Luteal function in pregnant and non-pregnant non-cycling inseminated cows ("Phantom" cows)

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ABSTRACT

Cows that do not return to oestrus within 24 days of insemination but are retrospectively diagnosed as not pregnant (non-return, non-pregnant or "Phantom" cows), frequently have a functional corpus luteum (CL) that does not regress. In this study, luteal function around the time of pregnancy diagnosis was compared between pregnant cows and "Phantom" cows with a CL. Cows in two dairy herds that had not returned to oestrus following first insemination were pregnancy tested by ultrasound 5 weeks after insemination. Final analyses included 17 pregnant cows that were cycling normally before being inseminated (CYCP); 22 pregnant cows that had been treated for anoestrus (ANP); and 11 "Phantom" (PC) cows that had not cycled since first insemination and were not pregnant. Blood and CL samples that were collected from each cow between 34 and 38 days after first insemination were assayed for progesterone (P4). Luteal P4 concentrations were similar in pregnant and PC cows (101.6 ± 9.9, 100.7 ± 8.9 and 98.9 ± 12.4 ng/mg for CYCP, ANP and PC cows). Plasma P4 concentrations were also similar (8.5 ± 0.5, 7.6 ± 0.4 and 7.1 ± 0.6 ng/mL) for CYCP, ANP and PC cows. The absence of a return to oestrus in this group of PC cows was most likely related to pregnancy recognition being followed by embryo death and then associated with a failure of luteolysis.

Keywords: cows; pregnant; Phantom; luteal progesterone; plasma progesterone.

INTRODUCTION

The InCalf project set up to study reproductive performance in Australian dairy herds identified a proportion of cows that were not re-inseminated within 24 days of their first insemination and were retrospectively diagnosed as not pregnant. These cows were defined as "Phantom Cows" (Cavalieri *et al*., 2000). They are a potential cause of economic loss as 21% of these cows were not pregnant at the end of a 21-week breeding period (Nation *et al*., 2001).

A major breeding goal is to increase the proportion of cows that become pregnant in the first six weeks of a seasonally intensive breeding period. Although high submission rates to insemination are a key to achieving this goal (Morton, 2004), they decline as the breeding period progresses (Cavalieri *et al.*, 2000; Morton, 2004). A study in ten Victorian dairy herds found that submission rates declined from 96% to 51% among non-pregnant cycling cows over three successive rounds of oestrus synchronisation (Cavalieri *et al*., 2000). Overall, 13% of the cows in these herds failed to return to oestrus even though they were not pregnant. This equates to a 13% incidence of the "Phantom cow" syndrome over all cows. A second Victorian study found that 19% of all of the inseminated cows in four herds became "Phantom cows" (Nation *et al*., 2001).

Pino (2004) found that plasma concentrations of progesterone (P4) and corpus luteum (CL) size between 25 and 37 days after insemination were similar in pregnant and "Phantom" cows. This result suggested that the function of the persistent CL in "Phantom" cows was similar to the function of normal CLs in pregnant cows with a similar pre-insemination history. The current study was conducted to investigate luteal function in pregnant and "Phantom" cows using biopsied CL tissue to estimate concentrations of P4 in luteal tissue.

MATERIAL AND METHODS

Ethics and care of animals

All experimental procedures were carried out under the guidelines of the University of Melbourne Animal Experimentation Ethics Committee Protocol # 03174. Procedures were carried out in two separate herds, Herd A and Herd B, located in the Macalister Irrigation District within a 20 km radius of Maffra. The experimental animals were run with their herd-mates throughout the experimental period. Any experimental interventions were carried out during or after milking times and animals were returned to the herd no later than two hours after the end of milking.

Oestrus events were monitored during the 30 days before the start of the breeding period by applying tailpaint. The paint was checked at every milking and especially from 18 to 24 days after first insemination (Macmillan *et al.*, 1988). Those cows not detected in oestrus by 8 days before the start of the breeding period were presented for a veterinary examination. Those diagnosed as anoestrus were treated with a CIDR device (Genetics Australia, Bacchus Marsh, Victoria) for 8 days and oestradiol benzoate (ODB;CIDIROL; Genetics Australia, Bacchus Marsh, Australia) injected concurrently with CIDR insertion (2 mg ODB) and again 24

hours after device removal(1 mg ODB). Animals were inseminated following detection of oestrus. Treated anoestrous animals were resynchronised for their subsequent oestrus (8-day re-used CIDR with 1 mg ODB at CIDR insertion, and at 24 hours after device removal) with insertion occurring from 13 to 15 days after first insemination.

