Original Article

Eur Arch Med Res 2018

FSH Induced in Vitro Growth of Small Antral Follicles are not Impacted by GnRH Agonist or Antagonist Treatment in Mice

Yılmaz Güzel

Department of Obstetrics and Gynecology, İstanbul Aydin University School of Medicine, İstanbul, Turkey

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 5 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 qRT-PCR. 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 *in vitro* growth of the follicles compared to those cultured without FSH after 5 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, FSH, GnRH agonist, GnRH antagonist

ORCID IDs of the authors:
Y.G. 0000-0002-5631-8638

Cite this article as:
Güzel Y. FSH Induced in Vitro Growth of Small Antral Follicles are not Impacted by GnRH Agonist or Antagonist Treatment in Mice. Eur Arch Med Res 2018

Corresponding Author:
Serdar Yüksel

E-mail:
yguzel47@gmail.com

Received: 18.08.2018

Accepted: 21.10.2018

DOI: 10.5152/eamr.2018.94840

INTRODUCTION

Our knowledge about the role of extra-pituitary GnRH and its receptors in the ovarian follicle development in human and other species is very restricted. In the mouse ovary, small antral follicles express the cognate receptors for GnRH and FSH (1-3). But their role in the FSH-driven growth of these follicles is unknown. Previous studies showed that although the systemic administration of GnRH agonist (GnRHa) was efficient to disrupt the estrus cycles, it failed to inhibit the follicular development, irrespective of the doses and injection sites (subcutaneous or intramuscular). Approximately 20% of healthy growing follicles were still observed during the 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 difficult to interpret the intra-ovarian actions of the GnRH analogs when they are administered systemically due to their effect on the hypothalamic–pituitary–ovarian axis. One *in vitro* study showed that the GnRH agonist 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 the apoptosis of granulosa cells regardless of the stages of follicular growth (5). It is unclear whether the *in vitro* GnRH analog treatment has any impact on the FSH-induced growth in *in vitro* conditions of small antral follicles isolated from mice and expressing the FSH and GnRH receptors. We aimed to address if the GnRH agonist and antagonist have any effects on the FSH-induced *in vitro* growth of early antral follicles in mice, expressing cognate receptors for FSH and GnRH.

METHODS

Study Design

We designed this study to investigate in *in vitro* conditions if the GnRH agonist and antagonist treatment has any impact on the FSH-induced growth of isolated early antral follicles in mice that are 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 5 days.

Follicle Isolations From Mice

All experiments were conducted on 21-day-old C57BL/6 mice. The Institutional Animal Care and Use Committee of Koç University 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 stereomicroscope (Olympus SZX12, Olympus America Inc., Center Valley, PA, USA) and randomly assigned to the groups to be cultured for 5 days.

Culture Medium Formulation

Isolated follicles were cultured in the (HEPES)- buffered Dulbecco modified eagle medium-F12 (DMEM-F12) culture medium with 10% fetal bovine serum supplemented with and without 100 mIU/mL of recombinant FSH, 3 mg/mL BSA, and 1% (v/v) Penicillin-G, streptomycin, and amphotericin-B cocktail at 37°C and 5% CO_2 in air.

The GnRH agonist leuprolide acetate (50ng/mL) and antagonist cetrorelix acetate (5ng/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

The 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. The isolated follicles were then placed in a one-follicle-for-each-well fashion and placed in the incubator. Once the matrigel solidified 30 mins after the incubation, 100uL of complete media was added on top of it and replenished every day. The images of the follicle diameter were taken every day using the Olympus IX71 microscope, and the follicle diameter was measured using a special software (Olympus DPS, USA).

Gene Expression Analysis by qRT-PCR

The RNA isolation from isolated follicles was performed by Quick-RNA MicroPrep (Zymo Research) following manufacturer's guidelines. The RNA quantification was completed by spectrophotometric read at 260 nm by Nanodrop (Thermo Scientific). A 1000 ng cDNA synthesis was performed by reverse transcription of RNA using a M-MLV Reverse Transcriptase (Invitrogen). A Light Cycler 480 SYBR Green I Master (Roche) was used to perform the quantification of relative mRNA expression levels of GnRH-R and FSH-R genes.

