

Ovarian and hormonal response of female goats to active immunization against inhibin

M S Medan^{1,2}, G Watanabe^{1,3}, K Sasaki⁴, Y Nagura⁵,
H Sakaime⁵, M Fujita⁵, S Sharawy² and K Taya^{1,3}

¹Laboratory of Veterinary Physiology, Tokyo University of Agriculture and Technology, 3-5-8 Saiwai-cho, Fuchu, Tokyo 183-8509, Japan

²Department of Theriogenology, Faculty of Veterinary Medicine, Suez Canal University, Ismailia, Egypt

³Department of Basic Veterinary Sciences, United Graduate School of Veterinary Sciences, Gifu University, Gifu 501-1193, Japan

⁴Hitachi Ltd, Central Research Laboratory, Tokyo 185-8601, Japan

⁵National Livestock Breeding Centre, Nagano Station, Nagano, Japan

(Requests for offprints should be addressed to K Taya; Email: taya@cc.tuat.ac.jp)

Abstract

This study was conducted to evaluate the effect of active immunization against inhibin on hormonal levels and the ovulation rate in goats. Ten adult Shiba goats (*Capra hircus*) in two groups were used in this study. The first group was injected with inhibin vaccine (immunized, $n=5$) and the second group was injected with Freund's adjuvant (control, $n=5$) followed by three booster injections at 4-week intervals. After the third booster injection, three consecutive periods of oestrus were induced using prostaglandin $F_{2\alpha}$ at intervals of 11 days. Blood samples were collected at 2–6 h intervals and the ovaries were monitored using B-mode ultrasonography. All inhibin-immunized goats generated antibodies that bound ¹²⁵I-labelled bovine inhibin and their FSH concentrations were significantly

higher than corresponding values in the control group. Also, inhibin-immunized goats had significantly higher preovulatory oestradiol-17 β ($P<0.01$) and higher concentrations of progesterone in the luteal phase ($P<0.05$). Immunization of goats against inhibin resulted in a significant ($P<0.01$) increase in ovulation rate (control: 1.7 ± 0.3 vs immunized: 7.6 ± 1.1).

These results demonstrate that active immunization against inhibin enhances ovarian follicular development and ovulation rate by promoting an increase in pituitary FSH secretion. Therefore, immunization against inhibin may be a useful alternative to the conventional approach of superovulation in goats.

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Introduction

In domestic animals, the induction of multiple ovulations is possible by potentiating the stimulatory effects of the endogenous gonadotrophin by administering hormones with follicle-stimulating hormone (FSH)-like activity or by removing the inhibitory action of ovarian hormones on gonadotrophin release by the hypothalamus–pituitary axis. Considerable interest has been placed on inhibin, which by a synergistic action with oestradiol is involved in the negative feedback regulation of FSH at the anterior pituitary (Findlay & Clarke 1987, Findlay *et al.* 1992, Taya 1993, Taya & Watanabe 1999) thereby controlling ovulation rates. Purification and partial characterization of the inhibin molecule revealed that both inhibin forms (A and B) share a common α -subunit, but different β -subunits (Ling *et al.* 1985). Also, the β -subunits were found to exist in dimeric forms (activins), often with opposite biological activity to inhibin (Ling *et al.* 1986, Vale *et al.* 1986). These observations suggest

that the α -subunit, but not the β -subunit, could be suitable as an immunogen to neutralize the activity of both inhibin forms. Domestic and laboratory animals have been immunized against a variety of inhibin preparations, and increases in ovulation rate have been reported in mares (McCue *et al.* 1992), sheep (Mizumachi *et al.* 1990, Wrathall *et al.* 1990, 1992, Wheaton *et al.* 1992, Anderson *et al.* 1998), cattle (Glencross *et al.* 1994), goats (Dietrich *et al.* 1995, Hennies *et al.* 2001) and guinea pigs (Shi *et al.* 2000). Although most studies revealed that passive immunization against inhibin increased FSH levels (Campbell & Scaramuzzi 1995, Kusina *et al.* 1995, Akagi *et al.* 1997, Takedomi *et al.* 1997, Nambo *et al.* 1998, Shi *et al.* 1999), there were conflicting results about the effect of active immunization against inhibin on FSH secretion.

