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ULTRASTRUCTURAL MORPHOMETRIC ANALYSIS OF THE UTERINE EPITHELIUM DURING EARLY PREGNANCY

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Scott Edward Smith

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ABSTRACT

Stereologic techniques were used to quantify ultrastructural changes in ovine uterine epithelial cells in cyclic and early pregnant ewes. Three time periods were examined during early pregnancy. These included pre-attachment period (day 13), peri-attachment period (day 16) and post-attachment period (days 19 and 22). Tissue from day 13 nonpregnant ewes was used as a control. 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 secretory 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 statistical difference between pregnant and non-pregnant uterine epithelium at day 13, a time prior to attachment, was lower S_V values of smooth endoplasmic reticulum (SER) in pregnant ewes. The mean S_V of SER increased from day 13 of gestation to its highest value on day 16 of gestation followed by a significant decrease to its lowest value on day 22 of gestation.

These changes in SER may reflect alterations in SER activities.

Lipid droplet V_V was significantly decreased between days 13 and 16 of gestation. Implantation in the ewe does not occur until later in gestation. Lipid droplets, containing mainly triglycerides, may be used as a source of nutrition for the developing embryo. Utilization of the lipid droplets may be the reason for a significant reduction in the V_V of this component.

There was a significant increase between days 13 and 16 of gestation in the S_V of rough endoplasmic reticulum (RER), however the S_V of RER was significantly lower between days 19 and 22 of gestation, which may indicate a decrease in RER activity.

Qualitative results confirm previous data indicating secretory granules are present in the syncytial mass. In this study, secretory granules were not observed in uterine epithelium during the pre and peri-attachment periods but were observed during the post-attachment period. The volume fraction of secretory granules was significantly increased from zero between days 16 and 19 of gestation. Surface per unit volume of golgi complex did not differ significantly between treatment groups, suggesting that secretory granules may be from trophoblastic binucleate cells.

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1. INTRODUCTION

In ruminants, embryonic mortality is a major cause of reproductive failure. Estimates have ranged up to 30% in sheep (Hawk, 1979). Other reports indicate reproductive failures in first service heifers occur as a result of early embryonic death. In dairy cattle there is approximately 13% fertilization failure and about a 15% embryonic failure (Ayalon, 1978). Maintenance of early pregnancy is very important in the livestock industry. It is important for advancements in animal breeding technology, such as: in vitro fertilization, embryo manipulation, sexing and genetic engineering of embryos, since evaluation of these procedures are ultimately dependent upon the successful transfer and development of embryos.

Interactions between the maternal tissues and the developing conceptus are essential to the establishment and maintenance of pregnancy. The presence of the blastocyst in the uterus results in alteration in maternal endocrinology, immunology, metabolism, and uterine cell morphology. This phenomenon has been coined maternal recognition of pregnancy. Pregnancy failure occurs when the the embryo or maternal tissues fail to produce and/or respond to the appropriate signals.

In large domestic farm animals maternal recognition of pregnancy occurs before the trophoblast of the conceptus attaches to the maternal epithelium. It has been demonstrated in sheep by embryo transfer studies that if the conceptus must be present in the uterus by day 12-13 for the corpus luteum (CL) to remain functional and pregnancy be maintained (Moor, 1976). If the embryo is not in the uterus by the specific day mentioned above, then the uterus secretes a luteolytic hormone, prostaglandin (PGF2a) which causes the CL to regress (Goding, 1974). In sheep, it is believed, the conceptus prolongs the life of the CL by producing a factor(s) which may reduce the release of the luteolysin. One factor that has been identified and shown to be involved in this process is ovine trophoblast protein 1 (OTP-1) (Godkin et al., 1982; Godkin et al., 1984b; Vallet et al., 1987; Salamonsen et al., 1988).

Biochemical alterations occur in uterine epithelium as a result of fetal-maternal interactions. Among these biochemical alterations include the production of prostaglandin F2a and prostaglandin E2 (Findlay, 1981). Alterations also occur in uterine neutral lipid content in pregnant uterine epithelial cells (Boshier, 1987).

During early pregnancy, uterine protein synthesis is also increased, which may be due, in part, to trophoblastic secretions or increased uterine responsiveness to steroids (Findlay, 1981). Protein synthesis is greater in caruncular and intercaruncular tissue of day 15 pregnant ewes when compared to day 15 non-pregnant ewes (Findlay et al., 1981).

As a result of these fetal-maternal interactions, ultrastructural changes in the uterine epithelium have been described. Changes in the uterine epithelium do not occur until day 14 of gestation (Boshier, 1969; Guillomot et al., 1981). At this time, the caruncles are characterized by a folded surface, and a depressed center. Also appearing on epithelial cells are grossly rounded, irregularly shaped microvilli (Guillmont et al., 1981). On day 16 uterine epithelial cell modification includes cell death which is associated with nuclear swelling, vacuolation, and disintegration (Boshier, 1969). Interdigitation of trophoblast and uterine epithelium was observed on day 20 by Davis and Wimsatt (1966) but may occur on day 18 (Boshier, 1969; King et al., 1982).

Syncytial mass formation may be the result of trophoblast binucleate cell migration and fusion with uterine epithelial cells (Amoroso, 1952; Wooding, 1984) or as a result of strong acid phosphatase activity which occurs between the fetal and maternal tissue (Boshier, 1969).

In this study stereologic techniques were used to quantify ultrastructural changes in ovine caruncular epithelium during the pre- (day 13), peri-(day 16) and post attachment (days 19 and 22) periods of implantation. The objective was to relate observed morphological changes with known biochemical interactions which occur between the conceptus and uterine epithelium.

2. LITERATURE REVIEW

ESTROUS CYCLE OF THE EWE

Sheep are seasonally polyestrous animals in which reduced photoperiods induce, in part, reproductive cyclicity. The reproductive response is mediated by the pineal gland through the secretion of melatonin which codes for day length (for review of seasonality in the ewe see: Bittman, 1983; Karsh et al., 1986; English et al., 1986). The mechanism by which melatonin modulates gonadotropin secretion is not well understood. Episodic LH secretion initiate seasonal cyclicity in response to decreasing day lengths.

The length of the estrous cycle, in ewes, is 16-17 days with estrus lasting for 24-36 hours (review by Hulet and Shelton, 1980). The estrous cycle is divided into four periods; estrus, metestrus, diestrus and proestrus but it is best to describe the cycle in terms of ovarian function which occurs in 2 phases: follicular and luteal. The estrous cycle is controlled by follicle stimulating hormone (FSH), lutenizing hormone (LH), estrogen (E2), and progesterone (P4) interactions. Behavioral estrus is initiated when the level of progesterone declines, due to regression of the corpus luteum (CL) followed by a rapid rise in estrogen concentration. High levels of estrogen and a background of low progesterone, cause the ovulatory

surge of LH. At the time of behavioral estrus, LH and estrogen levels have already declined. Several studies, reviewed above, have increased our understanding of these biological processes (for review see Kaltenbach and Dunn, 1980).

Gonadotropin releasing hormone (GnRH) is released intermittently by the hypothalmic neurons in response to effects of ovarian steroids, estrogen and progesterone. Because of the intermittent release of GnRH the ovary is subjected to pulses of FSH and LH, which are released by the anterior pituitary as reviewed by (Karsch, 1984).

The steroidal effects on GnRH secretions have been defined as short-term negative feedback, positive feedback, and long-term negative feedback (Clarke, 1987). In short-term negative feedback, estrogen blocks the anterior pituitary response to GnRH. This was proven when ovariectomized ewes with disconnected hypothalmo-pituitary were given estrogen during pulsatile GnRH treatment. The results of the study demonstrated estrogen exerts its negative feedback at the pituitary (Clark and Cummins, 1984).

