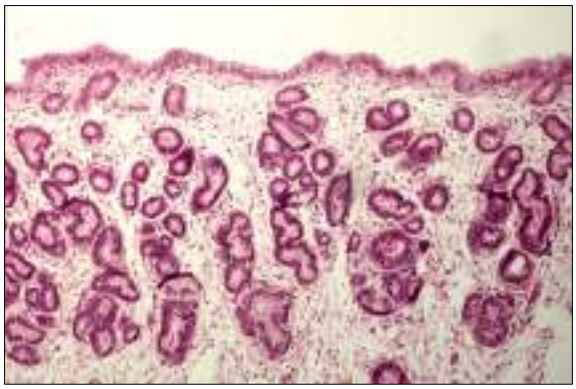
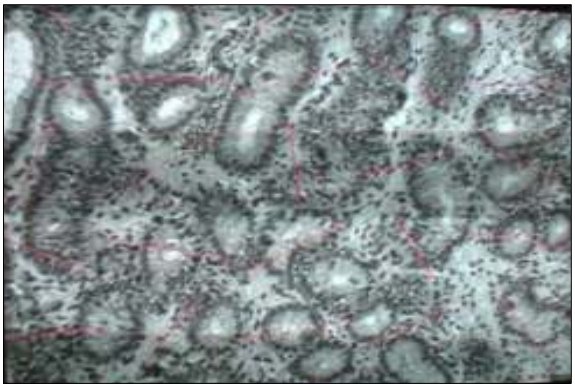
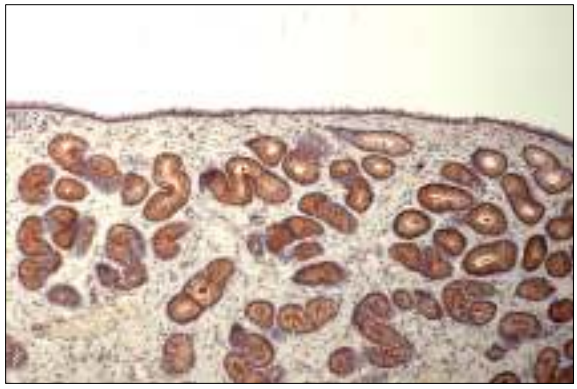
The effect of progesterone on equine endometrial morphology, gland surface density and uterocalin secretion







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Summary

Previous studies have shown that the equine endometrium changes morphologically under the influence of progesterone. It also begins to secrete the 19 kDa protein uterocalin, which is believed to act as a carrier or transport protein to provide the conceptus with essential vitamins and minerals during the early stages of development.
In the present study endometrial biopsies were used to assess and compare the effects of both endogenous and exogenous progesterone on endometrial morphology, the surface density of endometrial glands and their secretion of uterocalin. An attempt was also made to relate these findings to the duration of progesterone dominance. Therefore, endometrial biopsies were taken at several time points after ovulation and after exogenous progesterone administration. These were processed and sectioned, after which they were either stained with haematoxylin and eosin (H&E) for the morphometric study, or labelled immunohistochemically for the detection of uterocalin.
Morphometric analysis indicated that the endometrial changes are comparable after both endogenous and exogenous progesterone dominance and were most noticeable in the epithelium lining the endometrial glands, where the height of the epithelial cells had increased significantly by 3 – 5 days after ovulation and after 3 days of exogenous progesterone treatment. Glandular surface density, however, did not increase after ovulation, nor after treatment with exogenous progesterone. Immunohistochemical staining of the endometrial biopsies showed significant uterocalin secretion after 5 days in both normal dioestrus and after the administration of exogenous progesterone.
This suggests that the changes in the morphology of the endometrium are progesterone dependent and that gland surface density is either not progesterone dependent or might need more days of progesterone dominance before significant changes can be seen. It can be concluded that the secretion of uterocalin is initiated by progesterone and that the rate of secretion is sufficient by the time the equine embryo enters the uterine lumen on day 6 after ovulation.

Introduction

Cyclicity in the mare

The mare is a seasonal polyoestrous animal having an oestrous cycle with an average length of 19 – 22 days (Daels and Hughes, 1993). Hormones produced by the pineal gland, hypothalamus, pituitary gland, ovaries and endometrium control the oestrous cycle which can be divided into two phases, the follicular and the luteal phase.

The follicular phase includes pro-oestrus and oestrus. Pro-oestrus begins when progesterone levels start to decline as a result of luteolysis of the corpus luteum. Luteolysis occurs, between 13 and 16 days post-ovulation, as a consequence of endometrial release of prostaglandin F2α (Daels and Hughes, 1993).
Throughout the oestrous cycle a basal quantity of the gonadotropin releasing hormone (GnRH) is released from the hypothalamus, stimulating the synthesis and release of the gonadotropins follicle stimulating hormone (FSH) and luteinizing hormone (LH) from the anterior pituitary into the systemic circulation. At the level of the ovaries FSH is responsible for follicular recruitment, whereas LH is responsible for follicular maturation and oestrogen production by the maturing follicles. When oestrogen, in the presence of a low systemic concentration of progesterone, as occurs during oestrus, reaches a threshold level increasing levels of GnRH are released (Senger, 1999). This results in a pre-ovulatory LH-surge, leading to ovulation of the dominant follicle(s) (Daels and Hughes, 1993; Niswender and Nett, 1993; Senger, 1999; Blanchard *et al*. 2003).

Ovulation marks the onset of the luteal phase, which includes metoestrus and dioestrus. Metoestrus continues until a functional corpus luteum, governed by LH, reaches its maximum size after 2 – 2.5 days (Daels and Hughes, 1993).
The corpus luteum consists of progesterone-secreting luteal cells, originating mainly from the granulosal cells of the ovulatory follicle (Van Niekerk *et al*. 1975). Its lifespan depends on the endogenous release of prostaglandin F2α from the endometrium. The release of prostaglandin F2α may be prevented by an embryonic signal (the maternal recognition of pregnancy) or other factors, for example, endometrial pathology. However, in general, luteolysis occurs 13 – 16 days after ovulation resulting in the start of pro-oestrus and therefore a new oestrous cycle.

Early pregnancy

Hormonally, the first 14 days of pregnancy are very similar to dioestrus (Blanchard *et al*. 2003). The corpus luteum secretes progesterone, a steroid hormone that exerts a negative feedback on the production of GnRH in the hypothalamus after ovulation that inhibits maturation of preovulatory follicles. In addition, progesterone stimulates secretion by the endometrial glands, which contributes to a uterine environment that can provide support for the development of an embryo (Squires, 1993).
The single difference between hormone secretion during pregnancy and dioestrus is that during pregnancy the endometrial secretion of prostaglandin F2α is inhibited by a maternal

recognition or pregnancy signal. Hence, the corpus luteum does not regress and pregnancy can be maintained by the dominance of progesterone.

Endometrial morphology

The uterus of the mare is composed of 3 layers, the outer perimetrium (serosa), the middle myometrium and the inner endometrium (mucosa) (Kenney, 1978; Bacha and Bacha, 2000). The endometrium is made up of 2 layers, the luminal epithelium lining the uterine lumen and the lamina propria that extends from the basement membrane of the luminal epithelium to the myometrium. The lamina propria contains numerous endometrial glands, surrounded by stellate stromal cells in the part of the lamina that is called the stratum compactum and by a loose arrangement of interconnecting cells in the stratum spongiosum. The endometrial glands open into the uterine lumen and are lined by glandular epithelium (Kenney, 1978; Ginther, 1992; Bacha and Bacha, 2000).