The objective of this study was to determine the effect of active immunization against inhibin on gonadotrophins, oestradiol or progesterone secretion and ovulation rate in goats.

Materials and Methods

Preparation of the immunogen

The immunogen used (inhibin vaccine) was a synthetic peptide, corresponding to the N-terminal sequence (1–26) of the α -subunit of porcine inhibin conjugated to rabbit serum albumin as described previously (Konishi *et al.* 1996). For immunization, 100 μ g synthetic peptide per dose were dissolved in 1 ml PBS and emulsified with an equal volume of Freund's complete adjuvant.

Animals and treatment

Ten adult Shiba goats (*Capra hircus*) were used in this study. The animals were housed under natural daylight and fed 700 g/animal of hay cubes daily. Mineral salt blocks and water were freely available. Oestrous cycles were synchronized with two injections of 125 μ g of a synthetic analogue of prostaglandin $F_{2\alpha}$ (PGF_{2 α}) (Estrumate; Schering Plough Animal Health, NJ, USA) 11 days apart. Oestrus was detected using an aproned mature buck every 6 h. On day 10 of the oestrous cycle, animals were allocated to two groups: the immunized group ($n=5$) treated with s.c. injections of 1 ml inhibin vaccine emulsified in 1 ml Freund's complete adjuvant into four different sites followed by three booster injections at 4-week intervals and the control group ($n=5$) treated with s.c. injections of 1 ml saline emulsified in 1 ml Freund's complete adjuvant. After the third booster injection, oestrous cycles were synchronized by i.m. injection of 125 μ g PGF_{2 α} . Two further PGF_{2 α} injections were given at 11-day intervals to shorten oestrous cycles by inducing luteolysis. 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.

Blood samples for inhibin antibody titres and hormone concentrations

For examination of titres of inhibin antibodies, blood samples were collected by jugular venepuncture once per week. After the third booster immunization, blood samples were collected every 6 h for 2 days before PG-induced luteolysis, every 2 h from 48 to 72 h after PG injection to detect the luteinizing hormone (LH) surge, then every 6 h until the next PG injection. The same regimen of sampling was repeated during the next cycle to determine gonadotrophin level. Oestradiol-17 β and progesterone were determined in daily blood samples. Blood samples were collected into heparinized Vacutainer tubes (Venoject II; Terumo, Tokyo, Japan), centrifuged at 1200 g for 15 min, and plasma was separated and stored at -20°C until assayed for hormones.

Assessment of inhibin antibody titres

Changes in inhibin binding activity in plasma were determined by measuring the binding of ¹²⁵I-labelled inhibin (5000 c.p.m.) as reported previously (Kaneko *et al.* 1993). Plasma samples were diluted 1:10 with PBS containing 5% BSA. PBS (100 μ l) was added to each aliquot (100 μ l) of diluted plasma and incubated for 24 h at 37 $^{\circ}\text{C}$ with ¹²⁵I-labelled bovine 32 kDa inhibin. Bound tracer was then separated by adding 100 μ l PBS containing 1% bovine gamma globulin and 500 μ l PBS containing 25% polyethylene glycol (molecular mass 6000 Da), mixing for 3 min, centrifuging at 1200 g for 30 min at 4 $^{\circ}\text{C}$ and then counting the radioactivity in the precipitate. Inhibin-binding activity was expressed as a percentage of the total counts added.

Ultrasound examination

The ovaries were monitored using a B-mode scanner (ECHOPAL ultrasound scanner; Hitachi Medical Corporation, Tokyo, Japan) equipped with a 7.5 MHz transducer transrectally every 12 h from PG injection until the end of oestrus and on day 7 post-ovulation to count corpora lutea. The occurrence of ovulation was assessed as the disappearance of large antral follicles present at the previous ultrasonography examination confirmed by detection of corpora lutea on day 7 post-ovulation. Ovulation rate was determined by matching the number of large antral follicles that disappeared with the number of corpora lutea detected (Pierson & Ginther 1988).

