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Differential Effects of Gonadotropin-Releasing Hormone (GnRH) Agonist on Ovarian Function in Early and Late Follicular Phase of Pregnant Mare Serum Gonadotropin(PMSG)-Pretreated Immature Rats

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PMSG로 전처치한 미성숙 래트의 초기 및 후기 난포기에 있어서 GnRH Agonist가 난소 기능에 미치는 상이 효과

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요 약

본 연구에서는 GnRH가 과배란 처치된 래트의 초기 난포기와 후기 난포기에서 난소기능에 어떠한 영향을 미치는지를 이해하기 위해서, 30IU PMSG와 10IU hCG로 전처치된 미성숙 래트에 있어서 배란반응, 배란 난자의 형태학적 이상 유무 및 핵 성숙도, 난소 중량, 난소의 조직학적인 변화 및 혈중 스테로이드 호르몬 (17β-estradiol, progesterone 및 testosterone) 농도에 대하여 GnRH agonist의 효과를 검사하였다. GnRH agonist는 PMSG 전처치 후 초기 난포기 (PMSG 투여 후 6시간부터) 또는 후기 난포기(PMSG 투여 후 54시간부터)에 4시간 동안 20분 간격으로 경정맥 카테타를 통해 혈관내로 투여하였다. 각 실험동물은 혈중 스테로이드 호르몬의 변화를 측정하기 위하여 PMSG 투여 후 54시간, 72시간에 혈액을 채취하고 72시간에 희생시켰다. PMSG로 전처치한 미성숙 래트의 초기 난포기에 GnRH agonist의 투여는 GnRH agonist를 투여하지 않은 군(대조군)에 비해 과배란 억제, 형태학적 비정상 배란난자의 증가, 난소 중량의 감소, 난포폐쇄의 증가 및 혈중 스테로이드 호르몬의 농도 감소가 보였다. 한편 후기 난포기에 GnRH agonist의 투여는 대조군에서의 반응과 전반적으로 유사하였다. 이상의 결과, PMSG 및 hCG 처치로 과배란된 래트의 초기 난포기에 GnRH agonist의 투여는 난소기능을 전반적으로 억제하지만, 후기 난포기에 GnRH agonist의 투여는 난소기능에 영향을 미치지 않았다.

(Key words : GnRH agonist, ovarian function, PMSG, superovulation, rat)

INTRODUCTION

Superovulation technique, using pregnant mare serum gonadotropin (PMSG), has been widely used in many laboratories and livestock industries to obtain multiple oocytes and embryos.

However, these superovulation protocols have been reported to result in reduced fertility in large domestic (Armstrong and Evans, 1983; Evans and Robinson, 1980) and small laboratory animals (Yun and Kwun, 1984; Sherman *et al.*, 1982; Miller and Armstrong, 1981; Greenwald, 1976; Beaumont and Smith, 1975). In immature rats, superovulatory doses of PMSG lead to a progressive failure in early stages of pregnancy (Yun and Kwun, 1984). This failure was shown to be due to excessive estrogenic stimulation of genital tract following ovulation and asynchrony in embryonic and uterine development by disturbances in the oviductal and uterine environment (Walton and Armstrong, 1982). Early defects following superovulation with PMSG could primarily occur in the process of follicular development and oocyte maturation before ovulation by hyperstimulation of ovarian tissues (Moon *et al.*, 1990; Yun *et al.*, 1989). Since PMSG which has long biological half-life due to high glycosylation induces excessive ovarian steroidogenesis, pre- or periovulatory endocrine aspects including the excessive secretion of follicular steroids are related to affect the final maturation, subsequent fertilization, and developmental capability of ovulated oocytes (Moon *et al.*, 1990).

The use of hypothalamic regulator or pituitary gonadotropin preparations with shorter biological half-life might overcome some of the endocrine problems associated with the use of PMSG. It is reported that the employment of gonadotropin-releasing hormone (GnRH) agonists in combination with exogenous gonadotropin stimulation had gained multiple oocytes and embryos (Hardy and Hornstein, 1995; Martin *et al.*, 1993; Penzias *et al.*, 1993; Feldberg *et al.*, 1990; Palermo *et al.*, 1988; Neveu *et al.*, 1987) in human *in vitro* fertilization and embryo tranfer (IVF & ET) program. This protocol of ovulation induction reduces the risk of a premature luteinizing hormone (LH) surge and when performed for IVF & ET (Filicori *et al.*, 1991), increases the average number of oocytes obtained at retrieval (Penzias *et al.*, 1993). Besides, administration of GnRH agonists in late follicular phase of IVF patients increases serum levels of endogenous LH and follicle stimulating hormone (FSH) similar to a spontaneous gonadotropin surge with subsequent retrieval of mature oocytes which are capable of fertilization, implantation, and normal pregnancy (Segal and Casper, 1992; Testart *et al.*, 1989).

Therefore, the objective of this study is to investigate the GnRH action in early and late follicular phase on the ovarian function of superovulated immature rats treated with PMSG. In detail, this study examined the effects of GnRH agonist on the ovulatory response, the morphological normality and nuclear maturation of ovulated oocytes, the ovarian weight, the ovarian histology, and the circulating steroid hormone (17β-estradiol, progesterone and testosterone) levels in these rats.

