Effects of Estradiol on the Paracrine Regulator Expression of *In Vitro* Matured Murine Ovarian Follicles

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Abstract The preservation of female germ cells is important in the individuals with ovarian dysfunction and failure. For this purpose, ovarian follicle *in vitro* maturation (OFIVM) is an important technology for the retrieval of mature oocytes. In the *in vivo* follicular development, paracrine factors such as angiotensin (AT) and anti-Müllerian hormone (AMH) play important roles. We attempted to add estrogen during the OFIVM and to assess their expression on the follicular cells. The ovaries and pre-antral follicles were collected from 13-day C57BL/6 mice and cultured *in vitro* with estradiol (E₂) treatment for up to two weeks. In the whole ovaries, the expression of AT II was decreased and the expression of AMH was similar between control and E₂-treated ovaries after *in vitro* culture. Although there was no difference in the survival, ovulation, maturation and fertilization rates between control and E₂-treated groups, the expression of AT II in the follicular cells was down-regulated after E₂ treatment at mRNA level, and AMH showed similar expression. In conclusion, adding E₂ in OFIVM may regulate paracrine factors and their receptors that are related to follicular development. Further investigations are necessary to elucidate the roles of various sex hormones in the regulation of AT and AMH expression during the OFIVM.

Keywords Estrogen · Ovary · Angiotensin · Anti-Müllerian hormone

1 Introduction

The preservation of female germ cells such as oocytes is important in the patients with ovarian dysfunction and failure. Currently, in clinical practice, *in vitro* fertilization (IVF) procedures are widely used to retrieve oocytes after injecting gonadotropins in infertile women, however, the

yield is very limited in some cases. For this purpose to be served, ovarian follicle *in vitro* maturation (OFIVM) can be employed as an important technology for the retrieval of mature oocytes from fresh or frozen-thawed ovarian tissues.

Previous clinical reports revealed an efficacy of adding estradiol (E₂) in terms of improved ovarian stimulation, improved pregnancy and implantation rates, and greater uniformity in follicular development during IVF cycles [1, 2]. The other group reported a similar IVF outcomes of E₂ treatment to the control therapeutic option [3, 4]. However, the underlying molecular mechanism for these observed findings are not yet clarified.

In vivo and *in vitro* follicular development is supported and controlled by various paracrine factors [5]. Among these, angiotensin (AT) and anti-Müllerian hormone (AMH) as paracrine factors are well-known for their roles during folliculogenesis [6]. Estrogen level is related with the expression of AT system members [7]. Ovariectomy

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results in a decrease in AT receptors [8]. It increases AT production [9], and its increased secretion is associated with increased expression levels of AT receptor mRNA and protein [10–12].

There has been a controversy on the effects of estrogen on the levels of AMH. Estrogen treatment inhibited AMH mRNA expression in rat pre-antral and small antral follicles [13]. However, in small bovine follicles, E₂ treatment with 150 ng/mL showed no change in AMH protein levels [14]. In addition, in murine granulosa cells, E₂ inhibited AMH expression through estrogen receptor (ER)- α and ER- β respectively [15].

Despite quite a few reports suggesting the roles of estrogen in the regulation of AT and AMH expression, its impact on the expression of these factors during murine OFIVM has not yet been elucidated. In this study, we tried to evaluate the effects of E₂ treatment on the expression of AT, AMH and their receptors on the *in vitro* cultured follicular cells during murine OFIVM.

2 Materials and methods

2.1 Animal care and collection of ovaries and ovarian follicles

This study was approved by the Institutional Animal Care and Use Committee of Seoul National University Hospital (IACUC no. 15-0016-S1A0). C57BL/6 mice were used: mother mice were kept on a 12-hour light and 12-hour darkness cycle under SPF environment with mouse chow unrestrictedly available. Baby mice were allowed to stay with their mothers until sacrificed.

