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

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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®) (Odlind, 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 amenorrheic 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 Pipelle® biopsy (in women with irregular bleeding or >40 years old). Endometrial abnormality, such as hyperplasia, infection or the presence of polyps, was excluded by histopathological examination.

At the second appointment the severity of the complaint of menorrhagia was further assessed by more detailed questioning and a range of available therapies was discussed. Women who then opted for hysterectomy (most of whom had already tried medical treatment to control their symptoms), and agreed to participate in the study, were randomly allocated into LNG-IUS or control groups (using a table of computer-generated random numbers, Minitab v13, Minitab, Inc., State College, PA, USA) whilst they were on the waiting list for surgery. Following randomization, patients were interviewed by means of a detailed questionnaire and height and weight were recorded. Women in the LNG-IUS group had the system inserted for between two and four menstrual cycles prior to surgery. Women in the control group were asked to discontinue current medication for two and four menstrual cycles prior to surgery. Following hysterectomy, the unfixed uterus was taken immediately to the pathology department where the pathologist cut a transverse mid-sagittal section. The blocks taken from the anterior and posterior wall were further divided for light microscopy. Tissue for wax embedding was obtained. The specimens taken for routine examination were assessed independently by local pathologists and reports were obtained.

Specimen collection, processing and assessments
Following hysterectomy, the unfixed uterus was taken immediately to the pathology department where the pathologist cut a transverse mid-corpus slice (5–10 mm). This slice was then further divided into full thickness blocks from the anterior and posterior walls and lateral fornices. The blocks taken from the anterior and posterior wall were further divided for light microscopy. Tissue for wax embedding was fixed in 4% (v/v) buffered formaldehyde [Chemix (UK) Ltd, Standish, UK]. All specimens collected for research were examined by a consultant pathologist (C.J.R.S.) who was unaware of the group from which they were obtained. However as the effect of the LNG-IUS is so profound, the pathologist was not truly oblivious to the treatment. The specimens taken for routine examination were assessed independently by local pathologists and reports were obtained.

Immunocytochemistry
Serial sections (5 μm) were mounted on glass slides coated with 3-aminopropyltriethoxysilane (Sigma–Aldrich, Poole, UK). Vascular endothelium was identified in wax-embedded tissue sections (5 μm) using an antibody to CD31 (Clone JC 70A, dilution 1:500; Dako, Ely, UK). Smooth muscle cytoskeletal components, α-smooth muscle actin (Clone 1A4, dilution 1:500; Sigma) and myosin (Clone HSM-V, dilution 1:150; Sigma) were localized (Kohnen et al., 2000). Primary antibodies were diluted in 2% horse serum (Sigma) in phosphate-buffered saline pH 7.5. Antigen retrieval was used for detection of CD31 and myosin. Those sections were microwaved in 0.01 mol/l citrate buffer pH 6 for 8 and 45 min respectively (Kohnen et al., 2000). Non-specific binding sites were blocked with 20% horse serum and 20% human serum in PBS for 30 min at room temperature. Endogenous peroxidase activity was blocked by immersing slides in 0.5% H2O2 in methanol. In the case of CD31, inactivation was carried out before microwaving. For myosin detection, blocking 0.5% H2O2 was used after addition of the secondary antibody. Antibody binding was detected using an avidin–biotin complex (ABC) peroxidase kit (Vector Laboratories, Peterborough, UK) and diaminobenzidine substrate tablets according to the manufacturer’s instructions (Sigma). Negative controls were used in which the primary antibody was replaced with a mouse monoclonal IgG1 to Aspergillus niger glucose oxidase (Dako). Nuclei were counterstained with Harris’ haematoxylin (BDH, Lutterworth UK).

Microscopy and stereology
Sections were examined by bright field microscopy (BX50; Olympus, London, UK) and digital images were captured with a 3-chip colour camera (JVC, London, UK) and the ImagePro Plus version 4 image analysis program (Media Cybernetics, Des Moines, IA, USA). Stereology was undertaken using a mixture of computer-assisted and manual methods. Microscope fields were selected in a tessellated pattern either laterally (for specimens with a smooth surface) or vertically (on specimens with an irregular surface where the former approach would have been problematic) beginning with a random starting point. In order to prevent bias, the entire height of the tissue was sampled. In the case of vascular surface density, a map image was produced by scanning the sections with a 35 mm film scanner (Coolscan III, Nikon, Kingston upon Thames, UK). Orthogonal sampling grids tailored in shape to each section were applied with an image analysis program (ImagePro). The location of the sampling points was chosen with respect to the grid at low magnification in order to prevent sampling bias. Analysis was then carried out at higher magnification. Volume fraction was measured on 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 (Gra ticules 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

