Augmentation of luteal function in the lactating ewe after induction of ovulation with a gonadropin releasing hormone

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Numerous studies have shown that in the acyclic ewe, the first corpus luteum (CL) after administering gonadotropin releasing hormone (GnRH) will lead to inadequate progesterone (P_4) concentrations or will be short-lived. An attempt was made to promote the function of the GnRH-induced CL in lactating ewes (n = 70) either by increasing endogenous LH release through administration of naloxone (4 injections at 1 mg/kg body mass every 2 hrs over 3 days) or via an exogenous supply of gonadotropin (PMSG, 100 I.U., twice daily for 3 days). Treatments were applied in an effort to promote maturation of the preovulatory follicle or to stimulate the CL. Naloxone, either before or after, and PMSG prior to GnRH (Day 0) did not increase plasma P_4 concentrations. PMSG on Days 3–5 after GnRH was more effective in promoting luteal function (compared to untreated controls) than similar treatment on Days 6–8 or 9–11. This was evidenced by a greater ($P < 0,05$) total area under the P_4 curve (34,2 ± 2,9 vs 25,7 ± 2,9 units), an increased ($P < 0,05$) number of days for which P_4 concentrations exceeded 2,0 ng/ml (6,6 ± 1,5 vs 2,5 ± 1,1 days) and a higher ($P < 0,05$) P_4 level on Days 8–12. Only the P_4 concentrations on Days 10 and 11 were improved ($P < 0,05$) by PMSG on Days 6–8, while PMSG on Days 9–11 was without significant effect. Luteotropic support of the CL for a short period prior to mid-cycle was thus more beneficial to luteal function than stimulation at any other stage.

Verskeie studies met anestrus ooie het getoon dat die eerste corpus luteum (CL) na toediening van gonadotropiese vrystellingshormoon (GnVH) onvoldoende progesteroon (P_4) afskei, of 'n kort lewensduur het. 'n Poging is aangewend om die funksionering van 'n GnVH-verwekte CL by 70 lakterende ooie te verbeter. Endogene vrystelling van LH is bevorder deur middel van naloxonetoediening (4 inspuitings elke 2 uur vir 3 dae). Hierdie behandeling is met 'n eksogene bron van gonadotrofien (DMSG twee keer per dag vir 3 dae) vergelyk. Die behandelings is toegepas om, of volwassendheid van die follikel te bevorder of die CL na ovulasie te stimuleer. Naloxone, voor of na, en DMSG voor GnVH-toediening het nie die plasma-progesteroon waardes verhoog nie. Waar DMSG-behandeling op die derde dag na GnVH-inspuiting begin is, was die af skeiding van P_4 höer as waar toediening op of die sesde of negende dag begin is. Hierdie gevolgtrekking word gestaaf deur die groter ($P < 0,05$) area onder die P_4-kurwe (34,2 ± 2,9 vs 25,7 ± 2,9 eenhede), 'n verhoogde ($P < 0,05$) aantal dae waarop plasma P_4–konsentrasies 2,0 ng/ml oorskry het (6,6 ± 1,5 vs 2,5 ± 1,1 dae) en 'n höer ($P < 0,05$) P_4-waarde 8–12 dae na GnVH-toediening, in vergelyking met die kontrole. Slegs die DMSG-toedienings op Dae 6 tot 9 het die P_4 konsentrasies op Dae 10 en 11 verhoog ($P < 0,05$) terwyl DMSG-toediening op Dae 9 tot 11 geen effek gehad het nie. Die mees voordelige stadium om die funksie van die CL te bevorder, is dus kort na ovulasie.

Keywords: Lactating ewes, luteal function, opioids, progesterone, PMSG.

Introduction

Ovulation can be induced in the majority of postpartum or seasonally anoestrous ewes by treatment with gonadotropin releasing hormone (GnRH). However, the ensuing luteal function is subnormal, being characterized by reduced blood levels of progesterone (Hunter *et al.*, 1987) or a corpus luteum (CL) which regresses prematurely (Legan *et al.*, 1985).

