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Research

Effects of passive immunization of goats against inhibin on follicular development, hormone profile and ovulation rate

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This study was conducted to investigate the effect of immunoneutralization against endogenous inhibin on FSH secretion and ovulation rate, with the aim of developing a new superovulation method using inhibin antiserum in goats. Two groups of goats received an i.v. injection of either 10 ml normal goat serum (control; $n = 6$) or 10 ml inhibin antiserum developed against [Tyr^{30}]-inhibin α (1–30) ($n = 6$) 48 h before treatment with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). Blood samples were collected at 6 h intervals and ovaries were examined each day using a B-mode ultrasound scanner equipped with a 7.5 MHz transducer during the experimental period. Immunization against inhibin resulted in a four- to fivefold increase ($P < 0.01$) in plasma concentrations of FSH. After luteolysis, plasma concentrations of oestradiol increased markedly to reach a preovulatory peak, which was about two times higher ($P < 0.01$) than that of the controls. The treatment was accompanied by a significant increase in the total number of follicles of $\geqslant 3$ mm in diameter at 24 (8.2 ± 0.4 in inhibin antiserum group versus 4.8 ± 0.3 in control group) and 96 h later (13.5 ± 1.0 in inhibin antiserum group versus 5.3 ± 0.6 in control group). The ovulation rate was significantly ($P < 0.01$) higher in goats treated with inhibin antiserum (4.2 ± 0.5; $n = 6$) than in control goats (1.8 ± 0.3; $n = 6$). These results indicate that inhibin is an important factor in the regulation of FSH secretion in goats and demonstrate that passive immunization against inhibin at 48 h before treatment with $PGF_{2\alpha}$ induces the development of more follicles and increases ovulation rate. Thus, inhibin antiserum treatment may be an alternative to the use of exogenous gonadotrophins for induction of superovulation in goats.

Introduction

Inhibin is a heterodimeric glycoprotein hormone that selectively inhibits the secretion of FSH by the pituitary gland (Burger, 1988). A negative relationship between plasma concentrations of FSH and inhibin occur in several mammalian species (Taya, 1993; Taya and Watanabe, 1999). The finding that immunoneutralization of endogenous inhibin results in a significant increase in peripheral FSH indicates that inhibin is an important factor in the inhibitory regulation of FSH secretion in domestic as well as laboratory animals (Mann *et al*., 1989; Rivier and Vale, 1989; Wrathall *et al*., 1990; Glencross *et al*., 1994; Campbell and Scaramuzzi, 1995; Campbell *et al*., 1995; Kaneko *et al*., 1995a, 1997; Kishi *et al*., 1996; Nambo *et al*., 1998; Shi *et al*., 2000). However, ultrasound images of the ovary correlate with hormone profiles and demonstrated that an increase in plasma FSH precedes the emergence of each follicular wave (Adams *et al*., 1992; Sunderland *et al*., 1994; Kaneko *et al*., 1995b; Evans *et al*., 1997) and a decrease in FSH is coincident with functional selection of follicles (Sunderland *et al*., 1994; Kulick *et al*., 1999), indicating that the fluctuation in peripheral FSH concentration is a trigger for growth, selection and atresia of follicles. Multiple ovulations have been induced successfully by passive immunization against endogenous inhibin in species such as rats (Rivier and Vale, 1989), sheep (Wheaton *et al*., 1992, 1996), hamsters (Kishi *et al*., 1996), cows (Akagi *et al*., 1997; Takedomi *et al*., 1997), horses (Nambo *et al*., 1998), guinea-pigs (Shi *et al*., 2000) and mice (Wang *et al*., 2001). Thus, these initial results indicate that immunization of animals against endogenous inhibin to induce superovulation through increased endogenous FSH secretion is an alternative method to the current exogenous gonadotrophin protocols.

