

Ovarian Response and FSH Profile in Cows Following Injection of Various Doses of Inhibin Antiserum

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ABSTRACT. Dose effect of inhibin antiserum on ovarian response and hormonal profiles were investigated. On day 12 of the estrous cycle (day 0=estrus), 14 of 19 cows were given a single i.v. injection of 25 ml (n=4), 37.5 ml (n=5) or 50 ml (n=5) antiserum against inhibin produced in a castrated male goat. The other 5 animals were given 50 ml castrated male goat serum (control serum). The animals in each group received a single i.m. injection of 0.5 mg prostaglandin F2 α analogue (PG) 48 hr following the serum injection. The population of follicles and ovulation rate (estimated by the number of corpora lutea) were examined by ultrasonography. Administration of inhibin antiserum consistently resulted in a significant ($p<0.01$) increase in plasma concentrations of follicle-stimulating hormone (FSH) in inhibin-neutralized groups, although the increased FSH levels were sustained longer in 50-ml group than in the 25- and 37.5- ml groups. Levels in the circulating inhibin antibody titer were positively correlated with dosage of inhibin antiserum. A large number of antral follicles (≥ 4 mm in diameter) developed similarly after hypersecretion of FSH in all neutralized groups, coupled with a rise in plasma estradiol levels, while the number of large follicles (≥ 10 mm in diameter) on estrus showed a dose-dependent increase. Multiple ovulation (2 to 4) was recorded in all animals after injection of 50 ml inhibin antiserum, however all cows in the 25-ml group experienced only one ovulation and injection of 37.5 ml resulted in a variable number of ovulations (1 to 5). These results demonstrated that administration of inhibin antiserum on day 12, followed by injection of PG, was able to induce hypersecretion of FSH and subsequently multiple ovulations. The number of large follicles on estrus day and ovulations were affected by dosage of inhibin antiserum and were correlated with persistence of increased FSH levels or circulating antibody levels. — **KEY WORDS:** cattle, follicular growth, FSH, inhibin (passive immunization), ovulation rate.

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Knowledge about inhibin biology in female cattle has progressed in the last decade. Injection of highly purified bovine inhibin [2] or bovine follicular fluid [2, 12, 22, 34], a rich source of inhibin, can suppress FSH secretion without altering secretion of luteinizing hormone (LH) in ovariectomized and intact heifers. The recent findings [10, 14–16], that immunoneutralization of endogenous inhibin produced a significant elevation of peripheral FSH, offer evidence that inhibin is an important factor in the inhibitory regulation of FSH secretion. On the other hand, ultrasonographic observation of the ovary correlated with hormonal profiles demonstrated that an increase in plasma FSH preceded emergence of each follicular wave [1, 13, 17, 31] and a decrease in FSH was coincident with functional selection of follicles [31], suggesting that the fluctuation in peripheral FSH level is a trigger for growth, selection and atresia of follicles. As a new approach for the induction of multiple ovulation, active immunization of heifers against synthetic peptides of the bovine inhibin α subunit [10, 23, 29] or against ovine recombinant inhibin α subunit [26] has been carried out. However, in these previous reports, the relationship between peripheral FSH profile and coincident

inhibin binding or subsequent ovulation rate has not been fully demonstrated. In this study, using various doses of inhibin antiserum, we determined changes in plasma FSH levels and the number of ovulation or large follicles, to examine the relationship between FSH profile and ovarian response.

MATERIALS AND METHODS

Experimental design: Eight Japanese black, nine Japanese brown and two Japanese black \times Japanese brown cattle, clinically normal with regular estrous cycle, were used. Animals were randomly divided into four groups. On day 12 of the estrous cycle (day 0=estrus), 14 of 19 cows were given a single i.v. injection of 25 ml (n=4; 2 Japanese black and 2 Japanese brown), 37.5 ml (n=5; 2 Japanese black and 3 Japanese brown) or 50 ml (n=5; 2 Japanese black, 2 Japanese brown and 1 Japanese black \times Japanese brown) of inhibin antiserum. The other 5 animals (2 Japanese Black, 2 Japanese Brown and 1 Japanese black \times Japanese brown) were given 50 ml castrated male goat serum (control serum). The inhibin antiserum used in this study was produced in a castrated Saanen goat against purified bovine 32 kDa inhibin as described previously [14, 15]. The time of injection of serum was defined as 0 hr. At 48 hr, all the animals then received a single i.m. injection of 0.5 mg prostaglandin F2 α .

