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Ovarian Response and Hormonal Profiles in Heifers After Immunization and Re-immunization Against Inhibin α -Subunit

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Abstract. To explore a strategy for superovulation in heifers, effects of immunization and re-immunization of Japanese beef heifers against inhibin on ovarian response and hormonal profiles were investigated. On day 9 of the estrous cycle (day 0=day of estrus), ten heifers were primary injected intramuscularly (i.m.) either with inhibin vaccine (recombinant ovine inhibin α -subunit in oil emulsion, 125 μ g/ml) or a placebo (Montanide:Marcol adjuvant alone) followed by two booster injections at one estrous cycle intervals (immunized group). One year later, 3 injections of inhibin vaccine were given the same as in the first year (re-immunized group). Inhibin antibody titers in plasma of immunized heifers increased after the second injection of inhibin vaccine and in 3 out of 5 immunized heifers and multiple-ovulation (2.4 ± 0.6 , $n=5$) was induced after the third injection. On the other hand, in the re-immunized heifers, the plasma inhibin antibody titers increased sharply after the first injection of inhibin vaccine and in 4 out of 5 re-immunized heifers multiple ovulation (4.4 ± 1.1 , $n=5$) was induced after the first injection of inhibin vaccine. In all re-immunized heifers multiple-ovulation was induced after the third injection of inhibin vaccine (5.0 ± 1.1 , $n=5$). The results also showed that the ovulation rates were positively correlated to circulating inhibin antibody titers and plasma levels of FSH. These results confirmed that active immunization against inhibin α -subunit induced multiple ovulation mainly by attenuation of the suppressive effect of inhibin on FSH secretion in heifers and indicated that re-immunization against inhibin was a good way to induce multiple-ovulation in cattle.

Key words: Heifer, Active immunization, Inhibin, Superovulation, Cattle

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It is reported that immunoneutralization of inhibin to induce superovulation provides an alternative to current follicle stimulating hormone

(FSH) or equine chorionic gonadotropin (eCG) treatment protocols for the production of transferable embryos in mammals [1–8]. Several studies have shown that active immunization of heifers against inhibin α -subunit [9–13] and passive

immunization of cows against inhibin [5] increased ovulation rates, which is generally considered to be a result of neutralization of the suppressive effect of inhibin on the secretion of FSH, but the response of inhibin-immunized cattle varied greatly and was unpredictable. Therefore, further studies are needed to investigate how active immunization against the inhibin α -subunit can consistently sustain optimal levels of inhibin antibody titer for with consistency in cattle. On the other hand, although ultrasonographic examination has revealed that follicular development occurs in two or three waves related to FSH profiles during the normal estrous cycle [14–16], the relationships between follicular dynamics in each wave of follicular growth and FSH profiles after active immunization against inhibin have not been fully documented.

In the present study we therefore investigated the changes in plasma concentrations of FSH and follicular dynamics and ovulation rates in Japanese beef cattle after active immunization and re-immunization against the inhibin α -subunit, and tried to find a good way to induce superovulation in cattle.

Materials and Methods

Experimental design

Eight Japanese black heifers and two Japanese brown heifers between 5 and 9 years of age and weighing 455–510 kg, clinically normal with a regular estrous cycle and non pregnant, were used. They were randomly divided into immunized and control groups. On day 9 of the estrous cycle (day 0 = day of estrus), the heifers were injected intramuscularly (i.m.) either with inhibin vaccine (recombinant ovine inhibin α -subunit in oil emulsion, 125 μ g/ml) or a placebo (Montanide:Marcol adjuvant only) followed by two booster injections at estrous cycle length intervals. One year later, 3 injections of vaccine were given, as in the first year. All the heifers received a single i.m. injection of prostaglandin $F_{2\alpha}$ analogue (PG; Estrumate, Sumitomo Pharm., Osaka, Japan) on day 18 of the estrous cycle after the 3rd injection. The number of different sized follicles and corpora lutea were examined by ultrasonography. To examine the plasma inhibin antibody titers, blood samples were collected via venipuncture on day 9

(immediately before immunogen injection) and on day 18 of three consecutive estrous cycles after primary injections. To study hormone profiles, blood samples were collected every 12 h just after the 2nd booster and stored at -40°C until plasma concentrations of FSH, luteinizing hormone (LH), estradiol and progesterone were measured.

Inhibin vaccine

The α -subunit of ovine inhibin produced in *E. coli* by the recombinant DNA method [17] was used as an immunogen. Immunizations were given at a dose of 1 ml of the immunogen (125 μ g/ml) in Montanide 888:Marcol 52 (1:9) each time.

