Effects of neutralization of pregnant mares' serum gonadotrophin (PMSG) shortly before or at the preovulatory LH surge in PMSG-superovulated heifers on follicular function and development

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Normally cyclic heifers ($n = 34$) received 2500 iu pregnant mares' serum gonadotrophin (PMSG) i.m. at day 10 of oestrus, and 15 mg prostaglandin (PG) i.m. at day 12. Thereafter, a monoclonal antibody against PMSG was administered i.v. before ($n = 24$), at ($n = 6$) or shortly after ($n = 4$) the preovulatory LH surge. Peripheral blood concentrations of LH and oestradiol were compared; follicular development was monitored by daily ultrasound scanning; and the numbers of preovulatory-sized follicles and ovulations were counted 96 h after injection of PG following death. Anti-PMSG treatment before the LH surge inhibited the LH surge in 16 heifers (67%). In these heifers, the initial increase in oestradiol concentration upon PMSG stimulation to $167.5 \pm 35.0 \text{ pmol l}^{-1}$ was terminated immediately after anti-PMSG treatment and decreased rapidly to basal values, while the number of preovulatory-sized follicles remained constant until 68 h after PG injection; on average 0.4 ± 0.1 ovulations were counted. In the remaining eight heifers, five animals showed an immediate, but temporary, 20–60% drop in oestradiol concentration after anti-PMSG treatment. In all eight heifers 25% of the preovulatory-sized follicles ovulated. Treatment with anti-PMSG at or shortly after the LH surge did not affect the pattern of oestradiol concentration, but a significantly higher ovulation rate was observed in the animals treated shortly after the LH surge: 20.3 ± 2.6 versus 6.3 ± 2.3 in animals treated at the LH surge, which corresponded to 76% and 24% of the preovulatory-sized follicles monitored shortly before the period of multiple ovulations. Thus, neutralization of PMSG at any time before the maximum concentration of the preovulatory LH surge severely suppresses the functionality of the majority or all of the stimulated follicles, which is dependent on the time of injection of anti-PMSG. Although the follicles retain their size, they lose the potential to ovulate. It is concluded that interfollicular asynchrony of development is present at the time the LH surge occurs.

Introduction

Superovulation in cattle still produces rather variable results with respect to ovulation rate and yield of transferable embryos. In recent years pregnant mares' serum gonadotrophin (PMSG) superovulation procedures have included the administration of antibodies against PMSG around oestrus to suppress the adverse effects of residual PMSG by neutralizing it (Dhondt *et al.*, 1978; Bouters *et al.*, 1983). Administration of anti-PMSG shortly after the preovulatory LH surge that initiates final follicular and oocyte maturation improves the ovulation rate (Dieleman and Bevers, 1987) and the yield of transferable embryos (Dieleman *et al.*, 1989).

In practice, however, accurate determination of when the preovulatory LH surge occurs is difficult. In the majority of trials, anti-PMSG was administered at fixed times in relation to the time of the injection of prostaglandin (PG) or in relation to behavioural signs of oestrus (for review, see Dieleman *et al.*, 1993). When this method was used anti-PMSG was probably also administered before or at the time of the preovulatory LH peak as well as after this peak. In superovulated cows, the preovulatory LH peak occurs at intervals after injection of PG and onset of oestrus. These intervals vary considerably: 33–49 h (Bevers and Dieleman, 1987) after PG injection and –9–32 h (Callesen *et al.*, 1992) after onset of oestrus.

It is not clear what effect PMSG neutralization has shortly before or at the preovulatory LH surge on follicular function and development. Preliminary experiments (Vos *et al.*, 1991)

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indicated a suppression of follicular function. Administration of anti-PMSG about 24 h after the injection of PG reduced the number of ovulations to between none and one per cow (Bouters *et al.*, 1983; Saumande *et al.*, 1984; Boryczko *et al.*, 1992); this can be assumed to be well before the occurrence of the LH surge.

In this study, we investigated follicular function and development in PMSG-superovulated cattle after neutralization of PMSG before or shortly after the preovulatory LH surge.

