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# Comparative Analysis of the Effects of Gonadotropin-Releasing Hormone Agonist on the Proliferative Activity, Apoptosis, and Steroidogenesis in Cultured Porcine Granulosa Cells at Varying Stages of Follicular Growth

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**This study was conducted to analyze comparative effects of gonadropin-releasing hormone (GnRH) agonist on the proliferation, apoptosis, and differentiated function of cultured porcine granulosa cells from varying follicle stages. Comparative analyses of porcine granulosa cells from varying follicle stages to respond to GnRH agonist were performed in terms of proliferating cell nuclear antigen (PCNA) expression, occurrence of apoptosis, and 17β-estradiol ($E_2$) and progesterone (P) secretion. PCNA expression was examined by the avidin/biotin immunoperoxidase method with a monoclonal antibody to PCNA, and apoptosis was assessed by *in situ* DNA 3'-end labeling method and DNA fragmentation analysis. $E_2$ and P were measured by radioimmunoassays. The PCNA positive rate of granulosa cells cultured in the presence of GnRH agonist ($10^{-9}$ *M*) was lower compared with that of cells cultured in the absence of GnRH agonist. However, the apoptosis positive rate was higher, and $E_2$ and P secretion by cultured granulosa cells was lower in the presence of GnRH agonist ($10^{-9}$ *M*) compared with that in the absence of GnRH agonist. The inhibitory effect of GnRH agonist on PCNA positive rate of cultured cells was prominent in granulosa cells from small and medium but not from large follicles. By contrast, the inhibitory effect of GnRH agonist on $E_2$ and P secretion by cultured cells was prominent in granulosa cells from large but not small and medium follicles. The stimulatory effect of GnRH agonist on apoptosis positive rate of cultured cells was, however, uniform regardless of the stages of follicular growth. These results demonstrate that GnRH agonist exerts diverse actions on granulosa cells over the course of follicular growth. One downregulates granulosa proliferation in immature follicles as well as steroidogenesis in mature follicles, and the other upregulates apoptosis of granulosa cells regardless of the stages of follicular growth.**

**Key Words:** Granulosa cell; gonadotropin-releasing hormone; agonist; proliferating cell nuclear antigen; apoptosis; steroidogenesis; porcine.

## Introduction

In the late 1980s, gonadotropin-releasing hormone (GnRH) agonist was introduced as a means of downregulating the pituitary to prevent premature ovulation, which, in the past had necessitated canceling in vitro fertilization cycles *(1,2)*. The downregulating effects of GnRH agonist, as opposed to the stimulatory effects of GnRH, are related to the frequency of administration and the prolonged occupation of GnRH receptors by the agonist *(3)*. Since this introduction of GnRH agonist, pregnancy rates have increased because of the opportunity to retrieve cycles that would have been lost to early ovulation and because of the increase in the number of oocytes obtained in GnRH agonist cycles *(4)*. In addition to the downregulating effects, much evidence suggests direct effects of GnRH and its agonist on the ovary. GnRH and its agonist affect steroidogenesis in granulosa cells and luteal cells *(5–9)*. The direct effects of GnRH and its agonist on the ovary have been supported by the demonstration of specific, high-affinity binding sites for GnRH and its agonist *(10–12)*.

During ovarian follicular development, only limited numbers of follicles are selected for ovulation, whereas the remaining majority of follicles undergo atresia. The morphological sign of follicular atresia is typified by the formation of pyknotic nuclei within granulosa cells. It is now evident that follicular atresia is associated with internucleosomal fragmentation of DNA of granulosa cells *(13–16)*. Homeostatic control of follicle growth is thought to be the result of the dynamic balance between cell proliferation and cell death. Proliferating cell nuclear antigen (PCNA) is a cell cycle-related nonhistone nuclear protein

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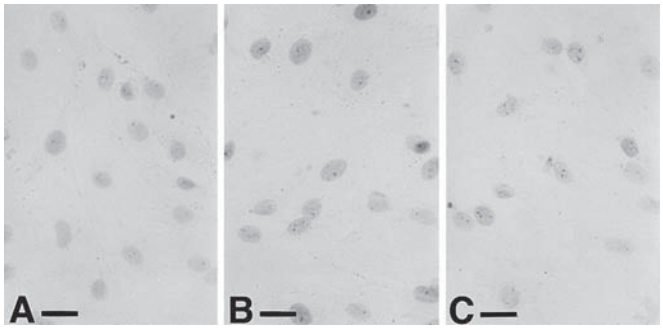


**Fig. 1.** Immunocytochemical staining of PCNA in porcine granulosa cells from small follicles cultured for 48 h under serum-free conditions. **(A)** Without any hormonal treatment; **(B)** in the presence of FSH (50 ng/mL); **(C)** in the presence of GnRH agonist ($10^{-9}$ *M*). Bars represent 5 μm. Original magnification: ×400.

with a molecular weight of 36 kDa. Elevated levels of PCNA appear in the late G1 phase and become maximal during the S phase of proliferating cells but are not detectable in resting cells *(17)*. Immunocytological PCNA labeling has proven useful in evaluating the proportions of proliferating cells *(18)*. On the other hand, *in situ* DNA 3'-end labeling with nonradioactive digoxigenin-dideoxy-UTP (dig-ddUTP) and DNA fragmentation assay have been used to evaluate the occurrence of apoptosis *(16,19,20)*.

