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## The Effects of Ovariectomy on Ghrelin Expression in the Rat Uterus\*

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A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation; D – writing the article; E – critical revision of the article; F – final approval of article; G – other

### Abstract

**Background.** Ghrelin is a hormone which has effects on the secretion of growth hormone, the gastrointestinal system, the cardiovascular system, cell proliferation and the reproductive system.

**Objectives.** The aim of this study is to investigate structural changes in the uterine tissue and to assess ghrelin immunoreactivity in the endometrium as a result of bilateral ovariectomy of rats.

**Material and Methods.** In this study, 28 adult female albino Wistar rats were used. The rats were randomly divided into four groups. Group I was the control group; Group II was the placebo group; Group III was ovariectomized; and Group IV was ovariectomized with 2mg/kg estrogen administered per day. Age-matched diestrous intact rats were used as controls. At the end of the experiment, the rats were decapitated 1, 3, 5, 7, and 15 days after ovariectomy under ketamine anesthesia and their uterine tissue was removed.

**Results.** In the ovariectomized rats, reductions in the sizes of both the uterine epithelium and the endometrial glands were observed, as well as a loss of connective tissue. Ghrelin-positive cells in the endometrial surface and the gland epithelium were visualized by immunohistochemistry. After ovariectomy, ghrelin expression was found to be decreased in a time dependent manner.

**Conclusions.** Bilateral ovariectomy leads to histological changes in the uterine tissue. Ovariectomy was observed to decrease endometrium ghrelin immunoreactivity (*Adv Clin Exp Med* 2014, 23, 3, 363–370).

**Key words:** endometrium, ghrelin, immunoreactivity, ovariectomy, rats.

Ghrelin is known to be an endogenous ligand for the GH-secretagogue receptor (GHS-R) [1]. Both ghrelin and the GHS-R are also expressed in quite a lot of tissues other than the hypothalamus and pituitary, including the brain [2], kidney [3], stomach [4] and uterus [5]. This molecule was originally noticed for its ability to elicit GH secretion *in vivo* and *in vitro* in various species, such as humans and rodents [6–8]. This unique expression strongly indicates that ghrelin, which can be produced locally, is also involved in hormone specific actions such as paracrine and autocrine effects, as well as in the actions of the gut-derived peptide [9]. Previous studies have demonstrated that ghrelin is involved in hormone-specific roles, including endocrine and non-endocrine reactions.

These reactions involve corticotropic, lactotropic, and gonadotropic axes controls, the cardiovascular and gastrointestinal systems, as well as carbohydrate metabolism via pancreatic insulin secretion, cell growth and proliferation in tissues and tumors [10, 11].

A mounting body of evidence indicates that ghrelin has a role in the control of reproductive physiology by two distinct, probably overlapping actions: (a) by means of systemic release of the stomach-derived peptide, which has effects on the reproductive system; and (b) through biological actions on reproductive organs via locally expressed ghrelin [12, 13]. More recently, circulating ghrelin has been reported to act at different levels on the rat hypothalamic-pituitary gonadal

\* This work was supported by a research grant from the Erciyes University Scientific Research Projects Unit (EUBAP, TST-08-365).

axis (HPGA), affecting gonadotropin releasing hormone (GnRH) pulsatility, as well as follicle stimulating hormone (FSH) and luteinizing hormone (LH) production and secretion [14, 15]. Ghrelin administration stops LH secretion centrally in ovariectomized (OVX) female rats [16], monkeys [17] and sheep [18].

The mammalian ovary is known to be a complex endocrine organ. It is responsible for oocyte release during ovulation and hormonogenesis. Ovarian steroids are one of the most important factors influencing uterine morphology and motility. Thus, the aim of this study was to investigate alterations in the ghrelin producing cells of the uterus induced by ovariectomy.

