Expression of Ghrelin in The Fetal Stomach

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***Abstract* — Ghrelin, an endogenous ligand of the growth hormone secretagogue receptor (GHS-R), is present in oxyntic glands of fundus of stomach. Ghrelin, in addition to its role in regulating GH secretion, it has direct orexigenic effect. We observed the expression and location of ghrelin in the developing human stomach by immunohistochemistry during prenatal period. The study was conducted on 8 aborted human fetuses of varying gestational ages ranging from 12-34 weeks. Immunostaining with monoclonal anti-ghrelin antibody showed ghrelin positive cells in the gastric glands; initially they are arranged as cluster of cells in the surface epithelium, gradually they were present in the basal part of gastric glands. In late gestation, they are arranged as individual cells, localised in base of the gastric glands. The cells are located close to the basement membrane as closed type of cells. The ghrelin positive cells are seen in the fundic and body region of stomach and very less frequently in pylorus. The very early appearance of ghrelin cells in the stomach during the entire period of prenatal development suggest that ghrelin is involved in local control of growth, differentiation, functioning of cell tissue in which they are localized.**

***Index Terms*— Anti-Ghrelin Antibody, Gastric glands in stomach, Ghrelin, Immunohistochemistry**

I. INTRODUCTION

Ghrelin is a peptide that was discovered as an endogenous ligand for the growth hormone secretagogue receptor (GHSR-1a) through which it stimulates Growth Hormone (GH) release from the anterior pituitary[1]. Ghrelin is predominantly synthesized in the stomach and secreted into the circulation. The X/A-like cells of gastric oxyntic glands of the stomach are the most abundant source of circulating ghrelin, the small intestine also synthesizes ghrelin to a lesser extent[2]. Additionally, it has been demonstrated that ghrelin is expressed in many tissues such as the lung, heart, pancreas, kidney, testis, pituitary, and hypothalamus[3].

Ghrelin has a unique structure with 28 amino acids and a n-octanoyl ester at its third serine residue, which is essential for its potent biological activity at the GHS-R1a. The ghrelin gene is transcribed as a pre pro-ghrelin mRNA isoform, which leads to the translation of a 117 amino-acid peptide. This prohormone is cleaved into pro-ghrelin, a 94 amino acid signaling peptide. Pro-ghrelin then requires posttranslational acylation with n-octanoic acid or n-decanoic acid at the third serine residue for its biological activity at the GHS-R1a. Ghrelin O Acyltransferase (GOAT) is the enzyme responsible for pro-ghrelin acylation[4] and it is also found predominantly in the stomach and digestive tract[4,5].

In the stomach and duodenum, GOAT co-localizes with ghrelin expressing cells[6], where it readily acylates newly synthesized pro-ghrelin. Pro-ghrelin is further processed by prohormone convertase to produce the 28 amino- acid mature ghrelin peptide[7]. Ghrelin cells are small, round shaped, represented as closed type cells in stomach. In all other parts of the gastrointestinal tract, they are represented by so-called open-type cells that are elongated, bottleshaped, with characteristic apical cytoplasmic processes that are in contact with the lumen[8]. Ghrelin mRNA and protein were identified in the fetal pancreas outnumbering the fetal stomach.

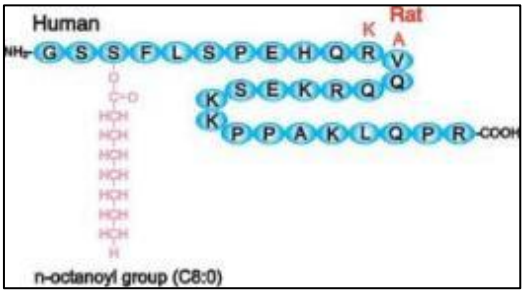

Figure 1. Structures of human and rat ghrelin[10].

