INTRAUTERINE MIGRATION OF THE PORCINE EMBRYO—INTERACTION OF EMBRYO, UTERINE FlushINGS AND INDOMETHACIN ON MYOMETRIAL FUNCTION IN VITRO

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Summary

Twenty-four pregnant Landrace and Yorkshire gilts were utilized to examine the relationship between myometrial activity and intrauterine embryo migration. On d 2 (1st day of estrus = d 0), embryos were flushed from one oviduct and transferred to the opposite oviduct. Uterine horns were ligated at the uterotubal junction on the flushed side and 40 and 50 cm posterior to the uterotubal junction on each side. On d 6 or 9 or 12 (n = 8), embryo migration was determined by flushing segments of the excised uterus. Strips of myometrium from the pregnant and nonpregnant horn of each gilt were subsequently removed and assigned to one of three in vitro experiments to examine the effects of the embryo and uterine flushings on myometrial contractility. Myometrial contractility increased concomitantly with embryo migration through the uterine ligations (day x side interaction, P<.10). Uterine flushings from pregnant horns contained a short-acting substance that mimicked, in part, the stimulatory influence(s) of the in situ embryo on in vitro myometrial contractility. However, only flushings from the uterine segment containing the d 12 embryos could overcome the in vitro inhibitory effects of indomethacin (P<.01) on myometrial contractility. The porcine embryo coincubated with myometrial strips could not directly stimulate contractions of the myometrium. Results of these experiments indicate that in gilts the stimulatory influence(s) of the migrating embryo on myometrial function may involve a "hormonal" factor of short half-life that does not directly affect the smooth muscle cell. (Key Words: Porcine, Embryo, Intrauterine Migration, Myometrium, Indomethacin.)

Introduction

Overcoming the barrier imposed by uterine capacity (Bazer et al., 1969) is a prerequisite to increasing the number of offspring born at each parturition. All litter-bearing species attempt to reduce this barrier by spacing their embryos before attachment or implantation. Patten (1948), Dhindsa et al. (1968), Waite and Day (1967), Polge and Dziuk (1970) and Anderson (1978) determined that intrauterine migration of porcine embryos occurs between d 7 and 12 (d 0 = 1st day of estrus). A number of hypotheses have been proposed to explain intrauterine migration of embryos. These postulations range from the existence of uterine refractory zones to embryo attachment (Mossman, 1937), differential growth of the uterus (Defeo and Kleinfeld, 1971) and repulsive electrical charge between embryos (Clemetson et al., 1971) to the more popular hypothesis of peristaltic contractions of the myometrium (Corner, 1921; Keye, 1923; Wislocki and Guttmacher, 1924; Kho-Seng Lim and Chao, 1927; Böving, 1971; Pusey et al., 1980). This latter hypothesis, however, is confounded by the reported quiescent nature of the myometrium when under the influence of progesterone such as during the time of intrauterine migration of embryos (Keye, 1923; Wislocki and Gutta-
macher, 1924; King and Young, 1957; Zerobin, 1968). If the peristaltic contraction of uterine smooth muscle is involved, then it would seem that porcine embryos can overcome the effect of progesterone and stimulate myometrial activity.

The present experiments were conducted to determine whether the presence of the porcine embryo increased myometrial activity in vitro and the mechanisms by which the embryo affects the function of the myometrium.

Materials and Methods

Twenty-four Landrace and Yorkshire gilts were assigned in equal numbers to one of three groups as they were detected in estrus (d 0). On d 2 of gestation gilts were anesthetized with sodium pentobarbital and halothane and subjected to mid-ventral laparotomy. Embryos were flushed from one oviduct chosen at random by use of medium effluxed from the uterotubal junction towards the cannulated ampulla and then transferred to the ampulla of the opposite oviduct. Single ligatures using umbilical tape (.32 cm wide) were subsequently placed firmly around the uterine horn at the uterotubal junction of the flushed oviduct and 40 and 50 cm posterior to the uterotubal junction of each horn. This procedure created a "pouch" in the tip of each uterine horn such that the effects of the embryo on uterine physiology were initially concentrated on one side.