## Material and Methods

### Animals

The study was conducted at the Erciyes University Hakan Çetinsaya Experimental and Clinic Research Center. Ethical approval was obtained from the Erciyes University Animal Research Local Ethics Committee and all procedures conformed to the "Guide for the Care and Use of Laboratory Animals". Twenty-eight adult female Wistar rats, 200–250 g in weight at the beginning of the experiments, were used. They were housed in a quiet and temperature- and humidity-controlled room ( $21 \pm 3^\circ\text{C}$  and  $60 \pm 5\%$ , respectively) in which a 12-h-light/dark cycle was maintained (light from 7:00 am to 7:00 pm). The rats were randomly divided into four groups. Group I: Control ( $n = 4$ ); Group II: Sham ( $n = 4$ ); Group III: OVX ( $n = 10$ ); and Group IV: 2 mg/kg/day estrogen given after OVX ( $n = 10$ ).

### Experimental Procedure

The OVX procedure was performed when the animals were 12 weeks old. The rats were anesthetized with a combination of intra-peritoneal ketamine (21.2 mg/kg) and xylazine (4.2 mg/kg). The OVX was preceded by a midline dorsal skin incision approximately 3 cm long. The ovarian vessels were then clamped and the ovaries removed. Afterwards, the uterine tubes were ligated and the muscles and skin were sutured [19]. For the replacement study, estrogen was administered to OVX rats intraperitoneally. Synthetic estrogen 17  $\beta$ -estradiol (Sigma Chemical Co., Steinheim, Germany) was dissolved in a mix of ethyl alcohol and sesame oil (Sigma Chemical Co., Steinheim, Germany) and 2 mg/kg/0.1 mL injection doses were prepared. After the surgery, the Group IV rats were given subcutaneous

2 mg/kg/day/0.1 mL 17  $\beta$ -estradiol. The placebo group was given 0.1 mL sesame oil subcutaneously. The animals were sacrificed by decapitation 1, 3, 5, 7 and 15 days after OVX. Age-matched diestrous intact rats were used as controls.

### Immunohistochemistry

The immunohistochemistry procedure was performed as previously described [20]. Briefly; five to six micrometer thick sections were cut by microtome, dewaxed and rehydrated following routine protocol, rinsed in deionized water and treated with 3%  $\text{H}_2\text{O}_2$  in methanol for 10 min to inhibit endogenous peroxidase activity. The sections were pre-incubated with normal rabbit serum (NRS) (S20-100, Chemicon International, Hampshire, UK), diluted 1 : 5 in phosphate-buffered saline (PBS) for 20 min at room temperature in a humidified chamber. The primary polyclonal antibody rose against ghrelin (Ghrelin goat polyclonal IgG, Santa Cruz Biotechnology, California, USA) was diluted 1 : 100 in 20% NRS. The sections were incubated with primary antiserum overnight at  $41^\circ\text{C}$ . The sections were incubated with secondary antibody (rabbit anti-goat IgG, Santa Cruz Biotechnology, California, USA) for 30 min at room temperature in a humidified chamber. The sections were exposed to streptavidin horseradish peroxidase (HRP) conjugate (43-4323, Zymed Laboratories Inc., South San Francisco, CA, USA), diluted 1:200, for 30 min. The substrate reaction was visualized with 0.5 mg/mL 3,30-diaminobenzidine (DAB) (Liquid DAB Plus Substrate Kit; 00-2020, Zymed Laboratories Inc., South San Francisco, CA, USA) plus 0.1% hydrogen peroxide for 3 min. All dilutions and thorough washes between steps were performed using PBS, pH 7.6, unless otherwise specified. The sections were counterstained with hematoxylin, dehydrated through a graded ethanol series and mounted with EntellanR (Merck, Darmstadt, Germany). For the negative controls, incubation with the primary antibody was omitted. Stomach tissue was used as the positive control.

