



Follicle-stimulating Hormone Induced *In Vitro* Growth of Small Antral Follicles are not Affected by Gonadotropin-releasing Hormone Agonist or Antagonist Treatment in Mice

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Abstract

Objective: In this study, we aimed to investigate if the gonadotropin-releasing hormone (GnRH) agonist and antagonist have any effect on the follicle-stimulating hormone (FSH)-induced-growth of early antral follicles of mice, cultured *in vitro*, and expressing cognate receptors for FSH and GnRH. For this purpose, small antral follicles were isolated from mouse ovaries and randomly assigned to the groups as control, FSH only, FSH + GnRH agonist, and FSH + GnRH antagonist, and they were cultured for five days.

Methods: The ovaries of C57BL/6 mice (n=24), which were 21 days old, were removed after euthanasia. Small antral follicles measuring ~200µ in diameter were mechanically isolated after the enzymatic digestion of the ovaries with collagenase and DNase-I. The expression of FSH and GnRH receptors in these follicles was validated by quantitative real time-polymerase chain reaction. Isolated follicles were randomly assigned into four different groups, each consisting of 20-30 follicles: control, FSH only, FSH + GnRH agonist, and FSH + GnRH antagonist.

Results: The FSH treatment significantly enhanced the *in vitro* growth of the follicles compared to those cultured without FSH after five days of the culture period. The antrum formation was markedly enhanced, and cumulus-oophorus complexes were more easily visible in the FSH-treated follicles compared to control follicles. The mean diameters of follicles treated with the FSH + GnRH agonist or the FSH + GnRH antagonist were not significantly different from those treated with FSH only, but they were significantly greater than control follicles.

Conclusion: These results may suggest that the GnRH agonist and antagonist do not appear to adversely affect the FSH-induced proliferation of mitotic non-luteinizing granulosa cells and the growth of early antral follicles of mice *in vitro*.

Keywords: Antral follicle, culture, matrigel, follicle-stimulating hormone, gonadotropin-releasing hormone agonist, gonadotropin-releasing hormone antagonist

INTRODUCTION

Our knowledge about the role of extra-pituitary gonadotropin-releasing hormone (GnRH) and its receptors in ovarian follicle development in humans and other species is very restricted. In the mouse ovary, small antral follicles express the cognate receptors for GnRH and follicle-stimulating hormone (FSH) (1-3). However, their role in FSH driven growth of these follicles is unknown. Previous studies showed that although systemic administration of GnRH agonist (GnRHa) was efficient to disrupt estrus cycles, it failed to inhibit follicular development, irrespective of the doses and injection sites (subcutaneous or intramuscular). Around 20% of healthy growing follicles were still observed during GnRHa treatment, and serum FSH levels

were not reduced either by antagonist or agonist treatment, suggesting that GnRHa does not suppress follicular growth even beyond the gonadotropin-dependent stage in mice (4). Also, it might be challenging to interpret the intra-ovarian actions of GnRH analogs when they are administered systemically due to its effect on the hypothalamic-pituitary-ovarian axis. One *in vitro* study showed that GnRHa exerts diverse actions on granulosa cells over the course of follicular growth. One down-regulates granulosa cell proliferation in immature follicles as well as steroidogenesis in mature follicles, and the other upregulates apoptosis of granulosa cells regardless of the stages of follicular growth (5). It is unclear whether *in vitro* GnRH analog treatment has any impact on FSH induced growth in *in vitro* conditions of



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small antral follicles isolated from mice and expressing FSH and GnRH receptors. We aimed to address if GnRHa and antagonist has any effects on FSH induced *in vitro* growth mouse early antral follicles expressing cognate receptors for FSH and GnRH.

METHODS

Study Design

We designed this study to investigate *in vitro* conditions if GnRHa and antagonist treatment have any impact on FSH induced growth of isolated mouse early antral follicles expressing cognate receptors for FSH and GnRH. For this purpose, small antral follicles were isolated from mouse ovaries and randomly assigned to the groups as control, FSH only, FSH + GnRHa, and FSH + GnRH antagonist and cultured for five days.

