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Ghrelin-Producing Cells and Ghrelin Receptor GHSR-1 in the Small Intestine of Human Embryos 6th Gestation Week and the Role of Ghrelin in Gastrointestinal Tract Differentiation

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Abstract: *Cell and tissue differentiation in the developing gastrointestinal tract during the embryonic period is carried out through complex intercellular signaling. Common biological signaling pathways between the differentiating tissues such as BMP, Hedgehog, PDGF, TGF-β, and Wnt signaling have been ascertained. The participation of enteroendocrine cells and their hormones in these processes is only hypothetical. The studies on enteroendocrine cells in the prenatal period is related mainly to the time of their occurrence. Links between hormonal production of enteroendocrine cells and tissue differentiation, which in adults express receptors for their hormones, have not been traced.*

Aim: *The aim of the present study is to ascertain the occurrence of ghrelin-producing cells in the early embryonic period of human and to study their relation to the other cells in the developing small intestine through the presence of ghrelin receptors GHSR-1 in them.*

Material and methods: *Fragments of small intestine of 6th- gestation-week human embryos are studied through transmission electron microscopy (TEM) and immunihistochemical reactions by the ABC method for ghrelin and ghrelin receptor GHSR-1.*

Results: *During 6th gestation week in the small intestine of human embryos we ascertain the presence of enteroendocrine cells. Ultrastructural characteristic reveals that they are of G-, D-, EC1- and EC2-type. Immunohistochemically we ascertained the presence of single ghrelin-producing cells among the crypt epithelium. In these areas the expression of ghrelin receptor GHSR-1 has been localised.*

Conclusion: *During 6th gestation week, in the small intestines of human embryos there is presence of highly differentiated enteroendocrine cells including ghrelin–producing ones. In the early embryonic period, when digestion is lacking, in the developing small intestine there is personal production of gastrin, somatostatin, serotonin and ghrelin. Through its receptors ghrelin can perform the function of an ontogenetic factor and to influence directly cell proliferation and differentiation in the maturation of the gastrointestinal tract.*

Key words: *human embryos, gastrointestinal tract, enteroendcrine cells, ghrelin, GHSR-1*

I. Introduction

The differentiation of cells and tissues in the developing gastrointestinal tract during the embryonic period occurs through complex intercellular signaling. Common biological pathways between the differentiating endoblast and mesenchyme derivatives such as BMP, Hedgehog, PDGF, TGF-β, and Wnt signaling have been ascertained (Mc Lin et al., 2009; Spence et al., 2011). For example, through the processes of Hedgehog and PDGF signaling the intestinal endoderm influences the surrounding mesenchyme and regulates the differentiation of mesenchyme myofibroblasts in smooth muscle cells (Karlsson et al., 2000; Van den Brink et al., 2007).

The participation of enteroendocrine cells and their hormones in these processes is only hypothetical. Most studies on enteroendocrine cells in the developing gastrointestinal tract are related only to the time of their occurrence (Stein et al., 1983; Ivanova et al., 1999; Mitrović et al.,2012). Enteroendocrine cells in human embryos are described in 8th gestation week (Stein et al., 1983). Larsson found that some regions of foetal gastrointestinal tract contain a large amount of enteroendocrine cells while the corresponding sector in the body of an adult lacks these cells. The author describes these cells as 'transient cell populations' and assumes that they play a role in the development and differentiation of the gastrointestinal tract, exerting trophic activity (Larsson, 1980). While studying endoblast-mesenchyme interactions in the developing duodenum of human embryos, a presence of enteroendocrine cells in it also in the 8th gestation week has been found. Due to the presence of secretory granules in them the authors assume participation of endocrine cells in the processes of

differentiation of the duodenal wall (Matsumoto et al., 2002).

Ghrelin is a recently discovered hormone. It is an oligopeptide of 28 aminoacid residues isolated for the first time in a rat stomach. Its presence was announced for the first time by Masayasu Kojima et al. in 1999 (Kojima et al., 1999). Salzet et al. Found that serum levels of ghrelin in thin rats are higher than those in fat ones (Salzet et al., 2003). In humans the stomach is also the main source of ghrelin (Ariyasu et al., 2001; Gnanapavan et al 2002). Numerous studies prove that ghrelin plasma levels increase on an empty stomach and after eating they are restored to normal levels (Tschöp et al., 2000; Asakawa et al., 2001; Toshinai et al., 2001). The basic target of ghrelin is the diencephalon. By connecting with specific receptors in the hypothalamic nuclei, ghrelin participates in the formation of acute feeling of hunger. Through receptors in the adenohypophysis it releases growth hormone secretion. The effects of ghrelin on the metabolic processes are due to its function as a ligand for the secretory receptors of the growth hormone in the adenohypophysis (Takaya et al., 2000).