Materials and Methods

Experimental design

The study was performed between December and April on 34 Dutch-Friesian heifers, which were housed indoors in groups of 4–5 animals under conditions which ensured normal ovarian cyclicity as described by Dieleman *et al.* (1983a). Experiments were started when at least one oestrous cycle was observed indoors. All animals were clinically examined and only heifers with a normal genital tract were selected. Progestagen ear implants (Crestar: Intervet International BV, Boxmeer) were applied to synchronize the oestrous cycle and were removed after 9 days. The heifers received 2500 iu PMSG i.m. (Folligon: Intervet International BV) on day 10 at 14:00 h, and 15 mg PG i.m. (Prosolvin: Intervet International) 48 h later (day 12) (day 0: first day of first oestrus after removal of the ear implants).

Blood samples were collected from the jugular vein as described by Dieleman *et al.* (1983a) at 07:00 h once a day from day 1 until day 12 of the synchronized cycle, then every 2 h starting 1 h before injection of PG until 91 h after PG injection.

Anti-PMSG (monoclonal 36A, Neutra-PMSG: Intervet International BV) was administered i.v. at a dose (5 ml) sufficient to neutralize 2500 iu PMSG within 1 h (Dieleman and Bevers, 1987). The monoclonal anti-PMSG 36A possesses no binding affinity to bovine LH and FSH, as no binding activity could be detected using this anti-PMSG with [125 I]-labelled highly purified bovine LH (bLH7981) and bovine FSH (bFSH HS-2-17). The first group of heifers ($n = 9$) was treated with anti-PMSG 24 h after the injection of PG. A second group consisting of 25 heifers was injected with anti-PMSG 36 h after PG injection to obtain animals treated with anti-PMSG shortly before, at, or shortly after the preovulatory LH surge. In PMSG-treated heifers the mean interval between PG injection and the maximum of the LH surge is 43.9 ± 7.9 h (mean $\pm$ SD, $n = 28$; range 33–49 h) (Bevers and Dieleman, 1987).

Ultrasound scanning and collection of ovaries

In all heifers ($n = 34$), the ovaries were examined using a real-time ultrasound scanner equipped with a 7.5 MHz intra-rectal linear array probe (Aloka, type SSD-248; Tokyo) to estimate the number and size of follicles in the categories: 2–5, 5–8 and > 8 mm; the category > 8 mm was defined as preovulatory-sized (Vos *et al.*, 1990). Ultrasound scanning was carried out on day 10 before PMSG administration and at 20, 44 and 68 h after PG injection. In general, multiple ovulations start 68 h after PG injection (Dieleman and Bevers, 1987).

The ovaries were recovered upon death 96 h after PG injection and the numbers of preovulatory-sized follicles and ovulations were counted.

Radioimmunoassays

Concentrations of progesterone and oestradiol were estimated by validated solid-phase 125 I radioimmunoassay methods (Coat-A-Count TKPG and TKE, respectively; Diagnostic Products Corporation, Los Angeles) as described by Dieleman and Bevers (1987). The sensitivity was 0.15 nmol progesterone l^{-1} and 7.5 pmol oestradiol l^{-1} ; the interassay coefficients of variation were 11% and 8.9%, respectively.

Concentrations of LH were estimated by a validated radioimmunoassay method as described by Dieleman *et al.* (1983b, 1986). The intra- and interassay coefficients of variation were $< 9\%$. The sensitivity was 0.4 μ g NIH-LH-B4 l^{-1} . Cross-reactivity of PMSG (highly purified PMSG PM23-2P; Intervet International) was 0.2%.

All concentrations of PMSG were estimated in duplicate in one assay with a homologous double-antibody radioimmunoassay method as described by Dieleman and Bevers (1987). Before radioimmunoassay, samples were incubated with donkey anti-mouse antibody-coated cellulose suspension as described by Dieleman and Bevers (1987) to remove monoclonal antibody against PMSG and its complexes with PMSG. The lowest detectable amount of PMSG was 0.3 μ g l^{-1} plasma; samples with a non-detectable concentration were arbitrarily assigned a value of 0.3 μ g l^{-1} . The intra-assay coefficient of variation was 7.1%.

Statistical analysis

Differences between the means of samples were tested for significance by analysis of variance according to Scheffé (1959). χ^2 analysis for heterogeneity was used to test the effect of time of treatment on the number of ovulations compared with the number of preovulatory-sized follicles. Data were considered to be significantly different at $P < 0.05$.

