Plasma concentrations of gonadotrophins, preovulatory follicular development and luteal function associated with bovine follicular fluid-induced delay of oestrus in heifers*

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Summary. In Exp. 1, injections of 10 ml bovine follicular fluid (bFF, i.v. or s.c.), given twice daily for 3 days after injection of a luteolytic dose of PGF-2 α , delayed the onset of oestrus in 3 of 6 heifers to 8 or 9 days after PGF-2 α , as compared with 2 or 3 days after PGF-2 α in control heifers. Mean plasma concentrations of FSH and LH during the injection period were not different from those in saline-injected heifers. In Exp. 2, i.v. injections of 20 ml bFF twice daily for 3 days uniformly delayed oestrus to 8 days after PGF-2 α (N=4) and injections of 20 ml bFF i.v. every 6 h for 24 h on the day of PGF-2 α injection delayed oestrus to 5.0 ± 0.6 days after PGF-2 α as compared with 2.8 ± 0.3 days for control heifers. In both treatment groups, plasma concentrations of FSH were suppressed during the injection period and increased transiently after treatment, but plasma concentrations of LH during the injection period were not different from those of control heifers. Plasma levels of oestradiol in heifers given bFF remained basal for 2 or 3 days after treatment, then increased several days before the delayed oestrus, in a manner similar to that in control heifers, and elicited normal preovulatory surges of LH and FSH. Plasma concentrations of progesterone and the length of the next oestrous cycle were normal, indicating formation of functional corpora lutea. Therefore, bFF treatments appear to delay oestrus by selectively suppressing plasma FSH, without affecting LH, and delaying the development of the preovulatory follicle. These results suggest that FSH may be critical to support the growth and development of the preovulatory follicle after luteolysis in cows.

Introduction

FSH and LH are essential for growth and maturation of ovarian follicles (Richards, 1980), but their relative roles during the final stages of follicular development in cattle have not been clearly defined. In cattle and sheep final growth and maturation of preovulatory follicles occur during a short follicular phase lasting several days after luteolysis (Hansel & Convey, 1983). After luteolysis, basal plasma concentrations of LH and the frequency of pulsatile LH release increase (Rahe, Owens, Fleeger, Newton & Harms, 1980; Goodman *et al.*, 1981; Schallenberger, Schams, Bullermann & Walters, 1984). Increased stimulation by LH is thought to promote preovulatory follicular development (McNatty, Gibb, Dobson & Thurley, 1981; McNeilly, Fraser & Baird, 1984). In contrast to changes in plasma concentrations of LH, concentrations of FSH decline after luteolysis in cows (Butler, Katz, Arriola, Milvae & Foote, 1983; Schallenberger *et al.*, 1984) and

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sheep (L'Hermite, Niswender, Reichert & Midgley, 1972), suggesting that increases in FSH are not needed for the final stages of follicular development. In addition, ovulation can be induced in anoestrous ewes by injections of LH (McNeilly, O'Connell & Baird, 1982). These results indicate that plasma levels of FSH during anoestrus are sufficient to allow preovulatory follicular development, but that LH support is inadequate. These studies have supported the concept that LH has a critical function in stimulation of preovulatory follicular development after luteolysis, whereas the role of FSH is more permissive. However, direct evidence for this idea is lacking.

Follicular fluid from all species examined contains a protein, inhibin or folliculostatin, which selectively depresses plasma concentrations of FSH and inhibits secretion of FSH by pituitary cells *in vitro* (Channing *et al.*, 1982; Grady, Charlesworth & Schwartz, 1982). Injections of bovine follicular fluid during the follicular phase of the oestrous cycle in heifers and ewes lengthened the interval from luteolysis to oestrus (Miller, Critser, Rowe & Ginther, 1979) and subsequent studies with ewes showed that plasma concentrations of FSH decreased during treatment (Miller, Critser & Ginther, 1982; McNeilly, 1984). Injections of bovine follicular fluid to ovariectomized heifers, prepubertal heifers and cyclic heifers during the late luteal phase of the oestrous cycle decreased plasma concentrations of FSH, but did not alter plasma concentrations of LH (Ireland, Curato & Wilson, 1983; Braun, Schams, Buck & Leidl, 1983; Johnson, Smith & Elmore, 1985). The aim of the present study was to determine whether treatment of heifers with bovine follicular fluid during the follicular phase of the oestrous cycle would selectively suppress plasma concentrations of FSH and, if so, whether this would alter preovulatory follicular development and/or subsequent luteal function.

