The absence of corpus luteum formation alters the endocrine profile and affects follicular development during the first follicular wave in cattle

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Abstract

We previously established a bovine experimental model showing that the corpus luteum (CL) does not appear following aspiration of the preovulatory follicle before the onset of LH surge. Using this model, the present study aimed to determine the profile of follicular development and the endocrinological environment in the absence of CL with variable nadir circulating progesterone (P_4) concentrations during the oestrous cycle in cattle. Luteolysis was induced in heifers and cows and they were assigned either to have the dominant follicle aspirated (CL-absent) or ovulation induced (CL-present). Ultrasound scanning to observe the diameter of each follicle and blood collection was performed from the day of follicular aspiration or ovulation and continued for 6 days. The CL-absent cattle maintained nadir circulating P_4 throughout the experimental period and showed a similar diameter between the largest and second largest follicle, resulting in co-dominant follicles. Oestradiol (E_2) concentrations were greater in the CL-absent cows than in the CL-present cows at day -1, day 1 and day 2 from follicular deviation. The CL-absent cows had a higher basal concentration, area under the curve (AUC), pulse amplitude and pulse frequency of LH than the CL-present cows. After follicular deviation, the CL-absent cows showed a greater basal concentration, AUC and pulse amplitude of growth hormone (GH) than the CL-present cows. These results suggest that the absence of CL accompanying nadir circulating P_4 induces an enhancement of LH pulses, which involves the growth of the co-dominant follicles. Our results also suggest that circulating levels of P_4 and E_2 affect pulsatile GH secretion in cattle.

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Introduction

Monovular species including cattle have a restricted ovulation quota; therefore, selection of the dominant follicle is the most drastic event during follicular development to decide its fertility. Follicular selection is characterized by the appearance of differences in growth rate between a future dominant follicle and future subordinate follicles in the same cohort, termed follicular deviation (Ginther *et al.* 1997). In cattle, deviation occurs when the largest follicle reaches an average of 8.5 mm in diameter (Ginther *et al.* 1996). Although the detailed mechanisms of follicular selection have not been completely clarified, recent studies indicate that specific changes of the intrafollicular insulin-like growth factor (IGF) system and the enhanced capacity of oestradiol (E_2) production in the future dominant follicle play a critical role in the selection

of a dominant follicle accompanied by a decline of circulating follicle-stimulating hormone (FSH) towards the nadir level (Austin *et al.* 2001, Rivera & Fortune 2001).

Increasing evidence suggests that progesterone (P_4) secreted from the corpus luteum (CL) has a regulatory effect on follicular selection and on the number of dominant follicles in cattle. P_4 is a crucial factor to negatively regulate pulsatile luteinizing hormone (LH) release from the pituitary gland and inhibits maturation and ovulation of the dominant follicle during the bovine oestrous cycle (Kinder *et al.* 1996). Administration of P_4 to decline circulating LH concentrations decreases the diameter of the dominant follicle and its ability to produce E_2 during first follicular wave in cattle (Ginther *et al.* 2001a). In addition, several studies suggest that P_4 secreted from the CL may have a regulatory role in the mechanism of follicular selection through modulation of LH secretion in cattle. It has been

reported that cows that developed co-dominant follicles during the first follicular wave had a lower P_4 concentration and a higher LH concentration than cows that developed a single dominant follicle (Lopez *et al.* 2004). The incidence of co-dominant follicles increased in the first follicular wave with developing CL rather than in the second follicular wave with the mature CL (Kulick *et al.* 2001). However, because there is still no suitable experimental model, the effect on the dynamics of follicular development by altering the LH pulse when P_4 secretion from the CL is completely suppressed during the oestrous cycle in cattle is not known.

Growth hormone (GH) is known as one of the important modulators for follicular development by acting both directly and indirectly, in combination, via the liver IGF1 (Lucy 2000). Exogenous GH treatment in cows increases the number of recruited follicles, and as a result, there is an increase in the number of growing follicles (Gong *et al.* 1993a, Kirby *et al.* 1997, Jimenez-Krassel *et al.* 1999). Several studies examined the release of GH during the oestrous cycle of goats (Yonezawa *et al.* 2005) and sheep (Landefeld & Suttie 1989), in which the release of GH was suppressed at the luteal phase and stimulated at the follicular phase according to the changes in circulating steroid hormone levels. On the other hand, these studies also indicated the effect of exogenous steroid hormone treatment on GH release (Landefeld & Suttie 1989, Yonezawa *et al.* 2005), where treatment of E_2 or P_4 for ovariectomized goats altered the GH secretory pattern: GH pulsatility was enhanced by E_2 and inhibited by P_4 (Yonezawa *et al.* 2005). In addition, a long-term (45 days) treatment of E_2 in ovariectomized cows showed an increase in the plasma GH concentration and an amplitude in the GH pulse (Simpson *et al.* 1997). These studies suggest that sex steroid hormones (P_4 and E_2) from the ovary have the ability to modulate pulsatile secretion of GH and that this feedback system may involve the regulation of ovarian function. However, it is not known whether this hypothesis applies to the oestrous cycle in cattle. In addition, an increase in the circulating IGF1 concentrations at the follicular phase has been reported in goats (Yonezawa *et al.* 2005) and cattle (Kawashima *et al.* 2007b). Since GH directly affects IGF1 secretion, changes in secretion pattern of steroid hormones during the oestrous cycle may alter not only GH pulsatility but also IGF1 secretion.

