Pregnancy-specific protein B, progesterone concentrations and embryonic mortality during early pregnancy in dairy cows

P. Humblot, S. Camous*, J. Martal*, J. Charlery, N. Jeanguyot, M. Thibier and R. G. Sasser†

Laboratoire d'hormonologie, U.N.C.E.I.A., B.P. 65, 94703-Maisons-Alfort, France;

**INRA, Domaine de Vilvert, 78350 Jouy-en-Josas, France; and*

†Department of Animal Science, University of Idaho, Moscow, ID 83843, U.S.A.

Summary. Pregnancy-specific protein B (PSPB) and progesterone concentrations were determined by RIAs in venous plasma during early pregnancy after 177 artificial inseminations (AI) performed in 76 cows and 71 heifers. The females were bled at 24, 26, 30–35 days and ~70 days (for non-returns to oestrus) after AI. In non-pregnant females without extended CL maintenance (progesterone < 1.5 ng/ml on Day 24) and or showing a normal time of return to oestrus (Group 1, N = 63), PSPB concentrations were undetectable whatever the stage after AI except in 2 cows. In pregnant animals (N = 83; Group 2) progesterone concentrations were > 10 ng/ml from Day 24 to the time of rectal palpation and PSPB concentrations rose continuously from 0.42 ± 0.07 (s.e.m.) ng/ml (Day 24) to 4.06 ± 0.3 ng/ml (time of rectal palpation). No coefficient of correlation between PSPB and progesterone concentrations was significant whatever the day of gestation studied. In cows with extended luteal function and subsequently found to be non-pregnant (late embryonic mortality) PSPB was undetectable (N = 21; Group 3) or detectable (N = 10; Group 4) at Days 24, 26 and/or 30–35 of pregnancy. At 24 and 26 days after AI progesterone concentrations were intermediate between those of Groups 1 and 2. At Day 24 females of Group 4 had higher progesterone concentrations than those of Group 3 ($P < 0.05$), but no differences between these two groups existed at subsequent stages after AI. Animals of Group 4 had lower PSPB concentrations than those of Group 2 between Days 24 and 30–35 ($P < 0.025$) but at the time of rectal palpation PSPB values fell to undetectable levels in all but 1 cow of Group 4. We conclude that (1) most pregnancy failures in cows are due to non-fertilization or early embryonic death and if AI is performed after 70 days *post partum* > 95% of these females have no detectable PSPB concentrations; (2) peripheral progesterone concentrations are lower at Days 24–26 after AI in cows with late embryonic mortality than in pregnant cows; (3) only 30% of non-pregnant females with extended luteal function (late embryonic mortality) have detectable PSPB levels which are lower than in pregnant cows; and (4) in pregnant animals there is no correlation between PSPB and progesterone concentrations. This suggests that under physiological conditions PSPB has no major effect on progesterone production or *vice versa*.

Keywords: PSPB; progesterone; pregnancy; embryonic death; cow

Introduction

The bovine conceptus produces numerous signals during early pregnancy. These include steroids, prostaglandins and proteins (Sauer, 1979; Shemesh *et al.*, 1979; Gadsby *et al.*, 1980; Lewis *et al.*, 1982; Eley *et al.*, 1983; Bartol *et al.*, 1984; Knickerbocker *et al.*, 1986).

In-vivo studies (Northey & French, 1980; Betteridge *et al.*, 1980; Humblot & Dalla Porta, 1984) have shown that the presence of a conceptus after Day 16 was necessary for maintenance of the corpus luteum (CL). Evidence that the signal originated from trophoblastic cells was supported by the effect of intrauterine deposition of trophoblastic extracts (Humblot & Dalla Porta, 1984) or vesicles (Heyman *et al.*, 1984). Helmer *et al.* (1985) and Knickerbocker *et al.* (1986) have further shown that the transient antiluteolytic action of the embryo was mediated by protein(s) similar to those previously found in sheep (Martal *et al.*, 1979; Godkin *et al.*, 1982, 1984a, b).