Fifteen of thirteen-day-old female mice were sacrificed using cervical dislocation. The ovaries were retrieved and kept in Hank's balanced salt solution supplemented with 10% fetal bovine serum (FBS; Hyclone, Morgan, Utah, USA). For IVF, an eight-week-old C57BL/6 male mouse was sacrificed by cervical dislocation, and the cauda epididymis and vas deferens were dissected.

2.2 OFIVM and estrogen treatment

The methods used for OFIVM have been modified from previous studies [16, 17]. For the experiment of OFIVM, early stage pre-antral follicles were mechanically separated using a syringe needle. Follicles sized approximately 100 μ m in diameter with a centrally located oocyte were selected for the *in vitro* culture. Each single follicle was placed in a 20- μ L droplet of culture media on a 60-mm culture dish, one follicle per droplet, and twenty-five droplets per dish. In the control group, OFIVM culture media were composed of MEM alpha (Invitrogen, Waltham, MA,

USA) supplemented with 5% FBS (Hyclone, Morgan, Utah, USA), insulin-transferrin-selenium (Invitrogen), penicillin/streptomycin (Invitrogen), and recombinant follicle stimulating hormone (FSH) and luteinizing hormone (LH). In the E₂-treated group, E₂ was added into culture media at 10⁻⁷ mol/L. The selected pre-antral follicles in a culture dish were incubated at 37 °C in 5% CO₂. Half volume of the medium was changed every other day up to two weeks. Morphological changes of growing follicles were observed under an phase-contrast microscope (Nikon, Tokyo, Japan).

2.3 Ovulation induction and *in vitro* fertilization

After 12 days of culture, 1.5 IU/mL of recombinant human chorionic gonadotropin (rhCG: Ovidrel®, Merck Serono, Geneva, Switzerland) supplemented with 5 ng/mL human epidermal growth factor (EGF; Invitrogen) were added to full-grown follicles with antral expansion to induce ovulation. After 16–18 h, ovulation rates and oocyte maturation rates were assessed.

Ovulated follicle was defined as microscopic visualization of extrusion of oocyte surrounded by cumulus granulosa cell complex. Ovulation rate was defined as the number of ovulated follicles divided by the number of growing follicles. After mechanical denudation of the ovulated oocyte from surrounding granulosa cells using a 28-gauge syringe needle, the released oocyte was classified as a germinal vesicle (GV) oocyte when the GV was present; as metaphase I (MI) when the GV was broken down; and as metaphase II (M II) when the first polar body was extruded. Oocyte maturation rate was defined as the number of M II oocytes divided by the number of ovulated oocytes.

For *in vitro* fertilization (IVF), fresh sperm were squeezed out from the dissected cauda epididymis and vas deferens with microforceps and incubated in human tubal fluid (HTF) medium (Millipore, Billerica, MA, USA) for an hour at 37 °C. Sperm motility and concentration were measured using an aliquot of diluted sperm suspension. More than 1 $\times$ 10⁶ sperm/mL were assessed as the optimal motile sperm concentration. After fresh sperm was transferred to the fertilization dish, M II oocytes were transferred into the dish with a wide-bore pipette tip. After incubation at 37 °C in HTF medium under 5% CO₂ for 4 to 6 h, oocytes were washed in serial drops of medium to remove excess sperm and debris. Using an inverted microscope, fertilization was assessed by the presence of 2 pronuclei (2PN) and the extruded second polar body in fertilized oocytes. Fertilization rate was defined as the number of 2PN divided by the number of M II oocytes added to the sperm.