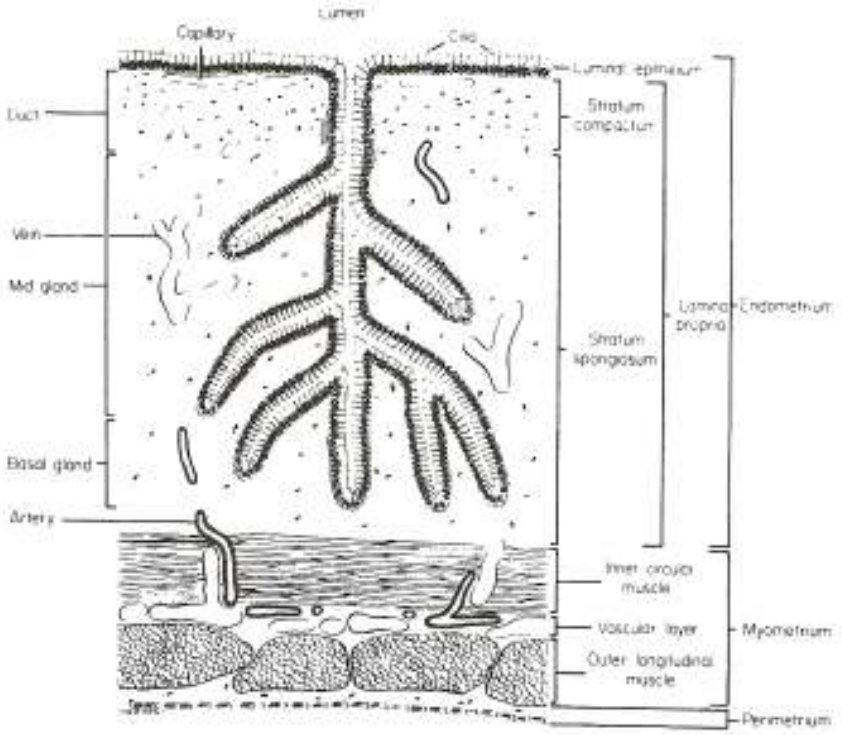

Figure 1. Kenney's diagram of a cross-section of the equine uterine wall. The relative thickness of the myometrium was greatly reduced for illustrative purpose. *From: Kenney (1978)*

The morphology of the endometrium changes under the dominance of both oestrogen and progesterone. As these hormones change throughout the oestrous cycle variations occur in the type and height of the luminal epithelium and the epithelium lining the endometrial glands, and the branch diameter, configuration and depth of the endometrial glands.

During anoestrus, when the endometrium is not under the dominance of either oestrogen or progesterone, the luminal epithelium can be cuboidal (Kenney, 1978; Gross and LeBlanc, 1984; Doig and Waelchli, 1993) to squamous (Doig and Waelchli, 1993) with an average height of 10µm (Kenney, 1978). The glandular epithelium can, besides cuboidal or squamous, also be columnar in shape (Kenney, 1978; Doig and Waelchli, 1993). As an indication of atrophy, the glands have contracted lumens (Gross and LeBlanc, 1984), a straight (Kenney, 1978; Doig and Waelchli, 1993) or non-tortuous configuration (Kenney, 1978) and a relatively low density (Gross and LeBlanc, 1984).

Oestrus is characterised by a tall columnar (Ricketts, 1975; Kenney, 1978; Ginther, 1992; Doig and Waelchli, 1993) or pseudostratified luminal epithelium (Ricketts, 1975) with cellular height of 20 – 50µm, whereas the glandular epithelium is tall columnar (Ricketts, 1975; Doig and Waelchli, 1993). The diameter of the glands is large as a result of the tall columnar epithelial cells lining the glands (Doig and Waelchli, 1993), and on longitudinal section the glands appear straight or non-tortuous (Ricketts, 1975; Ginther, 1992; Doig and Waelchli, 1993) with the smallest gland density (Ginther, 1992) compared to other stages (Kenney, 1978), possibly as a result of oedema in the lamina propria.

After oestrogen administration to anoestrous mares the glandular epithelium and lamina propria take on the characteristics of those described for mares in oestrus. Namely, the glandular epithelium becomes tall and columnar and oedema is apparent in the lamina propria (Hamer *et al*. 1985).

During dioestrus when the endometrium is under the dominance of progesterone the luminal epithelium can vary in size, from low columnar (Ricketts 1975, Kenney 1978) or tall columnar (Kenney, 1978) to cuboidal (Ricketts, 1975; Doig and Waelchli, 1993) and pseudostratified (Keenan *et al*. 1987), with a height varying from 10 – 20µm (Kenney, 1978; Keenan *et al*. 1987). The glandular epithelium is columnar with a height ranging from 16 – 19µm, and glandular diameter increases from 35µm during the first 5 days after ovulation to 51µm 9 days after ovulation, the latter being a result of the secretory activity of the endometrium (Keenan *et al*. 1987). The configuration of the glands is straight (Keenan *et al*. 1987) or tortuous (Kenney, 1978; Keenan *et al*. 1987; Ginther, 1992; Doig and Waelchli, 1993) and the glandular depth in the endometrium is 0.8 – 0.9mm (Keenan *et al*. 1987). Gland density appears greater in dioestrus due to a reduction in oedema in the lamina propria compared to that in oestrus (Kenney, 1978; Ginther, 1992), and Keenan *et al*. (1987) found a quantative density of 39 – 61 tubules per 0.5cm^2.

Progesterone administration to anoestrous mares resulted in both the luminal and glandular epithelium, and gland configuration being consistent with those described for mares in dioestrus. The epithelia had a height with a range of 10 – 20µm and the glands were tortuous (Hamer *et al*. 1985).

During the first nine days of pregnancy the luminal as well as the glandular epithelium has a columnar shape. The height of the luminal epithelium ranges from 19 – 23µm, the height of the glandular epithelium ranges from 17 – 19µm and the diameter of the glands is 38 – 56µm. The glands have a tortuous appearance with a depth of 1.0mm in the endometrium (Keenan *et al*. 1987) and a gland density of 57 – 59 tubules per 0.5cm^2 (Keenan *et al*. 1987).

Table 1 is a summary of the morphology during the different stages of the cycle and early pregnancy.

Table 1: Summary of differences in endometrial morphology

		Stage of cycle			
Parameter		**Anoestrus**	**Oestrus**	**Dioestrus**	**Early pregnancy**
Luminal epithelium	Description	Cuboidal/ squamous	Tall columnar/ pseudostratified	Low columnar/ tall columnar/ pseudostratified	Columnar
	Size (µm)	10	20 – 50	10 - 20	19 - 23
Glandular epithelium	Description	Cuboidal/ squamous/ low columnar	Tall columnar	Columnar	Columnar
	Size (µm)	-	-	16 - 19	17 - 19
Glandular diameter	Description	Contracted lumen	Large diameter	Large lumen	Dilated lumen
	Size (µm)	-	-	35 - 51	38 - 56
Glandular configuration	Description	Straight/ non-tortuous	Straight/ non-tortuous	Straight/ tortuous	Tortuous
Gland depth	Size (mm)	-	-	0.8 - 0.9	1.0
Glandular density	Description	Low	Minimal/ less than other stages	Increased compared to oestrus	-
	Tubules/0.5cm^2	-	-	39 - 61	57 - 59

Secretion of uterocalin from the endometrium

The equine endometrium secretes a multitude of proteins from its endometrial glandular and epithelial cells, also known as histotrophe or uterine milk. The most abundant of these proteins is uterocalin.

