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Original Research Article

The determination of Ghrelin immunoreactivity in gastric mucosa of fundus during pregnancy in mice

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Abstract

Ghrelin, called as appetite or hunger hormone, is mainly produced by gastric glands, and it stimulates appetite. Because of increasing food intake during pregnancy due to augmented metabolic demands, the gastrointestinal tract problems have more serious clinical importance in this period. The aim of this study was to determine the effects of pregnancy on ghrelin immunoreactivity in the gastric mucosa of fundus in mice. For this purpose a total of 20 Swiss albino female mice at 12–14 weeks of age were used. The tissue samples were collected on days 3 (early-E), 10 (mid-M), and 17 (late-L) of pregnancy. Tissue samples were taken from the great curvature of stomach. Ghrelin was demonstrated immunohistochemically using peroxidase-labelled streptavidin biotin complex method. H-score was used for semi quantitatively analysis of ghrelin-positive cells. Although the ghrelin-immunoreactive cells were scattered throughout the fundic glands, it was observed that they mainly located at the lower portion of these glands. Immunoreactivity was diffuse pattern in the cell cytoplasm. The ghrelin positive cells increased during pregnancy compared to non-pregnant control animals while the mean H-score, reflects the number and intensity of immunoreactive cells, was found the most higher at early and mid- gestational periods (C: 321,50±8,35; E: 419,20±11,24; M: 423,50±15,12; L: 378,05±17,07, $p<0,05$). It was concluded that these findings obtained from this study may suggested a new explanation for the relation to between the pregnancy and gastrointestinal disturbances.

Keywords: Ghrelin immunohistochemistry, fundic gland, H-score, pregnant mouse.

1. Introduction

Ghrelin, known as “hunger" or "appetite" hormone, is an endogenous ligand for the growth hormone secretagogue receptors. It was found that ghrelin not only induces growth hormone (GH) release [1-3] but also stimulates appetite, feeding and body weight gain independent of GH via affecting on the hypothalamic arcuate nucleus, known as the control centre of food intake. Thus, ghrelin has important role in the regulation of food consumption [4-6]. It was shown that the circulating ghrelin levels elevated before meal and fell to the lowest level within 1 h after eating in humans [7].

Ghrelin producing cells are endocrine cells of the gastro-intestinal tract especially located in the gastric gland cells [2, 3, 8]. The circulating ghrelin is mainly derived from gastro-intestinal tract, although the studies performed by using immunohistochemistry have shown that the ghrelin positive cells widely demonstrated in stomach, liver, kidney, pituitary gland, hypothalamus, pancreas, placenta [4]. It is also claimed that an important source of ghrelin is the fundus than in the other gastric region [6]. Langer *et al* [9] reported that the resection of gastric fundus by sleeve gastrectomy caused to decrease the plasma ghrelin levels following surgery.

Pregnancy may trigger some physiological changes involved an increase food intake and accumulation of fat in adipocytes [10]. Increasing appetite and food

intake are very important for the increased metabolic demands during pregnancy and lactation period. It was thought that plasma ghrelin concentration may involve in regulation of food intake, energy expenditure, and fetal development during pregnancy [4]. Szczepankiewicz *et al* [11] have demonstrated that serum ghrelin and growth hormone levels increased during pregnancy. Similarly, Palik *et al* [12] demonstrated that serum acylated ghrelin levels were significantly increased around the mid-gestation. However Makino *et al* [1] observed decreased ghrelin plasma concentration in normal pregnant women at the third trimester compared to non-pregnant and post-partum women.

The results of studies about ghrelin levels during pregnancy are controversial. The reasons of the contradictious results may related to different protocols applied such as measurement of the total [11] or acylated ghrelin [12] in serum, or deacyl ghrelin in plasma [10]. There are limited studies about the effects of pregnancy on ghrelin producing cells in fundic mucosa. The aim of this study was to determine the effects of pregnancy on ghrelin immunoreactivity in the gastric mucosa of fundus in pregnant mice.

2. Materials and methods

2.1. Ethical approval

All experimental procedures were performed after approving by the Ethical Committee of S.U. Medicine Faculty, Konya, Turkey.

2.2. Animal materials

For this purpose a total of 20 Swiss albino female mice at 12–14 weeks of age were used. Mice kept under optimal and controlled light/dark conditions and allowed food and water *ad-libitum*. After five animals separated as non-pregnant mice, the other females were allowed to mate with male mice. Mating female mice checked daily for presence of vaginal plug. In addition of the vaginal plug, the detection of sperm in vaginal smear at the next morning, female was considered to be day 0 of pregnancy.