### Semi-Quantitative Evaluation of Staining

In this study, only the cytoplasmic staining was scored. The intensity was checked by two histologist using the same microscope as a blind test. Intensity was scored in a range of 0–3 (0 = negative, 1 = light, 2 = moderate, and 3 = intense). Epithelium staining was also evaluated in terms of the percentage of the area (0 = negative,  $< 25\% = 0.1$ ,

26–50% = 0.4, 51–75% = 0.6, 76–100% = 0.9). They were all analyzed individually and the histoscores were obtained as area  $\times$  intensity [21, 22].

## Statistical Analysis

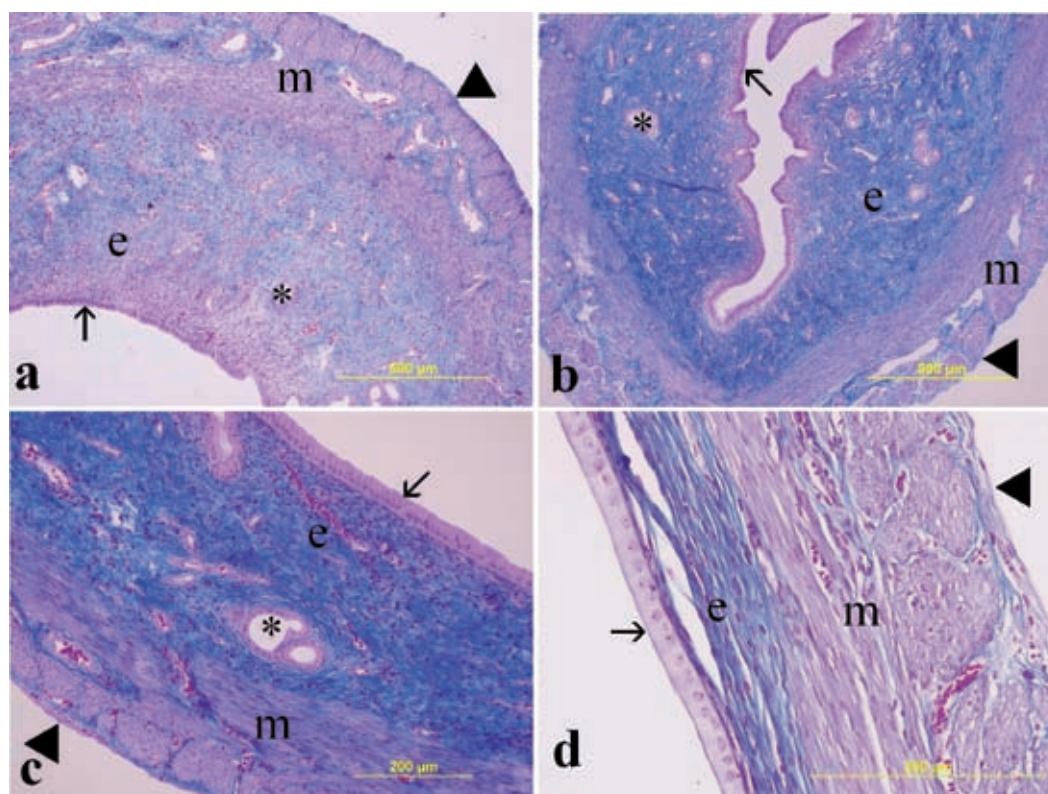
All analyses were performed using a statistical software package (SPSS for Windows®, v. 11.5). The data were expressed as mean  $\pm$  SEM. The data were analyzed statistically using one-way analysis of variance (ANOVA). Post hoc analyses were carried out with the Tukey test. Statistical significance was set at  $P < 0.05$ .

