Experimental Animals
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Effect of Cetrorelix administration on ovarian stimulation in aged mice

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Abstract: In mice, ovarian stimulation via hormone administration is an effective method for obtaining many ova simultaneously, but its effect is reduced by the influence of aging. In this study, we demonstrate that this problem can be improved by administering the gonadotropin-releasing hormone antagonist Cetrorelix prior to ovarian stimulation. Before 12-month-old female mice were injected with 5 IU pregnant mare serum gonadotropin and 5 IU human chorionic gonadotropin, we administered 5 μg/kg Cetrorelix for 7 consecutive days (7 times) or 3 times once every 3 days. As a result, 8.7 ± 1.9 (mean ± SEM, n=10) and 9.8 ± 1.3 (n=10) oocytes were obtained, respectively, as opposed to 4.7 ± 1.2 oocytes (n=9) in the case of no administration. Collagen staining of ovarian tissue showed that Cetrorelix administration reduced the degree of fibrosis, which improved ovarian function. In addition, equivalent fertilization and fetal development rates between control and Cetrorelix-treated aged mouse-derived oocytes were confirmed by *in vitro* fertilization and embryo transfer (Fertilization rate; control: 92.2% vs. 3 times: 96.9%/7 times: 88.5%, Birth rate; control: 56.4% vs. 3 times: 58.3%/7 times: 51.8%), indicating the normality of the obtained oocytes. It is concluded that Cetrorelix improved the effect of superovulation in aged mice without reducing oocyte quality. This procedure will contribute to animal welfare by extending the effective utilization of aged female breeding mice.
Key words: aging, fibrosis, murine, ovarian stimulation

Introduction

The female reproductive cycle is controlled by the hypothalamic-pituitary-gonadal axis [1–3]. Both this cycle and superovulation can be activated by treatment with gonadotropin. Superovulation is an effective method for collecting large numbers of meiosis II oocytes, which have the potential to undergo normal fertilization and development, from various experimental animals [4–8]. Superovulation has been induced in rats and mice by the administration of pregnant mare serum gonadotropin (PMSG) to stimulate follicular growth and the maturation of ovarian follicles, followed by treatment with human chorionic gonadotropin (hCG) to induce ovulation. In sexually mature mice, approximately 10 oocytes can be collected during natural ovulation, while 20–30 oocytes can be collected during superovulation [9]. Therefore, the use of superovulation has reduced the number of mice needed for experiments.

In female mice, reproductive capacity declines with aging, and various phenomena related to this decline are observed, such as delayed estrous cycle, decreased fertility rate and litter size after natural mating, reduced fertility rate after in vitro fertilization (IVF), decreased number of superovulated oocytes, and increased embryonic mortality after implantation [10, 11]. In humans, the follicular phase is shortened and the length of the menstrual cycle is increased with aging [12]. As aging proceeds in humans, serum follicle-stimulating hormone (FSH) levels tend to rise [13–15], which impairs the responsiveness of follicular development during ovarian aging and decreases the secretion of inhibin by reducing

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the number of follicles [12]. Although rising serum FSH levels increase the number of growing follicles, almost all of them are thought to regress before follicular maturation [16]. With aging, there is an accumulation of extracellular matrix in ovarian stroma, which decreases ovarian function as a result of fibrosis [17].

Gonadotrophin-releasing hormone (GnRH) analogs (agonists and antagonists), which control the secretion of gonadotropin, have been used for infertility treatment in assisted reproductive technology [18]. In addition, GnRH antagonists have been used to control ovulation in humans and mice [19, 20]. The administration of a GnRH antagonist to a mouse model of aging reduced ovarian fibrosis and increased the number of ovulated oocytes and pups [17]. It was also reported that the estrous cycle could be synchronized by progesterone and prostaglandin administration, and that the number of normal superovulated oocytes was increased by the administration of anti-serum inhibin (AIS) [21, 22].

