ULTRASTRUCTURAL AND SECRETORY ACTIVITY OF THE PORCINE UTERUS DURING THE ESTROUS CYCLE AND EARLY PREGNANCY: EFFECTS OF PREMATURE EXPOSURE TO EXOGENOUS ESTROGEN

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CHAPTER I

INTRODUCTION

On the average, pigs ovulate 14-16 ova, but litter size averages only about ten pigs per litter (Perry and Rowlands, 1962; Pope and First 1985). Embryonic mortality, characterized by the difference between the number of corpora lutea (CL) and the number of live embryos present, averages approximately 30% prior to day 40 of pregnancy with an additional loss of 10-20% evident after day 40 of gestation (Flint et al., 1982; Pope and First, 1985).

Maternal recognition and establishment of pregnancy involves the prolongation of the functional lifespan of the CL. In the pig, maintenance of CL function and the establishment of pregnancy depends upon conceptus estrogen production (Bazer and Thatcher, 1977; Heap et al., 1979). Estrogen functions to alter the direction of PGF2a secretion by the uterus from an endocrine (towards the uterine vasculature) to an exocrine (towards the uterine lumen) direction, thus preventing PGF2a from reaching the ovary and causing luteolysis. Estrogen release by the conceptus signals the maternal system on approximately day 11-12 thus extending CL function and maintaining pregnancy.

During early development in swine, blastocysts undergo a rapid, morphological transformation from spherical to filamentous form by day 12 of pregnancy (Anderson, 1978; Geisert et al., 1982b). Following trophoblast elongation and initiation of estrogen production, conceptus attachment occurs between days 13 and 18 of gestation (King et al., 1982).

Placentation in the pig involves a noninvasive interdigitation of uterine and trophoblastic microvilli (Dantzer, 1985) as well as alterations in the uterine epithelial glycocalyx. The glycocalyx is a carbohydrate coat present on the surface of most cells and has been implicated in numerous cell-cell interactions (Alberts et al., 1983). Dantzer (1985) demonstrated that porcine uterine epithelium is covered by a thick glycocalyx which becomes reduced in thickness during attachment and microvillar interdigitation. The uterine epithelial glycocalyx appears to function during placentation by extending fibers towards the trophoblast, thus anchoring the conceptus (Keys and King, 1990). A reduction in cell surface negativity of the mouse trophoblast (Jenkinson and Searle, 1977) and the endometrial epithelium of the rat (Hewitt et al., 1979) occur during implantation. Therefore, the uterine epithelial glycocalyx appears to play an important role in placental attachment.

As mentioned earlier, conceptus-derived estrogen is the signal for maternal recognition of pregnancy in swine,

however premature exposure of gilts to estrogen results in early embryo death. Long and Diekman (1986) demonstrated that addition of zearalenone, an estrogenic mycotoxin, to the feed of sows between days 7-10 of gestation resulted in complete embryonic mortality. Pope et al. (1986) reported that administration of exogenous estrogen to gilts on days 9 and 10 of gestation also resulted in complete embryo death by day 30 of pregnancy. Further studies by our laboratory have revealed that administration of estradiol valerate to gilts on days 9 and 10 of gestation resulted in degenerating conceptuses by day 16 of pregnancy (Morgan et al., 1987; Greis et al., 1989).

The purpose of the this thesis is (1) to examine surface and ultrastructural changes in uterine luminal epithelium during trophoblast attachment and characterize alterations associated with embryonic mortality due to early exogenous estrogen administration and (2) to characterize changes in uterine secretory activity which may be associated with the establishment of pregnancy in the pig.

CHAPTER II

LITERATURE REVIEW

Anatomy and Histology of the Sexually Mature Gilt Reproductive Tract

The reproductive system of the gilt consists of paired ovaries, oviducts, and uterine horns which open into a single uterine body, cervix, and vagina. The organs of the reproductive tract are suspended within the abdominal cavity by the broad ligament which consists of three major regions: the mesovarium, the mesosalpinx, and the mesometrium which support the ovaries, oviducts, and the uterus, respectively. The following section of this literature review will describe the functional anatomy and histology of the gilt reproductive system.

The Ovaries

The ovary functions as both an exocrine (development and release of oocytes) and endocrine (synthesis and secretion of steroid and protein hormones) organ. Since

pigs are multiovulators (Corner, 1921; Perry and Rowlands, 1962), the ovaries appear as a cluster of numerous protruding follicles and corpora lutea (Hafez, 1987). All functional ovaries contain numerous small (2-5 mm) follicles and approximately 10-20 of these enlarge to a preovulatory size of 8-12 mm in diameter during the proestrous and estrous phases (Corner, 1921; Anderson, 1987).

Akins and Morrissette (1968) reported that there are three major morphological phases during the ovarian cycle. During Phase 1, which extends from ovulation through day 8, corpora lutea (CL) develop and attain maturity while corpora albicans (CA) from the previous cycle continue to regress. Follicular development is also evident during this phase. In Phase 2, days 9 through 14, CL attain maximum size while follicular size remains unchanged. Phase 3 begins approximately at day 15 when CL become slightly ischemic. Regression of CL and increased follicular development are characteristic of this phase. The results of Akins and Morrissette (1968) support the earlier results of Corner (1921) and Perry and Rowlands (1962).

Histologically, the ovary consists of an inner medulla and an outer cortex and is covered by a simple cuboidal surface epithelium called the germinal epithelium. The medulla is loose connective tissue consisting of collagen and elastic fibers. It contains blood vessels, lymphatics, and nerves as well as some embryonic remnants (Banks, 1986; Hafez, 1987). The embryonic remnants, the rete ovarii, are

cords of epithelial cells (Banks, 1986). During the estrous cycle, the cortex of the porcine ovary contains numerous follicles, corpora hemorrhagica, corpora lutea, and corpora albicans (Hafez, 1987).

The mature ovarian follicle (Graafian follicle) is a fluid filled structure containg the primary oocyte. The follicle is composed of three major cell layers: theca externa, theca interna, and granulosa cells. The granulosa cell layer is approximately 6-9 cells and 0.13-0.17 mm in thickness while the theca interna is about 0.09-0.10 mm thick (Corner, 1919). The two theca layers are separated from the granulosa layer by a basement membrane. The primary oocyte is immediately surrounded by a single layer of simple columnar cells, the corona radiata, and is supported by a mound of granulosa cells, the cumulus oophorus. For a more complete histological description of the follicle see Corner (1919).

At ovulation, the oocyte and follicular fluid are expelled resulting in collapse of the follicle. With collapse of the follicle, the cavity becomes filled with blood thus forming the corpus hemorrhagicum (Banks, 1986; Anderson, 1987).

Within 6-8 days, corpora lutea have become 8-11 mm in size and weigh approximately 350-450 mg (Anderson, 1987). The CL consist of two distinct cell types, the large and small luteal cells. Corner (1919) first demonstrated that large and small porcine luteal cells originate from the

granulosa and theca interna cells, respectively. The transformation of granulosa and theca interna cells into luteal cells is characterized by hypertrophy of the cells, changes in cytoplasmic organelles, and accumulation of lipid and lutein pigment (Peters and McNatty, 1980).

If pregnancy is not established, corpora lutea regress and are replaced by connective tissue, and become corpora albicans. Regression of the CL allows increased follicular growth and a return to estrus.

The Oviduct

The porcine oviduct is approximately 30-40 cm long (Rigby, 1968), extends from the anterior tip of the uterine horn to the ovary and may be divided into four major regions: the infundibulum, ampulla, isthmus, and utero-tubal junction (Beck and Boots, 1974).

The infundibulum is the funnel-shaped opening to the oviduct. Finger-like projections, the fimbria, surround the opening of the infundibulum and function to direct recently ovulated oocytes into the infundibulum (Hafez, 1987). The epithelium of the fimbria consists of numerous ciliated cells (Stalheim et al., 1975; Wu et al., 1976) which aid in movement of oocytes. The infundibulum merges posteriorly with the remainder of the oviduct, the ampulla and isthmus. These two areas are readily distinguished due to the dilated lumen of the ampulla compared to the constricted lumen of

the isthmus. The isthmus is connected to the uterus by the utero-tubal junction (UTJ). The UTJ of the pig is characterized by mucosal folds of the isthmus that end in narrow villi with their tips oriented toward the uterus (Anderson, 1928; Beck and Boots, 1974). Villi of uterine origin are also present and in combination with the oviductal villi form an arrangement of mucosal folds around the opening to the oviduct (Anderson, 1928; Beck and Boots, 1974). The wall of the oviduct consists of the tunica mucosa, tunica muscularis, and the tunica serosa. The lamina epithelialis and the lamina propria is a highly vascularized layer of loose connective tissue devoid of glands. The lamina epithelialis consists of a single layer of columnar cells, although this layer may also be pseudostratified in the pig (Banks, 1986). Ciliated, non-ciliated, and peg cells are present within this layer (Beck and Boots, 1974; Anderson, 1987). The tunica mucosa is highly folded, exhibiting primary, secondary, and tertiary folds in the infundibulum and ampulla. However, little folding is evident in the isthmus. The tunica muscularis consists of an inner circular and an outer longitudinal layer of smooth muscle. This layer is poorly developed in the ampulla, whereas the isthmus exhibits a highly developed tunica muscularis (Beck and Boots, 1974; Banks, 1986). The tunica serosa surrounds the oviduct and consists of mesothelium and connective tissue (Beck and Boots, 1974; Banks, 1986).

The Uterus

The uterus of the gilt is classified as bicornuate since it consists of two long uterine horns and a single short uterine body. Previous reports have demonstrated that the uterine horns are approximately 1.5 m in length while the uterine body measures about 5 cm long (Rigby, 1968; Corner, 1921). Similar to the oviduct, the uterus consists of the tunica mucosa, tunica muscularis, and tunica serosa. However, these layers are referred to as the endometrium, myometrium, and perimetrium, respectively in the uterus.

The endometrium is comprised of the lamina epithelialis and the lamina propria (Banks, 1986). The lamina epithelialis consists of two populations of simple, cuboidal epithelium: luminal and glandular epithelial cells (Corner, 1921; Stroband et al., 1986; Stroband and Van der Lende, 1990). Few ciliated cells are evident in the luminal epithelium, whereas ciliated cells become more numerous in the glandular epithelium (Corner, 1921; Stroband et al., 1986). Stroband et al. (1986) reported that epithelial cell height ranged from 20-30 mm, but did not show any variation during the estrous cycle. However, Keys and King (1989) demonstrated a range in cell height from 17-25 um between days 10-19 of the gilts estrous cycle with a significant increase in cell height from day 16 to day 19. The ultrastructure of the porcine uterine luminal epithelium between days 10-16 of the estrous cycle exhibit changes

indicative of increasing synthetic and secretory activity with a decrease in activity at day 19 (Stroband et al., 1986; Keys and King, 1989).

Uterine glands are branched, tubular structures which extend into the lamina propria where they may become extensively coiled at their terminal ends (Banks, 1986; Hafez, 1987). Similar to the luminal epithelium, the glandular epithelial cells exhibit characteristics of increased synthetic activity as the estrous cycle and pregnancy progress (Perry and Crombie, 1982; Geisert et al., 1982a; Stroband et al., 1986).

The lamina propria, or endometrial stroma, is a loose connective tissue consisting of fibroblasts, macrophages, neutrophils, and plasma cells (Corner, 1921). The connective tissue immediately below the surface epithelium is vascularized by a subepithelial capillary plexus which becomes extensive during pregnancy (Heuser, 1927). The peripheral connective tissue of the lamina propria is less cellular and contains the terminal ends of the uterine glands and large blood vessels.

The myometrium is arranged in a similar manner as described in the oviduct. The myometrium is composed of an inner circular and an outer longitudinal layer of smooth muscle. Between these two muscle layers is a network of blood vessels, lymph vessels, and nerves called the stratum vasculare (Banks, 1986; Hafez, 1987).

The perimetrium consists of mesothelium and a thin

layer of connective tissue. The perimetrium is continuous with the mesometrium.

The Cervix

The cervix of the gilt is approximately 10 cm long and separates the uterus from the vagina. It is primarily composed of dense, fibrous connective tissue with only small amounts of smooth muscle (Eckstein and Zuckerman, 1952; Hafez, 1987). The cervix of the gilt is characterized by a constricted lumen and interdigitating cervical pads arranged in a corkscrew fashion similar to the spiral tip of the boar's penis (Eckstein and Zuckerman, 1952).

The cervical epithelium consists of ciliated and non-ciliated secretory, columnar epithelial cells (Hafez, 1987) and is a non-glandular tissue (Eckstein and Zuckerman, 1952). During estrus, the cervical epithelium produces large amounts of thin, watery mucus in response to estrogen, whereas the cervical mucus becomes highly viscous and forms a cervical plug during pregnancy (Eckstein and Zuckerman, 1952; El-Banna and Hafez, 1972; Hafez, 1987).

The cervix functions as a sperm resevoir, facilitates sperm transport, and prevents bacterial invasion during pregnancy (El-Banna and Hafez, 1972; Hafez, 1987).

The Vagina, Vestibule, and External Genitalia

The vagina has a well developed tunica muscularis that consists of an inner circular and an outer longitudinal layer of smooth muscle. The surface epithelium is highly folded and is composed of non-glandular, stratified squamous epithelial cells (Banks, 1986; Hafez, 1987). In the gilt, the surface epithelium of the vagina increases in thickness at estrus and becomes thinnest at days 12-16 of the estrous cycle (Hafez, 1987). The vagina functions as the female organ of copulation, prevents bacterial invasion, and serves as the birth canal during parturition.

The vestibule is caudal to the vagina and the junction between these two regions is marked by the urethral opening. Similar to the vagina, the surface epithelium of the vestibule is stratified squamous (Banks, 1986). The vestibule also contains the glands of Bartholin which are branched, tubular mucus glands and produce a viscous fluid (Banks, 1986; Hafez, 1987).

The external genitalia, vulva, consist of the labia majora, labia minora, and the clitoris. The clitoris is homologous to the male penis and is composed of cavernous tissue, adipose tissue, and smooth muscle (Banks, 1986). During proestrus and estrus, the vulva redden and swell in response to estrogen production.

Endocrinology of the Estrous Cycle

Attainment of sexual maturity (puberty) occurs at approximately 7 months of age, but may occur as early as four months of age (Corner, 1921; Anderson, 1987). Onset of puberty is characterized by recurrent periods of sexual receptivity, estrus, which lasts approximatley 40-60 hours and is characterized by lordosis (Signoret, 1970). Behavioral changes associated with estrus are due to high levels of estradiol produced from the preovulatory follicle. Van de Wiel et al. (1981) indicated that prolactin may also be involved in the behavioral aspects of estrus. The gilt is polyestrous, cycling throughout the year, with cycle lengths ranging from 18-23 days with a mean interestrous interval of 21 days. Ovulation occurs 38-42 hours after the onset of estrus and requires about four hours (du Mesnil du Buisson et al., 1970). The estrous cycle can be divided into four stages: proestrus and estrus which comprise the follicular phase and metestrus and diestrus which compose the luteal phase.

During the late luteal and early follicular phases, the ovary contains a pool of approximately 50 follicles between 2-5 mm in diameter (Anderson, 1987). Experiments involving electrocautery of follicles (Clark et al., 1979) and unilateral ovariectomy (Coleman and Daily, 1979; Clark et al., 1982) have demonstrated that recruitment of preovulatory follicles occurs between days 14-16 of the

estrous cycle. Grant et al. (1989) demonstrated a progressive decrease in follicle numbers from days 16-21 of the estrous cycle supporting previous data (Clark et al., 1973) which suggested the presence of a physiological block during the follicular phase which prevents replacement of atretic follicles in the proliferating pool. Foxcroft and Hunter (1985) also indicated that the dominant follicles on day 16 of the estrous cycle have elevated granulosa aromatase activity, luteinizing hormone (LH) binding to granulosa and theca cells, and are already producing estrogen.

Studies on steroidogenesis in porcine preovulatory follicles (Evans et al., 1981; Haney and Schomberg, 1981) support the two-cell theory first proposed for the rat (Falck, 1959). Unlike the rat, porcine thecal cells synthesize and secrete estradiol (Evans et al., 1981; Haney and Schomberg, 1981) and due to the close proximity of the theca to ovarian capillaries, thecal estradiol production may be an important source of peripheral plasma estradiol (Evans et al., 1981). Porcine theca cells produce estradiol in quantities comparable to granulosa cells (Evans et al., 1981; Haney and Schomberg, 1981). The addition of androgens increased theca cell production of estrogen, but not to the extent observed in the granulosa cells (Evans et al., 1981; Stoklosowa et al., 1982; Tsang et al., 1985). Estrogen production was greater in theca and granulosa cell co-cultures compared to the estrogen produced from the

individual cell types (Evans et al., 1981; Haney and Schomberg, 1981; Stoklosowa et al., 1982). Granulosa cells are the primary source of progesterone but are unable to synthesize androgens (Evans et al., 1981; Tsang et al., 1982), while theca interna cells produce large quantities of androgens (Evans et al., 1981; Haney and Schomberg, 1981; Stoklosowa et al., 1982). Tsang et al. (1987) demonstrated that granulosa cells secreted predominantly progesterone, whereas theca interna cells secreted mainly 17*a*-hydroxyprogesterone and androstenedione. Inhibition of progesterone production resulted in the conversion of exogenous progesterone to 17*a*-hydroxyprogesterone and androstenedione in theca interna, but not granulosa cells. These results suggest that the enzymes required for conversion of progesterone to androstenedione (17*a*-hydroxylase and C17,20-lyase) reside within the theca interna cells, while aromatase activity is substantially higher in the granulosa cells (Tsang et al., 1987). These data support the original work by Bjersing and Carstenson (1967) which demonstrated a lack of 17*a*-hydroxylase in porcine granulosa cells. Therefore, current research supports the two-cell theory of follicular estrogen production in which androstenedione is produced in theca interna cells and transferred to granulosa cells where it is aromatized to estradiol.

Estradiol production by the dominant preovulatory follicles results in the LH surge and ovulation. Coincident

with declining progesterone levels and maturation of preovulatory follicles, peripheral plasma estradiol concentrations begin to increase between days 16-18 of the estrous cycle reaching a peak of approximately 40 pg/ml about 2 days prior to estrus (Guthrie et al., 1972). Flowers et al. (1991) also demonstrated an estradiol increase concomitant with declining progesterone, but prior to increasing LH concentrations suggesting that the decrease in progesterone may initiate follicular maturation, not the increase in LH. This sustained level of estradiol exerts a positive feedback on the hypothalamus and anterior pituitary resulting in the LH surge (Van de Wiel et al., 1981). The surge release of LH from the anterior pituitary occurs about 40-42 hours prior to ovulation (Liptrap and Raeside, 1966; Foxcroft and Van de Wiel, 1982) and reaches a peak level of 4-6 ng/ml approximately 12 hours after the initiation of the LH surge (Van de Wiel et al., 1981).

Between days 16-20 of the porcine estrous cycle, increasing levels of estradiol are associated with decreasing concentrations of follicle stimulating hormone (FSH) (Van de Wiel et al., 1981). The concentration of FSH becomes minimal approximately 1 hour before estradiol attains its maximum concentration; the subsequent decrease in estradiol results in a preovulatory surge of FSH 1-9 hours after the initial rise in LH (Van de Wiel et al., 1981). Maximum FSH levels ranged from 11-19 ng/ml between 1-5 hours after maximum LH concentration were attained. A

secondary rise in FSH occurs on day 2-3 of the estrous cycle and reaches peak levels of approximately 20 ng/ml coinciding with minimal estradiol concentrations.

Ovulation occurs 38-42 hours after the onset of estrus and may involve prostaglandins (for review see Ainsworth et al., 1990). Ainsworth et al. (1975) and Tsang et al. (1979) demonstrated a dramatic increase in intrafollicular levels of prostaglandins E_2 (PGE_2) and F_{2a} (PGF_{2a}) shortly before ovulation and reached a maximum near the time of follicle rupture. It has been shown that both theca interna and granulosa cells produce PGE_2 and PGF_{2a} *in vitro* (Evans et al., 1983; Ainsworth et al., 1984). Inhibition of prostaglandin synthesis with indomethacin suppressed the preovulatory rise in prostaglandins and blocked ovulation (Ainsworth et al., 1979). Therefore, evidence suggests that prostaglandins play an important role in ovulation. For a more detailed review of follicular synthesis and intrafollicular actions of prostaglandins see Ainsworth et al. (1990).

Upon ovulation, the oocyte, follicular fluid, and the cumulus mass are expelled resulting in collapse of the follicle (Corner, 1919; Anderson, 1987). Under the influence of LH, the granulosa and theca interna cells of the recently ruptured follicle are transformed into luteal cells to form the corpus luteum (Corner, 1919; Anderson, 1987). The transformation of granulosa and theca cells into luteal cells involves cell hypertrophy, changes in cellular

organelles, and accumulation of lipid (Corner, 1919; Peters and McNatty, 1980). Within 6-8 days, the corpus luteum becomes fully formed attaining a diameter of 8-11 mm (Corner, 1919) and weighing approximately 350-450 mg (Anderson, 1987). Two distinct luteal cell types are present in the porcine corpus luteum: small and large luteal cells. Corner (1919) demonstrated that small luteal cells originated from theca interna cells, whereas large luteal cells derive from granulosa cells. Lemon and Loir (1977) demonstrated that both luteal cell types synthesize and secrete progesterone, but that large luteal cells produced significantly greater quantities of progesterone compared to small luteal cells *in vitro*. When LH was added to the luteal cell cultures, both cell types responded with increased progesterone production, however small luteal cells exhibited a greater response suggesting that small luteal cells contained a greater abundance of LH receptors (Lemon and Loir, 1977). Although LH is necessary for ovulation and luteinization, Brinkley et al. (1964) and du Mesnil du Buisson (1966) indicated that secretion of LH after ovulation does not appear necessary for normal CL development and function until after day 12.

Progesterone secretion increases with CL development, reaching a peak level of 35 ng/ml on day 12 of the estrous cycle (Guthrie et al., 1972). Plasma concentrations of progesterone closely correspond to the progesterone secretion into the ovarian venous blood throughout the

length of the estrous cycle (Masuda et al., 1967). During the luteal phase, when progesterone levels remain elevated, plasma concentrations of estrogen, FSH, and LH remain low (Guthrie et al., 1972; Parvizi et al., 1976). If pregnancy is not established, progesterone levels decline beginning on day 15 of the estrous cycle (Masuda et al., 1967) and reach basal levels (< 1 ng/ml) by day 17-18 (Guthrie et al., 1972). As progesterone levels decline, histological evidence of luteal regression becomes more apparent (Cavazos et al., 1969).

Loeb (1923) first demonstrated, via hysterectomy of guinea-pigs during the late luteal phase of the estrous cycle, that the uterus was the source of a luteolytic substance which caused regression of the CL since hysterectomy resulted in prolonged CL maintenance. Hysterectomy of gilts also resulted in prolonged CL maintenance (Spies et al., 1958; Anderson et al., 1961) suggesting that the luteolytic substance in pigs also originated from the uterus. Destruction or congenital absence of the uterine endometrium also resulted in maintenance of CL function (Anderson et al., 1969), indicating that the endometrium was the source of this luteolytic agent. Melampy and Anderson (1968) identified prostaglandin F_{2a} (PGF_{2a}) as the luteolytic agent originating from the uterine endometrium. Further research supported the hypothesis that PGF_{2a} is the luteolytic agent in swine. Administration of PGF_{2a} after day 12 of the

estrous cycle to cyclic (Hallford et al., 1974), pregnant (Diehl and Day, 1974), and hysterectomized (Moeljono et al., 1976) gilts resulted in regression of the CL, however administration of PGF_{2a} prior to day 12 of the cycle resulted in no luteolytic activity (Diehl and Day, 1974; Hallford et al., 1974). The porcine CL do not require additional pituitary support after the ovulatory LH surge until day 12-13 of the estrous cycle (du Mesnil du Buisson, 1966). This autonomous nature of the porcine CL may explain their refractoriness to PGF_{2a} prior to day 12 of the cycle. Henderson and NcNatty (1975) indicated that the CL remain refractory to PGF_{2a} until after day 12 at which time LH begins to dissociate from its luteal cell membrane receptors; prior to day 12, LH remained tightly bound to its receptors. Sensitivity of CL to PGF_{2a} is also enhanced by an increased number of high-affinity PGF_{2a} receptors on large luteal cells after day 12 (Gadsby et al., 1988, 1990).

Endometrial production of PGF_{2a} has been shown to increase after day 12 of the estrous cycle, corresponding to the increased sensitivity of the corpora lutea. Patek and Watson (1976, 1983) indicated that mid to late luteal phase endometrial tissue produced the highest levels of PGF_{2a} *in vitro*. Guthrie and Rexroad (1980) also demonstrated that endometrial PGF_{2a} secretion increased dramatically between days 14 and 16 of the estrous cycle. The increase in endometrial secretion of PGF_{2a} (Patek and Watson, 1976,1983; Guthrie and Rexroak, 1980) and plasma concentrations of

PGF_{2a} in the utero-ovarian vein (Gleeson et al., 1974; Moeljono et al, 1977) coincides with declining plasma progesterone levels from day 14-17 of the estrous cycle.

Due to the close apposition of the ovarian artery and the uterine vein, PGF_{2a}, produced by the uterine endometrium and secreted into the uterine venous system, is transferred into the ovarian artery (Del Campo and Ginther, 1973; Ginther, 1974). Kotwica (1980) also indicated that the lymphatic system may also be involved in this transfer in the pig. In the gilt, PGF_{2a} not only has a local effect upon the ipsilateral ovary via counter-current transfer, but also has a systemic effect resulting in luteolysis of the CL on the contralateral ovary (du Mesnil du Buisson, 1961; Anderson et al., 1966). The lower metabolism of PGF_{2a} in the lungs of swine compared to ruminants (Davis et al., 1979) may explain this systemic effect.

Henderson and McNatty (1975) demonstrated that binding of PGF_{2a} to its luteal cell receptor inactivates the adenyl cyclase system thus preventing continued progesterone production and activates lysosomal enzymes resulting in degradation of the corpora lutea. Morphological changes associated with CL regression include an increase in lysosomes, cytoplasmic disorganization, and invasion of connective tissue (Cavazos et al., 1969).

Pregnancy

Endocrinological Response During Early Pregnancy

Maintenance of pregnancy in the gilt is dependent upon the continued synthesis and secretion of progesterone from the corpora lutea. Removal of the ovaries at any stage of gestation results in pregnancy termination (du Mesnil du Buisson and Dauzier, 1957; Nara et al., 1981). Experiments utilizing luteectomy demonstrated that pregnancy can be maintained with as few as 2 to 5 CL present (Martin et al., 1977; Thomford et al., 1984). Similar studies have demonstrated that the CL are the primary source of progesterone during pregnancy. Nara et al. (1981) reported a decrease in plasma concentrations of progesterone following removal of CL, while Nase et al. (1985) demonstrated that luteectomy did not result in an increase in progesterone secretion by the placenta. Utilizing ovariectomized gilts, Ellicott and Dziuk (1973) demonstrated that a minimum of 6.0 ng/ml of progesterone is necessary to maintain pregnancy. Plasma progesterone concentrations increase to peak levels ranging from 20-30 ng/ml on day 12-14 of gestation and decline to 13-19 ng/ml by day 27-28 (Tillson et al., 1970; Robertson and King, 1974; King and Rajamahendran, 1985). Concentrations of progesterone remain constant through day 100 of pregnancy then decline to approximately 4.0 ng/ml at parturition

(Robertson and King, 1974; Knight et al., 1977).

Although the porcine corpus luteum does not require pituitary support until day 12-13 of the estrous cycle (du Mesnil du Buisson, 1966), hypophyseal luteotropic support is necessary for CL maintenance during pregnancy. Hypophysectomy after day 12-13 of gestation results in the termination of pregnancy (du Mesnil du Buisson et al., 1964; du Mesnil du Buisson and Denamur, 1969; Kraeling and Davis, 1974). Spies et al. (1967) demonstrated that administration of anti-ovine LH to gilts between days 25-29 of gestation resulted in CL regression and termination of pregnancy. Plasma concentrations of LH increase between days 13-18 of pregnancy to 2-3 ng/ml and then decline to basal levels for the duration of pregnancy (Parlow et al., 1964; Melampy et al., 1966). These results demonstrate that even though plasma concentrations of LH are low during pregnancy, LH is necessary for CL maintenance and for successful maintenance of pregnancy.

Plasma concentrations of estrogen differ between cyclic and pregnant gilts. Moeljono et al. (1977) reported that utero-ovarian venous plasma concentrations of estradiol were greater in pregnant compared to nonpregnant gilts between days 12-17. Guthrie et al. (1972) demonstrated that plasma concentrations of estrogen ranged from 16-28 pg/ml from days 3-24 of pregnancy. Peripheral plasma concentrations of estrone and estradiol remained low through day 70 of gestation at which point the concentrations of these

hormones increased to peak levels of 2-5 ng/ml and 400 pg/ml just prior to parturition, respectively (Robertson and King, 1974). Similar profiles of estrone and estradiol are evident in uterine venous plasma, although a small increase in these hormones was observed between days 20-30 of gestation (Knight et al., 1977; Robertson et al., 1985). Concentrations of estrone and estradiol were also higher in uterine lymph of pregnant compared to nonpregnant gilts between days 11-15 (Magness and Ford, 1982).

Conjugated estrogens appear to be more prevalent during gestation compared to the unconjugated estrogens. Estrone sulfate is the major conjugated estrogen present in the maternal peripheral plasma during pregnancy (Robertson and King, 1974). The plasma concentration of estrone sulfate exhibited fluctuating levels during gestation. Concentrations of estrone sulfate initially increase about day 16-20 of gestation, reach peak levels of approximately 3.0 ng/ml by day 30, decline to low levels of 35 pg/ml around day 50 of pregnancy followed by a second peak at 3.0 ng/ml a day before parturition (Robertson and King, 1974; Robertson et al., 1985). In contrast, estradiol-17b sulfate remained low throughout pregnancy with a slight rise in uterine venous plasma levels on days 26 and 28 of gestation. The uterine endometrium is the source of the conjugated estrogens. It has been shown that estrogens are localized in both the trophectoderm and yolk sac endoderm between days 10-16 with the yolk sac endoderm in the region of the

embryonic disc producing the greatest amounts (King and Ackerley, 1985; Bate and King, 1988). Estrone and estradiol produced by the porcine conceptus are metabolized to their conjugated forms by 17b-estradiol dehydrogenase and estrogen sulfotransferase activity present in the uterine endometrium and subsequently released into the maternal circulation (Pack and Brooks, 1974).