2.4 Immunocytochemical staining

In order to evaluate the expression of target genes, follicles were seeded individually onto multi-well dish (Nunc, Denmark) and cultured. To analyze the samples, media was discarded and the follicles were washed several times with PBS to remove the remaining mineral oil. And then, the cells were fixed with 4% paraformaldehyde (PFA; Sigma-Aldrich, St. Louis, MO, USA) for 15 min at room temperature (RT) and blocked with 3% bovine serum albumin solution for 12 h at 4 °C. Then, the cells were incubated with each primary antibody (1:100), rabbit anti-mouse angiotensin II (Novus Biologicals, Littleton, CO, USA) and mouse anti-FSH receptor (Abcam, Cambridge, UK) for overnight at 4 °C, and washed three times with TBS. Secondary antibodies, goat anti-rabbit 594 IgG (Molecular Probes, Grand Island, NY, USA) and goat anti-mouse 488 IgG (Molecular Probes) was treated for 50 min at 37 °C and washed three times with TBS. Finally, 10 µg/mL of DAPI (Sigma-Aldrich) was treated and the images were assessed under fluorescence microscope (EVOS-FL, Life Technologies, Grand Island, NY, USA).

2.5 Quantitative reverse transcription and polymerase chain reaction (qRT–PCR)

Total RNAs were extracted from the cultured follicles using Trizol reagent (Invitrogen). cDNAs were synthesized from 1 µg total RNAs using Accu premix (Bioneer, Daejeon, Korea), and used for the qRT–PCR reactions. Each specific primer (Table 1) and QuantiTect SYBR green premix were added to the cDNAs and amplified under the following conditions: incubation for 10 min at 95 °C, denaturation for 15 s at 95 °C, annealing for 40 s at 58 °C and 20 s for 72 °C for extension. All the reactions were

performed in triplicate and the Ct value was calculated based on the GAPDH expression.

2.6 Western blot of estrogen-treated ovaries

For the experiment of the whole ovaries, MEM alpha medium was used with supplementation with 5% FBS, ITS, penicillin/streptomycin (Invitrogen) in control group. In the E₂-treated group, E₂ was added into culture media at 10^{−7} mol/L. The whole ovaries of both groups were cultured for two days. Twenty microgram of total protein was mixed with SDS sample buffer and boiled for 3 min, separated by 10% SDS PAGE gel. Prepared samples were transferred onto polyvinylidene difluoride (PVDF) membranes (Immobilon; Millipore, Bedford, MA, USA). These membranes were blocked for 2 h at RT using 2% BSA solution and incubated for 12 h at 4 °C, with 1:500 diluted antibodies, rabbit anti-mouse angiotensin II and rabbit anti-mouse AMH (Abcam), and washed with TBS with triton x100 (TBST; Sigma-Aldrich). Then, the membranes were incubated for 1 h with the second antibodies, biotinylated goat anti-rabbit IgG (1:500) and biotinylated goat anti-mouse IgG (1:500), and washed with TBST. The protein bands were visualized with SuperSignal® West Dura Extended Duration Substrate (Thermo Scientific, Waltham, MA, USA). The membranes were exposed to Kodak film and visualized.

2.7 Statistical analysis

The experiments were repeated at least three times. Means and standard deviations were compared using the Student's *t* test. The outcomes of OFIVM was evaluated using Fisher's exact test. All the differences were considered statistically significant when *P* < 0.05. All data were

Table 1 Primer sequences used for the experiments

Gene	Sequences (5' → 3')	Sequences (3' → 5')
GAPDH	GAAGGTCGGTGTGAACGAAT	TTTGATGTTAGCGGGGTCTC
AT II receptor type I	CCATTGTCCACCCGATGAA	TGACTTTGGCCACCAGCAT
AT II receptor type II	CCTGGCAAGCATCTTATGTAGTTC	CCGGAAATAAAATGTTGGCAAT
AMH receptor II	TGTGTTTCTCCCAGGTAATCCG	AATGTGGTTCGTGCTGTAGGC
LH receptor	CCAGAGTTGTTCAGGGTCGCGC	AGCATCTGGTTCTGGAGTAC
FSH receptor	GCTCACCAAGCTTCGAGTCA	GCCTTAAATAGACTTGTTC
IGF receptor I	ACCCAGGCTCATAGCATACC	GCAAAGCACCTGCAACTGTA
IGF receptor II	CAATGGGGAAGTCGATGCTG	CTTGGCGAGCACGTGAC
AT II	CCGCATTTAAGTCTCACACA	ATCATGTAGTAGAGAACAGGAATTGCTT
AMH	GCAGTTGCTAGTCCTACATC	TCATCCGCGTGAAACA GCG
VEGF	CAGGCTGCTGTAACGATGAA	CAGGAATCCCAGAAACAACC