Results

Hormone concentrations

On average the mean PMSG concentration increased from being undetectable before injection of PMSG to 8.25 ± 0.44 (SEM) μ g l^{-1} ($n = 34$) on day 12. The PMSG concentration decreased markedly by at least 85% within 1 h after the administration of anti-PMSG; the PMSG concentration was then already less than or equal to the detection limit of the radioimmunoassay method.

The mean progesterone concentration on day 10 (i.e. before PMSG administration) was 12.5 ± 1.0 nmol l^{-1} ($n = 34$), which increased to 22.5 ± 1.5 nmol l^{-1} by day 12, i.e. before the injection of PG. Luteolysis was completed within 30 h after PG administration when the progesterone concentration was less than 3.2 nmol l^{-1} .

Fig. 1. Concentrations of (●) oestradiol and (○) LH in the peripheral blood of a heifer superovulated by pregnant mares' serum gonadotrophin (PMSG) treated with anti-PMSG 36 h after injection of prostaglandin (PG). No LH surge was observed.

Analysis of the LH concentrations showed a marked reduction in the number of heifers with a preovulatory LH surge. During the 92 h sampling period after the injection of PG an LH surge occurred in 18 (53%) animals.

Animals without LH surge

After the injection of anti-PMSG no LH surge was observed in seven of nine heifers treated 24 h after the injection of PG, and in nine of 25 heifers treated 36 h after PG injection.

In these heifers ($n = 16$) without an LH surge the oestradiol concentration increased from 11.9 ± 0.6 pmol l^{-1} before PMSG administration to 167.5 ± 35.0 pmol l^{-1} before anti-PMSG administration, after which it decreased immediately. Within 5 h, the oestradiol concentration dropped to 40% of the value before injection, and the concentration continued to decrease (Fig. 1).

The number of preovulatory-sized follicles increased significantly after PMSG treatment as observed by ultrasound scanning on day 10 and after the injection of PG (Table 1). Concurrently, the number of small follicles (2–5 mm) decreased significantly. Neutralization of PMSG did not decrease the number of preovulatory-sized follicles at 20 and 44 h after PG injection. Even at 68 h after PG administration the number of preovulatory-sized follicles remained constant. On average 14.4 ± 3.4 follicles > 8 mm and 0.4 ± 0.1 ovulations per heifer were counted 96 h after the injection of PG. In seven heifers of this group, one ovulation was observed in each animal.

Animals with LH surge

An endogenous preovulatory LH surge was observed in two and 16 heifers treated with anti-PMSG 24 and 36 h after injection of PG, respectively. The animals treated at 36 h received the anti-PMSG injection before ($n = 6$), on ($n = 6$) or after ($n = 4$) the LH surge. The maximum concentration of the LH surge of these groups and the intervals between this maximum and the injection of PG and of anti-PMSG are shown (Table 2).

The oestradiol concentration increased from 14.1 ± 1.9 pmol l^{-1} before PMSG administration to the high concentrations before anti-PMSG administration given in Table 3. Injection of anti-PMSG before the LH surge ($n = 8$ animals) resulted, in five heifers, in a temporary 20–60% fall in oestradiol concentration immediately after treatment; in the other three animals, no temporary decrease was observed. In the animals treated with anti-PMSG on or after the LH surge, the oestradiol concentration decreased after the preovulatory LH surge (Fig. 2).

The animals treated with anti-PMSG either before, on or after the LH surge showed a similar distribution of the follicles over the three size categories on day 10 before the administration of PMSG. The number of small (2–5 mm) follicles was predominant over that of the 5–8 and > 8 mm categories; the averages were 22.2 ± 1.8 , 2.0 ± 0.6 and 1.1 ± 0.1 ($n = 18$), respectively. As in the heifers without an LH surge, the number of preovulatory-sized follicles significantly increased 20 h after the injection of PG concurrent with a decrease in the number of small follicles.

