

Ultrastructural morphometric analysis of the uterine epithelium during early pregnancy in the sheep*

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Summary. Stereological techniques were used to quantify ultrastructural changes in the caruncular epithelium during the pre- (Day 13), peri- (Day 16) and post- (Days 19 and 22) attachment periods of placentation. Tissues from Day-13 non-pregnant ewes were used as controls. Uteri for stereological evaluation were perfused via the uterine artery with 3% glutaraldehyde and separated into proximal, middle and distal regions. Tissues from caruncular areas were processed for electron microscopy. Volume fractions (V_v) of nuclei, mitochondria, lipid and cytoplasmic granules were estimated by point-counting volumetry. Surface areas per unit tissue volume (S_v) of mitochondrial membranes and cristae, Golgi, plasmalemma, endoplasmic reticulum and nuclear membranes were estimated by line-intersection counting.

The only significant difference between pregnant and non-pregnant uterine epithelium at Day 13, a time before attachment, was a lower S_v of smooth endoplasmic reticulum (SER) in tissue from pregnant ewes. This value returned to control (non-pregnant Day 13) levels at Day 16, and was again significantly reduced at Days 19 and 22. The V_v of lipid decreased significantly at Day 16 and remained at reduced levels thereafter. These changes may reflect the effects of conceptus products on lipid storage and mobilization. The S_v of rough endoplasmic reticulum (RER) significantly increased on Day 16 of gestation, and remained elevated on Day 19. These results may reflect increased synthesis of protein for export at these times. In general, several of the values measured which may be indicative of cellular metabolism were reduced at Day 22 of pregnancy, perhaps suggesting diminished metabolism by the uterine epithelium after attachment of the trophoblast.

Large cytoplasmic granules were not observed in uterine epithelium at the pre- and early peri-attachment stage (Days 13 and 16); they were first observed at Day 19 and their V_v increased dramatically at Day 22. At this time, the S_v of uterine cellular organelles associated with protein synthesis (Golgi complex, RER, SER) was either unaltered or declining. These results support the concept that the large secretory granules may be derived from an extrauterine epithelial source such as the trophoblast.

Keywords: sheep; early pregnancy; uterine epithelium; ultrastructure; stereology

Introduction

The maintenance of early pregnancy is dependent upon fetal–maternal interactions which result in altered metabolism of the gravid uterus when compared to uterine metabolism during the oestrous cycle. These alterations include diminished episodic release of prostaglandin (PG) F-2 α , changes in neutral lipid content of endometrial epithelial cells (Boshier *et al.*, 1987) and increased protein synthesis by the uterus (Findlay, 1981). Morphological studies have provided comparisons of

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gravid and non-pregnant sheep uteri (Boshier, 1969; Guillomot *et al.*, 1981). No differences were observed before Day 14. At this time, gravid uteri were characterized by folded surfaces and depressed centres of caruncles. Epithelial cells exhibited rounded, irregularly shaped microvilli (Guillomot *et al.*, 1981). On Day 16 uterine epithelial cell modifications included cell death which was associated with nuclear swelling, vacuolation, and disintegration (Boshier, 1969). Definitive attachment of the conceptus occurred by interdigitation of trophoblast microvilli and uterine epithelium at Day 18 of gestation (Boshier, 1969; King *et al.*, 1982).

Formation of syncytial masses in the uterine epithelium was observed at the time of implantation and has been suggested to be the result of trophoblast binucleate cell migration and fusion with uterine epithelial cells (Amoroso, 1952; Wooding, 1984).

The objectives of the present study were to use stereological techniques to quantitate ultra-structural changes in the caruncular epithelium of sheep during the pre- (Day 13), peri- (Day 16) and post- (Days 19 and 22) attachment periods of placentation and, when possible, correlate the observed temporal structural alterations with known changes in uterine metabolism during early pregnancy.

