

DIŞI SIÇAN MİDESİNDE GHRELİN EKSPRESYONU ÜZERİNE OVARIKTOMİNİN ETKİLERİNİN ANALİZİ

Analysis of Ovariectomy Effects on Ghrelin Expression in Female Rat Stomach

Birkan YAKAN, Arzu YAY, Züleyha DOĞANYIĞIT, Tuba RIHTIM

ÖZET

Ghrelın, büyüme hormonu salgılanması, iştah, besin alımı, karbonhidrat metabolizması, gastrointestinal sistem, kardiovasküler sistem, hücre proliferasyonu ve üreme sistemi üzerine etkileri olan bir hormondur. Biz bu çalışmada bilateral ovariektominin mide dokusunda neden olduğu yapısal değişiklikleri, bu değişikliklerin sonucunda mide mukozasındaki ghrelın immünreaktivitesinin nasıl etkilendiğini belirlemeyi amaçladık.

Bu çalışmada 36 adet dişi Wistar-albino siçan kullanılmıştır. Bu siçanlar, yedi eşit gruba ayrılmıştır. Daha sonra deney grupları östrojen uygulanan ve uygulanmayan gruplara ayrılmıştır. Deneyin sonunda siçanların mide dokuları çıkarılmıştır. Dokulara rutin hematoksilin-eozin(HE) ve avidin-biyotin metodu ile ghrelın immünohistokimya boyama prosedürleri uygulanmıştır.

Östrojen uygulanmayan ovariektomize siçanlarda morfolojik farklılıklar gözlenmemiştir. Ovariektomiden sonraki 3. ve 5. gün gruplarının gastrik oksintik mukozalarında ghrelın pozitif hücrelerin sayısı önemli derecede artmıştır. Ovariektomiden sonraki 7.gün grubunda gastrik mukozada ghrelın pozitif hücrelerin sayısı azalmıştır. Bu azalma 17-β östrodiol uygulanmasından kaynaklanmış olabilir.

Bu sonuçlar; bilateral ovariektomi gastrik mukozada histolojik değişikliklere neden olmazken, gastrik ghrelın immünreaktivitesi üzerine etkili olduğunu göstermektedir.

Anahtar Sözcükler: *Mide; Ghrelın; İmmünohistokimya; Ovariektomi*

ABSTRACT

Ghrelin is a hormone, which has effects on growth hormone secretion, appetite, food intake, carbohydrate metabolism, gastrointestinal system, cardiovascular system, cell proliferation and reproductive system. In this study, we have intended to observe that bilateral ovariectomy will bring structural changes in the gastric tissue and as a result of these changes, affect how ghrelin immunoreactivity in the gastric mucosa.

In study, 36 adult female Wistar-albino rats were used. These 36 rats were divided into seven groups. Later, experiment groups were divided into groups of estrogen-administrated and non-estrogen-administrated. At the end of the experiment, the rats' gastric tissues were removed. Tissues are administrated ghrelin immunohistochemical staining with avidin-biotin-peroxidase and routine Hematoxylin –Eosin (HE) method.

In the groups of ovariectomized rats that are not administrated estrogen were not monitored with morphological differences. With the groups of the third and the fifth day after ovariectomy, the number of ghrelin positive cells in the gastric oxyntic mucosa significantly increased. With the group of the seventh day after ovariectomy, the number of ghrelin positive cells in the gastric mucosa was decreased. Decrease in ghrelin positive cells with ovariectomy may be caused by administration of 17 β - estradiol.

These results suggested that while bilateral ovariectomy causes no histological changes in the gastric mucosa, it is effective on gastric ghrelin immunoreactivity

Keywords: *Stomach; Ghrelin; Immunohistochemistry; Ovariectomy*

¹Erciyes University, Faculty of Medicine, Dept of Histology&Embryology, University of Erciyes, Kayseri, TURKEY

²Bozok University, Faculty of Medicine, Dept of Histology&Embryology, University of Bozok, Yozgat, TURKEY

Birkan YAKAN, Prof. Dr.
Arzu YAY, Doç. Dr.
Züleyha DOĞANYIĞIT, Yrd.Doç. Dr.
Tuba RIHTIM, Uzm.