Luteinizing hormone (LH) is required for preovulatory maturation of follicles (Haresign & Lamming, 1978) and this hormone may be needed to maintain CL function (Hansel & Convey, 1983). Consequently, inadequacies of the GnRH–induced CL could be attributed to deficiencies in the release of LH which is suppressed during the postpartum anoestrous period (Wright *et al.*, 1983). The low frequency of episodic LH release during the early postpartum period has been attributed to a prolongation in the time interval between GnRH releases from the hypothalamus (Wright *et al.*, 1981).

Where fertilization is successful, but the CL does not function normally, pregnancy is unlikely to continue. It has even been suggested that the embryo will not develop unless progesterone concentrations exceed a threshold level (Staples & Hansel, 1961). This is supported by the finding that administration of gonadotropins within a few days after mating, in order to stimulate the CL (Gamboni *et al.*, 1984), greatly improved pregnancy rates (Kittock *et al.*, 1983).

Endogenous opioid peptides (EOP) inhibit GnRH release from the hypothalamus of cattle (MacDonald *et al.*, 1986) and sheep (Matthews & Murdoch, 1985; Stansfield *et al.*, 1987). However, direct effects of EOP on the pituitary of these species have also been demonstrated (Matteri & Moberg, 1985; Chao *et al.*, 1986). LH secretion in the cycling (Brooks *et al.*, 1986) and postpartum ewe (Gregg *et al.*, 1986) thus appears to be modulated by EOP.

In view of the foregoing this study focussed on two main aspects: 1) When the lactating ewe is induced to ovulate by the administration of GnRH, will an increase in endogenous

LH levels (tonic LH) via administration of an opioid antagonist such as naloxone (NAL) promote maturation of follicles destined to ovulate. This may then result in improved luteal function as has been achieved by administering PMSG (Haresign & Lamming, 1978; Grobbelaar *et al.*, 1989). When naloxone is applied after ovulation, the increase in frequency of LH release can be expected to improve the secretory function of the newly formed CL. 2) When exogenous luteotropic support is provided in the form of PMSG, at what stage postovulation should administration occur in order to maximize the beneficial effects on luteal function?

Materials and methods

Experimental design

Seventy, lactating, South African Merino ewes, ranging in age from two to five years were used in the spring lambing season. Ewes lambed over a period of three weeks and in order to reduce seasonal effects, were assigned to one of three blocks so that all ewes that lambed within a period of a week were blocked together. Numbers within a block were thus dictated by the date of parturition. Within a block, ewes were assigned randomly to seven different treatment groups, irrespective of the age of the ewe or the number of lambs born (single or twin lambs). The layout of the treatment schedule is listed in Figure 1. At 35 ± 4 days postpartum (Day 0) all ewes received 4,2 g buserelin acetate (GnRH, Receptal, Hoechst) in a single im. injection. PMSG (UpJohn) treatments were administered twice daily for 3 consecutive days. Ewes received im. injections of 100 IU PMSG at 12–hourly intervals at 06:00 h and 18:00 h. Naloxone (Sigma International) was dissolved in 0,9% saline to a concentration of 0,05 g naloxone/2ml saline. NAL was injected by jugular venepuncture every 2 h at 0800 h, 1000 h, 1200 h and 1400 h for 3 consecutive days, either before (NAL–) or after (NAL+) GnRH. Ewes received 0,2 g Naloxone/day (approximately 1 mg naloxone/kg mass of the ewe per injection) during the experiment. Progesterone levels were measured in jugular blood samples collected every two days and drawn prior to the first administration of naloxone or PMSG for that day.

