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Human Recombinant Interleukin-3 (hrIL-3) Stimulates FSHR and LHCGR Expression in Ovaries of Female Rats

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Abstract

To investigate the impact of human recombinant interleukin-3 (hrIL-3) on ovulation in female rats ovaries. We used forty eight Wister rats were divided randomly into four equal groups twelve female each. All rats undergone superovulation protocols using pregnant mare serum gonadotropin (PMSG) 10IU intra-peritoneally. Final maturation of oocytes achieved after 48hours using human chorionic gonadotropin (hCG) 10IU intra-peritoneally. We used hrIL-3 in three doses: 15ng, 30ng, and 45ng/rat after superovulation. Concomitantly with hCG, intravenous injection of hrIL-3 was used in three doses o the three treated groups, while the control group received normal saline at the same time of hCG injection. The animals of each group were divided into two subgroups six female each According to the time of sacrificing after 12h and 36h of hCG injection. Samples of ovaries were obtainedv from all animals for RT-qPCR gene expression of fshr and lhcgr. There was a significant increase ($P<0.05$) of fshr gene after 12h of treatment in 15ng and 45ng hrIL-3 compared with control. However, there were no significant differences among the other groups compared to control. There is significant increase ($P<0.05$) of lhcgr gene after 12h of treatment in 15ng hrIL-3 compared with control, while there were no significant differences among the other groups. After 36h, lhcgr gene showed significant increase ($P<0.05$) of 45ng compared with control while there were no significant differences among the other groups.hrIL-3 with a dose of 45ng at 36h after hCG injection stimulates rat ovaries via increase gene expression of FSHR and LHCGR in ovarian tissues.

Keywords: hrIL-3, FSHR, LHCGR, Ovary, Female rats

Introduction

Ovary is a dynamic organ par excellence, performing diverse functions and undergoing marked changes regularly each reproductive cycle. Ovary is the reservoir of oocytes from dormant primordial state as well as formation of growing follicles and selection of dominant oocyte to ovulate (1). Ovulation is a complicated process that involves numerous common mediators of inflammatory reactions in addition to gonadotropins and steroid hormones (2). The gonadotropins LH and FSH, act on the theca cells and granulosa cells, respectively, in conjunction with a number of ovarian and endocrine factors, are essential for terminal folliculogenesis (3, 4). The LH pulses will increase in intensity as more time elapses from the previous event of ovulation cycle due to luteolysis of the corpus luteum and diminishing progesterone levels and eventually culminate in a sufficient surge to induce ovulation .(6 ,5)

Ovulation is a process occurs after the LH surge under the effect of hypothalamus and pituitary. The peri-ovulatory LH surge activates ovarian enzymes that make the follicle wall weaker so that the oocyte can be expelled more easily and ovulation occurs. In ovary, the dominant follicle required estrogens as final products to development properly (7). Estrogens also boost the responsiveness of theca and granulosa cells to FSH and LH by increasing development of receptors in granulosa cells. The previous studies confirmed that cytokines play a role in both follicular responsiveness to gonadotropins being stimulated and inhibited (8). The gonadotropin surge starts the

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www.qu.edu.iq/journalvm

ovulatory process by programming genes' transcriptional patterns of many follicles .(9)

It is possible that ovary can initiate inflammatory processes by itself. The cellular pool of the follicular fluid contains macrophages in amounts ranging from 5 to 15%. (10). The exact regulation of the immuno-endocrine system is carried out by autonomous functional ovarian cells such as theca cells and granulosa cells (11). Ovulation have been referred as acute inflammatory response due to the involvement of leucocytes, a well-known inflammatory mediators, and proteolytic enzymes in this biochemical process (12). It is widely acknowledged that granulosa cells are where the ovulatory process first begins, according to findings from studies in mice (13). In this study, we aimed to investigate the possible role of interleukin-3 in ovaries of female rats. We explored the gene expression study for LHCGR and FSHR genes from ovarian tissues to assess the aims of our study .

Materials and Methods

Ethical approval

The work procedures were approved by the College of Veterinary Medicine, University of Al-Qadisiyiah.