With in the embryonic mouse islet, ghrelin-producing cells are termed as epsilon cells that represent an endocrine population[11].These were identified very early during human fetal life from 11 weeks in the stomach, duodenum, and pancreas. In human fetuses and infants, ghrelin-IR cells were present throughout the gastrointestinal tract, most frequently in the pancreas and corpus mucosa of the stomach, and rarely in the antrum, duodenum, and cardia[12].
The aim of the present study is to assess the location and expression of ghrelin in the developing human stomach during prenatal period in the Indian population.

II. REVIEW OF LITERATURE

The name Ghrelin is based on 'ghre' a word root in Proto-Indo-European languages meaning 'grow' in reference to its ability to stimulate GH release. This growth hormone secretagogue (GHS) is expressed in many tissues such as pancreas pituitary gland, thyroid, testis, and placenta[1]. Initial studies of ghrelin focused on its role as a circulating orexigenic signal[13].

Approximately 60–70 % of circulating ghrelin is secreted by the stomach, with most of the remainder originating in the small intestine in adults. In the stomach, the ghrelin-containing cells are more abundant in the fundus than in the pylorus originally termed X/A-like cells. These ghrelin cells account for approximately 20 % of the endocrine cell population in adult oxyntic glands. However, the number of ghrelin cells in the fetal stomach is very low and increases after birth. As a result, the ghrelin concentration of fetal stomach is also very low and gradually increases after birth until 5 weeks of age[14].

EXPERIMENTAL STUDIES ON IDENTIFICATION OF GHRELIN

A synthetic Met5 enkephalin analog, Tyr-D-Trp-Gly-Phe Met-NH2, stimulated Growth hormone (GH) release from rat pituitary glands in vitro at concentrations of 1-100 µg/ ml. The effect was specific for GH and other pituitary hormones (LH, FSH, TSH, PRL, and ACTH) were not affected[15]. Later, through computer modelling techniques, a more potent hexapeptide i.e. His-D-Trp-Ala-Trp- D-Phe-Lys-NH2 (GHRP-6) was designed and synthesized [16].

GHRP-6 caused dose-dependent GH release both in vivo and in vitro without affecting the release of other hormones. Although both GHRP-6 and GHRH stimulated GH release from the pituitary gland, there were differences between these two peptides in their actions both invivo and invitro. It was demonstrated that synergistic effects of GHRP6 on GHRH stimulated both GH release and intracellular AMP accumulation in rat pituitary cell culture. By antagonist studies they studied that both GHRP6 and GHRH act on different receptors but have synergistic effect[17]. The hypothalamic & pituitary localisation of the GHSR was consistent with its role in GH release[18].

In 1999, Kojima et al. identified the protein ligand i.e., acting on GHSR, found in rat stomach and named it as GHRELIN. They identified its amino acid sequence and its octanoylation of serine at its third position by calcium influx assay. Ghrelin produced in stomach may reach act on the anterior pituitary via the blood circulation because its intravenous administration tom rats rapidly increased the plasma GH concentration. It is also synthesized in the hypothalamic arcuate nucleus, but the number of ghrelin neurons are small, and this hypothalamic ghrelin might act on the anterior pituitary via portal circulation to release GH[1].

Two major molecular forms of ghrelin are found, one which is biologically active form octanoylated ghrelin and inactive form des acyl ghrelin by radioimmunoassay (RIA) combined with HPLC[19].

The effect of ghrelin on urethane anesthetized rats showed increase in gastric acid secretion and gastric motility through an activation of vagal nerve in rats and inhibited by atropine administration and vagotomy. The stimulatory effect on gastric function by ghrelin is induced independently of its GH releasing effect[20].