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analogue (PG; Estrumate, Sumitomo Pharm., Osaka, Japan). Estrous behavior (standing estrus) was checked twice a day (9:00 and 15:00) after the PG treatment. Blood samples were collected via venipuncture every 12 hr just before immunization (0 hr) to 2 days after PG-induced estrus. When animals came into estrus, blood samples were collected every 4 hr for 24 hr in order to detect preovulatory LH and FSH surges. Plasma was removed after centrifugation and stored at -40°C until required assays for FSH, luteinizing hormone, estradiol and inhibin-binding activity.

Determination of ovarian response: The populations of ovarian follicles in each group were examined at 24 hr intervals from the day of immunization to 2 days after PG-induced estrus using an ultrasound scanner (SSD-650CL, Aloka, Tokyo, Japan) as described previously [17]. Number of ovulations was estimated by counting corpora lutea using ultrasonography between 7 and 9 days after the estrus. Follicles were divided into three groups according to their mean diameter (small; $\geq 4 < 7$, medium; $\geq 7 < 10$, large; ≥ 10 mm in diameter). In these Japanese beef cows, follicles larger than 10 mm in diameter corresponds to ovulatory follicle in the normal estrous cycle.

Radioimmunoassays (RIAs): Plasma concentrations of FSH were measured by RIA [4] using anti-bovine FSH β -subunit antiserum (USDA-5-pool), USDA-FSH-BP3 for radioiodination and USDA-FSH-B1 as a reference standard. Plasma concentrations of LH were measured by RIA [7] using anti-ovine LH serum (YM#18), USDA-bLH-I-1 for radioiodination and USDA-bLH-B-5 as a reference standard (YM#18 was supplied by Dr. Y. Mori, Department of Veterinary Medical Science, Faculty of Agriculture, The University of Tokyo, Japan and the other RIA materials for bovine FSH and LH were supplied by Dr. D. J. Bolt, USDA, Beltsville, MD, U.S.A.). The sensitivities of the assays for LH and FSH were 0.006 ng/tube (0.06 ng/ml) and 0.24 ng/tube (1.2 ng/ml) respectively, and the intraassay coefficients of variation were 6.5% for LH and 5.2% for FSH respectively.

Plasma concentrations of estradiol and progesterone were determined as described previously [33] using antiserum to estradiol-17 β (GDN244 [21], supplied by Dr. G. D. Niswender, Department of Physiology and Biophysics, Colorado State University, Fort Collins, CO, U.S.A.) and progesterone (GDN337 [8], supplied by Dr. G. D. Niswender). In the assay for estradiol, substances that interfered with the estradiol assay was removed from plasma samples with a mixture of 2 ml n-hexane and 0.5 ml acetonitrile [25]. The sensitivities of the assays for estradiol and progesterone were 0.32 pg/tube (0.32 pg/ml) and 2.5 pg/tube (25 pg/ml) respectively. The intraassay coefficients of variation were 4.2% for estradiol and 5.8% for progesterone, respectively.

Changes in inhibin-binding activity in plasma, at a final dilution of 1:30, were determined by measuring the binding of ^{125}I -labelled inhibin as described previously [15].

Statistics: Hormone data were subjected to analysis of

variance for repeated measures [9]. Data about the number of large follicles on estrous day or ovulations were subjected to analysis of variance. When a significant effect was obtained with analysis of variance, the significance of the difference between means was determined by Duncan's Multiple Range test. Calculation was carried out using the General Linear Model Procedure of the Statistical Analysis Systems [28]. A value of $p < 0.05$ was considered to be significant.

RESULTS

Estrous behavior (Table 1): Time of the onset of estrous behavior (standing estrus) in the control and neutralized animals were shown in Table 1. The animals in the 37.5- and 50-ml groups had a tendency to show estrous behavior earlier than the control animals.

