Decreased follicular size during late lactation caused by treatment with charcoal-treated follicular fluid delays onset of estrus and ovulation after weaning in sows

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ABSTRACT: The weaning to estrus and weaning to ovulation intervals in sows are controlled by ovarian follicular growth after weaning. Longer intervals could be caused by smaller diameter follicles at weaning that take more time to reach a preovulatory size. We addressed this hypothesis by decreasing the diameter of follicular populations before weaning and then measuring follicular development and interval to estrus and ovulation after weaning. The posterior vena cava, cranial to the entry of the ovarian vein, was cathetered for blood sampling and infusion in 20 sows at 12 ± 1 d after farrowing. Sows were assigned randomly to receive either 30 mL of charcoal-treated follicular fluid (FF, n = 9; a treatment known to decrease serum FSH and follicular diameter) or 30 mL of saline (n = 11) by venous infusion thrice daily (0700, 1500, and 2300 h) for 96 h beginning at 14 ± 1 d after farrowing. Sows were weaned 48 h after the last infusion. Blood samples were collected for FSH analysis thrice daily beginning on the day of catheterization and continuing until ovulation. Follicular diameter was determined once daily by transrectal ultrasonography. A treatment × time interaction was detected for serum FSH (P < 0.001) and follicular diameter (P < 0.001) because serum FSH and the diameter of follicular populations decreased in FF sows during the infusion period. After the infusion period, serum FSH rebounded in FF sows, and follicles resumed growth but grew at the same rate as those of saline-treated sows, thus failing to achieve equivalent diameters relative to saline-treated sows on a given day after weaning. As a result, sows treated with FF had longer (P < 0.05) weaning to estrus (6.1 ± 0.4 d) and weaning to ovulation (8.6 ± 0.5 d) intervals compared with saline-treated sows (4.7 ± 0.4 d and 7.2 ± 0.4 d, respectively). We conclude that the diameter of the follicular population at weaning is one factor that controls interval to estrus and ovulation in sows. Small follicles at weaning cannot undergo compensatory growth and require additional time to reach a preovulatory size.

Key words: follicle, sow, weaning

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INTRODUCTION

Sows are weaned 14 to 21 d after farrowing and generally return to estrus within 4 to 7 d after weaning. Although the 4 to 7 d interval is typical, the return interval can extend beyond 7 d, particularly during periods of seasonal infertility (Almond, 1992). Britt et al. (1985) reported that there is considerable variability for the population of ovarian follicles in sows at weaning. The weaning to estrus interval for individual sows, therefore, may depend partially on the population of follicles (smaller vs. larger diameter) on the ovary at weaning. For example, sows with a synchronized population of smaller diameter follicles at weaning may have a long weaning to estrus interval because their follicles take more time to reach a preovulatory size (Lucy et al., 2001). In a previous study, we found that sows with small follicles on d 3 after weaning returned to estrus and ovulated later than sows with medium or large follicles on d 3 after weaning (Bracken et al., 2003). The differences in follicular size on d 3 after weaning that we observed may have been established before weaning; i.e., the sows with smaller folli-
cles on d 3 might have had smaller follicles when they were weaned. Our hypothesis was that sows with smaller follicles at weaning would have longer weaning to estrus and weaning to ovulation intervals. Instead of retrospectively assigning sows to follicular size groups (as we did in the past; Bracken et al., 2003), we addressed the hypothesis by suppressing the diameter of follicular populations before weaning using an infusion of charcoal-treated porcine follicular fluid (FF; a treatment known to suppress follicular growth; Guthrie et al., 1988, 1997; Larson et al., 1991) and then measured follicular development and interval to estrus and ovulation in FF-compared with saline-treated sows. In this way we were able to assess the developmental capacity of small follicles on the ovaries of otherwise normal sows. Once-daily ultrasonography was used so that follicular development could be measured on each day of the experiment.

