Two oestrous cycles

Ten days insulin treatment reduced ovarian leptin expression of rat

Kemal Ozbilgin, MD, *Naci K. Kuscu*, MD.

ABSTRACT

Objective: Previous studies have reported a role for insulin in leptin secretion from rat ovarian cells although the time dependent effect of insulin has yet to be obtained. The study aim is to determine the time dependent effect of insulin on leptin staining intensity in the rat ovary.

Methods: Normally cycling rats were investigated in a spontaneous cycle (control, n=6), one cycle with insulin treatment 2U/day for 5 days (D5) (n=6), and 2 cycles with insulin treatment 2 U/day for 10 days (D10) (n=6) in the Department of Histology and Embryology, Celal Bayar University, Manisa, Turkey, from May 2004 to August 2004. Histological structures and leptin staining of the ovarian cells were investigated with immuno-histochemical technique and evaluated by a semi-quantitative scoring system.

Results: Number of follicles and stromal cells decreased with longer insulin treatment. No leptin staining was observed in granulosa cells of all groups. Staining intensity and H-score of the controls, D5 and D10 groups were (+++)/204, (++)/80 and (+)/9.5 for theca externa cells; (+++)/289.5, (++)/126 and (+)/65 for stroma; (++)/140, (++)/70 and (+)/21 for corpus luteum. The difference between H-scores of control and D10 groups was statistically significant in all tissue types ($p<0.01$).

Conclusion: Insulin treatment for 10 days (2 estrous cycles) reduced leptin-staining intensity in various tissues of the rat ovary and decreased follicle development in a time dependent manner.

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Leptin, a hormone encoded by the obesity gene, is exclusively expressed in white adipose tissue and various tissues. The ovary has recently been reported as another production site.[1-4] The endocrine or paracrine effects of leptin in the ovary are implied by the expression of functional leptin receptors on the surface of ovarian follicular structure including granulosa, theca and interstitial cells.[3,4] Leptin gene expression is regulated by a variety of hormones, growth factors, and cytokines.[5,6] Estrogens induce[7] whereas androgens

suppress leptin production.[8] Glucocorticoids and pro-inflammatory cytokines, such as tumor necrosis factor- and interleukin-1, may also directly induce leptin gene expression.[9] Insulin may be involved in the regulation of the leptin production, but the interaction between leptin and insulin in the ovary is still unclear. Body mass index seems to be the major correlating factor of serum leptin level in both normal women and patients with polycystic ovary syndrome (PCOS),[10] but these patients may still have higher serum leptin levels and suffer untoward

Address correspondence and reprint request to: Dr. Kemal Ozbilgin, Head, Department of Histology and Embryology, Celal Bayar University, School of Medicine, Manisa 45020, *Turkey*. Tel. +90 (236) 2331920. Fax. +90 (236) 2331466. E-mail: kemalozbilgin@yahoo.com

effect.[11] High leptin levels have been reported in the wall of polycystic ovarian follicles,[4] and this high local level has been speculated to have a deleterious effect in follicle-stimulating hormone (FSH) stimulation of steroid production. Insulin resistance and resultant hyperinsulinemia are important confounding factors in PCOS,[12] and hyperinsulinemia not only induces ovarian androgen synthesis,[13] but also acts as a risk factor in the development of glucose intolerance.[14] Insulin is known to have various effects on ovarian cell types and is a stimulating factor for androgen production by cultured theca cells,[15] as well as estrogen and progesterone production by cultured granulosa cells (GCs).[16] We have recently showed that insulin treatment for 4 weeks reduced ovarian leptin expression in rat ovary.[17] As leptin is known to antagonize insulin's effect in ovarian steroid synthesis, we aimed to search time dependent interactions between insulin and leptin production in the current study.

