THE EFFECTS OF ALTERED UTERINE SECRETION AND UTERO-BLASTOCYST SYNCHRONY ON BLASTOCYST DEVELOPMENT OF THE PIG

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CHAPTER I

INTRODUCTION

The pig is one of man's most prolific domesticated species. The average sow ovulates 17 ova of which approximately 16 begin embryonic development, however on the average, only 9 live piglets are usually farrowed (Self, 1962). These figures indicate that the prenatal loss of potential piglets occurs subsequent to actual fertilization of ova which is reported to be over 90 percent in this species (Perry and Rowlands, 1962, Oxenreider and Day, 1965).

Tess (1981) reported that the expected costs of swine production could be reduced more by improving the number of piglets born alive than by improving rate of body weight gain, feed conversion efficiency, lean growth rate or age of puberty onset. Thus an understanding of the mechanism(s) responsible for the relatively high rate of early embryonic loss in the pig (35 to 45 percent) would form the basis to any future prospects of increasing the number of piglets farrowed.

Previous studies have demonstrated that the majority (75 percent) of embryonic mortality occurs before day 30 of gestation (Perry and Rowlands, 1962, Bazer et al., 1969). Bazer et al. (1969) proposed that prenatal mortality in swine occurred in two phases, between days 7 to 40 and 40 to term. A clearer understanding of mortality is available regarding the mechanism of prenatal death in the later phase than the earlier period (Bazer et al., 1969, Knight et al., 1977).

It was over 60 years ago that Hammond (1921) proposed the concept of a limited capacity of the porcine uterus to support placental and foetal development as an explanation for embryonic loss. Numerous studies (Dziuk, 1968, Fenton et al., 1970, Knight et al., 1974, Knight et al., 1977) have clearly confirmed Hammond's original proposal. However, the mechanism of limited uterine capacity is only operative in late gestation and does not account for the high embryonic loss occurring before 30 days.

Studies by Perry and Rowlands (1962) and Bazer et al. (1969) indicated that a large proportion of embryonic loss appeared to occur at the time in gestation when the porcine blastocyst was undergoing dramatic structural and biochemical synthetic changes. These changes in the embryo form part of the embryonic signal to the maternal system alerting the latter of its presence and thus preventing luteolysis and a return to oestrus (see review by Bazer and Thatcher, 1977)

More recent studies have focused on this critical period of embryonic development and it is now postulated that certain embryos have a survival advantage over their littermates (Pope et al., 1982a, Pope and First, 1985, Wilmut et al., 1985). Just what is responsible for this survival advantage of morphologically advanced embryos is uncertain. However, Pope et al. (1982a) hypothesized that the more developed embryos had more synthetic activity and were able to advance and thereby alter the intra-uterine biochemical environment to such an extent that the less developed embryos could no longer survive. Whether embryonic death is due to an inability to utilize the nutrients available, secretion of a factor(s) which inhibit further development of the more immature blastocysts or to some other mechanism is unknown.

The following literature review will focus attention on the embryonic-maternal interrelationships that are established in normal pregnancy in the pig. Placentation, protein and steroid synthesis by the endometrium and blastocyst and the significance of synchrony between blastocyst development and uterine environment will be discussed as to probable roles in accounting for a significant proportion of early embryonic mortality in the pig.

CHAPTER II

LITERATURE REVIEW

Endocrine Profiles of the Oestrous Cycle and Pregnancy in the Pig

Oestrous Cycle

The length of the oestrous cycle in domestic species is determined by the functional lifespan of the corpus luteum (CL). In the pig, the necessity for luteotrophic support to maintain the CL during the first 12 days of the cycle is questioned because neither hypophysectomy early in the cycle (du Mesnil du Buisson and Leglise, 1963) nor administration of anti-luteinizing hormone antisera (Spies et al., 1967) caused alterations in plasma progesterone concentrations. However, after day 12, the CL were shown to be undergoing regression. In addition, exogenous administration of the known luteolysin (prostaglandin $F_{2\alpha}$) in swine, fails to cause regression of the CL until after day 12 of the cycle (Diehl and Day, 1974, Hallford et al., 1974). Thus, the CL of the pig is frequently referred to as being 'autonomous' for the first 12 days of the oestrous cycle.

In the absence of pregnancy, the CL begins to regress resulting in a rapid decline in plasma progesterone (P_4) from peak levels of 35 ng/ml on day 14 to less than 1 ng/ml on day 18 of the oestrous cycle (Guthrie et al., 1972). The decrease in plasma progesterone at

this time appears to be the result of the luteolytic action of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) released into the uterine venous vasculature (Moeljono et al., 1976, Bazer and Thatcher, 1977). Ginther (1979) demonstrated that uterine $PGF_{2\alpha}$ absorbed into the uterine venous vasculature could be transferred to the ovarian artery by counter-current exchange as the uterine veins drain through the utero-ovarian pedicle. The mechanism by which $PGF_{2\alpha}$ stimultes CL regression is unclear. Administration of gonadotrophins in an attempt to rescue the regressing CL are ineffective (Anderson, 1966) suggesting perhaps a loss of luteinizing hormone (LH) receptors on the luteal cell membrane. Ziecik et al. (1980) reported that there was a decrease in the number of occupied LH receptors on the luteal cell membrane after day 12. Tucker et al. (1979) described an increase in the quantity of a specific inhibitor to LH binding to its receptor as the CL ages. Henderson and McNatty (1975) suggested that until LH begins to dissociate from the luteal cell membrane, the pig CL remains refractory to $PGF_{2\alpha}$. However, when dissociation does begin at around day 12, conformational changes in the luteal cell membrane occur facilitating the binding of $PGF_{2\alpha}$. This in turn alters the adenylate cyclase system inhibiting progesterone synthesis while simultaneously activating lysosomal enzyme systems causing morphological regression of the luteal cells. In the ewe, there is also good evidence that $PGF_{2\alpha}$ is the agent responsible for normal regression of the CL (see Goding, 1974 for review) but Diekman et al. (1978) concluded that $PGF_{2\alpha}$ did not cause a reduction in the number of luteal LH receptors during initial luteolysis when serum progesterone levels decreased by 63 percent in 5-7 hours. A number of other possible actions of $PGF_{2\alpha}$ have been proposed to explain its

luteolytic action including reduced ovarian blood flow (Niswender et al., 1976), uncoupling of the LH receptor from adenylate cyclase (Evrard et al., 1978) and a direct cytotoxic effect (Silvia et al., 1984). Thus, some or all of these actions of $PGF_{2\alpha}$ maybe important for normal regression of the CL in the domestic species.

Plasma oestrogen levels remain relatively low in cyclic animals until about 3 days before oestrus when there is a major increase to peak oestradiol concentrations of about 38 pg/ml (Guthrie et al., 1972, Van deWiel, 1981) followed by a decline on the day of oestrus to less that 5 pg/ml. Zavy et al. (1980) reported that plasma oestradiol levels increased slightly in non-pregnant gilts between days 12 and 15. The source of oestrogens is the large antral follicles that become apparent at the end of the oestrous cycle. Hansel and Fortune (1978) reported that cholesterol was converted to androstenedione via pregnenolone in the theca interna of the follicle. The androstenedione is in turn metabolized to testosterone in the granulosa cells of the follicle and testosterone is subsequently converted to oestradiol. Follicles which are destined to ovulate are only those actively synthesizing oestrogen as demonstrated in sheep (Carson et al., 1981). However, the initial triggering mechanism to begin the follicular development and subsequent oestrogen synthesis is unknown. Foxcroft and Van deWiel (1982) suggested that P_4 may directly block final maturation of follicles during the luteal phase of the cycle or that qualitative changes in the pattern of LH/FSH secretion rather than a simple quantitative change following the initial decline in P_4 as the CL regresses, might be responsible for follicular maturation.

The rising plasma oestrogen concentration has been shown to have

a temporal relationship with the pre-ovulatory surge of LH and FSH (Guthrie et al., 1972). The latency period between the onset of rising plasma oestrogen and the onset of the pre-ovulatory surge of LH/FSH is approximately 50 hours. During the initial stages of this period, the rising oestradiol level completely suppresses episodic LH release (Foxcroft, 1978) while the effects on FSH are delayed somewhat with minimal FSH levels being attained immediately before the onset of the pre-ovulatory gonadotrophin surge (Van deWiel et al., 1981).

After peak oestrogen levels have been attained, there is approximately a 8-15 hour delay before LH levels peak, rising from 1.8 ng/ml the day before oestrus to a peak of 4-6 ng/ml shortly after the onset of oestrus. This is followed by a decline to basal levels of approximately 1 ng/ml 12 hours after oestrus onset (Niswender et al., 1970, Guthrie et al., 1972). During the surge of LH, there is generally an accompanying surge of FSH (Rayford et al., 1974).

The immediate consequences of the rise in LH include luteinization of the theca and granulosa cells of the follicle, a rapid decline in oestradiol production followed by a gradual increase in progesterone synthesis and ultimately ovulation (Foxcroft and Van deWiel, 1982). Following ovulation, there is a marked increase in the release of FSH for the first few days of the cycle while in contrast LH levels are consistently low (Van deWiel, 1981). The reason for the post-ovulatory rise in FSH secretion may result from the loss of inhibin in the follicular fluid after ovulation (Channing, 1979) and in view of the specific negative feedback of inhibin on FSH secretion this would provide a satisfactory explanation for the lack of an LH response at this same time. The functional significance of the second increase in FSH may be to stimulate follicle recruitment for ovulation

at the subsequent oestrus.

Pregnancy

During early pregnancy, the plasma endocrine profiles are similar to those reported during the first 10 to 12 days of the oestrous cycle. As a result of the conceptus maintenance of functional corpora lutea, plasma P_4 levels remain elevated until the end of pregnancy. However, the plasma concentration of P_4 does not remain at the 30 to 40 ng/ml levels reported on day 14 of the oestrous cycle but rather there is a 30 to 70 percent decrease in maternal plasma progestin concentrations between days 14 and 30 of gestation (Guthrie et al., 1972, Robertson and King, 1974). It has been suggested that the decline in plasma P_4 may reflect initiation of P_4 metabolism by either the uterine endometrium and/or conceptuses. Fischer et al. (1985) reported that endometrial tissue from pseudopregnant gilts did not convert $[H^3]P_4$ to oestrone or oestradiol while endometrial tissue from day 16 and day 25 pregnant gilts did produce substantial amounts of both oestrogens. In addition, Hendricks and Tindall (1971) reported metabolism of P_4 by pig endometrium to at least ten metabolites. The porcine blastocyst itself has been demonstrated to contain P_4 in the blastocoel fluid and Seamark and Lutwak-Mann (1972) suggested that the progestogens in blastocysts might be conveyed to them from the endometrial secretions. Furthermore, Perry et al. (1973) indicated that the blastocyst possessed an array of enzymes including those which could metabolize P_4 to oestrogens.

With respect to plasma oestrogen concentrations, Meoljono et al. (1977) reported that plasma oestradiol concentrations measured in the utero-ovarian vein were greater than 20 pg/ml between days 12 and 17

in pregnant gilts and less than 5 pg/ml in nonpregnant animals. The rise in plasma oestradiol concentration in pregnant gilts at this time may reflect the onset of oestrogen synthesis by the pig blastocyst. Heap and Perry (1974) reported that both oestrone and oestradiol were produced by pig blastocysts and that these 'free' oestrogens are acted upon by uterine endometrial enzymes such as sulphotransferase as they move towards the maternal circulation. Therefore conjugated forms of oestrogen would be expected in the maternal plasma. Robertson and King (1974) confirmed that oestrone sulphate (E_1S) is detectable in maternal plasma by day 16 (60 pg/ml) of pregnancy and in a more recent study Bazer et al. (1982) detected an increase in maternal plasma E_1S on day 12 of pregnancy, coinciding with the onset of blastocyst oestrogen secretion (Heap et al., 1979, Gadsby et al., 1980). By day 30 of pregnancy, the E_1S concentration increases to 3 ng/ml followed by a decline to 35 pg/ml on day 46 and then a gradual increase again to 3 ng/ml by parturition. Unconjugated plasma oestrogen concentrations have reportedly similar patterns of change throughout pregnancy (Robertson and King, 1974).

Ovulation, Fertilization and Early Cleavage in the Pig

The average number of ova ovulated in swine is affected by sexual age (Zimmerman et al., 1957), breed (Kirkpatrick et al., 1967a) nutrition (Kirkpatrick et al., 1967b) and other variables. In general, sows ovulate from 15-24 ova while gilts ovulate an average range of from 12-15 oocytes (Perry, 1954). The process of ovulation begins approximately 36-42 hours after the onset of oestrus (Oxenreider and Day, 1965) with ovulation occurring over a variable

and extended period of time due to the multiple follicles involved. Burger (1952) and Betteridge and Raeside (1962) determined the duration of this time period to range between 4 and 6 hours.

Ova are shed as secondary oocytes with the first polar body extruded and the haploid chromosomes arranged at the second metaphase spindle (Hancock, 1962). Primary oocytes are rarely seen in naturally ovulating pigs but the incidence of such ova increases when gonadotrophic hormones are used to either increase the number of ovulations (superovulation) or to induce ovulation (Polge and Dziuk, 1965). At the time the ova are shed from the follicle, they are surrounded by the cummulus oophorous cells which are dispersed prior to fertilization (Hunter, 1974).

Dziuk (1970) determined the optimum time for inseminating gilts or sows was approximately 12 hours before ovulation. Although spermatozoa can be found at the site of fertilization in the ampullary region of the oviduct within a few minutes of deposition, they are incapable of fertilization because they require an additional 2-3 hours to undergo the process of capacitation (Hunter and Dziuk, 1968). Actual attachment of spermatozoa to the zona pellucida encasing the oocyte occurs 1 to 2 hours after ovulation (Hancock 1972, 1974). Sperm penetration of the zona pellucida occurs through the invigorated progressive motility and release of lytic enzymes within the sperm acrosome (Hunter, 1982). The eventual fusion of the spermatozoa with the egg plasma membrane (vitelline membrane) results in activation of the oocyte and release of the cortical granules, a crucial step to block polyspermy (Austin, 1956). Even after the egg is activated, spermatozoa continue to attach and partially penetrate the zona but are incapable of fusing with the vitelline membrane.

Subsequent to incorporation of the spermatozoa into the egg cytoplasm (ooplasm), the oocyte completes the second metaphase resulting in extrusion of the second polar body and formation of the female pronucleus. Finally, the male and female pronuclei fuse restoring the diploid chromosome number (Austin and Short, 1982).

The first cleavage (mitotic) division resulting in the two cell embryo was reported to occur as early as 21 hours after mating by Hancock (1961). However, Oxenreider and Day (1965) indicated that 67 of 77 embryos examined were still at the one cell stage 30-54 hours after the onset of oestrus. These same authors indicated that the two cell stage did not begin until 60 hours after oestrus onset with the majority of 3 and 4 cell stages being present between 66 to 90 hours. Perry and Rowlands (1962) reported that four to five days after the onset of oestrus, 80 percent of the embryos were either at the 8 cell stage or further (morula, blastocyst) in development with all embryos reaching the morula and blastocyst stage by day 6 to 7. These studies clearly demonstrate that in the polyovulating species such as the pig,there is a wide variation in early morphological development between embryo littermates which can be partially attributed to individual differences in the time of ovulation relative to the onset of oestrus (Perry and Rowlands, 1962). Another explanation accounting for this early embryonic variation in development is the recent finding that specific genes regulate the rate of cleavage (Goldbard et al., 1982). In a recent study by Goldbard and Warner (1982), it was found that the rate of preimplantation mouse embryo development was influenced by genes both within and outside the histocompatability - 2 (H-2) complex. These authors described an H-2 associated gene which governed either fast or slow embryonic development. The timing of

early mammalian development is important because the embryo and uterus must have coordinate development so that the uterus is physiologically prepared for the embryo. Also in the mouse, the maternal immune system is maximally suppressed at exactly the time of implantation so as to prevent rejection of the embryos (Clark and McDermott, 1981). This implies therefore, that the timing of embryo development may be very important in allowing the embryo to escape immune rejection.

While these early cleavage divisions are occurring in the embryo, it is concurrently being transported, in a hormonally controlled manner, along the oviduct to the uterus by cilial and muscular activity, Blandau (1973). In this respect, the pig differs from several other domestic species in that transport into the uterus occurs at an early stage in embryo development. Oxenreider and Day, (1965) located intra-uterine embryos 66 hours after the onset of oestrus which concurred with the earlier reports by Pomeroy (1955) and Perry and Rowlands (1962).

Blastocyst Formation, Migration and Elongation

Majority of porcine blastocysts enter the uterus at the 4 to 8 cell stage of development. After continuing through several mitotic divisions, the ball of embryonic cells called a morula undergo the process of compaction. Compaction is a phenomenom whereby the cells change from spherical to wedge shape and flatten against one another so as to maximize the amount of cell-to-cell contact. This occurs through development of special junctional complexes (VanBlerkom and Motta, 1979). This whole process gives the cells polarity for the first time in that each now has an apical and basal surface. In

addition to establishing polarity, the outer surrounding layer of cells isolates the inner cell mass from the maternal environment.

The compact morula is soon transformed into a blastocyst in which the fluid cavity is referred to as the blastocoele (Heuser and Streeter, 1929). The blastocyst stage marks the first overt sign of cellular differientiation in the developing embryo with the formation of at least two cell types (Perry, 1981). The outer single layer of blastocyst cells is termed the trophectoderm, while the embryo itself forms from a thicker portion beneath the trophectoderm called the embryonic disc (embryoblast). Differientiation is complete by day 6 (Hunter, 1974). The blastocyst hatches from the zona pellucida on day 7 (Hunter, 1974) followed by rapid growth to 1 to 2 mm in diameter on day 8. A second tissue type forms by tangential division of a few cells from the embryonic disc which eventually forms a complete layer on the inner face of the trophectoderm. The second layer, termed endoderm, is formed between days 8 and 10 (Heuser and Streeter, 1929) and when complete bilaminar trophoblast is formed.

The blastocyst maintains its bilaminar spherical form while continuing to increase in diameter to approximately day 12 when the diameter ranges from 5 to 10 mm (Heuser and Streeter, 1929, Anderson, 1978, Geisert et al., 1982a). During the development period from day 6 to 12 of pregnancy, the blastocysts progressively occupy more and more of the uterine horns by migrating away from the utero-tubal junction (Dhindsa et al., 1967). There is eventually intermixing of the embryos from each horn so that by day 12, the entire uterus is occupied by blastocysts spaced equidistant from each other. Perry (1981) reported that overlapping of adjacent blastocysts rarely occurs. Migratory activity of the blastocysts is common in litter

bearing species and in the pig, is probably due to rhythmic myometrial contractions as described by Pope et al. (1982a, 1982b). Oestrogen and prostaglandin production by the blastocyst (Perry et al., 1973) may be responsible for stimulating local myometrial contractions and hence assist in uterine spacing. Equidistant spacing is maintained even if embryos are restricted to a short uterine segment by ligation (Dziuk, 1968). The migration of the blastocysts and their spacing is important not only for utilization of the entire uterine space, but it is essential for recognition and maintenance of pregnancy (see Bazer et al., 1982 for review).

On day 12 of pregnancy, the migratory activity of the blastocyst is complete (Polge and Dziuk, 1970), probably as a result of decreased myometrial contraction (King et al., 1982) and because the trophoblast layer undergoes rapid elongation filling the space within the uterine lumen (Perry, 1981). The pig blastocyst begins a rapid change in morphological structure on approximately day 11 to 12 of pregnancy (Heuser and Streeter, 1929, Anderson and Parker, 1976, Anderson, 1978, Perry, 1981, Geisert et al., 1982b).