| Table I. Characteristics of the control and treated groups |
|---------------------------------|--------|--------|
| No. recruited into group        | LNG-IUS| Control|
| No. completing study            | 16\(^{ab}\) | 23\(^c\) |
| Age (years)                     | 38.6   | 39.9   |
| Parity                          | 2.3    | 2.5    |
| Body mass index (kg/m\(^2\))    | 28.03  | 28.84  |
| Smokers                         | 4/16   | 12/23  |
| No. previously treated for menorrhagia | 10/16 | 16/23 |
| Mothers or sisters with hysterectomy | 7/16  | 7/23   |

\(^{a}\)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

\(^{b}\)Three women in the control group chose to withdraw from the study after randomization.

Figure 1. Tissue morphology after exposure to the levonorgestrel intrauterine system (LNG-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 micropolypoidal, 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 \(\mu\)m, B = 50 \(\mu\)m, C = 90 \(\mu\)m, D = 45 \(\mu\)m.
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 $\alpha$-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 $\mu$m, control 37.1 $\mu$m). 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 $\mu$m, control 174.2 $\mu$m, $P = 0.0001$, 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).
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.
implant also support this conclusion (Hickey et al.) through hysteroscopic observations of the endometrium after treatment with an LNG intrauterine system (LNG-IUS) and lumens was significantly greater following treatment ($P = 0.0004$). (B) The maximum value of the largest luminal diameter for each specimen was increased after exposure to the levonorgestrel intrauterine system (LNG-IUS) ($P = 0.0001$). (C) The number of luminal cross-sections per unit area was greater after treatment with LNG-IUS ($P = 0.01$). (D) No significant difference was observed in the vascular surface density (area per unit volume) between treated and control specimens.

It would appear after exposure to LNG-IUS that most of the vascular tree remains structurally intact after exposure to LNG-IUS; however, occasional larger diameter superficial thin-walled vessel segments develop. It is possible that these thin-walled vessels are more susceptible to breakdown during the course of events which lead to breakthrough bleeding. These findings are consistent with the previous findings of Hourihan et al. (1986) that revealed the presence of large vessels following oral progestogen exposure. Hysteroscopic observations of the endometrium after treatment with an LNG implant also support this conclusion (Hickey et al., 1998). Hysteroscopy suggests that the large cross-sections observed in the present study are equivalent to the superficial lesions seen macroscopically within the endometrium. There may be common mechanisms which generate this type of lesion in women exposed to progestogen-only contraceptives and who suffer breakthrough bleeding.

Comparison of the median vessel diameter between the treated and control groups showed that these were almost identical. The normal size of most vessels also provides evidence that although there may be a specific lesion, there are no significant gross effects on vascular morphology. The observation that the arterioles also appeared well-formed reinforces the concept that the vascular tree is largely normal. This view is further supported by the fact that total vascular surface area is not altered. Despite this, there appeared to be an increase in vessel volume fraction and vessel cross-sectional number. These increases could explain the change in vascular volume density. However, the absence of a difference in vascular surface density is difficult to reconcile with an increase in these other parameters. It may be that the presence of the very large occasional vessel segments may contribute to the increase in volume fraction without producing gross changes in total surface area. Normally the observed increase in the number of luminal cross-sections would be expected to reflect change either in the length density (length per unit volume) or the branch pattern. The use of vertical sections in the present study, however, precluded the calculation of length density (Howard and Reid, 1998). In this study the entire endometrium was examined quantitatively, but qualitative observations suggest that changes may be more superficial. The quantitative effect may therefore be diluted by inclusion of the basal layer in the analysis.

Similar conclusions about the effects of exposure to levonorgestrel on vascular development and maturation were drawn with Norplant (Rogers et al., 2000). These authors classified the degree of vessel maturity by grading the amount of circumferential smooth muscle actin staining. Women treated with Norplant who also suffered breakthrough bleeding had an increased percentage of vessel cross-sections not circumscribed with $\alpha$-actin. This finding suggested that exposure to levonorgestrel can modulate vascular development or maturation.

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 Brechin 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;
Kohnen et al., 1998). 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 progesterogens 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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