# Research

# Superovulation, fertilization and *in vitro* embryo development in mice after administration of an inhibin-neutralizing antiserum

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**The present study was conducted to investigate the effect of immunoneutralization against endogenous inhibin on oocyte and embryo production in adult and immature mice. At 12:00 h on day 2 of oestrus (day 1 of dioestrus), a single i.p. injection of inhibin antiserum (50, 100, 200 or 400 μl per animal) or equine chorionic gonadotrophin (eCG; 10 or 20 iu per animal) or control goat serum (100 μl per animal) was administered to adult female mice. After 48 h, the mice in each of the three groups were given a single i.p. injection of hCG (10 iu per animal). At 42 h after hCG injection, ova were collected from oviducts and cultured in KSOM solution. Treatments with both inhibin antiserum–hCG and eCG–hCG induced superovulation in all the animals tested. The number of oocytes in animals treated with inhibin antiserum was significantly higher (*P* < 0.05) compared with the control group, and the number of oocytes ovulated in animals treated with 200 or 400 μl inhibin antiserum was significantly (*P* < 0.05) higher than that in animals treated with 10 or 20 iu eCG. The superovulated oocytes that were fertilized normally *in vivo* were able to form blastocysts *in vitro*. The rate of blastocyst development for animals treated with 50–200 μl inhibin antiserum was significantly (*P* < 0.05) higher than that of the eCG-treated animals. Irrespective of the day of the oestrous cycle, 200 μl inhibin antiserum administered at 12:00 h on each of 4 days induced superovulation in all the animals tested. The rates of oocyte and embryo production by these animals were significantly (*P* < 0.05) higher than in the control groups. Furthermore, administration of inhibin antiserum at doses of 50, 100, 200 or 400 μl produced similar results in 26-day-old immature mice. These results indicate that passive immunoneutralization of endogenous inhibin α-subunit induces superovulation in immature and adult mice. The superovulated oocytes obtained by administration of inhibin antiserum have normal embryonic developmental competence. Thus, it is concluded that this inhibin antiserum method is a new practical alternative for induction of superovulation in mice instead of the more commonly used eCG–hCG protocol.**

## Introduction

Advanced reproductive techniques enable greater numbers of oocytes or embryos of high quality to be used to conduct much needed research into basic reproduction. Mice are used widely in the laboratory, especially to produce transgenic animals and also in embryo assay quality control tests for human *in vitro* fertilization (IVF) programmes (Scott *et al.*, 1993). In most studies, the use of a combination of equine chorionic gonadotrophin (eCG) and hCG has been the most common method to induce superovulation in mice. This method yields a relatively large number of oocytes and embryos. However, a disadvantage of these protocols is the long half-life of eCG, which interferes with normal fertilization and embryo development (Fraser, 1977; Sato and Marrs, 1986; Edgar *et al.*, 1987; Lehtonen and

Kankondi, 1987; Fossum *et al.*, 1989; Ertzeid *et al.*, 1993). It is increasingly accepted that oocyte and embryo quality may be affected by hyperstimulation with exogenous gonadotrophins (Legge and Sellens, 1994; Ma *et al.*, 1997). Thus, it is necessary to establish an alternative simple method for induction of superovulation in mice to overcome these problems.

Inhibin is an essential hormone in the regulation of FSH secretion in various mammals (de Jong, 1988). In previous studies, a negative relationship between plasma concentrations of FSH and inhibin has been established in several mammalian species (Taya, 1993; Taya and Watanabe, 1999). FSH is one of the important endocrine hormones in the regulation of ovarian folliculogenesis. Multiple ovulations have been induced successfully by passive immunization against endogenous inhibin in several species such as hamsters (Kishi *et al.*, 1996), rats (Rivier and Vale, 1989), guinea-pigs (Shi *et al.*, 2000), cows (Akagi *et al.*, 1997) and mares (Nambo *et al.*, 1998). Thus, these

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initial results indicated 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 for the production of valuable embryos for use in IVF.