MATERIALS AND METHOD

Immature female Sprague-Dawley rats were kept under temperature- and light-controlled conditions (20~25℃, 12L : 12D) and fed standard rat chow and water *ad libitum* throughout the experimental period. One day before the experiment (on the age of 27 days), the animals were installed with chronically indwelling catheters using the method described by Harms and Ojeda (1974). Briefly, a catheter made of silastic tubing (Dow-Corning Corp., Midland, MI) was slid into the external jugular vein to enter or approach the right atrium under pentobarbital anaesthesia (35mg/kg body wt.). To

prevent clot formation in the catheter, catheter was filled with dilute 25IU/ml heparin- saline solution.

On the following day (day 28 of age), the rats received a single subcutaneous dose (30IU/0.2 ml saline) of PMSG (Equinex, Ayerst, Montreal, Quebec, Canada) for superovulation induction. Following PMSG administration, the animals were treated with 100ng or 500ng GnRH agonist (pGLU-HIS-TRP- SER-TYR-$_D$-ALA-LEU-ARG-PRO-NHEt, Sigma Chemical Co., St. Louis, MO) starting at 6hr (in early follicular phase) and 54hr (in late follicular phase) after PMSG injection. GnRH agonist was intravenously injected via the jugular vein catheter every 20min for 4hrs in 0.1ml of sterile saline. The females were subcutaneously injected with 10IU human chorionic gonadotropin (hCG, Sigma Chemical Co., St. Louis, MO) in 0.2 ml sterile saline at 54hr to ensure ovulation. In order to analyze circulating steroid hormone (17β-estradiol, progesterone and testosterone) concentration, 0.5ml of whole blood was collected from females via the jugular catheter at 54hr and 72hr. The blood was allowed to stand at room temperature for 6~8hr to clot. The samples were centrifugated at 2,000rpm for 20min to separate serum. The sera were stored at -20℃ for subsequent assay of steroid hormones using radioimmunoassay (RIA).

Animals were sacrificed at 72hr after PMSG injection. To measure ovarian weight, ovaries dissected free from oviducts were cleaned of ovarian bursae, connective tissue and fat. They were then dried, paired and weighed in mg tissue. At the same time, to count oviductal oocytes, oviducts were separated from uterine horns at the uterotubal junction and flushed with a few drops of Dulbecco's phosphate-buffered saline (DPBS) under a stereo dissecting microscope (10×magnification, Nikon, SMZ-U), as described previously by Yun (1994). Subsequently, in order to facilitate the oocyte counting, the extracoronal cumulus cells surrounding the oocytes were dispersed after being exposed to DPBS solution containing 0.1% of hyaluronidase (type I-S: from bovine testes, Sigma Chemical Co., St. Louis, MO) for 10~15min. The recovered oocytes were counted under the stereo dissecting microscope (40× magnification), and were subjected to assess the occurrence of fragmentation and other degenerative changes. Briefly, those eggs showing an irregular cell mass with debris and empty zona pellucida were classified as abnormal.

To assess oocyte nuclear maturation, the recovered normal appearing oocytes were stained as described previously (Yun, 1994). Briefly, only normal appearing oocytes were placed into a 10×35mm petri dish containing 1.0% hypotonic sodium citrate and swollen at room temperature for 10min. Subsequently, the oocytes were transferred onto a grease-free slide with a thin coat of Mayer's albumen. The oocytes were allowed to dry over a hot plate (45~53℃) to enhance chromosome spreading. Then the dried oocytes were fixed with acetic alcohol (one part of glacial acetic acid and two parts of absolute ethyl alcohol) for 45min, and stained with 2% aceto-orcein for 30min. A stock solution of this stain had been made up in a fumehood by dissolving 2.2g of orcein(BDH Chemical Ltd. Toronto, Ontario) in 45ml of hot glacial acetic acid with continuous stirring for 30min and by adding 55ml of 0.9% NaCl solution. A working staining solution was prepared immediately before use by mixing two parts of the stock solution with three parts of 0.9% NaCl solution and by filtering with 0.8μm syringe filter. Finally, the stained oocytes were

allowed to a serial dehydration through 50%, 60%, 80% and 100% ethyl alcohol followed by xylene for 5min in each step, and subjected to a microscopic evaluation of nuclear maturation. Various stages of meiosis were identified according to the criteria described previously (Yun, 1994).

To observe ovarian histology, ovaries obtained at 72hr after PMSG injection were immidiately fixed in Bouin's solution (75% saturated picric acid, 20% buffered formalin and 5% glacial acetic acid) for about 6hr and washed for 12hr in 70% ethanol to remove excess fixation. The ovarian tissue was subsequently dehydrated in sequential concentrations of ethanol (70%, 80%, 90% and 100%), cleared, and embedded in paraffin wax. Then serial sections of the tissue block were cut 5 μm thick and stained with hematoxylin and eosin. All sections were examined for evidence of precocious ovulation or follicular atresia, and representative sections were taken for photomicroscopy (Olympus, BX50, Japan). Advanced stages of follicular atresia were defined by nuclear pyknosis and a loss of homogeneity of the granulosa cell layers in conjunction with thecal hypertrophy and varying degrees of oocyte degeneration.

