Insulin improves ovarian function during the ovarian cycle in adult mice

Ali Younesi[1,2*], Mohammad Hossein K. Godaneh[1,2*], Mohammad Mahdi Gheibi[1,2], Mohammad A.T. Zavareh[1], Sanaz Ziaeipour[1], Abbas Aliaghaei[1], Amirhosein Hasani[1], Amirreza Khosravi[1], Vahid Ebrahimi[3], Amir Raoofi[4], Shabnam Abdi[5], Mohammad-Amin Abdollahifar[1,2]

[1] *Cellular and Molecular Biology Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran*

[2] *Department of Biology and Anatomical Sciences, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran*

[3] *Department of Anatomy and Cell Biology, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran*

[4] *Leishmaniasis Research Center, Department of Anatomy, Sabzevar University of Medical Sciences, Sabzevar, Iran*

[5] *Department of Anatomical Sciences and Cognitive Neuroscience, Faculty of Medicine, Tehran Medical Sciences, Islamic Azad University, Tehran, Iran*

SUMMARY

Folliculogenesis is controlled by numerous inside and outside ovarian factors such as endocrine, paracrine, and autocrine signals. Among these factors, insulin is an important molecule that regulates processes in the female reproductive system. So, the purpose of this study was to determine the impact of insulin treatment on ovary tissue during the ovarian cycle. For this probe, 18 adult female NMRI mice were randomly divided into two groups: control and insulin (100 µL with a 72-hour interval by intraperitoneal injection for 30 days). Blood samples for hormonal evaluation were obtained from the heart, and the serum levels of FSH, LH, progesterone, and estradiol were measured. Then, right and left ovaries were extracted for real-time PCR and stereology, respectively. According to our results, insulin administration increased mRNA levels of INS-R, Ki67, while reduced Caspase-3, IGF-1, and FOXO-1. Insulin administration augmented the concentrations of female reproductive hormones. Histological analyses showed that the total volume of ovary in mice receiving insulin was significantly reduced. Likewise, a significant decrease in the number of antral follicles, Graafian follicles, and the number of corpora lutea were seen following the use of insulin. In contrast, the results showed a significant growth in the number of primordial and primary follicles in the insulin group compared with the control group. Briefly, insulin administration is able to increase the expression of genes related to insulin receptor and cell proliferation, as well as the amount of female reproductive hormones, but simultaneously, has a dual effect on the histological parameters of the ovary.

Corresponding author:
Mohammad-Amin Abdollahifar, Cellular and Molecular Biology Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran and Department of Biology and Anatomical Sciences, School of Medicine, Shahid Beheshti University Tehran, Iran. Arabi Ave, Daneshjoo Blvd, Velenjak, Tehran, Iran. Post Code: 19839-63113. Phone: +98 21 22 43 97 70. Orcid: 0000-0001-6947. E-mail: m_amin58@yahoo.com / abdollahima@sbmu.ac.ir

Submitted: March 7, 2022. **Accepted:** April 4, 2022

https://doi.org/10.52083/NJHH1910

*These two authors contributed equally to this work.

Key words: Ovarian function – Ovarian cycle – Insulin – Adult mice

INTRODUCTION

An oocyte is a female germ cell which is required in female reproduction. The oocyte develops during the process of oogenesis (folliculogenesis) in the ovarian follicle, a specialized unit of the ovarian cortex (Virant-Klun et al., 2015). It is commonly accepted that human ovaries consist of a fixed number of primordial follicles which are formed before birth, decrease with age and are depleted in menopause (Bendsen et al., 2006; Forabosco and Sforza, 2007). In general, oogenesis, as well as folliculogenesis, are regulated by a large number of intra-ovarian factors and extra-ovarian factors. On the other hand, follicular development as well as oocyte maturation are regulated by the highly complex coaction of endocrine, paracrine, and autocrine signals. Different organs, such as the hypothalamus, the anterior pituitary gland, the gonads and also the hypothalamic-pituitary-gonadal axis have a pivotal role in the follicular development as well as oocyte maturation (Nagahama and Yamashita, 2008; Edson et al., 2009). Among the effective factors in female reproductive system, insulin is one of the most effective molecules that regulate ovarian development and oogenesis (Saltiel and Kahn, 2001).