Materials and Methods

Uterine tissue was obtained from crossbred ewes (Finn, Suffolk, Dorset or Hampshire breeding). The ewes were examined daily for signs of oestrus with vasectomized rams, and at oestrus (Day 0) the ewes were mated to intact rams. On each of Days 13, 16, 19 and 22 of pregnancy 3 ewes were used and 3 control ewes were used on Day 13 of the oestrous cycle. Day 13 of the oestrous cycle was chosen as a control because hormone profiles are not different from those of Day-13 pregnant sheep and are also similar to those of sheep at Days 16, 19 and 22 of pregnancy.

Maternal tissue was collected by the following surgical procedure. Ewes were anaesthetized with chloral hydrate (88 mg/kg) and the reproductive tract was exposed by a midventral abdominal incision. Uteri from all animals were perfused *in situ* through the uterine artery with 50 ml 3% glutaraldehyde in 0.1 M-cacodylate buffer, pH 7.4 delivered over a period of 3 min. Immediately after uterine fixation, animals were killed with an i.v. injection of 10 ml T-61, Euthanasia Solution (Hoechst-Roussel Agri-Vet Company) and the reproductive tract removed before secondary fixation.

For transmission electron microscopy, uterine horns were cut with a razor blade into proximal, middle and distal regions and the tissue was placed in fixative (3% glutaraldehyde in 0.1 M-cacodylate buffer, pH 7.4). Subsections of 2 mm that contained the uterine epithelium-trophoblast interface were dissected from the caruncles. Tissues were post-fixed in 1% osmium in 0.1 M-cacodylate buffer, dehydrated in an alcohol series and embedded in Epon following a standard processing schedule (Glauert, 1975). Thick sections (2 μ m) were cut from randomly selected blocks and stained with methylene blue-azure II before thin sectioning to determine the location of uterine epithelium and trophoblast attachment. Ultrathin sections (50–80 nm) were cut using an LKB-IV ultra microtome. Areas chosen for thin sectioning included maternal epithelium and its junction to trophoblastic cells. Sections were mounted on copper grids and stained with 2% uranyl acetate and lead citrate (Venable & Coggeshall, 1965). Tissues were examined using a Philips 201 transmission electron microscope operated at 60 kV. Micrographs of uterine epithelium used for volume fraction (V_v) measurements of nucleus and lipid were made at $\times 2000$ magnification. Micrographs for V_v of cytoplasmic granules and mitochondria and for surface area per unit tissue volume (S_v) measurements of mitochondrial membranes and cristae, Golgi complex, plasmalemma, rough and smooth endoplasmic reticulum and nuclear membranes were made at $\times 15000$ magnification.

The V_v values of the nucleus, mitochondria, lipid droplets and cytoplasmic granules were estimated by point-counting volumetry. Data were collected by randomly placing a grid of equidistant lines over the sections of uterine epithelial tissue. Line intersections were regarded as sampling points, each of which represented a specific area surrounding the point. The sampling area of each point was determined by the point spacing and magnification (Fig. 1). The point fraction $P(p)$ of each cellular component was estimated as the number of points falling on that component $P(a)$ divided by the total number of points falling on all components $P(t)$. Point fraction has been shown to be equivalent to the area density $A(a)$, which in turn, has been shown to represent an unbiased estimate of the volume fraction (V_v) (Weibel, 1979a).

S_v expresses the relative surface area of cellular components contained within a given tissue volume (Weibel, 1979a). S_v measured in this study included mitochondrial membranes and cristae, Golgi complex, endoplasmic reticulum and nuclear membranes. S_v was estimated by the line-intersection method. A grid of equidistant lines was placed over the cell profiles in each tissue section (Fig. 1). The line intersections with the selected membranes were counted and used as data points. S_v was then calculated using the following formula (Tomkeieff, 1945),

$$S_v = 2I$$

where S_v = surface/unit volume and $2I$ = twice the number of intersections/length of line.