Recently, we established a bovine experimental model that showed the suppression of CL formation and continuance of nadir circulating P_4 concentrations for approximately 1 week following the aspiration of the preovulatory follicle at the follicular phase (Hayashi *et al.* 2006). To our knowledge, this is the first experimental model to completely suppress the development of CL during the oestrous cycle in cattle. By using this experimental model, the present study aimed to determine the profile of follicular development and endocrinological environment (P_4 , E_2 , FSH, LH pulse, GH pulse and total IGF1) in the absence of CL with nadir circulating P_4 concentrations during the oestrous cycle in cattle. We

hypothesized that the absence of CL formation during the first follicular wave alters the endocrine profile and affects follicular development; thus, the first follicular wave following follicular aspiration (CL-absent) was compared with the first follicular wave following ovulation during the oestrous cycle (CL-present). In experiment 1, we observed the differences of follicular diameter and circulating P_4 and E_2 concentrations during the first follicular wave between the CL-absent and CL-present heifers and cows. In experiment 2, we determined the profiles of circulating P_4 , E_2 , FSH, total IGF1 and the pulsatile release of LH and GH during the perifollicular deviation period in the CL-absent and CL-present cows.

Results

In both experiments 1 and 2, induction of luteolysis by prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) injection was observed using transrectal ultrasound scanning by monitoring ovulation in the CL-present group and by follicular aspiration in the CL-absent group, thereby confirming luteolysis in all animals used in this study. Ovulation and subsequent CL formation by gonadotrophin-releasing hormone (GnRH) injection following $PGF_{2\alpha}$ treatment were induced in all CL-present animals. In experiment 2, one CL-absent cow was removed from the study because this cow did not develop co-dominant follicles.

Experiment 1

Follicular diameter at day 3 and day 6

In the CL-present cows (Fig. 1A), the diameter of the largest follicle (F1) and the second largest follicle (F2) was similar at day 3 (F1, 9.9 ± 0.8 ; F2, 7.8 ± 0.7 mm) but the diameter of F1 was greater than that of F2 at day 6 (F1, 14.6 ± 0.5 ; F2, 7.5 ± 0.8 mm). In the CL-absent cows, F1 and F2 showed a similar diameter at both day 3 (F1, 8.2 ± 0.9 ; F2, 7.3 ± 0.9 mm) and day 6 (F1, 12.9 ± 0.7 ; F2, 11.2 ± 1.1 mm) after follicular aspiration. In heifers (Fig. 1B), as well as in cows, the diameter of F1 was greater than that of F2 in the CL-present heifers at day 6 (F1, 11.9 ± 0.3 ; F2, 7.1 ± 0.6 mm) but there was no difference in diameter between F1 and F2 in the CL-absent heifers (F1, 11.7 ± 0.7 ; F2, 9.8 ± 1.0 mm). As shown in Table 1, the number of CL-absent cattle that developed co-dominant follicles at day 6 was four out of five in both cows and heifers, including one cow and one heifer that showed double ovulation. At day 9, the number of CL-absent cattle showing double ovulation was four out of five cows and two out of five heifers.

Changes in plasma P_4 and E_2 concentrations during first follicular wave

Figure 2 shows changes in the plasma P_4 concentrations in cows (Fig. 2A), P_4 concentrations in heifers (Fig. 2B) and E_2 concentrations in heifers (Fig. 2C). Plasma P_4

Figure 1 Mean follicular diameter of two largest follicles (F1, largest follicle; F2, second largest follicle) for (A) cows and (B) heifers at day 3 and day 6. Day 0 was defined as the day of follicular aspiration for the CL-absent groups (cows, $n=5$; heifers, $n=5$) or the day of ovulation for the CL-present groups (cows, $n=5$; heifers, $n=3$). Data are shown as the mean $\pm$ S.E.M. of each time period. a, b, c: Values with different letters are significantly different within cows ($P<0.05$). x, y: Values with different letters are significantly different within heifers ($P<0.05$).

concentrations in cows and heifers, and E_2 concentrations in heifers showed an interaction between group and day ($P<0.01$). The CL-absent cows had a lower P_4 concentration than the CL-present cows at days 3 and 6 ($P<0.05$). The CL-absent heifers had a lower P_4 concentration than the CL-present heifers after the second half of day 1 ($P<0.05$), and had a greater E_2 concentration than the CL-present heifers during the second half of day 4 and the first half of day 5 ($P<0.05$).

