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GHRELIN ENDOCRINE CELLS IN THE HUMAN STOMACH DURING PRENATAL AND EARLY POSTNATAL DEVELOPMENT

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Abstract - The aim of this study was to investigate the appearance, localization and density of ghrelin cells in the human stomach during prenatal development. For this purpose the antrum and corpus of embryos, fetuses and infants are stained immunohistochemically by the streptavidin-biotin technique. The presence of P/D1 cells at 11 weeks of fetal development, their highest density during the first detection and higher density in the corpus than in antrum, and their localization in the glandular base of stomach gland, all suggest that ghrelin plays a major role in the early stages of the developing stomach.

Keywords: Human stomach, prenatal development, ghrelin endocrine cells, localization of ghrelin cells, density of ghrelin endocrine cells

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INTRODUCTION

Ghrelin is an acylated peptide produced predominantly by P/D1 stomach endocrine cells in the human fetus and adult (Kotunia and Zabielski, 2006). This peptide plays important roles in growth hormone release and appetite stimulation. Moreover, ghrelin exerts a control over gastric acid secretion and motility and provides a gastro protective action (Kotunia and Zabielski, 2006). In human fetuses and infants, ghrelin-IR cells were present throughout the gastrointestinal tract, most frequently in the corpus mucosa of the stomach, and rarely in the antrum, duodenum and cardia (Rindi et al., 2004, Kotunia and Zabielski, 2006). Ghrelin endocrine cells were identified very early during human fetal life from 10 weeks in the stomach, duodenum and pancreas (Rindi et al., 2002). In corpus and antrum stomach mucosa the ghrelin cells were observed mostly in the glandular base and a small number of cells were observed in the glandular neck: on the other hand, in the duodenum they were detected in the epithelia of crypts and villi (Gronberg et al., 2008). Only in the corpus mucosa are ghrelin cells small, round shaped, represented as closed type cells. In all other parts of the gastrointestinal tract they are represented by so-called open-type cells that are elongated, bottle–shaped, with characteristic apical cytoplasmic processes that are in contact with the lumen (Sakata et al., 2002). According to ultrastructural and light microscopy investigations, ghrelin cells in the human corpus mucosa represent a separate cell type from other functionally characterized endocrine cells such as somatostatin (D), serotonin (EC) or histamine enterochromaffin-like (ECL) cells, and in the mouse stomach, ghrelin cells colocalize with serotonin and somatostatin (Rindi et al., 2002). The results of a previous study sug-

gested that ghrelin-IR cells are most abundant in the corpus gland, while in the antrum and duodenum gland they are infrequent (rare). According to Rindi et al. (2002), the ghrelin endocrine cells in the human corpus mucosa account for 20-30 % of the entire endocrine cell population.

The aim of this study was to investigate the first appearance, distribution and density of ghrelin cells in the human stomach during prenatal and infant development.

MATERIAL AND METHODS

The tissue samples, complete wall of antrum and corpus from 38 fetuses, 2 embryos and 3 infants of different ages and both sexes (Table 1) were collected from legal abortions, premature deliveries and immediate postnatal deaths, according to the principles of the Ethical Committee. The ages of embryos were determined by measuring crown - rump length by the method of Carnegie (O'Rahilly et al., 1981), while the fetal age was determined by measuring the length of the foot, given that this measure correlates well with the gestation period. The examined samples of fetal origin were divided into three groups based on affiliation to a particular trimester of pregnancy (the first, the second and the third trimester).

Table 1. Embryos, infants and fetuses classified according to trimester of pregnancy

The examined samples	trimester of pregnancy	Number
Embryos (n=2)		2
Fetuses (n=38)	I trimester	3
	II trimester	24
	III trimester	11
Infants (n=3)		3
Σ		43

Stomach tissue specimens from each embryo and fetus were fixed in 10% buffered formalin and paraffin-embedded. The study was approved by the ethics committee of the Faculty of Medicine of University of Niš. Stomach wall morphology was normal in all cases which was confirmed by routine histopathological examination of hematoxylin- and eosin-stained sections.