GAPDH: Glyceraldehyde-3-Phosphate Dehydrogenase, AT II: angiotensin II, AMH: anti-Müllerian hormone, LH: Luteinizing hormone, FSH: Follicle-stimulating hormone, IGF: Insulin-like growth factor, VEGF: Vascular endothelial growth factor

analyzed using the Statistical Package for the Social Sciences for Windows (version 12.0, SPSS Inc., Chicago, IL).

3 Results

3.1 AT and AMH expression after whole ovary culture

To evaluate the effect of E_2 on whole ovaries, the expression of AT II and AMH was assessed at the protein level (Fig. 1). The expression of AT II and AMH was similar between control and E_2 -treated ovaries. The expression of AT II was slightly down-regulated in E_2 -treated ovaries, however, no difference was observed in the expression of AMH on whole ovaries after short *in vitro* culture.

3.2 Outcomes of OFIVM

E_2 -treated group (Fig. 2B) showed similar morphological changes with control group (Fig. 2A) during the OFIVM. Follicles in both groups showed typical development, ovulation and produced mature oocytes. There was no difference in survival (76.0% vs. 75.0%), ovulation (59.2% vs. 58.7%), maturation (73.3% vs. 72.7%) and fertilization (66.7% vs. 65.6%) rates between control and E_2 -treated groups ($P > 0.05$ in all) (Table 2).

3.3 Gene expression of dominant follicle after OFIVM

To assess the protein expression of AT II and FSH receptors (FSH-R) on the *in vitro* cultured follicles before and after treatment of E_2 , immunocytochemical staining was performed. Follicular AT II expression after OFIVM was down-regulated in the E_2 -treated group compared to control group. FSH-R was similarly expressed between the two groups (Fig. 3).

Fig. 1 The expression of paracrine factors in E_2 -treated mouse ovary (Western blot). CTL: control, E_2 : estradiol, AT II: angiotensin II, AMH: anti-Müllerian hormone

A Control group

B E_2 -treated group

Fig. 2 Murine follicular morphology after E_2 treatment. **A** control group, **B** E_2 -treated group. *a* Preantral follicle, *b* culture day 5, *c* culture day 12 (hCG treatment), *d* ovulated follicle. Typical development, antral formation and ovulation of follicle were shown in both groups during OFIVM. E_2 : estradiol, hCG: recombinant human chorionic gonadotropin, OFIVM: ovarian follicle *in vitro* maturation

To evaluate the expression of the receptor genes in the dominant follicular cells, qRT-PCR analysis was performed. The expression of AMH-Receptor (AMH-R) II, LH-Receptor (LH-R) and IGF-Receptor (IGF-R) I, were up-regulated in E_2 -treated group compared to control group (Fig. 4A). AT II-R I, AT II-R II, FSH-R and IGF-R II showed similar expression between the two groups. The expression of AT II was decreased and that of vascular endothelial growth factor (VEGF) increased in E_2 -treated group compared to control group (Fig. 4B). There was no difference in the expression of AMH between the two groups.

