An Ultrastrutural Study of Human Luminal Endometrial Cells Following Different Doses of Oestrogen Replacement Therapy

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Abstract

Introduction: The effects of different doses of oestrogen on the endometrium of women with premature ovarian failure have been examined in this study.

Materials and Methods: Four groups of women of reproductive age were studied; 1 normal fertile controls 2. patients given a standard, variable hormone replacement therapy (HRT) 3. a group given a fixed daily dose of 1 mg of oestrogen and 4. a group given a fixed daily dose of 4 mg of oestrogen. Endometrial diposises were taken at about 5-6 days after ovulation and tissue was prepared for light and electron microscopy. Morphometry was used to evaluate quantitatively various features of endometrial luminal epithelial cells. The volume fraction (Vv) of nucleus to cell in the standard group was significantly larger than the 4 mg group.

Results: The Vv of euchromatin to nucleus was larger in the controls and 4 mg group than the 1 mg subjects. The Vv of mitochondria to cell was largest in the control group. The ratio of desmosomes to surface membrane was increased ($P < 0.05$) in the 1 mg subjects.

Conclusion: These results suggest that, while standard HRT is generally a good mimic of controls, the 1 mg fixed dose delayed some membrane features and the fixed 4 mg group showed advancement in some organelle growth.

Key Words: Implantation, Oestrogen, Luminal epithelium, Morphometry, Human

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Introduction

The luminal epithelium of the endometrium is the first point of contact between the mother and the embryo. Its normal development is dependent on adequate amounts of ovarian hormones. In many cases women with POF (premature ovarian failure) can be treated by exogenous steroid hormones, which closely mimic those seen to produce a normal endometrium. Indeed, this hormone replacement therapy (HRT) can be so successful that endometrium from these women could receive a fertilized donor egg and deliver a healthy child (1-7). However, the detailed ultrastructure and histology of luminal epithelium from women treated with HRT has not been reported previously.

Li et al. (8) investigated the endometrial response of stroma and gland to oestrogen at the light microscopic level using morphometric techniques. They compared a standard variable dose regime 2 with three different daily fixed 1, 2, and 4 mg oestrogen regimes. They reported that normal endometrium could be achieved using a variable dosage and that a daily dose of 1 mg oestrogen was suboptimal. There was no noticeable effect using higher doses.

The present study was designed to examine the effects of three different programmes of HRT; a standard variable dose, a daily fixed 1 mg and, a fixed 4 mg dose of oestrogen on a group of women with premature ovarian failure at the ultrastructural level in order to extend the previous study of Li et al. (8) and to examine luminal epithelium in particular. In addition it was intended to compare the data obtained from the HRT groups with a group of well-characterised normal fertile women (9) in order to determine the various effects of oestrogen on luminal epithelium around the time of implantation.

Materials and Methods

* *Subjects*

Infertile Subjects

Infertile women with premature ovarian failure attended the outpatient clinic of the university department of Obstetrics and Gynaecology at the Jessop hospital for Women, Sheffield, UK. There were 18 women with premature ovarian failure (POF), defined as women with at least 6 months amenorrhoea, at the same time as having low plasma oestradiol (<100 pmol/l) and elevated plasma follicle stimulating hormone (FSH)> 20 IU /l inwomen under 40 years of age (10).

Fertile Subjects

Five normal fertile women (age 18-40yrs) were recruited; these are the same controls, which have been reported previously by Sarani, et al. (9). Normal fertile women were defined 11 as those who had regular menstrual cycles of between 25 and 35 days with no evidence of menstrual disorder and those who had not used any steroid hormonal contraception or intrauterine contraceptive device for at least 3 months prior to hospitalization andthose who had at least one successful pregnancy. All biopsies in the control subjects were timed by reference to the LH surge (designated day LH 0), which was determined by LH assays on daily samples of either morning urine or of plasma (11).

In the POF groups the study was a crossover design with each woman being allocated to her group at random. The first six women received standard hormone replacement therapy (HRT) in one cycle (2-8) and then changed to a fixed 1 mg oestrogen dose in the next cycle. The second six women received 1 mg fixed dose in the first cycle followed by the standard HRT in the next cycle. A third group of six women were treated in the same way as the first group but, received 4 mg of oestrogen in the second cycle. From each group a biopsy specimen was taken once at day 19 of each cycle by using a Sharman s Curette (Dow s Surgical Ltd, Sheffield, UK).