After developing to approximately 10 mm in spherical diameter, the porcine blastocyst transforms through an ovoid to tubular and finally a very long and thin filamentous form within 4 to 6 hours. Geisert et al. (1982b) estimated blastocyst growth occurred at 0.25 mm/hour when the spherical diameter was 4 to 9 mm which increased to 30 to 45 mm/hour after attaining a spherical diameter of 10 mm. Elongating blastocysts reportedly obtain lengths of >150 mm within just a few hours (Geisert et al., 1982b). Perry (1981) surmised that because the changes occurred so rapidly, the transformation was due to cellular deformation rather than cellular hyperplasia. Geisert et

al., (1982b) confirmed this by describing a cellular remodelling process to account for the very rapid elongation.

The elongated blastocysts are always found aligning along the mesometrial border of the uterus in which a hyperaemic area can be seen on the uterine luminal surface where the blastocysts are lodged (Perry and Rowlands, 1962, Anderson, 1978, Keys, King and Kennedy, 1986).

Despite the elongated blastocysts measuring one meter or more in length by day 16, each blastocyst will occupy only 20 to 30 cm of actual uterine horn because of the immense folding of the uterine mucosa (Perry, 1981).

Another important feature of blastocyst development is that not all embryos attain a spherical diameter of 10 mm simultaneously and therefore elongation is not synchronous among the littermate embryos(Perry and Rowlands, 1962, Dhindsa et al., 1967, Anderson, 1978). Hence, spherical, ovoid, tubular and filamentous forms can all be found occupying the same uterine environment (Anderson, 1978).

It is worth noting at this point, that Anderson (1978) reported filamentous blastocysts recovered on day 11 of pregnancy contained more protein than spherical, ovoid or tubular blastocysts on the same day, suggesting that elongated blastocysts maybe either more efficient in the uptake of uterine protein secretions or more synthetically active at this stage.

In addition to the blastocysts elongating on day 12 of pregnancy, Perry and Rowlands (1962) reported that the uterine horns increased from an average length of 190 cm on day 3 to 360 cm on days 13 to 18. Along with this increase in uterine length, Anderson (1978) also reported an increase in uterine weight per unit length between days 16

to 18.

Development of the Foetal Membranes and Blastocyst Attachment in the Pig to Day 30

Development of the Foetal Membranes

In the immediate post elongation period (Day 12), a layer of mesodermal cells expands from the embryonic disc and begins to extend laterally between the trophectoderm and extra-embryonic endoderm layers of the trophoblast (Heuser and Streeter, 1929, Perry, 1981). This layer, known as the extra-embryonic mesoderm is important in the formation of the foetal placental membranes. Mesodermal cells are also incorporated into the ventral portions of the embryonic disc and eventually form tissues within the embryo.

As the extra-embryonic mesoderm spreads away from the embryonic disc, it splits into two layers thus forming a cavity lined only by mesoderm which is termed the extra-embryonic coelom. The outer mesodermal layer and the overlying trophectoderm begin to form a rising fold immediately lateral to the embryonic disc. This ridge, known as the amniotic fold, continues to move over the embryo eventually fusing together to form a membrane covering (amnion) and cavity (amniontic cavity) surrounding the embryo.

The mesoderm continues to spread laterally away from the embryonic disc and surrounds the extra-embryonic endoderm thus forming the yolk sac. The yolk sac occupies the entire length of the blastocyst by day 14 to 15 of pregnancy (Patten and Carlson, 1974). The functions of the yolk sac include haemotopoiesis (Jordan, 1916), protein synthesis and nutrient transfer from the uterine lumen through the chorion to the ventral portion of the yolk sac that lies in

contact with the chorion, (Morriss, 1975). This placental arrangement is classified as the choriovitelline placenta (Heuser and Streeter, 1929, Patten and Carlson, 1974). The yolk sac continues development to approximately day 18 after which it rapidly regresses becoming vestigial by day 25 (Perry, 1981).

As the extra-embryonic coelom enlarges, the outer trophectoderm becomes lined with mesoderm which makes up the chorion. The chorion functions to provide nutrient, gaseous and electrolyte exchange between the developing embryo and uterine endometrium (Amoroso, 1952).

On approximately day 14 of pregnancy, a small out pouching of the embryonic hindgut splanchnopleure (mesoderm and endoderm) just posterior to the yolk sac begins development (Patten and Carlson, 1974). This developing membrane is termed the allantois and ultimately forms part of the true placenta of the pig. The allantois becomes highly vascularized and through its continued enlargement due to fluid accumulation (Goldstein et al., 1980) is forced into apposition with the chorion (Heuser and Streeter, 1929, Steven and Morriss, 1975). By day 26, almost the entire inner surface of the chorion is in contact with the allantois with the exception of the extreme ends of the chorion which ultimately form the necrotic tips. Thus, the chorioallantoic placenta is fully functional by day 25 to 30 of pregnancy in the pig.

Goldstein et al. (1980) suggested that the mechanism for transfer of water across the chorioallantois was an active process involving activation of the Na+/K+ ATPase system in the chorionic membrane. Goldstein et al. (1980) reported that the sodium concentration in allantoic fluid on day 20 was 135 mEq/L compared with a maternal plasma sodium concentration of 152 mEq/L. However, by day 30, the

sodium concentration in allantoic fluid was reduced to 9 mEq/L. Bazer et al, (1981a) speculated that the progesterone to oestrogen steroid ratio regulated the allantoic fluid accumulation through synthesis and/or activation of transport enzymes. This speculation was based on the fact that allantoic fluid accumulates during the two periods of pregnancy when the progesterone to oestrogen ratio is decreasing, i.e. days 15 through 30 and between days 50 to term. Allantoic fluid volume reportedly increases from approximately 4 ml on day 20 to 189 ml on day 30. Thus, as Na+ ions move down their concentration gradient from the maternal to allantoic fluid compartments, water and carbohydrates move into the allantoic cavity and are trapped while the Na+ ATPase pump actively transfers sodium ions back across the chorionic epithelium. The allantoic fluid is now known to contain electrolytes, carbohydrates, and uterine proteins (Goldstein, 1980, Bazer et al., 1981a) suggesting that it serves a function as an available reservoir for nutrient support (Bazer et al., 1975).

A similar accumulation of fluid occurs in the amnion but accumulation begins about day 30 increasing from approximately 1 ml to a peak of about 200 ml on day 85 followed by a steady decline to 26 ml at term (Knight et al., 1977). The true functions of the amnion remain unresolved but they may involve a nutritive role as well as providing a fluid environment and protective cushion surrounding the foetus.

Blastocyst Attachment

Purpose of the physical attachment of the chorion to the uterine epithelial surface is to establish communication between the vascular system of the foetus and that of the dam allowing for an efficient

means of exchanging nutrients and wastes. Within the class Mammalia, there is a great diversity in the actual type of implantation established (Amoroso, 1952, Wimsatt, 1975). Additionally, the factors regulating and controlling implantation also vary widely.

Despite the diverse forms of implantation in the different mammalian species, the process does have several unifying steps. First, the majority of mammalian blastocysts implant either superficially or interstitially (Perry, 1981). In the former, there is no penetration of the uterine luminal epithelium and the blastocyst remains within the uterine lumen. Some authors have suggested attachment might be a better description than implantation in these species (Cook and Hunter, 1978).

Attachment occurs in most of the domestic species including the horse and pig. In the cow and sheep, attachment can also be considered the appropriate term despite some superficial loss of uterine epithelial cells (King et al., 1982).

Interstitial implantation is characterized by penetration of the uterine luminal epithelium and embedding of the blastocyst in the stromal tissues which is characteristic of primate and rodent embryos (Finn, 1982).

Implantation in most mammalian species is preceeded by a loss of the zona pellucida so that the blastocyst is free to adhere to the uterine epithelium (Perry, 1981). In species with a interstitial type of implantation, apposition and adhesion of the blastocyst usually occur shortly after hatching while blastocysts of species with superficial attachment continue to develop without attaching to the uterine surface for days or weeks.

In the pig, the initiation of attachment is signified by a

cessation in migratory activity on day 12. This is known as the apposition stage and is characterized by an increased adhesiveness of the blastocyst (Perry, 1981, King et al., 1982). Within the uterine environment the porcine blastocyst does not invade the underlying uterine stroma. Nevertheless, the trophoblast cells retain the potential to be very invasive as demonstrated by their proteolytic activity when transplanted to ectopic sites such as the kidney or lens capsule (Samuel, 1971). Definitive attachment between trophoblast and uterine epithelium begins at day 14, but the connections are very tenuous as gentle flushing with saline readily removes the blastocysts off the uterine epithelium. The process of true attachment continues over a period of days and involves microvillous interdigitation between the uterine epithelial cells and those of the trophoblast (Hafez and Jainudeen, 1974, King et al., 1982).

Structural changes were noted by King et al. (1982) in both the uterine epithelial and trophoblast during the attachment phase. These authors described the uterine epithelial cells on day 11 and 12 as being columnar with flat surfaces and basal or central nuclei. Ultrastructurally, microvilli were seen sparsely scattered over the apical surface of the epithelial cell. On day 14 to 16, the epithelium was taller and the nuclei were located either centrally or apically. In addition, rounded protruberances began to appear on the apical surfaces of the epithelial cells at this stage, which continued to spread over the entire uterine surface from day 16 to 20. By day 18 to 20 the nuclei had returned to a central location and microvilli were reportedly very numerous on the apical surface. The chorionic cells appeared to begin attachment by molding themselves over the protruberances on the apical surfaces of the epithelial cells.

Cytoplasmic projections extended down from the chorionic cells between adjacent uterine epithelial cells almost one-third the distance to the basal lamina of the epithelium. Following the initial molding process of the chorionic cells, firm attachment was established by interdigitation of the opposing microvilli on the chorionic and uterine epithelial cells. This process reportedly spreads rapidly from the area of the embryonic disc peripherally (Perry, 1981) and is complete by day 24 (Amoroso, 1952, Perry, 1981). It is during this period that the allantoic sac is expanding and accumulating fluid, probably assisting in establishing the intimate contact necessary. A further modification of the placental attachment begins at discrete areas of the placenta on day 17 (Friess et al., 1981). These circular modifications, termed areolae, represent areas of the trophoblast overlying the mouth of the uterine glands. Areolae become diffusely spread over the entire attached trophoblast and are well developed by the fourth week of gestation (Perry, 1981). Histologically, the areolae appears as a dome-shaped formation in which the chorionic epithelium overlying the opening of the uterine gland is raised. As pregnancy progresses the chorionic surface of the areolae is thrown up into numerous folds thereby significantly increasing its internal surface area and enhancing the absorptive capacity of the trophoblast. The areolae which are surrounded by a capillary network and have an absorptive function as Chen et al. (1975) localized uteroferrin, a specific uterine secretory protein, within the areolae. Thus, it appears areolae serve the function of absorbing uterine gland secretions into the foetal-placental circulation.

The mechanism(s) involved in actual attachment of the blastocyst to the uterine epithelium are not well understood. However, it is a

gradual process that appears to be strictly controlled relative to time and position. Attachment is probably mediated through specific ultrastructural changes in the plasma membrane surfaces of the two opposing tissues (Mullins et al., 1980a). In the pig, studies on the protein and glycoprotein composition of the uterine epithelial cell plasma membrane were reported to be relatively constant throughout the oestrous cycle and early pseudopregnancy (Mullins et al., 1980a). This finding suggested that neither the altered hormonal status nor the obvious increased secretory capacity of the epithelial cells led to major alterations in the physical structure of the epithelial surface. These authors did suggest however, that despite not finding major changes in the polypeptide make-up of the plasma membrane, this did not rule out the possibility of a change in topographical distribution of the polypeptides on the microvilli. Other researchers have suggested that alteration of the glycoprotein coatings on the trophoblast and epithelial cell plasma membrane may be responsible for the very close apposition of microvilli interdigitation in other species (Enders and Schlafke, 1974, Schlafke and Enders, 1975, Jenkinson and Searle, 1977). The changes within the uterine epithelial surface may allow exposure of underlying electrostatic charges facilitating closer apposition of the two plasma membranes. Enders and Schlafke (1972) indicated that the glycocalyx was removed at specific regions of the trophoblast of the ferret, suggesting indeed the possibility exists that exposure of differing electrostatic sites might be responsible for holding the microvillous attachments together. Surface membrane associated enzymes could play a significant role in localized modifications of the glycoprotein layer (Mullins et al., 1980a).

While attachment is proceeding, the placenta itself is expanding and lengthening in part due to accumulation of allantoic fluid between days 20 and 30 (Knight et al., 1977). From day 30 to 60, there is a continued slower increase in placental size and after day 60, little change in placental size is recorded. However, this development appears to be sufficient for and preceeds the period of rapid foetal growth in the last trimester of pregnancy.

Blastocyst - Endometrial Interaction During Early Pregnancy in the Pig

In domestic species such as the pig, cow, horse, and sheep there is complete separation of the foetal and maternal circulatory systems by chorionic and uterine epithelium. Under these circumstances, the conceptus must rely extensively upon the nutrient (histotroph) transfer from the endometrial glandular and surface epithelium to the chroioallantois (Roberts and Bazer, 1980). Uterine secretions provided might reasonably be regarded as comprising a specialized culture medium designed for support of the embryo through most of pregnancy.

In contrast, rodent and primate species have an invasive (Schlafke and Enders, 1975) type of implantation therefore, uterine secretions may be important only during the preimplantation period (Bazer and Robert, 1983).

The composition of the uterine secretion is not static, varying primarily due to the maternal plasma oestrogen to progesterone ratio and with secretions of the conceptus itself (Knight et al., 1973, Bazer et al., 1981b, Bazer and Roberts, 1983). Briefly, steroids produced by the ovary during the oestrous cycle are the stimulus for preparation of the endometrium for the anticipated pregnancy (Aitken,

1979). Aitken (1979) grouped the endocrine control of endometrial secretion in three phases. In the first phase, plasma oestrogens at the time of ovulation are responsible for secretions that provide an environment for the transport, metabolism, and capacitation of sperm and, through the synthesis of progesterone receptors (Janne et al., 1978), primes the endometrium for its subsequent progestational proliferation. In the second phase, progesterone stimulation of the endometrium is responsible for secretions that provide an environment appropriate for blastocyst growth and implantation. During this stage, secretions from the blastocyst itself may locally modify/control the release of certain uterine secretions. Finally, in the third phase, endometrial secretions contribute to the continued nutritional requirements of the growing conceptus.

In the domestic species, it is essential to have synchronous development between the uterine secretory function and blastocyst development if pregnancy is to be achieved as evidenced by the failure of embryos to develop beyond the blastocyst stage if confined to the oviductal environment (Murray et al., 1971). This finding implies that some uterine factor(s) is essential for conceptus development.

Embryo transfer has also demonstrated that synchrony of the uterine environment of the donor and recipient animal is essential (Rowson and Moor, 1966, Webel et al., 1970), implying that precisely timed and regulated interactions must occur between the endometrium and blastocyst.

Endometrial Protein Secretion

Zavy et al. (1984) reported that total recoverable protein in uterine flushings was significantly greater in pregnant gilts compared

to non-pregnant animals. Furthermore, in both pregnant and non-pregnant gilts, total recoverable protein is affected by the day of pregnancy and oestrous cycle. These authors indicated that total recoverable protein increased between day 6 and 16 and then decreased by day 18 in association with luteal regression and the beginning of pro-oestrus in non-pregnant gilts. In contrast, in pregnant gilts, total recoverable protein increased from day 6 to 16 with levels being maintained through day 18. These findings concur with previous studies by Murray et al. (1972) and Bazer (1975). Knight et al. (1973) demonstrated that the administration of progesterone to ovariectomized gilts stimulated synthesis and secretion of uterine proteins with the quantity released being influenced by the level of progesterone administered. Roberts and Bazer (1980) reported similar findings with progesterone and also found that oestrogen alone had no stimulatory effect on uterine protein synthesis but when administered at low dosages in combination with progesterone, the recoverable protein in the uterine flushings was markedly increased, suggesting that oestrogen might modulate the release of uterine protein. Thus it is clear that ovarian progesterone is primarily responsible for synthesis and/or secretion of the endometrial proteins. Bazer et al. (1980) established unilateral pregnancies in gilts and found that endometrial explants from the gravid horn were significantly more active in protein synthesis compared with the non-gravid horn from the same animal. It therefore appears that although the nature of the secretion produced by the pregnant uterus is a consequence of maternal hormonal regulation alone, the tissue underlying a conceptus is quantitatively more active than that from unoccupied regions of a uterus, implying a local influence of the conceptus. Fischer et al.

(1985) reported a similar finding with respect to endometrial steroid synthesis. Qualitatively, Basha et al. (1980) were unable to demonstrate differences in the proteins secreted by endometrium from pseudopregnant animals and from the gravid and non-gravid horns of unilaterally pregnant sows. Among the proteins identified as specific uterine proteins were four acidic polypeptides of low molecular weight (Mr $\simeq$ 21000) and isoelectric points (pI) of 6.1 and 6.3. These polypeptides appear as two doublets following two dimensional polyacrylamide gel electrophoresis (2D-PAGE) and staining with coomassie blue. In addition to these acidic proteins, Basha et al. (1980) identified several basic proteins including uteroferrin (Mr 32000, pI 9.7) and lysosyme which were synthesized by short-term culture explants of endometrium from pregnant and pseudopregnant sows. While investigating the secretion of plasmin inhibitor by the endometrium, Fazleabas et al. (1983) also identified the same basic proteins reported by Basha et al. (1980) and in addition was able to localize a group of low molecular weight basic proteins (Mr $\simeq$ 14000, pI, 7.3 to 7.5) that he described as a group of plasmin isoinhibitors.

Several uterine proteins secreted by the pig endometrium have been identified such as a purple acid phosphatase termed uteroferrin (see Roberts and Bazer, 1980 for review), retinol binding protein (Adams et al., 1981), leucine aminopeptidase (Basha et al., 1978, Zavy et al., 1984), several cathepsins (A, B, C_1, C and E), lysosyme (Bazer, 1975, Roberts et al., 1976) and an inhibitor of plasminogen activator (Mullins et al., 1980, Fazleabas et al., 1981, 1983). Of the uterine proteins identified, uteroferrin has received the majority of attention. This glycoprotein gives uterine flushings recovered from gilts on days 12 and 16 a purple colour (Squire et al., 1972).

It comprises 10 to 15 percent of the total protein in the uterine flushings from progesterone treated gilts (Schlosnagle et al., 1974) and its site of synthesis as determined by cyto-immunofluorescent studies is the uterine glandular epithelium (Chen et al., 1975). Zavy et al. (1984) measured the concentration of uteroferrin in the uterine flushing on days 6 to 18 of the oestrous cycle and pregnancy and found that the acid phosphatase activity (a measure of uteroferrin content) increased significantly in both physiological states from days 12 to 18 when compared to days 6 to 10. The specific activity (acid phosphatase/mg protein) of the uteroferrin was greatest in pregnant gilts on day 12 followed by a decline to day 18 perhaps indicating utilization by the blastocyst or inhibition of synthesis and/or secretion due to high blastocyst oestrogen synthesis (Knight et al., 1973, Basha et al., 1979). In contrast, a similar increase in uteroferrin content in non-pregnant gilts did not occur until day 15.

Geisert et al. (1982) found that oestrogen of blastocyst origin initiated a synchronized release of protein and other endometrial components from the secretory vesicles in the glandular and surface epithelial cells on approximately day 12 of pregnancy. This probably accounted for the earlier peak concentrations of uteroferrin on day 12 in pregnant gilts versus day 15 in non-pregnant gilts in the study of Zavy et al. (1984).