The present in vitro study was conducted to analyze the comparative effects of GnRH agonist on the proliferative potential, apoptosis, and steroidogenic activity of granulosa cells at varying follicle stages by using a porcine model.

## Results

Immunocytochemical examination of granulosa cells cultured for 48 h under serum-free conditions demonstrated that PCNA label was immunolocated exclusively in the nuclei of some cultured granulosa cells (Fig. 1). In small follicle granulosa cells cultured in the presence of follicle-stimulating hormone (FSH) (Fig. 1B), PCNA-positive nuclei were more abundant in comparison to untreated granulosa cells (Fig. 1A). However, in small follicle granulosa cells cultured in the presence of GnRH agonist ($10^{-9}$ *M*), PCNA-positive nuclei were less abundant than those in untreated granulosa cells (Fig. 1C).

Determination of the mean percentage of PCNA positive nuclei of granulosa cells from small, medium, and large follicles cultured in the absence or presence of FSH or GnRH agonist for 48 h revealed that the addition of GnRH agonist decreased in a dose-dependent manner the PCNA positive rate of granulosa cells from small and medium follicles in comparison with that in untreated cultures (Fig. 2). Although FSH treatment significantly increased the PCNA positive rate of cultured granulosa cells from small and medium follicles, concomitant treatment with GnRH agonist ($10^{-9}$ *M*) in the presence of FSH resulted in a significant decrease in the PCNA positive rate of small follicle granulosa cells compared to treatment with FSH alone. Large follicle granulosa cells did not respond to GnRH agonist in terms of PCNA expression (Fig. 2).

*In situ* DNA 3'-end labeling with dig-ddUTP on granulosa cells cultured for 48 h under serum-free conditions showed that apoptosis-positive nuclei were apparent in some cultured granulosa cells, and that in granulosa cells cultured in the presence of FSH apoptosis-positive nuclei were less abundant compared with those in granulosa cells in untreated control cultures (Fig. 3). However, in granulosa cells cultured in the presence of GnRH agonist ($10^{-9}$ *M*), apoptosis-positive nuclei were more abundant than in untreated granulosa cells (Fig. 3).

Determination of the mean percentage of apoptosis-positive nuclei of granulosa cells cultured in the absence or presence of FSH or GnRH agonist for 48 h revealed that the addition of GnRH agonist increased the apoptosis positive rate of granulosa cells from small, medium, and large follicles (Fig. 4). Treatment with GnRH agonist in a concentration higher than $10^{-9}$ *M* significantly increased the apoptosis positive rate of cultured granulosa cells compared with that in untreated control cultures. Although FSH treatment significantly reduced the apoptosis positive rate of cultured granulosa cells from small and medium follicles, concomitant treatment with GnRH agonist ($10^{-9}$ *M*) in the presence of FSH resulted in a significant increase in the apoptosis positive rate of cultured granulosa cells from small, medium, and large follicles compared to treatment with FSH alone.

Furthermore, DNA fragmentation analysis of granulosa cells from small follicles cultured for 48 h without any treatment showed a laddering pattern of nucleosome-size fragments (Fig. 5). FSH treatment resulted in a slight decrease in the appearance of DNA laddering compared with that in untreated control cultures. By contrast, treatment with GnRH agonist alone caused an increase in the appearance of DNA laddering compared with that in untreated control cultures (Fig. 5). Concomitant treatment with FSH and GnRH agonist resulted in an increase in the appearance of DNA laddering compared to treatment with FSH alone (Fig. 5).

Figure 6 shows secretion levels of 17β-estradiol ($E_2$) and progesterone (P) by porcine granulosa cells cultured for 48 h in the absence or presence of GnRH agonist ($10^{-9}$ *M*) or FSH. The addition of FSH significantly increased $E_2$ secretion in cultured granulosa cells from small, medium, and large follicles, whereas an FSH-induced increase in P secretion by cultured granulosa cells was noted only in granulosa cells from large follicles. By contrast, the addition of GnRH agonist ($10^{-9}$ *M*) significantly decreased $E_2$ and P secretion of granulosa cells from large follicles but not from small and medium follicles, compared with that in untreated control cultures. Concomitant treatment with