The aim of the present study was to develop a practical superovulation protocol in immature and adult mice by immunoneutralization of endogenous inhibin. Furthermore, *in vitro* development of preimplantation embryos was evaluated.

## Materials and Methods

### Animals

Immature (26-day-old) and adult (3-month-old) female mice of the ddY strain (Sankyo Experimental Animal Supply Co., Tokyo) were used. The mice were housed in metal cages and maintained in a room with controlled illumination (14 h light : 10 h dark, lights on at 05:00 h) and temperature (22–24°C), with free access to commercial pellets and tap water available *ad libitum*. The mice were checked daily for 4 day oestrous cyclicity by examination of vaginal smears. The day of ovulation, as judged by the presence of cornified cells in the vaginal smear, was designated day 1 of the oestrous cycle. All procedures were carried out in accordance with the guidelines established by the Department of Veterinary Medicine, Tokyo University of Agriculture and Technology, for use of laboratory animals.

### Preparation of inhibin α-subunit antiserum

The inhibin α-subunit antiserum was obtained from a castrated goat immunized against [Tyr30]-porcine inhibin-α(1–30)-NH$_2$ conjugated to rabbit serum albumin (kindly provided by N. Ling, Neurocrine Biosciences Inc., San Diego, CA). The conjugate (3.6 mg) was dissolved in 1 ml saline and mixed with an equal volume of Freund's complete adjuvant. A castrated goat was given 2 ml of the suspension (including 3.6 mg of the conjugate) s.c. at each immunization. First, second and third immunizations were performed at 2 week intervals and, thereafter, monthly. Blood samples were obtained 2 weeks after each injection. The sera were collected and examined for inhibin antiserum titre, and titres of the antisera were checked in the following way. The antisera were diluted with 0.05 mol PBS l$^{-1}$ (pH 7.4) containing 1% (w/v) BSA, and the diluted samples were incubated with 5000 c.p.m. [$^{125}$I]-labelled bovine 32 kDa inhibin (325 Ci mmol$^{-1}$) at 4°C for 24 h in a total volume of 200 µl. Bound radioligands were separated by adding 100 µl of 1% (w/v) bovine gamma globulin in PBS and 500 µl of 25% (w/v) polyethylene glycol in PBS, and agitating the mixture for 3 min. After centrifugation at 1700 *g* at 4°C for 30 min, the radioactivity of the precipitate was counted in a gamma counter. The serum used in the present experiment had a titre of 1 : 1 × 10$^6$ as defined by final dilution of the antiserum required to bind 50% of added [$^{125}$I]-labelled bovine 32 kDa inhibin. The *in vivo*

efficiency of the antiserum was ensured by an increase in plasma concentrations of FSH after an i.v. injection of six doses (6.25–200.00 µl) of the antiserum at 11:00 h on day 1 and day 2 of dioestrus. A dose-related increase in the basal secretion of FSH was observed at 24 h after the injection, and the maximum response was noted when 100 µl of the antiserum was injected. The capacity of the antiserum to neutralize the inhibin bioactivity of rat inhibin (ovarian homogenate) was also examined *in vitro* using a dispersed anterior pituitary cell bioassay system. The secretion of FSH from cultured rat anterior pituitary cells was suppressed in a dose-dependent manner by rat ovarian homogenate, and the maximum suppression of the ovarian homogenate could be reversed by addition of increasing dosages of the antiserum. Human transforming growth factor β (TGF-β) and activin showed no crossreactivity with the inhibin antiserum. Control serum was obtained from a castrated goat immunized against BSA.

### Experiment 1: effect of different doses of inhibin antiserum administered on day 2 of the oestrous cycle on oocyte production, fertilization and embryo development

Female adult mice were primed at 12:00 h on day 2 of the oestrous cycle (day 1 of dioestrus) with a single i.p. injection of different doses of inhibin antiserum (50, 100, 200 or 400 µl per animal) or eCG (10 or 20 iu per animal) (Sankyo Zoki Co. Ltd, Tokyo) or control goat serum (100 µl per animal). After 48 h (day 4 of the oestrous cycle), the mice were given an injection of hCG (10 iu per animal) (Sankyo Zoki). The individual female mice were mated with a 3-month-old fertile male mouse of the ddY strain immediately after hCG administration. At 42 h after the hCG injection (day 2 after mating), five animals from each group were killed by decapitation and the oviducts were incised and separated for collection of embryos.