In the study of Chen et al. (1975), uteroferrin was found to localize in the lumen of the uterine glands between days 9 and 13 of the oestrous cycle. However, on day 14, uteroferrin was located in the endometrial stroma, ie. movement was away from the uterine lumen toward the basement membrane of the uterine glands. In contrast, during pregnancy, uteroferrin was absent in the stroma but was

localized within the glandular epithelial cells, the lumen of the glands and after day 30 was present within the placental areolae. Thus, throughout pregnancy, uterine protein is secreted in a direction that makes it available as a nutrient source to the developing foetus. This movement maybe directed by the local effect of blastocyst oestrogen on the endometrium, however, the mechanism involved is unknown.

The function of uteroferrin is believed to be the transport of iron to the foetus (Bazer and Roberts, 1980). This is achieved through absorption of secreted uteroferrin by the areola of the chorion. Uteroferrin may be absorbed into the capillaries of the areolae and therefore could be either transported directly to the foetus and/or transferred to the allantois where it has been found to accumulate after day 30 of gestation (Bazer et al., 1975) and would possibly be serving as a reservoir of iron.

Of the other uterine proteins identified and previously mentioned, the inhibitor of plasminogen activator has been well studied. Mullins et al. (1980) demonstrated that by day 12 of pregnancy, porcine blastocysts cultured in vitro released large quantities of plasminogen activator (PA) into the culture media. Fazleabas et al. (1983) demonstrated that plasminogen (the zymogen substrate for PA) was present within the uterine flushing from gilts between days 10 and 16. It was assumed that plasminogen was derived as a serum transudate. Thus, the potential to produce plasmin, a potent proteolytic enzyme exists within the pregnant uterus of the gilt through the action of PA on plasminogen.

Plasminogen activator has been implicated in the migratory and invasive spread of both malignant (Pollock et al., 1974) and normal

(Laug et al., 1980) cell types. Fazleabas et al. (1983) reported that the release of blastocyst PA was biphasic, occurring first at the time of blastocyst elongation on day 10 to 12 and second, from day 14 to 16 when early attachment to the uterine epithelium began. The potential invasiveness of porcine blastocysts has been demonstrated by transplanting them to ectopic sites (Samuel, 1971). Therefore, under normal circumstances, production of large quantities of plasmin within the uterine lumen must be prevented. Mullins et al. (1980) and Fazleabas et al. (1982, 1983) have demonstrated that a PA inhibitor was present in the uterine flushings during the luteal phase of the oestrous cycle and from pregnant gilts between days 10 and 16. Thus, it is concluded that the inhibitor(s) protects the uterine epithelium from potentially erosive products that could be formed by the action of blastocyst PA on secreted uterine plasminogen. It is believed that the inhibitor(s) coat the trophoblastic surface of the blastocyst thus preventing plasminogen activation to plasmin. Additionally, the inhibitor(s) may protect other macromolecules from enzymatic destruction (eg. uteroferrin). The purpose for which the potential to produce plasmin exists at all is unknown but plasmin may be important in trophoblast remodeling during elongation (Geisert et al., 1982b) as well as uterine development during pregnancy. Plasmin has also been found in the mammary gland during tissue remodeling in this organ (Ossowski et al., 1979).

With regard to the functions of the other known proteins, retinol binding protein is thought to transport Vitamin A to the foetus, lysosyme may be involved in the implantation process as well as having a bacteriostatic function and the functions of the remaining proteins, leucine aminopeptidase and cathepsin activities remain unknown.

However, these are probably cell surface or membrane associated enzymes which function in the breakdown of many varied substrates for use by the embryo (Bazer et al., 1981b).

Endometrial Steroid Metabolism

In addition to the protein synthetic and secretory activity of the uterine endometrium several studies have clearly demonstrated that the endometrium is very active in the metabolism of steroids, particularly maternal progesterone and foetal oestrogens (Hendricks and Tindall, 1971, Amoroso, 1973, Fisher et al., 1985).

Knight et al. (1977) observed that the uterine artery - uterine vein (A-V) difference in plasma progesterone concentration was positive at all stages of pregnancy from day 20 to 100 while in contrast, the A-V difference in oestrone and oestradiol was negative. These findings imply that progesterone is being taken up by the endometrium or its contents and is being metabolized possibly to oestrogens by the foetus or placenta. The effect of viable conceptuses on the plasma progesterone concentration was demonstrated in a study in which the plasma progesterone levels were significantly lower (7 to 26.5 ng/ml) at day 60 of gestation in ovariectomized pregnant gilts supplemented with injections of progesterone to maintain the pregnancy than similarly treated gilts without viable conceptuses (163-428 ng/ml) implying that the conceptus could metabolize progesterone and/or induced the endometrium to do so (Bazer et al., 1982).

A recent study by Fischer et al. (1985) suggested that the conceptus itself does influence the metabolism of [^{3}H] progesterone to oestrone and oestradiol by the endometrium. These authors found

that the endometrium from pseudopregnant gilts did not convert [H^3] progesterone to oestrone or oestradiol while endometrial tissue from day 16 and 25 pregnant gilts (contamination of the explants with trophoblast cells was considered negligible) produced substantial amounts of oestrone and oestradiol. This would imply that prior to separation of the trophoblast from the endometrium, the former had altered the enzymatic capacity of the endometrium.

Bazer et al. (1982) hypothesized that the endometrial tissues could metabolize progesterone to precursors for oestrogen production by the blastocyst itself. However, Fischer et al. (1985) could not substantiate this hypothesis which concurred with the finding by Hendricks and Tindall (1971) that the endometrium converts progesterone to 5α-reduced metabolites which cannot be aromatized to oestrogens. Fisher et al. (1985) proposed that this pathway may rapidly deplete the pool of progesterone and progesterone-metabolites available for conversion to oestrogens by the blastocyst.

Zavy et al. (1980) used uterine flushing to measure changes in the uterine luminal steroids during the oestrous cycle and early pregnancy in the pig. From these studies, it was reported that the uterine luminal content of progesterone was higher in non-pregnant animals than pregnant gilts again implying altered metabolism of maternal progesterone by the pregnant endometrium and/or the conceptuses. With respect to the total recoverable oestrogens, Zavy et al. (1980) reported that in non-pregnant gilts, both oestrone (E_1) and oestradiol (E_2) levels remained relatively constant from days 6 to 16 of the cycle but E_2 increased on day 18 reflecting the pro-oestrous increase in folliculogenic, oestrogen synthesis, and the pre-ovulatory rise in plasma E_2. In contrast, total recoverable

oestrogen levels were higher in pregnant gilts versus non-pregnant animals and furthermore, significant day trends occurred with both E_1 and E_2. Oestrone concentrations were high on day 8 and then declined to day 10 followed by a dramatic increase from days 12 to 18. In contrast E_2 concentrations were shown to increase between days 10 and 12 and then to decline to day 15 followed by a steady increase from days 15 to 18. The increasing concentrations of E_1 and E_2 in uterine flushings of pregnant gilts after day 10 coincides with the initiation of oestrogen synthesis by the blastocyst (Heap et al., 1979). An explanation for the steady increase in E_1 from days 12 to 18 while E_2 declined between days 12 to 15 might reflect endometrial conversion of E_2 to E_1 as demonstrated by Flood (1974).

The endometrium actively metabolizes blastocyst oestrogens as the physiologically active free forms of E_1, E_2, and oestriol (E_3) do not alter significantly in the maternal plasma in early pregnancy (Guthrie et al., 1972, Robertson and King, 1974, Zavy et al., 1980). However, the conjugated forms of these oestrogens increase in the maternal plasma particularly oestrone sulfate (E_1SO_4) as demonstrated by Robertson and King (1974). Heap and Perry, (1974) suggested that oestrogens were conjugated as they moved through the endometrium towards the maternal circulation by the action of sulphatransferase, a progesterone dependent enzyme whose activity in the endometrium has been shown to change in parallel with the circulating progesterone concentration (Pack and Brooks, 1974, Dwyer and Robertson, 1980, Heap et al., 1981).

Geisert et al. (1982a) reported on the concentrations of conjugated oestrogens in the uterine flushings of pregnant and non-pregnant gilts between days 10 and 14. These authors found that

E_1SO_4 did not change significantly between days 10 and 12 of the oestrous cycle but did increase on day 14, perhaps reflecting conjugation in the endometrium of oestrogen produced in the ovary. In pregnant gilts, both E_1SO_4 and E_2SO_4 increased significantly on day 12 resulting from conjugation of the oestrogens synthesized by the blastocysts as they begin to elongate.

Other Endometrial Secretions

Geisert et al (1982a) demonstrated that total calcium (Ca) increased in uterine flushing of pregnant gilts containing tubular and filamentous blastocysts. The increased Ca detected on day 12 had declined by day 14. In non-pregnant gilts, no such change in luminal Ca was found suggesting that the blastocyst's presence was responsible for the release of the Ca. The release of Ca coincides with the onset of oestrogen secretion by the blastocyst. Pietras and Szego (1975) have previously shown that oestrogens influence the movement of Ca in cells of the rat endometrium. In addition, Ca is known to influence the release of secretory vesicles from the cells of several tissues including adrenal medulla and pancreas (see review by Rasmussen and Barrett, 1984). Rubin and Laychock (1978) proposed that Ca, released from the plasmalemma of adrenal cortex when surface receptors are activated, is involved directly with activation of phospholipase A_2 to induce exocytosis of secretory vesicles and indirectly to enhance prostaglandin synthesis. Thus, blastocyst oestrogens may function in a similar manner bringing about synchronized release of the contents of the secretory cells of the uterine epithelium (Geisert et al., 1980a). Not only does uterine luminal Ca level increase at this time but so does the content of protein including uteroferrin and

plasminogen activator inhibitor, prostaglandin $F_{2\alpha}$, and prostaglandin E_2 (Fazleabas et al., 1983, Geisert et al., 1982a). Thus, this synchronized release of the epithelial cell contents could play a significant role in such processes as blastocyst elongation (Geisert et al., 1982b), implantation, maternal recognition of pregnancy (Bazer and Thatcher, 1977) and blastocyst nutrition (Roberts and Bazer, 1980).

Finally, Murray et al. (1980) demonstrated that riboflavin was secreted by the porcine endometrium. On day 8 in both pregnant and non-pregnant animals, the levels of riboflavin were reportedly much greater in uterine fluids than in other body fluids. Whether or not a functional relationship exists between uterine secretory riboflavin and embryonic development is unknown.

Blastocyst Steroid Metabolism

The porcine blastocyst is very active in the synthesis and metabolism of several steroids but its ability to synthesize and secrete large quantities of oestrogens appear to be crucial to preventing regression of the corpus luteum and to ensuring secretion of histotroph for continued blastocyst growth and development (Perry et al., 1973, Perry et al., 1976, Gadsby et al., 1980, Flint, 1981, Heap et al., 1981).

Perry et al. (1973) demonstrated that incubation of H^3 labelled androstenedione and dehydroepiandrosterone (DHA) with blastocyst tissue recovered at certain stages of early pregnancy resulted in the incorporation of these labelled androgens into both oestrone (E_1) and oestradiol-17β (E_2). These same authors reported that some labelled progesterone but not labelled cortisol or pregnenolone was

incorporated into oestrogens. Also, incubation of labelled oestrone with blastocysts confirmed the presence of a 17α-oxidoreductase enzyme system within the blastocyst tissue because an appreciable amount of radioactivity was subsequently incorporated into oestradiol-17β. During the same study, Perry et al. (1973) found that the blastocyst tissue could rapidly hydrolyze labelled oestrone sulphate (E_1SO_4) to the unconjugated form. However, there was little evidence for a sulphotransferase enzyme system that could catalyze the reverse reaction, i.e. form sulphated oestrogens as has been demonstrated in the endometrium of the pregnant and non-pregnant gilt (Pack and Brooks, 1974, Dwyer and Robertson, 1980).

These initial studies by Perry et al. (1973) have been subsequently confirmed and extended in further investigations (Perry et al., 1976, Flint et al., 1979) such that there is evidence that the porcine blastocyst can synthesize oestrone, oestradiol and oestriol from acetate, cholesterol, progesterone, DHA, androsteredione and conjugated oestrogens.

The onset of blastocyst oestrogen synthesis has been detected by day 12 post-breeding, as it appears to be closely associated with the time of rapid blastocyst elongation (Heap et al., 1981, Geisert et al., 1982a). A concentration gradient of over 100 had been shown to exist between the concentration of oestradiol-17β in blastocysts at days 12 and 13 and the endometrium (Gadsby and Heap, 1978). In addition, high levels of progesterone have been found in day 12 and 13 blastocysts as well as the enzyme 3β-hydroxysteroid dehydrogenase (Gadsby et al., 1976) which can convert pregnenolone to progesterone and DHA to androsteredione, both of which can be metabolized to oestrogens.

Since progesterone is essential for embryonic development to proceed normally (pregnancy fails if ovariectomized pigs are not given progesterone) it was hypothesized that circulating progesterone was the major substrate for blastocyst oestrogen synthesis. However, in experiments in which ovarian progesterone secretion was eliminated by ovariectomy and the pregnancy maintained with a non-aromatizable synthetic progestogen, medroxyprogesterone acetate (MPA), pregnancy proceeded normally (Flint et al., 1979, Heap et al., 1981). Heap et al. (1981) found no [H^3]-MPA was incorporated into oestrogen by blastocysts and therefore, since oestrogens are luteotrophic in the pig (Gardner et al., 1963), oestrogens must have been synthesized from precursors other than MPA. This finding also demonstrates that maternal ovarian progesterone is not critical to blastocyst oestrogen synthesis. Ultimately it appears that the critical enzyme in the pathway which is responsible for blastocyst oestrogen synthesis is aromatase which is able to convert androstenedione and testosterone to oestrone and oestradiol respectively (Perry et al., 1973, Heap et al., 1981).

The production of blastocyst oestrogens begin at about the 10 mm spherical stage on day 10.5 to 11 (Geisert et al., 1982a). Based on oestrone sulphate concentrations in maternal plasma, oestrogen secretion by the pig conceptuses is triphasic with major detectable increases between days 10 and 12 (Stoner et al., 1981) days 16 and 30 (Stoner et al., 1981, Robertson and King 1974) and day 60 to term (Knight et al., 1977).

The mechanism(s) regulating blastocyst oestrogen synthesis remain unknown. However, as will be discussed in the section on maternal recognition of pregnancy, oestrogens appear to be the embryonic signal

to the maternal system for this phenomenon. Recently, Mondeschein et al. (1985) reported that porcine blastocysts have the capacity to convert phenolic oestrogens to their 2- and 4- hydroxylated metabolites thus forming the catechol oestrogens 2- and 4- hydroxyoestradiol (E-2/4- H). The activity of E-2/4-H appears as a brief surge between days 10 and 14 of pregnancy coinciding with blastocyst elongation, blastocyst spacing, initial blastocyst attachment, maintenance of the corpora lutea and increased uterine blood flow. These authors hypothesized that because the catechol oestrogens are potent stimulators of prostaglandin synthesis and because peak activity of E-2/4-H coincided with initial peak aromatase activity, the catechol oestrogens may play a vital role in regulating some of the peri-attachment physiological changes in the blastocyst and endometrium. For example, because of the ability of catechol-oestrogens to interact with alpha and beta receptors on cells, the binding of catechol-oestrogens to alpha receptors on the smooth muscle of uterine arterioles leads to vasodilation and increased blood flow which may be one of the regulators of maternal recognition of pregnancy (Ford, 1982).

A further suggested function of blastocyst oestrogen synthesis proposed by Flint (1981) was that it may be involved in the control of endometrial production of blastocyst growth inhibitors or promoters as has been suggested to occur in diapausing rodents (Weitlauf and Kressling, 1981, Surani and Fishel, 1981). Embryonic diapause, which is essentially a dormant state of the blastocyst, is a time period of variable duration depending on the species (see Aitken, 1977 for review). Flint (1981) suggested that in those species in which blastocysts produce oestrogen before implantation, diapause may not be

possible. This hypothesis is based on the fact that the termination of delayed implantation in the rat and mouse is dependent on oestrogen. In support of this hypothesis, Flint (1981) reported that uterine luminal fluid from progesterone treated pigs contains compounds that stimulate and inhibit blastocyst DNA synthesis as determined by incorporation of labelled thymidine into DNA. This raises the possibility that oestrogen production by the porcine blastocyst maybe involved in the control of blastocyst growth by preventing uterine production of growth inhibitors. The fact that oestrogen synthesis is detectable in the blastocyst shortly before the onset of rapid trophoblast growth lends support to this possibility.

Thus it is apparent that the pig blastocyst has the capacity to synthesize oestrogens, primarily oestrone and oestradiol and limited amounts oestriol, from several different substrates. Maternal ovarian progesterone, although essential to maintain the pregnancy, it is not a mandatory substrate for oestrogen synthesis by the blastocyst. The physiological functions of the blastocyst oestrogen include stimulation of a synchronized release of the uterine glandular and epithelial cell contents, possible regulation of blastocyst growth, changes in uterine blood flow and is probably the blastocyst signal to the mother regulating maternal recogition of pregnancy.

Polypeptide Synthesis by the Porcine Blastocyst

Despite considerable evidence that oestrogens from the blastocyst are responsible for prolonged luteal maintenance and maternal recognition of pregnancy in the pig, studies have been undertaken in an attempt to identify polypeptides of conceptus origin which may be associated with pregnancy maintenance as has been reported in the ewe

(Godkin et al., 1982a). Studies on the concentration and occupancy of the LH receptor in the corpus luteum of cyclic and early pregnant pigs (Ziecik et al., 1980) have demonstrated a dramatic decline in LH occupancy, but a simultaneous rise in the receptor concentration between days 20 and 30 of pregnancy. One explanation for this is the existance of a luteotrophic substance which interacts with the LH receptor, but does not cross-react with the radioimmunoassay for LH. Flint (1981) reported that a conceptus extract had been partly purified and did not cross react in the radioimmunoassay for LH but did in the receptor assay.

Godkin et al. (1982b) demonstrated the release of radiolabelled protein macromolecules into the culture medium following incubation of pig blastocysts in the presence of radiolabelled leucine. The proteins, identified by two dimensional polyacrylamide gel electrophoresis and fluorography, were found to change with duration of pregnancy. Spherical (4-9 mm), tubular (10-50 mm) and early filamentous blastocysts (>100 mm length) recovered between days 10.5 and 12 of pregnancy all released, as their major products, a group of low molecular weight (Mr) acidic polypeptides (Mr ≃ 20,000-25,000, pI 5.6-6.2). By day 13 the pattern of protein synthesis changed markedly in that in addition to the low molecular weight acidic proteins, the major proteins detected were basic with a molecular weight range of 35,000-50,000. After day 18 these authors reported a further change in the secretory pattern with a new group of polypeptides appearing with molecular weights between 50,000 and 70,000. This latter group of proteins was found to correspond in electrophoretic mobilities to foetal serum proteins, including transferrin, alpha-foetoprotein and foetuin. At day 25 and 30, chorioallantoic tissues cultured in the

absence of any embryonic tissue released three other distinctive proteins. Between days 13 and 16, an additional very high molecular weight glycoprotein (Mr >650,000) was also released.

Godkin et al. (1982b) noted that the initial low molecular weight acidic proteins released by the expanding porcine blastocyst were similar in their physical properties to ones secreted by the expanding sheep blastocyst. In the sheep, these proteins become associated with the uterine epithelium of the pregnant ewe (Godkin et al., 1984). These authors suggested that since these are the first proteinaceous products released in major amounts by the pig and sheep blastocysts, they may have similar functions. Additionally, both the sheep and pig blastocysts produce the large molecular weight glycoprotein but sheep conceptuses did not secrete the basic polypeptides between days 13 and 18 as was found in the pig (Godkin et al., 1982a,b).

Thus, it has been demonstrated that the patterns of proteins released by the developing pig conceptus change markedly during early pregnancy even prior to attachment. However, despite the similarity of some of these products to those detected in other species, little is known about their function. It is possible that one of these products is the LH - like material reportedly extracted from pig blastocysts by Saunders et al. (1980).