İletişim:

Züleyha Doganyigit,
MSc, PhD, Dept. of Histology and Embryology, University of Bozok, Yozgat, Turkey
Tel: 05357465772
e-mail:
zuleyha.doganyigit@gmail.com

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INTRODUCTION

Ghrelin is a peptide hormone produced by the gastrointestinal system and is functional in regulation of eating habit and body weight through central effect. Kojima et al. defined ghrelin in the gastrointestinal fundus of rats first in 1999 (1). Ghrelin is basically a hormone with a lipopeptide structure of 28 amino acids produced by X(A) cells which have endocrinal functions in the gastrointestinal fundus, and, is the legend of Growth Hormone Secretion- Receptor (GHS-R) (1,2). It is monitored in the studies done through gene expression on humans and rats that ghrelin and its receptor are present in a wide range of body parts such as heart, kidneys, liver, lungs, pancreas, placenta, brain, pituitary and intestines (3-5). Ghrelin takes part at the secretion of growth hormone, energy balance, food intake and regulation of body weight, and shows its effect by binding to GHS-R type 1 (1).

Ghrelin and GHS-R are found in reproductive organs and placenta (6,7). The expression of ghrelin peptide and ghrelin mRNA is shown in both human and rat placentas. It is known that human and rat placentas have a strong correlation in terms of ghrelin expression and pregnancy periods. In the case of human placenta, ghrelin as an immunohistochemical can be monitored to be expressed in main cytotrophoblasts and very little in syncytiotrophoblasts in the first trimester. However, in the term, there has been observed no ghrelin as an immunohistochemical. On the other hand, with pregnant rats, while there has been no proof of ghrelin mRNA expression in the early period of pregnancy, we have seen a prominent increase on the 16th day of pregnancy, later decreasing in the continuing period (6).

It is stated that ghrelin and its functional receptor GHS-R 1 is expressed in adult human testicles. Ghrelin immunoreactivity has been seen in normal testicles, Leydig cells and lesser in Sertoli cells (8). Ghrelin is also expressed in ovary. While it is expressed highly in functional phase in corpus luteum, it is defined that it is expressed in lower levels in regression period (9). Observation of intense and specific ghrelin immunopositivity in steroidogenic luteal cytoplasm also supports this finding (10). Functional ghrelin receptor is found in

follicular and luteal surface epithel of the ovary, and in interstitial hilus cells. Ghrelin and GHS-R expression is also seen in the endometrium. It is thought that ghrelin has paracrine and oocrine effects in embryonic implantation causing many mediators act in synergy, and has very complex effects (11).

The borders of the direct effects of ghrelin on reproductive system are not yet known. It is stated that ghrelin has extragonadal effects on the reproductive system. It is shown that it suppresses LH secretion and lessens the LH response to GnRH invitro (12).

In summary, ghrelin regulates local or systemic balance between energy level and reproductive system (13,14). There are many unanswered question about the possible reproductive effects of ghrelin. In order to shed light on these unanswered questions, we have aimed at defining, by using immunohistochemical method, how the ghrelin expression in the gastrointestinal mucosa of ovariectomized female rats with lack of estrogen may be affected.

MATERIAL AND METHODS

In our study at Erciyes University Hakan Çetinsaya Experimental and Clinical Researches Center, we have used 36 non-pregnant young adult female Wistar-albino rats with an age of 12 weeks and with an average weight of 250 gr after receiving the legal permission from the Ethical Board. The environmental and experimental conditions are supplied by the center, the rats are kept in a room where the lightning is 12 hours on, and 12 hours off (dark between 19.00 and 07.00 hours) and the air conditioning is 60-70% humidity with 20-24 ° C temperature. These rats are randomly taken to seven different groups. The distribution of groups and test subjects are administrated in Table I. There has been administrated no surgical operation on the control group. All the rats in the experimental groups were under surgical operation after intraperitoneal ketamine (25mg/kg) + xylazine (5mg/kg) anesthesia. Prior to surgery, stretch reflex and blink reflex are checked. Ventral abdomen wall of our rats are shaven. After that, midline incision and sub-abdominal cavity exploration are done for taking out the two ovaries (Fig.1 a and b). In the Sham operation

midline incision and sub-abdominal cavity exploration are done, and the incised part is closed without action.

