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Embryo–uterine interactions in pigs during week 2 of pregnancy

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The second week of pregnancy is a particularly critical period for embryonic survival in pigs. Within that time, conceptus oestrogen synthesis is initiated, spacing and final placement of conceptuses is completed, and the signal for extending the functional lifespan of the corpora lutea is received by the mother. There is also a marked increase in blood flow to the uterus and the uterine endometrium produces and secretes nutrient histotrophe. Conceptus-derived oestrogen has been implicated in many of these events. It is also during this period that the trophoblast elongates dramatically and the inner cell mass starts to differentiate into the embryo proper. Here, we critically review the evidence that oestrogen is the sole factor initiating long-term corpus luteum maintenance in pigs. We also review the functions and general properties of the major secretory proteins in histotrophe and the role of oestrogen in controlling their expression. It is now generally accepted that asynchrony within a litter underlies much of the losses of conceptuses that are otherwise genetically normal, but which are lagging in their development; however, the underlying mechanisms remain unclear. Here we hypothesize that oestrogenic compounds derived from more advanced conceptuses or provided prematurely, either by injection or in the diet, trigger a massive increase in uterine expression and secretion of retinol-binding protein laden with retinol. We propose that less developed, smaller conceptuses are least able to contend with the sudden exposure to this potential teratogen at a time when they are particularly susceptible to imbalance in retinol supply. Hence, even though their growth proceeds for a few days, their developmental potential is irrevocably compromised.

Introduction

The intent of this paper is to review current information on the biochemical interactions that occur between conceptuses and the maternal endometrium during early pregnancy. We concentrate on the second week after conception, a period that is particularly critical to embryo survival and in which the mother must make appropriate adjustments in her physiology if the pregnancy is to continue. Some of the events that occur during week 2 of pregnancy in swine are listed (Table 1). Not surprisingly, this period is associated with considerable embryonic loss (see below). It is also clear that many of the phenomena listed are sequelae to the production of conceptus oestrogen. This latter topic is therefore discussed in some detail. Finally, porcine embryos rely heavily for their growth and development on provision of uterine secretory proteins, yet cannot tolerate an out-of-phase uterine environment. Since the control of uterine secretory activity and the function of individual protein components have long been an interest of this laboratory, these subjects also receive emphasis in this review.

Table 1. Major events of week 2 of pregnancy

Phenomenon	Day of pregnancy	Comments	References
Conceptus oestrogen synthesis	10–12	Precedes most phenomena described below	Perry *et al.*, 1976
Migration, spacing and placement of conceptuses	7–12	Probably mediated in part by conceptus oestrogen	Pope *et al.*, 1982; Dziuk, 1985; Laforest and King, 1992
Signal for maternal recognition of pregnancy*	12	Mediated by conceptus oestrogen and possibly other factors	Dhindsa and Dziuk, 1968; Frank *et al.*, 1978
Elongation of conceptuses	12	Considerable asynchrony between littermates; onset follows initiation of oestradiol synthesis and coincides with release of uterine proteins	Patten, 1948; Perry and Rowlands, 1962; Anderson, 1978; Geisert *et al.*, 1982a, b; Pope *et al.*, 1988; Xie *et al.*, 1990a
Increased uterine blood flow	13	Mediated by conceptus oestrogen	Ford *et al.*, 1982
Uterine secretory activity begins	12	Requires progesterone; modulated by oestrogen	Geisert *et al.*, 1982a, c; Roberts and Bazer, 1988
Secretion of retinol-binding proteins by conceptus	10–15	Initiated before conceptus elongation	Harney and Bazer, 1989; Godkin *et al.*, 1982; Trout *et al.*, 1992
Primitive streak formation and initiation of gastrulation and neurulation	9–14	Beginning of embryogenesis	Patten, 1948; Anderson, 1978; Jainudeen and Hafez, 1987
Secretion of type I and type II interferons	12	Modulation of maternal immune responses	LaBonnardière *et al.*, 1991
Firm adhesion of trophoblast to uterine epithelium	14	Appears defective in gilts or sows exposed to oestrogens prematurely	Keys and King, 1990; Morgan *et al.*, 1987

*Maternal recognition of pregnancy here refers to the extension of the functional lifespan of the corpora lutea.

Basis of Embryonic Loss in Pigs

The majority of corpora lutea present on the ovaries of gilts are represented by viable blastocysts at day 9 to 10 of pregnancy (Polge, 1982; Xie *et al.*, 1990a). However, only about 70–80% of these blastocysts are estimated to survive until day 25 (Pope and First, 1985). Losses are higher in sows than in gilts and are greatest when large numbers of ovulations occur (Perry, 1960). A recent study (Lambert *et al.*, 1991) indicated that most embryonic loss occurred before day 10 of pregnancy, but the observations were made on young, first cycle gilts, and peripubertal gilts tend to have an extended pro-oestrus with a prolonged oestrogen peak. Consequently, the oocytes of first cycle gilts may become developmentally defective owing to overexposure to the steroid (Archibong *et al.*, 1987). Nevertheless, whatever the timing of embryonic death, it remains poorly explained and has a major impact on productivity and hence profitability in the industry.