Inhibin-binding activity in the circulation following injection of inhibin antiserum (Fig. 1): Following injection of inhibin antiserum, a significant ($p < 0.001$) increase in the plasma inhibin-binding activity was noted by 12 hr in each group ($58.8 \pm 0.6\%$ (mean \pm S.E.M.) in the 25-ml group, $59.0 \pm 1.1\%$ in the 37.5-ml group, $62.0 \pm 0.6\%$ in the 50-ml group). Inhibin binding activity in the 50-ml group significantly decreased at 72 hr, showing a steady decline thereafter. There were significant ($p < 0.01$) differences in the levels of plasma inhibin binding activity among the three groups.

Plasma concentration of FSH and LH (Fig. 2): Following injection of control serum, concentrations of plasma FSH did not significantly change before the onset of the LH surge (0 to 72 hr after injection of control serum) (Fig. 2a). In contrast, injection of the three doses of inhibin antiserum produced a significant ($p < 0.01$) increase in plasma FSH during the same period compared to control animals. The FSH responses between 0 and 48 hr were not significantly different among the three neutralized groups. Between 60 and 72 hr, the increased FSH level was sustained in the 50-ml group while FSH levels in the 25- and 37.5-ml groups declined to the control levels. Coincident with the LH surge, a rise in plasma FSH was noted in all groups and there were no significant difference in the magnitude of the FSH surge among the four groups.

Concentrations of plasma LH in the control animals ranged from 0.5 to 1.0 ng/ml before the onset of the LH surge and showed no significant difference compared to the levels in the inhibin-neutralized animals (Fig. 2b). After the onset of PG-induced estrus, the preovulatory LH surge was noted in each group (124.4 ± 3.3 hr in the control group, 144.0 ± 16.2 hr in the 25-ml group, 108.0 ± 13.9 hr in the 37.5-ml group and 108 ± 12.8 in the 50-ml group). The peak value of the LH surge was 18.0 ± 3.4 ng/ml in the control group, 20.4 ± 4.5 ng/ml in the 25 ml-group, 15.5 ± 3.6 ng/ml in the 37.5 ml-group and 18.8 ± 4.7 in the 50 ml-group, respectively, there were no significant differences among the four groups.

Plasma concentrations of estradiol and progesterone

Table 1. Ovarian response and estrous behavior in cows receiving a single i.v. bolus injection of 50 ml control serum, 25 ml, 37.5 ml or 50 ml inhibin antiserum on day 12 of the estrous cycle (day 0=estrus)

Dose of inhibin antiserum	Cow number	Time of estrus	Number of large follicles on estrus	Number of ovulations ^{a)}
0 ml ^{b)} (control)	1	120 ^{c)} (72) ^{d)}	1	1
	2	116 (68)	1	1
	3	122 (74)	1	1
	4	122 (74)	2	1
	5	120 (72)	2	1
		120.0 ± 1.1 ^{e)} (72.0 ± 1.1)	1.4 ± 0.2 ^{af)}	1
25 ml	6	96 (48)	4	1
	7	144 (96)	2	1
	8	168 (120)	2	1
	9	120 (72)	2	1
		132.0 ± 15.5 (84.0 ± 15.5)	2.5 ± 0.5 ^{a)}	1
37.5 ml	10	96 (48)	6	5
	11	100 (52)	1	1
	12	96 (48)	3	3
	13	96 (48)	5	5
	14	120 (72)	1	1
		101.6 ± 4.7 (53.6 ± 4.7)	3.2 ± 1.0 ^{ab)}	3.0 ± 0.9
50 ml	15	96 (48)	8	3
	16	96 (48)	4	4
	17	144 (96)	6	2
	18	96 (48)	4	4
	19	96 (48)	4	4
		105.6 ± 9.6 (57.6 ± 9.6)	5.2 ± 0.8 ^{b)}	3.4 ± 0.4

a) Estimated by the number of corpora lutea.

b) Injected with 50 ml control serum.

c) Hours after injection of control serum or anti inhibin serum.

d) Hours after PG injection.

e) Means ± S.E.M.

f) Means without common characters are significantly ($P < 0.05$) different in each treatment (Duncan's Multiple Range test).