Despite the numerous studies related to ghrelin there is still a number of issues to consider. Up to now there is no definitive answer to the question which type of enteroendocrine cells produce this hormone. Whether is synthesized in a specific endocrine cell of a new type unknown so far? Whether it is produced by one, but already familiar type of endocrine cell? The possibility that different types of enteroendocrine cells synthesize ghrelin, along with other familiar to them hormones, also exists. Depending on the morphology and hormonal activity of cells, according to some authors it is produced by A type cells (glucagon), according to others- by D-type (somatostatin), G (gastrin), ECL (histamine), (Date et al., 2002; Sakata et al., 2010). Some claim that X-type (A-like cells), with unknown function are ghrelin-producing (Rindi et al., 2002), and according to other authors this hormone is produced by a new, unknown type of cells (Svenson et al., 2002; Yabuki et al., 2004). There are also authors who change their opinion with time. While studying ghrelin secretion from the islets of Langerhans in the pancreas in their previous studies they consider that ghrelin is produced by a separate type of enteroendocrine cell (Wierup al., 2002; Wierup al., 2004). In later studies the same authors report that one and the same pancreatic endocrine cell secretes ghrelin as well as other hormones (Wierup et al., 2013).

A no less debatable question is the one on the occurrence of differentiated ghrelin-producing cells in the developing gastrointestinal tract. In the human prenatal period ghrelin producing cells are found in 11^{th} gestational week. In this period ghrelin-producing cells, called epsilon cells, appear initially as single cells among the other externally secreting cells of the immature pancreas. In the following gestational weeks, epsilon cells form clusters and in 21^{st} gestational week they form ring-like structures around the other endocrine cells of the forming islets of Langerhans (Andralojc et al., 2009). A similar occurrence of ghrelin-producing cells in the human fetus stomach is described also in 11^{th} gestation week. In the stomach as well single cells occur initially which are located in the fundi of the immature gastric glands and after that they increase their number and ascend to the neck of the glands (Mitrović et al., 2012).

Ghrelin carries out its functions through binding with a specific GHS-R1 receptor. Two isoforms of the ghrelin receptor have been synthesized so far: GHS-R1A and GHS-R1B. GHS-R1A is a mature polypeptide of 366 amino acid sequences with 7 transmembrane domains. GHS-R1B is an immature polypeptide with 289 amino acid residues and 5 transmembrane domains (Albarrán-Zeckler, 2013). Studies on ghrelin receptor expression in embryonic organs and tissues are scarce. Ghrelin receptor GHS-R1A and GHS-R1B expression was registered in 11^{th} and 16^{th} gestation week in the stomach and duodenum of human embryos and fetuses (Mitrović et al. 2014).

Having in mind the intensive processes of cell proliferation and differentiation in the early embryonic period, it is important to know whether there is personal ghrelin production in the developing embryo or whether it is transferred in the embryo through the mother's blood. Proving the existence of ghrelin receptors in embryonic tissues would prove the direct participation of ghrelin in their maturation, in the organogenesis and body weight control in the prenatal period.

Aim: The aim of the present study is to ascertain the occurrence of ghrelin-producing cells in the early embryonic period in human and to investigate their relations with the other cells of the developing small intestine through the presence of ghrelin receptors GHSR-1 in them.

II. Material and methods

Fragments of 6^{th} gestation week human embryo small intestine are studied by transmission electron microscopy (TEM) and immunohistochemical reactions for ghrelin and ghrelin receptor GHSR-1. The immunohistochemical analysis (IHC) is by the ABC method through rabbit ABC Staining System (Santa Cruz Biotechnology, USA) with the corresponding primary antibody (Table № 1.). Human embryonic material is prepared through vacuum extraction in artificial discontinuation of regular pregnancy at the Clinic of Obstetrics and Gynecology at UMHAT 'Sv. Georgi'.

Determinatio of gestational week is performed not by anamnestic data from the patients but by morphological characteristics of the fragments from the extremities of the aborted fetuses (fig. 1).

Antibody	Catalogue number	Dilution in PBS	Localization of expression	Granule colour
ghrelin	goat polyclonal ghrelin antibody (C-18): sc-10368 - Santa Cruz Biotechnology USA	1:100	cytoplasmic	Black granules
Ghrelin receptors GHSR-1	goat polyclonal antibody GHSR-1: sc-10351 - Santa Cruz Biotechnology USA	1:100	cytoplasmic	Fine brown granules

Table 1. A list of the primary antibodies used in the immunohistochemical study.