Primers Used

Gene		*Sequence*
GnRH-R	F	5'-GGCTGCCTCTTCATCCCCCT-3'
	R	5'-CGTTCTCAGCCGAGCTCTTGGG-3'
FSH-R	F	5'- ACACAACTGTGCATTCAACGG-3'
	R	5'-GACTTGTTGCAAATTGGATGA-3'
GAPDH	F	5'-ACAGTCAAGGCCGATAATGG-3'
	R	5'-TCTCCATGGTGGTGAAGACA-3'

Statistical Analysis

Follicle diameter is a continuous datum and is expressed as the mean±standard deviation. Comparison of the groups was made using the analysis of variance and multiple comparison Tukey post-hoc test.

RESULTS

We first conducted a validation experiment to assess if the experimental design was appropriate to test the effects of GnRH agonist and antagonist on *in vitro* growth of early antral follicles in mice. For this purpose, small follicles that were 200-300 μ in diameter and had antrum formation were selected for the study. The expression of the GnRH and FSH receptors in these follicles were verified with qRT-PCR (Figure 1). Then, isolated follicles were randomly assigned into four groups: no FSH (control), FSH only, FSH+GnRH agonist, 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 at the beginning of 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 greater than that of their counterparts incubated without FSH (657±62 vs. 325±30 respectively, $p<0.01$). Follicles treated with the FSH+GnRH agonist (545±61 vs. 657±62 respectively, $p>0.05$) or with the FSH+GnRH antag-

onist (615±46 vs. 657±62 respectively, p>0.05) were not different from those treated with FSH only (Figure 2) with regard to the mean of the diameter. When the percentage of growth was compared among the groups, it appeared that the control follicles treated without FSH achieved a growth rate of 61% at the end of the 5-day culture period, which was significantly lower compared those treated with FSH (204%, p<0.01), FSH+GnRH agonist (162%, p<0.01), and FSH+GnRH antagonist (186%, p<0.01). We observed no considerable differences between the FSH, FSH+GnRH agonist, and FSH+GnRH antagonist groups in terms of the follicular growth rate.

DISCUSSION

We performed this study to investigate if the GnRH agonist and antagonist treatment under *in vitro* conditions has any impact on the FSH-induced growth of isolated early antral follicles in mice expressing cognate receptors for FSH and GnRH.

Our results suggest that in mice, the FSH-driven *in vitro* growth of small antral follicles is not adversely affected by the GnRH agonist or antagonist co-treatment *in vitro*. It has been shown by different groups that mouse ovaries express the GnRH receptor (1-4). In rats, comparison of the GnRH receptor expression at different follicular stage demonstrated that these mRNA levels of the GNRH-R gene vary depending on the 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 the GnRH-R gene expression (3, 8, 9). In human ovary, GnRH receptors are localized in the granulosa cells from the antral stage and in the corpus luteum (10).

Exploring intra-ovarian actions of the GnRH analogs is hampered by the fact that the systemic administration of the 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, the GnRH agonist leuprolide acetate and antagonist cetrorelix acetate on the FSH-driven *in vitro* growth of small antral follicles in isolated culture, which express the FSH and GnRH receptors.

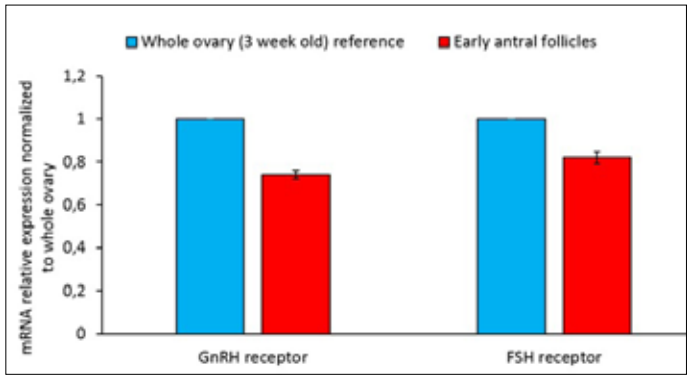

Figure 1. The mRNA expression of the GnRH and FSH receptors in small-size antral follicles of mice by quantitative RT-PCR in comparison to a whole ovary (3 weeks old) as a reference