It was reported that insulin-signaling is essential for ovulation, differentiation of granulosa cell, and female fertility (Sekulovski et al., 2020). And it is also accepted that in humans, hypoinsulinemia or hyperinsulinemia is in connection with ovarian function (Chang et al., 2005). Based on previous studies, the insulin receptor is a member of the ligand-activated receptor as well as tyrosine kinase family of transmembrane-signaling proteins which basically have a substantial role in the regulation of cell differentiation, cell growth and metabolism (Lee and Pilch, 1994).

Insulin receptor is expressed both in the thecal granulosa compartment as well as the oocyte surface, and it is right to point that, signaling pathways which are dependent on Insulin receptor promote oocyte maturation and follicular development (Das et al., 2016).

It has been reported that insulin is a key regulator of female reproduction, and insulin-sensitizing drugs are actually the only choice to reverse reproductive dysfunctions associated with metabolic disorders, such as insulin resistance, diabetes, and obesity (Das and Arur, 2017). On the other hand, polycystic ovary syndrome is one of the most prevalent systemic reproductive endocrine diseases as well. Recent studies have demonstrated that the main etiology and primary endocrine disorder of PCOS are hyperandrogenemia as well as insulin resistance (Wang et al., 2019). Based on other studies, insulin and insulin-like growth factors play a pivotal role in modification of the FSH-dependent cellular differentiation related to human granulosa cells (Garzo and Dorrington, 1984), and both insulin and somatotrophin can also lead to increase the level of IGF-I production by ovarian follicles. So that even insulin is significantly effective in reducing atresia and increasing progesterone during follicular culture (Cox et al., 1997). It has been reported that insulin, IGF-I and IGF-II are survival factors for early stage of human follicular development (Louhio et al., 2000).

The connection between the female reproductive system and the beneficial effects of insulin has been reported in previous studies, but more detailed understanding of insulin's action in regulation of oogenesis and folliculogenesis may be utilizable in opening the door to important medical applications. Thus, the aim of this study is evaluation of the effects of exogenous insulin on follicular development and some genes' expression related to this issue in adult female mice.

MATERIALS AND METHODS

Animals

For this study, 18 adult female NMRI mice weighing 25-30 g were attained from the Pasteur Institute, Tehran, Iran. All animals were cared under animal house standard conditions, including limitless access to water and food, 12-12-hour light/dark cycle, and normal room temperature (22±2°C). Then, the mice were randomly divided into two main control and insulin groups.

Treatments and sampling

Insulin (Sigma, St. Louis, MO, USA) was prepared by diluting in water. Each animal in experimental group received 100 µL of insulin with a 72-hour interval by intraperitoneal (IP) injection for 30 days. Control mice also were treated with similar amounts of water.

Hormonal measurement

Following deep anesthesia, blood samples for hormonal evaluation were gained from the heart of animals. Then, the blood samples were centrifuged at 6000 g at 4°C for 5 min before keeping them at -80°C until ready to be used. Subsequently, specific ELISA kit (EIA: Enzyme Immunoassay) for mice was applied to measure the blood serum levels of FSH, LH, progesterone, and estradiol using, according to the manufacturer's instructions. Results of FSH and LH were reported as Iu/mL, while progesterone as ng/mL and estradiol as pg/mL.

Total RNA extraction and real-time PCR

At the end of the treatment period, right ovaries were extracted for real-time PCR. For this purpose, whole RNA samples were extracted from ovary tissue, by means of the High Pure RNA Isolation kit, based on the manufacturer's instructions (Roche, Basel, Switzerland) and treated with DNase I (Roche, Basel, Switzerland) to eliminate genomic DNA contamination. Next, cDNA was synthesized in a total volume of 20 µl using a kit (Fermentas, Lithuania) at 42 °C for 60 min. Then, applied real-time PCR (TaqMan) was used based on QuantiTect SYBR Green kit (Takara Bio Inc, Japan) aimed at quantification of mRNA expression levels of INS-R, Ki67, Caspase-3, IGF-1, and FOXO-1 among two groups. All reverse and forward primer pairs were designed according to Primer 3 Plus software in exon-exon junction way to discriminate cDNA and genomic DNA. Beforehand, PCR primers were checked by Primer-Blast tool (Ebrahimi et al., 2019).