Experiment 2

Characteristics of follicular dynamics during first follicular wave

In the CL-absent cows, follicular deviation occurred 2 days after the follicular aspiration in two cows and 3 days after aspiration in the other two cows. In the

CL-present cows, follicular deviation occurred the day following ovulation in two cows and 2 days after ovulation in the remaining two cows.

Figure 3 shows the profile of follicular growth in the CL-absent and CL-present cows from day -1 to day 2 by daily ultrasound observation. In the CL-absent cows (Fig. 3A), follicular diameter in F1 was not significantly different from that in F2 from day -1 to day 2, resulting in co-dominant follicles. Following follicular deviation, the diameter of F1 and F2 was greater than that in F3 and F4 ($P<0.05$), and that of F2 in the CL-absent cows was greater than that of the CL-present cows ($P<0.05$). The diameter of F1, F2, F3 and F4 at day 2 was 11.8 ± 0.4 , 11.2 ± 0.2 , 7.3 ± 0.6 and 4.9 ± 0.1 mm respectively. In the CL-present cows (Fig. 3B), the diameter of F1 was greater than that of F2, F3 and F4 after follicular deviation ($P<0.05$); thus, only F1 continued to grow as a dominant follicle. The diameter of F1, F2, F3 and F4 at day 2 was 12.0 ± 0.1 , 7.6 ± 0.8 , 6.3 ± 0.5 and 5.6 ± 0.6 mm respectively.

Changes in plasma P_4 , E_2 and FSH concentrations during first follicular wave

Figure 4 shows changes in the plasma P_4 concentrations (Fig. 4A), E_2 concentrations (Fig. 4B) and FSH concentrations (Fig. 4C). Plasma P_4 and E_2 concentrations showed an interaction between group and day ($P<0.05$). The CL-absent cows had a lower P_4 concentration than the CL-present cows after day 0 ($P<0.05$). The E_2 concentration of the CL-absent cows was greater than that of the CL-present cows in the second half of day -1 , the second half of day 0 and the first half of day 1 and day 2 ($P<0.05$). Plasma FSH concentrations showed only a main effect of day ($P<0.01$).

Characteristics of LH pulses during first follicular wave

Figure 5 shows the characteristics of the LH pulses during the first follicular wave in the CL-absent and CL-present cows. Mean LH concentrations (data not shown) showed a main effect of group ($P<0.05$) and tended to show a main effect of day ($P<0.06$). The CL-absent cows had greater mean LH concentrations than the CL-present cows during the experimental period (CL-absent, 2.0 ± 0.04 ng/ml; CL-present, 1.6 ± 0.06 ng/ml; $P<0.01$). Basal LH

Table 1 The number of corpus luteum (CL)-absent cattle that showed co-dominant follicles and double ovulation in experiment 1.

	<i>n</i>	Co-dominant follicles at day 6	Double ovulation by day 9
Cows	5	4 ^a	4
Heifers	5	4 ^a	2

^aOne cow and one heifer already showed double ovulation. Day 0 = day of follicular aspiration.

Figure 2 Changes in the plasma concentrations of (A) progesterone for cows, (B) progesterone for heifers and (C) oestradiol for heifers. Blood samples were collected every 3 days in cows and twice daily in heifers from the day of follicular aspiration for the CL-absent group (cows, $n=5$; heifers, $n=5$) or the day of ovulation for the CL-present group (cows, $n=5$; heifers, $n=3$). Data are shown as the mean $\pm$ S.E.M. of each time period. A significant interaction between group and day was observed in all data. Asterisks indicate significant differences between groups (* $P<0.05$, *** $P<0.001$).

concentrations (Fig. 5A) showed a main effect of group ($P<0.05$) and tended to show a main effect of day ($P<0.06$). The CL-absent cows had a greater basal LH concentration than the CL-present cows during the experimental period ($P<0.01$). LH pulse amplitude (Fig. 5B) tended to show an interaction between group and day ($P<0.1$). The CL-absent cows had a higher LH

Figure 3 Follicular profiles of the four largest follicles for (A) CL-absent and (B) CL-present cows. Transrectal ultrasound scanning was performed daily from the day of follicular aspiration for the CL-absent cows ($n=4$) or the day of ovulation for the CL-present cows ($n=4$). Data were normalized to the day of follicular deviation (day 0) and are shown as the mean $\pm$ S.E.M. of each time period. In the CL-absent cows, the diameter of F2 was not different as compared with F1 from day -1 to day 2. In addition, the diameter of F1 and F2 was greater than F3 and F4 at day 1 and day 2 ($P<0.05$). In the CL-present cows, diameter of F1 was greater than that of F2, F3 and F4 at day 1 and day 2 ($P<0.05$). The CL-absent cows had greater F2 than the CL-present cows at day 1 ($P<0.05$) and day 2 ($P<0.01$).