On either d 6, 9 or 12 of gestation gilts were again laparotomized and the uterine horn, to which the embryos had been transferred, was flushed from the external bifurcation to the most posterior ligation (50 cm from the uterotubal junction). This procedure permitted detection of any embryos that had migrated through the ligations. Subsequently, the anterior one-half of both horns was excised and transferred to the laboratory in oxygenated Krebs-Ringer-Bicarbonate solution (39°C; DeLuca and Cohen, 1964). At the laboratory, the middle of each pouch was clamped with a hemostat and the resulting anterior and posterior halves of each pouch flushed (35 ml; 30.3 ± .3 ml recovered) to recover the embryos. The area between the 40- and 50-cm ligations was also flushed. Samples of myometrial tissue were immediately removed for utilization in one of three experiments.

Medium used for transferring embryos, flushing the uterus and incubation (Exp. 3) was Modified Ham's F-10 (Kane and Foote, 1970).

Exp. 1. Circumferential strips of myometrium (.5 × 4.0 cm) were excised from the uterine segment containing the most embryos (pregnant horn) and the corresponding segment of the nonpregnant horn (P and NP muscle strips, respectively). Muscle strips were suspended in baths containing 60 ml oxygenated Krebs-Ringer-Bicarbonate solution (39°C) and allowed 30 min to equilibrate to a base tension of 1 g. Changes in tension due to isometric contractions were monitored for frequency and amplitude with Grass Force-Displacement Transducers (FT10C) and recorded on a Gould Brush 2400 two-channel chart recorder. A contraction was defined as an increase in tension following a relaxation. The initial activity of the muscle strips was monitored for 10 min after the equilibration period. Intramural nerves were subsequently subjected to electrical field stimulation through two bipolar platinum electrodes situated parallel to the muscle strips (30 s, 70 V, 10 Hz and 1 ms duration). After 5 min the muscle was exposed to a bolus injection (80 μl) of isoproterenol (a β-adrenergic agonist, concentration in the bath 80 ng/ml) and monitored for 10 min. The baths were then flushed twice and refilled with Krebs-Ringer-Bicarbonate solution and the muscle strips allowed 15 min to recover to the initial activity. Subsequently, the muscle strips were exposed to propranolol (a β-adrenergic antagonist, 200 μl bolus; 200 ng/ml of medium) and Levophed bitrate (norepinephrine, an α- and β-adrenergic agonist, 20 μl bolus; 20 ng/ml of medium) and the activity monitored for another 10 min. Results of a preliminary trial using tissue from four gilts demonstrated that antagonism of β-adrenergic responses of myometrium with propranolol (200 ng/ml) did not alter myometrial contractility. The concentrations of drugs used in this experiment were predetermined to be within the linear portion of the dose-response curve for each respective drug. Finally, the tissue was washed and the base tension was increased from 1 to 5 g. Increasing the base tension from 1 to 5 g provided an indication of the work performance of the muscle (King, 1927).

Data were expressed as either frequency (the number of contractions during a 10 min period) or Montevideo units (frequency times amplitude; Caldeyro-Barcia and Poseiro, 1960). Due
to heterogeneity of variance, data were subjected to a logarithmic transformation and analyzed by use of a split-split-plot analysis of variance. Transformed means were compared by a protected Least Significant Difference test.