Each endometrial specimen was fixed immediately in 3% glutaraldehyde and processed for light and electron microscopy as described previously (9). From JB-4 blocks, 2 μm thick section were cut using glass knives on an Anglia Scientific AS500 microtome (Anglia Instruments, Cambridge, UK) and stained with 1% acid fuchsin and 0.05% Toluidine blue. Semi-thin sections (0.5 μm thick) were cut from Epon blocks using a Rechent (OMU3, Sweden) ultramicrotome and stained using 0.05% Toluidine blue. Ultra thin-sections (approximately 70 nm thick) were double stained with aqueous uranyl acetate and lead citrate (9). The ultra-thin sections were examined on a Philips 301-transmission electron microscope at a range of magnifications, which were determined with the aid of a grating replica.

* *Morphometry*

Estimation of volume fraction (Vv)

Five to ten non-overlapping micrographs of luminal epithelium were taken for each subject in a systematic random pattern at an initial magnification of 2200×. Measurements were made by using a projecting microscope (Carl Zeiss-Germany) to magnify negatives to a final magnification of about 22000. The images were superimposed with a lattice of 20-mm squares (0.9-μm apart on the tissue) and the volume fraction of nucleus, mitochondria, "vesicular system" (including Golgi, smooth endoplasmic reticulum and vesicles) and rough endoplasmic reticulum (RER) to cell and of euchromatin to nucleus were obtained.

Basement membrane arithmetic and harmonic mean thickness

Using a projecting microscope the negatives were projected at a magnification of 50000× over on a square lattice, each square of which was 50-mm apart (1μm on the tissue). Two perpendicular lines were drawn, one on the inner surface of the basement membrane and another on the outer surface of the basement membrane. These lines were orthogonal to the line of the square lattice. The length (l) between these two perpendicular lines was measured using a common ruler. By dividing the sum of all lenghths by the number of observations the arithmetic mean lenght was obtained ($\bar{\ell}$ a). Basement membrane arithmetic mean thickness (T_a) was measured according to the formula: $T_a = \bar{\ell}_a \times \frac{\pi}{4} \times \frac{10^6}{M}$ (12).

Basement membrane harmonic mean thickness was measured in the same way as arithmetic mean thickness, but instead of an ordinary ruler a logarithmic ruler was used. In this case the formula was: $T_h = \ell_h \times \frac{8}{3\pi} \times \frac{10^6}{M}$ (12).

* *Surface ratio*

Sing the 20 mm square lattice and recording the intersections of horizontal and vertical lines on the lattice and the projected images, the ratios of microvilli and desmosomes to cell membrane were estimated. In addition an arbitrary straight line drawn a cross the apical surface of the cell, along the base of microvilli of luminal epithelial cells, was used to allow estimation of the amplification of cell apical surface due to microvilli.

* *Nuclear profile dimensions*

Measurements were made on semi-thin sections with the aid of a drawing tube attached to an Olympus (BH-2) microscope and a microcomputer based digitizer using previously written software. The major (a) and minor (b) axes, mean profile diameter ($\sqrt{a \times b}$) and axial ratio (major axis/minor axis) of 50 longitudinally sectioned nuclear profiles were obtained from each subject.

* *Cell height*

The height of 10 randomly sampled, luminal epithelial cells that were cut longitudinally were measured using a drawing tube and ruler in one randomly selected JB-4 section per subject. Estimations were made on cells with clearly visible apical and basal borders using an oil immersion objective at a final magnification of 1050 times.

* *Linear nuclear density*

The linear nuclear density (number of nuclear profiles per unit length of epithelium) was estimated under oil immersion at a magnification of 1050 times. The length of four randomly sampled segments of the luminal epithelium, where cells were cut longitudinally, were examined per subject. The number of nuclear profiles within that length of line were counted and divided by the total number of nuclear profiles by the total length of epithelium to give the number of nuclear profile per-unit length of epithelium.