Biopsy material for TEM was fixated in 4% glutaraldehyde, NaPO4 buffer 0,1M, with subsequent postfixation in 4% OsO4 and 0,2M S'collin buffer. It was dehydrated in ascending alcohol series and was included in durcopan. It was warmed in gelatin capsules at 56˚C for 48h. gelatin capsules are removed with warm water and the material is cut into thick slices- – 0,5 mm. Cuts are mounted on glass slides, stained with methylene blue, covered and observed with a light microscope to determine the area of cutting. What follows is the cutting of ultrathin slices with thickness of 300 nm and their mounting on platinum nets. The mounted cuts are contrasted with 5% uranyl acetate, diluted 1:1 with 100% alcohol for 80 min. in dark. They are washed with 50% alcohol and Reinold's solution (water solution of Pb(NO3)2, Na(CoH5O7)2H2O for 20 min. and bid H2O. Observation and microphotography we performed on TEM 'Philips CM 12'.

Biopsy material for the immunohistochemical study we fixated in Buen solution for 24 hours and it included paraffin. Paraffin cuts with thickness of 5μm were deparaffinated and incubated for 30 min. in 2% H2O2 methanol for inactivation of the endogenous peroxidase.

The primary antibody for ghrelin (goat polyclonal ghrelin antibody: sc-10368 - Santa Cruz Biotechnology USA) is diluted in PBS in 1:100 ratio. The primary antibody for ghrelin receptor GHSR-1 (goat polyclonal antibody GHSR-1: sc-10351 - Santa Cruz Biotechnology USA), is diluted in PBS in 1:100 ratio.

The incubation of the cuts with the corresponding antibody is performed at 4◦C for 12 hours in damp camera. What follows is an incubation with the biotinylated secondary antibody for 30 min. in ABC complex for 15 min. and visualization with DAB chromogen. Deparaffinated and included in Vecta mount cuts are observed under microscope and photographed.

We used semi-quantitative estimation method for the obtained results. Positive reaction for ghrelin is found in the presence of black granules in the secretory granules of cells. Positive reaction for ghrelin receptor GHS-R1 is reported in the presence of fine brown granulation in the cell cytoplasm.

The specificity of immunihistochemical reactions for every studied antigen is confirmed by negative controls in which the specific antibodies are substituted with a buffer (PBS) or a normal non-immune serum. In them there is complete lack of product of the corresponding reaction.

We performed the observation and photographing of microscopic preparations with digital photomiscroscopy camera of light microscope "Olympus BX51".

III. Results

Presence of immature intestinal wall is found in 6th –gestation- week human embryos observed under a light microscope with HE staining. Forming short and wide villi can also be seen. The covering epithelium of the villi is single and cylindrical in some areas and in other it is still stratified. The villous stroma is built by wide mesenchymal layer in which the axial bundle of smooth muscle cells is still missing. There is no lamina muscularis mucosae formed. In between some villi the epithelium folds and outlines shallow crypts. The mesenchyme forms the rest of the intestinal wall. In its periphery a thin layer of elongated smooth muscle cells in a circular position of two-three layer can be seen (fig. 2).

TEM As early as 6th gestation week, under microscope, in the endoblast of the developing small intestine enteroendocrine cells type G, D, EC1 and EC2 can be found. Their morphological characteristics is not different from the one of enteroendocrine cells of an adult. These types of enteroendocrine cells are assumed to be ghrelin secreting, i.e. they are potentially ghrelin-producing cells.

Enteroendocrine cells in the embryonic small intestine are located between the endoblast epithelium cells of the forming glands as well as at the bottom of shallow Lieberkuhn cells. They have oval or pyramid-like shape with the enlarged basal part of the cell lies upon the basal membrane. The cytoplasmic matrix of the enteroendocrine cell is lighter in colour than that of the adjacent endoblast cells. The nucleus is oval, rich in euchromatin. Cellular organelles are similar to those of endoblast cells. Mitochondria are relatively small and elongated, evenly distributed in the cytoplasmic matrix. The amount of GER is different in the different types of enteroendocrine cells. The Golgi apparatus is located perinuclearly. The specific secretory granules in some of

the cells are located in the basal region of the cells but more commonly they are multiple, filling the whole cytoplasm (fig. 3). One type of the enteroendocrine cells are of the 'closed' type. They are rounded with multiple secretory granules and do not reach the mucosal surface (fig. 4). Other enteroendocrine cells are of the 'open' type. These cells have elongated bottle-like shape. The extended basal part lies on the basal lamina. There is presence of secretory granules in it (fig. 5). The narrowed apical part of the cell reaches the lumen. In this region of the enteroendocrine cell we can see pinocytic vesicles (fig. 6).