Although it is still not completely clear exactly when embryos unaccounted for at the end of the first month of pregnancy die, it has been proposed that the loss results from asynchronous development among littermates, a phenomenon that leads to poorly coordinated biochemical interactions between the conceptuses and the maternal systems (Pope, 1988; Pope *et al.*, 1990). Specifically, smaller, less advanced conceptuses are presumed to be lost because they become out of phase with the uterine environment. It is well established, for example, that embryos transferred to more advanced uteri quickly die (Jarrell *et al.*, 1990; Geisert *et al.*, 1991). Thus, some early losses may simply result from the inability of embryos to maintain their rates of development in the face of an increasingly hostile uterine environment, although the nature of the toxic response remains poorly understood. The situation is a little clearer at day 10 to 12 of pregnancy. Here most investigators have suggested that production of oestrogen by the larger conceptuses, as well as being responsible for extending the functional lifespan of the corpus luteum, causes a sudden change in the uterine milieu that cannot be tolerated by those conceptuses already lagging in development (Pope and First, 1985; Morgan *et al.*, 1987; Pope, 1988). This concept is discussed in greater detail later.

It seems unlikely that the more slowly developing conceptuses die because they are all genetically defective since both small and large day 7 blastocysts transferred to separate, surgically isolated uterine horns of a recipient female at day 6 of her cycle survive and appear normal 6.5 days later. However, attrition of small blastocysts is high if a similar transfer is made to a day 7 recipient (i.e. a synchronous, surrogate mother) with larger blastocysts placed in the contralateral horn (Table 2; Wilde *et al.*, 1988). Such an experiment illustrates two points. First, as stated above, genetically normal embryos probably comprise the majority of those lost during early pregnancy in the pig, although a selection against lagging embryos may be very useful in minimizing any possibility of a sow bearing defective young. Second, the embryos in one uterine horn can influence the development of others in the contralateral horn. Thus, an explanation for the findings is that the synchronous transfer of day 7 blastocysts to a day 6 uterus temporarily retards the more advanced embryos and allows the smaller ones to catch up. In the asynchronous transfer, however, the larger embryos retain their advantage, secrete oestrogen earlier and, by mechanisms still unclear, trigger changes in the other uterine horn.

In a normal pregnancy, considerable developmental asynchrony can be readily noted at about days 11–12 of pregnancy. It is not uncommon, for example, to find small spherical blastocysts < 5 mm in diameter along with tubular and filamentous forms (Perry and Rowlands, 1962; Anderson, 1978; Pope *et al.*, 1988). There are several possible causes of such variability. For example, different embryos may be genetically programmed to develop at different rates. Another possibility is that the oocytes shed last during ovulation, a process that occurs over several hours in pigs, give rise to the smaller embryos noted 12 days later (Pope *et al.*, 1988). This last hypothesis has been based on three observations: heterogeneity in follicle/oocyte maturation right before ovulation (Ainsworth *et al.*, 1980; Xie *et al.*, 1990b, Biggs *et al.*, 1993), skewed ovulation with a few oocytes shed later during the process of ovulation (Pope *et al.*, 1988) and slower cleavage embryos being the less developed conceptuses on day 11 (Xie *et al.*, 1990a). However, the relationship between the later matured follicles/oocytes and the later ovulators has not been established; nor has the developmental potential of the later matured oocytes been evaluated. Evidence has also emerged that ovulation intervals are not the only causes for embryo diversity (Pope, 1992; Soede

Table 2. Embryonic survival (mean ± SEM) after synchronous (day 7 recipient) and asynchronous (day 6 recipient) transfer of small and large day 7 littermate blastocysts

	Embryonic survival (%)	
Type of transfer	Small day 7 blastocysts	Large day 7 blastocysts
Synchronous ($n = 9$)	38.3 ± 5.8[a]	73.9 ± 5.8[b]
Asynchronous ($n = 7$)	75.4 ± 6.6[b]	70.7 ± 6.6[b]

[a,b]Means with different superscripts are significantly different ($P < 0.01$).
Data from Wilde *et al.* (1988) with permission.

et al., 1992). Nevertheless, there seems little doubt that it is these more poorly developed embryos that are subsequently lost.

Timing and Extent of Conceptus Oestrogen Production

As discussed in a later section, oestrogen, first produced by pig conceptuses at about day 11 of pregnancy, is considered to be critical for ensuring that the corpora lutea do not regress as they do at the end of a non-fertile oestrous cycle (Bazer *et al.*, 1982). By day 11, spherical blastocysts 5–7 mm in diameter can synthesize oestrogen from a variety of substrates (Perry *et al.*, 1976; Heap *et al.*, 1979; Fischer *et al.*, 1985), although a pathway involving Δ^4-3-ketosteroids, and particularly progesterone, seems most likely (see Conley *et al.*, 1992 for discussion). The oestrogen is secreted transiently, with maximal output per mass of tissue detected at the time of early elongation. This burst of activity is correlated with a transient increase in concentrations of oestrogens (oestradiol and oestrone) and particularly oestrone sulfate in maternal plasma (Stoner *et al.*, 1981; Bazer *et al.*, 1982). Oestradiol is oxidized to oestrone in a reaction catalysed by a specific dehydrogenase. Free oestrone is probably then sulfated by endometrial sulfotransferase. The activity of both enzymes reaches peak values in porcine endometrial tissue between days 5 and 13 (Pack and Brooks, 1974).