In most studies, a combination of equine chorionic gonadotrophin (eCG) and hCG has been the most

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common method to induce superovulation in goats. However, a disadvantage of these protocols is the long half-life of eCG which interferes with normal fertilization and embryo development (McIntosh *et al*., 1975; Armstrong *et al*., 1983; Ertzeid *et al*., 1993) and repeated eCG treatments induce anti-eCG antibodies that clearly have negative effects on reproduction of goats (Roy *et al*., 1999). Thus it is necessary to establish an alternative simple method for induction of superovulation in goats to overcome these problems. The aim of this experiment was to determine the effect of passive immunization against inhibin at 48 h before luteolysis on FSH secretion and ovulation rate in goats.

Materials and Methods

Experimental animals

Twelve goats (*Capra hircus*) housed under natural daylight and fed a maintenance diet of 700 g of hay cubes per animal per day were used in this study. Clean water and mineralized salt licks were available *ad libitum*. Oestrous cycles were synchronized with two injections of 125 μg of a synthetic analogue of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) (Estrumate, Schering Plough Animal Health, NJ) administered 11 days apart. Oestrous behaviour was examined at 6 h intervals with an aproned mature buck throughout the experimental period. On day 10 of the oestrous cycle, animals were allocated to one of two groups: (i) control, treated with i.v. injection of 10 ml normal goat serum ($n = 6$) and (ii) inhibin-immunized, treated with i.v. injection of 10 ml inhibin antiserum ($n = 6$). The time of the injection of serum was defined as 0 h. At 48 h, all animals were injected with 125 μg $PGF_{2\alpha}$ to induce oestrus and ovulation. Blood samples were collected, at 6 h intervals from 24 h before treatment until 120 h after treatment and at 2 h intervals from 48 to 72 h after $PGF_{2\alpha}$ injection, into heparinized Vacutainer tubes (Terumo Venoject II, Tokyo). An additional blood sample for progesterone determination was collected on day 7 after ovulation. Blood samples were centrifuged at 1200 ***g*** for 15 min at 4°C and plasma was separated and stored at –20°C until assayed for hormones. The experimental protocol was approved in accordance with the Guide for the Care and Use of Laboratory Animals prepared by Tokyo University of Agriculture and Technology.

Ultrasonography and determination of ovarian response

The follicle populations of the animals were monitored each day starting 24 h before treatment until the end of the experiment and at 12 h intervals at the time of ovulation using a B-mode scanner (ECHOPAL ultrasound scanner, Hitachi Medical Corporation, Tokyo) equipped with a 7.5 MHz transducer. A slightly arched plastic rod (30 cm in length and 20 mm in diameter) was fastened to the transducer to manipulate the probe externally into the rectum. All follicles $\geqslant 3$ mm in diameter were measured in two planes and the mean was calculated for each follicle. The occurrence of ovulation was assessed by the disappearance of large antral follicles that were present at the previous transrectal ultrasonography examination, and confirmed by detection of corpora lutea as described by Rajamahendran *et al*. (1989). The ovulation rate was determined by matching the number of large antral follicles that were no longer apparent with the number of corpora lutea detected by ultrasonography (Pierson and Ginther, 1988). Follicles were divided into three arbitrary groups according to their mean diameter (small: < 3.5 mm; medium: 3.5–5.0 mm; and large: > 5 mm).

Antisera

The antiserum against inhibin used in the present study was raised in ovariectomized goats to a synthetic peptide of 1–30 amino acid sequence of the N-terminal of the α-chain of pig inhibin conjugated to rabbit serum albumin as described by Araki *et al*. (2000).

Determination of inhibin binding activity

Changes in inhibin binding activity in plasma of the inhibin-immunized goats were determined by measuring the binding of ^{125}I-labelled inhibin (5000 c.p.m.) as reported by Kaneko *et al*. (1993). Plasma samples obtained at various timepoints after injection of the inhibin antiserum were diluted 1:10 with PBS containing 5% BSA. PBS (100 μl) was added to each aliquot (100 μl) of diluted plasma and incubated for 24 h at 37°C with ^{125}I-labelled bovine inhibin of 32 kDa. Bound tracer was then separated by adding 100 μl PBS containing 1% bovine gamma globulin and 500 μl PBS containing 25% polyethylene glycol (molecular weight 6000) for 3 min, followed by centrifugation at 1200 ***g*** for 30 min at 4°C. The radioactivity in the precipitate was counted. The binding activity of inhibin was expressed as a percentage of the total counts added. The intra-assay coefficient of variation was 3.9%.