Follicle Isolations from Mice

All experiments were conducted 21-day-old C57BL/6 mice. The Institutional Animal Care and Use Committee of Istanbul Aydın University Faculty of Medicine approved the protocol (issue number: 2015-20). Animals were euthanized by cervical dislocation, and ovaries were removed, minced into two or three fragments in pre-warmed Dulbecco modified eagle medium-F12 (DMEM-F12) culture medium. Then the pieces were disassociated with collagenase, DNase-I in DMEM-F12 supplemented with 5% bovine serum albumin (BSA) for 30 minutes at 37 °C. Small antral follicles were mechanically isolated under a stereomicroscope (Olympus SZX12, Olympus America Inc., Center Valley, PA, USA) and randomly assigned to the groups to be cultured for five days.

Culture Medium Formulation

Isolated follicles were cultured in (HEPES)-buffered DMEM-F12 culture medium with 10% fetal bovine serum supplemented with and without 100 mIU/mL recombinant FSH, 3 mg/mL BSA, and 1% (v/v) Penicillin-G, streptomycin, amphotericin-B cocktail at 37 °C and 5% CO₂ in air. GnRHa leuprolide acetate (50 ng/mL) and antagonist cetrorelix acetate (5 ng/mL) were used at the concentrations that were previously shown to have *in vitro* activity on granulosa cells and ovarian tissue samples (6).

3D Culture on Matrigel

Growth factor reduced matrigel was used in the study, we described previously (7). In brief, matrigel was thawed at 4 °C slowly, and then diluted with DMEM-F12 medium in a 1:1 ratio and added as 100 uL volume in each well of the 96 well culture plate and kept at 4 °C. Then isolated follicles were placed in one follicle for each well fashion and placed in the incubator. Once the matrigel is solidified 30 minutes after incubation, 100 uL of

complete media was added on top of it and replenished every day. The images of the follicle diameter were taken every day using an Olympus IX71 microscope, and follicle diameter was measured using specialized software (Olympus DPS, USA).

Gene Expression Analysis by Quantitative Real Time-Polymerase Chain Reaction

RNA isolation from isolated follicles was performed by Quick-RNA™ MicroPrep (Zymo Research) following the manufacturer's guidelines. RNA quantification was completed by spectrophotometric read at 260 nm by Nanodrop (Thermo Scientific). 1000 ng cDNA synthesis was performed by reverse transcription of RNA using moloney murine leukemia virus reverse transcriptase (Invitrogen). Light Cycler® 480 SYBR Green I Master (Roche) was used to quantify relative mRNA expression levels of GnRH-R and FSH-R genes.

Primers Used

Gene	Sequence
GnRH-R	R 5'-GGCTGCCTTTCATCCCCCT-3'
	R 5'-CGTTCTCAGCCGAGCTCTTGGG-3'
FSH-R	F 5'-ACACAAGTGTGCATTCAACGG-3'
	R 5'-GACTTGTGGCAAATTGGATGA-3'
GAPDH	F 5'-ACAGTCAAGGCCGATAATGG-3'
	R 5'-TCTCCATGGTGGTGAAGACA-3'

Statistical Analysis

Follicle diameter is continuous data and stated as the mean ± standard deviation. The comparison of the groups was made using ANOVA and multiple comparison Tukey post hoc tests.

RESULTS

We first conducted a validation experiment to assess if the experimental design is appropriate to test the effects of GnRHa and antagonist on *in vitro* growth of early antral follicles in mice. For this purpose, small follicles that were 200-300µ in diameter and had antrum formation were selected for the study. The expression of GnRH and FSH receptor in these follicles were verified with quantitative real time-polymerase chain reaction (Figure 1). Then, isolated follicles were randomly assigned into four groups: no FSH (control), FSH only, FSH + GnRHa, and FSH + GnRH antagonist, each group consisting of 23-25 follicles. The results shown below are the mean values of three independent replicates of the experiments. The initial diameter of the follicles