### Experiment 2: effect of a single injection of 200 µl inhibin antiserum administered on each of 4 days of the oestrous cycle on oocyte production, fertilization and embryo development

A dose of 200 µl inhibin antiserum administered at 12:00 h on day 2 of the oestrous cycle was found to be the most effective dose for induction of superovulation. Accordingly, in subsequent experiments, the dose of 200 µl inhibin antiserum was used to induce (super)ovulation in mice. Injections of inhibin antiserum (treated group) or 100 µl goat serum (control group) were administered i.p. at 12:00 h on each day of the 4 day oestrous cycle. The rest of the protocol was similar to that used in Expt 1.

### Experiment 3: effect of different doses of inhibin antiserum on oocyte production, fertilization and embryo development in immature mice

Female mice aged 26 days were treated using the same protocol as that used in Expt 1. Doses ranging from 50 µl to

**Table 1.** Effect of inhibin antiserum or equine chorionic gonadotrophin (eCG), administered i.p. to adult mice on day 2 of the oestrous cycle (day 1 of dioestrus), on the number of ovulating mice and the number of ovulated oocytes per animal

| Treatment | Number of mice that ovulated | Number of ovulated oocytes (range) |
|---|---|---|
| Control serum | 5/5 | $18.0 \pm 0.8$ (15–20) |
| 50 μl inhibin antiserum | 5/5 | $44.2 \pm 7.1$ (26–66)* |
| 100 μl inhibin antiserum | 5/5 | $59.0 \pm 4.9$ (48–77)*** |
| 200 μl inhibin antiserum | 5/5 | $73.0 \pm 7.4$ (60–94)***[a] |
| 400 μl inhibin antiserum | 5/5 | $63.2 \pm 4.9$ (52–80)***[a] |
| 10 iu eCG | 5/5 | $37.4 \pm 3.1$ (31–47)*** |
| 20 iu eCG | 5/5 | $41.6 \pm 3.4$ (30–49)*** |

Values are mean $\pm$ SEM ($n = 5$).
*$P < 0.05$ and ***$P < 0.005$ versus control group that received goat serum.
[a]$P < 0.05$ versus eCG-treated groups.

400 μl inhibin antiserum (treated group) were used to induce superovulation, whereas the animals in the control group received 100 μl goat serum.

*Embryo collection and culture* in vitro

At 06:00 h on day 2 after mating, fertilized (two-cell stage embryos) oocytes and also unfertilized and degenerated oocytes were harvested by flushing the excised oviducts with embryo culture medium–KSOM solution (containing NaCl (5.55 g $l^{-1}$), KCl (0.185 g $l^{-1}$), glucose (1 g $l^{-1}$), $KH_2PO_4$ (0.047 g $l^{-1}$), $MgSO_4$ (0.024 g $l^{-1}$), lactate (1.88 ml), pyruvate (0.022 g $l^{-1}$), $NaHCO_3$ (2.1 g $l^{-1}$), $CaCl_2 \cdot 2H_2O$ (0.25 g $l^{-1}$), L-glutamine (0.146 g $l^{-1}$), EDTA (0.0029 g $l^{-1}$), penicillin–streptomycin (0.075 and 0.050 g $l^{-1}$, respectively)) with 0.5% (w/v) BSA (Fraction V; Sigma Co., St Louis, MO) (Ho *et al.*, 1995; Summers *et al.*, 1995). The numbers of fertilized and unfertilized oocytes were recorded to evaluate the rates of oocyte production and fertilization. The two-cell stage embryos were harvested and cultured at 37°C in fresh KSOM solution in 5% $CO_2$ in air, 95% relative humidity for 54 h (96 h after hCG administration). The hatched blastocysts were counted at the end of the culture period.