Maternal Recognition of Pregnancy in the Pig

The oestrous cycle of the pig, as in the case of the other farm animal species, is characterized by a regular return to oestrus at 18 to 24 day intervals in the absence of pregnancy. This regularity is determined by failure of the corpora lutea (CL) to continue

functioning (i.e. producing progesterone) after day 15 of the cycle (du Mesnil du Buisson and Leglise, 1963). The demise of the CL facilitates another opportunity for the female to become pregnant. Thus, in the absence of viable conceptus there is a natural mechanism to cause regression of the CL and allow a return to oestrus. Loeb (1923) found that hysterectomy of guinea pigs during the luteal phase of the oestrous cycle prolonged the functional lifespan of the CL. This implies that the uterus naturally produces a substance(s) that induces regression of the CL i.e. the uterus produces a luteolytic factor(s).

Removal of the CL at anytime during pregnancy in the pig results in abortion indicating that progesterone from the CL is essential to maintenance of pregnancy (du Mesnil du Buisson and Dauzier, 1953). Therefore, if CL progesterone is essential for the maintenance of pregnancy, normal regression of CL around day 15 which occurs during the oestrous cycle must be prevented. The presence of conceptuses must alter the normal chain of events which occurs during CL regression. Process(es) by which the embryo prevents luteolysis in mammals has been described as the "maternal recognition of pregnancy" (Short, 1969).

Anderson et al. (1966) demonstrated that either the destruction or absence of endometrium resulted in prolonged maintenance of the CL, suggesting that the source of the luteolytic substance was in fact the endometrium itself. Moeljono et al. (1977) found that luteolysis in the pig was accompanied by a significant increase in the content of prostaglandin $F_{2\alpha}$ (PGF) in the utero-ovarian vein. It was also found that if exogenous PGF was administered to gilts on days 12 or 13 of the oestrous cycle the cycle length was shortened. However, this

was not the case if PGF was administered before day 12 of the cycle (Moeljono et al., 1976). The reason for this is that the CL of the pig are autonomous for the first 12 days of the oestrous cycle as previously described in this review. The autonomy of the porcine CL was demonstrated in a study by Caldwell et al. (1969), in which CL induced with HCG on day 6 of the oestrous cycle regressed along with the natural CL. However, accessory CL induced on days 8, 10, and 16 did not regress until 2, 3, and 7 days, respectively, after regression of the natural CL. Patek and Watson (1976) reported that in vitro PGF production was significantly greater in endometrial tissues taken from mid to late luteal phase gilts than tissue obtained earlier in the cycle. Furthermore, by incorporating indomethacin in the culture medium, these same researchers found that PGF synthesis could be blocked. Kraeling et al. (1981) confirmed the finding that indomethacin (a prostaglandin synthetase inhibitor) blocked PGF production and if administered to gilts between days 13 and 17 of the cycle resulted in prolonged CL function. The effect of indomethacin could be overcome by injecting PGF intramuscularly indicating that the indomethacin treatment did not effect the sensitivity of the CL to the luteolytic effect of exogenous PGF. Guthrie and Rexroad (1980) utilizing in vitro culture techniques also provided indirect evidence that endometrial PGF is a potential luteolytic agent in the pig.

Several researchers have demonstrated a temporal relationship between elevated PGF concentrations in utero-ovarian vein plasma of pigs and declining plasma progesterone concentrations associated with CL regression (Gleeson et al., 1974, Moeljono et al.,1977, Frank et al., 1977). In all these studies, there was a significant increase in

PGF concentration in the utero-ovarian vein in cyclic gilts at the time of luteal regression (days 13 to 17). A similar increase in PGF did not occur in pregnant gilts.

Ford and Christenson (1979) suggested that the utero-ovarian vein PGF concentration was lower in pregnant gilts between days 12 and 16 of pregnancy because of the dilution effect subsequent to increased uterine blood flow stimulated by blastocyst oestrogen secretion. Uterine arterial blood flow increases from 50 ml/min on day 11 to an average 100 to 150 ml/min on day 14 (Ford and Christenson, 1979). To ensure that the measured differences in the uterine vein plasma concentration of PGF between cyclic and pregnant gilts was not a reflection of the dilution effect of increased uterine arterial blood flow, Shille et al. (1979) measured the peripheral plasma concentration of the PGF metabolite, 13, 14-dihydro-15-keto-PGF (PGFM) and found that it increased significantly between days 13 and 15 in cyclic gilts while it remained at basal levels during the same period in pregnant gilts. Thus, there is very strong evidence indicating that PGF is indeed the primary uterine luteolysin in the pig. During pregnancy, the CL are maintained suggesting that the presence of conceptuses somehow alters the synthesis and/or release of PGF. The importance of conceptus number on maintenance of pregnancy was demonstrated by Polge et al. (1966) who indicated that unless at least two viable embryos were present in each horn before day 18, pregnancy was not maintained to day 40. Similarly, Anderson (1966) reported that the presence of a non-gravid horn usually resulted in CL regression while if increasing proportions of the non-gravid horn were surgically removed, the number of gilts remaining pregnant increased (Anderson et al., 1966). du Mesnil du Buisson (1961) found that leaving as little

as one-fourth of the uterine horn in non-pregnant gilts resulted in bilateral CL regression. This implies that a local and a systemic pathway exists whereby PGF can reach the ovaries. In contrast, the presence of a contralateral non-gravid horn during pregnancy in both the cow and sheep does not bring about CL regression implying that luteolysis in these species is limited to a local pathway.

Therefore in the pig, the conceptus must interact locally with a major portion of the uterine endometrium if pregnancy is to be established. Additionally, the importance of the conceptuses in prolonging the CL lifespan is evident by the fact that removal of the blastocysts before day 12 does not maintain the CL while the lifespan is extended if removal occurs after day 12. This implies that maternal recognition of pregnancy in swine occurs at approximately this stage of gestation.

Perry et al. (1976) suggested that oestrogen from the trophoblast of the porcine blastocysts is the luteotrophic/luteostatic agent in swine. This is consistent with the finding that the onset of oestrogen synthesis and secretion by the blastocysts begins around day 12 (Perry et al., 1976) and secondly, daily administration of 5 mg oestradial valerate from days 11 through 15 of the oestrous cycle results in long term maintenance of the CL (Gardner et al., 1963, Frank et al., 1977).

Based on the following studies, Bazer and Thatcher (1977) proposed a theory of maternal recognition of pregnancy in swine in which they postulated that oestrogen controlled the secretion of PGF by the uterine endometrium.

In their theory it was assumed that the synthesis of PGF was stimulated by progesterone and that synthesis and secretion occurred

in the epithelial cells of the endometrium in a similar fashion as previously described for the purple glycoprotein, uteroferrin. In addition, Bazer and Thatcher (1977) assumed that PGF and uteroferrin could be secreted into the uterine lumen (exocrine secretion) or into the uterine stroma towards the venous drainage (endocrine secretion). Furthermore, the direction of movement of uteroferrin and PGF was determined by the local concentration of oestrogen established by the trophoblast in the pregnant pig.

There is substantial data to support the endocrine-exocrine theory for CL maintenance and hence maternal recognition of pregnancy in the pig. First, a minimum of four viable embryos must be present (two in each horn) during the first three weeks of pregnancy in order to prevent luteal regression (Polge et al., 1966). This implies that there is sufficient endocrine secretion of PGF from areas of the endometrium not in contact with blastocysts to bring about CL regression and that exocrine secretion of histotroph and PGF is stimulated only locally around the oestrogen secreting elongated blastocysts. Second, Zavy et al. (1980) found that total recoverable PGF was greater in the uterine flushings from pregnant gilts (22,688 ng) on day 18 compared to non-pregnant gilts (454 ng) on the same day supporting secretion of PGF away from the uterine vasculature in pregnant animals (i.e. exocrine direction). Frank et al. (1978) demonstrated a similar response when she compared uterine luminal PGF levels between cyclic gilts and gilts induced into pseudopregnancy following oestradiol valerate treatment. This exocrine release of PGF concurs with the lowered number of PGF spikes, average concentration of PGF spikes and average overall PGF concentration in the utero-ovarian vein plasma in pregnant versus non-pregnant gilts

between day 12 and 20 after oestrus onset (Moeljono et al., 1977). An important difference was noted by Bazer et al. (1982) in that pseudopregnant gilts contained less than one-half the total PGF per uterus than did pregnant gilts, suggesting that the porcine blastocysts themselves synthesize and secrete additional PGF. This is supported by in vitro PGF synthesis and release by porcine blastocysts (Walker et al., 1977). Finally, the study of Chen et al. (1975) clearly demonstrated the difference in the pattern of endometrial secretion between cyclic and pregnant gilts. While studying uteroferrin, these authors found that in non-pregnant gilts, the glycoprotein was secreted into the lumen of the uterine glands through day 13 of the oestrous cycle. However, on day 14, the protein began to localize in the endometrial stroma surrounding the basement membrane of the uterine glands. This indicated that the protein could enter the interstitial fluid and vascular system within the endometrial stroma. In contrast to this pattern of movement of uteroferrin in non-pregnant gilts, the glycoprotein was not found in the endometrial stroma at any stage of pregnancy studied ranging from day 6 through 90. Ogra et al. (1979) using immunofluorescent antibody procedures, described a similar pattern of secretion with respect to PGF in oviductal epithelium of women.

In summary, maternal recognition of pregnancy in the pig is the result of progesterone induced synthesis and secretion of PGF into the uterine lumen at the time of onset of blastocyst elongation and oestrogen production. Because PGF secretion is in an exocrine direction away from the blood vasulature system, the corpora lutea are maintained as are plasma progesterone levels and continued secretion of uterine histotroph. How blastocyst oestrogen synthesis and release

stimulates the differential pattern in PGF movement is unknown at this time. However recent data has shown that to prolong the functional lifespan of the CL for long term (greater than 30 days) in cyclic gilts by administration of oestradiol, requires a biphasic exposure (Geisert et al., 1984). These authors reported that a single oestrogen treatment of cyclic gilts on days 9.5, 11, 12.5, 14, 15.5, or multiple treatments on days 11 and 15 or 14-16 extended the inter-oestrus period to approximately 30 days. However, a biphasic treatment on day 11 and then days 14-16 or continuous treatment from days 11-15 extended the inter-oestrus period to greater than 60 days. It has also been demonstrated that the pig exhibits a similar biphasic pattern of oestrogen release during pregnancy (Zavy et al., 1980, Stoner et al., 1981, Geisert et al., 1982a). Thus, it appears that to fully maintain CL function throughout pregnancy requires a biphasic pattern of oestrogen stimulation of the endometrium, the first being around day 11 coincident with blastocyst elongation and the second at approximately day 14 to 17.

Early Embryonic Death in the Pig

The pig experiences a natural 35 to 40 percent prenatal mortality during gestation (Perry and Rowlands, 1962, Bazer et al., 1969, Anderson, 1978). These authors have demonstrated that 75 percent or more of this loss occurs within the first 25-30 days of pregnancy. Bazer et al. (1969) hypothesized that a large portion of early embryonic death resulted from mechanisms which predetermined the number of embryos the uterus could support, i.e. "uterine capacity" was limited. Indeed, several experiments have proven this to be true but its effect is not operative until after day 30 (Dziuk, 1968,

Fenton et al., 1970, Webel and Dziuk, 1974).

Fenton et al. (1970) conducted a study in which one ovary and the ipsilateral uterine horn was removed from sexually mature gilts. These researchers found that following surgery, the remaining ovary compensated in terms of ovulation rate in accordance with the finding of Brinkley et al. (1964) and that on day 25 of pregnancy there was no difference in the number of corpora lutea or live embryos per gilt between intact control animals and the unilaterally ovario-hysterectomized (UHOX) gilts. However, on day 105 of gestation, the foetal survival rate in the intact gilts was signifcantly greater than UHOX gilts. The conclusion was that the quantity of uterine tissue did not limit uterine capacity until after 25 days of gestation. Similar studies in which the uterine space per embryo was decreased either by uterine horn ligation, superovulation (Dziuk, 1968, Webel and Dziuk, 1974) or by superinduction (Dziuk, 1968, Pope et al., 1972) have also demonstrated that overcrowding is not a major factor limiting litter size in swine during early pregnancy but is significant after day 30 of gestation. Foetuses recovered under these conditions have been found to have shorter crown-rump lengths, weigh less than normal contemporaries, have less placental surface area and lower total number of areolae (Knight et al., 1977). It is unclear why in this crowded environment some foetuses are so adversely affected while others are not. This is perplexing if one considers the porcine uterus to be functioning as a single physiological unit. Under this condition the blood supply and provision of nutrients to all foetuses would be expected to be distributed equally. One explanation might be that placental size differs among littermates and this may stem from the fact that not all blastocysts elongate

simultaneously and rarely do they overlap but rather they elongate until contact is made with adjacent embryos (Perry and Rowlands, 1962, Perry, 1981). Indeed, Knight et al. (1977) demonstrated that placentae were significantly longer in small litters versus those with a large number of conceptuses.

There are two mechanisms operative in establishing a normal pregnancy in swine that attempts to reduce crowding of individual foetuses. The first is that the blastocysts migrate throughout the uterus spacing themselves equidistant from each other as previously described. Secondly, the uterus itself undergoes a lengthening process thus allowing a final adjustment of the embryo spacing (Perry and Rowlands, 1962). Uterine lengthening appears to occur as a result of two mechanisms, an early endocrine induction probably as a result of blastocyst oestrogen synthesis and release (Pope and First, 1985) and a later lengthening due to distension by accumulating foetal allantoic and amniotic fluids (Bazer et al., 1981). Nevertheless, it is clear that the majority of embryonic loss occurs in the first few weeks of the pregnancy. Until recently, this period of embryonic loss had not been studied to any extent other than to eliminate certain postulates. First, fertilization failure was shown not to be a factor (Perry and Rowlands, 1962) as was insufficient luteal function (Webel et al.,1975). McFeely (1967) studied chromosomal anomalies in early pig embryos and concluded that such anomalies might account for about 10 percent of early embryonic deaths but in his conclusion he did state that some of the abnormalities might not be lethal.

Bazer et al. (1969) indicated that overcrowding per se did not account for the high incidence of early embryonic death in swine. These authors postulated that early embryos might be competing for

some unknown uterine secretory substance(s) critical to development and survival. However, more recent studies have indicated that there may exist a placental difference in survival capacity between early embryo littermates in the pig. Utilizing embryo transfer techniques, Wilmut et al. (1985) reported that when recovered embryos were divided into groups according to their stage of develolpment and then returned to ligated regions of the uterus, least developed embryos were more likely to die by day 13 to 15 than the most advanced groups in the same uterus. Pope et al. (1982c) surgically recovered embryos from different coloured breeds of gilts on day 5 or 7 of pregnancy. Recipient gilts then received either all day 5 embryos of the same breed, all day 7 embryos of the same breed or half day 5 embryos from one coloured breed and half day 7 embryos from a different coloured breed on day 6 of their cycle. The results indicated no difference in survival capacity between either day 5 or day 7 embryos when transferred alone to day 6 recipients but when day 5 and day 7 embryos were cohabitting the same uterus, significantly more day 7 embryos survived (as determined by hair colour) to day 60 of gestation, there was no apparent difference in survival rate between cohabitting day 5 and day 7 embryos to day 13 of gestation . The conclusion from this experiment was that morphologically more developed embryos had acquired a survival advantage over their more immature littermates. Considering the extreme variation that exists in the morphological stage of development between littermates at least to day 12 of pregnancy under normal circumstances, Anderson (1978), it is possible that natural biological selection against immature blastocyst forms may exist in the pig. Pope et al. (1982c) hypothesized from their unique experiments that the older embryos had more synthetic activity

and thereby altered the biochemical environment within the uterus to such an extent that the less developed embryos could no longer survive. In an attempt to further demonstrate the survival advantage of morphologically advanced embryos, additional embryos were transferred into already pregnant recipients, a procedure known as superinduction. In this experiment, day 6 and 7 pregnant sows received day 7 and 6 embryos respectively. This protocol should result in proportionately more embryos being recovered from the day 6 recipients if day 7 embryos have a survival advantage. Indeed, this was the case with significantly more day 6 recipients being superinduced (i.e. contained more embryos than corpora lutea) at day 30 of pregnancy (Pope and First, 1985). Thus the survival advantage of morphologically advanced embryos is apparently real and subsequent studies have begun to explore explanations for this phenomenom.

The early relationship that exists between the blastocyst and uterine environment is very fragile. This is clearly demonstrated by the necessity for a fine degree of synchrony between donor and recipient animals when transferring embryos. This has been demonstrated in many species including the rabbit (Adams, 1973), sheep (Rowson and Moor, 1966), cow (Rowson et al., 1969), and pig (Webel et al., 1970).

The most dynamic changes in the intra-uterine environment with respect to its biochemical make-up occur at the time of maternal recognition of pregnancy. This environmental change is induced by oestrogen synthesized and secreted by the sufficiently matured blastocysts on about day 11-12 of pregnancy (Geisert et al., 1982a). Exogenous administration of oestradiol valerate between days 11 and 15 of the cycle will similarly induce intra-uterine environmental changes

and result in maintenance of the CL (Gardner et al., 1963, Frank et al., 1978).

Utilizing this latter approach, Pope and First (1985) reported that if pregnant gilts were administered 2.0 mg of oestradiol valerate on day 9 and 10 of pregnancy, the gilts failed to remain pregnant at day 30. However, if the injections of oestrogen were initiated on day 12 and 13, all gilts remained pregnant at day 30. Therefore alteration of the uterine environment before blastocysts are sufficiently mature to secrete their own oestrogen proved to be embryocidal. However, administration of exogenous oestrogen after the onset of endogenous secretion of blastocyst oestrogen had no effect on embryo survival.

The overall conclusion that can be drawn from these recent experiments is that early embryonic death in the pig maybe due to an inherent variation between blastocysts in their survival capabilities particularly around the time of maternal recognition of pregnancy. It is during this time period that several critical events occur in both the blastocyst and the uterus.

Based on the experimental findings of these more recent studies and knowledge of the porcine blastocyst-endometrium interrelationships, two experiments were designed in an attempt to define more precisely when and to provide some insight as to why 30 percent of the fertilized ova in the pig fail to survive the first 2 to 3 weeks of pregnancy. The experimental designs, results, and conclusions from these two studies are presented in the following two chapters.

CHAPTER III

DEVELOPMENT OF PORCINE BLASTOCYSTS IN A UTERINE ENVIRONMENT ADVANCED BY EXOGENOUS OESTROGEN

Introduction

Recent studies have suggested that during early pregnancy in the pig, morphologically advanced blastocysts have a greater survival potential than their less developed littermates (Pope, Maurer, & Stormshak, 1982c, Pope & First, 1985, Wilmut, Sales & Ashworth, 1985). Considering the wide variation which can occur between embryos within a litter regarding the degree of morphological development on Day 12 of pregnancy, as indicated by embryos ranging from 2 to 10 mm in diameter (Anderson, 1978, Geisert, Brookbank, Roberts & Bazer, 1982b) a potential survival advantage of more advanced embryos is not surprising. Pope et al. (1982c) hypothesized that the biochemical synthetic activity of more developed embryos stimulated a marked alteration in the biochemical make-up of the uterine luminal environment. It was in turn suggested that an altered uterine environment was embryocidal to those embryos not capable of adapting to the change in uterine secretion. Porcine blastocysts increase their biochemical and physiological activity after attaining a spherical diameter of approximately 10 mm on Day 11.5 of pregnancy. This is evidenced by the onset of oestrogen synthesis (Heap, Perry, Gadsby, & Burton, 1975, Gadsby, Heap, & Burton, 1980, Fischer, Bazer,

& Fields, 1985) polypeptide synthesis (Godkin, Bazer, Lewis, Geisert & Roberts, 1982), and concurrent rapid elongation of the blastocyst from a spherical to filamentous form (Geisert et al., 1982b). Geisert, Renegar, Thatcher, Roberts & Bazer (1982a) demonstrated that the onset of blastocyst oestrogen synthesis was followed by a significant increase in intraluminal calcium content and a synchronized release of secretory vesicles from uterine glandular epithelial cells resulting in radical changes in the biochemical milieu within the uterine lumen.