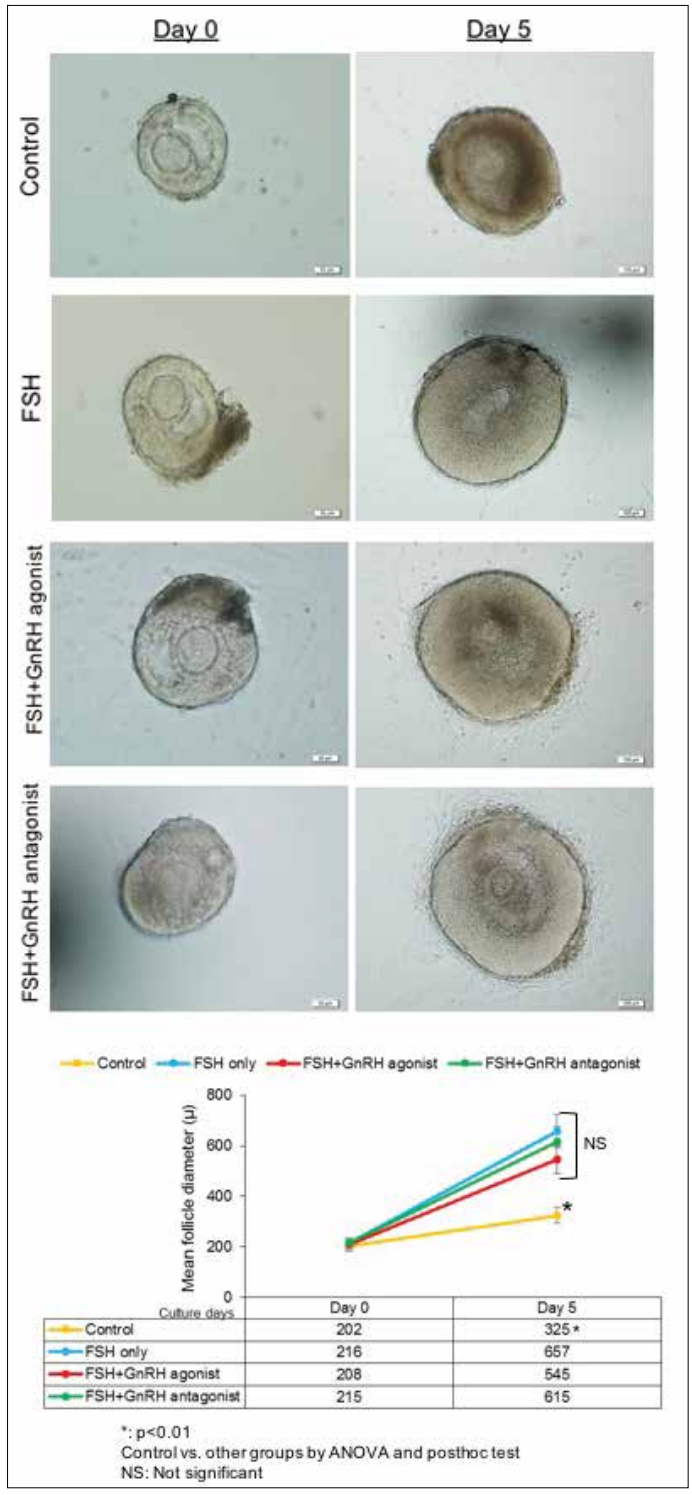

Figure 2. The growth of the small antral follicles isolated and cultured individually in three-dimensional extracellular matrix protein matrigel. The FSH treatment significantly enhanced in vitro growth of the follicles compared to those cultured without FSH at the end of the 5-day culture period. The mean diameters of the follicles treated with the FSH+GnRH agonist (545±61 vs. 657±62, respectively, p>0.05) or FSH+GnRH antagonist (615±46 vs. 657±62, respectively, p>0.05) were not significantly different from those treated with FSH only, but were significantly greater than control follicles

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 the FSH treatment together either with a GnRH agonist or antagonist. It is unknown whether the GnRH analogs have any effect on the growth kinetics of the small antral follicles that have not reached a diameter of 2-10mm, and therefore, are not visible on ultrasound.