After deparaffinization in xylene, followed by dehydration with a descending series of alcohol and rehydration in distilled water, the corpus and antrum sections (5 μm thick) were heated in a microwave oven (at 680 W, in 10 mmol/L citrate buffer pH 6.0, for 20 min) for epitope retrieval.

Tissue sections were treated with 0.3% H_2O_2 solution in PBS to block endogenous peroxidase activity. The sections were then incubated with appropriately diluted primary antisera against ghrelin (polyclonal goat antibody, Santa Cruz Biotechnology, USA, sc-10368, dilution 1:100), and chromogranin A (monoclonal mouse antibody, DAKO A/S Denmark, M 0869, dilution 1:50) in a humidity chamber for 60 min at room temperature. Immunostaining was performed using the streptavidin-biotin technique (LSAB+/HRP Kit, Peroxidase Labeling, K0690, DAKO Cytomation, Denmark). The immunoreactivity complex was visualized with DAKO Liquid DAB+ Substrate/ Chromogen System (Code No. K3468), and counterstained with Mayer's hematoxylin (Merck). The corpus and antrum sections in which the primary antibody had been omitted were used as staining control.

Statistical analysis

Chromogranin A- and ghrelin-IR cells were counted using a computer-supported imaging system connected to a light microscope (Olympus AX70) with an objective magnification of ×10. The number of CgA-IR and P/D1-IR cells was expressed per mm^2 of epithelium. The results were expressed as mean ± SD. Values were compared by nonparametric Mann-Whitney U test using the program SPSS 10 for Windows. Differences of $p<0.05$ were accepted as the level of significance.

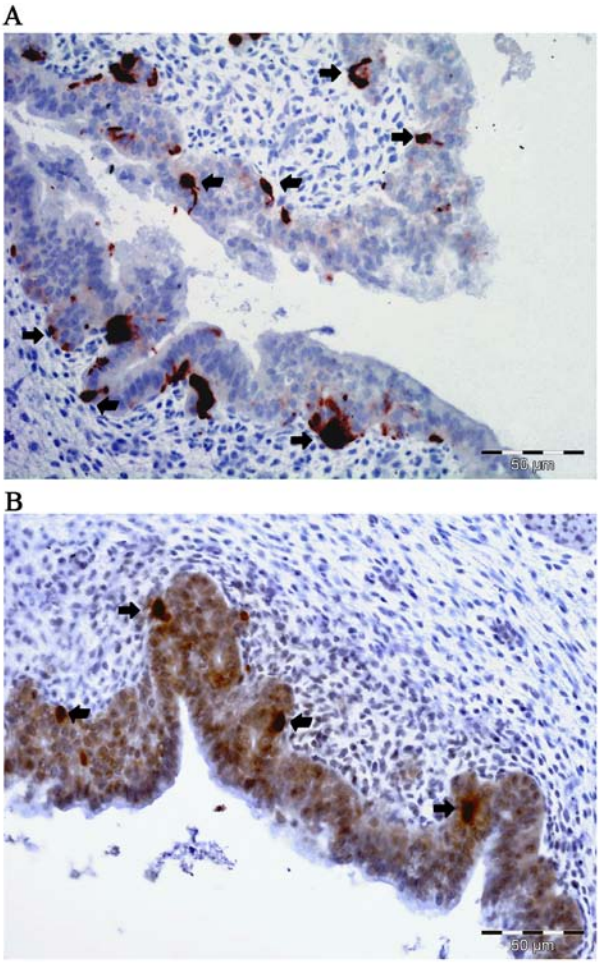

Figure 1. Chromogranin A and ghrelin-IR endocrine cells in the corpus mucosa of a 12-week-old fetus. Chromogranin A-IR cells either isolated or in small clusters in a developing corpus of the stomach (A). Ghrelin immunoreactive cells grouped in small clusters in a developing corpus (B). → - immunoreactive cells.