*Statistical analysis*

Data are presented as mean $\pm$ SEM. Significance of differences between two means was tested by Student's *t* test if the variance was uniform or by Cochran–Cox test if the variance was not uniform. The level of significance was $P < 0.05$.

**Results**

*Experiment 1: effect of different doses of inhibin antiserum administered on day 2 of the oestrous cycle on oocyte production, fertilization and embryo development in cyclic mice*

All the mice tested in each experimental group were superovulated (Table 1). Administration of various doses of inhibin antiserum (50–400 μl per animal) or eCG (10 or

20 iu per animal) at 12:00 h on day 2 of the oestrous cycle significantly ($P < 0.05$) increased the number of ovulations compared with that in the control group treated with goat serum (Table 1). The most effective dose to induce superovulation was 200 μl inhibin antiserum. The oocyte production in mice treated with 200 μl or 400 μl inhibin antiserum was also significantly ($P < 0.05$) higher than that in the two groups of eCG-treated mice.

The number of two-cell stage embryos and the rate of fertilization for each treatment group are shown (Table 2). The early embryos were harvested by flushing the excised oviducts with KSOM solution at day 2 after mating. The production of two-cell stage embryos in the groups treated with either inhibin antiserum or eCG was significantly ($P < 0.05$) higher than that of the control group (Table 2). The number of two-cell stage in the group treated with 200 μl inhibin antiserum was significantly ($P < 0.005$) higher than in either eCG-treated group. However, the rate of fertilization was not different between the inhibin antiserum- or eCG-treated groups and the control group (Table 2).

The effects of inhibin antiserum and eCG on development of hatched blastocysts are shown (Table 2). The number of hatched blastocysts for the groups treated with either inhibin antiserum or eCG was significantly ($P < 0.05$) higher than in the control group (Table 2). Furthermore, there was no difference in the rate of blastocyst development between groups treated with 50, 100 or 200 μl inhibin antiserum and the control group. The rates of blastocyst development in the groups treated with 50, 100 or 200 μl inhibin antiserum were significantly ($P < 0.05$) higher than those in both of the eCG-treated groups (10 or 20 iu) (Table 2).

*Experiment 2: effect of a single injection of 200 μl inhibin antiserum administered on each of 4 days of the oestrous cycle on oocyte production, fertilization and embryo development*

When mice were treated with 200 μl inhibin antiserum on each day of the oestrous cycle, all the animals were

**Table 2.** Effect of inhibin antiserum or equine chorionic gonadotrophin (eCG), administered i.p. on day 2 of the oestrous cycle (day 1 of dioestrus), on production of fertilized oocytes (two-cell stage embryos), rate of fertilization *in vivo*, production of hatched blastocysts and rate of blastocyst development in adult mice

| Treatment | Number of fertilized oocytes (range) | Fertilization rate (%) | Number of hatched blastocysts (range) | Rate of blastocyst development (%) |
|---|---|---|---|---|
| Control serum | 15.2 ± 0.7 (13–17) | 84.6 ± 1.6 | 13.8 ± 0.5 (12–15) | 90.9 ± 1.3 |
| 50 µl inhibin antiserum | 35.2 ± 5.2 (21–47)* | 80.2 ± 2.8 | 30.6 ± 4.7 (19–42)* | 86.9 ± 1.5 [b] |
| 100 µl inhibin antiserum | 49.6 ± 4.8 (37–63)*** | 83.9 ± 3.7 | 42.6 ± 3.6 (33–54)***[a] | 86.4 ± 1.7[a] |
| 200 µl inhibin antiserum | 60.2 ± 3.7 (52–72)***[b] | 83.9 ± 4.2 | 53.0 ± 4.3 (41–67)***[b] | 87.7 ± 2.4[a] |
| 400 µl inhibin antiserum | 48.4 ± 6.2 (29–62)** | 75.6 ± 5.8 | 39.2 ± 5.8 (21–52)* | 80.0 ± 2.4*** |
| 10 iu eCG | 29.0 ± 1.4 (25–32)*** | 78.6 ± 3.9 | 21.2 ± 1.0 (19–24)*** | 73.2 ± 2.2*** |
| 20 iu eCG | 32.8 ± 2.6 (26–41)*** | 79.3 ± 6.6 | 22.0 ± 2.1 (17–27)** | 67.2 ± 4.1** |