After neutralization of PMSG, ultrasound scanning 44 h after PG injection showed an increase in the number of preovulatory-sized follicles that was not significant; the numbers of the other size categories remained similar to those observed shortly before anti-PMSG treatment (Table 4). At about the time of multiple ovulations 68 h after injection of PG, the number of preovulatory-sized follicles decreased significantly only in the heifers treated with anti-PMSG after the LH surge. Accordingly, in this group the number of ovulations per heifer (20.3 ± 2.6) was significantly higher than that of heifers treated with anti-PMSG before the LH surge (3.5 ± 2.5 and 7.3 ± 2.5 , 24 and 36 h after PG injection, respectively) or on the LH surge (6.3 ± 2.3). Although the numbers of preovulatory-sized follicles 44 h after PG injection were not significantly different for the heifers treated before or after the LH surge (Table 4), a difference in response to PMSG stimulation seemed to occur. The effects of this difference in response on the difference in the number of ovulations was avoided by performing χ^2 analysis on the number of ovulations versus the number of preovulatory-sized follicles observed 44 h after PG injection. A significant effect of treatment with anti-PMSG before the maximum concentration of the preovulatory LH surge was found in comparison to heifers treated after this maximum was reached. The number of preovulatory-sized follicles observed by ultrasonography 68 h after PG injection (Table 4) was not significantly different from that counted upon death 96 h after PG injection for the heifers treated with anti-PMSG before (20.0 ± 0.0 and 12.2 ± 2.5 , 24 and 36 h after PG injection, respectively), on (20.8 ± 4.3) or after (9.7 ± 1.0) the preovulatory LH surge.

Discussion

Injection of monoclonal anti-PMSG neutralized circulating PMSG immediately, as reported by Dieleman and Bevers (1987). This antibody can therefore be used to investigate the effects of deprivation of PMSG at specific times during the phase of follicular development preceding the maximum concentration of the preovulatory LH surge.

Table 1. Number of follicles per size category as observed by ultrasound scanning at different times during superovulation in all heifers without the preovulatory LH surge upon administration of anti-pregnant mares' serum gonadotrophin (PMSG) 24 or 36 h after prostaglandin (PG) injection

Follicle category (mm)	Day 10	Time (h) after PG injection		
		20	44	68
2–5	16.6 ± 2.9 ^a	8.5 ± 1.0 ^b	6.6 ± 0.9 ^b	6.3 ± 0.8 ^b
5–8	4.0 ± 1.4	9.7 ± 1.6	7.6 ± 1.6	7.8 ± 1.5
> 8	1.4 ± 0.2 ^a	8.8 ± 2.2 ^{ab}	14.0 ± 2.5 ^b	12.5 ± 2.6 ^b

Values are means ± SEM; number of heifers = 16.

Values within a row with different superscripts are significantly different.

Follicles > 8 mm: preovulatory-sized follicles.

Table 2. Intervals and maximum concentration of the LH surge of heifers treated with pregnant mares' serum gonadotrophin (PMSG) and injected with anti-PMSG at 24 or 36 h after prostaglandin (PG) injection

Time of anti-PMSG treatment	Interval PG – LH	Interval anti-PMSG – LH	Maximum concentration of the LH surge ($\mu\text{g l}^{-1}$)
24 h after PG	37.0 ± 4.0 (<i>n</i> = 2)	13.0 ± 4.0	6.8 ± 1.1
36 h after PG:			
Before LH surge	44.3 ± 1.1 (<i>n</i> = 6)	8.3 ± 1.1	13.0 ± 2.4
On LH surge	38.7 ± 0.3 (<i>n</i> = 6)	2.7 ± 0.3	12.3 ± 2.8
After LH surge	32.5 ± 1.3 (<i>n</i> = 4)	– 3.5 ± 1.3	12.1 ± 1.7

Values are means ± SEM.

n = number of heifers.

Table 3. Oestradiol concentrations at specific times in the peripheral blood of heifers treated with pregnant mares' serum gonadotrophin (PMSG) and injected with anti-PMSG at 24 or 36 h after prostaglandin (PG) injection

Time of anti-PMSG treatment	Oestradiol concentration 5 h before anti-PMSG treatment (pmol l^{-1})	Oestradiol concentration 5 h after anti-PMSG treatment (pmol l^{-1})	Oestradiol concentration at LH peak (pmol l^{-1})
24 h after PG	235.3 ± 47.7 (<i>n</i> = 2)	116.3 ± 63.9	132.5 ± 79.1
36 h after PG:			
Before LH surge	219.3 ± 80.0 (<i>n</i> = 6)	197.6 ± 84.4	228.0 ± 82.1
On LH surge	329.1 ± 92.9 (<i>n</i> = 6)	325.6 ± 64.6	346.9 ± 85.8
After LH surge	488.8 ± 112.9 (<i>n</i> = 4)	259.2 ± 90.7	488.8 ± 112.9

Values are means ± SEM. *n* = number of heifers.

