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THE EFFECTS OF INSULIN AND PMSG ON DEVELOPMENT OF FOLLICLES, GRANULOSA CELLS DIAMETER AND MRNA EXPRESSION OF CYTOCHROME P450 AROMATASE IN CULTURED OVARIES

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ABSTRACT

The objective of this study was to determine the actions of different concentrations of insulin alone or in association with follicle-stimulating hormone (FSH) in organ culture and evaluated follicles growth ,granulosa cells diameter and mRNA expression of cytochrome P450 aromatase (CYP19A1). Ovaries were cultured for 5 days in the absence or presence of insulin (0.2, 1, 5 and 10 µg/ml) alone or plusPMSG (10 ng/ml). In the medium with insulin 0.2 and 1 µg/ml in combination PMSG, follicles size ($P<0.01$), granulosa cells diameter ($P<0.05$) and levels of p450 aromatase ($P<0.05$) were significantly increased compared with other treatments. While in high dose of insulin (5, 10 µg/ml)alone or in combination with PMSG percentage of morphologically normal follicles decreased during 5 days of in vitro culture ($P<0.05$). In conclusion, 0.2 and 1 µg/ml insulin plus PMSG was more efficient in stimulating follicular development and increasing expression of the CYP19A1 genes.

Keywords: Insulin, PMSG, organ culture, granulosoa cells, cytochrome P450 aromatase (CYP19A1)

INTRODUCTION

Ovarian follicles and oocytes development, depends upon the expression of genes , hormones, and growth factors (1). Steroidogenic capacity of granulosa cells is one of the known markers for healthy antral follicles.(2) A critical step during the estrous cycle that leads to the expression of estrus, is increase secretion of estradiol by ovulatory and non-ovulatory follicles (3). Oestradiol is a principal steroid produced by granulose cells (4). In ruminants and humans, granulosa cells convert theca-derived androgens to oestrogens with the enzyme cytochrome P450 aromatase (5). The expression of Cyp19 mRNA is stimulated by follicle-stimulating hormone (FSH) (6). FSH is not the sole stimulator of Cyp19 expression/ activity,several other factors have been shown to interact with FSH to enhance oestradiol secretion in various species. Insulin can promote FSH-stimulated oestradiol production in granulosa cells (7, 8, 9), and can induce Cyp19 expression and/or oestradiol secretion granulosa cells in the absence of FSH (9, 10).

Insulin, a polypeptide produced by pancreatic b cells wich in addiction to regulate serum glucose concentrations , involved in the regulation of cell growth and development in many tissues such as ovaries (11).In the ovary, insulin mediate follicules development, steroidogenesis and oocyte maturation (12).

Actions of insulin are mediated through insulin receptors (INSR), which it was shown that are placed throughout all ovarian tissues, such as the granulosa and theca cells and stromal tissue (13). Various reproductive processes, such as regulating the neurotransmitter synthesis of GnRH, and control of gonadotropin secretion are mediated with insulin (14) Insulin also promote FSH-stimulated oestradiol production in granulosa cells(6). In mice, the principal regulators of cell proliferation, differentiation, and survival of ovarian follicles are gonadotropins,(luteinizing Hormone [LH] and FSH) and insulin (15).Synergistically action of insulin with FSH induce granulosa cell differentiation and proliferation. Production of FSH-dependent steroid and LH receptor induction also facilitates with insulin in cultured granulosa cells (16,17).Insulin is able to the produce estrogen and progesterone in the presence of FSH in women granulosa cells (18). FSH can increase both the production of estradiol and the expression of CYP19A1 mRNA in bovine granulosa cells cultured in serum-free

medium (19)and the presence of insulin has been shown to enhance this effect (20).

Thus, according to action of insulin on many organs, such as ovary, and uses of it,as a critical supplement in vitro culture of preantral follicles, we evaluated different concentrations of insulin, alone or in combination with PMSG with or without FSH during the organ culture of ovary and its effect on development of follicles and expression of CYP19A1 mRNA .

MATERIALS AND METHODS

Culture of mouse ovaries

Mouse ovaries of 5 weeks were cultured at 37°C with 5% CO2 in 500 µl tissue-specific medium on 32-well plates (21). The next day, 250 µl of fresh medium was added into each well, and half of the total medium (250 µl) was replaced with fresh medium every other day. Following day 4, the basic medium was replaced by 500 µl of fresh medium every other day. The day when ovaries were placed in culture was marked as day 0. The basic medium for fetal ovarian culture consists of Dulbecco's modified Eagle's medium (DMEM)/F12 plus a-minimal essential medium (a-MEM) (1:1) (Gibco-BRL, Carlsbad, CA, USA) with 3% (w/v) BSA, 1 mg/ml of Fetuin (Sigma, St. Louis, MO, USA), 0.23 mmol/l pyruvic acid,100 IU/ml of penicillin G, and 100 mg/ml of streptomycin sulphate (Gibco-BRL) in the absence or presence of insulin (0.2,1,5 and 10 µg/ml) alone or in combination with PMSG (10 ng/ml).