Enteroendocrine G cells Enteroendocrine G cells have an oval shape, large basally located nucleus. Secretory granules are round or oval with distinct limiting membrane and reveal subnuclear location. Granules are various in size (180 – 300 nm) and various electronic density. The matrix of the granules is filamentous or with small granules and between it and the limiting membrane there is light and narrow halo. Enteroendocrine G cells in adults secrete gastrin as well as enkephalins and endorphins (fig. 7).

Enteroendocrine D cells Eneroendocrine D cells have large granules with size between 250 and 400 nm. They are homogenous with moderately dense core. The membrane of some secretory granules is interrupted. Secretory product of D cells in adults is somatostatin (fig. 8).

Enteroendocrine EC cells Enteroendocrine EC cells have secretory granules which are polymorphous. They are with rod-like or biconcave form and narrow, light halo. The secretory product has high electron density. We found presence of EC cells types EC1 and EC2. The granules of EC1 type are polymorphous, mainly elongated with size of 200 – 300 nm. In adult they secrete serotonin and P substance (fig. 9.). EC2 type have cytoplasm, filled with oval or irregular in shape granules with size of 200 – 400 nm. The content of the granules is serotonin and motilin (fig. 10).

Immunohistochemical reaction for ghrelin and ghrelin receptor GHS-R1 Positive immunohistchemical expression of ghrelin and ghrelin receptor GHS-R1 we found also in the digestive tube of 6^{th}-gestation-week human embryos. Ghrelin positive cells are single, located at the bottom of the shallow crypts (fig. 11, fig. 12.). In their cytoplasm we observed dense clustering of black granules. In the endoblast cells in these regions we observed also positive expression of ghrelin receptor GHS-R1, visualized through fine brown granulation in them (fig. 13).

IV. Discussion

We found that during 6^{th} gestation week in the small intestine of human embryos there is presence of enteroendocrine cells. Their number is minimal - single cells scattered among other epithelial cells. The ultrastructural characteristics of these cells indicates that they are of the following types: G cells, gastrin-producing; D cells – somatostatin producing, EC1 type, serotonin and motilin producing and EC2 type, serotonin and pancreatic polypeptide producing cells. During 6^{th} gestational week of the development when digestion has not developed, enteroendocrine cells, which we found, are highly differentiated. They reveal a morphological characteristic with presence of specific secretory granules identical to those of enteroendocrine cells of adults. The embryonic enteroedocrine cells, as well as those of adults, are 'open' and 'closed' type. The cells of the 'open' type have extended basal and elongated apical region which reaches the intestinal lumen. These cells have the ability to analyze the chemical composition of food. Enetroendocrine cells of the 'closed' type are oval, lie with a broad base on the basal lamina and do not reach the lumen. They can react to mechanical stretching of the small intestinal wall and to blood stimuli.

According to data from other authors, the earliest time enteroendocrine G and D cells were found in the gastric antrum and fundus during 8^{th} gestation week. During 10^{th} gestation week in the same regions of the stomach A cells- glucagon-producing – occur. EC cells are described during 11^{th} gestation week (Stein et al., 1983). There is a report on the occurrence of enteroendocrine cells of the immature gastric epithelium during the 9^{th}, 10^{th} gestation week (Oberg K, 1998). Later, durng 11^{th} gestation week enteroendocrine cells were found in the large intestine of human embryos (Kostiukevich et al., 1996).

During studies with anti-chromogranin A antibody, a common marker for enteroendocrine cells, some authors find the occurrence of enteroendocrine cells during 10^{th} gestation week as well. According to them in 10^{th} gestation week somatostatin cells (D cells) occur which secrete glucagon (A cells) occur in 12^{th} gestation week (Mitrović et al 2012). In the later stages of foetal development, enteroendocrine cells are more numerous and clustered in groups. These are cells, mainly D,G and ECL cells (stomatostatin-, gastrin- and histamine-producing cells) (Radu, 1994).