Materials and Methods

Experiment 1. Nine Holstein heifers with normal oestrous cycles were injected with 25 mg prostaglandin (PG) F-2 α i.m. (Lutalyse; Upjohn Co., Kalamazoo, MI, U.S.A.) on Day 14, 15 or 16 of the oestrous cycle (Day 0 = oestrus) to induce luteal regression. Heifers were injected with 10 ml of charcoal-extracted bovine follicular fluid (bFF, preparation described below) or saline (9 g NaCl/l) every 12 h for 3 days beginning at the time of PGF-2 α injection. Treated heifers were injected with bFF i.v. (N=3) or s.c. (N=3), and control heifers (N=3) were injected with saline i.v. Heifers were checked for oestrous activity twice daily. Blood samples were collected by jugular venepuncture 72 and 48 h before injection of PGF-2 α , and then every 6 h from 24 h before injection of PGF-2 α until 48 h after heifers were in oestrus. Blood samples were collected daily throughout the oestrous cycle after treatment. Plasma was stored at -20°C until assayed for FSH, LH and progesterone by radioimmunoassays (RIA).

Experiment 2. Twelve Holstein heifers with normal oestrous cycles were injected with 25 mg PGF-2 α on Day 14 or 15 of the oestrous cycle. At 2 days before injection of PGF-2 α , a Silastic medical-grade cannula (i.d. 0.10 cm; o.d. 0.22 cm; Dow Corning Corp., Midland, MI, U.S.A.) was inserted into the jugular vein of each heifer. Heifers were treated with i.v. injections of 20 ml charcoal-extracted bFF (N=4) or saline (N=4) every 12 h for 3 days beginning at the time of PGF-2 α injection (total of 6 injections). Another group of heifers was treated with i.v. injections of 20 ml bFF every 6 h for 24 h beginning 6 h before PGF-2 α injection (N=4, total of 5 injections) to test the hypothesis that FSH is most critical for follicular development at the beginning of the follicular phase. Blood samples were taken from the cannulae every 2 h from 24 h before until 74 h after PGF-2 α treatment and then every 4 h until 48 h after oestrus. Cannulae were filled with heparin-saline (200 i.u./ml) after each sample. Cannulae were then removed and blood samples were collected daily by jugular venepuncture until the next oestrus. Heifers were checked for oestrus twice daily. Plasma concentrations of FSH, LH, progesterone and oestradiol-17 β were determined by RIA.

Preparation of charcoal-extracted bFF. Bovine follicular fluid was collected at a local slaughterhouse by aspiration of all visible follicles <2.5 cm diameter (to exclude obviously cystic follicles). The follicular fluid was frozen on solid CO₂ for transport to the laboratory and then stored at -20°C. Steroids were removed from bFF by a modification of the procedure used by Miller *et al.* (1979). The follicular fluid was stirred with 50 mg charcoal/ml (Norit-A, Fisher Scientific, Fair Lawn, NJ, U.S.A.) for 45 min at room temperature and centrifuged at 2000 *g* for 30 min at 4°C. The supernatant was centrifuged at 12000 *g* for 30 min at 4°C and filtered through glass-fibre filter paper to remove charcoal particles. A different batch of bFF was used in each of the two experiments: charcoal extraction reduced concentrations of progesterone in bFF from 60.6 to 0.3 ng/ml and from 62.5 to 0.1 ng/ml in the batches of bFF used in Exps 1 and 2, respectively. Oestradiol concentrations in bFF were reduced from 61.4 to 0.06 ng/ml and from 39.6 to 0.04 ng/ml in Exps 1 and 2, respectively.

Hormone assays. The limit of sensitivity of each RIA described below was determined by the concentration of hormone which depressed binding of the radioligand to 90% of the binding in buffer control tubes (100% binding). The intra-assay coefficient of variation (CV) was calculated by averaging the CV for duplicate determinations of 20 randomly chosen samples in 4 or 5 assays for each hormone. Inter-assay CV was calculated by averaging the CV for 2 or 3 plasma pools run in duplicate in each assay.