Animals and Experimental design

Forty-eight virgin Wister female rats were explored in this experiment. Aged 10 weeks and weighted between (150-165) gram. The animals were housed in the animal house reared to the Ethics of Veterinary Medicine at AlQadisiyiah University. The animals allowed to feed ad. libitum in well ventilated cages and adapted for 14 days before t experimentation. After adaptation, all animals were subjected to superovulation protocol achieved according to (14). Briefly, by intraperitoneal injection of 10 international unit (IU) of pregnant mare serum gonadotropin (PMSG) (Ovejero de, Mexico). Final maturation of oocytes undertaken after 48h by injection of hCG 10 IU I/P (Msd Vet co, USA).

The human recombinant interleukin-3 (hr-IL-3) (Sigma Aldrich, UK) was used in three upward increased doses (15ng, 30ng, and 45ng/rat) (15). At the same time of hCG injection, animals were divided randomly into four groups each group had 12 rats At the same time of hCG injection, the control group received normal saline intravenously, whereas treated groups received 15, 30, and 45 ng of hrIL-3/rat intravenously.. Each group was furtherly divided into two subgroups each contained six female rats according to the time of sacrifaction including two subgroups after 12h and 36h of hCG injection, respectively. Ovulation will expected to occur about 12-15h after hCG injection .(14)

Gene expression of fshr and lhcgr

This approach was carried out according to the comparative Ct approach (ΔΔCt) with normalization to the level of the control group in the presence of the transcript levels to those of gapdh mRNA according to the recommendation of (16). For this purpose, amplifying of fshr and lhcgr genes were carried out using the following primers: these were recruited from .(17)

Table 1: primers (sequences) of the genes utilized in the study.

Primer name	Sequence '5---------3'	Target gene
LHCGR-F	TCCAATGTGCTCCAGAACCAGATGCT	*lhcgr*
LHCGR-R	GCCACTCCCTGTCTGCCAGTCTATG	
FSHR-F	CCTGGTCTCCTTGCTGGCATTCTTGG	*fshr*
FSHR-R	TCGGTCGGAATCTCTGTCACCTTGCT	
GAPDH-F	GAAGATCAAGATCATTGCTCCT	*gapdh*
GAPDH-R	TACTCCTGCTTGCTGATCCA	

Al-Qadisiyah Journal of Veterinary Medicine Sciences
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Quantitative Reverse Transcription Real-Time PCR (RT-qPCR):

RNA extraction:

Ovarian tissue was cut off about 50 mg that was lysed by adding 400 µl of Lysis Buffer, 4 µl ß-mercapto-ethanol and 20 µl Proteinase K (20 mg/ml) solution to a 1.5 ml micro-centrifuge tube with sample then homogenized by pestle and mix by vertexing. These were incubated at 56°C for 10 minutes and centrifuge at 13,000 rpm for 3 minutes. The supernatant was tenderly transferred into the upper reservoir of the spin column 1 (white ring) with 2.0ml collection tube which then centrifuged at 13,000 rpm for 30 sec meanwhile the flow-through was saved. Binding Buffer was added (400 µl) to the sample flow-through in a collection tube, mixed well, and centrifuged at 13,000 rpm for one minute after pulse-vertexing for 10 seconds. A fresh 1.5ml microcentrifuge tube was used to transfer 600µl of supernatant. The binding buffer and 200µl of 100% ethanol were then added and mixed thoroughly. 600 µl of lysate was transferred into the upper reservoir of the spin column 2 (green ring) using 2.0ml collection tube without wetting the rim. Centrifuge at 13,000 rpm for 10sec. Then the flow-through was removed and assemble the spin column with the 2.0 ml collection tube.

The previous two steps were repeated using remained lysate followed by adding 500µl of washing 1 solution to spin column with collection tube, then centrifugated at 13,000 rpm for 10sec. The flow-through had pour off and spin column assembled with the 2.0 ml collection tube. In a RNase-free tube, 10 µl of DNase (1 U/µl), 40µl of DNase I Reaction Buffer were added and mixed. The mixture added directly on column matrix then incubated at room temperature for 15 minutes. Then, we added 500µl from washing 1 solution was added to spin column with collection tube followed by 1 minute centrifugation with 13,000 rpm. Then, the flow-through had pour off and assembled spin column with 2.0 ml collection tube. We added 700 µl of washing 2 solution to the spin column with collection tube, then centrifuged for 1 minute at 13,000 rpm before pouring off the flow-through and assembling spin column with the 2.0ml collection tube. Additional 13,000 rpm centrifugation for 1 minute was used to dry ethanol residualed in spin column, which was then transferred to the new 1.5ml micro-centrifuge tube. The RNA was eluted by adding 50µl of elution solution to spin column with a microcentrifuge tube and centrifuging at 13,000 rpm for 1 minute .