Table 1 Number of ewes which cycled spontaneously prior to treatment or which reovulated shortly after ovulation was induced by GnRH on Day 0

Treatment relative to GnRH	Group	Cycled spontaneously	Reovulated	Were anoestrus
Control	Control	1	2	7
Before ovulation:				
Naloxone Days –3 to –1	NAL-	1	4[a]	5
PMSG Days –3 to –1	PMSG-	2	4[a]	4
After ovulation:				
PMSG Days 3 to 5	PMSG3	0	2[b]	8
PMSG Days 6 to 8	PMSG6	1	2[b]	7
PMSG Days 9 to 11	PMSG9	0	3[b]	7
Naloxone Days 2 to 4	NAL +	0	1[b]	9

Numbers with different superscripts differ significantly ($P < 0,05$)

Sampling continued for 18 days after the GnRH injection. Ewes which had cycled spontaneously prior to the onset of the experiment were identified by measuring the progesterone concentration of a sample taken five days prior to GnRH administration.

Hormone determination

Serum concentrations of P_4 were determined by radioimmunoassay (Butcher, 1977). Intra- and inter-assay co-efficients of variation were 13,56% (Pool 1), 19,07% (Pool 2) and 9,35% (Pool 1), and 7,8% (Pool 2), respectively. Recovery of ^{3}H-progesterone added to the serum before extraction was 89,64 ± 3,95% with a co-efficient of variation of 4,4%.

Statistical analysis

Initially, hormonal data were analysed as a split-plot design with 'treatment effects' as the whole plot and 'time' as the subplot stratum. Homogeneity of the variance-covariance structures for each treatment and pooled treatments were

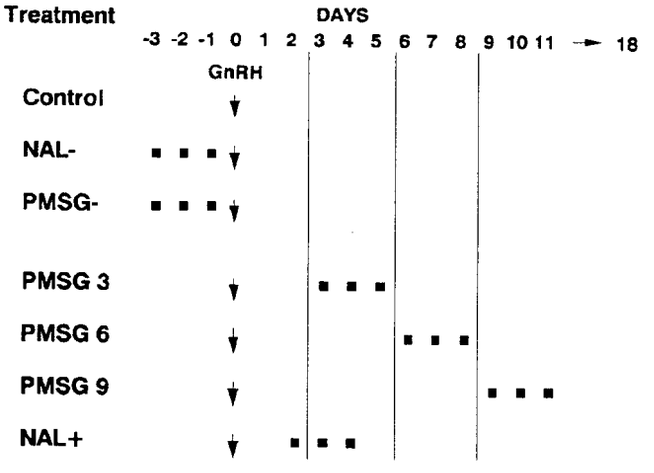

Figure 1 Diagrammatic representation of treatments applied and the time of administration in relation to GnRH injection (Day 0).

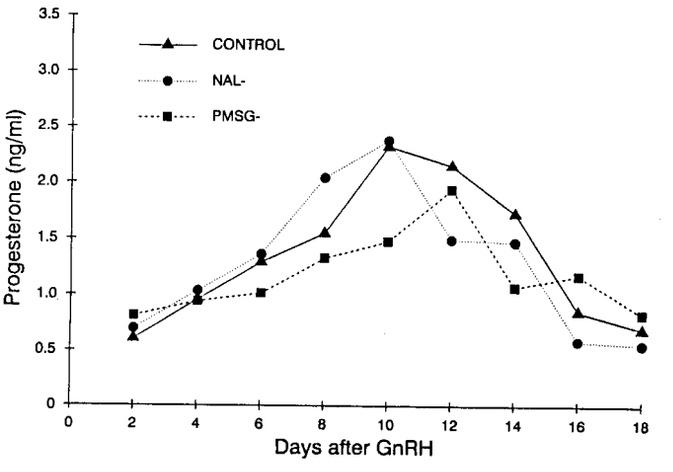

Figure 2 Effect of treatment with naloxone or PMSG prior to ovulation on subsequent luteal function in ewes.

assessed using the repeated measures macro of the Genstat Package. A correction factor was estimated and applied to the degrees of freedom (DF) in the F test when compound symmetry was not present. However, when homogeniety of the error variance fails to hold, the univariate splitplot tests of treatment means are inconclusive (Gill & Hafs, 1971). The analysis then proceeded with a combination of orthogonal comparisons of nth order regression curves for each treatment (Clarke, personal communication) and by partitioning of the treatment x period interaction to permit sensitive comparisons of treatments. These were evaluated with Bonferroni t statistics (Gill, 1986). Treatment effects on the proportion of ewes which exhibited a short-lived CL were tested by chi-square.