* *Volume weighted mean volume $\bar{V}$*

Vertical semi-thin sections were cut and displayed on a Quantimet Q970 image analyser (Cambridge

Instrument Ltd) using a Polyvar microscope at a magnification of about 2600 times (a graticule was used to determinate exact magnification). The outlines of every luminal epithelial cell nucleus on ten fields of vertical sections were drawn on acetate sheets using a fine point felt tip pen. A test system of parallel lines 10 mm apart was superimposed on the luminal epithelial cells. The outlines of nuclei were oriented so that their long axis was parallel to the vertical line of the guide "direction finder" on the test system (13-14). When a point hit a nucleus; a line was drawn through this point. These lines produced point sampled intercepts whose lengths (which were measured by a ruler in mm corrected for magnification), raised to the third power multiplied by $\pi/3$ and then averaged over all intercepts, give an unbiased estimation of $\overline{V}v$ (15). About 100 nuclear profile were traced and measured from each subject.

∗ *Data analysis*

Data was collected from each individual and the mean and standard error calculated per group (n=number of subjects). Where necessary, log transformations of ratio data were calculated for each group before statistical testing. Finally groups were compared using Student's *t* test. The means were considered to differ significantly if the P value was less than 0.05.

Results

Volume fraction data for the various features of endometrial epithelial cells examined are presented in table I. The volume fraction of nucleus to whole cell was higher (P< 0.05) in the fixed 4 mg, 0.3.2±0.023 (mean±SE) than the standard group (Fig. 1).

The ratio of euchromatin to whole cell in the fixed 1 mg group (0.711±0.020) was smaller (P< 0.05) than both controls and the fixed 4 mg group. The proportion of mitochondria to whole cell was higher (P< 0.05) in the controls (0.076±0.010) than both the fixed 4 mg and standard groups (Table I). There was no difference in the proportion of vesicular system and rough endoplasmic reticulum to whole cell among these groups.

Table I: Volume fraction data for luminal epithelial cells

Feature	Control group n=5	Fixed 1 mg group n=6	Fixed 4 mg group n=6	Standard group n=6
Nucleus: cell	0.246±0.037	0.241±0.021	0.235±0.009[b]	0.302±0.023
Euchromation: nucleus	0.783±0.016	0.711±0.020[a]	0.791±0.024[c]	0.690±0.049
Mitochondria: cll	0.076±0.010	0.051±0.005	0.050±0.004[d]	0.050±0.005[e]
"Vesicular system" :cell	0.023±0.005	0.036±0.007	0.039±0.010	0.035±0.004
RER: cell	0.017±0.003	0.029±0.007	0.022±0.003	0.015±0.003

Results are mean±standard error

n= number of indivinuals

a. P<0.05 control group versus fixed 1 mg group

b. P<0.05 standard group versus fixed 4 mg group

c. P<0.05 fixed 1 mg group versus fixed 4 mg group

d. P<0.05 control group versus fixed 4 mg group

e. P<0.05 control group versus standard group

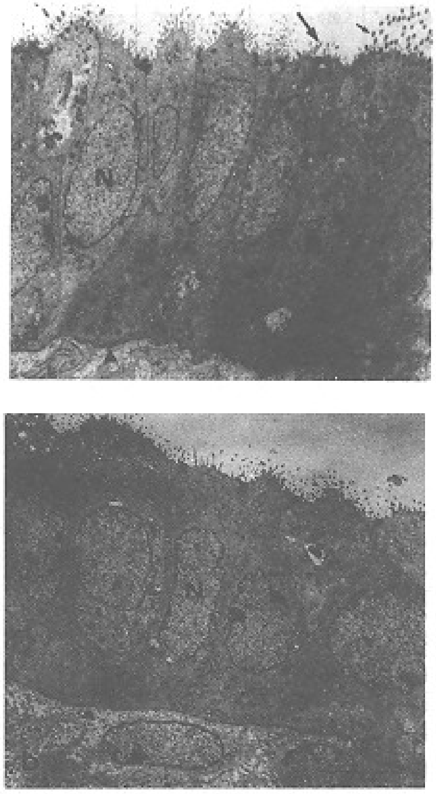

Fig. 1: a.b: Luminal epithelial cells from a fertile control women at day LH+6 (a) and (b) in POF women treated with fixed daily dose 4-mg oestrogen at day 20 of menstrual cycle. Mitochondria (empty arrow), microvilli (large filled arrow), epithelial cell nucleus (N) nodeous (empty arrowhed) and basement membrane (filled arrowhead). Qualitaitively there are few differences between the 4 mg and control tissue. Both show active, secretory cells with developed apical microvilli and euchromatic nuclei. Satining: Uranyl Acetate-Lead Citrate

(a) Bar represent 3.2 μm

(b) Bar represent 5.3 μm

The amplification of luminal epithelial cell apical surface due to microvilli (3.142±0.539) was smaller (P< 0.05) in the fixed 1 mg group than the controls (table 2). The proportion of microvilli to whole cell surface was smaller (P< 0.05) in the fixed 1 mg (0.250±0.025) than both the control and the standard groups. The ratio of desmosomes to whole cell surface (0.068±0.009) was highest (P< 0.01) in the fixed 1 mg when compared with the control and the standard groups (Table 2).