The C57BL/6J inbred mouse strain has been used in a wide range of research fields as a major experimental animal. In the reproductive research field, it is difficult to continually collect a sufficient number of oocytes from mice, even when superovulation is induced, because reproductive efficiency declines with age. Generally, aged mice are treated as retired animals by breeders and researchers aiming to collect oocytes. Given that aged mice exhibit high levels of endogenous FSH, which inhibits the formation of mature oocytes [23]. We hypothesized that responsiveness to administered gonadotropins could be improved by inhibiting the secretion of FSH. Therefore, in the present study, we investigated whether a GnRH antagonist, Cetrorelix, could improve the effect of superovulation treatment in aged mice.

Materials and Methods

Animals

C57BL/6J Jcl and Jcl:ICR mice purchased from CLEA Japan, Inc. (Tokyo, Japan) and bred at the Kasumi Animal Center of the Natural Science Center for Basic Research and Development at Hiroshima University. To examine superovulation, 16- and 52-week-old C57BL/6J females were used as young and aged mice, respectively. To prepare pseudopregnant females for the embryo transfer procedure, mature females and vasectomized males of the ICR strain were used. All experiments were carried out with the permission of the Committee of Animal Experimentation at Hiroshima University (approval number: A14-7).

Superovulation and oocyte collection

To collect mature meiosis II oocytes, female mice were injected intraperitoneally with 5 IU PMSG (ASKA Pharmaceutical, Tokyo, Japan) approximately 17:00 followed by injection with 5 IU hCG (ASKA Pharmaceutical) 48 h later. At 16 h after hCG injection, the mice were sacrificed by cervical dislocation, and ovulated oocytes were collected from the oviduct ampulla in 250 μl human tubal fluid (HTF) medium. After IVF, oocytes without fragmentation or degeneration were considered as normal.

Cetrorelix administration

To investigate the effect of a GnRH antagonist on the number of ovulated oocytes, 5 μg/kg Cetrorelix (Nippon Kayaku, Tokyo, Japan) was injected intraperitoneally at approximately 17:00 once a day or once every 3 days for 1 week before PMSG and hCG administration. The experimental design of this study is shown in Fig. 1.

Sperm collection and IVF

Sperm were collected from the cauda epididymis using a 26 G syringes into 250 μl HTF medium which was modified with a 1/3 concentration of 1.7 mM $CaCl_2$ and without sodium lactate, and were incubated for 1.5 h at 37°C in 5% CO_2 to induce capacitation. After incubation, the collected oocytes were inseminated with 1.0×10^5 sperm/ml in HTF medium for 6 h at 37°C in 5% CO_2. To allow embryonic development to proceed, the zygotes were washed and incubated at 37°C in 5% CO_2 in KSOM medium.

Day	1	2	3	4	5	6	7	8	9	10	11
Administration	Cetrorelix							PMSG		hCG	
Control	-	-	-	-	-	-	-	●	-	●	Examination (Oocyte collection, IVF, Embryonic transfer)
Cetrorelix 3 times	●	-	-	●	-	-	●	●	-	●	
Cetrorelix 7 times	●	●	●	●	●	●	●	●	-	●	

Fig. 1. Time course of ovarian stimulation with and without Cetrorelix. Cetrorelix was injected at approximately 17:00, and pregnant mare serum gonadotropin (PMSG) was injected 24 h later. At 48 h after PMSG injection, human chorionic gonadotropin (hCG) was injected. At 16 h after hCG injection, oocytes were collected and examined.

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Embryo transfer

The embryos that had developed to the 2-cell stage by IVF were transferred into the oviducts of day 1 pseudo-pregnant ICR strain mice under anesthesia. On day 20, live offspring were confirmed by natural birth or cesarean section.

Frozen sections

After washing the ovaries in phosphate-buffered saline (PBS), they were fixed with 4% paraformaldehyde in PBS overnight at 4°C. The ovaries were cryoprotected by incubation in 30% sucrose in PBS overnight at 4°C. Next, they were embedded in optimal cutting temperature compound (Sakura Seiki, Tokyo, Japan) and frozen in liquid nitrogen. Sections (7 μm thick) were cut with a CM1800 cryostat (Leica, Nussloch, Germany), and the sections were placed on CREST coated glass slides (Matsunami Glass, Osaka, Japan) and dried.