Hypothesis that alteration of uterine secretion by morphologically mature blastocysts might create an unfavorable environment for less developed littermates may explain a significant portion of the high (20-40 percent) embryonic loss within the first 2-3 weeks of pregnancy in the pig (for review see Pope & First, 1985).

The importance of synchrony between blastocyst development and uterine environment has been demonstrated through the application of embryo transfer techniques in several species including the sheep (Rowson & Moor, 1966), cow (Rowson, Moor & Lawson, 1969) and pig (Polge, 1982). In these species, the most successful transfer of embryos occurs when synchrony of donor and recipient is within 24 h. The fact that porcine blastocysts can range from 2 to 10 mm spherical structures to filamentous forms measuring over 15 cm within the same uterine horn on Day 12 of pregnancy (Anderson, 1978), indicates that under normal circumstances there can be, based on blastocyst growth rates determined by Geisert et al.(1982b), greater that a 24 h difference in blastocyst development.

Geisert, Thatcher, Roberts & Bazer (1982c) indicated that oestrogen administration to gilts on Day 11 of the oestrous cycle

stimulated a uterine secretory response similar to that initiated by blastocyst oestrogen synthesis. Therefore, a study was designed to determine if porcine blastocysts would develop in a environment in which endometrial secretion had been prematurely advanced from its normal synchrony with blastocyst elongation.

Materials and Methods

Animals. Sexually mature crossbred gilts of similar age (7-8 months), weight (100 to 130 kg) and having exhibited at least two oestrous cycles of normal duration (18 to 22 days) were utilized in all experiments. Gilts were observed for oestrus twice daily (0800 and 1800 h) in the presence of intact boars with day of onset of oestrus designated Day 0. Gilts assigned to be bred were mated to fertile boars at the start of oestrus, and 12 and 24 hours from the initiation of oestrus.

Experimental design. Embryos were surgically recovered from donor gilts (n=12) on Day 5 and transferred to either a Day 5 synchronous group or one of two Day 6 asynchronous groups (n=4 per group). In the latter two groups the uterine environment was 24 hours in advance of embryonic development. On Day 11 gilts in the synchronous group and one asynchronous group received a 5 mg intramuscular injection of oestradiol valerate (EV) while the other served as a vehicle (sesame oil) treated control.

On Day 14, all recipient gilts were ovario-hysterectomized via mid-ventral laparotomy following induction of anaesthesia with thiopentol sodium (1.0 g, IV) and maintenance on a closed circuit system of halothane (2-5 percent) and oxygen (1.0 L/min.). Embryos were recovered on Day 14 of pregnancy to allow sufficient time for

transferred blastocysts to have undergone the elongation process. Following removal of the uterus, each horn was flushed with 20 ml sterile 0.9 percent saline and the flushing examined for the presence of embryos. Embryos were visually evaluated for size, form, and intactness. The saline flushing volume recovered was centrifuged (12,000 g) for 20 minutes at 4° C. The supernatant was stored at -20° C until analyses could be performed.

Embryo transfer procedures were performed following induction of anaesthesia as described previously. The reproductive tract of the donor was exposed via mid-ventral laparotomy. After isolation of the oviduct, a small puncture was made into the lumen of the isthmus with a cutting needle 1.0 cm distal from the utero-tubal junction. A modified polyvinyl catheter with two side ports cut 1.5 cm from the tip was inserted into the oviduct and gently threaded 3 to 4 cm into the uterine horn. The uterine horn was clamped between finger and thumb about 20 cm from the uterine body while 20 ml of recovery media (modified Dulbecco's PBS containing 10 precent (v/v) fetal calf serum) was infused into the horn. The fluid was gently massaged down the horn and then back through the catheter into a sterile dish. The dish was immediately examined under a binocular microscope and embryos identified and classified (degenerated/intact and morula/blastocyst) before transfer to short term culture media (modified Dulbecco's PBS containing one percent (v/v) fetal calf serum) in a second dish. Embryos were washed three times in the second media at room temperature prior to transfer. Recipient gilts were prepared for surgery in the same manner as above. Only the tip of one uterine horn was exposed and a puncture incision made in a similar manner to the recovery procedure. An average of 10 (range 7-13) transferable

embryos were drawn into the tip of a sterile siliconized fire polished pasteur pipette attached to a sterile glass syringe in a volume of less than 0.5 ml of media. The tip was gently passed through the puncture wound and threaded into the uterine horn for at least 4.0 cm whereupon the embryos were deposited. The laparotomy incision was closed in routine fashion. All gilts undergoing surgical procedures were injected with procaine penicillin G im (20,000 IU/kg).

Uterine calcium analysis. Calcium (Ca) concentrations in uterine flushings were measured with a Calcette calcium analyser (Precision Systems, Inc., Sudbury, MA). The system utilized ethylene glycol tetraacetic acid for fluorometric titration of calcium in aqueous solutions (Alexander, 1971). The sensitivity of the analysis was 10^{-4} ug/ml.

Uterine protein and acid phosphatase activity. Protein concentration in uterine flushings was determined by the method of Lowry, Rosebrough, Farr & Randall (1951). Acid phosphatase (AP) activity was determined, using p-nitrophenylphosphate as the substrate as previously described by Basha, Bazer & Roberts (1979). One unit of activity was defined as the capacity to release one mole p-nitrophenol per minute at pH 4.9 in 0.1 M acetate buffer.

Two dimensional polyacrylamide gel electrophoresis (2D-PAGE). Qualitative changes in protein collected in the uterine flushings were analysed by 2D-PAGE. A volume of uterine flushing containing 2 mg of protein was dialysed (molecular weight cutoff 3500) against several volumes of 1 mM Tris-HCl buffer (pH 8.2). After lyophilisation, the dialysed uterine protein was prepared for 2D-PAGE by directly dissolving in 1 ml of 5 mM K_2CO_3 containing 9.4 M urea, two percent (v/v) nonident P-40 and 0.5% (w/v) dithiothreitol. The

2D-PAGE was performed (300 ug protein loaded) for acidic and basic proteins according to the method described by Basha et al. (1979).

Prostaglandin F Analysis. Concentration of prostaglandin F (PGF) in uterine flushings was measured by a double-antibody radioimmunoassay using the procedures previously described by Geisert, Rasby, Minton, & Wettemann (1986). The $PGF_{2\alpha}$ antisera (gift from K.T. Kirton, Upjohn Company) cross-reacted <.1 percent with PGA, PGA_2, PGB, PGB_2, PGE_2, PGE_3, <1 percent for 15-keto $PGF_{2\alpha}$ and 8 percent for $PGF_{1\alpha}$. Therefore, data are expressed as immunoreactive PGF. All samples were measured in a single assay which had an intra-assay coefficient of variation of 2.9 percent.

Oestradiol-17β Analysis. Concentrations of oestradiol-17β (E_2) in uterine flushings were determined by radioimmunoassay using procedures previously described by Hoagland & Wettemann (1984). Intra- and inter-assay coefficients of variation were 24.9% and 18.0% respectively.

Plasminogen Inhibitor Analysis. Plasminogen inhibitor (PI) activity in uterine flushings was determined by using ^{125}I fibrin plate assay previously described by Fazleabas, Geisert, Bazer & Roberts (1983). A range of uterine protein concentrations was added at 4° C to previously prepared ^{125}I - fibrin coated microtitre plates. A constant amount of activated plasminogen (4 ug) was added and the plates incubated at 37° C for three hours. Inhibition was determined by comparing the release of ^{125}I with control values of activated plasminogen run in the absence of inhibitor. One inhibitory unit (IU) was defined as the ability to inhibit one relative unit (RU) of enzyme activity.

Statistical Analysis. Data were analysed by least squares

analysis variance using the General Linear Models procedures of the Statistical Analaysis System (Barr, Goodnight, Sall, Blair & Chilko, 1979). Orthogonal contrasts of means were made as follows Asynchronous control vs synchronous EV, asynchronous EV vs asynchronous control and synchronous EV.

Results

Embryo survival is not affected when donor embryos are transferred 24 hours out of synchrony with the recipient's oestrous cycle (Polge, 1982). Therefore, the asynchronous vehicle treated group was expected to exhibit normal embryo development while the asynchyronous EV treated gilts would have uterine endometrial secretion stimulated in advance of the expected time of blastocyst elongation. The treatment of synchronous transferred gilts with EV on Day 11 served the purpose of showing that exogenous oestrogen treatment itself at the time of normal blastocyst elongation and oestrogen release had no detrimental effect on the development and survival of embryos.

The proportion of gilts which contained filamentous blastocysts after flushing the uterine horns on Day 14 is presented in table 1. Embryos from asynchronous recipient gilts treated with EV on Day 11 failed to survive to Day 14 (except one) while filamentous blastocysts were present in all asynchronous recipient control gilts and synchronous recipient gilts treated with EV on Day 11. One spherical blastocyst flushed from an asynchronous EV treated gilt measured 20 mm in diameter which was classified as abnormal since pig blastocysts rapidly transform into tubular and filamentous morphology after reaching 10 mm in diameter (Geisert et al., 1982b).

Least square means for total recoverable Ca, AP and specific activity of AP in uterine flushings are summarized in table 2. While total protein in uterine flushings was similar between treatment groups, oestrogen administration on Day 11 lowered total calcium ($P<.02$) and stimulated an increase ($P<.10$) in total acid phosphatase activity in uterine flushings. Oestrogen treatment tended to increase specific acid phosphatase activity in uterine flushings to almost twice that measured in gilts treated with vehicle on Day 11.

Total recoverable E_2, PGF and PI content in uterine flushings are summarized in table 3. Oestradiol content in uterine flushings was significantly ($P<.10$) higher in asynchronous vehicle and synchronous oestradiol recipient gilts both of which contained intact filamentous blastocysts. In contrast, the asynchronous oestradiol treated recipient gilts in which embryos did not survive had low levels of recoverable oestradiol.

Although no significant differences were found between the three treatment groups with respect to recoverable PGF, the asynchronous oestradiol treated gilts tended to have lower PGF levels with the exception of one gilt from which an elevated concentration of PGF was recovered.

Plasminogen inhibitor levels were greater ($P<.10$) in the asynchronous oestradiol treated gilts compared to the synchronous oestradiol and asynchronous vehicle treated recipients.

Two dimensional electrophoresis of acidic and basic polypeptides in uterine flushings from different treatments showed similar qualitative polypeptide profiles. The polypeptides present were similar to those described by Basha, Bazer, Geisert & Roberts (1980b). A representative profile of the acidic and basic polypeptides is

presented in figures 1 and 2 respectively.

Discussion

Pope et al. (1982c) demonstrated morphologically advanced blastocysts had a better chance for survival than their less developed counterparts when transferred to the same uterine environment. These investigators hypothesized that larger embryos had more synthetic activity and hence advanced the biochemical environment within the uterus to such an extent that it became unfavorable to less developed embryos.

Geisert et al. (1982a) demonstrated that uterine glandular epithelial cells accumulate secretory vesicles until about Day 11 or 12 of pregnancy and that the contents of these vesicles are released during the period of blastocyst elongation and oestrogen production. In pregnant pigs, these contents are released in a "synchronized" pattern while the process is protracted in cyclic gilts. A similar synchronized release of uterine histotrophe can be induced by administering exogenous oestrogen to cyclic gilts on Day 11 (Geisert et al., 1982c). Administration of oestrogen to cyclic gilts stimulates an increase in uterine luminal calcium, protein, PGF, and PGE. A more recent study has indicated that endometrial sensitivity to oestrogen does not occur until after Day 10 of the oestrous cycle (Geisert, Zavy, Wettemann, & Biggers, 1984).

The results from the present study demonstrate that oestradiol treatment on Day 11 advances uterine secretion with respect to calcium and acid phosphatase activity and that advancing uterine secretion in the presence of embryos that are 24 hr behind in development (relative to their uterine environment) is embryocidal. Neither the degree of

asynchrony between donor and recipient gilts nor the oestradiol treatment itself caused embryonic loss as evidenced by recovery of viable filamentous blastocysts from asynchronous control and synchronous oestradiol treated gilts.

The viability of the blastocysts in the asynchronous control and synchronous oestradiol recipients was indicated by the presence of elongated blastocysts and the increase in recoverable oestradiol-17β in both groups compared to asynchronous oestradiol treated gilts, indicating that blastocysts were synthetically active (Gadsby et al., 1980, Fischer et al., 1985). Higher recoverable oestradiol-17β from the asynchronous control gilts in contrast to the synchronous oestradiol treated gilts, whose blastocysts were 24 hr older than those in the asynchronous control group, may be explained by the biphasic pattern of oestrogen synthesis by porcine blastocysts during early pregnancy (Zavy, Bazer, Thatcher, & Wilcox, 1980, Geisert et al., 1982a). The onset of blastocyst oestrogen synthesis results in an initial increase in recoverable oestrogen in the uterine flushings on Day 12 followed by a decline to Day 15 followed by a secondary increase. Thus, the recovery of less oestrogen from synchronous oestradiol treated gilts on Day 14 may simply reflect the normal declining synthesis in these older blastocysts after initial elongation.

Prostaglandins F and E_2 increase in the uterine lumen of pregnant gilts on Day 12 coinciding with the period of rapid elongation and oestrogen production (Geisert et al., 1982a). In contrast, administration of exogenous oestrogen to cyclic gilts on Day 11 does not stimulate an increase in luminal PGF until approximately Day 15 (Frank, Bazer, Thatcher & Wilcox, 1978). Thus, although

recoverable PGF content in both asynchronous control and synchronous oestradiol treated gilts containing viable blastocysts were not significantly greater than in asynchronous oestradiol treated gilts, this may have been due to the recovery of an unexplained elevated level of PGF from one gilt in the asynchronous oestradiol treated group. Nevertheless, it appeared that synthesis of PGF by blastocysts in the asynchronous control and synchronous oestradiol treated groups was occurring normally.

Plasminogen inhibitors (PI) which are synthesized and released from the uterine luminal surface epithelium (Fazleabas, Bazer, Hansen, Geisert, & Roberts, 1985) are thought to protect the uterine epithelial surface from proteases released by the conceptus during elongation and attachment, in particular, the protease plasminogen activator (Fazleabas, Geisert, Bazer & Roberts, 1983). Both asynchronous control and synchronous oestradiol treated recipient gilts had lower recoverable levels of PI in the uterine flushings perhaps because of the loss of PI through its coating of blastocysts in these groups in contrast to the absence of embryos in the asynchronous oestradiol treated gilts. Fazleabas et al. (1983) indicated that several washings are required to remove PI from blastocysts in culture.

It is apparent that early embryonic death may occur in the pig when blastocysts are presented a uterine environment which has been advanced with oestrogen 24 hr before they can undergo elongation. If blastocysts are left to develop at their own rate and allowed to stimulate the uterine environmental change at the onset of elongation, blastocyst development continues normally as demonstrated in the asynchronous control group. This would imply, that it is the

blastocysts which regulate the time when appropriate changes occur in the uterine environment relative to their morphological development with the uterus serving a permissive role.

The failure of blastocysts to survive in asynchronous oestradiol treated gilts may have been through loss of their ability to undergo elongation as evidenced by failure to recover blastocyst tissues from the uterine flushings. The process of elongation is not well understood but appears to involve cellular remodelling and not hyperplasia (Perry, 1981, Geisert et al., 1982b). Geisert, Rasby, Minton & Wettemann(1986) recently demonstrated that inhibition of blastocyst prostaglandin synthesis did not prevent blastocysts from elongating and suggested the release of prostaglandin may represent a secondary response resulting from cellular remodelling in the blastocyst.

Geisert et al. (1982a) indicated that total recoverable calcium increased in uterine flushings of pregnant gilts containing tubular and early filamentous blastocysts on Day 12. The endometrial release of calcium into the uterine lumen becomes insensitive to additional stimulation with oestradiol following the initial elevation and decline of calcium on Day 12 (Geisert et al. 1982c). Although the role of calcium in the elongation process of the blastocyst is unclear, this ion is known to play a key role in several physiological responses including the release of secretory vesicles and activation of phospholipase A_2 (Rubin & Laychock, 1978, Rasmussen & Barrett, 1984). Also, involvement of calcium in regulating intracellular alterations in the microfilaments and tubules of blastocyst during elongation is another possibility.

In rats and mice, stage specific proteins have been isolated from

uterine fluids immediately before implantation (Aitken, 1977, Tzartsos & Surani, 1979) when there is, as in the pig, an alteration of the uterine environment. Embryos unable to respond to the altered uterine environment do not implant and are lost. In the rat, uterine proteins were found to bind to the cell membranes of blastocysts (Tzartsos & Surani, 1979) but how these proteins influence the pattern or rate of embryo development is unknown. The role of pig endometrial proteins in regulating blastocyst development is unknown. It is possible that morphologically immature blastocysts lack receptors to recognize changes in regulatory proteins released by the endometrium or exposed on the uterine surface epithelium after oestrogen stimulation and therefore do not undergo developmental changes necessary for survival. The fact that endometrial calcium release becomes insensitive to further oestrogen stimulation after its initial response (Geisert et al., 1982c) would support such an idea.

In conclusion, this study has defined a critical period in porcine blastocyst development in which blastocysts are unable to survive an environment advanced with oestrogen on Day 11, perhaps through a failure to undergo normal elongation. It is not known why elongation may have failed but it is clear that blastocysts must attain a certain degree of morphological maturity before they can respond and survive in the altered uterine environment that is normally induced on Day 11 to 12 of pregnancy. In light of the wide variation in degree of morphological maturity between littermate embryos (Perry & Rowlands, 1962, Anderson, 1978) the failure of immature embryos to survive the change in the intrauterine environment, could account for a significant portion of early embryonic death in the pig.

Table 1. Proportion of gilts having normal elongated blastocysts present on Day 14

Group	Number of filamentous blastocysts
Asynchronous Control	4/4
Asynchronous EV 11	0/4[a]
Synchronous EV 11	4/4

[a] One gilt had a single 20 mm spherical blastocyst present while no blastocysts were found in the remaining three gilts.

Table 2. Means for total calcium, protein, acid phosphatase activity and specific acid phosphatase activity in uterine flushings of recipient gilts treated with oestradiol valerate or vehicle on day 11 of pregnancy

Treatment Group	Calcium (mg)	Protein (mg)	Acid Phosphatase activity (μmPi/min)	Specific activity (μmPi/min/mg prot.)
Asynchronous Control	0.69[a]	201	697[c]	3.60
Asynchronous EV	0.11[b]	217	1256[d]	6.10
Synchronous EV	0.00[b]	261	1464[d]	5.90
Overall S.E M.	± 0.10	± 29	± 295	± 1.8

[a,b] Means in columns not having a common superscript differ (P<.02).
[c,d] Means in columns not having a common superscript differ (P<.10).

