Induction of Superovulation by Immunoneutralization of Endogenous Inhibin in Immature Rats

Harumichi ISHIGAME1,2), Mohamed S MEDAN1,3), Maiko KAWAGUCHI4), Atsushi FUKUDA1), Gen WATANABE1,5), Koji Y ARAI2) and Kazuyoshi TAYA1,5)

1)Laboratory of Veterinary Physiology, and 2)Department of Tissue Physiology, Faculty of Agriculture, Tokyo University of Agriculture and Technology, Fuchu, Tokyo 183–8509, Japan, 3)Department of Theriogenology, Faculty of Veterinary Medicine, Suez Canal University, Ismailia, Egypt, 4)Department of Toxicology and Pharmacology, Faculty of Pharmacy, Musashino University, Tokyo 202–8585, Japan, 5)Department of Basic Veterinary Science, The United Graduate School of Veterinary Sciences, Gifu University, Gifu 501–1193, Japan

Abstract. The effects of passive immunoneutralization of endogenous inhibin on ovulation rate in immature rats were investigated. Efficiency of superovulation on production of fertilized oocytes was compared between the inhibin antiserum (inhibin-AS) and equine chorionic gonadotropin (eCG) protocols. Immature female Wistar strain rats were superovulated with a single injection of 100–200 µl inhibin-AS, with and without an injection of human chorionic gonadotropin (hCG). A total of 77.8% of the 26–30-day-old rats treated with a single injection of 100–200 µl inhibin-AS ovulated 72 h after treatment, while rats given normal goat serum (NGS; 200 µl) did not ovulate. At 28 days of age, all of the inhibin-AS treated rats ovulated when additional hCG treatment was given, whereas the number of ovulated oocytes was not affected. The number of ovulated oocytes in the inhibin-AS-hCG treated groups was significantly higher than that of the NGS-hCG treated group. Plasma concentrations of FSH in the inhibin-AS-hCG treated group significantly increased compared with the NGS treated group. While the percentage of mated rats in the 200 µl inhibin-AS-hCG treated group was significantly lower than that of the 15 IU eCG-hCG treated group, the fertilization rate was comparable between the two groups. The number of fertilized oocytes in the 200 µl inhibin-AS-hCG treated group was significantly higher in comparison with the 15 IU eCG-hCG treated group. These results suggest that immunoneutralization of endogenous inhibin could be a reliable method for induction of superovulation to collect a large number of normally fertilized oocytes in immature rats.

Key words: Immature rat, Immunoneutralization, Inhibin, Superovulation (J. Reprod. Dev. 51: 559–566, 2005)
been reported that high doses of eCG may have a detrimental effect on oocyte quality, such as impaired fertilization [5] and preimplantation development [6–8]. These adverse effects are thought to be a result of excessive follicular stimulation because of the inherent long biological half-life of eCG.

Another approach to induce superovulation is the administration of a purified preparation of FSH. It is reported that FSH treatment induced a higher number of ovulated oocytes than eCG protocol in several animals, including immature rats [9–12]. However, one of the disadvantages of this treatment is that FSH has a short half-life, which requires repeated injections in 12 h intervals or constant infusion via subcutaneously implanted osmotic minipumps. Therefore, using FSH for induction of superovulation is more complicated and expensive.

Inhibin is a heterodimeric glycoprotein hormone that selectively suppresses FSH secretion from the pituitary gland [13, 14]. A negative relationship between plasma concentration of FSH and inhibin has been established [14]. Successful multiple ovulations have been observed by passive immunization against endogenous inhibin in several species [15–23]. Recently, we have reported that immunization against inhibin induced a higher rate of ovulation compared to the eCG-hCG protocol in adult rats and both adult and immature mice [19, 22, 23]. These studies also showed that inhibin immunization protocol could produce large numbers of normally developed oocytes.

The aim of this study was to examine the efficiency of immunoneutralization of endogenous inhibin in immature rats, in terms of ovulation rate and the competence of fertilization, to be used in the reproductive-developmental research.

Materials and Methods

Animals

Immature female Wistar strain rats (26–30 days of age), which generally first ovulate at about 35 days of age, were used. They were weaned at 21 days of age and kept in a room with controlled lighting (14 h light : 10 h dark, lights on 0500 h) and temperature (25 ± 2 C), and free access to food and water ad libitum. All procedures were carried out in accordance with the guidelines established by the Tokyo University of Agriculture and Technology.