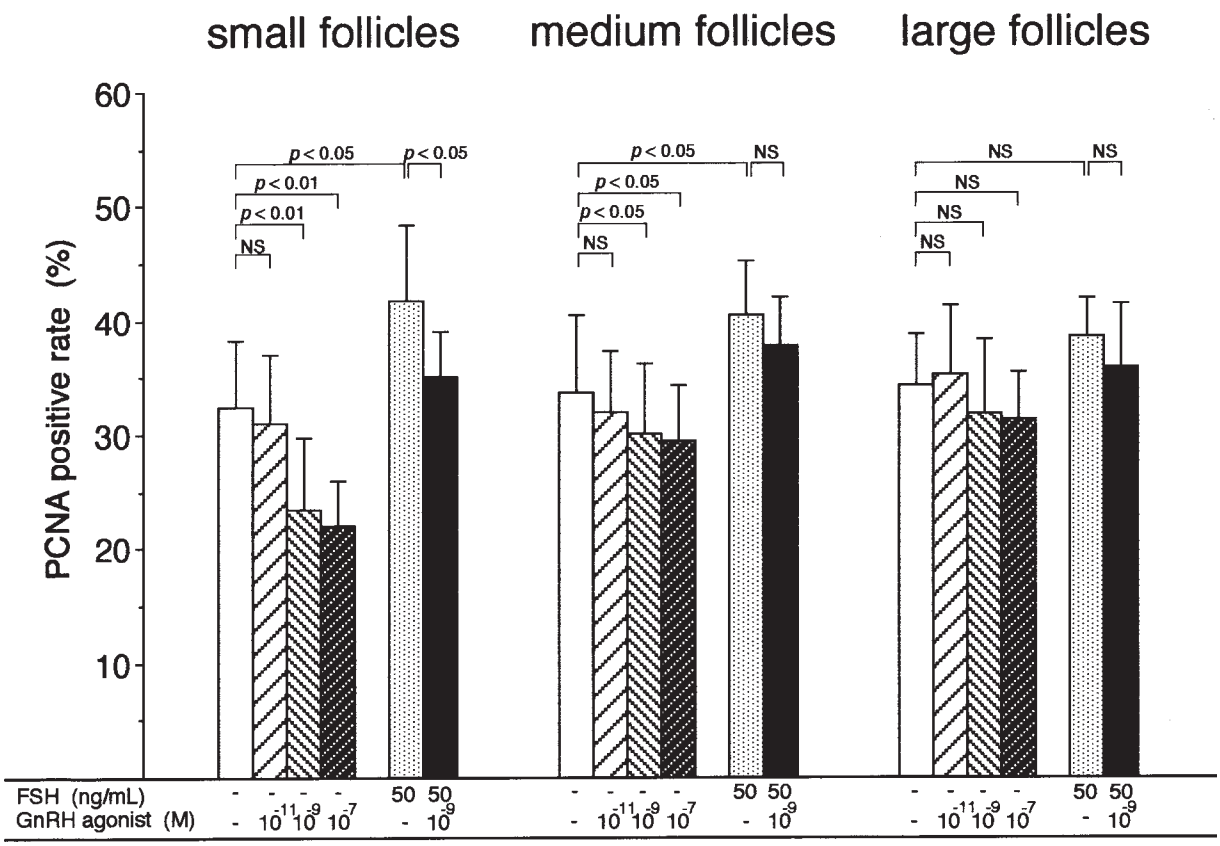


**Fig. 2.** PCNA positive rate of porcine granulosa cells from varying sizes of follicles cultured for 48 h under serum-free conditions. Results represent the mean ± SD of four determinations.

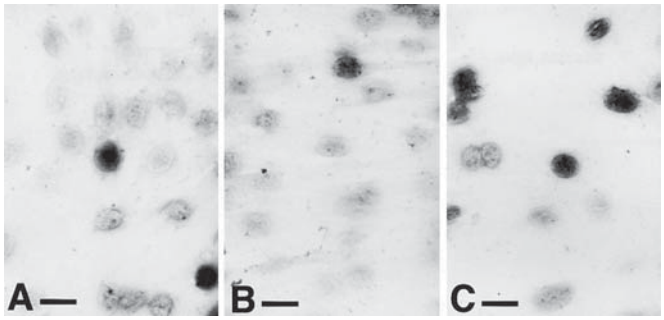


**Fig. 3.** *In situ* DNA 3'-end labeling with dig-ddUTP on porcine small follicle granulosa cells cultured for 48 h under serum-free conditions. **(A)** Without any hormonal treatment; **(B)** in the presence of FSH (50 ng/mL); **(C)** in the presence of GnRH agonist ($10^{-9}$ *M*). Bars represent 5 μm. Original magnification: ×400.

FSH and GnRH agonist resulted in a significant decrease in $E_2$ and P secretion of granulosa cells from large follicles compared to treatment with FSH alone.