In order to determine serum steroid hormones (17β-estradiol, progesterone and testosterone), 0.5ml aliquots of sera were extracted with 3ml ethyl ether by vigorously vortexing. These extracts were evaporated for 60min and reconstituted in 1ml GPBS (buffer). 100μl aliquots of the extracts were assayed in duplicate for steroid hormones by specific RIA using the antisera. In the assay procedures, approximately 6,000 cpm of tracer (^{3}H) was added to each tube. The unbound steroid was removed by the 0.2ml cold dextran coated charcoal, and the bound steroid was counted in a liquid scintilation analyzer (TRI-CARB 2300 TR, Packard). The binding efficiency (%B_0) of the steroid antibodies was 40~50% and %NSB was less than 5%. The coefficient of variation (*CVs*) was 9~10% for 17β-estradiol, 8~9% for progesterone and 7~9% for testosterone, respectively. Hormone concentrations were expressed as ng/ml sera.

The significance of differences between the means of treatment groups were evaluated statistically by analysis of variance employing the LSD (Least Significant Difference) of Student's T test.

RESULTS

Oocyte count obtained after treatment with 500ng GnRH agonist in early follicular phase of superovulated rats (1.00±0.68 oocytes/rat) was significantly ($p<0.01$) decreased, compared to that from the superovulated rats of control group (51.50±4.66 oocytes/rat) that was not treated with GnRH agonist. However, the count obtained after treatment with 500ng GnRH agonist in late follicular phase of superovulated rats (49.29±7.17 oocytes/rat) was comparable to controls (Fig. 1).

As shown in Figure 2, the percentage of normal-appearing oocytes recovered from the rats treated with 500 ng GnRH agonist in early follicular phase (43.34±23.34%) was significantly ($p<0.05$) decreased, compared to that from control rats (86.79±2.43%). In contrast, morphological normality in the rats treated with 500ng GnRH agonist in late follicular phase (73.86±7.20%) was comparable to that obtained from controls.

The ovarian weight obtained in early follicular phase of superovulated rats treated with 500ng GnRH agonist (8.60±0.49mg) was significantly ($p<0.05$) decreased, compared to

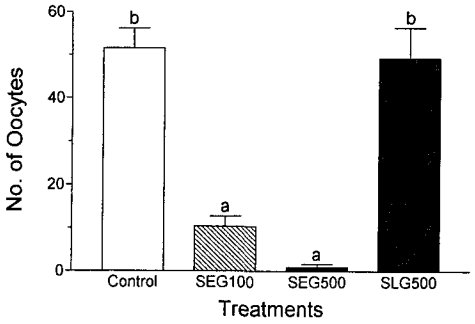


Fig. 1. Ovulatory responses after treatments with GnRH agonist in early or late follicular phase of immature rats pretreated with 30IU PMSG and supplemented with 10IU hCG. GnRH agonist was injected intravenously every 20min for 4hr from 6hr in early follicular phase (100ng GnRH agonist, SEG100 and 500ng GnRH agonist, SEG500) or from 54hr in late follicular phase (500ng GnRH agonist, SLG500) after PMSG pretreatment. Control rats were administered with saline at the same time of GnRH treatment. The number of oocytes recovered from oviduct at 72hr after PMSG pretreatment, is expressed as the mean±SE (n=8). The mean values with no superscripts in common are significantly different ($p<0.01$).

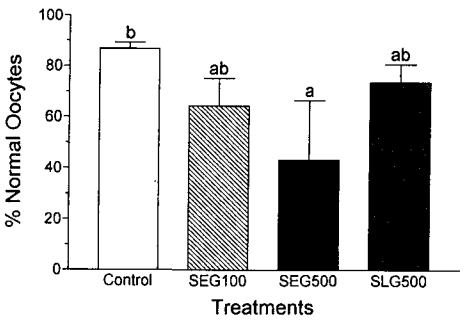


Fig. 2. Morphological normalities of recovered oocytes after treatments with GnRH agonist in early or late follicular phase of immature rats pretreated with 30IU PMSG and supplemented with 10IU hCG. GnRH agonist was injected intravenously every 20min for 4hr from 6hr in early follicular phase (100ng GnRH agonist, SEG100 and 500ng GnRH agonist, SEG500) or from 54hr in late follicular phase (500ng GnRH agonist, SLG500) after PMSG pretreatment. Control rats were administered with saline at the same time of GnRH treatment. The results of % normal oocytes recovered from oviducts at 72hr after PMSG pretreatment, are expressed as the mean±SE (n=8). The mean values with no superscripts in common are significantly different ($p<0.05$).

that from the rats of control group (21.58± 3.81mg). However, the ovarian weight obtained from superovulated rats treated with 500ng GnRH agonist in late follicular phase (19.30± 1.77mg) was comparable to that from control group (Fig. 3).