RESULTS

During the embryonic period of human development we did not find the existence of chromogranin A (CgA) or ghrelin (P/D1) immunoreactive (IR) endocrine cells in the human stomach. In the antrum and corpus stomach, CgA immunoreactivity, which served as a common marker of all endocrine cells, was revealed in 10th week of gestation, while P/D1-IR endocrine cells were found during the 11th week of fetal development.

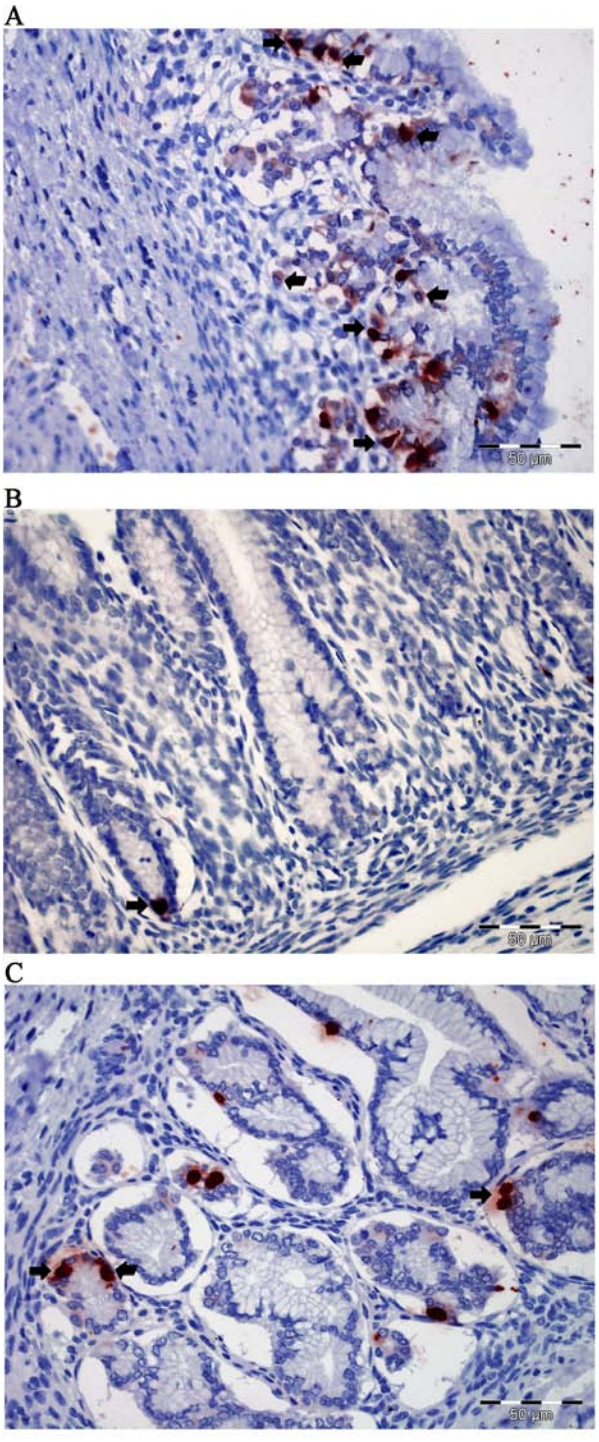

Figure 2. Ghrelin endocrine cells in the corpus (A), antrum (B) and cardia (C) of the human fetus during the second trimester of gestation (24/25 gestational week). A relatively higher number of ghrelin cells was found in the corpus than in the antrum. → - immunoreactive cells.