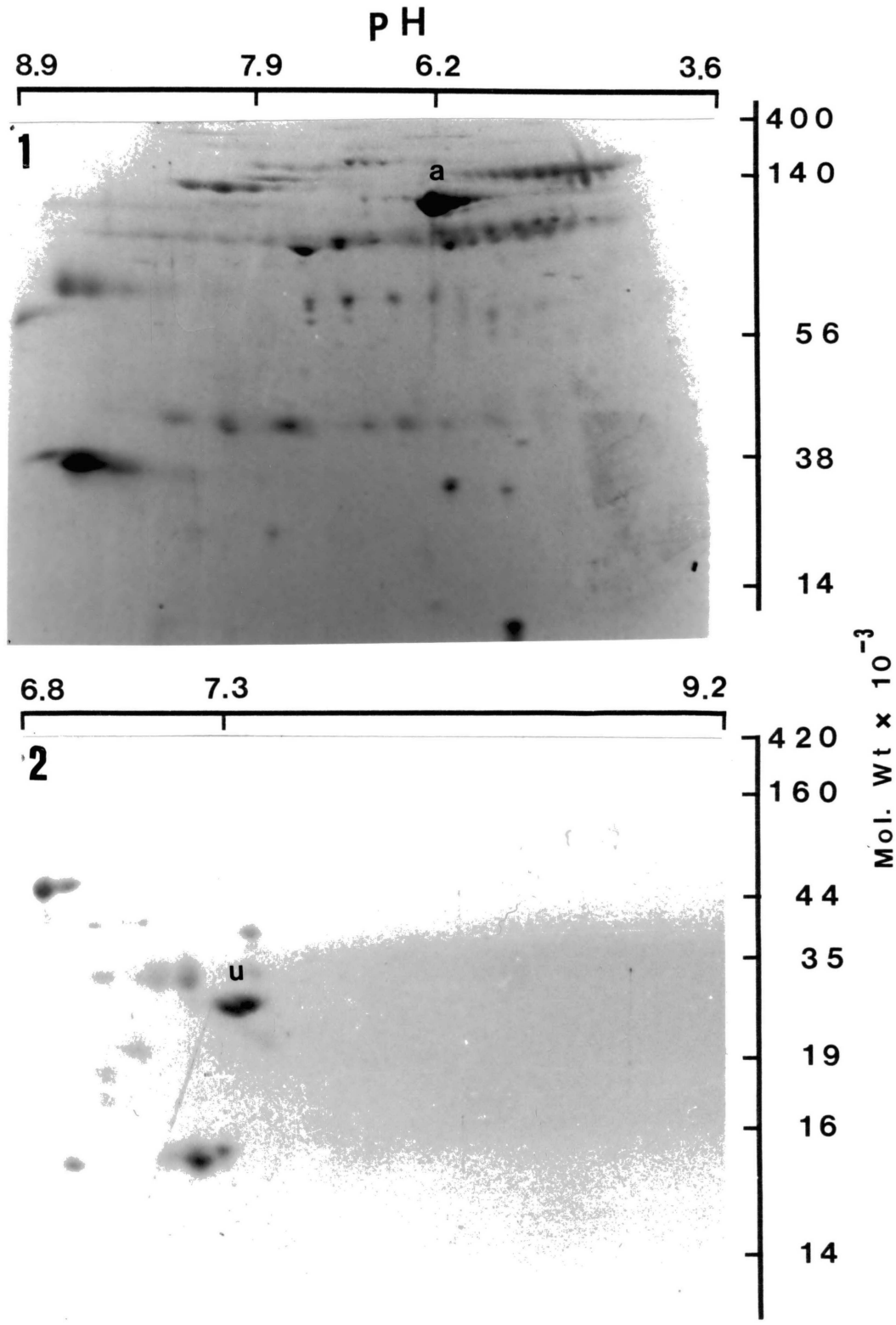
Table 3. Means for total oestradiol, prostaglandin F and plasminogen inhibitor activity in uterine flushings of recipient gilts treated with oestradiol valerate or vehicle on day 11 of pregnancy

Treatment Group	Oestradiol (pg)	Prostaglandin (ng)	Plasminogen Inhibitor (IU)
Asynchronous Control	2093[a,c]	198	56688[d]
Asynchronous EV	280[b]	180	333297[c]
Synchronous EV	899[d]	361	44257[d]
S.E.M	± 423	± 177	± 107131

[a,b] Means in columns not having a common superscript differ (P<.01).
[c,d] Means in columns not having a common superscript differ (P<.10).

Fig. 1. Two dimensional gel electrophoresis of acidic polypeptides in day 14 uterine flushings of an asynchronous gilt treated with oestradiol valerate on day 11. a = albumin

Fig. 2. Two dimensional gel electrophoresis of basic polypeptides in day 14 uterine flushings of an asynchronous gilt treated with oestradiol valerate on day 11. u = uteroferrin.


pH
8.9
7.9
6.2
3.6
1
a
400
140
56
38
14
6.8
7.3
9.2
2
u
420
160
44
35
19
16
14
Mol. Wt x 10^{-3}

CHAPTER IV

DEVELOPMENT AND SURVIVAL OF PORCINE BLASTOCYSTS ON DAY 16 OF PREGNANCY FOLLOWING OESTROGEN ADMINISTRATION ON DAY 9 OR DAY 9 AND 10

Introduction

Prenatal death may result from abnormal embryonic development, inappropriate intrauterine environment or failure of the embryo to properly signal its presence (Wilmut, Sales & Ashworth, 1986). Establishment and maintenance of pregnancy involves intricate communication between the developing conceptus and dam, particularly with respect to ovarian and uterine responses (Heap, Flint & Gadsby, 1979, Bazer, Geisert, Thatcher & Roberts, 1982).

At a biochemical level, interactions between the preimplantation blastocyst and endometrium are likely to be very complex, the purpose of which is to establish a dialogue to ensure that intrauterine conditions are appropriate for implantation and embryonic growth. To successfully support these events it is mandatory to have sufficient nutritional support, suppression of the maternal immune response to prevent rejection, properly timed embryonic migration, spacing, and attachment as well as continued maintenance of luteal function (Flint, 1981, Bazer & Roberts, 1983).

Utilizing embryo transfer, several studies have clearly demonstrated the necessity for close synchrony between stage of

embryonic development and uterine environment (Moore & Shelton 1964, Wilmut & Sales, 1981, Polge, 1982). On Day 12 of pregnancy, porcine blastocysts begin to synthesize and secrete large amounts of oestrogen (Perry, Heap, & Amoroso, 1973, Perry, Heap, Burton, & Gadsby, 1976, Geisert, Renegar, Thatcher, Roberts, & Bazer, 1982a) coincidental to the onset of rapid elongation resulting in a marked alteration of the biochemical make-up of the intrauterine environment. Pope & First (1985), reported that gilts injected with oestradiol valerate on Day 9 and 10 of pregnancy failed to remain pregnant at Day 30. However, if oestrogen was administered on Day 12 and 13 of pregnancy, i.e. presumably after blastocysts have elongated and initiated their own synthesis and secretion of oestrogen, pregnancy was maintained. This finding indicated that oestrogen stimulation of the endometrium and/or blastocysts prior to a critical period occuring during normal development, resulted in pregnancy failure.

Porcine blastocysts begin to secrete oestrogen and elongate soon after attaining a spherical diameter of 10 mm on approximately Day 11.5 of gestation (Gadsby, Heap & Burton, 1980, Geisert et al. 1982a). The uterine secretory response induced by blastocyst oestrogen release can also be mimicked by exogenous oestrogen administration (Geisert, Thatcher, Roberts & Bazer, 1982c). Therefore the uterine secretory response normally induced by blastocyst oestrogen release could possibly be advanced by administering exogenous oestrogen on Day 9 or Day 9 and 10 (Geisert, Biggers, Wettemann & Zavy 1984, Geisert, Zavy, Wettemann & Biggers, submitted for publication).

The following study was designed to establish the period in which early embryonic mortality occurs after gilts are administered exogenous oestrogen before Day 11 of pregnancy, and to determine

possible causes of early mortality when blastocysts are presented a uterine environment which is advanced relative to their normal development.

Materials and Methods

Animals. Sexually mature crossbred gilts of similar age (7 to 8 months), weight (100 to 130 kg) and having exhibited at least two oestrous cycles of normal duration (18 to 22 days) were utilized in both experiments. Gilts were observed for oestrous behaviour twice daily (0800 to 1800 h) in the presence of intact boars with the initial day of oestrus designated Day 0. Gilts were mated to fertile boars at the onset of oestrus and 12 hours and 24 hours from the initiation of oestrus.

Experiment 1 Twelve gilts were utilized to determine the effect of administering exogenous oestradiol valerate, prior to the period of rapid blastocyst elongation on Day (D) 11 and 12. Gilts were randomly assigned (n=4) to receive one of the following treatments control, intramuscular injection (4 ml) of vehicle (Sesame oil) on D 9 and 10 of pregnancy, EV 9, single intramuscular injection of 5 mg oestradiol valerate (R.J. Legere & Co., Scottsdale, AZ, USA) on D 9 of pregnancy, EV 9 and 10, intramuscular injection of 5 mg oestradiol valerate on D 9 and 10 of pregnancy.

On D 11 of pregnancy, gilts were hemi-hysterectomized following induction of anaesthesia with a 5 percent solution of thiopental sodium (Abbott laboratories, Chicago, IL, USA) and maintenance on a closed circuit system of halothane (2-5 percent, Aveco Co., Inc., Fort Dodge, IA, USA) and oxygen (1.0L/min).

Following exposure by midline laparotomy, one uterine horn and

the ipsilateral ovary were removed and placed on ice. The incision site was then closed in a routine manner. The uterine horn was trimmed free of mesometrium and flushed with 20 ml of 0.9 percent sterile saline to recover the uterine luminal contents. Uterine flushings were immediately examined for blastocysts whose number and size were recorded. Each flushing was centrifuged at 12,000 g for 20 minutes at 4° C and supernatant obtained was stored at -20° C until analysed for calcium, protein (quantitative and qualatative analysis), acid phosphatase activity, prostaglandin F, oestradiol 17B, and plasminogen inhibitor content.

Based on the size of blastocysts recovered from the first horn on D 11, an estimation was made as to when blastocyst elongation would occur in the remaining horn (Geisert, Brookbank, Roberts, & Bazer, 1982b). The second uterine horn was then removed, and flushed in the same manner as previously described.

Experiment 2. Twelve gilts were randomly assigned (n=4) to the three treatment groups described in Experiment 1. Total hysterectomy was performed on Day 16 with each uterine horn trimmed of mesometrium and flushed as indicated in Experiment 1. Recovered blastocyst, if present, were evaluated for intactness (nondegenerating/degenerating). Following centrifugation, uterine flushings were stored until analyzed as in Experiment 1.

Uterine Calcium Analysis. Calcium (Ca) concentrations in uterine flushings were measured with a Calcette calcium analyser (Precision Systems, Inc., Sudbury, MA). The system utilized ethylene glycol tetraacetic acid for fluorometric titration of calcium in aqueous solutions (Alexander, 1971). The sensitivity of the analysis was 10^{-4} ug/ml.

Uterine protein and acid phosphatase activity. Protein concentration in uterine flushings was determined by the method of Lowry, Rosebrough, Farr, & Randall (1951). Acid phosphatase (AP) activity was determined, using p-nitrophenylphosphate as the substrate as previously described by Basha, Bazer, & Roberts (1979). One unit of activity was defined as the ability to release one umole p-nitrophenol per minute at pH 4.0 in 0.1 M acetate buffer.

Two dimensional polyacrylamide gel electrophoresis (2D-PAGE). Qualitative acidic and basic protein changes in the uterine flushings were analysed by 2D-PAGE. A volume of uterine flushing containing 2 mg of protein was dialysed (molecular weight cutoff $\simeq$ 3500) against several volumes of 1 mM Tris-HCl buffer (pH 8.2). After lyophilization, dialysed uterine protein was prepared for 2D-PAGE by directly dissolving in 1 ml of 5 mM K_2CO_3 containing 9.4 M urea, 2% (v/v) Nonidet P-40 and 0.5 percent (w/v) dithiothreitol. The 2D-PAGE was performed (300 ug protein loaded) for acidic and basic proteins according to the method described by Basha et al. (1979).

Prostaglandin F Analysis. Prostaglandin F (PGF) concentrations in uterine flushing was measured by a double-antibody radioimmunoassay using the procedures previously described by Geisert, Rasby, Minton, & Wettemann (1986). The $PGF_{2\alpha}$ antisera (gift from K.R. Kirton, Upjohn Company) cross-reacted <.1 percent with PGA, PGA_2, PGB, PGB_2, PGE_2, PGE_3: <1 percent for 15-Keto $PGF_{2\alpha}$ and 8 percent for $PGF_{1\alpha}$. Therefore, data are expressed as immunoreactive PGF. All samples were measured in a single assay which had an intraassay coefficient of variation of 2.9 percent.

Oestradiol-17β Analysis. Concentrations of oestradiol - 17β (E_2) in uterine flushings were determined by radioimmunoassay using procedures

previously described by Hoagland & Wettemann (1984). Intra-and inter-assay coefficients of variation were 24.9% and 18.0% respectively.

Plasminogen Inhibitor Analysis. Plasminogen inhibitor (PI) activity in uterine flushings was determined by using ^{125}I fibrin plate assay previously described by Fazleabas, Geisert, Bazer, & Roberts (1983). A range of uterine protein concentrations was added at 4° C to prepared ^{125}I fibrin coated microtitre plates. A constant amount of activated plasminogen (4 ug) was added and plates incubated at 37° C for three hours. Inhibition was determined by comparing the release of ^{125}I with control values of activated plasminogen run in the absence of inhibitor. One inhibitory unit (IU) was defined as the ability to inhibit one relative unit (RU) of enzyme activity.

Statistical Analysis. Data were analysed by least squares analyses of variance (Steel & Torrie, 1960). The overall statistical model for analyses of uterine flushings in Experiment 1 included effects of treatment, Day of horn removal, and treatment by Day interaction. Orthogonal contrasts of means were made as follows· Vehicle versus EV 9, EV 9 and 10, EV 9 versus EV 9 and 10. In Experiment 2, orthogonal contrasts of treatment means for the uterine flushing parameters were made as follows Vehicle versus EV 9, EV 9 and 10, EV 9 versus EV 9 and 10.

Results

Experiment 1. The average diameter of spherical blastocysts recovered on Day 11 was 7 mm with a range of 2 to 11 mm. Administration of EV did not affect normal blastocyst development as gilts in each treatment contained normal appearing filamentous blastocysts following

removal of the second horn.

Least square analysis of variance for means for total recoverable calcium, protein, acid phosphatase activity, and specific activity (acid phosphatase activity/mg uterine protein) in uterine flushings from EV and vehicle treated gilts are presented in table 4. A significant treatment by Day interaction was detected for acid phosphatase activity ($P<.08$) while total uterine protein content was affected by treatment ($P<.01$) and Day ($P<.01$). Day effects ($P<.01$) were detected for specific activity. Although nonsignificant, oestradiol valerate treatment on D 9 and D 9 and 10 stimulated an increase in uterine calcium on D 11 followed by a decline on D 12, whereas, in vehicle treated gilts the calcium levels increased four-fold from D 11 to D 12. It is interesting that although the calcium content recovered from two EV 9 and 10 treated gilts on D 11 and D 12, was very low to undetectable, blastocyst elongation appeared to occur normally. Oestradiol valerate treatment increased uterine protein content approximately two-fold ($P<.01$) from D 11 to D 12 while in contrast only a small increase occurred in vehicle treated gilts. Acid phosphatase activity was greater on D 11 and D 12 in the uterine flushings of both oestradiol valerate treated gilts compared with controls.

Least square means for total recoverable oestradiol 17 , prostaglandin F and plasminogen inhibitor are presented in table 5. A significant treatment by day interaction was detected for plasminogen inhibitor ($P<.04$) with increased amounts recovered from uterine flushings in the second horn of EV 9 and 10 treated gilts. This may reflect an increased stimulation of release due to oestradiol treatment or decreased binding of PI to the blastocysts. Total

recoverable oestradiol was significantly affected by Day ($P<.01$) with E_2 content being greater in D 12 uterine flushings (D 11,0.90 ng vs D 12, 4.0 ng) after blastocyst elongation.

Although nonsignificant, recoverable levels of PGF decreased from D 11 to D 12 in both oestrogen treated groups in contrast to an increase in the controls. Whether this is an indication that blastocyst development and biochemical activity is being affected is not known.

Basha, Bazer, Geisert & Roberts (1980) have previously described the acidic and basic polypeptides in porcine uterine flushings following 2D-PAGE analysis. Profiles of acidic polypeptides present in the D12 uterine flushing from a vehicle and EV treated gilt are illustrated in figure 3A and 3B. Two endometrial proteins ($Mr \simeq 18000$, pI = 6.6 - 6.3) appear on D 11 and 12 in EV and control gilts, however intensity of staining was noticeably increased in the EV treated gilts. Two additional acid polypeptides ($Mr \simeq 22000$ and 40000, pI = 5.3) appeared in EV gilts on D 12, but were absent in vehicle treated gilts.

Basic polypeptides from uterine flushings which were detected by 2D-PAGE are presented in figure 4. Uteroferrin ($Mr \simeq 35000$, pI = 9.7) indicated by the letter U and two other basic endometrial proteins ($Mr \simeq 50,000-60,000$, pI 9.7) appear on D 12. Although similar qualitative changes occurred in vehicle and EV gilts between D 11 and 12 the staining on D 12 increased in EV treated gilts compared to vehicle animals. The increase in intensity would be consistent with a greater content of acid phosphatase activity since over 90% of acid phosphatase activity in the uterus is due to uteroferrin. Oestradiol valerate treatment did not appear to markedly affect plasmin

isoinhibitors (figure 4).

Experiment 2. Effect of EV administration on D 9 or D 9 and D 10 on embryo survival at D 16 of pregnancy is presented in table 6. In comparison to the large mass of intact filamentous blastocysts present in uterine flushings of vehicle treated gilts, conceptus tissues in oestrogen treated gilts were fragmented and degenerative. Flushings from these gilts contained excessive amounts of cellular debris with only a few intact filamentous blastocysts present. Least square means for total recoverable calcium, protein, acid phosphatase activity and specific acid phosphatase activity are presented in table 7. Oestradiol valerate treatment did not affect total recoverable calcium or protein present in uterine flushings, but did decrease ($P<.03$) total recoverable acid phosphatase and specific acid phosphatase activity approximately five-fold.

Least square means for total recoverable oestradiol 17β, prostaglandin F and plasminogen inhibitor concentrations are presented in table 8. Oestradiol valerate on D 9 and D 9 and 10 resulted in lower ($P<.01$) amounts of recoverable oestradiol in D 16 uterine flushings while recoverable prostaglandin F and plasminogen inhibitor were unaffected by EV treatment.

Although no quantitative differences were detected for total amount of recoverable protein oestrogen administration stimulated quantitative changes in selected polypeptides present in uterine flushings on D 16 (figure 5 and 6) as determined by staining intensity on 2D-PAGE gels. Oestradiol valerate administration altered the staining intensity of a number of acidic and basic polypeptides. One group of very acidic polypeptides ($Mr \simeq 40000$, $pI = 5.4$) were especially intensified in uterine flushings from EV treated gilts

(figure 5B) whereas the same polypeptides were barely discernible in profiles of control gilts (figure 5A).

The appearance of uteroferrin (Mr ≃ 35000, pI = 9.7), and two other non serum proteins (Mr ≃ 50000-60000, pI = 9.7) located above uteroferrin (figure 6A and 6B) were greatly attenuated in D 16 flushings from EV treated gilts. Decreased staining intensity of uteroferrin is also reflected by the lower acid phosphatase activity and specific acid phosphatase activity from uterine flushings of EV treated gilts (table 7). Although EV administration appeared to decrease the intensity of staining for the latter basic proteins, the complex of low molecular weight (Mr ≃ 14000) plasmin isoinhibitors (Fazleabas, Geisert, Bazer & Roberts, 1983) were unaffected by EV treatment.

Discussion

Experiment 1 demonstrated that oestradiol valerate (EV) administered intramuscularly on either Day 9 or Day 9 and 10 of pregnancy advanced endometrial secretion as indicated by the increased content of protein and uteroferrin (acid phosphatase activity) recovered from flushings on Day 11 compared with the vehicle treated gilts. Although not significant, release of calcium also appeared to be altered as indicated by the lower uterine content during elongation in oestrogen treated gilts, compared with the elevated levels which normally occur during elongation in control animals.