Plasma concentrations of PGF_{2a} in the utero-ovarian vein increase concomitantly with declining plasma progesterone levels from day 14-17 of the estrous cycle (Gleeson et al., 1974; Moeljono et al., 1977; Frank et al., 1977). In contrast, PGF_{2a} concentrations in the utero-ovarian vein were reduced in pregnant gilts (Moeljono et al., 1977). Frank et al. (1977) reported that induction of pseudopregnancy via administration of estradiol valerate to nonpregnant gilts between days 11-15 resulted in a similar pattern of PGF_{2a} secretion evident in pregnant gilts. Plasma concentrations of the PGF_{2a} metabolite, 15-keto-13,14-dihydro PGF_{2a} (PGFM) are highly correlated to levels of PGF_{2a}. Guthrie and Rexroad (1981) demonstrated an increase in PGFM during days 15-19 of the estrous cycle, whereas PGFM remained at basal levels during early pregnancy.

Embryonic Migration

Intrauterine migration of porcine embryos prior to

attachment to the uterine endometrium is critical for embryo survival. Porcine embryos move to the ampullary-isthmic junction, the site of fertilization, within a few hours after ovulation where they remain for approximately 36 hours (Hunter, 1974). The embryos enter the tips of the uterine horns about 48 hours after ovulation and are then at the 4-cell stage of development (Hunter, 1974). Transport of the porcine embryos through the oviduct appears to be hormonally mediated. It has been suggested that estrogen restricts passage of the embryo by constricting the isthmus; declining estrogen and rising progesterone levels after ovulation allow transport of the embryo through the isthmus (Dziuk, 1985). Once in the uterus, intrauterine migration of the porcine blastocysts occurs between day 7-12 of pregnancy with migration between horns first evident around day 8-9 of gestation (Dhindsa et al., 1967). By day 12 of gestation, the embryos fully occupy both uterine horns (Dhindsa et al., 1967) and are evenly dispersed (Dziuk, 1985). Polge and Dziuk (1970) demonstrated that embryonic migration ceases on day 12 of pregnancy.

The precise mechanism(s) of intrauterine migration of the porcine embryos remains unknown, however peristaltic contractions of the uterine myometrium appear to be involved. The contractile activity of the uterine myometrium may be influenced by conceptus secretory products. Pope and Stormshak (1981) first indicated that estradiol may be involved in embryo migration since

estradiol-impregnated silastic beads migrated further than cholesterol-impregnated beads (384 ± 96.1 versus 68.8 ± 28.6 cm, respectively). Further research also demonstrated that estradiol-impregnated beads migrated farther than beads containing cholesterol (Pope et al., 1982b, 1986). Increased blastocyst estrogen synthesis coincided with an increased frequency of myometrial contractions in vitro suggesting that blastocyst-derived estrogen may be responsible for intrauterine embryo migration (Pope et al., 1982a). Conceptus produced estrogen may also act upon the uterine myometrium indirectly via prostaglandins (Pope et al., 1982a). Pope and co-workers (1982b) have also suggested that histamine produced by porcine blastocysts may influence myometrial contractility thereby facilitating embryonic migration.

The equidistant distribution of the porcine blastocysts in the uterus by day 12 of gestation is essential for the the establishment of pregnancy. Polge et al. (1966) reported that a minimum of four embryos (two in each horn) must be present in the uterus to maintain pregnancy. Dhindsa and Dziuk (1968a) further demonstrated that embryos must be present in both uterine horns between days 10 and 12 of gestation in order to maintain pregnancy, however when embryos were flushed from one uterine horn between days 12-20, unilateral pregnancies (19/38) could be maintained after day 12 of gestation through at least day 30 of gestation. Studies utilizing uterine ligations have demonstrated that

as the proportion of the uterus that remains unoccupied increases, the less likely that the pregnancy will be maintained. Dhindsa and Dziuk (1968b) demonstrated that 50-60% of the pregnancies can be maintained when only one-eigth of the uterus is unoccupied, however when one-fourth is unoccupied only 20-30% can be maintained and when one-half of the uterus is unoccupied pregnancy fails to be established.

These data indicate that migration and the presence of porcine blastocysts by day 12 of gestation is critical for the establishment and maintenance of pregnancy.

Early Conceptus Development

Fertilization of porcine embryos occurs at the ampullary-isthmic junction with approximately 95-98% of the ova being fertilized (Perry and Rowlands, 1962; Oxenreider and Day, 1965). Two-celled embryos can be seen 17-20 hours after ovulation, although this stage of development is relatively short, lasting only 6-8 hours (Hunter, 1974). However, after the second cleavage, the embryo remains in the four-cell stage for 20-24 hours (Hunter, 1974). The embryo enters the uterus during the four-cell stage approximately 46-50 hours after ovulation (Oxenreider and Day, 1965; Hunter, 1974). Up to this stage of development, little RNA synthesis by the embryo is evident. At the eight-cell stage of development, nucleoli and increased

ribosome numbers can be observed (Stroband and Van der Lende, 1990) while RNA synthesis appears to commence during the four-cell stage (Freitag et al., 1988). These developmental changes indicate activation of the embryonic genome. Initial signs of compaction become evident at the eight-cell stage (Hunter, 1974) with junctional complexes forming at the compacted morula stage around day 5 of pregnancy (Barends et al., 1989).

In an extensive review of mouse early embryonic development, Johnson (1981) reported that a large amount of evidence suggests that the inner cells of cleavage and morula stage embryos will differentiate into the embryo proper, while outer cells become the trophectoderm. A similar pattern of development is observed in porcine embryos. Papaioammou and Ebert (1988) demonstrated that inner and outer cells can be distinguished as early as the 12-16 cell stage of develoment with the inner cells comprising approximately 14% of the cells at the morula stage of development.

By day 6-7 of pregnancy, porcine embryos exhibit a conspicuous blastocoele cavity and inner cell mass and thus have attained the blastocyst stage of development (Perry and Rowlands, 1962; Hunter, 1974) with the cell number at this stage being approximately 32-64 cells or greater (Hunter, 1974; Papaioannou and Ebert, 1988). Blastocysts hatch from the zona pellucida around day 7-8 of gestation (Perry and Rowlands, 1962; Hunter, 1974; Papaioannou and Ebert, 1988).

Just prior to hatching, inner cells attain a maximum of 25% of the total cell number (Papaioannou and Ebert, 1988).

After hatching, porcine blastocysts undergo distinct morphological changes. It has been reported that spherical, tubular, and filamentous embryos can be observed within the same uterine horn (Perry and Rowlands, 1962; Anderson, 1978; Geisert et al., 1982b). Geisert et al. (1982b) described the changes that occur in the porcine conceptus during the morphological transformation from the spherical to filamentous forms. Once the embryo hatches from the zona pellucida, the blastocyst increases in size to 10 mm in diameter by day 11-12 of gestation at a rate of .25 mm/hour. Shortly after attaining the 10 mm spherical stage of development, the porcine blastocyst rapidly elongates to a filamentous form at a rate of 30-45 mm/hour and reaches up to 150 mm in length within 2-3 hours. Geisert et al. (1982b) demonstrated that the increase in blastocyst diameter up to 10 mm involves cellular hyperplasia as indicated by an increase in the mitotic index, whereas elongation of the porcine conceptus to its filamentous form involves cellular remodeling. This cellular reorganization within the trophectoderm and endoderm results in the formation of an elongation zone, a dense band of cells which extends from the inner cell mass to the ends of the conceptus (Geisert et al., 1982b). Filapodia of endodermal origin are present between the endoderm and trophectoderm (Geisert et al., 1982b; Barends et al., 1989; Stroband and

Van der Lende, 1990) and may be involved with elongation of the porcine conceptus (Geisert et al., 1982b). Albertini (1987) suggested that the endoderm may induce the formation of actin filaments within the trophoblast which may then contribute to cellular reorganization during rapid elongation. Stage-specific changes in actin distribution within the trophectoderm have been shown to occur between days 8-13 of pregnancy (Mattson et al., 1990). Mattson et al. (1990) have suggested that these actin filaments may be involved in microvillar reorganization, epithelial transport, and elongation of the trophectoderm. Barends et al. (1989) also suggested that the endoderm induces the formation of the basal lamina by the trophectoderm. Richoux et al. (1989) demonstrated the presence of fibronectin and laminin during early conceptus development. These researchers first detected fibronectin, located at the interface of the trophectoderm and the endoderm, prior to endodermal cell migration, while laminin is produced by the endodermal tissue and accumulates between these two cell layers. Richoux and co-workers (1989) suggested that fibronectin allowed migration of endodermal and mesodermal cells, whereas laminin probably functioned to stabilize interactions between the extraembryonic cell layers. Fibronectin has also been shown to increase the development of individual blastomeres from 8-cell porcine embryos through the blastocyst stage of development *in vitro* (Saito and Niemann, 1991). The porcine conceptus continues to

elongate and will reach up to 1 meter in length by day 16-18 of gestation (Perry and Rowlands, 1962; Anderson, 1978). For a more detailed description of early embryonic develpment in the gilt, see Stroband and Van der Lende (1990).

Conceptus Secretory Activity

In order to establish pregnancy in the gilt, the corpora lutea must be protected from the luteolytic effects of PGF_{2a}. In the gilt, maintenance of pregnancy and nourishment of the conceptus depends upon endometrial secretions and selective transport of serum factors. Steroids, proteins, and prostaglandins produced by the developing porcine conceptus are essential for the establishment and maintenance of pregnancy in the gilt.

Steroids. The production of estrogens by the porcine conceptus was first reported by Perry et al. (1973), who demonstrated the production of estrone and estradiol-17b from androstenedıone and dehydroepiandrostenedione (DHA) between days 14-16 of gestation. These results provided evidence for the presence of aromatase, 17,20-desmolase, and 3-sulfatase within the porcine conceptus (Perry et al., 1973). Further research also demonstrated the conversion of androstenedıone, DHA, and testosterone to estrone and estradiol-17b by day 14-18 porcine conceptuses (Heap et al., 1975; Gadsby et al., 1980). Pig blastocycsts can also

utilize progesterone to synthesize estrogens. Fischer et al. (1985) indicated that porcine conceptuses, ranging from 7 mm spherical to tubular and filamentous forms, are able to convert progesterone to estrone and estradiol. Estrogen synthesis by the porcine conceptus has been localized to the trophectoderm and the yolk-sac endoderm with the yolk-sac endoderm producing substantially greater quantities (King and Ackerley, 1985). Estrone and estradiol-17b production by the yolk-sac endoderm is greatest in the region of the embryonic disc (Bate and King, 1988). Dantzer and Svenstrup (1986) reported that development of smooth endoplasmic reticulum observed in the basal region of the trophoblast was related to high levels of estrone, estradiol-17b and estrone sulfate.

The ability of the porcine uterine endometrium to produce only limited amounts of estrogen (Fischer et al., 1985) indicates that estrogen present in the uterine lumen reflects synthesis and secretion by the conceptus. Estrogen content in uterine flushings is greater in pregnant compared to cyclic gilts between days 12-18 (Zavy et al., 1980; Ford et al., 1982a; Geisert et al., 1982a; Stone and Seamark, 1985). The content of estrogen in uterine flushings durine early pregnancy exhibits a biphasic profile. Zavy et al. (1980) reported that estrogen concentrations in uterine flushings increase between days 10 and 12 of gestation, decline by day 15, and increase again on day 18 of pregnancy. A similar pattern of conceptus estrogen

production was demonstrated by Ford et al. (1982a) who reported that estrogen content peaked at day 13 and then declined by day 15 of gestation. Although levels of estrogen were lower on day 15 compared to day 13 of pregnancy, nadir concentrations of estrogen were actually observed on day 14 with levels increasing on day 15 of gestation (Stone and Seamark, 1985). Geisert et al. (1982a) demonstrated that uterine luminal content of estrogen increased with elongation of the porcine conceptus. Uterine flushings with tubular blastocysts had approximately a 4-fold greater content of estrogen compared to uterine flushings containing spherical blastocysts. Uterine estrogen content was even greater in flushings which contained filamentous conceptuses on day 12 of pregnancy, but sharply declined by day 14 (Geisert et al., 1982a). Similar concentrations of conjugated estrogens, estrone sulfate and estradiol sulfate, were also evident in uterine flushings from pregnant females (Geisert et al., 1982a). The sustained increase in estrogen secretion by the porcine conceptus after day 14 of gestation appears to be necessary for the maintencance of CL function beyond day 30 of gestation (Geisert et al., 1990 for review).

Porcine conceptuses are also capable of synthesizing and releasing catecholestrogens, the hydroxylated metabolites of estradiol. Mondschein et al. (1985) demonstrated that estrogen-2/4-hydroxylase (E-2/4-H) converts estradiol to its hydroxylated forms, 2-OHE_2 and 4-

OHE_2. Production of catechol estrogens from estradiol occurs between days 10-14 of pregnancy (Mondschein et al., 1985) during the period of conceptus elongation and establishment of pregnancy. The profile of catechol estrogen synthesis and release is similar to that observed for estradiol production during early pregnancy. Production of catechol estrogens increases from day 12-14 of gestation (Mondschein et al., 1985), declines by day 15 and then increases between days 16-18 of gestation (Chakraborty et al., 1989). It has recently been reported that porcine conceptuses are also capable of an alternative pathway for hydroxylation of estradiol. Chakraborty et al. (1990a) demonstrated the conversion of estradiol to 15*a*-hydroxyestradiol via estrogen 15*a*-hydroxylase. The profile of blastocyst estrogen 15*a*-hydroxylase is similar to that previously shown for E-2/4-H (Mondschein et al., 1985); estrogen 15*a*-hydroxylase activity is greatest on days 12-13 and lowest on day 15 of gestation (Chakraborty et al., 1990a). These authors suggest that 15*a*-hydroxyestradiol may be involved in the maternal recognition of pregnancy and implantation since the enzyme is localized primarily in the extraembryonic membranes and is active during these time periods. Since catecholestrogens are rapidly metabolized in the peripheral circulation, Mondschein et al. (1985) has suggested that they may act locally upon the uterus and/or conceptus. Decreased activity of catechol-O-methyltransferase in the conceptus during increased E-2/4-H

activity on days 11-13 is consistent with a possible role for catecholestrogens in the establishment of of pregnancy in swine (Chakraborty et al., 1990b).

Although previous studies suggested that the porcine conceptus does not produce progesterone, since removal of the ovaries or CL resulted in termination of pregnancy (du Mesnil du Buisson and Dauzier, 1957; Martin et al., 1977; Nara et al., 1981; Thomford et al., 1984), recent experiments suggest that the porcine placenta is capable of producing progesterone. Knight and Kukoly (1990) demonstrated that porcine placental tissue was capable of producing progesterone *in vitro*. Progesterone levels increased linearly between days 25-40, plateaued between days 40-50, rose to peak levels at day 100, and then decreased abruptly at day 110 of gestation. The production of progesterone by the porcine placenta appears to be limited by the amount of pregnenolone available since supplementation of pregnenolone to porcine placental tissue cultures enhances progesterone production (Knight and Jeantet, 1991). Heap et al. (1975) demonstrated that the porcine conceptus lacks the necessary enzyme, C_{20-22} desmolase, to convert cholesterol to pregnenolone, explaining the lack of placental progesterone production *in vivo*.

Proteins. Porcine conceptuses recovered during early pregnancy and cultured *in vitro* synthesize and secrete a wide variety of proteins. From days 10-12 of gestation, the

porcine conceptuses primarily produce a group of low molecular weight, acidic proteins (Godkin et al., 1982a; Gries et al., 1989). These proteins are characterized by a molecular weight of 20,000-25,000 daltons and an isoelectric point (pI) ranging from 5.6-6.2. Gries et al. (1989) demonstrated that this group of proteins was present through day 18 of pregnancy, but in much reduced quantity. Harney et al. (1990) has recently demonstrated that porcine conceptuses synthesize and secrete retinol-binding protein (Mr=19,000-22,000; pI=5.6-6.5) between days 10-15 of gestation. This conceptus derived retinol-binding protein (RBP) is a major secretory product during this time period. Utilizing immunocytochemical localization, Harney et al. (1990) has shown that RBP is present primarily in the trophectoderm and yolk sac. Expression of RBP messenger ribonucleic acid (mRNA) present in the porcine trophoblast increased from day 12 to day 21 and was greater than RBP mRNA in the chorioallantoic placenta which was present at low levels from days 30-90 and absent between days 105-112 of pregnancy. Harney and co-workers (1990, 1991) suggest that secretion of RBP by the porcine conceptus may be important for local transport of retinoids to the developing conceptus and may play an important role in conceptus development throughout pregnancy. Cross and Roberts (1988) have demonstrated that porcine conceptuses secrete a group of proteins with a molecular weight of Mr=24,000 daltons and a pI=5.2-5.6. Characterization of this latter group of

proteins revealed the presence of antiviral activity and a sequence homology to alpha and gamma interferons (La Bonnardiere et al., 1991). Ovine (Godkin et al., 1982b) and bovine (Helmer et al., 1987) conceptuses also produce a similar group of proteins during early pregnancy. The ovine and bovine polypeptides, ovine (oTP-1) and bovine (bTP-1) trophoblast protein-1, have a high sequence homology with alpha-interferons (Imakawa et al., 1987,1988) and are involved in the establishment of pregnancy in ruminant species. Mirando et al. (1990) demonstrated that proteins with antiviral activity are produced by porcine conceptuses from days 10-15 of pregnancy. These proteins were low during the period of establishment of pregnancy, days 10-12, but increased at days 14-15. La Bonnardiere et al. (1991) has recently shown that conceptus produced antiviral proteins are a combination of interferon-alpha and interferon-gamma like proteins. It does not appear that the conceptus derived antiviral proteins are involved with the establishment of pregnancy and extension of CL lifespan in the pig since the intrauterine infusion of porcine conceptus secretory proteins did not lengthen the interestrous interval in gilts (Harney and Bazer, 1989) and porcine antiviral proteins are produced after the period of maternal recognition of pregnancy in the gilt (Mirando et al., 1990). Since interferon-gamma is an immune interferon produced by T lymphocytes (Stobo et al., 1974), the apparent production of this interferon by the porcine conceptus suggests that these

proteins may be involved in some immunological aspects of pregnancy (La Bonnardiere et al., 1991).

After day 13 of gestation, the pattern of protein secretion by the porcine conceptus is altered. The major proteins secreted between days 13-16 of pregnancy exhibit molecular weights ranging from 35,000-50,000 daltons and have pI's in the basic range (Godkin et al., 1982a; Powell-Jones et al., 1984). Gries et al. (1989) reported that porcine conceptuses produce two major groups of polypeptides during early gestation. One of these proteins (Mr=40,000; pI=7.9-9.0) increases between days 14-18 of gestation; while the other polypeptide (Mr=22,000; pI=6.2) was present in diminishing quantities from days 12-18 of gestation. Baumbach et al. (1988) demonstrated that day 14-17 porcine conceptuses produce a basic protein with a Mr=43,100 and a pI>7. Utilizing anti-sera to this protein, Baumbach et al. (1988) showed that this basic protein was present in the conceptus trophectoderm at day 11 of gestation. It has been recently been demonstrated that this basic protein has sequence similarity to human cellular fibronectin and is antigenically similar to both human plasma and porcine embryonic fibronectin (Baumbach et al., 1991).

The serine protease, plasminogen activator (PA) has been demonstrated to be released by porcine blastocysts in culture (Fazleabas et al., 1983). Fazleabas et al. (1983) reported that the release of PA is biphasic in nature; conceptus production of PA increased from days 10-12,

declined to day 13, and again increased from days 14-16 of gestation. Plasminogen activator has been implicated in tumor invasiveness and migration (Ossowski et al., 1973) as well as tissue remodeling (Beers et al., 1975; Ossowski et al., 1979). The high level of PA production by the porcine conceptus coincides with early tissue remodeling and a later phase of tissue proliferation suggesting that PA may be involved in conceptus elongation in swine (Fazleabas et al., 1983).

The presence of growth factors in porcine conceptus tissue has also been reported. Insulin-like growth factor-1 (IGF-1) content in porcine conceptus and uterine flushes increased from day 8, reaching a peak at day 12, and then declined at day 14 of gestation (Letcher et al., 1989). Levels of IGF-1 mRNA were also evident in conceptus tissue between days 12-16 of gestation, however no temporal changes were evident in the mRNA levels (Letcher et al., 1989). The amount of IGF-1 and IGF-1 mRNA present in conceptus tissue was much lower than that observed in the uterine endometrium. Since conceptus IGF-1 mRNA remained constant, the changes in IGF-1 levels in uterine flushings primarily reflect endometrial synthesis and secretion whereas the change in conceptus IGF-1 content may reflect uptake of IGF-1 from uterine secretions (Letcher et al., 1989). Porcine conceptuses respond to IGF-1 in culture with an increase in cytochrome P_{450} aromatase activity, though this response is stage dependent since day 12 conceptuses respond while day

10 conceptuses fail to respond (Hofig et al., 1991). These results suggest that IGF-1 may act through an autocrine/paracrıne pathway to modulate conceptus steroidogenesis. Zhang et al. (1991) reported that porcine conceptuses contain high affinity epidermal growth factor binding sites between days 9-13 of gestation. The possible role(s) of growth factors upon conceptus growth and development in the piq require further elucidation.

Prostaglandins. Both PGF_{2a} and PGE_2 are produced by porcine conceptuses. Watson and Patek (1979) reported that porcine embryos secrete PGF_{2a} *in vitro* between days 16-22 of gestation. Utilizıng radiolabelled arachidonic acid, Lewis and Waterman (1983) demonstrated that day 16 porcine conceptuses synthesize and secrete PGF_{2a}, PGE_2, and PGFM *in vitro* with PGE_2 being the predominant prostaglandin produced. Similar results were observed by Davis et al. (1983) who demonstrated that the content of PGE was greater than PGF on every day examined. Davis et al. (1983) also reported that phospholipase A_2, the rate limiting enzyme in prostaglandin synthesis, increases with conceptus age. Stone and co-workers (1986) reported that porcine blastocysts between days 4-8 of pregnancy produce primarily PGE_2, but also produce low quantities of PGF_{2a} and PGH_2. Culture of embryonal membranes from days 13, 16, and 19 of gestation produced more PGF_{2a} per gram of tissue than uterine endometrıal tissue indicating that the conceptus may also actively produce PGF_{2a} *in vivo* (Guthrie and Lewis,

1986).

It has been suggested that the synthesis and secretion of prostaglandins by the porcine conceptus may be involved in embryonic migration and conceptus elongation as well as alterations in uterine vascularity and blood flow during early pregnancy (Davis et al., 1983; Lewis and Waterman, 1983). Kraeling et al. (1985) indicated that treatment of gilts with indomethacin, an inhibitor of prostaglandin synthesis, from days 10-25 of pregnancy resulted in the inhibition of pregnancy. However, Geisert et al. (1986) demonstrated that indomethacin failed to block embryo development and elongation when administered between days 10-13 of gestation. These results suggest that prostaglandins may be involved in placental attachment after day 13 of gestation, but do not appear to play a role in early conceptus development and elongation (Geisert et al., 1986).

Maternal Recognition of Pregnancy

Prolongation of the functional lifespan of the CL is dependent upon a signal produced by the developing porcine conceptus. This phenomenon was originally described by Short (1969) and termed the "maternal recognition of pregnancy". Since then, maternal recognition and establishment of pregnancy in swine has been extensively reviewed (Bazer et al., 1982, 1984, 1986, 1989; Geisert et

al., 1990).

The period when porcine conceptuses signal the maternal system, thus extending CL function, has been determined by flushing embryos from the uterus on various days post-estrus. Dhindsa and Dziuk (1968a) reported that flushing of porcine conceptuses from one uterine horn on or before day 10 of gestation resulted in termination of pregnancy. However, flushing of embryos from day 12-20 of gestation resulted in maintenance of pregnancy suggesting that the critical period for pregnancy maintenance is between day 10 and 12 of gestation (Dhindsa and Dziuk, 1968a). Ford et al. (1982a) demonstrated that flushing conceptuses from the uterine horns on days 13 and 15 of gestation increased the interestrous interval by approximately five days, whereas flushing the uterus on day 11 failed to increase interestrous intervals. The time period critical for CL maintenance and establishment of pregnancy was further defined when flushing porcine conceptuses from the uterus on days 11, 12, and 13 resulted in extension of CL function for 3-13 days while flushing the uterus on day 10 did not extend CL function (van der Meulen et al., 1988). Therefore, the porcine conceptus signals the maternal system on approximately day 11-12, thus extending CL function and maintaining pregnancy.

Coincident with this time period, day 11-12 of gestation, the porcine embryo undergoes a rapid elongation from spherical to tubular and filamentous forms (Geisert et

al., 1982b) and initiates estrogen production (Geisert et al., 1982a). Estrogen, produced by the developing porcine conceptus, has been implicated as the signal for maternal recognition of pregnancy in swine. Several studies have demonstrated that administration of estrogen, either intrauterine or systemic, results in extension of CL function (Ford et al., 1982b; Saunders et al., 1983; Frank et al., 1977; Geisert et al., 1987; King and Rajamahendran, 1988). Intrauterine administration of estradiol benzoate between days 10-14 of the estrous cycle increased interestrous intervals to approximataely 28 days (Saunders et al., 1983). Placement of silastic beads containing estradiol-17b into the uterine lumen of gilts also resulted in estrous cycles of 24-28 days in duration (King and Rajamahendran, 1988). In a previous experiment, Ford et al. (1982b) demonstrated that CL weight and utero-ovarian venous progesterone concentrations on day 18 of the estrous cycle were greater in sows treated with estradiol compared to control sows, thus providing evidence that estradiol maintains luteal function. Although estradiol administration increases the interestrous interval to approximately 24-28 days, these estradiol treatments failed to extend CL function beyond 30 days. As previously discussed, conceptus estrogen production is biphasic in nature. Estrogen production by the porcine conceptus increases from day 10.5-12 of pregnancy, decreases between days 13 and 14, and increases again after day 15 of

gestation (Fischer et al., 1985). This biphasic profile of estrogen release is reflected in the estrogen content of uterine flushings (Zavy et al., 1980; Geisert et al., 1982a; Stone and Seamark, 1985). To determine the role biphasic estrogen production has in the duration of the interestrous interval, Geisert et al. (1987) administered estradiol benzoate at various times during the estrous cycle. Estradiol treatment on day 11 or days 14-16 extended the interestrous interval to approximately 28 days, whereas estradiol treatment on day 11 and days 14-16 extended CL function beyond 60 days (Geisert et al., 1987). Similar results were reported by Frank et al. (1977) who demonstrated that administration of estradiol from days 11-15 resulted in interestrous intervals greater than 60 days. During the secondary rise in conceptus estrogen production, the uterine endometrium exhibits an increase in the number of cells that express estrogen receptors on day 18 of gestation (Geisert et al., 1990). These results suggest that the secondary increase in estradiol production by the porcine conceptus is essential for complete establishment of pregnancy (Geisert et al., 1990). In contrast to these data, van der Meulen et al. (1991) reported that intrauterine administration of physiological doses of estradiol between days 11-15 did not extend CL function indicating that other substances may also be required for CL maintenance in the pig.

Maternal recognition of pregnancy in swine has been

hypothesized to be controlled by an estrogen-induced alteration of PGF_{2a} secretion (Bazer and Thatcher, 1977). This theory proposes that conceptus-derived estrogen alters the direction of PGF_{2a} secretion from an endocrine direction, towards the uterine vasculature, to an exocrine direction, towards the uterine lumen, thus preventing PGF_{2a} from reaching the CL and causing luteolysis. Studies designed to measure uterine luminal content of PGF_{2a} have further supported this theory. Frank et al. (1977) demonstrated that gilts treated with estrogen had higher levels of PGF_{2a} present in the uterine lumen compared to cyclic gilts between days 14-20 of the estrous cycle. Prostaglandin F_{2a} concentrations in uterine flushings were greater in pregnant compared to cyclic gilts suggesting that PGF_{2a} is sequestered in the uterine lumen of pregnant gilts (Zavy et al., 1980). Utilizing a bilateral perifusion system, Gross et al. (1988) was able to measure PGF_{2a} secretion by the luminal and myometrial surfaces of the porcine endometrium. Gross et al. (1988) demonstrated that PGF_{2a} secretion was greater from the luminal surface for day 12 and 14 pregnant gilts, whereas PGF_{2a} secretion was higher from the myometrial surface for day 10 pregnant and day 14 cyclic gilts, thus indicating that the shift in PGF_{2a} secretion from an endocrine to an exocrine direction occurs between days 10 and 12 of pregnancy. The shift in PGF_{2a} secretion is concomitant with initiation of conceptus estrogen production (Geisert et al., 1982a), however

alteration of PGF_{2a} secretion is not entirely a result of conceptus estrogen production.

Alteration of PGF_{2a} secretion toward the uterine lumen appears to involve interactions between estrogen and prolactin as well as possible interactions between estrogen and porcine conceptus secretory proteins (pCSP). Prolactin receptors have been dectected in the uterine endometrium of pigs (Dehoff et al., 1984; Young and Bazer, 1987, 1989). Young et al. (1990) demonstrated that prolactin receptors remain similar between days 8-15 in cyclic gilts. In contrast, prolactin receptors were similar between days 8-11 of gestation, increased on day 12, and remained elevated between days 14-30 of pregnancy (Young et al., 1990). Prolactin has been shown to enhance the uterine secretory response to exogenous estrogen administered on day 11 of the estrous cycle (Young and Bazer, 1988). This may be the result of an increase in prolactin receptors since administration of estradiol valerate increases endometrial prolactin receptors within six hours prior to the increase in uterine secretory activity (Young et al., 1990). Gilts treated with both prolactin and estradiol had higher levels of uteroferrin, glucose, and PGF_{2a} in uterine flushings compared to gilts that received only estrogen (Young et al., 1989). Similar results were observed by Gross et al. (1990) who demonstrated that neither estrogen nor prolactin individually altered PGF_{2a} secretion, however endometrium from estrogen-treated gilts perifused with prolactin

exhibited a shift in PGF_{2a} secretion toward the uterine lumen. Gross et al. (1990) also demonstrated that perifusion with calcium ionophore shifted secretion of PGF_{2a} toward the luminal surface suggesting that endometrial calcium release may be involved with alteration in PGF_{2a} secretion. Bazer et al. (1989) reported that endometrium removed from gilts 6-12 hours after estrogen treatment and then perifused with prolactin responded with a shift in PGF_{2a} secretion from an endocrine to exocrine direction within 30 minutes. These authors suggest that estrogen treatment may induce prolactin receptors in the uterine endometrium which allow prolactin to act upon the endometrium and induce calcium cycling across the epithelium.