Uterocalin, also known as P19, is a protein with a molecular mass of approximately 19 kDa (Crossett *et al*. 1996). It is secreted from the endometrial glandular and epithelial cells (Crossett *et al*. 1996; Crossett *et al*. 1998; Stewart *et al*. 2000[a]; Stewart *et al*. 2000[b]) and is a member of the lipocalin protein family. This family consists of small secretory proteins with very similar tertiary structures and the ability to bind and transport sparingly soluble or chemically sensitive hydrophobic ligands (Flower, 1996).
Uterocalin binds to the capsule surrounding the equine embryo (Stewart *et al*. 1995) acting as a carrier or transport protein for such nutrients as essential lipids and amino acids. These provide the embryo with some of the vital components required for early embryonic development (Suire *et al*. 2001; Kennedy, 2003; Kennedy, 2004). This histotrophic nutrition of the developing embryo is essential prior to the establishment of haemotrophic nutrition by the allantochorionic placenta.

From available data it can be concluded that uterocalin is not being secreted during oestrus (Stewart *et al*. 1995; Crossett *et al*. 1996; Crossett *et al*. 1998; Stewart *et al*. 2000[a]).
During dioestrus Crossett *et al*. (1998) localized uterocalin in 2 endometrial biopsies that were taken 2 days after ovulation, Stewart *et al*. (1995; 2000[a]) found uterocalin in the uterine

flushings from mares 10 days after ovulation and Crossett *et al*. (1996) detected it in endometrial biopsies at day 14 of the cycle.
The data concerning the appearance of uterocalin during early pregnancy are not consistent. Stewart *et al*. (1995) detected uterocalin in the uterine flushings taken at day 10, but not in those taken at day 7 of pregnancy. Crossett *et al*. (1996), Crossett *et al*. (1998) and Stewart *et al*. (2000[a]) all detected uterocalin at 14 days of pregnancy.
The secretion of uterocalin appears to be progesterone dependent, as its presence in uterine flushings correlates with peripheral serum progesterone concentrations (Stewart *et al*. 1995). Furthermore, its secretion can be induced by administering exogenous progesterone to anoestrous mares for several days (Stewart *et al*. 1995).

Aims of the project

The aims of the project were:
a) to assess and compare the changes in morphology and gland surface density and expression of uterocalin in the endometrium of the mare under the dominance of a post ovulation rise in progesterone or after the administration of progesterone to oestrous pony mares;
b) to relate changes in morphology, gland surface density and uterocalin expression to the length, in days, of the dominance of natural or exogenous progesterone.

Materials and methods

Animals and monitoring of the oestrous cycle

The oestrous cycle of 14 Thoroughbred and pony mares (aged 7 – 17 years) were monitored by daily transrectal realtime ultrasonography of the ovaries and uterus to assess follicular development, uterine oedema and the day of ovulation (day 0). Ovulation was also confirmed by measuring a rise in the progesterone concentration in peripheral serum samples using an amplified enzyme-linked (AELIA) assay for progesterone (Allen and Sanderson, 1987).

Tissue samples

The endometrial biopsies were taken during oestrus (n = 5), at day 1 (Ov + 1; n = 5), 3 (Ov + 3; n = 6), 5 (Ov + 5; n = 10), or 8 (Ov + 8; n = 3) after ovulation and at day 1 (P_4 + 1; n = 4), 3 (P_4 + 3; n = 6) or 5 (P_4 + 5; n = 4) after administration of exogenous progesterone using a Yeomans biopsy forceps passed through the cervix. (Ricketts 1975) A jugular blood sample was taken from each mare at the time of the biopsy to determine the serum progesterone concentration. At the time of the biopsy or at a minimum of 5 days post ovulation all mares received an injection of cloprostenol (Estrumate™) intramuscularly.

When the serum progesterone concentration fell below 1 ng/ml and oedema was noted in the uterus but no follicle larger than 35mm in diameter was present, the mares received daily injections of exogenous progesterone in oil (100mg/ml, 3ml 1dd) until the day of biopsy, the first day of administration taken as Progesterone + 0.
The biopsies were fixed overnight in formal saline, than processed in an automated tissue processor, embedded in paraffin wax and stored at room temperature until used.

Samples for morphology and stereology

The wax-embedded tissues were sectioned at 4 μm using a microtome, placed onto microscope slides and dried overnight at 37˚C. They then were dewaxed in Histoclear for 30 minutes, rehydrated through an alcohol gradient, washed in running tap water for 5 minutes and stained in Mayers' haematoxylin for 10 minutes. After removing excess stain by washing in running tap water for 10 minutes, the tissue was stained in Eosin for 12 minutes after which the tissue was washed again in running tap water for 30 seconds to remove excess eosin solution. The sections were then dehydrated through an alcohol gradient, washed briefly in Citroclear and finally mounted in DPX.

Samples for immunohistochemistry

The wax-embedded tissue samples were sectioned at 4 μm. Two sections of each tissue sample were placed onto positively charged microscope slides and dried overnight at 37˚C. Prior to use the sections were dewaxed in Xylene, rehydrated through an alcohol gradient and

washed in running tap water. After antigen retrieval by boiling the sections in a 0.01M sodium citrate buffer pH 6.0, the slides were washed again in running tap water. Next endogenous peroxidase was blocked in a 0.3% H_2O_2 in methanol solution and non-specific binding of the antibodies was blocked by adding normal horse serum (2,5% diluted, Vector RTU Vectastain® kit) onto the slides and incubate them in a moist chamber. After 30 minutes either P19 antibody (diluted 1:10,000 in Tris Buffered Saline pH 7.6) or pre-immune serum (diluted 1:1,000 in TBS pH 7.6) was added to the sections. Both were incubated in a moist chamber for 1 hour after which the antisera were visualised using the biotinylated reagent and the Vectastain elite ABC reagent from the RTU kit and the DAB peroxidase substrate kit (Vector cat. no. SK-4100), washing them thoroughly in between in a 1% Tween in TBS buffer pH 7.6. Finally the sections were counterstained with Mayers' haematoxylin, washed in running tap water to remove excessive stain, dehydrated through an alcohol gradient, cleared in Citroclear and finally mounted in DPX.

Morphology

Tissue sections were examined under a light microscope with the 40x objective to determine the height of the luminal epithelium and the glandular epithelium and the diameter of the gland lumen The sections also were examined with the 10x objective to determine the depth of the glands in the tissue using a eyepiece graticule that was calibrated with a stage micrometer.