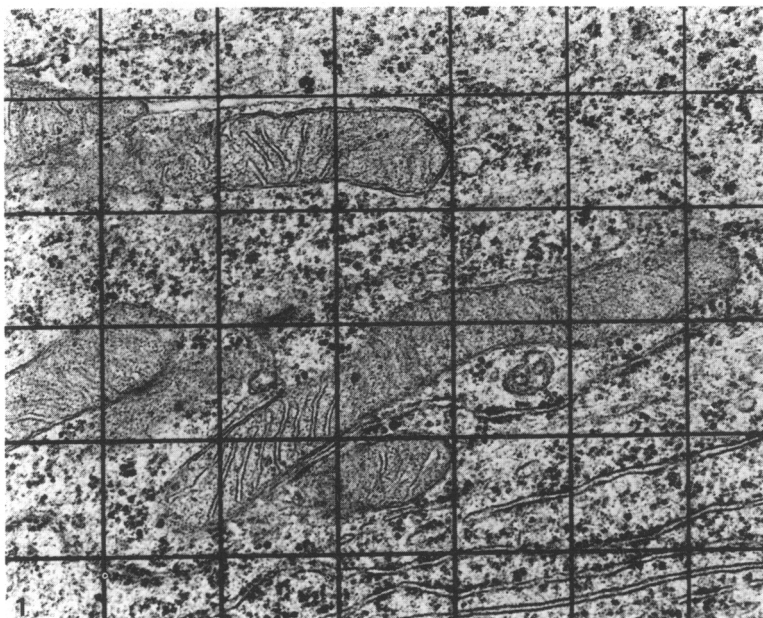


Fig. 1. Photomicrograph of equidistant lines over a tissue section used for stereological evaluation.

One-way analysis of variance was used to test differences in mean V_v and S_v among uterine regions per animal, between animals and between gestational groups. Differences among organelle means were computed using orthogonal contrasts (Ostle & Mensing, 1975).

To achieve a standard error of <10% of the mean a preliminary study was performed. The results of this study determined the number of samples to be taken from each region and the number of micrographs to be taken from each sample (Weibel, 1979b). It was concluded that 3 samples from each region would be processed for electron microscopy and 15 micrographs would be taken from each sample. The total number of electron micrographs used in this study was 2025.

Results

The appearance of the smooth and rough endoplasmic reticulum and Golgi complex was similar between all treatment groups. Therefore, they will be described together. The smooth endoplasmic reticulum contained a dilated lumen and numerous transport vesicles were associated with them. Rough endoplasmic reticulum also contained a distended lumen and was associated with an abundance of ribosomes and polyribosomes. The Golgi complex was well developed and was also associated with numerous transport vesicles.

No gross anatomical differences between Day 13 of the oestrous cycle (not shown) and Day 13 (Fig. 2) of gestation were observed, therefore they will be described together. The cells of the epithelial layer appeared columnar with many microvilli, which were short and regularly distributed. Nuclei were basally located, with chromatin uniformly dispensed and containing prominent nucleoli. Lipid droplets were numerous and randomly distributed while mitochondria were randomly located in the cell. Crystalline bodies were also present in some sections of epithelium on Day 13 of gestation.

Uterine epithelium of Day-16 pregnant ewes was columnar and lateral separations between cells were observed (Fig. 3). This separation was probably not fixation or processing artefact, since lateral cell separation did not occur in tissue prepared from Day 13 of gestation. Microvilli were longer, stood upright and were regularly distributed. Nuclei contained uniformly dispersed