Table 2 The outcomes of OFIVM

	Survival rate	Ovulation rate	Maturation rate	Fertilization rate
Control group	76.0% (76/100)	59.2% (45/76)	73.3% (33/45)	66.7% (22/33)
E ₂ -treated group	75.0% (75/100)	58.7% (44/75)	72.7% (32/44)	65.6% (21/32)

$P > 0.05$ in all. E₂: estradiol, OFIVM: ovarian follicle *in vitro* maturation

Fig. 3 The expression of AT II and FSH-R on the follicular cells after OFIVM (immunocytochemical staining). AT II expression on granulosa cells of *in vitro* matured follicle was down-regulated in the E₂-treated group compared to control group. FSH-R expression was similar between the two groups. E₂: estradiol, AT II: angiotensin II, DAPI: 4',6-diamidino-2-phenylindole, dihydrochloride, FSH-R: follicle stimulating hormone receptor, OFIVM: ovarian follicle *in vitro* maturation

Fig. 4 Relative expression of receptor and paracrine factor genes on dominant follicle of E₂-treated group compared to control group (Quantitative RT-PCR). **A** The expression of receptor genes on dominant follicle of E₂-treated group. **B** The expression of paracrine factor genes on dominant follicle of E₂-treated group (* $P < 0.05$). AT

3.4 Comparison of gene expression between dominant and atretic follicles after OFIVM with E₂ treatment

qRT-PCR analysis of the receptor genes expression on the atretic follicular cells revealed that the expression of AT II-R I was down-regulated and that of AMH-R II, FSH-R and IGF-R II was up-regulated in E₂-treated group compared to control group (Fig. 5A). AT II-R II, LH-R and IGF-R I showed similar expression between the two groups. Regarding the comparison of paracrine factors, the expression of AT II and AMH were decreased in E₂-treated group compared to control group (Fig. 5B). There was no difference in the expression of VEGF between the two groups.

The comparison between dominant and atretic follicles revealed that the expression of AT II-R I, LH-R and IGF-R I was up-regulated and that of FSH-R and IGF-R II down-regulated in dominant follicles compared to atretic follicles (Fig. 6A). AT II-R II and AMH-R II were similarly expressed between the two groups. Dominant follicles showed lower AT II expression and higher AMH expression than atretic follicles (Fig. 6B). The expression of VEGF was similar in the two groups.

II: angiotensin II, AMH: anti-Müllerian hormone, LH: luteinizing hormone, FSH: follicle-stimulating hormone, IGF-R: insulin-like growth factor receptor, VEGF: vascular endothelial growth factor, RT-PCR: reverse transcription polymerase chain reaction

Fig. 5 Relative expression of receptor genes and paracrine factor genes on atretic follicles of E₂-treated group compared to control group (Quantitative RT-PCR). **A** The expression of receptor genes on

the atretic follicles of E₂-treated group. **B** The expression of paracrine factor genes on the atretic follicles of E₂-treated group (**P* < 0.05)

Fig. 6 Relative expression of receptor genes on dominant follicle compared to atretic follicle in E₂-treated group (Quantitative RT-PCR). **A** Relative expressions of receptor genes on dominant follicle compared to atretic follicle in E₂-treated group. **B** Relative expression of paracrine factors on dominant follicle compared to atretic follicle

in E₂-treated group (**P* < 0.05). AT II: angiotensin II, AMH: anti-Müllerian hormone, LH: luteinizing hormone, FSH: follicle-stimulating hormone, IGF-R: insulin-like growth factor receptor, VEGF: vascular endothelial growth factor, RT-PCR: reverse transcription polymerase chain reaction

4 Discussion

OFIVM is an important technology to be used for the long-term preservation of oocytes or oocyte-containing follicles in the patients with impending ovarian failure. It can also be used as an analyzing tool for investigating the molecular regulation mechanism occurring during the follicular maturation *in vitro*. Numerous reports maintained beneficial effects of supplementing estrogen and observed improved ovarian stimulation, pregnancy and implantation rates, more synchronized follicular development during IVF treatment cycles [1, 2]. In spite of these roles of estrogen in the folliculogenesis, the relevant molecular mechanism is currently unknown. In this present study, OFIVM was conducted using single follicular culture, so it could mimic the micro-environment of individual follicle under the cross-talk between multiple follicular systems *in vivo*.

