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Effects of kisspeptin on the maturation of human ovarian primordial follicles *in vitro*

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Summary

At this time, with advances in medical science, many cancers and chronic diseases are treatable, but one of their side effects is infertility. Some women also want to delay pregnancy for personal reasons. There has been some evidence that kisspeptin activates broad signals by binding to its receptor, suggesting that the role of kisspeptin in direct control of ovarian function includes follicle growth and steroid production. In this study, the effect of kisspeptin on improving the quality and results for human ovarian follicles was investigated. A section of ovary was removed laparoscopically from women between 20 and 35 years of age ($n = 12$). Pieces were divided randomly into two groups, control and treatment (with 1 μM kisspeptin). Real-time PCR was performed for *GDF9*, *BMP15* and *mTOR* gene expression assessments. Western blotting was carried out to measure AKT and FOXO3a protein expression. Data were analyzed using one-way analysis of variance (ANOVA) and Tukey's test; means were considered significantly different at a P-value < 0.05. During treatment with the kisspeptin group, maturity genes are expressed. Therefore, kisspeptin is an effective substance to improve the quality of the human ovarian medium as it increases the maturity of follicles.

Introduction

At this time, one strategy to maintain fertility in women who are facing the risk of ovarian function loss, including women with cancer (in which loss is due to chemotherapy and radiation therapy), is to freeze ovarian tissue and transplant it after recovery. However in many cases, especially in women with leukaemia, there are malignant cells in the ovary, so re-transplantation of ovarian tissue is not a good option if the follicles inside the tissue can potentially grow *in vitro* (Jadoul *et al.*, 2010; Donnez *et al.*, 2017; Rivas Leonel *et al.*, 2019). Primary follicles have special benefits for premenopausal girls who have undergone chemotherapy. Currently, the only preservation option for girls before puberty is ovarian tissue storage and transplantation after complete recovery (Wallace *et al.*, 2014; Shi *et al.*, 2017). Although the follicle matures in the connective tissue, it has little ability to fertilize or implant so, to date, only 60 live births worldwide have been reported from transplanted frozen ovarian tissue (Ramezani *et al.*, 2017; Taghizabet *et al.*, 2018). There is also the possibility of cancer cells re-emerging if ovarian tissue is transplanted. Conversely, it has been clinically proven that the duration of activity of a thawed frozen ovary that is transplanted to a person is from 9 months to 3 years, while there is a two-thirds reduction in follicles due to ischaemia resulting from transplantation. Therefore, transplantation of thawed frozen ovarian tissue is not a suitable method for some patients. For these women, *in vitro* culture of follicles derived from ovarian tissue is recommended as the majority of ovarian cortex follicles are inactive primordial follicles (Choi *et al.*, 2007; Mazoochi *et al.*, 2009; Kona *et al.*, 2016). Recent research has shown that culture of the human ovary cortex containing primordial follicles significantly changes inactive primordial follicles to existing follicles and growth occurs in a short period of 6–10 days.

When the primordial follicles in the ovarian cortex begin to grow, they reach the multilayered stage but do not survive. There are several culture media for follicle (egg) growth in the laboratory. Among them, there has been only one successful report for the human primordial follicle to the stage of meiosis puberty, but in this report the meiotic spindle was defective and fertilization did not occur (Kotani *et al.*, 2001; Ebrahimi *et al.*, 2010; Trapphoff *et al.*, 2016).

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In one animal study on mice, there was only one live birth but this exhibited defects such as obesity; however, proof of concept showed that full development of human eggs could occur *in vitro* (Skorupskaite *et al.*, 2014) Further optimization and morphological evaluation are needed to determine whether the eggs were normal. Therefore, any study to optimize culture media will be valuable. Because kisspeptin (metastin) is a neuropeptide produced in the hypothalamic axis and made by two populations of neurons in the hypothalamus, it affects egg and ovarian maturation *in vivo*. Its gene (*KISS1*) is located on chromosome 1q32.11 and modulates the expression of the antioxidant enzymes against oxidant agents; it also regulates puberty and reproductive activities through its membrane receptors coupled with G protein (Kotani *et al.*, 2001). Kisspeptin triggers the hypothalamic–pituitary–gonadal axis, inducing gametogenesis by releasing follicle-stimulating hormone (FSH) and luteinizing hormone (LH) through the pituitary gland. Many studies have demonstrated the role of kisspeptin in ovarian function control, such as follicular development, steroidogenesis, oocyte maturation, and ovulation (Aslan *et al.*, 2017; MacManes *et al.*, 2017).

