Endocrine, *vol. 24, no. 2, 115–119, July 2004* 0969–711X/04/24:115–119/$25.00

Immunization Against Endogenous Inhibin Increases Normal Oocyte/Embryo Production in Adult Mice

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The objectives of the present study were to induce superovulation in adult mice by immunoneutralization of endogenous inhibin and to investigate embryo development in vivo and in vitro. Adult female mice of the ddY strain at 3 mo of age were superovulated with a single ip injection of inhibin antiserum (inhibin-AS; 50, 100, or 200 µL) at 12:00 h on metestrus of the 4-d estrous cycle. The control group was treated with 100 µL normal goat serum (control serum). Five mice were sacrificed every 6 h and blood samples were collected for hormonal assay. Individual female mice were bred in the afternoon of proestrus with individual fertile males. Embryos were collected at 08.00 h on d 2 postcoitus and cultured in KSOM solution. Treatment with inhibin-AS significantly increased the concentrations of plasma FSH. Furthermore, there was a significant elevation in plasma concentrations of estradiol-17β and progesterone in the inhibin-immunized groups in comparison with controls. The superovulated oocytes that were fertilized normally in vivo were able to develop normally to blastocysts in vitro. The number of oocytes and blastocysts developed in animals treated with inhibin-AS was significantly higher than that for mice treated with control serum. Moreover, the rate of fertilization and the rate of blastocyst development were similar in inhibin-AS treated and control groups. These results indicate that immunoneutralization of endogenous inhibin induces superovulation in adult mice without additional treatment with human chorionic gonadotropin (hCG). In addition, the superovulated oocytes obtained by administration of inhibin-AS have normal embryonic developmental competence.

Received April 12, 2004; Revised May 25, 2004; Accepted June 7, 2004.

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Key Words: Mice; inhibin antiserum; superovulation; embryo development.

Introduction

Mice are commonly used animal models in reproductive and developmental research and a large number of ova must be made available. The common method for induction of superovulation is treatment with a combination of equine chorionic gonadotropin (eCG) and human chorionic gonadotropin (hCG). However, several studies have reported high rates of pre- and postimplantation losses in animals superovulated with eCG *(1–3)* in addition to a reduced response to repeated injections of gonadotropins, which may be attributed to an immunologically mediated ovarian refractoriness *(4–6)*.

Inhibin is an essential hormone regulating FSH secretion in various mammals *(7)*. A negative relationship between plasma concentration of FSH and inhibin has been established *(8–13)*, and superovulations have been induced successfully by passive immunization against endogenous inhibin in several species including mice *(14)*, rats *(15,16)*, hamsters *(17)*, cows *(18,19)*, mares *(20)*, and goats *(21)*. Furthermore, several studies *(14,16,19,22,23)* have shown that oocytes superovulated with immunization against inhibin have the ability to develop normally. Although we succeeded in inducing superovulation in mice by using inhibin-AS and hCG, further studies are needed to investigate the ability of inhibin-AS to induce superovulation without administration of hCG. The present study aims to induce superovulation in adult cyclic mice by using a single injection of inhibin-AS without additional treatment with hCG and to investigate embryonic developmental competence.

Results

Effect of Inhibin–AS Administered IP into Adult Mice on Metestrus on Hormonal Profile

Following the injection of control serum, plasma concentrations of FSH did not significantly change. However,

