Effect of different cyclical sequential progestins on endometrial vascularity in postmenopausal women compared with the natural cycle: a morphometric analysis

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The effect of trimegestone-based and norethisterone-based hormone replacement therapy (HRT) regimens on the endometrial vascularity compared with that of the endometrium of the natural cycle were evaluated using immunohistochemical techniques. Endometrial vascular space area, diameter and number were defined in the functionalis layer of the endometrial samples from postmenopausal women who either completed a randomized, double blind, dose-ranging study of continuous oral micronized oestradiol 2 mg daily with trimegestone 0.05, 0.1, 0.25 and 0.5 mg/day from day 15–28 for six treatment cycles or were given cyclical sequential norethisterone (NET)-based HRT together with continuous 2 mg oral oestradiol valerate for three cycles. The control samples were LH-dated endometrial biopsies. NET-based HRT was associated with a higher number of smaller vascular spaces compared with the trimegestone-treated endometrium or that of the natural cycle. There was no dose-dependent effect in the four dose groups of trimegestone. In conclusion, norethisterone may exert a different effect on angiogenesis to that of trimegestone on endometrial vascular development.

Key words: CD34/endometrium/hormone replacement therapy/norethisterone/trimegestone

Introduction

Postmenopausal sex steroid administration is associated with initiation of withdrawal bleeding. In 50% of instances, such bleeding is irregular (Habiba et al., 1996). This irregular bleeding may respond to an increase in the dose of progestogen administered, but with the majority of progestogens, such dose-dependent modulation of uterine bleeding is associated with a higher incidence of adverse effects, such as bloatedness, mastalgia, fluid retention and mood swings. About 50% of women on hormone replacement therapy (HRT) discontinue treatment due to withdrawal bleeding (Nachtingall, 1990).

Progesterone inhibits endothelial cell proliferation (Vazquez et al., 1999), and in constant physiological levels, it affects the mitotic activity of the endometrial vasculature (Tsai and O’Malley, 1994), which may lead to vascular fragility and irregular bleeding patterns.

Morphological changes in the endometrial blood vessels may be one of the factors responsible for bleeding. Gaps start to appear between the endothelial cells in the premenstrual phase (Sixma et al., 1980), the spiral arterioles become larger and longer in the secretory phase (Johannisson, 1990) and the lumen of the subepithelial capillaries increases in diameter in the secretory phase (Sheppard and Bonnar, 1980).

Endometrial blood vessels undergo different morphological changes under the effect of different sex steroids. Norethisterone administered orally, in a dose of 300 µg per day, for 2 months led to an increase of the plasmalemmal vesicles of the endothelial cells, reflecting an increase in endothelial permeability (Johannisson et al., 1982). Levonorgestrel administered through an intra-vaginal ring (20 µg/day) induced a reduction in the number of endometrial arterioles, with an increase in the endothelial gaps and haemostatic plugs (Hourihan et al., 1991a). Given orally, levonorgestrel (30 µg/day) induced a similar reduction in the number of spiral arterioles and an increase in the number of dilated subepithelial venules (Hourihan et al., 1991b), while subdermal levonorgestrel implants (Norplant) induced an increase in vascular density (Rogers et al., 1993).

There is controversy in the literature regarding the vascular density in the natural cycle. Authors reported no variation in the vascular density with different phases of the natural cycle, and therefore concluded that there is no correlation between vascularity and stromal development (Shaw et al., 1979; Hourihan et al., 1986; Rogers et al., 1993). On the other hand, a significant increase was found in the vascular surface area, diameter and total number of capillaries in the secretory phase compared with the proliferative phase (Ota et al., 1998).

In a recent study (Al-Azzawi et al., 1999), it has been demonstrated that there are dose-dependent changes in the pattern of bleeding in women treated with four doses of sequentially administered trimegestone (0.05, 0.1, 0.25, and 0.5 mg/day), for 6 months. Women on cyclical sequential norethisterone (NET), exhibited two patterns of bleeding: (i) early bleeders who bled before the completion of the progestogen phase of the sequential HRT cycle, and had a wide variability of the day of onset, heavier and more prolonged bleeding; (ii) late bleeders who bled after the completion of the progestogen phase of sequential HRT, whose cycles were less variable, shorter and lighter (Habiba et al., 1996), suggestive of different responses of the endometrial vasculature to the same progestogen.