## Discussion

The data demonstrate, for the first time, that in the porcine model GnRH agonist exerts follicular maturation, stage-dependent, diverse actions on granulosa cells: one inhibits the proliferative activity of granulosa cells in immature follicles, as well as the steroidogenic activity in granulosa cells in mature follicles, and the other stimulates apoptosis of granulosa cells regardless of follicular maturation stage. Interestingly, the inhibitory effect of GnRH agonist on the proliferative activity is prominent in immature granulosa cells from small and medium follicles, whereas the inhibitory effects of GnRH agonist on steroidogenic activity is prominent in mature granulosa cells from large follicles. The stimulatory effect of GnRH agonist on the occurrence of apoptosis, however, is pronounced in both immature and mature granulosa cells. Furthermore, the data indicate that FSH treatment protects granulosa cells isolated from small and medium follicles from undergoing GnRH-mediated apoptosis, but fails to have a protective effect in granulosa cells from large follicles, and that FSH treatment stimulates estrogen synthesis in granulosa cells from all follicle sizes, but stimulates only progesterone synthesis in the large follicles.

GnRH agonists have received considerable attention as therapeutic agents for synchronizing follicle growth during ganadotropin stimulation for in vitro fertilization. The major improvement attributed to the use of GnRH agonist has been the suppression of endogenous luteinizing hormone (LH) surges and higher yields of oocytes in cycles in

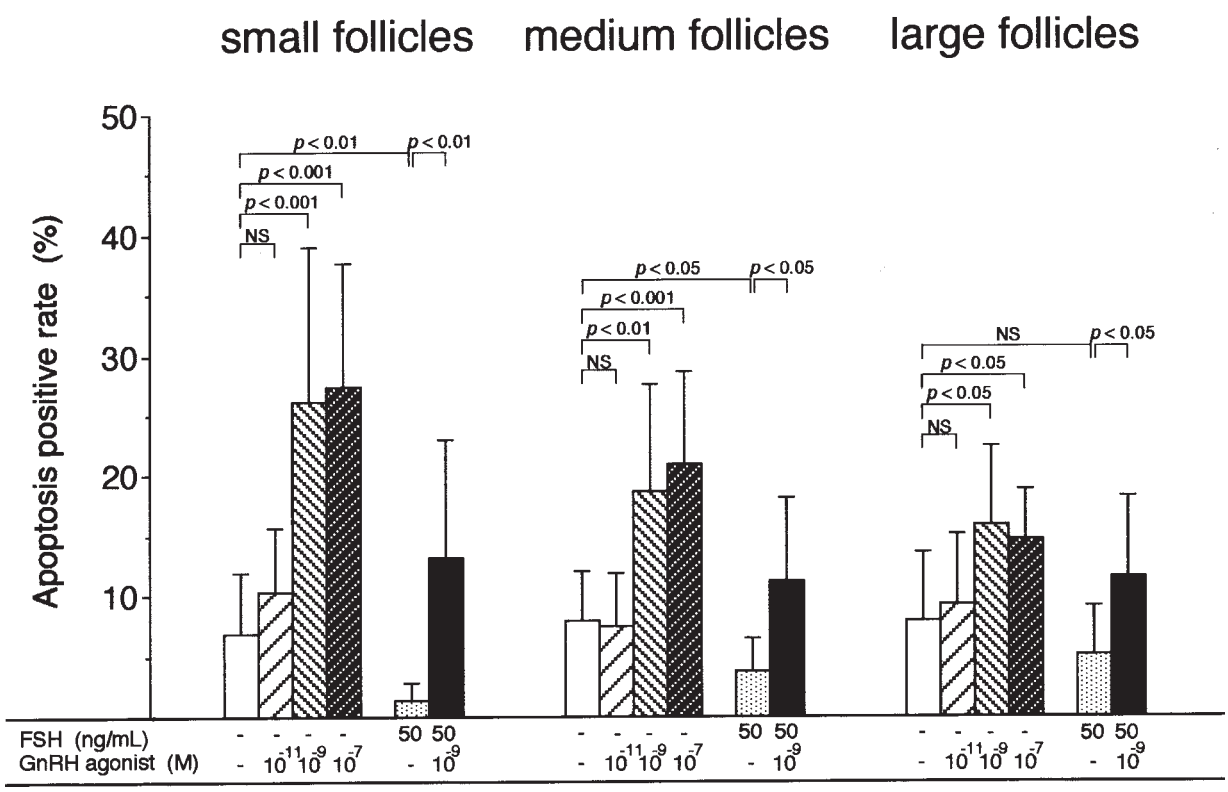


**Fig. 4.** Apoptosis positive rate of porcine granulosa cells from varying sizes of follicles cultured for 48 h under serum-free conditions as assessed by the *in situ* DNA 3'-end labeling method. Results represent the mean ± SD of four determinations.