A pregnancy-specific protein (named bovine PSPB) isolated from cow placental membranes (Butler *et al.*, 1982) is secreted by binucleate trophoblastic cells (Reimers *et al.*, 1985). Development of a radioimmunoassay (RIA) has allowed determination of concentrations of PSPB in cow serum throughout pregnancy (Sasser *et al.*, 1986) and, unlike antiluteolytic protein(s) (Martal *et al.*, 1979; Godkin *et al.*, 1982), PSPB seems to be secreted continuously throughout gestation with concentrations rising from the beginning to the end of pregnancy (Sasser *et al.*, 1986). In the cow, pregnancy failure results mainly from embryonic mortality which may or may not result in prolongation of luteal function (Ayalon, 1978; Diskin & Sreenan, 1980; Humblot, 1986). Plasma progesterone concentration at 22–24 days after insemination (Humblot, 1986) can help to distinguish early from late (after Day 16) cases of embryonic mortality, because the former induces no prolongation of progesterone secretion (Humblot & Dalla Porta, 1984).

The objectives of this study were to determine (1) the change in PSPB concentrations in cow plasma during early pregnancy, (2) the plasma concentrations of PSPB with respect to late embryonic mortality as diagnosed by progesterone concentration and (3) the relationships between plasma concentration of PSPB and progesterone in early pregnancy.

Materials and Methods

Animals and treatments. The 76 cows (1–8 lactations) and 71 heifers (18–24 months old) were of the French Friesian breed and kept in free stable areas. The cows were inseminated at natural oestrus; only those inseminated after 70 days *post partum* were selected to avoid interference with PSPB present in the peripheral circulation during the post-partum period (Ruder & Sasser, 1986). Heifers were systematically inseminated at 72 and 96 h after two i.m. injections of 25 mg prostaglandin tromethamine salt (Dynolytic N.D.: Upjohn, Courbevoie, France) administered at 12-day intervals. Among these females, 17 cows and 9 heifers were used after two successive inseminations and 2 additional cows after three inseminations for a total of 177 sampling sequences after AI.

Samples and assay procedures. The females were bled systematically at 24, 26 and between 30 and 35 days after AI for determination of plasma progesterone and plasma PSPB concentrations. Rectal palpation was performed on cows or heifers not seen in oestrus at 70.5 ± 9 days after AI to confirm pregnancy ($N = 120$). An additional blood sample was taken at time of rectal palpation. Peripheral blood (15 ml) was collected from the jugular vein into heparinized vacutainers (Beckton Dickinson, Grenoble, France). Plasma was separated by centrifugation (1000 g) and stored at -20°C until assayed.

An assay procedure previously described and validated by Thibier & Saumande (1975) was used to determine progesterone concentration. Antiserum 111/6 (Specific Antisera Ltd, Wilmslow, Cheshire, U.K.) was raised in a goat against progesterone-11 α -succinyl-BSA and was used at a final dilution of 1:12 000. This antiserum displayed <13% cross-reactivity with 17 α -hydroxyprogesterone. Progesterone and 17 α -hydroxyprogesterone were separated before RIA by Sephadex LH20 microcolumn chromatography. The accuracy and efficiency of this procedure were satisfactory as previously reported (Thibier *et al.*, 1973). The sensitivity of the assay was 0.05 ng/ml. Inter- and intra-assay coefficients of variation were 6.8% and 5.9% when duplicate estimates were run in the assays (7 assays) for a 1.5 ng/ml (1.47 ± 0.13) reference plasma sample, and 7% and 5.7% respectively when duplicate estimates were run (8 assays) for a 4 ng/ml (4.27 ± 0.37) sample of reference plasma.

Validation criteria for the RIA for bovine PSPB have been described by Sasser *et al.* (1986). Assays were performed on 200 μl samples of plasma. Rabbit antiserum to bovine PSPB (RGS 38-1) was used at a final dilution of 1:300 000. It has been shown previously that there is minimal cross-reactivity (<0.05%) in the assay with bovine LH and FSH and no reactivity with other hormones such as prolactin, TSH or GH. Mean sensitivity of the standard curve (standard preparation R-37) was 135 pg per assay tube. Intra- and inter-assay coefficients of variation were 6.8 and 20.4% respectively.