The embryonic small intestine in 6^{th} gestation week that we observed in our study is in the process of maturation. It already has formed short and broad villi and between them- presence of shallow crypts of Lieberkuhn. It is in the epithelium of these crypts that we found positive immunohistochemical ghrelin

expression. Our results show that the first ghrelin-positive cells are single, located at the bottom of the shallow crypts. The studies of other authors show analogical localization of ghrelin-producing cells in the duodenum of an adult. They ascertain a considerable number of ghrelin- immmunoreactive cells deep at the bottom of the crypts of Lieberkuhn. Single cells in the gland of Brunner also express ghrelin (Grönberg et al., 2008).

In a study of Mitrović et al., ghrelin-producing cells are found in the corpus of the stomach during 11th gestation week. The number of ghrelin-producing cells in the human fetus stomach after 11th till 16th gestation week increases. It is largest during the second trimester of pregnancy and during the third trimester it reaches minimal values. Immediately antepartum the number of all enteroendocrine cells id highly reduced (Mitrović et al., 2012). In a later study the same authors found ghrelin-producing cells in the duodenum of human embryo in the 10th and 11th gestation week. Ghrelin-producing cells are located singly or in clusters at the bottom of the crypts of Lieberkuhn (Mitrović et al., 2014). This data correspond to our results which were obtained in an earlier stage of embryonic development- namely in 6th gestation week.

In human, immunoreactive ghrelin has been studied in the umbilical cord during 20th gestational week and it has been found that its concentration in the umbilical cord is higher than that in the blood of adults (Cortelazzi et al., 2003). Other authors also report that the total ghrelin concentration in the blood from the umbilical cord is higher than that in the blood vessels of the mother (Makino et al., 2002; Bellone et al., 2004).

The source of foetal ghrelin has not yet been identified with a certainty. According to numerous studies, in this period ghrelin is produced mainly in the foetal pancreas (Wierup et al., 2002; Chanoine et al., 2004; Prado et al., 2004; Wierup et al., 2004;). Authors such as Wierup et al., found immunohistochemically numerous ghelin-producing cells in the foetal pancreas during 18th -22nd gestation week. Other authors such as Chanoine et al. assume that during the foetal period ghrelin is produced by the pancreas and participates in the programming of the energy balance mechanisms, orexigenic pathways and adipogenesis. Despite the lack of full characteristic of the receptor dependent effect of ghrelin on the isle cells, these authors state that ghrelin has a distinct role in β-cellular differentiation, survival and functions (Chanoine et al., 2004).

Dispersed single ghrelin-producing cells (epsilon cells) are described in the primitive exocrine pancreas of human fetus in the 13th gestation week. During the following weeks these cells form clusters. In 21st gestation week ghrelin-producing cells for a ring of almost uninterrupted peripheral layer around the other endocrine cells of the isles of Langerhans. The authors of this study ascertain a prenatal ghrelin production by the pancreas (Andralojc et al., 2009).

In the foetal period a ghrelin source are also the lungs. According to Volante et al.(2002), the major amount of ghrelin during the foetal period in human is produced by the pancreas and lungs and a small amount – by the foetal stomach.

Our immunohistochemical studies of ghrelin receptor GHS-R1 in 6th-gestation-week human embryos show positive receptor expression in the developing small intestine. It is localized in the epithelial cells from the fundus of the forming crypts.

Mitrović et al. report of ghrelin receptor expression during the 11th and 16th gestation week in the stomach and duodenum of human fetuses. During the first trimester of pregnancy, there are high levels of GHS-R1a and GHS-R1b expression in the gastric antrum as well as in the duodenal villi, crypts and Brunner's glands. During the second trimester, the expression is also positive in the gastric corpus (Mitrović et al., 2014).

We believe that the positive ghrelin receptor expression in the earliest stages of the embryonic development, i.e. 6th gestation week, ascertained by us shows the participation of ghrelin through its receptors in the processes of differentiation in the gastrointestinal tract. A similar effect of ghrelin is presented in a study on the cellular structures of the mesodermal cells of human embryos. The authors ascertain that ghrelin stimulates the differentiation of human embryonic stem cells into cardiomyocytes and this process is mediated by ghrelin receptor GHS-R1A (Yang et al., 2011).

V. Conclusion

As a conclusion, our study found that in the 6th gestation week in the developing intestine of human embryo there is presence of differentiated enteroendocrine cells. The ultramicroscopic characteristic of these cells shows presence of G cells – gastrin producing, D cells- somatostatin producing, EC1 and EC2 cells-serotonin producing. We proved immunohistochemically the presence of ghrelin-producing cells. The evidenced positive expression of ghrelin and ghrelin receptor GHS-R1 in the small intestine during the 6th gestation week of the early embryonic period of human shows the ability of ghrelin to perform the function of a specific ontogenic factor which participates in the differentiation of the gastrointestinal tract. Histogenetic processes in the digestive tube are not isolated from the same processes in the whole body. The early differentiation of enteroendocrine cells in the epithelium of the digestive tube assumes their endocrine effect on the developing cells and tissues in other organs and systems.