Table 2: Surface feature data for luminal epithelial cells

Feature	Control group n=5	Fixed 1 mg group n=6	Fixed 4 mg group n=6	Standard group n=6
Ration of microvilli:whole cell surface	0.394±0.062	0.250±0.028	0.316±0.046	0.398±0.039[c]
Amplification of apical membrane* due to microvilli	5.959±0.957	3.142±0.539[a]	4.289±0.641	5.126±0.831
Ration of desmosomes: whole cell surface	0.039±0.006	0.068±0.009[b]	0.046±0.006	0.037±0.003[d]

Results are mean±standard error

n= number of indivinuals

1 & 2. P<0.05 control group versus fixed 1 mg group

3. P<0.05 standard group versus fixed 1 mg group

4. P<0.05 standard group versus fixed 1 mg group

In the nuclear profile dimensions only the nuclear profile major axis was shorter (P< 0.05) in the controls (3.68±0.19 μm) than the standard group (table 3).

Table 3: Cell profile dimensions of epithelial cells

Feature	Control group n=5	Fixed 1 mg group n=6	Fixed 4 mg group n=6	Standard group n=6
Major axis of nuclear profile (μm)	8.69±0.64	8.56±0.56	8.36±0.19	8.65±0.66
Minor axis of nuclear profile (μm)	3.68±0.19	3.90±0.23	4.24±0.19	5.07±0.481
Mean diameterof nuclear profile (μm)	5.54±0.18	5.82±0.22	5.84±0.06	6.14±0.20
Axial ration of nuclear profile	2.65±0.29	2.67±0.27	2.18±0.19	2.10±0.17
Cell height (μm)	19.01±1.31	20.38±1.54	22.47±2.39	20.07±2.33
Nuclear profile linear density μm^{-1}	204±7	,253±24	198±10	200±10
Basement membrane harmonic mean thickness (nm)	62±4.8	72±5	75±6	70±5
Basement membrane arithmetic mean thickness (nm)	60±7	79±14	80±6	99±22

Results are mean±standard error

n= number of indivinuals

1. P<0.05 control group versus standard group

The basement membrane harmonic mean thickness (range 62±4.76 to 75±6.00 nm) did not differ between groups (table 4).

Table 4: Volume weighted mean volum data (μm^3) for luminal epithelial cell components

Cell component	Control group n=5	Fixed 1 mg group n=6	Fixed 4 mg group n=6	Standard group n=6
Volume of cell μm^c	871±172	883±115	1000±60	715±40[d]
Volume of nucleuse μm^c	190±15	202±11	234±15	215±10
Volume of euchhromation μm^c	149±13	145±11[b]	184±10	146±9[e]
Volume of mitochondria μm^c	69±19	46±8	51±6	35±2[f]
Volume of vesicular system μm^c	18±3	31±6	38±9	25±3
Volume of RER μm^c	13±1[a]	25±6[c]	23±4	10±2[g]

Results are mean±standard error

n= number of indivinuals

a. P<0.05 control group versus fixed 4 mg group

b. P<0.05 fixed 1 mg group versus fixed 4 mg group

c. P<0.05 fixed 1 mg group versus standard group

d. P<0.05 fixed 4 mg group versus standard group

e & f. P<0.05 fixed 4 mg group versus standard group

Also the basement membrane harmonic mean thickness (ranging from 60±6.51 to 99±21.57nm) followed a similar pattern (Fig. 2). Mean cell heights with a range from 19.01±1.13 μm to 22.47±2.39 μm also remained unchanged (table 5).