Interactions between estrogen and pCSP may also be involved in the maternal recognition of pregnancy in swine. Initial studies indicated that pCSP do not appear to interact with estrogen to prevent luteolysis since intrauterine infusion of pCSP to gilts treated with a low level of estrogen between days 12-15 of the estrous cycle did not result in extended interestrous intervals (Harney, 1988; Harney and Bazer, 1989). In contrast to these results, Dubois and Bazer (1988, 1991) indicated that pCSP alter endometrial PGF_{2a} secretion. Perifusion of endometrium from cyclic and pseudopregnant gilts with pCSP resulted in greater luminal secretion of PGF_{2a} compared to myometrial secretion with the greatest response evident in

endometrium from pseudopregnant gilts. These results indicate that pCSP may enhance the ability of estrogen to shift PGF_{2a} secretion from an endocrine to an exocrine direction (Dubois and Bazer, 1988, 1991).

Present evidence supports the exocrine versus endocrine theory of maternal recognition of pregnancy in swine as originally proposed by Bazer and Thatcher (1977). Data indicate that prolactin and pCSP may mediate the role of conceptus-derived estrogen in the reorientation of PGF_{2a} secretion.

Comparative Placentation

Placentation is defined as the intimate fusion or apposition of fetal membranes with the maternal uterine endometrium for the exchange of nutrients (Mossman, 1937). Histological classification of the mammalian placenta (Grosser, 1909; cited by Steven, 1975) is based upon the number of tissue layers separating the fetal and maternal vasculature. In the simplest classification, the epitheliochorial placenta, the following six tissue layers are present: (1) fetal capillary endothelium, (2) fetal connective tissue, (3) fetal chorionic epithelium, (4) maternal uterine epithelium, (5) maternal connective tissue, and (6) maternal capillary endothelium. Based upon this classification scheme (Grosser, 1909), mammalian placentae are classified as either epitheliochorial, syndesmochorial,

endotheliochorial, or haemochorial. Steven (1975) noted that, unlike most of the other organs of the body, the placenta shows a wide variety of species-specific structural modifications. The purpose of the following review will be to briefly examine placentatıon in rodents (mice and rats), ruminants (cattle and sheep), and swine.

Rodents

Placentation. Utilizing Grosser's (1909) classification scheme, placentation in mice and rats is described as haemochorıal. Haemochorial placentation is characterized by the loss of all three maternal tissue layers while all three fetal layers remain intact. Observations by Enders (1965) demonstrated that the placenta of rodents may be more correctly classified as haemotrichorıal since three layers of trophoblast separate maternal blood from fetal capillaries. The trophoblast tissue layers enclose maternal blood spaces thus providing a labryinthine appearance of the placenta (Amoroso, 1952).

Implantation in rodents occurs eccentrically. This form of ımplantatıon involves the movement of the embryo into the crypts and folds within the uterine endometrium (Mossman, 1937; Amoroso, 1952). During the apposition phase of implantation, the embryo aligns itself in the proper orientation. The blastocyst implants in an antimesometrial orientation with the ınner cell mass oriented mesometrially

(Mossman, 1937; Amoroso, 1952). After apposition to the uterus, the blastocyst continues development of extraembryonic membranes and attaches to the uterus, while the uterine endometrium undergoes the process of decidualization.

Extraembryonic Membranes. Development of the rodent extraembryonic membranes is characterized by inversion of the embryonic germ layers. Rodent embryos develop both functional yolk-sac and chorio-allantoic placentae (Amoroso, 1952; Steven, 1975).

The yolk-sac placenta consists of the visceral and parietal walls and Reichert's membrane (Amoroso, 1952). The parietal wall, the bilaminar omphalopleure, consists of a layer of parietal endodermal cells and a layer of trophoblast, whereas the visceral wall, the vascular splanchnopleure, is composed of a layer of endoderm and mesoderm tissue (Wislocki and Padykula, 1953). In a recent review, Jollie (1990) described the development of the yolk-sac placenta in rodents. At day 7 of gestation, the endoderm differentiates and proliferates from the inner cell mass and lines the blastocoele cavity of the embryo, thus forming the parietal wall of the yolk sac. Expansion of the amniotic cavity separates the inner cell mass from the developing ectoplacental cone on day 8 of pregnancy. Formation of the visceral wall of the yolk sac results from inversion of a portion of the yolk sac that contains mesodermal tissue and movement of the embryonic disc away

from the ectoplacental cone. The mesodermal tissue proliferates between the layers of the visceral wall of the yolk sac to form a trilaminar structure. The area between the parietal and visceral walls of the yolk sac becomes the yolk sac cavity. By day 10 of pregnancy, the visceral wall of the yolk sac separates to form an extraembryonic coelom. On day 7-8 in mice (Batten and Haar, 1979) and day 10 in rats (Lambson, 1966), the mesodermal layer undergoes angiogenesis to vascularize the visceral wall of the yolk sac by a peripheral vitelline circulation.

Reichert's membrane has been defined as a hyaline membrane between the trophoblast and the endoderm of the bilaminar omphalopleure (Mossman, 1937). In an extensive histochemical study of Reichert's membrane in rats, Wislocki and Padykula (1953) demonstrated that Reichert's membrane is composed of mucopolysaccharides or glycoproteins and exhibits properties similar to compacted collagenous fibers. Further research has shown that Reichert's membrane is the basal lamina of the parietal yolk sac endodermal epithelium (Clark et al., 1975; Minor et al., 1976). Welsh and Enders (1987) reported that by mid-gestation in the rat, the peripheral trophoblast is extremely thin and highly fenestrated. Therefore, only Reichert's membrane separates maternal circulation from the yolk sac cavity suggesting a possible path for the transport of substances from the maternal circulation to the visceral endoderm (Welsh and Enders, 1987).

On days 8-10 of pregnancy in rodents, the allantois differentiates and evaginates from the hindgut region of the embryo into the extraembryonic coelom (Steven and Morriss, 1975; Jollie, 1990). The developing allantois fuses with the chorion of the ectoplacental cone and the allantoic blood vessels invade the chorionic endoderm thus forming the haemochorial chorio-allantoic placenta (Steven and Morriss, 1975; Jollie, 1990). The yolk-sac placenta decreases in significance as the chorio-allantoic placenta becomes established.

Two populations of trophoblast are present in the rodent conceptus, polar trophoblast and mural trophoblast. The haemochorial chorio-allantoic placenta is established from the polar trophoblast cells. The trophoblast cells at the embryonic pole of the embryo differentiates and proliferates to form the ectoplacental cone (Amoroso, 1952; Steven and Morriss, 1975; Peel and Bulmer, 1977). On day 7 of gestation, the ectoplacental cone invades into the uterine endometrium and destroys vascular endothelial cells (Amoroso, 1952). Invasion of the ectoplacental cone into the uterine endometrium results in maternal blood becoming enclosed in the trophoblastic tissue, therefore the ectoplacental cone contains lacunae of maternal blood (Amoroso, 1952; Steven and Morriss, 1975; Peel and Bulmer, 1977). Fusion of the allantois to the chorion and loss of the ectoplacental cavity results in the formation of the chorio-allantoic placenta.

The peripheral, mural, trophoblast cells of the rodent embryo are involved in the adhesion and invasion of the embryo into the uterine endometrium (Enders and Schlafke, 1967; Potts, 1968; Tachi et al., 1970). The mural trophoblast cells are initially extremely thin with flattened nuclei; they are subsequently transformed into giant cells (Enders and Schlafke, 1967; Potts, 1968). Bevilacqua and Abrahamsohn (1988) demonstrated that transformation of flat trophoblast cells into giant cells is accompanied by an increase in their content of ribosomes, rough endoplasmic reticulum, golgi complexes, lysosome-like bodies, and heterophagosomes. Transformation into giant cells is associated with their ability to invade the uterine endometrium (Bevilacqua and Abrahamsohn, 1988).

Attachment. Attachment of the embryo to the uterine endometrium involves two processes, adhesion and invasion. Several comprehensive studies have examined the attachment phase of implantation in rodents (Enders and Schlafke, 1969; Tachi et al., 1970; Schlafke and Enders, 1975).

Adhesion of the trophoblast to the uterine luminal epithelium is one of the initial events involved in implantation. Using estrogen-conditioned uterine luminal epithelium which is necessary for implantation in rats, Nilsson (1967) first suggested that an increased adhesiveness of an unspecific type may be involved in attachment of mouse and rat embryos. Pinsker and Mintz (1973) originally suggested that changes in the cell surface

glycoproteins of mouse embryos may be involved in adhesion to the uterine luminal epithelium. These authors demonstrated that recovery of labeled surface material from blastocyst stage embryos was greater than that recovered from cleavage stage embryos and that the label was incorporated into the higher molecular weight components. These results suggest that an early alteration of the cell surface near the blastocyst stage of development may be responsible for the stage-specific ability of mouse embryos to attach to the uterine endometrium. Glycoproteins present on most cell surfaces are involved in cellular recognition and adhesion (Luft, 1976). The glycocalyx, a carbohydrate rich coat that covers the surface of most cells, has been implicated in cell-cell interactions (Alberts et al., 1983). Glycoproteins associated with the glycocalyx appear to be involved with trophoblast attachment to the uterine endometrium (Chavez and Enders, 1982; Richa et al., 1985; Chavez, 1986). Utilizing lectin binding techniques, several studies have demonstrated that significant alterations occur in the blastocyst and endometrial cell surface carbohydrate composition prior to implantation in mice (Chavez and Enders, 1981, 1982; Lee et al., 1983; Chavez and Anderson, 1985; Azuma et al., 1991). Temporal changes in the binding of Ricinus communis agglutinin-I (RCA-I) and peanut agglutinin (PNA) were observed on mouse embryos, however these changes could not be conclusively implicated in adhesion of the embryo to the uterus (Chavez and Enders,

1981). Chavez and Enders (1982) subsequently demonstrated the dissappearance of Dolichos biflorus agglutinin (DBA) binding sites from mouse trophoblast during the time of blastocyst adhesion. Recent results have demonstrated that certain galactose and/or N-acetylglucosamine glycoproteins on the cell surface of mouse trophoblast giant cells change from day 8.5 to day 10.5 of gestation in association with implantation (Azuma et al., 1991). Lee et al. (1983) and Chavez and Anderson (1985) demonstrated that the uterine luminal epithelium also undergoes alterations in cell surface glycoproteins prior to implantation and during pregnancy.

Alterations in the surface charge of the blastocyst and endometrium in rodents has also been implicated in blastocyst adhesion to the uterine epithelium. Jenkinson and Searle (1977) demonstrated that the mouse trophoblast contains negatively charged surface groups which are reduced at implantation. Similar results were observed by Hewitt et al. (1979) on the rat uterine epithelial surface. These authors demonstrated a reduction of polycationic ferritin binding between days 2 and 6 of gestation in rats. A reduction of blastocyst and endometrial cell surface negativity may facilitate adhesion of the embyro to the uterine luminal epithelium.

Glycosyltransferases may also be involved in adhesion (Roseman, 1970). Glycosyltransferases, enzymes that catalzye the addition of sugars to oligosaccharadies, would

function as a bridge between the surface they reside on and the apposing sugar on the adjacent cell surface (Roth et al., 1971, Roth and White, 1972). Dutt et al. (1987) have demonstrated that cultured uterine epithelial cells from mice synthesize lactosaminoglycans and that these polysaccharides appear to be involved in a galactosyltransferase-dependent cell adhesion system in the uterus. The lactosaminoglycans are synthesized in response to estrogen stimulation (Dutt et al., 1988), however estrogen alone appears insufficient to induce synthesis of lactosaminoglycans by immature mouse uterine epithelial cells (Carson and Tang, 1989). It has been recently reported that fucosylated lactosaminoglycans are present on the apical surfaces of mouse uterine luminal epithelium and may be involved in the adhesion of mouse blastocysts to the uterine endometrium (Kimber et al., 1988; Lindenberg et al., 1988).

Heparan sulfate proteoglycans are also present on the cell surface of preimplantation blastocysts and uterine epithelium in mice (Farach et al., 1987; Tang et al., 1987). Farach et al. (1987) demonstrated that heparan sulfate proteoglycans can mediate attachment and outgrowth of mouse embryos on uterine epithelial cells. Subsequent research has shown that mouse uterine epithelial cells cultured in vitro express high-affinity binding sites that bind heparin/heparan sulfate (Wilson et al., 1990). These authors demonstrated that these heparin-binding sites were

associated with firmly attached, cell surface components and that the majority of these binding sites were located basally with only 9-14% present on the apical surface of polarized epithelial cells.

The second process of attachment involves invasion of the blastocyst into the uterine endometrium. In the early stages of attachment in the rat, day 5 of gestation, the trophoblast cells exhibit an intimate association with uterine epithelial cells as shown by the presence of interdigitating microvilli (Enders and Schlafke, 1967). By day 6 of pregnancy, microvilli were absent from uterine epithelial cells and the formation of tight junctions maintained contact between the trophoblast and the uterine epithelial cells (Enders and Schlafke, 1969; Tachi et al., 1970). Schlafke and Enders (1975) described implantation in the mouse and rat as "Displacement Implantation" since invasion of the trophoblast results in displacement of uterine epithelium. At approximately day 7 of gestation, the displaced uterine luminal epithelial cells show varying degrees of degeneration (Enders and Schlafke, 1967). El-Shershaby and Hinchliffe (1975) demonstrated that degeneration of mouse uterine epithelial cells was first noticeable at the ultrastructural level at 113 hours post coitum. This initial degeneration was characterized by the presence of autophagosomes, vacuoles that contained granular material, membranous structures, and possibly deteriorated mitochondria. At 105 hours post mating, an increase in the

size of dense lysosomal bodies was evident in viable epithelial cells (El-Shershaby and Hinchliffe, 1975). These results indicate that uterine epithelial cells undergo autolytic breakdown around the invading blastocyst. Parr et al. (1987) demonstrated that degenerating uterine epithelial cells at implantation sites were characterized by surface blebbing, shrinkage, cell fragmentation, condensation of chromatin, and ındentation and fragmentation of nuclei. These characterıstics suggest that uterine epithelial cell death during implantatıon in mice and rats is due to apoptosis and not necrosis (Parr et al., 1987). The degenerating uterine luminal epithelial cells of mice and rats are phagocytosed by the embryonic trophoblast (Finn and Lawn, 1968; Enders and Schlafke, 1969; Tachi et al., 1970; Schlafke and Enders, 1975). Bevilacqua and Abrahamsohn (1988) reported that transformation of flat trophoblast cells into giant trophoblast cells in mice is associated with cell activation and acquistion of phagocytic capabilities. In the early stages of invasion, day 7 of gestation, the trophoblast does not penetrate beyond the basal lamina of the uterine luminal epithelial cells (Enders and Schlafke, 1967, 1969; Tachi et al., 1970; Schlafke and Enders, 1975). By day 8 of gestation, the basal lamina was absent and trophoblast cells were in intimate contact with uterine stromal cells (Tachi et al., 1970). Schlafke and Enders (1975) have suggested that this pause in trophoblast invasion may represent a tıme when trophoblast growth is

undergoing alterations. Schlafke et al. (1985) demonstrated the presence of decidual cell flange-like processes that penetrate the basal lamina and form a flattened expansion of cytoplasm beneath the rat trophoblast. These data suggest that the decidual cells are responsible for disruption of the basal lamina and expansion of the implantation chamber (Schlafke et al., 1985).

It has been hypothesized that the action of proteases may be involved in implantation (see Denker, 1980, 1981, 1982 for reviews). Several trypsin- and chymotrypsin-like enzymes have been detected in mouse blastocysts (Dabich and Andary, 1976). Dabich and Andary (1974) also demonstrated that intrauterine administration of proteinase inhibitors resulted in embryonic loss suggesting that proteinases may be involved in implantation in mice. The plasminogen activator-plasmin system may also be involved in mouse embryo implantation. Strickland et al. (1976) reported that plasminogen activator increased in cultured mouse trophoblast during implantation. These authors demonstrated that maximal plasminogen activator activity was present at a time equivalent to epithelial penetration (Stickland et al., 1976). Another group of proteolytic enzymes that may be involved in rodent implantation is the cathepsins. Cathepsins are lysosomal cysteine proteinases that may be the most active proteinases in the body (Barrett and Kirschke, 1981). Elangovan and Moulton (1980a) demonstrated that the level of cathepsin D in rat uterine luminal

epithelium decreased during blastocyst implantation. The rate of cathepsin D synthesis in implantation sites was only half of the rate observed in inter-implantation areas (Elangovan and Moulton, 1980a). Elangovan and Moulton (1980b) also demonstrated that progesterone specifically increased the synthesis of cathepsin D to maximal by 6 hours post-injection and that progesterone stimulated an increase in cathepsin D within the uterine endometrium as observed via immunohistochemical localization. Together, these data suggest that lysosomal cathepsin D may be involved in autolysis of the uterine luminal epithelium during blastocyst implantation in rats (Elangovan and Moulton, 1980a,b). Cathepsins have also been implicated in blastocyst implantation in species other than rodents. Recent studies have demonstrated that cathepsin L is present in uterine glandular epithelial cells (Verhage et al., 1989) and in uterine flushings (Li et al., 1991) of the cat. These results (Verhage et al., 1989; Li et al., 1991) and the proteolytic activity of cathepsin L as well as its affinity for collagen (Kirschke et al., 1982) and elastin (Mason et al., 1982) suggest that cathepsin L may be involved in implantation in cats.

During adhesion and invasion of the blastocyst into the uterine endometrium, the uterine stroma undergoes decidualization. This process involves differentiation of stromal cells in preparation for blastocyst implantation.

Decidualization. Initiation of decidualization occurs

on approximately day 6 of gestation, about 24 hours after attachment of the blastocyst to the uterus (Enders and Schlafke, 1967; Finn, 1982). One of the first signs of decidualization is edema in the uterine stroma as a result of an increase in vascular permeability (Finn, 1982). Proliferation of the decidua results primarily from cell division and enlargement of stromal fibroblasts as they transform into decidual cells (Abrahamsohn, 1989). Enders and Schlafke (1967) demonstrated that stromal fibroblasts accumulate fibrillar material and glycogen during decidualization. The decidua can be separated into antimesometrial and mesometrial decidua. The antimesometrial decidua is first evident below the basal lamina of the implantation sites (Welsh and Enders, 1985). The antimesometrial decidua reaches maximal development at day 8-9 in mice and day 10-11 in rats after which time the decidua regresses and is sloughed into the uterine lumen around day 16 of gestation (Welsh and Enders, 1985; Abrahamsohn, 1989). For a more detailed examination of the antimesometrial decidua, see Welsh and Enders (1985). The mesometrial decidua is composed of decidual cells and granular cells, however these decidual cells are smaller than those observed in the antimesometrial decidua and are often binucleate (Abrahamsohn, 1989).

The process of decidualization is hormonally mediated. Psychoyos (1963) demonstrated that the uterus must be exposed to progesterone for a minimum of two days followed

by exposure to estrogen in order to become receptive to the blastocyst. Blastocyst implantation induces the decidual reaction and continuous secretion of progesterone and estrogen is necessary to maintain the decidual tissue (Yoshinaga, 1982).

In a short review of rodent implantation, Finn (1982) briefly discussed possible functions of decidual tissue. The three primary functions hypothesized for decidual tissue were (1) protection of the fetus from immunological rejection, (2) protection of the uterus from the invasive properties of the trophoblast, and (3) communication and movement of molecules between individual decidual cells.

Ruminants

Placentation. Placentation in cattle and sheep involves apposition, adhesion, and attachment of the conceptus to both caruncular and intercaruncular regions of the uterine endometrium. The majority of ruminant conceptuses initially attach approximately one-third the way up the uterine horn from the cervix (Lee et al., 1977). The conceptuses attach centrally such that they remain within the uterine lumen and do not invade into the uterine stroma (Steven and Morriss, 1975). The conceptus becomes immobilized at the area of attachment by extension of trophectodermal papillae into the uterine glands (Guillomot et al., 1981; Guillomot and Guay, 1982; Wooding et al.,

1982). Based on the number of maternal and fetal tissue layers present, placentation in cattle and sheep was initially classified as syndesmochorial (Grosser, 1909). However, Steven (1975) indicated that placentation in ruminants is actually epitheliochorial in nature. Early in implantation in ruminants (days 18-20), binucleate cell migration into and fusion with the maternal epithelium results in the formation of a syndesmochorial placenta (Wooding, 1982). The syndesmochorial placenta is maintained in the placentomes of the ewe, however in the entire cow placenta and in the interplacentome regions of the ewe placenta the uterine epithelium is regenerated resulting in the formation of the epitheliochorial placenta (Wooding, 1982). Placentation in cows and ewes is initially diffuse through the first month of gestation with attachment becoming more intimate with the formation of placentomes at day 30-33 (King et al., 1982).

Extraembryonic Membranes. Elongation of the bovine conceptus begins at approximately days 12-13 of gestation and rapidly increases over the next several days (Betteridge et al., 1980). By day 21 of pregnancy in the cow, contact between the chorion and the uterine endometrium is evident in both the ipsilateral and contralateral uterine horns (King et al., 1982).

Ruminant conceptuses initially develop a chorio-vitelline placenta early in gestation. As the yolk sac develops, it fuses with the expanding chorion and

establishes a vitelline circulation which remains functional until about day 17 of gestation (Amoroso, 1952). As the allantois develops from the hindgut region of the embryo and expands, the yolk sac starts to regress. By day 17 of pregnancy, the yolk sac has separated from the chorion (Amoroso, 1952). Expansion of the allantois and its subsequent fusion with the chorion results in the formation of the allanto-chorionic membrane.

The allantois fuses with the entire surface of the chorion except in the region of the amnion and the tips of the chorion. The allantois of sheep and cattle conceptuses does not encompass the amnion, but instead is formed in a T-shaped cavity (Amoroso, 1952; Steven and Morriss, 1975). The stalk of the allantois runs up one side of the amnion and separates into two arms of unequal length. The longer section of the allantois extends into the non-gravid uterine horn (Steven and Morriss, 1975). In the areas where the allantois does not separate the amnion from the chorion, these two membranes fuse to form the amniochorion (Amoroso, 1952; Steven and Morriss, 1975).

The extraembryonic membranes of sheep and cattle conceptuses develop modifications that facilitate attachment of the chorio-allantois to the uterine endometrium. The first of these modifications is the formation of trophoblast papillae. The chorion of the ovine conceptus develops trophoblast papillae around day 13 of gestation which extend into the lumen of uterine glands by the third week of

gestation (Guillomot et al., 1981; Wooding and Staples, 1981; Wooding et al., 1982). The penetration of the trophoblast papillae into the uterine glands appears to function in immobilizing the conceptus within the uterine lumen. Another modification of the fetal membranes of sheep and cattle, compared to the sow and mare, is the presence of trophoblastic binucleate cells. Binucleate cells differentiate within the trophectoderm on approximately days 14-15 of gestation in sheep and days 17-18 of gestation in cattle (Wooding and Morgan, 1989). The binucleate cells migrate out of the trophectoderm and form a syncytium with the uterine epithelial cells (Wooding, 1982). These cells have been implicated in the transport of placental lactogen to the maternal system (Wooding, 1984, 1987). The third modification of ruminant extraembryonic membranes is the formation of cotyledons. Cotyledons are distinct villous areas present on both the allanto-chorion and the amnio-chorion (Amoroso, 1952; Steven and Morriss, 1975) which interlock with the crypts of uterine endometrial caruncles to form structures called placentomes (Amoroso, 1952; Steven and Morriss, 1975; Perry, 1981). Placentomes begin to develop during the fourth week of gestation in sheep (Wimsatt, 1950) and between days 30-33 in cattle (King et al., 1979).

Attachment. Leiser (1975) reported that the apposition and attachment of bovine conceptuses commenced in the region near the embryo on approximately days 18-19 of gestation,

extending throughout the entire uterine endometrium by day 27. King et al. (1982) observed initial attachment of the chorion to caruncular and intercaruncular uterine epithelium at day 19 of gestation, however discrete areas of contact were not evident throughout the uterus until day 21 of pregnancy. Similarly, Wathes and Wooding (1980) first reported contact between the chorion and the uterine epithelium at day 20 of gestation. Therefore it is apparent that attachment of the bovine conceptus to the uterine epithelium occurs on days 18-20 of gestation. Attachment of the sheep conceptus follows a similar pattern, but begins at an earlier stage of gestation. In sheep, adhesion between the conceptus and the uterine caruncular epithelium develops between days 16-18 of gestation (Guillomot et al., 1981) with the interdigitation of microvilli becoming evident around day 18 (King et al., 1982) to day 20 (Davies and Wimsatt, 1966). Attachment of ruminant conceptuses occurs in both caruncular and intercaruncular regions of the uterine endometrium. Ultrastructural changes in the conceptus and uterine epithelium during implantation in sheep and cattle have been extensively studied (Wimsatt, 1950; Bjorkman, 1954; Davies and Wimsatt, 1966; Boshier, 1969; Wathes and Wooding, 1980; Guillomot et al., 1981; Guillomot and Guay, 1982; King et al., 1981, 1982), therefore only a brief description of these changes will be included in this review.

Early in gestation (day 17-19 in cattle and day 14-16

in sheep), the uterine epithelium is characterized by tall columnar epithelium while the chorion consists of cuboidal to low columnar epithelial cells (Wathes and Wooding, 1980; Guillomot et al., 1981; King et al., 1981,1982). As gestation progresses, a reduction in epithelial cell height becomes evident so that by day 18 in the ewe (King et al., 1982) and day 21-23 in the cow (Wathes and Wooding, 1980; King et al., 1981,1982) the uterine epithelium consists of cuboidal to low columnar epithelial cells. By day 14-15 of gestation in sheep and day 17-18 in cattle, binucleate cells differentiate from uninucleate trophoblast cells (Wooding and Morgan, 1989). Initially, the binucleate cells have no contact with the basement membrane or apical tight junctions (Boshier and Holloway, 1977; Wathes and Wooding, 1980; Wooding, 1984). At day 16 in the ewe, binucleate cells were only occassionally evident in the chorion and none were evident in the uterine epithelium (Boshier, 1969; King et al., 1982). In contrast, binucleate cells compose approximately 6% and 3% of the cell population in the chorion and uterine epithelium, respectively, at day 18 of pregnancy in the cow (Wathes and Wooding, 1980). Migration of trophoblast binucleate cells involves the extension of a large, smooth pseudopod through the apical tight junction to contact and fuse with the uterine epithelium (Wathes and Wooding, 1980). By day 20 of gestation in the cow, binucleate cells constitute approximately 20% of the chorionic epithelium and are located adjacent to maternal

giant cells (Wathes and Wooding, 1980; King et al., 1981). Migration and fusion of trophoblast binucleate cells to uterine epithelial cells results in the formation of a syncytium which consists of trinucleate and multinucleate cells in both cattle and sheep (Amoroso, 1952; Boshier, 1969; Wathes and Wooding, 1980; King et al., 1982). Studies utilizing phosphotungstic acid staining (Wooding and Wathes, 1980) and autoradiography (Wooding et al., 1981) indicated that migration and fusion of binucleate cells are the main source of new syncytial nuclei. The cells of the syncytium contain an odd number of nuclei due to the fusion of binucleate cells with an odd number of uninucleate uterine epithelial cells. Migration of the binucleate cells and the subsequent syncytium formation is accompanied by the degeneration of maternal epithelial cells which are phagocytized by the chorionic epithelium (Davies and Wimsatt, 1966; Boshier, 1969; Wathes and Wooding, 1980; Wooding, 1982). By days 26-27 of gestation in the cow, binucleate and multinucleate cells make up about 70% of the uterine luminal epithelium (Wathes and Wooding, 1980). From days 28-40 of gestation in the cow, the trinucleate and multinucleate cells disappear and are replaced by residual uninucleate epithelial cells (Wathes and Wooding, 1980; Wooding, 1982). Wooding and Morgan (1989) reported that binucleate cell migration continues throughout gestation, but the trinucleate cells that are formed are only transient. In the ewe, binucleate cells migrate in larger

quantities which may account for the persistence of the syncytium throughout gestation in sheep (Wimsatt, 1951).

It has been suggested that binucleate cells may have several roles in placentation in ruminants (King et al., 1982). Davies and Wimsatt (1966) hypothesized that the syncytium was formed for immunological protection, however Wooding and Morgan (1989) believe that the syncytium would not provide an adequate immunological barrier since it is noncontinuous and, in the cow, only transient. Although binucleate cell migration and fusion continues throughout gestation in the cow, the resulting trinucleate cells are short lived (Wooding and Morgan, 1989). It has been demonstrated that cytoplasmic granules of sheep binucleate cells contain ovine placental lactogen (Wooding, 1981; Morgan et al., 1987). Since binucleate cell granules are carried into the syncytium with the binucleate cells and then exocytosed into the maternal stromal tissue (Wooding, 1984, 1987), binucleate cell migration and syncytium formation may function to transport fetal products to the maternal system. King et al. (1982) suggested that binucleate cell migration may also function to stabilize the fetal membranes until interdigitation of microvilli develops.

Placentation in ruminants also involves the formation of placentomes. Placentomes are formed by microvillous interdigitation between the fetal membrane cotyledons and the uterine caruncles. In the cow the caruncle is knob-

shaped and the cotyledon attaches over it, whereas in the ewe the caruncle is cup-shaped and the chorionic tissue fits within it (Perry, 1981). According to Wimsatt (1950), placentomes begin initial development during the fourth week of pregnancy in sheep. King et al. (1982) reported that by day 14 of pregnancy in sheep, depressions were evident in some caruncles with ridges forming around day 15 of gestation. Although no junctions were evident between the chorion and the uterine endometrium, King et al. (1982) suggested that adhesion had taken place in caruncular regions by day 16 of gestation. In the cow, placentomes are recognizable on the uterine surface by day 20 of gestation as discrete oval structures (King et al., 1980). King et al. (1980) demonstrated that placentome size and attachment between the fetal membranes and the uterine endometrium increased by day 29, however chorionic villi and caruncular crypts had not yet formed in the placentomes. King et al. (1979) demonstrated that villous attachment in the placentomes developed between days 30-33 with the formation of villi and crypts becoming evident by day 33 of gestation. Placentomes become larger and more complex as gestation progresses (King et al., 1979).