The rate-limiting enzyme during this first period of conceptus oestrogen production is probably 17α-hydroxylase cytochrome P_{450}, the expression of which reaches peak values during the tubular phase of development and declines as conceptuses become filamentous (Conley *et al.*, 1992). Oestradiol concentrations in conceptus tissues are approximately fifty-fold higher at day 12 than at day 14 of development (67.5 *versus* 1.3 pg in 100 μg protein, respectively) (Conley *et al.*, 1992). Moreover, total content of oestrogens in flushings from uterine horns containing early elongating conceptuses at about day 12 of pregnancy is considerably higher than at day 14, when all the conceptuses are filamentous (about 20 ng *versus* about 2 ng) (Geisert *et al.*, 1982a).

It is difficult to determine exactly how much oestrogen such conceptuses are elaborating *in vivo*. Measurements from tissue extracts and uterine flushings do not take into account how efficiently steroid is removed from the uterine lumen. Estimates made on production by cultured conceptuses are also likely to be low since the tissues are in general metabolic decline and do not necessarily have access to all the precursor molecules needed to produce oestrogens efficiently.

Nevertheless, Wilde and Pope (1987) incubated individual day 11.5 conceptuses for only 6 h. Large quantities (20–40 ng) of oestradiol were produced by both tubular and filamentous conceptuses. If a litter contains 10–15 conceptuses, 1–3 μg oestradiol will be produced daily. These data are somewhat in

agreement with those of Ford *et al.* (1982), who calculated both total blood flow to the uterus and the difference between concentrations of oestradiol and oestrone in uterine vein and uterine artery. On the basis of their data, it can be estimated that approximately 1.4 μg of free oestrogens leave the uterus daily at about day 13 of pregnancy. Export from the uteri of nonpregnant gilts was negligible. However, differences of 100 pg ml^{-1} in utero–ovarian concentrations of oestrone sulfate, which was not measured by Ford *et al.* (1982), have been observed between pregnant and nonpregnant gilts at day 11–12 of gestation (Stoner *et al.*, 1981; Bazer *et al.*, 1982). Similarly, Stone and Seamark (1985) reported that plasma concentrations of oestrone sulfate in mated gilts were 100–200 pg ml^{-1} higher than those in unmated gilts at about this stage of gestation. If the blood volume of these gilts is assumed to be 7 litres, the total content of water soluble oestrone sulfate at any time would exceed 700 ng. As oestrone sulfate would be expected to have a half-life of minutes in serum rather than hours, it seems likely that during this stage of pregnancy large amounts of sulfated oestrone are being fabricated. The question arises, therefore, as to whether the conceptuses are the source of all this oestrogen. Conceivably some is of uterine or even ovarian origin, although the ability of the former to synthesize oestrogen during early pregnancy has been reported to be limited (Fischer *et al.*, 1985). Clearly these data on oestrogen concentrations in blood need to be re-evaluated, particularly as there are inconsistencies in some of the values that have been obtained for individual steroids and their sulfated derivatives.

Conceptus Oestrogens and the Signal for Maternal Recognition of Pregnancy

The conceptus signal that initiates luteal maintenance in pregnant sows must be produced by at least day 12 of pregnancy and conceptuses must also occupy each uterine horn by that time for the signal to be effective (Dhindsa and Dzuik, 1968). There seems little doubt that $PGF_{2\alpha}$, originating from the uterine endometrium, is the luteolytic substance that limits the lifespan of the corpora lutea during the oestrous cycle. This topic has been reviewed extensively elsewhere (Bazer *et al.*, 1986, 1989) and will not be covered here. However, the hypothesis that a relatively brief exposure of the uterus to conceptus-derived oestrogen at about day 11–14 provides the protective basis for this extension of the lifespan of the corpus luteum (Bazer and Thatcher, 1977) deserves some further consideration.

Exogenous oestrogens administered in large amounts to nonpregnant gilts at about day 12 have long been known to be luteotrophic (Gardner *et al.*, 1963), and pseudopregnancy accompanied by prolonged bilateral maintenance of corpora lutea can be induced by daily injections of milligram quantities of oestradiol between days 11 and 15 after the onset of oestrus (see Frank *et al.*, 1978; Bazer *et al.*, 1982). However, such doses cannot reasonably be regarded as physiological. Provision of oestradiol at the site where it is presumed to be most effective, i.e. in the uterus, in amounts designed to mimic those released by conceptuses during this period have had little or no effect on luteal lifespan. For example, van der Meulen *et al.* (1991) observed that injection of 380 ng of oestradiol every 6 h from day 11 to day 15 into both uterine horns resulted in a slight but nonsignificant increase (21.7 ± 1.0 days *versus* 20.5 ± 1.5 days in controls) in inter-oestrous interval. Similar data were obtained in an unpublished study cited by Ford *et al.* (1982) in which gilts receiving 375 ng oestradiol every 6 h on days 10–14 experienced a 3-day delay in luteolysis and then returned to oestrus.