Exp. 2. Two additional muscle strips from the nonpregnant uterine horn were excised from the same location as in Exp. 1 and placed into Krebs-Ringer-Bicarbonate solution (4 C) until they could be placed into the muscle baths. As the myometrium warmed to 39 C, contractile activity commenced (5 to 10 min), after which the muscle strips were allowed 30 min to equilibrate to 1 g of tension. The initial activity of the muscle strips was monitored for 10 min after the equilibration period. Subsequently, 5 ml of Krebs-Ringer-Bicarbonate solution in the muscle bath was replaced with 5 ml of flushings (39 C) from either the uterine segment containing the most embryos (P flushings) or the corresponding segment of the nonpregnant horn (NP flushings). The flushings were centrifuged at 800 x g for 20 min to remove cellular debris and kept at 39 C until utilized. The remaining flushings were refrigerated (4 C) until utilized in Exp. 3. The flushings were allowed 5 min to affect myometrial activity after which they were removed and the muscle strips were washed. Fifteen minutes after washing, indomethacin in 100 /~1 ethanol was placed into the muscle baths for 10 min (final concentration, 10 /~g/ml). Indomethacin, an inhibitor of prostaglandin synthesis, was included because the porcine embryo, at the time of migration, causes an increase in the concentration of intrauterine prostaglandins (Zavy et al., 1980). The tissue was washed after exposure to indomethacin and allowed 15 min to recover before flushings were again added. In the presence of the flushings, indomethacin was again added to the muscle baths and the response of the muscle monitored for another 10 min.

Due to the consistency of amplitudes, data were expressed only as frequency of contractions. The initial frequency was subtracted from all the subsequent responses and the resulting data analyzed as in Exp. 1.

Exp. 3. Uterine tissue from the nonpregnant horn was removed circumferentially at a width of .5 cm, trimmed of endometrium and incubated with or without embryos and either P or NP flushings resulting in an experiment of 2 x 2 factorial design. The incubation conditions were optimized in a series of preliminary trials. These conditions consisted of maintaining a neutral pH under an atmosphere of 95% O2-5% CO2 (3 mm pressure). Tissue was incubated (39 C) for 20 h in Modified Ham's F-10 at a volume of 5 and 15 ml (the latter containing 3 ml of either P or NP flushings; final concentration of flushings = 20%) for the first 6 and the remaining 14 h, respectively. Incubations were terminated by opening the uterine tissue mesometrially and transferring these muscle strips to baths as described above. Contractile activity was then observed for 20 min at 1 g of tension.

Data were expressed as Montevideo units and analyzed by use of a split-split-plot analysis of variance with the sub-subplots partitioned into a 2 x 2 arrangement (embryo x flushings).

Estradiol-17ß Assay. The remaining uterine flushings and the media following the 20-h incubation were subsequently analyzed for estradiol-17ß by radioimmunoassay procedures previously described by Redmer and Day (1981). This procedure was modified by extracting 5 to 10 ml of flushing or medium three times with 10 ml of diethyl ether (extraction efficiency, >95%). The antiserum used by Redmer and Day (1981) was diluted to a final concentration of 1:60,000. Intra-assay coefficient of variation (n = 7) was 11.4%. Final amounts of estradiol in NP flushings, P flushings and incubation medium were subjected to a logarithmic transformation and compared by use of an analysis of variance. Differences between transformed means were tested for significance by a protected Least Significant Difference test.

Results

Examination of the uterine flushings revealed limited intrauterine migration of d 6 embryos (figure 1). However, d 9 embryos had begun to migrate through the ligations and by d 12 some of the embryos had migrated sufficiently past the ligatures to be recovered in the posterior portion of the uterine horn.

The mean content of estradiol-17ß in uterine flushings from the P and NP horns increased by d 12, but the estradiol content of P flushings of d 12 gilts was greater than that of NP flushings (day x side interaction, P<.01, figure 2).
Figure 1. Distribution of embryos within the uterine horns of gilts on d 6, 9 and 12 (1st day of estrus = d 0) of gestation. For illustrative purposes the left horn contains the embryos.
but NP muscle strips failed to increase until d 12 of gestation (day x side x tension interaction, P<.01). At 5 g base tension the frequency of contractions of P and NP muscle strips did not increase until d 12 (figure 3). Similarly, when the muscle activity was expressed as Montevideo units (figure 4), a day x side interaction (P<.10) was evident at both base tensions. The pattern of myometrial activity was similar at both base tensions; activity (Montevideo units) of the P muscle strips increased in a linear fashion from d 6 while that of the NP muscle strips did not increase until d 12.