Values are mean ± SEM ($n = 5$).
*$P < 0.05$, **$P < 0.01$ and ***$P < 0.005$ versus control group that received goat serum.
[a]$P < 0.05$ and [b]$P < 0.005$ versus eCG-treated groups.

**Table 3.** Effect of 200 µl inhibin antiserum, administered i.p. into adult mice on each day of a 4 day oestrous cycle, on the number of ovulating mice and the number of ovulated oocytes per animal

| Stage of oestrous cycle | Treatment | Number of mice that ovulated | Number of ovulated oocytes (range) |
|---|---|---|---|
| Day 1 of oestrous cycle (oestrus) | Control serum | 5/5 | 13.8 ± 2.2 (8–20) |
| | 200 µl inhibin antiserum | 5/5 | 63.4 ± 5.4 (49–78)*** |
| Day 2 of oestrous cycle (day 1 of dioestrus) | Control serum | 5/5 | 18.0 ± 0.8 (15–20) |
| | 200 µl inhibin antiserum | 5/5 | 73.0 ± 7.4 (60–94)*** |
| Day 3 of oestrous cycle (day 2 of dioestrus) | Control serum | 5/5 | 14.2 ± 0.6 (13–16) |
| | 200 µl inhibin antiserum | 5/5 | 66.2 ± 8.2 (41–91)*** |
| Day 4 of oestrous cycle (pro-oestrus) | Control serum | 4/5 | 12.0 ± 2.0 (0–16) |
| | 200 µl inhibin antiserum | 5/5 | 48.4 ± 7.0 (33–74)* |

Values are mean ± SEM ($n = 5$).
*$P < 0.05$ and ***$P < 0.005$ versus corresponding control group that received goat serum.

superovulated (Table 3). The rates of oocyte and embryo production were significantly ($P < 0.05$) higher in the inhibin antiserum-treated animals than in the control animals (Tables 3 and 4). However, the rates of oocyte and embryo production were not different among groups treated with inhibin antiserum at 12:00 h on different days of the oestrous cycle.

*Experiment 3: effect of different doses of inhibin antiserum on oocyte production, fertilization and embryo development in immature mice*

All the animals tested in each experimental group were superovulated (Table 5). Administration of various doses of inhibin antiserum (50–400 µl per animal) significantly ($P < 0.01$) increased oocyte production compared with that in the control group (Table 5). The number of two-cell stage embryos in the inhibin antiserum-treated animals was significantly ($P < 0.005$) higher than that for the control group (Table 5). However, the rate of fertilization was not different between the inhibin antiserum-treated groups and the control group (Table 6). Owing to significant differences in

production of two-cell stage embryos between the inhibin antiserum-treated groups and the control group, the number of hatched blastocysts for each of the inhibin antiserum-treated groups was significantly ($P < 0.01$) higher than that for the control group (Table 6). There was no difference in rates of blastocyst development among different treatments (Table 6).