Using higher doses of oestradiol (100 μg day^{-1}; days 10–14), which were either infused into the uterine lumen or injected subcutaneously, Saunders *et al.* (1983) observed that both treatments gave a modest 4–5 day extension of the oestrous cycle. Finally, Laforest and King (1992) inserted Silastic beads impregnated with oestradiol or oestradiol benzoate into the uterine lumen of gilts on day 10 of the oestrous cycle. The beads released enough oestradiol to day 16 to maintain intraluminal concentrations at or above those observed in pregnant gilts. These authors also observed only a 2–6 day extension of the oestrous cycle in these gilts.

The above results may at first appear to contrast with the results of Ball and Day (1982), who developed the unilaterally pregnant pig model to examine the mechanisms of luteal maintenance. These authors successfully maintained bilateral corpora lutea to day 19 in 67% of unilaterally pregnant gilts infused with conceptus extracts. The extracts, containing 15 ng total oestrogens ml^{-1} were introduced infused (15 ml day^{-1}) into the unoccupied contralateral horn on days 12–19. Adsorption of steroids with charcoal completely nullified this effect. However, it is unclear whether these unilateral pregnancies would

have been maintained beyond day 20. It seems likely that the bilateral maintenance of corpora lutea to day 19 resulted from a 3–5 day delay in luteolysis as noted above.

Glossop and Foulkes (1988) pointed out that in commercial breeding units there are two distinct periods when sows return to oestrus after service. The first was at about day 20 (range day 17–23), and presumably represents animals in which fertilization failed or in which embryos were lost before day 12. The second occurs at about day 26 (range day 24–31). These animals may have been exposed to conceptus oestrogen and exhibit the short delay in luteolysis noted above (van der Meulen, 1991). Indeed, Geisert *et al.* (1987) suggested that the conceptus generates two waves of oestrogen for maintaining the function of the corpus luteum, the first at about day 11, the second between day 14 and day 16. Again, however, pharmacological doses of oestradiol were used to test the hypothesis.

It is difficult to reconcile many of these observations with the view that conceptus-derived oestrogens are the only biologically active substances involved in maintenance of corpora lutea in the pig. However, there are few clues as to what the other factors might be, particularly since conceptus secretory proteins infused into the uterine lumen between day 12 and day 15 failed to provide oestrous cycle extension in nonpregnant gilts given a 1 mg injection of oestradiol on day 11 (Harney and Bazer, 1989).

Localized Effects of Oestrogen on Uterine Tissue

In the previous section, we argued that one aspect of maternal recognition of pregnancy in pigs, namely maintenance of the corpora lutea, may not be mediated by conceptus-derived oestrogens alone. However, small amounts of oestrogen comparable to those likely to be released by embryos at day 11–12 do have marked effects that are undoubtedly important in pregnancy (Table 1). In particular, uterine blood flow is increased eight- to ten-fold within 12 h of an infusion of small amounts of oestradiol (375 ng) into the uterine lumen of gilts at day 11 of their oestrous cycle (Ford *et al.*, 1982). A comparable increase occurs during pregnancy (Ford and Christenson, 1979). These effects may, in fact, be mediated by catechol oestrogens, short-lived oestrogen metabolites known to be synthesized by porcine trophoblast tissue (Mondschein *et al.*, 1985). There is also evidence that conceptus-derived oestrogen is responsible for a rapid release of calcium and uterine secretions into the uterine lumen (Table 1), a phenomenon that must markedly alter the environment to which conceptuses are exposed (Geisert *et al.*, 1982a). These uterine secretions have generally been considered to involve materials already synthesized and stored intracellularly in secretory granules. However, as will be discussed later, oestradiol can markedly upregulate the mRNA for at least two secretory proteins, although others are less affected.

Magness and Ford (1982) have pointed out that oestrogen content of lymph as well as of the utero–ovarian vein draining the uterus is high at days 11, 13 and 15 of pregnancy and that this steroid and its metabolites might well reach the postganglionic sympathetic vasoconstrictor nerves, which are believed to control blood flow to both the uterus and ovary, by such a route. Thus, onset of production of oestradiol at day 11 might influence ovarian as well as uterine physiology.

Embryocidal Effects of Oestrogen and Oestrogen Analogues

Zearalenone is a metabolite of the fungus *Fusarium roseum* and acts as a potent oestrogen. It is commonly found in mouldy corn, which, when consumed by sows, causes a range of physiological responses, including abortion during early pregnancy and extended inter-oestrous intervals in which the corpora lutea are bilaterally maintained (Long and Diekman, 1984). Feeding zearalenone in a controlled manner (1 mg day^{-1}) to sows between days 7 and 10 after mating has been observed to lead to abnormalities in the organization of the embryonic disc by day 12 in early elongating conceptuses and obvious degeneration of the entire litter or filamentous conceptuses by day 13 (Long *et al.*, 1992). Few, if any, abnormalities have been noted in the uterine endometrium or in the serum profiles of pituitary hormones, the synthesis of which could potentially be responsive to oestrogen. Identical degenerative changes and complete pregnancy loss result when pregnant gilts are injected with fatty acid esters of oestradiol before day 10.5 of pregnancy (Morgan *et al.*, 1987; Gries *et al.*, 1989). Both treatments result in premature release of endometrial secretions into the uterine lumen.