Data analysis. Females were declared non-pregnant when plasma progesterone concentrations were <1.5 ng/ml at 24 days after AI. At all stages of pregnancy studied PSPB concentrations were considered significantly different from

Table 1. Groups of animals and interpretation according to progesterone (Day 24) and PSPB concentrations, return to oestrus and rectal palpation

Group	Progesterone conc. (ng/ml)	PSPB	Time of oestrus (days)	Rectal palpation	No. (%) of cows*	Interpretation
1†	< 1.5	Not detectable‡	≤ 24	—	63 (35.6)	Fertilization failure or early embryonic mortality
2	> 1.5	Detectable§	None	Pregnant	83 (46.9)	Gestation
3	> 1.5	Not detectable	> 24¶	Not pregnant	21 (11.9)	Late embryonic mortality (after Day 16)
4	> 1.5	Detectable	> 24¶	Not pregnant	10 (5.7)	Late embryonic mortality (after Day 16)

*At the end of the trial.

†Includes 25 animals with progesterone > 1.5 ng/ml, but observed in oestrus before 24 days.

‡Only 2 cows had detectable concentrations on Day 24.

§On Day 24, 26 or 30–35.

¶Or no return to oestrus.

zero when the two B/Bo replicates were < 95% of buffer control. Based upon the progesterone and PSPB concentrations, return to service, and results of rectal palpation, animals were assigned to one of four groups (Table 1). At each stage after AI, data were analysed by non-orthogonal analysis of variance (Snedecor & Cochran, 1956). Multiple comparisons between groups were further studied by the method of Scheffé (Lellouch & Lazar, 1974). Data are presented as mean $\pm$ standard error of the mean (s.e.m.).

Results

Progesterone and PSPB concentrations in non-pregnant animals (Group 1)

In non-pregnant animals without extended CL maintenance and/or showing return to oestrus at or before 24 days after AI (N = 63), progesterone concentrations (Fig. 1) increased from 2 ± 0.32 ng/ml on Day 24 to 6.2 ± 0.5 ng/ml on Day 30–35. This illustrates that a new oestrous cycle had begun before 24 days after AI. In these females, PSPB concentrations remained below the limit of detection of the assay, except in 2 cows. Those 2 cows had detectable PSPB values on Day 24 (0.32 and 0.31 ng/ml) but these remained low (< 0.5 ng/ml).

Progesterone and PSPB concentrations in pregnant animals (Group 2) and females exhibiting late embryonic mortality (Groups 3 and 4)

In pregnant animals (Group 2), from Day 24 to the time of rectal palpation, progesterone concentrations (Fig. 1) were above 10 ng/ml. There was no change in progesterone concentration with age of gestation ($r = 0.11$; ($P > 0.05$). Within each individual, progesterone concentrations at Days 24 and 26 were highly correlated with each other and, to a lesser extent, to concentration at Day 30–35 (Table 2). No relationship was found between progesterone concentrations at the beginning of pregnancy and at the time of rectal palpation.

Mean progesterone concentrations in females from Groups 3 and 4 (combined) were significantly lower ($P < 0.001$) at all stages studied when compared to those of pregnant cows. The multiple comparison analysis revealed that animals with non-detectable PSPB concentrations (Group 3) always had lower ($P < 0.001$) progesterone concentrations than did pregnant animals. In the 10 females with elevated PSPB concentrations (Group 4) the same observation was made at Days 26 and 30–35 but progesterone concentrations at Day 24 were not significantly different from

Fig. 1. Progesterone (a) and PSPB (b) concentrations after AI in cows in Groups 1–4 (see Table 1). Values are mean $\pm$ s.e.m.

those of pregnant cows and heifers. At each stage studied the large variation in progesterone concentrations suggests that luteal regression occurred at various times in individuals of these two groups.