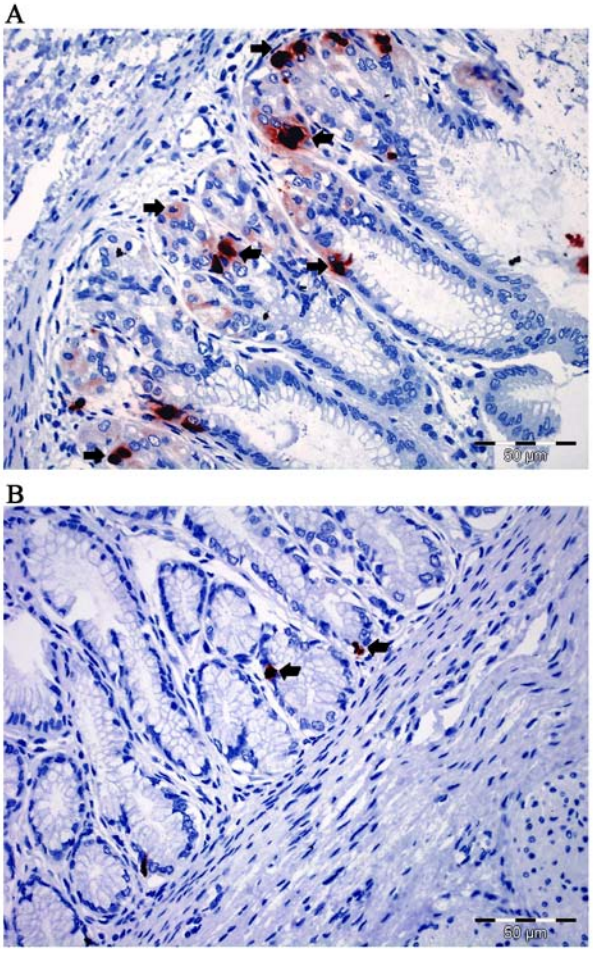

Figure 3. Ghrelin endocrine cells in corpus (A) and antrum (B) of human fetal stomach during the third trimester of gestation (27/28 gestational week). In the antral mucosa ghrelin-IR cells were present in much smaller numbers than in the corpus mucosa. → - immunoreactive cells.

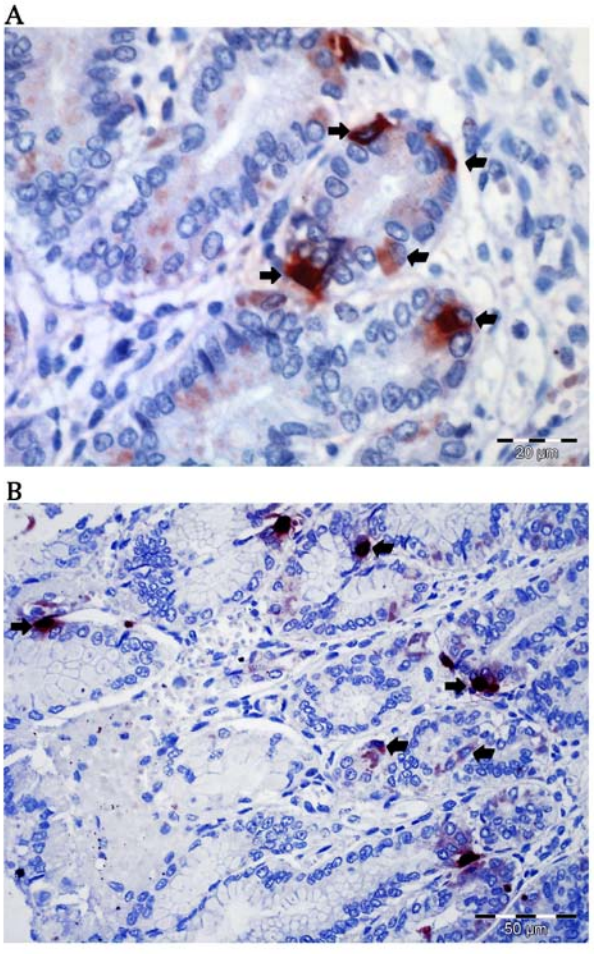

Figure 4. Ghrelin endocrine cells in corpus (A) and antrum (B) of human stomach infants two weeks of age. Note that ghrelin-IR cells in the corpus glands are located in the base and body of the corpus gland (A), while in antral gland ghrelin-IR cells are located in the deeper parts of the antral mucosa (B). → - immunoreactive cells.