Electrical field stimulation produced no alterations in contractile activity of the myometrium. The addition of isoproterenol reduced (P<.05) the frequency of contractions of both P and NP muscle strips (table 1). Reduction in the number of contractions was indicative of β-adrenergic stimulation because the base tension was below 1 g. Furthermore, the response of the myometrium of both horns to the inhibitory effect of isoproterenol increased (P<.05) by d 9 and 12 of gestation. Although this increase in β-adrenergic responsiveness did not correspond with the initial activity at 1 and 5 g tension, it may be consistent with the

Figure 2. Quantity of estradiol-17β (pg) within the P and NP flushings of d 6, 9 and 12 gilts. Each point represents the mean of eight gilts. The transformed error mean squares for day and side (main and subplot) are .99 (21 df) and .80 (21 df), respectively.

Figure 3. Frequency of contractions of myometrial strips excised from d 6, 9 or 12 pregnant gilts at 1 and 5 g tension. Each point represents the mean of eight gilts. The transformed error mean squares for day, side and tension (main, subplot and sub-subplot, respectively) are .0036 (21 df), .0003 (21 df) and .0003 (42 df), respectively.
Figure 4. Activity (Montevideo units) of myometrial strips excised from d 6, 9 or 12 pregnant gilts at 1 and 5 g tension. Each point represents the mean of eight gilts. The transformed error mean squares for day, side and tension (main, subplot and sub-subplot, respectively) are .0224 (21 df), .0055 (21 df) and .0041 (42 df), respectively.

The quiescent nature of the uterine nervous system at this time. Exposure to propranolol (at a dosage sufficient to antagonize the β-adrenergic receptors) and norepinephrine increased the base tension and the frequency of contractions (P<.05) relative to the initial activity.

Exp. 2. Results of this experiment demonstrated that only the addition of P flushings recovered on d 12 increased (P<.01) the frequency of contractions of NP muscle strips of d 12 gilts above basal levels (figure 5). Indomethacin reduced the frequency of contractions of NP muscle strips (d 6, 9 and 12) to the same extent regardless of whether previously exposed to P or NP flushings. Reintroducing flushings after indomethacin failed to alter the frequency of contractions from the initial activity. In combination with indomethacin,

TABLE 1. PERCENTAGE CHANGE IN FREQUENCY OF UTERINE CONTRACTIONS FOLLOWING EXPOSURE TO α- AND β-ADRENERGIC AGONISTSA,b

<table>
<thead>
<tr>
<th>Item</th>
<th>Day 6</th>
<th>Day 9</th>
<th>Day 12</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P</td>
<td>NP</td>
<td>P</td>
</tr>
<tr>
<td>ISO (%)c</td>
<td>45.3e</td>
<td>40.7e</td>
<td>82.7f</td>
</tr>
<tr>
<td>NE (%)d</td>
<td>33.3</td>
<td>23.5</td>
<td>22.1</td>
</tr>
</tbody>
</table>

aNumbers represent the mean of eight myometrial strips.
bThe transformed error mean squares for day, side and agonist (main, subplot and sub-subplot, respectively) are .010 (21 df), .005 (21 df) and .011 (42 df), respectively.
cIsoproterenol (80 ng/ml).
dPropranolol (200 ng/ml) plus levophed bitartrate (norepinephrine, 20 ng/ml).
e,fMeans with different superscripts differ (P<.05).
only P flushings of d 12 gilts overcame the suppressive effects of the drug on frequency of contractions of the myometrium (P<.01).

Exp. 3. Results of this experiment are presented in table 2. Activity of P and NP muscle strips, though depressed after the 20-h incubation in the presence of corresponding flushings and in the presence and absence of the embryos, was greater (P<.05) for d 9 gilts than either d 6 or 12 gilts. There were, however, no side differences (P vs NP) in muscle activity. Likewise, neither the addition of flushings nor the embryo altered muscle activity.