The oocytes recovered from oviducts after treatments with GnRH agonist in superovulated rats were classified for each stage of nuclear maturation. The proportion of normal-appearing oocytes analyzable for the classification of each stage was actually 90~100%, since some oocytes were lost or scattered their chromosomes by occasional rupture of cell membrane during the process of preparation and staining. Further more, the stained oocytes were actually not possible to distinguish between metaphase I and metaphase II, because of rapid deterioration or inconsistent formation of typical polar body. The percentages of oocyte nuclear maturation after treatments with GnRH agonist in early follicular phase of the control rats pretreated with PMSG are presented in Fig. 4. The nuclear maturation of superovulated oocytes recovered from the control rats pretreated with PMSG was characterized with prematurity and asynchronization: 20.30% in prophase I stage, 6.67% in anaphase I stage, 0.61% in telophase I stage, and 62.73% in metaphase I/II stage. The meiotically aberration phenomena of superovulated control oocytes were not altered by treatments with 100ng GnRH agonist. On the

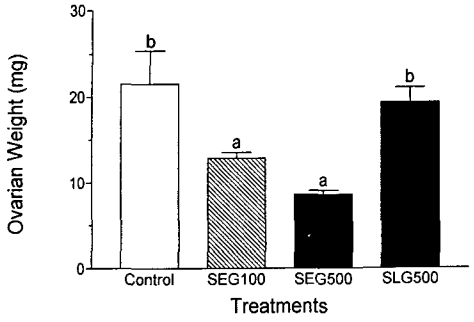


Fig. 3. Ovarian weights after treatments with GnRH agonist in early or late follicular phase of immature rats pretreated with 30IU PMSG and supplemented with 10IU hCG. GnRH agonist was injected intravenously every 20min for 4hr from 6hr in early follicular phase (100ng GnRH agonist, SEG100 and 500ng GnRH agonist, SEG500) or from 54hr in late follicular phase (500ng GnRH agonist, SLG500) after PMSG pretreatment. Control rats were administered with saline at the same time of GnRH treatment. The results of dried and paired ovarian weight measured at 72hr after PMSG pretreatment, are expressed as the mean±SE (n=8). The means with no superscripts in common are significantly different ($p<0.05$).

other hand, the oocytes recovered from 500ng GnRH agonist-treated rats consistently exhibited the stage of prophase I, while a total of only two normal-appearing oocytes were analyzed because of high recovery of abnormal oocytes. The nuclear maturation following the treatment with 500ng GnRH agonist in late follicular phase of PMSG-pretreated rats was also characterized with prematurity and asynchronization. However, the asynchronization of nuclear maturation found in this group was less pronounced than that from the rats treated with 500ng GnRH agonist in early follicular phase. Each percentage of oocyte nuclear maturation in this group was 41.67% in prophase I stage, 4.17% in anaphase I stage, 0.00% in Telophase I stage, and 54.17% in Metaphase I/II stage.

In ovarian histology, a majority of the follicles in superovulated control rats pretreated with 30IU PMSG were in tertiary stage of growth (Fig. 5). The follicles exhibited not only healthy corpora lutea but a few signs of atresia. In contrast, ovarian morphology after treatment with GnRH agonist in early follicular phase of superovulated rats displayed lots of atretic follicles with granulosa cells containing pronounced pyknotic nuclei (Fig. 6). The granulosa layers of the many atretic follicles were characterized to be thinned with the distinct signs of degeneration. There was also a marked loss of structural integrity in the cumulus oophorus granulosa cells. Some large Graafian follicles had become luteinized with non-ovulated and fragmented oocytes. On the other hand, the follicles obtained from the rats treated with 500ng GnRH agonist in late follicular phase were found to be tertiary stage of growth undergoing a few signs of atresia. However, the atresia found in this group was less pronounced than that from the rats treated with 500ng GnRH agonist in early follicular phase (Fig. 7).

The concentrations of steroid hormones (17β-estradiol, progesterone and testosterone) in blood of the rats treated with GnRH agonist in early and late follicular phase during preovulatory period are presented in Fig. 8. The serum levels of steroid hormones after treatment with 500ng GnRH agonist in early follicular phase of PMSG-pretreated rats were measured at 54hr after PMSG. The mean levels of serum steroid hormones in the rats treated with 500ng GnRH agonist were significantly ($p<0.05$) decreased, compared to those from control group; the levels of 17β-estradiol, progesterone and testosterone were 0.23±0.07ng/ml, 32.86±1.58ng/ml and 4.26±0.94ng/ml in control rats, and 0.08±0.03ng/ml, 12.70±3.47ng/ml and 2.23±0.67ng/