Experimental design

The potentially suitable cows were inseminated on the first two days of the artificial insemination program. Paint strips were monitored routinely with returns to service being re-inseminated. In addition, the anoestrous cows that did not display oestrus after the resynchronised oestrus each had a blood sample taken within 3 days of the expected oestrus to identify cows with concentrations of P4 similar to those found in the luteal phase of an oestrous cycle or early pregnancy. Previously cycling cows that had no tailpaint removed were not blood sampled at this time as it was considered unlikely that they would become anoestrus.

Potentially suitable cows from each herd were presented for pregnancy diagnosis by transrectal ultrasound (Aloka, Tokyo, Japan) 35 days after the start of artificial insemination. Cows were enrolled if they had their first insemination in the first two days of the artificial insemination period; and had not been detected in oestrus following insemination. The enrolled animals were categorised depending on pregnancy status and their cycling status before first insemination. The groups were:

1. Pregnant cows that were cycling before first insemination (CYCP).
2. Pregnant cows that were anoestrus before first insemination (ANP).
3. Non-pregnant or "Phantom" cows (PC). Any cows with low plasma P4 concentrations in samples taken at about Day 24 were excluded.

Corpus luteum biopsy samples were collected between 34 and 38 days after the start of the period of artificial insemination (Days 34 to 38 of pregnancy). Two luteal tissue samples were collected from each cow from no more than three attempts using the procedure described by Borman (2004). Each tissue sample was divided into two and snap frozen in dry ice. Some samples were subsequently found to be unsuitable. The maximum cross sectional diameters of each CL were also measured and blood sampled for P4 analysis. The final number of cows in the CYCP, ANP and PC categories were 17, 22 and 11 respectively.

Progesterone assays

Plasma and luteal tissue samples were analysed in two separate assays for each herd. Luteal tissue was extracted using the method described by Borman (2004) and luteal P4 concentration for each animal was estimated as the mean concentration of 2 to 4 tissue samples. Total P4 was calculated by multiplying luteal P4 concentration (ng/mg) by the estimated volume of the CL (cm^3) assuming that 1 cm^3 weighed 1 g. The volume was derived from the formula $^4/_3\pi r^3$ where r was determined by ultrasound examination. The average intra-assay CV was 4.09% across all assays, and the inter-assay CVs were 6.78, 7.84 and 13.53% for QCH, QCM and QCL respectively.

Statistical analyses

Data were analysed by a one way analysis of variance (univariate ANOVA; SPSS for Windows v12.0.1) with the model including herd and group as fixed effects. All main effects and their interactions were included in the initial model and the interaction was removed when not significant ($P > 0.1$). Only significant interactions have been reported.

RESULTS

Experimental animals

Animals in both herds had similar average intervals from calving ($P = 0.86$) and daily milk yields (22.0 ± 1.1 vs. 25.6 ± 1.0 L/day for Herd A vs. Herd B; $P = 0.16$). Table 1 summarises these parameters for the different groups of animals in both herds.

Luteal function

Plasma and luteal P4 concentrations between 34 and 38 days after first insemination were similar for pregnant and PC cows in both herds (Figure 1; $P > 0.05$).

TABLE 1: Descriptive parameters for pregnant cows that were previously cycling (CYCP), pregnant cows that were previously anoestrus (ANP) and non-pregnant cows that were not re-inseminated (Phantom cows; PC) in two seasonal dairy herds.

Herd	Group	Interval from calving (d)	Milk yield (L/d)
Herd A	CYCP	87.0 ± 5.1	20.5 ± 1.5
	ANP	57.9 ± 4.1	22.5 ± 1.3
	Phantoms	63.3 ± 6.8	22.5 ± 1.8
	All cows	*69.4 ± 3.2*	*21.8 ± 1.1*
Herd B	CYCP	70.3 ± 4.3	24.2 ± 1.4
	ANP	62.2 ± 4.3	26.2 ± 1.4
	PC	73.4 ± 5.1	26.3 ± 1.6
	All cows	*68.6 ± 2.7*	*25.6 ± 1.0*

FIGURE 1: Concentrations of progesterone (P4) in the plasma (a) and corpus luteum (b) between 34 and 38 days after first insemination in CYCP (■), ANP (□) and PC (Cross hatch) cows.

