

Influence of Experimental Intrauterine Infusion of *Arcanobacterium pyogenes* Solution on Ovarian Activity in Cycling Cows

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(Received 24 May 2007/Accepted 18 September 2007)

ABSTRACT. To examine the ovarian response to *Arcanobacterium pyogenes* (*A. pyogenes*) in uterus, bacterial solution was infused into the uteri of cows, and the follicle and corpus luteum (CL) development were monitored with a real-time ultrasound instrument. In addition, the plasma concentrations of progesterone (P₄) and 13, 14-dihydro-15-keto-PGF_{2α} (PGFM) were determined. A 10 ml bacterial solution that contained *A. pyogenes* (8 to 15 × 10⁸ CFU/ml) was infused into the uterus of eight cows transcervically three days after natural ovulation. As a control, sterile physiological saline was infused into 4 other cows. The dominant follicle developed normally in 8 cows after bacteria inoculation. In 4 of these 8 cows, the developing CL regressed, and the first wave dominant follicles, which normally become atretic, ovulated after the inoculation. In the remaining 4 cows, the CL did not regress. The PGFM concentration increased transiently in all 8 cows after the infusion. The mean PGFM concentration of the cows with a regressed CL was significantly lower (P<0.01) than that of the cows whose CLs did not regress. In the control cows, there was no regression of developing CLs, no ovulation of first wave dominant follicles and no transient increase in PGFM after the infusion of sterile physiological saline. These results show that infusion of *A. pyogenes* into the uterus did not affect folliculogenesis and might have induced PGF_{2α} production from the uterus.

KEY WORDS: cattle, endometritis, ovarian activity, PGFM, progesterone.

J. Vet. Med. Sci. 70(1): 77–83, 2008

The onset of normal ovarian cyclic activity is one of the most important events for dairy cows to regain maximum breeding potential following parturition. However, there is a high prevalence of ovarian disturbances during this period. The most common disturbances are delayed cyclicity or ovulation [23], a prolonged luteal phase [23, 24] and cystic ovaries [3]. The risk factors for ovarian disturbances after parturition include calving season, length of the dry period, body condition, housing system, parity and general health problems like mastitis, lameness or pneumonia and intrauterine infection [3, 14, 19, 23, 24, 26]. For instance, endometritis delays the initiation of folliculogenesis and increases the interval from calving to first ovulation [19, 26]. Bosu *et al.* [3] and Mateus *et al.* [19] demonstrated that the development of cystic ovaries is related to infection of the uterus. Furthermore, a prolonged luteal phase is induced by uterine abnormalities [19, 22, 24]. On the other hand, Peter *et al.* [25] reported that postpartum uterine infection might contribute to the early demise of the CL.

Although many ovarian abnormalities occur in cows after delivery, the mechanism through which uterine infection affects ovarian function has not been clearly determined because of several factors, such as endometrial damage, the presence of lochia, low pituitary sensitivity to GnRH and the beginning of milk production. *Arcanobacterium pyogenes* (*A. pyogenes*) is a significant persistent pathogenic bacteria recovered from the uterus after parturition [21, 37]. In this study, a bacterial solution containing *A. pyogenes* was infused into the uteri of healthy non-lactating cows, and

their ovaries were monitored to clarify the mechanism through which *A. pyogenes* affects ovarian activity.

MATERIALS AND METHODS

Animals: Twelve Holstein cows at least 4 years of age were used. They were housed in a tie-stall barn at Azabu University. All cows had not been inseminated after their last parturition because they were kept for education of students. The parities of the cows were unknown. Furthermore, all were at least two years postpartum, none had been milked and all had exhibited two normal estrous cycles prior to the start of the experiment.

Ultrasound scanning: The experiment began on Day 1 of the estrous cycle (Day 0 = the day of natural ovulation). Ovulation on Day 0 was confirmed by rectal palpation and a real-time ultrasound instrument (Model SSD-500, Aloka Co., Ltd., Tokyo, Japan) equipped with a 5 MHz transrectal linear transducer. Follicular development and the changes in the sectional area of the corpus luteum (CL) were monitored daily between 9:00 am and 12:00 am using the ultrasound instrument. All visible follicles (≥4 mm in diameter) were recorded, and the dominant follicle of the wave was defined as the follicle reaching the largest diameter retrospectively. In regard to the CL, its area was recorded using the area measuring function of the ultrasound instrument. With this measuring function, the area is calculated from the length of the CL circumference. When a central cavity was present in the CL, the area of the cavity was subtracted from the whole area [16]. Ovulation was diagnosed when the pre-ovulatory follicle disappeared. All observations were performed until Day 30.