Although these observations do not rule out a direct toxic effect of oestrogens on conceptuses just before the time they elongate, it seems more probable that the treatments cause acute changes in the uterine milieu that the conceptus, and particularly the embryo proper, cannot tolerate. This sudden exposure to a more hostile environment leads to embryonic death, not immediately, as noted in asynchronous transfers in the day 5–7 or 6–8 periods noted earlier (Geisert *et al.*, 1991), but rather within a few days. Oestrogen administered only slightly later, that is day 11 to 12, is without any embryotoxic effect. It is a major puzzle why uterine secretions provide such a narrowly permissive environment and what components can be lethal one day yet without apparent harm on the next. We speculate later that the embryotoxic component may be retinol.

Components of Histotrophe and Their Likely Functions

Several reviews, including Roberts and Bazer (1988) and Roberts *et al.* (1993), have dealt with the composition and function of the progesterone-responsive components of uterine secretions of the pig. Davis (this supplement) also discusses their production by primary cultures of uterine epithelial cells. The topic will therefore be covered only briefly here.

Histotrophe or uterine milk is so called because it is assumed to nurture the developing conceptus *in utero*. In species such as the pig, the trophoblast of which is noninvasive and fails to make direct contact with the maternal blood supply, a reliance on uterine secretions to provide macromolecules with a nutrient or protective role is assumed to be more complete than in species such as mice or humans in which the trophectoderm immediately invades after blastocyst hatching. As a consequence, the pig uterine endometrium begins to secrete very large quantities of several secretory proteins in response to progesterone (Table 2). The most abundant of these secretory components is the purple acid phosphatase, uteroferrin, which transports iron across the placenta (Roberts *et al.*, 1986; Roberts and Bazer, 1988) and which may also be a potent growth factor on pig haematopoietic progenator cells (Bazer *et al.*, 1991). Uteroferrin has been fully sequenced (Hunt *et al.*, 1987), its cDNA (Simmen *et al.*, 1988; Ling and Roberts 1993) and its gene (Simmen *et al.*, 1989) cloned and it has been shown to be identical to an intracellular tartrate-resistant acid phosphatase normally sequestered in lysosomes (Ling and Roberts, 1993). Purple uteroferrin-like molecules are also secreted into the uterine lumen of the horse (McDowell *et al.*, 1982; Zavy *et al.*, 1982) and cow (C. Ketcham, W. Clark, F. W. Bazer and R. M. Roberts, unpublished results), but an involvement in iron transport in these species, although suspected, has not been confirmed. Uteroferrin expression is responsive not only to steroids, but possibly also to prolactin (Young *et al.*, 1989; Fliss *et al.*, 1991).

In addition to uteroferrin, several other major progesterone-responsive polypeptides of porcine uterine secretions have by now been purified, and their cDNA cloned. They include a trio of basic glycoproteins most probably derived from a common precursor molecule by differential post-translational modification (Murray *et al.*, 1989; Malathy *et al.*, 1990). They are members of the widespread serpin superfamily of proteins, many of whose members are proteinase inhibitors. No function, however, has yet been ascribed to these uterine serpins, although similar molecules are secreted by the uterus of sheep (Ing and Roberts, 1989) and cows (N. Mathialagan and R. M. Roberts, unpublished).

A group of smaller basic proteins that are known to be highly effective inhibitors of plasmin, trypsin and chymotrypsin have also been described. Recently their cDNA have been cloned (Stallings-Mann and Trout, 1993), demonstrating conclusively that they belong to the Kunitz family of inhibitors, whose best known member is bovine pancreatic trypsin inhibitor (aprotinin or Trasylol®). Such inhibitors probably serve to limit any potential damage initiated by proteolytic enzymes released from the conceptus itself and may even control trophoblast invasiveness (Mullins *et al.*, 1980; Fazleabas *et al.*, 1982, 1984). In addition, it is conceivable that they may play a role in decreasing local inflammatory responses to the presence of conceptus tissue by neutralizing proteinases released by immune cells.

Lysozyme, which presumably has a bacteriostatic role, is a fairly minor component of uterine flushings. Its activity increases in response to progesterone, but only in rough proportion to the increase in total protein secreted (Hansen *et al.*, 1985). It therefore remains unclear whether it is responsive to progesterone.