In a study in 2018, convincing evidence showed that kisspeptin activated broad signals by binding to its receptor, suggesting a role for kisspeptin in the direct control of ovarian function. The results obtained from this study showed that the intraovarian kisspeptin/KISS1R system modulated granulosa cell proliferation and apoptosis, oocyte maturation, ovulation, and steroidogenesis by regulating the mitogen-activated protein kinase (MAPK) signalling pathway. In addition to the MAPK pathway, the PI3K/Akt pathway also functions in both granulosa cells and oocytes (Hu *et al.*, 2017; Cao *et al.*, 2019).

Materials and methods

Twelve healthy ovaries from women aged 25–40 years who had undergone hysterectomy and had their fallopian tubes closed were used after pathology examination to ensure the health of ovarian tissue. Ovarian tissue was transferred to a culture medium containing sodium pyruvate (2 mM), glutamine (2 mM), human serum albumin (3 mg/ml), penicillin G (75 μg/ml), and streptomycin (50 μg/ml).

Ovarian tissue culture

Ovarian tissue was transferred to fresh culture medium, observed under a microscope, and extra tissue and blood clots were removed. The resulting tissue was divided into 1 × 1 × 0.5 mm square pieces. Finally, the samples were cultured in 24-well plates. Here 300 μl of culture medium containing HEPES, glutamine, penicillin, streptomycin, transferrin, selenium, and human insulin in the sham group w added to each well. In groups treated with kisspeptin, doses of 35 or 40 pmol of kisspeptin were added to the culture medium. The culture plates were incubated for 37 days at 37°C and 5% CO_2. After 8 days, growing follicles were observed on the surface of the tissue.

Evaluation of phosphorylation of FOXO3/Bax and ATK by western blotting

At the end of the ovarian tissue treatment period, the surface of the Petri dishes was then washed twice with cold PBS, and then 100 μl of lubricating buffer containing 2.25 ml RIPA buffer, 50 μl protease inhibitor, 50 μl orthovanadate, and 250 μl sodium fluoride were added to each Petri dish. With the help of a scraper, the tissue was collected and transferred into a microtube. The details of the protocol were carried out according to the study by Qin *et al.* (2022). Antibody cat. nos.: AKT, #9272; FOXO3, SC-48348; GAPDH, GTX100118; secondary antibody (rabbit), BA1054-2; secondary antibody (mouse) SC-516102.

Investigation of gene expression for mTORC, BMP15, GDF9 and MOS by RT-PCR

Expression levels of genes *mTORC*, *BMP15*, *GDF9* and *MOS* was evaluated by RT-PCR in four groups, using RT-PCR, and after mRNA extraction and cDNA synthesis. RNA was extracted from each replicate of the treatments using a Qiagen microkit according to the manufacturer's recommended instructions. cDNA synthesis was performed using a Qiagen cDNA synthesis kit according to the manufacturer's instructions. The primers were designed using Primer Design software and their specificity was verified by the NCBI site. After replication efficiency, gene expression was assessed using specific primers and SYBR green. Threshold cycle values of target genes compared with host genes were corrected and the relative expression of the gene was calculated using the $2^{-\Delta\Delta Ct}$ method.