Microscopic Study

After 5 days in culture, four ovaries from each treatment were fixed in 4% paraformaldehyde for 4 h and then embedded in paraffin. Serial sections (thickness, 5 µm) were cut and stained with hematoxylin-eosine.Each nucleus ofoocyte from each follicles were chosen for follicle/oocyte counts, and the average was used as the follicle/oocyte number of one ovary .To assess the progression of follicle formation, we counted follicles/oocytes (primordial, preantral , antral ,graaf) in ovarian sections.For measuring the diameter of ovarian follicle in each developmental stage, 45 microscopic fields were randomly chosen in each mice. Then, using the ocular micrometer of a light microscope (Olympus EH, America Inc.), at a magnification of 40×, the diameter of each ovarian follicle, oocyte, granulosa cells, interna and externa theca cells were measured.

RNA preparation and Real Time PCR (RT –PCR) Total RNA was prepared from frozen ovary extraction of RNA and reverse transcription of RNA to cDNA was

performed using RNX-Plus (Cinna gen, Karaj, Iran) and 2-steps RT-PCR kit (Vivantis, UK), respectively, due to manufacturer instructions. Reverse transcriptions were performed with oligo-d(T) primer at 50 8C for Hsd17b1, and with oligo-d(T) and gene-specific primer for Cyp19 (50-GACTCTCATGAATTCTCCATACATCT-30) (Fu¨ rbass et al. 1997) at 42 8C. For PCR, the primers were 50-CTGAAGCAACAGGAGTCCT AAA TGTACA-30 (sense) and 50-GGACTAGTAATGAGGGGCCCAATTCCC AGA-30 (antisense) for Cyp19 (22). Real time-PCR performance using SYBR Green PCR Master Mix (Amplicon) and Rotor-Gene 6000 Series software version 1.7.65 .Cycling conditions for Cyp19 were denaturing at 94 8C for 3 min, followed by 27 cycles of 94 8C for 45 s, 60 8C for 30 s and 72 8C for 1 min. Preliminary experiments were performed to verify that PCR product intensity increased with amount of RNA in the reverse transcription reaction, and that PCR intensity increased with cycle number. Amplification was performed within the linear range for each primer pair, and PCR products were visualised on agarose gels after staining with ethidium bromide (22).

Statistical Analysis

The data were analyzed using SPSS, version 16 for Windows. The diameter of follicles and granulosa cells and also expression of CYP19A1 mRNA in the control and cultured groups were compared by one-way analysis of variance (ANOVA) and Tukey's post hoc test. The differences were considered to be significant when $P<0.05$.

RESULTS

In the first set of experiment ovaries were cultured for 5 days in Dulbecco's modified Eagle's medium (DMEM)/F12 plus a-minimal essential medium (a-MEM) (1:1) with 3% (w/v) BSA, 1 mg/ml of Fetuin, 0.23 mmol/l pyruvic acid,100 IU/ml of penicillin G, and 100 mg/ml of streptomycin sulphate in the absence or presence of insulin (0.2,1,5 and 10 µg/ml) alone or in combination with PMSG (10 ng/ml).

Follicular and granulosa cells diameter

The follicle and granulosa cells diameters were evaluated and shown in figure 1and 2. After 5 day of culture, a significant increase in follicular diameter was observed in 0.2 and 1 µg/ml insulin plus PMSG 10ng/ml compared to the control groups. In general, the tissues cultured in the presence of 0.2 and 1 µg/mL of insulin in combination PMSG showed significantly larger follicle and granulosa cells diameters than the other

treatments in the same period ($p<0.01$). The average diameter of preantral and antral follicles was decreased in high concentration insulin (5 , 10 µg/ml) alone and combination with PMSG ($p<0.05$)

There were no significant differences between mean follicles diametere of preantral and antral follicles in the controls and PMSG group .Antral follicles were not seen in insulin 5,10 experiment groups. There were no significant differences between the means of primordial and primary follicles in diameter in the control and treatment groups . The mean for preantral and antral follicles diameter decreased significantly ($P<0.05$) in high dose insulin groups(5,10 µg/ml) plus PMSG in comparison with the controls. Moreover, significant ($P<0.05$) decreases were seen between the mean of graaf follicle diameter in the between 0.2,1µg/ml insulin plus PMSG culture and other group .