Plasma concentrations of LH were measured by RIA (Niswender, Reichert, Midgley & Nalbandov, 1969) using anti-ovine LH antiserum (GDN No. 15) supplied by Dr G. D. Niswender, highly purified ovine LH (LER-1056-C2) for radioiodination donated by Dr L.E. Reichert, Jr and bovine LH (NIH-LH-B9) as standard. The second antibody was goat anti-rabbit immunoglobulin G (Miles Laboratories, Elkhart, IN, U.S.A.). The sensitivity of the assay was 0.12 ng LH/assay tube. The intra-assay CV was 7.0% and the inter-assay CV was 13.8% (18 assays; average for 3 pools at 91, 73 and 20% on the standard curve).

Plasma concentrations of FSH were measured by RIA (Bolt & Rollins, 1983; Butler *et al.*, 1983) using reagents obtained from the USDA Animal Hormone Program. Reagents included (1) specific antiserum directed against the β -subunit of highly purified bovine FSH (USDA-FSH-BP1 β), (2) purified bovine FSH (USDA-FSH-BP3) for radioiodination (using chloramine-T; Bolt & Rollins, 1983), and (3) a standard preparation of bovine FSH (USDA-FSH-B1, with biological potency $1.7 \times$ NIH-FSH-B1). The second antibody was the same as that used in the RIA for plasma LH. The sensitivity of the assay was 0.9 ng FSH/assay tube (3.0 ng/ml). The intra-assay CV was 9.4% and the inter-assay CV was 12.8% (18 assays; average for 2 pools at 54% and 40% on the standard curve).

Plasma concentrations of progesterone were measured by a specific RIA (Beal, Milvae & Hansel, 1980) with a sensitivity of 16 pg/assay tube, an intra-assay CV of 8.7% and an inter-assay CV of 13.4% (9 assays; average for 2 pools at 73% and 32% on the standard curve). Progesterone was extracted from 100 μ l plasma with petroleum ether. Recovery of labelled progesterone added to cow plasma was $84.3 \pm 0.8\%$ (s.e.m.; $N = 50$) and the solvent blank was negligible. Values were not corrected for recovery.

Plasma concentrations of oestradiol-17 β were measured using a modification of the extraction, purification, and RIA procedure of England, Niswender & Midgley (1974). The antibody is highly specific for oestradiol-17 β (Korenman *et al.*, 1974). Oestradiol was extracted from 2 ml plasma by two successive extractions with 5 ml diethyl ether. Extracts were chromatographed on Sephadex LH-20 columns equilibrated with benzene:methanol (85:15 v/v). Recovery of [³H]oestradiol-17 β (1200 c.p.m.) added to each sample before extraction was determined and used to correct for procedural losses. The recovery of labelled oestradiol was $82.3 \pm 0.9\%$ (mean $\pm$ s.e.m.; $N = 150$) and the solvent blank was always <0.5 pg/ml. The radioligand used in the assay was oestradiol-17 β -[¹²⁵I]tyrosine methyl ester (Micromedex Systems, Horsham, PA, U.S.A.). Donkey anti-sheep gamma globulin (Cambridge Medical Diagnostics, Inc., Billerica, MA, U.S.A.) was used as the

second antibody. The assay sensitivity was 0.6 pg/assay tube (0.9 pg/ml). The intra-assay CV was 8.1% and the inter-assay CV was 8.5% (4 assays; average of 3 pools at 42, 27 and 12% on the standard curve).

Statistical analyses. The effects of treatments on the interval from injection of PGF-2 α to oestrus and to the preovulatory surges of LH and FSH and on length of the oestrous cycles after treatments were assessed by analysis of variance followed by linear contrasts to determine differences between individual treatments. Plasma concentrations of FSH, LH, oestradiol and progesterone in different treatment groups were compared by split-plot analysis of variance for repeated measurements over time (Gill, 1979). Analysis of variance was used to compare average concentrations of FSH before injection of PGF-2 α (mean concentrations in samples -24 h to 0 h from PGF-2 α) to mean concentrations 24 to 48 h after PGF-2 α and mean concentrations after termination of bFF injections.

Results

Experiment 1

Treatment of heifers with 10 ml injections of bFF delayed oestrus from 2.3 ± 0.3 days (mean $\pm$ s.e.m.) after PGF-2 α in control heifers to 8 days after PGF-2 α in 2 of 3 heifers given bFF injections i.v., and to 9 days in 1 of 3 heifers given bFF injections s.c. (Table 1). The remaining bFF-treated heifers were in oestrus 3 or 4 days after PGF-2 α . Oestrus was therefore delayed in only 3 of the 6 heifers treated with bFF.