Table 3. Progesterone-stimulated uterine secretory proteins of pigs

Protein name or description	Molecular weight and other properties	Features of mRNA	Cell type in which expressed	Presumed function
Uteroferrin	35 000 glycoprotein; purple with an Fe–Fe center	about 1.5 kb; 340 codons	Uterine glandular epithelium	Iron transport to conceptus
Uterine serpins	42 000–50 000 glycoproteins (extensively processed). Feature of serine proteinase inhibitors	about 1.5 kb; 417 codons	Uterine glandular epithelium	Unknown
Uterine Kunitz inhibitor	M_r 14 000 glycoprotein (?); contains a 6-cysteine Kunitz domain at its NH_2-terminus	0.8 kb; 122 codons	Surface (predominantly) and glandular epithelium	Inhibits plasmin, trypsin and possibly other serine proteinases
Retinol-binding protein	21 000 protein; identical to serum retinol-binding protein	about 1 kb; 201 codons	Surface and glandular epithelium	Vitamin A transport to conceptus
Lysozyme	about 14 000 protein; identical to porcine spleen and stomach enzyme	about 1.4 kb; 119 codons	Unknown	Bacteriostatic

The final component of histotrophe that will be discussed in any detail is retinol-binding protein (RBP). RBP is seen after analysis of two-dimensional polyacrylamide gel electrophoresis as two to three low molecular weight (M_r about 22 000) acidic (pI about 6.0) polypeptides. Although the uterine RBPs were once considered to be a group of unique molecules with only partial sequence similarity to serum RBP (Clawitter *et al.*, 1990), more detailed analyses and most recently the cloning of their cDNA have confirmed that they are probably identical in sequence to the form secreted by liver. The presence of isoforms probably results from deamidination or other post-translational modifications. RBP is presumed to provide vitamin A to the conceptuses, and, as expected, there is a marked increase in retinol content of uterine flushings associated with RBP secretion. Thus, the RBP in uterine flushings is at least partially loaded with retinol.

Finally, examination of two-dimensional gels on which uterine secretions have been analysed reveals a variety of more minor proteins that have yet to be identified. These probably include growth factors, hormones and hormone-binding proteins that are themselves hormonally responsive (Simmen *et al.*, 1992, 1993). Serum proteins, including albumin and immunoglobulins, are invariably present in various amounts, presumably as a transudate from serum as they do not appear to be synthesized by the endometrium (Basha *et al.*, 1980a,b). In part, their presence may have arisen as an artefact of the flushing process, for unlike uteroferrin, the uterine serpins and RBP, there is no evidence that serum proteins, including transferrin, albumin or immunoglobulins, are taken up by the conceptus at any time during pregnancy (Buhi *et al.*, 1983).

Oestrogen and Control of Uterine Secretory Activity

All of the major components of uterine histotrophe, with the possible exception of lysozyme, the localization and steroid responsiveness of which has not been examined closely, are synthesized by the surface and glandular epithelial cells of the endometrium of ovariectomized gilts in response to prolonged daily treatment with progesterone (Table 3). Oestrogens clearly have a modulatory role. At low daily doses, for example, total protein in uterine flushes increases markedly. At higher amounts, however, secretion of progesterone-induced proteins is markedly depressed (Roberts and Bazer, 1980).

During the oestrous cycle, the progesterone induced components are not secreted in significant quantities until the late luteal phase (day 14–16) (Geisert, 1982a). As with progesterone treatment of ovariectomized gilts, this delay probably represents the time required for the uterine epithelium to develop a fully functional secretory capacity. In pregnancy, however, the pattern of secretion is somewhat different in that there is an abrupt release of secretions into the lumen at the time the conceptuses begin to elongate and to produce oestrogen, i.e. on or about day 12. This release of histotrophe can be mimicked in nonpregnant gilts by a single injection of oestrogen at day 11 (Geisert *et al.*, 1982c). Until recently it was believed that the action of oestrogen was solely on the secretory rather than the biosynthetic limb of the pathway of histotrophe production. This view may have to be modified as it appears that injections of oestradiol markedly upregulate the content of mRNA for some of the secretory proteins, for example RBP and uterine serpin (Trout *et al.*, 1992) but not others, for example uteroferrin (W. E. Trout and M. Stallings-Mann, unpublished; Simmen *et al.*, 1991). Concentrations of RBP and uterine serpin mRNA are markedly higher in uterine horns of day 12 gilts carrying filamentous conceptuses than in horns in which none of the blastocysts has elongated (Fig. 1; Trout *et al.*, 1992).

Protein Composition of Conceptus Secretions at the Time of Maternal Recognition of Pregnancy

LaBonnardière (this supplement) has reviewed the evidence that pig conceptuses secreted both a type I, i.e. interferon α (IFN-α)-like and type II, i.e. IFN-γ-like interferon, during the early elongation phase of development. However, these cytokines have not been implicated in protecting the corpus luteum from the luteolytic effects of uterine-derived $PGF_{2\alpha}$ as has been demonstrated for the IFN-τ (also a type I IFN) in cattle and sheep. For example, neither Harney and Bazer (1989) nor ourselves (K. Kramer, J. C. Cross and R. M. Roberts, unpublished) have been able to extend luteal lifespan by intrauterine infusion of