MATERIALS AND METHODS

Animals

Multiparous crossbred (Duroc, Landrace, and Yorkshire composite) sows (n = 20) were used. Approximately 3 d before farrowing, sows were brought into the University of Missouri Animal Science Research Center and placed in farrowing crates. The farrowing room within the Animal Science Research Center was air-conditioned (22 to 25°C) with a photoperiod of 16 h of light and 8 h of dark. Sows were fed grain (corn-soybean diet) and had ad libitum access to water. Within 24 h of farrowing, litters were standardized to 10 pigs per litter. Sows were weaned at 20 ± 1 d after farrowing. The University of Missouri Animal Care and Use Committee approved the experimental procedures.

Catheterization and Blood Sampling

Sows were fitted with a lateral saphenous vein catheter 12 ± 1 d after farrowing by using the method of Benoit and Dailey (1991). The depth of the catheter (65 cm; positioned within the posterior vena cava and cranial to the ovarian vein) was validated in a preliminary experiment in which a catheter was inserted into a luteal phase sow and blood concentrations of progesterone were used to determine the minimal depth to ensure that the end of the catheter was cranial to the entry of the ovarian vein (approximately 53 cm; Bracken, 2003).

Beginning on the day of catheterization, blood samples were collected thrice daily at 0700, 1500, and 2300 h, and sampling continued until ovulation. Approximately 2 mL of blood was removed from the catheter and discarded. Blood samples were then collected into serum collection tubes (9 mL of Monovette Z, Sarstedt Inc., Newton, NC). The catheter was flushed with 3 to 5 mL of heparinized-saline solution (40 units per mL) after samples were collected. Samples were stored at 4°C for 8 h to allow for coagulation. Coagulated samples were centrifuged at 2,000 × g for 15 min at 4°C. Serum was recovered and stored at −20°C until assayed.

Preparation of Follicular Fluid for Infusion

Follicular fluid was aspirated from ovaries that were collected from approximately 180-d-old prepubertal pigs and then frozen at −20°C. The frozen FF was thawed at room temperature, and a FF-charcoal mixture was made [5% (wt/vol) C-170 Carbon Decolorizing Neutral, Fisher Scientific, Hampton, NH]. The mixture was stirred for 60 min and then centrifuged for 15 min at 2,400 × g. The low-speed supernatant was decanted and centrifuged for 60 min at 20,000 × g. The high-speed supernatant was strained through multilayered cheesecloth and frozen at −20°C. The batches of charcoal-treated FF were thawed and combined into a single pool that was used for all infusions. Aliquots of the pool were thawed and filter-sterilized (0.2-µm diam. pore size). The concentration of estradiol in the sterile, charcoal-treated FF pool was less than 3 pg/mL.

RIA

Concentrations of estradiol and FSH were measured by using validated RIA. The estradiol assay was originally validated for bovine plasma (Kirby et al., 1997) and was later validated for porcine follicular fluid (Liu et al., 2000) and for porcine serum and plasma using similar procedures. The estradiol assay was sensitive to 1.25 pg/mL, with an intraassay CV of 14% and an interassay CV of 19%.

Serum concentrations of FSH were measured by validated RIA. Antiserum and ligand were kindly donated by A. F. Parlow (National Hormone and Pituitary Program, Torrance, CA). Serum (200 µL) was incubated with 200 µL of antiovine FSH (AFP-C5288113; 1:50,000 dilution in protein assay buffer (PAB; 0.1% gelatin, 0.01% thimersol, 0.01 M PO4, 0.9% NaCl, pH 7.2) with normal rabbit serum (1:300]) at 4°C for 24 h. On d 2, 100 µL of PAB containing approximately 20,000 cpm of [125I]-pFSH (AFP-10640B) were added,
and the incubation continued for an additional 24 h at 4°C. Precipitation of the antiserum complexes was performed on d 3 with the addition of 100 μL of goat anti-rabbit antiserum (1:50 dilution in PAB; Antibodies Inc., Davis, CA) and 200 μL of a solution of 12.5% polyethylene glycol (average 8,000 molecular weight). The tubes were incubated for 1 h at room temperature. Final centrifugation was at 1,500 × g for 30 min at 20°C. Supernatant was decanted, and the pellet was counted for 1 min. Concentrations of FSH in unknown samples were estimated from a standard curve (0.02, 0.04, 0.08, 0.16, 0.31, 1.25, 2.50, 5.0, and 10 ng per tube) using pFSH (USDA pFSH I2). Increasing volumes of porcine plasma (100, 200, and 300 μL) resulted in a displacement curve that was parallel to the standard curve. Addition of different masses of pFSH to the assay (0.06, 0.125, and 0.25 ng per tube) resulted in an average recovery of 116 ± 2%. The FSH assay was sensitive to 0.4 ng/mL, with an intraassay CV of 9% and an interassay CV of 14%.