Phalloidin staining

Ovarian fibrosis was detected by phalloidin staining (Life Technologies, Carlsbad, CA) [17]. All ovaries of control mice and mice administered Cetrorelix 7 times were weighed at 2 days after PMSG administration, and sectioned. After washing the frozen sections twice in PBS, they were permeabilized with 0.2% Triton X-100 (Fujifilm Wako Pure Chemicals, Osaka, Japan) in PBS for 10 min and treated with 3% bovine serum albumin for 30 min. Phalloidin staining was performed as described previously [7]. The phalloidin positive areas in the ovaries were measured by the area measurement tool of a BZ9000 fluorescence microscope (Keyence, Osaka, Japan).

Statistical assessment

The data shown in Table 1 and Fig. 2 were analyzed using Student's *t*-test, whereas as the data shown in Tables 2 and 3 were analyzed using one-way analysis of variance followed by Dunnett's *post hoc* test. $P<0.05$ was considered statistically significant.

Results

Comparison of the number of superovulated oocytes between young and aged mice

To investigate whether superovulation was affected in aged C57BL/6J mice, we compared the number of oocytes collected from young (16 weeks old) and aged (52 weeks old) mice after the administration of PMSG and hCG. The results showed that the number of mice in which ovulation was induced was not significantly different between both groups, but the numbers of collected oocytes and normal oocytes were significantly reduced in aged mice compared with young mice (Table 1, $P<0.05$). This indicated that superovulation was reduced with aging in C57BL/6J mice.

Analysis of the effects of Cetrorelix on superovulation

To examine whether Cetrorelix contributed to the recovery of archegonium function in aged mice, we investigated the number of oocytes collected after the administration of Cetrorelix following treatment with PMSG and hCG. An average of 6.6 oocytes per mouse were collected in the control non-administration group, whereas 13.2 and 11.4 oocytes per mouse were collected in the mice injected with Cetrorelix 3 times and 7 times in 1 week, respectively ($P<0.05$ compared with the control group). Next, we examined the rate of morphologically normal oocytes collected following Cetrorelix administration using microscopy. Control mice and mice administered Cetrorelix 3 and 7 times showed 71.2%, 74.2%, and 76.3% normal oocytes, respectively, indicating that Cetrorelix did not induce any remarkable morphological changes. However, the number of normal oocytes was significantly increased in mice administered Cetrorelix 3 and 7 times compared with control mice ($P<0.05$). We also examined the effect of Cetrorelix on the number of normal ovulated oocytes in young mice. The result showed that there was no significant difference between the control mice and mice administered Cetrorelix 7 times (control, 23.5 ± 11.6 oocytes [n=6] vs. 7 times, 20.9 ± 1.0 oocytes [n=10]). These data suggest that Cetrorelix may improve archegonium function and increase the number of ovulated oocyte having developmental

Table 1. Effects of superovulation on the number of collected oocytes in young (16 weeks) and aged (52 weeks) mice

Age (week)	No. of females	No. of oviducts with ovulated oocytes (%)	Mean ± SEM no. of oocytes		
			Total	Normal (%)	Abnormal (%)
16	16	32 / 32 (100)	28.3 ± 3.1	23.4 ± 2.7 (82.8)	4.9 ± 0.8 (17.2)
52	9	16 / 18 (88.9)	6.6 ± 1.0*	4.7 ± 1.2 (71.2)*	1.9 ± 0.2 (28.8)

* $P<0.05$ compared with 16 weeks of age. Oocyte collection of young and aged mice was performed using 4 mice and 3 mice per sampling, respectively.

competency after superovulation (Table 2).

Effect of Cetrorelix on oocyte fertilization and early development

More than 70% of oocytes collected following Cetrorelix administration were morphologically normal (Table 2). To examine whether these oocytes had normal fertility and early developmental ability, we performed IVF and transferred 2-cell stage embryos into recipient ICR mice. The rate of 2-cell stage development control oocytes and oocytes collected after the administration of Cetrorelix 3 and 7 times in aged mice was 92.9%, 96.9%, and 88.5%, respectively, indicating that Cetrorelix had no major effect on fertilization (Table 3). The offspring rate of control 2-cell stage embryos and embryos following the administration of Cetrorelix 3 and 7 times was 56.4%, 58.3%, and 51.8%, respectively (Table 3), indicating that Cetrorelix did not have an effect on early development.