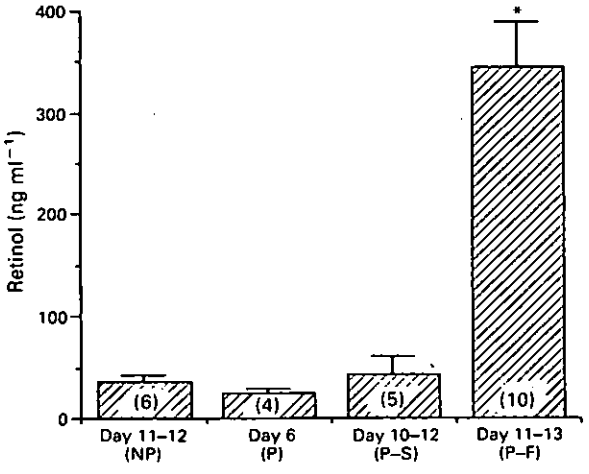

Fig. 1. Concentrations (means ± SEM) of vitamin A (retinol) in uterine flushings obtained from gilts on days 11–12 of the oestrous cycle (NP), on day 6 of pregnancy (P) and on days 10–13 of pregnancy. Flushings obtained on days 10–13 of pregnancy were subclassified depending upon whether all of the embryos in the litter were spherical (P–S) or whether some embryos had reached the filamentous stage (P–F) of development. The number of gilts within each group is indicated within each bar. *Significantly different from all other groups ($P < 0.001$).

conceptus secretory proteins presumably containing the two types of IFN. We have also found that recombinant bovine IFN-α was also ineffective in this regard. Nevertheless, the local production of such potent cytokines within the uterine lumen is unlikely to proceed unnoticed by the mother, and it would be surprising if they did not initiate some form of physiological response.

The major proteins released by the conceptuses during the critical day 10–12 period are not, however, IFN but retinol-binding proteins apparently identical in sequence to those produced by the liver and endometrium (Harney *et al.*, 1990; Trout *et al.*, 1991). Presumably, the RBP is secreted as the apo-form, that is carrying no retinol, as mammalian embryos do not store vitamin A. Production of RBP by the conceptus appears to precede uterine RBP mRNA expression in the uterus by at least a day (Trout *et al.*, 1991, 1992). As development progresses, several other proteins, the majority of which have not been characterized, can be identified on polyacrylamide gels (Godkin *et al.*, 1982). Their contributions to maternal–conceptus interactions remain completely unknown.

Vitamin A and its Effects on Reproduction and Embryonic Development

A requirement for vitamin A in reproduction has long been known. Both males and females on a vitamin A-deficient diet become sterile, and lack of vitamin A during pregnancy often causes abortion. In addition, various malformations have been noted in progeny of female rats on a low vitamin A diet (Thompson *et al.*, 1964). Retinoic acid is not an adequate substitute for retinol in preventing these disorders of pregnancy and is most likely not transported across the placenta. Dietary vitamin A, provided in the form of retinol, its esters or precursors such as β-carotene, seems to be required to allow normal rates of cell division, proper organ development, and for growth of the placenta in rats and most probably all mammals (Thompson *et al.*, 1964).

However, the diffuse epitheliochorial nature of the pig placenta is thought to place considerable limitations on the amount of water-insoluble nutrients, such as retinol, that can reach the conceptuses, since such compounds normally require protein chaperones in order to circulate in maternal blood. The

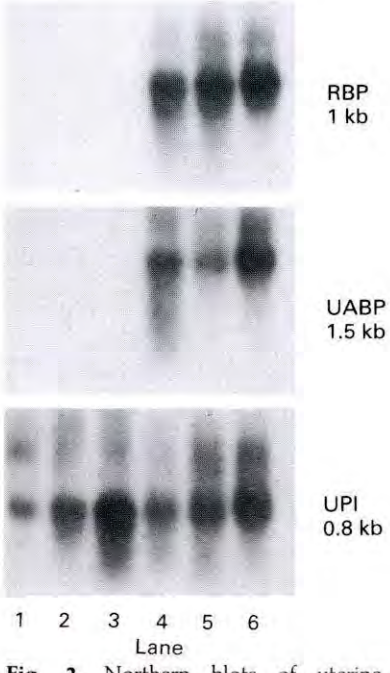

Fig. 2. Northern blots of uterine endometrial RNA (40 μg) following hybridization with porcine cDNA probes for retinol-binding protein (RBP), uterine serpin (UABP) and uterine plasmin/trypsin inhibitor (UPI). RNA was isolated on day 12 of pregnancy from gilts bearing spherical (lanes 1–3) or filamentous (lanes 4–6) conceptuses. Note the absence of signal for the mRNA of RBP and the serpin from uteri of gilts with only spherical conceptuses, but the high expression of these mRNA where elongating conceptuses are present. By contrast, UPI mRNA is expressed in all day 12 gilts. The cDNA probe for UPI was provided by M. Stallings-Mann.