During the preovulatory LH surge, responsiveness of the pituitary gland to GnRH increases (Reeves et al., 1971). Due to the increase responsiveness of the pituitary to GnRH, LH concentrations increased 2-3 fold in plasma (Clark and Cummins, 1984). A rise in GnRH secretion is associated with the LH surge as studies in rats (Sarkar et al., 1976), monkeys (Neill et al., 1977), and women (Miyake et al., 1980) indicate. In sheep, the generation of the LH surge is due to a rise in estrogen secretion as studies by Goding et

al., (1969) indicate. It was concluded (Levine et al., 1985; Schillo et al., 1985; Clarke, 1987) that estrogen exerts a positive feedback effect on the pituitary making it more responsive to GnRH. It appears the ovulatory surge of LH stimulates proteolytic enzymes including collagenase which causes the breakdown of the follicular walls connective tissue (Kaltenbach and Dunn, 1980). Failure of the preovulatory surge of LH and FSH is a result of desensitization of the pituitary gland to GnRH (Kesner and Convey, 1982) and not to the depletion of LH and FSH content (Convey et al., 1981).

During the luteal phase of the estrous cycle both estrogen and progesterone exert a negative feedback on gonadotropin secretion (Goodman and Karsch, 1980; Martin et al., 1983). Goodman et al., (1981) used lower progesterone doses which had no effect on LH secretion. When given in combination with estrogen it was able to reduce pulse frequency. It can therefore be concluded from the studies described above that estrogen and progesterone are able to exert negative feedback effects on GnRH secretion.

Occuring also during the luteal phase is the process of folliculogenesis. Folliculogenesis involves three processes: recruitment, selection and dominancy (for review see Ireland, 1988). Recruitment is the period of time when follicles gain the ability to respond to gonadotropins (Goodman and Hodgen, 1983). The primary protein hormones involved in folliculogenesis are FSH and LH (Hisaw, 1947). Therefore, it is thought recruitment may develope during this stage of folliculogenesis (Ireland, 1987). The second period is the selection process when only a few recruited follicles are

selected to escape atresia. The final period is dominancy in which only ovulatory or dominant follicules escape atresia (Goodman and Hodgen, 1983).

Estradiol is the key steroid in folliculogenesis (Richards, 1980). Production of estrogen can best be described by the two cell theory. This steroid is produced from the granulosa and theca cells in response to FSH and LH activity. When FSH and/or LH bind their receptor a steroidogenic enzyme is enhanced which activates the cAMP-dependent process and steroid synthesis occurs (Richards, 1980). The end of the luteal phase occurs when the CL regresses and progesterone production declines.

In sheep, luteolysis occurs as a result of prostaglandin F2a (PGF2a) (Goding, 1974; McCracken, 1981). Prostaglandin F2a is secreted from the uterus at the end of the estrous cycle (Wiltibank and Casida, 1956) and is transported to the ovary by a local counter current transfer mechanism.

Numerous prostaglandins have been associated with reproduction, prostaglandin F2a (PGF2a) and prostaglandin E2 (PGE2), have received the most attention and are the most throughly researched. During the estrous cycle in ewes, it is accepted that PGF2a is the natural occuring luteolysin (McCracken, et al., 1972; Goding, 1974). If an embryo is not present in the uterus by day 13 post estrous, regression of the corpus luteum will occur (Moor and Rowson, 1964, 1966). Most researchers (Pexton et al., 1966; Ellinwood et al., 1979; Nett et al., 1976b; Lewis et al., 1977) agree there is no difference in the concentration of PGF2a secreted by the uterus

comparing pregnant and nonpregnant ewes. Although (Silvia, 1984) reported that higher dosages of PGF2a in pregnant ewes was necessary to inhibit progesterone secretion when compared to nonpregnant ewes.

Corpra lutea in pregnant ewes are resistant to the luteolytic effect of PGF2a which may be due to the increased secretions of PGE2 during the critical period of maternal recognition of pregnancy. Prostaglandin E2, is luteotrophic and functions to protect luteal tissue from the actions of PGF2a (Silvia et al., 1984). These biological processes may be accomplished by increasding the blood flow to the corpus luteum, since PGE2 is a vasodialator (Bergstrom et al., 1968) or cause inhibition of luteolytic factors from the large luteal cells by stimulating adenylate cyclase in the small luteal cells to produce more progesterone (Silvia et al., 1984).

Prostaglandins are synthesized from a common precursor, arachidonic acid, which is a 20-carbon fatty acid. Arachidonic acid is found in the plasma membrane as a component of phospholipids, specifically, phosphatidlyinositol (Holub et al., 1970). The enzyme phospholipase c hydrolyses phosphatidlyinositol-bisphosphate into diacyl glyceral (DAG) and inositol-triphosphate (IP3) (for review see Berridge and Irvine, 1984). Diacyl glycerol (DAG) lipase can cleave arachidonic acid direction from DAG. Diacyl glycerol can act as a second messenger to activate protein kinase C, which will phosphorylate specific protein substrates to activate or inactivate enzymes (Niwhizuka et al., 1984). Inositol-triphosphate can also act as a second messenger by causing an increase in calcium (Ca++) in the cytosol. Calcium is released from the endoplasmic reticulum

and can activate phospholipase A which can directly cleave arachidonic acid from phospholipids (Van den Bosch, 1980). Once arachidonic acid is liberated from phosphotidylinositol it is acted upon by cyclooxygenase a heme-containing deoxygenase which is bound to the smoooth endoplasmic reticulum. Cyclooxygenase functions to convert arachidonic acid to PGG2, which is then acted upon by several other enzymes to produce other prostaglandins (Huslig et al., 1979).

Prostaglandin (PGF2a) is released in a pulsatile manner, varying from 5ng to 22ng per ml of plasma (Thornburn et al., 1973; Baird et al., 1976), into the uterine vein and is transported directly into the ovarian artery which is in close apposition with the uterine vein, which then carries it to the ovary (McCraken, 1972). It is believed that PGF2a inhibits the ability of receptor bound LH to stimulate the adenylate cyclase system (Thomas et al., 1978: Khan and Rosberg, 1979; Fletcher and Niswender, 1982) or it may cause a reduction in fluidity of the plasma membrane. This may hinder the interaction of the LH receptor complex with adenylate cyclase (Buhr et al., 1979; Carlson et al., 1982; Goodsaid-Zulduondo et al., 1982).

Oxytocin is synthesized in large luteal cells and plays a role in the ovarian cycle in ruminants by causing release of PGF2a from the uterus (Sharma and Fitzpatrick, 1974). Oxytocin receptor number increases on both caruncular and intercaruncular endometrium at the time of luteolysis (Sheldrick and Flint, 1985). Other studies, including immunization against oxytocin, supports the role of

oxytocin acting as a luteolysin (Sheldrick et al., 1980; Schramms et al., 1983).

Maternal Recognition of Pregnancy

Continued progesterone production by the CL is necessary for the maintenance of pregnancy in all placental mammals (Amoroso, 1952). In successful pregnancies, the presence of a viable conceptus in the uterus alters maternal endocrinology resulting in luteal maintenance. In sheep the embryo must be in the uterus by day 13 in order for the CL to be maintained past day 14 (Moor and Rowson, 1966). If the embryo is removed prior to day 13 the interestrous intervals is not effected, but removal of the embryo after this time prolongs luteal maintenance and interestrous intervals. Transfer studies show blastocysts transferred to syncronized recipients prior to maternal recognition of pregnancy are successful where as recipients receiving embryos after this time returned to estrus. It is believed in sheep and cattle that conceptus proteins regulate maternal recognition of pregnancy. This was demonstrated in studies when conceptus homogenates, (Rowson and Moore, 1967), secretory proteins (Godkin et al., 1984b) or extracts (Martal et al., 1979) prolonged luteal maintenance when infused into cycling animals. It is believed that conceptus proteins alter PGF2a metabolism, resulting in maintenance of CL function.