Bacterial inoculation: Ten ml of bacterial solution was

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transcervically infused into the uteri of 8 cows on Day 3. This bacterial solution contained 8 to 15×10^8 colony forming units/ml of *A. pyogenes* in physiological saline, and these numbers of *A. pyogenes* were enough to induce pyometra in the cows [7]. The *A. pyogenes* used in this study was isolated from a cow with postpartum endometritis using a previously reported method [15]. Four remaining cows were infused with 10 ml of sterile physiological saline as a control.

Hormone analysis: The plasma progesterone (P_4) and 13, 14-dihydro-15-keto-PGF $_{2\alpha}$ (PGFM) concentrations were determined. The blood samples were collected each day from the tail using with a vacuumed heparinized tube. The plasma was separated by centrifugation (2,000 g for 10 min), and it was stored at -20°C for P_4 and at -80°C for PGFM until determination of the respective concentrations. The P_4 concentration was measured by radioimmunoassay without extraction using commercial kits (Diagnostic Products Corporation, Los Angeles, CA, U.S.A.). Recovery was 80–120% when measuring standard sera containing known amounts of steroids. The cross-reactivity of the anti- P_4 antibody for progesterone, 5α -pregnane-3-20-dione, 17α -hydroxyprogesterone, 5β -pregnane-3-20-dione, 20α -dihydroprogesterone, testosterone, 5β -pregnane-3 α -ol-20-one, androstenediol and 17β -estradiol were 100, 9, 3.4, 3.2, 0.2, 0.1, 0.05, <0.05 and <0.05%, respectively. The intra- and interassay coefficients of variation were 8.8% and 9.7%, respectively, and the detection limit was 0.02 ng/ml. An enzyme immunoassay kit (Cayman Chemical Corporation, Ann Arbor, MI, U.S.A.) was used to quantify PGFM without extraction [6]. The cross-reactivity of the anti-PGFM

antibody for 13,14-dihydro-15-keto PGF $_{2\alpha}$, 13,14-dihydro-15-keto Prostaglandin E $_2$, 15-keto Prostaglandin F $_{2\alpha}$, Prostaglandin D $_2$, Prostaglandin E $_2$, Prostaglandin F $_{1\alpha}$, 2,3-dinor-6-keto Prostaglandin F $_{1\alpha}$, 6-keto Prostaglandin F $_{1\alpha}$ and Prostaglandin F $_{1\alpha}$ were 100, 2.7, 1.8, <0.01, <0.01, <0.01, <0.01, <0.01 and <0.01% respectively. The intra- and interassay coefficients of variation were 4.3% and 10.8%, respectively, and the detection limit was 8.2 pg/ml.

Data analysis: The PGFM concentration data was analyzed using repeated measures ANOVA (treatment \times time) followed by Fisher's protected least significant difference (PLSD) test. The level of significance was established as $P < 0.05$.

RESULTS

Control cows: In the 4 control cows (Cows 1 to 4), the first wave dominant follicles that developed following infusion of sterile physiological saline regressed without ovulation, and the second wave dominant follicles ovulated on Day 23 (Cows 1 and 2), Day 20 (Cow 3) or Day 24 (Cow 4, Table 1). Representative time courses for all response parameters of the control cows are shown in Fig. 1. The CL developed, and we observed a normal P_4 secretion pattern. Although there was no change in PGFM after infusion of sterile physiological saline, there was a rise when the high P_4 value declined.