Measurement of RNA concentration

The concentration of RNA concentration was performed using (Quantus™ Fluorometer, USA). 20X TE Buffer (pH 7.5)was diluted with nuclease-free water to produce 1X TE buffer. With the aid of the latter buffer, the QuantiFluor® Dye working solution was prepared. 10µl of QuantiFluor® Dye had mixed with 3,990µl of 1X TE buffer to make a 1:400 dilution. By adding 200µl of QuantiFluor® to the blank sample and RNA Dye to a 0.5ml PCR tube, the QuantiFluor® ONE RNA System was ready. Additionally, 200µlof the QuantiFluor® Dye working solution was added to a 0.5 ml PCR tube to prepare the blank sample for all other QuantiFluor® Systems. The given standard volume and the volume of 1X TE buffer were used to generate the nucleic acid standard in a 0.5 ml PCR tube (5 l of standard to 200 l of working solution). RNA samples had measured in accordance with the standard measurement established using the Quantus Fluorometer reading.

cDNA synthesis

A total of RNA (about 100ng) was reversed transcribed to cDNA using the kit from (ADDBio, Korea) as following: (H2O 3µl, 2X add script cDNA 10µl, dNTPs 2µl, random oligos hexamer 1µl, and RNA 4µl), the total volume was 20µl. The thermal

Al-Qadisiyah Journal of Veterinary Medicine Sciences
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www.qu.edu.iq/journalvm

conditions were as following : (25°C for 10 min, 50°C for 60 min, 80°C for 5 min, and 4°C on hold) for priming, reverse transcriptase (RT), RT inactivation, and store cDNA, respectively.

Quantitative Reverse transcriptase PCR (RT-qPCR) Preparation

RT-qPCR amplification :

Initially, the amplification was achieved using AddScript RT-qPCR Syber master (AddBio, Korea).

The reaction was including: 4µl of H2O, 10µl of AddScript RT-qPCR, forward primer (0.05 pmol/20 µl), reverse primer (0.05 pmol/20 µl), and cDNA, 2µl of each one. The total amount was 20µl. This was carried out for the internal reference gene (gapdh) in same components .

RT-qPCR data normalization

The delta-delta Ct method was used to normalize transcript levels to those of 12S-rRNA mRNA as mentioned by (16); in which the following formula was employed:

ΔΔ-2 CT = [(CT gene of interest- CT internal control) sample A- (CT gene of interest-CT internal control) sample B)]. Sample A means one certain group, Sample B means another certain group.

Statistical analysis

Statistical analysis of the lhcgr and fshr genes after 12 hours and 36 hours achieved by one-way analysis of variance (ANOVA) test. While the statistical analysis of the lhcgr and fshr genes from comparing the 12 hours and 36 hours treatment was analyzed using two-way analysis of variance (ANOVA) test. We used Graph Pad Prism-Version 5 to analyze these data (SAS Institute, Inc., USA). Values considered statistically significant when ($P < 0.05$).

Results

Analysis of the RT-qPCR gene expression data of FSHR

The fold change comparison between the groups expressed fshr gene after 12 hours of treatment was presented in (Figure 1A). This figure showed significant increase ($P<0.05$) of the 15ng dose compared with control. Similar result was noticed in the group of 45ng compared with control. However, no any significant differences among the other groups in comparison with control. The fold change comparison between the groups expressed fshr gene after 36 hours of treatment was presented in (Figure 1B). This figure showed significant increase ($P<0.05$) of the 45ng dose compared with control. However, there were no any significant differences among the other groups in comparison with control. The fold change comparison between the groups expressed fshr gene (12 hours compared with 36 hours) was

Analysis of the RT-qPCR gene expression data of LHCGR

The fold change comparison between the groups expressed lhcgr gene after 12 hours of treatment was presented in (Figure 1D). This showed significant increase ($P<0.05$) of the 15ng dose compared with control group, while there were no any significant differences among the other groups. The fold change comparison between the groups expressed lhcgr gene after 36 hours of treatment was presented in (Figure 1E). This revealed significant increase ($P<0.05$) of the 45ng dose compared with control group, while there were no any significant differences among the other groups. The fold change comparison between the groups expressed lhcgr gene (12 hours compared with 36 hours) was presented in (Figure 1F). This figure showed significant increase ($P<0.05$) of the 15ng dose after 12 hours compared with the same does after 36 hours. However, there were no any significant differences among the other groups. presented in (Figure 1C). This figure showed no significant differences among the compared groups.

