

## Effect of the Conceptus and Lack of Effect of Uterine Space on Endometrial Protein Secretion during Mid-Gestation in Swine<sup>1</sup>

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### ABSTRACT

The effect of the conceptus and of reduced uterine space on endometrial protein secretion was examined on Days 40, 60, and 80 of gestation in white crossbred gilts. Twenty-nine gilts were checked daily for estrus, and 15 were given 5 mg estradiol valerate daily from Days 11 to 15 (Day 0 = day of estrus) of the estrous cycle to induce pseudopregnancy. The remaining 14 pigs were mated during estrus. All pigs were laparotomized on Day 4, and one uterine horn was ligated to produce one crowded and one roomy uterine environment. Pigs were killed on Days 40, 60, and 80 of pregnancy or pseudopregnancy. The reproductive tracts were collected, and placental tissues from pregnant pigs and endometrial tissues from all pigs were cultured in the presence of <sup>3</sup>H-leucine to evaluate protein secretion. Conditioned medium was dialyzed, measured for incorporation of radioactivity into nondialyzable macromolecules, and then subjected to two-dimensional (2D)-PAGE to determine the effect of uterine space and day of pregnancy or pseudopregnancy on overall protein secretion rate and secretion of specific proteins. Fetal survival, fetal weight, and placental weight were decreased ( $p < 0.01$ ) in the crowded uterine environment compared to the roomy uterine environment. Incorporation of <sup>3</sup>H-leucine into nondialyzable macromolecules by endometrial tissue in culture was not affected by uterine space. Secretion of nondialyzable macromolecules by endometrium from pregnant pigs was not different from that by endometrium from pseudopregnant pigs on Day 40 but was greater ( $p < 0.01$ ) on Days 60 and 80. Results of 2D-PAGE indicated that secretion of two endometrial proteins appeared to be increased ( $M_r$  13 000, pI 7 and  $M_r$  13 000, pI 5.5), and secretion of one protein appeared to be decreased ( $M_r$  14 000, pI 6.2), in pregnant vs. pseudopregnant pig endometrial cultures. Decreasing uterine space did not affect incorporation of <sup>3</sup>H-leucine by placental tissue in culture. Examination of 2D-PAGE fluorographs of proteins secreted by placental tissue indicated that uterine space had no consistent effect on the array of proteins observed. However, secretion of a protein of  $M_r$  35 000, pI 4 increased by Day 60. Another protein ( $M_r$  45 000, pI 4) increased by Day 80. Two other proteins ( $M_r$  12 500, pI 4.6 and  $M_r$  13 000, pI 4.8) increased in some placental cultures by Day 60 of pregnancy and were consistently present in cultures from Day 80 placenta. These data indicate that uterine space does not influence endometrial or placental protein secretion on Days 40-80 of pregnancy. Secretion of specific proteins was greater by endometrium from pregnant compared to pseudopregnant pigs as early as Day 40 of pregnancy, and total endometrial protein secretion (measured as incorporation of <sup>3</sup>H-leucine into nondialyzable macromolecules) was greater for pregnant compared to pseudopregnant pigs by Day 60 of pregnancy; these findings suggest the possibility that the conceptus can alter endometrial protein secretion. The increase in endometrial protein secretion coincides with changes in secretion of specific placental proteins. These proteins or other compounds secreted by the conceptus may stimulate endometrial protein secretion during pregnancy.

### INTRODUCTION

The endometrium and placenta are the maternal and fetal components of the interface between the dam and the conceptus. Nutrients that are required for growth and development of the fetus must pass through the endometrium, either by diffusion or by facilitated transport. Some required nutrients are delivered as part of, or are bound to, proteins secreted by uterine endometrial glands [1]. Uteroferrin [2] and retinol-binding protein [3] are two such proteins that have been well characterized [4-7]. The array of proteins secreted by the endometrium during pregnancy is controlled predominantly by progesterone; the proteins secreted by endometrium from ovariectomized, progesterone-treated pigs are the same as those secreted by endometrium collected during pregnancy [8,9]. There is also