Stereology

The surface density (the surface area per unit volume; Sv) of the endometrial glands was estimated using a modified stereology technique (Digital Stereology 2.3), a method previously described by Lefranc and Allen (2007[a]). Vertical sections of endometrium, positioned with the luminal epithelium aligned parallel to the upper border of the screen, were digitised using a 10x objective and projected onto a computer screen. A grid of 5 rows and 5 columns of test points were used to estimate the endometrial surface density. Only the test points that overlaid the endometrium were entered into the computer programme (Figure 2), before a total of 25 isotropic needles were positioned randomly onto the screen (Figure 3). Per biopsy 1 section was examined and this section was subdivided into a superficial and a basal part, the superficial part being right underneath the luminal epithelium and the basal part being one field underneath the superficial part. Both parts were examined with 8 fields, counting the number of intersections between the needles and the secretory surface of the glandular epithelium and with 8 fields counting the number of intersections between the needles and the basement membrane of the glandular epithelium. In this way a total of 32 fields were examined per biopsy, estimating the secretory surface area of the endometrial glands and the surface area underneath the epithelial cells, i.e. the outer border of the endometrial glands. The computer calculated the surface density automatically and the estimation of Sv was considered to be sufficiently precise when the coefficient of error fell below 5%.

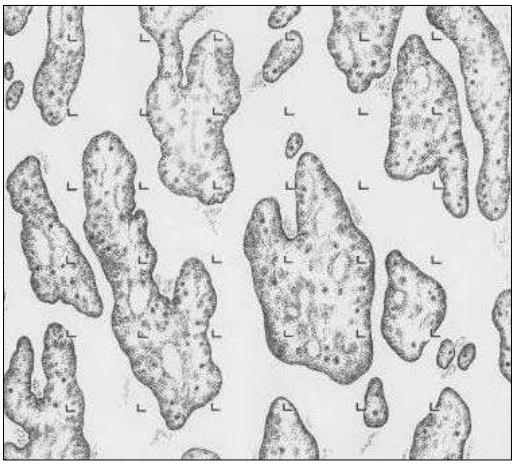

Figure 2. Test system grid of 36 test points overlaid onto a vertical histological section. *From: Lefranc and Allen (2007[a])*

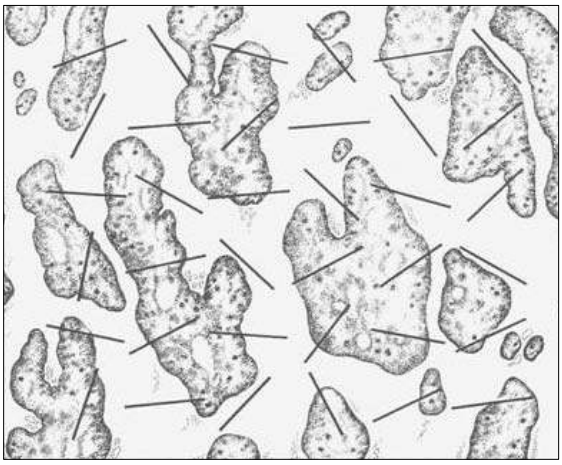

Figure 3. A total of 36 linear probes ('needles') overlaying the section, with 35 intersections counted. *From: Lefranc and Allen (2007[a])*

Immunohistochemistry

All sections were examined under the microscope to score the amount of positive staining in the luminal epithelium, the glandular epithelium and the glandular lumen. This was subjectively scored as negative (0) if there was no staining visible and light (1), medium (2) or strong (3) according to the intensity of the staining.

Statistics

Mean ± s.e.m. are presented throughout. For skewed data, median and range values are presented. Statistical analysis for morphology, gland surface density and immunohistochemistry were performed using a one-way ANOVA with Tukey's *post hoc* test, or for skewed data, a one-way ANOVA on ranks with Dunn's *post hoc* test (Sigma-Stat v.2.0). Data were considered significant when $P<0.05$.

Results

Morphology

The histological observations are summarized in Table 2 and presented as the mean ± s.e.m.

Table 2. Histological observations.

Parameters		**Stage of cycle or days post progesterone treatment**							
		Oestrus	**Ov + 1**	**Ov + 3**	**Ov + 5**	**Ov + 8**	**P_4 + 1**	**P_4 + 3**	**P_4 + 5**
Luminal epithelium	Height (μm)	15.0 ± 1.3	16.1 ± 1.7	19.4 ± 1.6	17.7 ± 1.2	13.6 ± 0.4	17.7 ± 1.2	16.9 ± 1.2	17.9 ± 1.7
Superficial									
Glandular epithelium	Height (μm)	12.8 ± 0.8^{a,b,c,d}	16.1 ± 0.4	18.5 ± 0.1^{a}	18.8 ± 0.7^{b}	15.9 ± 0.9	15.4 ± 0.6	17.9 ± 0.9^{c}	18.6 ± 1.6^{d}
Glandular diameter	Size (μm)	9.5 ± 1.3	10.9 ± 0.7	15.4 ± 2.1	10.9 ± 1.5	16.5 ± 3.4	14.9 ± 0.9	11.3 ± 1.7	13.9 ± 2.3
Basal									
Glandular epithelium	Height (μm)	15.1 ± 0.7^{a,b,c,d}	19.0 ± 0.7	19.3 ± 0.7	20.8 ± 0.9^{a}	20.1 ± 0.8^{b}	18.5 ± 0.9	19.9 ± 0.9^{c}	20.0 ± 1.1^{d}
*Glandular diameter**	Size (μm)	8.9 (7.5-14.2)	12.4 (9.6-19.3)	10.4 (9.8-13.6)	10.5 (10.3-12.0)	10.4 (10.2-11.0)	12.9 (9.2-14.2)	10.9 (9.8-13.3)	12.9 (11.0-19.4)
Glandular depth	Size (mm)	0.70 ± 0.04	0.98 ± 0.08	1.01 ± 0.08	1.1 ± 0.1	1.3 ± 0.3	0.57 ± 0.04	0.79 ± 0.09	1.0 ± 0.1

[a-d] Significant difference between different groups are indicated by different letter superscripts (at least $P < 0.05$)
* For skewed data results are presented as median and range of the data

There were no statistically significant differences in the height of the luminal epithelium at different stages of the oestrous cycle. However, in the superficial portion of the glands the height of the epithelium was significantly less during oestrus than when compared to either 3 or 5 days post ovulation or progesterone treatment ($P < 0.05$ in all cases).
Mean superficial or basal glandular diameter did not alter significantly during the different stages of the oestrous cycle or after the administration of progesterone. Despite these findings, the height of the epithelium in the basal portion of the glands was significantly lower during oestrus than when the endometrium had been under the dominance of progesterone for several days, namely, ≥ 5 days post ovulation or ≥ 3 days post progesterone treatment ($P < 0.05$).
No significant differences were observed between the depths of the glands during the oestrus cycle or between different time points after progesterone administration.

Stereology

The stereological measurements are summarised in Table 3 and presented as the mean ± s.e.m.

Table 3. Stereological measurements.