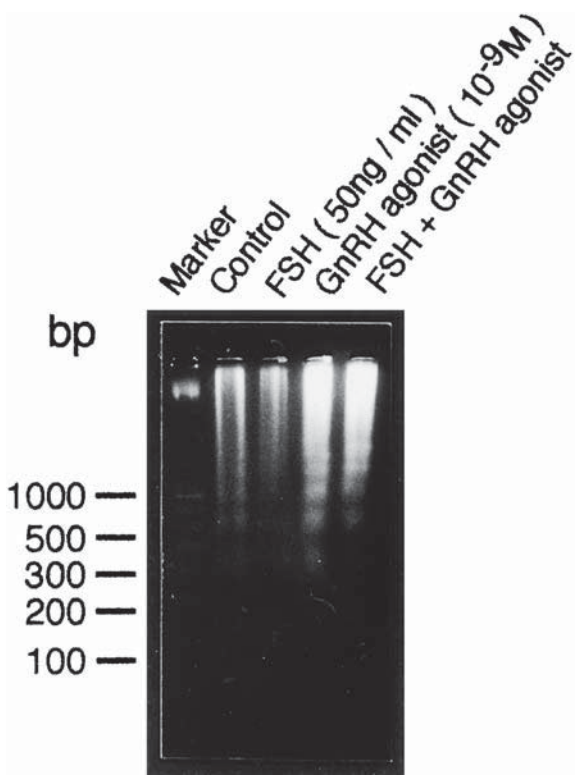


**Fig. 5.** DNA fragmentation analysis in porcine small follicle granulosa cells cultured for 48 h under serum-free conditions. This experiment was repeated four times with similar results.

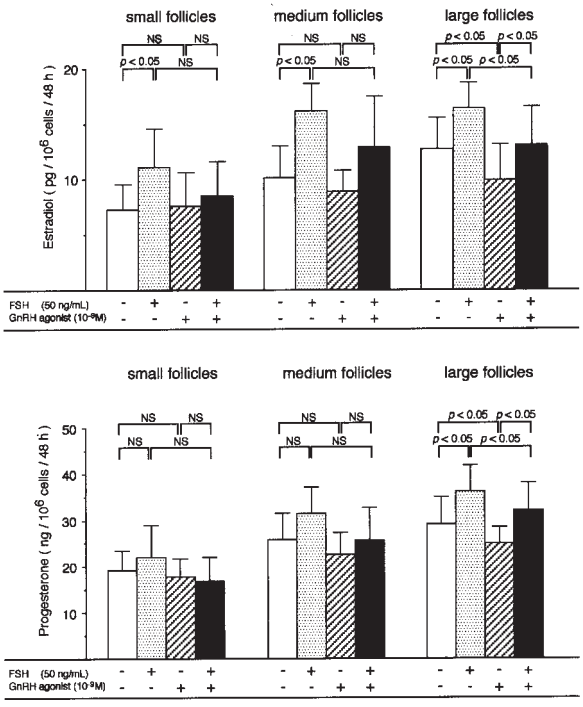


**Fig. 6.** Effect of treatment with GnRH agonist in the absence or presence of FSH on $E_2$ and P secretion by cultured porcine granulosa cells from varying sizes of follicles. Porcine granulosa cells harvested from small, medium, and large follicles were cultured in the presence or absence of FSH (50 ng/mL) or GnRH agonist ($10^{-9}$ *M*) for 48 h. Results represent the mean ± SD of four determinations.

which pituitary suppression preceded ovarian stimulation *(21,22)*. On the other hand, Polson et al. *(23)* reported that the use of GnRH agonist neither increased the number of oocytes obtained nor improved the quality of oocytes compared to treatment with hMG alone. Benadiva et al. *(24)* reported that pretreatment with GnRH agonist resulted in a remarkable decrease in the ovarian response to human menopausal gonadotropin (hMG) stimulation. Furthermore, Brzyski et al. *(25)* reported that more atretic and fewer preovulatory oocytes were obtained when GnRH agonist was used concurrently with FSH than when FSH was used alone. Their findings seem to be closely consistent with the stimulatory effect of GnRH agonist on granulosa cell apoptosis regardless of the stage of follicular maturation observed in the present study.

Several groups have demonstrated direct inhibitory effects of GnRH and its agonist in granulosa cells, including aromatase activity, LH/human chorionic gonadotropin receptor induction, and steroidogenesis *(26,27)*. Recently, Yano et al. *(28)* reported that GnRH agonist stimulated prostaglandin $E_2$ and P production by rat ovarian follicles, but not $E_2$ production. They also described that GnRH agonist inhibited proliferation of granulosa cells through apoptosis. Several studies also suggested that GnRH and its agonist may act as an atretogenic factor for follicular development and increase atresia of follicles *(16,20,29)*. In the ovary, >99% of the follicles undergo a degenerative process called atresia during reproductive life. Despite the overwhelming occurrence of atresia in the ovary, the cellular and molecular events underlying this phenomenon remain poorly understood. Yet, extending earlier morphological analysis, it is now evident that apoptotic cell death is the molecular mechanism underlying follicular atresia *(13,14,16)*. The factors that trigger apoptosis are cell specific, and different cell types may follow diverse early steps, ultimately leading to a common activation of endonuclease and the irreversible apoptotic cell death. Gonadotropins, insulin-like growth factor-I, epidermal growth factor, transforming growth factor-α, basic fibroblast growth factor, and estrogen have been identified as folliuclar survival factors capable of suppressing follicle apoptosis *(19,30,31)*. By contrast, androgens, interleukin-6, and GnRH are potential atretogenic factors capable of inducing follicular apoptosis *(19,20,32)*. Collectively, these findings suggest that the regulation of apoptosis during follicular atresia is extremely complex and dependent on the actions and interactions of pituitary-derived gonadotropin, hypothalamic hormone, and locally produced ovarian factors.