The ovariectomized rats are randomly separated into groups of A and B, and are taken to a heated room in cages in order to prevent hypothermia. In order to maintain postoperative analgesia 25-75mg/kg and 2mg/ml parasetamol is added to their drinking water. A solution of ethyl alcohol and sesame oil 1/3 mixed with 17 β -estradiol (E2) 2mg/kg (15) is administrated subcutaneously to rats in B groups considering the operation day as zero. For the Sham group, a solution of ethyl alcohol and sesame oil is administrated subcutaneously as placebo.

At the end of the experiment, rats are sacrificed by giving ketamine + xylazin. Control and sham groups are sacrificed after determination of diestrous phase of estrous cyclous. Later on, the stomach tissues of these animals are taken and embedded into paraffin blocks after examining under routine microscope fixed in 10% neutral formalin solution. Sections of 5-6 μ m are taken from these paraffine blocks and they are examined through immunohistochemistry for Hematoxylin-Eosin and ghrelin expressions.

H&E staining

The samples were deparaffinized and rehydrated with distilled water. They were then ablated in 1% hydrochloric acid alcohol solution for 30 seconds after staining with hematoxylin for 7 minutes and then were washed with distilled water. Samples were stained with eosin for 2 min, dehydrated and immersed in xylene for 15 min. Finally the samples were mounted.

Immunohistochemistry

Avidin-Biotin-Peroxidase method is used in order to determine the ghrelin immune reactivity in the gastric tissue. The staining method is presented in Table IV. The sections taken from paraffin blocks in width of 5 μ m are transferred to polyisin slides. Tissues that are deparaffinized are exposed to H₂O₂ after dehydration of different stages alcohol serials in order to prevent endogen peroxidase. To avert the background staining, these tissues are incubated in with primary antibody (Ghrelin goat polyclonal IgG, Santa Cruz Biotechnology,

California, U.S.A) in a room with a temperature of +4 oC and normal humidity after being exposed to 1,5% regular rabbit serum. The next day, after administrating DAB (Diaminobenzene) chromogene, streptavidin HRP (Horse Radish Peroxidase) and secondary antibody (goat-rabbit IgG, Santa Cruz Biotechnology, California, U.S.A), Gill hematoxylin negative staining is done. For the negative control of prepared tissues, PBS (phosphate buffer saline) is used instead of primary antibodies while other steps are performed as figured as before. Processed with alcohol and xylol, the tissues are surfaced with entellan. The preparations are photographed after monitoring with the microscope (Olympus BX51).

Statistical analysis

In our study, the effects of ovariectomy on the ghrelin immunopositive cells in gastric tissue are analyzed statistically. The normal distribution of the data is checked with Shapiro-Wilk test. Single direction variance analysis is used for normal distributions while Kruskal-Wallis analysis is used for abnormal distributions. For multiple comparisons of parametric and nonparametric values, we have used Student-Newman-Keuls method. The statistical consideration value was chosen as $p < 0.05$.

Morphometrical analysis

In order to determine the ghrelin positive cell density in the rat gastric mucosa, the number of ghrelin positive cells calculated in each section with the help of a digital camera after they are photographed under light microscope (Olympus BX51). A computer assisted visual analysis program is used for this process. This analysis software is supplied by Ankara University School of Medicine Department of Histology and Embryology. Thanks to this visual analysis program, we could calculate the number of mucosal ghrelin positive cells in each section.