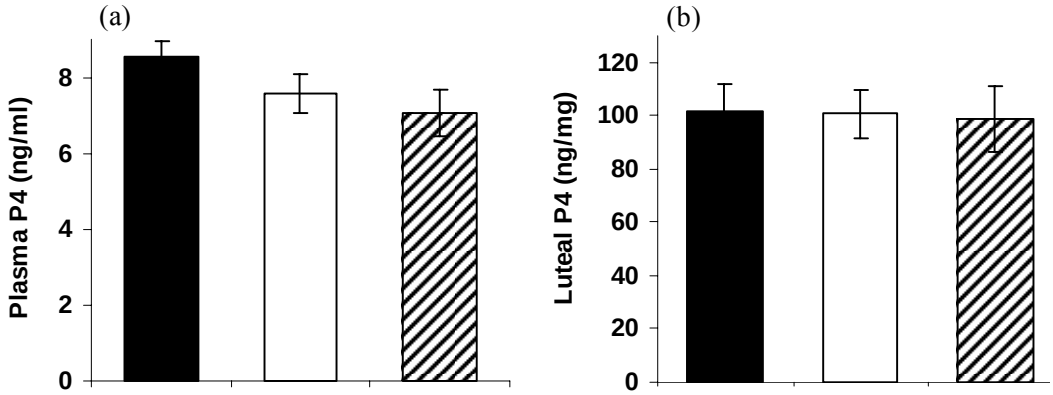
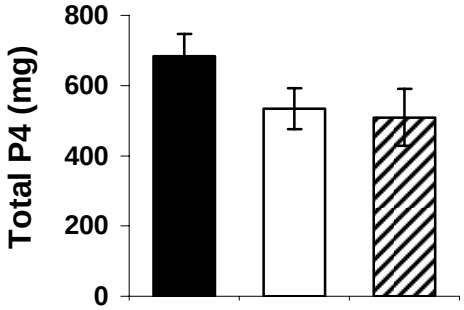


FIGURE 2: Total progesterone (P4) between 34 and 38 days after first insemination in the corpus luteum of CYCP (■), ANP (□) and PC (Crosshatch) cows. (P = 0.15).



The total estimated P4 in the CL of pregnant and PC cows was similar (P = 0.15) but differed between herds (Figure 2; P <0.01). Total P4 was lower in animals from Herd A compared with those in Herd B (386.2 ± 59.0 vs. 764.5 ± 51.7 mg for Herd A vs. Herd B).

DISCUSSION

Luteal function was similar in pregnant and PC cows between 34 and 38 days after first insemination in both herds, and was not affected by cycling status before insemination. Concentrations of P4 in the plasma and CL were similar, as was estimated total luteal P4.

Due to the nature of the syndrome, PC cows were diagnosed retrospectively. This provided a challenge to experimental design because investigators do not know which cows would be "Phantoms" before pregnancy diagnosis was carried out. For this reason PC cows in the current experiment comprised a mixture of animals that cycled spontaneously or were treated for anoestrus before the start of the breeding period. Anoestrous cows were targeted because treatment for anoestrus is a risk factor for developing the "Phantom" cow syndrome.

This experiment is the first occasion that CL biopsies were collected from pregnant cows in a commercial herd. The technique had been previously validated in 8 pregnant non-lactating cows that had CL's biopsied on four occasions between Days 32 and 53 of pregnancy (Borman, 2004). Collection of multiple tissue samples at weekly intervals did not alter luteal cell dynamics and was not associated with any incidence of embryonic loss in that preliminary study. Lactating cows in a commercial environment are under the influence of different stressors and there was a possibility that the technique would disrupt the CL or pregnancy and cause embryonic loss. Only three of the 21 pregnant animals in Herd A and 2 of the 18 pregnant cows in Herd B were found to be not pregnant at a subsequent pregnancy diagnosis eight weeks after biopsy, representing a loss rate of 10.3% between 35 and 90 days (5 and 13 weeks) of gestation.

The current experiment supports evidence from other studies to suggest that luteal function in PC cows is similar to that in pregnant cows. The CLs of the PC cows produced as much P4 per unit weight as those of pregnant cows and plasma concentrations of P4 were also similar. It was not possible to determine if early embryonic loss occurred before the pregnancy diagnosis on Day 35. In a previous study, only 10% of "Phantom" cows were pregnant at Day 28 and subsequently lost an embryo (Nation, 2002). None of the "Phantom" cows in the current experiment had evidence of a resorbing/deceased embryo or uterine fluid, but the possibility of embryonic loss cannot be ruled out.

The results of this study combined with those by Cavalieri *et al*. (2000), Nation *et al*. (2001) and Pino *et al*. (2006) indicate that the "Phantom" Cow Syndrome involves inseminated cows that each have apparently normal CL function as reflected by

plasma and luteal concentrations of P4, but have an extended luteal phase that is not associated with the presence of an identifiably viable embryo at least by Day 35. Pino *et al.* (2006) reported that the incidence was most frequent in early lactation when milk yield would be close to its peak but body tissue was still being mobilised. The extended luteal phase seems most likely to be the sequel to an embryonic death occurring after pregnancy recognition but before placentation.

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