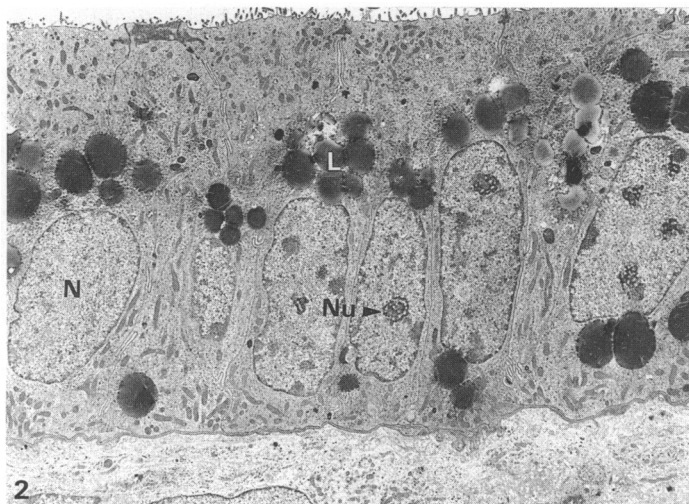


Fig. 2. Day 13. No gross morphological differences were observed between gravid and non-gravid uterine epithelium. Note columnar uterine epithelium, with nuclei (N) containing uniformly dispersed chromatin, prominent nucleoli (Nu), and numerous lipid droplets (L). $\times 3000$.

chromatin with distinct, prominent nucleoli. Lipid droplets were less numerous than at Day 13 and mitochondria were randomly distributed throughout the epithelial cells.

At Day 19 of gestation, different sections of caruncular epithelium contained areas where trophoblast was attached (Fig. 4) and other areas where it was not attached. In areas where attachment had not occurred uterine epithelium had morphology similar to that described at Day 16. Lateral separations between cells were apparent, crystalline bodies were present in some cells and the number of lipid droplets appeared to be diminishing progressively. Distinct differences in uterine epithelial morphology were observed in sections where trophoblast was attached. Here, uterine cells appeared to be in a progressive reorganization from the columnar morphology observed on Day 16 to a thin, flattened and often syncytial morphology observed at Day 22. The epithelium was less organized and distinct cell boundaries were difficult to discern in some sections. Cytoplasmic granules were observed only in cells attached to trophoblast. Cytoplasmic granules were round, densely stained and variable in size. Lipid droplets appeared less abundant and microvilli were regularly arranged and longer than those found on previous days.

On Day 22, the epithelial layer attached to the trophoblastic layer was extremely thin, and was in close proximity to maternal blood vessels (Fig. 5). Cytoplasmic granules appeared more numerous, although they were not present in all sections. In some sections cell boundaries were indistinguishable and the epithelial layer appeared as a multinucleated syncytium. Caruncular epithelium not connected to trophoblast was low columnar with a majority of nuclei located basally.

Mean V_v values for nuclei, lipid droplets, mitochondria and cytoplasmic granules for the various treatment groups are shown in Table 1. The mean V_v values of nuclei at Day 13 of the oestrous cycle and Day 13 of gestation were not significantly different. However, a significant decrease in nuclear V_v occurred between Days 13 and 16 of gestation. The mean V_v values for nuclei were similar at Days 16, 19 and 22 of gestation. The V_v of lipid was not significantly different between Day 13 of the cycle and Day 13 of gestation. A significant decrease was found between Day 13 and Days 16, 19 and 22 of gestation. Cytoplasmic granules were not observed before Day 19 of gestation. Hence their V_v was not different from zero on Days 13 and 16 and increased significantly on Days 19 and 22. Mitochondrial mean V_v values were similar among all treatment groups.