Fig. 1. Photograph of fragment upper extremity (arm) of human embryo 6th gestational week

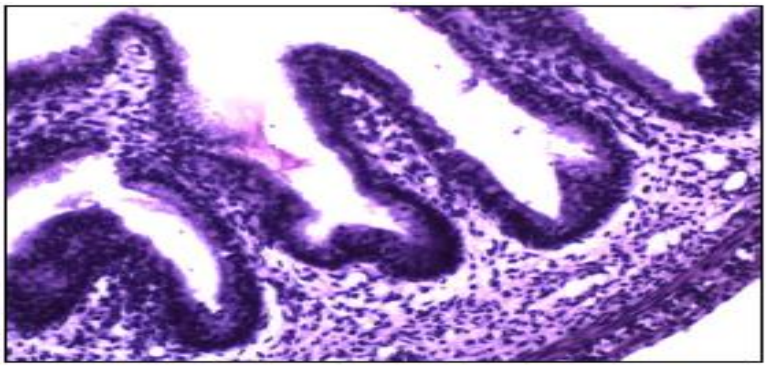

Fig. 2. Small intestine of human embryo 6th gestational week. Immature wall with forming villi and crypts. Paraffin preparation HE staining x 20.

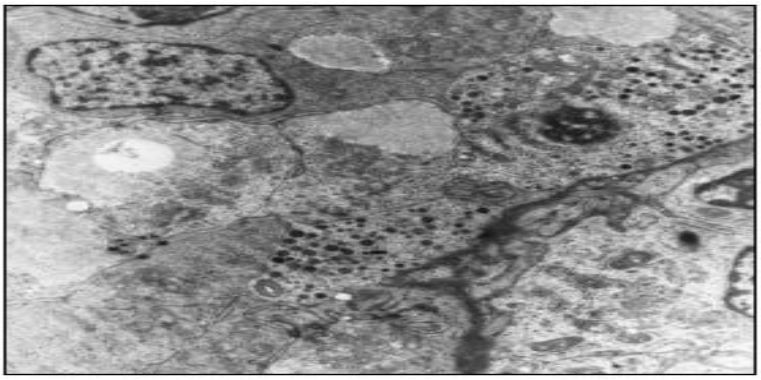

Fig. 3. Small intestine of human embryo 6th gestational week. Adjacent enteroendocrine G cells located between endoblast cells. TEM. Micr. Magn. x 1850; Photogr. Magn. x 9250.

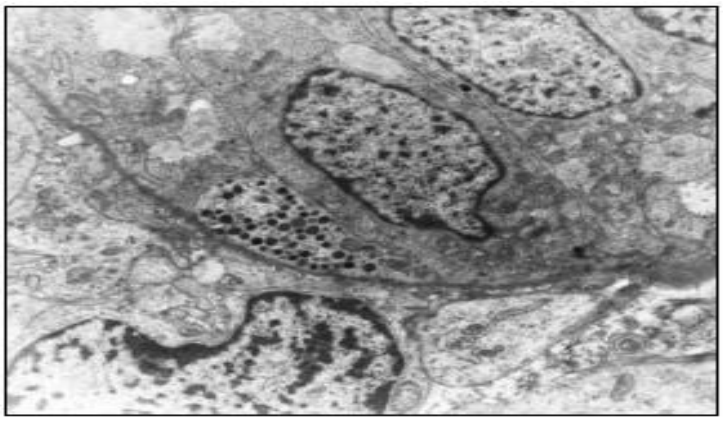

Fig. 4. Small intestine of human embryo 6th gestational week. Enteroendocrine cell of the 'closed' type-fragment from basal section of G cell. TEM. Micr. Magn. x 1850; Photogr. Magn. x 9250.