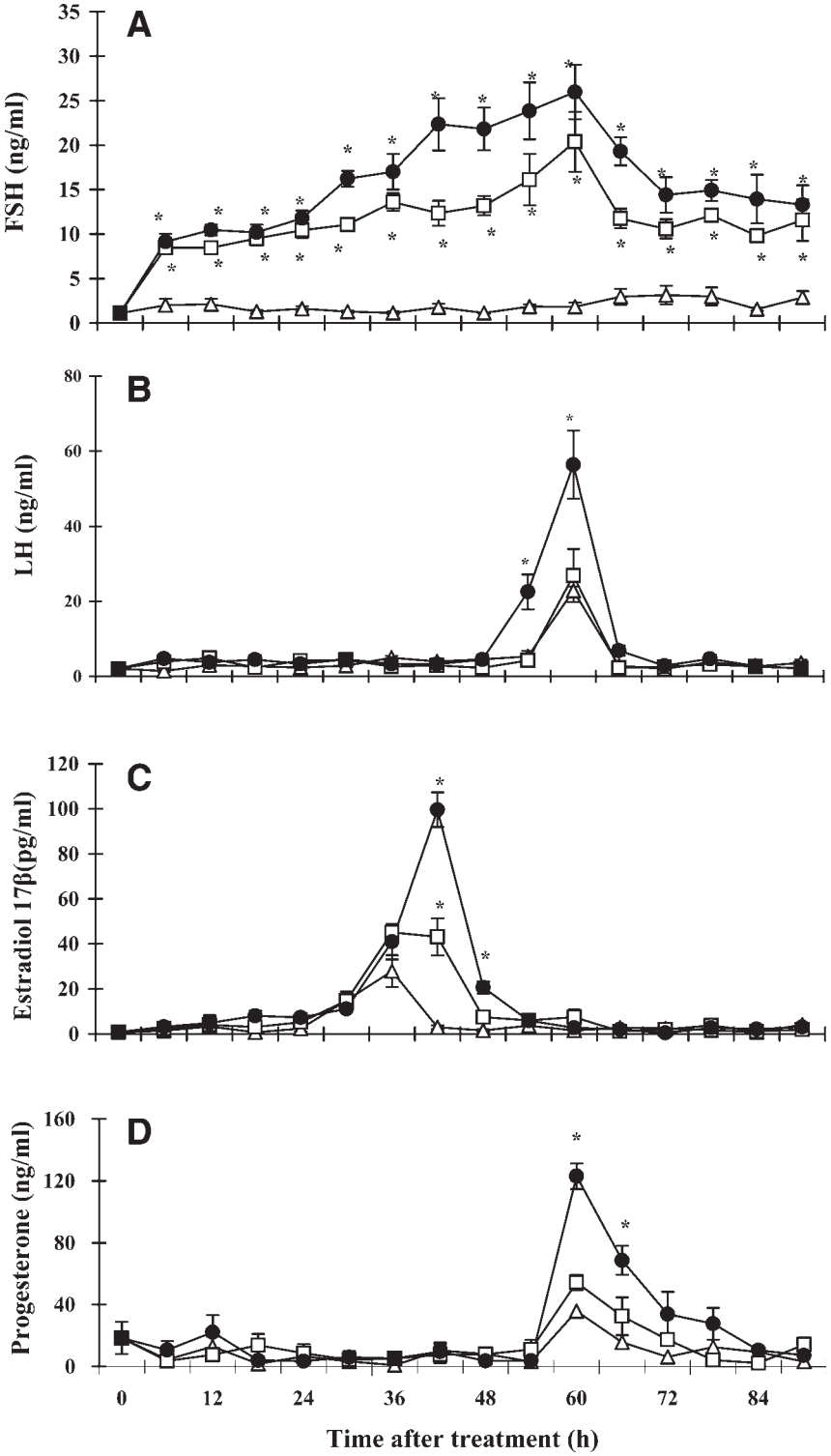

Fig. 1. Changes in plasma concentrations of FSH (**A**), LH (**B**), estradiol-17β (**C**), and progesterone (**D**) in adult cyclic mice treated with 50 μL inhibin-AS (□); 200 μL inhibin-AS (●), or control serum (△) on metestrus. Each point represents five mice. *$p < 0.05$ versus control group.

treatment with the inhibin-AS resulted in a significant increase in plasma concentrations of FSH starting 6 h after treatment and lasting for 90 h (Fig. 1A). The LH surge was observed in control and inhibin-AS treated groups 60 h after treatments. Furthermore, LH surge concentrations were significantly higher in the group treated with 200 μL inhibin-AS in comparison with other groups (Fig. 1B).

The plasma concentrations of estradiol-17β started to increase at 30 h in control and inhibin-AS treated groups, and the peak levels were significantly higher in the group

Table 1
Effect of Inhibin-AS Administered IP into Adult Cyclic Mice on Metestrus on the Number of Ovulating Mice and the Number of Oocytes per Animal

Treatment	Number of mice that ovulated	Number of ovulated oocytes*
Control serum	5/5	17.2 ± 1.7^{a}
50 µL inhibin-AS	5/5	42.2 ± 5.2^{b}
100 µL inhibin-AS	5/5	58.6 ± 7.1^{bc}
200 µL inhibin-AS	5/5	70.0 ± 7.3^{c}

*Values are mean ± SEM ($n = 5$).
^{abc}Means with different superscripts in the same column are significantly different ($p < 0.05$).