evidence that low concentrations of estrogens can synergize with progesterone to increase protein secretion by the endometrium [8,10] while high concentrations of estrogen may be inhibitory [9]. Comparing the secretion of proteins into culture by endometrium from the pregnant and the nonpregnant horn of unilaterally pregnant pigs has led to the conclusion that the conceptus increases the rate of protein secretion by the endometrium, but does not change the array of proteins secreted by the endometrium [11]. In swine, pseudopregnancy can be induced by injecting pigs with 5 mg/day estradiol valerate on Days 11-15 of the estrous cycle [12]. The proteins secreted by endometrium from pseudopregnant pigs and by endometrium from the pregnant and nonpregnant horns of unilaterally pregnant pigs are not different [11]. Presumably, the conceptus secretes factors that increase the endometrial protein secretion rate. Although the identity of the putative factor(s) is unknown, it is known that the conceptus secretes steroids [13], prostaglandins [14], and proteins [15]. Furthermore, the stage of pregnancy at which the conceptus becomes capable of stimulating the endometrium is unknown; in their experiments Basha et al. [11] reported data from Day 60 of preg-

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<sup>1</sup>Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the same by USDA implies no approval of the product to the exclusion of others that may also be suitable.

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nancy only. Finally, whether the conceptus-induced stimulation of secretion of endometrial proteins is responsive to restriction of uterine space is also unknown.

Because proteins secreted by the endometrium provide nutrients to the developing conceptus, factors capable of stimulating endometrial protein secretion might be used to increase the number of fetuses that the uterus can maintain until farrowing (i.e., uterine capacity). The objectives of the present experiment were to characterize the effect of the presence of the conceptus on endometrial protein secretion with respect to 1) stage of pregnancy and 2) uterine space. A further objective was to determine whether there are changes in secretion of specific placental proteins that are temporally associated with the onset of conceptus-induced enhancement of endometrial protein secretion.

## MATERIALS AND METHODS

Twenty-nine crossbred gilts were observed daily for estrous behavior. Pigs either were mated at estrus (pregnant group) or received i.m. injections of 5 mg estradiol valerate in corn oil on Days 11–15 (Day 0 = first day of estrus) of the subsequent cycle (pseudopregnant group). On Day 4, all pigs were laparotomized and the number of CL and length of both uterine horns were recorded; double ligatures were placed on one uterine horn, using procedures described by Dzuik [16], to create roomy and crowded uterine environments. With this procedure, the space for potential number of fetuses (based on number of CL) in the crowded uterine environment of pregnant pigs is one half that of the roomy uterine environment. Ligatures were similarly placed in pseudopregnant pigs to control for nonspecific effects of surgery. Pregnant and pseudopregnant pigs were killed on Days 40 ( $n = 3$  and  $n = 5$ , respectively) 60 ( $n = 5$  and  $n = 5$ , respectively), and 80 ( $n = 6$  and  $n = 5$ , respectively). These days were chosen so that changes in endometrial and placental protein secretion occurring during midpregnancy could be examined and the observations of Basha et al. [11] extended. Fetuses and placentae were weighed, length of each uterine environment was measured, and number of CL was recorded for each pig. Placental and adjacent endometrial tissues from three conceptuses within each uterine environment for pregnant pigs, and endometrial tissue from two random sites per uterine environment for pseudopregnant pigs, were collected into ice-cold minimum essential medium (MEM) (20 ml) having 0.1 times the normal amount of leucine among other modifications [17]. Endometrial and placental tissues (200 mg) were cultured in 0.1-strength leucine MEM (5 ml) in the presence of 50  $\mu$ Ci  $^3$ H-leucine for 24 h at 37°C under an atmosphere of 50% N<sub>2</sub>, 45% O<sub>2</sub>, and 5% CO<sub>2</sub>. Medium was separated from tissue by centrifugation (2500  $\times$  g for 10 min) and then frozen at -20°C until processed further. Only endometrial and placental tissues from pigs having conceptuses in both uterine environments were cultured.

Conditioned medium from endometrial and placental tissue cultures was dialyzed against 10 mM Tris, pH 8.2 (three 4-liter changes, each incubated overnight); then 100  $\mu$ l was used to determine incorporation of  $^3$ H-leucine into nondialyzable macromolecules by scintillation counting. Dialyzed samples of endometrial and placental tissue culture medium (2 ml) were lyophilized and then subjected to two-dimensional (2D)-PAGE and fluorography [18] for examination of proteins synthesized by the tissues in culture. Because all cultures contained the same tissue weight and volume of medium, results of 2D-PAGE and fluorography are directly comparable.