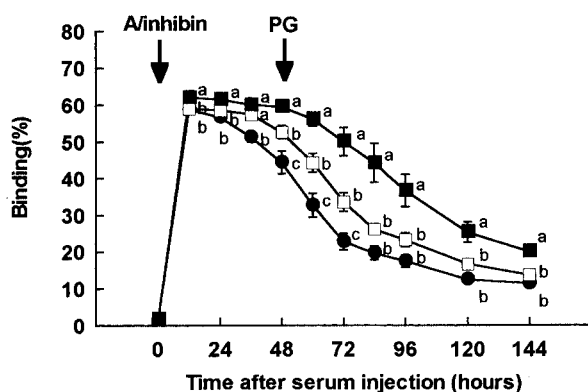


Fig. 1. Inhibin-binding activity in plasma, at a final dilution of 1:30, of cows receiving a single i.v. bolus injection of inhibin antiserum at dosage of 25 ml (□), 37.5 ml (○) or 50 ml (△) on day 12 of the estrous cycle (day 0=estrus). Arrows indicate injections of inhibin antiserum (A/inhibin) or PG. Values are means ± S.E.M. Means without common characters are significantly ($p < 0.05$) different from each other at the same time (Duncan's Multiple Range test).

(Fig. 3): In the control animals, plasma estradiol increased significantly ($p < 0.05$) after PG injection and reached a peak (13.8 ± 1.6 pg/ml) around the day of estrus (120 hr) (Fig. 3a). Treatment with 37.5 or 50 ml inhibin antiserum produced a further increase in plasma estradiol for 2 days after PG injection compared to the control animals, although the response in the 50-ml group was greater ($p < 0.05$) than in the 37.5-ml group. Estradiol levels in these two groups decreased thereafter. Following injection of 25 ml antiserum, plasma estradiol showed a transient increase compared to the control groups but this was not significant.

Concentrations of plasma progesterone ranged from 6 to 10 ng/ml before PG injection and dropped sharply within 12 hr after PG treatment, remaining low thereafter. There were no significant changes in plasma progesterone among the four groups (Fig. 3b).

Ovarian response (Table 1 and Fig. 4): After injection of control serum, there were several small and medium follicles in the ovary (Fig. 4a). Administration of inhibin antiserum, at various doses, resulted in a significant ($p < 0.01$) increase in the number of various sized follicles compared to the

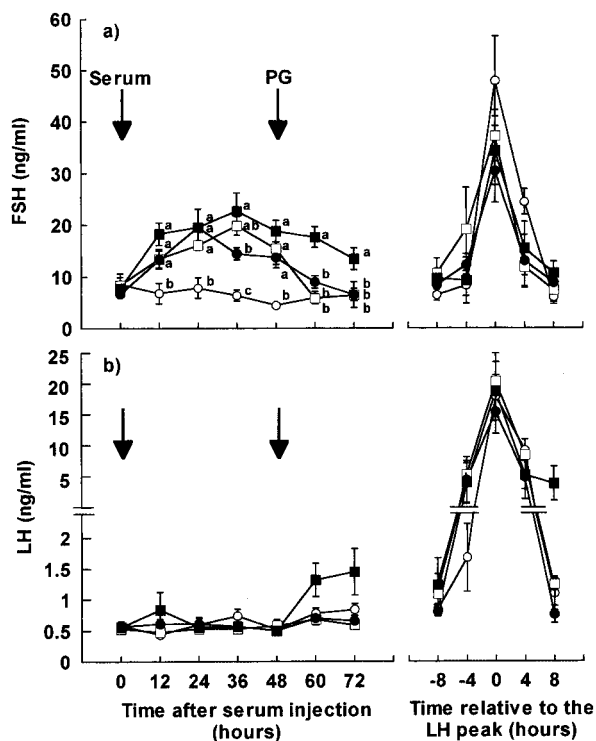


Fig. 2. Changes in plasma concentrations of (a) FSH and (b) LH in cows receiving a single i.v. bolus injection of 50 ml control serum (○), 25 ml (□), 37.5 ml (△) or 50 ml (●) inhibin antiserum on day 12 of the estrous cycle (day 0=estrus). Arrows indicate injections of sera or PG. Values are means \pm S.E.M. Means without common characters are significantly ($p < 0.05$) different from each other at the same time (Duncan's Multiple Range test).