**Ultrasonography and Analyses of Ovarian Follicular Populations**

Beginning at 0700 h on the morning after catheterization, transrectal ultrasonography was performed once daily with an Aloka 500V ultrasound machine (Corometrics Medical Systems Inc., Wallingford, CT) and a 7.5-MHz linear transducer attached to a polyvinyl carbonate handle. Ultrasonography continued until sows ovulated. Three sows did not ovulate (n = 2 FF and n = 1 saline-treated) and were evaluated until d 8 (FF sow) or d 9 (FF sow; control sow) after weaning.

For all sows, images of both ovaries were recorded on videotape. Videotapes were reviewed using Adobe Premiere (Adobe Systems Inc., San Jose, CA), and representative clips (a single clear ultrasonographic sweep through the ovary) for both ovaries were saved as digital images. The digital ovarian video clips were edited to 5 individual frames for each ovary. The individual frames included different planes of the ovary. The size of follicles was determined by analyzing the images with ImageJ (National Institutes of Health, Bethesda, MD). The calibrated scale within ImageJ was standardized to the resident scale on the ultrasound image (10 mm demarcation). A line was scribbled vertically from the top edge to the bottom edge of each follicle. Follicles with clear and well-defined borders were measured. The follicular diameter measurements were saved to a spreadsheet file and used for statistical analyses. The average follicular diameter and the number of follicles in individual size classes were determined for each day. Follicular data were collected for both ovaries and combined so that a single observation per sow was used on each day (e.g., total number of ovarian follicles, average follicular diameter).

**Estrus Detection**

Estrus detection was conducted twice daily (0900 and 1500 h) beginning 2 d after weaning using fence line contact with a boar. Sows were considered to be in estrus when they exhibited the standing reflex in the presence of the boar. Estrus detection continued until ovulation was detected by ultrasound.

**Statistical Analyses**

The summary procedure of SAS (Version 6.12. 1998, SAS Inst. Inc., Cary, NC) was used to generate average follicle diameter and number of follicles in specific class sizes. Class 1 follicles were 1 mm to less than or equal to 3-mm diam.; class 2 follicles were greater than 3-mm and less than or equal to 5-mm diam., and class 3 follicles were greater than 5-mm diam. Data were analyzed as repeated measures using the PROC MIXED procedures of SAS (1998). The model for all analyses included the main effects of treatment, time, and treatment × time interaction. The appropriate covariance structure for each variable was determined by testing several different covariance structures and then choosing the covariance structure with the lowest fit statistics. Serum FSH, serum estradiol, and follicular class data were analyzed using an autoregressive covariance structure. Data for average follicular diameter were analyzed using a heterogeneous autoregressive covariance structure. The effects of treatment on weaning to estrus interval and weaning to ovulation interval were tested by using a model that included treatment. A type I error rate of P > 0.10 was considered nonsignificant unless stated otherwise. Data are expressed as least squares means ± SEM.

**RESULTS**

**Serum Concentrations of FSH and Estradiol**

Serum concentrations of FSH were similar in both groups of sows before infusion; however, after infusion serum FSH remained constant in saline-treated sows but decreased in FF sows (Figure 1A, time × treatment, P < 0.001). After weaning, serum FSH rebounded in FF sows to concentrations greater than in saline-treated sows. Serum concentrations of estradiol were low before weaning and increased after weaning (Figure 1B; time, P < 0.001). The effect of treatment and treatment × time interaction were not significant for serum estradiol.