## Statistical Analysis

Length of uterus per embryo, embryo survival (number of fetuses/number of CL), fetal weight, and placental weight were analyzed using a model that included effects of day of pregnancy, pig within day of pregnancy, uterine environment, the uterine environment by day of pregnancy interaction, and the uterine environment by pig within day of pregnancy interaction. Two pigs on Day 80 of pregnancy had no fetuses in the crowded horn, so data from these pigs were excluded from analysis of fetal and placental weights and protein secretion in culture of endometrial and placental tissue. Fetal and placental weights were log transformed to alleviate heterogeneity of variance. Endometrial incorporation of  $^3$ H-leucine into nondialyzable macromolecules was analyzed using a model that included effects of reproductive status (pregnancy or pseudopregnancy), day (Day 40, 60, or 80 of pregnancy or pseudopregnancy), status by day interaction, pig within status by day interaction, uterine environment (roomy or crowded), status by uterine environment interaction, status by uterine environment by day interaction, and uterine environment by pig within status by day interaction. Because a significant status by day interaction was detected, data were further analyzed using the following orthogonal contrasts: 1) Day 60 pregnant vs. Day 80 pregnant, 2) Day 60 pseudopregnant vs. Day 80 pseudopregnant, 3) Day 40 pseudopregnant vs. Day 40 pregnant, 4) Days 60 and 80 pregnant combined vs. Days 60 and 80 pseudopregnant combined, and 5) Day 40 pregnant and pseudopregnant combined vs. Days 60 and 80 pregnant and pseudopregnant combined. Data for placental incorporation of  $^3$ H-leucine into nondialyzable macromolecules were examined by analysis of variance using a model that included effects of day of pregnancy, pig within day of pregnancy, uterine environment, the uterine environment by day of pregnancy interaction, and the uterine environment by pig within day of pregnancy interaction. In each analysis, error terms appropriate with the effect of pig as a random effect were used for tests of significance.

## RESULTS

Least squares means for length of uterus per embryo, fetal survival, fetal weight, and placental weight are illus-