Determination of ovarian response

The populations of ovarian follicles in each group were examined at 24 h intervals from the day of the second booster by using an ultrasound scanner (SSD-650CL, Aloka, Tokyo, Japan) as described previously [18]. The ovulation rate was estimated by counting the number of corpora lutea by ultrasonography between 7 and 9 days after estrus. Follicles were arbitrarily 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 cattle, follicles larger than 10 mm in diameter were considered to be preovulatory follicle in the normal estrous cycle [16].

Radioimmunoassays (RIAs)

Plasma concentrations of FSH were measured by RIA [19] with anti-bovine FSH β -subunit antiserum (UCB Bioproduct, S.A. Braine l'Alleud, Belgium), USDA-FSH-BP3 for radioiodination and USDA-FSH-B1 as a reference standard. Plasma concentrations of LH were measured by RIA [20] with 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, 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 intra- and inter-assay coefficients of variation were 3.0% and 9.8% for FSH and 6.5% and 11.5% for LH, respectively.

Plasma concentrations of estradiol and progesterone were determined as described previously [21] with antiserum to estradiol-17 β (GDN244), and progesterone (GDN337) (supplied

by Dr. G. D. Niswender, Animal Reproduction and Biotechnology Laboratory, Colorado State University, Fort Collins, CO, U.S.A.). In the assay for estradiol, substances that interfered with the estradiol assay were removed from plasma samples with a mixture of 2.0 ml n-hexane and 0.5 ml acetonitrile [22]. The intra- and inter-assay coefficients of variation were 4.2% and 9.8% for estradiol and 5.8% and 13.4% for progesterone, respectively. Plasma inhibin antibody titers were determined by measuring the binding of 125 I-labeled inhibin at a final dilution of 1:12 as described previously [23].

Statistics

Results were subjected to analysis of variance for repeated measures [24]. When a significant effect was obtained with analysis of variance, the significance of the difference between two means was determined by Student's *t*-test. Calculation was carried out by the General Linear Model Procedure of the Statistical Analysis Systems [25]. A value of $P < 0.05$ was considered to be significant.

Results

Inhibin-binding activity in the circulation (Fig. 1)

In the first year experiment, inhibin antibody titers in the plasma of the immunized group increased gradually after the first booster, and reached a peak level at 9 days after the second booster (Fig. 1, a). In the second year experiment, inhibin antibody titers in plasma of the immunized group increased rapidly and reached a peak level after the first injection and remained at peak levels during the second and third injections (Fig. 1, b). On the other hand, the placebo injection had no effect on inhibin antibody titers in plasma during the two consecutive years.

Ovarian response (Table 1 and Fig. 2)

The mean number of follicles from the start of the follicular wave are shown in Fig. 2. All heifers in the experiment had two waves of follicular development after the second booster injection except one control heifer which had only one preovulatory wave. The emergence and duration of follicle waves in immunized heifers were similar to those in control animals. In the preovulatory wave, the numbers of follicles of different size in

Fig. 1. Inhibin antibody titer in plasma of heifers after immunization (a) and re-immunization (b) against the inhibin α -subunit. Note: (a) immunization in the first year, (b) re-immunization in the second year. Arrows represent injections of placebo (○) or inhibin vaccine (□). Values are shown as the means $\pm$ SEM in five observations. * $P < 0.05$ compared with the value for the respective control (Student's *t*-test).

immunized heifers were greater than those in control heifers. There were no significant differences between immunized and control groups in the maximum diameters of dominant follicles in each waves; they were 8.2 ± 0.5 mm ($n=4$; control group) and 8.8 ± 0.5 mm ($n=5$; immunized group) in the first wave after the second booster injection, and 11.8 ± 0.3 mm ($n=5$; control group) and 11.3 ± 0.5 mm ($n=5$; immunized group) in the preovulatory wave, respectively.

In three out of five immunized heifers multiple ovulations were induced after the third booster injection in the first year experiment (Table 1). In the second year experiment, in 4 out of 5 heifers multiple ovulations were induced after the first injection. In all heifers multiple ovulations were

Table 1. Ovulation rates in heifers after immunization and re-immunization against inhibin

Group	Number	Immunization			Re-immunization		
		1st	2nd	3rd	1st	2nd	3rd
Treatment	A	1	1	3	6	4	6
	B	1	1	4	7	1	8
	C	1	1	3	5	1	2
	D	1	1	1	3	5	6
	E	1	1	1	1	4	3
	Mean $\pm$ SEM	1	1	2.4 $\pm$ 0.6	4.4 $\pm$ 1.1	3.0 $\pm$ 0.8	5.0 $\pm$ 1.1
Control	F	1	1	1	1	1	1
	G	1	1	1	1	1	1
	H	1	1	1	1	1	1
	I	1	1	1	1	1	1
	J	1	1	1	1	1	1
	Mean	1	1	1	1	1	1

Numbers in the table are the numbers of corpora lutea in ovaries between day 7 and day 9 after each estrus.

successfully induced after the third injection in the re-immunized group. All heifers with multiple ovulations exhibited high levels (over 25%) of plasma inhibin antibody titers. The numbers of antral follicles and ovulations were closely correlated with the inhibin antibody titers (follicles $r=0.59$, $P<0.05$; ovulation $r=0.73$, $P<0.01$).