Intrauterine bacteria infusion: In 4 of the 8 cows infused with bacterial solution, the first wave dominant follicles that were developing at the time of bacteria inoculation ovulated on Day 11 (Cow 5), Day 10 (Cow 6) or Day 8 (Cows 7 and

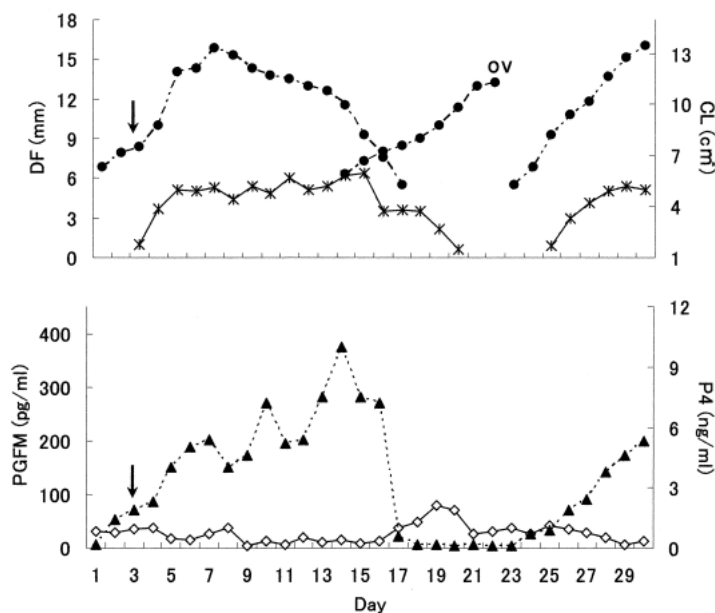


Fig. 1. Profiles of the dominant follicle, corpus luteum, progesterone and PGFM in a representative control cow (Cow 1). Ov: ovulation. \downarrow : saline inoculation. \bullet : diameter of the dominant follicle. $*$: area of the corpus luteum. \diamond : PGFM concentration. \blacktriangle : progesterone concentration.

Table 1. The change of dominant follicle diameter in each cow

			Day																																																	
Dominant follicle			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30																				
Control cows	Cow 1	1	6.8	7.9	8.4	10	14	14	16	15	14	14	14	13	13	12	9.3	7.6	5.5																																	
		2															6.3	7.3	8	8.5	9	10	11	13	13	OV																										
		3																								5.5	6.8	9.3	11	12	14	15	16																			
	Cow 2	1	4.8	7	7.5	8.3	8.4	8.7	8.9	9	9.8	10	10	11	8.5	7																																				
		2															4.6	6.1	7.4	8.3	9	10	12	13	OV																											
		3																									5.2	7.8	7.9	11	12	14	15																			
	Cow 3	1	5.2	6.3	11	12	14	14	15	15	14	14	14	12	12	12	10	9.9	9.5	9.1	6																															
		2										5.7	7.3	6.9	9	11	14	15	17	18	OV																															
		3																									5.2	6.6	10	13	13	14	14	15	13																	
	Cow 4	1	5	7.5	7.9	10	11	11	12	12	12	12	12	12	12	12	12	12	13	12	12	12					5.2	6.6	10	13	13	14	14	15	13																	
		2																			5.9	7	10	11	12	OV																										
		3																										4.2	6.5	7	8	9.7	10	11																		
Group OV	Cow 5	1	5	6.5	8.5	9.1	9.6	13	14	15	17	OV																																								
		2												4.8	6.3	7.3	7.8	7.8	7.9	7.9	8	8.2	6.3	5.3																												
		3																								4	5	6	7.2	8.4	12	13	14																			
	Cow 6	1	6	7.8	9.5	12	13	14	15	15	OV																																									
		2												4.3	7.3	7.9	9.3	11	11	12	12	12	12	10	9.7	9.7	9.6	9.4	9	8.1	7	5.3																				
		3																												5	6.2	12	13																			
	Cow 7	1	8.2	9	9.8	13	14	15	17	OV																																										
		2									6	7.8	8.4	9.6	9	11	11	12	12	12	13	14	12	11	11	8.2	5.3																									
		3																								4.2	6.3	6.8	7.2	9.8	11	13	15	16	17	OV																
	Cow 8	1	7.3	12	14	15	16	17	OV																																											
		2									7	8.8	11	13	14	15	15	16	16	16	17	16	16	16	15	14	11	11																								
		3															8.3	8.6	9	9.5	9.7	9.8	10	10	10	10	11	11	11	12	OV																					
Group NOV	Cow 9	1	5.6	8.4	9.9	9.7	11	13	11	10	9.6	8.3	7.8	7.3	6.4	5.8	4.9	4.3																																		
		2																																																		
		3																		5.8	8	9.5	11	12	14	OV																										
	Cow 10	1	6	6.8	7	8.5	9.5	11	11	12	11	11	6.8	6.2	5.4												6.3	6.8	7.2	8.9	12	12																				
		2													4.8	5	6.8	8.7	11	13	14	14	OV																													
		3																									5.3	8.3	10	12	12	13	13	13	12	12																
	Cow 11	1	4.8	6.8	8.1	8.7	9.4	11	11	12	11	11	9.8	9.7	9.5	7.5																																				
		2										5.3	6.3	7.6	8	8.5	8.9	9	9.1	9.6	9.8	10	10	10	13	OV																										
		3																																																		
	Cow 12	1	7.6	8.5	11	12	13	14	15	17	17	16	14	13	12	12	11	9.8	9.3	7.9																																
		2											5	6.8	9	12	13	15	16	OV																																
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																					5	7.2	9.3	12	13	13	13	13	13	13	12																					

