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Effect of regular exercise on reproductive function of aged female in mouse model

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Summary

Purpose: This study examined whether regular exercise can improve ovarian angiogenic factors expression, oocyte quality, and fertility in aged female mice. *Materials and Methods:* Aged C57BL female mice (30-32 weeks) were treated with regular exercise or without exercise for six weeks and then mated with male mice. After mating, the pregnancy outcome was examined. Female mice of two age groups (6-8 and 30-32 weeks) were regularly exercised for six weeks. After superovulation, mating, and zygote retrieval, ovarian VEGF and eNOS expression, and ovarian apoptosis were examined. *Results:* The number of pregnant mice, offspring, and embryo development rate in aged mice were significantly higher in the exercise group compared with controls. Ovarian VEGF and eNOS expression was increased, but ovarian apoptosis was decreased in the exercise group. *Conclusions:* This study demonstrates that regular exercise in aged mice improves oocytes quality and fertility, possibly by regulating ovarian eNOS and VEGF expression.

Key words: Exercise; Ovarian angiogenic factors; Oocyte quality; Fertility; Aged female.

Introduction

Nowadays many women have delayed first childbearing to advanced ages [1, 2]. Female age is one of THE significant factors affecting a fertility because of ovarian aging, decrease in sexual behavior, and increase in risks of other disorders affecting fertility such as fibroids, tubal diseases, and endometriosis [3]. This trend for age-related decline of fertility is clearly shown in assisted reproductive technique (ART) cycle as well as in natural cycle [4-7]. Likewise, female age is an impact factor in fertility and advancing age is an important factor contributing to an increasing incidence of infertility. Nevertheless, female reproductive aging remains a difficult problem in infertility treatment.

Oocyte aging is stimulated as the ovary ages [8]. It results in gradual decreases in the quality and quantity of oocytes, including decreased number of ovulated oocytes, decreased viability of preimplantation embryos, and increased percentages of abnormal/degenerating oocytes [9, 10]. Therefore, oocyte aging is a common cause of ART failures [11]. However, the reason for the oocyte aging-related decline in oocyte quality is not clearly understood, although it seems that endocrine, paracrine, genetic, and metabolic factors are effective. Chromosomal aneuploidy and injury of mitochondrial DNA of oocyte and embryo may be mainly considered as causes of the age-related decline in oocyte quality [1, 10, 12]. However, the finding of Tatone *et al.* that age-related nuclear and cytoplasmic damage may occur as a result of inadequate ovarian angiogenesis in primordial follicles, as well as in ovarian stroma vessels, suggests that dysfunction in ovarian angiogenesis can be an important cause of oocyte aging [13].

The ovary and uterus have cyclic angiogenic processes to facilitate follicular development and implantation processes. Especially, in ovarian angiogenesis, vascular density markedly increased in follicles undergoing development from preantral to antral stage [14]. Gaulden proposed that a deficient microvasculature develops around the dominant follicles with aging, resulting in the hypoxia of follicles [15]. Atretic follicles have a decreased vascularity during follicular development, while the selected follicles show a more elaborate microvasculature and the mature follicles have an increased vascular density [16, 17]. These results indicate that ovarian angiogenesis plays a critical role in follicular development processes that require neovascularization, including follicular growth, selection of dominant follicles, inhibition of follicular atresia, ovulation, and corpus luteum formation [18, 19]. Vasculature of the follicle plays an important role in the preferential delivery of gonadotropin, oxygen, and nutrients essential for follicular development [20]. Indeed, dysfunction in ovarian angiogenesis may be an important cause of anovulation, polycystic ovarian syndrome (PCOS), pregnancy loss, and possibly infertility. Therefore, it can be postulated that the activation of ovarian an-