Ovine Trophoblast Protein-1

Ovine trophoblast protein 1 (OTP-1) is a low molecular weight acidic polypeptide, Mr 17,000-21,000, which is secreted by the sheep conceptus between days 13-21 of pregnancy but not beyond day 21 (Godkin et al., 1982). It is secreted at a time when the blastocyst must be present in the uterus to maintain the CL (Moore, 1968). Ovine TP-1 is produced by trophoblast cells of the conceptus and is taken up by caruncular and intercaruncular epithelial cells. Immunocytochemical studies show OTP-1 localization in surface uterine epithelial cells in pregnant ewes. This was not found in the case of nonpregnant ewes, proving the source of this protein must be coming from the blastocyst (Godkin et al., 1984). Three possible functions of OTP-1 have been described by Godkin et al., (1984a). The first function is stimulation of the endometrium to secrete proteins to nourish the blastocyst since it is unattached at this time. Another possibility is by controlling the synthesis, release or sequestration of PGF2a. A final possibility is that OTP-1 may cause the endometrium to secrete selected proteins, which may act on the ovary or in an autocrine fashion on the uterus.

Recently, OTP-1 was shown to have significant amino acid homology with human and bovine interferon alpha (Imakawa et al., 1987; Stewart et al., 1987) Ovine TP-1 and recombinant human interferon alpha (Roferon) were demonstrated to have identical actions on uterine cell protein and prostaglandin metabolism (Salamonsen et al., 1988). This data indicates that OTP-1 may have

functions within the immunology of pregnancy as well as maternal recognition of pregnancy.

Endometrial Secretions During Early Pregnancy

Ovarian steroid hormones released from the ovaries influence endometrial activities. Studies have demonstrated protein metabolism changes during early pregnancy and during the estrous cycle (Miller and Moore, 1976; Zamiri and Blackshaw, 1979; Baird, 1978). Several studies suggest endometrial protein synthesis is enhanced by estrogen or by the presence of the conceptus (Shutt and Cox, 1972; Miller et al., 1977a; Koligian and Stormshak, 1977). A study performed by Salamonsen et al., (1985) states that day 13 pregnant ewe epithelial endometrial cells incorporate more 35 S-methionine into secreted proteins than do day 13 estrous ewes. Nonpregnant ewe endometrial epithelial cell protein synthesis and secretion can mimic pregnant ewe endometrial epithelial cells when blastocyst secretions are added to the culture.

Protein synthesis was found to be greater in caruncular and intercaruncular tissues of pregnant animals compared day 15 nonpregnant animals. The difference was significant in the caruncular tissue (Findlay et al., 1981). Evidence suggests caruncular areas have greater activity of lysosomal enzymes, acid phosphatase and beta glucuronidase than intercaruncular areas (Murdoch and Which, 1968; Murdoch, 1970; Findlay et al., 1981). It has been suggested by Murdoch (1970) that enzyme activity in the

uterine caruncules may be important for implantation and attachment of the bastocyst to the maternal unit.

Fertilization

Fertilization occurs in the oviduct, where the zygote spends 72 hours near or at the ampullary-isthmic junction prior to entering the uterus (review by Bazer and First, 1983). The zygote surrounded by the zona pellucida, a mucopolysaccharide coat, divides into a mass of cells known as the morula (Perry, 1981). The morula, (16-32 cell stage), is a compact group of wedged shaped cells packed inside the zona pellucida (McLaren, 1980). The time it takes for the fertilized egg to enter the uterus is not uniform. In some species, such as the ovine, the morula reaches the uterus on the fourth day of gestation (Guillomot et al., 1981). The morula then developes a fluid-filled cavity with a single peripheral layer of flattened cells known as trophoblast and a groups of smaller cells known as inner cell mass to one side of the central cavity. This group of cells known as inner cell mass or embryonic disc will give rise to the body, while the cells of the trophoblast will give rise to extraembryonic membranes (Perry, 1981). Sheep blastocysts are spherical between days 4 and 10 of gestation. They then begin to elongate by day 12 and day 14 (Bindon, 1971) may reach a length of 20cm before attaching to the uterine epithelium (Austin and Short, 1982).

Ultrastructure of the Uterine Epithelium

In sheep, changes in the endometrium appear similiar in pregnant and non-pregnant ewes until day 13. The surface of the caruncles are smooth, convex, and are covered with uniformly distributed microvilli (Guillomot et al., 1981; King, 1982).

On day 14 of gestation, the first changes occur on the caruncules in both uterine horns, but these changes do not occur on all of the caruncles at the same time (Boshier, 1969; Guillomot et al., 1981). Changes are characterized by folded surfaces and depressed centers, which disappears after day 16. Appearing on caruncular epithelial cells are grossly rounded, irregulary shaped microvilli (Guillomot et al., 1981; King, 1982). There is no difference in intracaruncular areas between pregnant and cyclic ewes (Guillomot et al., 1981).

Three different types of cell contact were described by Guillomot, et al., (1981) and King, (1982). First, and most frequently, the apices of uterine epithelial microvilli are apposed to plasma membrane of trophoblast cells. Second, where conceptus and uterine epithelial microvilli were deficient their membranes were discontinuously apposed. Third, the appearance of the trophoblastic projections occured, when the uterine epithelium pushed against the plasma membrane of the trophoblast cells.

According to King, (1982) contact between the conceptus and carunclar epithelium occured by day 14. Adhesion developed between days 16 and 18 of pregnancy (Guillomot et al., 1981). Modification

of the caruncular epithelium was first apparent on day 16 of pregnancy. Reduction in the number of epithelial cells was observed and proposed to be a result of cell death within the columnar uterine epithelium (Boshier, 1969). Cell death was usually associated with nuclear swelling, vacuolation and disintegration (Boshier, 1969). According to Guillomot et al., (1981) the uterine epithelium appeared flat and syncytial masses were formed. Although (King, 1981) demonstrates carcunclar and intercaruncular epithelium consisted of flat, cuboidal, or low columnar cells. Interdigitation between the maternal and fetal unit was observed on day 20 (Davis and Wimsatt, 1966) but probably began about day 18 (Boshier, 1969; King, 1982).

Ultrastructure of the Trophoplast

Trophoblast of spherical sheep blastocyst consisted of flattened cells, which are connected by unilateral desmosomes. The trophoblast is not attached to the uterine lumen, and the embryonic disc appears as a cell mass protruding from the trophoblast. A dense coating is present on the trophoblast, which is completely covered with microvilli (Carnegie et al., 1985). Expansion of the blastocyst occurs around day 13. Between days 14 and 15, most microvilli disappear, and the cells become spindle shaped as apposition occurs (Guillomot et al., 1981; King, 1982). The trophoblast in apposition to intercaruncular area carries villous digitations on days 15 through 18, and villi increase in number and

size on days 16 through 18. The villi near the uterine glands stand upright on the trophoblastic regions (Boshier, 1969).

Trophoblastic binucleate cells first appear during the 16th day of gestation and increase in number thereafter. According to Boshier, (1969) they are most numerous in the distal trophoblast apposed to the maternal epithelium, but were not present in the uniform caruncular epithelium. Another source states binucleate cells first appear in trophoblast at day 14 of gestation (Wooding, 1984).

Binucleate Cells

Studies by Wooding, (1984) suggest binucleate cell formation may start as soon as elongation begins. By day 16 they form 15-20% of the trophectoderm which is apposed to caruncular epithelium. Ultrastructural evidence indicates that binucleate cells migrate upto the uterine epithelial microvilli and fuse with individual uterine epithelial cells to produce trinucleate cells by 16 days post coitum. Additional binucleate cells fuse with trinucleate cells to form syncytial plaques which are thinner than the uterine epithelium they replace which may aid in the delivery of binucleate cell granules to the maternal blood stream. Using phosphotungstic acid (PTA) staining of binucleate cells it was demonstrated that the granules disappear after morphological evidence of cell fusion is lost. It has been suggested that most granules disappear into the connective tissue (Wooding, 1984).

Binucleate cell granules have been shown by immunocytochemical techniques to contain ovine placental lactogen at mid pregnancy (Marteal et al., 1977; Reeding and Watkins, 1978). Ovine placental lactogen was undectable earlier than day 22 (Wooding, 1984). Although Reeding and Watkins,(1978) were able to detect ovine placental lactogen at day 16 and Carnegie et al., (1982) at day 14 by immunofluorescent localization.