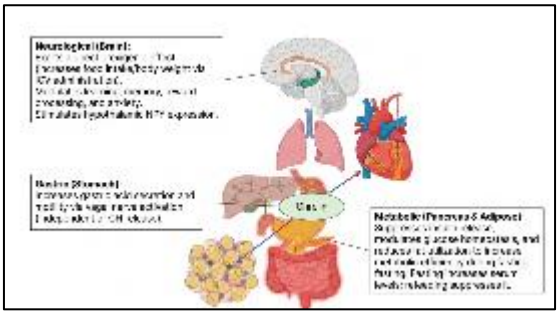

Figure 2. Physiological functions of ghrelin

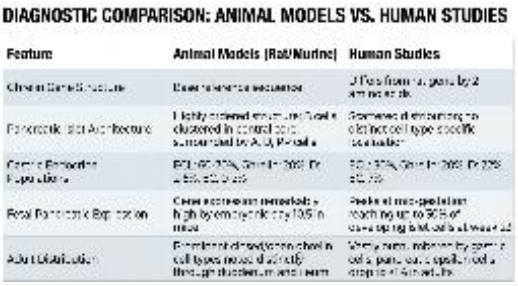

Fig 3. Differences in Ghrelin expression in animal Vs. Human studies

III. MATERIALS AND METHODS

Six fetuses with gestational ages more than 24 weeks were obtained from spontaneous abortions and rest of the two fetuses were below 24 weeks, obtained from cases coming for Medical Termination of Pregnancy (MTP). The parameters as Crown-Rump length, Bi-parietal diameter and foot length were measured in all the procured fetuses.The gestational age of the fetuses were determined by evaluating the Crown-Rump length. After procuring the fetus, a median incision was given on the anterior abdominal wall for immediate fixation of the gut. The fetus was then immersed for fixation in 10% formalin. After 24 hours, stomach was dissected and removed en-block from the lower end of esophagus up to the pylorus and preserved in fresh fixative for two weeks. Specimens that showed any degree of autolysis were not considered for the study. The dissected stomach was labeled and processed for paraffin embedding keeping the long axis of the stomach as the cutting surface. Seven micron thick serial sections were generated on a rotary microtome. Each longitudinal tissue sections had the cardia, fundus, body and pylorus.

Staining

Sections were stained with haematoxylin and eosin (H&E) stain to see the morphology of the developing stomach. In each fetal stomach, few sections were processed for immunohistochemistry (IHC). Deparaffinised sections were incubated in citrate buffer and the endogenous peroxidase activity was blocked using 70% methanol and 6% H2O2. After washing with working solution of phosphate buffer with 0.1% Triton X, slides were treated with Super Block (SkyTek) for 10 minutes for blocking the nonspecific antigen. The sections were then incubated with monoclonal anti-ghrelin antibody [EPR20502] (ab 209790) at a dilution of 1:100, at 4°C overnight. Slides were then treated with Ultra-tek antipolyvalent (SkyTek) for 10 minutes followed by Ultra-tek HRP (red cap), washing with 0.01M PBS-TX thrice after each step. The reaction was observed using diaminobenzidene (DAB) as chromagen.

The sections were then examined under a BX 61 motorised microscope and the images were captured with Olympus DP71 camera. Processing of images was done using the Image Pro plus MC 6 software.

IV. RESULTS

12 weeks:

The surface epithelium of fundic and body region of stomach was stratified cuboidal ciliated epithelium (3-4 layers thick). At the lower of gastric pits, few cluster of differentiated cells were seen as acinar glands.

Few ghrelin positive cells were located in the surface epithelium and gastric glands of the fundic region of stomach. There is also diffuse expression of ghrelin in the mucosa and muscularis externa.

14 weeks: The surface epithelium of fundic and body region of stomach was stratified cuboidal epithelium but reduced to 2 to 3 cell layers. Gastric glands were observed as simple tubules. The glands were with cells differentiated into mucus cells at neck, parietal cells at body, and chief cells at base of the gland.

Ghrelin positive cells were located in basal part of gastric gland as cluster of cells of the fundic region of stomach. There were also seen as isolated cells close to the basement membrane.