Expression CYP19A1

The effects of insulin alone or associated with FSH were evaluated using CYP19A1 mRNA expression levels by real-time PCR (Fig. 3). The mRNA expression levels for CYP19A1 were detected in all the treatments. After 5 days of culture the addition of insulin (0.2 and 1 µg/ml) plus PMSG significantly affect the CYP19A1 mRNA expression . Interestingly, we found that the addition of insulin alone to the culture medium significantly reduced CYP19A1mRNA expression compared

with insulin plus PMSG treatments. Finally, the highest levels of mRNA expression for CYP19A1 were observed when FSH was associated with low dose of insulin (P , 0.05).

DISCUSSION

Results of the present study suggest that insulin and FSH have synergic effects on aromatase activity of granulosa cells. Previous in vitro studies have shown that insulin can enhance estradiol production granulosa cells. This is the first report comparing the effects of insulin and FSH on ovarian granulosa cells aromatase activity in mice. Results of the present study also indicate that aromatase activity increases with low concentration of insulin treatment.

The effect of insulin and PMSG on granulosa cells is likely physiologically relevant.

Average diameter of follicles and granulosa cells in low concentrations of insulin (0.2,1µg/ml) and PMSG increase compared with the control-free insulin group.

Insulin injections have shown that increase estradiol concentrations in follicular fluid of cattle in vivo. Thus,according previous studies and our results, insulin and FSH may

be physiologically relevant regulators of ovarian follicular estradiol production(3). In this study showed the highest levels of mRNA expression for CYP19A1is in medium of PMSG and insulin (0.2,1 µg/ml), which were associated with the highest diameter of granulosa cells. CYP19A1 is a steroidogenic enzyme that catalyze estrogen from androgens in the ovary (24) .FSH is the first stimulator for CYP19A1 expression and enzyme activity in ruminants (25,26).

However, some study reported that insulin has an essential role for CYP19A1 expression.

Insulin is modulate the response of granulose cells to gonadotropins through its own receptor

and enhances estradiol production in granulosa cells at physiological concentrations (18).

Insulin in combination with FSH has been shown to induce theca and granulosa cell steroidogenesis in monkeys (27).

In conclusion, low concentrations of insulin with FSH were more efficient for follicles development. In fact ,the combination of these hormones stimulated follicular development and maintained follicular survival as well as increased estradiol secretion. In addition, the interaction of the two hormones (FSH and insulin) positively influenced diameter of follicles and granulose cellsa Thus, we have revealed combining PMSG and low concentration of insulin, can influence the development of follicles and increase mRNA expression .Further research will be required to determine if exogenous insulin could be used to enhance reproductive efficiency in mice.

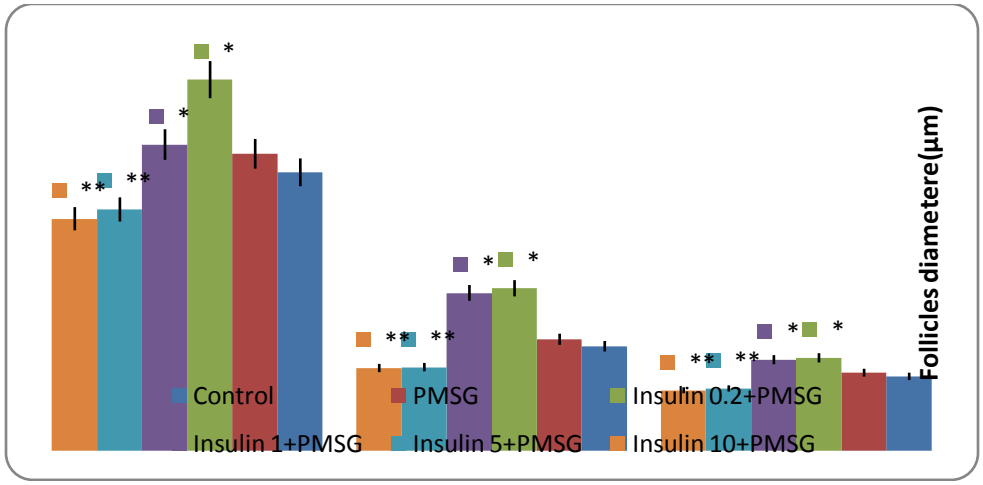

FIG 1.The diameter(µm) of ovarian follicles with normal morphology after in vitro culture in medium in the presence of different concentrations of insulin (0.2,1, 5, and 10 µg/ml) plus PMSG (0.1 mIU/ml) ,PMSG alone and control. * There were significant increase diameter between 0.2,1µg/ml insulin plus PMSG culture with other groups(P<0.01). There were significant decrease diameter between 5,10µg/ml insulin plus PMSG culture with other groups(P<0.01)**