CD34 is a glycoprotein expressed on haematopoietic...
progenitor cells, in the basement membrane of the vascular endothelium (Traweek et al., 1991), and on the luminal surface of the endothelial cells (Schlingemann et al., 1990). The CD34 antigen was chosen for detection, although not an exclusive marker of endothelial cells, in preference to Von Willebrand factor, which gives a weaker staining, or the less specific antigen Ulex europaeus (Traweek et al., 1991).

The aim of this study was to establish the vascular morphometric changes using the endothelial cell marker, CD34, (i) in the natural cycle, (ii) in response to trimegestone-based HRT in a dose ranging study, and (iii) to compare the changes in vascular morphometry between two sequential HRT regimens: trimegestone-based and the widely used, NET-based.

Materials and methods

**Endometrium of the natural cycle**

The control samples were deep endometrial biopsies, which included functionalis and basalis layers, obtained from healthy, regularly menstruating women aged 27–50 years, undergoing laparoscopic sterilization using sharp curette or from hysterectomy specimens. The clinical indications for the hysterectomies were: cervical intraepithelial neoplasia, premenstrual tension syndrome and benign ovarian cysts. None of these women had received any hormonal treatments for 2 months prior to the procurement of the specimens (n = 30). All women were given urinary LH surge detection kit tests (First Response; Carter Wallace Limited, Folkestone, UK), which were used during the month preceding the endometrial biopsy or hysterectomy. The technique used to obtain endometrial samples from hysterectomy specimens was as follows: the uterus was opened in the coronal section and the posterior wall of the uterine cavity was sliced vertically from fundus to isthmus, each slice being 1 cm wide and numbered alphabetically A–E from right to left. The study materials were from slice B. Endometrial samples were taken from a specific area of the uterus to maximize consistency, since the fundus is recognized to be the most hormone responsive area of the endometrium. Area B was specifically chosen as this avoided the lateral edge of the fundal endometrium, which may sometimes undergo tubal epithelial metaplasia.

The control specimens were fixed immediately in 10% formal saline, embedded in paraffin wax, and 5 μm sections were stained with haematoxylin and eosin (H&E) for histological assessment of the phase of the endometrial development.

These biopsies were dated both by LH surge and the date of the last menstrual period, and were examined by two independent pathologists who were blinded to the LH surge and menstrual dates. They characterized the specimens according to the criteria of Noyes (Noyes et al., 1980). Where all agreed, the specimen was included as a control sample. The histological diagnoses were proliferative (n = 7), early luteal (n = 9), late luteal (n = 7) and menstrual (n = 7).

**HRT-treated endometrial samples**

This study involved women on two progestogen regimens:

(i) A total of 176 postmenopausal women were given oral trimegestone (0.05, 0.1, 0.25 and 0.5 mg per day) from day 15–28, with continuous micronized oestradiol tablets 2 mg daily (Hoechst Marion Roussel, Romainville, France), in a randomized, double blind, dose-ranging study, for six treatment cycles. The protocol was approved by the local ethics committees and all patients signed an informed consent. This cohort of 176 women recruited in our centre was part of a multicentre double blind dose-ranging study population of 256 women.

Included in the study were healthy women aged 45–65 years (mean ± SD, 52.5 ± 5.1) with intact uterus, who were at least 6 months postmenopausal, had had HRT for ≥2 years, or had been on HRT for at least 1 year with pretreatment FSH and oestradiol concentrations in the postmenopausal range. None had received any form of sex steroid treatment for 6 weeks before the commencement of study medications. Those who had ever used oestradiol implants were excluded. Tests for liver and renal function were performed and those women with abnormalities were excluded. All women over the age of 50 years had a normal mammogram within 3 years and normal cervical smears within the previous 6 months. General, breast and pelvic examinations were conducted to confirm normality.

Endometrial biopsies were obtained using a vabra curette, and sampling only the functional layer, on day 24 of the last treatment cycle.