**Follicular Growth and Development**

Follicular diameter was initially similar but became less for FF sows compared with saline-treated sows after the infusion period (Figure 2; treatment × time interaction, P < 0.002). Average follicular diameter increased after weaning, but follicles in FF sows re-
Follicular growth in sows

Figure 1. Least squares means (± SEM) for serum concentrations of FSH (A) and estradiol (B) before and after weaning in sows. Sows were either infused thrice daily with saline (control; ○) or charcoal-treated porcine follicular fluid (●) beginning 144 h (6 d) before weaning and ending 48 h (2 d) before weaning (horizontal black bar in A denotes infusion period). There was an effect of treatment × time ($P < 0.001$) for serum concentrations of FSH and an effect of time ($P < 0.001$) for serum concentrations of estradiol.

mained smaller compared with follicles in saline-treated sows.

The number of class 1 follicles (1- to 3-mm diam.) was similar before infusion in control and FF sows (Figure 3A). By the end of infusion, however, the number of class 1 follicles had decreased in FF sows (treatment × time interaction, $P < 0.008$). The relatively low numbers of class 1 follicles in FF sows persisted until 1 d after weaning when the number of class 1 follicles increased in FF sows. The number of class 1 follicles began to decrease in FF sows by 2 d after weaning. The decrease in the number of class 1 follicles in FF sows occurred about 1.5 d after the decrease in class 1 follicles in saline-treated sows.

The number of class 2 follicles (>3- to 5-mm diam.) decreased in FF sows during the infusion period (treatment × time interaction, $P < 0.001$). Fewer class 2 follicles in FF sows were present until 2 d after wean-
Figure 2. Least squares means (± SEM) for average follicular diameter in sows that were either infused thrice daily with saline (control; ○) or charcoal-treated porcine follicular fluid (●) beginning 6 d before weaning and ending 2 d before weaning (horizontal black bar denotes infusion period). There was a treatment × time interaction (P < 0.002).

ing when the number of class 2 follicles increased in FF sows. The number of class 2 follicles peaked 2 d later (4 d after weaning) in FF sows. The number of class 2 follicles was similar for FF and control by 7 d after weaning.

The number of class 3 follicles (>5-mm diam.) increased 1 d after weaning and reached a near maximum by 5 d after weaning in saline-treated sows. The increase in the number of class 3 follicles was delayed in FF sows (treatment × time interaction, P < 0.001). For FF sows, the number of class 3 follicles increased 3 d after weaning and reached a near maximum by 6 d after weaning.

Weaning to Estrus and Ovulation Intervals

One control sow failed to show estrus and ovulate. Two FF sows showed estrus but had not ovulated by the end of the ultrasonography period. Among estrus and ovulatory sows, there was an effect of treatment on weaning to estrus and weaning to ovulation intervals. The weaning to estrus interval for control and FF-treated sows was 4.7 ± 0.4 and 6.1 ± 0.4 d, respectively (treatment, P < 0.013). The weaning to ovulation intervals for control and FF-treated sows was 7.2 ± 0.4 and 8.6 ± 0.5 d, respectively (treatment, P < 0.034).

DISCUSSION

In this study of weaned sows, a treatment was applied that reduced average follicular diameter, and the regression as well as regrowth of ovarian follicles were studied daily by using ovarian ultrasonography. Charcoal-treated porcine FF has been shown to decrease serum concentrations of FSH and decrease the number of small and medium follicles in pigs (Guthrie et al., 1988; Knox et al., 1991; Knox and Zimmerman, 1993), and FF was efficacious for this purpose in our hands as well. Serum FSH began to decrease within 8 h after the beginning of infusion and reached a nadir within 36 h after the beginning of infusion. The decrease in FSH that we observed was probably a consequence of inhibin found in high concentrations within porcine FF (Guthrie et al., 1997). Porcine FF may also act directly on the follicle and inhibit its development (Guthrie et al., 1988; Larson et al., 1991).

We undertook this study because we wanted to address the developmental capacity of small ovarian follicles after weaning. The ovarian follicular population at weaning is diverse, consisting of follicles of various sizes and statuses (healthy or atretic; Britt et al., 1985). We have suggested in the past that a synchronous population of follicles contributes to the preovulatory pool (Lucy et al., 2001). If true, then the stage of development of this pool of follicles (smaller vs. larger) may partially control the interval to estrus. This would only be true if small follicles cannot undergo compensatory growth after weaning. A decrease in follicular diameter occurs in undernourished sows, but follicular development in these sows is confounded by the hormonal effects of undernutrition (Quesnel et al., 1998). Hence, this study assessed the capacity of small follicles to develop after weaning in sows that were not undernourished.