2.3. Tissue samples

The tissue samples were collected from pregnant mice on days 3 (early-E), 10 (mid-M), and 17 (late-L) of pregnancy whereas non-pregnant female mice were also sacrified at the end of the study. All samples were taken from the great curvature of stomach. The samples were fixed in 10% neutral buffered formalin for immunohistochemistry applications for 24 hour at room temperature. After fixation process, the samples were dehydrated, cleared, and embedded in paraffin. Ghrelin was demonstrated immunohistochemically using peroxidase-labelled streptavidin biotin complex method [13, 14].

Semi quantitative analyses of ghrelin immunoreactivity was performed according to Godbole *et al* [15] using the H-score method which is based on intensity of staining. Briefly, randomly selected three sections from each animals were evaluated and scored for the staining intensity (1, 2, 3, and 4) corresponding the presence of weak, intermediate, strong, and very strong, respectively. H-score was calculated for each animal by adding the numbers generated by using the formula below;

$$\Sigma\ (1+i) \times pi$$

1: Constant value;
i: Intensity score;
pi: Percentage of intensity

3. Results

Ghrelin cells were characterised by round shaped. Although the ghrelin-immunoreactive cells were scattered throughout the fundic glands, it was observed that they mainly located at the lower portion of these glands. Immunoreactivity was diffuse pattern in the cell cytoplasm. The ghrelin positive cells increased during pregnancy compared to non-pregnant control animals while the mean H-score was found the most higher at early and mid- gestational periods (Figure 1, Table 1, $p<0,05$).

Table 1: H-scores of ghrelin immunreactivity in fundic glands during pregnancy

	Non-pregnant control	Early Pregnancy (Day 3)	Mid Pregnancy (Day 10)	Late Pregnancy (Day 17)
H-score (mean±SE)	321.50 ± 8.35^{c}	419.20 ± 11.24^{ab}	423.50 ± 15.12^{a}	378.05 ± 17.07^{b}

[a-c:] Values within the same line with no common superscripts are significantly ($P<0.05$) different.

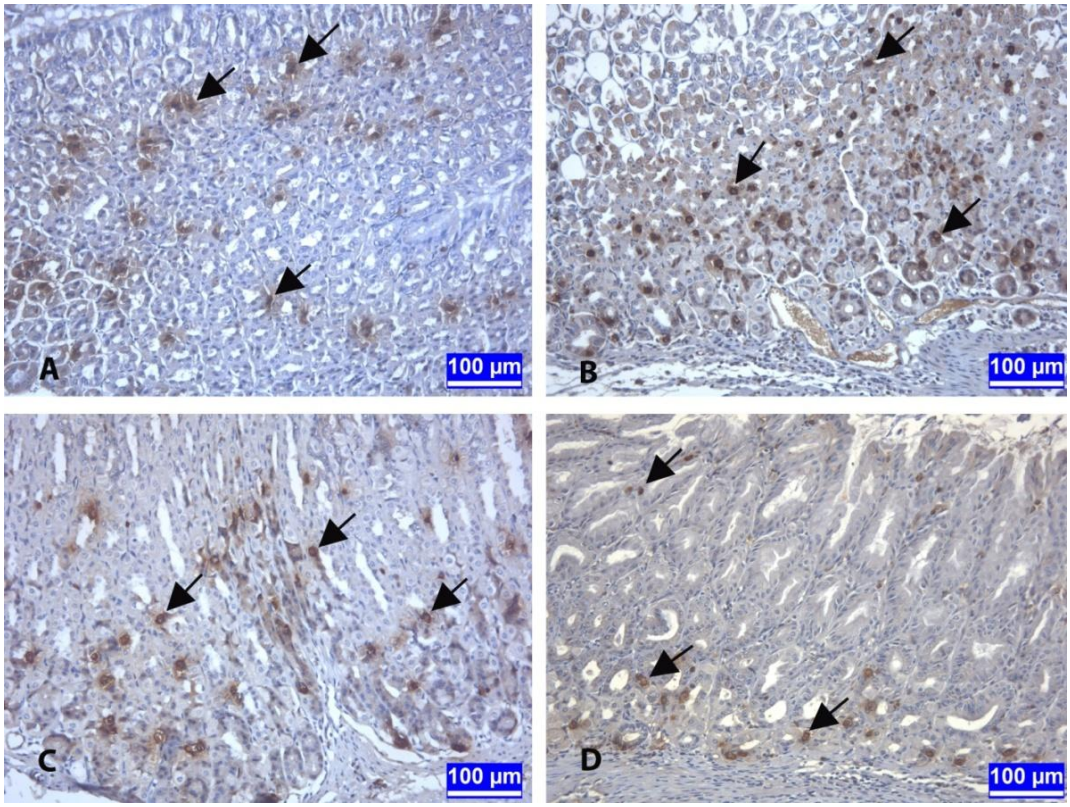

Figure 1: Ghrelin immunopositive cells (arrows) in mouse stomach. Ghrelin cells are scattered throughout the fundic glands.
A. Non-pregnant control, B. Early, C. Mid-, and D. Late gestational stage. Ghrelin Iimunohistochemical staining.