(ii) Twenty-five postmenopausal women aged 48–80 years (mean ± SD, 62.9 ± 9.3) who were scheduled for vaginal hysterectomy as part of their treatment for uterine prolapse, were given 2 mg of oestradiol valerate daily with norethisterone 1 mg/day from day 17–28 for three cycles (Novartis, Camberley, Surrey, UK). Included in the study were healthy women aged >50 years with intact uterus who were at least 6 months postmenopausal. The exclusion criteria and the safety parameters were as in group (i). The tissue specimens were obtained from the corpus region, and the hysterectomy was performed between day 11 and 21 of the commencement of norethisterone phase of the third treatment cycle.

**Immunohistochemistry**

Sections 5 μm thick were obtained from paraffin wax-embedded specimens were incubated in microwave oven (750 W) in citrate buffer at pH 6.0, for 30 min, for antigen retrieval. Sections were then incubated with 6% hydrogen peroxide for 10 min to block endogenous peroxidase. Normal rabbit serum was then applied (NRS; Dako, Glostrup, Denmark) to minimize non-specific reactivity. The sections were then incubated with the primary antibody, 1:100 (2 μg/ml) mouse monoclonal against the CD34 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), overnight at room temperature. Specimens were washed with phosphate buffered saline (PBS) for 20 min between the steps. Endometrial sections were incubated with biotin-linked secondary antibody (Dako), Vectastain ABC peroxidase (Elite; Vector Laboratory, Peterborough, UK) and DAB substrate (diaminobenzidine; Vector Laboratory) according to manufacturer’s recommendations. Controls were included using mouse immunoglobulin (IgG) (Sigma, Poole, Dorset, UK) instead of the primary antibody. To test for the binding specificity of the secondary antibody, the primary antibody was omitted. The positive control was stained with CD34 and there was no background reaction, while the negative control did not show any staining.

**Assessment of the endometrial sections**

The pattern of the distribution of the positively stained vascular spaces was assessed in the endometrial sample, and then 10 randomly selected fields (Hamilton, 1995) per slide (×200) were captured to evaluate the total vascular space area, diameter and number per field. All fields examined were restricted to the functionalis layer. All the vascular spaces positive for CD34 were measured, and that included the collapsed blood vessels since they were lined by CD34 positive endothelium. The actual measurements of the vascular diameter included the distance spanned from the outer edge of the CD34 positive membrane of one side of the vessel to the outer edge of the CD34 positive membrane of the other side. The measurements were averages of the maximum and minimum diameter.

Images were captured using Axioplan microscope (Carl Zeiss,
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Oberkochen, Germany), and a colour video camera (Sony CCD/RGB). The corresponding areas of an image captured with $\times200$ was $0.121\, \text{mm}^2$ using a measurement graticule, and therefore the data presented are per unit area. The measurements were evaluated using the KS300 image analysis programme (Kontron Imaging Systems, Thame, Oxfordshire, UK).

Statistics
The data did not fulfill the assumptions necessary for using the analysis of variance and $t$-test, therefore the non-parametric Kruskal–Wallis and Mann–Whitney tests were used.

Results
In the trimegestone-based HRT group, 176 women were randomized to one of the four dose groups and 131 completed the study; seven women did not start treatment after randomisation and were withdrawn from the study: lost to follow-up ($n = 3$), protocol violation ($n = 3$) and adverse events ($n = 1$). Thirty-eight women did not complete the study, of whom only nine withdrew due to irregular bleeding. There was no statistically significant difference between the number of patients who were assigned to, or those who withdrew from, each trimegestone dose group.

The total number of the endometrial samples obtained at the end of the study was 129, as two women declined to have a biopsy at the end of the study. Endometrial biopsies of women who bled before the day of the biopsy ($n = 12$) were excluded for consistency (particularly as the endometrium would start the healing-proliferation phase post-menses), and 10 other samples were too scanty for meaningful analysis. One hundred and seven endometrial biopsies were available for morphometric assessment with anti-CD34 antibody ($n = 23, 28, 29$ and $27$ in the $0.05, 0.1, 0.25$ and $0.5\, \text{mg}$ trimegestone dose groups respectively).

In the NET-based HRT, 25 women completed 3 months of treatment and all had regular withdrawal bleeding prior to their scheduled hysterectomy.