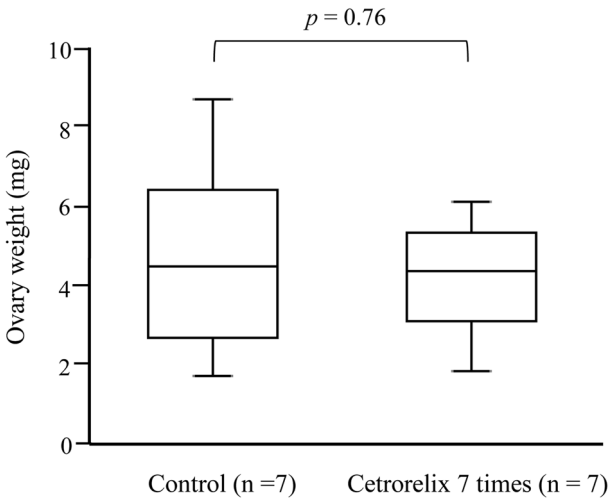

Fig. 2. Ovary weight of control and Cetrorelix-administered aged mice. Fourteen ovaries of these groups were derived from 7 mice. There was no difference in ovary weight between the groups (P=0.76).

Phalloidin staining of ovary tissues

Because Cetrorelix was found to improve ovarian archegonium function in aged mice, we investigated how it affected the internal structure of ovarian tissue. Cetrorelix administration did not have a significant effect on ovary weight compared with the control (Fig. 2). Next, to determine how Cetrorelix affected aging-associated ovarian fibrosis, the ovaries were stained using phalloidin. Fibrosis was not detected in sections from control and young mice administered Cetrorelix (Fig. 3A and B). In contrast, most of the sections in control aged mice showed strong focal staining, but sections from the mice administered Cetrorelix showed a very low level of phalloidin staining, indicating that Cetrorelix significantly decreased the formation of fibrosis in aged ovaries (Fig. 3C–E).

Discussion

Mammalian reproductive function declines with age. In this study, we used aged and young mice to investigate reproductive function. We collected significantly fewer ovulated oocytes from aged mice than from young mice after both groups were subjected to superovulation (Table 1). In contrast, superovulation became more effective in aged mice when they were treated with the GnRH antagonist Cetrorelix, which suppresses FSH and luteinizing hormone (LH) secretion, and is considered effective for treating infertility. Cetrorelix significantly increased the number of normal oocytes collected without an increase in morphologically abnormal oocytes in the 3- and 7-times-weekly treatment regimens (Table 2). Although a previous study showed that the number of

Table 2. Results of superovulation in aged mice treated with Cetrorelix

Treatment	No. of females	No. of oviducts with ovulated oocyes (%)	Mean ± SEM no. of oocytes		
			Total	Normal (%)	Abnormal (%)
Control	9	16 / 18 (88.9)	6.6 ± 1.0	4.7 ± 1.2 (71.2)	2 ± 0 (28.8)
Cetrorelix 3 times	10	18 / 20 (90.0)	13.2 ± 2.9*	9.8 ± 1.3* (74.2)	3 ± 2 (25.8)
Cetrorelix 7 times	10	20 / 20 (100)	11.4 ± 3.3*	8.7 ± 1.9* (76.3)	3 ± 1 (23.7)

*P<0.05 compared with control. Sampling of each experimental group was repeated 3 times.

Table 3. Development of superovulated oocytes using Cetorelix in aged mice

Treatment	No. of females	No. of oocytes	No. of 2-cell embryos (%)	No. of embryos transferred	No. of offspring (%)
Control	9	42	39 (92.9)	39	22 (56.4)
Cetrorelix 3 times	10	98	95 (96.9)	60	35 (58.3)
Cetrorelix 7 times	10	87	77 (88.5)	56	29 (51.8)

Sampling of each experimental group was repeated 3 times.