There were no differences in plasma concentrations of FSH or LH in bFF-treated and control heifers during the injection period ($P > 0.05$, data not shown). All heifers had preovulatory surges of FSH and LH on the day of oestrus; in 3 heifers, therefore, preovulatory surges of FSH and LH were also delayed to 8 or 9 days after PGF-2 α treatment (Table 1). The length of the oestrous cycle (Table 1) and plasma concentrations of progesterone (data not shown) after treatment were similar

Table 1. Effect of 10 ml injections of bovine follicular fluid (bFF, i.v. or s.c.) or saline (i.v.) every 12 h for 3 days (Exp. 1) on time to oestrus, time to the preovulatory LH/FSH surge, and length of the oestrous cycle after treatment in individual heifers

Treatment	Day of oestrus†	Time from PGF-2 α to LH/FSH surge (h)	Length of oestrous cycle after treatment (days)
Saline	2	72	25
	3	72	20
	2	54	19
		*66.0 $\pm$ 6.0	*21.3 $\pm$ 1.9
bFF, i.v.	4	90	18
	8	192	21
	8	186	27
		*156.0 $\pm$ 33.0	*22.0 $\pm$ 2.6
bFF, s.c.	4	96	30
	3	90	20
	9	222	23
		*136.0 $\pm$ 43.0	*24.3 $\pm$ 3.0

*Mean $\pm$ s.e.m. There were no significant differences amongst means in the same vertical column ($P > 0.05$)

†Days after injection of PGF-2 α .

in bFF-treated heifers and control heifers ($P > 0.05$), indicating that functional corpora lutea were formed in animals that exhibited delayed oestrus.

The inconsistent results obtained in this experiment led us to perform Exp. 2, in which the dose of bFF was doubled and blood samples were collected more frequently.

Experiment 2

Effects of bFF on the length of the oestrous cycle. In heifers treated with 20 ml injections of bFF for 3 days, oestrus was uniformly delayed to 8 days after PGF-2 α treatment and preovulatory surges of LH and FSH occurred on the same day (Table 2). Oestrus and gonadotrophin surges were also delayed in heifers given more frequent injections of 20 ml bFF for 1 day but the response was more variable (5.0 ± 0.6 days, mean $\pm$ s.e.m.; Table 2). One heifer in that group showed continuous oestrous activity between 5 and 9 days after PGF-2 α treatment and plasma concentrations of oestradiol (see below) suggested abnormal development of the preovulatory follicle. Oestrus was not detected in one heifer, but preovulatory surges of LH and FSH occurred between 6 and 7 days after PGF-2 α .

Table 2. Effect of i.v. injections of 20 ml bovine follicular fluid (bFF) or saline every 12 h for 3 days or 20 ml bFF every 6 h for 1 day (Exp. 2) on time to oestrus, time to the preovulatory LH/FSH surge, and length of the oestrous cycle after treatment in individual heifers

Treatment	Day of oestrus†	Time from PGF-2 α to LH/FSH surge (h)	Length of oestrous cycle after treatment (days)
Saline	3	68	22
	2	60	20
	3	78	19
	3	74	22
		*70.0 $\pm$ 3.9 ^a	*20.8 $\pm$ 0.8 ^a
bFF, 3 days	8	190	19
	8	202	21
	8	206	23
	8	202	20
		*200.0 $\pm$ 3.5 ^c	*20.8 $\pm$ 0.9 ^a
bFF, 1 day	6	146	16
	Not detected	166	18§
	4	94	23
	5‡	138	26¶
		*136.0 $\pm$ 15.2 ^b	*19.0 $\pm$ 2.1 ^a

*Mean $\pm$ s.e.m. Means in the same vertical column with different superscripts are different, $P < 0.005$ (ANOVA).

†Days after injection of PGF-2 α .

§Days from preovulatory LH surge to next oestrus.

‡Heifer showed oestrous behaviour 5, 6, 7, 8 and 9 days after PGF-2 α .

¶A corpus luteum was not formed; plasma concentrations of progesterone remained < 1 ng/ml. Not included in the mean.