Stereological evaluation

Mice were profoundly anesthetized and sacrificed by IP injection of sodium pentobarbital. Subsequently, transcardial perfusion was made using chilled normal saline and fresh fixative solution, containing 4% paraformaldehyde (PFA) in 0.1 M phosphate-buffered saline (PBS). Finally, the left ovary samples of animals were extracted, placed in 10% formalin solution, sectioned into 5µm slices, and prepared for histological hematoxylin and eosin (HandE) staining and stereological analysis. The following factors were estimated in ovarian tissue: volume of ovary and the total number of primordial, primary, antral, Graafian follicles, and finally, the total number of corpora lutea. The total volume of the ovary was estimated using the Cavalieri method and following formula:

$$V\_total = \sum P \times \frac{a}{p} \times t$$

In this formula, Σp is the total number of points superimposed on the microscopic photos, and (a/p) is the area associated with each point, and t is the thickness of the microscopic sections.

Besides, the total number of ovarian cells and corpora lutea was measured via the optical dissector technique and also succeeding principles: The numerical density (N_v) was considered as:

$$N_v = \frac{\sum Q}{\sum P \times h \times \frac{a}{f}} \times \frac{t}{BA}$$

In this formula, ΣQ is the number of the cell nuclei, ΣP is the total number of the unprejudiced calculating frame in all fields of ovarian tissue, h is the height of the dissector, ratio a to f (a/f) is the frame area, t is the actual slice thickness measured in every field of tissue using the microcator, and BA is the block advance of the microtome which was fixed for 10 µm.

Statistical analysis

The results were analyzed by Kruskal Wallis test, using the SPSS software version 19.00 (IBM Corp., Armonk, NY, USA). $P<0.05$ was considered as significant differences.

RESULTS

Insulin administration increased mRNA levels of INS-R, Ki67, while reduced Caspase-3, IGF-1, and FOXO-1 during ovarian cycle

According to real time-PCR data, insulin treatment significantly improved the expression of mRNA levels of insulin receptor (INS-R), as well as proliferation marker (Ki67) in ovarian tissue compared to the control group (in both cases, $P<0.05$, Fig. 1). On the other hand, insulin administration significantly decreased the expression of mRNA levels Caspase-3 as a typical marker of apoptosis as well as IGF-1 and FOXO-1 in ovarian tissue compared to the control group (in the case of all three genes, $P<0.05$, Fig. 1).

Table 1. Primers design.

Genes	Primer sequences
INSR	F: GAGAGTGGTGGAGTTGAGTTGG R: TGTGGAGGATGGAGGAGGAG
Ki 67	F: AGGAAAGTAGATAGGAAGGAAG R: AGGGAGTGGTGATAGAAAGAG
FOXO1	F: AACTGAGGAGCAGTCCAAAGATG R: AACTGAGGAGCAGTCCAAAGATG
IGF1	F= GGAAGCTATGGAGTGGGAAAAG R= CCGAGAGGTGGAGTGATTTGA
Caspase3	F= AGTGGGACTGATGAGGAGATGG R= AGTGGAGTACAGGGAGAAGGA
β-actin	F= TCAGAGCAAGAGAGGCATCC R= GGTCATCTTCTCACGGTTGG

Insulin administration augmented the concentrations of reproductive hormones during ovarian cycle

Based on hormonal analysis, insulin treatment significantly improved the concentration of FSH ($P<0.05$, Fig. 2A), LH ($P<0.05$, Fig. 2A), and estradiol ($P<0.01$, Fig. 2B). However, this increase was not significant for progesterone (Fig. 2B).

Insulin administration altered histological parameters during ovarian cycle

At the end of the experiment, histological analyses showed that the total volume of ovarian tissue in mice receiving insulin was significantly reduced compared to mice in the control group ($P<0.05$, Fig. 3B).