Table 1. The primer sequences of target genes

Target genes	Primer sequencing	Annealing temperature (°C)
GAPDH	F: CATGGTCTACATGTTCCAGTATGATTC R: TCACCATCTTCCAGGAGCG	56
BMP15	F: TGACGCAAGTGGACACCCTA R: GAAGAAGGAAAGTGATTGGTTGGG	56
GDF9	F: AGGCATACGTACCAAGGAGG R: CCACAACAGTAACACGATCCAGG	58
mTOR	F: TGTGATGGCTGTGAAGATCC R: TATTCACCTCCTGCCTCACC	56
MOS	F: CGGTTTTTCTGGGACAACAA R: AAAAACGTGTCTCGCCTTTC	58

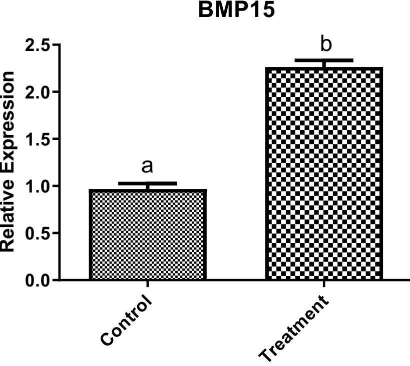

Figure 1. mRNA expression levels of BMP15 in experimental groups. Different lowercase letters (a and b) indicate a significant difference between groups.

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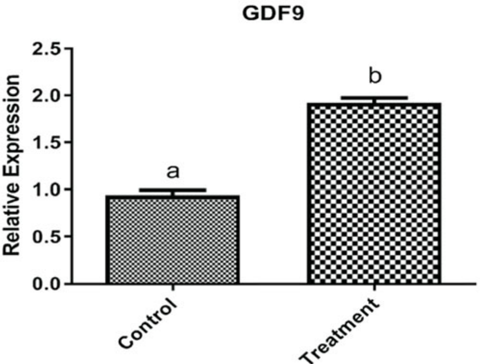

Figure 2. mRNA expression levels of GDF9 in experimental groups. Different lowercase letters (a and b) indicate a significant difference between groups.

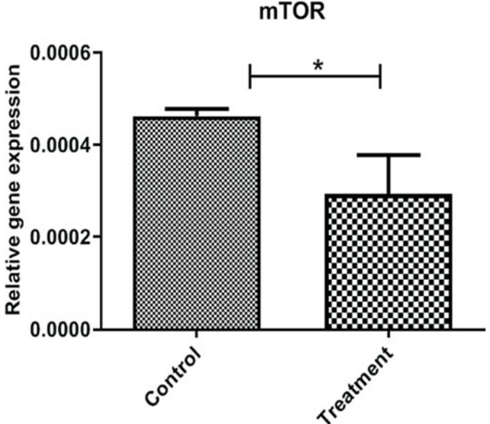

Figure 3. mRNA expression levels of mTOR in experimental groups. '*' indicates a significant difference between groups at $P < 0.05$.

Results

Gene expression

BMP15

As can be seen in Figure 1, in the treatment group, the expression level is also higher than in the control group ($P < 0.05$; Figure 1).

GDF9

Real-time PCR was used to evaluate the effect of kisspeptin on the expression of follicle maturity genes. The characteristics of the primers used are given in Table 1. In the treatment group, the expression level was higher than in the control group and its difference compared with all groups was significant ($P < 0.05$; Figure 2).

mTOR

In the treatment group, the expression levels of mTOR and MOS were lower than in the control group ($P < 0.05$; Figures 3 and 4).

Protein expression

The expression of AKT protein in the treatment group was lower than in the control group. In the treatment group the expression

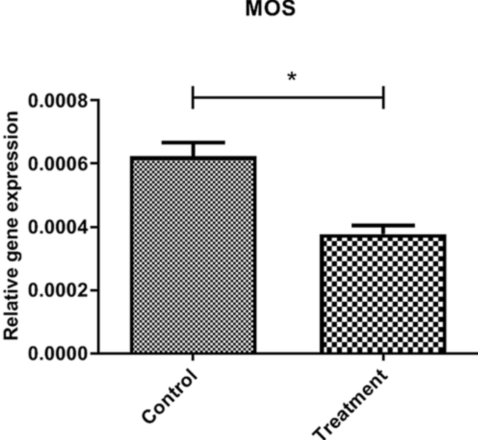

Figure 4. mRNA expression level of MOS in experimental groups. '*' indicates a significant difference between groups at $P < 0.05$.

levels of FOXO3a and Bax were significantly higher than in the control group ($P < 0.05$; Figure 5).