It has been demonstrated that the entire caruncule is covered by plaques of syncytium by 20-24 days of gestation. The caruncular epithelium syncytium contains no nuclear division indicating nuclei must be from binucleate cell migration as reviewed by Wooding, (1981).

There are conflicting views on syncytial mass formation in the ewe. Amoroso, (1952) and Wooding, (1984) agree that implantation and syncytial mass formation is a result of binucleate cell migration. Whereas others (deDuve, 1963; Gaham, 1967; Boshier, 1969) believe syncytial mass formation may be due to increased acid phosphatase activity which occurs between the trophoblast and uterine epithelium or to lysosomal enzyme activity which causes death of the cell or its modification.

3. MATERIAL AND METHODS

TISSUE COLLECTION

Uterine tissue was obtained from crossbred ewes (Finn, Suffolk, Dorset, or Hampshire breeding). The ewes were checked daily for signs of estrus with vasectomized rams, and at estrus, the ewes were bred to intact rams. Four gestational groups, three days apart beginning at day thirteen were chosen: 13, 16, 19 and 22 days. A total of fifteen ewes were studied with three in each gestational group and three in a control group (day 13 estrous). Maternal tissue was collected by the following surgical procedure. Ewes were anesthetized with chlorohydrate and the reproductive tract was exposed by a midventral abdominal laparotomy. Uteri from both gestational and estrous groups were perfused through the uterine artery with 50ml of 3% glutaraldehyde in .1M cacodylate buffer, PH 7.4. Day 13 pregnant uteri were also perfused via the uterine artery after the embryos were flushed from the uterus using the following standard procedure. The cervix was first clamped and a 25cm length of sterile silastic tubing was inserted into the pregnant horn. The embryo was then flushed from the uterus by injecting 20ml of .1M cacodylate buffered 3% glutaraldehyde into the

nonpregnant horn. The glutaraldehyde was massaged through the uterus, and embryos were collected in a serum bottle. Ewes were terminated following these procedures.

ELECTRON MICROSCOPY

Fixed uterine horns were cut with a razor blade into proximal, middle and distal regions and the tissue was placed in fixative (3% glutaraldehyde in .1M cacodylate buffer). Two millimeter subsections including the uterine epithelium were dissected from the caruncular regions, where the trophoblast was still attached. Both pregnant and non-pregnant tissue was post-fixed in 1% osmium in 0.1M cacodylate buffer, dehydrated in an alcohol series and embedded in epon following a standard processing schedule. Two micron thick sections were cut and stained with methylene blue-azure II prior to thin sectioning to determine the location of uterine epithelium and trophoblastic tissue. Ultrathin sections (50-80nm) 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 Venable Coggeshalls lead citrate. Tissues were examined using a Philips 201 transmission electron microscope operated at 60 KV. Micrographs for volume fraction (V_V) measurements of nucleus and lipid were made at 2,000 magnification. Micrographs for volume fraction measurements of secretory granules and mitochondria and for surface area per unit volume (S_V)

measurements of mitochondrial membranes and cristae, golgi complex, plasmalemma rough and smooth endoplasmic reticulum and nuclear membranes were made at 15,000 magnification.

STEREOLOGY

Stereology "estimates three-dimensional parameters of biological structures from two-dimensional pictures obtained in the electron microscope. The structural data can be combined with biochemical measurements to relate quantitatively cellular structures with their biochemical function in vivo" (Schwerzmann and Hoppeler, 1985).

Volume fractions of nucleus, mitochondria, lipid droplets and secretory granules were estimated by point counting volumetry (Weibel, 1969). Data was 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 (Plate 1). The area fraction (Aa) of each cellular component was estimated as the number of points falling on that component P(a) divided by the number of total points falling on the tissue P(t). This point fraction P(p) has been shown to be equivalent to the A(a), which in turn, has been shown to represent an unbiased estimate of the volume fraction (V_V) (Weibel, 1979).

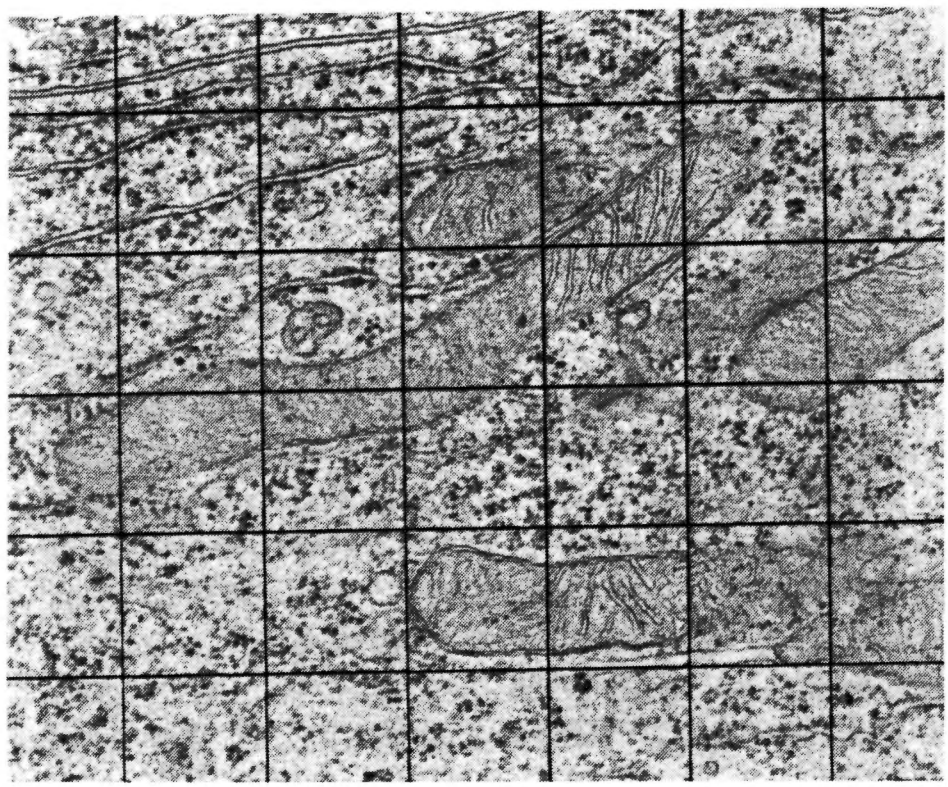


Plate 1. Grid of Equidistant Lines. Photomicrograph of equidistant lines over a tissue section.

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

$$Sv = 2I$$

where, Sv= surface/ unit volume

2I= twice the number of intersections/ length of line

One-way analysis of variance was used to test differences in mean volume fractions and surface densities among uterine regions per animal, animals and gestational groups. Differences among organelle means were compared using orthogonal contrasts.

4. RESULTS

ULTRASTRUCTURAL OBSERVATIONS

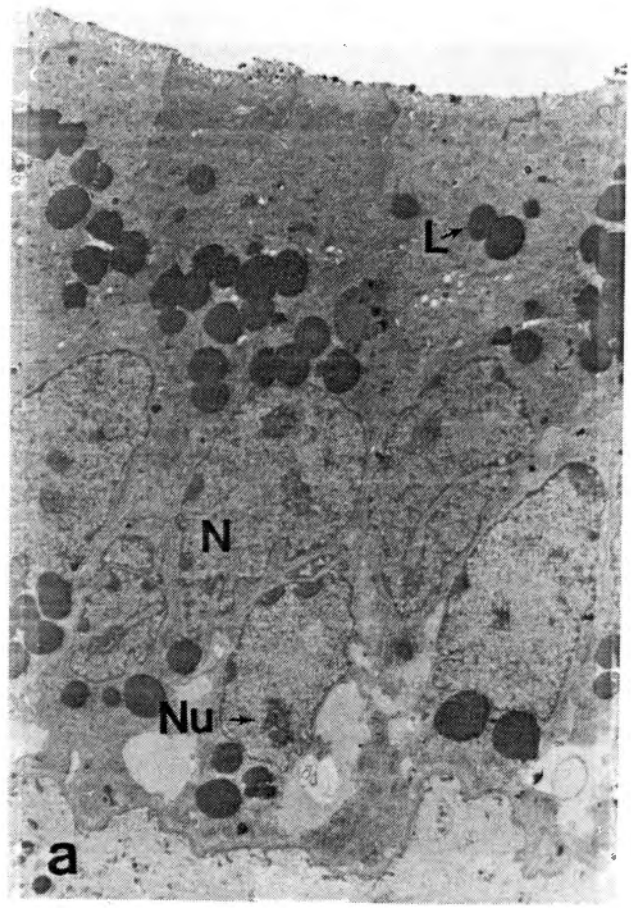
Day 13 of Estrous and Day 13 of Gestation