nutrient carrier protein complex must cross several maternal cell layers, including an intact uterine epithelium to reach the pig conceptus from uterine capillaries. In the case of iron, the endometrium synthesizes uteroferrin as an intermediary and not the iron transport protein of serum, transferrin (Roberts and Bazer, 1988). Possibly as a consequence of this indirect mechanism of transport, piglets are almost devoid of iron stores at birth. With retinol, however, the transport protein synthesized by the endometrium appears identical to the serum retinol-binding protein released by the liver (Stallings-Mann *et al.*, 1993). Nevertheless, it seems likely that some of the same limitations exercised over iron transport may still be operative, particularly during a period of vitamin A need. At about day 12 the amount of retinol in uterine flushings during a normal pregnancy rises 10- to 50-fold within hours (Fig. 1) and large amounts of RBP mRNA are detected in the endometrium (Fig. 2; Trout *et al.*, 1992). The actual concentration of retinol to which the embryos are exposed is difficult to estimate because the secretions are diluted by a large excess of saline during uterine flushing, but they must exceed 10 μmol l^{-1}. Thus, the conceptuses themselves appear to signal uterine secretion of retinol and RBP, suggesting that high amounts of retinol may be required at this stage of development.

Paradoxically, such high amounts of retinol may also pose a threat to embryonic survival because in excess, the compound is embryotoxic (Thompson *et al.*, 1964). Moreover, vitamin A derivatives (Isotretinoin or 11-*cis*-retinoic acid and etretinate) cause abortion and severe teratogenesis in humans and various experimental animals (Lammer *et al.*, 1985). The basis of the teratogenesis possibly lies in the ability of the synthetic retinoids and various byproducts of retinol metabolism to occupy both retinoic acid receptors and the more recently discovered retinoid X receptors (RxRα, -β and -γ). The latter bind 9-*cis*-retinoic acid, but their affinities for *trans*-retinoic acid are low (Levin *et al.*, 1992; Heyman *et al.*, 1992). Both types of receptor function somewhat similarly to steroid receptors (Evans, 1988; Beato, 1989). Once occupied, they act as transactivators in a dimeric association to specific nucleotide sequences (*cis*-enhancer elements), which are usually placed upstream of the transcription start sites of responsive genes, and either increase or decrease transcriptional rates. The situation is, however, quite complex, since there are many kinds of receptor with different tissue distributions. Moreover, the RxR form heterodimeric associations with both the various *trans*-retinoic acid receptors and the receptors for thyroid hormone and vitamin D (Green, 1993). These complex interactions probably provide for graded responsiveness towards retinoic acid and its derivatives in a very large group of genes. Excess retinoic acid or the presence of unusual retinoic acid homologues probably alters such a balanced response or distorts the normal function of the receptors.

At this stage, it is worth speculating why retinoic acid might be so potent in pigs at the time of blastocyst elongation. First, the considerable change in the general morphology of the conceptus requires extensive tissue remodelling and growth of the extra-embryonic membranes (Geisert *et al.*, 1982b). Retinoic acid is likely to be important in this regard because it is known to influence production of several components of the extracellular matrix (Vasios *et al.*, 1989; Schüle *et al.*, 1990), cell surface adhesive molecules (Agura *et al.*, 1992) and at least one proteinase (urokinase-type plasminogen activator) and its associated inhibitor that are key players in control of matrix breakdown (Tienari *et al.*, 1991). Interestingly, plasminogen activator is released in large amounts as pig blastocysts elongate (Mullins *et al.*, 1980; Fazleabas *et al.*, 1983). A second reason why retinoic acid may be important at day 11 is that porcine embryos are at a critical developmental stage. Formation of the primitive streak and embryo plate are initiated at about this time (Patten, 1948; Jainudeen and Hafez, 1987). Since retinoic acid is known to control genes involved in embryonic organization in chickens (Brickell and Tickle, 1989; Yokouchi *et al.*, 1991), amphibians (López and Carrasco, 1992) and mice (Simeone *et al.*, 1990), it would be surprising if it were not also involved in pigs.

Retinol-binding Protein, Oestrogen and Embryonic Loss in the Pig: a Hypothesis

Here we wish to speculate on the interrelated functions of oestrogen and retinol-binding protein during early pregnancy in pigs. First, as discussed in the previous section, we propose that retinol concentrations within the uterine lumen may be limiting at the time of blastocyst elongation. Indeed, provision of retinol and other components released from the endometrium at about day 12 may be required for conceptus elongation. Competent conceptuses probably signal their need for these secretions by synthesizing oestradiol. However, because no retinol storage capacity has been described for the pig uterus, the uterine epithelium must presumably acquire the retinol it needs from the blood circulation and thus may not be able to deliver all of the retinol required for optimal conceptus survival, particularly in the case of large litters. Competition for retinol may therefore be one cause of embryonic loss.

We also propose that advanced conceptuses, which are the first to synthesize oestrogen, are also the first to secrete large quantities of apo-retinol-binding protein, possibly as a result of an autocrine effect of oestradiol. Advanced conceptuses may therefore be able to protect themselves from exposure to the high concentrations of retinol in the uterus at that time. The least advanced conceptuses, which may not secrete sufficient apo-RBP to protect themselves, then die owing to premature and inappropriate gene expression resulting from intracellular conversion of retinol to retinoic acid and its derivatives. The observations pertaining to embryonic death caused by injections of oestradiol at days 9–10.5 of pregnancy or by exposure to zearalenone during the period preceding elongation are entirely consistent with the above hypothesis, although other explanations are clearly possible.

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