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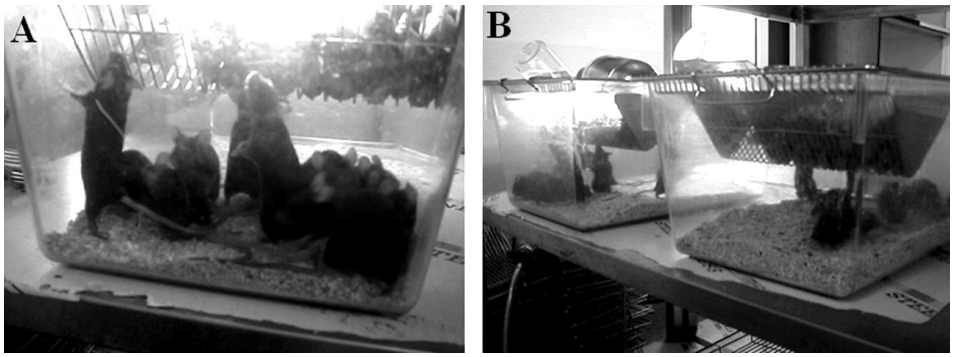

Figure 1. — Exercise treatment protocol. Aged female mice (30-32 weeks) are illuminated with incandescent lights (60 Watts, 220V), placed on the top of the cage, starting at 11:00 a.m. for 30 minutes daily (A). This illumination increases the temperature within the cage by causing heat and finally stimulates physical activity of mice compared to mice of control group (B).

giogenesis may be a good strategy for the improvement of age-related decline in oocyte quality.

Vascular endothelial growth factor (VEGF) is well known to be a critical regulator of angiogenesis and it is expressed not only in follicles, but also in the stroma of the ovary, and it plays an important role in follicular development and ovulation [21]. Nitric oxide (NO), known to be a potent vasodilator and angiogenic factor, plays an important role in ovarian angiogenesis during folliculogenesis and ovulation, including in the determination of oocyte quality and embryo developmental competence and the inhibition of atresia and apoptosis of the growing follicles [22, 23]. NO also mediates the vascular permeability of VEGF [24].

Previous several studies have revealed the relation between exercise and fertility. Female athletes with intense physical activities can result in menstrual dysfunction and infertility [25, 26]. Morris *et al.* reported that regular exercise before IVF negatively affects pregnancy outcomes, especially in women who exercised four or more hours per week for 1-9 years [27]. However, some data has suggested that exercise for weight loss in overweight women improves ovulation and subsequent fertility [28, 29]. Loucks contradicted that the observational design of the Morris *et al.* study does not warrant any conclusion because they did not consider energy intake and the expenditure of IVF patients [30, 31]. It has been recommended that pregnant women should engage in moderate intensity exercise for at least 30 minutes on most if not all days of the week in the absence of any contraindications [32]. Moderate activity may be beneficial to implantation by increasing insulin sensitivity [33]. Recently, Palomba *et al.* showed that implantation, clinical pregnancy, and live birth rates are significantly increased in women who performed physical activity regularly compared with those who did not [34]. Likewise, the relation between exercise and fertility remains controversial because it is complex, absolutely depends on the type, intensity, and duration of exercise, and difficult to define exercise intensity affecting fertility potential.

Some studies have reported the relation between exercise and angiogenesis [35, 36]. A single bout of moderately intense treadmill running upregulates VEGF expression of skeletal muscle in rats [37] and upregulation of VEGF mRNA also occurs during exercise in humans in both normal healthy individuals and patients with heart failure [35, 38]. Bloor *et al.* reviewed the possibility that exercise can induce angiogenesis process by stimulating the expression of angiogenetic factors such as VEGF [39]. However, there is no study on the effect of regular exercise on reproductive outcome in aged female. Therefore this study aimed to examine whether regular exercise can improve the expression of ovarian anigogenic factors, oocyte quality, and fertility potential in aged females using the mouse model.

Materials and Methods

This study was approved by the institutional review board of Pusan National University Hospital.

In all experiments, C57BL inbred mice were used and purchased from Korea Experimental Animal Center (Daegu, South Korea). Mice were maintained on a light-dark cycle, with light on at 5:00 a.m. and off at 7:00 p.m., and with food and water available ad libitum.

Forty C57BL female mice of 30-32 weeks were divided into the two groups. One group (n=20) were regularly exercised to stimulate physical activity by illuminating incandescent lights (60 Watts, 220V), placed on the top of the cage, starting at 11:00 a.m. for 30 minutes daily (Figure 1). The other group (n=20) served as control without the induction of exercise.