Table 1. Distribution of rats according to groups

Group	N	Process
GroupI	3	Control group, no action.
GroupII	3	Sham group, sutured after the incision, placebo administrated.
GroupIIIA	3	Uterus tissue is taken out on the first day after the ovariectomy.
GroupIIIB3	3	Ovariectomy and E2 administrated, dissected on the first day.
GroupIVA	3	Uterus tissue is taken out on the third day after the ovariectomy
GroupIVB	3	Ovariectomy and E2 administrated, dissected on the third day.
GroupVA	3	Uterus tissue is taken out on the fifth day after the ovariectomy.
GroupVB	3	Ovariectomy and E2 administrated, dissected on the fifth day
GroupVIA	3	Uterus tissue is taken out on the seventh day after the ovariectomy.
GroupVIB	3	Ovariectomy and E2 administrated, dissected on the seventh day
GroupVIA	3	Uterus tissue is taken out on the fifteenth day after the ovariectomy.
GroupVIIB	3	Ovariectomy and E2 administrated, dissected on the fifteenth day

RESULTS

At the analysis with light microscope, in the diestrous phase of control group and in gastric tissue section taken mainly from cardia and fundus of experiment group, gastric layers are monitored as normal (Fig. 2a). Epithel was mono-layered simple prismatic while lamina propria was in a structure of loose connective tissue. In gastric tissue samples taken from Sham group, the staining is observed almost the same as it was in control group. There has been no histological change observed in gastric tissues of groups which estradiol is administrated and not administrated on the first day after the ovariectomy. Gastric epithel is observed mono-layered simple prismatic while lamina propria, submucosa and muscle layers are normal (Fig. 2b). There has been no histological change observed in gastric tissues of groups which estradiol is administrated and not administrated on the third day after the ovariectomy (Fig. 2c). There has been no histological change observed in gastric tissues of groups which estradiol is administrated and not administrated on the fifth day after the ovariectomy (Fig. 2d). There has been no histological change observed in gastric tissues of groups which estradiol is administrated and not administrated on the seventh day after the ovariectomy (Fig. 2e). There has been some thickening in the mucosa layer of gastric tissue sections and vascular increase in gastric tissues in groups of

which estradiol is administrated and not administrated on the fifteenth day after the ovariectomy (Fig. 2f).

In histochemical staining, contrary to the observations on other groups, there has been an increase in the number of ghrelin positive cells on the third and the fifth day after the ovariectomy (Fig. 3a). On the other hand, in the number of stained ghrelin positive cells on the seventh and the fifteenth day after the ovariectomy, there has been a decrease (Fig. 3b).

In the morphometric analysis density of ghrelin-positive cells were detected. According to groups respectively (from G7B to G1) mean cell counts in mm² defined as 78, 75, 98, 82, 116, 105, 125, 118, 96, 92, 84 and 81. Respectively, group 1 (81,75,78), group 2 (72,79, 74), group 3A (97,102, 95), group 3B (85,79, 82), group 4A (99, 102,147), group 4B (107,95, 113), group 5A (115,132,128), group 5B (127,109,118), group 6A (99,105,86), group 6B (90,101,85), group 7A (79,85,88) and group 7B (78,80,85) reflected the number of ghrelin positive cells in mm².

According to statistical analysis, on the day 7 (G6A) and the day 15th (G7A) after the ovariectomy were found to constitute the highest difference mean cell counts among the groups ($p < 0,05$). There was the most significant difference in the groups that are not estrogen and

sham group (G2) in terms of average number of ghrelin-positive stained cells ($p<0,05$). In ovariectomized rats was occurred a big difference between the day 5th

(G5A ve 5B) and the day 15th (G7A ve 7B) in terms of mean ghrelin-positive cell counts ($p<0,05$).