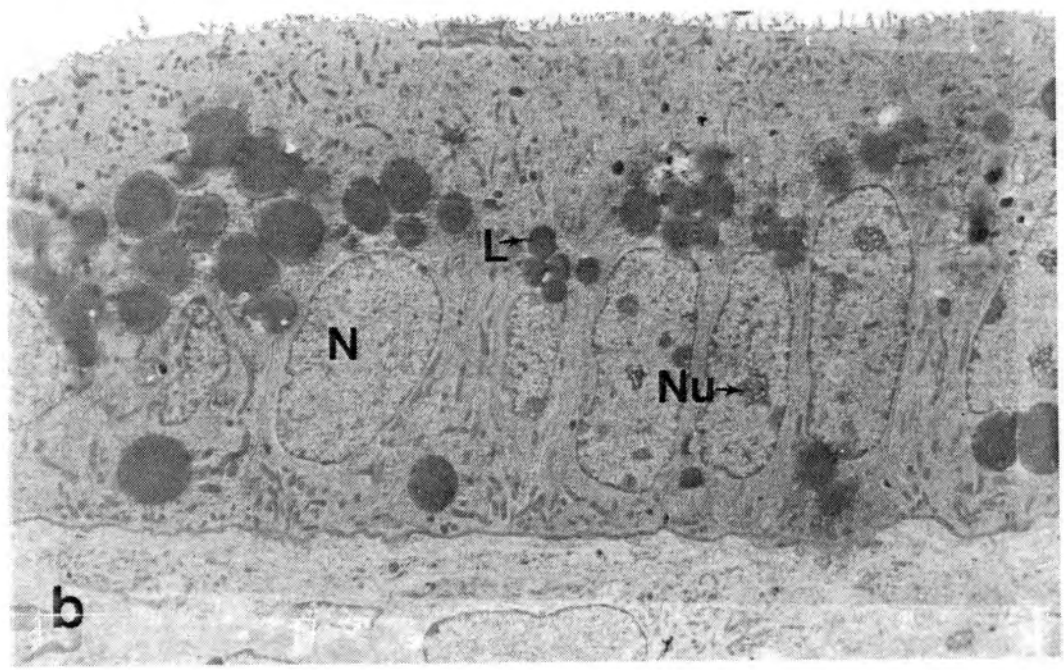
No gross anatomical differences between day 13 of the estrous cycle and day 13 of gestation were observed, therefore they will be described together. The cells of the epithelial layer appeared columnar with many microvilli, which were short and regularly distributed. Nuclei were euchromatic and contained prominent nucleoli. Lipid droplets were numerous and randomly distributed while mitochondria were concentrated apically and basally of the basally located nuclei. Crystalline bodies were also present in some sections of epithelium on day 13 of gestation (plate 2).

Day 16 of gestation

Uterine epithelium of day 16 pregnant ewes was columnar although lateral separations between the cells were observed. Microvilli were longer, stood upright and were regularly distributed. Nuclei, were euchromatic with distinct, prominent nucleoli (plate 3). Lipid droplets, which were less numerous

Plate 2. Photomicrographs of Uterine Epithelium at Day 13 of the Estrous Cycle (a) and Day 13 of Gestation (b) (4,800x). Photomicrographs of similiarities between day 13 pregnant and non-pregnant columnar epithelium, euchromatic nuclei (N), with prominant nucleoli (Nu), numerous lipid droplets (L).


L
N
Nu
a


L
N
Nu
b

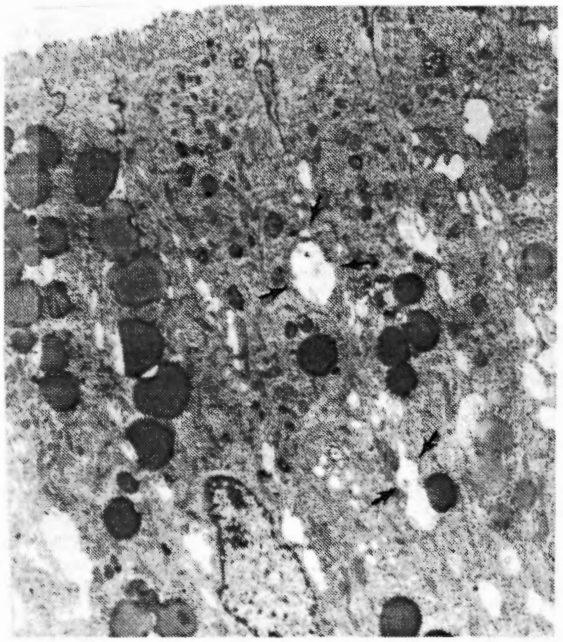


Plate 3 Photomicrograph of Uterine Epithelium at Day 16 of Gestation (4,200). Columnar uterine epithelium showing lateral cell separation (arrows).

than at day 13, and mitochondria were randomly distributed throughout the epithelial layer.

Day 19 of Gestation

In sections where trophoblast was attached to uterine epithelium, the latter was less organized, contained granules and distinct cell boundries were not observed. Microvilli were longer than those found on previous days studied, although their regular arrangement appeared to be the same as previously described. Lipid droplets appeared less abundant than on the previous days. Mitochondria were randomly dispersed throughout the epithelial layer and crystalline bodies were present in some sections of epithelium (plate 4). Sections not apposed to trophoblast had the same morphology as described in day 16 animals (plate 5).

Day 22 of gestation

On day 22, the uterine epithelial layer attached to the trophoblastic layer was extremely thin and cell boundries were difficult to distinguish (plate 6). Caruncular epithelium not connected to trophoblast was columnar with a majority of nuclei located basally (plate 7). Blood vessels in connective tissue were much larger than those observed during the pre and peri-attachment periods.