## Results

Light microscopic examinations showed normal uteruses in the control group (Fig. 1a). In both the placebo group and the first-day group (Fig. 1b) no histological changes were observed after OVX. The endometrium, which was composed of simple columnar epithelium and myometrium, appeared normal. Decreases in the height of surface and glandular epithelium, connective tissue and the number of glandula in the *lamina*

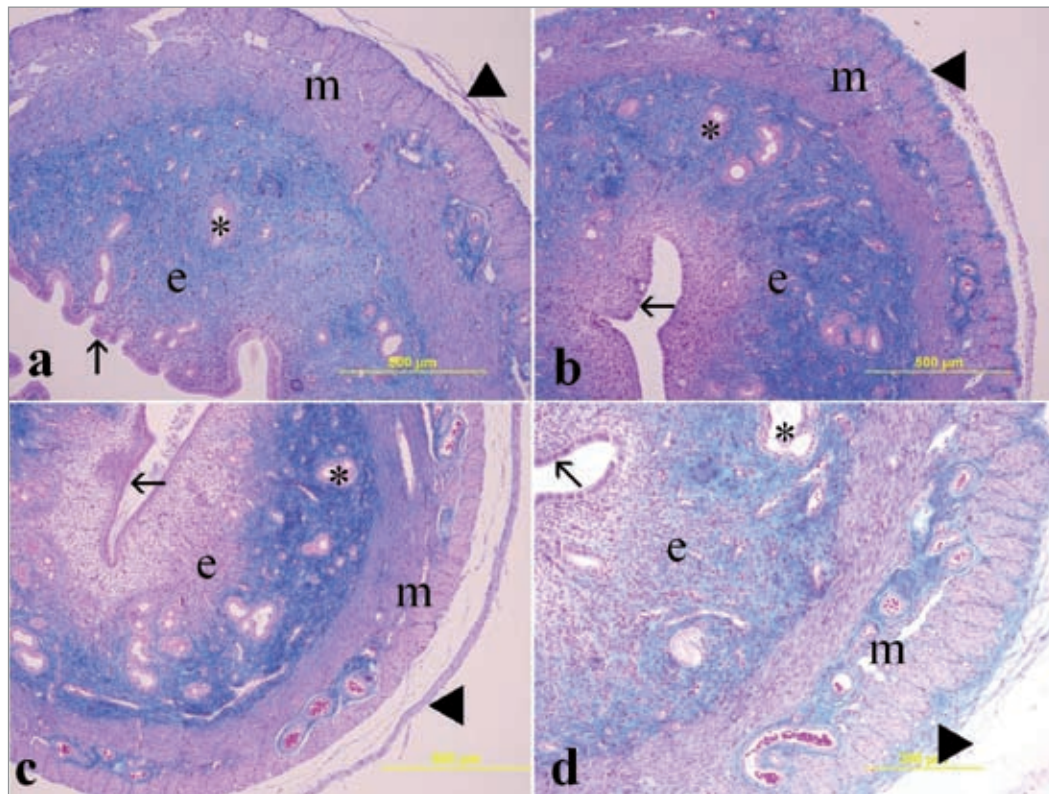
*propria* were observed in the third-day group after OVX. In addition, thinner uterine walls were noted. Moreover, the endometrium was composed of low columnar epithelium, and thinning of the *lamina propria* was observed on the 5<sup>th</sup> (Fig. 1c) and 7<sup>th</sup> day after OVX. The thickness of the uterine walls was also decreased. On the fifteenth day after OVX, thinning of the uterine walls was more evident (Fig. 1d). The endometrium was composed of simple cuboidal epithelium and a thin *lamina propria*. A reduction in collagen staining and a decrease in the number of glandula in the *lamina propria* was observed. No histological disorder was observed in the experimental group that was administered estrogen after OVX (Fig. 2). The measured thicknesses of uterine walls are presented in Fig. 5. Uterine wall thickness was shown to decrease after OVX and this decreases was significantly different than in the group that was administered estrogen ( $p < 0.05$ ) (Fig. 5B).