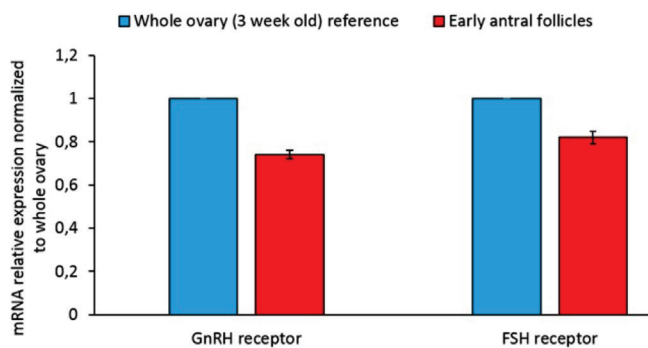


Figure 1. mRNA relative expression normalized to whole ovary FSH: Follicle-stimulating hormone, GnRH: Gonadotropin-releasing hormone at the beginning of the experiment was comparable among the groups (Figure 2). At the end of the culture period, the mean follicle size (μ) of the follicles treated with FSH was notably higher than their counterparts incubated without FSH (657 ± 62 vs. 325 ± 30 respectively, $p < 0.01$). Follicles treated with FSH + GnRHa (545 ± 61 vs. 657 ± 62 respectively, $p > 0.05$) or with FSH + GnRH antagonist (615 ± 46 vs. 657 ± 62 respectively, $p > 0.05$) were not different from those treated with FSH only (Figure 2) in the mean of diameter. When the percentage of growth was compared among the groups it appeared that control follicles treated without FSH achieved a growth rate of 61% at the end of 5 day culture period, which was significantly lower compared those treated with FSH (204%, $p < 0.01$), FSH + GnRHa (162%, $p < 0.01$) and FSH + GnRHa (186%, $p < 0.01$) groups. We observed no considerable differences between FSH, FSH + GnRHa, and FSH + GnRH antagonist groups in terms of follicular growth rate.

DISCUSSION

We performed this study to investigate *in vitro* conditions if GnRHa and antagonist treatment have any impact on FSH induced growth of isolated mouse early antral follicles expressing cognate receptors for FSH and GnRH.

Our results suggest that in mice, FSH-driven *in vitro* growth of small antral follicles is not adversely affected by GnRHa or antagonist co-treatment *in vitro*. It has been shown by different groups that mouse ovaries express the GnRH receptor (1-4). In rats, the comparison of GnRH receptor expression at different follicular stages demonstrated that these mRNA levels of the *GnRH-R* gene vary depending on follicles degree of maturation as well as the estrous cycle stage. While follicles in preantral and small antral stages and corpora lutea showed lower expression, Graafian and atretic follicles had the highest level of *GnRH-R* gene expression (3,8,9). In human ovary, GnRH receptors are localized in granulosa cells from the antral stage and in the