Unit is mm. OV means ovulation.

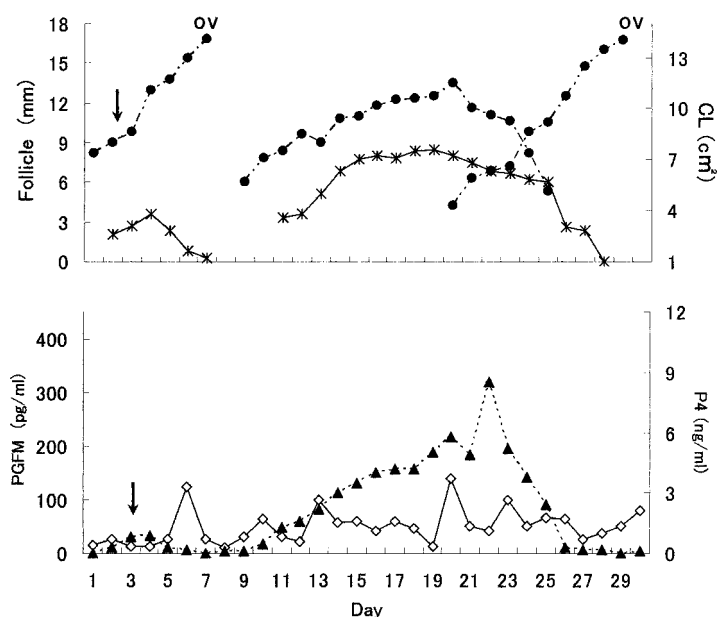


Fig. 2. Profiles of the dominant follicle, corpus luteum, progesterone and PGFM in a representative cow (Cow 7) from Group OV. Ov: ovulation. ↓: bacteria inoculation. ●: diameter of the dominant follicle. *: area of the corpus luteum. ◇: PGFM concentration. ▲: progesterone concentration.

8) without regression (Group OV, Table 1). Representative time courses for all response parameters of Group OV are shown in Fig. 2. After bacteria inoculation, the developing CL regressed and the increasing level of P_4 suddenly decreased to a value of less than 1 ng/ml. Development of the CL after ovulation of the first wave dominant follicle was normal. PGFM increased transiently on Day 6 after bacterial inoculation. In Cows 7 and 8, the next ovulation was observed on Days 30 and 28, respectively. Discharge of transparent mucus from the vulva was observed in Cow 7 for 4 days after bacteria inoculation. However, no purulent mucus indicating severe endometritis was discharged from any of the 4 cows. Furthermore, no abnormal fluid accumulation in the uterine lumen was observed by ultrasound scanning.

In the 4 remaining cows that received the bacteria inoculation (Cows 9 to 12, Group NOV), the first wave dominant follicles that developed following inoculation regressed without ovulation, and the second wave dominant follicles ovulated on Day 23 (Cow 9), Day 20 (Cow 10), Day 24 (Cow 11) or Day 18 (Cow 12, Table 1). Representative time courses for all response parameters of Group NOV are shown in Fig. 3. The developing CL did not regress, and the P_4 secretion pattern was normal. PGFM increased transiently on Day 4 after bacteria inoculation. It then returned to the original value and increased again on Day 12 when the high P_4 value declined. In Cows 9 and 10, discharge of transparent or subtly cloudy mucus from the vulva was observed for 3 or 4 days after bacterial inoculation. However, no purulent mucus was discharged from any of the 4

cows. Furthermore, none was observed in the uterine cavity by ultrasound scanning.