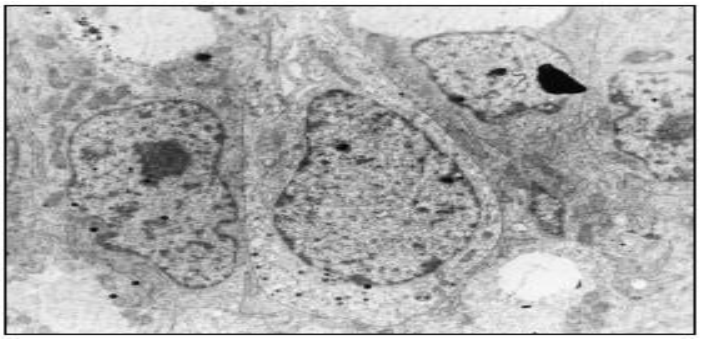

Fig. 5. Small intestine of human embryo 6th gestational week. Enteroendocrine cell of the 'open' type. Basal section of enteroendocrine G cell with a small number of secretry granules. TEM. Micr. Magn. x 1500; Photogr. Magn. x 7500.

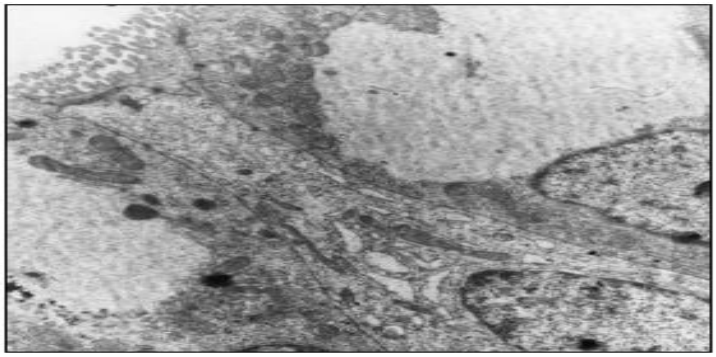

Fig. 6. Small intestine of human embryo 6th gestational week. Enteroendocrine cell of the 'open' type. Apical section of the same enteroendocrine G cell reaching the lumen with a single granule in it. TEM. Micr. Magn. x 2350; Photogr. Magn. x 11250.

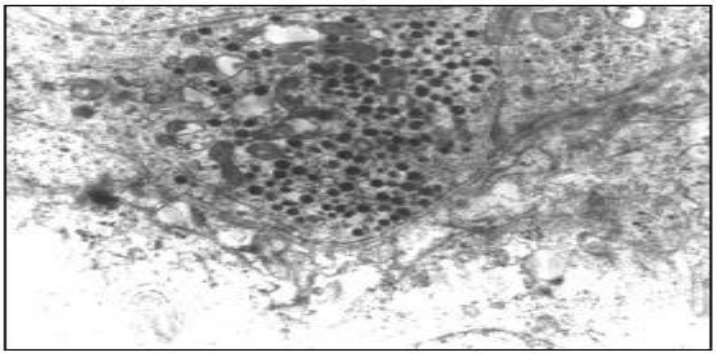

Fig. 7. Small intestine of human embryo 6th gestational week. Fragment of enteroendocrine G cell. TEM. Micr. Magn. x 3900; Photogr. Magn. x 19500

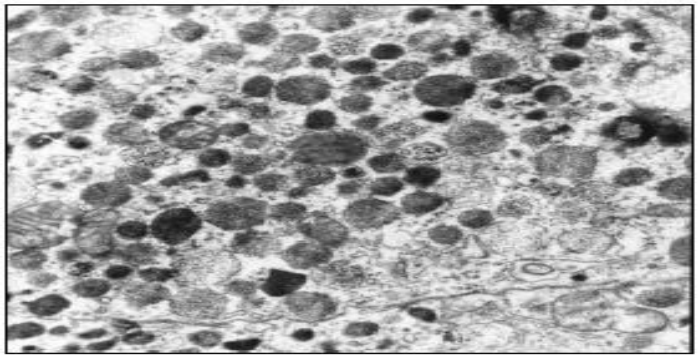

Fig. 8. Small intestine of human embryo 6th gestational week. Fragment of enteroendocrine D cell. TEM. Micr. Magn. x 5000; Photogr. Magn. x 25000

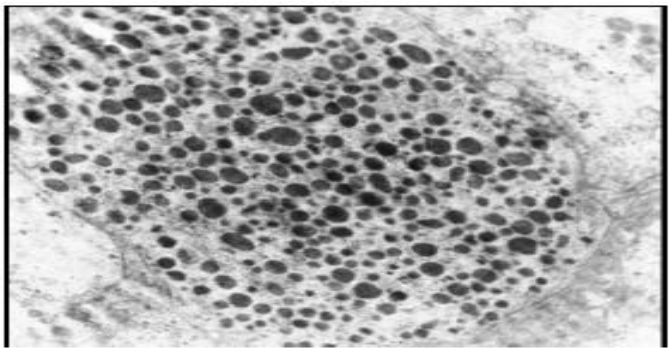

Fig. 9. Small intestine of human embryo 6th gestational week. Fragment of enteroendocrine EC1 cell. TEM. Micr. Magn. x 3900; Photogr. Magn. x 19500