Effects of bFF on plasma concentrations of LH and FSH. Plasma concentrations of FSH in control heifers started to decline after luteolysis and were 20% lower between 24 and 48 h ($P < 0.05$) after injection of PGF-2 α when compared to pre-treatment concentrations (Figs 1a, 2a). In heifers treated with bFF for 3 days plasma levels of FSH were 28% lower ($P < 0.05$) than those in control heifers by 6 h after PGF-2 α (the time of the first injection of bFF). In heifers treated with bFF for 3

Fig. 1. Changes in plasma concentrations (mean $\pm$ s.e.m.; $N=4$) of FSH (a) and LH (b) after injection of PGF-2 α (0 h) in heifers treated with i.v. injections of 20 ml saline or bovine follicular fluid (bFF) every 12 h for 3 days. Times of injections are indicated by arrows. Data for each treatment are shown until just before the time of preovulatory surges of gonadotrophins.

days plasma concentrations of FSH were 29% lower between 24 and 48 h after than before injection of PGF-2 α (Fig. 1a). Plasma concentrations of FSH remained lower in bFF-treated heifers than in control heifers throughout the injection period ($P<0.05$), except for the period between 36 and 48 h after the beginning of treatment when differences were marginally significant ($P<0.10$). Plasma concentrations of FSH in heifers treated with bFF for 1 day were decreased relative to values in control heifers between 8 and 20 h after the first injection of bFF (2–14 h after PGF-2 α ; $P<0.05$), then increased before the last injection of bFF and were no different from control values between 22 and 46 h (16–40 h after PGF-2 α ; $P>0.05$; Fig. 2a). In heifers treated with bFF for 1 or 3 days, concentrations of FSH increased by 24 h after the last injection of bFF and remained elevated for 24–36 h. During this 24–36 h period FSH values were similar to those before injection of PGF-2 α ($P>0.05$) in heifers treated with bFF for 3 days, but were higher than those before injection of PGF-2 α ($P<0.05$) in heifers treated with bFF for 1 day. After injection of PGF-2 α , plasma concentrations of LH increased in a similar manner in the heifers treated with bFF and in control heifers (Figs 1b, 2b).

Fig. 2. Changes in plasma concentrations (mean $\pm$ s.e.m.; $N=4$) of FSH (a) and LH (b) after injection of PGF-2 α (0 h) in heifers treated with i.v. injections of 20 ml saline (as in Fig. 1) or with 20 ml bovine follicular fluid (bFF) every 6 h for 1 day. Times of injections are indicated by arrows. Data for each treatment are shown until just before the time of preovulatory surges of gonadotrophins.

Plasma concentrations of FSH and LH in control and bFF-treated heifers normalized to the time of the preovulatory LH surge are shown in Figs 3(a) and 3(b). Plasma concentrations of FSH around the time of the gonadotrophin surges were similar in control heifers and heifers treated with bFF for 3 days but were higher and more variable 2 days before the surge in heifers treated with

Fig. 3. Changes in plasma concentrations (mean $\pm$ s.e.m.; $N = 4$) of FSH (a) and LH (b) relative to the preovulatory peaks of LH (0 h) in heifers treated with i.v. injections of 20 ml saline or bovine follicular fluid (bFF) every 12 h for 3 days or 20 ml bFF every 6 h for 1 day.

Fig. 4. Changes in plasma concentrations of oestradiol after PGF-2 α -induced luteolysis (Day 0) in individual heifers treated with i.v. injections of 20 ml saline every 12 h for 3 days (a) or 20 ml bovine follicular fluid (bFF) every 6 h for 1 day (b) or 20 ml bFF every 12 h for 3 days (c). Times of injections are indicated by arrows. Heifers were in oestrus within 24 h of peak levels of oestradiol. One heifer (b, $\circ$ — $\circ$) was in oestrus 5, 6, 7, 8 and 9 days after PGF-2 α .

bFF for 1 day ($P < 0.025$). In all groups, plasma levels of FSH increased again within 17.5 ± 1.1 h (mean $\pm$ s.e.m.; $N = 11$) after the preovulatory peak of FSH and remained elevated for 21.5 ± 2.1 h (mean $\pm$ s.e.m.; $N = 11$). Concentrations of LH between 48 and 4 h before the surge were 1.4 times higher in heifers treated with bFF for 1 or 3 days than in control heifers ($P < 0.05$). However, concentrations of LH during and up to 48 h after the surge were not different among treatments ($P > 0.05$).