In the control group (Fig. 3a) ghrelin expression was seen in the surface epithelium (++) , glandular epithelium (++) , stroma (+) and muscle layer (+). No difference in ghrelin expression was observed between the placebo group and the first-day group after OVX (Fig. 3b). In these groups, ghrelin

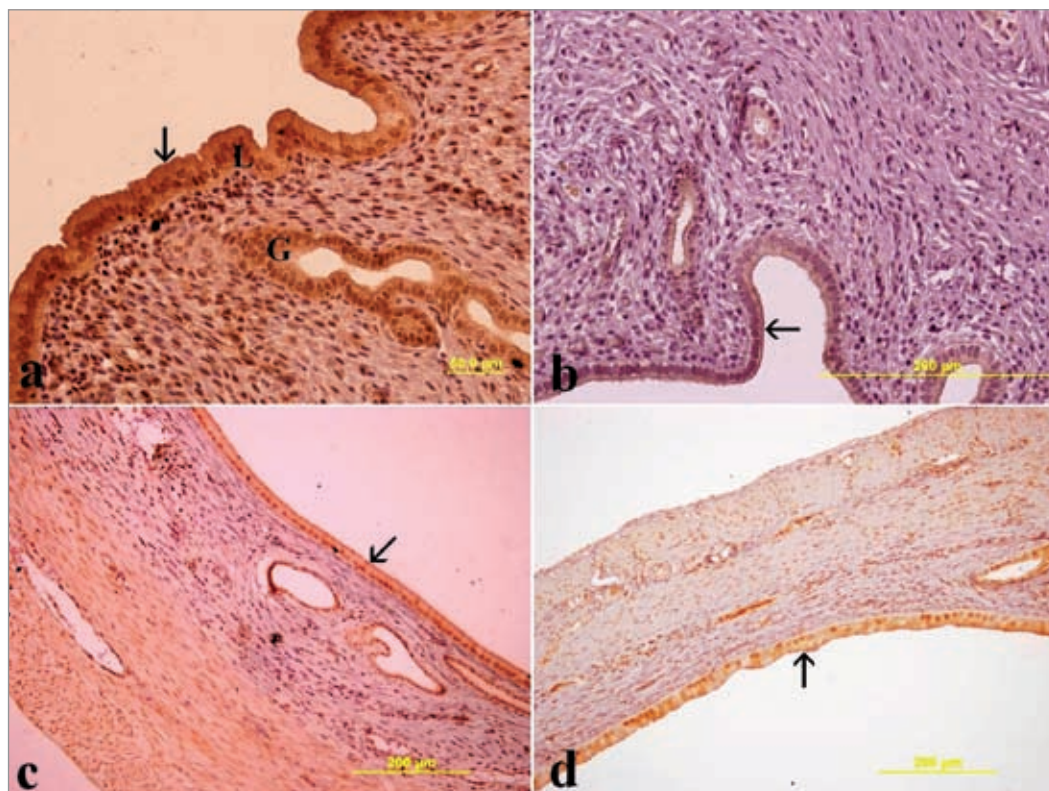


**Fig. 1.** The effects of ovariectomy on uterus morphology: (a) Group I (control), (b) Group III (1<sup>st</sup> day after ovariectomy), (c) Group III (5<sup>th</sup> day after ovariectomy), (d) Group III (15<sup>th</sup> day after ovariectomy). The endometrium (e), myometrium (m) and perimetrium (arrow head) is normal in (a) and (b), however, the uterine wall thickness and the cell height of the luminal (arrow) and glandular epithelium (\*) are reduced over time after OVX in (c) and (d). Sections stained with Masson's Trichrome