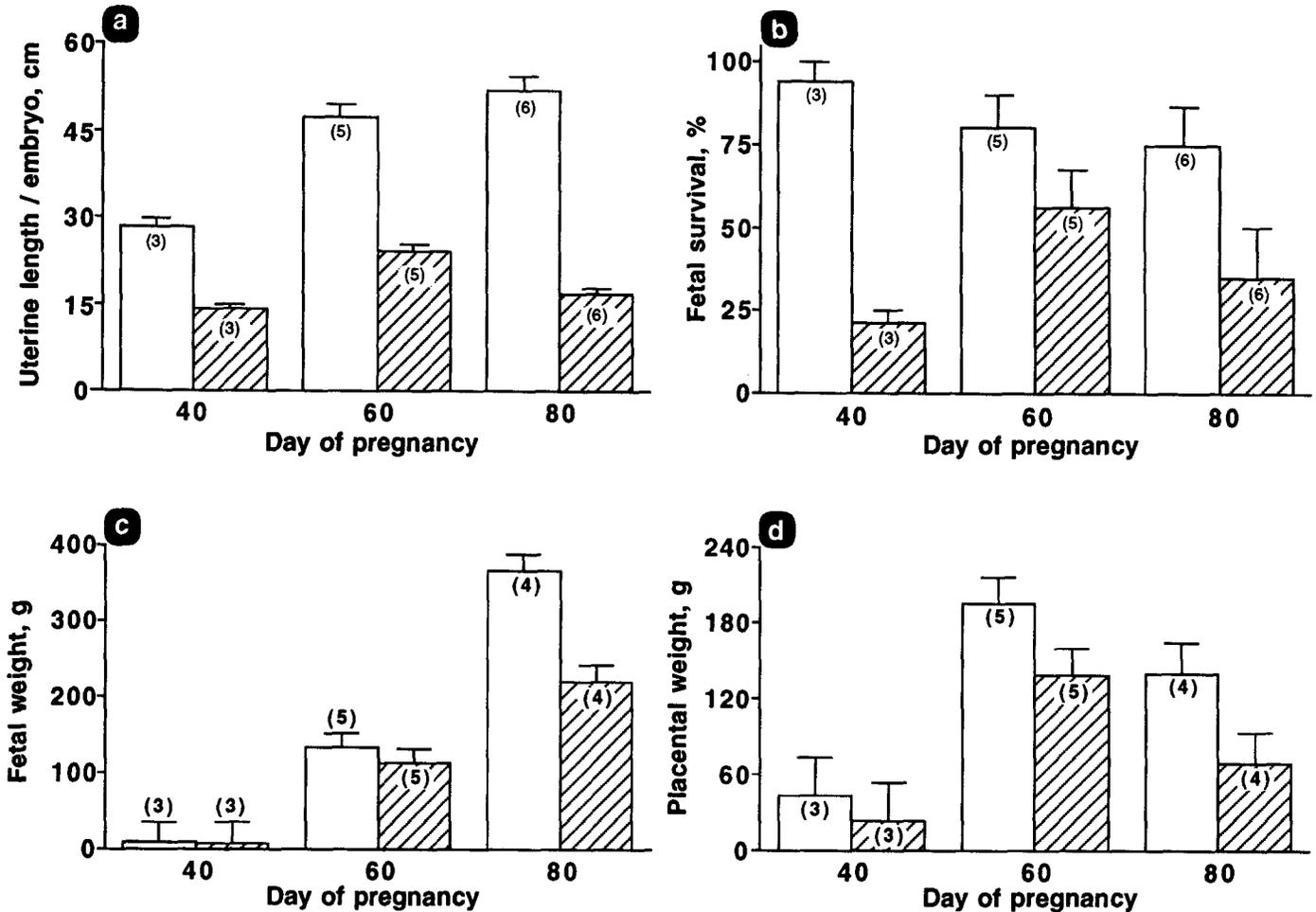


FIG. 1. Means ( $\pm$  SEM) for uterine length per embryo (a), fetal survival (fetus/number of CL) (b), fetal weight (c), and placental weight (d) for roomy (open bars) and crowded (hatched bars) uterine environments from pigs on Days 40, 60, or 80 of pregnancy are illustrated. Number in parentheses is number of observations (pigs) for each mean. Main effects of day of pregnancy ( $p < 0.05$ ) and uterine environment ( $p < 0.01$ ) were detected for (a), of uterine environment ( $p < 0.01$ ) for (b), of day of pregnancy ( $p < 0.01$ ) and uterine environment ( $p < 0.01$ ) for (c), and of day of pregnancy ( $p < 0.01$ ) and uterine environment ( $p < 0.01$ ) for (d).

trated in Figure 1. Length of uterus per embryo was reduced (effect of uterine environment,  $p < 0.01$ ) in the crowded uterine environment compared to the roomy uterine environment. No effect of day of pregnancy or interaction between day of pregnancy and uterine environment was detected. Fetal survival was also reduced ( $p < 0.01$ ) in the crowded vs. the roomy uterine environment. No effect of day of pregnancy or interaction of day of pregnancy with uterine environment was detected, indicating that the effect of uterine environment on fetal survival did not differ during the period examined.

Effects of day of pregnancy ( $p < 0.01$ ) and uterine environment ( $p < 0.01$ ) were detected for fetal and placental weights. No interaction between uterine environment and day of pregnancy was detected for fetal weight or placental weight.

Table 1 presents a summary of least squares means for endometrial incorporation of  $^3\text{H}$ -leucine into nondialyzable macromolecules in culture for the different treatment groups.

No main effect of uterine environment and no interaction with uterine environment was detected for endometrial incorporation of  $^3\text{H}$ -leucine into nondialyzable macromolecules. An interaction between reproductive status (pregnancy or pseudopregnancy) and day was detected ( $p < .01$ ) for endometrial incorporation of  $^3\text{H}$ -leucine into nondialyzable macromolecules. Contrasts indicated that incorporation of  $^3\text{H}$ -leucine on Day 40 of pregnancy was not different from incorporation on Day 40 of pseudopregnancy. Endometrial incorporation of  $^3\text{H}$ -leucine into nondialyzable macromolecules on Day 60 of pregnancy was not different from that on Day 80 of pregnancy. Likewise, incorporation on Day 60 of pseudopregnancy was not different from incorporation on Day 80 of pseudopregnancy. Incorporation of  $^3\text{H}$ -leucine on Days 60 and 80 of pregnancy combined was greater ( $p < 0.01$ ) than incorporation on Days 60 and 80 of pseudopregnancy combined. These results indicate that the conceptus stimulates endometrial protein secretion on Days 60 and 80 but not on Day 40 of pregnancy.