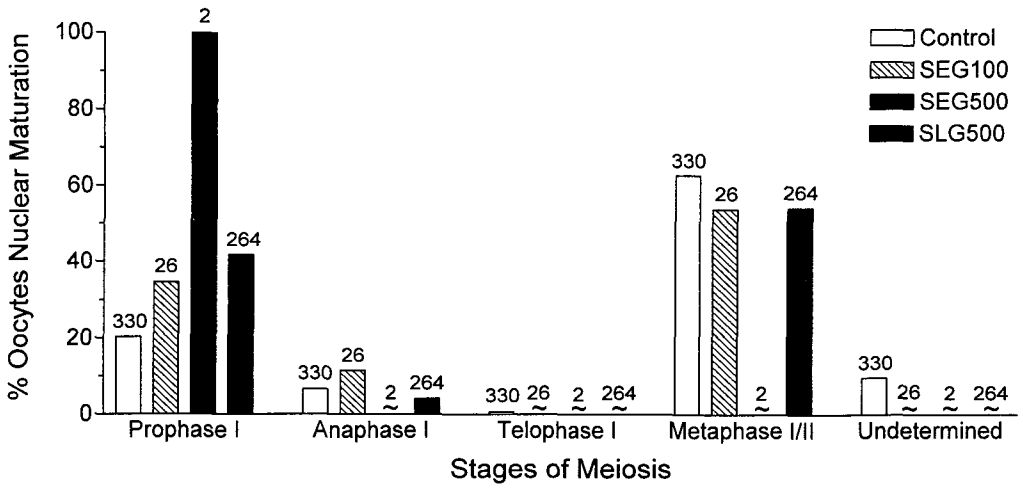
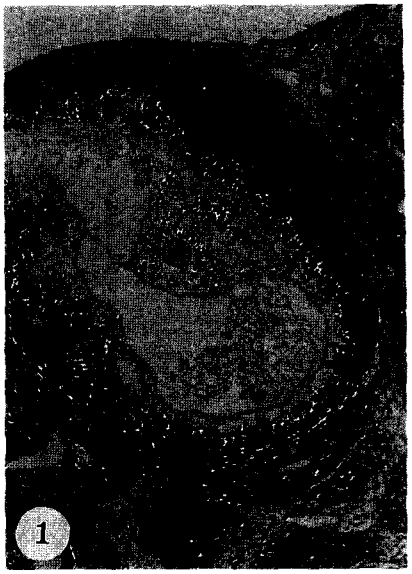


Fig. 4. Nuclear maturation of recovered oocytes after treatments with GnRH agonist in early or late follicular phase of immature rats pretreated with 30IU PMSG and supplemented with 10IU hCG. GnRH agonist was injected intravenously every 20min for 4hr from 6hr in early follicular phase (100ng GnRH agonist, SEG100 and 500ng GnRH agonist, SEG500) or from 54hr in late follicular phase (500ng GnRH agonist, SLG500) after PMSG pretreatment. Control rats were administered with saline at the same time of GnRH treatment. The number above each bar represents oocyte counts examined; the oocytes recovered with visible signs of degeneration were excluded. ~denotes 0%.

ml in the rats treated 500ng GnRH agonist in early follicular phase. On otherwise the mean levels of serum steroid hormones in the rats treated with 500ng GnRH agonist in late follicular phase was comparable to that from control group; the levels of 17β-estradiol, progesterone and testosterone in this group were 0.16±0.06ng/ml, 31.94±1.66ng/ml and 4.29 ±0.71ng/ml. The levels of steroid hormones (17 β-estradiol, progesterone and testosterone) in circulating blood during postovulatory period are presented in Fig. 9. The serum levels of steroid hormones after treatment with 500ng GnRH agonist in early follicular phase of PMSG-pretreated rats measured at 72hr after PMSG. The mean levels of 17β-estradiol and progesterone in the rats treated with 500ng GnRH agonist were comparable to that from control group; the levels of 17β-estradiol and progesterone were 0.14±0.01ng/ml and 35.34± 5.79ng/ml in control rats, and 0.14±0.02ng/ml and 37.94±4.71ng/ml in the rats treated 500ng GnRH agonist in early follicular phase. However, the concentration of testosterone in the rats treated with 500ng GnRH agonist was significantly ($P<0.05$) decreased, compared to that from control group; the level of testosterone was 3.42±0.45ng/ml in control rats and 1.21±0.13ng/ml in the rats treated with 500ng GnRH agonist in early follicular phase. On otherwise the mean levels of serum steroid hormones in the rats treated with 500ng GnRH agonist in late follicular phase, except for 17β -estradiol, were significantly ($P<0.05$) decreased, compared to those from control group; the levels of 17β-estradiol, progesterone and testosterone in this group were 0.20±0.08ng/ml, 10.04±2.65ng/ml and 0.38±0.10ng/ml.

Fig. 5. Light microscopic analysis of ovarian morphology obtained at 72hr after 30IU PMSG supplemented with 10IU hCG in the immature r The sections (5μm) were stained with hematoxyl and eosin. ① Ovary showing a large tertiary follicle. Magnification, ×100. ② Normal oocyte. Magnification, ×400.



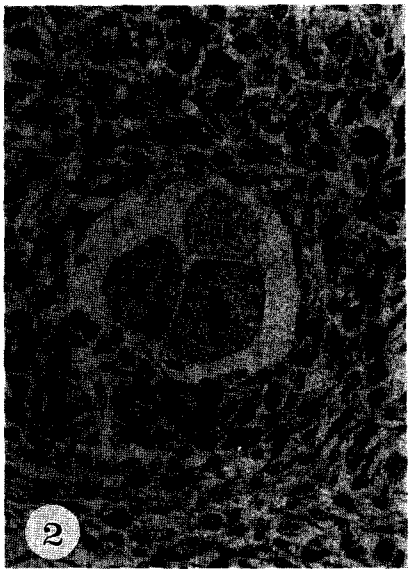


Fig. 6. Light microscopic analysis of ovarian morphology obtained at 72hr after treatment wit 500ng GnRH in early follicular phase of the immature rat pretreated with 30IU PMSG a supplemented with 10IU hCG. Sections (5μm) w stained with hematoxylin and eosin. ① Atretic antral follicle with typical atretic signs. Magnification, ×100. ② Corpus luteum with non-ovulated and fragmented oocyte. Ma fication, ×400.