Results

On examination of the individual plasma P_4 profiles it became evident that some ewes had ovulated prior to the commencement of treatments ($P_4 > 0,5$ ng/ml) whereas others had undergone a short cycle and had re-ovulated approximately 6–8 days after the GnRH injection. In many such ewes the P_4 concentration on day 18 had not declined below 2 ng/ml. These ewes (Table 1) were eliminated for further analysis of the results. Definitions of normal (McLeod *et al.*, 1983; Brown *et al.*, 1988) and abnormal luteal function (Hunter *et al.*, 1988) were applied in eliminating animals. A greater proportion ($P < 0,05$) of ewes exhibited a short-lived CL in those groups treated before GnRH (PMSG–, NAL–) than where treatments commenced after ovulation (Table 1). The mean P_4 concentration, for ewes within each treatment based on samples drawn every two days, is depicted in Figures 2 and 3. It is clear (Figure 2) that neither PMSG nor naloxone, when administered prior to ovulation, resulted in an improved luteal function (compared to the untreated controls). In contrast, provision of lutcotropin on Days 3–5 after ovulation markedly stimulated the secretory activity of the CL, as evidenced by the significant ($P < 0,05$) increase in the total area under the P_4 curve, from Day 0 to 18 after GnRH (Table 2). No significant response to the immediate stimulatory effect of PMSG from Days 3–5 of the induced cycle (measured by the area under the P_4 curve from Days 4 to 6) was detected (Table 2). However, by Days 7–9 the effect of PMSG early in the cycle (PMSG 3) was stimulatory (Table 2). In contrast, PMSG administration either on Days 6–8 or 9–11 did not alter significantly, either the area under the P_4 curve for the whole cycle or shortly after such treatments commenced (Table 2). Although ewes treated with PMSG after GnRH administration exhibited higher peak P_4 concentrations than untreated controls or those given PMSG prior to ovulation (Table 3), the differences were not significant. The augmentation of luteal function by PMSG administration on Days 3–5 is illustrated further by the significantly ($P < 0,05$) greater number of days during which P_4 levels remained above 2 ng/ml than in Control ewes and those treated before GnRH administration (Table 3). Application of the repeated measures analysis indicated a highly significant effect of both 'time' and of the interaction between treatment and time. The epsilon value used to reduce DF for the F test was 0,6972. A significant time × treatment interaction occurred and this suggested that treatment differences might exist. Preplanned orthogonal contrasts of the fitted response curves, using 'time' to the 4th degree and 'ewes within each treatment', supported the earlier conclusion that neither provision of naloxone nor PMSG (CONTR vs PMSG– and NAL–; PMSG– vs NAL–) before ovulation were beneficial as regards luteal function. This analysis also suggested that exogenous luteotropic support was stimulatory (CONTR *vs* PMSG 3, PMSG 6 and PMSG 9; $P < 0,01$), but that stage of the cycle during which it was provided was perhaps of lesser importance (PMSG 3 *vs* PMSG 6 and PMSG 9: not significant, PMSG 6 *vs* PMSG 9: not significant). Preplanned non-orthogonal comparisons between treatment means (Bonferroni's test) for specific days indicated that PMSG on Days 3–5 and Days 6–8 significantly ($P < 0,05$) increased plasma P_4 levels on Days 8–12 and 10–11, respectively (Figure 4), whereas when injected on Days 9–11 no significant effect occurred.