TABLE 1. Nondialyzable radioactivity (DPM  $\times 10^7$ /g tissue) in cultures of endometrial and placental tissue collected from roomy (R) or crowded (C) uterine environments on Days 40, 60, and 80 of pregnancy or pseudopregnancy.

	Day of pregnancy or pseudopregnancy					
	40		60		80	
	R	C	R	C	R	C
Endometrium-pregnant <sup>a</sup>	2.80 $\pm$ 0.41 (3) <sup>b</sup>	3.32 $\pm$ 0.41 (3)	4.96 $\pm$ 0.29 (5)	4.48 $\pm$ 0.29 (5)	3.72 $\pm$ 0.34 (4)	4.91 $\pm$ 0.34 (4)
Endometrium-pseudopregnant	2.08 $\pm$ 0.29 (5)	2.35 $\pm$ 0.29 (5)	2.67 $\pm$ 0.29 (5)	2.62 $\pm$ 0.29 (5)	2.52 $\pm$ 0.29 (5)	2.39 $\pm$ 0.29 (5)
Placenta <sup>c</sup>	1.59 $\pm$ 0.20 (3)	1.15 $\pm$ 0.20 (3)	1.40 $\pm$ 0.14 (5)	1.38 $\pm$ 0.14 (5)	1.56 $\pm$ 0.16 (4)	1.37 $\pm$ 0.16 (4)

<sup>a</sup>Mean incorporation was greater ( $p < 0.01$ ) by endometrium from pregnant pigs than by endometrium from pseudopregnant pigs on Day 60 and 80 but not Day 40.

<sup>b</sup>Least Squares Means  $\pm$  SEM from analysis of variance. Number of observations (pigs) for each mean is in parentheses.

<sup>c</sup>No effect of day, uterine environment or day, by uterine environment interaction was detected.

Representative fluorographs showing the radiolabeled proteins present in endometrial tissue culture medium from Days 40, 60, and 80 of pregnancy and pseudopregnancy are illustrated in Figure 2. No consistent effect of uterine space was observed on the pattern of proteins observable via 2D-PAGE and fluorography (not shown). Endometrial secretion of a protein of  $M_r$  13 000, pI 7 was enhanced in pregnant vs. pseudopregnant pigs on all days examined (protein 2, Fig. 2). Also, endometrial secretion of another protein ( $M_r$  13 000, pI 5.5; protein 3, Fig. 2) was enhanced for pregnant pigs in a day-specific manner, with the greatest difference between pregnant and pseudopregnant pigs observed on Day 60 of pregnancy. Finally, endometrial secretion of another protein ( $M_r$  14 000, pI 6.2; protein 1, Fig. 2) was decreased in pregnant pigs compared to pseudopregnant pigs. These observations are evidence of conceptus-induced changes in secretion of specific proteins.

Placental incorporation of radioactivity into nondialyzable macromolecules (Table 1) was not affected by day of pregnancy or uterine environment. No interaction between uterine environment and day of pregnancy was detected.

Radiolabeled proteins secreted into the culture medium by placental tissue from Days 40, 60, and 80 are illustrated in Figure 3. Increased secretion of a protein of  $M_r$  35 000, pI 4 (protein 1, Fig. 3) was consistently observed on fluorographs of proteins secreted by Day 60 compared to Day 40 placental tissue. Two proteins with  $M_r$  13 000, pI 4.8 and  $M_r$  12 500, pI 4.6 (proteins 2 and 3, Fig. 3) were observed on some fluorographs from Day 60 of pregnancy and were consistently observed on fluorographs from Day 80 of pregnancy. Also, on Day 80 of pregnancy, another protein ( $M_r$  45 000, pI 4; protein 4, Fig. 3) was increased. The secretion of other proteins was not affected consistently by day of pregnancy. No consistent effect of uterine environment on the array of proteins secreted by placental tissue in culture was observed (not shown).