Parameters		Stage of cycle or days post progesterone treatment							
		Oestrus	**Ov + 1**	**Ov + 3**	**Ov + 5**	**Ov + 8**	**P_4 + 1**	**P_4 + 3**	**P_4 + 5**
Superficial									
Outer border glands	Sv (μm^{-1})	0.027 ± 0.001[a]	0.032 ± 0.003	0.035 ± 0.002	0.035 ± 0.001	0.032 ± 0.004	0.032 ± 0.003	0.040 ± 0.002[a]	0.034 ± 0.002
Secretory surface area	Sv (μm^{-1})	0.008 ± 0.001	0.010 ± 0.001	0.011 ± 0.001	0.010 ± 0.0004	0.008 ± 0.001	0.010 ± 0.001	0.011 ± 0.001	0.009 ± 0.001
Basal									
Outer border glands	Sv (μm^{-1})	0.028 ± 0.001	0.028 ± 0.001	0.029 ± 0.002	0.029 ± 0.001	0.027 ± 0.002	0.034 ± 0.003	0.030 ± 0.004	0.027 ± 0.001
Secretory surface area	Sv (μm^{-1})	0.008 ± 0.001	0.008 ± 0.001	0.008 ± 0.0004	0.008 ± 0.001	0.005 ± 0.001	0.007 ± 0.001	0.010 ± 0.001	0.007 ± 0.001

[a] Significant difference between groups is indicated by letter superscript (at least $P < 0.05$)

The surface density of the outer border of the glands in the superficial portion of the gland showed a trend to increase under the action of endogenous or exogenous progesterone. However, this was only statistically significant when oestrus was compared to P_4 + 3 ($P = 0.003$). Likewise, the basal portion of the glands also showed no significant difference in mean surface density values between the different groups ($P = 0.379$).
Similarly, although the action of progesterone resulted in a slight increase in the mean surface densities of the secretory surface of the superficial portion of the glands, this was not apparent in the basal portion. However, no significant difference was found ($P = 0.130$).

Immunohistochemistry

The figures mentioned below are presented in Attachment I.

During oestrus no staining was evident in either the luminal or glandular epithelium. However, some light to medium staining was observed in the glandular luminae, mainly in the basal portion. (Figure 1) Fibrosed portions of the glands, known as gland nests, which were evident in two biopsies examined, showed medium staining.
At one day post ovulation (Ov + 1) there was no detectable staining in both the luminal and glandular epithelium. Medium to strong staining was detected in the basal lumen of the glands, but not in the superficial lumen. (Figure 2) Three of the biopsies showed medium to high staining in the gland nests.
At three days post ovulation (Ov + 3) no staining was evident in the luminal epithelium. (Figure 3) The glandular epithelium stained light to medium in the superficial portion and medium in the basal portion of the glands. In the superficial lumen the staining was light, while in the basal portion the staining was medium. Three of the biopsies had gland nests that all showed medium staining.
By five days post ovulation (Ov + 5) staining of the luminal epithelium was evident in all the biopsies examined. The intensity of the staining was light in 4 of the biopsies examined and

strong in the remainder. The glandular epithelium and basal portion of the gland lumen also stained strongly. In addition, the superficial lumen also showed medium to strong reactivity with the antibody. (Figure 4) The intensity of staining apparent within gland nests varied greatly.
At eight days post ovulation (Ov + 8) all the biopsies showed intense strong staining of the luminal epithelium, glandular epithelium and glandular lumen. (Figure 5)
After one day of exogenous progesterone treatment (P_4 + 1), there was no detectable staining in the luminal epithelium. The glandular epithelium and the superficial lumen all showed light staining. In the basal lumen of the gland there was light to medium staining. (Figure 6)
From the biopsies taken after 3 days of progesterone treatment (P_4 + 3) staining was variable with no staining was seen in 3 biopsies, but strong staining in the remaining 2. The glandular epithelium and the lumen of the glands both showed medium to high staining. (Figure 7)
At five days post progesterone treatment (P_4 + 5) all the biopsies showed strong immuno-reactivity to the antibody in the luminal epithelium, the glandular epithelium and the basal lumen of the glands. Staining was medium to strong in the superficial lumen. (Figure 8) As with the post-ovulatory biopsies, staining of the gland nests in the animals treated with exogenous progesterone was inconsistent.
No detectable staining in the tissues was evident when the primary antiserum was replaced by an equivalent dilution of pre-immune control serum.

The results of the immunohistochemical localization of uterocalin are summarized in Table 4 and are presented as mean ± s.e.m.

Table 4. Immunohistochemical localization.

Parameters	**Stage of the cycle or days post treatment with progesterone**							
	Oestrus	**Ov + 1**	**Ov + 3**	**Ov + 5**	**Ov + 8**	**P_4 + 1**	**P_4 + 3**	**P_4 + 5**
Luminal epithelium	$0^{a,b,c}$	$0.2 \pm 0.2^{d,e,f}$	$0.5 \pm 0.3^{g,h,i}$	$2.3 \pm 0.3^{a,d,g,j}$	$3.0 \pm 0^{b,e,h,k}$	$0.5 \pm 0.5^{j,k,l}$	1.2 ± 0.7	$2.8 \pm 0.3^{c,f,i,l}$
Superficial								
*Glandular epithelium**	0 (0-1)	1 (0-2)	1.5 (0-3)	3 (3-3)	3 (3-3)	1 (0.5-1)	3 (1.75-3)	3 (3-3)
*Lumen**	0 (0-1.25)	0 (0-1.25)	1 (0-2)	2 (2-3)	3 (3-3)	1 (1-1)	3 (1-3)	2.5 (2-3)
Basal								
Glandular epithelium	$0.4 \pm 0.3^{a,b,c,d}$	1.2 ± 0.6	1.7 ± 0.5	2.5 ± 0.2^{a}	3.0 ± 0^{b}	1.3 ± 0.6	2.4 ± 0.4^{c}	2.8 ± 0.3^{d}
*Lumen**	1 (0.75-1.25)	1 (1-2.25)	2 (1-3)	3 (3-3)	3 (3-3)	1.5 (1-2)	3 (1.75-3)	3 (2.5-3)

$^{a-l}$ Significant differences between groups are indicated by different letter superscripts (at least $P < 0.05$)
* Results are presented as median and range of the data

The intensity of the staining in all parts of the tissue showed a trend to increase under the action of both endogenous and exogenous progesterone, although these were not significant in case of the lumen of the glands and the epithelium in the superficial portion of the glands.

In the luminal epithelium, however, the staining was significantly less during oestrus, 1 and 3 days after ovulation and 1 day after the administration of progesterone than when compared to ≥ 5 days after ovulation and 5 days after progesterone administration ($P < 0.05$ in all cases).
In the basal epithelium the staining increased significantly under the dominance of progesterone for several days, namely ≥ 5 days after ovulation and ≥ 3 days after progesterone treatment. However, the statistical analysis on the intensity of the staining should be interpreted with caution because there is a bias towards positive staining and the interpretation of the intensity of the staining is subjective.

Discussion

Morphology

The endometrial morphology of the mare has been described by several authors. For example, both Kenney (1978) and Keenan (1987) evaluated normal endometrial morphology in the cyclic mare, with the latter author providing a detailed morphometric study of the epithelium and gland structure of the endometrium in relation to days post-ovulation. The effects of exogenous progesterone on endometrial architecture in acyclic mares have also been investigated (Hamer *et al*. 1985).
The present study was conducted to see if and how the different morphological structures of the endometrium change under the dominance of both endogenous and exogenous progesterone and how long this takes. The results indicated that the changes in endometrial morphology seen after treatment with exogenous progesterone were similar to those observed after ovulation (i.e. during dioestrus) and concur with those previously noted by Hamer *et al*. (1985). Changes were most noticeable in the glandular epithelium and after 3 days of either exogenous progesterone treatment or endogenous progesterone following ovulation the epithelium in the superficial portion of the glands had increased significantly in height from that observed during oestrus. Likewise, the epithelium lining the basal part of the glands increased significantly in height after 3 days of exogenous progesterone. However, in those mares ovulating naturally, it was not until 5 days after ovulation, and the influence of endogenous progesterone from the corpus luteum, that a significant heightening of the basal epithelial cells was apparent.