Al-Qadisiyah Journal of Veterinary Medicine Sciences
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www.qu.edu.iq/journalvm

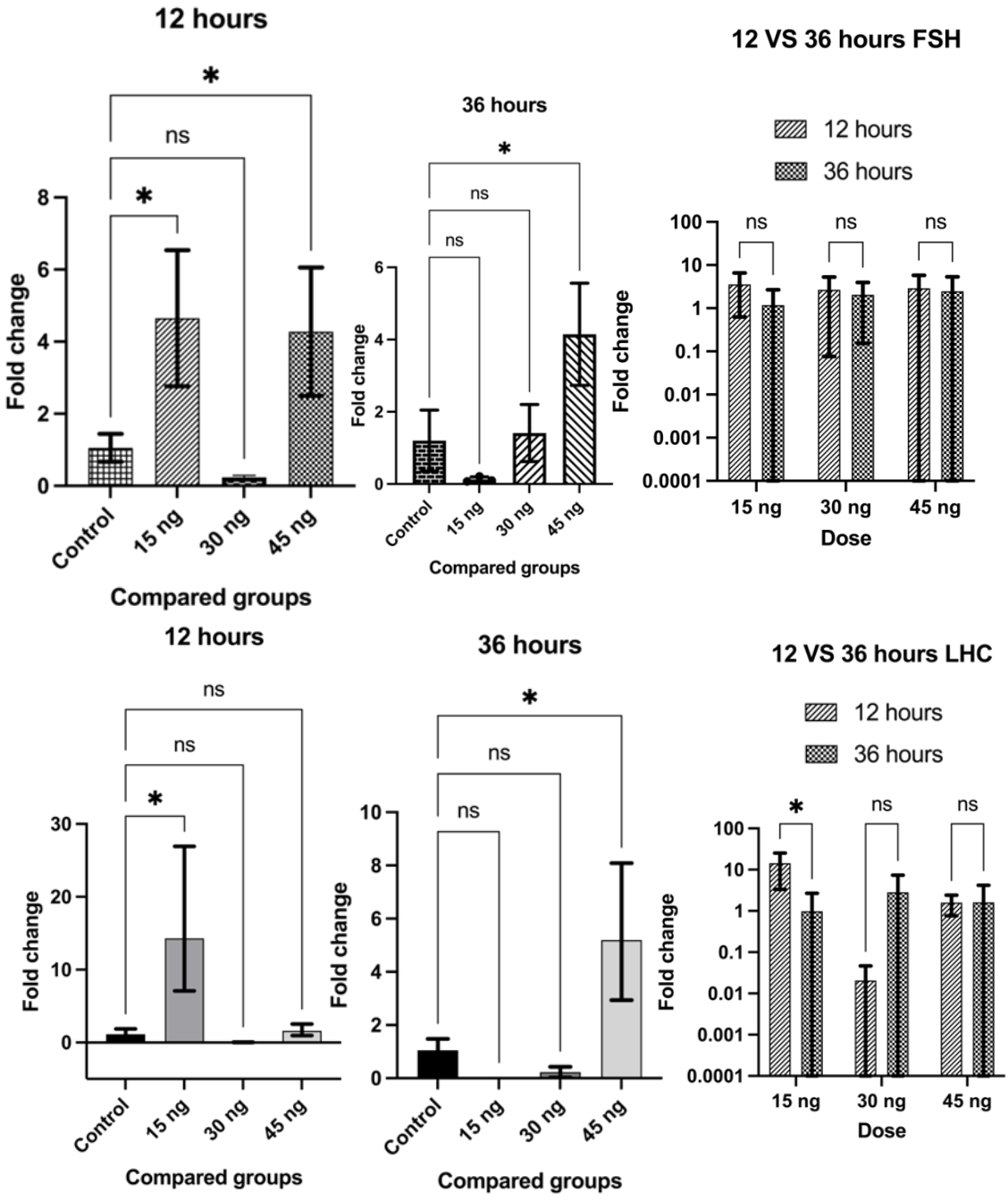

Figure 1: Fold change comparisons between the groups expressed *fshr* gene (upper row: A to C) and *lhcgr* gene (Lower row: D to F). A. After 12 hours of treatment. B. After 36 hours of treatment. C. 12 hours compared with 36 hours. D. after 12 hours of treatment. E. After 36 hours of treatment. F. 12 hours compared with 36 hours.