The inital adhesion of the chorion to the uterine endometrium appears to involve cell surface glycoconjugates. Wordinger and Amsler (1980) reported that the apical surface of the uterine luminal epithelium in the cow is covered by a thick glycocalyx. These authors demonstrated that the

uterine epithelial glycocalyx is composed of acidic glycoproteins with sulfated and sialic acid side groups with the sulfated residues being the predominate type. Wordinger and Amsler (1980) did not observe any variation in the uterine epithelial glycocalyx with the stage of the estrous cycle. Utilizing different staining techniques, Guillomot et al. (1982) demonstrated the presence of a glycocalyx on the trophoblast and uterine endometrial surfaces in sheep. Similar to results in the cow (Wordinger and Amsler, 1980), Guillomot et al. (1982) did not observe any changes in the uterine endometrial glycocalyx during the estrous cycle or during attachment of the conceptus during pregnancy in the ewe. However, alterations in the trophoblast glycocalyx were evident at day 15 of gestation concomitant with trophoblast attachment (Guillomot et al., 1982). These authors observed a more uniform distribution of the glycocalyx on the trophoblast surface via ruthenium red and cationic ferritin staining. Utilizing lectin staining procedures, changes in the carbohydrate composition of the glycocalyx could be demonstrated (Whyte and Robson, 1984; Munson et al., 1989). In sheep, Whyte and Robson (1984) reported that trophoblast reacted strongly with the *Tetragonolobus purpureas* (TP) lectin on both days 14 and 17 of gestation indicating the presence of fucose residues on the trophoblast surface. Wheat germ agglutinin (WGA) staining was not evident on day 14 of pregnancy, but by day 17 of gestation intense WGA staining was present. This

increase in WGA reactivity occured during the time of implantation and was indicative of the production of N-acetylglucosamine residues on the sheep conceptus. Sheep uterine endometrium showed only weak reactivity with WGA and Concanavalin-A (Con-A) (Whyte and Robson, 1984). Using a wide variety of lectins, Munson et al. (1989) observed differences in lectin binding on the chorion and uterine endometrium during pregnancy in the cow. The intense binding of WGA and PNA to bovine endometrium and chorion observed by Munson et al. (1989) was similar to the WGA binding evident in the sheep trophoblast (Whyte and Robson, 1984). Munson et al. (1989) demonstrated high intensity binding of several lectins to the arcade region of placentomes as well as the intercotyledonary chorion suggesting the possible presence of cell adhesion molecules in areas of the placenta which are not anchored by the villous interdigitation seen in the placentomes. Thus alterations in the carbohydrate composition of the trophoblast and endometrial glycocalyx may be involved in the initial adhesion of the ruminant conceptus to the uterine endometrium.

Swine

Placentation. Like the ruminant species, the porcine conceptuses align themselves along the mesometrial region of the uterus and attach centrally such that they remain within

the uterine lumen and do not invade into the uterine stroma (Amoroso, 1952; Steven and Morriss, 1975). In the pig, all six (three fetal and three maternal) tissue layers separate the fetal and maternal vasculature. Therefore, based upon Grosser's (1909) classification scheme, the porcine placenta is epitheliochorial in nature. Since attachment of the porcine conceptus to the uterine epithelium extends over the entire surface of the chorion with the exception of the areas over uterine glands and at the tips of the chorion, the porcine placenta is referred to as diffuse (Perry, 1981). Therefore, the pig develops a diffuse, epitheliochorial placenta.

Extraembryonic Membranes. The development of the porcine fetal membranes (chorion, amnion, allantois, and yolk sac) has been the subject of extensive research (Heuser, 1927; Heuser and Streeter, 1929; Amoroso, 1952; Steven and Morriss, 1975; Perry, 1981).

The amnion develops from the folding of the chorion over the embryo (Heuser and Streeter, 1929; Perry, 1981). The embryonic disc remains exposed until after the initial development of the primitive streak and early neural fold (Heuser and Streeter, 1929). Cranial and caudal folds in the chorion raise above the embryo at the same time that lateral folds are developing. These amniotic folds fuse dorsally over the embryo (Heuser and Streeter, 1929; Perry, 1981). By day 18 of gestation, the amniotic folds have fused together and the amnion separates from the chorion

(Perry, 1981).

The yolk sac is initially formed by the proliferation of endoderm along the inner surface of the trophoblast ectoderm (Heuser, 1927; Steven and Morriss, 1975). On days 11-12 of gestation, the porcine conceptus undergoes a rapid elongation from spherical to tubular and filamentous forms that involves cellular remodeling rather than cellular hyperplasia (Geisert et al., 1982b). Shortly after trophoblast elongation, mesodermal tissue proliferates from the embryonic disc and migrates between the ectoderm and endoderm (Perry, 1981). Following proliferation, a split develops in the mesoderm such that one layer underlies the ectoderm while the other layer overlies the endoderm. These layers become the chorion and the yolk-sac splanchnopleure, respectively (Perry, 1981). The areas where the trilaminar omphalopleure, which consist of the yolk-sac endoderm, mesoderm, and trophoblast ectoderm, remain intact constitutes the yolk-sac placenta which appears to have absorptive and secretory capabilities (Heuser, 1927). The cavity formed by the split in the mesoderm is the extraembryonic coelom. As the extraembryonic coelom expands, the yolk sac becomes separated from the trophoblast around day 18 of gestation and is displaced by the developing allantois (Perry, 1981).

The allantois becomes apparent at approximately day 14 of gestation as an evagination from the hindgut region of the embryo and is composed of outer mesodermal and inner

endodermal tissue layers (Steven and Morriss, 1975; Perry, 1981). The allantois expands rapidly and by day 17-18 of gestation extends towards the ends of the chorion (Steven and Morriss, 1975; Perry, 1981). At approximately day 19 of gestation, the allantoic mesoderm fuses to the mesoderm lining the chorion. The fusion of the allantois to the chorion initially occurs in the region opposite the allantoic stalk and subsequently spreads over the rest of the chorion (Heuser, 1927). The resulting allantochorionic membrane consists of inner endoderm, outer ectoderm, and intervening mesoderm. The mesodermal layer gives rise to the allantoic vasculature (Mossman, 1937; Amoroso, 1952). Expansion of the allantois presses the amnion against the chorion. Fusion occurs between the amnion and chorion where these two membranes are in contact with each other (Heuser, 1927; Amoroso, 1952). Fusion of the allantois to the chorion continues through day 24-26 of gestation at which time contact is established over the entire surface except in the extremities of the chorion and where the amnion has fused to the chorion (Heuser, 1927; Amoroso, 1952; Steven and Morriss, 1975). The allantois does not extend to the distal tips of the chorion which subsequently degenerates due to the lack of vascularization (Amoroso, 1952; Steven and Morriss, 1975). Fusion of the allantois and the chorion results in the formation of the chorio-allantoic membrane which attaches to the uterine epithelium.

Areolae are specialized structures that develop on the

allanto-chorion at approximately the third to fourth week of gestation (Brambel, 1933). On day 30 of gestation, the areolae appear as small white spots on the allanto-chorion (Brambel, 1933; Heuser, 1927; Friess et al., 1981). The areolae initially develop in the central region and subsequently spread over the rest of the allanto-chorion by day 50 of gestation (Brambel, 1933). Two morphologically distinct types of areolae are present on the allanto-chorion. The regular areolae are circular, opaque, and elaborately folded whereas the irregular areolae are translucent, have an irregular outline, and do not exhibit any complex folding (Amoroso, 1952; Perry, 1981). The irregular areolae are approximatley 15 times greater in diameter than regular areolae (Heuser, 1927; Brambel, 1933). Unlike the extensive folding of the chorion evident in regular areolae, formation of irregular areolae involves alterations in the uterine epithelium (Perry, 1981). Friess et al. (1981) reported that the lumen of the areolae contains uterine secretions, histotroph, and that ultrastructural examination indicates that the chorionic epithelium has a high absorptive capacity. It has also been demostrated that the areolae are the sites of absorption of uteroferrin secreted by the uterine glands (Chen et al., 1975). Therefore, the areolae, which lie over the openings of uterine glands, appear to function in the absorption of uterine secretions for the nourishment of the developing conceptus.

Attachment. Corner (1921) first demonstrated that attachment of the porcine conceptus to the uterine epithelium is initiated on day 13 of pregnancy. More recent examinations of the conceptus-uterine interface confirm that attachment commences at approximately days 13-14 of gestation and is initiated in the region of the embryo and then progresses towards the extremities of the allantochorion (Perry et al., 1976; Dantzer, 1985; Keys and King, 1990). On day 13 of gestation, the uterine epithlial cells develop rounded protuberances on their apical surface (King et al., 1982; Dantzer, 1985; Keys and King, 1990). Initial attachment of the conceptus involves the apposition of the chorion to these epithelial protuberances. The epithelial protuberances may therefore serve to immobilize the porcine conceptus until a more intimate association can be established (Dantzer, 1985). At this stage of pregnancy, the chorion also extends cytoplasmic projections between the adjacent uterine epithelial cells (King et al., 1982; Dantzer, 1985). These cytoplasmic projections do not appear to penetrate further than the maternal epithelial junctional complexes (King et al., 1982; Dantzer, 1985) and appear to be involved in the pinocytotic uptake of histotroph (Stroband et al., 1984). Dantzer (1985) demonstrated that the maternal epithelium was covered by a thick glycocalyx, whereas a thin glycocalyx was observed on the trophoblast. The maternal glycocalyx becomes reduced during attachment and microvillous interdigitation (Dantzer, 1985). Keys and

King (1990) demonstrated that short microvilli and a thick glycocalyx evenly covered the uterine epithelium, but were scattered unevenly over the trophoblast. Initial apposition of the trophoblast to uterine epithelium appeared to be restricted to the smooth surface that developed due to the formation of epithelial protuberances and loss of microvilli (Keys and King, 1990). Extension of uterine epithelial glycocalyx fibers toward the trophoblast in these areas appeared to aid in anchoring the conceptus (Keys and King, 1990). Therefore, the uterine epithelial glycocalyx may function in conceptus-uterine interactions to facilitate attachment early in gestation.

By days 15-16 of gestation, the epithelial protuberances are reduced and interdigitation of uterine epithelial and trophoblast microvilli is developing (Dantzer, 1985). Keys and King (1990) reported that various stages of attachment could be observed at day 18 of gestation. In those areas exhibiting more advanced attachment, microvillous interdigitation was well developed and apical doming had become less prominent. By day 24-26 of gestation, attachment and microvillous interdigitation had spread over the majority of the conceptus-uterine interface (Amoroso, 1952; Dantzer, 1985). Further reinforcement of conceptus attachment is accomplished by the extensive folding of the uterus. King et al. (1982) demonstrated that the allantochorionic membrane at days 28-30 of gestation develops numerous secondary ridges which

penetrate into endometrial troughs. Dantzer (1984) reported that as gestation progresses from days 20-100, the uterine endometrium undergoes extensive macroscopic and microscopic folding. The allanto-chorion develops non-permanent ridges and troughs that are complementary to the endometrial folds such that the allanto-chorion is 2.5-4.0 times the length of the corresponding endometrium (Dantzer, 1984).

As previously mentioned, the uterine epithelial glycocalyx appears to function in conceptus attachment early in gestation. Alterations in the carbohydrate compostion of the glycocalyx may facilitate its role in attachment. Whyte and Robson (1984) demonstrated that porcine trophoblast reacted strongly with TP lectin on days 9 and 14 of gestation indicating that the trophoblast surface contains high levels of fucose. Day 9 and 14 porcine conceptuses did not bind Con-A or Ricinus communis types I and II lectins (RCA-I and RCA-II), however day 14 conceptuses did bind WGA. These results suggest that the porcine conceptus contains fucose residues at both days 9 and 14 of gestation and N-acetylglucosamıne resıdues at day 14, but do not appear to contain galactose or N-acetylgalactosamine residues (Whyte and Robson, 1984). The porcine endometrium did not react with TP, RCA-I, or RCA-II, but reacted weakly with WGA and Con-A. Rober and Holtz (1988) demonstrated that conceptus and endometrial tissue contained galactose, galactosamine, and fucose residues prior to conceptus attachment, but not after attachment was established. Further research will be

necessary to determine the changes that occur in the carbohydrate composition of the epithelial glycocalyx and the role these carbohydrates may have during attachment.

The porcine conceptus is noninvasive within the uterus, however when transplanted to ectopic locations it exhibits invasive properties (Samuel, 1971; Samuel and Perry, 1972). It has been demonstrated that the porcine conceptus produces several proteolytic enzymes including lysozyme, leucine-aminopeptidase, and cathepsins B_1, D, and E (Roberts et al., 1976) as well as plasminogen activator (Mullins et al., 1980; Fazleabas et al., 1983) and two glycosidases, b-hexosaminidase and b-galactosidase (Hansen et al., 1985) Proteases, especially plasminogen activator, have been impicated in implantation in rodents (Strickland et al., 1976; Denker, 1980, 1981, 1982). Plasminogen activator functions through the conversion of plasminogen to plasmin which can then hydrolyze connective tissue, basement membrane components, and fibrin (Werb et al., 1980). Plasmin inhibitors are produced by the porcine uterus during the luteal phase of the estrous cycle and during pregnancy (Mullins et al., 1980; Fazleabas et al., 1983) and during pseudopregnancy (Fazlaeabas et al., 1982). Fazleabas et al. (1984) demonstrated that the major isoform of the plasmin inhibitor is predominantly synthesized by the uterine luminal epithelium. These uterine plasmin inhibitors may function to control proteolytic activity within the uterine lumen, thus protecting the uterine epithelium from the

invasive properties of the porcine conceptus. Lysozymes are hydrolytic enzymes that cleave the b1,4-glycosidic linkages of bacterial peptidoglycans (Roberts and Bazer, 1988). Roberts et al. (1976) demonstrated that lysozyme activity is present in porcine uterine secretions and that they may be progesterone-induced proteins. It has been hypothesized that these lysozymes have an antibacterial function in the uterine lumen to pigs (Roberts and Bazer, 1988). Roberts et al. (1976) also demonstrated that cathepsin B_1, D, and E activities are present in uterine flushings of ovariectomized, progesterone-treated gilts, but not in gilts that did not receive progesterone treatment. These data suggest that the cathepsins may be progesterone induced. Cathepsins are lysosomal cysteine proteases (Barrett and Kirschke, 1981) that have been implicated in blastocyst implantation in rats (Elangovan and Moulton, 1980a,b) and cats (Verhage et al., 1989; Li et al., 1991). Farmer et al. (1989) demonstrated that the porcine uterus also produces antileukoproteinase, a protease inhibitor that inhibits the activities of elastase and cathepsin G (Seemuller et al., 1986). Simmen and Simmen (1990) suggested that antileukoproteinase may function to maintain placental membrane integrity. In the gilt, cathepsins are only a minor component of uterine flushings (Roberts et al., 1976). The role, if any, that cathepsins play in conceptus attachment in the pig is presently unknown.

Early Embryonic Mortality

Embryonic mortality in gilts occurs at a higher frequency during the early stages of pregnancy. Embryonic mortality, characterized by the difference between the number of live embryos present and the number of CL, ranges from 20-40% between days 10-40 of pregnancy and averages approximately 30% (see Perry, 1954; Flint et al., 1982; Pope and First, 1985 for review). An additional loss of 10-20% is evident after day 40 of gestation. The majority of embryonic loss occurs prior to day 25 (Perry, 1954), during the time period of conceptus elongation and establishment of pregnancy.

As previously discussed, conceptus-produced estrogen is the signal for maternal recognition of pregnancy in swine, however premature exposure of gilts to estrogen or estrogenic compounds results in early embryo death. Long and Diekman (1986) demonstrated that addition of zearalenone, an estrogenic mycotoxin produced by *Fusarium roseum* mold, to the diet of sows between days 7-10 of gestation resulted in complete embryonic mortality. At autopsy, day 30 of gestation, 3 of 4 sows treated with zearalenone between days 7-10 of gestation exhibited complete embryo loss and CL were regressing. When sows received zearalenone between days 2-6 or days 11-15 of gestation, pregnancy was uneffected (Long and Diekman, 1986). These authors indicated that embryonic loss was not

associated with changes in serum concentrations of LH and FSH. Administration of zearalenone to sows between days 7-10 of gestation did not alter the number or spacing of embryos when examined on days 9 and 11 of pregnancy, however alterations in the uterine environment were evident (Long et al., 1987). Concentrations of magnesium and zinc in uterine flushings were higher on days 11 and 13 of gestation in zearalenone treated compared to control sows, whereas calcium was similar between treatments on days 9 and 13, but lower on day 11 in zearalenone treated sows (Long et al., 1987). Pope et al. (1986) demonstrated that early administration of estradiol-17b also resulted in embryo death by day 30 of gestation. Dosages of 2.0 mg estradiol-17b per day administered on days 9 and 10 of pregnancy caused embryonic death in the majority of gilts while dosages of 8.0 mg/day or greater resulted in complete embryo loss in all gilts by day 30 of gestation (Pope et al., 1986). Administration of estradiol to gilts on days 9 and 10 of gestation resulted in degenerating conceptuses by days 14-16 of pregnancy (Morgan et al., 1987; Gries et al., 1989). Gries et al. (1989) reported that embryonic death was associated with uterine secretory alterations. A group of basic polypeptides (Mr=30,000; pI=7.9-9.0) became attenuated with embryo death. The function of these proteins is currently unknown, but they may be involved with placental attachment (Morgan et al., 1987; Gries et al., 1989). These data suggest that embryonic mortality as a

result of premature estrogen exposure may be due to a failure in placental attachment.

The establishment of pregnancy in swine involves a multitude of interactions between the conceptus and the uterus. Estrogen plays a critical role in the establishment of pregnancy in the pig, however premature exposure to estrogen during early pregnancy is detrimental to conceptus survival. The purpose of this dissertation was to characterize alterations in the uterine ultrastructure and secretory environment concomitant with embryonic death resulting from early administration of exogenous estrogen.

CHAPTER III

ENDOMETRIAL SURFACE AND SECRETORY ALTERATIONS ASSOCIATED WITH EMBRYONIC MORTALITY IN GILTS ADMINISTERED ESTRADIOL VALERATE ON DAYS 9 AND 10 OF GESTATION

Introduction

The glycocalyx is a carbohydrate rich peripheral zone at the surface of most eukaryotic cells which, owing to its composition and exposed position on the cell surface, has been implicated in numerous cell-cell interactions (Alberts et al., 1983). Such interactions exist between the developing embryo and the maternal endometrium for the establishment of pregnancy. Working with mice, Finn and Martin (1984) found that cell adhesion involving the uterus and embryo was influenced by the concentration of estrogen and progesterone. In addition, attachment of the conceptus appears to involve glycoproteins present on the trophoblastic and uterine epithelial cell surfaces (Chavez and Enders, 1982; Richa et al., 1985). Utilizing methods such as differential binding to lectins and cationic dyes, Schlafke and Enders (1975) and Sherman and Wudl (1976)

demonstrated that significant alterations occur in the composition of endometrial cell surface carbohydrates prior to implantation. Similarly, Lee et al. (1983) used fluoresceinated lectin binding to show carbohydrate groups exposed on the uterine cell surface change during the preimplantation period. Dutt et al. (1987) have shown that cultured uterine epithelial cells from mice synthesize lactosaminoglycans. These oligosaccharides appear to function in a galactosyltransferase-dependent cell adhesion system within the uterus and are synthesized in response to estrogen stimulation (Dutt et al., 1988). Based on these observations and from those of Nelson et al. (1977) which showed the presence of cell surface galactosyl-transferase on syncytial trophoblast tissue of human placenta, Dutt et al. (1987) suggested that this system may be functional in the uteri of many species. Agents that would perturb galactosyl-transferase function, such as proteases, also interfere with epithelial cell adhesion and cause a release of lactosaminoglycans from endometrial epithelium (Dutt et al., 1987). It was also shown that lactosaminoglycans are partially protected from proteolysis by the presence of divalent cations.

Placentation in the pig is associated with a reduction of the uterine glycocalyx as well as interdigitation of trophoblastic and uterine microvilli (Dantzer, 1985). The level of cell surface negativity has also been implicated in conceptus attachment. A reduction of trophoblast cell

surface negativity at the time of blastocyst adhesion (Jenkinson and Searle, 1977) and a reduction of anionic sites on rat endometrial epithelium occur during implantation (Hewitt et al., 1979). Thus, the uterine glycocalyx appears to play a vital role during placental attachment.

During early development in swine, blastocysts undergo a rapid morphological transformation from spherical to filamentous form by day 12 of pregnancy (Anderson, 1978; Geisert et al., 1982b). In addition to these morphological changes, porcine blastocysts attaining a diameter of 10 mm on days 12 and 13 of gestation begin to synthesize and release large quantities of estrogen (Perry et al., 1973; Gadsby et al., 1980; Fischer et al., 1985) and polypeptides (Godkin et al., 1982). Following embryo elongation and the initiation of estrogen production, conceptus attachment occurs between days 13 and 18 of gestation (King et al., 1982).

In experiments designed to examine the effect of a premature estrogen stimulation on embryonic development, Pope et al. (1986) demonstrated that gilts receiving exogenous estrogen on days 9 and 10 of gestation had complete embryonic death by day 30 of gestation. Further studies by our laboratory have revealed that administration of estradiol valerate on days 9 and 10 of gestation results in degenerating conceptuses by day 16 of pregnancy (Morgan et al., 1987; Greis et al., 1989).

The purpose of the present study was to examine uterine endometrial surface and secretory changes occurring in the endometrial epithelium of the pig during the period of trophoblast attachment in an attempt to characterize alterations associated with embryonic death due to early exogenous estrogen administration.

Materials and Methods

Animals

Mature cyclic crossbred gilts were observed for estrous behavior twice daily (07:00 and 17:00) in the presence of intact boars. After displaying two estrous cycles of normal duration (17-22 days), gilts were bred naturally at the onset of estrus (day 0) and 12 and 24 hours later.

Gilts were randomly assigned to receive one of the following two treatments: control (n=10), intramuscular injection (.25 ml) of sesame oil on day 9 and 10 of gestation or estrogen (n=10), intramuscular injection (5.0 mg) of estradiol valerate on day 9 and 10 of gestation. Following random assignment to treatment groups, one uterine horn from each gilt was removed on either day 12 or day 16. In gilts from which the uterine horn was excised on day 12 the remaining horn was removed on day 14, whereas in gilts which had the first horn removed on day 16 the remaining horn was excised on day 18.

Surgical Procedures

Gilts were unilaterally hysterectomized after induction of anaesthesia with a 5% solution of thiopentone sodium and maintained on a closed circuit system of halothane (5% Fluothane) and oxygen (1.0 liters/min.). The uterus was exposed via midventral laparotomy and a randomly selected uterine horn, and its ipsilateral ovary, excised. After routine closure of the incision, gilts were postsurgically treated with antibiotics.

General

Following removal of a section of uterine horn for analysis by scanning (SEM) and transmission (TEM) electron microscopy, the remaining portion of the uterine horn was transported on ice to a sterile laminar flow hood. The ovary and broad ligament were trimmed from the uterine horn and the horn was flushed with 20 ml of sterile 0.9% physiological saline. Blastocysts recovered from control and estrogen treated gilts were evaluated for viability (i.e. intact vs. degenerative/fragmented embryos) under a binocular dissection microscope. Uterine flushings were centrifuged at 12,000 x g for 15 min at 4 °C. The supernatant was decanted and stored at -20 °C until analyzed

for antiviral activity (AVA) and proteins secreted as determined by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE).

The pellet resulting from centrifugation of the uterine flushing was resuspended in 5.0 ml of 0.9% saline. An aliquot (20 ml) of the cell suspension was added to a trypan blue solution, consisting of 40 ml of 0.9% saline and 40 ml of 0.2% Trypan Blue dye, and mixed thoroughly. A 10 ml aliquot of this suspension was transferred to a hemacytometer chamber, and leukocytes were counted.

Endometrial tissue from control and estrogen treated gilts was dissected away from the myometrium, cut into 2-3 mm explants, and placed in Eagle's minimum essential medium (MEM). Media was prepared with L-glutamine reduced to one-tenth of normal concentration, filtered through a 0.20 micron membrane and stored at 4 °C. Antibiotic and antimycotic solution containing penicillin (100,000 U/ml) and streptomycin (10 mg/ml) was added to media prior to culture. Endometrial explants (200 mg) were cultured in 7.0 ml MEM supplemented with 25 uCi [^{3}H]-glucosamine (specific activity = 40 Ci/mmole) to detect de novo glycoprotein synthesis. Explants were cultured for 24 hours at 37 °C in an atmosphere of 5% CO_2, 45% N_2, and 50% O_2. After incubation, culture media was separated from tissue and centrifuged at 12,000 x g for 15 min. The supernatant was decanted and stored at -20 °C until analyzed for [^{3}H]-glucosamine incorporation by 2D-PAGE and fluorography.

Two-Dimensional Polyacrylamide Gel Electrophoresis. Media from endometrial cultures were dialyzed (Spectra/Por, molecular weight cut-off = 3,500; Spectrum Medical Industries, Inc.) against 10 mM tris-HCl buffer. An aliquot (100 ml) from each dialysate was used to determine the total dpm of [^{3}H]-glucosamine retained. Uterine flushings and dialyzed culture media were lyophilized and redissolved in 5 mM K2CO3 containing 9.3 M urea, 2% (v/v) Nonidet P-40, and .5% (w/v) dithiothreitol. Reconstituted culture media and uterine flushings were subjected to 2D-PAGE (Basha et al., 1979).

A volume of culture media containing approximately 200,000 dpm was loaded onto each gel. Gels were stained with Coomassie Blue, impregnated with sodium salicylate, dried onto filter paper, and overlaid with Kodak XAR x-ray film. Fluorographs were developed after approximately 10 weeks of exposure at -70 °C.

Approximately 300 mg of protein from uterine flushings was loaded onto gels. Gels were silver stained as previously described by Wray et al. (1981).

Antiviral Activity. Antiviral activity (AVA) in uterine flushings was determined as described by Fulton et al. (Fulton et al., 1986). Culture media containing a volume of uterine flushing was added to established monolayers of Madin-Darby bovine kidney (MDBK) cells in 24-well tissue culture plates. Vesicular stomatitis virus (0.1 ml, VSV) was added and the culture incubated for 24 hours at

37 °C. Antiviral activity was assessed by inhibition of cytopathic effects of VSV by counting the number of plaque forming units (PFU). The amount of antiviral activity in flushings was expressed as the reciprocal of the dilution that resulted in 50% inhibition of plaque formation.

Tissue Preparation for Electron Microscopy. Immediately upon removal of the uterine horn, a 7-10 cm section approximately 10-20 cm anterior to the uterine body was excised and opened along the antimesometrial border. Endometrial tissue explants (6-10 mm sections) from control and estrogen treated gilts were dissected away from the myometrium and immersed in modified Karnovsky's fixative (2% glutaraldehyde and 1.6% paraformaldehyde; KF) as described by Karnovsky (1965). Endometrial explants were cut into 1-2 mm pieces, immersed in fresh fixative, and placed on a rotating platform for 2 h at room temperature. After fixation, tissue samples were rinsed with 0.1 M cacodylate buffer and post-fixed with 1% osmium tetroxide in 0.1 M cacodylate buffer for 1 hour. Following post-fixation, tissue was rinsed with 0.1 M cacodylate buffer, dehydrated in a graded series of alcohol, and embedded in epon-araldite (Mollenhauer, 1964).

Ruthenium Red Staining. Endometrial tissue samples were fixed in KF + 0.2% ruthenium red (Polysciences, Inc.) for 2 h at room temperature on a rotating platform. Endometrial tissue was subsequently rinsed in 0.1 M cacodylate buffer and post-fixed in 1% osmium tetroxide +

0.05% ruthenium red for 1 h. Following post-fixation, tissue was stained en block with 1% uranyl acetate, rinsed repeatedly with distilled water, dehydrated in a graded series of alcohol solutions, and embedded in epon-araldite. To determine the specificity of ruthenium red staining, endometrial tissue was treated with trypsin. Trypsin (Sigma, St. Louis) was diluted to 5.0 mg/ml in 0.1 M sodium phosphate buffer (pH=7.3). Tissue samples were immersed in enzyme solution for 30 min and then processed for TEM as described above.

Cationic Ferritin Staining. Following primary fixation with KF, tissue samples were rinsed with 0.1 M cacodylate buffer and incubated in cationic ferritin (CF, Polysciences Inc.) at a concentration of 0.4 mg/ml for 1 h on a rotating platform. Endometrial samples were subsequently rinsed with 0.1 M cacodylate buffer, post-fixed in osmium tetroxide, dehydrated, and embedded. A sample of the endometrial tissue was treated with trypsin as described previously to determine the specificity of cationic ferritin binding. Electron micrographs were analyzed by image analysis to quantify the number of cationic ferritin granules bound to the epithelial glycocalyx.

Transmission Electron Microscopy (TEM). Tissue samples were sectioned on a Porter-Blum MT-2 ultramicrotome. Thin sections (60-90 nm) were collected on 200 mesh uncoated copper grids. Ruthenium red stained samples were post-stained with Sato's lead stain (1967) while CF treated

tissue remained unstained after sectioning. Thin sections were observed on a JEOL 100 CX TEMSCAN transmission electron microscope at 80 KV.

Scanning Electron Microscopy (SEM). Following dehydration, endometrial tissue samples were dried in a Samdri PVT-3 critical point dryer. Tissue samples were then mounted on aluminum studs and coated with gold-palladium (200 A°) in a Techniques Hummer II. Specimens were observed on a JEOL JSM-35 scanning electron microscope at 25 KV.

Statistical Analysis. Data were analyzed by least squares analyses of variance using the General Linear Models procedures of SAS (1979). Antiviral activity, leukocyte counts, cationic ferritin binding, and dpm [^{3}H]-glucosamine/mg tissue wet weight from explant cultures were each analyzed for differences between treatment, day, and treatment by day interactions.

Results

Intact filamentous embryos were recovered from a uterine horn of control gilts on all days (5/5, 4/5, 5/5, and 5/5 on days 12, 14, 16, and 18 of gestation, respectively), whereas, intact embryos were recovered from estrogen treated gilts only on day 12 (4/4) of pregnancy. Conceptus tissue from estrogen treated gilts on days 14, 16, and 18 of gestation was degenerating and fragmented in all instances (4/4, 4/4, 3/3, respectively). One control gilt

exhibited degenerating conceptus tissue on day 14 of gestation, however the ovary ipsilateral to the uterine horn from which the embryos were recovered contained what appeared to be several follicular cysts and was thus classified as abnormal, subsequently this gilt was excluded from the analyses. Embryos were not recovered in four estrogen treated gilts, one on each day studied. One uterine horn recovered on day 18 from a treated animal had developed a uterine infection and was excluded from analysis.