Geisert et al. (1982c) demonstrated that administration of oestrogen on Day 11 of the gilts oestrous cycle would induce endometrial secretion similar to that which occurs during blastocyst oestrogen synthesis and elongation between Day 11 and 12 of pregnancy.

Recent data has indicated that the uterine endometrium is not responsive to oestrogen until after the tenth day of the oestrous cycle (Geisert, Zavy, Wettemann, & Biggers, submitted for publication). However, an earlier release of uterine secretion was achieved on D 11 and 12 by administration of oestrogen on D 9.5 of the oestrous cycle. The alteration of calcium, acid phosphatase and protein in pregnant gilts after EV administration in the present study are consistent with the changes reported in cyclic gilts.

The failure of calcium to increase between D 11 and 12 in EV gilts would question the necessity of this response for blastocyst elongation to occur. Although the factors regulating elongation are unclear, it had been proposed that calcium may be involved, perhaps through activation of phospholipase A_2 and subsequent methylation of membrane phospholipids (Geisert et al. 1986). The fact that elongated blastocysts were recovered from all gilts upon removal of the second horn on Day 12, indicated that the uterine advancement in protein and acid phosphatase secretion also did not interfere with the mechanism(s) regulating blastocyst elongation. However gel electrophoresis did indicate qualitative as well as some selected quantitative changes in the acidic polypeptide profiles of uterine flushings between vehicle and oestrogen treated gilts from D11 to D12. Similar changes in polypeptides were observed in cyclic gilts administered oestrogen on D 9.5 (Geisert, Zavy, Wettemann & Biggers submitted for publication). Therefore, the polypeptides appear to be reflective of uterine changes and not blastocyst degeneration. The role of these polypeptides in blastocyst development are not known, however, they appear to be related to conceptus degeneration on D 16.

It is apparent from the second experiment that advancing the

uterine environment by administering EV on D 9 or D 9 and 10 created a situation which was unfavorable to elongated blastocysts between D 12 to 16. This was evidenced by the recovery of cellular debris and degenerating filamentous fragments from 6 of 8 EV treated pregnant gilts. Geisert et al.(1984) indicated that treatment of gilts with EV on D 9.5 of the oestrous cycle extended the interoestrous interval to approximately 28 days. Thus, loss of progesterone support is not involved with the embryonic mortality observed in this study. Oestradiol has also been shown not to be directly embryocidal, since gilts will maintain pregnancy when oestrogen is administered after D 11 (Pope & First, 1985).

The fact that embryos were degenerated by Day 16 in the present study concurs with Pope & First (1985) who found gilts treated with oestrogen in a similar fashion were not pregnant at Day 30. Besides the appearance of cellular debris, the recovery of significantly lower concentrations of oestradiol 17β on Day 16 from uterine flushings of EV 9 and EV 9 and 10 treated gilts would also indicate that blastocyst degeneration was occurring. Normally, blastocyst oestrogen synthesis has a biphasic pattern of secretion increasing from Day 11 to 12 then declining to Day 15 followed by a second sustained increase from Day 16 to 30 (Robertson & King, 1974, Zavy, Bazer, Thatcher & Wilcox, 1980).

Following elongation, the next crucial step in establishing pregnancy is the attachment of the trophoblastic membrane to the uterine luminal surface epithelium. In the pig, attachment occurs between D 13 and 18 of pregnancy (King, Atkinson & Robertson, 1982). Failure of attachment to occur must be considered as one possible explanation for the demise of embryos when given oestrogen on D 9 and

D 10.

The mechanism(s) involved in actual attachment of blastocysts to the uterine epithelium are not well understood. However, attachment is probably mediated through specific ultrastructural and biochemical changes in the plasma membrane surfaces of the two opposing tissues, the trophoblast and uterine surface epithelium (Schlafke & Enders, 1975). In the pig, which has a superficial placentation (Perry, 1981), studies on the protein and glycoprotein composition of the uterine epithelial cell plasma membrane were reported to be relatively constant throughout the oestrous cycle and early pseudopregnancy suggesting that neither the altered hormonal status nor the obvious increased secretory capacity of the epithelial cells led to major alterations in the physical structure of the epithelial cell surface (Mullins, Horst, Bazer & Roberts, 1980). However, the possible "unmasking" of polypeptides which act as receptors on the plasma membrane for the attachment process cannot be ruled out. Anderson, Olson & Hoffmann (1986) have indicated that stage-specific alterations of protein and saccharide composition occur on the apical surface of the rabbit endometrium at the time of implantation. Other researchers have suggested that alteration of the glycoprotein coatings on both the trophoblast and epithelial cell plasma membrane may be responsible for the very close apposition necessary for interdigitation of microvilli (Enders & Schlafke, 1974, Jenkinson & Searle, 1977)

The close association of blastocyst degeneration with the appearance of an intensified group of acidic (pI = 5.4) polypeptides (Mr 40000) after EV treatment makes their involvement in conceptus development an interesting possibility. Similar polypeptides have been reported in the uterine flushings on D 12 from cyclic gilts

injected with 5mg oestradiol benzoate on Day 9.5 of the cycle (Geisert, Zavy, Wettemann & Biggers, submitted for publication). These researchers indicated that this group of polypeptides did not appear in uterine flushings from cyclic gilts injected with oestradiol on Day 11 or 14 implying that oestrogen treatment before Day 11 alters the quantitative and qualitative secretion of uterine proteins released on Day 11 to 12. The function(s) of these proteins is unknown but they are of uterine and not blastocyst origin. It is possible that these proteins are associated with the uterine epithelial surface and that EV treatment before D 10 causes their release from the surface thus preventing normal blastocyst attachment.

During early pregnancy, in the rat, the uterus attains sensitivity to the deciduogenic stimulus of the blastocyst for a limited period of time. Lejeune, Van Hoeck & Leroy (1981) found that the effect of oestrogen on the pretreated progesterone rat uterine luminal epithelium controlled the capacity of the latter to respond to deciduogenic stimuli and to transmit stimuli from the uterine lumen to the underlying stroma. Changes in the apical membrane of these cells in particular are thought to be important for the transduction of deciduogenic stimuli (Moulton & Koenig, 1986). Although the superficial type of placentation in the pig is markedly different from the invasive implantation in the rat, it was not unreasonable to speculate that ultrastructural and biochemical changes occur in the plasmalemma of the uterine surface epithelium in response to oestrogen for blastocyst attachment. If oestrogen advanced changes in the endometrial cell surface as well as uterine glandular secretion, i.e. uteroferrin, prior to the time of normal blastocyst attachment, the uterine environment may no longer be permissive to blastocyst

attachment at D 14 to 16.

The results from our study have demonstrated that uterine secretion can be advanced by administering exogenous oestradiol on D 9 or D 9 and 10 of pregnancy. There are significant qualitative changes in the recoverable uterine proteins following such treatment but the functional importance of this change remains unknown. Advancing uterine secretion does not appear to prevent blastocyst development through elongation. However, blastocysts become degenerative by D 16 of pregnancy. Early mortality could be due to a failure of blastocysts to undergo normal attachment, perhaps due to loss of polypeptides or receptors from the luminal surface epithelium. If the pig is similar to other species, attachment can only occur during a relatively defined period of uterine sensitivity to blastocyst signals. In the pig, blastocyst oestrogen appears to be the primary signal to the dam for pregnancy recognition and the sequencing of all subsequent events including attachment. Premature exposure of the endometrium to oestrogen may alter this sequencing to such an extent that blastocyst attachment and survival is impossible.

Table 4. Means for total calcium, protein, and acid phosphatase activity in unilateral day 11 and day 12 uterine flushings from pregnant gilts treated with oestradiol valerate or vehicle on day 9 and 10 of pregnancy

Treatment	Day of Unilateral Hysterectomy	Calcium (mg)	Protein+* (mg)	Acid Phosphatase Activity** (µm Pi/min)	Specific Activity* (µm Pi/min/mg Prot.)
Vehicle	11	0.30	25	20	0.82
	12	1.17	36	59	1.84
EV 9	11	1.10	46	104	2.32
	12	0.73	70	274	4.00
EV 9 and 10	11	0.53[a]	39	100	2.10
	12	0.16	81	178	2.31
Overall S.E.M.		± 0.33	± 7	± 26	± 0.44

* Day effect (P<.01)
** Treatment x Day interaction (P<.08)
\+ Treatment effect (P<.01)
a Abnormally low levels were recovered in uterine flushings on D 11 and D 12 from two gilts containing normal elongated blastocysts on D 12.

Table 5. Means for total oestradiol, prostaglandin F and plasminogen inhibitor in unilateral day 11 and day 12 uterine flushings from pregnant gilts treated with oestradiol valerate or vehicle on day 9 and day 9 and 10 of pregnancy

Treatment	Day of Unilateral Hysterectomy	Oestradiol+ (pg)	Prostaglandin F (ng)	Plasminogen Inhibitor* (IU)
Vehicle	11	867	8.03	35876
	12	2780	12.81	14474
EV 9	11	915	15.18	23523
	12	5987	3.77	27079
EV 9 and 10	11	935	0.64	25001
	12	3139	0.49	54844
Overall S.E.M.		± 927	± 6.37	± 7760

* Treatment x Day Interaction ($P<.04$)
\+ Day Effect ($P<.01$)

Table 6. Proportion of gilts with either normal or degenerating embryos on day 16 following oestrogen administration on day 9 or day 9 and 10

Group	Number of gilts with normal embryos	Number of gilts with degenerating embryos
Control	4/4	0/4
EV 9	2/4[a]	2/4[b]
EV 9 and 10	0/4	4/4[b]

[a] Considerable amount of cellular debris was present in the flushing with the filamentous blastocysts.

[b] Cellular debris was present with small fragments of blastocyst membranes which were degenerating.

Table 7. Means for total calcium, protein and acid phosphatase activity in day 16 uterine flushings of pregnant gilts treated with oestradiol valerate or vehicle on day 9 and day 9 and 10 of pregnancy

Treatment	Calcium (mg)	Protein (mg)	Acid Phosphatase Activity (μmPi/min)	Specific Activity (μmPi/min/mg protein)
Vehicle	0.20	172	1877[a]	14.60[a]
EV 9	0.35	170	525[b]	3.40[b]
EV 9 and 10	0.29	160	250[b]	3.40[b]
Overall S.E.M.	± 0.12	± 29	± 265	± 3.60

[a,b] Values with different superscripts are different (P<.03)

Table 8. Means for total oestradiol, prostaglandin F, and plasminogen inhibitor in day 16 uterine flushings of pregnant gilts treated with oestradiol valerate or vehicle on day 9 and 10 of pregnancy

	Oestradiol (pg)	Prostaglandin F (ng)	Plasminogen Inhibitor (IU)
Vehicle	1182[a]	3.56	57823
EV 9	584[b]	4.80	86453
EV 9 and 10	311[b]	2.26	70812
S.E.M.	± 161	± 1.23	± 15683

a,b Values with different superscripts are different ($P<.01$).

Fig. 3. Two dimensional gel electrophoresis of acidic polypeptides in day 12 uterine flushings

A) Vehicle treated gilt

B) Gilt treated with 5 mg oestradiol valerate on day 9 and 10.

Note: The increase in staining intensity of two progesterone induced polypeptides (solid arrowhead) in the flushing of the oestrogen treated gilt. Also, note the appearance of two polypeptides (open arrowheads) which are not present in the vehicle treated gilt flushings. (a = serum albumin)

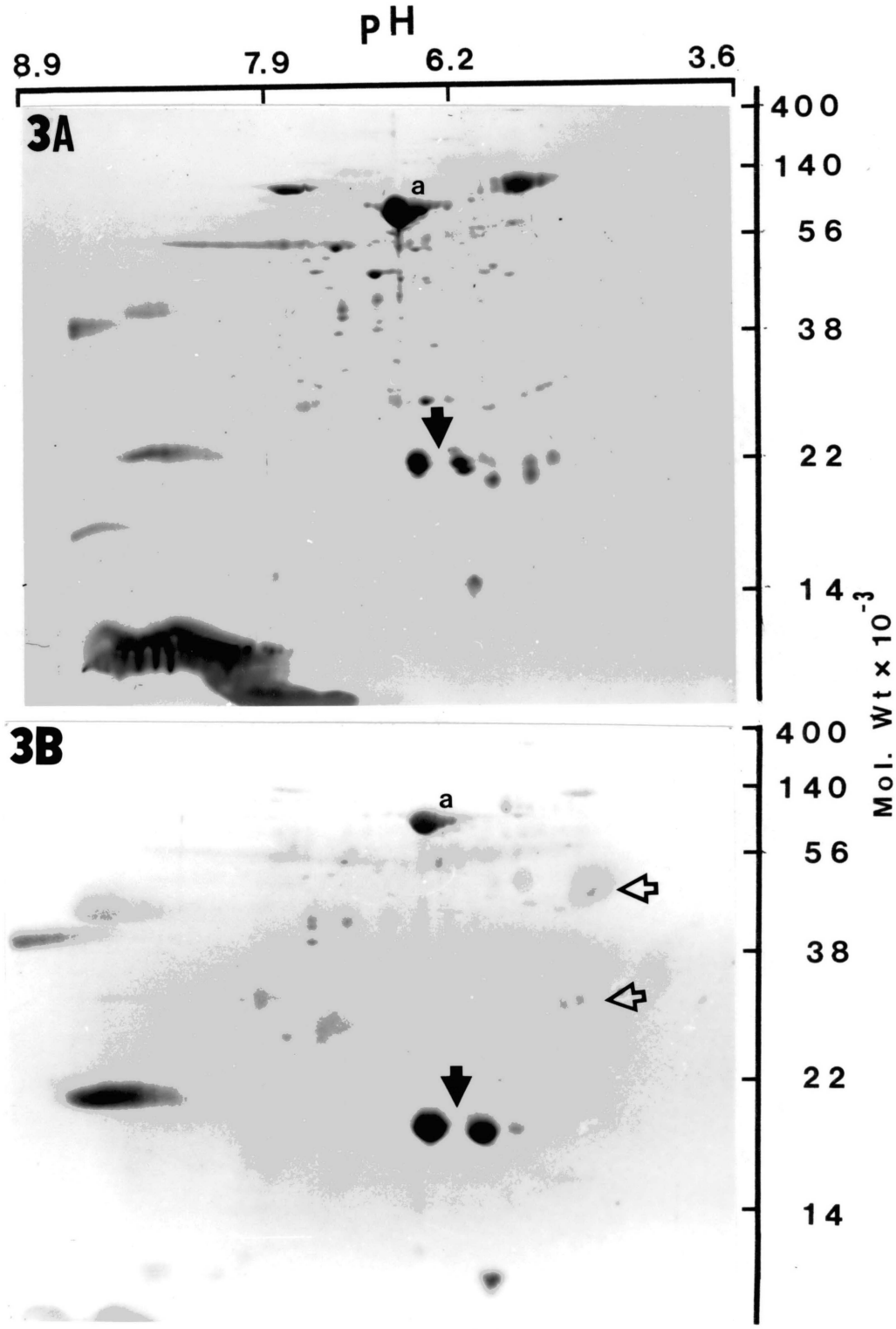

pH
8.9
7.9
6.2
3.6
3A
a
400
140
56
38
22
14
3B
a
400
140
56
38
22
14
Mol. Wt x 10-3

Fig. 4. Two dimensional polyacrylamide gel electrophoresis of basic polypeptides in day 12 uterine flushings

(A) Vehicle treated gilt

(B) Gilt treated with EV on D 9 and 10

(u = uteroferrin, arrow = plasmin isoinhibitors)

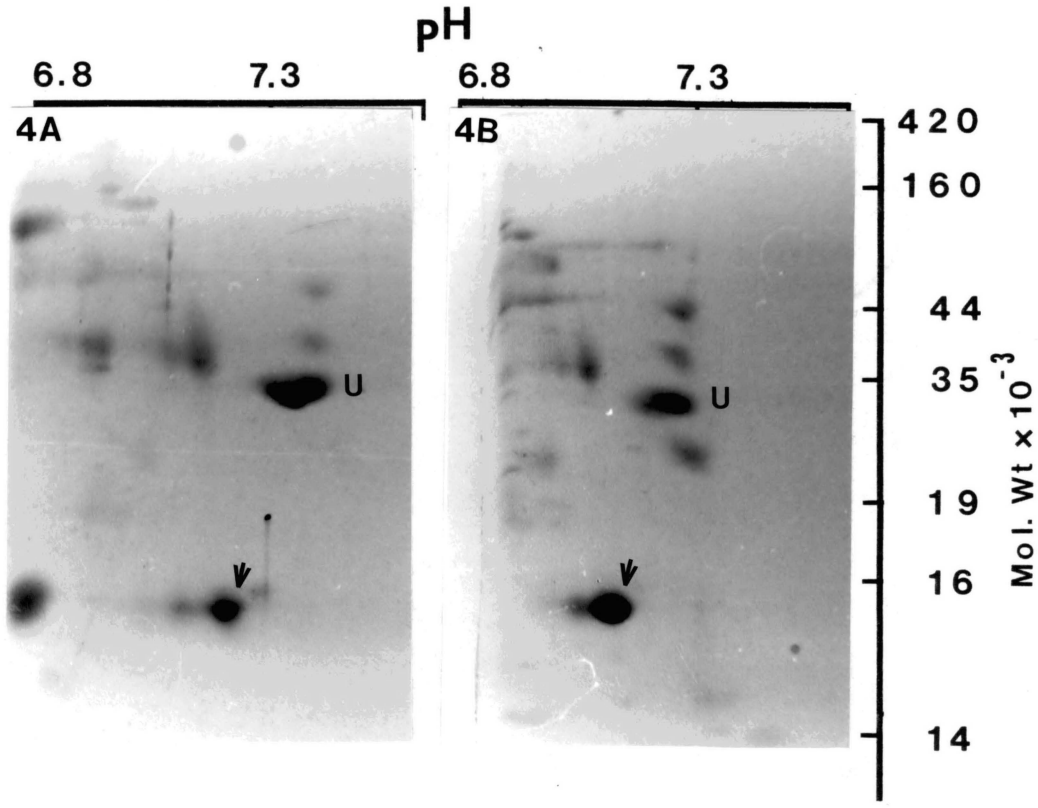

pH
6.8
7.3
4A
U
6.8
7.3
4B
U
420
160
44
35
19
16
14
Mol. Wt x 10^-3

Fig. 5. Two dimensional polyacrylamide gel electrophoresis of acidic proteins in day 16 uterine flushings from

(A) Vehicle treated gilt

(B) Gilt treated with EV on D 9 and 10.