18 weeks: As the development of acinar glands is seen by invagination of surface epithelium, the ghrelin positive cells were seen in the basal part of the glands. There is also diffuse expression of ghrelin in the submucosa and lamina propria. Blood vessels were showing ghrelin positive immunoreactivity.

20 weeks: Few ghrelin positive cells were seen in cut sections of glands in lamina propria as cluster of cells in a single gland. They were located in the basal part of the gland towards basement membrane and the nucleus of the cell was round in shape. Localised expression is seen from this gestation.

24 weeks: In fundic and body region of the stomach, numerous tubular glands were seen opening into pits. Parietal cells increased in size and in number in gastric glands. These cells have broad base bulging towards the lamina propria and narrow apex

projecting towards the lumen of the gland. These were in the body of the gland and few at the base.
Ghrelin positive cells were seen at central part of the gland as individual cells towards basement membrane. There was also diffuse expression in the muscularis externa layer.

30 weeks: Mucosa of the fundus and body shows increased thickness with increased number of gastric pits and gastric glands in the lamina propria. Increased glandular tissue was seen along with increase in length of glands. The Ghrelin positive cells were located at various locations in a gastric gland, close to the neck and towards the central part of the gland.

a) H&E b) Immunostaining

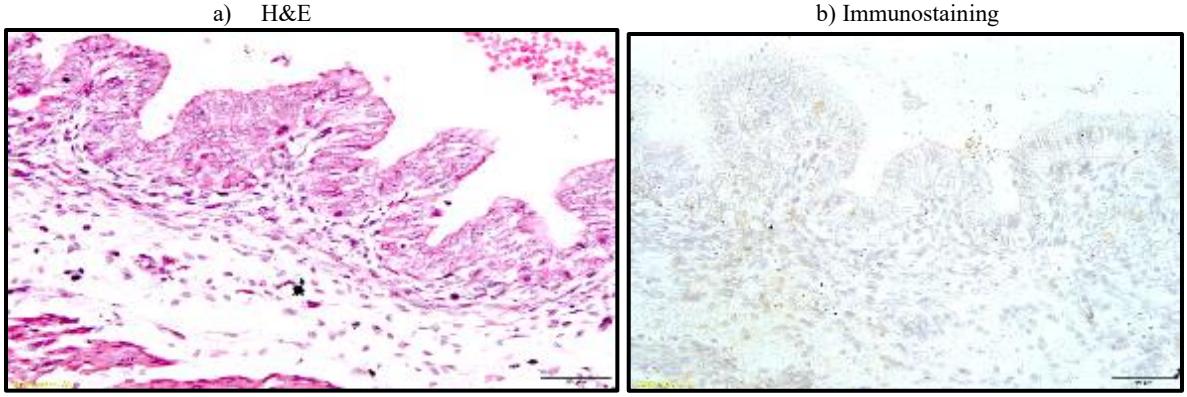

Fig.4 : a) Haematoxylin & Eosin (H&E) staining showing lining of the developing fundus part of stomach at 14 weeks containing surface epithelium (SE), lamina propria (LP), submucosa (SM) Scale Bar: 50µm. b) Immunostaining in the stomach demonstrates ghrelin positive cells showing a positive reaction with anti-ghrelin antibody (brown colour). These are located mainly in the surface epithelium

32 weeks:
Numerous long tubular glands were seen to occupy the mucosa extending from the base to the muscularis mucosa. Glands show increase in branching pattern. Ghrelin positive cells showed very faint expression in basal part of the

34 weeks:
The glandular features resembled the adult pattern. About 3-4 glands opening into one gastric pit. Each gastric gland showing at neck mucous cells, parietal cells in the body region and chief cells at the base/fundus of the gland. Ghrelin positive cells were in the basal part of the gastric gland. There shows increased number of ghrelin positive cells. There was also diffuse expression of ghrelin in the muscularis externa layer.