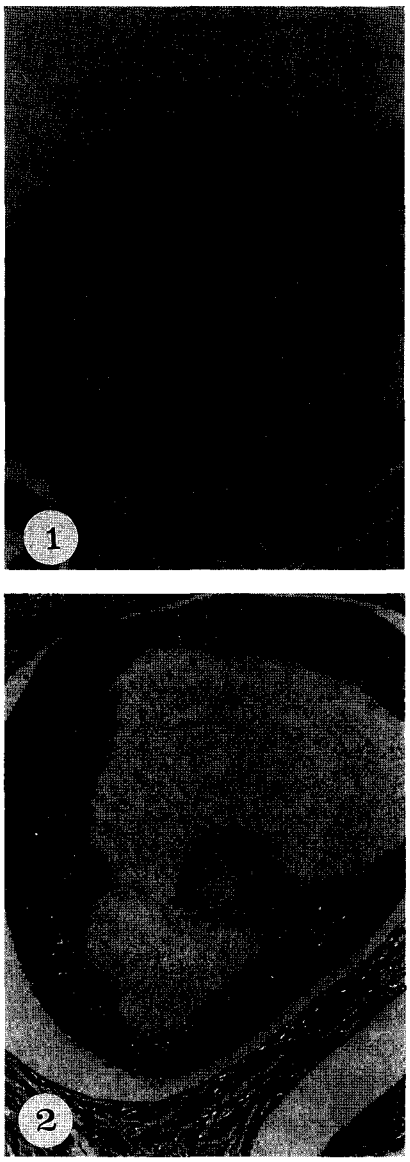


Fig. 7. Light microscopic analysis of ovarian morphology obtained at 72hr after treatment wit 500ng GnRH in late follicular phase of the immature rat pretreated with 30IU PMSG a supplemented with 10IU hCG. The sections (5μ were stained with hematoxylin and eosin. ① Healthy antral follicle. Magnification, ×100. ② Healthy corpus luteum. Magnification, ×100.

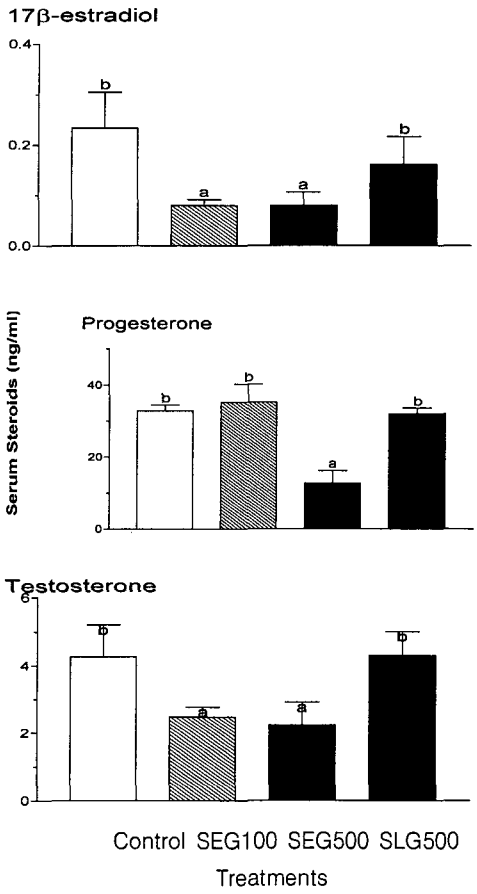


Fig. 8. Preovulatory serum levels of 17β -estradiol, progesterone and testosterone after treatments with GnRH agonist in early or late follicular phase of immature rats pretreated with 30IU PMSG and supplemented with 10IU hCG. GnRH agonist was injected intravenously every 20min for 4hr from 6hr in early follicular phase (100ng GnRH agonist, SEG100 and 500ng GnRH agonist, SEG500) or from 54hr in late follicular phase (500ng GnRH agonist, SLG500) after PMSG pretreatment. Control rats were administered with saline at the same time of GnRH treatment. The levels of serum steroids measured at 54hr after PMSG pretreatment, are expressed as the mean±SE (n=5). The mean values with no superscripts in common are significantly different ($p<0.05$).