Endometrium of the natural cycle (Figure 1a)
There was a significant difference in the mean percentage vascular space area between the phases ($P = 0.007$; Kruskal–Wallis test), with smaller area in the early luteal phase. The mean percentage vascular space area was significantly lower in the early luteal phase compared with the late luteal and menstrual phases of the natural cycle ($P = 0.009$ and $P = 0.001$ respectively; Table I, Figure 2a). The average diameter of the vascular space was lower in the early luteal phase compared with the late luteal and menstrual phases ($P = 0.03$ and $P = 0.009$ respectively; Table I, Figure 2b), while there was a higher mean number of vascular spaces in the menstrual phase, but this was not statistically significant in comparison with the other phases of the natural cycle (Figure 2c).

Trimegestone-treated endometrium (Figure 1b)
The mean percentage vascular space area and average vascular diameter were higher in the high dose group ($0.5\, \text{mg}$); however, there was no statistically significant difference between the four dose groups (Figure 2a,b). There was a lower mean number of vascular spaces the higher the dose of trimegestone, but this trend was not statistically significant (Figure 2c).

There was no statistically significant difference in the vascular parameters studied between women who bled on the day of the biopsy compared with those who had not bled by then (data not shown). There was no evidence of a difference in the endometrial vascularity between the trimegestone-treated endometrium and the natural cycle.
Table I. Mean and SD of the vascular parameters in the endometrium of the natural cycle, trimegestone-treated or norethisterone-treated (NET) cycles

<table>
<thead>
<tr>
<th>Vascular parameters</th>
<th>Endometrium of the natural cycle</th>
<th>Trimegestone dose group</th>
<th>NET</th>
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<tbody>
<tr>
<td></td>
<td>P</td>
<td>EL</td>
<td>LL</td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>n</td>
<td>7</td>
<td>9</td>
</tr>
<tr>
<td>% Vascular space area (per mm²)</td>
<td>2.3</td>
<td>1.9</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td>(0.6)</td>
<td>(0.2)</td>
<td>(0.4)</td>
</tr>
<tr>
<td>Vascular space diameter (per mm²)</td>
<td>15.0</td>
<td>13.1</td>
<td>15.4</td>
</tr>
<tr>
<td></td>
<td>(3.3)</td>
<td>(1.0)</td>
<td>(2.6)</td>
</tr>
<tr>
<td>No. of vascular spaces per mm²</td>
<td>114.7</td>
<td>110.8</td>
<td>113.4</td>
</tr>
<tr>
<td></td>
<td>(28.7)</td>
<td>(20.1)</td>
<td>(26.6)</td>
</tr>
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P = proliferative; EL = early luteal; LL = late luteal; M = menstrual.

Norethisterone-treated endometrium (Figure 1c)

The mean percentage vascular space area was higher in the NET group, but significant only in comparison with the natural cycle ($P = 0.03$, Figure 2a). The average diameter of the vascular spaces was lower in the NET group and there was a significant difference between the NET-treated endometrium compared with the trimegestone-treated endometrium or the natural cycle ($P = 0.003$, $P = 0.004$ respectively; Table I, Figure 2b). The mean number of vascular spaces was significantly higher in the NET group compared with the trimegestone-treated endometrium or the natural cycle ($P = 0.000$, $P = 0.000$ respectively; Table I, Figure 2c). There was no evidence of apparent difference in the vascular parameters studied between women who had bled by the day of the biopsy and those who had not (data not shown). The timing of the uterine specimen in relation to NET administration had no effect on the endometrial vascularity.

Discussion

In this study, the morphometric features of endometrial microvasculature of the functionalis layer in four phases of the natural cycle and in endometrial samples obtained from