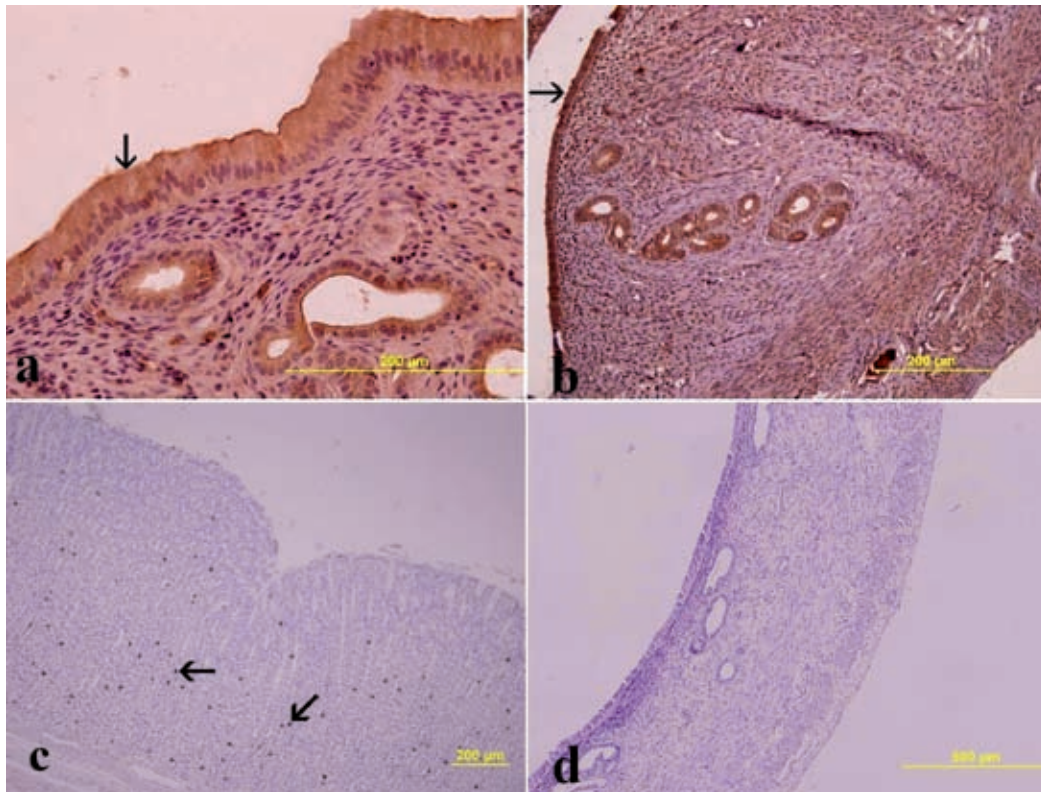




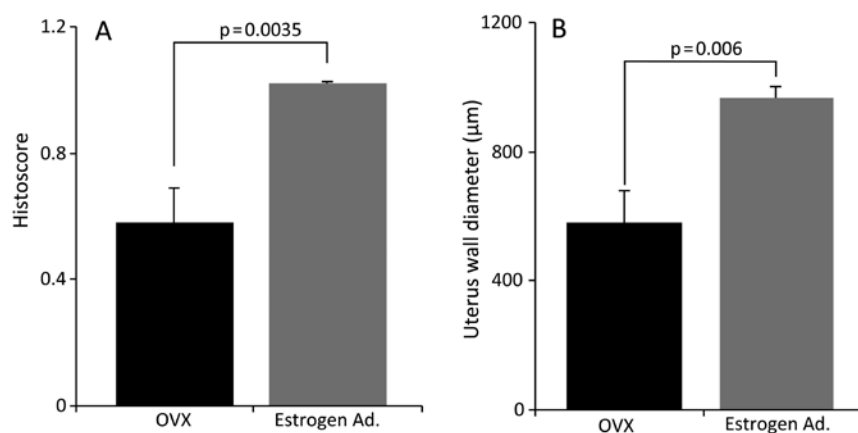
**Fig. 2.** The effects of estrogen supplementation on the uteruses of ovariectomized rats: (a) Group IV (3<sup>rd</sup> day after ovariectomy), (b) Group IV (5<sup>th</sup> day after ovariectomy), (c) Group IV (7<sup>th</sup> day after ovariectomy), (d) Group IV (15<sup>th</sup> day after ovariectomy). Endometrium (e), myometrium (m) and perimetrium (arrow head) are normal in the group that was administered estrogen after ovariectomy. Sections stained with Masson's Trichrome