**Discussion**

Many studies involving superovulation have been conducted in mice (Christenson and Eleftheriou, 1972; Spindle and Goldstein, 1975; Cosby *et al.*, 1989; Garcia *et al.*, 1993; Ziebe *et al.*, 1993; Munoz *et al.*, 1995). In these studies, an eCG–hCG protocol has been used because this combination can produce a relatively large number of ovulations. However, preimplantation embryo development and successful implantation of superovulated oocytes induced by eCG–hCG appear to be interfered with by high concentrations of oestradiol, probably because of long-lasting high circulating concentrations of eCG (Fraser,

**Table 4.** Effect of 200 μl inhibin antiserum, administered i.p. into adult mice on each day of a 4 day oestrous cycle, on production of fertilized oocytes (two-cell stage embryos), rate of fertilization *in vivo*, production of hatched blastocysts and rate of blastocyst development

| Stage of oestrous cycle | Treatment | Number of fertilized oocytes (range) | Fertilization rate (%) | Number of hatched blastocysts (range) | Rate of blastocyst development (%) |
|---|---|---|---|---|---|
| Day 1 of oestrous cycle (oestrus ) | Control serum | 11.8 ± 2.2 (6–18) | 83.8 ± 0.3 | 10.2 ± 2.0 (5–16) | 85.9 ± 1.1 |
| | 200 μl inhibin antiserum | 53.6 ± 5.0 (37–64)*** | 84.4 ± 3.2 | 44.2 ± 5.9 (26–59)*** | 81.3 ± 4.3 |
| Day 2 of oestrous cycle (day 1 of dioestrus) | Control serum | 15.2 ± 0.7 (13–17) | 84.6 ± 1.6 | 13.8 ± 0.5 (12–15) | 90.9 ± 1.3 |
| | 200 μl inhibin antiserum | 60.2 ± 3.7 (52–72)*** | 83.9 ± 4.2 | 53.0 ± 4.3 (41–67)*** | 87.7 ± 2.4 |
| Day 3 of oestrous cycle (day 2 of dioestrus) | Control serum | 12.4 ± 0.2 (12–13) | 87.6 ± 0.2 | 10.2 ± 0.4 (9–11) | 82.2 ± 1.8 |
| | 200 μl inhibin antiserum | 54.6 ± 8.0 (31–74)** | 81.6 ± 8.9 | 45.2 ± 7.9 (21–61)* | 81.1 ± 4.1 |
| Day 4 of oestrous cycle (pro-oestrus) | Control serum | 10.5 ± 1.8 (6–14) | 87.0 ± 4.6 | 9.3 ± 1.7 (5–12) | 87.6 ± 1.9 |
| | 200 μl inhibin antiserum | 43.0 ± 6.7 (30–68)* | 88.5 ± 3.2 | 37.6 ± 6.0 (26–60)* | 87.2 ± 0.1 |

Values are mean ± SEM (*n* = 5).
*P < 0.05, **P < 0.01 and ***P < 0.005 versus corresponding control group that received goat serum.

**Table 5.** Effect of inhibin antiserum, administered i.p. into 26-day-old immature mice, on the number of ovulating mice and the number of ovulated oocytes per animal

| Treatment | Number of mice that ovulated | Number of ovulated oocytes (range) |
|---|---|---|
| Control serum | 5/5 | 14.4 ± 1.1 (11–17) |
| 50 μl inhibin antiserum | 5/5 | 67.8 ± 8.2 (44–89)*** |
| 100 μl inhibin antiserum | 5/5 | 87.4 ± 10.9 (72–101)*** |
| 200 μl inhibin antiserum | 5/5 | 102.8 ± 9.8 (81–137)*** |
| 400 μl inhibin antiserum | 5/5 | 67.4 ± 11.2 (47–109)** |

Values are mean ± SEM (*n* = 5).
**P < 0.01 and ***P < 0.005 versus control group that received goat serum.

**Table 6.** Effect of inhibin antiserum, administered i.p. into 26-day-old immature mice, on production of fertilized oocytes (two-cell stage embryos), rate of fertilization *in vivo*, production of hatched blastocysts and rate of blastocyst development