During this period, CgA (Fig. 1A) and ghrelin (Fig. 1B) cells were larger than the surrounding non-endocrine cells in the stomach mucosa and grouped in small clusters of 3 - 4, closely located cells. During the first trimester of gestation development, most of the ghrelin cells were present in the glandular base and body of the stomach, while in the glandular neck ghrelin-IR cells were rare (Fig.1B).

During the second trimester of gestation, P/D1-IR cells were mainly observed as individual cells (Fig. 2A, B), localized in the basal and central parts of the stomach gland. In the same period of prenatal development, ghrelin-IR cells in the basal part of the cardiac gland were also detected (Fig. 2C). During the third trimester of gestation, the distribution and density of P/D1-IR cells in the corpus (Fig. 3A) and antrum (Fig. 3B) were the same as in the second trimester.

In infants, the ghrelin cells in the corpus (Fig. 4A) were relatively large compared to the surrounding epithelial cells, and were mostly round or irregularly circular in shape, while in the antrum (Fig. 4B)

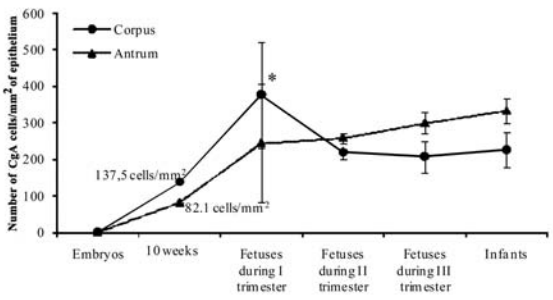

Figure 5. The relation between the number of chromogranin A cells in the corpus and antrum of the human stomach during embryonic, fetal periods of development, as well as in infants. The results are present as mean ± SD; p<0.05 the first vs. second and third trimester.

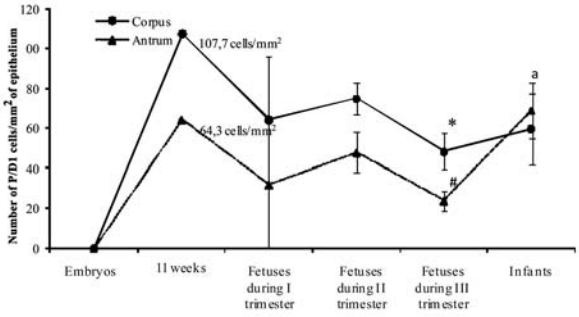

Figure 6. Relation of the ghrelin cell number between the corpus and antrum of human stomach during embryonic, fetal periods of development, as well as in infants. The results are presented as mean ± SD; corpus: *p<0.05 the second vs. third trimester; antrum: #p<0.05 the second vs. third trimester; [a]p<0.05 the third trimester and postnatal life.

they were irregular, round or triangular shaped, with pronounced connections with the lumen.

Morphometric analysis

Number of Chromogranin A-IR cells: The number of CgA positive cells in the corpus of the fetal stomach at 10 weeks of development amounted to 137.5/mm^2 mucosal surface epithelium. After that, their number increased (Fig. 5) and during the first trimester the number of CgA-IR cells reached a maximal value (377.0 cells/mm^2 mucosal surface epithelium). In the second and third trimester, the number of CgA-IR cells was significantly lower($p<0.05$) than in the first trimester, but higher than at 10 weeks of gestation (Fig. 5). The slight increase in density of endocrine cells immediately after birth does not reach the level achieved in the first trimester of gestation (Fig. 5).