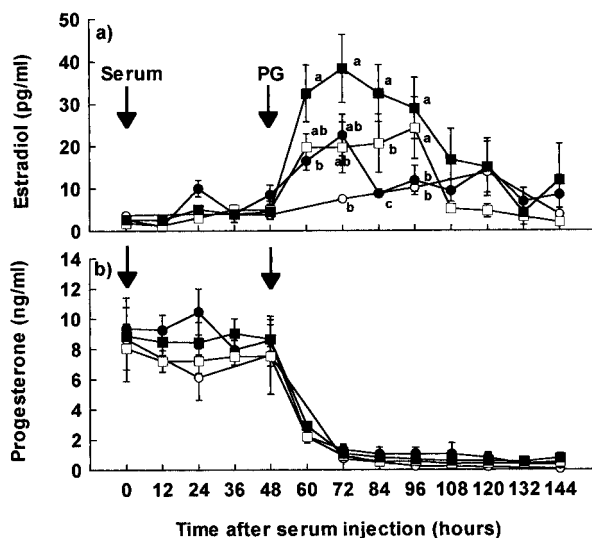


Fig. 3. Changes in plasma concentrations of (a) estradiol and (b) progesterone in cows receiving a single i.v. bolus injection of 50 ml control serum (○), 25 ml (□), 37.5 ml (△) or 50 ml (●) inhibin antiserum on day 12 of the estrous cycle (day 0=estrus). Arrows indicate injections of sera or PG. Values are means \pm S.E.M. Means without common characters are

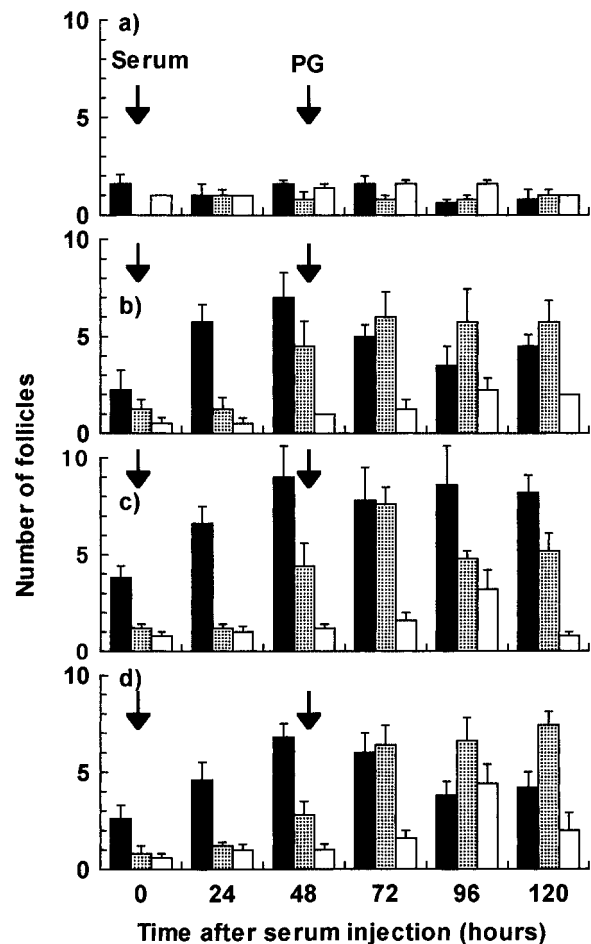


Fig. 4. Mean number of follicles in cows receiving a single i.v. bolus injection of (a) 50 ml control serum, (b) 25 ml, (c) 37.5 ml, or (d) 50 ml inhibin antiserum on day 12 of the estrous cycle (day 0=estrus). Small follicles ($\geq 4 < 7$ mm in diameter) are represented by solid bars, medium follicles ($\geq 7 < 10$ mm) by shaded bars, and large follicles (≥ 10 mm) by open bars. Arrows indicate injections of sera or PG. Vertical bars represent S.E.M.