The profiles of PGFM: The time courses of the mean PGFM concentrations of each group are shown in Fig. 4. The PGFM concentration of Group OV was significantly lower than that of Group NOV ($P < 0.01$) during the observation period. In Group OV, PGFM increased on Day 5, remained at this higher level until Day 8 and then returned to the original value. The peak value was similar to the original value of Group NOV and the control cows. In Group NOV, PGFM increased on Day 4, remained at this high level until Day 5 and then returned to the original value. The value for Group OV on Days 3, 4, 9 and 15 were significantly lower than those of Group NOV ($P < 0.05$).

DISCUSSION

When cows develop endometritis after parturition, *Escherichia coli* (*E. coli*) and *A. pyogenes* are usually the most prominent bacteria present in uterine lochial secretions [37], and the endometritis delays folliculogenesis or ovulation after parturition [14, 19, 23, 24, 26]. The endotoxin originating from *E. coli* in the uterus can affect secretion of GnRH or LH and delay resumption of ovarian activity after parturition [1, 2, 4, 20, 28, 36]. However, *A. pyogenes* does not produce endotoxin [37]. Furthermore, in this study, normal folliculogenesis occurred in all eight cows after bacteria inoculation, and the first wave dominant follicles, which normally regress, ovulated in 4 of the 8 treated cows. These findings suggest that there may be another way in which *A.*

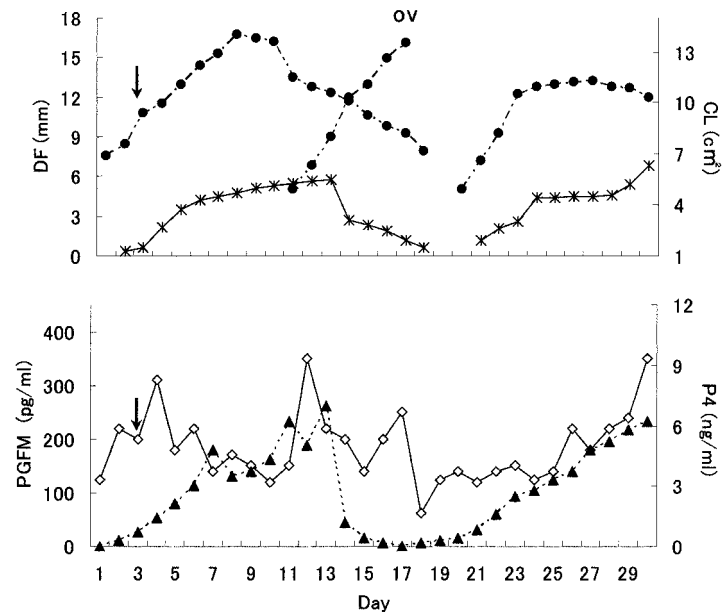


Fig. 3. Profiles of the dominant follicle, corpus luteum, progesterone and PGFM in a representative cow (Cow 12) from Group NOV. Ov: ovulation. ↓: saline inoculation. ●: diameter of the dominant follicle. *: area of the corpus luteum. ◇: PGFM concentration. ▲: progesterone concentration.