## DISCUSSION

Results indicate that the presence of the conceptus caused changes in the secretion of specific proteins by the endometrium between Days 40 and 80 of pregnancy, a result that contrasts with the results of Basha et al. [11]. Findings

further demonstrate changes in secretion of specific placental proteins that occur coincidentally with the onset of conceptus-induced enhancement of endometrial protein secretion. The temporal association of increased secretion of these placental proteins with increased endometrial protein secretion suggests, but does not prove, the possibility that one may cause the other.

Results of the current experiment confirm and extend some of the observations of Basha et al. [11]. These results indicate that the effect of the conceptus on endometrial protein secretion is not present on Day 40 of pregnancy but is present on Days 60 and 80 of pregnancy. Day 60 of pregnancy is approximately the time when the placenta reaches its maximum weight [19] and fetal growth accelerates [19]. It is tempting to speculate that the enhancement of endometrial function is an adaptation of the placenta and endometrium to the cessation of placental growth and the onset of rapid fetal growth.

Basha et al. [11] concluded that the pattern of proteins synthesized by pregnant and pseudopregnant pig endometrium was similar and also that only the overall rate of protein secretion was affected by the conceptus. In contrast, results of the present study indicate that secretion of specific proteins appears to be increased in the presence of the conceptus and that one protein is secreted by pseudopregnant pig endometrium but not by pregnant pig endometrium. The difference among results may be due to methodology. Basha et al. [11] used <sup>35</sup>S-methionine to radiolabel proteins secreted in culture. Methionine is a less common amino acid in proteins than leucine. Therefore, some proteins that we were able to detect using leucine may not have been detected by Basha. Furthermore, the 2D-PAGE fluorographs presented by Basha et al. [11] were not exposed as long as those in the current experiment. In addition, the proteins observed in the present experiment are low-molecular-weight proteins that might have been below the molecular weight range of the 2D-PAGE gels of Basha et al. [11] despite their use of similar 2D-PAGE methodology. The fact that these specific proteins may be controlled by the conceptus suggests the possibility that they may be important in some aspect of conceptus growth or development during this period of pregnancy. Because se-