Hormone analysis

Plasma concentrations of FSH were measured by RIA as described by Araki *et al.* (2000) using anti-ovine FSH, National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK)-FSH-I-1 for radioiodination, and NIDDK-oFSH-RP-1 as a reference standard. Plasma concentrations of LH were measured by RIA as described by Mori & Kano (1984) using anti-ovine LH (YM No. 18), NIDDK-oLH-I-3 for radioiodination, 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 of oestradiol-17 β and progesterone were determined by a double-antibody RIA system using ¹²⁵I-labelled radioligands as described previously (Taya *et al.* 1985). Antisera against oestradiol-17 β (GDN 244) and progesterone (GDN 337) were kindly provided by Dr G D Niswender (Animal Production and Biotechnology, Colorado State University, Fort Collins, CO, USA). The intra- and interassay coefficients of variation were 5.7 and 7.4% for oestradiol-17 β and 8.2 and 9.2% for progesterone respectively.

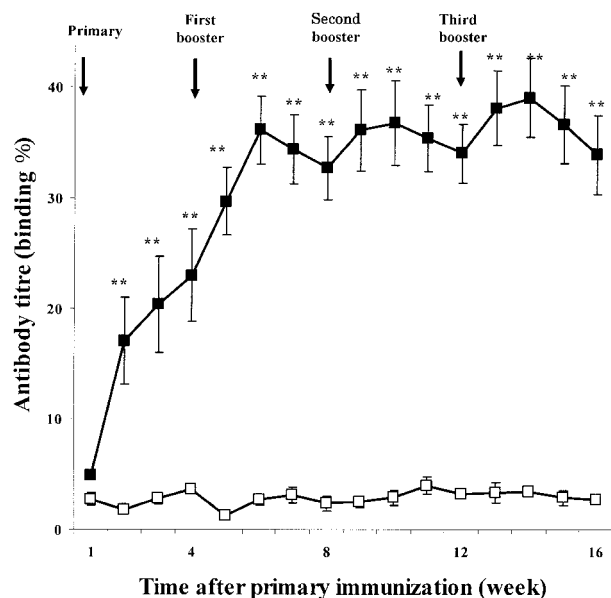


Figure 1 Plasma levels of inhibin binding in goats immunized against inhibin α -subunit (■; $n=5$) or control goats (□; $n=5$) in plasma diluted 1:10. Arrows indicate time of immunization. Values are means \pm S.E.M. Inhibin antibody titres are expressed as the percentage of ^{125}I -labelled bovine 32 kDa inhibin bound at a plasma dilution of 1:10. ** $P<0.01$ compared with corresponding values in controls.

Statistical analysis

Mean values \pm S.E.M. 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 inhibin antibody titres and ovulation rate.

Results

Plasma anti-inhibin titres

The time course of antibody development in immunized animals as determined by binding of ^{125}I -labelled bovine inhibin is shown in Fig. 1. Antibodies that bound ^{125}I -labelled bovine inhibin were produced by all goats receiving the vaccine. Titres rose after primary immunization reaching $36.2 \pm 3.1\%$ at 1:10 dilution within 6 weeks. In contrast, antibody titres in the control group remained low ($<3.0\%$).

Plasma concentrations of FSH and LH

The overall mean basal FSH concentrations for the 30-day period after the third booster immunization (PG-shortened cycles) were significantly higher ($P<0.05$) in

inhibin-immunized goats (9.2 ± 0.2 ng/ml, $n=132$) than in the controls (5.0 ± 0.2 ng/ml, $n=132$). In both groups, PG injection was followed by a preovulatory surge in FSH (immunized: 27.1 ± 2.3 vs control: 19.9 ± 0.8 ng/ml; $P<0.05$) coincident with the LH surge (Fig. 2a). Plasma concentrations of LH did not differ significantly between inhibin-immunized and control goats (Fig. 2b). Overall, mean plasma LH values were 0.9 ± 0.1 ng/ml in the immunized group and 1.1 ± 0.1 ng/ml in the control group. The mean time interval between $\text{PGF}_2\alpha$ injection and the occurrence of the preovulatory LH surge was significantly ($P<0.05$) shorter in the inhibin-immunized group than the control group (53.9 ± 1.5 h in the immunized group and 61.3 ± 1.8 h in the control group).