control group (Fig. 4b-d). Although there were no significant difference in the total number of antral follicles (≥ 4 mm in diameter) among neutralized groups, the number of large follicles on estrus was 2.5 ± 0.5 in the 25-mg group, 3.2 ± 1.0 in the 37.5-mg group and 5.2 ± 0.8 in the 50-mg group (Table 1). There was a dose-related increase in the number of large follicles after inhibin immunization. Multiple ovulation, estimated by the number of corpora lutea, was noted in 3 of 5 animals (3.0 ± 0.9) in the 37.5-mg group and all animals (5/5: 3.4 ± 0.4) in the 50-mg group, while the animals in both control and 25-mg groups experienced only one ovulation (Table 1). The number of large follicles on estrus or ovulations was significantly correlated with dosage of inhibin antiserum (large follicle

significantly ($p < 0.05$) different from each other at the same time (Duncan's Multiple Range test).

number on estrus: $r=0.67$, $p<0.01$, ovulation rate: $r=0.65$, $p<0.01$).

DISCUSSION

The present study demonstrated that the relationship between dosage of inhibin antiserum and FSH profile or subsequent ovarian response. FSH profile and ovarian response was apparently affected by the amount of inhibin antiserum in the circulation.

Injection of various doses of inhibin antiserum resulted in a consistent rise in plasma FSH levels without altering LH secretion, while the FSH response to inhibin immunization in the 50-mg group lasted longer than in the 25- and 37.5-mg groups. The results confirm previous findings that passive [14–16, 32] or active [10] immunization against inhibin produced a hypersecretion of FSH in female cattle, indicating that inhibin is an important negative regulator of FSH secretion. The difference in the FSH response among the neutralized groups correlated with the difference in the levels of the circulating inhibin antibody, in other words, the difference in the capacity to immunoneutralize circulating inhibin. The increased FSH secretion returned to normal levels until the onset of the preovulatory gonadotropin surge, probably due to neutralization of the antibody in plasma by an increase in the secretion of inhibin [18, 27] and of the free α subunits [19, 20] derived from a large number of growing antral follicles.

Neutralization of endogenous inhibin, using various dose of inhibin antiserum, consistently stimulated the growth of a large number of antral follicles (≥ 4 mm) coupled with a rise in plasma estradiol levels, confirming previous reports in cattle [14–16, 32]. The observation that rise in plasma FSH preceded the emergence of new follicles leads to the conclusion that hypersecretion of FSH stimulates multiple growth of follicles. The number of large follicles on estrus showed a dose dependent increase. Ovulation rate, estimated by the number of corpora lutea, increased in all cows receiving 50 mg inhibin antiserum, while all cows treated with 25 mg antiserum experienced one ovulation whereas injection of 37.5 mg antiserum resulted in a variable number of ovulations (1 to 5). In our previous report [32], administration of 75 mg inhibin antiserum raised FSH secretion for 72 hr and induced multiple ovulation of 6.6 ± 2.5 . Considering the FSH profile in the present and previous [32] studies, a persistence of increased FSH levels is likely to be involved in the maintenance or stimulation of growth of large follicles. On the other hand, recent results obtained from active immunization [3, 24] or repeated injections of inhibin antiserum [5, 6] suggest that ovarian response to inhibin immunization is not only mediated by the rise in circulating FSH levels but also by neutralization of local actions of inhibin on follicular growth. The mature form of inhibin (32 kDa) enhanced thecal androgen production [11, 35] while suppressing estradiol production in granulosa cells [36] *in vitro*, and precursors of α -inhibin seem to act as a

competitor of FSH receptors in granulosa cells [30]. In this study, levels of inhibin binding activity in the circulation of each group are apparently correlated with dosage of inhibin antiserum. The result suggests that difference in the persistence of circulating inhibin antibody among the three groups accounts for the difference of the number of large follicles or ovulations by attenuating these inhibitory actions of inhibin on follicular growth.

In summary, the present results indicate that administration of inhibin antiserum on day 12, followed by injection of PG, resulted in hypersecretion of FSH and subsequently multiple growth of follicles and multiple ovulation. The number of large follicles and ovulations were affected by dosage of inhibin antiserum and correlated with persistence of increased FSH levels or circulating antibody levels. These results suggest that ovarian response after passive immunization against inhibin are dependent on not only the rise in plasma FSH levels but also direct effects of inhibin immunization.

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