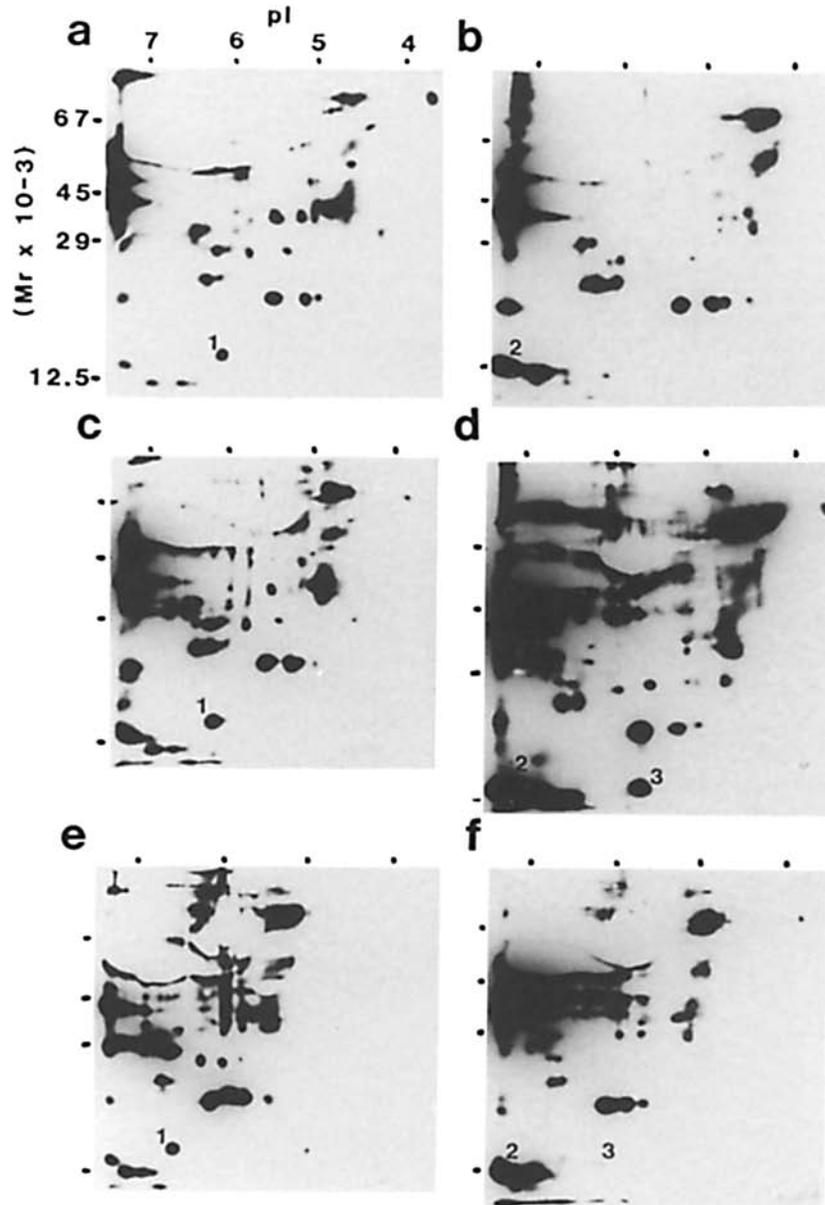


FIG. 2. Representative fluorographs of 2D-PAGE gels of proteins secreted in culture by endometrial tissue from pseudopregnant (a, c, e) and pregnant (b, d, f) pigs collected on Day 40 (a, b), 60 (c, d), or 80 (e, f) of pregnancy or pseudopregnancy. Protein 1 was consistently increased in pseudopregnant pigs. Protein 2 was consistently increased in endometrium from pregnant compared to pseudopregnant pigs on all days examined. Protein 3 was increased in pregnant pigs compared to pseudopregnant pigs primarily on Day 60 and to a lesser extent on Day 80 of pregnancy. No consistent changes in other proteins were observed.

cretion of these proteins is not influenced by uterine space, the decrease in placental weight would mean a decrease in access to endometrial surface area when uterine space per conceptus is decreased. This could limit the amount of these proteins available to the developing conceptus, resulting in decreased growth of the conceptus. Identification of these endometrial proteins and their function during this period of pregnancy therefore warrants further investigation.

The mechanism for the increase in endometrial protein secretion in the presence of the conceptus is not known.

However, changes in conceptus secretion of steroids, prostaglandins, proteins, or many other biologically active compounds may be responsible. The results of Basha et al. [11], which indicated that the conceptus effect was present only in the gravid horn of unilaterally pregnant gilts, rule out the possibility of a systemic effect caused by the presence of the conceptus. Differences in progesterone secretion between pseudopregnant and pregnant pigs is a possible explanation of the conceptus effect; however, progesterone secretion in pregnant and pseudopregnant pigs is similar