Oestradiol-17 β and progesterone

Plasma concentrations of oestradiol-17 β rose after PG-induced luteolysis to reach a peak value which was significantly ($P<0.01$) higher in immunized goats (47.8 ± 2.9 pg/ml, $n=15$) than in controls (24.8 ± 1.7 , $n=15$). The concentration then fell and remained relatively low until the next PG injection, with the exception of a smaller peak 4–5 days after oestrus (Fig. 3a). Three to four days after ovulation (ovulation was determined by ultrasonography as sudden disappearance of large follicles) the expected rise in the plasma concentrations of progesterone was observed in all goats, confirming that ovulation had occurred. Progesterone values were significantly ($P<0.05$) higher in immunized compared with respective values in controls (Fig. 3b).

Ovarian activity and ovulation rate

After injection of $\text{PGF}_2\alpha$, all goats exhibited oestrus and ovulated. The interval from $\text{PGF}_2\alpha$ injection to onset of oestrus was significantly ($P<0.05$) shorter in the immunized group (46.8 ± 1.8 and 54.4 ± 2.5 h in actively immunized and control goats respectively). Ovulation rates were recorded over three PG-shortened consecutive oestrous cycles after the third booster immunization against inhibin. There was around a 4-fold increase in ovulation rate in goats actively immunized against inhibin (Fig. 4). The mean ovulation rate was 1.7 ± 0.3 and 7.6 ± 1.1 in control and immunized groups respectively. There was a positive correlation ($r=0.9$, $P<0.001$) between inhibin antibody titre and ovulation rate. Figure 5 shows ultrasound images of ovaries of immunized goats containing many preovulatory follicles during the follicular phase and many corpora lutea during the luteal phase in the limited ovarian space, confirming multiple ovulation compared with control goats.

Discussion

In the present study, we have clearly demonstrated the efficacy of immunization against inhibin α -subunit in