Angiotensin is a vasoactive factor which may have a direct effect on the ovarian follicular physiology. As autocrine/paracrine regulator, it has been known to be involved in the ovarian follicular development since the existence of prorenin, renin, angiotensinogen, angiotensin-converting enzyme, AT and its receptors were identified in the ovary. This ovarian renin-angiotensin system (RAS) plays an important role in such dimensions of ovarian follicular physiology as follicular development, steroidogenesis [18], oocyte maturation [19] and ovulation [20] through its receptors on granulosa cells. Its concentration increased in the follicular fluid of the dominant follicle [21]. In addition, previous reports revealed that AT II is a major factor in the regulation of function of atretic follicles [12]. Disorders occurred in the ovarian RAS are known to be the cause or the result of polycystic ovary syndrome, ovarian tumors, and hyperstimulation syndrome [19]. The roles of estrogen in the tuning of AT levels *in vitro* are

controversial. Various studies reported that supplementation of E_2 could induce increase [9, 11] or decrease [22, 23] of the expression of AT system member genes. In our results, E_2 -treated group showed lower expression of AT II than control group. This suggests that exogenous addition of E_2 during OFIVM could regulate the *in vitro* cultured follicle as in the case of subordinate follicles *in vivo* that are affected by E_2 produced by dominant follicle(s). *In vivo*, paracrine system in the ovary including AT II selects dominant follicle(s), supports its ovulatory maturation, and suppresses the other subordinate follicles. This present result was consistent with process of selecting dominant follicle during follicular maturation and ovulation process *in vivo*. Although E_2 -treated group showed similar outcomes of OFIVM compared to control group, the expression of paracrine factors and of their receptors was affected by adding E_2 as observed from our results.

The association between estrogen and AMH has been suggested in numerous *in vitro* and *in vivo* settings [24–26]. Previous studies reported that AMH inhibited E_2 production in ovarian follicle by suppression of aromatase [27, 28]. However, few studies reported the AMH profile after adding E_2 in an *in vitro* setting. Our present results showed a discrepancy in the level of expression between AMH and its receptors. E_2 treatment induced higher AMH-R II expression without significant effects on AMH expression compared to control on *in vitro* matured follicles. Although the relationship between AMH and AMH-R II were not fully revealed, it could be due to the roles of regulatory factors for the intra-follicular AMH/AMH-R II system. Further study should be necessary.

In our study, atretic follicles with E_2 treatment showed different expression profiles of receptor genes, although they showed similar trends in the profile of paracrine factors. In the comparison between dominant and atretic follicles during OFIVM with E_2 treatment, dominant follicles showed higher expression of AT II-R I, LH-R, IGF-R I and AMH; and lower expression of FSH-R, IGF-R II and AT II compared to atretic follicles. These results suggest that the effects of E_2 treatment could be more influential in dominant follicles compared to subordinate atretic follicles. Consistent with our results, paracrine factors and their receptors on ovarian follicles, via different processes, make a pivot role in follicular selection for ovulation *in vivo* [29, 30]. It can be proposed that multiple rather than single follicular culture be optimal experimental model for evaluating the competence of dominant follicles.

In spite of the aforementioned suggestive reports on the association between estrogen and AMH, only a few studies showed divergent findings regarding the effects of estrogen on the AMH levels in different cells. Estrogen treatment suppressed AHM mRNA expression in rat pre-antral and small antral follicles [13], but showed no change in AMH

protein levels in small bovine follicles [14]. In murine granulosa cells, estrogen showed different regulation of AMH expression according to the receptor subtype [15].

In conclusion, our study suggests that OFIVM may be an important experimental model mimicking the micro-environment of single follicle *in vivo*. Adding E_2 in OFIVM could regulate paracrine factors and its receptors related in follicular development. Further investigations are necessary to elucidate the role of various sex hormones in the regulation of AT and AMH expression during the OFIVM.

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Compliance with ethical standards

Conflict of interest The authors have no conflicts of interest.

Ethical statement The study was approved by the IACUC of the Seoul National University Hospital (IACUC no.15-0016-S1A0).

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