pulse amplitude than the CL-present cows at day -1 and day 1 ($P<0.05$). LH pulse frequency (Fig. 5C) tended to show a main effect of group ($P<0.1$). The CL-absent cows had a higher LH pulse frequency than the CL-present cows during the experimental period ($P<0.05$). Area under the curve (AUC; Fig. 5D) showed a main effect of group ($P<0.05$) and tended to show a main effect of day ($P<0.08$). The CL-absent cows had a higher AUC than the CL-present cows during the experimental period ($P<0.01$).

Characteristics of GH pulses during first follicular wave

Representative 8 h plasma GH profiles in the CL-absent and CL-present cows are shown in Fig. 6. Figure 7 shows the characteristics of the GH pulses during the first follicular wave in the CL-absent and CL-present cows. Mean GH concentrations (data not shown) tended to

Figure 4 Changes in plasma concentration of (A) progesterone, (B) oestradiol and (C) FSH for the CL-absent and CL-present cows. Blood samples were collected twice daily from the day of follicular aspiration for the CL-absent cows ($n=4$) or the day of ovulation for the CL-present cows ($n=4$). Data were normalized to the day of follicular deviation (day 0) and are shown as the mean $\pm$ S.E.M. of each time period. A significant interaction between group and day was observed in progesterone and oestradiol. No significant interaction between group and day was observed in FSH. Asterisks indicate significant differences between groups (* $P < 0.05$, ** $P < 0.01$).

show a main effect of group ($P < 0.07$). The CL-absent cows had a greater mean GH concentration than the CL-present cows during the experimental period (CL-absent, 13.0 ± 0.8 ng/ml; CL-present, 9.6 ± 0.8 ng/ml; $P < 0.01$). Basal GH concentrations (Fig. 7A) tended to show an interaction between group and day ($P < 0.1$).

The CL-absent cows had a greater basal GH concentration than the CL-present cows at days 1 and 2 ($P < 0.05$). GH pulse amplitude (Fig. 7B) showed an interaction between group and day ($P < 0.05$). The CL-absent cows had a higher GH pulse amplitude than the CL-present cows at days 0 and 1 ($P < 0.07$). GH pulse frequency (Fig. 7C) showed neither a main effect of group and day nor their interaction. AUC (Fig. 7D) tended to show an interaction between group and day ($P < 0.1$). The CL-absent cows had a higher AUC than the CL-present cows at day 1 and day 2 ($P < 0.05$).

Changes in plasma total IGF1 concentration during first follicular wave

Changes in the plasma total IGF1 concentrations are shown in Fig. 8. Plasma total IGF1 concentrations showed an interaction between group and day ($P < 0.01$). Although there was no significant difference in the plasma total IGF1 concentration between the two groups, it increased in the CL-absent cows on the first half of day 2 compared with the first half of day 0, and the second half of day 2 compared with day -1 and the first half of day 0 ($P < 0.05$).

Discussion

The significant findings of this study were that: i) the absence of CL during the first follicular wave induced the development of co-dominant follicles and double ovulation and ii) GH secretion such as the basal concentration, pulse amplitude and AUC of GH, and plasma total IGF1 concentration increased under a circulating level of nadir P_4 and high E_2 .

A high incidence (80%) of co-dominant follicles was observed in the CL-absent cows and heifers. We also observed double ovulation within 9 days from follicular aspiration in all cows and half the heifers developed co-dominant follicles in experiment 1. Thus, our results suggest that both dominant follicles were healthy and had an ovulatory capacity in most of the CL-absent cattle. Our present results are consistent with a recent study in which the high incidence of co-dominant follicles was observed in the contralateral remaining ovary when the corpus haemorrhagicum was removed soon after ovulation (Gumen & Wiltbank 2005).

LH pulse amplitude was higher in the CL-absent cows than in the CL-present cows at day -1, whereas plasma P_4 concentrations did not differ between the two groups. Since the CL-absent cows had higher plasma E_2 concentrations in the second half of day -1 ($P < 0.05$) and a greater tendency than the CL-present cows in diameter of F1 ($P < 0.06$) at day -1, E_2 secreted from F1 may stimulate LH secretion in the CL-absent cows at day -1. To support this, treatment with E_2 benzoate increases the amplitude and basal concentrations of LH pulses in cattle (Austin *et al.* 2002). In addition, a second possibility could be considered that pituitary stores of LH and sensitivity to GnRH might differ