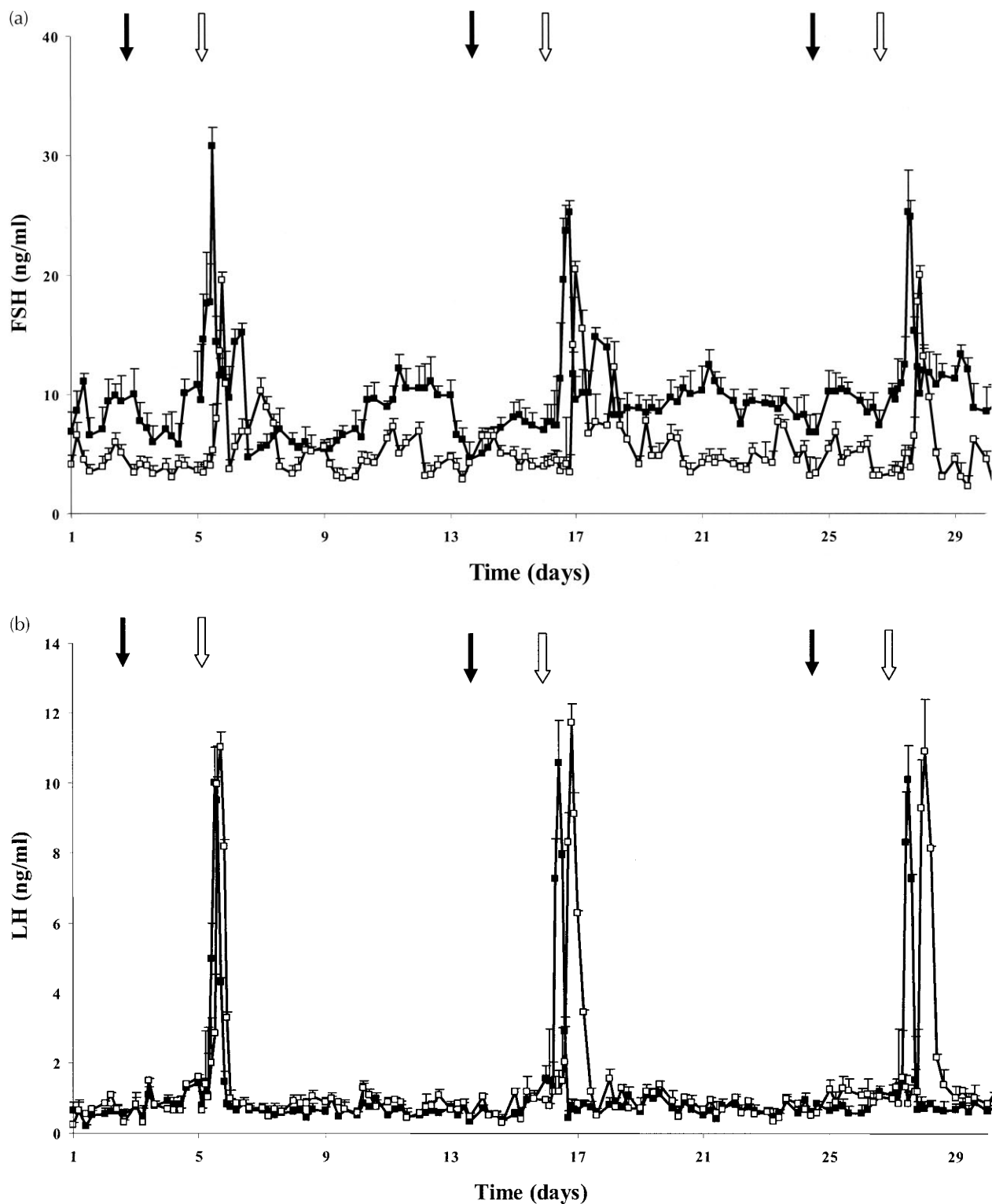


Figure 2 Plasma concentrations of FSH (a) and LH (b) in inhibin-immunized (■; $n=5$) and control goats (□; $n=5$) during 30 days encompassing three consecutive periods of oestrus. Black arrows indicate PG injection and white arrows indicate oestrus. Values are means \pm S.E.M.