The difference observed in the response of the basal epithelium to exogenous versus endogenous progesterone could have been influenced by several factors. Firstly, and likely the major factor, was the slower rise in circulating progesterone levels between the two groups after treatment or ovulation. Although no significant differences were observed in the mean progesterone serum concentrations between groups on day 1 (Table 5) in all likelihood levels of progesterone in the treated animals would have risen almost immediately after treatment and, hence, in effect by the end of day 0 (start of treatment). Unfortunately, in retrospect, jugular blood samples were taken prior to injection of the progesterone on the first day of treatment (day 0) and the mares were not re-sampled later on the first treatment day.

Table 5: Serum progesterone concentration at the moment of biopsy.

		Post ovulation	Post treatment
Day 0	(ng/ml)	0.4 (0.2 – 0,5)[a, b]	0.4 (0.2 – 0.5)
Day 1	(ng/ml)	0.9 (0.6 – 1.4)[c, d]	1.6 (1.2 – 1.9)
Day 3	(ng/ml)	3.6 (2.7 – 4.5)	3.2 (2.1 – 10.9)
Day 5	(ng/ml)	6.2 (5.2 – 9.4)[a, c]	3.5 (3.1 – 4.6)
Day 8	(ng/ml)	8.3 (7.4 – 16.0)[b, d]	-

Results are presented as median and range of the data
Day 0 refers to oestrus, Day 1 refers to either Ov + 1 or P_4 + 1, etc.
[a-d] Significant differences between groups are indicated by different letter superscripts (at least $P<0.05$)

In addition, the earlier increase in epithelial height in the basal portion of the glands after exogenous progesterone treatment compared to the naturally ovulating mares could have also been influenced by the fact that the ovulating animals were returned to oestrus by administration of a prostaglandin F2α analogue after biopsy and therefore never underwent a normal dioestrus length and a spontaneous return to oestrus. Time constraints prevented mares being rested between sampled cycles.

There is also the possibility that the use of exogenous progesterone induces atypical changes in the architecture and the function of the endometrium compared to endogenous progesterone, although this has not been thoroughly investigated.

When compared to previous published morphometric analysis of the endometrium the results from this present study did not concur entirely. For example, the height of the epithelium in both the luminal and basal portions of the glands during oestrus were marginally lower when compared to data obtained by Kenney (1978). This might be due to the low number of animals sampled in this study (n = 5) or to the timing of the biopsy, since Kenney (1978) reported that the height of the epithelium can vary between 15 – 30µm and sometimes even 50µm during oestrus. During dioestrus there was no difference between the findings of this study and the data presented by Kenney (1978). Although direct comparisons are difficult, since Kenney (1978) took the first 7 days after ovulation as a whole and Keenan *et al*. (1987) grouped dioestrus measurements as either between 1 – 5 or 6 – 9 days after ovulation, the present study established lower values for the height of the luminal epithelium in the first 5 days after ovulation, but marginally higher values for days 6 – 9 after ovulation. Measurement of the endometrial epithelium after the administration of exogenous progesterone was performed by Hamer *et al*. (1985). The average height is similar to that measured in this study, although Hamer *et al*. (1985) did not differentiate between the luminal and the glandular epithelium and obtained his data 22 days after the administration of the progesterone.

The only author to report on the luminal diameter of the glands was Keenan *et al*. (1987). His values were greater than those obtained in this present study, most likely because he measured both lumen and epithelial cells lining the lumen as a whole.

Variations in the histomorphometric findings between this study and those of other authors could be accounted for by the variation in fixative used for the tissue under examination. Fixation in formal saline, as used in the present study, has been shown to cause less tissue shrinkage than Bouin's fixative (Gerstenberg, 1994) which was used in the studies undertaken

by Kenney (1978) and Keenan *et al*. (1987). Differences in the location of the biopsy site are not likely to account for any observed differences, since both the height of the epithelium and the distribution of the glands are reported to be uniformly spread through the uterus (Bergman and Kenney, 1975; Ricketts, 1975; Blanchard *et al*. 2003; Lefranc and Allen, 2007[a]).

Stereology

The surface density of endometrial glands in the mare has been described by several authors. Ginther (1992), Kenney (1978) and Keenan *et al*. (1987) all discussed gland density during different stages of the oestrous cycle and Lefranc and Allen (2007[a]) measured and compared the surface density of the endometrial glands in Welsh Pony and Thoroughbred mares. However, while all of these previous studies measured the changes in Sv-values after normal ovulation, none of them examined the effects of treatment with exogenous progesterone.

In the present study the surface density of equine endometrial glands was measured to determine the effect of both endogenous and exogenous progesterone on the secretory surface and the surface area underneath the epithelial cells, i.e. the outer border of the glands. The results indicated that gland surface density does not increase after either ovulation or treatment with exogenous progesterone. An increase in gland surface density seen in the outer border of the superficial portions of the endometrial glands after 3 days of progesterone treatment could not be explained, as no such increase could be detected after 5 days of progesterone treatment, or at 3, 5 and 8 days after ovulation.
The results did not concur with those of Ginther (1992), Kenney (1978), Keenan *et al*. (1987) and Lefranc and Allen (2007[a]). All these authors noted that, as dioestrus advanced, gland density (Ginther, 1992; Kenney, 1978; Keenan *et al*. 1987) and gland surface density (Lefranc and Allen, 2007[a]) increased as a result of increasing tortuosity and branching of the glands under the influence of luteal progesterone. Lefranc and Allen (2007[a]), however, noted a significant increase in gland density only after 8 days post ovulation, while Keenan *et al*. (1987) could only detect an increase in gland density at 12 – 14 days after ovulation. Furthermore, the Sv-values reported by Lefranc and Allen (2007[a]) were appreciably higher than those found in the present study, despite the fact that both studies measured the secretory surface area of the endometrial glands.

The differences in gland surface density between the present study and that of Lefranc and Allen (2007[a]) could have been due to several factors.
Firstly, the biopsies examined in the present study came from a mixture of Pony and Thoroughbred mares and Lefranc and Allen (2007[a]) have demonstrated a lower endometrial gland surface density in Pony than in Thoroughbred mares. Nevertheless, this breed difference cannot explain the apparent lack of any increase in gland surface density during the cycle, since Lefranc and Allen (2007[a]) noted that Sv-values in both groups of equids increased after ovulation.
Secondly, the higher values found by Lefranc and Allen (2007[a]) could have stemmed from their use of Bouins' fluid as the tissue fixative, which is well known to cause more shrinkage of the tissue (Gerstenberg, 1994) than the formal saline fixative used in the present study.

Thirdly, although both studies employed the same 3 dimensional computer assisted morphometric analysis technique to obtain the data, Lefranc and Allen (2007[a]) employed 5 times as many test points per biopsy than the figure of 200 per biopsy recommended by Gundersen and Jensen (1987) and used in the present study.
Nevertheless, despite these minor methodological differences the results of the present study contradict those of Lefranc and Allen (2007[a]). Namely, no increase in endometrial gland surface density occurred in mares after ovulation or after treatment with exogenous progesterone.

Immunohistochemistry

Uterocalin, a 19 kDa protein, is secreted by the epithelial cells that line the endometrial glands and the lumen of the uterus of mares and other equids. Stewart *et al*. (1995, 2000[a]) and Crossett *et al*. (1996, 1998) evaluated the appearance, persistence and disappearance of uterocalin during the oestrous cycle and pregnancy and although they observed a decline in secretion rate from about 16 days post ovulation in the cycling mare, and from day 20-23 in pregnancy, they remained unsure of the time of its first appearance after ovulation.