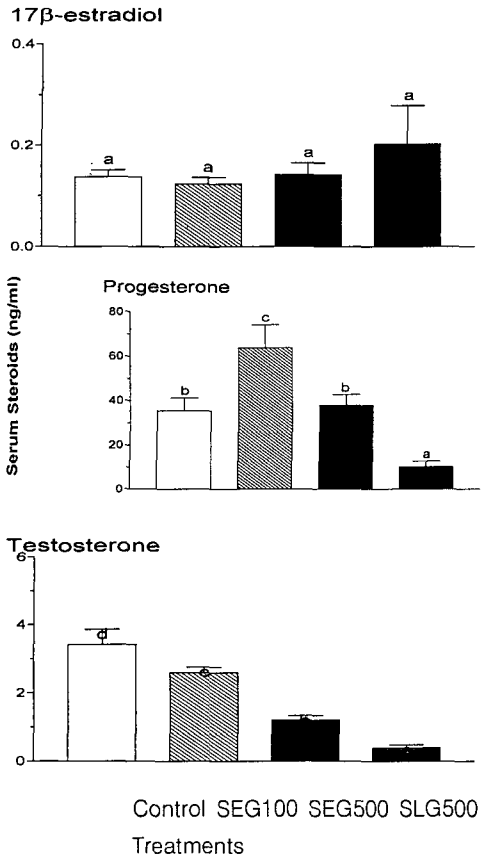


Fig. 9. Postovulatory serum levels of 17β-estradiol, progesterone and testosterone after treatments with GnRH agonist in early or late follicular phase of immature rats pretreated with 30IU PMSG and supplemented with 10IU hCG. GnRH agonist was injected intravenously every 20min for 4hr from 6hr in early follicular phase (100ng GnRH agonist, SEG100 and 500ng GnRH agonist, SEG500) or from 54hr in late follicular phase (500ng GnRH agonist, SLG500) after PMSG pretreatment. Control rats were administered with saline at the same time of GnRH treatment. The levels of serum steroids measured at 72hr after PMSG pretreatment, are expressed as the mean±SE (n=5). The mean values with no superscripts in common are significantly different ($p<0.05$).

DISCUSSION

In the current experiment, the ovulatory responses of immature rats treated with 30IU PMSG and supplemented with 10IU hCG were quite similar to the results reported previously (Yun *et al.*, 1987). This hormone treatment certainly induced superovulation at the average of 51 oocytes per rat. Besides of superovulation, PMSG treatment increased the proportion of abnormal appearing oocytes, and caused premature or asynchronous nuclear maturation of ovulated oocytes as confirmed previously (Yun *et al*, 1989; Yun *et al.*, 1987).

The results of this study have shown that treatment with GnRH agonist in early follicular phase of the immature rats superovulated with PMSG markedly inhibited ovulatory response, compared to that from control rats that was not treated with GnRH agonist. In addition, treatment with GnRH agonist in PMSG- pretreated immature rats greatly increased the percentage of abnormal oocytes. These results were further confirmed by a marked decrease of ovarian weight and histological finding of ovaries characterized by a follicular atresia with pyknosis in granulosa cells. Furthermore, the treatment with GnRH agonist in the rat superovulated with PMSG distinctly decreased serum steroid hormones (17β-estradiol, progesterone and testosterone) during preovulatory period. These results indicate that *in vivo* the treatment of GnRH agonist in early follicular phase has an overall inhibitory effect on the ovarian function in immature rats superovulated with PMSG. Vickery (1986) reported that continuous administration of GnRH agonist decreased ovarian estrogens, progesterone and androgens production by interfering with ovarian function. A GnRH-induced decrease in estrogen production offers an explanation for

blocking effect of GnRH on various estrogen-dependent ovarian functions including folliculogenesis and ovarian weight gain. As for the possible mechanism involved in the inhibitory effect of GnRH in early follicular phase, GnRH be relate to a decrease intracellular cyclic adenylate monophosphate (cAMP) levels by inhibiting adenylate cyclase activity and elevating phosphodiesterase activity(Erickson *et al.*, 1994; Knecht *et al.*, 1982). The adenylate cyclase system plays an important role in the hormonal activation of steroidogenesis in granulosa cells (Knecht *et al.*, 1981). Therefore, antigonadal action of GnRH and its agonists are probably expressed through inhibition of cyclic nucleotide production, especially, cAMP production. Thus, GnRH and its agonist seem to have adverse effects on steroidogenesis *in vivo* and consequently interfere with follicular development.

The ovulatory response and morphological normality of ovulated oocytes were not changed by GnRH agonist treatment in late follicular phase of immature rats pretreated with PMSG. In addition, the treatment of GnRH agonist in late follicular phase of PMSG-pretreated rats did not show any significant change ovarian weight and ovarian morphology, compared to those from control rats. These results had been further evidenced by the concentrations of circulating steroid hormones during preovulatory period that were similar to those from control rats. These results indicate that inhibitory action of GnRH on ovarian functions occurrs in early follicular phase but not in late follicular phase. Knecht *et al.* (1981) have reported that almost complete inhibition of FSH action and granulosa cell differentiation was occurred in early follicular phase after FSH treatment followed by GnRH agonist. However, the treatment of GnRH agonist in mid- and late-follicular phase caused only cessation of granulosa cell maturation. GnRH stimulates not only progesterone and androgens secretion but causes a general stimulation of follicular steroidogenesis in isolated preovulatory rat follicles (Hillenjo *et al.*, 1984). The mechanism for the GnRH stimulation of steroid production is not fully understood. It is demonstrated that GnRH stimulates certain steroidogenic enzymes in granulosa cells of rat preovulatory follicle, notably on 3β-hydroxysteroid-dehydrogenase (3β-HSD) (Jones and Hsueh, 1981) and 20α-hydroxysteroid-dehydrogenase (20α-HSD) (Jones and Hsueh, 1982). Furthermore, GnRH agonist stimulates 20α-OH-progesterone accumulation in isolated preovulatory rat follicular cells (Hillenjo *et al.*, 1984). Those enzymes and this hormone play an important role in the follicular steroidogenesis. Thus, in the present study, the treatment of GnRH agonist in late follicular phase seems to have no adverse effects on ovarian functions *in vivo*.