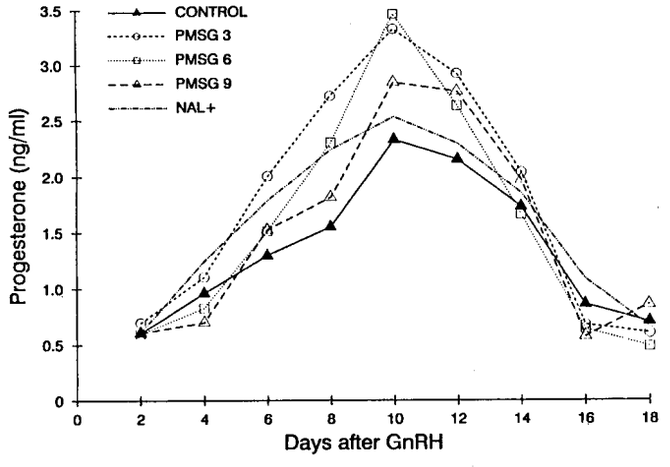

Figure 3 Influence of exogenous or endogenous luteotropic support on progesterone production in ewes.

Table 2 Area under the progesterone curve after GnRH-induced ovulation in ewes treated to stimulate luteal function

Treatment group	n	Mean area ± SEM Days 0–18	4–6	7–9	10–12
Control	7	25,7 ± 2,4^{a}	3,0 ± 0,3^{a}	4,4 ± 0,5^{a}	6,6 ± 1,0^{ab}
NAL-	5	27,4 ± 3,7^{ab}	3,3 ± 0,6^{a}	5,4 ± 1,1^{ab}	6,2 ± 0,7^{a}
PMSG-	4	24,9 ± 2,9^{ab}	2,9 ± 0,4^{a}	3,7 ± 0,8^{a}	5,5 ± 1,2^{a}
PMSG3	8	34,2 ± 2,9^{b}	4,3 ± 0,6^{a}	7,6 ± 0,9^{b}	9,4 ± 0,9^{b}
PMSG6	7	29,8 ± 4,1^{ab}	3,1 ± 0,5^{a}	6,4 ± 0,9^{ab}	9,2 ± 1,3^{ab}
PMSG9	7	28,6 ± 3,9^{ab}	2,9 ± 0,4^{a}	5,4 ± 0,8^{ab}	8,2 ± 1,5^{ab}
NAL +	9	27,4 ± 3,7^{ab}	3,7 ± 0,8^{a}	5,5 ± 1,2^{ab}	6,6 ± 0,9^{ab}

^{ab} Means within the same column without common superscripts differ ($P < 0,05$).

Table 3 Peak P_4 concentrations and number of days P_4 exceeded 2 ng/ml for ewes treated with PMSG or Naloxone before or after a GnRH-induced ovulation

Treatment	Peak P_4 level (ng/ml)±SEM	No of days P_4>2ng/ml ±SEM
Control	2,04 ± 0,44[a]	2,5 ± 1,1[a]
NAL-	2,10 ± 1,30[a]	2,4 ± 1,5[a]
PMSG-	2,02 ± 1,10[a]	2,5 ± 1,2[a]
PMSG3	3,15 ± 0,33[a]	6,6 ± 1,5[b]
PMSG6	3,11 ± 0,44[a]	5,3 ± 1,2[ab]
PMSG9	2,77 ± 0,52[a]	3,8 ± 1,3[ab]
NAL+	2,20 ± 1,27[a]	4,6 ± 1,5[ab]

[ab] Means within the same column with different superscripts differ ($P < 0,05$).