The embryos remained viable under the experimental conditions because d 6 embryos hatched from the zona pellucida. Additionally, the 6, 9 and 12 embryos synthesized estradiol during the 20-h incubation (table 3) in quantities similar to those synthesized by comparable embryos in vivo (figure 2).

Discussion

Results of the first experiment demonstrated that intrauterine migration of the porcine embryo through uterine ligations coincided with increased myometrial activity. By d 9 of gestation embryos migrated through the first ligation (40 cm from the uterotubal junction), 3 d later, d 12, embryos had migrated through the second and most posterior ligation. Dhindsa and Dziuk (1968) observed that porcine embryos could migrate through double ligations placed one-third but not through similar ligations placed two-thirds the distance from the uterotubal junction to the uterine body. The activity, frequency of contractions or Montevideo units, of P muscle strips of d 9 and 12 gilts increased relative to P muscle strips of d 6 gilts. Muscle strips removed from an area of the uterus devoid of embryos (NP muscle strips) failed to increase in frequency of contractions or Montevideo units until d 12. Although it is not possible to conclude that embryo migration was due to increased myometrial function, these data do support the supposition of myometrial involvement in embryo migration set forth earlier (Corner, 1921; Keye, 1923; Wislocki and Guttamacher, 1924; Böving, 1971; Pusey et al., 1980).

The increase in frequency of contractions of NP muscle strips of d 12 gilts was not expected and is not consistent with the quiescent nature of the uterus as observed in gilts on d 12 of the estrous cycle (Keye, 1923; Wislocki and Guttamacher, 1924; King and Young, 1957; Zerobin, 1968). Perhaps by d 12, the stimulatory influence of the embryo becomes systemic. Alternatively, embryos may have migrated through the uterine body to a position posterior to the ligations on the nonpregnant horn, thereby, influencing myometrial function.

It does not appear from these data that the presence of the embryo stimulated muscle activity through the autonomic nervous system because the porcine uterine nerves were quiescent at this time. This apparent quiescence of intramural nerves was confirmed by an independent procedure. The addition of physiological (100 ng/ml) and then pharmacological doses (>10 μg/ml) of both imipramine and
TABLE 2. ACTIVITY (MONTEVIDEO UNITS) OF MYOMETRIAL STRIPS FOLLOWING INCUBATION WITH OR WITHOUT EMBRYOS AND FLUSHINGS\textsuperscript{a,b}

<table>
<thead>
<tr>
<th>Day</th>
<th>P tissue With embryo</th>
<th>P tissue Without embryo</th>
<th>NP tissue With embryo</th>
<th>NP tissue Without embryo</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>6.4</td>
<td>7.4</td>
<td>5.1</td>
<td>5.2</td>
</tr>
<tr>
<td>9</td>
<td>4.9</td>
<td>9.5</td>
<td>11.1</td>
<td>9.3</td>
</tr>
<tr>
<td>12</td>
<td>6.3</td>
<td>3.7</td>
<td>3.4</td>
<td>3.5</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Numbers represent the mean of eight myometrial strips.

\textsuperscript{b}Standard error based on the common estimate of variance = 5.5.

tyramine failed to significantly alter responses of the porcine myometrium. These drugs, in combination, concentrate the neurotransmitter present in the varicosities within the neurovascular junction.

Alpha- and \( \beta \)-adrenergic stimulation was sufficient to conclude that the porcine myometrium contains receptors for both these agonists. Response of P and NP muscle strips to the \( \beta \)-adrenergic agonist increased concomitantly with the intrauterine migration of the blastocysts. However, this response might be due to an increase in myometrial \( \beta \)-adrenergic receptors induced by the increased levels of estrogen being synthesized by the embryo or endometrium. Krall et al. (1978) demonstrated that estrogen increased \( \beta \)-adrenergic receptors in the myometrium of the rat.