Hormone analysis

Plasma concentrations of FSH were measured by a radioimmunoassay system, as described by Araki *et al*. (2000) using anti-ovine FSH, NIDDK-FSH-I-1 for radioiodination, and NIDDK-oFSH-RP-1 as a reference standard. Plasma concentrations of LH were measured by radioimmunoassay as described by Mori and Kano (1984) using anti-ovine LH (YM No. 18), NIDDK-oLH-I-3 for radio-iodination and NIDDK-oLH-RP-24 as a reference standard. The intra- and interassay coefficients of variation were 9.8 and 12.6% for FSH and 5.9 and 6.5% for LH, respectively. Plasma concentrations

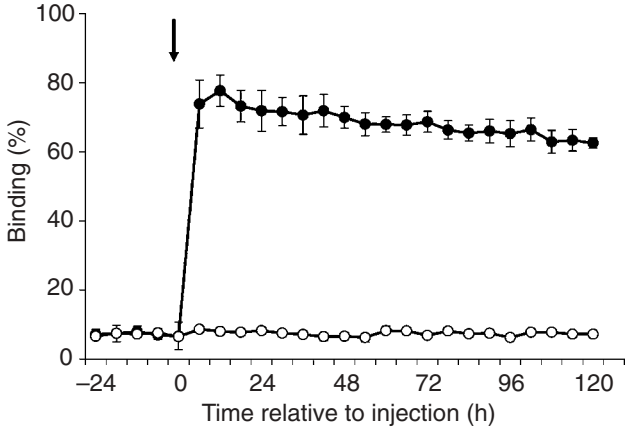

Fig. 1. Binding capacity of plasma inhibin at a dilution of 1:10 in goats that received an i.v. bolus injection of 10 ml normal goat serum (○; $n = 6$) or 10 ml inhibin antiserum (●; $n = 6$) at 0 h (arrow). Values are means ± SEM.

of oestradiol and progesterone were determined by a double-antibody radioimmunoassay system using ^{125}I-labelled radioligands as described by Taya *et al*. (1985). Antisera against oestradiol (GDN 244) and progesterone (GDN 337) were kindly provided by G. D. Niswender (Animal Production and Biotechnology, Colorado State University, Fort Collins, CO). The intra- and interassay coefficients of variation were 5.7 and 7.4% for oestradiol and 8.2 and 9.2% for progesterone, respectively.

Statistical analysis

Mean values (± SEM) were calculated and analysed using two-way ANOVA. Duncan's multiple-range test was used for detection of significant differences using the SAS computer package (SAS, 1987). Wilks' Lambda correlation was made between progesterone concentrations and number of corpora lutea.

Results

Binding capacity of plasma hormones

After immunization, there was an increase ($P < 0.01$) in the ability of plasma to bind inhibin in all immunized animals. At 6 h after animals were immunized against inhibin, the binding capacity of inhibin in plasma at a final dilution of 1:10 was 77.7 ± 6.9% compared with 8.0 ± 0.7 in the control group (Fig. 1). The binding capacity showed a steady decline ($P < 0.01$) over the course of the experiment in the immunized group.

Plasma concentrations of FSH and LH

After the injection of normal goat serum, plasma concentrations of FSH did not significantly change during the period before the FSH surge. In contrast, treatment