To examine whether the exercise treatment influences the pregnancy outcome of aged female mice (30-32 weeks-old), after four weeks, the female mice were mated with the same strained individual male mice (12 weeks-old) during two weeks, maintaining the exercise treatment protocol. Then, the pregnancy outcome was observed for subsequent two weeks (1st observation). Mice that were not pregnant were re-mated during further two weeks and re-examined the pregnancy outcome for the following two weeks (second observation).

To examine whether the exercise treatment improves ovarian response and oocyte quality, such as developmental competency of oocytes, female mice of the two age groups (6-8 weeks and 30-32 weeks) were treated with exercise protocol for six weeks, and then superovulated by intraperitoneal co-injection with 0.1 mL of 5 IU of pregnant mare's serum gonadotropin (PMSG), followed by injection of 5 IU of human chorionic gonadotropin (hCG) approximately 48 hours later. Then the mice were immediately

paired with an individual male. The following morning the mice were inspected, and those with a confirmed vaginal plug were considered fertilized. The control group was superovulated with PMSG and hCG without exercise treatment. Ten mice per each group were used.

Female mice with a confirmed vaginal plug were sacrificed by cervical dislocation 18 hours after the hCG injection, and zygotes (one-cell embryos) were retrieved from the oviductal ampulae. Cumulus-enclosed zygotes were denuded by incubation for one minute with 1 mg/mL hyaluronidase in Dulbecco's phosphate buffer saline (dPBS). Zygotes were pooled and washed three times in human tubal fluid (HTF) media supplemented with 10% human follicular fluid (hFF). Only healthy zygotes were cultured in 30-mL drops of media (HTF + 20% hFF) under paraffin-oil at 37°C in a 5% CO2 incubator for four days, and the media was changed daily. The number of zygotes retrieved and fragmented per mouse was counted, and embryo development to blastocyst stage was evaluated.

Just after the one-cell embryos were retrieved, both ovaries were collected and provided to examine the expression of VEGF and endothelial nitric oxide synthase (eNOS) by Western blot analysis and immunohistochemistry. Some portions of ovaries were provided in assessment of ovarian apoptosis.

Proteins were extracted by mechanical homogenization of ovaries in the presence of 200 mL ice-cold lysis buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, and 1 mM EDTA). The protein content of cell lysate was determined with Bradford reagent using bovine serum albumin (BSA) as the standard. The authors separated 20 mg of cell lysate by SDS-PAGE and transferred to PVDF (immobilon-P) membrane.

The transfer was performed at a constant voltage of 15 V for 30 minutes. For Western blotting, the membrane was incubated with anti-mouse VEGF antibody (1:1000 dilution) and anti-mouse NOS antibody (1:1000 dilution) in TBS containing 1% skim milk for four. hours and one hour, respectively, at room temperature. After washing three times with TBS-T (containing 0.04% Tween-20), the blotted membranes were incubated with horseradish peroxidase–conjugated goat antibody for 30 minutes at room temperature. After washing three times with TBS-T, the proteins were visualized by the enhanced chemiluminescence detection system according to the recommended procedure.

Serial sections (4 μm) of formalin-fixed, paraffin-embedded ovarian tissues were spread on the coated slides and placed in an oven at 60°C for one hour. Then the slides were deparaffinized in xylene and dehydrated in a graded series of ethanol. The endogenous peroxidase was quenched with 0.3% hydrogen peroxide at room temperature for five minutes, and then the tissues were rinsed four times for five minutes each time in PBS. The samples were incubated with normal serum and then incubated with rabbit anti-mouse VEGF polyclonal rabbit IgG antibody and anti-mouse eNOS rabbit IgG antibody at a dilution of 1:100 in PBS/BSA overnight at 4°C. After four washing with PBS for 15 minutes each time, the samples were incubated with biotinylated secondary antibody for 30 minutes at room temperature and washed three times. Then the samples were incubated with streptavidin–peroxidase conjugate in PBS for 30 minutes at room temperature and incubated with 3,30 diaminibenzidine chromogen. Counterstaining was performed with Mayer's hematoxylin. The results were assessed under a light microscope by two blinded pathologists.

Serial sections (4 μm) of formalin-fixed paraffin-embedded ovarian tissues collected from each age group were spread on the coated-slides, and placed in an oven at 60°C for one hour, and then the slides were deparaffinized in xylene and dehydrated in a graded series of ethanol.