Table 2
Effect of Inhibin-AS Administered IP into Adult Cyclic Mice on Metestrus on Production of Fertilized Oocytes (Two-Cell Stage Embryos) and Rate of Fertilization In Vivo*

Treatment	Number of fertilized oocytes	Fertilization rate (%)
Control serum	15.0 ± 1.7^{a}	87.7 ± 1.3^{a}
50 µL inhibin-AS	36.0 ± 4.9^{b}	85.3 ± 0.7^{a}
100 µL inhibin-AS	49.6 ± 5.8^{bc}	84.8 ± 1.0^{a}
200 µL inhibin-AS	59.2 ± 5.9^{c}	84.8 ± 1.0^{a}

*Values are mean ± SEM ($n = 5$).
^{abc}Means with different superscripts in the same column are significantly different ($p < 0.05$).

treated with 200 µL inhibin-AS compared with the group treated with 50 µL inhibin-AS or the control group (Fig. 1C). In addition, plasma concentrations of progesterone increased in inhibin-AS treated and control groups at 60 h after treatment. The peak levels of plasma progesterone were significantly higher in mice treated with 200 µL inhibin-AS in comparison with other groups (Fig. 1D).

Effect of Inhibin-AS Administered IP into Adult Mice on Metestrus on Oocyte Production, Fertilization, and Embryo Development

Administration of inhibin-AS (50, 100, or 200 µL per animal) on metestrus significantly increased the number of ovulations in comparison with the control group (Table 1). There was a dose-dependent effect with the highest ovulation rate in the 200-µL treated group. Table 2 shows the number of fertilized oocytes and fertilization rate. The production of two-cell stage embryos in all inhibin-AS treated groups was significantly higher compared with the control group. There was no difference in the fertilization rate between inhibin-AS treated and control groups. The developmental competence of embryos is shown in Table 3. There was no significant difference between the groups treated with inhibin-AS and the control group in the rate of blastocyst development. However, the number of hatched blastocysts for the groups treated with the inhibin-AS was significantly higher than for the control group.

Table 3
Effect of Inhibin-AS Administered IP into Adult Cyclic Mice on Metestrus on Production of Hatched Blastocysts and Rate of Blastocyst Development*

Treatment	Number of hatched blastocyst	Rate of blastocyst development
Control serum	13.2 ± 1.6^{a}	87.9 ± 1.0^{a}
50 µL inhibin-AS	30.0 ± 3.9^{b}	83.2 ± 0.7^{a}
100 µL inhibin-AS	42.2 ± 5.1^{bc}	84.9 ± 0.9^{a}
200 µL inhibin-AS	50.2 ± 4.9^{c}	84.9 ± 0.5^{a}

*Values are mean ± SEM ($n = 5$).
^{abc}Means with different superscripts in the same column are significantly different ($p < 0.05$).

Discussion

The results of the present study clearly showed that immunoneutralization of endogenous inhibin can be used effectively to induce superovulation in adult cyclic mice without additional treatment with hCG, making it more practical and efficient compared with the previous methods such as eCG–hCG *(24,25)* and recombinant FSH *(26)*. Immunoneutralization of endogenous inhibin increased plasma concentrations of FSH, which, in turn, led to stimulation of ovarian folliclular development and increased ovulation rate. In previous studies, a significant increase in plasma concentrations of FSH has been shown by the passive immunization against inhibin during the estrous cycle in domestic *(10,18,19,20,21,27,28)* and laboratory *(16,23,29)* animals, indicating the important role of inhibin in regulating FSH secretion.

In the present study, plasma concentrations of estradiol-17β increased significantly in the mice treated with inhibin-AS. These findings suggest that a high level of endogenous FSH stimulated follicular development and resulted in the production of a large amount of estradiol-17β. This induced the LH surge by a positive-feedback effect on the hypothalamus and pituitary axis, leading to induction of superovulation. Moreover, progesterone values were significantly higher in the immunized group compared to the control group, reflecting the increased number of preovulatory follicles as the granulosa and theca cells secreted progesterone under the effect of LH. During the growth and maturation of ovarian follicles, the expression of LH receptors in granulosa cells is increased in preovulatory follicles *(30, 31)* with increased ability to produce progesterone in response to gonadotropins *(32–35)*. Differentiated granulosa cells from preovulatory follicles readily up-regulate the expression of steroidogenic acute regulatory protein (StAR) and

produce large amounts of progesterone in response to LH. A previous study *(36)* demonstrated that inhibin immunoneutralization resulted in a significant increase in progesterone secretion from rat preovulatory follicles cultured in vitro.