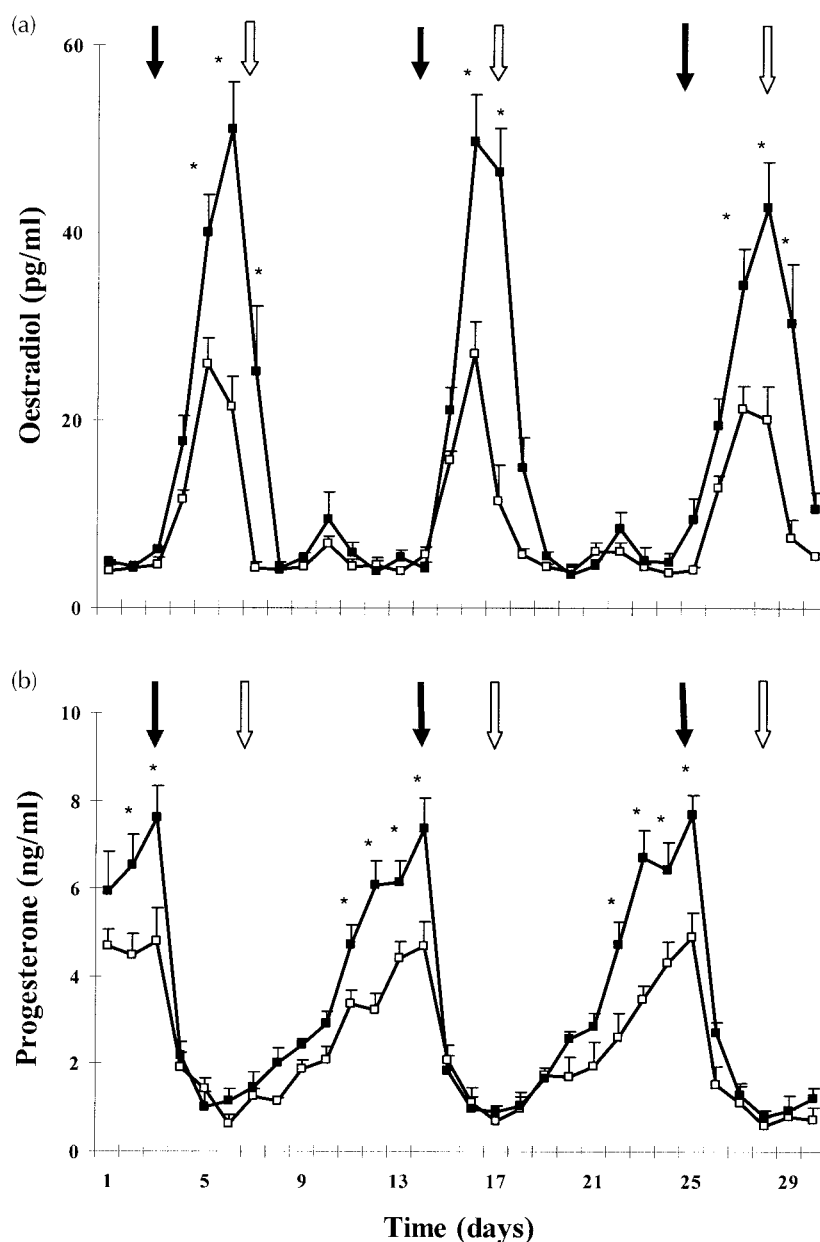


Figure 3 Plasma concentrations of oestradiol-17 β (a) and progesterone (b) in inhibin-immunized (■; $n=5$) and control goats (□; $n=5$) during 30 days encompassing three consecutive periods of oestrus. Black arrows indicate PG injection and white arrows indicate oestrus. Values are means \pm S.E.M. * $P<0.05$ compared with the control values.

increasing the ovulation rate in goats. We have also shown that superovulation is associated with elevated plasma concentrations of FSH. Immunization against inhibin stimulated an immune response and all immunized goats generated antibodies that bound ^{125}I -labelled inhibin. Antibody binding was significant, confirming that the increased ovulation rate after immunization with synthetic peptides based on the inhibin α -subunit was due to

immunoneutralization of endogenous inhibin. Mean plasma concentrations of FSH were significantly higher in immunized animals compared with control animals throughout 30 days (during PG-shortened oestrous cycles). This provides further evidence that immunization against inhibin increased plasma concentrations of FSH and then increased ovulation rate. Increased FSH levels in the immunized group were in agreement with those reported

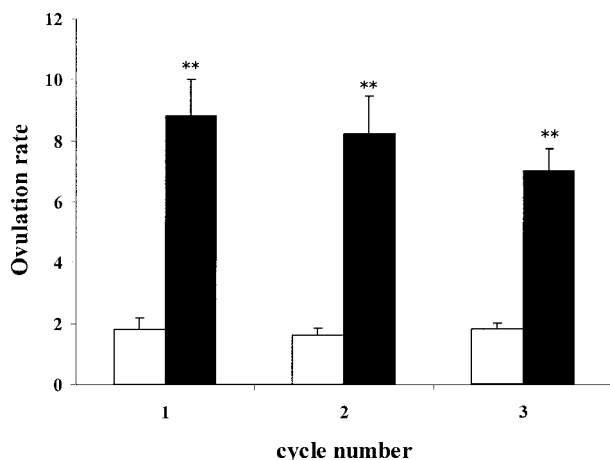


Figure 4 Ovulation rate in inhibin-immunized (solid bar; $n=5$) and control goats (open bar; $n=5$) during three consecutive PG-shortened oestrous cycles after the third booster immunization. Values are means \pm S.E.M. ** $P<0.01$.

in ewes (Mizumachi *et al.* 1990, Wrathall *et al.* 1990, 1992, Wheaton *et al.* 1992, Russell *et al.* 1994, Tannetta *et al.* 1997) and disagree with Hennies *et al.* (2001), who reported an increase in ovulation rate in inhibin-immunized goats without any change in FSH level. Immunoneutralization of endogenous inhibin was thought to result in diminished negative feedback on the anterior pituitary gland resulting in increased FSH secretion, subsequently increased follicular development and finally increased ovulation rate. In the present study, an approximately 4-fold increase in ovulation rate was observed following the third booster immunization. Similar findings were recorded in ewes (Anderson *et al.* 1998). In contrast to FSH, there was no marked change in the LH profile between the immunized and the control goats. The lack of an effect on LH secretion recorded in inhibin-immunized goats contrasts with the findings of Hennies *et al.* (2001) who reported that LH levels were reduced in inhibin-immunized goats. However, in the present study,