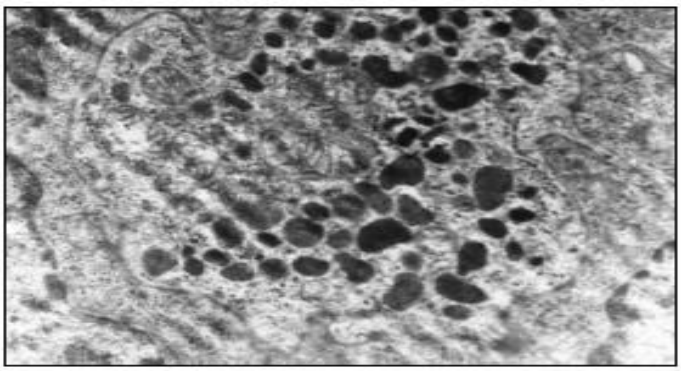

Fig. 10. Small intestine of human embryo 6th gestational week. Fragment of enteroendocrine EC2 cell. TEM. Micr. Magn. x 3900; Photogr. Magn. x 19500

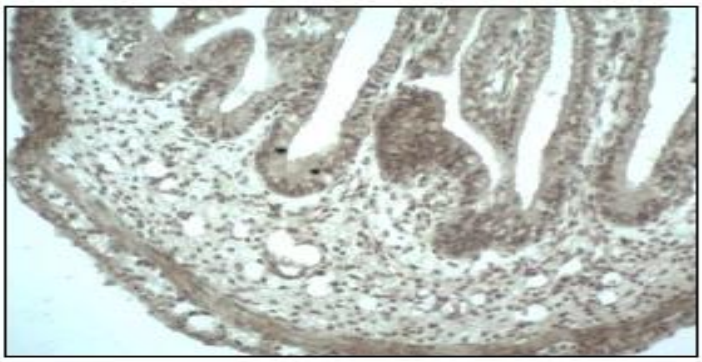

Fig. 11. Small intestine of human embryo 6th gestational week. Single ghrelin-positive cells in the forming crypts. IHC. Paraffin preparation. x 20.

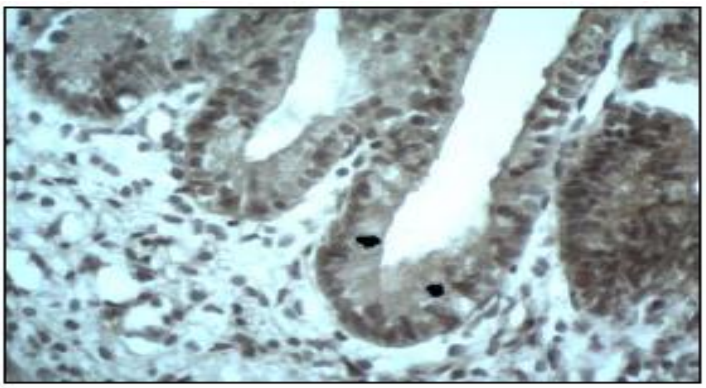

Fig. 12. Small intestine of human embryo 6th gestational week. Single ghrelin-positive cells in the forming crypts. IHC. Paraffin preparation. x 40.

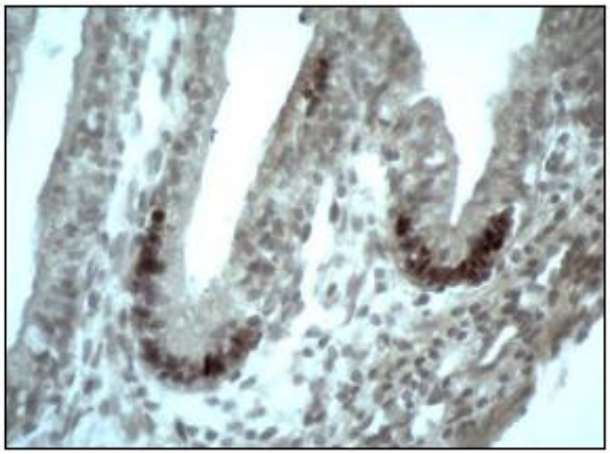

Fig. 13. Small intestine of human embryo 6th gestational week. Positive reaction for GHS-R1 in the endoblastic epithelium. IHC. Paraffin preparation. x 40.

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