Inhibin antiserum (inhibin-AS)

The inhibin α-subunit antiserum was obtained from castrated goats immunized against [Tyr30]-inhibin α (1–30)-NH2 conjugated to rabbit serum albumin. This conjugate was kindly provided by Dr. N. Ling (Neuroendocrine Biosciences, Inc., San Diego, CA, U. S. A.). The titer of the antiserum was determined as outlined in our previous reports [24]. The serum used in the present experiment had a titer of 1:1 000 000 as defined by the final dilution of the antiserum required to bind 50% of added 125I-labeled bovine 32-kDa inhibin. The in vivo efficiency of the antiserum was ensured by an increase in plasma concentration of FSH after an i.v. injection of the antiserum, as described previously [25].

Effects of treatment with inhibin-AS injected at 26–30 days of age on induction of ovulation and the number of oocytes

Immature female rats, 26–30 days of age, were injected at 0900 h with inhibin-AS (100–200 µl per animal) into the jugular vein under light ether anesthesia. Control animals were administered a normal goat serum (NGS; 200 µl per animal). After 56 h (at 1700 h on day 2 after injection), some rats injected with inhibin-AS or NGS were given an injection of 10 IU hCG (Sankyo Zoki Co. Ltd., Tokyo, Japan). The oviducts were recovered 72 h after treatment and the recovered oocytes were counted under a dissecting microscope.

Effect of treatment with inhibin-AS on the concentration of FSH

Female immature rats, 28 days of age, were administered a single i.v. injection of 200 µl of inhibin-AS or NGS at 0900 h into the jugular vein under light ether anesthesia. Inhibin-AS and NGS treated rats were decapitated every 6 and 12 h after treatment, respectively, and trunk blood samples were collected into heparinized centrifuge tubes. After 56 h, only rats injected with inhibin-AS were given an injection of 10 IU hCG. Blood samples were centrifuged immediately at 1700 g for 30 min at 4 C. Plasma was separated and stored at −20 C until assayed for FSH.
**Effects of treatment with inhibin-AS and eCG on production of fertilized oocytes**

When female immature rats were 28 days of age, they were administered a single i.v. injection of 200 µl inhibin-AS or an i.p. injection of 15 IU eCG (Sigma Chemical Co., St. Louis, MO, U. S. A.) at 0900 h. After 56 h, animals were given an injection of 10 IU hCG, and the individual female rats were mated with a fertile adult male rat of the same strain. The next day (on day 0 of pregnancy), rats were examined for the presence of a vaginal plug or spermatozoa in the vagina. Between 1500 and 1700 h on day 0 of pregnancy, the oviducts were removed and flushed with saline. The recovered oocytes were counted under a dissecting microscope and scored for the occurrence of fertilization by judging pronucleus formation and the presence of the second polar body.

**Radioimmunoassay of FSH**

The plasma concentration of FSH was measured using an NIDDK RIA kit (Bethesda, MD, U. S. A.) for rat FSH. Iodinated preparations were rat FSH-I-5. The antisera used were anti-rat FSH-S-11. Results were expressed as rat FSH RP-2. The intra- and inter-assay coefficients of variations were 4.8% and 11.4%, respectively.

**Statistics**

Mean values (± SEM) were calculated and analysed using one-way ANOVA. Duncan’s multiple range test was used for detection of significant differences using the SAS computer package [26]. The chi-square test was used to assess if there was any differences in the percentage of ovulated rats, the mating rate, and the fertilization rate. A probability of P<0.05 was considered to be significant.