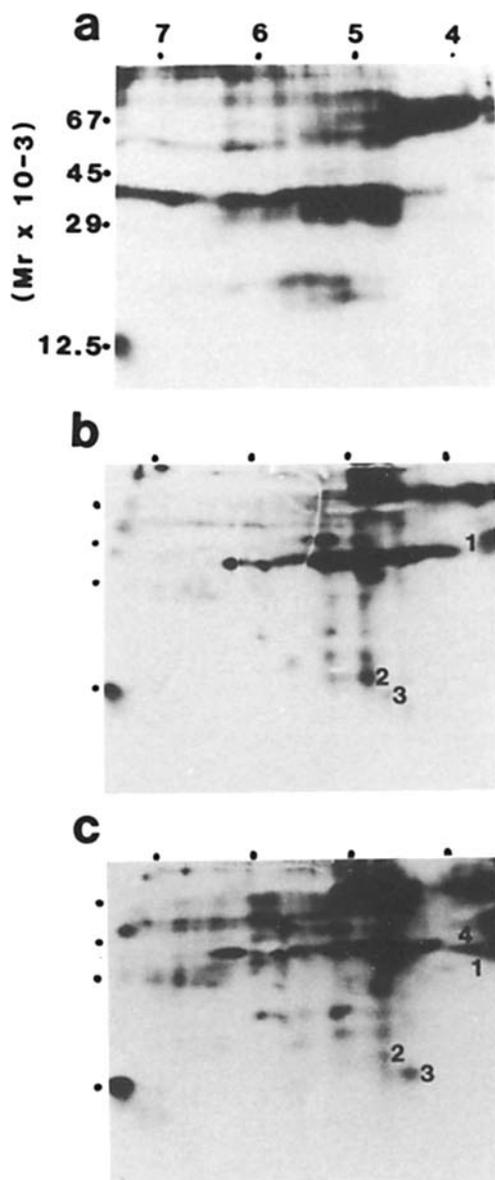


FIG. 3. Representative fluorographs of 2D-PAGE gels of proteins secreted in culture by placental tissue collected on Days 40 (a), 60 (b), and 80 (c) of pregnancy. Protein number 1 was consistently increased by Day 60 of pregnancy. Proteins 2 and 3 were increased in some cultures on Day 60 and were consistently present on Day 80. Protein 4 increased consistently between Day 60 and Day 80 of pregnancy. No consistent changes in other proteins were observed.

[12]. While the porcine conceptus does secrete progesterone, the amount secreted by the conceptus is insufficient to maintain pregnancy [20]. Therefore, differences in progesterone between pregnant and pseudopregnant pigs is not a likely explanation for the effect of the conceptus. The porcine placenta secretes estrogen [13], and estrogen has been reported to both enhance (low doses [8, 10]) and inhibit (high doses [9]) endometrial protein secretion. Secretion of estrogen by the porcine conceptus reaches a peak about Day 30, falls to a nadir near Day 40, and then in-

creases steadily throughout the remainder of gestation [21]. An inhibitory effect of estrogen does not seem a possible explanation because the presence of the conceptus, which secretes estrogen, enhanced rather than inhibited protein secretion. If estrogen is stimulatory, then the effect of the conceptus should reflect the increase in estrogen secretion from Day 60 to Day 80. However, the effect of presence of the conceptus on total endometrial protein secretion, as measured by incorporation of <sup>3</sup>H-leucine into nondialyzable macromolecules, did not differ on Day 60 compared to Day 80. It therefore seems unlikely that estrogen secretion by the pig placenta is the conceptus factor involved. Further experimentation will be required to determine the conceptus factor responsible for the effects observed here.

The molecular weight and isoelectric point of placenta-secreted proteins 1 and 4 are similar to those of placenta-secreted proteins whose secretion was enhanced in a crowded uterine environment on Days 25 and 35 of pregnancy [17]. Results of the present study indicated that these proteins are unaffected by the uterine environment on Days 40, 60, or 80 of gestation. It is possible that conditions that evoke the response on Days 25 and 35 are alleviated by Day 40 of pregnancy so that this effect of uterine space is no longer observed. One possibility is that the loss of conceptuses that occurred before Day 40 because of uterine crowding might have improved the ability of the remaining fetuses to survive. This hypothesis is supported by the observation that significant conceptus loss had already occurred by Day 40 of pregnancy in the present experiment and that no further conceptus loss occurred during later pregnancy. Alternatively, the conceptuses may respond to uterine crowding during only a limited period of gestation. The function of these proteins during normal pregnancy (Days 60 to 80) and under crowded uterine conditions (Days 25 to 35) warrants further investigation to reconcile the results of the current experiment with those of previous experiments.

In conclusion, the study reported here has demonstrated that the presence of the conceptus results in increased endometrial protein secretion in culture on Days 60 and 80 of pregnancy but not on Day 40. This effect is not strictly a general effect of the conceptus on overall endometrial protein secretion rate, because specific proteins are increased at the same time that others appear to be either unaffected or only marginally affected. The effect of the presence of the conceptus on endometrial protein secretion coincides temporally with increased secretion of four placental-secreted proteins, suggesting the possibility that these proteins could be involved in stimulating endometrial function. Further experimentation is required to elucidate the functions of the endometrial proteins that increase or decrease in the presence of the conceptus, as well as the role of the placental-secreted proteins that increase during this time. An understanding of the mechanism by which the conceptus stimulates endometrial protein secre-

tion may be useful in alleviating some of the effects of a crowded uterine environment and therefore may allow an increase in uterine capacity and litter size.

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