Previous studies involving superovulation have been conducted in mice *(37–39)*. In these studies, an eCG–hCG protocol has been used. However, detrimental effects of gonadotropin stimulation have been revealed possibly due to modified steroid profiles, altering the microenvironment of the developing follicles and their enclosed oocytes *(40–42)*. This possibility led us to consider an alternative method that could be used to induce superovulation in mice, and we already succeeded in establishing a new method using inhibin-AS and hCG *(14)*. In the present study, immunoneutralization of endogenous inhibin in adult mice induced superovulation without additional injection of hCG with comparable results to inhibin-AS and hCG as reported previously *(14)*. The production of oocytes by the mice treated with the inhibin-AS increased markedly in a dose-dependent manner. Inhibin-AS did not have any adverse effects on embryo quality as assessed by morphologic and developmental competence in mice. The superovulated oocytes could be fertilized normally in vivo, leading to the development of blastocysts.

In conclusion, passive immunization against inhibin increased FSH levels and ovulation rate without additional treatment with hCG. In addition, the superovulated oocytes have normal developmental competence in vitro. Thus, immunoneutralization of endogenous inhibin is an ideal alternative and provides a more efficient approach for induction of superovulation in mice.

Materials and Methods

Animals

Adult female mice of the ddY strain (Sankyo Experimental Animal Supply Co., Tokyo, Japan) were used at 3 mo of age. They were housed under controlled temperature and lighting (lights on from 05:00 to 19:00) and were provided with commercial pellets and tap water *ad libitum*. The 4-d estrous cycle was monitored by daily examination of the vaginal smears. The day of ovulation, as judged by the presence of cornified cells in the vaginal smear, was designated the day of estrus. Mice were used only after at least two successive normal 4-d estrous cycles. The care and use of the animals were in accordance with the requirements established under the *Guide for the Care and Use of the Laboratory Animals* by the Tokyo University of Agriculture and Technology.

Preparation of Inhibin α-Subunit Antiserum

The inhibin α-subunit antiserum used in the present study was obtained from a castrated goat immunized against [Tyr^{30}]-inhibin α(1-30) NH_2 conjugated to rabbit serum albumin. The conjugate was kindly provided by Dr. N. Ling (Neuroendocrine Biosciences Inc., San Diego, CA, USA). The titer of the antiserum was determined as in our previous reports *(29)*. In vivo efficacy of the antiserum was ensured by an increase in plasma concentrations of FSH after an iv injection of the antiserum, as described previously *(29)*. The control serum was obtained from a castrated goat immunized against bovine serum albumin (BSA).

Effect of Inhibin-AS Administered IP into Adult Cyclic Mice on Metestrus on Plasma Levels of FSH, LH, Estradiol-17β, and Progesterone

Inhibin-AS (50, 100, and 200 µL/animal) or control serum (100 µL/animal) was ip administered to individual animals at 12:00 h on metestrus. Five animals were sacrificed every 6 h both in inhibin-AS or control serum treated groups throughout a single estrous cycle (90 h after treatments). Blood samples were collected into heparinized centrifuge tubes and plasma was immediately separated by centrifugation and stored at –20°C until assayed for FSH, LH, estradiol-17β, and progesterone.

Effect of Inhibin-AS Administered Ip into Adult Mice on Metestrus on Oocyte Production, Fertilization, and Embryo Development

Female mice were primed at 12:00 h on metestrus with a single ip injection of different doses of inhibin-AS (50, 100, and 200 µL/animal) or control serum (100 µL/animal). At 17:00 h of the following proestrus, the individual female mouse was bred by a single fertile male. Five animals from each of the inhibin-immunized groups and the control group were sacrificed at 08:00 h on d 2 postcoitus. The oviducts were taken for the collection of embryos.

Embryo Collection and Culture In Vitro

At 8:00 h on the d 2 postcoitus, all ova including unfertilized, degenerated, and fertilized oocytes (cleaved oocytes are referred to as fertilized and uncleaved oocytes or those with shrunken or fragmented cytoplasm were referred to as unfertilized or degenerated) were harvested by flushing the excised oviducts with embryo culture medium—KSOM solution *(43,44)*. The number of fertilized and unfertilized oocytes was recorded to evaluate the oocyte production and fertilization rate. Two-cell stage embryos were picked and cultured at 37°C with fresh KSOM solution in a condition of 5% CO_2 in air, 95% relative humidity for 60 h. The hatched blastocysts were counted and photographed at the end of culture period.