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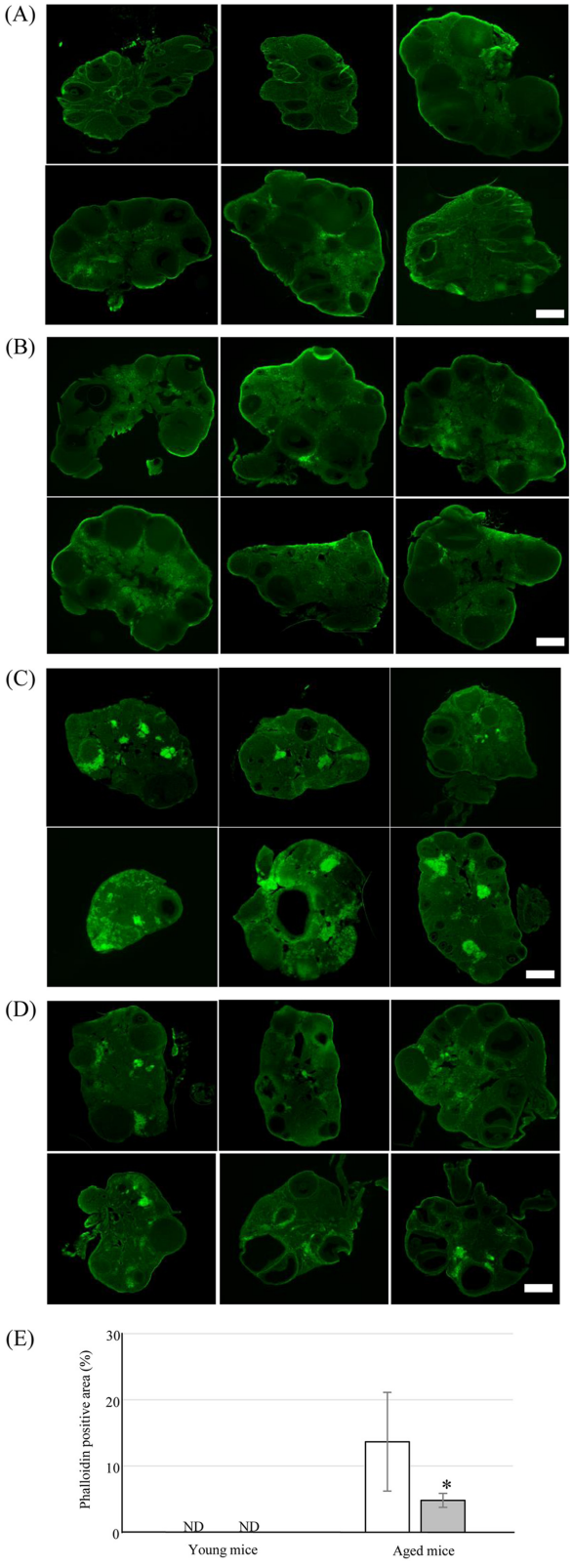

Fig. 3. Phalloidin staining of control ovaries (A) and Cetrorelix administered ovaries (B) in young mice, and control ovaries (C) and Cetrorelix administered ovaries (D) in aged mice. White scale bar, 200 μm. (E) The detection rate of phalloidin-positive areas in the ovary is shown in the bar graph (white bars, control; gray bars, Cetrorelix-administered ovaries). The areas were measured with the area measurement tool of a BZ9000 fluorescence microscope (*P<0.05). ND, not detected.

aneuploid oocytes was increased in aged mice [24], we found that oocytes collected from aged mice showed similar fertilization rates and offspring yields to those of oocytes collected from 10-week-old mice during our routine work (data not shown), confirming their normality (Table 3).