Before the injection of anti-PMSG, the patterns in the peripheral blood of the concentrations of progesterone and oestradiol are generally in accordance with the patterns reported by Bevers and Dieleman (1987) for PMSG/PG-treated cows. The high oestradiol concentration can be attributed to the number of preovulatory-sized follicles (Bevers and Dieleman, 1987). After neutralization of PMSG, the preovulatory LH surge occurring in only 53% of the heifers displayed an amplitude and extent that were normal for PMSG/PG-stimulated cows (Dieleman *et al.*, 1989). In these animals, the oestradiol concentration decreased to basal values after the maximum concentration of the LH surge was reached, in agreement with studies by Dieleman and Bevers (1987),

whereas in the heifers without an LH surge, the oestradiol concentration decreased rapidly, immediately after the anti-PMSG treatment.

Administration of anti-PMSG 36 h after the injection of PG resulted in the treatment of heifers shortly before, at the ascending limb of the LH surge or after this surge, owing to normal variation in duration of the interval between injection of PG and the LH surge (Bevers and Dieleman, 1987; Callesen *et al.*, 1992). As an inverse correlation has been reported between this interval and the number of preovulatory-sized follicles (Bevers and Dieleman, 1987), it could be expected that heifers treated with anti-PMSG before the LH surge would show a smaller response than would those treated after the

Fig. 2. Concentrations of (●) oestradiol and (○) LH in the peripheral blood of heifers treated with pregnant mares' serum gonadotrophin (PMSG). The patterns are representative for heifers in which an LH surge was observed upon anti-PMSG treatment (a, b) before [24 and 36 h after prostaglandin (PG) injection, respectively], (c) on or (d) after the LH surge.

LH surge. However, no significant difference in the number of preovulatory-sized follicles was observed by ultrasound scanning 44 h after the injection of PG, although a tendency could be distinguished for which the difference in oestradiol concentration 5 h before injection of anti-PMSG is also indicative. Counting the number of follicles using transrectal ultrasound scanning is probably less accurate for ovaries containing numerous large follicles present before and after the LH surge in PMSG-treated heifers (Vos *et al.*, 1992) than for ovaries during the unstimulated oestrous cycle (Pierson and Ginther, 1987). Nevertheless, ultrasound scanning clearly showed the stimulatory effect of PMSG treatment when the numbers of follicles in the three size categories were compared on day 10 and 20 h after the injection of PG.

Neutralization of PMSG before the eventual LH surge during the phase of selection and growth of follicles obviously suppressed the LH surge in most of the heifers in which no LH surge was detected. This group of animals comprised a markedly higher proportion (67%) of the PMSG/PG-treated heifers than did the general 14–20% proportion of animals in which no LH surge occurs upon superovulation with PMSG/PG (Callesen *et al.*, 1986; Dieleman *et al.*, 1989). The usual hormonal mechanisms regulating the release of the preovulatory LH surge (Bevers *et al.*, 1989) are probably inhibited in these animals owing to the rapid decrease in the oestradiol concentration immediately after the administration of anti-PMSG. Apparently, FSH-like support by PMSG is still

required for follicular oestradiol production during the phase of development preceding the LH surge in a large proportion of animals treated for superovulation. Although it can be assumed that all stimulated follicles lose their functionality, they obviously do not undergo morphological regression until 32–44 h after injection of anti-PMSG (as observed by ultrasound scanning).