a)H&E b) Immunostaining

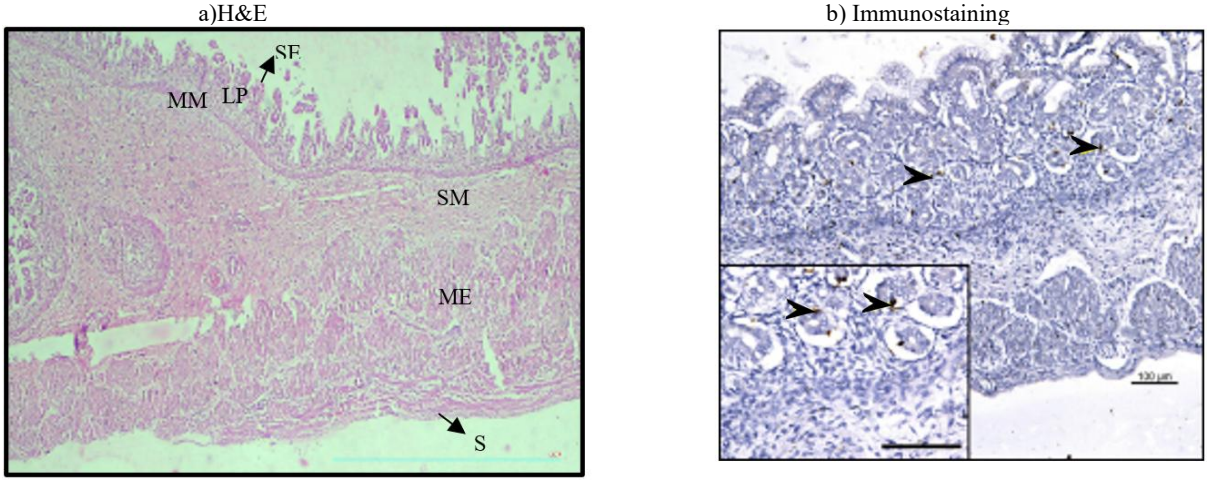

Fig.5 : a) H&E staining showing lining of the developing fundus part of stomach at 20 weeks containing SE, LP, muscularis mucosa (MM), SM, muscularis externa (ME), serosa(S) .Scale Bar: 1000µm. b) Immunostaining in the stomach demonstrates ghrelin positive cells showing a positive reaction with antighrelin antibody (brown colour). These are located mainly in cut sections of the gastric glands (arrows). Scale Bar: 100µm

a)H&E b) Immunostaining

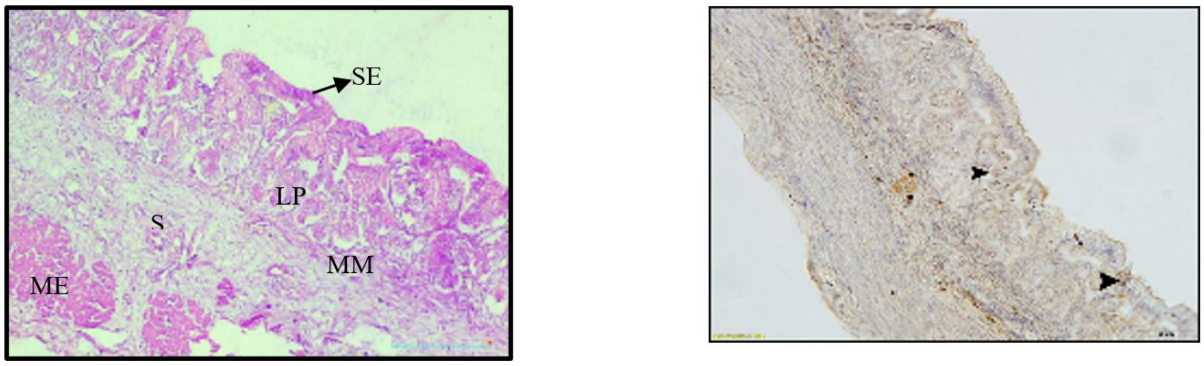

Fig.6 : a) H&E staining showing lining of the developing fundus part of stomach at 24 weeks containing SE, LP, MM, SM, ME, S .Scale Bar: 200µm. b) Immunostaining in the stomach demonstrates ghrelin positive cells (Black arrow). Scale Bar: 50µm