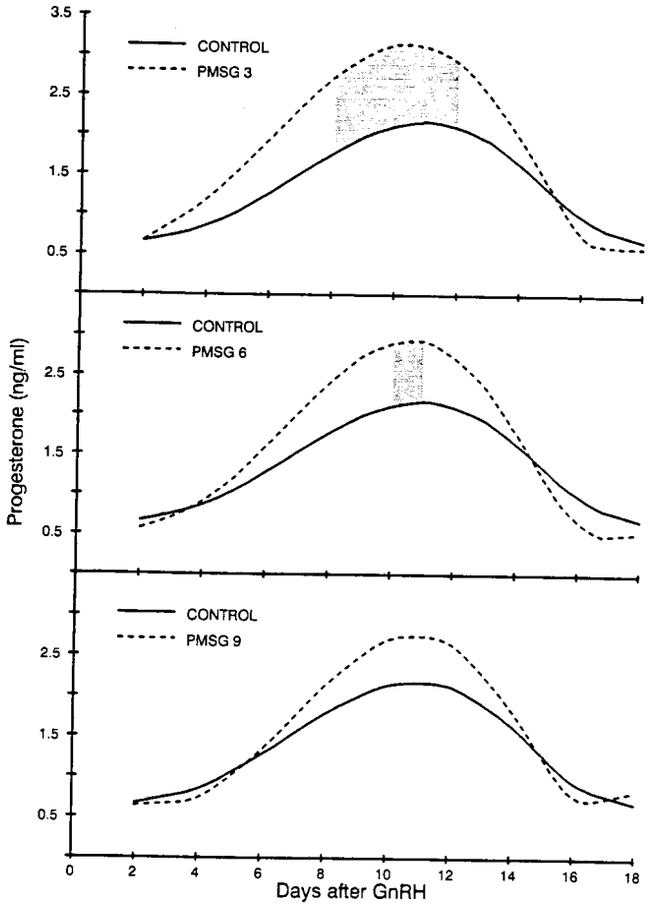

Figure 4 Estimated regression curves of progesterone concentration for ewes receiving PMSG for 3 days beginning on Days 3, 6 or 9 after GnRH administration. The shaded area indicates significant differences ($P < 0,05$) in mean daily concentration.

Discussion

Conclusions from both the present study and that of Grobbelaar *et al.* (1989) suffer from one important handicap. This was the failure to induce a high incidence of short-lived CL when the ewes (Controls) received GnRH and no other hormone treatment. This contrasts with studies in which more than 60% of the anoestrous ewes given GnRH as a single, large injection (Haresign *et al.*, 1975) or repeated small doses, with or without a bolus injection (McLeod *et al.*, 1982b; Hunter *et al.*, 1986; Hunter *et al.*, 1988) exhibited premature demise of the CL.

The data in Table 1 show that of all the ewes treated, only 36% exhibited a short-lived CL. Contrary to expectation, the highest incidence was in the groups treated with PMSG or naloxone prior to GnRH to stimulate follicle maturation (Table 1). This does not conform with reports where either PMSG (Haresign & Lamming, 1978) or repeated injections of LH (McNeilly *et al.*, 1982) or GnRH (McLeod *et al.*, 1982b) were administered prior to ovulation. When the GnRH dose was as large as 1 000 ng and administered every 2 h for 8 days, the luteal function was judged to be normal (McLeod *et al.*, 1982a) although the rise in P_4 was delayed until after the cessation of GnRH administration. The P_4 profiles described by McLeod *et al.* (1982a) are similar to many of those seen in the present study, particularly where PMSG was administered prior to GnRH. It appears that such secretory patterns are worthy of closer scrutiny since Legan *et al.* (1985), Southee *et al.* 1988) and Hunter *et al.* (1988) have recently demonstrated that the postovulatory progesterone rise may only slightly exceed 0,5 ng/ml and be of transient duration.

Except for the difference in season (spring *vs* autumn), there is no obvious reason why the results of the present study should differ notably from those reported by Grobbelaar *et al.* (1989). In the latter study, PMSG pretreatment improved the quality of luteal function to equal that of spontaneously cycling, non-lactating ewes. Continuous exposure to elevated levels of LH is suspected of causing desensitization of ovarian receptors (Clayton *et al.*, 1979). If this occurs, infusion of LH (Grobbelaar *et al.*, 1989) is likely to have been more detrimental than twice daily injections (present study). However, the results reported here do not support such an expectation.

A further possibility worth considering is that in cycling ewes the preovulatory follicle is exposed to episodic LH pulses. In contrast, ewes receiving twice daily injections of PMSG would be exposed to fairly constant levels of gonadotropin because of the extended half-life of this preparation (McIntosh *et al.*, 1975). The question as to whether or nor LH must be delivered in an episodic fashion in order to sustain maturation of follicles remains unanswered (McNatty *et al.*, 1982).