**Results**

**Effects of treatment with inhibin-AS injected at 26–30 days of age on induction of ovulation and the number of oocytes**

The percentage of ovulated rats and the number of ovulated oocytes after treatment with inhibin-AS are shown in Table 1. The groups injected only with NGS did not ovulate. The percentage of ovulated rats after treatment with hCG was gradually increased starting at 28 days of age in the rats given NGS. A total of 77.8% (28/36) of the 26–30-day-old rats treated with a single injection of 100–200 µl inhibin-AS were ovulated 72 h after treatment. The percentage of rats that ovulated was not statistically different regardless of the amount of inhibin-AS given and regardless of the

<table>
<thead>
<tr>
<th>Days of age</th>
<th>Group</th>
<th>No. of rats ovulated/ No. of rats examined (%)</th>
<th>No. of oocytes recovered (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>26</td>
<td>NGS 200</td>
<td>0/5 (0.0)</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>NGS 200-hCG</td>
<td>0/5 (0.0)</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Inhibin-AS 100</td>
<td>3/4 (75.0)</td>
<td>15.0 ± 3.1 (9–19)</td>
</tr>
<tr>
<td></td>
<td>Inhibin-AS 200</td>
<td>4/7 (57.1)</td>
<td>96.3 ± 5.5 (83–110)</td>
</tr>
<tr>
<td></td>
<td>Inhibin-AS 100-hCG</td>
<td>7/7 (100)</td>
<td>57.9 ± 11.5 (15–107)</td>
</tr>
<tr>
<td></td>
<td>Inhibin-AS 200-hCG</td>
<td>8/8 (100)</td>
<td>51.4 ± 13.2 (12–105)</td>
</tr>
<tr>
<td>28</td>
<td>NGS 200</td>
<td>0/5 (0.0)</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>NGS 200-hCG</td>
<td>3/5 (60.0)</td>
<td>11.7 ± 1.2 (10–14)</td>
</tr>
<tr>
<td></td>
<td>Inhibin-AS 100</td>
<td>5/6 (83.3)</td>
<td>85.3 ± 2.7 (69–72)</td>
</tr>
<tr>
<td></td>
<td>Inhibin-AS 200</td>
<td>4/6 (66.7)</td>
<td>73.0 ± 7.6 (52–88)</td>
</tr>
<tr>
<td></td>
<td>Inhibin-AS 100-hCG</td>
<td>5/5 (100)</td>
<td>69.8 ± 9.1 (43–90)</td>
</tr>
<tr>
<td></td>
<td>Inhibin-AS 200-hCG</td>
<td>7/7 (100)</td>
<td>81.1 ± 7.8 (46–102)</td>
</tr>
<tr>
<td>30</td>
<td>NGS 200</td>
<td>0/5 (0.0)</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>NGS 200-hCG</td>
<td>4/5 (80.0)</td>
<td>10.3 ± 2.6 (3–14)</td>
</tr>
<tr>
<td></td>
<td>Inhibin-AS 100</td>
<td>6/7 (85.7)</td>
<td>61.5 ± 6.2 (33–71)</td>
</tr>
<tr>
<td></td>
<td>Inhibin-AS 200</td>
<td>6/6 (100)</td>
<td>90.5 ± 14.5 (54–143)</td>
</tr>
</tbody>
</table>

Values are means ± SEM.

abcValues with different superscripts within the same age group are significantly different (P<0.05, Duncan’s multiple range test).
age at which inhibin-AS was injected. All of the inhibin-AS treated rats ovulated when additional hCG was injected.

A single injection of both amounts of inhibin-AS induced superovulation (ranged from 15.0 ± 3.1 to 96.3 ± 5.5) in 26–30-day-old rats. In 26-day-old rats, a high variability among individuals was observed in the groups given inhibin-AS-hCG. In contrast, the variability was relatively low in 28-day-old rats injected with inhibin-AS-hCG when compared to the 26-day-old rats. The difference in the number of ovulated oocytes between inhibin-AS-hCG and NGS-hCG treated groups was statistically significant. Administration of hCG did not increase the number of ovulated oocytes in the inhibin-AS treated 28-day-old rats.

Although most of the 30-day-old rats were induced to superovulate by a single injection of inhibin-AS, degenerated cumulus-free oocytes were obtained in one animal of each group 72 h later (33 oocytes for 100 µl inhibin-AS and 57 oocytes for 200 µl inhibin-AS). Because they were at the age just before the first ovulation, it seemed to be difficult to distinguish spontaneous first ovulation from inhibin-AS induced first ovulation. Therefore, we conducted the following experiments with a combined treatment of inhibin-AS and hCG in 28-day-old rats.