Treatment by day interaction ($P<0.01$) was detected for antiviral activity (Table 1). Antiviral activity (AVA) in uterine flushings peaked on day 16 of gestation in control gilts and then declined by day 18. In contrast, AVA in estrogen treated gilts was similar to controls on day 12 with an increase on day 14 but AVA did not increase from days 14-18 of pregnancy. Total content of AVA in uterine flushings from estrogen treated gilts was 10 fold less than controls on day 16 and 18.

No day or treatment effects were indicated for total leukocytes present in uterine flushings. Mean leukocyte numbers were not different between control (509,970 $\pm$ 175,999) and estrogen treated (297,588 $\pm$ 157,418) gilts ($P>.10$).

Fluorographs (Fig. 1) from 2D-PAGE gels of endometrial explants showed similar profiles of glycoprotein synthesis between treatment groups. A band of three distinct basic

glycoproteins (Mr=30,000; pI 7.9-9.0) was present in culture media of both control (Fig. 1A) and estrogen treated (Fig. 1B) animals. Two bands of acidic glycoproteins (Mr=47,000; pI 6.2-7.0 and Mr=33,000-44,000; pI 5.0-7.0) were similar between treatment groups. Analysis of de novo glycoprotein synthesis revealed no differences between control (9,626.28 + 1,480.13) and estrogen treated (12,045.59 + 1,569.92) gilts (P>.20)

SEM

At day 12 of gestation, uterine epithelium of control gilts exhibits a uniform surface with no apical doming evident (Fig. 2a&b). Uterine folding is also evident at this time in control gilts (Fig. 2b), though this folding is not extensive. Microvilli are abundant and cilia are often observed (Fig. 2a&b). Protrusion of epithelial cell apical domes was first evident on day 14 of gestation (Fig. 2c). The epithelial cells are densely covered with microvilli (Fig. 2c) and trophoblast tissue was often found within the uterine folds (Fig. 2d). By day 16 of gestation, individual epithelial cells were more clearly observed due to extensive protrusion of their apical surfaces (Fig. 3a). This protrusion was even more prominent on day 18 of gestation (Fig. 3c). In areas of trophoblast apposition on days 16 and 18 of gestation, epithelial cell apical domes were prominent and microvilli were extremely short and sparse

(Fig. 3b&d).

In estrogen treated animals, the uterine epithelium was similar to the epithelial surface of control gilts at day 12 of gestation (Fig. 4a&b). Initial differences between estrogen treated and control tissue were observed on day 14 of gestation. Although some areas of the uterine epithelial surface of estrogen treated gilts (Fig. 4c) were similar to controls (Fig. 2c) at day 14, other tissue areas exhibited cells that appeared ruptured or had only sparse numbers of microvilli present (Fig. 4d). Areas of the uterine epithelium exhibiting extensive loss of microvilli are more evident by day 16 (Fig. 5a). In many instances, individual epithelial cells appear indistinct (Fig. 5b) and did not exhibit the prominent apical dome protrusion observed in control gilts (Fig. 3a). The endometrial surface changes observed in estrogen treated gilts at day 16 of gestation were still evident at day 18 (Fig. 5c&d).

TEM

Treatment of endometrial tissue with trypsin prior to fixation resulted in the loss of ruthenium red staining (Fig.6a). At day 12 of gestation, the uterine glycocalyx is relatively thin in both control (Fig. 7a) and estrogen treated (Fig. 7b) gilts as indicated by ruthenium red staining. The glycocalyx progressively thickens and becomes more fibrous between days 14-18 of gestation (Fig. 7c and

Fig. 8a&c) in control gilts. On day 18 of pregnancy in control gilts, the glycocalyx thickens at the tips of the microvilli (Fig. 8c). The density of the glycocalyx observed in control gilts was not evident in estrogen treated gilts after day 12 of gestation. In contrast, the glycocalyx detaches from the epithelial microvilli on day 14 of gestation (Fig. 7d). The glycocalyx remains sparse on days 16 and 18 of gestation in estrogen treated gilts (Fig. 8b&d) compared to control gilts (Fig. 8a&c).

Treatment of endometrial tissue with trypsin prior to fixation and incubation with CF resulted in a reduction of the glycocalyx. Despite the thinning of the glycocalyx, CF binding remained intense (Fig. 6b). Both an increased number of ferritin granules bound (627.00 $\pm$ 31.13) and an increase in the counts/area (.273 $\pm$ .01) are evident in trypsin treated tissue. This increase in CF binding may be due to the exposure of more CF binding sites on the glycocalyx as suggested by Guillomot et al. (1982). Intense CF binding was evident on day 12 of gestation in control gilts (Table 2) with ferritin granules being evenly distributed over the microvilli (Fig. 9a). By day 14 of gestation, CF binding was still abundant but became more concentrated at the tips of the microvilli (Fig. 9c). A reduction in the intensity of CF binding was evident in estrogen treated gilts compared to controls on day 12 of pregnancy (Fig. 9b). This was more apparent by day 14 of gestation in estrogen treated gilts which exhibited sparse

CF binding (Fig. 9d). Cationic ferritin binding remained sparse through days 16 and 18 of gestation in both control and estrogen treated gilts (Fig. 10a-d). Although CF binding was sparse, no differences were apparent between days 14-18 in either control or estrogen treated gilts (Table 2). A reduction of the maternal glycocalyx was observed at areas of placental attachment (Fig. 11).

Discussion

Pope et al. (1986) first demonstrated that administration of estradiol-17ß to gilts on days 9 and 10 of gestation resulted in embryonic death prior to day 30, however no effect was observed when estradiol-17ß was administered on days 12 and 13. A similar effect was reported in sows fed zearalenone, an estrogenic mycotoxin (Long and Diekman, 1986). Sows fed zearalenone on days 7-10 of gestation demonstrated complete embryonic mortality by day 30, while normally developing embryos are present on days 30-32 of pregnancy in sows receiving zearalenone on either days 2-6 or days 11-15 of gestation (Long and Diekman, 1986).

Morgan et al. (1987) reported that intact conceptuses were present on days 11 and 12 of gestation in gilts administered estradiol valerate on days 9 and 10, but were degenerating by day 16 of pregnancy. More recently, intact conceptuses were recovered on days 12 and 14 of gestation

from gilts receiving estradiol valerate on days 9 and 10, while embryonic tissue was degenerating on days 16 and 18 of pregnancy (Greis et al., 1989). In the present study, intact embryos were recovered only on day 12 of gestation in estrogen treated gilts. Degenerating embryos were recovered on all other days which supports the findings of earlier studies (Morgan et al., 1987; Greis et al., 1987).

Media from porcine conceptus cultures have been shown to have antiviral activity (Cross and Roberts, 1988). Mirando et al. (1990) demonstrated that proteins with antiviral activity are secreted after day 10 of gestation, but do not appear to be involved in the establishment of pregnancy in the pig (Harney and Bazer, 1989). Since these proteins possess antiviral properties, they may function in maintaining conceptus viability and survival. Data from the present study has shown that AVA increased after day 12 to highest levels on day 16 and declined by day 18 in control gilts. Levels of AVA in the uterine fluid of estrogen treated and control gilts were similar only on day 12 when normal embryos were found in both treatments. However, AVA after day 12 in estrogen treated gilts was much lower than in control animals presumably due to a lack of viable embryos after day 12.

An examination of the uterine fluid from estrogen treated and control gilts on days 12, 14, 16, and 18 of pregnancy revealed no differences in the distribution of leukocytes when examined on a treatment or day basis,

suggesting that conceptus death was not associated with leukocyte invasion. It is not known if the leukocyte distribution within the endometrial tissue was affected by either treatment or day since this was not determined in the present study.

Greis et al. (1989) demonstrated differences in protein synthesis and secretion between control and estrogen treated gilts after estradiol valerate administration on days 9 and 10 of gestation. A distinct band of three basic polypeptides (Mr=30,000; pI 7.9-9.0) was present in all control gilts, but was attenuated in estrogen treated gilts as indicated by [^{3}H]-leucine incorporated endometrial explant cultures. A similar attenuation of an acidic polypeptide (Mr=100,000; pI 3.5-5.0) was evident in estrogen treated gilts compared to controls. In the present study, labeling with [^{3}H]-glucosamine demonstrated that many of these proteins were glycoproteins though no differences were evident between treatments. The function of these glycoproteins during pregnancy are currently unknown.

Luft (1971) used staining with ruthenium red to indicate the presence of acidic polysaccharides on cell surfaces. In the present study staining with ruthenium red demonstrated that the uterine glycocalyx progressively thickens between days 12-18 of gestation in control gilts. The glycocalyx thickens between days 13-19 of the estrous cycle and becomes thicker and more fibrous at corresponding stages of pregnancy (Keys and King, 1990). In the present

study a reduction of the maternal glycocalyx was observed at areas of placental attachment. Previous studies have also reported a reduction in the uterine glycocalyx upon attachment of the conceptus in the mouse (Enders and Schlafke, 1974; Chavez and Anderson, 1985), rat (Hewitt et al., 1979), rabbit (Anderson and Hoffman, 1984), and pig (Dantzer, 1985), suggesting that a reduction in the maternal epithelial glycocalyx may be an integral part of implantation in these species.

Administration of estradiol valerate on days 9 and 10 of gestation resulted in loss of the uterine glycocalyx beginning on day 14. The loss of the glycocalyx coincided with the onset of embryonic mortality in estrogen treated gilts. Glycoproteins present on most cell surfaces are involved in cellular recognition and adhesion (Luft, 1976). Glycoproteins associated with the glycocalyx have also been implicated in trophoblast attachment to the uterine epithelium (Chavez and Enders, 1982; Richa et al., 1985; Chavez, 1986). The glycocalyx appears to anchor the maternal and fetal epithelia together during placental adhesion (Keys and King, 1990). In rodents it is evident that estrogen induces synthesis of glycoproteins associated with the uterine glycocalyx (Takata and Terayama, 1979; Dutt et al., 1988; Morris et al., 1988; Takeda, 1988). From these studies and the present one, it is apparent that the maternal glycocalyx is involved in placental attachment.

Binding of CF demonstrated the presence of anionic

sites on the endometrial glycocalyx. In the present study, CF binding was intense and uniformly distributed over microvilli on day 12 of gestation in control gilts. After day 12, the intensity of CF binding decreased through day 18 of gestation indicating a reduction in epithelial cell surface negativity. These results are similar to those reported by Hewitt et al. (1979) who showed a reduction in polycationic ferritin (PCF) labeling between days 2-6 of gestation in rats. Evidence indicates that reduction of cell surface negativity enhances cell adhesion (Vicker and Edwards, 1972; Weiss et al., 1975; Grinnell, 1976), which suggests that a reduction of anionic sites on the uterine luminal epithelium may also facilitate trophoblast apposition in the pig.

Although ferritin binding was reduced at day 14 of gestation in control gilts, binding had become more localized toward the tips of the microvilli. This is also evident at day 5 of pregnancy in the rat (Hewitt et al., 1979) and has been previously reported at day 15 of gestation in the pig (Dantzer, 1985). These anionic sites could facilitate the initial contact of the trophoblast with the uterine surface epithelium as well as being involved in conceptus-uterine interactions during implantation.

Gilts receiving estradiol valerate on days 9 and 10 of gestation exhibited a reduction in CF binding as early as day 12 of gestation. Estrogen treated gilts display less CF binding than control gilts on day 12 of gestation, but no

differences were evident between control and estrogen treated gilts between days 14-18 of gestation.

In areas of trophoblast apposition, little or no CF binding was evident on the uterine epithelium at day 18 of gestation in control gilts. Anderson and Hoffman (1984) reported a lack of PCF labeling on the mesometrial and antimesometrial aspects of rabbit endometrium at presumptive areas of implantation as well as a lack of PCF binding at non-implantation areas. These results are similar to the report of Hewitt et al. (1979) which indicated no difference in the loss of anionic sites between presumptive implantation sites and other areas of the luminal epithelium in the rat. Similarly, Guillomot et al. (1982) observed no differences in CF between caruncular and intercaruncular areas of uterine epithelium in cyclic or pregnant ewes.

Pope et al. (1982) suggested that embryonic death in swine may be a result of alterations in uterine secretory activity following estrogen exposure during early pregnancy. Estradiol administration on day 11 of gestation advances uterine secretory activity (Geisert et al., 1982c; Morgan et al., 1987) resulting in asynchrony between the uterine environment and the developing conceptus which results in embryo death. Although embryonic death may be caused by different mechanisms, the results from these studies (Geisert et al., 1982c; Morgan et al., 1987) demonstrate the importance of synchrony between the conceptus and uterine environment.

Results from the present study indicate that the presence of the maternal glycocalyx and its subsequent modification during attachment appear to be essential for placental attachment and conceptus survival. Premature exposure of the uterus to estrogen stimulates alterations in the uterine epithelial surface resulting in failure of conceptus attachment and subsequent embryonic death.

TABLE I

ANTIVIRAL ACTIVITY OF UTERINE FLUID FROM GILTS TREATED WITH ESTRADIOL VALERATE OR VEHICLE ON DAYS 9 AND 10 OF GESTATION

	Treatment[a,b]	
Day of Gestation	Vehicle	Estradiol Valerate
12	< 200	< 200
14	1440 ± 919	726 ± 64
16	5960 ± 1206	693 ± 593
18	3713 ± 712	370 ± 8

[a] Treatment x Day interaction ($P < 0.05$)

[b] Mean ± S.E.; n=3

TABLE II

MEAN CATIONIC FERRITIN BINDING IN GILTS TREATED WITH VEHICLE OR ESTRADIOL VALERATE ON DAYS 9 AND 10 OF GESTATION

Day of Gestation	N	Number of Ferritin Granules[a]	Epithelial Surface Area (mm^2)	Counts/Area[a]
Control				
Day 12	11	223.27 ± 30.38	1976.27 ± 125.08	.117 ± .009
Day 14	14	97.79 ± 16.97	1779.80 ± 136.56	.053 ± .007
Day 16	7	35.43 ± 3.36	1147.07 ± 116.12	.033 ± .004
Day 18	4	53.25 ± 4.42	847.88 ± 117.08	.065 ± .004
Estradiol				
Day 12	5	74.40 ± 6.30	1917.40 ± 131.78	.040 ± .005
Day 14	5	45.40 ± 9.26	1380.20 ± 52.32	.033 ± .007
Day 16	6	39.50 ± 10.59	1307.92 ± 226.61	.028 ± .004
Day 18	4	104.50 ± 38.67	1440.85 ± 287.69	.066 ± .010
Trypsin				
Day 12	3	627.00 ± 31.13	2316.67 ± 164.87	.273 ± .012

[a]Treatment x Day interaction ($P < 0.05$)

Figure 1. Fluorographs from two-dimensional polyacrylamide gel electrophoresis analysis of polypeptides secreted in vitro by uterine endometrial explants from control (a) and estrogen treated (b) gilts on day 14 of gestation. The group of polypeptides (arrowhead) remain unchanged irrespective of treatment.

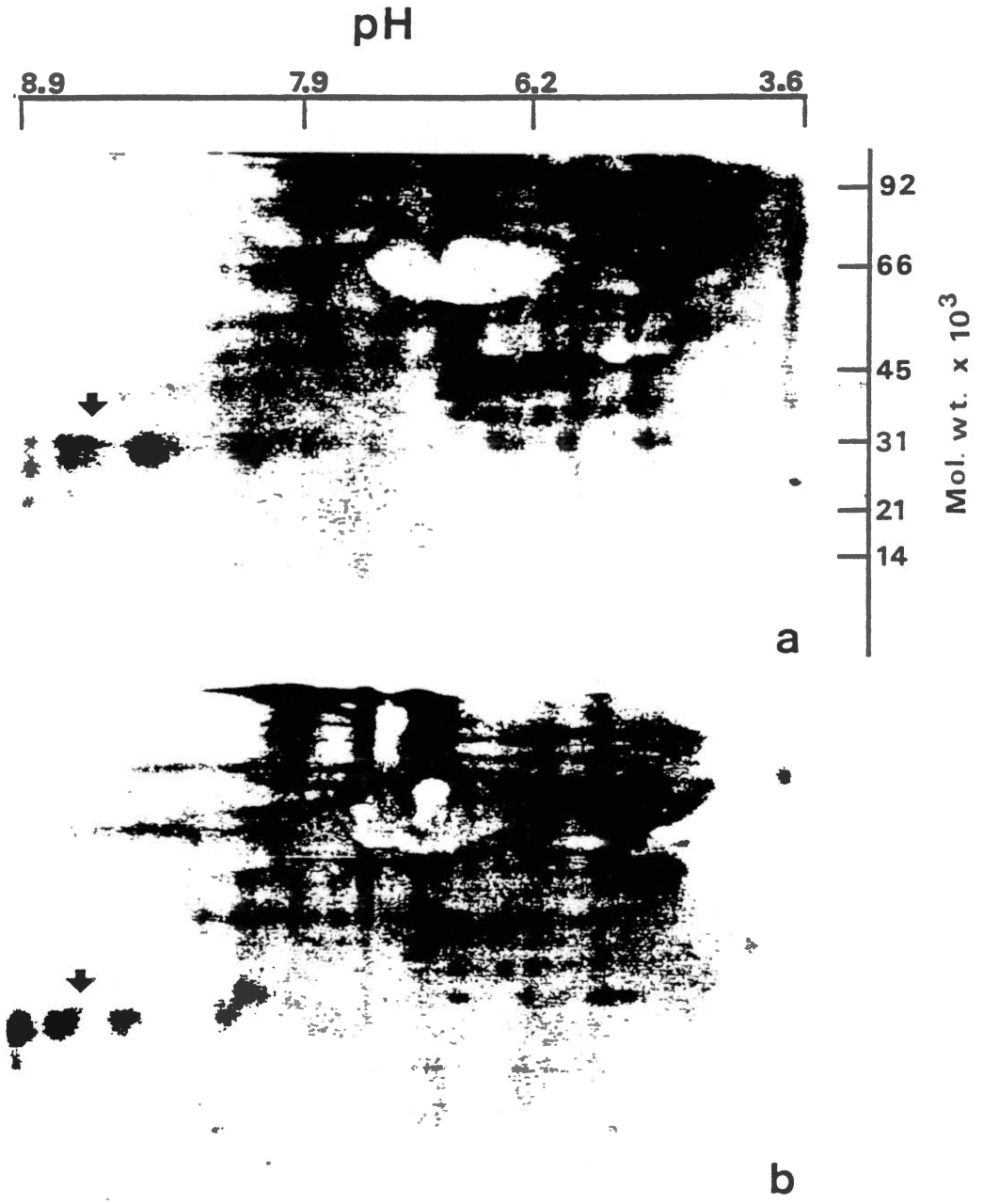

pH
8.9
7.9
6.2
3.6
92
66
45
31
21
14
Mol. wt. x 10^3
a
b

Figure 2. Scanning electron micrographs of uterine epithelial surfaces from control gilts on day 12 (a&b) and day 14 (c&d) of gestation. (a) Trophoblast (T) is apposed to uterine epithelium (E). x300. (b) Uterine folding is evident. Cell apical surfaces are smooth and stereocilia are present. x1200.

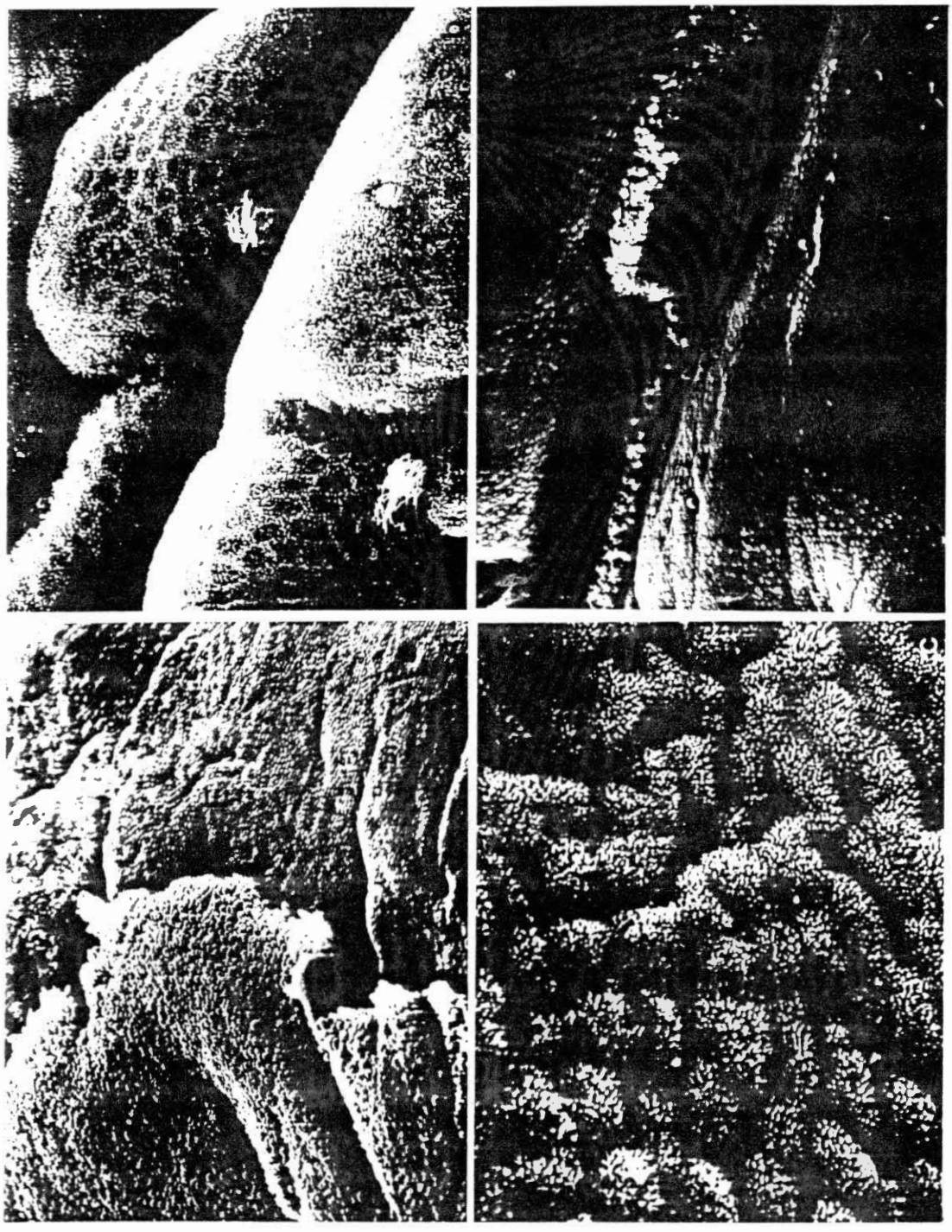


Figure 3. Scanning electron micrographs of uterine epithelial surfaces from control gilts on day 16 (a&b) and day 18 (c&d) of gestation. (a&b) Apical protrusion of epithelial cells are evident, microvilli are dense and trophoblast tissue is present (a, x5400; b, x940). (c&d) Microvilli appear less dense, apical domes are prominent and trophoblast is apposed to the uterine epithelium (c, x4000; d, x660).

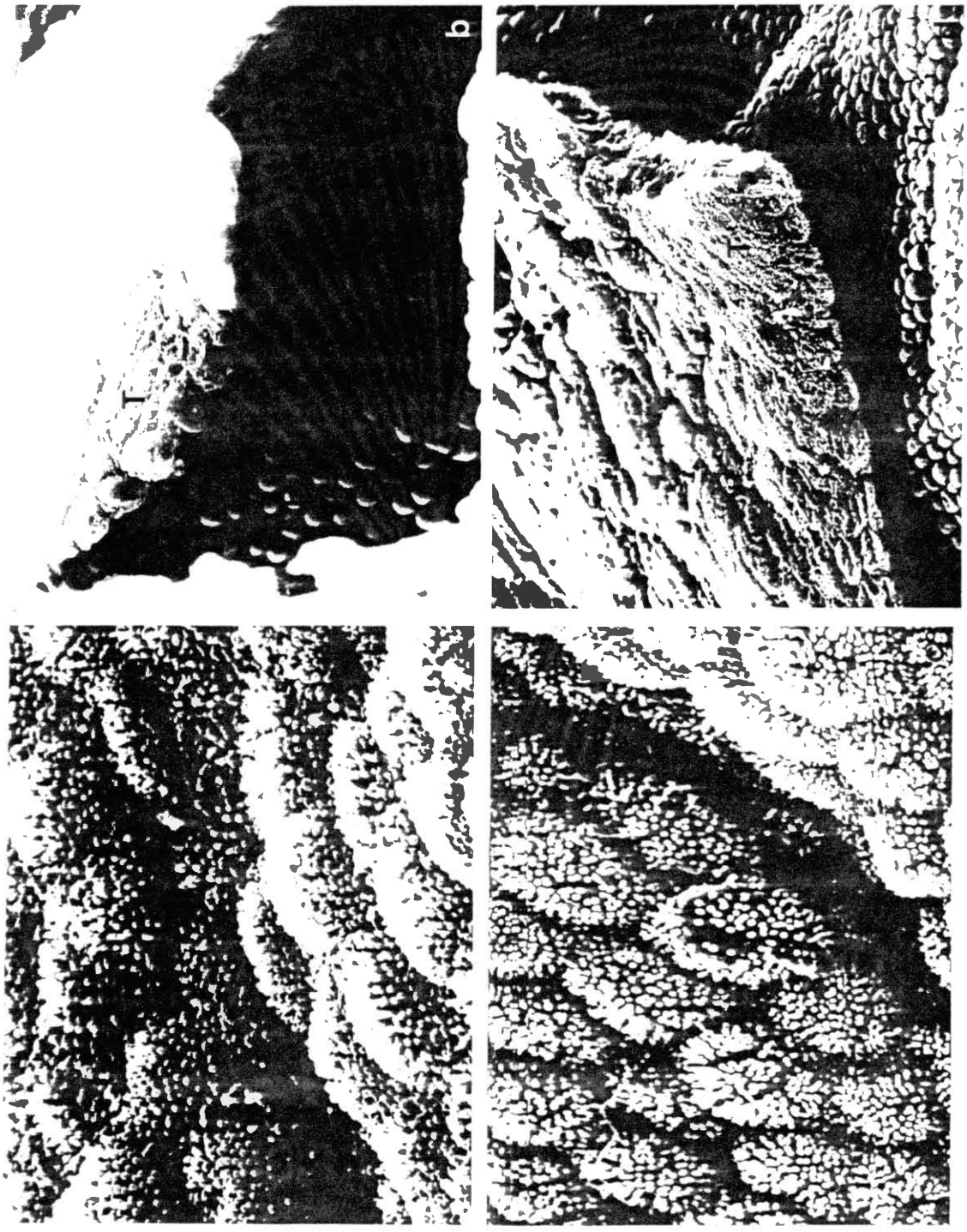

b

Figure 4. Scanning electron micrographs of uterine epithelial surfaces from estrogen-treated gilts on day 12 (a&b) and day 14 (c&d) of gestation. (a&b) Uterine surface morphology is similar to that observed on day 12 in control gilts (a, x1000; b, x2200). (c) Protrusion of apical surfaces and numerous microvilli are evident. x2200. (d) Epithelial cells appear degenerate and many cells are lacking microvilli. x2000.

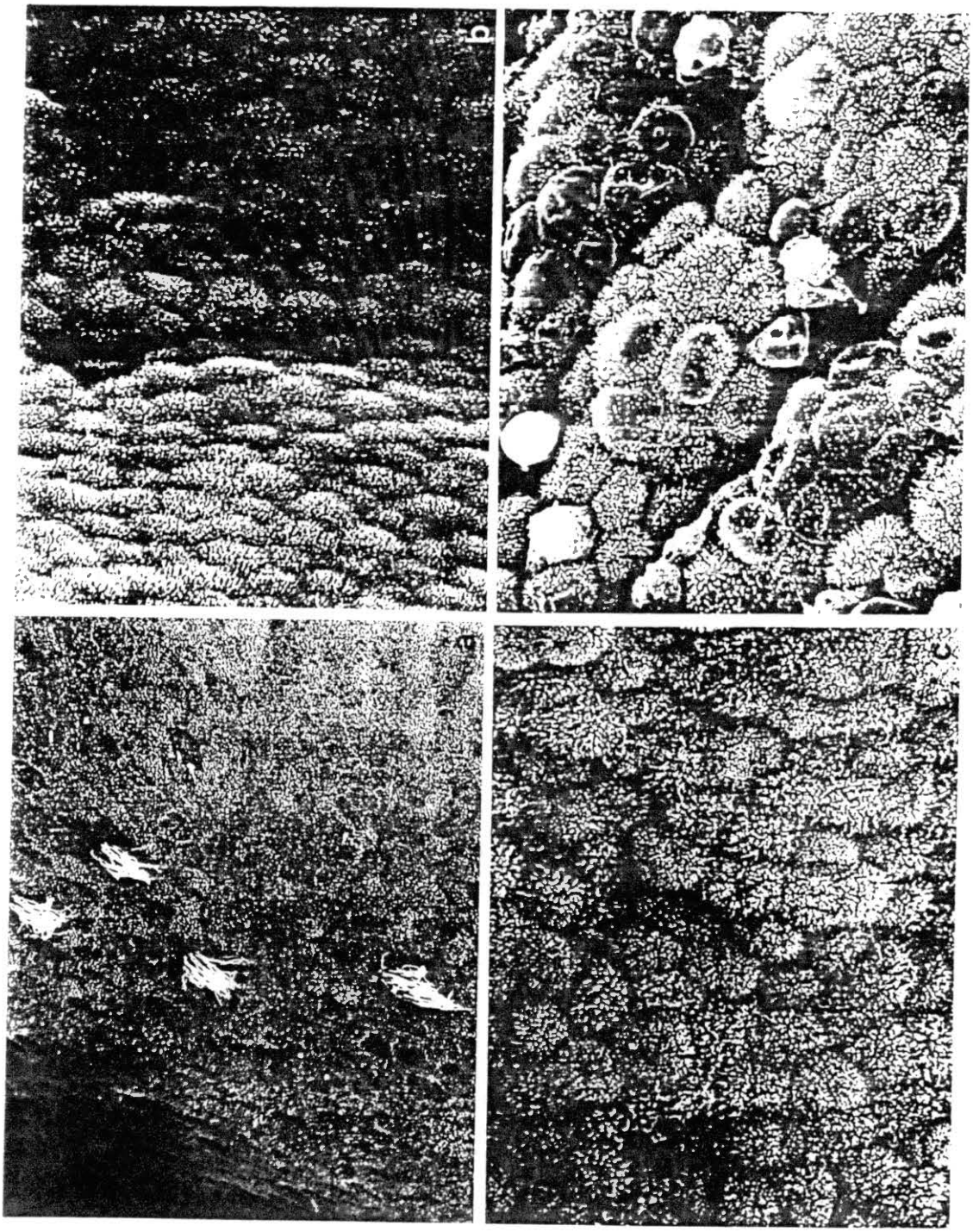


Figure 5. Scanning electron micrographs of uterine epithelial surfaces from estrogen-treated gilts on day 16 (a&b) and day 18 (c&d) of gestation. Loss of microvilli is extensive in some areas (a, x1800) while the uterine epithelium appears eroded and lacks apical doming in others (b, x6000). (c&d) The uterine epithelium exhibits similar morphology as on day 16 (c, x3000; d, x1300).