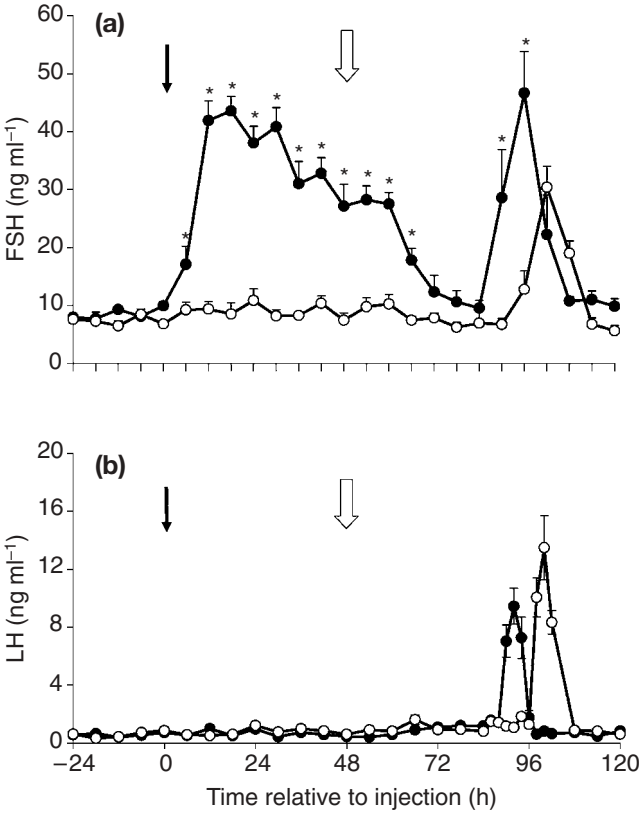

Fig. 2. Plasma concentrations of (a) FSH and (b) LH in goats that received an i.v. bolus injection of 10 ml normal goat serum (○; $n = 6$) or 10 ml inhibin antiserum (●; $n = 6$) at 0 h (solid arrow) and an i.m. injection of $PGF_{2\alpha}$ at 48 h later (open arrow). Values are means ± SEM. *$P < 0.05$ compared with the control values.

with the inhibin antiserum resulted in a marked increase ($P < 0.01$) in plasma concentrations of FSH compared with control values. In the immunized group, there was a four- to fivefold increase ($P < 0.01$) in plasma FSH concentrations within 12 h of treatment (Fig. 2).

Thereafter, mean plasma FSH concentrations remained relatively stable for about 24 h and then declined ($P < 0.01$). The preovulatory FSH peak in goats immunized against inhibin occurred about 6 h before that of the control group and the peak value of the FSH increase in the goats immunized against inhibin was significantly higher than in the control group. There was no significant difference in the basal plasma LH concentrations between immunized (0.79 ± 0.1 ng ml^{-1}) and control (0.86 ± 0.1 ng ml^{-1}) goats. However, the LH surge tended to be lower in the immunized group compared with the control group (9.4 ± 1.2 ng ml^{-1} ($n = 6$) and 13.5 ± 2.2 ng ml^{-1} ($n = 6$), respectively; Fig. 2).

Plasma concentrations of oestradiol and progesterone

In control animals, plasma concentrations of oestradiol increased significantly ($P < 0.05$) after treatment

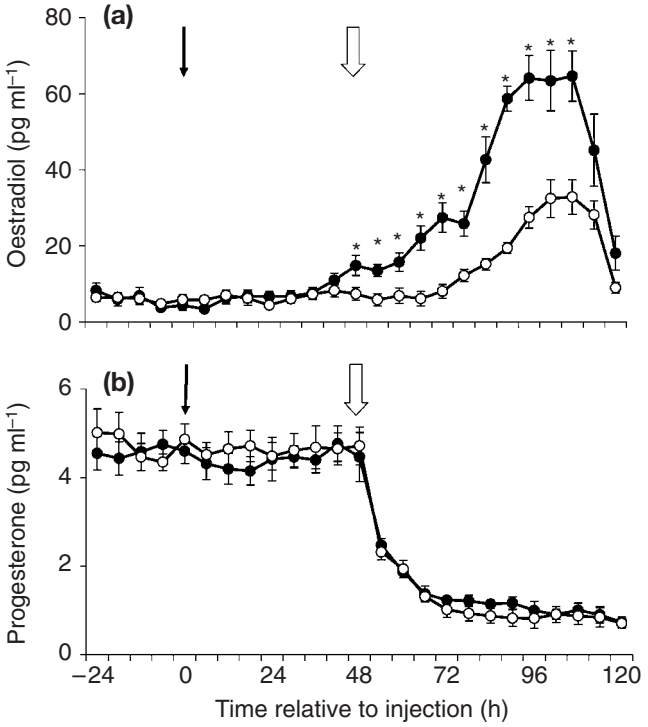

Fig. 3. Plasma concentrations of (a) oestradiol and (b) progesterone in goats that received an i.v. bolus injection of 10 ml normal goat serum (○; $n = 6$) or 10 ml inhibin antiserum (●; $n = 6$) at 0 h (solid arrow) and an i.m. injection of $PGF_{2\alpha}$ 48 h later (open arrow). Values are means ± SEM. $^*P < 0.05$ compared with the control values.