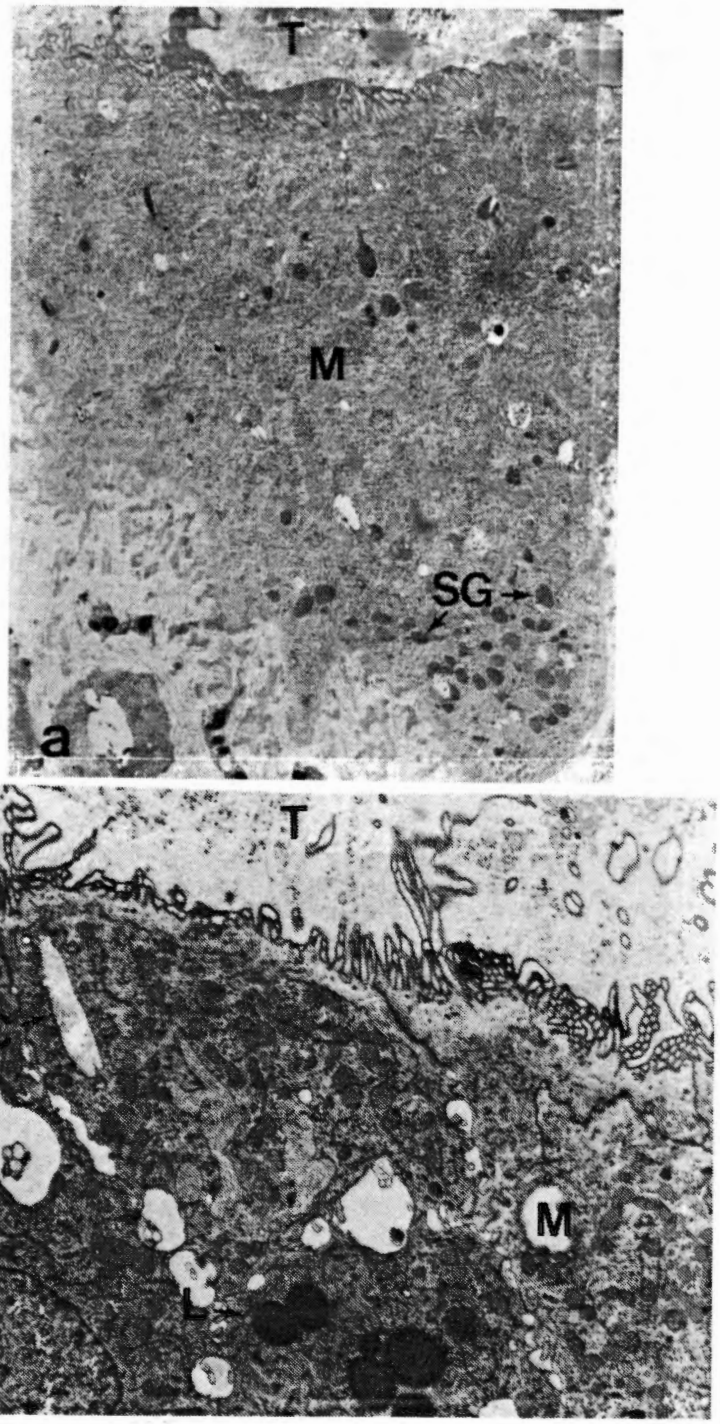


Plate 4 Photomicrograph of Uterine Epithelium at Day 19 of Gestation attached to Trophoblast (a,b) (48,000) (7,000). Maternal epithelium (M) attached to the trophoblast (T) appeared less organized and contained secretory granules (SG), lipid droplets (L) appeared throughout epithelial layer, and crystalline bodies (C) appeared in some sections.

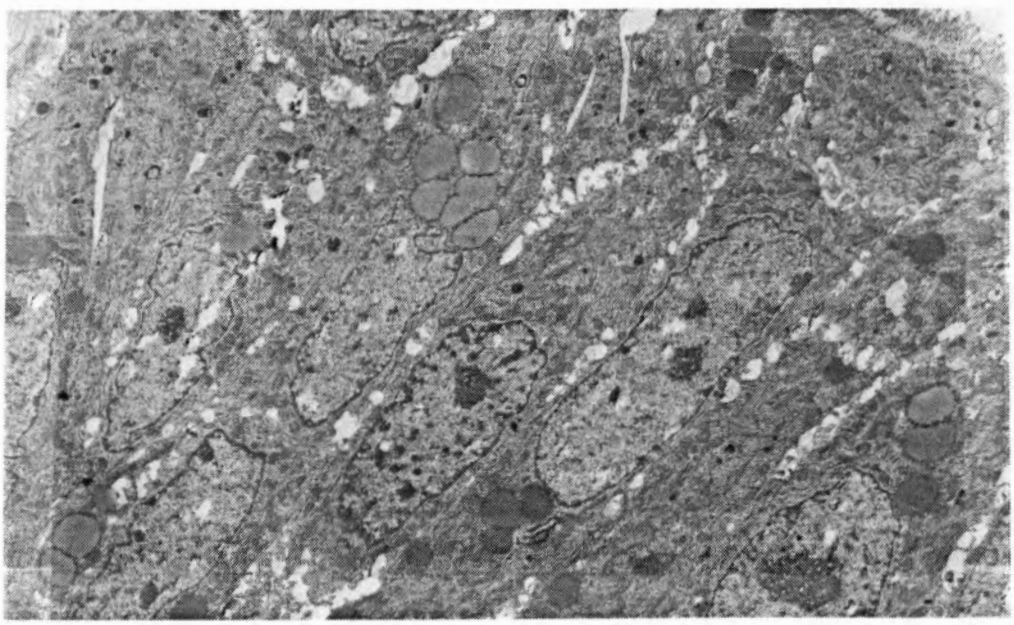


Plate 5 Photomicrograph of Uterine Epithelium at Day 19 of Gestation (4,000x). Unattached uterine epithelium showing the same morphology as day 16 of gestation.

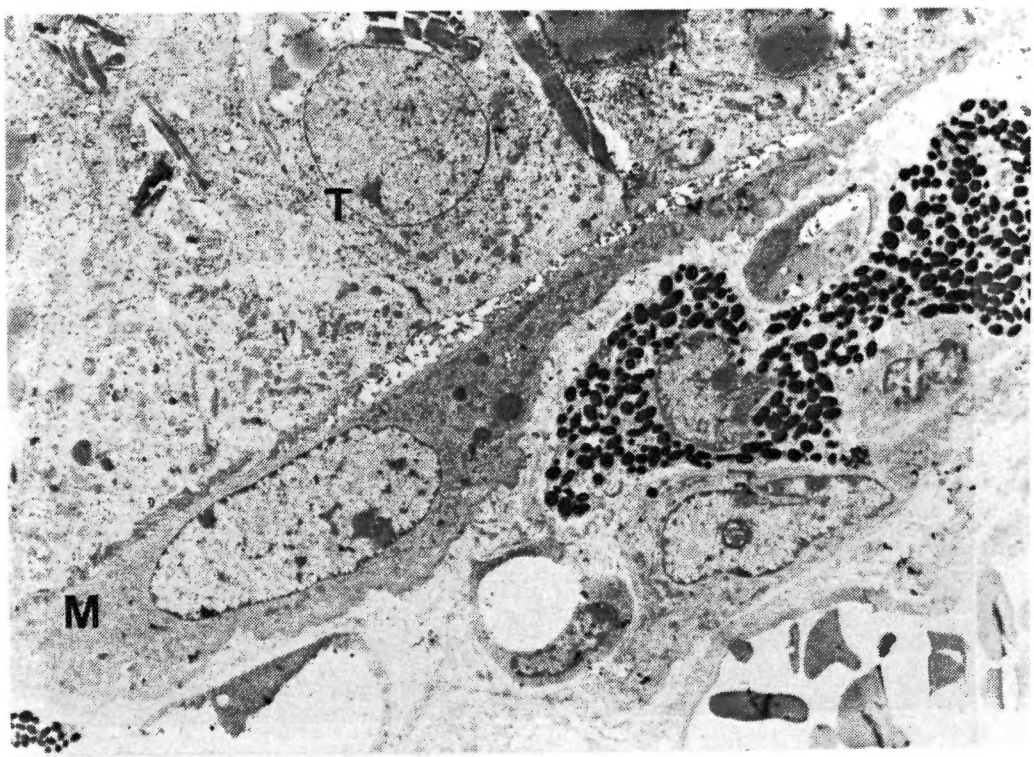


Plate 6 Photomicrograph of Uterine Epithelium at 22 Days of Gestation attached to Trophoblast (4,000x). Photomicrograph showing hard to distinguish cell boundries in maternal (M) epithelium when attached to trophoblast (T).