| Treatment | Number of fertilized oocytes (range) | Fertilization rate (%) | Number of hatched blastocysts (range) | Rate of blastocyst development (%) |
|---|---|---|---|---|
| Control serum | 11.8 ± 1.0 (9–14) | 81.8 ± 1.6 | 10.2 ± 0.7 (8–12) | 86.8 ± 1.1 |
| 50 μl inhibin antiserum | 55.0 ± 6.3 (36–71)*** | 81.4 ± 0.8 | 47.4 ± 5.2 (32–61)*** | 86.4 ± 1.1 |
| 100 μl inhibin antiserum | 72.8 ± 4.1 (59–84)*** | 83.3 ± 0.6 | 63.2 ± 4.0 (52–74)*** | 86.7 ± 1.7 |
| 200 μl inhibin antiserum | 86.0 ± 7.5 (68–112)*** | 83.9 ± 1.0 | 73.4 ± 5.3 (60–91)*** | 85.7 ± 1.2 |
| 400 μl inhibin antiserum | 51.6 ± 7.3 (36–78)*** | 77.6 ± 2.0 | 43.2 ± 6.3 (30–66)** | 83.5 ± 0.7 |

Values are mean ± SEM (*n* = 5).
**P < 0.01 and ***P < 0.005 versus control group that received goat serum.

1977; Sato and Marrs, 1986; Edgar *et al.*, 1987; Lehtonen and Kankondi, 1987; Fossum *et al.*, 1989; Ertzeid *et al.*, 1993; Legge and Sellens, 1994; Ma *et al.*, 1997). This possibility led us to consider an alternative method that could be used to induce superovulation and to obtain a population of oocytes with high rates of successful fertilization and embryo development. Thus, the aim of the present study was to develop a new superovulation protocol in mice using immunoneutralization of endogenous inhibin. This is the first report to show that passive immunization against the inhibin α-subunit increases the number of oocytes and rate of blastocyst production in both immature and adult mice.

The mice that received 50–400 μl inhibin antiserum on day 1 of dioestrus (day 2 of oestrus) were superovulated. The production of oocytes by the mice treated with the inhibin antiserum protocol increased markedly in a dose-dependent manner. Fifty microlitres of inhibin antiserum was sufficient to induce superovulation, whereas 200 μl inhibin antiserum was the most effective dose. Inhibin antiserum did not have any adverse effects on embryo quality as assessed by morphological and developmental

competence in mice. The superovulated oocytes could be fertilized normally *in vivo*, leading to the development of blastocysts in the same chronological progression as observed *in vitro* after normal ovulation of oocytes. Furthermore, as immature mice are used widely to obtain mature oocytes or embryos, similar experiments were conducted using 26-day-old mice. The data obtained with immature mice in the present study were comparable, in terms of both differences among doses and among different treatment groups, to those of adult mice. Many previous studies have shown that FSH plays a major role in the regulation of ovarian folliculogenesis. In several mammalian species, inhibin is an important negative feedback regulatory factor in determining the circulating concentration of FSH (Taya, 1993; Taya and Watanabe, 1999). Suppression of negative feedback of inhibin upon the pituitary gonadotrophs increases FSH secretion (Ireland *et al*, 1983; Quirk and Fortune, 1986; Beard *et al.*, 1990). In previous studies, it has been reported that passive immunization against inhibin increased the circulating FSH concentrations in rats (Rivier and Vale, 1989; Arai *et al.*, 1996), hamsters (Kishi *et al.*, 1996), guinea-pigs (Shi *et al.*, 1999), cows (Taya *et al.*, 1991; Kaneko *et al.*, 1993), ewes (Wheaton *et al.*, 1992; Wrathall *et al.*, 1992) and mares (Nambo *et al.*, 1998). However, the results of the present study do not provide direct evidence as to whether the improvement in oocyte production observed in mice immunized against inhibin α-subunit is due to increases in plasma FSH concentrations. Anti-inhibin IgG is able to enter the follicular fluid of antral follicles after passive immunization, indicating that inhibin antibodies play a local role affecting follicular development (Shi *et al.*, 2000). As plasma FSH concentrations were not measured in the present study, further studies are needed to elucidate the mechanism by which immunoneutralization of endogenous inhibin increases oocyte production in both immature and adult mice.