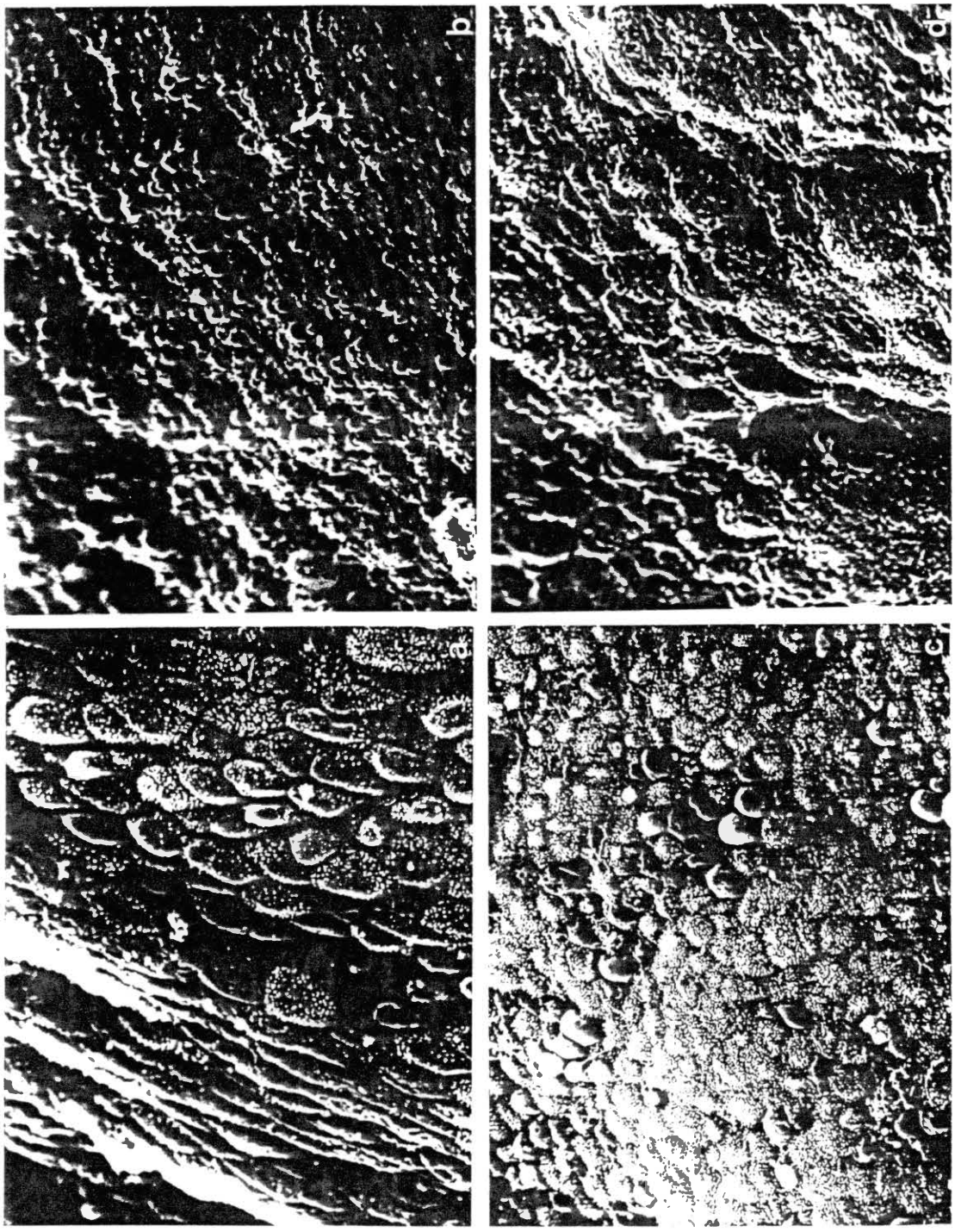
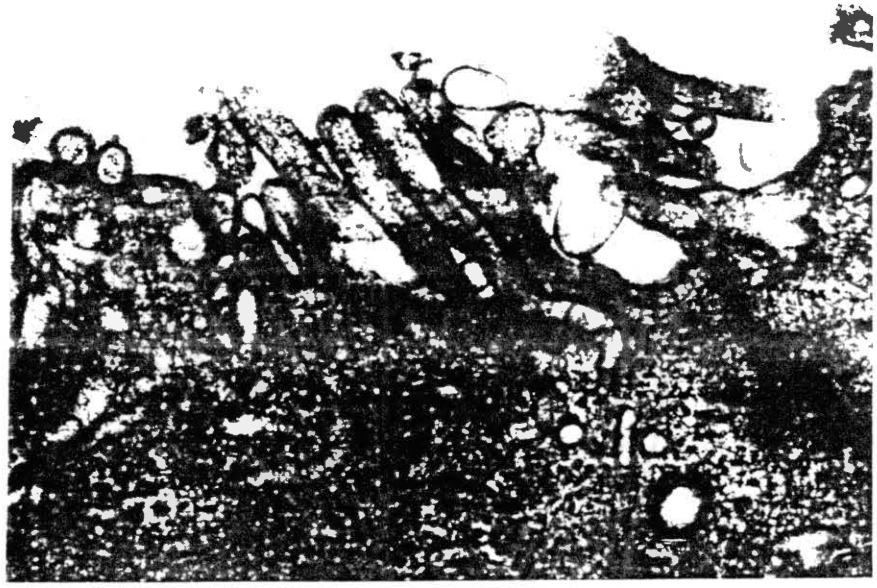

b
d
a
c

Figure 6. Transmission electron micrographs of uterine epithelial surfaces of control gilts at day 12 of gestation after treatment with trypsin. Although the endometrial glycocalyx is removed, as demonstrated by a lack of ruthenium red staining (a, x36000), cationic ferritin binding remains abundant (b, x36000).




b

Figure 7. Transmission electron micrographs of uterine epithelial surfaces stained with ruthenium red. At day 12 of gestation, both control (a, x48000) and estrogen-treated (b, x36000) gilts have a thin fibrous glycocalyx covering microvilli. At day 14 of gestation, control gilts (c, 36000) exhibit a thickening of the glycocalyx whereas the glycocalyx becomes detached from the microvilli of estrogen-treated gilts (d, x29000).

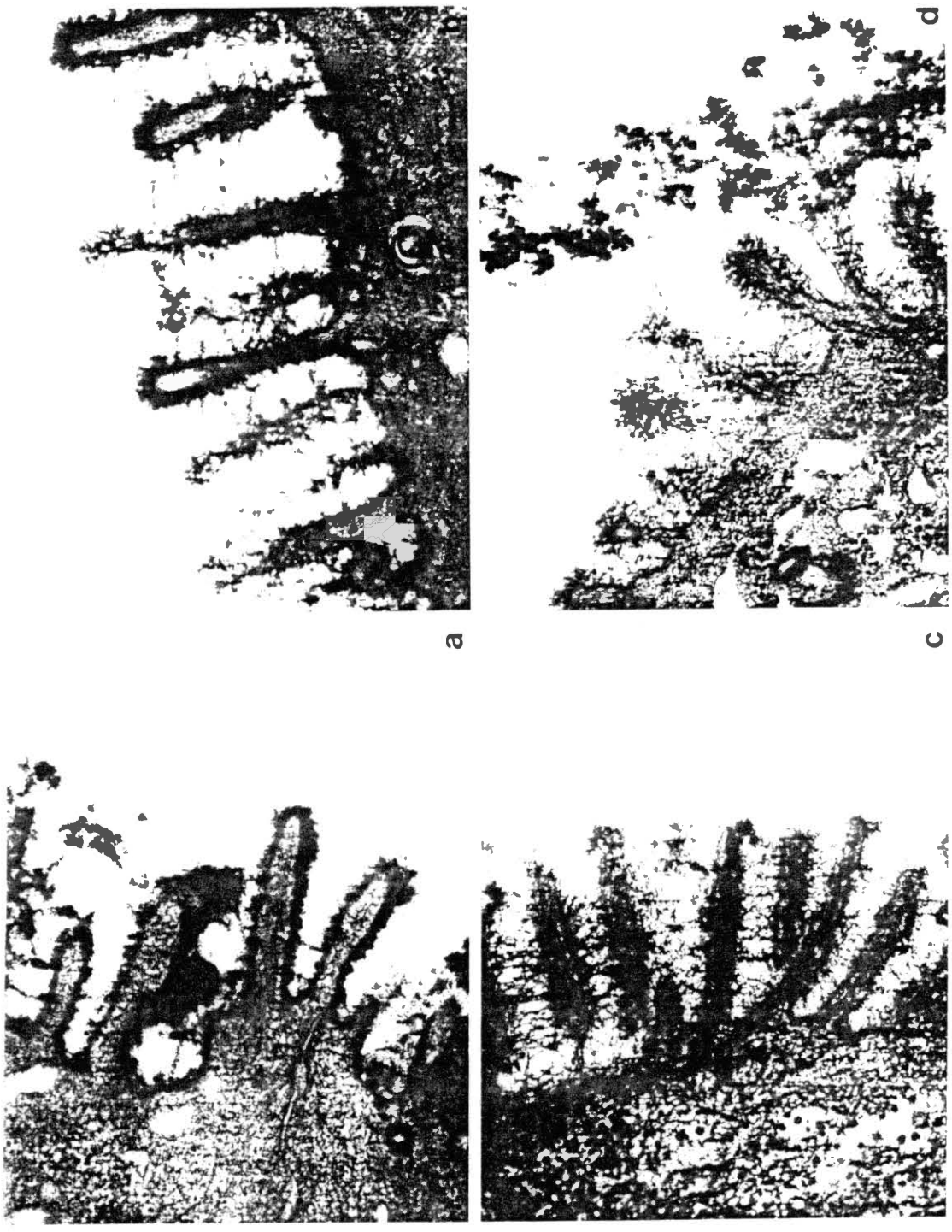

a
c

Figure 8. Transmission electron micrographs of uterine epithelial surfaces stained with ruthenium red. Control gilts (a, x36000) have a thick, fibrous glycocalyx compared to estrogen-treated (b, x36000) gilts which exhibit a sparse glycocalyx at day 16 of gestation. A similar morphology is evident at day 18 of gestation except that the glycocalyx is thicker at the tips of the microvilli in control gilts (c, x48000). The glycocalyx from day 18 estrogen-treated gilts remains sparse (d, x36000).


b
d
a

Figure 9. Transmission electron micrographs of uterine epithelial surfaces stained with cationic ferritin (CF). At day 12 of gestation, control gilts (a, x48000) exhibit abundant CF binding compared to estrogen-treated gilts (b, x48000) where CF binding is reduced. By day 14 of gestation CF binding is more abundant at the tips of the microvilli in control gilts (c, x48000), whereas CF binding is sparse in estrogen-treated gilts (d, x48000).


b
d
a
c

Figure 10. Transmission electron micrographs of uterine epithelial surfaces stained with cationic ferritin. Sparse CF binding is observed at both day 16 (a, x48000; b, x48000) and day 18 (c, x48000; d, x48000) with no apparent differences between control (a&c) and estrogen-treated (b&d) gilts.

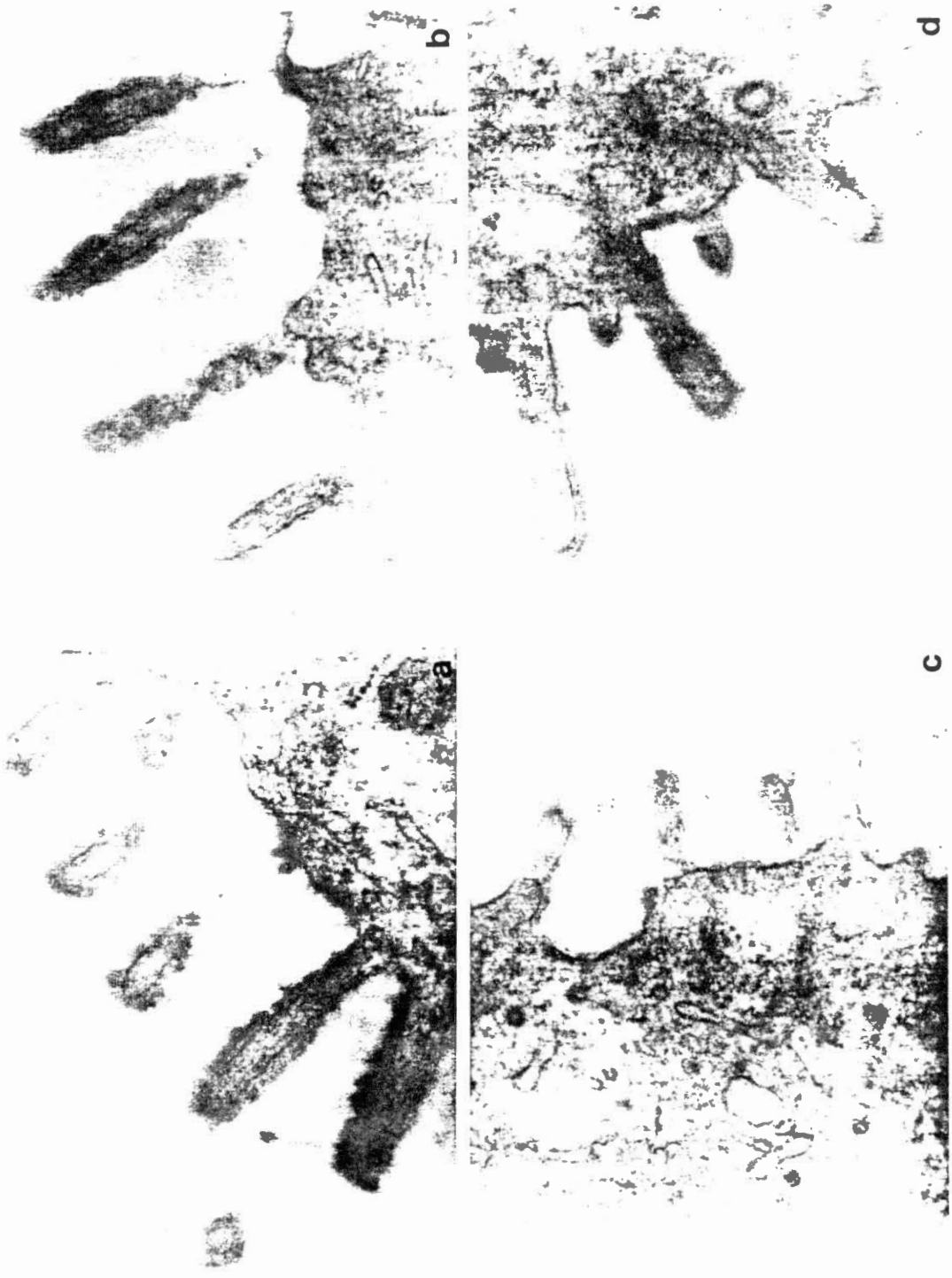
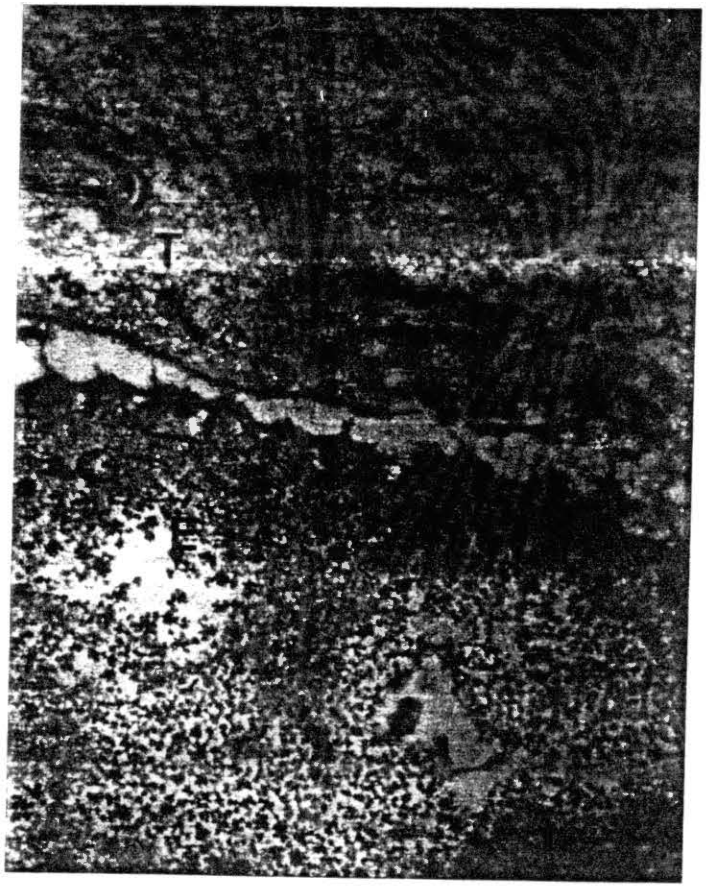

b
d
a
c

Figure 11. Transmission electron micrograph of trophoblast (T) apposed to uterine epithelium (E). Notice the reduced thickness of the glycocalyx and height of the microvilli. x29000.



CHAPTER IV

ULTRASTRUCTURAL EXAMINATION OF PORCINE UTERINE LUMINAL EPITHELIUM AFTER ESTROGEN ADMINISTRATION ON DAYS 9 AND 10 OF GESTATION

Introduction

Upon attaining a diameter of 10 mm on day 12 of pregnancy, porcine blastocysts undergo a rapid morphological transformation from spherical to filamentous form (Anderson et al., 1978; Geisert et al., 1982). During this period of trophoblast elongation, porcine blastocysts secrete large quantities of estrogen (Perry et al., 1973; Gadsby et al., 1980; Fischer et al., 1985) which are involved in the establishment of pregnancy in the pig (Bazer and Thatcher 1977; Heap et al., 1979). Following trophoblast elongation, conceptus attachment occurs between days 13 and 18 of gestation (King et al., 1982) beginning in the region of the embryonic disc and extending toward the periphery of the trophoblast by day 26 (Dantzer 1985). Placentation in the pig involves a noninvasive ınterdigitation of uterine

and trophoblastic microvilli as well as a reduction of the uterine glycocalyx in areas of trophoblast apposition (Dantzer 1985).

Keys and King (1988) indicated that estrogen administration between days 10 and 13 of the gilts' estrous cycle resulted in alterations in the uterine luminal epithelium. Estrogen administered systemically or into the uterine lumen resulted in increased uterine folding, glycogen accumulation, increased synthetic and secretory activity, and a thickening of the epithelial glycocalyx. These estrogen-induced alterations were similar to ultrastructural changes evident during early pregnancy (Stroband et al., 1986; Keys and King 1990).

Pope et al. (1986) demonstrated that administration of exogenous estrogen to gilts on days 9 and 10 of gestation (which is two days prior to normal estrogen synthesis and release by the conceptus) resulted in complete embryonic loss by day 30 of pregnancy. Further studies by our laboratory have revealed that administration of estradiol valerate to gilts on days 9 and 10 of pregnancy resulted in degenerating conceptuses by day 14 or 16 (Morgan et al., 1987; Greis et al., 1989; Blair et al., 1991). Blair et al. (1991) demonstrated that embyonic death following premature exposure of gilts to estrogen was associated with a loss of the glycocalyx on the microvilli of the uterine surface epithelium on day 14 of gestation.

The objective of the present study was to examine

ultrastructural changes in the uterine luminal epithelium of the pig during the period of trophoblast attachment and to characterize alterations which might by associated with the loss of the uterine epithelial glycocalyx and embryonic mortality due to early exogenous estrogen administration.

Materials and Methods

Animals

Mature, cyclic crossbred gilts were observed for estrous behavior twice daily (0700 and 1700 h) in the presence of intact boars. After exhibiting two estrous cycles of normal duration (17-22 days), gilts were bred naturally at the onset of estrus (Day 0) and 12 and 24 h later.

Gilts were randomly assigned to receive one of the following two treatments: intramuscular injection (0.25 ml) of sesame oil on days 9 and 10 of gestation (Control; n=6) or intramuscular injection (5.0 mg) of estradiol valerate on days 9 and 10 of gestation (n=6). Following assignment to treatment groups, gilts were scheduled to be unilaterally hysterectomized on either days 12 and 14 or days 16 and 18 of gestation.

Surgical Procedures

Gilts were unilaterally hysterectomized after induction of anesthesia with a 5% solution of thiopentone sodium as previously described (Blair et al. 1991). Anesthesia was maintained on a closed-circuit system of halothane (5% Fluothane; Halocarbon Industries Inc., North Augusta, SC) and oxygen (1.0 L/min). Once a surgical plane of anesthesia was attained, the uterus was exposed via mid-ventral laparotomy and a randomly selected uterine horn and its ipsilateral ovary were excised. After routine closure of the incision, gilts were treated post-surgically with antibiotics.

Tissue Preparation for Electron Microscopy

Immediately upon removal of the uterine horn, a 7-10 cm section approximately 10-20 cm anterior to the uterine body was excised and opened along the antimesometrial border. Endometrial tissue explants from control and estrogen-treated gilts were dissected from the myometrium and immersed in modified Karnovsky's fixative (2% glutaraldehyde and 1.6% paraformaldehyde; KF) as described by Karnovsky (1965). Endometrial explants were cut into 1-2 mm pieces, immersed in fresh fixative, and placed on a rotating platform for 2 h at room temperature. Following fixation, tissue samples were rinsed with 0.1 M cacodylate buffer and

post-fixed with 1% osmium tetroxide in 0.1 M cacodylate buffer for 1 h. Following postfixation, tissue was rinsed with 0.1 M cacodylate buffer, dehydrated in a graded series of alcohol, and embedded in epon-araldite (Mollenhauer 1964).

Transmission Electron Microscopy

Tissue samples were sectioned on a Porter-Blum MT-2 ultramicrotome (Sorvall Instruments, Norwalk, CT). Thin sections (60-90 nm) were collected on 200-mesh uncoated copper grids. Thin sections were subsequently poststained with a 7.5% uranyl acetate solution and Sato's lead stain (Sato 1967). Tissue samples were observed on a JEOL TEMSCAN transmission electron microscope (JEOL Ltd., Tokyo, Japan) at 80 KV.

Volume Density of Organelles

Volume density (V_D) values for glycogen deposits, mitochondria, golgi complexes, rough and smooth endoplasmic reticulum (RER and SER, respectively), clear vesicles and dense bodies were estimated by point counting volumetry (Weibel 1979). Point counts were performed by placing a grid of equidistant lines (0.5 mm^2) over electron micrographs of uterine epithelial tissue. Line

intersections were regarded as sampling points. For each organelle, point count data was pooled from all micrographs within treatments and days of gestation. Values of V_D were calculated as percentages of the cytoplasmic compartment where V_D = [points over organelle]/[(total points) - (nuclear points + points not on uterine epithelial tissue)].

Statistical Analysis

Volume density of organelles was analyzed by least squares analysis of variance using General Linear Models of SAS (1987). Data was analyzed for the main effects of treatment, day of gestation, and side (apical versus basal) as well as for interactions between the main effects. Polynomial contrasts were utilized to determine differences over days of gestation.

Results

On all days studied, uterine luminal epithelial cells were characterized by the presence of vesicular nuclei, prominent golgi complexes, and abundant mitochondria. Table 1 provides the mean V_D of organelles from porcine uterine luminal epithelial tissue from Control and E-treated gilts. In the present study, conceptuses were degenerated by day 14 of gestation in E-treated gilts. Conceptus degeneration was

similar to that reported in previous studies by our laboratory (Morgan et al. 1987; Gries et al. 1989; Blair et al. 1991).

Glycogen

Figure 1 demonstrates the accumulation of glycogen deposits in the uterine epithelium. The amount of glycogen accumulated on day 12 of gestation was greater ($P < 0.05$) in E-treated compared to Control gilts while glycogen content on day 16 of gestation was greater ($P < 0.05$) in Control compared to E-treated gilts (Table 1). However, glycogen content was similar between treatments on days 14 and 18 of pregnancy. The accumulation of glycogen increased ($P < 0.05$) between days 12, 14, and 16 of gestation in Control gilts and then decreased on day 18 of gestation to levels similar to those observed on day 14 of gestation. In E-treated gilts, glycogen content increased ($P < 0.05$) from day 12 to day 14 of pregnancy and then remained constant through day 18 of gestation. Glycogen was present in greater amounts ($P < 0.05$) in the basal aspect compared to the apical aspect of uterine luminal epithelial cells in Control and E-treated gilts on days 16 and 18 of gestation.

Mitochondria

Mitochondria were abundant in both Control and E-treated gilts on all days examined (Fig. 2). The mean V_D of mitochondria (Table 1) was similar between treatments on days 12, 14, and 18 of pregnancy, whereas mitochondria comprised a greater ($P < 0.05$) proportion of the uterine epithelial cytoplasm in E-treated compared to Control gilts on day 16 of gestation. In Control gilts, the mean V_D of mitochondria was similar between days 12-16 of gestation, but increased on day 18 ($P < 0.05$). In E-treated gilts, mean V_D increased ($P < 0.05$) between days 12 and 14 of gestation and then remained similar through day 18 of gestation. No differences were evident in the mean V_D of mitochondria in the apical and basal aspects of the uterine epithelium.

Golgi Complex

Golgi complexes had a supranuclear orientation (Fig. 2), and were well developed and associated with vesicles on all days examined. No differences in V_D were seen between treatments or between the days of gestation studied.

RER and SER

Volume density of rough endoplasmic reticulum was not different between Control and E-treated gilts on days 12-18 of gestation (Fig. 3). In Control gilts, V_D of rough endoplasmic reticulum was similar from days 12-16 of gestation, however day 12 was lower than day 18 of gestation ($P < 0.05$). In E-treated gilts, V_D of rough endoplasmic reticulum was higher ($P < 0.05$) on days 16 and 18 of gestation compared to day 12 of gestation. On each day examined, V_D of the rough endoplasmic reticulum was similar between the apical and basal compartments of the uterine epithelium, however when pooled over the days studied V_D was greater ($P < 0.05$) in the apical compared to the basal aspect of the uterine luminal epithelium. The V_D of rough endoplasmic reticulum was similar between apical and basal regions in Control gilts, whereas in E-treated gilts the V_D was greater ($P < 0.05$) in the apical compared to the basal part of the cell.

Smooth endoplasmic reticulum comprised only a small proportion of the uterine epithelial cytoplasm. The V_D of smooth endoplasmic reticulum was greater ($P < 0.05$) in Control gilts compared to E-treated gilts on day 12 of gestation, however the V_D was similar between treatments on days 14-18 of pregnancy. On day 12 of gestation, V_D of smooth endoplasmic reticulum was greater ($P < 0.05$) in the apical aspect of the uterine luminal epithelium compared to

the basal aspect in both Control and E-treated gilts; the V_D was similar between the apical and basal portion of the cells on days 14-18 of gestation.

Vesicles and Dense Bodies

On day 12 of gestation, V_D of clear vesicles was greater ($P < 0.05$) in Control compared to E-treated gilts (Fig. 4), however one gilt did exhibit a large number of vesicles underlying the apical cell membrane of the uterine luminal epithelium at day 12 of gestation (Fig. 6). Clear vesicles were similar between treatments on days 14-18 of gestation. In Control gilts, clear vesicles were more abundant on day 12 of pregnancy compared to days 14-18 ($P < 0.05$) whereas V_D of clear vesicles was similar between days 12-18 in E-treated gilts. On day 12 of pregnancy, vesicles were more abundant ($P < 0.05$) in the apical aspect of the uterine epithelium compared to the basal aspect. On all other days of pregnancy, V_D of clear vesicles were similar between compartments.

No treatment or day differences were evident in the V_D of dense bodies. However, dense bodies were more abundant ($P < 0.05$) in the basal compared to the apical portion of the uterine epithelium in Control and E-treated gilts (Fig. 5).

Discussion

Several recent reports have described ultrastructural changes of the porcine uterine luminal epithelium during the estrous cycle and early pregnancy (Dantzer 1985; Stroband et al., 1986; Keys and King 1989, 1990; Stroband and Van der Lende 1990). Previous studies (Morgan et al., 1987; Gries et al., 1989; Blair et al., 1991) have demonstrated that administration of estradiol valerate on days 9 and 10 of gestation resulted in embryonic death by day 14-16 of pregnancy. In a previous report (Blair et al., 1991) we indicated that embryonic death in gilts prematurely exposed to estrogen was associated with a loss of the uterine epithelial glycocalyx on day 14 of gestation.

In the present study, treatment of gilts with estradiol valerate increased the amount of glycogen accumulation in uterine surface epithelium on day 12 of gestation, but the glycogen content was similar between treatment groups on days 14-18 of pregnancy. Accumulation of glycogen in the uterine luminal epithelium increased between days 12, 14, and 16 of gestation and then decreased on day 18 of gestation in Control gilts. Previous studies have also reported a rapid increase in the amount of glycogen after day 12 of pregnancy (Dantzer 1985; Stroband et al., 1986; Keys and King 1990). Keys and King (1988) reported that estrogen stimulates an increase in glycogen in cyclic gilts suggesting that conceptus-derived estrogen may act to direct

glycogen deposition in the pig. Estrogen has also been shown to increase glycogen accumulation by uterine epithelial cells in the rat (Boettinger 1946; Walaas 1952). In the present study, no differences in glycogen content were evident between days 12-18 of gestation in E-treated gilts. Lack of a difference in E-treated gilts is most likely due to a preemptive estrogen-induced increase in glycogen accumulation on day 12 of pregnancy compared to Controls.