Radioimmunoassays (RIA) for FSH, LH, Estradiol-17β, and Progesterone

Plasma concentrations of FSH and LH were determined by double-antibody radioimmunoassy using an NIDDK kit for rat FSH and LH. The iodinated preparations were rat FSH I-8 and LH I-8, and the antisera used were anti-rat FSH (S-11) and anti rat LH (S-9). Results were expressed in terms of NIDDK rat FSH (RP-2) and LH (RP-2). The

intra- and interassay coefficients of variation were 4.6% and 14.2% for FSH and 6.5% and 8.7% for LH, respectively. The minimum detection limit was 0.3 ng/mL for FSH assay and 0.02 ng/mL for LH assay. Plasma concentrations of estradiol-17β and progesterone were determined by double-antibody radioimmunoassay using ^{125}I-labeled radioligands as described previously *(45)*. Antisera against estradiol-17β (GDN 244) and progesterone (GDN 337) were kindly provided by G. D. Niswender (Animal Reproduction and Biotechnology, Colorado State University, Fort Collins, CO, USA). The intra- and interassay coefficients of variation were 3.9% and 6.0% for estradiol-17β and 6.3% and 15.2% for progesterone, respectively. The minimum detection limit was 5 pg/mL for estradiol-17β assay and 0.04 ng/mL for progesterone assay.

Statistics

All data are expressed as mean ± SEM. Statistical analysis was performed using one-way ANOVA. Significant differences were determined using Tukey's honestly significant difference (HSD) test for multiple comparisons between different treatment groups. Statistical difference was confirmed at $p < 0.05$. Statistical analyses were performed using SAS Software (SAS Institute Inc., Cary, NC).

Acknowledgments

We express our gratitude to Dr. Reinhold J. Hutz, (Department of Biological Science, University of Wisconsin-Milwaukee, Milwaukee, WI, USA) for reading the original manuscript and for valuable suggestions. We are grateful to the Rat Pituitary Hormone Distribution Program and Dr. A.F. Parlow, NIDDK, NIH, Bethesda, MD, USA for providing RIA materials of rat LH and FSH, and Dr. G. D. Niswender (Animal Reproduction and Biotechnology Laboratory, Colorado State University, Fort Collins, CO, USA) for providing antisera to estradiol-17β (GDN 244) and progesterone (GDN 337). This work was supported in part by 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 and a Grant-in-Aid for Scientific Research (No. 03338) from the Japan Society for the Promotion of Science.