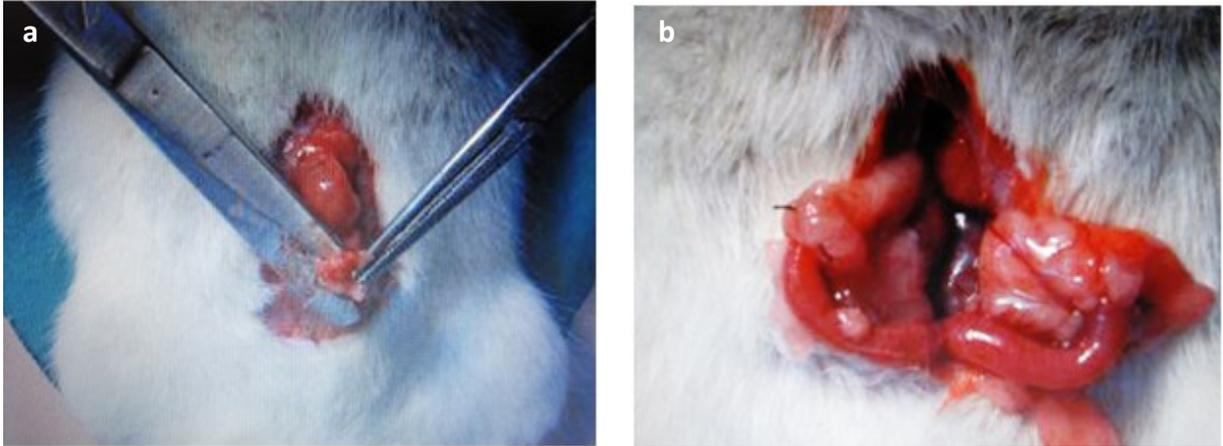


Figure 1. (a) Rats with midline incision and ready for ovariectomy. (b) Vision of ovariectomized rat before the abdomen is closed.

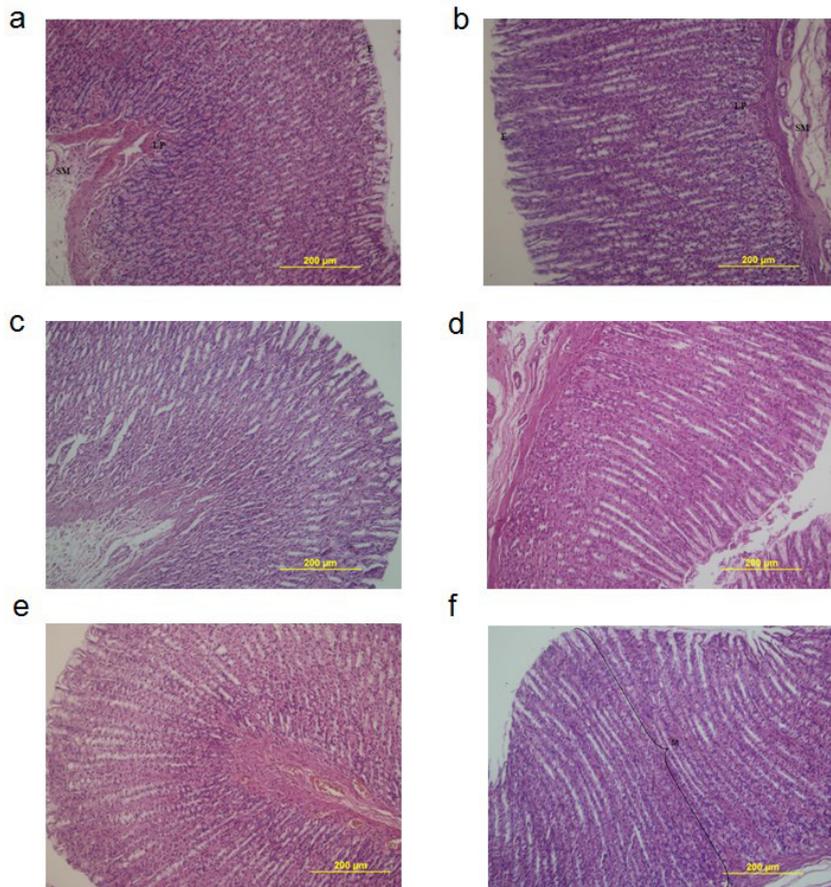
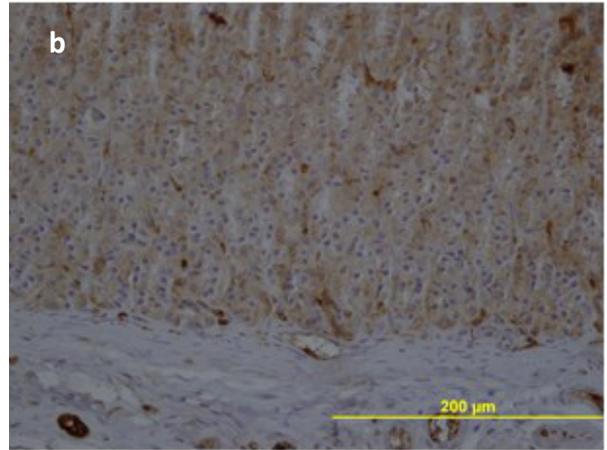
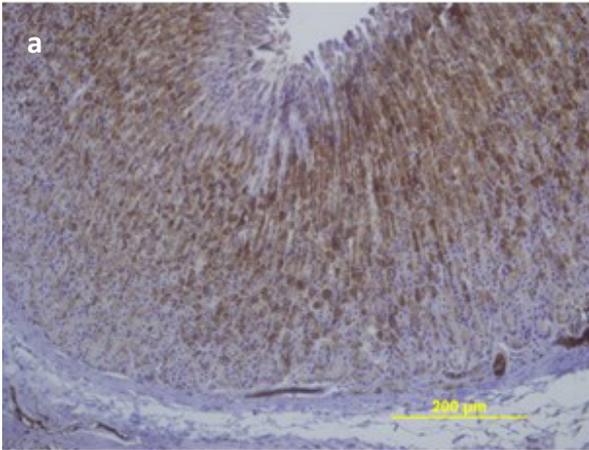