Mitochondria, which were abundant in the uterine luminal epithelium of Control and E-treated gilts, were evenly dispersed between the apical and basal regions of the uterine epithelium. Mitochondria increased between days 16-18 of gestation in Control gilts and from day 12 to day 14 of gestation in E-treated gilts. This early increase in mitochondria in E-treated gilts suggests a hastening in the metabolic activity of the uterine luminal epithelium compared to Control gilts.

Rough endoplasmic reticulum content was similar between Control and E-treated gilts, however rough endoplasmic reticulum was more abundant on day 18 of gestation compared to day 12 in both treatment groups. Stroband et al., (1986) demonstrated an increase in rough endoplasmic reticulum after day 8 of pregnancy whereas Keys and King (1989) showed extensive rough endoplasmic reticulum after day 13 of pregnancy. In addition, the present study has shown that rough endoplasmic reticulum was significantly more abundant

in the apical region compared to the basal region by day 18 of gestation.

Dense bodies were present in greater amounts in the basal region compared to the apical region of the uterine luminal epithelium. Stroband et al., (1986) indicated increasing amounts of these structures after day 11 of gestation, however in the present study no differences in dense bodies were evident between days 12-18 of gestation. These dense bodies appear to be associated with secretion (Dantzer et al., 1981; Friess et al., 1981) as Stroband et al. (1986) has previously indicated that at least some of these structures are secretory granules.

Clear vesicles are abundant between days 8 and 10 of gestation in porcine uterine luminal epithelium, but are reduced after day 10 (Stroband et al., 1986; Keys and King 1990). In the present study, clear vesicles were not very abundant in the uterine luminal epithelium between days 12-18. A release of these vesicles from the surface epithelium during the period of trophoblast elongation in a similar fashion to release occurring from glandular epithelial cells (Geisert et al., 1982a) may explain the low numbers later in gestation. Conceptuses from the gilt exhibiting large numbers of vesicles were still at the spherical stage of development, a stage of conceptus development which produces only low levels of estrogen, which may explain the numerous amount of vesicles still present within the epithelium.

The results of the present study show no major

alterations in the ultrastructure of the uterine luminal epithelium which were coincidental with the loss of the uterine epithelial glycocalyx or embryonic death. The morphological changes observed are consistent with increased synthetic and secretory activity of the uterine luminal epithelium as gestation progresses. Estrogen appeared to increase this secretory activity early in E-treated compared to Control gilts as indicated by earlier increases in glycogen accumulation, mitochondria and rough endoplasmic reticulum. These morphological results support previous data (Geisert et al., 1982c; Morgan et al., 1987) which demonstrated that exogenous estradiol administration advances uterine secretions.

TABLE III

Volume density of cellular organelles of porcine uterine luminal epithelium after estrogen administration on days 9 and 10 of gestation[a]

	Day of Gestation							
	Control				E2-Treated			
Organelle	12	14	16	18	12	14	16	18
Apical								
Glycogen	1 15±0 62	9 71±3 86	15 92±5 71	10 51±2 95	6 34±4 72	13 52±4 28	7 85±1 61	11 18±3 81
Mitochondria	6 31±1 58	7 00±1 02	7 11±0 68	9 08±1 13	5 86±0 33	7 41±0 11	8 84±0 04	8 82±0 96
Golgi	1 80±0 74	1 66±0 69	2 27±1 06	1 01±0 03	1 00±0 30	1 99±0 32	1 42±0 72	1 48±0 34
RER	1 38±0 17	1 97±0 54	1 99±0 18	2 54±0 20	1 35±0 36	2 08±0 39	2 49±0 32	2 18±0 07
SER	1 57±0 49	0 94±0 22	0 68±0 33	0 89±0 08	0 59±0 05	0 71±0 08	1 09±0 07	0 91±0 02
Vesicles	2 68±1 41	0 64±0 23	0 95±0 16	0 65±0 16	0 58±0 17	0 63±0 19	0 50±0 05	0 39±0 10
Dense Bodies	0 75±0 10	0 49±0 13	0 94±0 43	0 62±0 23	0 49±0 05	0 62±0 22	1 10±0 40	0 80±0 53
Basal								
Glycogen	3 82±1 58	17 19±1 87	26 73±4 05	20 67±1 50	16 15±5 72	17 39±1 09	22 58±3 28	18 27±3 99
Mitochondria	6 48±0 94	7 51±0 83	6 21±0 88	8 54±0 34	6 06±0 18	8 51±0 79	9 66±0 27	9 65±0 85
RER	1 31±0 23	1 90±0 35	1 79±0 35	1 81±0 50	1 17±0 28	1 55±0 44	1 72±0 08	1 62±0 28
SER	0 64±0 10	0 69±0 19	0 58±0 23	0 63±0 14	0 25±0 03	0 23±0 08	0 83±0 17	0 73±0 24
Vesicles	0 30±0 05	0 04±0 02	0 23±0 02	0 22±0 05	0 15±0 02	0 11±0 04	0 28±0 05	0 28±0 02
Dense Bodies	1 45±0 20	2 33±0 98	2 00±0 69	2 65±0 38	2 69±0 24	2 31±0 48	2 32±0 42	2 17±1 35

[a] Volume density represented as a percent of cytoplasm

Figure 12. Representative photomicrograph of porcine endometrium showing the basal portion of the surface epithelium from control gilts. A dramatic increase in glycogen (GL) occurred between days 14 (a, x13,300) and 16 (b, x12,500) in Control gilts. *Bar*: 1.0 um.

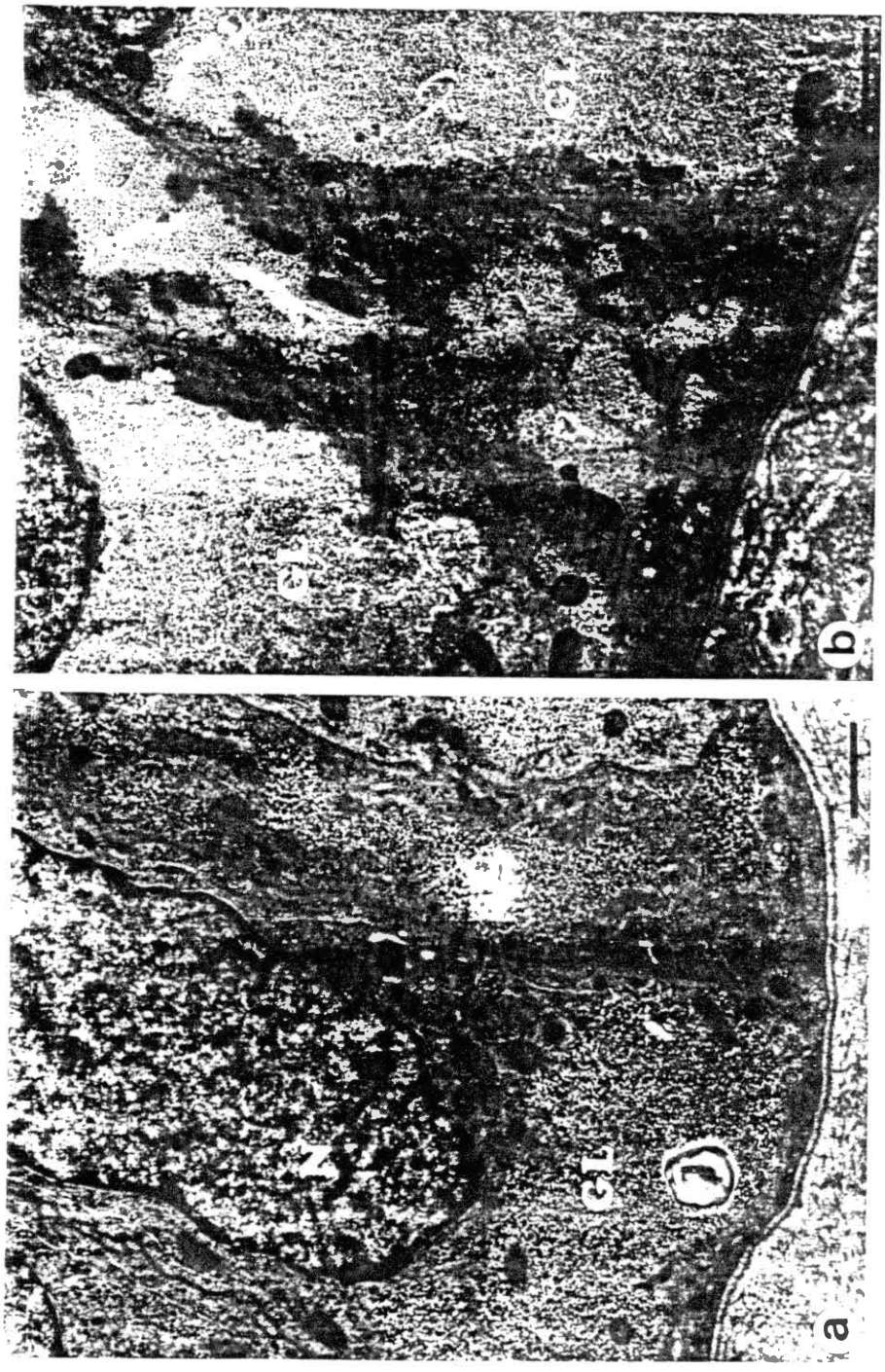

a
b

Figure 13. Mitochondria (M) were abundant on all days studied and were evenly dispersed between apical and basal regions of the uterine luminal epithelium. Golgi complexes (G) were prominent and located in a supranuclear orientation. (x14,300). *Bar*: 1.0 um.

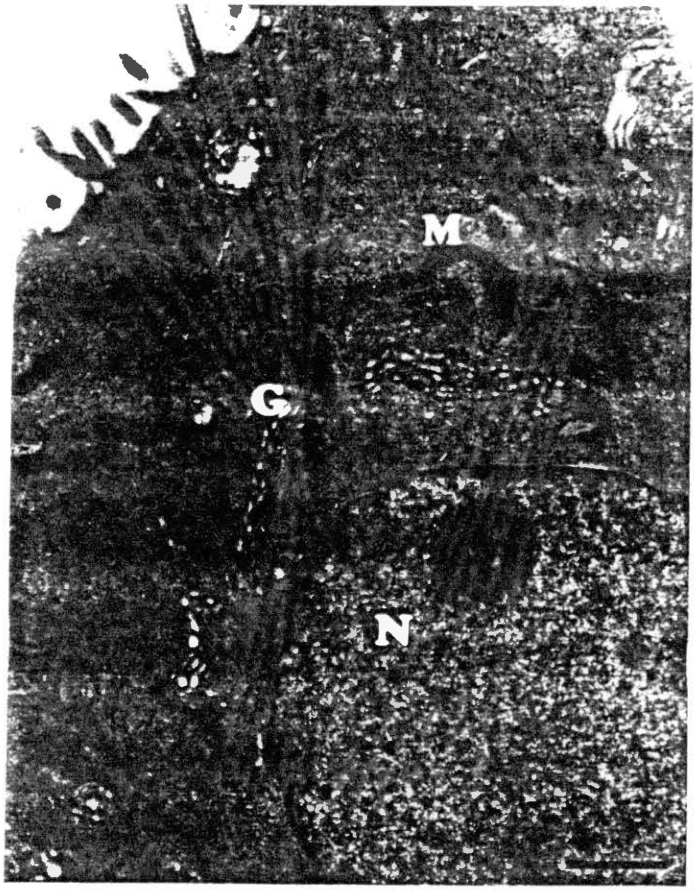

M
G
N

Figure 14. Rough endoplasmic retıculum (*arrows*) increased durıng the later days of gestation in both Control and E-treated gılts. (x13,800). *Bar*: 1.0 um.

Figure 15. Clear vesicles (*arrow*) are located in the apical aspect of the uterine luminal epithelial cells. At day 12 of gestation, the clear vesicles are more numerous in Control (a, x14,000) compared to E-treated (b, x13,300) gilts in which few vesicles are evident. *Bar*: 1.0 um

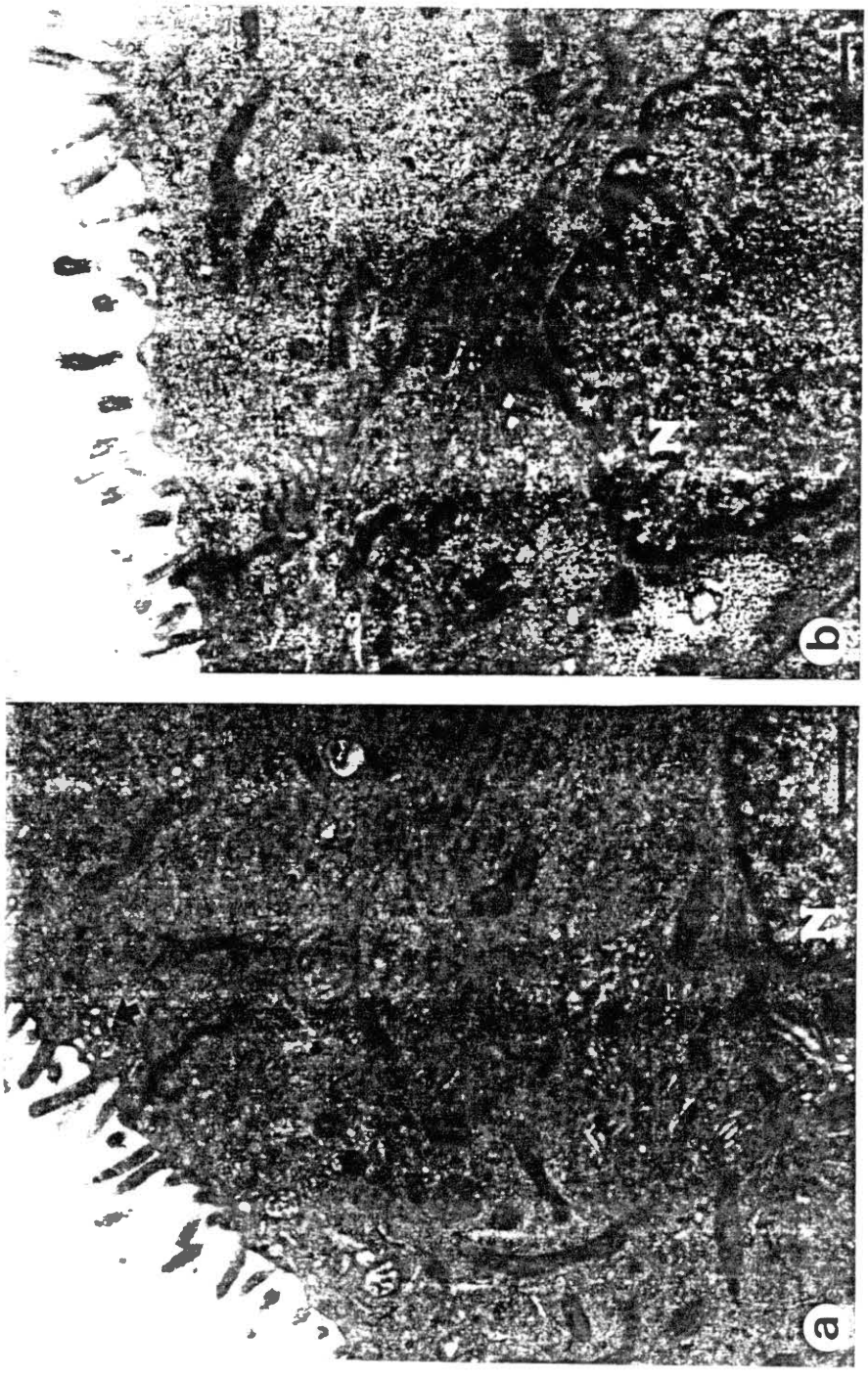
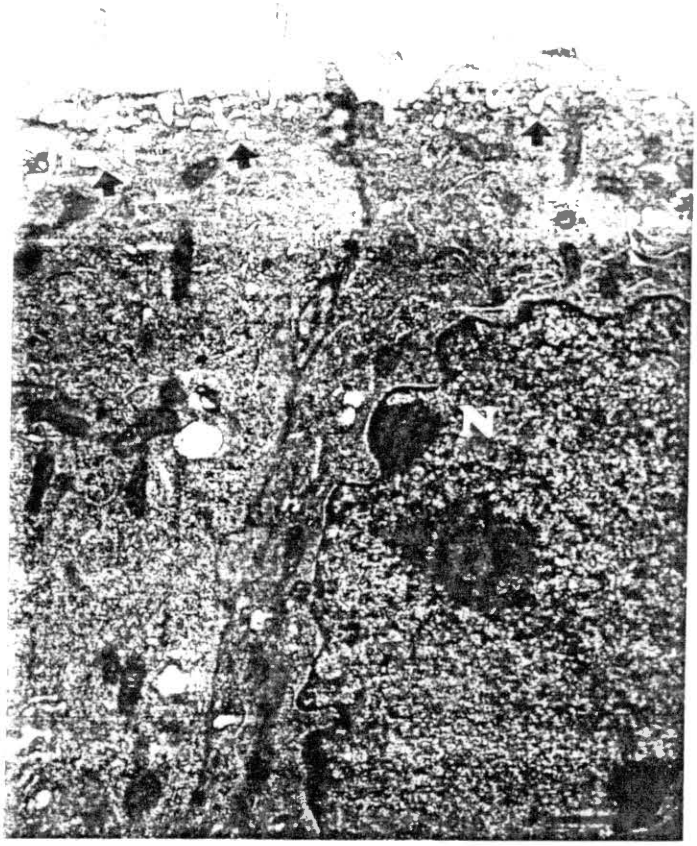

b
Z
a
Z

Figure 16. Dense bodies (*arrows*) were evident on all days studied, but were located predominantly in the basal region of the uterine luminal epithelium. (x13,750). *Bar*: 1.0 um.

Figure 17. Numerous clear vesicles are evident immediately below the apical cell membrane. Conceptuses in this gilt were still at the spherical stage of development. x15,000. *Bar*: 1.0 um


N

CHAPTER V

CHARACTERIZATION AND PROTEOLYTIC ACTIVITY OF CATHEPSIN L IN ENDOMETRIUM AND UTERINE FLUSHINGS OF CYCLIC AND PREGNANT GILTS

Introduction

Cathepsins are lysosomal cysteine proteases (Barrett and Kirschke, 1981) that have been implicated in blastocyst implantation in rats (Elangovan and Moulton, 1980 a,b) and cats (Verhage et al., 1989; Li et al., 1991). Jaffe et al. (1989) demonstrated that a progesterone-dependent protein secreted by the feline uterine endometrium has high nucleotide and amino acid sequence similarity to human, rat, and mouse cathepsin L. Recent studies have demonstrated that feline cathepsin L is present in uterine glandular epithelial cells (Verhage et al., 1989) and in uterine flushings (Li et al., 1989). The proteolytic activity of cathepsin L as well as its high affinity for collagen (Kirschke et al., 1982) and elastin (Mason et al., 1986) suggest that cathepsin L may be involved in implantation in

cats.

It has been demonstrated that several lysosomal enzymes are also produced by the uterine endometrium and secreted into the uterine lumen of pigs (Roberts et al., 1976), cattle (Roberts and Parker, 1974), and sheep (Roberts et al., 1976). Some of these lysosomal enzymes include lysozyme, leucine-aminopeptidase, and cathepsin B_1, D, and E (Roberts et al., 1976) as well as two glycosidases, ß-hexosaminidase and ß-glucosidase (Hansen et al., 1985). Both lysozyme and leucine-aminopeptidase are accumulated in the allantoic fluid. Lysozyme is believed to have a bactericidal function (Galask and Snyder, 1970; Sutcliffe, 1975) due to its ability to cleave the ß1,4-glycosidic linkages of bacterial peptidoglycans (Roberts and Bazer, 1988). The function of leucine-aminopeptidase in the uterus remains unclear, however Basha et al. (1978) has hypothesized that aminopeptidase may function to hydrolyze small peptides for uptake by the conceptus. Roberts et al. (1976) reported that the enzyme activities of cathepsins B_1, D, and E are present in the uterine flushings of ovariectomized, progesterone-treated gilts, but not in gilts that did not receive progesterone treatment suggesting that porcine uterine cathepsins may also be progesterone induced. Cathepsins appear to comprise only a minor component of porcine uterine secretory proteins. The function of cathepsins during conceptus attachment in the pig is currently unknown.

The objective of the present study was to determine whether cathepsin L was present in porcine uterine endometrium and uterine flushings and to characterize changes in cathepsin L and cathepsin L mRNA expression during the estrous cycle and early pregnancy.

Materials and Methods

Animals

Crossbred gilts were observed for estrous behavior twice daily (0700 and 1700 h) in the presence of an intact boar. After exhibiting two estrous cycles of 17-22 days in duration, gilts were randomly assigned to remain cyclic or be mated at the onset of estrous (day 0) and 12 and 24 h later. Cyclic gilts (n=16) were hysterectomized on days 5, 10, 12, 15, and 18 of the estrous cycle (3 gilts/day) with an additional gilt hysterectomized at estrus (day 0). Pregnant gilts (n=12) were hysterectomized on days 10, 12, 15, and 18 (3 gilts/day).

Gilts were hysterectomized after induction of anesthesia with a 5% solution of thiopentone sodium and maintained on a closed-circuit system of halothane (5% Fluothane; Halocarbon Industries Inc., North Augusta, SC) and oxygen (1.0 L/min). Once a surgical plane of anesthesia was attained, the uterus was exposed via midventral laparotomy and the entire uterus and both ovaries were

excised. After routine closure of the incision, gilts were post-surgically treated with antibiotics.

Immediately upon removal, the uterus was trimmed free of the ovaries and broad ligament. Uterine horns were flushed individually with 20 ml of sterile, 0.9% physiological saline. In pregnant gilts, uterine flushings were examined for the presence of conceptuses to confirm pregnancy. Uterine flushings were centrifuged at 12,000 x g for 15 min at 4 ^{o}C. The supernatant was decanted and stored at -20 ^{o}C until analyzed.

After flushing, the uterus was opened along the antimesometrial border. Endometrial tissue was dissected away from the myometrium, washed in sterile, 0.9% physiological saline, blotted on sterile surgical sponges and frozen in liquid nitrogen. Endometrial samples were stored at -75 ^{o}C until analyzed for mRNA content.

Endometrium from two gilts (one day 15 cyclic gilt and one day 15 pregnant gilt) and the conceptuses recovered from the pregnant gilt were placed in culture. For endometrial cultures, a section of the uterus was placed on ice, transported to the laboratory, and processed in a sterile laminar flow hood. Endometrial tissue was dissected from the myometrium, cut into 2-3 mm explants and placed in Eagle's minimum essential medium (MEM). Endometrial explants (200 mg) were cultured in 7.0 ml MEM for 24 h at 37 ^{o}C in an atmosphere of 5% CO_2, 45% N_2, and 50% O_2. Following incubation, culture media was separated from the

endometrial tissue and centrifuged at 12,000 x g for 15 min at 4 ^{o}C. The supernatant was decanted and stored at -20 ^{o}C until analyzed. Conceptuses recovered from the pregnant gilt were cultured using the same procedure utilized for endometrial explants.

Polyacrylamide Gel Electrophoresis (PAGE) and Western Blotting

Uterine flushings and media from endometrial and conceptus cultures were dialyzed (Spectra/Por, molecular weight cut-off = 12,000-14,000, Spectrum Medical Industries, Inc.) against 10 mM tris-HCl buffer. Dialyzed uterine flushings and culture media were lyophilized and redissolved in 5mM K_2CO_3 containing 9.3M urea, 2% (v/v) Nonidet P-40, and 0.5% (w/v) dithiothreitol. Reconstituted samples were subjected to one-dimensional (1-D, 100 mg protein) and two-dimensional (2-D, 300 mg protein) sodium dodecyl sulfate (SDS)-PAGE.

Proteins resolved by SDS-PAGE were subsequently transferred to Immobilon-PVDF transfer membranes (Millipore Corp., Bedford, MA) with a Milliblot-SDE Transfer System (Millipore Corp.). Transfer membranes were incubated with rabbit antisera to feline cathepsin L (produced and generously provided by Dr. Harold Verhage). Immunoreactive proteins were visualized with an Immuno-Blot horseradish peroxidase assay kit (Bio-Rad, Richmond CA). Incubations

were done with a 1:1000 dilution of the primary antiserum.

RNA Extraction and Analysis

Total RNA was extracted from 1 g of porcine uterine endometrium by acid guanidinium thiocyanate-phenolchloroform extraction (Puissant and Houdebine, 1990).

For slot blot analyses, 5, 10, and 20 mg samples of total RNA were immobilized on nitrocellulose membranes using a Bio-Dot SF Microfiltration Apparatus (Bio-Rad). Analysis of total RNA by Northern hybridization was accomplished using the method described by (Current Protocols In Molecular Biology, Vol. 1). Briefly, total RNA samples (10 mg) were denatured in 10X MOPS, 37% formaldehyde, and formamide by incubating for 15 min at 55 ^{o}C. Samples were subjected to electrophoresis through a 1.2% agarose gel containing 10X MOPS and formaldehyde. Following electrophoresis, RNA was transferred to a nitrocellulose membrane by Northern transfer. Nitrocellulose membranes were baked at 80 ^{o}C for 2 h in a vacuum oven. Membranes were prehybridized at 42 ^{o}C for 4 h followed by a 24 incubation at 42 ^{o}C in hybridization buffer containing 0.1 mg/ml feline cathepsin L cDNA (generously provided by Drs. Harold Verhage and Randall Jaffe; Jaffe et al., 1989). Feline cathepsin L cDNA had been nick translated and labeled with biotin using the BioNick Labeling System (Gibco BRL, Gaithersburg, MD). After hybridization, binding was

detected with the BluGene Nucleic Acid Detection System (Gibco BRL) according to the manfacturer. Membranes were washed twice (3 min/wash) in 2X sodium salt citrate (SSC)-0.1% SDS and twice (3 min/wash) in 0.2X SSC-0.1% SDS at room temperature followed by two 15 min washes in 0.16X SSC-0.1% SDS at 42 ^{o}C. Following post-hybridization washes, membranes were incubated for 1 h at 65 ^{o}C in blocking solution (3% bovine serum albumin in 0.1 M tris-HCl, 0.15 M NaCl; pH=7.5). After blocking, the membranes were incubated with streptavidin-alkaline phosphatase conjugate for 10 min. at room temperature. Hybridization signal was detected by incubating the membranes in the detection reagent for 2-3 h at room temperature.

Preparation of Cathepsin L cDNA Probe

The cathepsin L cDNA provided was used to transform competent cells of E. coli DH5a as described by Hanahan (1983). Transformants were selected on LBA containing 50 ug/ml ampicillin. Plasmid DNA was isolated from transformant E. coli cells by using the rapid screening procedure of Birnboim and Doly (1979). Three strains containing recombinants of the expected size were isolated for large scale preparation. Large scale isolation of plasmid DNA was performed by the cleared lysate method of Clewell and Helenski (1970) and the DNA was purified by CsCl-ethidium bromide density gradient centrifugation.

Gradient-purified plasmid DNA samples were extracted with isopropanol saturated with 5 M NaCl to remove the ethidium bromide, and desalted and concentrated in 10 mM Tris-HCl, 1 mM Na_2EDTA, pH=7.5, by using a Centricon-30 microconcentrator according to the manfacurer's instructions (Amicon Corp. Danvers, MA).

It was then necessary to remove the 430 bp insert from the plasmid DNA. In order to remove the cDNA insert, restriction endonuclease digestions were performed according to manfacturer's directions (Boehringer Mannheim Biochemicals, Indianapolis, IN), and to methods described by Maniatis et al. (1982). Restriction fragments were separated in horizontal 0.9% agarose gels run in Tris-borate buffer for 12 to 14 h at 55 V. Preparative agarose gels were loaded with 2.0 ml of digested plasmid DNA. The 430 bp DNA band of interest was located in the stained gel, cut out, and electroeluted from the agarose slice in an Elutrap chamber according to the manufacturer's instructions (Schleicher and Schuell Inc., Keene, NH).

The purified 430 bp cDNA fragment was biotinylated with the BioNick Translation Kit (Gibco, BRL).

Enzyme Assay

Proteolytic activity of cathepsin L in uterine flushings was assayed as described by Barrett and Kirschke (1981). Samples of uterine flushings containing 7.5 mg of

total protein was diluted to 500 ml in 0.1% Brij solution (Sigma, St. Louis, MO) followed by the addition of 250 ml of assay buffer (340 mM sodium acetate, 60 mM acetic acid, 4 mM disodium EDTA, and 8 mM dithiothreitol; pH = 5.5). The mixture was incubated in a 40 ^{o}C water bath for 1 min to activate the enzyme. The enzyme reaction was started by adding 250 ml of 20 mM substrate solution. The 20 mM substrate solution was prepared by diluting 1 mM Z-Phe-Arg-NMethylcuomarin HCl (Sigma) in dimethyl sulfoxide. After incubating for 10 min at 40 ^{o}C, the reaction was stopped by the addition of 1.0 ml of stopping reagent (100 mM sodium monochloroacetate, 30 mM sodium acetate, and 70 mM acetic acid; pH = 4.3). The fluorescence of the samples was determined by excitation at 370 nm and emmission at 460 nm in a Farrand MK2 spectrofluorometer (Farrand Optical Co., Inc.). Proteolytic activity of cathepsin L in uterine flushings was expressed as units of fluorescence/7.5 mg total protein.

Statistical Analysis

Data were analyzed by least square analysis of variance using General Linear Models of SAS (1987). Cathepsin L proteolytic activity was analyzed for differences between treatment, day, and treatment by day interactions.

Results

Enzyme activity of cathepsin L in porcine uterine flushings was analyzed during the estrous cycle and early pregnancy. In cyclic gilts, cathepsin L proteolytic activity remained low through day 12 and then increased on day 15 of the estrous cycle ($P<0.10$), whereas proteolytic activity increased on day 12 ($P<0.05$) and remained elevated through day 18 in pregnant gilts (Fig. 1). On days 10 and 15 proteolytic activity in uterine flushings was similar between cyclic and pregnant gilts, while pregnant gilts exhibited greater proteolytic activity ($P<0.05$) on days 12 and 18 compared to cyclic gilts.