Note the appearance of a group of very acidic polypeptides in the EV treated gilt which are barely detectable in the vehicle treated gilt flushings. (solid arrowhead) (a = serum albumin)

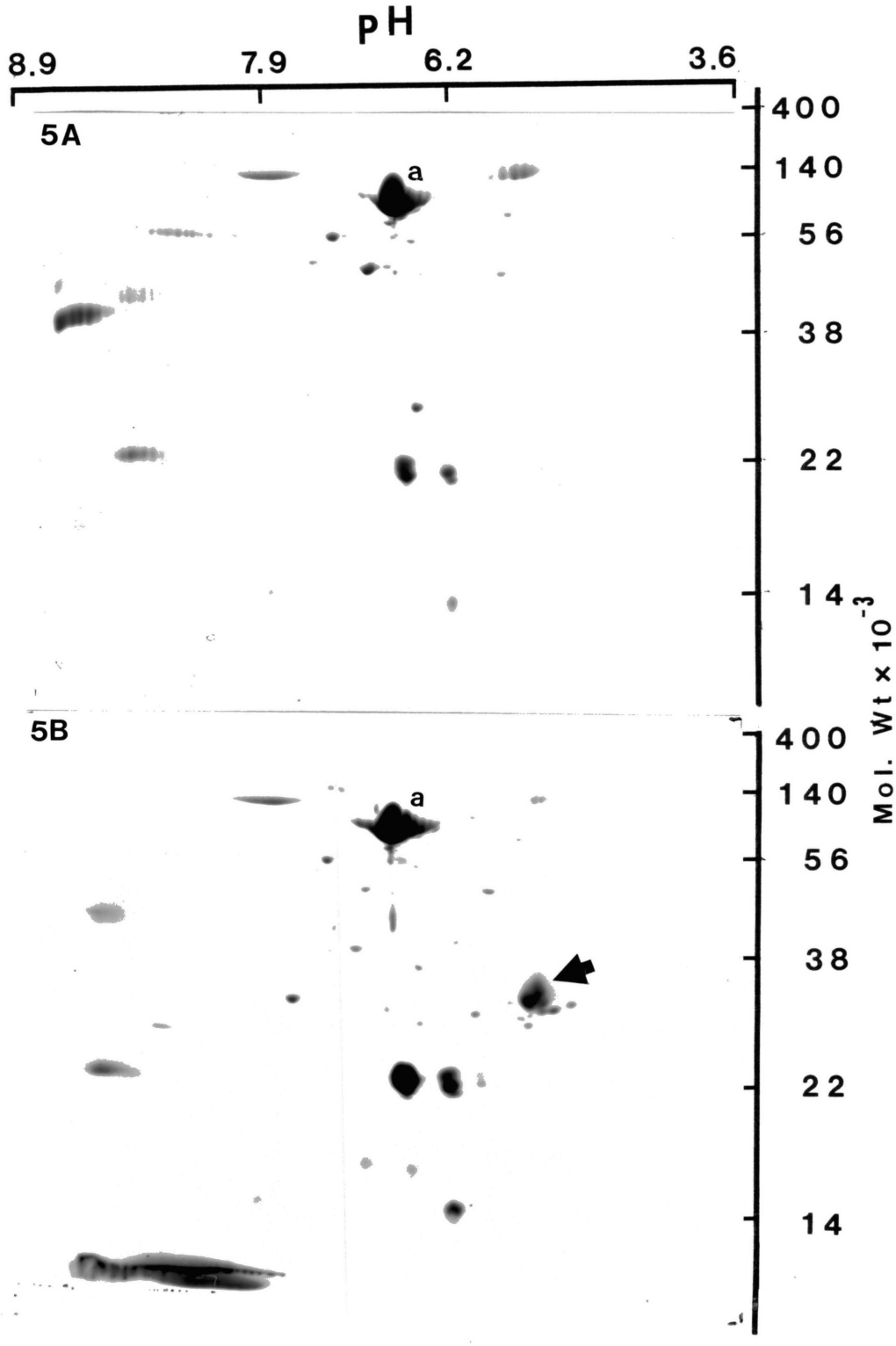
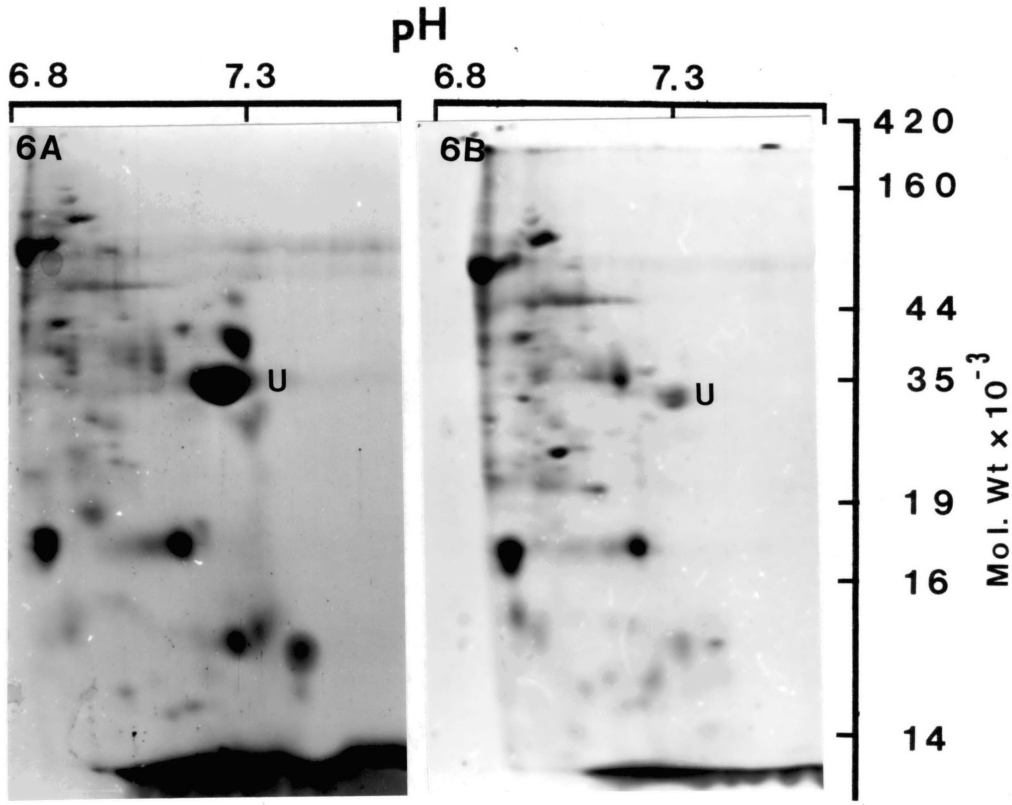

pH
8.9
7.9
6.2
3.6
5A
a
400
140
56
38
22
14
5B
a
400
140
56
38
22
14
Mol. Wt x 10^{-3}

Fig. 6. Two dimensional polyacrylamide gel electrophoresis of basic proteins in day 16 uterine flushings

(A) Vehicle treated gilt

(B) Gilt treated with EV on D 9 and 10. Note the decrease in staining intensity of uteroferrin (u) and the plasmin isoinhibitors (solid arrow) in the EV treated gilt.


pH
6.8
7.3
6.8
7.3
6A
6B
U
U
420
160
44
35
19
16
14
Mol. Wt x 10^{-3}

CHAPTER V

GENERAL DISCUSSION

Polge (1982) demonstrated that in swine a ± 24 h difference between onset of oestrus in the donor and recipient gilts resulted in an acceptable success rate when transferring embryos but as the degree of asynchrony widened, the success rate declined dramatically. This implies a certain degree of synchrony between development of the embryos and their uterine environment.

When the degree of asynchrony between donor and recipient is greater than 24 h it is not known whether the failure to maintain pregnancy is due to premature death of embryos and/or to inability of the embryos to signal the maternal system in adequate time to prevent regression of the corpora lutea.

Results from experiment 1, in which embryo transfer techniques were utilized to establish an asynchronous relationship between blastocyst development and uterine environment confirmed that a greater than 24 h asynchrony between blastocyst development and the uterine secretory response (Geisert et al., 1982a) is embryocidal. In experiment 1, transferring embryos to recipients whose uterine environment was 24 h in advance of the donor's from which the embryos were taken did not adversely affect embryonic development provided blastocysts were allowed to develop until they attained a morphological maturity whereby they could begin synthesis and secretion of oestrogen (Heap et al., 1975, Gadsby et al., 1980, Zavy

et al., 1980).

Geisert et al. (1982a) reported that the onset of oestrogen synthesis and secretion coincided with stage of embryonic development. Oestrogen synthesis is associated with elongation of the blastocyst which begins at approximately the 10 mm spherical stage on Day 11 of pregnancy. As the morphological changes in the blastocyst proceed through the tubular to the filamentous form, oestrogen secretion increases and then subsequently declines by Day 13. This is followed by a second increase in synthesis beginning around Day 16 (Robertson and King, 1974, Zavy et al., 1980). The fact that blastocysts which were asynchronous with their uterine environment appeared to develop normally provided the uterine secretory response was not stimulated by administering exogenous oestrogen on Day 11 (Geisert et al., 1982c) implies that the blastocysts determine when alteration of the uterine environment occurs and that they do so when they are sufficiently mature to respond to the change in uterine environment. Thus the onset of oestrogen synthesis by porcine blastocysts is dependent on the degree of morphological maturity which once attained, stimulates the appropriate alteration of the uterine environment which allows rapid blastocyst expansion. It would therefore appear that the role of the uterus in early embryonic development is permissive until this degree of developmental maturity has been reached by the blastocysts.

With the exception of the uterine glycoprotein, uteroferrin, a specific function has not been identified for the various components of the luminal fluid following its synchronized release from the endometrium after oestrogen stimulation. However, it is clear that stimulating uterine secretion before blastocysts have attained this apparently critical degree of morphological maturity, results in early

blastocyst mortality.

It is unclear from experiment 1 why the asynchronous embryos failed to survive following oestradiol administration on Day 11. The oestradiol treatment itself was not embryotoxic as normal elongated blastocysts were recovered from synchronous recipient gilts administered oestradiol on Day 11.

The failure to recover any evidence of blastocyst tissue on Day 14 from the uterine flushings of asynchronous gilts treated with oestradiol on Day 11 suggests that the blastocysts degenerated soon after oestradiol administration and therefore failure to undergo elongation must be considered as one possibility that accounts for blastocyst death.

The process of blastocyst elongation in the pig is poorly understood but appears to involve cellular remodelling of the trophoblastic membrane (Geisert et al., 1982b) rather than cellular hyperplasia (Perry, 1981). Regulatory factors associated with the phenomenon are unknown but calcium ions have been suggested to play a critical role. This suggestion stems primarily from the fact that calcium is released from the endometrium at the onset of blastocyst elongation (Geisert et al., 1982b), and that calcium is known to play an important role in altering membrane fluidity perhaps through the methylation of membrane phospholipids (Hirata & Axelrod, 1980). Calcium ions are also essential for the exocytosis of secretory vesicles from several secretory cell types (Rubin & Laychock, 1978, Rasmussen & Barrett, 1984) and calcium also activates phospholipase A_2 resulting in the formation of arachidonic acid and subsequent synthesis of various prostaglandins (Hirata & Axelrod, 1980). However, Geisert et al. (1986) recently demonstrated that

prostaglandins did not play an essential role in blastocyst elongation. Oestrogen treatment on Day 9 or Day 9 and 10 of pregnancy was shown to advance the uterine calcium surge so that calcium levels were declining during the time of blastocyst elongation. In two gilts administered oestradiol valerate on Day 9 and 10 of pregnancy, calcium was almost nondetectable when the first uterine horn was removed on Day 11 and again when the second uterine horn was removed on Day 12. Despite this finding, normal elongated blastocysts were recovered on Day 12. This suggests that calcium ions per se are not essential in the elongation process itself. Instead, the increase in Ca ions in uterine luminal fluid following oestrogen stimulation may simply reflect oestrogen stimulation of an efflux of calcium from endometrial epithelial cells following an increase in membrane fluidity and activation of Ca-ATPase. This enzyme stimulates an efflux of Ca ions from the cell (Hirata & Axelrod, 1980). Other regulatory factors which may be involved in blastocyst elongation might include specific uterine proteins which trigger the elongation process.

Tzartsos and Surani (1979) demonstrated the binding of specific uterine proteins to the cell membranes of rat blastocysts but they could not determine how these proteins influenced the pattern or rate of embryonic development. However, it is reasonable to suggest that oestrogen stimulated release of specific uterine proteins which might regulate blastocyst elongation is a possibility. Furthermore, if blastocyst development has not reached a critical morphological stage whereby receptors on the trophoblast plasma membrane are present to recognize these uterine secreted regulatory proteins, further blastocyst development would be unlikely.

Two dimensional polyacrylamide gel electrophoresis (2D-PAGE) did

not distinguish any significant qualitative differences in the uterine proteins present in the flushings from the three recipient transfer groups. Although the sensitivity of this analytical procedure is limited, it is unlikely that the uterine secretion from the oestradiol treated groups contained component(s) that the control group did not and which was in itself embryotoxic. It is concluded from experiment 1 that a certain degree of morphological maturity must be attained by porcine blastocyst before they can respond to and continue development in a uterine environment stimulated by oestrogen on Day 11 of pregnancy. The failure of blastocyst to develop cannot be determined from the present experimental design but an absence of membrane receptors specific for certain regulatory proteins of uterine origin is one possibility to account for the loss of blastocysts. Another possibility is that immature blastocysts in fact possess receptors for inhibitory factors secreted by the uterus and that these receptors are lost or modified with maturity, perhaps at the onset of oestrogen synthesis.

Whatever the explanation is for the demise of immature blastocysts within a normal Day 11 uterine environment, experiment 1 has provided clear evidence that immature blastocysts could be indirectly placed in an unfavorable uterine environment by their more advanced littermates who induce intra-uterine changes which are inappropriate to their developmental stage.

In experiment 2, the primary objectives were to determine what effect administering exogenous oestradiol to gilts on Day 9 or Day 9 and 10 of pregnancy had on the biochemical make-up of uterine secretions recovered on Day 11, 12 and 16 of pregnancy and what effect these secretions had on blastocyst elongation and attachment.

Pope and First (1985) had previously shown that pregnant gilts administered oestradiol on Day 9 and 10 of pregnancy, i.e. before the onset of blastocyst oestrogen synthesis on Day 11, resulted in pregnancy failure by Day 30. However, oestrogen administered on D 12 and 13 had no adverse effects on pregnancy.

The recovery of intact filamentous blastocysts from the second uterine horn on Day 12 of pregnancy indicated that oestradiol treatment on Day 9 and 10 did not prevent blastocyst elongation. However, when this same treatment was repeated and the uterus was removed on Day 16, blastocysts were degenerating.

The administration of oestradiol on Day 9 and Day 9 and 10 of pregnancy advanced the uterine secretory response such that higher levels of recoverable calcium, protein and acid phosphatase were found in the uterine flushings from the first horn removed on Day 11 compared with those levels in control gilts. Furthermore, in contrast to the normal pattern of Ca release between Day 11 and 12 in control gilts, Ca level declined in both oestradiol treated groups between Day 11 and 12. Nevertheless, elongation of the blastocysts occurred in all gilts during this period.

Two dimensional gel electrophoresis demonstrated a marked alteration in staining intensification of various acidic and basic polypeptides in the uterine flushings from oestradiol treated gilts on Day 11 and 12. Not only were these quantitative differences apparent in the oestradiol treated gilts but in addition, qualitative changes occurred suggesting that premature stimulation of the uterine endometrial secretory response, as a result of oestradiol administration on Day 9 or Day 9 and 10, alters the pattern of endometrial protein release. Of particular interest was the

appearance of a group of very acidic (pI=5.4) proteins with a molecular weight (Mr 40,000) which were first discernable on Day 12. However, by Day 16, the appearance of these acidic polypeptides was markedly increased and coincidently, the blastocysts which were normal in appearance on Day 12 following oestradiol treatment on Day 9 and 10, were now degenerating. Although the function of these polypeptides is unknown at present, it is possible that they may have an important role in blastocyst attachment.

Following elongation of the blastocyst, it is essential that attachment of the trophoblastic membrane to the endometrial surface epithelium begins soon thereafter if development of the blastocyst is to continue. Attachment is initiated in the pig around Day 13 and involves an interdigitation of microvilli on the two opposing plasma membranes (King et al., 1980). The regulatory factors associated with attachment are unknown, however, the appearance of these acidic polypeptides in conjunction with blastocyst degeneration suggests a possible role for these proteins in normal conceptus development. These proteins are known to be of uterine origin as they have been reported in Day 12 uterine flushings from cyclic gilts injected with oestradiol benzoate on Day 9.5 of the cycle (Geisert et al., submitted for publication). Perhaps of more significance is the fact that these same polypeptides do not appear in the uterine flushings of gilts treated with oestrogen on Day 11 or 14, indicating that stimulation of the uterine endometrium with oestrogen before Day 11 results in a significant alteration in the quantitative and qualitative secretion of uterine protein. Therefore, appropriate synchrony between uterine endometrial development and exposure to oestrogen appears to be an important factor in allowing continued blastocyst development.

In the pig, which has a superficial placentation (Perry, 1981), studies on the protein and glycoprotein composition of the uterine epithelial cell plasma membrane were reported to be relatively constant throughout the oestrous cycle and early pseudopregnancy (Mullins et al., 1980). However, Anderson et al. (1986) reported that the rabbit endometrial epithelium undergoes differentiation prior to the time of blastocyst implantation which includes loss of surface negativity and a change in glycocalyx morphology. These same authors provided evidence for stage-specific alterations in the protein and saccharide composition of the apical surface of endometrial epithelium prior to implantation. Furthermore, these authors indicated that the implanting blastocyst further modified the luminal surface. Other researchers have also suggested that alteration of the glycoprotein coatings of both the trophoblast and epithelial cell plasma membrane may be a prerequisite for the very close apposition necessary for interdigitation of micorvilli (Enders and Schlafke, 1974, Jenkinson, and Searle, 1977).

In light of these findings, it is interesting to speculate that the acidic polypeptides appearing in the uterine flushings as early as Day 12 in gilts administered oestrogen on Day 9 could possibly represent polypeptides shed from the surface of the uterine epithelium. Although these polypeptides were not detectable in the control gilts in Day 16 uterine flushings, this may be because these polypeptides coat the trophoblast of viable blastocysts in a similar fashion to the plasmin isoinhibitors. Another explanation for the shedding of these potential surface polypeptides would be to expose receptors sites to the trophoblast to facilitate attachment. However, it is apparent that premature shedding of the proteins is embryocidal

perhaps by preventing proper attachment. Although this hypothesis is purely speculative it should be noted that there is a restricted period of sensitivity in the rat to the deciduogenic stimulus from the blastocyst. Oestrogen controls the capacity of the uterus to respond to such stimuli and to transmit stimuli from the uterine lumen to the underlying stroma (Lejeure et al., 1981). Blastocysts which are relatively retarded at the time uterine sensitivity is attained, do not implant and are rapidly lost from the uterus (Dickman and Noyes, 1960). This latter finding emphasizes again that not only is proper timing of the uterine secretory response essential for continued blastocyst development but just as important is the degree of blastocyst morphological maturity attained so that it can respond to the uterine environmental changes.

In conclusion, the three experiments described in this thesis have identified two major components of embryonic mortality in the pig. Firstly, a degree of morphological maturity must be attained by the blastocyst before it can respond to and continue development in the altered uterine secretions induced by oestrogen on Day 11 of pregnancy. This finding could well account for a portion of the embryonic death that occurs naturally in pregnancy due to the fact that blastocysts do not develop synchronously, thereby resulting in a wide variation in morphological forms present at the time of uterine sensitivity to blastocyst oestrogen (Anderson, 1978).

The second conclusion is that exposure of the uterine endometrium to oestrogen before Day 11 in the pig modifies the pattern of uterine protein secretion. While this alteration does not affect the blastocysts ability to elongate, ultimately blastocysts fail to survive perhaps because of a failure to attach to the endometrial

epithelium. This failure may be the result of either an altered uterine environment or the premature loss of surface proteins from the endometrial epithelial cells. These could be proteins which unmask receptors facilitating attachment or they could form part of the receptor complex itself. Nevertheless, the appearanace of these polypeptides coincides with total embryonic loss.

Future studies in this area might be directed towards isolation and purification of the group of acidic polypeptides associated with mortality. Subsequently antibody production to the protein maybe utilized to locate the source of the uterine protein in the endometrial epithelial cell.

In an effort to reduce loss of morphologically immature blastocysts which cannot survive the oestrogen stimulated altered uterine environment, inhibition of oestrogen synthesis by mature blastocysts might be attempted thus delaying the uterine secretory response. This could allow further maturation of the more immature forms so that they might be able to survive in the altered uterine environment when eventually stimulated.

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