The proportion of females with progesterone concentrations < 1.5 ng/ml at Days 30–35 was very similar whether or not PSPB was detected (8/21, Group 3; 4/10, Group 4). Correlative analysis

Table 2. Correlation coefficients between progesterone and PSPB concentrations at the different days of pregnancy and at time of rectal palpation for animals (83) in Group 2

Stage of pregnancy	Progesterone conc.				PSPB conc.			
	Day 24	Day 26	Day 30–35	Rectal palpation	Day 24	Day 26	Day 30–35	Rectal palpation
Progesterone								
24	1							
26	0.454**	1						
30–35	0.236*	0.297**	1					
Rectal palpation	0.14	0.153	–0.01	1				
PSPB								
24	–0.114	0.155	0.08	0.124	1			
26	–0.184	0.101	0.006	0.110	0.937**	1		
30–35	–0.138	0.08	0.072	0.099	0.717**	0.794**	1	
Rectal palpation	0.101	0.108	0.08	0.04	0.183	0.211	0.257*	1

* $P < 0.05$; ** $P < 0.01$.**Table 3.** Concentrations of PSPB during the first 70 days after AI in cows in Group 2 or Group 4 (late embryonic mortality)

Group	Time after AI			
	Day 24	Day 26	Day 30–35	At rectal palpation
2	0.42 ± 0.07 (83)	0.87 ± 0.08 (83)	1.98 ± 0.13 (83)	4.06 ± 0.30 (77)
4	0.27 ± 0.07 (10)	0.53 ± 0.10 (10)	1.28 ± 0.34 (10)	0.2 ± 0.15 (6)

Values are mean ± s.e.m. for the no. of cows given in parentheses.

for cows showed that the mean interval between time of AI and return to oestrus was not significantly longer in Group 4 (48.5 ± 11.5 days, $N = 5$) than in Group 3 (38.4 ± 5.2 days, $N = 4$); however, those intervals differed ($P < 0.01$) from those of Group 1 (24.9 ± 2 days, $N = 35$).

Between 24 and 30–35 days after AI, pregnant females (Group 2) had higher ($P < 0.025$) PSPB concentrations than did those of Group 4 (Table 3). Figure 1 illustrates the differences ($P < 0.005$) in rate of increase of PSPB concentration in these two groups between 24 and 30–35 days after AI. Additionally, no interaction between status (pregnant or late embryonic mortality) and stage of pregnancy was found.

The large variation of PSPB concentrations at 30 days in Group 4 reflects the variation in the time of embryo death; only 2 cows had concentrations lower than the limit of detection of the assay. After 30 days, concentrations increased in pregnant cows (Group 2) and declined to undetectable levels in Group 4 females (Fig. 1). In Group 4 only 1 heifer had a detectable concentration of PSPB (i.e. 0.96 ng/ml) at the time of rectal palpation.

Relationship between progesterone and PSPB concentrations in females in Groups 2 and 4

In pregnant animals (Group 2), correlation coefficients between progesterone and PSPB concentrations were not significantly different from zero (Table 2) at any stage of pregnancy. At Day

Fig. 2. Individual progesterone and PSPB concentrations in Group 1 cows (●, pregnant) and Group 4 cows (□, late embryonic mortality) at 30–35 days after AI.

30–35, there was no relationship between progesterone and PSPB concentrations in animals in Groups 2 and 4 (Fig. 2). Figure 2 further illustrates the fact that most of the females in Group 4 (6/10) at 30 days had progesterone and PSPB concentrations very similar to those of pregnant ones.