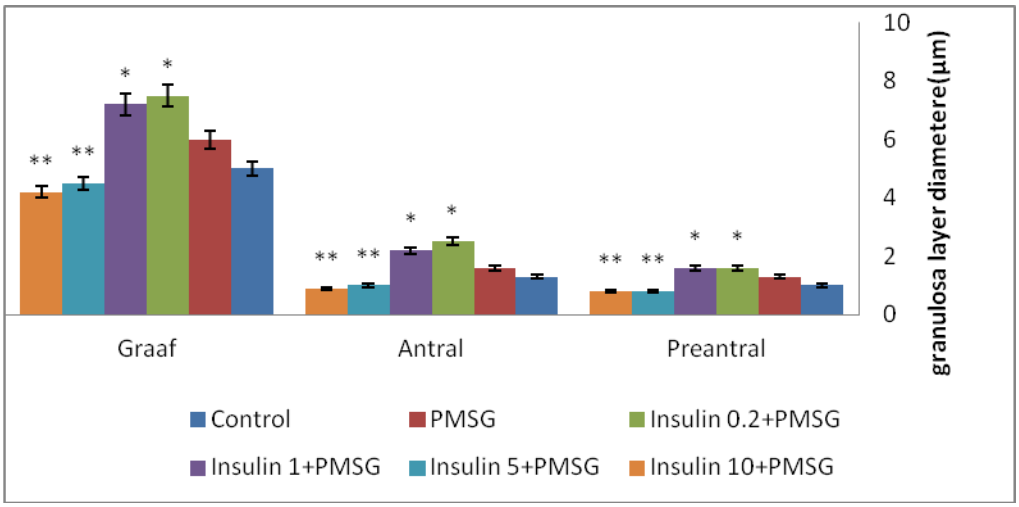

FIG 2.The diameter(µm) of granulosa cells with normal morphology after in vitro culture in medium in the presence of different concentrations of insulin (0.2,1, 5, and 10 µg/ml) plus PMSG (0.1 mIU/ml) ,PMSG alone and control. * There were significant increase diameter between 0.2,1µg/ml insulin plus PMSG culture with other groups(P<0.01). There were significant decrease diameter between 5,10µg/ml insulin plus PMSG culture with other groups(P<0.05)**

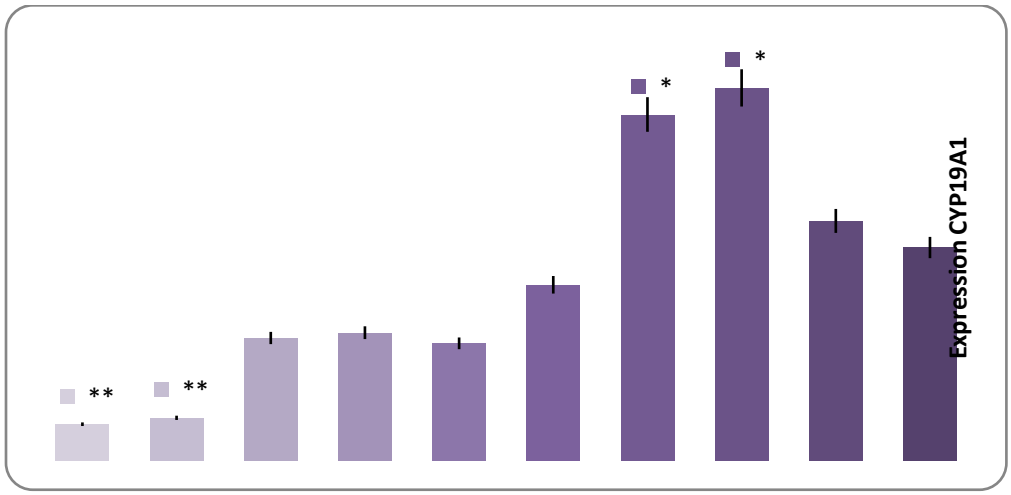

FIG 3 Expression of CYP19A1 mRNA in medium in the presence of different concentrations of insulin (0.2,1, 5, and 10 µg/ml) plus PMSG (0.1 mIU/ml) ,PMSG alone and control. * There were significant increase diameter between 0.2,1µg/ml insulin plus PMSG culture with other groups(P<0.05). There were significant decrease diameter between 5,10µg/ml insulin culture with other groups(P<0.05)**

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