4. Discussion

Ghrelin acts as short-term appetite inducer before diet intake and it also has important function for GH release, energy balance, food intake control, and the regulation of metabolic functions in vertebrates [5,16]. Although Rindi *et al*[17] demonstrated ghrelin cells in developing human fetal tissues and ghrelin producing cells are located in liver, kidney, pituitary gland, pancreas, placenta, hypothalamus, and stomach, it was claimed that the ghrelin in blood stream is mainly arised from by enteroendocrine cells in the mammalian gastric mucosa, especially in gastric fundus [18,19].

It is thought that gastric ghrelin has important multiple physiological functions and the functions of ghrelin cells are strickly controlled by many complicated mechanisms. In gastric mucosa, there is a close relation between the parietal cells and ghrelin producing cells [20]. Penkova & Atanassova [21] have demonstrated the ghrelin receptors in the epithelial cells of gastric glands and Lieberkuhn crypts of the duodenum in human. The investigators [21] claimed that ghrelin has paracrine effect on the gastro-intestinal cells. Besides, Date *et al*[19] have shown that the gastric acid secretion was increased by intracerebroventricular injection of ghrelin and they claimed that the ghrelin was the part of the central regulation of the gastric acid secretion by activating the vagus system.

Embryonic growth and fetal development is strongly affected by the maternal diet composition and nutritional consumption [22]. Decreases in the food consumption by pregnant mother during pregnancy cause to decrease the size of their offsprings [4]. It is also claimed that the plasma total ghrelin level was positively correlated with fetal development during pregnancy [22]. Słupecka *et al*[16] demonstrated that the maternal diet during pregnancy and lactation influences ghrelin level in both dams and their offspring. Nakahara *et al*[4] demonstrated that the body weight of the neonate born from mother treated with ghrelin during pregnancy were significantly greater than offsprings those of the ghrelin non-treated mothers.

The increasing protein demand may stimulate ghrelin production during the pregnancy. Erdmann *et al*[23] reported that the ghrelin production was induced by dietary protein but fat and carbohydrate intake caused reducing ghrelin production. Gao *et al*[24] reported that daily food intake was greater in pregnant rats fed a low protein diet than control group in early pregnancy. Moreover, the same investigators [24] found that the plasma ghrelin levels were increased in late pregnant animals fed low-protein diet. Fuglsang *et al*[25] observed the highest serum ghrelin

levels in the second trimester whereas the levels decreased at their lowest level in the third trimester in pregnant women.

However Shibata *et al*[26] reported that there was no significant change in ghrelin levels in the rat stomach during pregnancy. Besides although they pointed out that appetite increased gradually up to day 20 of pregnancy, it was emphasized the plasma ghrelin level was not directly related to the increased food intake during pregnancy. In the present study, although it was observed that H-score, reflects the number and intensity of immunoreactive cells, was modestly decreased at the late gestational period, the ghrelin immunopositive cells increased throughout the pregnancy compared to non-pregnant control animals while the mean H-score was found the most highest at early and mid- pregnancy (Table 1, $p<0,05$).

Because of increasing food intake during pregnancy and lactation period, some certain metabolic diseases and gastrointestinal tract problems have more serious clinical importance in these periods [27]. Plasma ghrelin levels, the number of ghrelin producing cells, and ghrelin mRNA expression levels have been studied in various gastric diseases [20].

It was thought that some certain diseases affecting the gastric mucosa like that *H. pylori* infection, chronic gastritis, gastric ulcer, gastric carcinoma, might effect on ghrelin production [28]. These problems may cause decreasing fetal weights via the lower absorbs ion of nutrients including protein, lipid, and carbonhydrate.

5. Conclusion

In conclusion, our study indicates that the staining intensity of ghrelin immunureactive cells in fundic glands are influenced by the pregnancy. These findings presented in here would help establish for understanding the physiological functions of ghrelin and its relation to some certain gastro-intestinal diseases during pregnancy. In addition, the presence of ghrelin producing cells and its receptors in gastro-intestinal tract may be clinical importance for a new therapeutic approach during pregnancy.

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