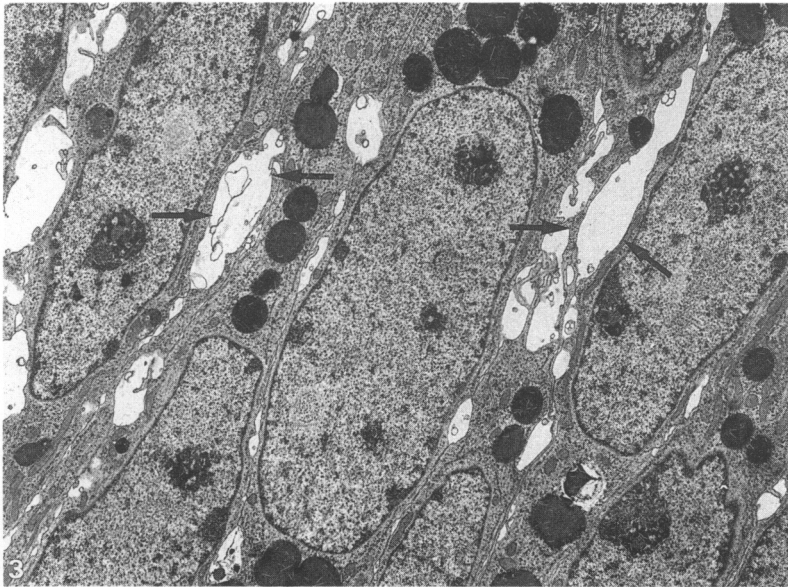


Fig. 3. Day 16 of gestation. Columnar uterine epithelium showing lateral cell separation (arrows). $\times 5000$.

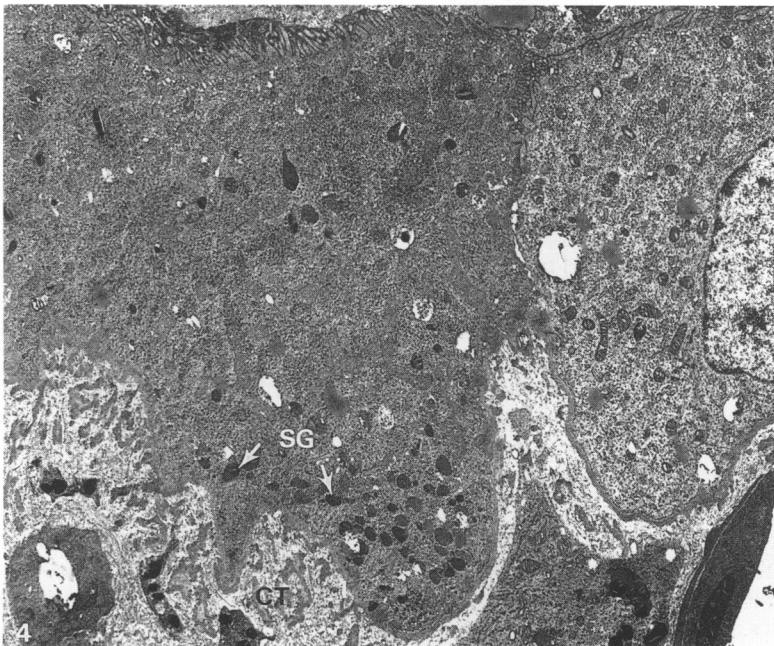


Fig. 4. Day 19 of gestation. Uterine epithelium attached to trophoblast. Note presence of cytoplasmic granules (SG) near maternal connective tissue (CT). $\times 5000$.