Discussion

In pregnant animals, mean progesterone concentrations at Day 24 and at subsequent stages of pregnancy were very similar to values reported by Eley *et al.* (1979), Bartol *et al.* (1981) and Knickerbocker *et al.* (1986). These levels were slightly higher than those observed at earlier stages (Days 18–21) by Glencross *et al.* (1973), Lukaszewska & Hansel (1980), Glencross & Pope (1981), Roche *et al.* (1985) and Lucy & Stevenson (1986). This confirms that, after an initial rise during the first 3 weeks of pregnancy, progesterone concentrations remain stable throughout the first third of gestation (Donaldson *et al.*, 1970; Heap *et al.*, 1973). The fact that, in pregnant animals, no relationship seemed to exist between progesterone concentrations at the beginning of pregnancy and the time of rectal palpation, at 70 days, suggests that after Day 30 progesterone production may be regulated by factors other than those involved in the antiluteolytic mechanism. It is possible that luteotrophic factors such as those described by Hickey & Hansel (1987) or found in allantoic fluid by Hickey *et al.* (1985) influence progesterone production by the CL later during pregnancy.

It is confirmed here, by analysis of progesterone concentration on Day 24 and by observed early return to oestrus, that about 60% of pregnancy failures result from non-fertilization or early embryonic death (Ayalon, 1978; Diskin & Sreenan, 1980; Humblot, 1986) before the moment (Day 16) at which the antiluteolytic signal is present (Northey & French, 1980; Humblot & Dalla Porta,

1984). In most of these early failures (>95%), an elevation of PSPB concentrations was not detectable at or after 24 days. This reflects the lack of major development of trophoblastic tissue and suggests that a few cases of early embryonic death (without any prolongation of CL function) are associated with a very low PSPB production. Moreover, among cows with a delayed luteolysis, the females with detectable PSPB concentrations had, at Day 24, higher progesterone concentrations than did those with undetectable PSPB concentrations. This suggests that the production of the antiluteolytic signal and of PSPB could be related at the beginning of pregnancy. This is consistent with the fact that both PSPB (Reimers *et al.*, 1985) and antiluteolytic protein(s) (Martal *et al.*, 1984; Heyman *et al.*, 1984) are released by trophoblastic cells.

The fact that no quantitative relationship between progesterone and PSPB concentrations exists in pregnant animals, whatever the stages of pregnancy, indicates that there is no effect of PSPB on progesterone production, or *vice versa*, under physiological conditions. At 24 days after AI, mean progesterone concentrations in animals expressing late embryonic death were lower than those from pregnant cows. Such a difference has been described in the first 18 days of pregnancy but in relation to early embryonic mortality or fertilization failure (Hansel, 1981). This confirms the report by Cavestany & Foote (1985) from milk progesterone values that high progesterone concentrations in the first 24 days of gestation are associated with pregnancy maintenance. At subsequent stages after AI among those cows expressing late embryonic mortality, no difference in progesterone concentrations was found between females whether or not they had elevated PSPB concentrations. Moreover, at 30 days after AI, the proportion of cows which had completed luteolysis was very similar in these two groups and no difference was found in terms of return to oestrus. This shows that, despite higher progesterone concentrations on Day 24 in cows with PSPB production, luteolysis was not delayed further.

As in a previous work (Sasser *et al.*, 1986) we observed a wide variation in the moment at which PSPB was first detected in pregnant animals. In this study it was only at 30 days after AI that all pregnant cows had elevated plasma PSPB concentrations. At this stage of pregnancy, there was a large range of PSPB concentrations. Almost all cows with detectable PSPB values in which late embryonic mortality was observed presented concentrations very similar to those found in cows which remained pregnant. Consequently, at 30 days it was impossible to differentiate those cows that were to remain pregnant from the few that did not by PSPB assay alone or even when combined with progesterone concentrations.

From 31 non-pregnant cows which had a delay in luteolysis only one third had detectable PSPB concentrations. This low rate of pregnancy failures associated with elevated PSPB concentrations explains the high accuracy of the RIA for PSPB when used as a pregnancy test (Sasser *et al.*, 1986; Sasser & Ruder, 1987; Humblot *et al.* 1988). Moreover, this shows that measurement of both progesterone and PSPB in peripheral plasma can be very useful to study the way in which various factors may chronologically affect embryonic mortality.

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