**Fig. 3.** The effects of ovariectomy on ghrelin expression in the uterus: (a) Group I (control), (b) Group III (1<sup>st</sup> day after ovariectomy), (c) Group III (5<sup>th</sup> day after ovariectomy), (d) Group III (15<sup>th</sup> day after ovariectomy). Ghrelin immunoreactivity (arrow) is strongly expressed in the luminal (L) and glandular (G) epithelium in (a) and (b). A decrease of ghrelin expression over time after OVX is visible in (c) and (d). Sections stained with immunoperoxidase



**Fig. 4.** The effects of ovariectomy on ghrelin expression in the uterus: (a) Group II (placebo), (b) Group IV (5<sup>th</sup> day after ovariectomy) (c) positive controls (ghrelin immunopositive cells in stomach tissue); (d) negative controls (Group III, 15<sup>th</sup> day after ovariectomy). Ghrelin expression (arrow) is at similar levels in both the control group and the group administered estrogen (Group IV). Sections stained with immunoperoxidase



**Fig. 5.** The effects of estrogen administered after ovariectomy: (A) The histoscore of ghrelin was increased in the group that was administered estrogen after ovariectomy. (B) The uterus wall thickness was increased in the group that was administered estrogen after ovariectomy

expression was noted in the surface and glandular epithelium, stroma and muscular layer. A decrease in ghrelin expression was observed over time after OVX in Group III (Fig. 3c and 3d). However, in the group that was administered estrogen after OVX, the ghrelin expression level was same as in the control group (Fig. 4). The expression of ghrelin was assessed according to the intensity and amplitude of expression. Figure 5 shows the expression levels of ghrelin in the luminal and glandular epithelium. Ghrelin expression in the OVX group was significantly different than that of the group administered estrogen ( $p < 0.05$ , Fig. 5a).

## Discussion

Ghrelin is an acylated polypeptide hormone secreted predominantly by the endocrine cells of the stomach [1, 23]. Quite a lot of evidence clearly shows that ghrelin is responsible for regulating many things, such as energy balance, GH release, food intake, G protein-coupled receptors and the GH secretagogue receptor (GHSR) type 1a [16]. Interestingly, ghrelin is also involved in reproduction, by influencing the synthesis and release of reproductive factors by the brain and the pituitary, and by influencing gonadal physiology [12, 13].



As noted above, ghrelin administration stops LH secretion centrally in ovariectomized rats [16]. Furthermore, Fernandez et al. [14] demonstrated that ghrelin is capable of inhibiting LH secretion *in vivo* in prepubertal males and in gonadectomized animals, both male and female, but FSH release was not affected.

A number of growth factors and cytokines secreted by the reproductive tract and the preimplantation embryo exert a paracrine and/or autocrine influence on the rate of embryo development [24]. Both ghrelin and GHS-R mRNAs have been detected in the morula and in further stages of embryo development; in addition, ghrelin protein is produced by the reproductive tract and released into the uterine fluid [25]. Ghrelin levels in uterine fluid were strikingly augmented over a fasting period in mice, and ghrelin stopped the development of preimplantation murine embryos *in vitro* [26]. Aghajanova et al. demonstrated that ghrelin and GHSR expression is less intense in the mid-secretory endometrium of infertile women than in fertile controls [27]. Furthermore, it was demonstrated that in rats, chronic ghrelin treatment throughout the first half of pregnancy decreases litter size [28].

Ghrelin also negatively modulates cell viability and proliferation [11]. While GHS-R1a mRNA is detected in the endometrium during the normal menstrual cycle, ghrelin expression is observed in the secretory endometrium, particularly in luminal and glandular epithelial cells. Since specific products secreted by these cells play an important role in embryo implantation, these data indicate a role for ghrelin as a probable paracrine/autocrine regulator of human endometrial receptivity. In this study, ghrelin expression was seen in the surface epithelium, glandular epithelium, stroma and muscle layer in the rat uterus. These findings agree with the distribution patterns and the possible paracrine/autocrine regulator role of ghrelin in the rat uterus that have been reported previously [27].