with $PGF_{2\alpha}$ and reached a peak (32.4 ± 5.0 pg ml^{-1}) at about the day of oestrus. Treatment with inhibin antiserum induced a marked increase in plasma oestradiol concomitant with the growth of a large number of follicles and reached a peak (64.1 ± 5.9 pg ml^{-1}) at about the day of oestrus (Fig. 3).

Passive immunization against inhibin had no effect on plasma progesterone concentrations and treatment with $PGF_{2\alpha}$ at 48 h after administration of inhibin antiserum or normal goat serum resulted in a rapid decrease ($P < 0.01$) in progesterone concentrations in both the immunized and control groups (Fig. 3). However, during the subsequent luteal phase (7 days after ovulation), plasma concentrations of progesterone were significantly higher ($P < 0.05$) in immunized goats compared with control goats (Table 1). There was a significant and positive ($r = 0.9$; $P < 0.01$) correlation between plasma concentrations of progesterone and the number of corpora lutea.

Oestrus and ovarian response

All goats showed signs of oestrus and ovulated. The interval from administration of $PGF_{2\alpha}$ to the onset of oestrus tended to be shorter in passively immunized goats compared with control goats (Table 1). After immunization, there was a significant ($P < 0.01$) increase in the total number of follicles (⩾ 3 mm in diameter) in animals immunized against inhibin compared with the control group. In the group immunized with inhibin antiserum, the total number of follicles increased from a mean of 5.0 ± 0.3 before immunization to a mean maximum of 13.5 ± 0.8. The number of small (< 3.5 mm), medium (3.5–5.0 mm) and large (> 5 mm) follicles at each scan in the two groups is shown (Fig. 4). In the control group, the mean number of small, medium or large follicles did not vary significantly over the time of the experiment. In goats immunized against inhibin, small follicles increased within 24 h and thereafter, the number of medium and large follicles increased. Goats treated with inhibin antiserum had significantly more preovulatory follicles within the limited ovarian space compared with the control group (Fig. 5). In addition, treatment with inhibin antiserum resulted in significantly more ($P < 0.01$) ovulations than in the control group (Table 1).

Discussion

The present study clearly demonstrates that immunoneutralization of endogenous inhibin in cyclic goats results in an increase in plasma concentrations of FSH that, in turn, leads to the stimulation of ovarian follicle development and increased ovulation rate. After treatment with inhibin antiserum, plasma FSH increased and reached the highest concentration within 12 h, but thereafter decreased to control values within 48 h. The preovulatory FSH concentration in the immunized group reached a peak shortly before the control group. This earlier peak in FSH may be attributed to an earlier peak in oestradiol in immunized goats which, in turn, induced GnRH release from the hypothalamus and release of LH and FSH. The decrease in FSH secretion could be attributed to FSH-stimulated oestradiol and inhibin secretion (Wheaton *et al*., 1992; Mann *et al*., 1993). The magnitude and quantitative nature of FSH secretory profiles induced by passive immunization support an endocrine role for inhibin in regulating FSH secretion in goats. Thus, inhibin, through regulation of FSH secretion by negative feedback, appears to be an important factor in regulation of follicular development. An FSH-mediated mechanism effect of inhibin immunization on ovulation rate is in agreement with that reported by O'Shea *et al*. (1994). In goats, a reciprocal relationship between circulating FSH and immunoreactive inhibin concentrations has been reported during the oestrous cycle (Medan *et al*., 2001), indicating that inhibin secreted from the ovary plays an important role as a regulator of FSH secretion. The increase in FSH concentration observed after immunization resulted in a marked stimulation of follicle growth within 24 h. In goats immunized against inhibin,

Table 1. Effect of passive immunization against inhibin on oestrus, ovarian response and plasma progesterone (day 7 after ovulation) in goats