Tilly et al. *(13,15)* reported that ovarian follicular atresia is correlated with extensive apoptosis within both the granulosa and thecal layers of regressing follicles, and that the presence of apoptosis in atretic follicles was associated with significant decreases in estrogen concentrations of follicular fluid compared to those in healthy follicles of the same size. In a clinical viewpoint, Nakahara et al. *(33)* reported that a higher number of oocytes retrieved and lower incidence of apoptotic bodies in mural granulosa cell masses were associated with a higher pregnancy rate. It is possible that apoptosis may occur initially in the granulosa cells during the course of atresia of antral follicle, and that a higher occurrence of apoptosis in granulosa cell layer results in low steroid concentration in follicular fluid and poor quality of the oocyte.

In the present study, we have demonstrated that GnRH agonist acts as an upregulator of apoptosis of granulosa cells regardless of the stage of follicular maturation. Because it is evident that apoptosis is the underlying mechanism of follicular atresia, our data reinforce the findings that GnRH agonist is one of the atretogenic factors. In addition, we have revealed that GnRH agonist exerts a dual action on granulosa cells during follicular maturation: one that downregulates granulosa cell proliferation in the early stage of follicular maturation, and one that downregulates granulosa cell steroidogenesis in the late stage of follicular maturation. This is consistent with clinical findings that the administration of GnRH agonist with gonadotropin for in vitro fertilization reduces the total number of oocytes retrieved and decreases the concentration of steroid hormones in the serum and follicular fluid compared to treatment to gonadotropin alone *(23,34)*.

## Materials and Methods

### *Materials*

Phenol red–free Dulbecco's modified Eagle's medium (DMEM) and antibiotics solution ($1 \times 10^5$ U/L of penicillin, 100 mg/L of streptomycin) were purchased from Gibco-BRL (Grand Island, NY). GnRH agonist (buserelin acetate) was a generous gift from Hoechst Marion Roussel (Tokyo, Japan). Monoclonal antibody (MAb) to PCNA was purchased from Oncogene Science (Cambridge, MA).

### *Monolayer Cultures of Porcine Granulosa Cells*

Porcine ovaries were obtained at a local slaughterhouse and placed into physiological saline containing $1 \times 10^5$ U/L of penicillin and 100 mg/L of streptomycin. Follicular fluids were harvested aseptically by the needle aspiration method *(35)* from small (1 to 2 mm), medium (3–5 mm), and large (6–11 mm) follicles as previously described *(36)*. Granulosa cells were separated from follicular fluids by centrifugation at 500*g* for 5 min at 4°C, and washed twice with Dulbecco's phosphate-buffered saline. Monolayers of porcine granulosa cells harvested from small, medium, and large follicles were cultured with approx $1 \times 10^6$ cells/cm$^2$ in 75-cm$^2$ flasks in 20 mL of DMEM supplemented with 10% fetal bovine serum for 120 h at 37°C in a humidified atmosphere of 5% $CO^2$-95% air. Thereafter, the cultured cells were stepped down to serum-free conditions by incubating in serum-free

DMEM in the presence or absence of porcine FSH (NIH-FSH-P1; 50 ng/mL) or GnRH agonist ($10^{-11}$ – $10^{-7}$ *M*) for the subsequent 48 h. The doses of GnRH agonist used were determined on the basis of maximum serum concentrations (117.3 ± 42.5 pg/mL) of GnRH agonist (buserelin acetate) after intranasal administration to humans in vivo *(37)*.

### *Immunocytochemical Staining*

Cultured granulosa cells were fixed in 90% ethanol. Immunocytochemical staining was performed employing the avidin/biotin immunoperoxidase method using a polyvalent immunoperoxidase kit (Omnitags, Lipshaw, MI) as previously described *(38)*. A mouse MAb to PCNA was used at a dilution of 1:80 as the primary antibody. To ensure the specificity of the immunological reaction, adjacent control cytological sections were subjected to the same immunoperoxidase method, except that the primary antibody was replaced by nonimmune murine IgG (Miles, Ekhardt, IN) at the same dilution as the specific antibody. In the control experiments, replacement of the specific primary antibody with nonimmune IgG resulted in a lack of positive immunostaining.

Immunostained cells were analyzed and scored in a blind fashion without knowledge of the experimental group. All stained nuclei were scored as positive for PCNA. The mean percentage of PCNA-positive nuclei (PCNA positive rate) for each group of cultured granulosa cells was determined by observing more than 1000 nuclei for each of the experimental samples and was used to evaluate the proliferating activity of the cells.