Effects of bFF on plasma concentrations of oestradiol and progesterone. Plasma concentrations of oestradiol in control heifers increased steadily between the time that PGF-2 α was injected and oestrus and were greater ($P < 0.025$) than in bFF-treated heifers by 18 h after PGF-2 α (Figs 4a, b, c). In heifers treated with bFF for 3 days, oestradiol did not increase until 4–6 days after PGF-2 α and peaked at oestrus. Similarly, in heifers treated with bFF for 1 day, oestradiol increased several days before oestrus and the preovulatory surges of LH and FSH. The magnitude of the delayed rise in plasma concentrations of oestradiol in bFF-treated heifers was not different from that in control heifers and resulted in relatively normal preovulatory surges of LH and FSH. One exception was a heifer treated with bFF for 1 day which showed persistent oestrous activity between 5 and 9 days after PGF-2 α , had two peaks in plasma oestradiol at 5 and 8 days (Fig. 4) and failed to form a corpus luteum (Table 2).

Treatments with bFF did not affect plasma concentrations of progesterone during the oestrous cycle after treatment ($P > 0.05$; data not shown) or alter the length of this oestrous cycle (Table 2), indicating that normal corpora lutea were formed (with the exception of one heifer described above).

Discussion

Treatment of heifers with injections of bovine follicular fluid after PGF-2 α -induced luteolysis delayed the normal preovulatory increase in plasma concentrations of oestradiol and the onset of oestrus, suggesting that development and/or function of preovulatory follicles was inhibited during treatment. Plasma concentrations of FSH were suppressed during the injection period but basal concentrations of LH were unaffected, increasing similarly after luteolysis in treated and control heifers. These results suggest that LH alone is not sufficient to stimulate the normal development and/or function of the preovulatory follicle after luteolysis, but that FSH also plays a critical role.

The results of Exp. 2 are consistent with other reports in cattle that injections of follicular fluid selectively suppress plasma concentrations of FSH (Ireland *et al.*, 1983; Braun *et al.*, 1983; Johnson *et al.*, 1985). In contrast, Johnson & Smith (1985) reported that plasma concentrations of FSH were not decreased significantly during treatment of heifers with injections of follicular fluid on Days 1 through 5 of the oestrous cycle. However, treatment did delay the onset of oestrus after injection of PGF-2 α on Day 6 and there was a transient increase in FSH after treatment ended. These authors suggested that inhibin activity in the follicular fluid injections may have been insufficient to decrease FSH or that blood samples were not taken frequently enough (twice/day) to detect a decrease in FSH. In Exp. 1 of the present study, failure of follicular fluid to suppress FSH levels and to consistently delay oestrus in all heifers was probably due to an inadequate dosage of follicular fluid, since using twice the dosage in Exp. 2 delayed oestrus in all heifers. In contrast, Miller *et al.* (1979) reported that dosages of follicular fluid similar to those used in Exp. 1 consistently delayed oestrus to 8 or 9 days after PGF-2 α in all heifers.

Plasma concentrations of FSH in control heifers decreased after PGF-2 α -induced luteolysis, in agreement with reports that levels of FSH decline during the follicular phase of normal oestrous cycles (Butler *et al.*, 1983; Schallenberger *et al.*, 1984). However, concentrations of FSH in follicular fluid-treated heifers were significantly lower and decreased earlier after PGF-2 α treatment than in control heifers. These findings are consistent with reports that treatment of cyclic ewes with bovine follicular fluid delayed the time from luteolysis to oestrus (Miller *et al.*, 1979) and decreased plasma

concentrations of FSH without changing basal concentrations and pulsatile secretion of LH (Miller *et al.*, 1982; McNeilly, 1984). In monkeys, relatively small decreases in plasma concentrations of FSH induced by injections of pig follicular fluid during the follicular phase of the cycle impaired follicular development (Stouffer & Hodgen, 1980; DiZerega *et al.*, 1981; Channing, Anderson, Hoover, Gagliano & Hodgen, 1981). It is likely that, in the present study, the small decrease in FSH during the follicular phase was sufficient to inhibit follicular development and delay oestrus despite the fact that basal plasma concentrations of LH increased normally after luteolysis and were slightly higher in follicular fluid-treated heifers than in control heifers during the 2 days before the preovulatory surges of gonadotrophins.