Changes in plasma levels of FSH in inhibin immunized and NGS treated groups

Plasma levels of inhibin-AS gradually decreased after injection of inhibin-AS (Fig. 1a). Plasma levels of FSH significantly increased within 6 h after administration of inhibin-AS when compared with those of the NGS treated group (Fig. 1b). The levels of FSH remained high throughout the experiment.

Effects of treatment with inhibin-AS and eCG on the production of fertilized oocytes

The mating rate in the inhibin-AS-hCG treated group (25.0%) was markedly lower than that of the eCG-hCG treated group (65.2%). However, the number of oocytes recovered (78.3 ± 6.2 vs. 46.0 ± 4.4) and the number of fertilized oocytes (64.8 ± 4.3 vs. 41.7 ± 4.1) in the inhibin-AS-hCG treated group were significantly higher when compared to the eCG-hCG treated group. There was no significant difference between groups in fertilization rate (87.6 ± 5.9 vs. 91.1 ± 2.6).

Discussion

In this study, the results clearly show that passive immunization against endogenous inhibin in immature rats increased plasma concentrations of FSH and induced superovulation. These results indicate that endogenous inhibin is a primary
factor in the regulation of species-specific ovulation rate, mainly through the control of FSH secretion as described previously in many species [14]. The results of the present study also confirm the previous findings that endogenous inhibin already plays a physiological role in the regulation of FSH secretion in late-prepubertal female rats. A similar observation was also reported that injection of inhibin-AS increased the ovulation rate in immature rats through induction of elevated plasma FSH levels [27]. It has also been reported that involvement of inhibin in modulating FSH secretion starts at 20 days of age in female rats [28, 29].

A single injection of inhibin-AS could successfully induce superovulation 72 h after treatment in most of the 26–30-day-old immature rats, as previously observed in adult rats and hamsters [16, 22], however, a few rats did not ovulate. Additional treatment with hCG increased the percentage of rats that ovulated, but not the number of ovulated oocytes in 26- and 28-day-old rats. Therefore, treatment with a combination of inhibin-AS and hCG is more effective to collect a large number of oocytes in immature rats. In an earlier study, administration of inhibin-AS in late-prepubertal rats did not influence the timing of first ovulation [27]. This finding was not in agreement with our results that advanced first ovulation was observed in most of the immature rats after a single injection of inhibin-AS. It is well known that first ovulation takes place when the hypothalamic-pituitary-ovarian axis becomes fully mature with age, and estradiol-17β, produced mainly by antral follicles, is one of the critical factors regulating first ovulation in immature rats. When the ovary becomes capable of producing estradiol-17β levels of sufficient magnitude, and for a sufficiently long period of time, the first preovulatory LH surge and the first ovulation will occur. Because production of estradiol-17β is largely dependant on the number of mature antral follicles [30], one possibility to account for this discrepancy is the period of elevated FSH levels after treatment with inhibin-AS. The present study showed that plasma concentrations of FSH remained high even 72 h after inhibin-AS treatment in 28-day-old rats, while in a previous study, FSH levels declined to normal levels 48 h after treatment [27]. Thus, the present results suggest that sustained high levels of endogenous FSH induced by inhibin-AS stimulate follicular development and result in production of large amounts of estradiol-17β that are sufficient to induce the advanced preovulatory LH surge by a positive feed back effect to the hypothalamus and pituitary axis, leading to induction of superovulation.

A single injection of eCG can also induce first ovulation in immature rats. The release of endogenous LH occurs 56–59 h following a low dose eCG (3 IU) injection [31]. However, it is reported that injection of a superovulatory dose of eCG (40 IU) in pre-pubertal rats induced the first ovulation as early as 24 h after treatment [5]. These effects may be attributed to the long half-life and the predominant LH-like activity of eCG in triggering advanced ovulation. Differences and contradictions concerning the influence of first ovulation may be caused by different strains, ages, doses, kinds, and qualities of hormone preparation used for the experiments. Further studies to examine the development of the hypothalamus-pituitary-ovarian axis will be necessary to evaluate.