Figure 2. (a) The mean percentage vascular space area in the four dose groups of trimegestone-based hormone replacement therapy (HRT) (0.05, 0.1, 0.25 and 0.5 mg), the norethisterone-based HRT and the four phases of the natural cycle, proliferative (P), early luteal (EL), late luteal (LL) and menstrual (M). Open circles are used for the mean percentage vascular space area in the individual women. *The percentage vascular space area was significantly lower in the early luteal phase compared with the late luteal and menstrual phases ($P = 0.009$, $P = 0.001$ respectively). (b) The mean vascular space diameter in the four dose groups of trimegestone-based HRT (0.05, 0.1, 0.25 and 0.5 mg), the norethisterone-based HRT and the four phases of the natural cycle. Open circles are used for the mean diameter of the vascular space in the individual women. *The mean vascular space diameter was smaller in the NET group compared with the trimegestone-treated endometrium or that of the natural cycle ($P = 0.003$, $P = 0.004$ respectively). (c) The mean number of the vascular spaces in the four dose groups of trimegestone-based HRT (0.05, 0.1, 0.25 and 0.5 mg), the norethisterone-based HRT and the four phases of the natural cycle. Open circles are used for the mean number of the vascular spaces in the individual woman. *The mean number of the vascular spaces was higher in the NET group compared with the trimegestone-treated endometrium or that of the natural cycle ($P < 0.0001$, $P < 0.0001$ respectively).
women treated with HRT, using two different progestogens are presented. The expression of CD34 in the endometrium of the natural cycle is at variance with our previous report on the endometrial vascularity using an H&E technique (Wahab et al., 1999). The use of CD34 staining technique is more accurate in identifying blood vessels compared with H&E staining, where it is difficult to identify the microvasculature, which when constricted or small may blend with the surrounding stromal cells (Shaw et al., 1979, 1981). CD34 antigen is also expressed on endothelial cells of lymphatics; however, there is no indication in the literature that sex steroids modify lymphangiogenesis.

A similar decrease in the number of vascular spaces in early luteal phase has been reported (Rogers et al., 1993), and an increase in the menstrual phase; however, their findings were not statistically significantly different from the other phases of the natural cycle. This may be due to the number of fields examined per biopsy, which they did not state, or due to the small number of specimens examined. Moreover, the endometrial samples in the control group of this study were highly characterized by the agreement of four parameters, viz. date of the last menstrual period, urinary LH surge, and the total agreement of two independent histopathologists to the dating of the tissue.

New vessel formation involves degradation of the basement membrane by the action of collagenase and plasminogen activators secreted by the endothelial cells. Endothelial cells then migrate through these openings formed in the basement membrane as a loose sprout. A lumen is formed by curvature of the endothelial cells, followed by division of the endothelial cells, and then canalization as the sprouts join each other (Findlay, 1986).

The major stimulus of angiogenesis is hypoxia (Adair et al., 1990), and increased metabolic demands (Reynolds and Redmer, 1992), but the exact mechanism of action is not known.

Capillaries, including those of the endometrium, are lined by a continuous single layer of endothelial cells arranged over a basement membrane. Pericytes surround some of these endothelial cells which form projections that make contact with endothelial cells. Capillaries lack muscle layer; however, capillaries and small vessels (consist of one or two endothelial cells) may stain for α-smooth muscle actin (α-SMA), which is an antibody for smooth muscle cells (Verbeek et al., 1994; Abberton et al., 1996). Ultrastructurally, endothelial cells show changes in activity and size according to the phase of the menstrual cycle (Roberts et al., 1992).

Oestrogen and progesterone receptors have not been found in endometrial endothelium (Critchley et al., 1993), which is at variance with the findings of others (Iruela-Arispe et al., 1999). Therefore, it is plausible to postulate that steroidial modulation of changes in cellular activity and morphology may be mediated indirectly through changes in extracellular matrix proteins (Ingber and Folkman, 1988), integrins (Vitola et al., 1996), or through the induction of cytokines such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor, bFGF (Ferriani et al., 1993), epidermal growth factor, EGF (Haining et al., 1991), or transforming growth factor alpha, TGFα (Horowitz et al., 1993).

VEGF exists in five isoforms expressed in the endometrium, but only VEGF165 and VEGF121 variants are shown to be modulated by sex steroids (Huang et al., 1998). Evidence of increased VEGF expression has been documented in polycystic ovariies (Ferrara et al., 1998), and in ovarian hyperstimulation syndrome (McClure et al., 1994). They are present in the extracellular matrix and attached to proteoglycans on the cell surface. VEGF may act in a paracrine/autocrine manner as small patches of immunostaining were found around some blood vessels, in the glandular epithelium and in the stromal cells (Gargett et al., 1999). VEGF acts synergistically with angiopoietin-1, to induce sprout formation (Koblizek et al., 1998).