Figure 5 Characteristics of LH pulses in the CL-absent and CL-present cows. (A) Basal concentration, (B) pulse amplitude, (C) pulse frequency and (D) area under the curve (AUC). Data were normalized to the day of follicular deviation (day 0) and are shown as the mean $\pm$ S.E.M. of each time period. Tendency of interaction between group and day was observed in pulse amplitude ($P < 0.1$). No significant interaction was observed for other data. Basal concentration, pulse frequency and AUC were greater in the CL-absent cows during experimental period compared with the CL-present cows. Asterisks indicate significant differences between groups ($P < 0.05$).

as a result of cows in the CL-present group experiencing an LH surge in the previous 2 days, whereas follicular aspiration presumably blocked the LH surge in the CL-absent cows. An increase in the circulating E_2 concentration and follicular diameter in the CL-absent cows at day -1 suggests a more rapid growth of follicles in these cows than the CL-present cows. It is well documented that FSH plays a critical role in bovine follicular development before selection, and is especially closely associated with an alteration of the intrafollicular IGF1 system to promote E_2 production (Rivera & Fortune 2003). Lopez *et al.* (2004) reported that cows that develop multiple dominant follicles show an increase in circulating FSH and E_2 concentrations before follicular deviation. Therefore, although there was no difference in plasma FSH concentrations between the two groups from day -1 to day 2, FSH may contribute to the development of co-dominant follicles.

One of the key characteristics of follicular selection is the acquisition of an LH receptor in granulosa cells. However, there are various reports about the timing of the acquisition of the LH receptor by granulosa cells during bovine follicular development (Xu *et al.* 1995, Bao *et al.* 1997, Evans & Fortune 1997, Beg *et al.* 2001). On the other hand, there was no difference in the LH receptor mRNA expression in theca cells between future dominant follicle and future subordinate follicles before follicular selection (Xu *et al.* 1995, Bao *et al.* 1997, Evans & Fortune 1997). Thus, it seems that theca cells of both F1 and F2 might be able to respond to LH before follicular deviation in the CL-absent cattle. In the present study, the basal LH concentration, pulse frequency and AUC of LH were greater in the CL-absent cows than in the CL-present cows during the experimental period. Several studies demonstrated that LH is involved in follicular selection, and that the incidence of co-dominant follicles is closely associated

Figure 6 Individual representative 8 h plasma GH profiles in the (A) CL-present and (B) CL-absent cows. Arrows indicate GH pulses.

Figure 7 Characteristics of GH pulses in the CL-absent and CL-present cows. (A) Basal concentration, (B) pulse amplitude, (C) pulse frequency and (D) area under the curve (AUC). Data were normalized to the day of follicular deviation (day 0) and are shown as the mean $\pm$ S.E.M. of each time period. Tendency of interaction between group and day was observed in basal concentrations and AUC ($P < 0.1$). Interaction between group and day was observed in pulse amplitude ($P < 0.05$). No significant interaction between group and day was observed in pulse frequency. Asterisks indicate significant differences between groups ($P < 0.05$).

with an increase in the level of circulating LH during the bovine follicular wave (Ginther *et al.* 1998, Kulick *et al.* 1999, 2001, Lopez *et al.* 2004). Suppression of LH secretion by P_4 treatment at the time of follicular deviation decreases follicular E_2 and free IGF1 concentrations and increases IGF-binding protein 2 (IGFBP2) concentration in the follicular fluid of the dominant follicle (Ginther *et al.* 2001b). Pulsatile LH infusion into GnRH agonist-treated cows and during the follicular wave of cyclic cows demonstrated that the LH is required for mRNA expression of steroidogenic enzymes in both granulosa and theca cells, which is responsible for a dramatic increase in the follicular fluid concentration of E_2 and further follicular growth (Crowe *et al.* 2001, Manikkam *et al.* 2001, Hampton *et al.* 2004). We postulated that enhanced LH pulses in the CL-absent cows permit accelerated F2 growth and thus stimulate E_2 production. Hence, F2 would reach a dominant status and override follicular selection together with F1, resulting in the development of co-dominant follicles.

A previous study that used ovariectomized goats indicated that E_2 treatment enhanced the GH pulse amplitude and AUC, whereas treatment with P_4 reduced them (Yonezawa *et al.* 2005). Our present results showed that the basal GH concentration and AUC of GH increased at days 1 and 2, and the pulse amplitude increased at days 0 and 1 in the CL-absent cows more than in the CL-present cows. These changes of pulsatile GH release in the CL-absent cows were observed in accordance with the appearance of differences in plasma P_4 and E_2 levels between the two groups, suggesting that the GH secretory profiles were altered by the circulating levels of ovarian sex steroid hormones. Therefore, our results strongly suggest that the ovarian sex steroid hormones have the ability to modulate pulsatile pattern of GH secretion in cows.