In situ TUNEL analyses were performed, according to the instructions of a commercial assay kit. Briefly, sections were first washed in equilibration buffer, incubated with TdT enzyme in a humidified chamber at 37°C for one hour, and then, the cells were washed and incubated at room temperature for 30 minutes in the dark with fluorescein-conjugated anti-digoxigenin. The washed specimens were counterstained with propidium iodide (1 μg/ml) and visualized with fluorescent microscope. Apoptotic cells show an intense yellow fluorescence, whereas normal cells appear red when stained with propidium iodide.

All data were presented as mean ± standard deviation. Statistical analysis was performed using the unpaired Student's *t*-test and chi-square test. $P < 0.05$ was considered statistically significant.

Results

In order to examine whether exercise treatment improves fertility ability in aged female, the authors firstly tested the pregnancy rate and the number of offspring delivered of aged female mice (30-32 weeks) after mating with male mice. The pregnancy of female was checked at the two time periods; two weeks (first observation) and four weeks (second observation) after mating. At the first observation, the pregnancy rate was significantly higher in the exercise group (45%) than the control group (20%) ($p < 0.05$). At second observation for the remaining female who did not achieve pregnancy, a significant higher pregnancy rate was also found in the exercised group (30%) compared to the control group (5%) ($p<0.05$). Overall, the total pregnancy rate of the first and second observation showed 75% in the exercise group and 25% in the control group ($p < 0.05$). The mean number of offspring delivered per mice was 9.2 and 6.3 in the exercise and control group, respectively with a significant difference ($p < 0.05$) (Table 1).

Next, in order to elucidate whether the beneficial effect of exercise treatment on fertility outcome is attributed to the improved oocyte quality, the authors examined developmental competence of oocytes of aged female mice (30-32 weeks) after the exercise treatment. The mean number of one-cell embryos (zygotes) flushed was 12.6 in the exercise group which was statistically significantly higher compared with 10.8 in the control group ($p < 0.05$). The embryo development rate to blastocyst was also statistically significantly increased in the exercise treatment (43.8%) compared with the control group (8.1%; $p < 0.05$). A noticeably larger number of developing embryos were arrested in the control group at the two-cell stage (Table 2).

Thirdly, the authors tested whether the beneficial effect of exercise treatment shown in aged mice can also be applied in young mice of 6-8 weeks. However, exercise effect was not found in young mice. As shown in Table 3, the number of zygotes retrieved and the embryo development rate was very higher than those of aged mice of 30-32 weeks shown in Table 2, but they were similar in both the exercise treatment and control groups.

Table 1. — *Effect of exercise treatment on the pregnancy outcomes in aged female mice.*

	Exercise group (n=20)	Control group (n=20)	*p*-value
No. of pregnant mice within first observation	9 (45%)	4 (20%)	<0.05
No. of pregnant mice within second observation	6 (30%)	1 (5%)	<0.05
No. of total pregnant mice	15 (75%)	5 (25%)	<0.05
No. of total offspring/mice (mean ± SD)	9.2±1.3	6.3±2.7	<0.05

Finally, the authors examined ovarian VEGF and eNOS expression by Western blot and immunohistochemistry whether the beneficial effect of exercise relates to the activation of ovarian angiogenesis. The authors also tested ovarian apoptosis by TUNEL apoptosis assay. VEGF and eNOS expression showed a remarkable increase in the exercise treatment group compared to the control group (Figures 2 and 3). Imunohistochemistry especially showed that VEGF and eNOS expressions were usually localized in granulosa cells, stromal cells, and endothelial cells. This result suggests that the exercise treatment induces an increase of VEGF and eNOS expression in ovarian cells.

Conversely, ovarian apoptosis was decreased in the exercise group compared to the control group. The remaining follicles after ovulation were shown and few apoptotic cells were detected (red color) in the ovary of exercise treatment, However, in the ovary of control group, no follicle were found, whereas apoptotic cells (yellow color) were appeared all over the ovary (Figure 4).

Discussion

The present study shows that exercise for the stimulation of physical activity improves ovarian function and oocyte quality, and finally fertility of aged female mice. As far as is known, this report is the first study aimed to examine the relation between fertility and exercise in aged female, although this study was done in the mouse model, not human design.