In mice, aging causes stromal fibrosis in ovarian tissue with the accumulation of extracellular matrices [25], as well as the oversecretion of LH and FSH [23]. The specific mechanism by which this fibrosis develops remains unclear; however, it may lead to changes in ovarian structure that inhibit follicle formation and decrease oocyte numbers. In this study, there is a possibility that follicle formation was disrupted in the aged mice due to the presence of fibrosis across the entire ovary (Fig. 3). However, ovary weight was not significantly different between Cetrorelix-treated aged mice (in which superovulation became more effective) and untreated controls, although we hypothesized that it would be heavier in the former (Fig. 2). On the Basis of these findings, we inferred that the ovulation-enhancing effect of Cetrorelix is achieved through an increase in the margin for follicle formation with a reduction in fibrosis, as well as through the promotion of follicle formation itself (Fig. 3). A previous study highlighted the role of LH receptor-positive cells, which secrete fibrosis-inducing extracellular matrices, in the ovarian stroma [17]. The authors reported that the increased number of LH receptor-positive cells in an aged mouse model was reduced by treatment with a GnRH antagonist, and accordingly demonstrated a close relationship between age-induced LH oversecretion and stromal cell-related fibrosis [17]. In this study, we did not find any effect of Cetrorelix on the cells in ovarian tissue; however, we surmise that the reduction in fibrosis was due to an effect on extracellular matrix-producing stromal cells. We will focus on elucidating the correlations between age-related fibrosis and altered stromal cell response in future research.

Efficient oocyte collection in aged mice is generally problematic even with the use of superovulation. Thus, mice retired from breeding are not utilized as a source of oocytes. We confirmed that combining superovulation with a GnRH antagonists enhanced the effect of PMSG and hCG in aged mice. Because AIS and GnRH antagonist have opposing effects on FSH secretion, they may not have synergistic effects on ovulated oocytes. Aged mice treated with a GnRH antagonist can recover archegonial function, and such mice can accordingly be expected to serve as a potential source of oocytes. The results of this study link Cetrorelix treatment with enhancing the value of aged mice as utilizable sources of oocytes, and suggest that this treatment contributes to

"minimizing the number of animals used for experiments as far as possible"—one of the principles of the 3Rs for animal use—thus yielding benefits from the perspective of animal welfare.