Likewise, the results obtained from the cell count indicated a significant decrease in the total number of ovarian antral follicles ($P<0.001$, Fig. 2A), Graafian follicles ($P<0.01$, Fig. 2B), and the total number of corpora lutea ($P<0.001$, Fig. 2B) following the use of insulin compared with the control group. On the contrary, the results revealed a significant rise in the total number of both ovarian primordial and primary follicles in insulin-treated mice compared to the control group ($P<0.001$ and $P<0.05$, respectively) (Fig. 2A).

DISCUSSION

In the present study, the data showed the level of mRNA expression in genes related to insulin receptor and Ki67 significantly increased, and the level of mRNA expression in genes related to executioner caspase-3, IGF-1 and FOXO-1 significantly decreased. On the other hand, the data of this study showed that, the Serum level of FSH, LH and Estradiol as well as the number of primordial and primary follicles significantly increased. It is right to point that, the insulin receptor is a tyrosine kinase receptor that upon binding insulin, catalyzes the phosphorylation of several intracellular substrates, including the insulin receptor substrate proteins (IRSs), GAB-1, Shc, APS, p60DOK, SIRPS, and c-Cbl. Each of these substrates interacts with a series of signalling proteins which lead to initiation of different signalling pathways (Saltiel and Pessin, 2002; Shaw et al., 2011). Following phosphorylation, the binding of the regulatory subunit of insulin receptor to IRSs forms an active heterodimer which phosphorylates membrane phosphatidylinositol residues in the 3′ position of PIP3 to form PDK1/2 and thereafter activates Akt/PKB (Dupont and Scaramuzzi, 2016). On the Oder hand, mTORC2 is activated by insulin and controls cell survival, metabolism, and cytoskeletal organization (Yoon et al., 2017). These pathways can lead to reduction of apoptosis and insulin may have reduced gene expression of executioner caspases-3 in these pathways.

As previous studies have shown, FSH can enhance cell proliferation via mTOR and/or ERK and inhibit apoptosis via Akt1 and FOXO3a