In the present study, there were no differences in the plasma total IGF1 concentration between the CL-absent cows and the CL-present cows; however, it increased after rather than before follicular deviation in the CL-absent cows. An increase in the total IGF1 concentration under circulating levels of high E_2 and nadir P_4 after selection of co-dominance in the CL-absent cows is similar to an increase in the circulating total IGF1 at the follicular phase during the bovine oestrous cycle (Kawashima *et al.* 2007b). In addition, Echternkamp *et al.* (1990) reported that cattle selected for the birth of twins have a greater IGF1 concentration than cattle not selected for such a birth. It has been reported that bovine liver expresses mRNA for E_2 receptor, but not P_4 receptor (Pfaffl *et al.* 2002). Thus, an

Figure 8 Changes in plasma concentration of total IGF1 for the CL-absent and CL-present cows. Blood samples were collected twice daily from the day of follicular aspiration for the CL-absent cows ($n = 4$) or the day of ovulation for the CL-present cows ($n = 4$). Data were normalized to the day of follicular deviation (day 0) and are shown as the mean $\pm$ S.E.M. of each time period. A significant interaction between group and day was observed. a, b: Values with different letters are significantly different within CL-absent cows ($P < 0.05$).

increase in total IGF1 concentration may be directly induced by high circulating E_2 secreted from co-dominant follicles. Additionally, since GH is known as a potent stimulator of liver IGF1 secretion, enhanced GH pulses in the CL-absent cows would indirectly stimulate an increase in total IGF1 secretion. Previous studies demonstrate that both GH and IGF1 have the ability to stimulate cell proliferation, steroidogenesis and expression of LH and FSH receptors in bovine follicular cells (Gong *et al.* 1993b, 1994, Spicer *et al.* 1993, Spicer & Echternkamp 1995, Sirotkin & Makarevich 1999). Therefore, enhanced GH pulses and increased total IGF1 concentration in the CL-absent cows may contribute to the growth of the co-dominant follicles.

In conclusion, our results suggest that the absence of CL accompanying nadir circulating P_4 during bovine first follicular wave induces the growth of the co-dominant follicles. Nadir circulating P_4 concentrations in the CL-absent cattle allow the enhancement of LH pulses, which may be involved in further growth of the second largest follicle together with the largest follicle and the appearance of co-dominance. Our results also showed that a condition of nadir circulating P_4 and increased circulating E_2 enhances the GH pulses, suggesting that sex steroid hormones from the ovary may affect the regulation of GH pulsatility in cows.

Materials and Methods

Animals

The animal experiment was carried out at the Field Center of Animal Science and Agriculture, Obihiro University, Japan. Experimental procedures complied with the Guide for Care and Use of Agricultural Animals of Obihiro University. Heifers and non-lactating cows were fed twice a day with corn silage, concentrate and timothy hay (54.1% dry matter (DM) for total dissolved nitrogen (TDN) and 12.5% DM for crude protein (CP). The lactating cows were fed twice a day with a total mixed ration consisting of grass, corn silage and concentrate (70.6% DM for TDN and 14.8% DM for CP). They had access to water *ad libitum*.

The animals had at least two oestrous cycles of normal length (21–23 days) before being used in the experiment. In all animals, the existence of CL in the ovary was confirmed at the mid-luteal stage of the oestrous cycle (10–15 days after oestrus) using transrectal ultrasound scanning; then, they received 500 μ g of a $PGF_{2\alpha}$ analogue (cloprostenol (Estrumate); Schering-Plough Animal Health K.K., Osaka, Japan) i.m. to induce luteolysis.

Experimental procedure

In both experiments 1 and 2, we set the starting point of the experiment as the time of the beginning of CL formation. The CL-present group was started from ovulation, and the CL-absent group was started from the time of disappearance of the dominant follicle by follicular aspiration since ovulation does not occur in this group.

Experiment 1

Ten lactating cows and eight heifers were used and randomly assigned to one of two groups. In the CL-absent cows ($n=5$) and heifers ($n=5$), follicular aspiration was performed at 42 h and 36 h after $PGF_{2\alpha}$ injection respectively. Our previous study confirmed that the dominant follicles were aspirated before exposure to the LH surge (Hayashi *et al.* 2006). The time of follicular aspiration was different between cows and heifers because some heifers showed oestrus at 42 h after $PGF_{2\alpha}$ injection in a preliminary experiment. In the CL-present cows ($n=5$) and heifers ($n=3$), ovulation of the preovulatory follicle was induced by a GnRH analogue (Fertirelin acetate 100 μ g; (Conceral); Schering-Plough Animal Health) injected 48 h after $PGF_{2\alpha}$. Ovulation of the preovulatory follicle was checked by ultrasound scanning, performed at a 24-h interval from the GnRH injection. The day of follicular aspiration (CL-absent groups) or ovulation (CL-present groups) was designated as day 0. Blood samples were obtained from day 0 to day 6 by caudal venipuncture just before each ultrasound scanning every 3 days in cows to determine P_4 and twice a day every 12 h in heifers to determine P_4 and E_2 . In both groups of cows and heifers, ultrasound scanning to monitor follicles was performed every 3 days at days 0, 3, 6 and 9.