	Control group	Immunized group
Number of treated goats	6	6
Number of goats detected in oestrus[a]	6	6
Number of goats ovulating[b]	6	6
Interval to oestrus[c] (h)	62.0 ± 4.6	50.0 ± 3.4
Ovulation rate[d]	1.8 ± 0.3	4.2 ± 0.5**
Mean maximum number of follicles ⩾3 mm in diameter	5.3 ± 0.8	13.5 ± 0.8**
Plasma progesterone (ng ml^{-1}) on day 7 after ovulation	4.6 ± 0.5	7.38 ± 0.9*

[a]Detected by mature bucks at 6 h intervals within 3 days after $PGF_{2\alpha}$ treatment.
[b]Detected by ultrasonography (large collapsed follicles and formation of corpora lutea at that site).
[c]The time (h) from $PGF_{2\alpha}$ treatment to onset of oestrus.
[d]The number of collapsed follicles or corpora lutea per goat ovulating.
*$P < 0.05$, **$P < 0.01$ significantly different compared with the control value in the same row.

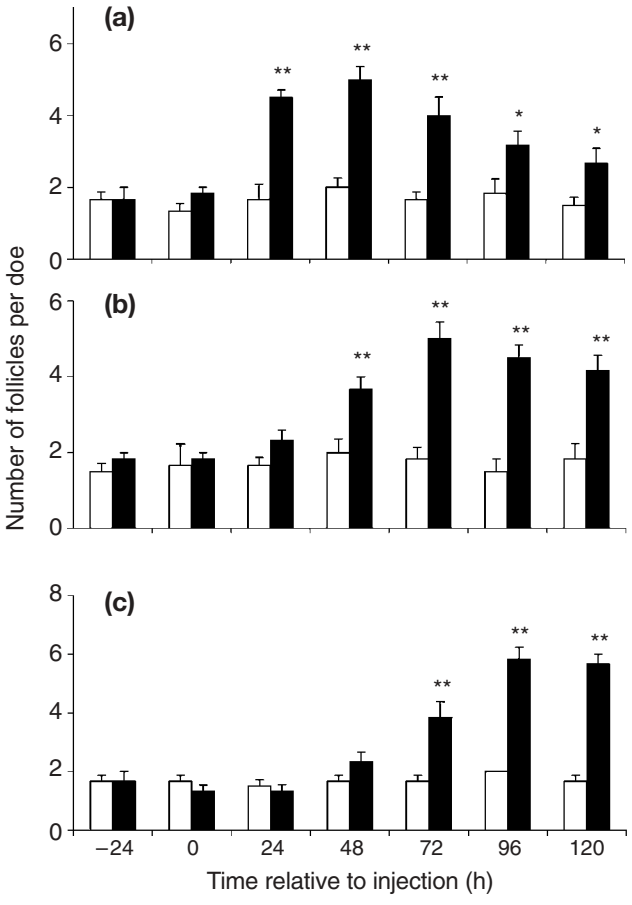

Fig. 4. Number of (a) small (< 3.5 mm in diameter), (b) medium (3.5–5 mm in diameter) and (c) large (> 5 mm in diameter) follicles in goats that received an i.v. bolus injection of 10 ml normal goat serum (□; $n = 6$) or 10 ml inhibin antiserum (■; $n = 6$) on day 10 of the oestrous cycle (0 h) and an i.m. injection of $PGF_{2\alpha}$ at 48 h later. *$P < 0.05$; **$P < 0.01$ compared with control values.