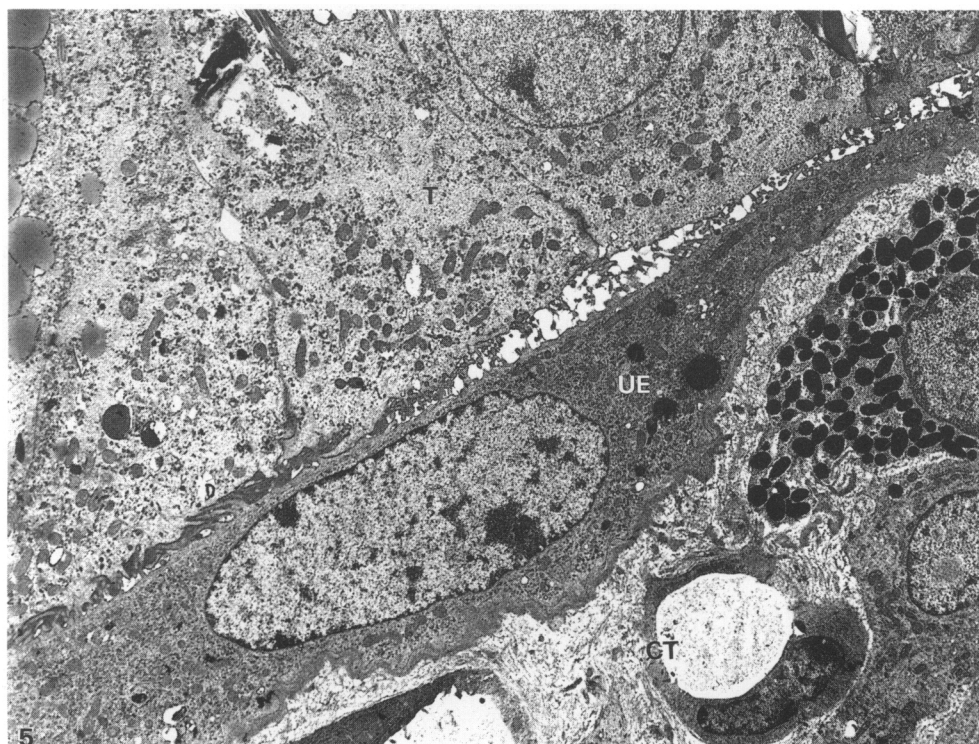


Fig. 5. Day 22 of gestation. Uterine epithelium (UE) and attached trophoblast (T). Note thin uterine epithelial layer with trophoblast in close proximity to maternal connective tissue (CT). $\times 5000$.

Table 1. Mean volume fractions* of cellular organelles in sheep endometrium epithelial cells during early pregnancy

Parameter	Day 13 of oestrous cycle	Day of pregnancy			
		13	16	19	22
Nucleus	26.42 (1.65) ^a	23.48 (1.23) ^a	15.94 (1.82) ^b	16.52 (0.87) ^b	13.49 (2.28) ^b
Lipid	10.12 (0.93) ^a	13.13 (2.46) ^a	5.50 (0.92) ^b	3.32 (1.62) ^b	6.95 (2.45) ^b
Cytoplasmic granules	0.0 (0.0) ^a	0.0 (0.0) ^a	0.0 (0.0) ^a	0.12 (0.051) ^b	0.56 (0.26) ^c
Mitochondria	5.82 (0.40) ^a	5.19 (0.72) ^a	5.52 (0.25) ^a	5.30 (0.44) ^a	3.89 (0.48) ^a

^{a,b,c}Means of volume fractions with different superscripts are significantly different ($P < 0.05$).

*Volume fraction expressed as % (standard error).

Average S_v values of membrane cell components are listed in Table 2. S_v values for mitochondrial cristae were similar among all treatment groups. Values for the outer mitochondrial membrane were similar on Day 13 of the cycle and Days 13–19 of gestation, and significantly decreased on Day 22. Nuclear membrane S_v was similar on Day 13 of the cycle and gestation followed by a significant decrease at Days 16 through 22. The mean S_v for plasmalemma was similar on Day 13 of the cycle and on Days 13 and 16 of gestation, but values on Days 19 and 22

were significantly lower. Rough endoplasmic reticulum (RER) S_v was significantly greater on Days 16 and 19 of gestation. On Day 22, the S_v value of RER decreased to pre-attachment (Day 13) levels. Mean S_v of SER was significantly reduced on Day 13 of gestation. The S_v was increased at Day 13 and 16 of gestation and decreased at Day 16 and Days 19 and 22. Surface per unit volume of Golgi complex was not significantly different between treatment groups.