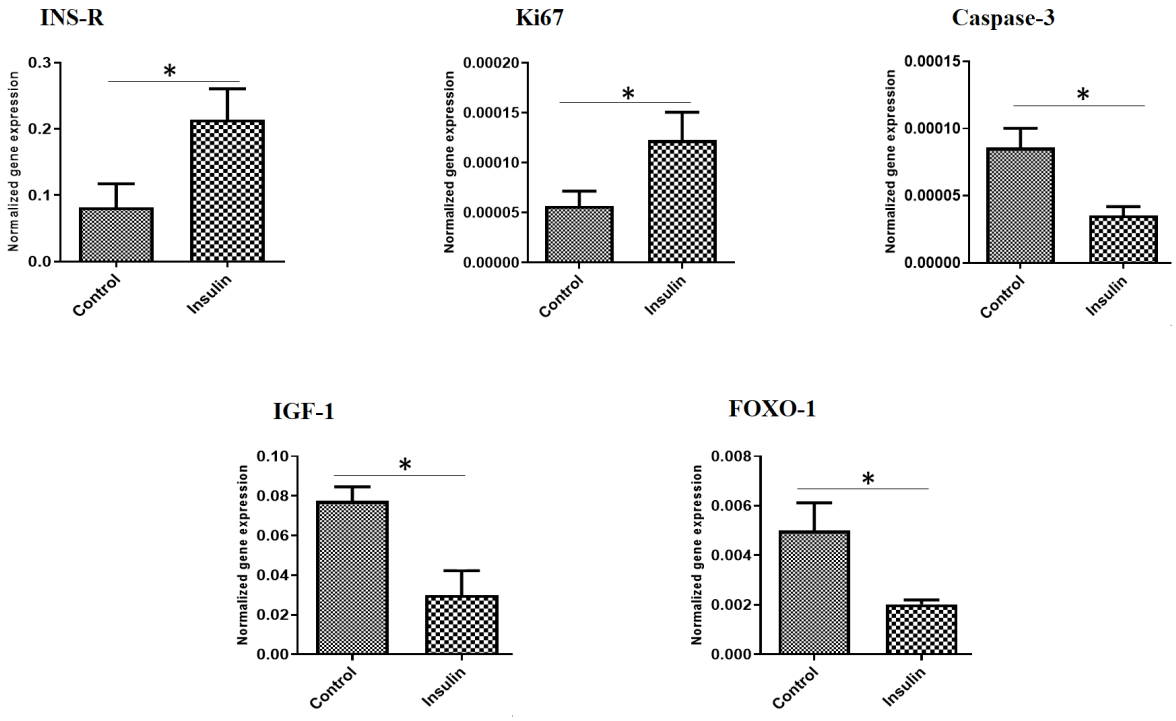

Fig. 1.- The effect of insulin administration on the expression of genes involved in the mechanism of insulin function, proliferation and apoptosis during ovarian cycle of NMRI mice Insulin injection significantly increased mRNA levels of INS-R (*P<005), Ki67 (*P<005), while significantly reduced caspase-3 (*P<005), IGF-1 (*P<005), and FOXO-1 (*P<005) in treated mice compared with the control mice.

(Dupont and Scaramuzzi, 2016). In this regard, in present study, the data showed that the serum level of FSH has increased in response to insulin so that it can be said, by increasing FSH, insulin has been able to increase proliferation and expression of Ki67 and also reduce apoptosis. Based on previous studies, it is reported that, the expression and activity of FOXO1 are modulated by LH and FSH via PI3K/Akt pathway (Liu et al., 2009). Therefore, it can be said that, increased level of LH and FSH have led to modulation of FOXO1 gene expression, after insulin injection.

On the other hand, insulin signalling via AKt1 and FOXO1 can increase the level of LH-stimulated steroidogenesis. Therefore, elevated level of LH can lead to increase steroidogenesis. There are

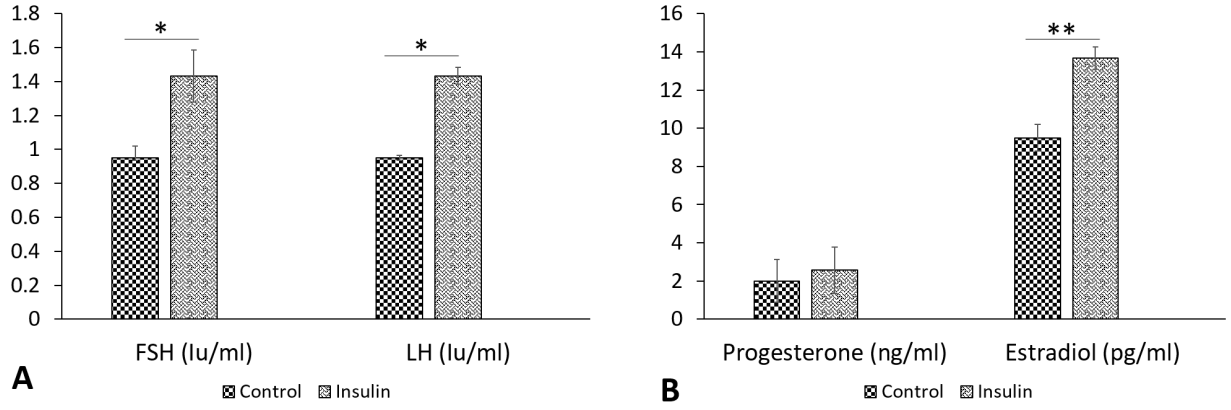

Fig. 2.- The effect of insulin administration on hormonal alterations during ovarian cycle of NMRI mice Insulin injection significantly improved the concentration of FSH (*P<005), LH (*P<005), and estradiol (**P<001). However, this increase was not significant for progesterone.