The FF infusion decreased the average size of ovarian follicles. This effect of FF has been observed in the
Figure 3. Least squares means (± SEM) for the number of follicles in class 1 (1- to 3-mm diam.; A), class 2 (>3- to 5-mm diam.; B) and class 3 (>5-mm diam.; C) before and after weaning in sows. Sows were either infused thrice daily with saline (control; ○) or charcoal-treated porcine follicular fluid (●) beginning 6 d before weaning and ending 2 d before weaning (horizontal black bar in A denotes infusion period). The treatment × time interaction was significant for class 1 ($P < 0.008$; A), class 2 ($P < 0.001$; B), and class 3 ($P < 0.001$; C) follicles. There were 2 saline-treated sows that ovulated on d 6 and thus were not included in the d 7 data.
past (using single or multiple-point slaughter data; Guthrie et al., 1988; Knox and Zimmerman, 1993), but to our knowledge this is the first time that the process has been studied dynamically in pigs using ultrasonography. The average follicular diameter reached the limit of ultrasonographic detection (1 mm) by 3 d after the beginning of infusion. Although serum concentrations of FSH increased almost immediately after the end of infusion, average follicular diameter did not increase until 3 d later. Infusion of FF decreased the number of class 1 (1 to 3 mm) and class 2 (>3 to 5 mm) follicles. The decrease in the number of follicles was relatively rapid for class 2, where follicle numbers were suppressed by d −4 (2 d after the beginning of infusion). The response for class 1 follicles was less, and this may reflect the regression of follicles out of class 2 and into class 1 (hence, increasing class 1 numbers). A wave-like pattern of follicular growth was observed after the end of infusion where follicles moved through the different classes in both FF and saline sows. The timing of the follicular events was delayed in FF sows. The delay in follicular development (diameter and number of follicles) in FF-treated sows led to longer weaning to estrus and weaning to ovulation intervals in FF sows.

Follicles in FF and saline-treated sows appeared to grow at the same rate (similar slope) after weaning although the time of development relative to weaning was delayed for FF sows. The pattern of follicular development that we observed (delayed growth in sows that ovulate later after weaning) was consistent with our previous observations (Lucy et al., 2001). Thus, preovulatory follicles that developed in FF sows were not compromised in terms of their developmental capacity. They were simply at a smaller size (1 mm for FF compared with 3 mm for control) when sows were weaned. If there is a synchronous population of healthy follicles on the sow ovary, then the size of this population will contribute to the interval to estrus after weaning.

Serum concentrations of FSH increased within 16 h after the end of infusion, and there was a rebound in serum FSH that persisted until 3 d after weaning. When data from individual sows were examined, we did observe, as has been reported by others (Cox and Britt, 1986; Kelly et al., 1988), an increase in serum FSH on the first day of estrus (presumably associated with the LH surge) and a secondary release of FSH near ovulation (data not shown). A rebound in concentrations of FSH was previously documented in pigs treated with FF (Knox et al., 1991). The FSH rebound experienced by the FF sows may have affected the rate of follicular development. Other methods could have been used to decrease blood FSH (immunization or GnRH antagonists), but each of these methods has its own caveats that limit the interpretation of follicular growth studies. The rebound in FSH may have actually increased the rate of follicular growth in the FF sows. The true weaning to estrus and weaning to ovulation intervals may have been longer in FF sows if the FF sows had not experienced the large increase in FSH during the rebound period. Other methods that control for the FSH rebound (including GnRH antagonists followed by FSH/LH replacement) could possibly be used in future studies of follicular growth in sows.

In conclusion, FF infusion decreased the average size of follicles and delayed follicular growth after weaning. The diameter of follicles at weaning is, therefore, one factor that controls interval to estrus and ovulation. Sows that recruit their entire population of preovulatory follicles from a small follicle pool at weaning will have longer intervals to estrus and ovulation because additional time is needed for follicles to reach the preovulatory size. It may be possible to shorten interval to estrus and ovulation by developing methods to ensure a synchronous population of large follicles at the time of weaning in sows.

**LITERATURE CITED**