Table 2. Mean surface area per unit volume* ($\mu\text{m}^2/\mu\text{m}^3$) of cellular organelles in sheep endometrium epithelial cells during early pregnancy

Parameter	Day 13 of oestrous cycle	Day of pregnancy			
		13	16	19	22
Outer mitochondrial membrane	1.51 (0.11) ^a	1.41 (0.064) ^a	1.49 (0.067) ^a	1.31 (0.12) ^a	1.01 (0.10) ^b
Mitochondrial cristae	0.93 (0.084) ^a	0.81 (0.061) ^a	1.16 (0.29) ^a	0.74 (0.089) ^a	0.68 (0.090) ^a
Plasmalemma	0.99 (0.049) ^a	0.94 (0.060) ^a	1.01 (0.090) ^a	0.76 (0.097) ^b	0.74 (0.067) ^b
Golgi complex	0.063 (0.011) ^a	0.039 (0.012) ^a	0.076 (0.019) ^a	0.073 (0.029) ^a	0.10 (0.25) ^a
Nuclear membrane	0.49 (0.022) ^a	0.48 (0.034) ^a	0.33 (0.018) ^b	0.32 (0.020) ^b	0.29 (0.029) ^b
Rough endoplasmic reticulum	0.22 (0.023) ^a	0.33 (0.076) ^a	0.56 (0.10) ^b	0.52 (0.11) ^b	0.33 (0.67) ^a
Smooth endoplasmic reticulum	0.23 (0.049) ^a	0.057 (0.018) ^b	0.24 (0.028) ^a	0.047 (0.023) ^b	0.022 (0.0081) ^b

^{a,b}Means of surface area per unit volume with different superscripts are significantly different ($P < 0.05$).

*Surface area per unit volume expressed as % (standard error).

Discussion

The ultrastructure of the sheep uterine epithelium during early pregnancy has been the subject of several previous reports (Davis & Wimsatt, 1966; Boshier, 1969; Guillomot *et al.*, 1981; Wooding, 1984). The present study is the first to utilize quantitative morphometry to analyse and compare alterations in subcellular components and organelles of the caruncular epithelium in tissue obtained during the luteal phase of the oestrous cycle, and the pre-, peri- and post-attachment periods of placentation.

The only statistically significant difference observed between epithelium from pregnant and non-pregnant animals at Day 13 was decreased mean S_v values of SER in the former. Values were also lower in tissue from pregnant animals on Days 19 and 22 but not Day 16. The physiological significance of these observations is unclear. Smooth endoplasmic reticulum plays a major role in lipid metabolism and houses enzymes for steroidogenesis. We are aware of no physiological events that would correlate with the observed anatomical alterations in SER values. The results probably do not reflect alterations in PGF-2 α metabolism since tissue concentrations of PGF-2 α are known to be higher in endometrium from pregnant animals during early pregnancy (Wilson *et al.*, 1972; Ellinwood *et al.*, 1979). Hence, one would expect higher rates of metabolism to be associated with increased but not decreased S_v values of SER.

Our studies demonstrated that the V_v of caruncular epithelial cells occupied by stored lipids was not different at Day 13 of pregnancy from Day 13 of the oestrous cycle but decreased significantly at Day 16 of pregnancy and remained low as pregnancy progressed (Days 16–22). These findings are in general agreement with those of Boshier *et al.* (1987), who demonstrated, by subjective evaluation, a significant decrease in the number of lipid droplets at Days 15–16 of pregnancy compared to that of Day 14–15 non-pregnant animals and a greater reduction in lipid droplets at Days 18–23.

Appearance and disappearance of neutral lipids during the oestrous cycle in uterine epithelium of rats, sheep and cattle have been well documented (Alden, 1947; Marinov & Lovell, 1968; Boshier & Holloway, 1973; Brinsfield & Hawk, 1973). It has been suggested that during the oestrous cycle progesterone facilitates lipid storage while oestrogen causes loss of neutral lipids via enhanced esterase activity (Boshier & Holloway, 1973; Boshier & Katz, 1975). It is clear from our study and those of others (Bassett *et al.*, 1969; Baird *et al.*, 1976; Boshier *et al.*, 1987) that loss of neutral lipids from the caruncular epithelium during early pregnancy is not a response to alterations in ovarian steroid production as seen during the oestrous cycle since plasma progesterone concentrations remain elevated and plasma oestrogen concentrations are low (Bassett *et al.*, 1969; Tsang, 1978). Our results support the contention expressed by Boshier *et al.*, (1987) that products from the expanded blastocyst result in the alteration of lipid metabolism in the caruncular epithelium.