Methods. The study was conducted in the Animal Research Laboratory of Celal Bayar University, School of Medicine, Manisa, Turkey from May 2004 to August 2004, and general guidelines of the university for the animal care were strictly followed. Eighteen adults, female Wistar rats with an average weight of 250 grams were divided into 3 groups to receive either saline (control group), human insulin 2U/day for 5 days (group D5) or human insulin 2U/day for 10 days (group D10). First day of the proestrous state was determined with histological smears obtained from each rat's vagina, and treatment was then begun. In the end of the treatment period, the rats were sacrificed, ovaries were removed and both histological structures and leptin staining intensities were determined. Tissue samples were fixed in 10% formalin for 24 hours, dehydrated in increasing concentrations of ethanol, followed by clearance of xylene and embedded in paraffin wax. For immunohistochemical staining, the samples were first incubated in 60⁰C overnight and then held in xylene for 30 minutes and rehydrated through a series of ethanol solutions for 2 minutes each, the sections were washed with distilled water and phosphate buffered saline (PBS) for 10 minutes. Then, they were held in 2% trypsin in tris buffer at 37⁰C for 15 minutes and washed with PBS 3 times for 5 minutes. The limits of sections were incubated in 3% hydrogen peroxidase for 15 minutes to inhibit endogenous peroxidase activity. The tissues were washed with PBS 3 times for 5 minutes each and stained with primary antibodies; anti-human leptin (human leptin DSL-10-23100 Texas,USA) for 18 hours. They were washed with PBS 3 times for 5 minutes each and incubated with anti-rabbit biotin. Having washed the secondary antibody with PBS 3

times for 5 minutes, the samples were stained with streptavidin. After washing the secondary antibody with PBS 3 times for 5 minutes, the sections were washed with Dako diaminobenzidine to detect immunoreactivity and then washed with Mayer's hematoxylin. They were covered with Dako mounting medium and were observed with an Olympus BX50 light microscope, and the pictures were taken by using 100 ASA Fuji color film. Control samples were processed in an identical manner in the absence of primary antibody. Staining intensities were determined by a semi-quantitative scoring system, such as (+) = denoting as mild with a value of 1, (++) = moderate with a value of 2 and (+++) = strong with a value of 3 by a blind observer. In addition, H-Score was calculated with the following equation:

$$\text{H-score} = \quad P_i \, (i+1)$$

i = intensity of staining with a value of (+), (++), (+++) and P_i = percentage of stained epithelial cells for each intensity, varying from 0-100%. Kruskal-Wallis with post-hoc Dunn's test was used for statistical analysis, $p<0.05$ was accepted as significant.

Results. Cuboidal cells constituted ovarian epithelium in the control group. Ovarian follicles were in different developmental stages. Many layers of GCs surrounded the follicles and scarce corpora lutea were seen. Stromal cells around the follicle and fibroblasts formed a sheath of connective tissue. In group D5, the epithelium was columnar, and reduced number of follicles and theca cells were observed. The number of corpora lutea increased, and the vessels were dilated. In group D10, the number of follicles extremely reduced while theca cells and stroma were also decreased. Corpus luteum structures increased and the vessels were dilated.

In the control group, no leptin staining was observed in GCs. Staining intensity and H-score were as follows: (+++)/204 for theca externa cells, (+++)/289.5 for stroma, and (++)/140 for corpus luteum (**Figures 1a & 1b**).

In D5 group, no leptin staining was observed in GCs. Staining intensity and H-score were as follows: (++)/80 for theca externa cells, (++)/126 for stroma and (++)/70 for corpus luteum (**Figures 2a & 2b**).

In D10 group, no staining was observed in GCs. Staining intensity and H-score were as follows: (+)/9.5 for theca externa cells, (+)/65 for stroma and (+)/21 for corpus luteum (**Figures 3a & 3b**).

Negative control staining for leptin antibody is shown in **Figures 4a and 4b**. The difference between H-scores of normal and D10 groups was statistically significant in all tissue types ($p<0.01$).
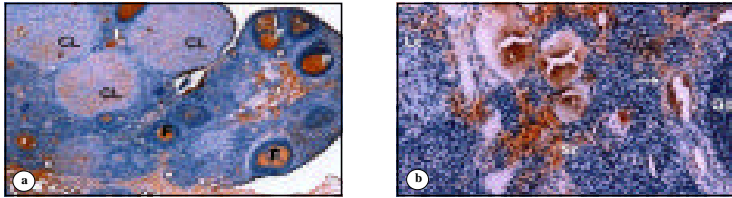
Figure 1 - Control group **a)** immunohistochemical detection of leptin expression in the rat ovarian cells. Panoramic views of rat ovary showing various developmental stage follicles (many primordial and primary with seven graafian follicles [F]). Corpus luteum (CL). Original magnification x40 and **b)** strong leptin staining is seen in luteinized cells (Lc). Stromal cells (Sc) and theca externa cells (arrow) shows a leptin immunostain similar to the luteinized cell. Furthermore, granulosa cells (Gc) is not stained with leptin. Original magnification x200