Because the stimulatory influence of the embryo was not neural, it was concluded that the factor(s) responsible for increasing muscle activity was \textit{"hormonal"} and affected the smooth muscle cells of the uterus. Results of Exp. 2 demonstrated that some short-acting component of the d 12 uterine flushings associated with the embryo increased the frequency of muscle contractions. This factor(s) found in the flushings mimicked, in part, the changes in intrinsic muscle activity observed in Exp. 1. The d 12 embryo was able to alter uterine flushings sufficiently to overcome the inhibitory effects of indomethacin. Vane and Williams (1973) observed the ability of indomethacin to reduce spontaneous activity of rat myometrium. Indomethacin has been shown to inhibit: 1) prostaglandin synthesis (Vane, 1971), 2) protein kinase (Catalán et al., 1980; Goueli and Ahmed, 1980), 3) prostaglandin binding to serum proteins, thus increasing the rate of oxidation of prostaglandin (Attallah and Lee, 1980), and 4) availability of calcium within the smooth muscle cell (Northover, 1977). Results of the present experiment suggest the nature of the active component in d

TABLE 3. QUANTITY OF ESTRADIOL-17\( \beta \) (PG) IN THE INCUBATION MEDIUM FOLLOWING EXPOSURE OF MYOMETRIAL STRIPS TO FLUSHINGS AND EMBRYOS\textsuperscript{a,b}

<table>
<thead>
<tr>
<th>Day</th>
<th>P tissue With embryo</th>
<th>P tissue Without embryo</th>
<th>NP tissue With embryo</th>
<th>NP tissue Without embryo</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>36.8</td>
<td>33.4</td>
<td>35.4</td>
<td>38.3</td>
</tr>
<tr>
<td>9</td>
<td>180.7</td>
<td>27.9</td>
<td>49.8</td>
<td>32.0</td>
</tr>
<tr>
<td>12</td>
<td>3,512.3</td>
<td>2,677.4</td>
<td>1,119.5</td>
<td>56.7</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Numbers represent the mean of eight observations.

\textsuperscript{b}The transformed error mean squares for day, side and incubation (main, subplot and sub-subplot, respectively) are 2.09 (21 df), .34 (21 df) and .20 (126 df), respectively.
12 flushings to be a prostaglandin. Zavy et al. (1980) observed an increase in the concentration of luminal prostaglandin $F_2\alpha$ in uteri of pregnant gilts beginning 12 d postmating.

Uterine flushings coincubated with myometrium for 20 h (Exp. 3) failed to alter muscle activity. However, flushings used immediately after recovery from the uterus were able to affect myometrial function within a 5 min exposure period (Exp. 2). Perhaps the reduced activity of the muscle strips after a 20-h incubation precluded any change in activity produced by the flushings. Alternatively, these observations suggest either some sort of destabilization of the factor(s) found in flushings or destabilization of an endometrial intermediate initially present in the uterine flushings. The increased activity following incubation of myometrium of d 9 gilts is assumed to be due to chance alone or some component of uterine physiology not investigated in the present experiment.

Estradiol synthesis by the blastocyst was also coincident with migration of the blastocyst through the ligations. The d 12 embryos coincubated with myometrium synthesized estradiol in culture but were unable to increase muscle activity as determined after coincubation. In a separate trial, a 20-h incubation of muscle strips of d 12 nonpregnant gilts either in the presence or absence of estradiol (1 ng/ml media) failed to increase muscle activity (n = 5, 12.8 ± 6.4 vs 19.6 ± 10.5 Montevideo units, respectively). These data suggest that either estradiol is not involved in increasing muscle activity or, under these conditions, could not directly stimulate smooth muscle cells. The latter supposition might involve estrogen stimulation of an intermediate(s) such as prostaglandin or histamine which in turn activates the myometrium.

Understanding the mechanism(s) involved in the descent and eventual distribution of embryos in polyovular species might prove valuable to increasing litter size. Results of these experiments suggest an involvement of myometrial function in displacing the porcine embryos through the uterine lumen. This stimulatory influence(s) of the embryo appears to be "hormonal" rather than neural. The effect of the embryo does not appear to be exerted directly on the smooth muscle cell, but rather may involve an intermediate of short half-life.

**Literature Cited**


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