Discussion

In this study, we explored three different doses of hrIL-3 at three upward increased doses (15ng, 30ng, and 45ng/rat). Female rats in 15ng group of hrIL-3 had significant increase ($P<0.05$) in gene expression of fshr and lhcgr genes at 12h. However, there were no any significant differences ($P>0.05$) in gene expression of these two genes at 36h.

Al-Qadisiyah Journal of Veterinary Medicine Sciences
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www.qu.edu.iq/journalvm

Regarding rats in 30ng group of hrIL-3, they had no any significant differences (P>0.05) among the other groups at both times 12h and 36h. Whereas female rats in 45ng group of hrIL-3 had significant increase (P<0.05) in expression of fshr gene only at 12h. while after 36h, both fshr and lhcgr genes were upregulated in this group .

Numerous studies have provided evidence that cytokines regulate physiological processes, in addition they participate during immune pathological reactions (18). The increase in fshr and lhcgr genes results in the stimulation of ovaries through several mechanisms. After secretion from the pituitary gland, FSH and LH bind to specific receptors in gonadal somatic cells, the FSH receptor (FSHR) and the LH receptor (LHR) (19, 20). Theca and granulosa cells, rather than oocytes, were the sites for somatic expression for FSHR and LHCGR. Granulosa cells express both FSHR and LHCGR and react to both gonadotropins, whereas theca cells only express LHCGR and react to LH stimulation (20). FSH stimulates ovarian follicle development and granulosa cell function via FSHR (20, 21). Binding of FSH to its receptor will activate granulosa cells in both a cAMP-dependent and cAMP-independent manner (20). By controlling the expression of about 500 target genes, FSHR activation promotes follicular maturation (22). One of the most important genes whose expression is induced by FSH is lhcgr (23). This gene encodes the luteinizing hormone/choriogonadotropin receptor (LHCGR). FSH stimulation is essential for LHR expression, but FSH alone is not enough for oocytes to respond to LH surge and undergo maturation and ovulation. Other factors produced from granulosa and theca cells including insulin like growth factor-1 (IGF-1), interleukin-6, and estrogen in response to these gonadotropins and act synergistically in an autocrine/paracrine manner to induce LHR expression (2). In mural granulosa cells, binding of LH to LHCGR determines the final stages of follicle maturation and ovulation. When LH binds to LHCGR, the granulosa cells will activated resulting in increases cAMP levels in mural granulosa cells (20). In addition, LHR expressed on mural granulosa cells activates ERK1/2 kinases and number of growth factors such as epiregulin and amphiregulin that are essential to achieve successful ovulation.(24)

In our study, hrIL-3 with a dose of 45ng at 36h is the best dose to enhance ovarian functions in female rats to stimulate development of ovarian follicles and ovulation. This is attributed to the ability of this dose in perseverance to upregulate both fshr and lhcgr genes which indicates upregulation of FSHR and LHCGR on rat ovaries both 12h and 36h, except the downregulation of lhcgr appeared at 12h in our study. This downregulation of lhcgr may be due to the marked decrease in LHR expression following LH surge (2, 25). This transient downregulation of LHR is attributed to the action of micro RNAs (miRNAs) .(2)

The differences in ovarian response to the different doses at different times may be attributed to the specific site transcription factors. In granulosa cells, there are subset of gonadotropin genes are essential for development and maturation of oocytes and critical for ovulation (20). At folliculogenesis, dynamic alterations in expression of several genes will occur to the oocyte, and these genes are regulated by transcription factors. Numerous germ-cell specific regulators of transcription are critical for the formation and the maintenance of follicle such as Solh1, Sohlh2, Nobox, Figla, and Lhx8.(26)

In conclusion, human rIL-3 with a dose of 45ng at 36h increases gene expression of both FSHR and LHCGR in ovarian tissues of female rats.

Conflict of interest

The work, here, has no conflict of interest as declared by the authors.

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