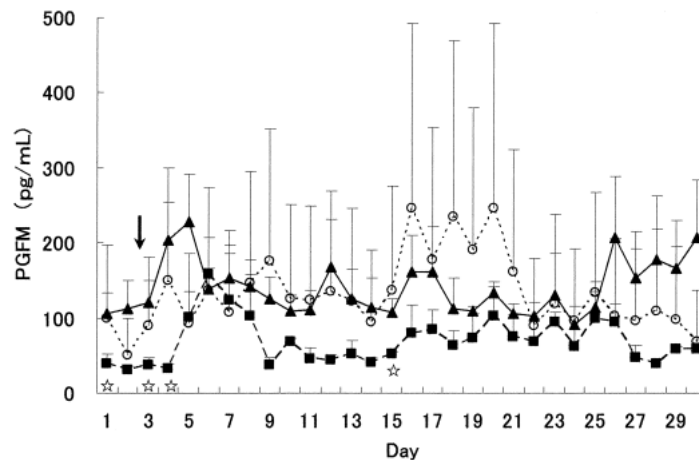


Fig. 4. The time courses of the mean PGFM concentrations of Group OV (■), Group NOV (▲) and the control cows (○). Values are shown as mean \pm SE. ↓: bacteria inoculation. ☆ $P < 0.05$, compared with the respective value for the Group NOV.

pyogenes affects ovarian activities other than disturbance of pulsatile GnRH and LH release.

In Group OV, the developing CL regressed and the P_4 level decreased prior to ovulation. This phenomenon shows that inoculation of *A. pyogenes* could induce regression of the CL, and as a result of this regression, the decreased level of P_4 induces ovulation of the first wave dominant follicle. When first ovulation occurs in the presence of a heavily contaminated uterus, the corpus luteum can persist [21]. On the

other hand, Peter *et al.* [25] reported that postpartum uterine infection induces release of $PGF_{2\alpha}$ and might contribute to early demise of the CL formed after the first postpartum ovulation. The results of this study support this previous report.

The PGFM concentration increased after bacterial inoculation in Group OV and Group NOV. Because $PGF_{2\alpha}$ is promptly metabolized to PGFM, the increased level of PGFM shows that $PGF_{2\alpha}$ was synthesized after *A. pyogenes*

inoculation. It has been reported that the peptidoglycan in gram-positive bacterial cell walls is capable of stimulating release of cytokines such as TNF- α or IL-1 β [33, 34] and that these cytokines stimulate the endometrium to produce PGF_{2 α} [5, 11, 32].

The mechanisms involved in luteolysis remain to be elucidated. In this study, the CL regressed in only 4 of 8 cows that received bacterial inoculation. In the 4 remaining cows, the CL did not regress, and the first wave dominant follicle became atretic without ovulation despite PGFM increasing after bacteria inoculation. In the case of pyometra, another type of uterine infection, the CL persists without regression even if the serum concentration of PGFM is high [18, 29, 35]. Uterine tissues can synthesize different types of prostaglandins, in particular PGE₂, during the inflammation reaction [10]. PGF_{2 α} and PGE₂ can have opposite effects, and PGE₂ may have antiluteolytic and/or luteotrophic properties [17]. Although we did not measure PGE₂ in this study, the extent of PGE₂ secretion might have a role in deciding whether the CL persists or regresses.

The mean concentration of PGFM in Group OV was significantly lower than that of Group NOV and the control cows during the observation period. Peter *et al.* [27] and Skarzynsky *et al.* [31] reported that there is a difference between the basal PGFM concentrations of cows with short estrous cycles and cows with normal estrous cycles and that sensitivity of the CL to PGF_{2 α} seems to be dependent on the plasma oxytocin level. In this study, the PGFM concentration of Group OV was significantly lower than that of Group NOV and that of the control cows. Even after bacteria inoculation, the peak value was only similar to the original value of the Group NOV and control cows. Because regression of the CL occurred at that peak value in Group OV, the sensitivity of the CL to PGF_{2 α} in Group OV might be higher than that of Group NOV. Furthermore, angiotensin II and endothelin-1, in addition to PGF_{2 α} , may have important roles during luteolysis in the bovine CL [8, 9, 12, 13, 30]. In the future, it is necessary to elucidate the mechanisms that determine whether a CL persists or regresses.

The conditions in this study might be different from those of the puerperal period because pituitary sensitivity to GnRH after parturition is low. However, there may be other mechanisms by which intrauterine infection affects ovarian function, such as the induction of CL regression by PGF_{2 α} shown in this study, in addition to impairment of reproductive neuroendocrine activity by endotoxin produced by *E. coli*.

The present study suggests that the presence of *A. pyogenes* in the uterus does not affect folliculogenesis but does stimulate PGF_{2 α} production from the uterus, and this PGF_{2 α} level is enough to induce regression of the CL in some cows.

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