### *In Situ DNA 3'-End Labeling*

DNA fragmentation in cytological sections was examined using a nonradioactive detection method *(16,19,20)*. The sections were treated with proteinase K (Gibco-BRL) for 30 min, followed by three washes in Tris buffer (100 m*M* Tris and 150 m*M* NaCl, pH 7.5). After incubation for 10 min in terminal transferase buffer (200 m*M* potassium cacodylate, 25 m*M* Tris, 0.25 mg/mL of bovine serum albumin, and 5 m*M* $CoCl_2$, pH 6.6) at room temperature, terminal transferase (1 U/μL) (Sigma, St. Louis, MO), dig-ddUTP (5 m*M*) Boehringer Mannheim, Indianapolis, IN), and ddATP (45 m*M*) (Pharmacia, Uppsala, Sweden) were added in the fresh buffer and incubated at 37°C for 1 h. After three washes in Tris buffer, the sections were incubated with blocking buffer (100 m*M* Tris, 150 m*M* NaCl [pH 7.5], and 0.5% [w/v] blocking reagent) (Pharmacia) for 30 min at room temperature before the addition of antidigoxigenin antibody conjugated to alkaline phosphatase (AP). After incubation with the antibody (1:1000 in 0.5% [w/v] blocking reagent, 100 m*M* Tris, 150 m*M* NaCl, pH 7.5) at room temperature for 2 h, the slides were washed three times in Tris buffer and equilibrated in AP buffer (100 m*M* Tris, 100 m*M* NaCl, and 50 mmol/L of $MgCl_2$, pH 9.5) before additing substrates (337.5 μg/mL of nitroblue tetrazolium and 175 μg/mL of 5-bromo-4-chloro-3-inodyl-phosphate) (Sigma) for AP. After 1–3 h, the color reaction was terminated with 10 m*M* Tris and 1 m*M* EDTA, pH 8.0. When the antibody conjugate was omitted from the procedure or when only ddATP was used as substrate for the terminal transferase, no color reaction was detected.

Cells that incorporated dig-ddUTP were analyzed and scored in a blind fashion without knowledge of the experimental group. All stained nuclei were scored as positive for apoptosis. The mean percentage of apoptosis-positive nuclei (apoptosis positive rate) for each group of cultured granulosa cells was determined by observing more than 1000 nuclei for each of the experimental samples and was used to evaluate cell death.

### *DNA Fragmentation Analysis*

At the termination of cultures, cultured cells were harvested and spaun at 400*g*. The pellet was resuspended in 35 μL of lysis buffer consisting of 20 m*M* EDTA, 100 m*M* Tris (pH 8.0), 0.5% sodium lauryl sarcosinate, and 7 mg/mL of proteinase K (Gibco-BRL). The samples were heated at 50°C for at least 2 h, and 10 μL of 10 mg/mL of RNase was added and then incubated for a further 2 h. Electrophoresis was carried out on a 1% agarose gel that was afterward stained with 0.1% ethidium bromide for 4 h before visualizing the DNA bands under ultraviolet light *(39,40)*.

### *Measurement of $E_2$ and P*

Concentrations of $E_2$ in medium were measured using an Estradiol RIA kit (Green Cross, Tokyo, Japan). The crossreactivity with estrone ($E_1$) in the Estradiol RIA kit was <0.50 %. Concentrations of progesterone P in medium were measured using a Progesterone RIA kit (Green Cross). The crossreactivity with pregnenolone in the Progesterone RIA kit was <0.12 %. These assays were performed as previously described *(36)*.

### *Statistical Analysis*

All experiments were repeated four times. Data are presented as means ± SD. Analysis of variance was used to determine statistical difference between effects of treatments as indicated in the text. A value of $p < 0.05$ was considered significant.

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## References

1. Droesch, K., Muasher, S. J., Brzyski, R. G., Jones, G. S., Simonetti, S., Liu, H. C., and Rosenwaks, Z. (1989). *Fertil. Steril.* **51,** 292–297.