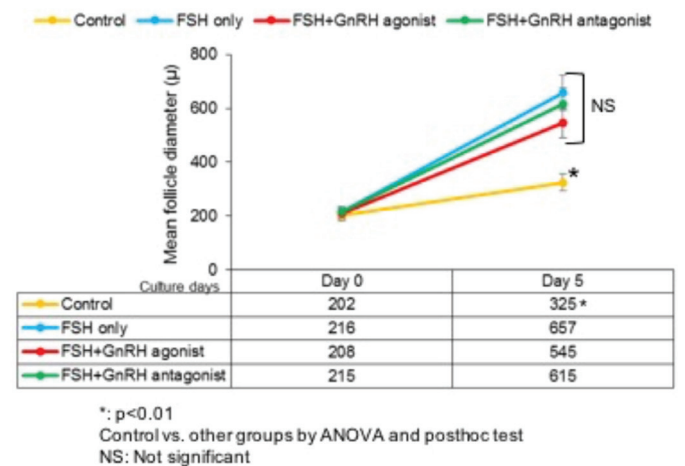
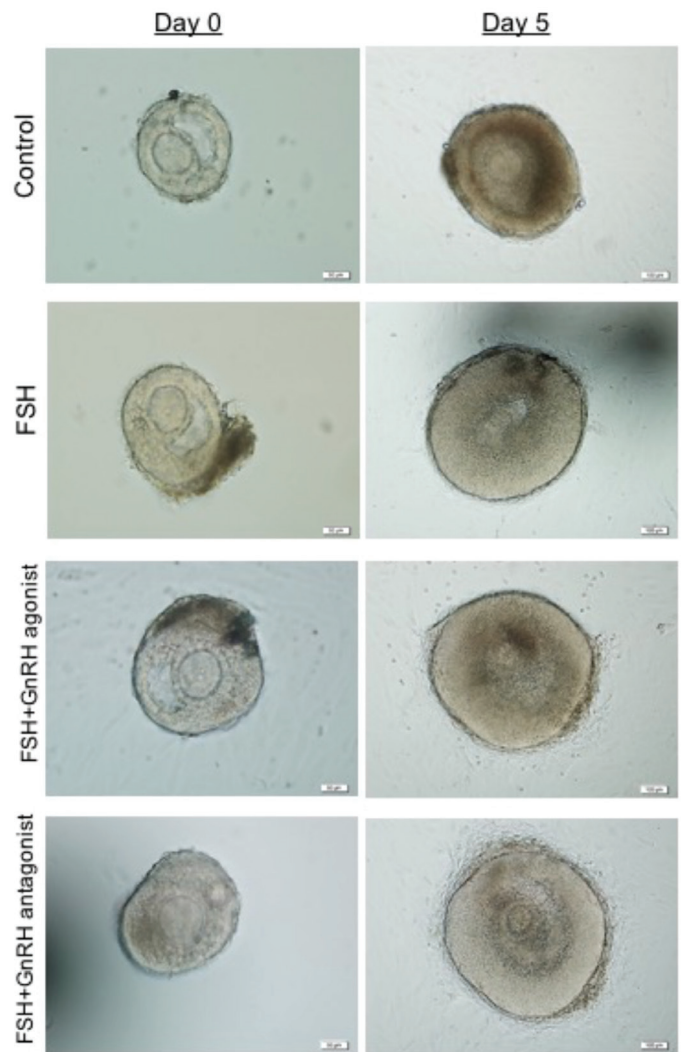


Figure 2. Control vs. other groups by ANOVA and posthoc test NS: Not significant, FSH: Follicle-stimulating hormone, GnRH: Gonadotropin-releasing hormone corpus luteum (10). Exploring intra-ovarian actions of GnRH analogs is hampered by the fact that the systemic administration of GnRH analogs affects the hypothalamic-pituitary-ovarian axis,

precluding one from investigating the sole intra-ovarian action of GnRH analogs on folliculogenesis. We, therefore, aimed to investigate the effect of GnRH analogs, namely, GnRHa leuprolide acetate and antagonist cetrorelix acetate on FSH driven *in vitro* growth of small antral follicles in an isolated culture that express FSH and GnRH receptors.

Although not conducted in human antral follicles, our data might be particularly important from the perspective of assisted reproduction in humans because controlled ovarian stimulation is carried out with FSH treatment together either with a GnRHa or antagonist. It is unknown whether GnRH analogs have any effect on the growth kinetics of small antral follicles that have not reached 2-10 mm diameter, therefore, are not visible on ultrasound.

Our study has several limitations as follows: First, this is an *in vitro* study on the individual culture of isolated small antral follicles. Therefore, the paracrine/autocrine interaction of locally produced growth factors in the intra-ovarian environment that may potentially affect or modify the actions of GnRH analogs cannot be assessed using this model. Second, matrigel contains some growth factors such as EGF, FGF, and TGF, in addition to its main components, extracellular matrix proteins collagen, laminin, and entactin, etc. They may change the response of the follicles to FSH with and without GnRH analogs. Third, the competence of these *in vitro* grown follicles for full growth, ovulation, mature oocyte yield, fertilization rate, and embryo development could not be assessed using this experimental methodology. Fourth, GnRHa and antagonists were not tested at different concentrations. After a certain threshold level, they may have a different action after they bind to their cognate receptors in the follicle.

Ethics

Ethics Committee Approval: The Institutional Animal Care and Use Committee of İstanbul Aydın University Faculty of Medicine approved the protocol (issue number: 2015-20).

Informed Consent: Not applicable.

Peer-review: External and internal peer-reviewed.

Financial Disclosure: The author declared that this study received no financial support.

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