Experiment 2

In this experiment, we performed frequent blood sampling to analyse the pulsatile release of LH and GH. Nine non-lactating cows were used. For frequent blood sampling, all cows had a 14-gauge catheter (Argyle CV Catheter Kit; Nippon Sherwood Medical Industries Ltd, Tokyo, Japan) fixed into the jugular vein 2 days before injection of $PGF_{2\alpha}$. In the CL-absent cows ($n=5$), follicular aspiration was performed at 42 h after injection of $PGF_{2\alpha}$. Frequent blood sampling (5 ml) was done every 15 min for successive 8 h in 24 h from just after follicular aspiration, and continued for 6 days. In the CL-present cows ($n=4$), ovulation of the preovulatory follicle was induced by a GnRH analogue injected 48 h after $PGF_{2\alpha}$. For FSH and E_2 determination, blood samples (20 ml) were collected every 6 h for 48 h after injection of GnRH. For determination of the LH surge, a 5 ml blood collection was done every 2 h from 2 h before to 8 h after GnRH injection. Ovulation of the preovulatory follicle was monitored by transrectal ultrasound scanning, performed at a 6 h interval from 24 h after GnRH injection until ovulation was observed. Frequent blood sampling (5 ml) began every 15 min for successive 8 h in 24 h from the time point at which ovulation was observed, and continued for 6 days. In both groups, blood collection (20 ml) for determination of P_4 , E_2 and FSH was done twice daily for 6 days and transrectal ultrasound scanning was performed daily immediately after the end of the frequent blood sampling for 6 days to monitor the number and diameter of each follicle.

Because the length of the time period from ovulation or follicular aspiration to follicular deviation varied among the cows, all the data (follicular diameter, circulating hormone concentrations and characteristics of LH and GH pulses) were normalized to the day of observed deviation based on the criteria established in a previous study (Ginther *et al.* 1996). The day of follicular deviation was defined as the day of beginning of the greatest difference in growth rates between the largest follicle and

the second largest follicle in the CL-present cows or between the co-dominant follicles and third largest follicle in the CL-absent cows during the retrospectively identified ultrasound observation. The day of follicular deviation was designated as day 0.

Follicular aspiration and transrectal ultrasound scanning

The procedure for follicular aspiration was done as previously described (Hayashi *et al.* 2006). Briefly, for ultrasound guidance of the aspiration needle, an ultrasound scanner (SSD-5500, Aloka Co., Tokyo, Japan) was equipped with a 7.5 MHz transvaginal convex transducer (UST-M15-21079, Aloka) attached to a stainless steel needle guide. Before follicular aspiration, cows received caudal epidural anaesthesia with 5 ml of 2% lidocaine ((Xylocaine); AstraZeneca Co., Osaka, Japan) to prevent straining, and then the vulva and perineal area were cleaned. The transvaginal convex transducer was inserted into the vagina, and the ovary containing the targeted follicle was positioned next to the transducer face by rectal manipulation so that the targeted follicle was displayed on the needle path of the monitor. An 18-gauge single-lumen stainless steel needle connected to a 5 ml disposable syringe was pushed into the needle guide and inserted into the antrum of the follicle through the vaginal wall. If the preovulatory follicle could not be identified by ultrasonography, all follicles with a diameter of ≥ 6 mm were aspirated.

To determine the number and size of developing follicles, the ovaries were scanned by transrectal ultrasonography using an ultrasound scanner (SSD-5500, Aloka) equipped with a 7.5 MHz convex transducer (UST-995-7.5, Aloka) in a standardized procedure as previously described in our laboratory (Acosta *et al.* 2002, 2005). Co-dominant follicles were defined as the difference in diameter between the F1 and F2, which was less than 2 mm at the last day of the experiment.

Hormone determinations

In both experiments 1 and 2, blood samples were collected using sterile 10 ml tubes containing 200 μ l (100 μ l for frequent sampling in experiment 2) of a stabilizer solution (0.3 M EDTA, 1% acid acetyl salicylic, pH 7.4). All tubes were immediately chilled in ice water, centrifuged at 2000 *g* for 15 min at 4 °C, and the plasma obtained was stored at -30 °C until hormone analysis. At the end of the experiment, the concentrations of P₄, E₂, FSH, LH, GH and total IGF1 in plasma were determined in duplicate by second-antibody enzyme immunoassay (EIA) using 96-well ELISA plates (Corning Glass Works, Corning, NY, USA). All procedures for each EIA were previously established in our laboratory (Mutayoba *et al.* 1990, Miyamoto *et al.* 1992, Watanabe *et al.* 1997, Acosta *et al.* 1998, Wijayagunawardane *et al.* 1998, Kawashima *et al.* 2007a, 2007c). Steroid hormone assays were carried out after extraction with diethyl ether described previously in our laboratory (Acosta *et al.* 1998). The extraction efficiency of E₂ and P₄ was 80 and 95% respectively. The EIA for P₄ was done as previously described (Miyamoto *et al.* 1992). The standard curve ranged from 0.05 to 50 ng/ml, and the effective dose (ED₅₀) of the assay was 3.2 ng/ml. The intra- and inter-assay coefficients of variation (CV) averaged 6.5 and 9.7%