The number of CgA-IR cells in the antrum of the fetal stomach at 10 weeks of gestation was 82.1 cells/mm^2 mucosal surface epithelium. The number of CgA-IR cells gradually increased during the prenatal period of development and in early postnatal period (Fig. 5).

The number of cells in the corpus and antrum during fetal development and early postnatal life shows an inverse proportionality. In fact, the number of CgA-IR cells in the corpus of the stomach was higher in the first trimester of fetal development, while in the second and third trimester, as well as in postnatal period, their number was smaller than the values obtained in the antrum (Fig. 5).

Number of Ghrelin-IR cells: The number of ghrelin-IR cells observed in the 11th week of fetal development in the corpus stomach (107.7 cells/mm^2 mucosal epithelium; Fig. 6) gradually decreased and reached the lowest value during the third trimester ($p<0.05$ the second vs. third trimester; Fig. 6). Thereafter, a slight increase of P/D1-IR cell numbers was detected in the early postnatal life.

In the antrum stomach at 11th week of gestation, the number of ghrelin-IR cells was 64.3 cells/mm^2. During fetal development the changes in the number of P/D1-IR cells shows a similar pattern as in the corpus. However, the increase in the number of these cells in the antrum during early postnatal life was statistically significant ($p<0.05$; Fig. 6).

Table 2 shows that the number of CgA-IR cells, at the time of first detection, was higher in the corpus than in the antrum. However, during fetal development, their number decreased in the corpus while in the antrum it increased, so that in the in-

Table 2. The number of chromogranin-A and ghrelin cell number in the corpus and antrum of stomach during embryonic, fetal and infants periods of human development

		Corpus	Antrum
HgA-IR cells			
Embryos		0 (n=2)	0 (n=2)
Fetuses			
	I trimester	377,0 ± 146,7 (n=3)	244,6 ± 162,5 (n=2)
	II trimester	219,2 ± 18,5 (n=22)	259,3 ± 13,1 (n=16)
	III trimester	207,7 ± 44,3 (n=8)	298,9 ± 29,5 (n=8)
Infants		226,0 ± 47,8 (n=3)	333,8 ± 33,3 (n=3)
Ghrelin-IR cells			
Embryos		0 (n=2)	0 (n=2)
Fetuses			
	I trimester	64,4 ± 32,8 (n=3)	32,1 ± 32,1 (n=2)
	II trimester	74,9 ± 8,4 (n=22)	47,9 ± 10,0** (n=16)
	III trimester	48,4 ± 9,7 (n=8)	23,9 ± 4,0 (n=8)
Infants		59,7 ± 18,2 (n=3)	69,1 ± 13,8 (n=3)

The results are presented as number of immunoreactive cells/mm^2 of epithelium ± SD; ** $p<0,05$ corpus vs. antrum.

fants the number of CgA-IR cells was higher in the antrum compared to the corpus. The number of P/D1-IR cells during fetal development was higher in the corpus than in the antrum, but their number in the group of infants was the approximate.

DISCUSSION

We have detected ghrelin cells for the first time in the antrum and corpus stomach mucosa in the 11th week of fetal development. After this point, the number of P/D1 cells gradually declines until the third trimester of fetal development. In infants their number is increased. The number of ghrelin cells in the corpus was higher than in the antrum during prenatal human development; however, the number of positive cells in the antrum and corpus of the infants was approximate. Ghrelin cells were also observed in the cardia during the second trimester of fetal development.