Western blot analysis of cathepsin L in uterine flushings using 2-D gels demonstrated that a group of proteins (Mr = 41,000; pI = 6.0-6.5) cross-reacted with feline cathepsin L antisera (Fig. 2). These polypeptides were low on days 0, 5, and 10 in both cyclic and pregnant gilts (Fig. 3). Immunoreactivity of uterine flushings to feline cathepsin L antisera increased on days 15 and 18 in both cyclic and pregnant gilts with pregnant gilts appearing to have a greater reactivity compared to cyclic gilts. Western blot analysis of 1-D gels on which endometrial and embryo culture media were run along side uterine flushings demonstrated that endometrium of both cyclic and pregnant gilts secrete proteins that cross-react with antisera to feline cathepsin L (Fig. 4). However, these proteins are of

a greater molecular weight (Mr = 45,000; pI = 6.0- 6.5) than those present in uterine flushings. Embryo culture did not produce any proteins that cross-reacted with feline cathepsin L (Fig. 4, lane 6).

Changes in cathepsin L mRNA expression were analyzed via slot blot hybridization. Cathepsin L mRNA content was low on day 0 and 5, increased slightly on day 10 and was readily apparent on day 12 of the estrous cycle (Fig. 5a). Day 15 mRNA content was similar to day 12, but increased slightly on day 18 of the estrous cycle. In pregnant gilts (Fig. 5b), cathepsin L mRNA levels were similar on day 10, 12, and 15 and were enhanced on day 18 compared to cyclic gilts.

Discussion

Several studies have reported that the porcine uterus secretes lysosomal enzymes into the uterine lumen (Roberts et al., 1976; Basha et al., 1978; Bazer et al., 1981; Hansen et al., 1985; Roberts and Bazer, 1988). Roberts et al. (1976) demonstrated that uterine flushings from progesterone-treated gilts contained lysozyme and leucineaminopeptidase as well as cathepsins B_1, D, and E. Cathepsins B_1, D, and E have been considered to be minor components of porcine uterine secretory proteins (Roberts et al., 1976; Bazer et al., 1981; Roberts and Bazer, 1988), however these cathepsins have only been detected in porcine

uterine flushings by their enzymatic activities.

In the present study, we have demonstrated that cathepsin L is present in porcine uterine flushings and cathepsin L mRNA is present in porcine uterine endometrium during the estrous cycle and early pregnancy. Cathepsin L proteolytic activity in porcine uterine flushings was low through day 12 in cyclic gilts and increased on day 15, whereas cathepsin L proteolytic activity increased on day 12 and remained elevated through day 18 of gestation in pregnant gilts. These changes in proteolytic activity correspond with the changes in cathepsin L observed by Western blot analysis. Immunoreactive polypeptides detected with antisera to feline cathepsin L were low through day 10 and then increased in both cyclic and pregnant gilts.

Western blot analysis revealed that cathepsin L in porcine uterine flushings have a Mr = 41,000 Da and a pI = 6.0-6.5. In contrast, Verhage et al. (1989) reported that the predominant isoform of cathepsin L in cat uterine flushings exhibited a Mr = 36,000 Da and a pI = 6.0-6.5. The other two isoforms of cathepsin L observed in the cat (Mr = 28,000, pI=5.5-6.0; Mr=41,000, pI=5.5-6.0) were both present in uterine flushings with the 28,000 Da isoform showing the least cross-reactivity (Verhage et al., 1989). Results from endometrial and conceptus cultures in the present study revealed that cathepsin L is synthesized and secreted from the uterine endometrium, but not porcine conceptuses. Unlike the results reported by Verhage et al. (1989), which

demonstrated that the 28,000 Da polypeptide was the predominant isoform in endometrial cultures while the 41,000 Da isoform was absent, results from the present study indicate that the cathepsin L isoform observed in porcine endometrial cultures had a higher molecular weight than the isoform observed in uterine flushings. The higher molecular weight isoform in endometrial cultures may be a precursor form of cathepsin L. In mouse NIH 3T3 fibroblasts, the precursor form of cathepsin L (Mr = 39,000) is secreted in response to growth promoting agents, not the mature lysosomal form (Mr = 20,000) (Prence et al., 1990).

Endometrial content of cathepsin L mRNA showed a slight increase on day 10 of the estrous cycle and was readily evident on day 12. Thus mRNA levels appeared to increase in cyclic gilts prior to the increase in uterine luminal content of cathepsin L and cathepsin L proteolytic activity. In pregnant gilts, cathepsin L mRNA was similar on days 10, 12, and 15 of gestation and then increased on day 18. These results are consistent with cathepsin L content and proteolytic activity in uterine flushings.

Cathepsins have been implicated in blastocyst attachment in rats (Elangovan and Moulton, 1980a,b) and cats (Jaffe et al., 1989; Verhage et al., 1989; Li et al., 1991). Elangovan and Moulton (1980a) demonstrated that the level of cathepsin D in rat uterine luminal epithelium decreased during blastocyst implantation and that the rate of cathepsin D synthesis in implantation sites was only half

that observed in inter-implantation areas. Elangovan and Moulton (1980b) also demonstrated that the synthesis of cathepsin D attained maximal levels within 6 hours after progesterone administration. Thus, cathepsin D synthesis appears to be mediated by progesterone and may be involved in autolysis of uterine luminal epithelium during blastocyst implantation in the rat (Elangovan and Moulton, 1980a,b). Jaffe et al. (1989) demonstrated that a major feline progesterone-dependent endometrial secretory protein (PDP) has high nucleotide and amino acid homology to human, rat, and mouse cathepsin L. Verhage et al. (1989) reported that PDP is not present in uterine flushings, endometrial cultures, and glandular epithelium until after a minimum of 2 days of progesterone treatment. Uterine content of cathepsin L, measured by enzymatic activity (Li et al., 1991), was highly correlated with the presence of PDP in cat uterine flushings as detected by Western blot analysis (Verhage et al., 1989). Uterine endometrial content of PDP mRNA was also detected in ovariectomized cats only after estrogen priming followed by treatment with estrogen and progesterone (Jaffe et al., 1989). Therefore, it is apparent that synthesis of cathepsin L by the cat uterus is progesterone dependent.

Uteroferrin, a lysosomal acid phosphatase secreted by the porcine endometrium in response to progesterone, is synthesized and secreted by glandular epithelium and functions in the transport of iron to the developing fetus

(Chen et al., 1975), not as an acid phosphatase. Therefore, porcine cathepsin L may also have a role in the uterine environment that is different from its role as a lysosomal protease.

Recent evidence indicates that mouse cathepsin L is the major excreted protein (MEP) of transformed mouse fibroblasts as well as nontransformed mouse fibroblasts stimulated with platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) (Frick et al., 1985; Chiang and Nilsen-Hamilton, 1986; Prence et al., 1990). Prence et al. (1990) demonstrated that PDGF alters the lysosomal protein transport system such that cathepsin L is secreted rather than stored within the lysosomes. These authors suggest that mouse fibroblast cathepsin L may be secreted to hydrolyze extracellular matrix components or cell surface proteins in preparation for cell growth. The porcine uterus produces several growth factors including insulin-like growth factor-I, -II, and an EGF-like peptide (see Simmen and Simmen, 1990 for review). Therefore cathepsin L might play a role in endometrial growth.

Previous research in our laboratory (Greis et al., 1989) demonstrated that a distinct band of three basic polypeptides (Mr = 30,000; pI = 7.9-9.0) are synthesized and secreted by the porcine endometrium. Secretion of these proteins was attenuated concurrent with embryonic death in gilts treated with estrogen on days 9 and 10 of gestation. Recent evidence (Geisert, unpublished observations)

indicates that this group of polypeptides exhibits approximately 40% amino acid sequence similarity with human procollagen. Interactions between this group of endometrial polypeptides and the laminin and fibronectin present in the developing porcine conceptus (Richoux et al., 1989) may function in conceptus attachment. Since collagen is one of the primary substrates for cathepsin L (Kirschke et al., 1982), it is possible that cathepsin L may also be involved the establishment of pregnancy in swine.

In conclusion, we have shown that the lysosomal enzyme, cathepsin L, is synthesized and secreted by the porcine uterine endometrium during the estrous cycle and early pregnancy. The role of cathepsin L in the pig uterus is presently unknown, but its secretion during important events of pregnancy is consist with a function during conceptus attachment and the establishment of pregnancy.

Figure 18. Cathepsin L proteolytic activity in porcine uterine flushings from cyclic and pregnant gilts

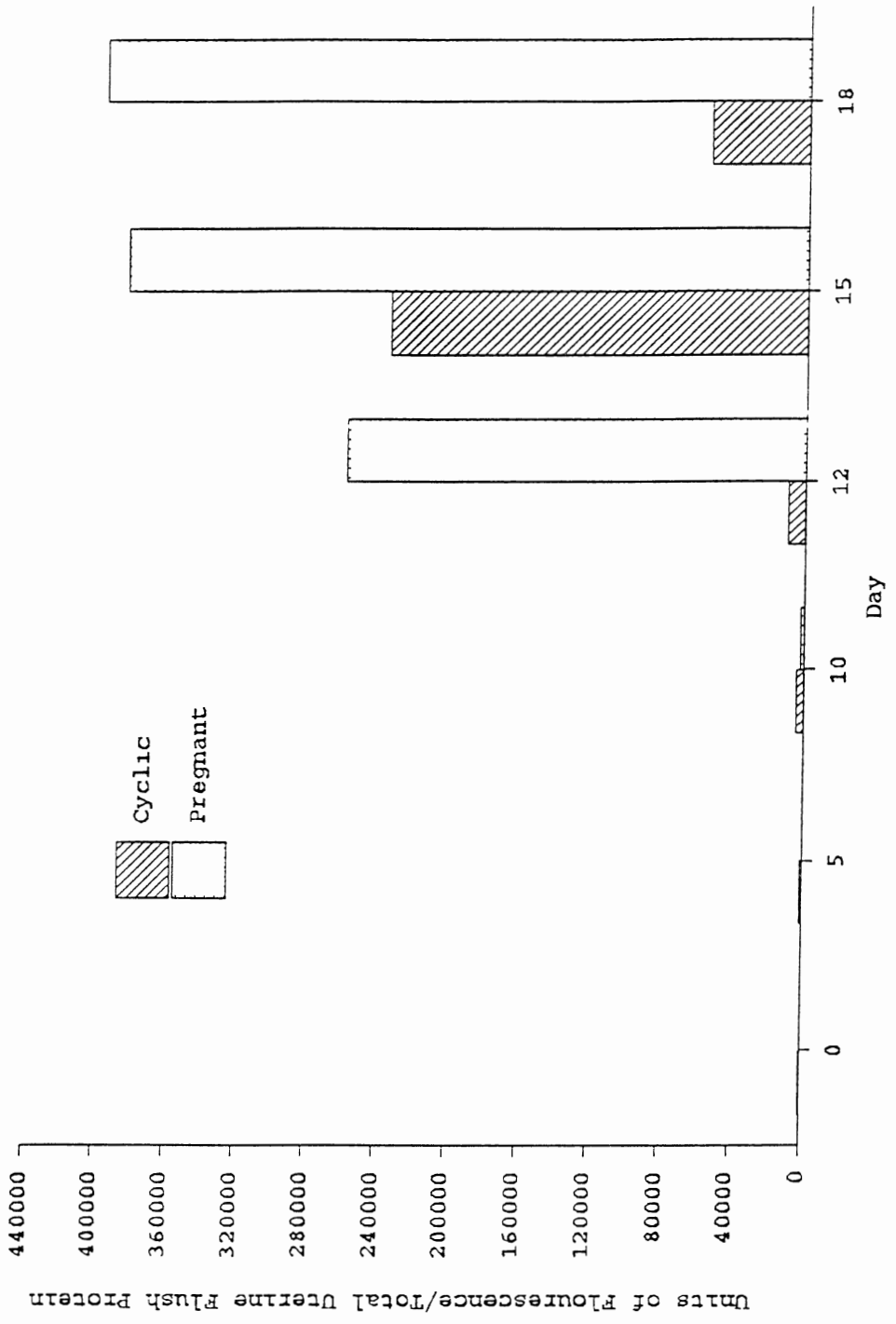
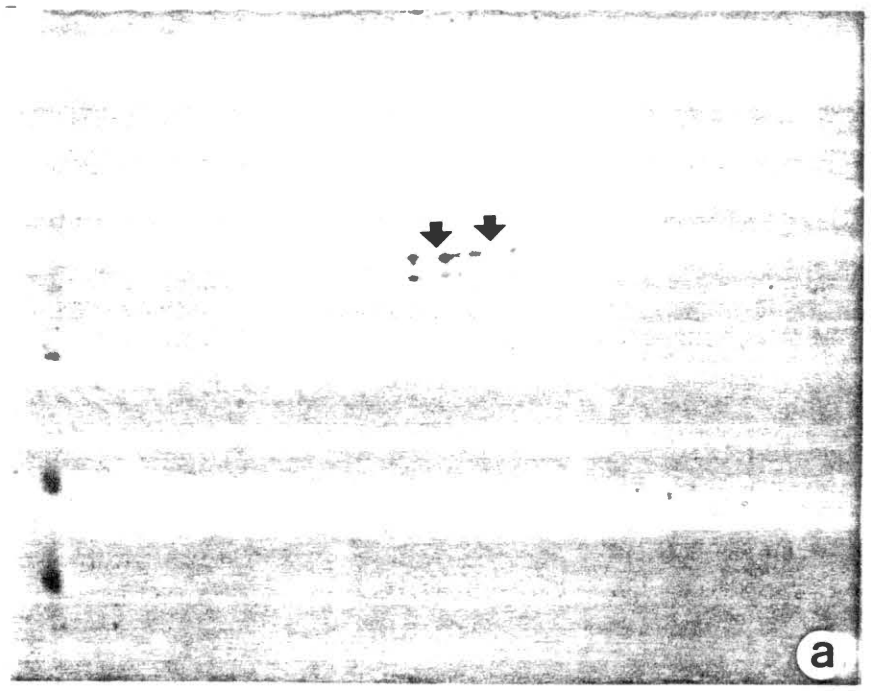

Units of Flourescence/Total Uterine Flush Protein
440000
400000
360000
320000
280000
240000
200000
160000
120000
80000
40000
0
0
5
10
12
15
18
Day
Cyclic
Pregnant

Figure 19. (A) Western blotting of 2-D SDS-PAGE of uterine flushings from a day 15 cyclic gilt. Polypeptides (Mr = 40,000-45,000; pI = 6.0-6.5) with immunoreactivity to antisera to feline cathepsin L are readily apparent (arrows). (B) Fluorograph of 2-D SDS-PAGE of endometrial culture media. Note the group of polypeptides (arrows) that correspond to the polypeptides which exhibit cathepsin L immunoreactivity.


a

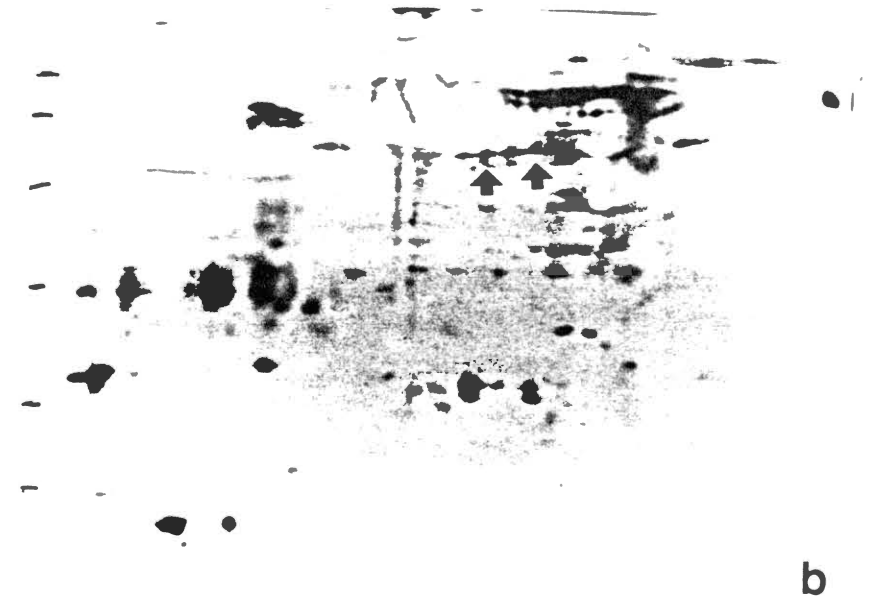

b

Figure 20. Western blot of 1-D SDS-PAGE of uterine flushings from cyclic and pregnant gilts collected on days 0 (lane 2), 5 (lane 3), 10 (lane 4), 12 (lane 6), 15 (lane 8), and 18 (lane 10) of the estrous cycle and days 10 (lane 5), 12 (lane 7), 15 (lane 9), and 18 (lane 11) of gestation. Lane 12 contains a sample of feline endometrial culture media as a positive control

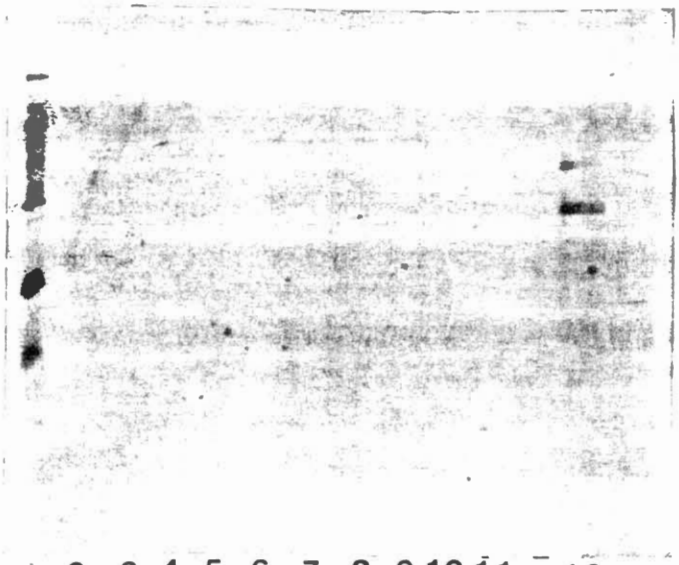

2 3 4 5 6 7 8 9 10 11 12
LANE

Figure 21. Western blot of 1-D SDS-PAGE of day 15 cylic (lane 2) and day 15 pregnant (lane 4) uterine flushings and day 15 cyclic (lane 3) and day 15 pregnant (lane 5) endometrial culture media as well as day 15 embryo culture media (lane 6). Lane 7 contains an aliquot of feline endometrial culture media as a positive control.

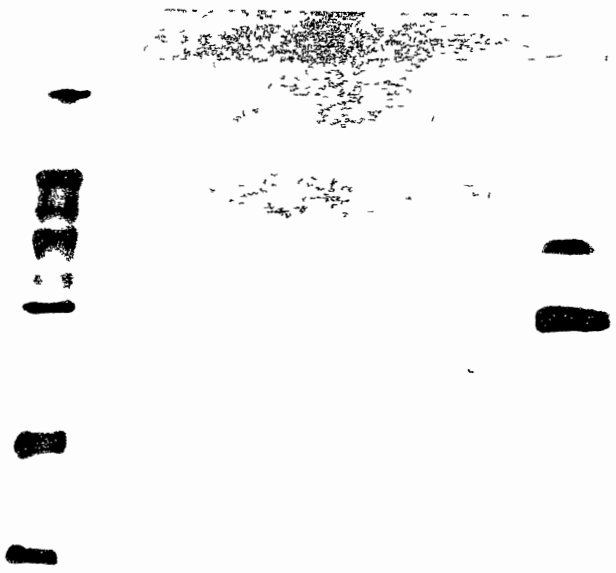
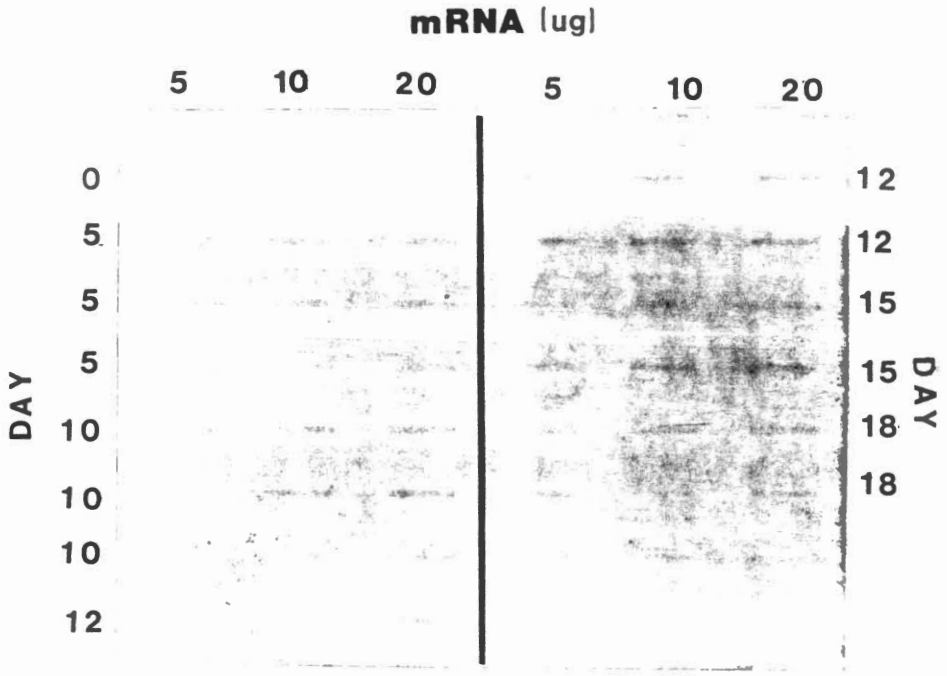

1 2 3 4 5 6 7
LANE

Figure 22. Slot blot analysis of cathepsin L mRNA from uterine endometrium of cyclic gilts.


mRNA (ug)
5
10
20
5
10
20
DAY
0
5
5
5
10
10
10
12
12
12
15
15
18
18
DAY

CHAPTER VI

GENERAL DISCUSSION

Placentation in the gilt occurs between days 13 and 18 of gestation (King et al., 1982). Conceptus attachment is initiated on approximately day 13-14 of gestation in the region of the embryonic disc and extends toward the extremities of the allanto-chorion (Dantzer, 1985; Keys and King, 1990). Placentation in the pig involves the noninvasive interdigitation of uterine epithelial and trophoblast microvilli (Dantzer, 1985). The uterine epithelial glycocalyx becomes reduced in areas of trophoblast apposition (Dantzer, 1985). Keys and King (1990) demonstrated that uterine epithelial glycocalyx fibers extend toward the trophoblast in areas of apposition suggesting that the glycocalyx may function to anchor the conceptus and uterine epithelia together during placental adhesion.

Maternal recognition and the establishment of pregnancy in the pig depends upon conceptus estrogen production (Bazer and Thatcher, 1977; Heap et al., 1979). However, premature exposure to estrogen or compounds containing estrogen activity results in early embryo death (Long and Diekman,

1986; Pope et al., 1986). Long and Diekman (1986) demonstrated that an estrogenic mycotoxin, zearalenone, fed to sows between days 7 and 10 of gestation resulted in complete embryonic loss by day 30 of gestation, however this loss was not the result of alterations in the number or spacing of embryos when examined on days 9 and 11 of pregnancy (Long et al., 1987). Pope et al. (1986) reported that administration of estradiol-17b on days 9 and 10 of gestation also resulted in complete embryo death by day 30 of pregnancy. It has been demonstrated that this embryonic loss occurs at approximately days 14-16 of gestation as characterized by the presence of degenerating conceptuses (Morgan et al., 1987; Greis et al., 1989). since embryonic death occurs during the critical period of conceptus attachment, it is likely that the observed embryonic loss may result from failure of the conceptus to attach to the uterine epithelium.

The present dissertation was designed to characterize surface and ultrastructural changes in the uterine luminal epithelium as well as alterations in the uterine secretory activity that may be associated with embryonic mortality due to early exogenous estrogen administration. As reported in previous studies (Morgan et al., 1987; Greis et al., 1989), the present study demonstrated that estrogen administration on days 9 and d10 of gestation resulted in embryonic death by days 14-16 of pregnancy. However, the present study demonstrated a loss of the uterine epithelial glycocalyx

concurrent with the incidence of embryonic death. This supports the possible involvement of the uterine glycocalyx in initial adhesion of the conceptus to the uterine epithelium as suggested by Keys and King (1990).

Alterations in the carbohydrate composition of the porcine uterine epithelial glycocalyx may facilitate its role in conceptus attachment, however little research has focused on the uterine glycocalyx during placental attachment in the pig. Whyte and Robson (1984) demonstrated that the porcine endometrium reacted weakly with WGA and Con-A lectins and did not react to the lectins TP, RCA-I, and RCA-II. Conflicting results reported by Rober and Holtz (1988) demonstrated that the porcine uterine epithelial glycocalyx contained galactose, galactosamine, and fucose residues prior to, but not after, conceptus attachment. The porcine trophoblast has been shown to contain N-acetyl-glucosamine (Whyte and Robson, 1984), galactose and galactosamine (Rober and Holtz, 1988), and fucose (Whyte and Robson, 1984; Rober and Holtz, 1988). The glycocalyx has been implicated in numerous cell-cell interactions (Alberts et al., 1983). Therefore, changes in the carbohydrate composition of the glycocalyx may well be important in conceptus attachment. A more complete characterization of the specific carbohydrate moieties that compose the uterine and trophoblast glycocalyx is necessary to more accurately clarify the nature of this carbohydrate-rich cell surface coat. Further characterization of the glycocalyx would

facilitate a better understanding of the changes that occur in the uterine glycocalyx during the estrous cycle and early pregnancy. Interactions between the trophoblast and uterine epithelial glycocalyces will require further elucidation before the role of the glycocalyx in conceptus attachment can be determined.

During the period of trophoblast elongation, porcine conceptuses synthesize and secrete large quantities of estrogen (Perry et al., 1973; Gadsby et al., 1980; Fischer et al., 1985). This conceptus-produced estrogen stimulates changes in the uterine ultrastructure and secretory activity. Keys and King (1988) demonstrated that administration of estrogen to cyclic gilts stimulated increased uterine folding, glycogen accumulation, increased synthetic and secretory activity, and a thickening of the uterine epithelial glycocalyx. The changes observed following estrogen administration are similar to the changes which occur during early pregnancy (Stroband et al., 1986; Keys and King, 1990). Estrogen also stimulates an increase in uterine secretory activity. Geisert et al. (1982c) demonstrated that estrogen administration results in an increase in calcium, protein, and prostaglandins in uterine flushings. These changes are similar to the uterine secretory response to conceptus-derived estrogen observed during early pregnancy.

The adminstration of estrogen on days 9 and 10 of gestation as described in the present study resulted in

morphological changes similar to alterations observed during early pregnancy (Stroband et al., 1986; Keys and King, 1990), however estrogen administration stimulated these changes to occur earlier than normal. Although the morphological changes did not appear to effect the uterine epithelial glycocalyx or embryonic death, the changes in the uterine ultrastructure are consistent with advanced uterine secretory activity induced by early estrogen administration (Geisert et al., 1982c; Morgan et al., 1987).

The porcine uterine endometrium produces several proteolytic enzymes including cathepsins B_1, D, and E (Roberts et al., 1976). Cathepsins are lysosomal cysteine proteases (Barrett and Kirschke, 1981) that have been implicate in blastocyst implantation in rats (Elangovan and Moulton, 1980 a,b) and cats (Verhage et al., 1989; Li et al., 1991) due to their high affinity for collagen (Kirschke et al., 1982) and elastin (Mason et al., 1986). However, cathepsins B_1, D, and E have only been considered minor components of porcine uterine secretions (Roberts et al., 1976; Bazer et al., 1981; Roberts and Bazer, 1988) and have only been detected in uterine flushings by their enzymatic activities.

Results from the present study demonstrate that cathepsin L is synthesized and secreted by the porcine uterine endometrium during the estrous cycle and early pregnancy. The secretory profile of cathepsin L suggests that cathepsin L is a progesterone-induced lysosomal protein

similar to uteroferrin.

Evidence in the mouse indicates that cathepsin L is a major secretory product of NIH 3T3 fibroblasts stimulated with PDGF and EGF (Frick et al., 1985; Chiang and Nilsen-Hamilton, 1986; Prence et al., 1990). Prence et al. (1990) suggested that cathepsin L may function to hydrolyze extracellular components in preparation for cell growth. Since the porcine uterus produces several growth factors (Simmen and Simmen, 1990), it is possible that one or more of these growth factors may stimulate the secretion of cathepsin L which may subsequently mediate the effects of growth factors upon endometrial growth.

Recent investigations (Geisert, unpublished data) indicate that a group of polypeptides secreted by the uterus exhibits approximately 40% amino acid sequence similarity with human procollagen. Secretion of this polypeptide (Mr = 30,000; pI = 7.9-9.0) becomes attenuated concurrent with embryonic death in gilts administered estradiol valerate on days 9 and 10 of gestation (Greis et al., 1989). Results from the present study identified these polypeptides as being glycosylated. At present, it remains unknown whether these glycoproteins are secreted from the uterine luminal or glandular epithelium or from the uterine stroma. Therefore, it is unclear whether these glycoproteins are components of the basement membrane or perhaps the uterine epithelial glycocalyx. If these glycoproteins are epithelial in origin and are integral components of the glycocalyx, thay may be

involved in interactions between the uterus and the attaching conceptus. Since collagen is a major substrate of cathepsin L (Kirschke et al., 1982) and results from the present study indicate that cathepsin L is secreted from the porcine uterus during the period of conceptus attachment, this enzyme may play a role in possible interactions between the uterine glycocalyx and laminin and fibronectin present in the developing conceptus (Richoux et al., 1989). Further research utilizing purified cell cultures would enable one to determine what cell type produces these glycoproteins. If a specific antibody can be produced to this glycoprotein, it would be possible, via immunocytochemical methods, to determine whether the glycoprotein is present on the uterine epithelial glycocalyx. If present on the glycocalyx, it would then be necessary to characterize any alterations that may occur during the estrous cycle and early pregnancy, thus providing insight to its possible role in the establishment of pregnancy.

It is apparent that the uterine epithelial glycocalyx is involved in placental attachment in pigs. Since placentation is noninvasive in the pig, the role of the glycocalyx may be critical for embryo attachment and survival. Further characterization will be necessary to determine the composition of this carbohydrate-rich coat. A greater understanding of the nature of the glycocalyx will facilitate further research toward discovering the role the glycocalyx plays in conceptus attachment and survival as

well as determining what substrates regulate changes in the glycocalyx during early pregnancy.

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