Figure 2. (a) Group I gastric tissue is epithel (E), lamina propria (LP) and submucosa (SM) layers are in normal structure. (b) Group III A and B: gastric tissues (E, LP, SM) are without change (c) Group IV A and B: stomach is in normal structure (d) Group V A and B: gastric tissues are observed normal. (e) Group VI A and B: gastric tissues are without change. (f) Group VII A and B: thickening in the mucosa layer of gastric tissue sections and vascular increase observed. H&E X20.

Figure 3. (a) Group IV: Ghrelin positive stained cells observed. (b) Group VII: Ghrelin positive stained cells observed. Immunoperoxidase X20.



DISCUSSION

The ovariectomy that is performed before the natural age of menopause dramatically affects the hormonal dynamics. In natural menopause, ovaries stay healthy and continue secreting androgens. These androgens containing testosterone and androstenedione may be transformed into a poor estrogen or oestron. On the other hand, the surgical menopause causes the total end for the secretion of androgen, estrogen and progesterone. Instead of a 5 or 10-year-progressive transformation, there happens the acute lack of androgen, estrogen and progesterone.

GHS-R and its related legend, ghrelin, are two of the newest members of GH group. The expression of ghrelin and GHS-R in brain, placenta, kidneys, uterus and small intestine, apart from hypothalamus and pituitary glands, tells us that ghrelin regulate, different functions of GHS-R, with a great effect over it, on these organs. In addition to its regulation of different kinds of physiological function both in vitro and in vivo, ghrelin also is a strong agent for GH secretion. Other effects of ghrelin over normal tissues are; stimulation of lactotropin and corticotropin, controlling energy consumption, and controlling gastric motility and acid secretion (16).

All we know about the energy metabolisms is of studies has been done especially on obesity for the last de-

cade. As long as obesity, prevalence of obesity and its complications are studied, there have been new mediators defined. As one of those new mediators, ghrelin has been studied intensely. All these studies have been done for a better understanding of the body that has been trying to regulate fat storage and energy balance with the help of central and peripheral hormones. The close relationship among energy metabolism, food intake and reproductive physiology, disorders about food intake, diseases (obesity, malnutrition, anorexia nervosa) and metabolic changes alter the role of gonadotrophins and gonadal hormones, and that is very important for fertility. The increasing body weight and fat tissue damages the menstruation pattern, and so affects the fertility potential. Obese women change the insulin resistance only by losing weight and this eases the fertility (17).