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References

1. Achermann JC, Jameson JL. Fertility and infertility: genetic contributions from the hypothalamic-pituitary-gonadal axis. Mol Endocrinol. 1999; 13: 812–818. [Medline] [CrossRef]
2. Cannon JG. Adaptive interactions between cytokines and the hypothalamic-pituitary-gonadal axis. Ann N Y Acad Sci. 1998; 856: 234–242. [Medline] [CrossRef]
3. Thackray VG, Mellon PL, Coss D. Hormones in synergy: regulation of the pituitary gonadotropin genes. Mol Cell Endocrinol. 2010; 314: 192–203. [Medline] [CrossRef]
4. Cole HH. Superfecundity in rats treated with mare gonadotropic hormone. Am J Physiol. 1937; 119: 704–712. [CrossRef]
5. Edgar DH, Whalley KM, Mills JA. Preimplantation development following in vitro fertilization of mouse oocytes: effects of timing of superovulation and preincubation in vitro. J In Vitro Fert Embryo Transf. 1987; 4: 111–115. [Medline] [CrossRef]
6. Gates AH. Viability and developmental capacity of eggs from immature mice treated with gonadotrophins. Nature. 1956; 177: 754–755. [Medline] [CrossRef]
7. Kanda A, Nobukiyo A, Yoshioka M, Hatakeyama T, Sotomaru Y. Quality of common marmoset (Callithrix jacchus) oocytes collected after ovarian stimulation. Theriogenology. 2018; 106: 221–226. [Medline] [CrossRef]
8. Mizoguchi H, Dukelow WR. Effect of timing of hCG injection on fertilization in superovulated hamsters. Biol Reprod. 1980; 23: 237–241. [Medline] [CrossRef]
9. Spearow JL, Bradford GE. Genetic variation in spontaneous ovulation rate and LH receptor induction in mice. J Reprod Fertil. 1983; 69: 529–537. [Medline] [CrossRef]
10. Nelson JF, Felicio LS, Osterburg HH, Finch CE. Altered profiles of estradiol and progesterone associated with prolonged estrous cycles and persistent vaginal cornification in aging C57BL/6J mice. Biol Reprod. 1981; 24: 784–794. [Medline] [CrossRef]
11. Suzuki H, Togashi M, Moriguchi Y, Adachi J. Relationship age-related decline in fertility and in vitro fertilization rate in IVCS mice. J Reprod Dev. 1994; 40: 107–116. [CrossRef]
12. Baird DT, Collins J, Egozcue J, Evers LH, Gianaroli L, Leridon H, et al. ESHRE Capri Workshop Group. Fertility and ageing. Hum Reprod Update. 2005; 11: 261–276. [Medline] [CrossRef]
13. Hansen KR, Thyer AC, Sluss PM, Bremner WJ, Soules MR, Klein NA. Reproductive ageing and ovarian function: is the early follicular phase FSH rise necessary to maintain adequate secretory function in older ovulatory women? Hum Reprod. 2005; 20: 89–95. [Medline] [CrossRef]
14. Klein NA, Battaglia DE, Fujimoto VY, Davis GS, Bremner WJ, Soules MR. Reproductive aging: accelerated ovarian follicular development associated with a monotropic follicle-stimulating hormone rise in normal older women. J Clin Endocrinol Metab. 1996; 81: 1038–1045. [Medline]
15. McTavish KJ, Jimenez M, Walters KA, Spaliviero J, Groome NP, Themmen AP, et al. Rising follicle-stimulating hormone levels with age accelerate female reproductive failure. Endocrinology. 2007; 148: 4432–4439. [Medline] [CrossRef]
16. Richardson SJ, Nelson JF. Follicular depletion during the menopausal transition. Ann N Y Acad Sci. 1990; 592: 13–20, discussion 44–51. [Medline] [CrossRef]
17. Umehara T, Kawai T, Kawashima I, Tanaka K, Okuda S, Kitasaka H, et al. The acceleration of reproductive aging in $Nrg1^{flox/flox}$; Cyp19-Cre female mice. Aging Cell. 2017; 16: 1288–1299. [Medline] [CrossRef]
18. Kiesel LA, Rody A, Greb RR, Szilágyi A. Clinical use of GnRH analogues. Clin Endocrinol (Oxf). 2002; 56: 677–687. [Medline] [CrossRef]
19. Janssens RM, Brus L, Cahill DJ, Huirne JA, Schoemaker J, Lambalk CB. Direct ovarian effects and safety aspects of GnRH agonists and antagonists. Hum Reprod Update. 2000; 6: 505–518. [Medline] [CrossRef]
20. Singh P, Krishna A. Effects of GnRH agonist treatment on steroidogenesis and folliculogenesis in the ovary of cyclic mice. J Ovarian Res. 2010; 3: 26. [Medline] [CrossRef]
21. Hasegawa A, Mochida K, Inoue H, Noda Y, Endo T, Watanabe G, et al. High-Yield superovulation in adult mice by anti-inhibin serum treatment combined with estrous cycle synchronization. Biol Reprod. 2016; 94: 21. [Medline] [CrossRef]
22. Pallares P, Gonzalez-Bulnes A. A new method for induction and synchronization of oestrus and fertile ovulations in mice by using exogenous hormones. Lab Anim. 2009; 43: 295–299. [Medline] [CrossRef]
23. Parkening TA, Collins TJ, Smith ER. Plasma and pituitary concentrations of LH, FSH and prolactin in aged female C57BL/6 mice. J Reprod Fertil. 1980; 58: 377–386. [Medline] [CrossRef]
24. Merriman JA, Jennings PC, McLaughlin EA, Jones KT. Effect of aging on superovulation efficiency, aneuploidy rates, and sister chromatid cohesion in mice aged up to 15 months. Biol Reprod. 2012; 86: 49. [Medline] [CrossRef]
25. Wynn TA, Ramalingam TR. Mechanisms of fibrosis: therapeutic translation for fibrotic disease. Nat Med. 2012; 18: 1028–1040. [Medline] [CrossRef]