The foregoing should be viewed in the light of the dramatic effect, on plasma P_4 levels, of luteotropic support reported here (Figure 3) and also observed by Kittok *et al.* (1983) and Grobbelaar *et al.* (1989). It appears that the mechanism which results in a short-lived CL is not the same as that responsible for subnormal progesterone secretion during a cycle of approximately normal duration. It has been suggested that in ewes destined to have a short-lived CL the premature regression of the CL is due to an early release of $PGF_2\alpha$ from the uterus (Legan *et al.*, 1985; Hunter *et al.*, 1989). However, Rahmanian & Murdoch (1987) have shown that in the cycling ewe the CL can have

an inherent limited lifespan that is not dependent on the presence of the uterus.

The suggestion that short-lived and inadequate CL are the result of different mechanisms is supported by Rahmanian & Murdoch (1987). They proposed that factors which influence the quality of CL function reside within the follicle. The follicle can thus be capable of ovulating before it acquires the capacity to form a normal CL (deZerega & Hodgen 1981; Murdoch *et al.*, 1983).

The possibility remains that inadequate luteal function could be due to deficiencies in maturation of the follicle prior to ovulation or the consequence of insufficient luteotropic support of the developing CL. Evidence favouring the latter is provided by the stimulatory effect of PMSG when administered after ovulation (Figure 3). However, just how inadequate luteal function is defined can modify the interpretation. Grobbelaar *et al.* (1989) suggested that P_4 levels need to rise above 2 ng/ml, whereas McLeod *et al.* (1983) have set a limit of > 1,5 ng/ml, while Southee *et al.* (1988) proposed that such a level needs to be exceeded for at least 8 days. If the latter criteria were to be applied to the present study then only one ewe would have been judged to have shown normal luteal function.

Gamboni *et al.* (1984) injected anoestrous ewes with 500 i.u. hCG on Day 5 after induced ovulation and recorded an increase in plasma P_4 on Day 10. This agrees with the response obtained from PMSG on Days 3–5 (Figures 3 and 4). The stimulatory effect of PMSG, when delayed until Day 9 after GnRH (Figure 2) is similar to that obtained where hCG was administered on Days 11–13 in lactating ewes (Kittok *et al.*, 1983).

Although there is little doubt that the administration of PMSG in the early part of an induced cycle will improve the function of the CL, the effect on pregnancy rates of ewes remains to be determined.

The failure of naloxone treatment (either before or after GnRH) to stimulate luteal function might be explained on the basis that endogenous LH levels were not increased by the treatment regime followed. This contention is based on the finding that when naloxone was given to rams on subsequent days the responsiveness of LH secretion changed (Ebling & Lincoln, 1985). Similarly, Currie & Rawlings (1989) observed a transient effect on LH pulse amplitude in cycling ewes. However, the enhancement of pulse frequency by naloxone continued over a 26-h infusion period (Currie & Rawlings, 1989). Seasonal effects were also possible since Ebling & Lincoln (1985) proposed that opioid inhibition of LH release may be highest when sheep are expected to be sexually active. The experiment reported here was conducted at the start of the breeding season.

Although Brooks *et al.* (1986) suggested that naloxone will increase LH levels only in the presence of raised P_4 concentrations, this is not supported by Currie & Rawlings (1989). Furthermore, naloxone has been shown to be capable of raising tonic LH concentration in lactating ewes (Gregg *et al.*, 1986; Newton *et al.*, 1988). In addition, constant infusion of 0,5 mg naloxone/kg/h for 24 h resulted in a slight increase in progesterone levels in cycling ewes (Currie & Rawlings, 1989). Accordingly, the sampling schedule employed in the present study was not designed to measure the effect on LH levels. However, Whisnant *et al.* (1986) noted a relationship between days postpartum and the dose of naloxone that was needed to improve tonic LH levels in lactating beef cows. The possibility thus exists that the dose injected and the duration of naloxone administration may have been inappropriate in the present study. Future studies will need to address this question as well as the possibility that enkephalins produced by the CL (Cupo *et al.*, 1987) may also exert a role.

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