Exercise has been known to have many health benefits, but the relation between exercise and fertility remains controversial because of difference in the study design for the type, intensity, and duration of exercise. Several studies reported that physical activity affects implantation: moderate activity can be beneficial to implantation, whereas vigorous activity negatively affects implantation by lowering leptin levels, which is an important factor in regulating embryo implantation and endometrial receptivity [33]. Some studies strongly suggest that frequent vigorous activity can be a risk factor as infertility [40, 41] and women who had a vigorous exercise were less likely to conceive and have a live birth than women without a history of exercising [27]. Recently Evenson *et al.* showed that an active lifestyle in the preceding year favorably impacted the IVF outcome [42]. Collectively these results indicate that moderate activity may be more beneficial in fertility than vigorous activity. In the present study, the fact that the present exercise protocol resulted in a beneficial effect on fertility means that the intensity of this exercise may be moderate for aged female mice.

Aerobic exercise led to a 40% higher rate of ovulation in comparison with dietary restriction (800 kcal/d), (25% *vs*. 65%, respectively) [43]. Physical activity can restore ovarian function by insulin sensitization [44, 45]. In a prospective study, individualized aerobic exercise of 16 weeks

Table 2. — *Effect of exercise treatment on ovarian function and oocyte developmental competency in female mice aged 30-32 weeks.*

Exercise	No. of offspring females	No. of zygotes flushed	No. of zygotes flushed/mouse	No. of zygotes fragmented (%)	No. of zygotes cultured	No. of 2-cell embryos (%)	No. of blastocyst (%)
− (Control)	10	107	10.8±2.7*	21 (19.6%)	86	22 (25.6%)	7 (8.1%)
+ (Exercise)	10	133	12.6±1.9†	12 (9.0%)†	121	77 (63.6%)†	53 (43.8%)†

*Mean±SD. †$p < 0.05$ (*vs.* control).

Table 3. — *Effect of exercise treatment on ovarian function and oocyte developmental competency in female mice aged 6-8 weeks.*

Exercise	No. of offspring females	No. of zygotes flushed	No. of zygotes flushed/mouse	No. of zygotes fragmented (%)	No. of zygotes cultured	No. of 2-cell embryos (%)	No. of blastocyst (%)
− (Control)	10	189	19.0±2.9*	6 (3.2%)	183	131 (71.6%)	94 (51.3%)
+ (Exercise)	10	198	19.3±3.3	7 (3.5%)	192	141 (73.4%)	97 (50.5%)

*Mean ± SD.

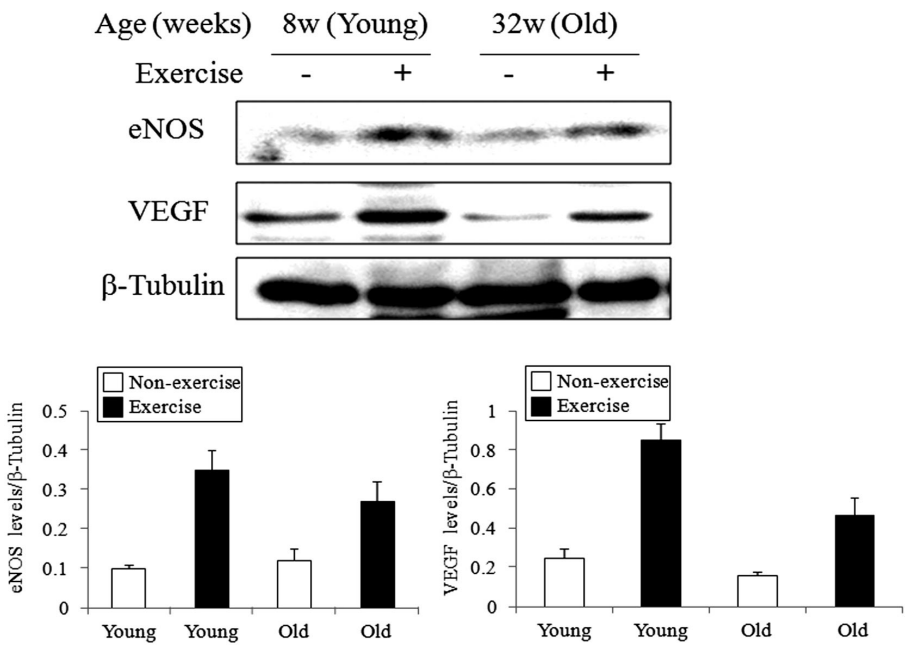

Figure 2. — Western blot analysis for VEGF and eNOS expressions in ovaries in young and aged female mice. Tubulin expression was used as control. Ovaries were isolated just after the flushing of zygotes 17-18 hours post-hCG injection.