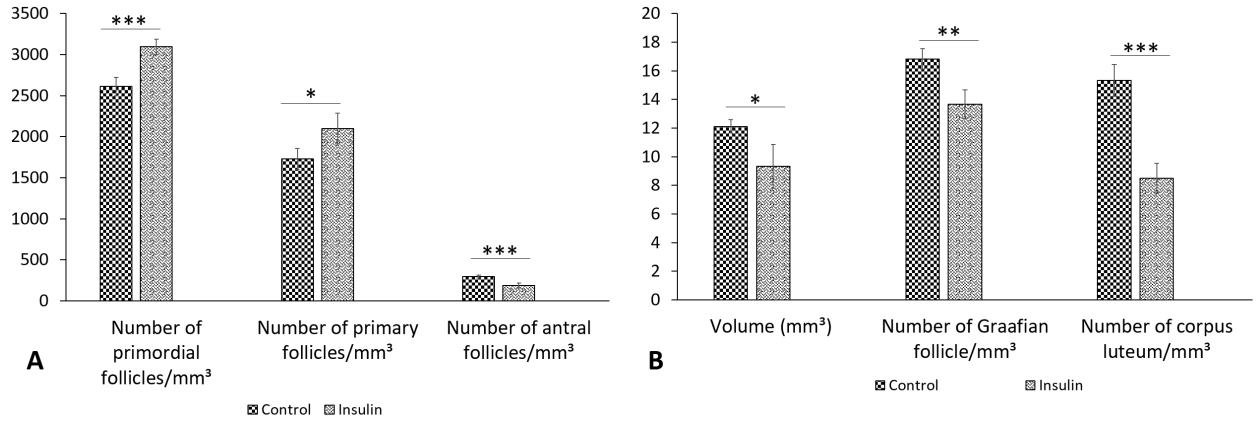

Fig. 3.- The effect of insulin administration on histological changes during ovarian cycle of NMRI mice Histological analyses showed that the total volume of ovarian tissue in group receiving insulin was significantly reduced compared to the control group (*P<005) Similarly, a significant decrease in the total number of ovarian antral follicles (***P<0001), Graafian follicles (**P<001), and the total number of corpus luteum (**P<0001) were observed following insulin usage compared with the control group In contrast, a significant rise in the total number of ovarian primordial and primary follicles in insulin-treated mice compared to the control mice (***P<0001 and *P<005, respectively).

many studies in which beneficial effects of Insulin on ovarian function were reported and they confirm the data of present study. For example it was reported that, the increase in the level of growth hormone may be involved in follicular development to enter the ovulatory phase, and the insulin system may support the maturation of preovulatory follicles (Shimizu et al., 2008). And it was also demonstrated that insulin, insulin-like growth factor I, and gonadotropins have significant role in granulosa cell proliferation, progesterone production, estradiol production, and insulin-like growth factor I production in vitro (Spicer et al., 1993), and following the increase in insulin-like growth factor I, this hormone is able to control tissue homeostasis, cell proliferation and apoptosis (Kooijman et al., 2006).

It was reported that insulin signalling can lead to increasing proliferation and hyperplasia of the ovarian surface epithelium, which has approximately 4–6 cell layers with a high rate of proliferation, as well as decrease in follicular integrity via upregulation of the PI3-kinase pathway (King et al., 2013). Furthermore, the data of the present study showed that the number of primordial and primary follicles in the insulin group are increased in comparison

