smooth muscle marker expression was also seen in the superficial part of the stroma (Figure 3D, E). Even within stromal regions of high smooth muscle marker expression, well-developed vessel walls were not evident (Figure 3F, G). Smooth muscle and myosin positive areas in the basalis co-existed with areas where the markers were solely restricted to the vessels with none in the surrounding stroma. Well-formed arterioles which would normally be found deeper within the tissue were sometimes present superficially where an irregular endometrial surface existed (Figure 3H, I). In control tissue, well-formed arterioles were restricted to the basal stroma (Figure 3J, K).

Quantitative assessment of vascular morphology

In view of the variability in the endometrial morphology and thickness (described above), it was not possible to stratify the tissue for analysis using defined rules. Data were therefore obtained independently of position. The volume fraction of tissue occupied by endothelial cells and vascular lumens was increased following treatment with LNG-IUS (median vessel volume fraction LNG-IUS 7.69%, control 3.85%, P = 0.0004, Figure 4A). Most vessels were of normal size (median of median values of vessel diameters LNG-IUS 38.7 μm, control 37.1 μm). However, a small number of vessels with a large luminal diameter were also present. The maximum value of the largest luminal diameter for each specimen was significantly increased after exposure to the LNG-IUS (median maximal diameter LNG-IUS 312.9 μm, control 174.2 μm, P = 0.0001, Figure 4B). There was a significant difference between the number of vessel cross-sections per unit area between the treated and control groups (median number per unit area LNG-IUS 55.5, control 24.6, P = 0.0003, Figure 4C). However, vascular surface density determined by cycloid grid intercept counts was not significantly different between the two groups (LNG-IUS 0.0136, control 0.0111 arbitrary units, P = 0.37, Figure 4D).

Figure 2. CD31 immunolocalization of the vascular endothelium within the endometrium of treated and control tissue. (A) Thin-walled, superficial vessel (arrow) within a decidualized area of superficial stroma after levonorgestrel (LNG) exposure. (B) Large diameter cross-sections of thin-walled vessels (arrows) are present within the superficial part of the tissue. (C) A range of vascular cross-sectional diameters including vessels of normal size can be observed within a given field (arrows). (D) Control tissue within which a normal microvascular network has developed. Scale bars: A = 50 μm, B = 100 μm, C = 110 μm, D = 100 μm.
Discussion
This study demonstrates a change in vascular morphology following short-term exposure to the LNG-IUS. The work was carried out on women who had elected to have a hysterectomy for the treatment of menorrhagia. Menorrhagia was not diagnosed objectively by measuring menstrual blood loss. However, whilst it is appreciated that some of the women may not have had an actual blood loss >80 ml per month (Hallberg et al., 1966; Cameron, 1989), the use of a control group (with random allocation of women to the treatment arm) permitted us to comment about short-term exposure to LNG in women with a robust clinical diagnosis of menorrhagia.

Figure 3. α-Smooth muscle actin and myosin in treated (A–I) and control (J, K) specimens shows great variability of expression. (A) A well-decidualized endometrium after levonorgestrel intrauterine system (LNG-IUS) exposure showing myosin distribution in a restricted number of perivascular cells (arrow). (B) A higher magnification image showing the presence of perivascular myosin positive cells (arrows). (C) A basal vessel in treated tissue showing the usual pattern of myosin expression in the myometrial smooth muscle. (D) An area of abundant α-smooth muscle actin expression within LNG-treated tissue in superficial stroma. (E) The corresponding area shows a more restricted pattern of myosin expression. (F) A higher magnification image from an area within the stroma showing periglandular (g) and perivascular (v) expression of actin. (G) A higher magnification image showing periglandular (g) and perivascular (v) expression of myosin. (H) α-Smooth muscle actin in well-formed arterioles close to an irregular surface after treatment. (I) Myosin distribution in the same vessels as that shown in (H) demonstrating the well-developed nature of the vessel walls. (J) α-Smooth muscle actin distribution in a control specimen showing well-developed basal arterioles and superficial stromal expression. (K) Myosin distribution in well-developed basal arterioles and smaller superficial vessels. Scale bars: A = 80 μm, B = 40 μm, C = 80 μm, D, E = 80 μm, F, G = 50 μm, H, I = 70 μm, J, K = 50 μm.
implant also support this conclusion (Hickey et al 1998). Observations of the endometrium after treatment with an LNG intrauterine system (LNG-IUS) might be the ‘expansion’ of the underlying stroma resulting in the glands to widen. A driving force behind these changes might be the loss of glandular structural integrity causing the openings of thin-walled vessels to develop. It is possible that these thin-walled vessel segments may contribute to the breakdown of the epithelium. Weakening of the epithelium might in turn result in local breakdown and shedding ensues and atrophy begins, the superficial part of the endometrium would be difficult, if not impossible, to obtain full thickness tissue specimens from women successfully treated with the LNG-IUS. It is noteworthy that the bleeding problems resolved after surgical removal of these polyps. It seems likely that these vascular abnormalities may not persist when the tissue becomes atrophic, and this is probably associated with the resolution of breakthrough bleeding. It would be difficult, if not impossible, to obtain full thickness tissue specimens from women successfully treated with the LNG-IUS over a protracted period. We can therefore only speculate that as localized breakdown and shedding ensues and atrophy begins, the superficial part of the endometrium may be replaced by a tissue without superficial lesions.