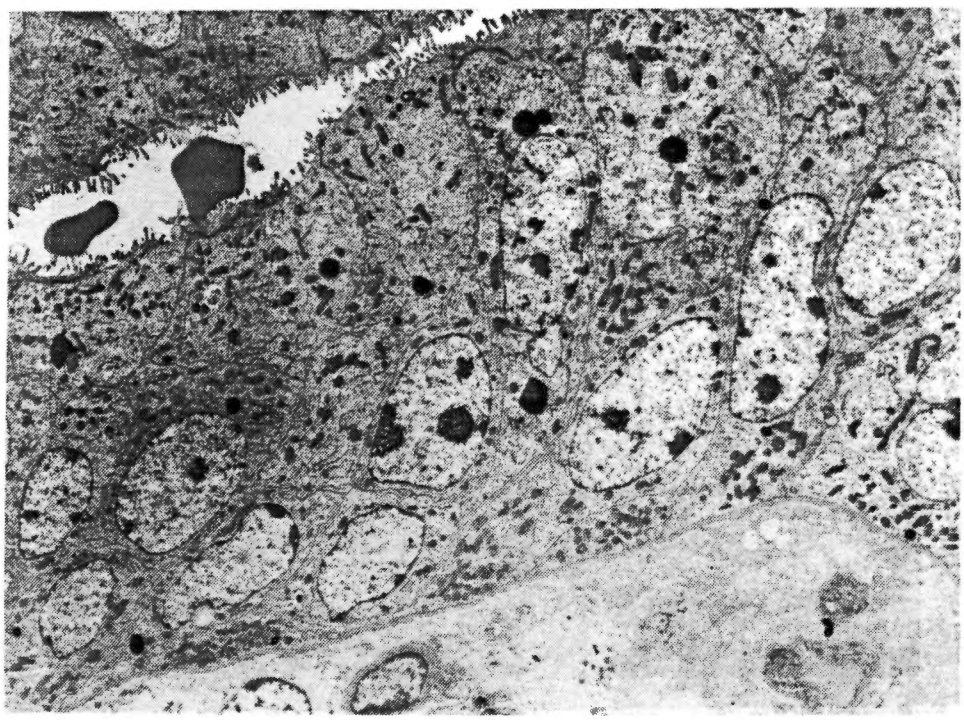


Plate 7 Photomicrograph of Uterine Epithelium at Day 22 of Gestation (3,750x). Caruncular epithelium not attached to trophoblast appears columnar with euchromatic nuclei.

STEREOLOGY

Mean volume fractions of nuclei, lipid droplets, mitochondria and secretory granules for the various treatment groups are shown in Table 1. The mean volume fraction of nuclei at day 13 of the estrous cycle and day 13 of gestation were not significantly different. A significant decrease in nuclear volume fraction occured between days 13 and 16 of gestation. The mean volume fraction of nuclei was similar at days 16, 19 and 22 of gestation. Volume fraction of lipid was not significantly different between day 13 of estrous and day 13 of gestation. A significant difference was noted between days 13 and 16 of gestation with the mean volume fraction decreasing significantly. Lipid mean volume fraction was similar for days 16, 19 and 22 of gestation. The volume fraction of secretory granules was not significantly different from zero for day 13 of estrous and days 13 and 16 of gestation. Secretory granules were also similar between days 19 and 22, where the Vv was significantly greater than zero. Mitochondrial mean volume fractions were similar between all treatment groups.

Average surface per unit volumes of membrane cell components are listed in Table 2. Surface per unit volume of mitochondrial cristae was similar between all treatment groups. Outer mitochondrial membrane was similar between day 13 of estrous and days 13 through 19 of gestation, and significantly decreased on day 22. Nuclear membrane surface per unit volume was similar on day 13

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

	Day of Pregnancy				
Parameter	d13**	d13	d16	d19	d22
Nucleus	26.42 (1.65)[a]	23.48 (1.23)[a]	15.94 (1.82)[b]	16.52 (.87)[b]	13.49 (2.28)[b]
Lipid	10.12 (.93)[a]	13.13 (2.46)[a]	5.50 (.92)[b]	3.32 (1.62)[b]	6.95 (2.45)[b]
Secretory Granules	0.0 (0)[a]	0.0 (0)[a]	0.0 (0)[a]	.12 (.051)[b]	.56 (.26)[c]
Mitochondria	5.82 (.40)[a]	5.19 (.72)[a]	5.52 (.25)[a]	5.30 (.44)[a]	3.89 (.48)[a]

* Volume fraction expressed as % (Standard Error)

** Day 13 Non-pregnant

Table 2. Mean surface area per unit volume* (μ^2/μ^3) of cellular organelles in ovine endometrium epithelial cells during early pregnancy.

	Day of Pregnancy									
Parameter	d13**		d13		d16		d19		d22	
Outer Mitochondrial Membrane	1.51	(.11)[a]	1.41	(.064)[a]	1.49	(.067)[a]	1.31	(.12)[a]	1.01	(.10)[b]
Mitochondrial Cristae	.93	(.084)[a]	.81	(.061)[a]	1.16	(.29)[a]	.74	(.089)[a]	.68	(.090)[a]
Plasmalemma	.99	(.049)[a]	.94	(.060)[a]	1.01	(.090)[a]	.76	(.097)[b]	.74	(.067)[b]
Golgi Complex	.063	(.011)[a]	.039	(.012)[a]	.076	(.019)[a]	.073	(.029)[a]	.10	(.025)[a]
Nuclear Membrane	.49	(.022)[a]	.48	(.034)[a]	.33	(.018)[b]	.32	(.020)[b]	.29	(.029)[b]
Rough Endoplasmic Reticulum	.22	(.023)[a]	.33	(.076)[a]	.56	(.10)[b]	.52	(.11) [b]	.33	(.067)[a]
Smooth Endoplasmic Reticulum	.23	(.049)[a]	.057	(.018)[b]	.24	(.028)[a]	.047	(.023)[b]	.022	(.0081)[b]

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

** Day 13 non-pregnant

of estrous and on day 13 of gestation; a significant decrease between days 13 and days 16 through 22. The mean surface per unit volume for plasmalemma was similar on day 13 of estrous and days 13 and 16 of gestation. Plasmalemma decreased significantly on days 19 and 22. Rough endoplasmic reticulum surface per unit volume was significantly lower on days 13 of gestation when compared to day 16 of gestation. Mean surface per unit volume of smooth endoplasmic reticulum was significantly different between day 13 of estrous and day 13 of gestation. The S_V was different between days 13 and 16 of gestation and between days 16 and 19, but not between days 19 and 22. Surface per unit volume of golgi complex was not significantly different between treatment groups.

5. DISCUSSION

In this study, no observed ultrastructural differences occured between days 13 of estrous and day 13 of gestation but differences did occur on day 16 of gestation. This is in agreement with studies by Boshier (1969) who did not detect differences in uterine epithelium until day 16 of gestation. Differences observed by Boshier (1969) included uterine cell breakdown, a reduction in the number of epithelial cells and cell death. The present study observed lateral cell separation between the uterine epithelial cells at points of attachment to the trophoblast.

Binucleate cell granules were observed in transformed uterine epithelium at day 19 of gestation and their volume fraction significantly increased by day 22 of gestation. This is in agreement with studies by Wooding (1984) who suggests uterine cell transformation is a result of binucleate cell granules.

Smooth endoplasmic reticulum plays a major role in lipid metabolism. Hypertrophy of smooth endoplasmic reticulum may indicate an increase in activity of SER, whereas a decrease in S_V may indicate decreased SER activity. This study may help explain the above findings (Fincher et al., 1986; Salamonsen et al., 1988) because of the significant decrease in mean S_V of smooth endoplasmic reticulum between days 13 of estrous and day 13 of gestation which

may indicates a decrease in smooth endoplasmic reticulum activity. This decrease in SER activities may indicate decreased prostaglandin synthesis from the caruncular epithelial cells.

A reason for decreased concentration of prostaglandin from the uterus during this time period may be due to conceptus interference. Conceptus secretions such as OTP-1 may cause inhibition of phospholipase A2 activity. Another reason may be the presence of OTP-1 which might control endoperoxidase intermediate which may inhibit phosphatidlyinositol turnover from the cell membrane, which would then decrease arachidonic acid formation. Significant increases in mean S_V of SER between days 13 and 16 of gestation may be due to increased SER activities. This study also demonstrates the highest mean S_V of SER between treatment groups occured on day 16. The significant decrease in mean S_V of SER between days 16 and 19 of gestation may be due to decreasing S_V of the whole cell and/or to the decrease in SER activities.