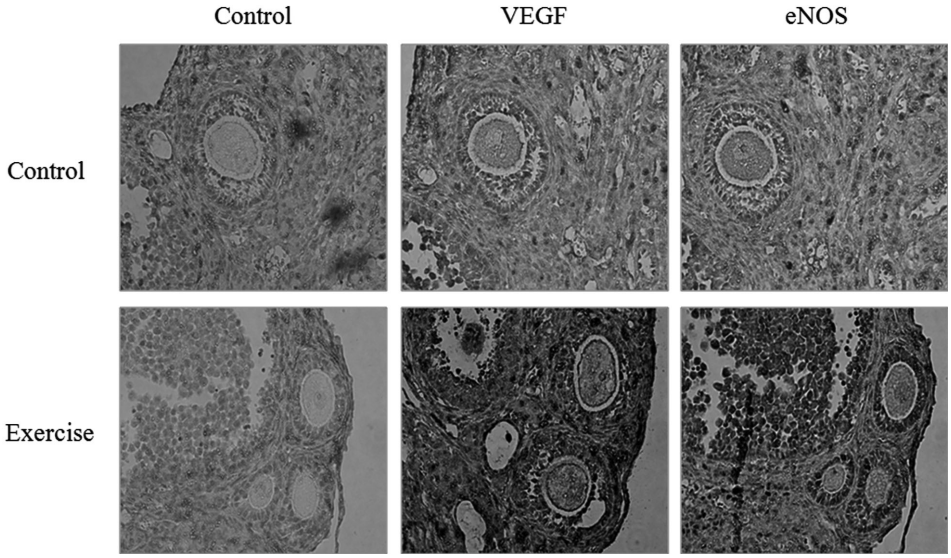

Figure 3. — Representative photomicrograph of VEGF and eNOS immunohistochemistry in ovaries in aged female mice. Ovaries were isolated just after the flushing of zygotes and fixed. Control was immunostained without primary antibody (purple color). Cells immunostained with VEGF and eNOS specific antibody showed brown color. G: granulosa cells; S: stroma cells; E: endothelial cells. Final magnification, ×400.

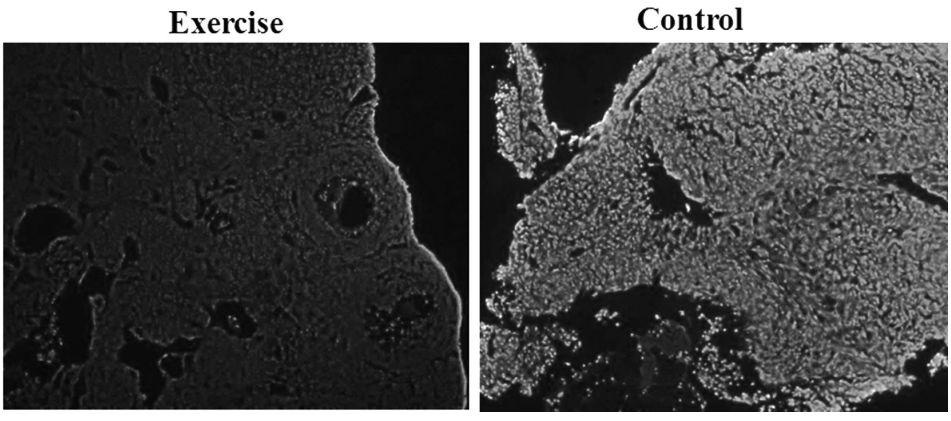

Figure 4. — Ovarian apoptosis using the TUNEL assay. In the ovary of exercise treatment, the remaining follicles after ovulation are shown and few apoptotic cells are detected (red color). However, in the ovary of control group, no follicles were found, whereas apoptotic cells (yellow color) were appeared all over the ovary.