Analysis of RER revealed that the mean S_v increased from Day 13 to 16 of pregnancy, remained elevated through Day 19 and declined afterwards. These results may be interpreted to suggest an increase in protein synthesis for export during the pre- and peri-attachment periods of placentation and decreased synthesis during the post-attachment periods. Our study provides morphometric evidence that supports the conclusion drawn from several biochemical studies that protein synthesis by ovine uterine epithelium is enhanced during the pre- and peri-attachment periods. Findlay (1981) demonstrated quantitative increase in the incorporation of radiolabelled leucine into cellular macromolecules *in vitro* by uterine epithelium from Day-15 pregnant ewes when compared to controls. Salamonsen *et al.* (1988) demonstrated an increased secretion of synthesized proteins from epithelial endometrial cells obtained from pregnant compared with non-pregnant animals at Day 13. Although we did not find significant differences in S_v of RER in endometrium from pregnant animals at Day 13, values were higher than those from non-pregnant animals and significant differences were observed in tissues taken from animals in the subsequent time period.

The stimulus for increased protein synthesis by endometrium from gravid uterus appears to be blastocyst secretions. Godkin *et al.* (1984) showed that ovine trophoblast protein 1 (OTP-1), a trophoblast interferon (Imakawa *et al.*, 1987) believed to be responsible for the maintenance of early pregnancy in the ewe, binds specifically to endometrium and stimulates synthesis and release of specific proteins by endometrial explants from non-pregnant animals. Subsequently, it was shown that blastocyst-conditioned medium, OTP-1 and recombinant human interferon α affected the release of specific proteins and PGE-2 and PGF-2 α from uterine epithelial cells (Salamonsen *et al.*, 1988). A significant reduction on the S_v occupied by RER was observed at Day 22 of pregnancy. This may be indicative of decreased synthesis of protein for export once placentation has been established.

Coincident with declining RER values, a dramatic increase in the presence of cytoplasmic granules was observed. One explanation for these apparently contradictory observations (i.e. reduced RER values but increased presence of cytoplasmic granules) may be that the cytoplasmic granules are produced from a cellular source other than the endometrial epithelium. This concept has been proposed by others (Assheton, 1906; Amoroso, 1952). In addition, Wooding (1984) provided convincing morphological evidence that trophoblast binucleate cells migrate into the maternal syncytium and release their granules by exocytosis into the maternal connective tissue. Additional morphometric evidence that supports the concept that syncytial cytoplasmic granules arise from a source outside the uterine epithelium is our observation that there was no change in the mean S_v of Golgi apparatus.

No significant differences in the V_v values for mitochondria or the S_v values for mitochondrial cristae and Golgi apparatus were observed between tissues from pregnant and non-pregnant animals or when the different time periods of pregnancy were compared. There are several possible explanations for these results, all of which are speculative. These include: (a) the method of analysis may not have been sufficiently sensitive to detect changes, (b) metabolic alterations in these organelles may not be reflected in alterations in morphology, and (c) the metabolic activity of these organelles may not change significantly during early pregnancy.

Reductions in values of S_v were observed in several organelles at Day 19 and especially Day 22. These included lower S_v values for plasmalemma, RER, SER and outer mitochondrial membrane. Lower V_v values for nuclei and lipid droplets were first noted at Day 16 and they remained low at Days 19 and 22. These results may indicate reduced metabolic activity of endometrium associated with syncytial formation or may be influenced by trophoblast contributions to the syncytium. It is understandable that uterine metabolic activity may decrease coincident with attachment when the conceptus is no longer free-living in the uterus and less dependent on histotroph for nutrition.

Supported by USDA Grant CRC 88-37242-4012. We thank Mrs Mary Cox for typing the manuscript.

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