The present study was performed to determine more accurately the time of first appearance of uterocalin in the endometrium after normal ovulation and after treatment with exogenous progesterone. The results indicated that uterocalin secretion is equivalently initiated by both endogenous and exogenous progesterone after about 5 days. This is in contrast to the results reported by Crossett *et al*. (1998), who observed faint positive staining for uterocalin in the glandular epithelium as early as 2 days after ovulation. In the present study two of the biopsies taken on the first day after ovulation also showed very light staining for uterocalin in the glandular epithelium, but in the other 3 biopsies taken at the same early stage of the cycle no uterocalin was detected.
The present results confirm other findings of Crossett *et al*. (1998), that the intensity of staining increases with the advancement of dioestrus. This was also reported by Stewart *et al*. (1995), who noted a peak of uterocalin secretion between days 12 and 14 after normal ovulation in non-pregnant mares. Together, these findings indicate that the ability of the endometrium to secrete uterocalin in response to rising progesterone levels is a gradual process, with quite marked individual differences between mares.
In the present study secretion of uterocalin could be initiated by the administration of exogenous progesterone for several days. Previously, Stewart *et al*. (1995) had concluded that uterocalin secretion is progesterone dependent, when they reported a strong association between uterocalin secretion and peripheral serum progesterone profiles.

In the lumenae of the basal portions of the endometrial glands uterocalin was seen during oestrus and on the first day after ovulation. This positively staining material is unlikely to be newly secreted uterocalin since serum progesterone levels are low during oestrus and mild secretory activity of the epithelium in the basal parts of the glands only became apparent at 3 days post ovulation. Stewart *et al*. (2000[a]) reported similar findings and concluded that this material was likely to be residual secretion which had not been completely expelled from the glands during the previous dioestrus. Furthermore, some of the biopsies used in the present

study contained so called ‘gland nests’ (Kenney and Doig, 1986). In these fibrosed portions of the glands the intensity of staining for uterocalin varied greatly during the oestrous cycle and showed a very different pattern from the non-fibrosed glands. This corresponds with the findings of Stewart *et al*. (2000[a]), who suggested that these unusual staining patterns in gland nests could be due to either a lack of synthesis or an impairment of the secretory mechanism of the damaged glands. They concluded that both reasons may contribute to reduced fertility in ageing mares.

Conclusions

The aims of the project were to assess and compare the changes in morphology and gland surface density and expression of uterocalin in the endometrium of the mare under the dominance of a post-ovulation rise in progesterone and after the administration of exogenous progesterone during oestrus.

Morphologically the height of the glandular epithelium in the superficial portions of the endometrial glands increased under the dominance of both endogenous and exogenous progesterone after 3 days. The basal epithelium lining the glands showed an increase in height at day 5 after ovulation, but already at day 3 after the start of the exogenous progesterone treatment. These results lead to the conclusion that the morphological changes in the endometrium are progesterone dependent and are similar for both endogenous and exogenous progesterone.

No increase in endometrial gland surface density was noted with advancing dioestrus, or after the administration of exogenous progesterone. Thus, it was concluded that surface density of the glands is either not influenced by progesterone, or more days of progesterone dominance are needed to induce changes.

Uterocalin secretion was most evident in the luminal epithelium and in the epithelium lining the basal portions of the endometrial glands. Although the staining density increased gradually after both ovulation and progesterone treatment, it took 5 days of progesterone dominance before staining for uterocalin became significant. Thus, when the young equine embryo arrives in the uterine lumen on day 6 after ovulation, there is already a significant level of secretion of uterocalin by the endometrial glands.

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Finally I want to thank everyone who worked at the former Equine Fertility Unit during my 3 months stay there. Their help and support made my stay in Newmarket truly unforgettable.