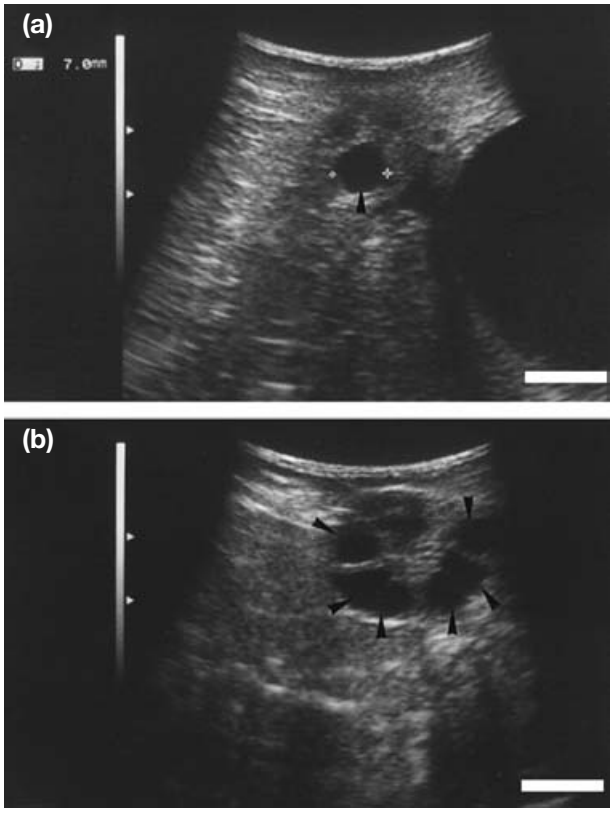

Fig. 5. Representative ultrasonography images of the goat ovary 4 days after treatment with (a) control serum or (b) inhibin antiserum. Note that many follicles are located within the limited ovarian space in the goat that had been passively immunized with inhibin antiserum (arrowheads). Scale bars represent 10 mm.

there was an increase in the number of small follicles at 24 h after treatment with inhibin antiserum. This finding indicates tha t the increase in FSH stimulated the development of a new cohort of small follicles. Similar results were reported in ewes (Mann *et al.*, 1993). The observation that the increase in plasma FSH preceded the emergence of new follicles leads to the conclusion that hypersecretion of FSH stimulates multiple growth of follicles. Presumably, this action, timed to coincide with $PGF_{2\alpha}$ treatment in the present study, enlarges the pool

of follicles that can be recruited, which gives rise to the greater ovulation rate in inhibin antiserum treated goats. From day 3 after injection of inhibin antiserum, the number of large follicles started to increase in association with declining plasma FSH. The results indicate that selection of large follicles occurred in the recruited follicles as a consequence of the decline in FSH secretion. With regards to LH concentrations, there was no marked change in basal LH concentrations between immunized and control goats. However, immunized goats displayed a lower LH surge than control goats. This difference might be explained by the increased oestradiol secreted by the large number of developing follicles in immunized goats. Kishi *et al*. (1996) reported that the preovulatory surge of plasma LH observed on the afternoon of pro-oestrus in cyclic golden hamsters was suppressed in groups treated with inhibin antiserum. In relation to these findings of a lower LH surge in inhibin immunized animals, Wrathall *et al*. (1990) reported that LH release induced by a GnRH challenge was lower in ewes actively immunized against inhibin. The marked increase in oestradiol observed in goats treated with inhibin antiserum, indicates that immunization against inhibin enhances follicular development and, in turn, secretion of oestradiol from the ovarian follicles. Similar results were recorded in cattle (Kaneko *et al*., 1995a; Takedomi *et al*., 1997). The higher progesterone concentration on day 7 after ovulation reflects a greater mass of luteal tissue (increased number of corpora lutea) as detected by ultrasonography.

It is well known that repeated use of eCG to induce superovulation in goats results in diverse effects because of the formation of anti-eCG antibodies (Roy *et al*., 1999). Moreover, a higher incidence of premature regression of corpora lutea in eCG-stimulated goats versus FSH-superovulated goats was recorded (Armstrong *et al*., 1983; Rosnina *et al*., 1992; Riesenberg *et al*., 2001). Therefore, passive immunization against inhibin which selectively increases FSH secretion may be a suitable and simple alternative to eCG for inducing superovulation in goats. However, this protocol requires further investigation using a large number of animals and study of the repeatability and long-term effects of its use.

In conclusion, the present study demonstrated that inhibin is an important factor in the regulation of FSH secretion in goats and that passive immunization against inhibin 48 h before $PGF_{2\alpha}$ treatment induced a marked increase in FSH, oestradiol, ovarian follicle population and ovulation rate. Therefore, the neutralization of inhibin bioactivity may be a potential method for inducing follicular development and increased ovulation rate in goats.

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