Study Limitations

First this is an *in vitro* study on an individual culture of isolated small antral follicles. Therefore, 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, entactin, etc. They may change the response of the follicles to FSH with and without the GnRH analogs. Third, the competence of these follicles grown *in vitro* for full growth, ovulation, mature oocyte yield, fertilization rate, and embryo development could not be assessed using this experimental methodology. Forth, the GnRH agonist and antagonist were not tested at different concentrations. After a certain threshold level, they may have a totally different action after they bind to their cognate receptors in the follicle.

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*.

Ethics Committee Approval: Ethics committee approval was received for this study from The Institutional Animal Care and Use Committee of Koç University (2015-20).

Peer-review: Externally peer-reviewed.

Conflict of Interest: The author has no conflicts of interest to declare.

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

REFERENCES

1. Torrealday S, Lalioti MD, Guzeloglu-Kayisli O, Seli E. Characterization of the gonadotropin releasing hormone receptor (GnRHR) expression and activity in the female mouse ovary. Endocrinology 2013; 154: 3877-87.
2. Mo Y, Peng P, Zhou R, He Z, Huang L, Yang D. Regulation of gonadotropin-releasing hormone (GnRH) receptor-I expression in the pituitary and ovary by a GnRH agonist and antagonist. Reprod Sci 2010; 17: 68-77.
3. Horicks F, Van Den Steen G, Gervy C, Clarke HJ, Demeestere I. Both in vivo FSH depletion and follicular exposure to Gonadotrophin-releasing hormone analogues in vitro are not effective to prevent follicular depletion during chemotherapy in mice. Mol Hum Reprod 2018; 24: 221-32.
4. Horicks F, Van Den Steen G, Houben S, Englert Y, Demeestere I. Folliculogenesis Is Not Fully Inhibited during GnRH Analogues Treatment in Mice Challenging Their Efficiency to Preserve the Ovarian Reserve during Chemotherapy in This Model. PLoS One 2015; 10: doi: 10.1371/journal.pone.0137164.
5. Huang J, Wang X, Li Z, Ma R, Xiao W. Effects of GnRH agonists on the expression of developmental follicular anti-mullerian hormone in varying follicular stages in cyclic mice in vivo. Mol Med Rep 2015; 12: 4305-13.
6. Bildik G, Akin N, Senbabaoglu F, Sahin GN, Karahuseyinoglu S, Ince U, et al. GnRH agonist leuprolide acetate does not confer any protection against ovarian damage induced by chemotherapy and radiation in vitro. Hum Reprod 2015; 30: 2912-25.
7. Oktem O, Buyuk E, Oktay K. Preantral follicle growth is regulated by c-Jun-N-terminal kinase (JNK) pathway. Reprod Sci 2011; 18: 269-76.
8. Bauer-Dantoin AC, Jameson JL. Gonadotropin-releasing hormone receptor messenger ribonucleic acid expression in the ovary during the rat estrous cycle. Endocrinology 1995; 136: 4432-8.
9. Whitelaw PF, Eidne KA, Sellar R, Smyth CD, Hillier SG. Gonadotropin-releasing hormone receptor messenger ribonucleic acid expression in rat ovary. Endocrinology 1995; 136: 172-9.
10. Maggi R, Cariboni AM, Marelli MM, Moretti RM, Andre V, Marzagalli M, et al. GnRH and GnRH receptors in the pathophysiology of the human female reproductive system. Hum Reprod Update 2016; 22: 358-81.