Attachment I

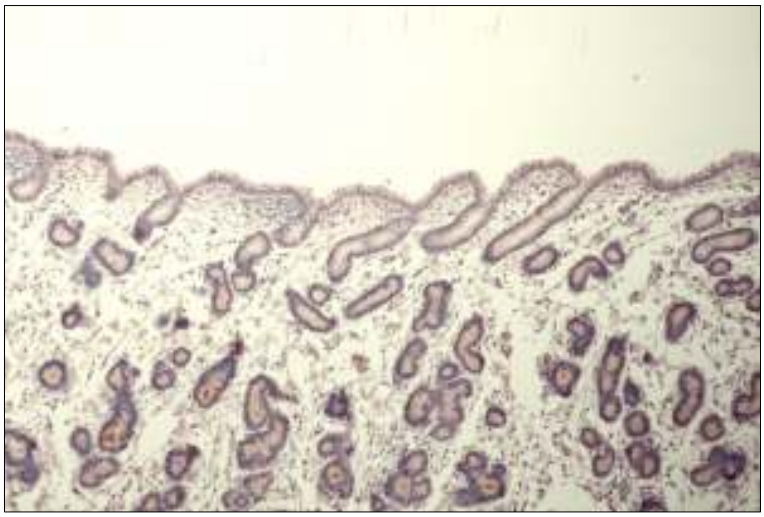

Figure 1. Oestrus

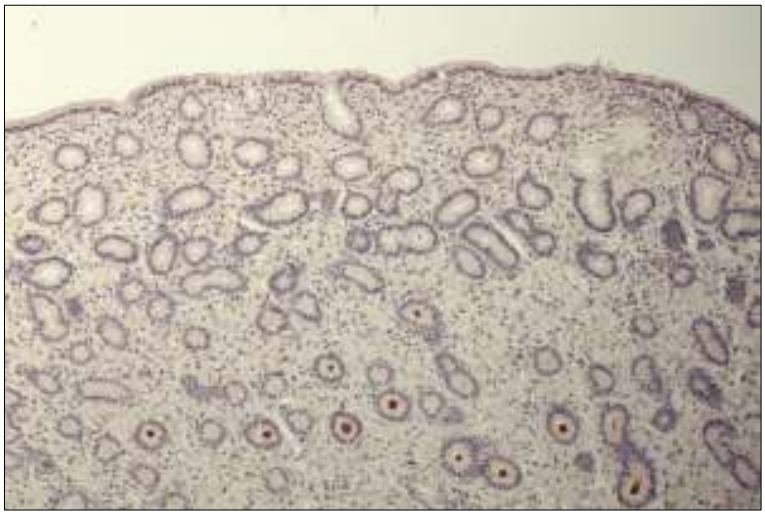

Figure 2. Ov + 1

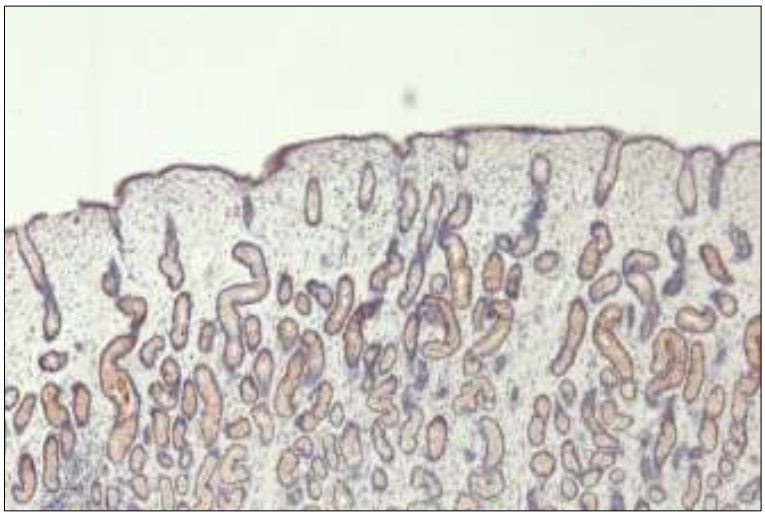

Figure 3. Ov + 3

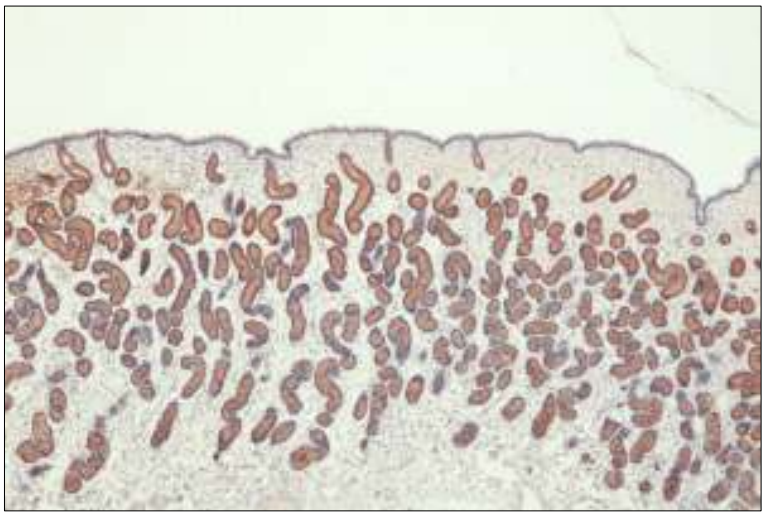

Figure 4. Ov + 5

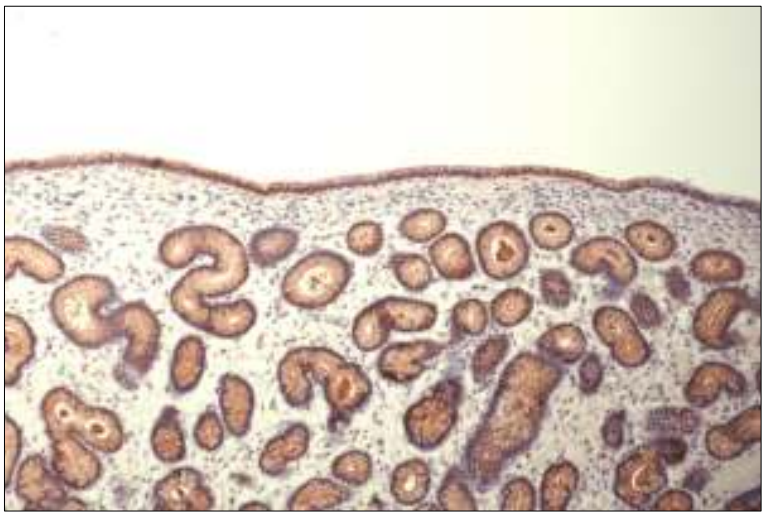

Figure 5. Ov + 8

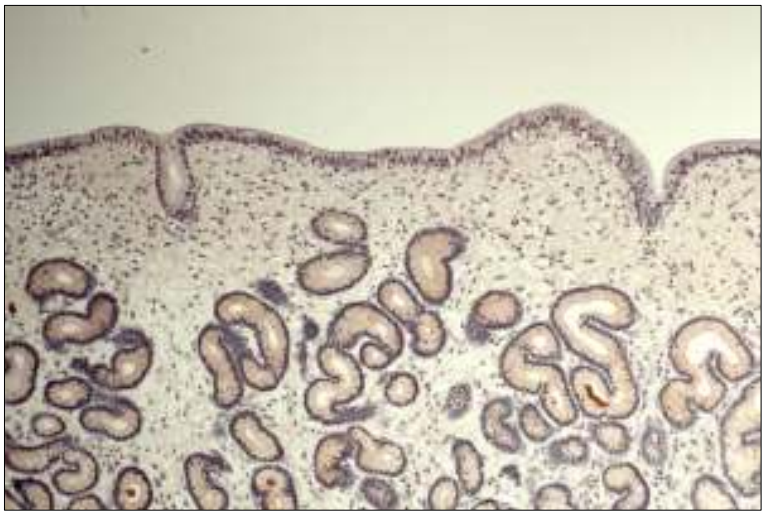

Figure 6. P4 + 1

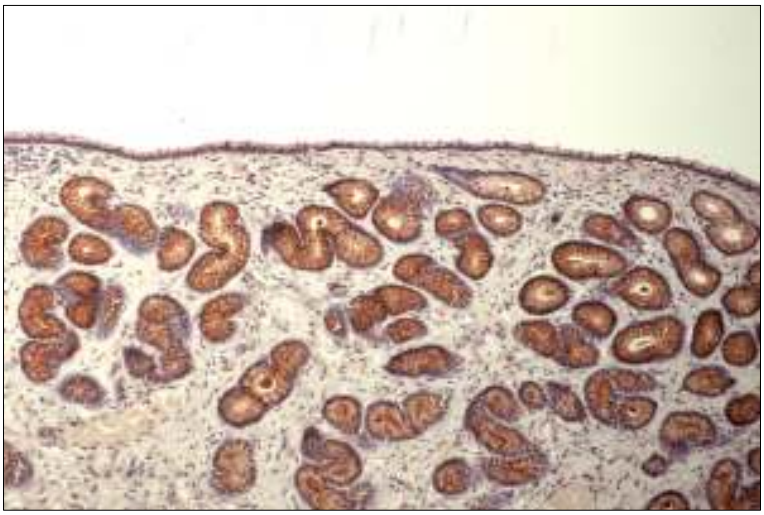

Figure 7. P4 + 3

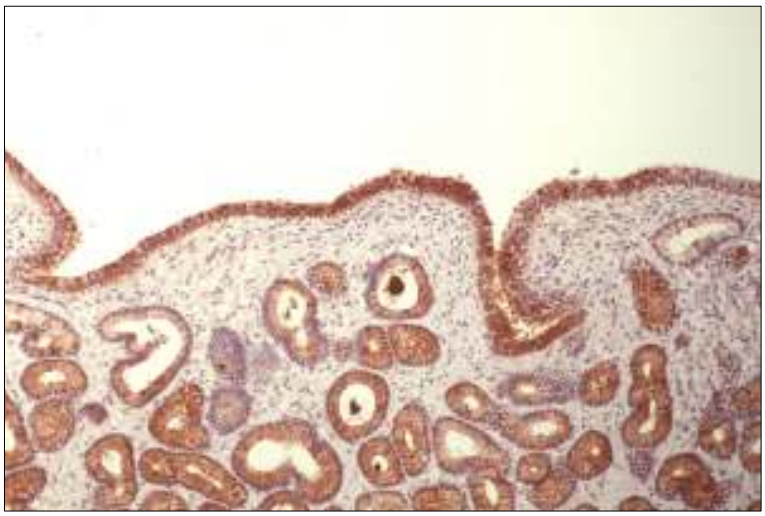

Figure 8. P4 + 5

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