Plasma concentrations of oestradiol remained basal until several days after the last injection of follicular fluid in heifers treated for 1 or 3 days, indicating that development and/or function of preovulatory follicles was inhibited during treatment. Normal ovulatory follicles developed after treatments ended, since the increase in plasma concentration of oestradiol before oestrus was similar to that in control heifers and relatively normal surges of LH and FSH occurred on the day of oestrus. In addition, the length of the next oestrous cycle and plasma concentrations of progesterone during the luteal phase were normal, suggesting formation of functional corpora lutea. Experiments *in vitro* have demonstrated that FSH stimulates aromatization of androgen to oestradiol by granulosa cells of a variety of species (Richards, 1980). The results of the current study provide evidence *in vivo* that FSH is required for the preovulatory rise in plasma oestradiol.

The delay to oestrus and the preovulatory peaks of plasma gonadotrophins was more uniform in heifers treated with follicular fluid for 3 days (8 days after PGF-2 α) than in those treated for 1 day (4, 5, 6 and 7 days after PGF-2 α). One possible explanation for this difference is that, during the 3-day treatment, populations of follicles at stages of development earlier than the preovulatory follicle became atretic, reducing the number of follicles from which the preovulatory follicle could be selected. Treatment with follicular fluid for 1 day may have been too short to inhibit uniformly the prospective preovulatory follicle and/or induce atresia in less developed follicles. Therefore, a preovulatory follicle might have required more variable times to develop.

Within 24 h after the last injection of bovine follicular fluid in heifers treated for 1 or 3 days, plasma concentrations of FSH increased and remained elevated for 24–36 h. Increased levels of FSH after treatment with follicular fluid have been reported in rats (DePaolo, Hirshfield, Anderson, Barraclough & Channing, 1979), sheep (Miller *et al.*, 1982; McNeilly, 1984) and monkeys (Channing *et al.*, 1981). DePaolo *et al.* (1979) suggested that endogenous production of inhibin may be low during treatment with follicular fluid due to inadequate stimulation of follicles with FSH, and that levels of FSH might increase after treatments are ended because the exogenous source of inhibin is removed. It was reported that in heifers the inhibin-like activity in follicular fluid of presumptive ovulatory follicles is higher than in atretic follicles and increases progressively during the 36 h after prostaglandin-induced luteolysis (Padmanabhan, Convey, Roche & Ireland, 1984). In the present study it is likely that endogenous production of inhibin by the ovaries was decreased due to inhibition of preovulatory follicular development and/or function. In heifers injected with bovine follicular fluid every 6 h for 1 day (Exp. 2), levels of FSH increased before the last injection of bFF, suggesting that the pituitary may have become refractory to inhibition. A refractory response to bovine follicular fluid was reported for ewes (McNeilly, 1984) and was postulated to be due to reduced concentrations of oestradiol in plasma which might normally synergize with follicular fluid to inhibit FSH secretion. This is not an explanation for the results of the present study since plasma concentrations of oestradiol were reduced similarly in heifers treated with follicular fluid for 1 and 3 days, but a refractory response only occurred in heifers treated for 1 day.

On the day after the preovulatory surges of LH and FSH, plasma concentrations of LH returned to baseline but levels of FSH increased again in follicular fluid-treated and control heifers. The secondary increase in plasma FSH on the day after the gonadotrophin surges is consistent with previous reports for heifers (Walters & Schallenberger, 1984), sheep (L'Hermite *et al.*, 1972;

Salamonsen *et al.*, 1973; Pant, Hopkinson & Fitzpatrick, 1977), hamsters (Bast & Greenwald, 1974) and rats (Daane & Parlow, 1971).

Although inhibition of preovulatory follicular development in heifers was clearly associated with decreased concentrations of plasma FSH, follicular fluid may have also inhibited folliculogenesis by a direct effect on the ovary. Follicular fluid contains a variety of substances which inhibit binding of FSH to granulosa cells *in vitro* (Fletcher, Dias, Sanzo & Reichert, 1982; Sato, Ishibashi & Iritani, 1982) and which inhibit follicular response to gonadotrophins in experiments *in vivo* (Kling *et al.*, 1984; Cahill, Driancourt & Findlay, 1984). From the results of the present study we cannot rule out the possibility that follicular fluid treatments may have had direct effects at the level of the ovary. However, the association of significant decreases in plasma FSH with a delay of oestrus and preovulatory follicular development suggests that in cattle FSH is required to support the growth and development of the preovulatory follicle.

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