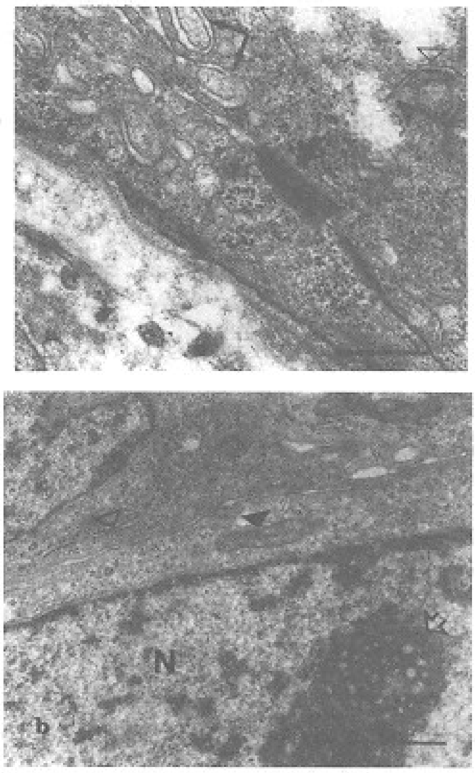

Figure 2.a,b: High power views from a normal fertile women on day LH+6 A nucleuse (N) lateral wall folding (small arrows), desmosome (larg arrow), mitochendria (small filled arrow), RER (empty arrowhead) and nuclear channel system (empty arrow). Nuclear channels are unique, helical tubural folding of the inner nuclear membrane. They accrue only during a well-defined period of the mid luteal phase in human. Also typical of this time in the extensive membrane trafficking that occurs during the mid-luteal phase.

The linear nuclear density also (ranged from 198±10 μm^{-1} to 253±24 μm^{-1}) did not differ between groups (table 3). Cell volume (1000±60 μm3) was larger ($P < 0.05$) in the fixed 4 mg group than the standard group (table 2). Nuclear volume ranged from (190±15 μm^3 to 234±15 μm^3) and did not differ between groups. Euchromatin volume in the fixed 1 mg group (145±11 μm^3) and in the standard group (149±9 μm^3) was smaller than the fixed 4 mg group. The volume of mitochondria was smaller ($P < 0.05$) in the standard group than the fixed 4 mg subjects (Table 4). There were no significant differences between any groups in the vesicular system volume. The volume of rough endoplasmic reticulum was higher ($P < 0.05$) in the fixed 4 mg group than both the control group and the standard group (Table 4).

Discussion

In the present study the higher volume fraction of euchromatin to nucleus in the control and fixed 4 mg dose groups compared with the fixed 1 mg dose group suggested a significantly reduced transcriptional activity in the fixed 1 mg group. This finding is in agreement with the study of Li et al. (8) who used the same tissue samples and reported that treatment with the fixed 1 mg dose oestrogen caused sub-optimal priming of endometrium and a significant decrease of supra and sub-nuclear secretory vacuoles in gland cells seen at the light microscope level. They suggested this would result in an increase in the volume fraction of glands occupied by gland cells.

Dockery et al. (16) reported that in the glandular epithelium of fertile women, the proportion of cell occupied by RER decreased between days LH +2 and LH +5. They also stated that on day 19 (approximately day LH +5) women with premature ovarian failure who were treated with sub-optimal doses of oestrogen, had cytoplasmic features, which were similar to those at day LH +3 of normal fertile women. It might be anticipated that the high volume fraction of euchromatin to nucleus in the control and the 4 mg groups in the present study would lead to there being more RER in these groups than in the fixed 1 mg group. However in the present study the volume fraction of RER was similar between the groups. It is possible that the increased transcription had not had time to pass the message to the RER for transcription at the time of biopsy. The proportion of mitochondria to cell is significantly smaller in the fixed 4 mg and in the standard groups when compared with the control group. Also this feature tended to be significantly smaller in the fixed 1 mg group than the control group. There are two suggestions as to why these changes in mitochondria might have been seen: 1, mitochondrial activity may be directly hormone dependent and increased when oestrogen levels increased. 2, mitochondria may not be directly hormone dependent but, when protein synthesis induced by increased oestrogen put a high demand for energy, mitochondria increased in response. For both hypotheses a high Vv ratio of mitochondria to cell would be predicted. According to Li et al. (16) plasma levels of oestradiol on day 15, 19 and 29 of women with premature failure that were treated with both standard HRT regime and the fixed 4 mg dose were much higher than those on the corresponding days of the natural cycle. This may suggest because of the smaller ratio of mitochondria to cell in both standard variable and 4 mg treated groups that these luminal epithelial cells were relatively advanced and already have passed the stage of DNA transcription, almost finished protein synthesis and so have reduced energy demand (and therefore lower Vv of mitochondria to cell). The small Vv ratio of mitochondria to cell in the fixed 1 mg group is likely to be due to a sub-optimal level of oestrogen and so caused a delay in cell development and so the mitochondria had not yet begun to increase in response to the cell demand for energy. These data suggest that these oestrogen regimes did not create a completely physiological menstrual cycle.