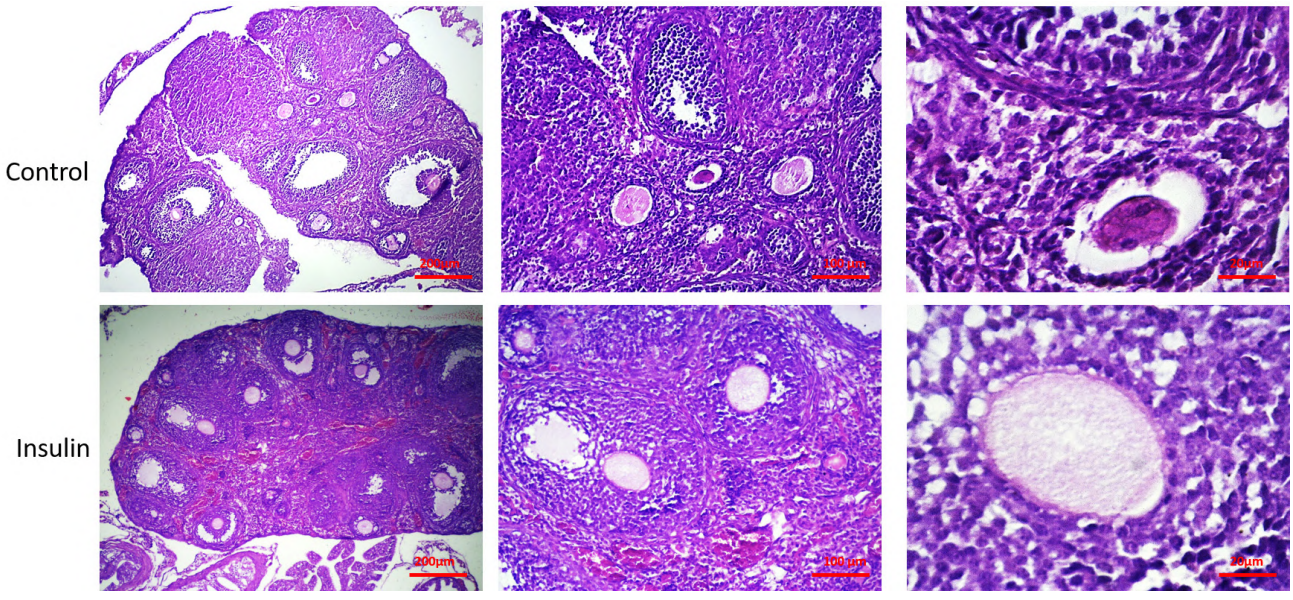

Fig. 4.- Photomicrograph of the ovaries stained with HandE (4x, 10x and 40x) in study groups.

with the control group, and there is no evidence of an increase in the number of Graffian follicle or in antral follicle while, based on the data of gene expression, it is predicted that in the gonadotropin-dependent stages of follicular development, insulin may play a significant role. Thus, it is suggested that, in future studies, the period of insulin injection should be longer for more accurate outcomes. In conclusion, based on the data of this study, it can be said that insulin improves the function of ovarian tissue during the ovarian cycle. Overall, it can be concluded that insulin administration is able to rise the expression of genes related to insulin receptor and cell proliferation, as well as the amount of female reproductive hormones, but at the same time has a dual effect on histological parameters of the ovary, including the volume of ovary as well as growing follicles.

ACKNOWLEDGEMENTS

This work has been performed at Department of Biology and Anatomical Sciences, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran (registration No: 24874). The study was funded by Cellular and Molecular Biology Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran (Grant number:1399-24874).

STATEMENT OF ETHICS

The authors declare that all experiments protocols were approved by the Ethics Committee, Deputy of Research, Shahid Beheshti University of Medical Sciences, Tehran, Iran (IR.SBMU.RETECH.REC.1399.1120). All methods were carried out in accordance with relevant guidelines and regulations.

REFERENCES

BENDSEN E, BYSKOV A, ANDERSEN CY, WESTERGAARD LG (2006) Number of germ cells and somatic cells in human fetal ovaries during the first weeks after sex differentiation. *Human Reprod,* 21: 30-35.

CHANG AS, DALE AN, MOLEY KH (2005) Maternal diabetes adversely affects preovulatory oocyte maturation, development, and granulosa cell apoptosis. *Endocrinology,* 146: 2445-2453.

COX NM (1997) Control of follicular development and ovulation rate in pigs. *J Reprod Fertil, Supplement,* 52: 31-46.

DAS D, ARUR S (2017) Conserved insulin signaling in the regulation of oocyte growth, development, and maturation. *Mol Reprod Develop,* 84: 444-459.

DAS D, NATH P, PAL S, HAJRA S, GHOSH P, MAITRA S (2016) Expression of two insulin receptor subtypes, insra and insrb, in zebrafish (*Danio rerio*) ovary and involvement of insulin action in ovarian function. *Gen Comp Endocrinol,* 239: 21-31.

DUPONT J, SCARAMUZZI RJ (2016) Insulin signalling and glucose transport in the ovary and ovarian function during the ovarian cycle. *Biochem J,* 473: 1483-1501.