Discussion. Insulin stimulates androgen production in theca cells,[15] estrogen and progesterone production in cultured GCs.[16] Leptin is produced in the ovary, another source besides adipose tissue.[1-4] Insulin and leptin are known to have antagonistic interaction in the ovary, but time and dose-dependent leptin suppression of insulin mechanism is not completely known yet. Our initial article showed that 4-week insulin treatment reduced leptin staining in rat ovaries,[17] and the second series of experiments were conducted to investigate time-dependent relationship of insulin treatment on leptin expression. In the current study, we observed that leptin staining intensity gradually diminished on thecal, luteal, and interstitial cells.

Leptin expression has been detected in histological sections of normal and polycystic human ovaries[4] and in murine ovary,[18] but there is no final agreement on the exact cellular distribution of this expression at each follicular stage. We observed that leptin staining was prominent in thecal, luteal, and interstitial cells in the control group and suggested that leptin might be a physiological regulator of follicular or luteal function of the ovary. Karlsson et al[3] first analyzed leptin receptor expression in the human ovary, and reported that leptin might have a direct effect for biological response. In addition, Zachow and Magoffin[19] suggested that ovarian follicular cells were regulated directly by leptin. Leptin has a structural similarity to that of the cytokine family, which acts via transmembrane receptors and regulates many signaling pathways, often in a tissue-specific manner, such as Type 2 diabetes.

Insulin stimulates estrogen and progesterone production by cultured GCs.[16] In this study, leptin expression was not observed on GCs of all groups. Archanco et al[1] have also found that leptin messenger ribonucleic acid (mRNA) was expressed in GCs of developing rat follicles but leptin protein was expressed only in mature GCs. These cells may express leptin mRNA from the beginning of follicular development but its translation may not occur until their differentiation during the luteal phase.[20] In this study, the rat ovaries staining with leptin antibody were in diestrus I (metestrus) stage. Thus, the contradiction may be explained by variations in ovarian leptin expression at different times of the rat estrous cycle during which leptin quantity of GCs may be insufficient to be observed with immunohistochemical staining.

Leptin suppressed progesterone production by luteinized human GCs in the presence of insulin at physiological (10 and 50 ng/ml), high physiological (100 ng/ml), and also very low levels (as little as 0.1 ng/ml).[21] Granulosa cell is very sensitive to leptin action and even low leptin dose may be sufficient enough to suppress to GC function.[22] This suppressive effect begins at 4 hours for physiological doses, and at 24-48 hours both high physiological and very low doses. This trial indicated that leptin might act in an acute manner.