Neutral lipid appearance and disappearance during the estrous cycle in the uterine epithelium of rats, sheep and cattle is well known (Alden, 1947; Marinov & Lovell, 1968; Brinsfield & Hawk, 1973; Boshier & Holloway, 1973). It has been demonstrated that progesterone facilitates lipid storage while estrogen causes the loss of neutral lipids via an enhanced esterase activity (Boshier & Holloway, 1973; Boshier & Katz, 1975). During the estrous cycle, lipid droplets appear by day 8 a time when progesterone levels are high (Boshier, 1987) and reach there maximum by day 14 or 15 (Brinsfield and Hawks, 1973; Boshier 1987). In this study mean

lipid volume fraction V_V was not significantly different between days 13 of estrous and 13 of gestation. This can be explained by the fact that progesterone concentration levels show little marked change in pregnant vs nonpregnant ewes during this time period (Bassett et al., 1969). On day 15 of the estrous cycle or about 12 hours before the onset of estrous progesterone levels are low (<0.2 ng/ml) (Bjersing et al., 1972; Pant et al., 1972).

The findings in this study are in partial agreement with studies by Boshier (1987) who demonstrated a significant decrease in the number of lipid droplets between days 15-16 whereas in this study a significant decrease in mean V_V of lipid droplets were found between days 13 and 16 of pregnancy. Disagreement occurs between treatment groups consisting of days 15-16 and days 18-19 in which Boshier (1987) found a significant decrease in the intraepithelial lipid scores between these treatment groups. This study finds no significant difference in V_V of lipid droplets between days 16 and 19 of gestation. The difference indicated here may be do to the difference in measuring techniques. This finding does not correlate with the theory of high progesterone concentrations causing lipid storage while estrogen causes lipid utilization. This decrease may however be due to estrogen which may be secreted from the trophoblast, enter the uterine epithelium and enhance esterase activity (Boshier, 1969). Lipid droplets may play a role in embryo nutrition since the trophoblast is not fully attached to the uterine epithelium during this peri-attachment stage. In sheep and rats the loss of neutral lipids occur as a result of blastocyst presence

(Boshier, 1976). Boshier (1969) found the pregnant uterine horn containing the expanded blastocyst had a lower lipid score when compared to the uterine horn of estrous during the same time period. This is in agreement with this study since the blastocyst at day 16 was expanded and the mean V_V of lipid droplets decreased significantly from day 13 of gestation to day 16.

After day 16 no significant differences were found in V_V of lipid droplets. This may be due to the higher levels of progesterone present and low levels of estrogen, which occur during pregnancy.

Studies by Salmonson (1988) measuring incorporation of 35 S-methionine demonstrated higher protein synthesis in day 13 epithelial tissue in the presence of a blastocyst when compared to day 13 of the estrous cycle. In this study there was no increase in mean S_V of RER between days 13 of estrous and day 13 of pregnancy. A hypertrophy of RER, which would indicate an increase in RER activity would have supported the studies by Salmonson (1986).

Findlay (1981) measured the rate of leucine incorporation in day 15 pregnant and day 15 estrous ewes. These findings suggest that protein synthesis increases in the presence of a blastocyst prior to implantation. Other reports also demonstrate increased protein production by the uterus when proteins were flushed from the uterus between days 13 and 18 of pregnancy (Roberts et al., 1976; Ellinwood, et al., 1979). Although these flushings may have included conceptus tissues. In this study there was a significant increase in $_Vv$ of RER between days 13 and 16 of gestation which also

disagrees with several other studies. Reasons why protein synthesis increased during early pregnancy may be increased uterine blood flow, enhanced responsiveness of the endometrium to stimulations by steroids or the presence of conceptus secretions such as proteins, prostaglandins or other secretions. The significant decrease in mean S_V of rough endoplasmic reticulum between days 19 and 22 of gestation may be related to syncytial mass formation and to the significant reduction of cell plasmalemma which occured between days 19 and 22 of gestation.

Cell plasmalemma S_V was not different amoung treatment groups until day 19 when there was a siginificant decrease. This significant decrease is probably due to the reorganization of the uterine epithelium into a syncytial mass. Qualitative results of this study suggest syncytial mass formation occurs after day 16 at which time secretory granules are observed in the syncytial mass.

Mean volume fraction of secretory granules between days 19 and 22 of pregnancy were significantly increased from zero which may suggest secretory granules are from an extra-uterine source. The granules are probably coming from the trophoblast since granules were not present in uterine tissue that was not attached to the trophoblast. This is in agreement with Wooding (1981) who has demonstrated with electron microscopy and staining of fetal binucleate cells that the binucleate cells migrate into the uterine epithelium release their granules, and cause uterine cell transformation. Granules being supplied by an extra-uterine source is further supported by no significant changes in S_V of golgi

complex present in the uterine epithelium, since mean V_V of secretory granules significantly increased from zero between days 19 and 22 of pregnancy. This study also supports demonstrations by Boshier (1969) who suggests epithelial transformation is due to increased esterse activity. Qualitative results of this study show lateral cell membrane separation which may be due to increase esterase activity (Boshier, 1969) since this was observed on sections which were attached and unattached to the trophoblast. Trophoblast secretions such as OTP-1 may cause this increased esterase activity since OTP-1 binds the uterine epithelium as early as day 13 of pregnancy (Godkin et al., 1984).

Mean volume fraction of mitochrondria and surface per unit volume of mitochondrial cristae were not significantly different among the uteri examined, which may indicate that these celluar organelle activities are not pregnancy dependent. A significant decrease between days 19 and 22 of pregnancy may not be of biological significance since surface volume of mitochondrial cristae and volume fraction of mitochondria did not change.

The significant decrease in mean S_V of nuclear membrane and V_V of nuclei between days 13 and 16 of gestation, is not due to increased cell volume, since the cell membrane S_V showed no signigicant changes until day 19 of gestation.

6. CONCLUSION

Biochemical and morphological changes occur in the uterine epithelium in response to early pregnancy. Biochemical changes in maternal caruncular tissue includes alterations in the metabolism of protein, lipid and prostaglandin. Qualitative results demonstrate no morphological difference in uterine epithelium between day 13 of estrous and day 13 of gestation. Both feature columnar epithelium, with euchromatic nuclei, numerous nucleoi, and randomly dispersed lipid droplets. On day 16 of gestation lateral cell membrane separation begins and by day 22 of gestation a majority of the fetal-maternal junction is composed of syncytial masses.

In this study mean V_V and S_V of nuclei and nuclear membranes significantly decreased between days 13 and 16 of gestation. This decrease is not due to increased cell volume because Sv of cell membranes did not change. If a change would have occured in Sv of cell membrane one could expect a change in the volume of the cell assuming surface area remained unchanged.

The significant decrease in Sv of smooth endoplasmic reticulum (SER) between days 13 of estrous and day 13 of gestation followed by a significant increase between days 13 and 16 of pregnancy may be indicative of changes in SER activity such as prostaglandin synthesis.

Surface per unit volume of rough endoplasmic reticulum increased significantly between day 13 and 16 of gestation which may indicate increased RER activity.

In the present study the decreasing volume fraction of lipid droplets between days 13 and 16 of gestation may be due to utilization of the triglyceride as a component of the histotroph which is used by the embryo as a source of nutrition, prior to implantation. Mitochondrial activity may not be pregnancy dependent since V_V of mitochondria and S_V of mitochondrial cristae did not change between treatment groups.

Qualitative results observed indicate syncytial mass formation does not occur prior to day 19 of gestation. Sections of pregnant day 19 caruncular epithelium not attached to the trophoblast showed lateral cell separation, while day 19 maternal epithelium attached to the trophoblast showed syncytial mass transformation. This study concludes from qualitative and quantitative results that the syncytium is formed in response to trophoblastic binucleate cell fusion with uterine epithelium. These granules were seen in micrographs of transforming cells.

Surface per unit volume of golgi complex within caruncular uterine epithelial cells did not change between treatment groups, indicating no increase in activity. Volume fraction of secretory granules significantly increased between days 16 and 19 of gestation and granules were present in micrographs of the syncytial mass. Therefore from stereological measurements and qualitative

observations, the orgin of secretory granules may be from trophoblastic binucleate cells.

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