Basic FGF is expressed in glandular epithelial and stromal cells, although there were no changes in expression across the phases of the natural cycle (Ferriani et al., 1993; Gold et al., 1994). However, there is increased glandular and stromal expression of bFGF in simple and complex hyperplasia, where it may stimulate the synthesis of plasminogen activator by the endothelial cells (Mignatti et al., 1989; Gold et al., 1994).

The involvement of extracellular matrix proteins (Ingber and Folkman, 1988) was highlighted by the finding of cycle regulation of thrombospondine-1 in the endometrium. Thrombospondine-1, a multifunctional extracellular matrix glycoprotein, is a suppressor of angiogenesis in vitro and in vivo, and is regulated by progesterone (Iruela-Arispe et al., 1996).

Increased microvascular density was noted in endometria of women treated with levonorgestrel subdermal implants (Rogers et al., 1993), and suggested uncoupling of microvascular and extravascular compartments. On the other hand, medroxyprogesterone acetate administration was found to suppress angiogenesis in endometrial cancer transplant experiments (Jikihara et al., 1992), and suppressed fibroblast growth factor activity in cultures of endometrial cancer cells (Fujimoto et al., 1989). Orally administered medroxyprogesterone acetate was shown to reduce microvascular density in women with endometrial hyperplasia (Abulafia et al., 1999).

A known difference between levonorgestrel and medroxyprogesterone acetate is that the former possesses much higher androgenic effect than the latter. In this study trimegestone, with the characteristic of very low androgen receptor binding, behaved in a manner similar to progesterone in the natural cycle, as reflected by the vascular morphometric parameters. The androgenic progestogen norethisterone, on the other hand, affected microvascular density in a manner analogous to subdermal levonorgestrel implants, although levonorgestrel administration was continuous and not cyclical. This suggests that androgen receptor activation probably plays an important role in modulating angiogenesis. An example has been demonstrated (Watson et al., 1998), when androgen receptor activation in stromal cell culture resulted in an increase in epidermal growth factor (EGF) production. Moreover, norethisterone may be converted to ethinylestradiol (Fotherby, 1994), thereby augmenting the oestrogenic stimulation of the endometrium.

It can be argued that the regional variation in endometrial development could underlay the difference in the endometrial
vascular parameters observed between trimegestone and nor-
ethisterone treated endometrium. Shaw et al. (Shaw et al.,
1979) reported a lower mean number of blood vessels in the
isthmus compared with the fundus, corpus or cornua; however,
they doubted the statistical significance of their finding. In
this study, the endometrial biopsies from the NET-based HRT were
obtained from the corpus region of the hysterectomy specimens,
while in the trimegestone groups, a vabra curettage was used,
which would sample tissues from different parts of the uterine
cavity. Therefore, such differences may be expected to result
in a lower number of vascular spaces compared with the NET-
treated endometrium, but as such does not explain the preva-
ence of smaller vascular diameter in the latter group.

There is a difference in the duration of the two types of
progestogens, as trimegestone was given for 6 months, while
cyclical NET was given for 3 months. There is no information
in the literature on vascular morphometry defined by CD34
overtime. However, it has been found (Hickey et al., 1999)
that most of the changes in the compartments of the endometrial
vascular basement membrane induced by Norplant revert to
normal after 12 weeks of treatment. Nevertheless, it should be
pointed out that the essential difference is the continuous
administration of levonorgestrel in a previous study (Hickey
et al., 1999) compared with the cyclical sequential
administration of the progestogens in this study.

The endometrial vascular morphometry as studied by CD34
did not explain the different patterns of bleeding in these two
HRT regimens, as there was no difference in the endometrial
biopsies obtained from women who bled on the day of the
biopsy and those who had not bled by then.

In conclusion, NET-based HRT was associated with a higher
number of smaller vascular spaces than in the trimegestone
treated endometrium or that of the natural cycle, and therefore
it opens the question for further assessment of the mechanism
of vascular development in the endometrium to help in the
optimization of future therapeutics.

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