The results of the present study demonstrate that 26-day-old immature mice appear to be more responsive to inhibin antiserum than do adult mice. All the doses of inhibin antiserum tested stimulated a relatively higher number of follicles to develop *in vivo* and subsequently to be ovulated in immature mice compared with adult mice. This finding indicates that immature mice aged at least 26 days have acquired a functional negative feedback control of pituitary FSH secretion by ovarian inhibin. A different responsiveness observed between cyclic and immature animals in response to the inhibin antiserum may be explained, at least in part, by differences in the concentrations of other negative regulatory factors of gonadotrophin secretion, such as oestradiol. In addition, Spearow *et al.* (1999) reported that several genes, such as Oriq, determine the ovulation rate. The expression of these genes is related to ovarian responsiveness to the gonadotrophins. Therefore, it is possible that the expression of these genes varies during development from the immature to the adult stage. However, it has not yet been determined whether the inhibin antiserum–hCG protocol is effective in other strains

of mice that are known to be poor responders to eCG–hCG treatment.

In the present study, eCG–hCG treatment increased oocyte and embryo production in adult mice of the ddY strain. The fertilization of oocytes appeared to be normal; however, the rate of blastocyst formation *in vitro* of the oocytes obtained from eCG–hCG-treated animals was significantly decreased compared with that of mice undergoing normal ovulation and mice undergoing superovulation after treatment with 50–200 μl inhibin antiserum. Furthermore, as the dose of eCG increased, the blastocyst developmental competence of the eggs tended to be affected more markedly. The results of the present study confirm that a high dose of eCG tends to increase the number of abnormal oocytes and embryos, as has been reported in many previous studies (Fraser, 1977; Sato and Marrs, 1986; Edgar *et al.*, 1987; Lehtonen and Kankondi, 1987; Fossum *et al.*, 1989; Ertzeid *et al.*, 1993). Therefore, use of the inhibin antiserum method for induction of superovulation in mice appears to be more advantageous in terms of both oocyte and embryo production and quality.

All the mice treated with 200 μl inhibin antiserum on each day of the oestrous cycle were superovulated. In mice with 4 day oestrous cycles, an LH surge occurs in the afternoon of pro-oestrus (day 4 of the oestrous cycle) and subsequent ovulation takes place in the early morning of oestrus (day 1) (Parkening *et al.*, 1982). When the inhibin antiserum protocol was performed chronologically as described earlier, higher numbers of oocytes and embryos were obtained. When 200 μl inhibin antiserum was administered to ddY mice on days 1, 3 or 4 of oestrus (day of oestrus, day 2 of dioestrus or day of pro-oestrus, respectively), the rates of oocyte and embryo production were decreased compared with mice that received inhibin antiserum on day 2 of oestrus (day 1 of dioestrus). However, the ovulation rate for these mice was still significantly higher than that of the control mice. Kishi *et al.* (1996) reported that inhibin antiserum administered on day 1, 2 or 3 of the oestrous cycle (oestrus, day 1 or 2 of dioestrus) increased the rate of ovulation in hamsters significantly. Superovulation could also be induced by administration of exogenous gonadotrophin on random days of the oestrous cycle in mice (Fowler and Edwards, 1957; Edwards and Fowler, 1960; Redina *et al.*, 1994). Thus, it seems that the inhibin antiserum–hCG protocol works in line with other protocols that are commonly used for the induction of superovulation, but that the inhibin antiserum protocol yields superior results in mice. Slight differences in oocyte and embryo production in response to administration of inhibin antiserum on different days of the oestrous cycle may be due to a different follicular population in the ovary at different stages of the cycle.

In conclusion, passive immunization against endogenous inhibin α-subunit induces superovulation in immature and adult mice. The superovulated oocytes have normal embryonic developmental competence *in vitro*. As it is relatively easy to obtain inhibin antiserum in large volumes

at a low cost through immunization of goats against inhibin α-subunit, the inhibin antiserum–hCG method is an ideal alternative for induction of superovulation in mice compared with the more commonly used eCG–hCG protocol.

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