respectively. The EIA for E₂ was carried out as previously described (Wijayagunawardane *et al.* 1998). The standard curve ranged from 2 to 2000 pg/ml, and the ED₅₀ of the assay was 3.3 pg/ml. The average intra- and inter-assay CVs were 6.3 and 9.5% respectively. The FSH concentrations were determined directly in duplicate as 30 μ l plasma samples using a sensitive EIA for FSH determination in bovine plasma based on the streptavidin-biotin technique by a modification of a method previously reported by Watanabe *et al.* (1997). The standard curve for FSH ranged from 0.18 to 12 ng/ml, and the ED₅₀ of the assay was 1.7 ng/ml. The intra- and inter-assay CVs were 10.1 and 16.2% respectively. The LH concentration was determined directly in duplicate as 15 μ l plasma samples using a sensitive EIA for LH determination in bovine plasma based on the streptavidin-biotin technique as described by Mutayoba *et al.* (1990). The standard curve for LH ranged from 0.09 to 50 ng/ml, and the ED₅₀ of the assay was 3.1 ng/ml. The intra- and inter-assay CVs were 8.3 and 11.2% respectively. The GH concentration was determined directly in duplicate as 15 μ l plasma samples using a sensitive EIA for GH determination in bovine plasma based on the streptavidin-biotin technique by a modification of the method previously described (Kawashima *et al.* 2007c). The standard curve for GH ranged from 0.78 to 100 ng/ml, and the ED₅₀ in this assay system was 6.2 ng/ml. The intra- and inter-assay CVs were 8.1 and 9.2% respectively. The EIA for total IGF1 was done as previously described (Kawashima *et al.* 2007a). Briefly, total IGF1 determination in plasma was performed by EIA after protein extraction by an acid-ethanol mixture (87.5% ethanol and 12.5% 2 M HCl) to minimize interference with IGF1s. The standard curve for IGF1 ranged from 0.39 to 50 ng/ml, and the ED₅₀ of the assay was 2.5 ng/ml. The intra- and inter-assay CVs were 6.9 and 7.5% respectively.

LH and GH pulse analysis

Characteristics of the LH pulses were determined by the PULSAR computer program (Merriam & Wacher 1982). The cut-off criteria for pulse determination G1, G2, G3, G4 and G5 were 3.0, 1.0, 0.8, 0.4 and 0.4 respectively (Yoshioka *et al.* 2001). The following pulse characteristics were calculated: 1) mean concentration, 2) basal concentration, 3) pulse amplitude, the difference between the peak and the preceding basal level, 4) pulse frequency, the mean number of pulses per 8 h and 5) AUC. GH pulses were determined for individual cows as described by Walters *et al.* (1984). Briefly, a pulse was defined as occurring when the value of the highest sample (peak) exceeded a preceding sample (basal) by at least four times the intra-assay CV of the EIA. There had to be at least one more value on the decreasing slope of a pulsatile hormone increase before basal concentrations were reached again. The amplitude was determined by subtracting the basal from the peak value. Mean and basal concentrations, pulse amplitude, pulse frequency and AUC were calculated.

Statistical analysis

In experiment 2, all data (follicular diameter, circulating hormone concentrations and characteristics of LH and GH pulses) were normalized to the day of observed follicular deviation.

In both experiments 1 and 2, normal distribution of the data was assessed by Kolmogorov–Smirnov test. The data of follicular diameter in experiment 1 were evaluated by one-way ANOVA following Tukey–Kramer’s test in both cows and heifers. The data of plasma concentrations of P_4 , E_2 , FSH and total IGF1, follicular diameter in experiment 2, and characteristics of the LH and GH pulses were analysed by two-way repeated-measures ANOVA. When an interaction between group and day was detected, mean values were calculated for each group and each sampling period, and the significant differences were analysed by Tukey–Kramer’s test. If a main effect of the group was detected, significant differences between the CL-absent and CL-present groups were analysed using the Student’s *t*-test throughout the experimental period. Results were presented as mean $\pm$ S.E.M. Statistical significant was considered to be at $P < 0.05$.

Declaration of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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