### Table 2. Effects of inhibin-AS or eCG on production of fertilized oocytes

<table>
<thead>
<tr>
<th></th>
<th>eCG 15 IU</th>
<th>Inhibin-AS 200</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of rats mated / No. of rats examined (%)</td>
<td>15/23 (65.2)</td>
<td>6/24(25.0)*</td>
</tr>
<tr>
<td>Total number of oocytes (n)</td>
<td>690 (15)</td>
<td>454 (6)</td>
</tr>
<tr>
<td>Mean number of oocytes (range; n)</td>
<td>46.0 ± 4.4 (29–80; 15)</td>
<td>78.3 ± 6.2 (62–99; 6)*</td>
</tr>
<tr>
<td>Total number of fertilized oocytes (n)</td>
<td>625 (15)</td>
<td>389 (6)</td>
</tr>
<tr>
<td>Mean number of fertilized oocytes (range; n)</td>
<td>41.7 ± 4.1 (20–75; 15)</td>
<td>64.8 ± 4.3 (52–82; 6)*</td>
</tr>
<tr>
<td>Mean fertilization rate (%)</td>
<td>91.1 ± 2.6</td>
<td>87.6 ± 5.9</td>
</tr>
</tbody>
</table>

Values are means ± SEM.
* Significantly different from the eCG treated group (P<0.05, chi-square test or student’s t-test).
this point.

Sander et al. reported that the maximum ovulation rate was observed when inhibin-AS was injected within the last 2–3 days before the first ovulation [27]. In agreement with this, it was also reported that inhibin-AS treatment was more effective in increasing ovulation rate in 5-day-cyclic adult rats when injected on the second day of diestrus, rather than on the first [32]. In the present study, the follicles capable of ovulating in response to hCG were not present in 26-day-old rats treated with NGS and these increased gradually with age. Accordingly, considerable variation in individuals was observed after inhibin-AS treatment in 26-day-old rats compared to that of the 28–30-day-old rats. The present results suggest that the effect of inhibin-AS treatment is much dependent on the pre-antral follicular population in the ovary at the time of the FSH increase, as observed previously [27, 32].

The results of present study also demonstrated that an equal number of oocytes collected from immature rats treated with inhibin-AS-hCG could be fertilized as oocytes retrieved from the eCG-hCG treated rats. In addition, these rats provided a higher number of fertilized oocytes compared to the eCG-hCG treated rats. Although we did not examine the developmental ability of oocytes superovulated with inhibin-AS, previous studies demonstrated that oocytes superovulated with immunization against inhibin had normal developmental competence in adult rats, mice, and guinea pigs [19, 22, 23, 33]. These results suggest that this is also applicable in the case of immature rats. Thus, the inhibin-AS-hCG protocol can be alternatively used to induce superovulation in immature rats. On the other hand, the mating rate was significantly lower in the inhibin-AS-hCG treated immature rats when compared with the eCG-hCG injected rats. This was not in agreement with our previous results that a normal mating rate was observed in adult rats and mice given inhibin-AS [19, 22, 23]. We do not know the reason why inhibin-AS treated immature rats did not mate as equally as the eCG treated rats, however, the application of assisted reproductive technologies such as in vitro fertilization could be helpful to obtain fertilized oocytes using the inhibin-AS-hCG protocol in immature rats. Further studies to measure circulating estradiol-17β in inhibin-AS or eCG treated rats will be necessary to evaluate the difference in mating rates between the two groups.

In conclusion, the present study demonstrates that passive immunization against endogenous inhibin α-subunit can induce superovulation, and that the ovulated oocytes have normal fertilization ability. Therefore, immunization of immature rats against inhibin could be used to induce superovulation to collect large numbers of normally fertilized oocytes for reproductive and developmental research.

Acknowledgements

We are grateful to Dr. A. F. Parlow and the Rat Pituitary Hormone Distribution Program (NIDDK, NIH, Bethesda, MD, U. S. A.) for providing RIA materials and to Dr. N. Ling (Neuroendocrine Inc., San Diego, CA, U. S. A.) for providing [Tyr^{30}]-inhibin α (1–30). This work was supported by JSPS Research Fellowship for Young Scientists (14.20179), a Grant-in-Aid for Scientific Research (No. 033338) from the Japan Society for the Promotion of Science, and a Grant-in-Aid for Scientific Research (The 21st Century Center of Excellence Program, E-1) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

References


30. Kishi H, Taya K, Watanabe G, Sasamoto S. Follicular dynamics and secretion of inhibin and