The absence of CgA-IR (a pan-neuroendocrine marker) and P/D1-IR cells during the embryonic period of development, presented in this paper, can be explained by the absence of these cells in the stomach or due to insufficient sensitivity of the method used to detect the low expression of the CgA and during this developmental period. Chromogranin A-positive cells in the corpus and antrum mucosa were detected during the early fetal period (10 weeks), whereas ghrelin-IR cells were detected one week later, unlike Rindi et al. (2002), who detected ghrelin-IR cells in the corpus during 10 weeks of human development. It was reported that ghrelin-IR cells in rats are expressed in the fetal stomach from pregnancy day 18 (Hayashida et al., 2002). According to Yabuki et al. (2004), X cells in the dog, A-like or X cells in the rat, and P/D1 cells in humans synthesize ghrelin. The presence of P/D1 cells in the cardia during middle fetal development is in line with the results of Grönberg et al. (2008).

During fetal and neonatal human development, the ghrelin-IR cells are small and round-shaped, located in the glandular base and body as small clusters of 3 – 4 cells during the first trimester, and are mainly present as individual cells. Hayashida et al. (2002) has reported that ghrelin-IR cells in rat fetal and neonatal stomach are round–shaped, with compact electron-dense granules, which indicates that the secretion of ghrelin from the fetal stomach is initiated during late pregnancy. Rindi et al. (2002) proved that secretory granules in ghrelin cells in the rat synthesize and store exclusively ghrelin, while in mice the

colocalization of ghrelin with serotonin and somatostatin in the same endocrine cells was confirmed. The colocalization of ghrelin and VMAT2 (Vesicular Monoamine Transporter 2) in the secretory granule of ghrelin cells was also reported (Rindi et al., 2004).

During fetal and early postnatal development, P/D1 cells represent 10-20% of the whole neuroendocrine cell population in the antrum, and 20-30% in the corpus, which is in accordance with the results of Date et al. (2000). The number of ghrelin-IR cells per mm^2 of corpus epithelium compared to the antrum during the prenatal period of human development, was twice as high in, except in the group of infants where the number of P/D1 cells was approximated. Moreover, Tanaka-Shintani and Watanabe (2005) showed that the number of ghrelin-IR cells per mm^2 in adult healthy humans was ten times higher in the epithelium of the corpus than in the antrum (60 cells/mm^2 in corpus; 7 cells/mm^2 in antrum). And almost identical numbers of ghrelin-IR cells were found in the corpus mucosa of adult rats (60 ± 10/mm^2 mucosal surfaces). Their number in the duodenum was ten times smaller. The number of ghrelin-IR cells in hamsters was significantly lower than in rat and mouse stomachs (110±20 cell/mm^2 compared to 50±10cell/ mm^2 of epithelium) (Yabuki et al., 2004). The density of ghrelin-IR cells in rat stomach during the postnatal period of development linearly increased and was higher in females than in males (Sakata et al., 2002).

In human and rat infants ghrelin cell density remains low until the weaning period when it is greatly expands. Fak et al. (2007) showed that delayed weaning prevents the normal rise in both corpus ghrelin expression as well as ghrelin plasma concentration. Ghrelin cell density, mRNA expression and plasma concentration were significantly lower in the group of rats with delayed weaning compared to the control group, where the mechanism underlying those findings could be related to the different nutrient compositions of milk and solid food. In human plasma the ghrelin concentration significantly increased after birth, reaching its maximum during the first two years of life, and then declines to the end of puberty. Ghrelin level demonstrates a negative correlation with the anthropometric parameters in healthy infants born at term and postnatally (Fak et al., 2007).

Based on the presented findings, we conclude that the very early appearance of ghrelin cells in the gastroenteropancreatic axis, and maintenance of a significant density of these cells during the entire period of prenatal development suggest that ghrelin hormones are involved in the local control of growth, differentiation and functioning of cell tissue in which they are localized.

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Abbreviations

IR – immunoreactive; P/D1 – ghrelin endocrine cells; CgA – chromogranin A; VMAT2 - Vesicular Monoamine Transporter 2; ECL - enterochromaffin-like; EC – serotonin endocrine cells; D – somatostatin endocrine cells.

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