Discussion

This study showed that adding kisspeptin to FSH-rich culture medium increases maturity in the cumulus–oocyte pig complex that can lead to increased expression of GDF9 and BMP15. Hu *et al.* (2017) stated that kisspeptin is secreted from puberty, increasing follicle maturation, reducing the number of immature follicles and defects in kisspeptin, and causing primary ovarian insufficiency (POF). Treatment of porcine cumulus–oophorus cells with kisspeptin in culture medium increased the expression of GDF9 and BMP15. Taniguchi *et al.* (2017) showed that the amount of follicular fluid kisspeptin was directly related to the increase in mature follicles and estradiol. Kisspeptin also increased in the pre-ovulatory and luteal periods and was expressed in growing follicles in granulosa and theca cells. Kisspeptin promoted the maturation of follicles and increased the number of secondary follicles. This finding confirmed previous studies that kisspeptin directly affected the maturity of follicles (Hu *et al.*, 2017). An earlier study showed that adding kisspeptin to sheep culture medium increased oocyte maturation.

In conclusion, the results of this study showed that kisspeptin has a stimulating effect on the maturation of human ovarian follicles in culture.

Ethics approval and consent to participate

The present study was extracted from a research project approved by the Men's Health and Reproductive Health Research Center, Shahid Beheshti University of Medical Sciences. Informed consent was received from all participants.

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Competing interests. In the present study, the authors did not have any conflict of interest.

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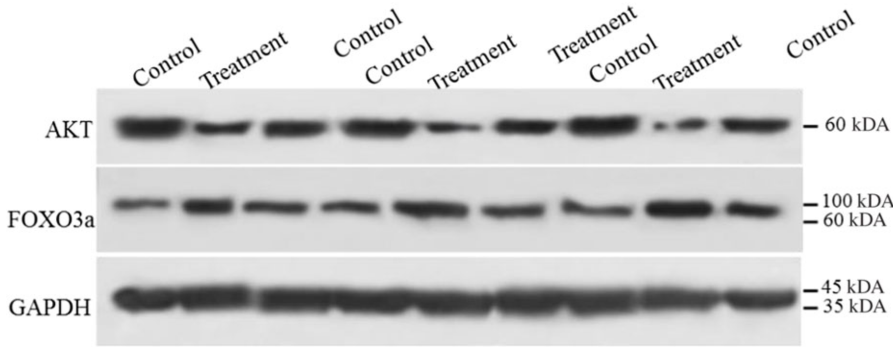

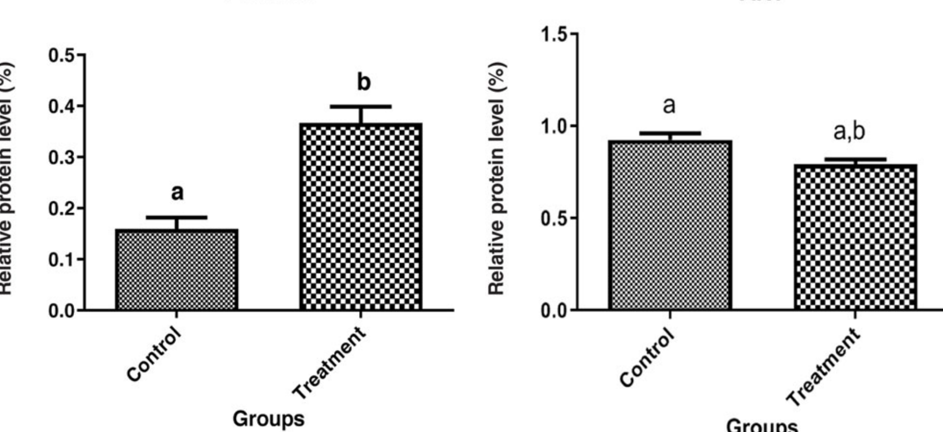

Figure 5. Western blot analysis of AKT and FOXO3a. Different lowercase letters (a and b) indicate a significant difference between groups.

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