Ovarian morphology after treatment with GnRH agonist in early follicular phase of superovulated rats displayed lots of atretic follicles. In this current experiment, a significant finding is that GnRH agonist induced pyknosis in granulosa cell. Since pyknosis is typical indicator of follicular atresia, it has been suggested that GnRH may act as an atretogenic factor for follicles. Billig *et al.* (1994) have demonstrated that GnRH acts as an atretogenic factor, because GnRH agonist increased apoptotic DNA fragmentation in the ovarian cells with a time- and dose-dependent manner. It is noteworthy that Erickson *et al.* (1994) have shown that GnRH treatment of hypophysectomized estrogen-primed rats caused a rapid and dramatic decrease in the mitotic index of granulosa cells of atretic as well as healthy antral follicles, followed by a stimulation of

granulosa cell pyknosis. Furthermore, they have suggested that autocrine and paracrine secretion of ovarian GnRH might cause atresia by mechanisms involving increased insulin-like growth factor-binding protein-4 (IGFBP-4) synthesis, a physiological marker of atresia; GnRH agonist stimulates the expression of IGFBP-4 protein in rat granulosa cells *in vitro*, and abolishes the ability of FSH to inhibit IGFBP-4 expression and to induce IGFBP-4 protease activity. However, this mechanism involved in GnRH action in the granulosa cells is not yet clear.

In summary, the overall results in this study indicate that GnRH agonist disturbs overall ovarian functions in early follicular phase but not in late follicular phase of superovulatory dose of PMSG-pretreated immature rats. This concept has been further evidenced by the findings of a great decrease in ovarian weight, increase in atresia of follicle, and a significant decrease in circulating steroid hormones (17β-estradiol, progesterone and testosterone) in immature rats treated with GnRH agonist in early follicular phase. These data provide a basis for explaining the inhibitory effects of GnRH on reproductive functions, and for future studies on the mechanism involved in GnRH regulation of the ovary.

CONCLUSION

In the present study, to understand how gonadotropin-releasing hormone (GnRH) affects ovarian functions in superovulated rats, we examined the effects of GnRH agonist treatment on the ovulatory response, the morphological normality and nuclear maturation of ovulated oocytes, the ovarian weight, the ovarian histology, and the circulating steroid hormone (17β-estradiol, progesterone and testosterone) levels in immature rats pretreated with 30IU pregnant mare serum gonadotropin (PMSG) and supplemented with 10IU human chorionic gonadotropin (hCG). GnRH agonist was intravenously injected via jugular vein catheter every 20min for 4hr in the early follicular phase (from 6hr after PMSG) or in the late follicular phase (from 54hr after PMSG) of superovulatory dose of PMSG-pretreated immature rats. All animals were sacrificed at 72hr after PMSG adminstration.

The multiple administration with GnRH agonist in early follicular phase of immature rats pretreated with superovulatory dose of PMSG caused a marked inhibition of ovulatory response. It was also found that treatment with GnRH agonist in early follicular phase increased the proportion of abnormal appearing oocytes (especially, in the rats of the group treated with 500ng GnRH agonist), compared to that from the rats of control regimen that was not treated with GnRH agonist. Furthermore, the treatment with GnRH agonist in early follicular phase caused a great decrease of ovarian weight, a marked promotion of follicular atresia, and a significant decrease in circulating steroid hormones (17β-estradiol, progesterone and testosterone) in preovulatory phase, compared to control group. On the other hand, the treatment with GnRH agonist in late follicular phase produced the ovulatory response and the morphological normality of ovulated oocytes similar to that from control group. As well, the ovarian weight in the rats treated with GnRH agonist in late follicular phase was comparable to that from control regimen. In ovarian morphology, a majority of follicles in the ovary obtained from GnRH agonist treatment in late follicular phase were tertiary follicles with a few atretic follicles. The nuclear maturation of oocytes recovered from oviducts in immature

rats from GnRH agonist treatment group was characterized by prematurity and asynchronization in both follicular phases, which were similar to control group. Accordingly, there was no significant difference in the effect of GnRH agonist treatment in both follicular phases examined in this study on the nuclear maturation of ovulated oocytes in immature rat.

The overall results in this study indicate that GnRH agonist disturbs ovarian functions in early follicular phase but not in late follicular phase of superovulation pretreated immature rats in terms of ovulatory response and morphological normality of ovulated oocytes. This concept has been further evidenced by the findings of a great decrease in ovarian weight, a marked promotion of follicular atresia, and an obvious decrease in circulating preovulatory steroid hormones (17β-estradiol, progesterone and testosterone) following GnRH agonist treatment in early follicular phase.

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