In the animals with a preovulatory LH surge, a temporary fall in the oestradiol concentration occurred immediately after the administration of anti-PMSG before the onset of the LH surge. A similar fall was not observed upon injection of anti-PMSG either at or after the LH surge. Probably, this drop was obscured by the normal decrease in the oestradiol concentration to basal values initiated by the preovulatory LH surge. The temporary fall may indicate that neutralization of PMSG affects the oestradiol production by all preovulatory-sized follicles. However, it is more likely that the oestradiol production is inhibited in only some of the preovulatory-sized follicles, which is similar to the process observed in the animals without an LH surge. The observed reduced number of ovulations in animals treated with anti-PMSG before the LH surge in comparison with that of animals treated after the surge supports this view. It is suggested that some of the preovulatory-sized follicles are less advanced and still require FSH-like support by PMSG shortly before the preovulatory LH surge at the end of the phase of selection and growth of follicular development. This indicates that there is interfollicular

Table 4. Number of follicles per size category as observed by ultrasound scanning during superovulation in heifers with a preovulatory LH surge upon administration of anti-pregnant mares' serum gonadotrophin (PMSG) at 24 or 36 h after prostaglandin (PG) injection

Time of anti-PMSG treatment	Follicle category (mm)	Time (h) after PG injection		
		20	44	68
24 h after PG (n = 2)	2–5	8.5 ± 3.5	10.5 ± 2.5	10.5 ± 4.5
	5–8	20.0 ± 5.0	16.0 ± 5.0	14.0 ± 4.0
	> 8	14.5 ± 1.5 ^a	26.5 ± 5.5 ^a	23.0 ± 3.0 ^a
36 h after PG:				
Before LH surge (n = 6)	2–5	12.2 ± 0.3	8.5 ± 1.0	9.5 ± 1.3
	5–8	11.3 ± 1.7	9.2 ± 2.3	7.3 ± 1.3
	> 8	11.3 ± 3.2 ^a	19.7 ± 4.9 ^a	16.8 ± 5.6 ^a
On LH surge (n = 6)	2–5	9.3 ± 1.0	8.8 ± 1.3	8.3 ± 0.8
	5–8	20.7 ± 2.4	12.0 ± 2.5	13.0 ± 2.2
	> 8	11.2 ± 7.2 ^a	29.0 ± 7.5 ^a	20.2 ± 7.1 ^a
After LH surge (n = 4)	2–5	8.5 ± 1.3	8.3 ± 2.3	8.8 ± 1.3
	5–8	15.5 ± 3.4	13.3 ± 5.2	14.0 ± 3.5
	> 8	25.0 ± 3.7 ^a	34.0 ± 4.3 ^a	6.3 ± 2.3 ^b

Values are means ± SEM; n = number of heifers; values within a row with different superscripts are significantly different; follicles > 8 mm: preovulatory-sized follicles.

asynchrony in the preovulatory follicular population. Evidently, interfollicular asynchrony is still present when the LH surge occurs.

Neutralization of PMSG at the ascending limb of the LH surge produced only 6.3 ovulations from the 29 preovulatory-sized follicles, as observed 44 h after the injection of PG, in comparison with the 20.3 ovulations in animals treated with anti-PMSG shortly after the maximum concentration of the LH surge. In PMSG/PG-treated heifers, multiple ovulations are generally completed 24–30 h after the maximum concentration of the LH surge (Dieleman and Bevers, 1987). It can therefore be assumed that the preovulatory-sized follicles observed by ultrasound scanning 68 h after the injection of PG will not have ovulated in the animals treated with anti-PMSG before, on or after the LH surge – as is also indicated by the number of ovulations in these animals. Moreover, the number of large follicles counted upon slaughter was not significantly different from that observed 68 h after PG injection.

It is possible that, upon superovulatory treatment, advanced follicles evoke the preovulatory LH surge, which occurs at too early a stage of development for the less advanced follicles. These less advanced follicles possibly do not react properly to the LH signal, which may lead to follicles that are abnormal a few hours before ovulation, either containing over four times higher oestradiol concentrations than those of the preovulatory follicle in non-stimulated heifers (Dieleman *et al.*, 1983a, 1988) or showing discrepancies between follicular and oocyte maturational stage (de Loos *et al.*, 1991; Hyttel *et al.*, 1991).

In conclusion, administration of anti-PMSG at fixed times in relation to the injection of PG or to the onset of oestrus may produce rather variable results with regards to ovulation rate and embryo yield.

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