Plasma concentrations of FSH, LH, estradiol and progesterone (Fig. 3)

Mean plasma concentrations of FSH (12.1 ± 0.4 ng/ml, $n=5$) in the immunized group were significantly ($P<0.01$) higher during the 11-day (days 9–19 of the estrous cycle) period after the second booster injection than the values (8.4 ± 0.3 ng/ml, $n=5$) in the control group. Concentrations of plasma LH ranged from 0.3 to 0.7 ng/ml ($n=10$) in both control and immunized groups and did not differ significantly from group to group (Fig. 3b). Mean FSH concentrations during the 11-day period after the second booster injection closely correlated with the incidence of multiple-ovulation ($r=0.83$, $P<0.01$).

In the control animals, concentrations of plasma estradiol increased significantly ($P<0.05$) 12 h after PG injection (Fig. 3c). Immunization against inhibin α -subunit produced a further increase in the plasma concentration of estradiol after PG injection compared to the control animals and the peak level in these animals was 18.4 ± 3.2 pg/ml ($n=5$), significantly ($P<0.05$) higher than the control value (9.4 ± 0.7 pg/ml, $n=5$).

Plasma concentrations of progesterone ranged

from 4 to 7 ng/ml before PG injection and dropped sharply within 12 h after PG treatment, remaining low thereafter. Plasma concentrations of progesterone before PG injection in the immunized group tended to be higher than in the control group (Fig. 3d).

Discussion

The present study clearly demonstrated that re-immunization with only one injection of inhibin vaccine sharply produced peak levels of plasma inhibin antibody titer in immunized heifers, and three injections with inhibin vaccine successfully induced multiple ovulation in all immunized heifers. These results indicated that active immunization against inhibin α -subunit is a long-acting and efficient method to induce multiple ovulations in cattle.

In the first year experiment, active immunization against inhibin α -subunit also significantly increased the number of follicles in the PG-induced preovulatory wave after the 2nd booster and subsequently induced multiple ovulations. The number of ovulations and antral follicles were positively correlated to the circulating inhibin antibody titers, and these results were in agreement with recent reports [9, 26–30]. In the present study, ovulation rates were also positively correlated with plasma levels of FSH. Mean plasma concentrations of FSH in multiple ovulating heifers were significantly (44%, $P<0.01$) higher during the 11-

Fig. 2. Numbers of follicles in ovaries of immunized heifers after the second booster injection of placebo (○) or inhibin vaccine (□) in the first year experiment. Note: (a) small follicles ($\geq 4 < 7$ mm in diameter), (b) medium follicles ($\geq 7 < 10$ mm in diameter), (c) large follicles (≥ 10 mm in diameter), (d) total number of follicles. Values are shown as the means $\pm$ SEM in five observations. * $P < 0.05$ compared with the value for the respective control (Student's *t*-test).

day period (days 9–19 of the estrus cycle) after the second booster injection than the values in the animals having a single ovulation, and this was consistent with a previous report [6]. Therefore, the results of the present study confirmed that active immunization against inhibin induced multiple ovulation mainly by attenuating of the suppressive effect of inhibin on FSH secretion in heifers.

Fig. 3. Plasma concentrations of FSH (a), LH (b), estradiol (c) and progesterone (d) in immunized heifers after the second booster injection of placebo (○) or inhibin vaccine (□) in the first year experiment. Values are shown as the means $\pm$ SEM in five observations. * $P < 0.05$ compared with the value for the respective control (Student's *t*-test).

Consistent with previous findings [31], plasma concentrations of estradiol rose immediately after PG injection in all heifers. The peak level of plasma concentrations of estradiol in the inhibin-immunized group was about twice high as that in controls. This difference in the plasma concentration of estradiol is in agreement with the increase in the numbers of preovulatory follicles.

In summary, the present study clearly demonstrated that active immunization against the inhibin α -subunit increased ovulation rates mainly

by attenuating of the suppressive effect of inhibin on FSH secretion in heifers and indicated that re-immunization against inhibin was a good way to induce multiple ovulations in cattle.

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