The studies done after the discovery of ghrelin showed that this molecule is effective on many systems in the organism. The major subject of studies on the effects of ghrelin are those on hormone secretion, appetite, food intake, carbohydrate metabolism, gastrointestinal system, cardiovascular system, cell proliferation and reproductive system.

Intravenous administration of ghrelin on rats increases the basal gastric acid secretion and gastric motility depending on the dose while subcutaneous administra-

tion has no effect on the basal gastric acid secretion (18,19). It is stated that the maximum response to intravenous ghrelin was as much as the increase to the response of subcutaneous histamine (3mg/kg) injection in terms of gastric acid secretion. These effects can be stopped by performing both bilateral cervical vagotomy and atropine. However, H₂-receptor antagonist of histamine cannot prevent this response. Therefore, it is thought that ghrelin has an effect on gastric functions through nervus vagus (20,21).

It is accepted that the appetite is controlled by the brain and food intake is regulated in central nervous system especially by complex mechanisms in the hypothalamus (22). The effect of ghrelin on appetite is in different figures (1). This effect, stimulating the appetite, is defined as ghrelin reaches the brain passing first through hypothalamic arkuat nucleus and then through blood-brain barrier by active transportation after its synthesis in the stomach. Besides this, ghrelin, which is synthesized peripherally, stimulates vagal afferent nerve endings and that causes the expression of GHS-R, and stimulates the hypothalamus through nucleus solitarius that has vagal connection. The ghrelin level in human circulation system increases during hunger and decreases when the stomach is full. The highest level of ghrelin during the day can be monitored between 2 and 4 hours (23). While hunger increases the ghrelin level, in 60-120 minutes after the food intake it decreases. Ghrelin, administrated as an exogen, causes increase in food intake, decreases fat consumption in the body, and as a result of this, triggers the increase in fat tissue. The effect of ghrelin on fat tissue and appetite increase is thought to be independent of its effect over growth hormone, and this is thought to be regulated by special neurons in the central nervous system in which leptin is an agent for this (24).

It is stated that ghrelin stimulates appetite because its level in blood serum increases before each meal, and that it inhibits the emptying of energy stocks and cachexy (25). It is shown that hunger increases ghrelin secretion in rats while carbohydrate intake decreases its secretion (26). The effects of ghrelin on energy homeostasis emerge at hypothalamus in the central nervous system. Therefore its effects are not restricted only in

the peripheral tissues where it is produced (27).

Deaths related to obesity are a major problem in today's world. The main approach about preventing obesity is the use of anti-obesity drugs. Using ghrelin vaccination for obesity is high on the agenda. That's because obesity is defined with low GH and ghrelin levels. However, many researchers reported that there is no coherence between these two approaches. Researches showed that ghrelin levels of obese individuals are quite low compared to levels of thin people. Weights lost with a diet have caused increase in the ghrelin levels in the circulation. Probably this situation of ghrelin related to the bodyweight is regulated with the help of insulin and is not affected by the amount of fat and fat distribution of the body. It is observed that the increase in the level of ghrelin before after the meal with obese individuals is higher than thin individuals and that n-octanyl ghrelin has no effect on the obese (13).

In the light of this information, we have intended to study the effects of ovariectomy and E2 placement on the immunoreactivity of ghrelin in gastric mucosa of rats.

The main source of ghrelin hormone is neuroendocrine cells in fundus and pylorus of stomach, and it is included in the circulation after being produced here (20). Ghrelin positive cells are situated close to capillary and it has no relation with oxyntic lumen. Therefore, this shows the secretion is through gastric veins, not to gastrointestinal canal. In this manner, it can circle the whole body system (28). Ghrelin is first found in the oxyntic glands in fundus (20). Oxyntic glands are found in the inner surface of corpus and fundus of stomach, and constitute 80% of stomach proximal (29). Under light and electron microscope observations, different types of cells are defined in the glandular mucosa of the stomach by using immunohistochemical methods and by taking the ultra structural characteristics of secretion granules into consideration (30). There are seven main types of endocrine cells that are defined in human and rat stomach. These are; enterochromaffin-like cells (ECL), P cells, D cells, enterochromaffin cells (EC), X/A cells, D1 cells and agranulated cells (31).