EBRAHIMI V, BOROUJENI ME, ALIAGHAEI A, ABDOLLAHIFAR MA, PIRYAEI A, HAGHIR H, SADEGHI Y (2020) Functional dopaminergic neurons derived from human chorionic mesenchymal stem cells ameliorate striatal atrophy and improve behavioral deficits in Parkinsonian rat model. *Anat Rec (Hoboken),* 303: 2274-2289.

EDSON MA, NAGARAJA AK, MATZUK MM (2009) The mammalian ovary from genesis to revelation. *Endocr Rev,* 30: 624-712.

FORABOSCO A, SFORZA C (2007) Establishment of ovarian reserve: a quantitative morphometric study of the developing human ovary. *Fertil Steril,* 88: 675-683.

GARZO VG, DORRINGTON J (1984) Aromatase activity in human granulosa cells during follicular development and the modulation by follicle-stimulating hormone and insulin. *Am J Obstet Gynecol,* 148: 657-662.

KING SM, MODI DA, EDDIE SL, BURDETTE JE (2013) Insulin and insulin-like growth factor signaling increases proliferation and hyperplasia of the ovarian surface epithelium and decreases follicular integrity through upregulation of the PI3-kinase pathway. *J Ovarian Res,* 6: 1-15.

KOOIJMAN R (2006) Regulation of apoptosis by insulin-like growth factor (IGF)-I. *Cytokine Growth Factor Rev,* 17: 305-323.

LEE J, PILCH PF (1994) The insulin receptor: structure, function, and signaling. *Am J Physiol Cell Physiol,* 266: C319-C334.

LIU Z, RUDD MD, HERNANDEZ-GONZALEZ I, GONZALEZ-ROBAYNA I, FAN H-Y, ZELEZNIK AJ, RICHARDS JS (2009) FSH and FOXO1 regulate genes in the sterol/steroid and lipid biosynthetic pathways in granulosa cells. *Mol Endocrinol,* 23: 649-661.

LOUHIO H, HOVATTA O, SJÖBERG J, TUURI T (2000) The effects of insulin, and insulin-like growth factors I and II on human ovarian follicles in long-term culture. *Mol Human Reprod,* 6: 694-698.

NAGAHAMA Y, YAMASHITA M (2008) Regulation of oocyte maturation in fish. *Development, Growth Different,* 50: S195-S219.

SALTIEL AR, KAHN CR (2001) Insulin signalling and the regulation of glucose and lipid metabolism. *Nature,* 414: 799-806.

SALTIEL AR, PESSIN JE (2002) Insulin signaling pathways in time and space. *Trends Cell Biol,* 12: 65-71.

SEKULOVSKI N, WHORTON AE, SHI M, HAYASHI K, MACLEAN JA (2020) Periovulatory insulin signaling is essential for ovulation, granulosa cell differentiation, and female fertility. *FASEB J,* 34: 2376-2391.

SHAW LM (2011) The insulin receptor substrate (IRS) proteins: at the intersection of metabolism and cancer. *Cell cycle,* 10: 1750-1756.

SHIMIZU T, MURAYAMA C, SUDO N, KAWASHIMA C, TETSUKA M, MIYAMOTO A (2008) Involvement of insulin and growth hormone (GH) during follicular development in the bovine ovary. *Animal Reprod Sci,* 106: 143-152.

SPICER L, ALPIZAR E, ECHTERNKAMP S (1993) Effects of insulin, insulin-like growth factor I, and gonadotropins on bovine granulosa cell proliferation, progesterone production, estradiol production, and (or) insulin-like growth factor I production in vitro. *J Animal Sci,* 71: 1232-1241.

VIRANT-KLUN I (2015) Postnatal oogenesis in humans: a review of recent findings. *Stem Cells Cloning,* 8: 49-60.

WANG J, WU D, GUO H, LI M (2019) Hyperandrogenemia and insulin resistance: the chief culprit of polycystic ovary syndrome. *Life Sci,* 236: 116940.

YOON M-S (2017) The role of mammalian target of rapamycin (mTOR) in insulin signaling. *Nutrients,* 9: 1176.