2. Meldrum, D. R., Wisot, A., Hamilton, F., Gutlay, A. L., Kempton, W., and Huynh, D. (1989). *Fertil. Steril.* **51,** 455–459.
3. Yen, S. S. C. (1983). *Fertil. Steril.* **39,** 257–266.
4. Hughes, E. G., Fedorkow, D. M., Daya, S., Sagle, M. A., Van de Koppel, P., and Collins, J. A. (1992). *Fertil. Steril.* **58,** 888–896.
5. Ying, S. Y. and Guillemin, R. (1979). *Nature* **280,** 593–595.
6. Hsueh, A. J. W. and Erickson, G. F. (1979). *Science* **204,** 854–855.
7. Clayton, R. N., Harwood, J. P., and Catt, K. J. (1979). *Nature* **282,** 90–92.
8. Hori, H., Uemura, T., and Minaguchi H. (1998). *Endocr. J.* **45,** 175–182.
9. Lin, Y., Kahn, J. A., and Hillensj T. (1999). *Hum. Reprod.* **14,** 885–888.
10. Latouche, J., Crumeyrolle-Arias, M., Jordan, D., Kopp, N., Augendre-Ferrante, B., Cedard, L. and Haour, F. (1989). *Endocrinology* **125,** 1739–1741.
11. Harwood, J. P., Clayton, R. N., and Catt ,K. J. (1980). *Endocrinology* **107,** 407–413.
12. Harwood, J. P., Clayton, R. N., and Catt, K. J. (1980). *Endocrinology* **107,** 414–421.
13. Tilly, J. L., Kowalski, K. I., Johnson, A. L., and Hsueh, A. J. W. (1991). *Endocrinology* **129,** 2799–2801.
14. Hughes, F. M. and Gorospe, W. C. (1991). *Endocrinology* **129,** 2415–2422.
15. Tilly, J. L., Kowalski, K. I., Schomberg, D. W., and Hsueh, A. J. W. (1992). *Endocrinology* **131,** 1670–1676.
16. Hsueh, A. J. W., Billig, H., and Tsafriri, A. (1994). *Endcr. Rev.* **15,** 707–724.
17. Kuki, P., Vanderlaan, M., Dolbeare, F., Gray, J., and Tan, E. M. (1986). *Exp. Cell Res.* **166,** 209–219.
18. Robbins, B. A., Vega, D. D. L., Ogata, K., Tan, E. M., and Nakamura, R. M. (1987). *Arch. Pathol. Lab. Med.* **111,** 841–845.
19. Billig, H., Furuta, I., and Hsueh, A. J. W. (1993). *Endocrinology* **133,** 2204–2212.
20. Billig, H., Furuta, I., and Hsueh, A. J. W. (1994). *Endocrinology* **134,** 245–252.
21. Tan, S. L., Kingsland, C., Campbell, S., Mills, C., Bradfield, J., Alexander, N., Yovich, J., and Jacobs, H. S. (1992). *Fertil. Steril.* **57,** 810–814.
22. San-Roman, G. A., Surrey, E. S., Judd, H. L., and Kerin, J. F. (1992). *Fertil. Steril.* **58,** 744–749.
23. Polson, D. W., MacLachlan, V., Krapez, J. A., Wood, C., and Healy, D. L. (1991). *Fertil. Steril.* **56,** 509–514.
24. Benadiva, C. A., Blasco, L., Tureck, R., Mastroianni, L. and Flickinger, G. L. (1990). *Fertil. Steril.* **53,** 479–485.
25. Brzyski, R. G., Muasher, S. J., Droesch, K., Simonetti, S., Jones, G. S., and Rosenwaks, Z. (1988). *Fertil. Steril.* **50,** 917–921.
26. Hsueh, A. J. W., Wang, C., and Erickson, G. F. (1980). *Endcrinology* **106,** 1697–1705.
27. Otani, T., Maruo, T., Ashitaka, Y., and Tojo, S. (1982). *Endocrinol. Japan* **29,** 597–605
28. Yano, T., Yano, N., Matsumi, H., Morita, Y., Tsutsumi, O., Schally, A. V., and Taketani, Y. (1997). *Horm. Res.* **48,** 35–41.
29. Erickson, G. F., Li, D., Sadrkhanloo, R., Liu, X. J., Shimasaki, S., and Ling, N. (1994). *Endocrinology* **134,** 1365–1372.
30. Chun, S. Y., Billig, H., Tilly, J. L., Furuta, I., Tsafriri, A., and Hsueh, A. J. W. (1994). *Endocrinology* **135,** 1845–1853.
31. Tilly, J. L., Billig, H., Kowalski, K. I., and Hsueh, A. J. W. (1992). *Mol. Endcrinol.* **6,** 1942–1950.
32. Gorospe, W. C. and Spangelo, B. L. (1993). *Endocr. J.* **1,** 3–9.
33. Nakahara, K., Saito, H., Saito, T., Ito, M., Ohta, N., Sakai, N., Tezuka, N., Hiroi, M., and Watanabe, H. (1997). *Fertil. Steril.* **67,** 302–308.
34. Filicori, M., Flamigni, C., Cognigni, G. E., Falbo, A., Arnone, R., Capelli, M., Pavani, A., Mandini, M., Calderoni, P., and Brondelli, L. (1996). *Fertil. Steril.* **65,** 387–393.
35. Channing, C. P. and Kammerman, S. (1973). *Endocrinology* **92,** 531–540.
36. Maruo, T., Hayashi, M., Matsuo, H., Yamamoto, T., Okada, H., and Mochizuki, M. (1987). *Endocrinology* **121,** 1233–1241.
37. Saito, S., Saito, H., Yamasaki, R., Hosoi, E., Komatsu, M., Iwahara, H., and Maeda, T. (1985). *J. Immunol. Methods* **79,** 173–183.
38. Maruo, T. and Mochizuki, M. (1987). *Am. J. Obstet. Gynecol.* **156,** 721–727.
39. Swat, W., Ignatowicz, L., and Kisielow, P. (1991). *J. Immunol. Methods* **137,** 850–856.
40. Yui, J., Garcia–Lloret, M., Wegmann, T. G., and Guilbert, L. J. (1994). *Placenta* **15,** 819–835.