Analyses using *in situ* hybridization and immunohistochemical methods have shown the localization of the ghrelin in rat and human reproductive tissues. Immunohistochemical and mRNA profiling studies have suggested that rat gonadal ghrelin expression is a function of the estrous cycle, and that its levels increase in the cytoplasm of luteal phase steroidogenic luteal cells [29]. A similar expression pattern has been found in the human ovary, where hilus interstitial cells and young and mature *corpus lutei* were found to be ghrelin-immunoreactive [30]. Ghrelin inhibits estradiol and progesterone secretion from granulosa cells of the human ovary, but this effect was blocked in the presence of a ghrelin receptor antagonist [31].

Sex steroid hormones in tissues can be targeted to analyze various functions in OVX animals. Murray et al. showed that OVX reduces the cell heights of the luminal and glandular epithelium in the sheep uterus. They also demonstrated that these changes are ameliorated by the co-administration of estrogen and progesterone after OVX [32]. Furthermore, the current authors investigated both structural changes and ghrelin expression in the rat uterus following OVX. The present study has demonstrated that an OVX decreases the cell heights of the surface and glandular epithelium, the connective tissue and the number of glandula in the lamina propria. However, these changes were not observed when estrogen replacement therapy was administered. Ghrelin expression was seen in the endometrium rather than in other parts of the uterus. A decrease in ghrelin expression was observed over time following the OVX. However, in the group of rats given supplemental estrogen administration after the OVX, ghrelin expression was found to be the same as the control group. The reduction in ghrelin expression following the OVX is likely to be a result of the loss of ovarian steroids after the ovariectomy. Tanaka et al. investigated the presence of ghrelin mRNA, its receptor mRNA and ghrelin peptide in the normal menstrual cycle and during early pregnancy. They reported that ghrelin expression increased during the interval between the proliferative phase and the secretory phase, and increased dramatically in the decidualized endometrium [5]. The decrease in ghrelin expression in the endometrium after the OVX in the current study is compatible with the findings reported in Tanaka's study. Gualillo et al. reported that no specific changes in ghrelin expression were found in the stomach of adult rats after OVX [33]. On the other hand, Matsubara et al. [34] used immunohistochemistry to examine the numbers of ghrelin-positive cells in OVX rat stomach. They showed the number of ghrelin-positive cells was increased at day 3 after OVX in rats. They also showed that this increase in the number of ghrelin cells caused by OVX was reversed by the administration of 17 $\beta$ -estradiol. Their study suggested that up-regulation of ghrelin expression was increased by a decline in the estrogen level in the stomach. Zhang et al. [35] demonstrated that OVX decreased uterus weights and increased serum ghrelin levels in ovariectomized rats compared to a control group. The increase in serum ghrelin levels after OVX in the study by Zhang et al. might have been due to the increase of ghrelin-positive cells in the stomach, as shown by Matsubara et al. However, in the present study ghrelin production in the uterine tissue after OVX was assessed directly, and decreased expression of ghrelin in the

endometrium was noted. This diversity suggests that the mechanism by which ghrelin expression is regulated is based on several factors including estrogen, age and tissue. The direct effect of the ovaries on the uterus might be responsible of the decrease of ghrelin expression in the endometrium.

In conclusion, the present study shows that (a) morphological changes were observed in the rat uterus after OVX; (b) ghrelin expression was

distributed throughout the endometrium; and (c) ghrelin expression was reduced in the endometrium following OVX, probably due to the decrease in the estrogen level. Overall, these data reinforce the concept that ghrelin may have a paracrine/autocrine regulatory role in the endometrium. However, further study is warranted to determine the factors involved in the regulation of the ghrelin expression.

**Acknowledgements.** The authors wish to thank Dr. Eser Kılıç, Dr.Cagrı Sakalar and Dr. Metin Aytekin for technical support.

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Conflict of interest: None declared

Received: 6.11.2012

Revised: 25.02.2013

Accepted: 9.06.2014