The irregular appearance of the endometrial surface after treatment with LNG-IUS is not specific to intrauterine delivery of levonorgestrel per se. Polypoidal structures are known to develop after treatment with tamoxifen and often manifest with breakthrough bleeding (Hann et al., 2001), which suggests that endometrial surface irregularity or micropolyps may contribute directly to bleeding patterns observed after exposure to the LNG-IUS. This view is supported by the hysteroscopic observations of Brenchin et al. (2000) who reported the presence of polyps in three women after long-term exposure to the LNG-IUS. It is noteworthy that the bleeding problems resolved after surgical removal of these polyps.

The presence of irregularities and micropolyps may be caused by changes within the epithelium and stroma (Figure 5). Thinning of the superficial and glandular epithelium noted here and previously observed in an ultrastructural study (Gu et al., 1995) may result in mechanical weakness. Weakening of the epithelium might in turn result in loss of glandular structural integrity causing the openings of the glands to widen. A driving force behind these changes might be the ‘expansion’ of the underlying stroma resulting from localized and patchy decidualization (Gu et al., 1995;
Localized shedding of superficial tissue could further exacerbate the development of irregularity. Exposure to the LNG-IUS causes changes to stromal cell phenotype and increased expression of cytoskeletal markers within some parts of the stroma. The presence of smooth muscle markers without the morphological characteristic of perivascular smooth muscle cells raises the question of whether these cells are differentiating in a non-physiological fashion due to the effect of local high-dose levonorgestrel. In our previous report (Kohnen et al., 2000) we suggested that expression of smooth muscle actin within the basal stromal cells identified cells that were myofibroblastic in nature. As myofibroblasts have been noted to appear during wound healing and other pathological situations, the presence of these cells may therefore be associated with the shedding and healing response of the tissue (Badid et al., 2000). The basal level of expression of the smooth muscle markers within the stroma was, however, greater within some patients in the control group in the present study than previously reported (Kohnen et al., 2000). This difference may reflect differences in case-mix between the two studies.

As there is a lack of nuclear receptors for estrogen and progesterone within the endometrial vasculature (Kohnen et al., 2000), steroid responsive genes expressed in the surrounding stromal cells may be of importance in the vascular response to levonorgestrel. It is not clear at the present time whether such genes would be involved in stromal decidualization, vascular growth, vascular wall maturation or in events that could lead to the breakdown of tissue. Previous studies have shown that the total volume of menstrual blood loss is reduced after exposure to LNG-IUS despite an increase in inter-menstrual bleeding (Andersson and Rybo, 1990; Irvine et al., 1998). This increase in inter-menstrual bleeding presents a significant constraint to compliance. However, counselling that informs the patient of the likely side-effects has been shown to be effective as an interim measure to improve continuation of treatment (Cameron, 2001). In this group of women who sought hysterectomy as a treatment for menorrhagia, the response to the LNG-IUS may have been somewhat different from women using the device for contraception or from those who do not experience breakthrough bleeding. Regardless of the reason for treatment, in the longer term it may be possible to devise strategies which modulate tissue growth and vessel development prior to insertion of the LNG-IUS. These strategies could possibly minimize the changes in vascular morphology that are seen in the first few months after the initiation of treatment.

In conclusion, morphological examination of the endometrial vascular system after short-term exposure to the LNG-IUS revealed changes within the superficial endometrium that may explain the observed high incidence of breakthrough bleeding. The irregularity of the endometrial surface before the onset of treatment-induced atrophy may also be linked to bleeding patterns. Similar changes reported after exposure to other progestogens or tamoxifen suggest a common underlying effect in treatment-induced inter-menstrual bleeding. These data suggest that future approaches should consider either pretreatment to make the endometrium ‘atrophic’ or ‘unresponsive’, or the concomitant administration of other agents (such as antiprogestogens or inhibitors of metalloproteinase activity) to limit these adverse effects of progestogen on the endometrial blood vessels.

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References


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