References

1 Ertzeid, G. and Storeng, R. (1992). *J. Reprod. Fertil.* **96,** 649–655.
2 Ertzeid, G., Storeng, R., and Lyberg, T. (1993). *J. Assist. Reprod. Genet.* **10,** 286–291.
3 Ertzeid, G. and Storeng, R. (2001). *Hum. Reprod.* **16,** 221–225.
4. Bavister, B. D., Dees, C., and Schultz, R. D. (1986). *Am. J. Reprod. Immunol. Microbiol.* **11,** 11–16.
5. Swanson, W. F., Roth, T. L., Graham, K., Horohov, D. W., and Godke, R. A. (1996). *Am. J. Vet. Res.* **57,** 302–307.
6 Roy, F., Maurel, M. C., Combes, B., et al. (1999). *Biol. Reprod.* **60,** 805–813.
7 de Jong, F. H. (1988). *Physiol. Rev.* **68,** 555–607.
8. Taya, K. (1993). *Curr. Trend. Experim. Endocrinol.* **1,** 97–116.
9 Kaneko, H., Nakanishi, Y., Taya, K., et al. (1993). *J. Endocrinol.* **136,** 35–41.
10 Kaneko, H., Nakanishi, Y., Akagi, S., et al. (1995). *Biol. Reprod.* **53,** 931–939.
11 Kishi, H., Okada, T., Kawazu, S., et al. (1997). *Reprod. Fertil. Dev.* **9,** 447–453.
12. Araki, K., Arai, K. Y., Watanabe, G., and Taya, K. (2000). *J. Andrology* **21,** 558–565.
13 Medan, M. S., Watanabe, G., Sasaki, K., Sharawy, S., Groome, N. P., and Taya, K. (2003). *Biol. Reprod.* **69,** 57–63.
14 Wang, H., Herath, C. B., Xia, G., Watanabe, G., and Taya, K. (2001). *Reproduction* **122,** 809–816.
15 Rivier, C. and Vale, W. (1989). *Endocrinology* **125,** 152–157.
16 Ishigame, H., Medan, M. S., Watanabe, G., et al. (2004). *Biol. Reprod.* **71,** 236–243.
17 Kishi, H., Okada, T., Otsuka, M., Watanabe, G., Taya, K., and Sasamoto, S. (1996). *J. Endocrinol.* **151,** 65–75.
18 Akagi, S., Kaneko, H., Nakanishi, Y., Takedomi, T., Watanabe, G., and Taya, K. (1997). *J. Vet. Med. Sci.* **59,** 1129–1135.
19 Takedomi, T., Kaneko, H., Aoyagi, Y., et al. (1997). *Theriogenology* **47,** 1507–1518.
20 Nambo, Y., Kaneko, H., Nagata, S., et al. (1998). *Theriogenology* **50,** 545–557.
21 Medan, M. S., Watanabe, G., Sasaki, K., et al. (2003). *Reproduction* **125,** 751–757.
22. D'Alessandro, A., Martemucci, G., and Iaffaldano, N. (1999). *J. Reprod. Fertil.* **115,** 185–191.
23 Shi, F., Mochida, K., Suzuki, O., et al. (2000). *Endocr. J.* **47,** 451–459.
24 Legge, M. and Sellens, M. H. (1994). *J. Assist. Reprod. Genet.* **11,** 312–318.
25 Lasserre, A., Espinosa, M. B., and Vitullo, A. (1998). *Biocell.* **22,** 157–162.
26 Rice, V. C., Zusmanis, K., Malter, H., and Mitchell-Leef, D. (1993). *Life Sci.* **53,** 31–39.
27 Mann, G. E., Campbell, B. K., McNeilly, A. S., and Baird, D. T. (1989). *J. Endocrinol.* **123,** 383–391.
28 Wheaton, J. E., Carlson, K. M., and Kusina, N. T. (1992). *Biol. Reprod.* **47,** 361–367.
29 Arai, K., Watanabe, G., Taya, K., and Sasamoto, S. (1996). *Biol. Reprod.* **55,**127–33.
30 Uilenbroek, J. T. and Richards, J. S. (1979). *Biol. Reprod.* **20,** 1159–1165.
31 LaPolt, P. S., Oikawa, M., Jia, X. C., Dargan, C., and Hsueh, A. J. (1990). *Endocrinology* **126,** 3277–3279.
32 Richards, J. S., Jonassen, J. A., Rolfes, A. I., Kersey, K., and Reichert, L. E. Jr. (1979). *Endocrinology* **104,** 765–773.
33 Park, O. K. and Mayo, K. (1991). *Mol. Endocrinol.* **5,** 967–978.
34 Natraj, U. and Richards, J. S. (1993). *Endocrinology* **133,** 761–769.
35 Conneely, O. M. (2001). *Endocrinology* **142,** 2194–2199.
36 Smyth, C. D., Gosden, R. G., McNeilly, A. S., and Hillier, S. G. (1994). *J. Endocrinol.* **140,** 437–443.
37 Spindle, A. I. and Goldstein, L. S. (1975). *J. Reprod. Fertil.* **44,** 113–116.
38 Ziebe, S., Guoliang, X., and Byskov, A. G. (1993). *Hum. Reprod.* **8,** 385–388.
39 Garcia, J., Kholkute, S. D., and Dukelow, W. R. (1993). *Lab. Anim. Sci.* **43,** 198–199.
40 Moor, R. M., Osborn, J. C., and Crosby, I. M. (1985). *J. Reprod. Fertil.* **74,** 167–172.
41 Szoltys, M., Galas, J., Jablonka, A., and Tabarowski, Z. (1994). *J. Endocrinol.* **141,** 91–100.
42 Ma, S., Kalousek, D. K., Yuen, B. H., and Moon, Y. S. (1997). *J. Assist. Reprod. Genet.* **14,** 162–169.
43 Ho, Y., Wigglesworth, K., Eppig, J. J., and Schultz, R. M. (1995). *Mol. Reprod. Dev.* **41,** 232–238.
44 Summers, M. C., Bhatnagar, P. R., Lawitts, J. A., and Biggers, J. D. (1995). *Biol. Reprod.* **53,** 431–437.
45. Taya, K., Watanabe, G., and Sasamoto, S. (1985). *Jpn. J. Anim. Reprod.* **31,** 186–197.