improves ovarian morphology independent of changes in body composition [46]. These results have suggested the possibility that the improved effect of physical activity on fertility may be related to the ovarian function as well as implantation processes of endometrium. However, there are very few studies on the direct relation between exercise and ovarian function and oocyte quality, especially in the aspect of female age. In this respect, another notable finding in the present study was that exercise for the stimulation of physical activity may increase fertility ability in aged female by improving ovarian function and oocyte quality, such as developmental competency

In addition, this result seems to be more meaningful considering the two facts: one is that many women undergoing fertility treatment make poor lifestyle choices that may affect treatment outcome [47] and the other is that advancing female age continuously increases as an important and fastidious infertility cause. Therefore our result indicates that the choice of favorable lifestyle such as a moderate physical activity or exercise may be very important for female with advancing age to achieve pregnancy.

This study did not clearly elucidate the mechanism that exercise treatment results to improve ovarian function and oocyte quality. However, this study showed the increased ovarian VEGF and eNOS expression and decreased ovarian apoptosis. This result is very consistent with the previous study that exercise upregulates VEGF and eNOS expression [35, 37, 39, 48]. It has been well known that NO production is an important element of VEGF signaling [49] and these factors play a critical role in angiogenesis. Several studies demonstrated an importance role of VEGF in follicular development: direct injection of VEGF gene fragment or VEGF into the ovary increases angiogenesis and the number of follicles to be ovulated [50-52] and suppression of follicular angiogenesis by the inhibition of VEGF inhibits antral follicular development and ovulation [53]. Thus, it is likely that VEGF regulation in the ovarian follicular phase can benefit ovarian stimulation for poor responders. Our study group also have previously demonstrated that the activation of ovarian angiogenesis improve ovarian function and oocyte quality in aged female mice [54-56]. Therefore it is speculated that the beneficial effect of exercise on ovarian function and oocyte quality may be attributable to the activation of ovarian angiogenesis through the increased expression of ovarian VEGF and eNOS.

However, in young mice, beneficial effect of exercise on fertility was not found although ovarian VEGF and eNOS expression was increased in the exercise group compared to in the control group. This result is somewhat expected because fertility ability of young female is enough high to counter balance the beneficial effect of exercise. In addition, we observed this similar result in our previous studies [54-56].

The current study also has several limitations. First, this study did not use a general exercise equipment or protocol, such as treadmill because it was not available. Thus the present authors designed an exercise method to stimulate physical activity by illuminating with incandescent lights (60 Watts, 220V), placed on the top of the cage because this illumination method has been widely used to collect circulating blood from tail vein of the mouse. This method increases physical activity enough to be dripping wet with sweat, and results to increase blood flow and extend blood vessel. As a result, this method facilitates the collection of blood. Another demerit of this method is not to standardize and quantify the intensity of exercise. This study did not compare the effect of exercise according to the intensity of illumination or exercise treatment time and used just a constant illumination for 30 minutes daily. Nevertheless, the present exercise protocol by illuminating with incandescent lights may fully show the exercise-like effect by increasing expression of ovarian angiogenic factors and by improving ovarian function and oocyte quality, and finally fertility ability in aged female mice. This result indicates that an optimal exercise treatment can improve the age-related decline in fertility. Second, this study did not examine the effect of exercise on implantation because many studies have shown that physical activity can affect implantation of endometrium [33]. This indicates that we cannot assess the possibility that the beneficial effect of this exercise treatment on fertility may be attributed to the improved implantation, as well as on the increased ovarian function and oocyte quality. Therefore further study is needed to warrant this possibility.

Conclusion

In conclusion, the present study demonstrates that regular exercise induced by illumination with incandescent lights in aged mice improves their reproductive outcomes by improving ovarian function and developmental competence of oocytes. This result suggests the possibility that the beneficial effect of an optimal exercise on oocyte quality and fertility may be associated with the activation of ovarian angiogenesis by increasing ovarian eNOS and VEGF expression. Finally this research provides a promising strategy that exercise could be effective in the treatment of age-related decline of fertility.

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