In the standard group, the volume fraction of nucleus to whole cell was significantly higher than in the 4 mg group. This may be because the fixed 4 mg subjects had a higher plasma oestrogen level than in the standard group, may causes an advance in protein synthesis in the 4 mg group.

Besides these changes inside the cell, alteration in the cell surface, such as changes in microvilli organization are essential for successful implantation. 17 The shape of uterine epithelial cell microvilli change from being long, at the time of oestrus, to thin, short, flat and irregular in shape at around 5/6 days of pregnancy (18). Therefore the estimation of microvilli ratio to cell membrane may be another useful indicator of endometrial development in relation to implantation.

The ratio of microvilli to whole cell membrane was significantly bigger in the standard group than in the fixed 1 mg group. This feature also tended to be bigger in the control group than the fixed 1 mg group. It has been reported that there are qualitative as well quantitative hormone dependent changes in the glycocalyx (both in thickness and morphology) on the apical surface of uterine luminal epithelial cells in the preimplantation period (19-22). The results of extensive biochemical analysis have reported strong evidence for hormone dependent changes in glycoproteins on uterine epithelial cells (23). Reports of (24-26) described production of specific glycoproteins including hyaluronate, lactosaminoglycans, glacotosyl-transferases, heparin/heparan sulphate proteoglycans and their proteins at the apical surface of the uterine epithelial cells around the time of implantation. These molecules are known to have the potential to be involved in cell recognition and embryo attachment to luminal epithelium.23 Amplification of apical membrane due to microvilli was significantly smaller in the fixed 1 mg group when compared with the control group. This feature even tended to be smaller in the fixed 1 mg group than the standard group. It has been reported by that the length and number of microvilli decrease around implantation time (day LH +6/7) (27, 28, 30). Murphy et al. also stated that these changes in microvillus morphology are essential for successful implantation and are accompanied by fundamental changes in the carbohydrate content of plasma membrane (17). Therefore the significant changes seen in the microvilli from subjects given daily 1 mg oestrogen are likely to have a harmful effect on implantation. Conversely, those subjects given daily 4 mg fixed oestrogen or standard doses HRT had microvilli close enough to controls subjects that, were an embryo present, these luminal cells might be able to allow implantation. It is believed that simultaneous to the microvilli changes there are some other important changes in cell membrane features that should be considered.

The ratio of desmosomes to whole cell was significantly bigger in the fixed 1 mg group than the standard and the control groups. Desmosomes enable epithelial cells to form strong structural units by connecting the cytoskeletal elements of adjacent cells together or to the extracellular matrix (31). The higher ratio of desmosomes to membrane in the fixed 1 mg group of the present study compared to the other groups may be due to the overall delay in epithelial cell development following sub-optimal amounts of oestrogen available to this group. This is suggested because normally, desmosomes decrease around day LH +6 but then rapidly increase again (9). Therefore any delay in development would make this decrease occur later, with a relatively higher level in desmosome to membrane ratio in the fixed 1 mg group than in groups where development was not delayed at the time of biopsy. Since this normal decrease around the time of implantation may assist in embryo penetration, such a delay in the 1 mg group would be likely hinder implantation.

The bigger average volume of euchromatin in the fixed 4 mg group than both the standard group and the fixed 1 mg group and also the numerically bigger volume than the control group probably indicate that transcription was higher in the fixed 4 mg group than the other. Furthermore generally bigger organelles and overall cell volume in the 4 mg group indicates that cell secretion was higher or prolonged in the fixed 4 mg group than other experimental groups. Again indicating a relative advance in the group.

In conclusion, the findings in the present ultrastructural study on luminal cells suggest that endometrial development in subjects treated with the daily fixed 1 mg oestrogen dose had significantly delayed membrane features when compared with controls, the fixed 4 mg group and those treated with the variable standard regime. However, the fixed 4 mg group showed evidence of advancement in some of the features examined. These features are likely to have a significant influence of implantation.

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