The X/A cells in the stomach are discovered by Davis (32) in 1954. Granules in these cells have been a secret until the discovery of ghrelin. After the discovery of ghrelin which has a characteristic effect on the GH secretion in the rat stomach by Kojima et al., the granules in X/A cells are defined by using molecular techniques and they are determined responsible for the synthesis of ghrelin. In the gastrointestinal system (GIS), both ghrelin and ghrelin mRNA expression is localized in X/A cells that are in oxyntic glands which produce acid in humans and rats. One of the other endocrinal cells of oxyntic mucosa, D cells, which secrete somastostatine, and enterocromaffin-like cells that are histamine-rich are not ghrelin positive (33). In addition, it is stated that ghrelin is found in the gastric endocrine cells of humans and rats (20). Ghrelin is synthesized along the digestive system in duodenum, ileum, caecum and colon. Among these ghrelin positive cells no difference has been observed in terms of immunoreactivity, but it is discovered that the number of ghrelin immunoreactivity cells decreases close to colon.

Small, circular shaped ghrelin immunopositive cells are defined as closed type cells. In addition to these, another type of cell that has a triangular shape and has apical cytoplasm that are related to lumen is defined as opened type (34). Sakata et al. (35) found in a study that they have done on rats that ghrelin producing cells are found mostly in GIS organ and stomach, and generally in characteristic of opened type ghrelin immunopositive cells.

In recent years, it is stated that ghrelin may be effective on hematological parameters. Narin et al. (36) claimed in a study that ghrelin that is administered in dose of 10 nmol/kg for five days probably may increase the number of lymphocyte by stimulating lymphopoiesis. In our study that we have aimed at finding answers to unanswered question, we have tried to determine by histochemical method how the ghrelin expression in gastric mucosa of female rats that are lacked of estrogen may be affected after ovariectomy. Besides these, we have performed H.E. staining method, morphometric analysis, and a comprehensive statistical analysis. With the help of H.E. staining method, we have observed that on the fifteenth day of ovariectomy, different than

other groups, mucosa was thickened and vascularization has increased in the sections of stomach tissues. As a result of the immunohistochemical staining and morphometric analysis the group that the highest number of ghrelin positive stained cells was G5A, which was not administered estrogen on the fifth day of ovariectomy. This finding shows coherence with the study that is done by Matsubara et al. (37). In addition, with the help of multiple comparison tests among groups for the number of ghrelin positive cells, the greatest difference ($q=5,692$) is observed in G6A, which were not administered estrogen on the seventh day after the ovariectomy, and G7A, which were not administered estrogen on the fifteenth day after the ovariectomy. When we compare the groups in terms of the relation between the criteria whether or not the estrogen is administered and the number of ghrelin positive cells, the biggest difference ($q=3,914$) was between Sham group (G2) and the group which estrogen is not administered. Besides, when we perform the multiple comparison test for the relationship between groups about whether or not the ovariectomy is performed and the number of ghrelin positive cells, the highest difference ($q=8,665$) was between the groups of the fifth day and the fifteenth day.

We did not observe a peculiar morphological difference with the groups that are not administered estrogen after the ovariectomy. The number of ghrelin positive stained cells in the oxyntic mucosa on the third day and the fifth day after the ovariectomy has noticeably increased. Seven days after the ovariectomy, the number of ghrelin positive stained cells decreased. This decrease in the number of ghrelin with ovariectomy is probably because of 17- β - estradiol administration. Consequently, bilateral ovariectomy causes no histological changes in the gastric tissue while it is observed that ovariectomy is effective on gastric ghrelin immunoreactivity.

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Conflict of interest

We declare that we have no conflict of interest.

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