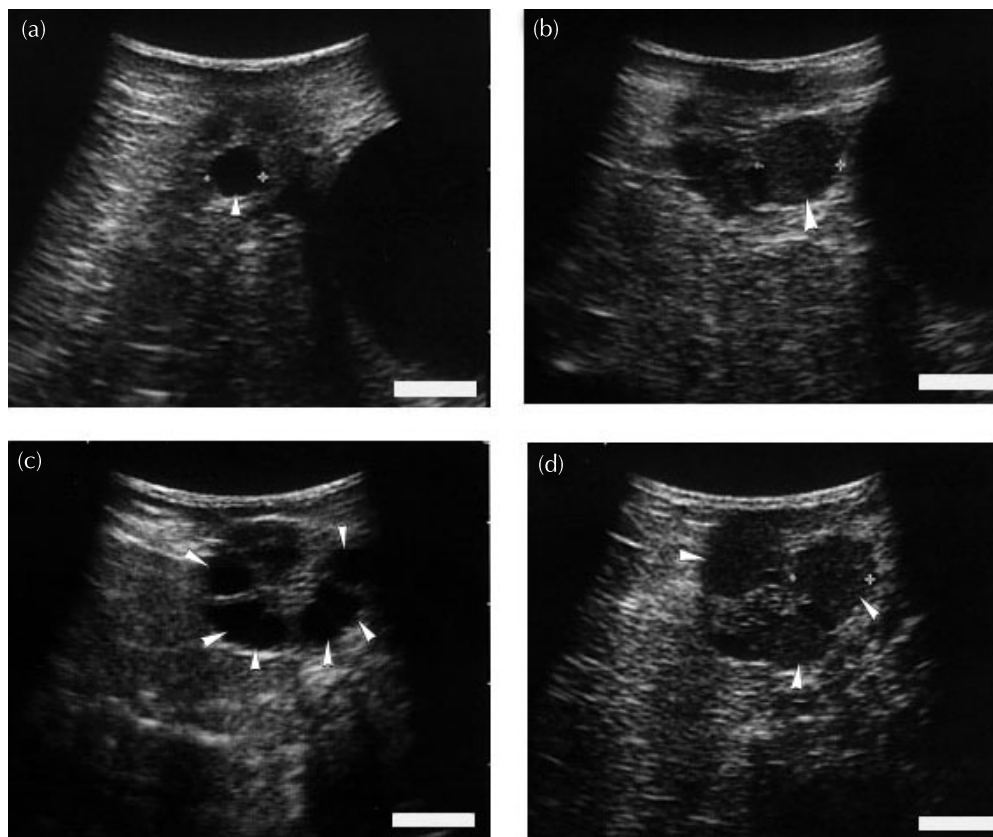


Figure 5 Ultrasound images of ovaries of goats produced by using a transrectal 7.5 MHz transducer and B-mode scanner. (a) The arrowhead indicates an antral follicle. (b) The arrowhead indicates a corpus luteum. (c) The arrowheads indicate multiple antral follicles. (d) The arrowheads indicate multiple corpora lutea. Note that there are many antral follicles and corpora lutea in inhibin-immunized goats (c and d) compared with control goats (a and b). Scale bar represents 10 mm.

immunized goats displayed an earlier preovulatory LH surge than control goats. This might be attributed to the increased amounts of oestrogen secreted by the large number of developing follicles in immunized goats (Campbell & Scaramuzzi 1995). Similar results were recorded in ewes immunized against inhibin (Anderson *et al.* 1998). Plasma concentrations of oestradiol rose immediately after PG injection in all goats. The peak level of plasma concentrations of oestradiol-17 β in the inhibin-immunized group was significantly higher than controls. This is similar to previous results recorded in goats (Hennies *et al.* 2001). The increase in plasma concentrations of oestradiol-17 β is probably due to an increased number of oestrogenic follicles destined to ovulate. The progesterone levels in inhibin-immunized goats were significantly higher than in controls. The higher progesterone levels most likely reflect the increased ovulation rate and increased corpora lutea numbers in immunized goats.

In conclusion, the present study demonstrates that active immunization against the inhibin α -subunit increases ovarian activity and ovulation rate, which is associated with elevated FSH secretion. Therefore, immunization of goats against inhibin could be used to generate increases in litter size or increased numbers of oocytes and/or embryos for multiple-ovulation embryo-transfer programmes.

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