It is known that the theca cell is another important source of leptin in the follicles and the number of cells containing leptin protein increases during maturation and decreases during regression.[1] We found a very strong immunoreactivity in theca cells of the control group. Although similar results were reported by Arcananco et al,[1] other authors have reported only weak positivity for leptin protein in thecal cells of human mature follicles[4] and mouse ovary.[18] This contradiction is explained by Archanco et al[1] that weak staining of these cells may be fibroblast-like cells derived from the thecal layer not in the steroid-producing cells. Leptin staining intensity of theca cells gradually reduced when treated with insulin. This variation might be due to a reciprocal interaction between insulin and leptin
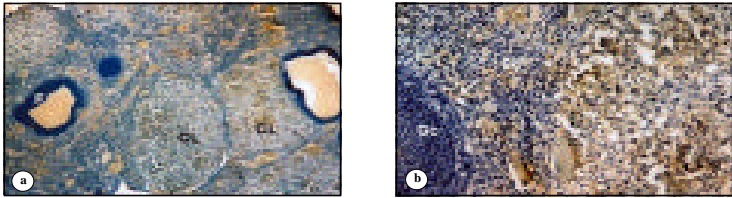
Figure 2 - D5 group **a)** immunohistochemical detection of leptin expression in the rat ovarian cells treated with insulin. Panoramic views of rat ovary showing decreased follicular number (F) and leptin staining intensity in the consecutive section. Corpus luteum (CL). Original magnification x40 and **b)** moderate leptin staining is seen in luteinized cells (Lc). Stromal cells (Sc) and theca externa cells (arrow) shows a leptin immunostain similar to the luteinized cell. Furthermore, granulosa cells (Gc) is not stained with leptin. Original magnification x200
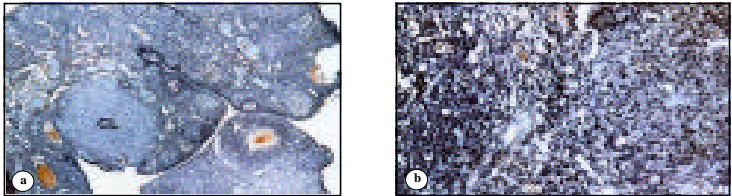

Figure 3 - D10 group **a)** immunohistochemical detection of leptin expression in the rat ovarian cells treated with insulin. Panoramic views of rat ovary showing dilated follicular cysts (F), decreased follicular number and leptin staining intensity in the consecutive section. Corpus luteum (CL). Original magnification x40 and **b)** faint leptin staining is seen in luteinized cells (Lc). Stromal cells (Sc) and theca externa cells (arrow) shows a leptin immunostain similar to the luteinized cell. Furthermore, granulosa cells (Gc) is not stained with leptin. Original magnification x200
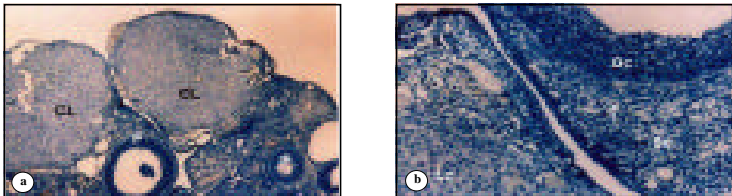

Figure 4 - Negative control staining for leptin **a)** corpus luteum (CL), Graffian follicle (F) Original magnification x40 and **b)** luteinized cells (Lc). Stromal cells (Sc), theca externa cells (arrow), granulosa cells (Gc). Original magnification x200

expression on theca cells. Spicer et al[20] have concluded that leptin, at physiological levels, can directly attenuate insulin-induced steroidogenesis of theca cells, such as progesterone and androstenedione. In a low leptin environment (namely poor nutrition), ovarian function is dictated primarily by gonadotropins and insulin/IGF-I. In a moderate to high leptin environment (namely obesity), the ovary is kept from "over" producing estradiol via leptin inhibition of insulin-induced androstenedione production by the thecal cells and aromatase activity by GCs.[20]

The growth of follicles was very less in number, numerous cysts developed within the cortex of ovaries and leptin staining intensity decreased on the follicle walls of D10 group. Leptin appears to antagonize both GCs and theca cells throughout the menstrual cycle[6,20-23] by directly or indirectly via the several growth factors (insulin-like growth factor I, transforming growth factor-) and hormones (insulin, glucocorticoids) on gonadotropin (FSH/luteinizing hormone). The quantity of both leptin expression and its receptors are known to change throughout the estrous cycle[1] and this effect may be mediated by steroid hormones. Thus, leptin may be the strongest signal for follicular development as a paracrine hormone. In addition, leptin may suppress estradiol production and interfere with the development of dominant follicles and oocyte maturation.[6]

In conclusion, our study indicated that insulin treatment reduced staining intensity of leptin in ovarian cells and this inhibition may be involved in reduced estrogen and increased androgen production. Thus, the inhibitor effects of insulin may act in a short time that their morphologic signs initiate on 5-days and complete on 10-days of the rat ovary.

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