a)H&E b) Immunostaining

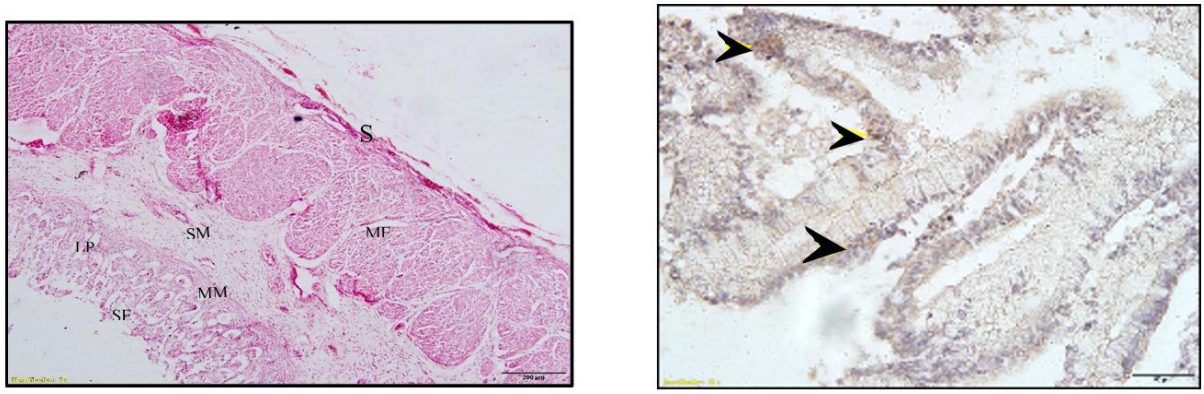

Fig.7: a) H&E staining showing lining of the developing fundus part of stomach at 30 weeks containing SE, LP, MM, SM, ME, S .Scale Bar: 200µm. b) Immunostaining in the stomach demonstrates ghrelin positive cells (Black arrow). Scale Bar: 50µm

V. DISCUSSION

The ghrelin cell of the neuroendocrine cells in the gastric gland has widespread actions. It is a potent orexigenic peptide that stimulates food intake and body weight gain, suggesting that ghrelin plays an important role in energy homeostasis. In prenatal life it helps in growth and development[12]. They are closed type with no continuity with lumen in rat stomach[21]. The expression of ghrelin is similar to the rat studies as it initially appears in gastric lining and gradually localised in the basal parts of the gastric glands. P/D1 (ghrelin) cells are around to ovoid cell located in the oxyntic glands.

During first trimester of gestation development, most of the ghrelin cells present in the glandular base of the body of stomach and rarely in glandular neck. In further gestations, they are located in basal and central part of glands[12]

Our study is similar to the previous studies done on localisation of ghrelin in animal and human stomach. Immunostaining with anti-ghrelin antibody showed ghrelin positive cells in the staining of gastric glands; initially 12- 18 weeks (1st trimester) they are arranged as cluster of cells in the surface epithelium, gradually they were present in the basal part of gastric glands.

During 20-24 weeks (2nd trimester) the number of ghrelin cells are increased and located at various areas of gastric gland. In late gestation, in 3rd trimester they are arranged as individual cells, localised in base of the gastric glands. The cells are located close to the basement membrane as closed type of cells that have no continuity with the lumen, suggesting that they respond to physical stimuli from the lumen and chemical stimuli from the basolateral side or both[8].

The ghrelin positive cells are seen in the fundic and body region of stomach and very infrequent in pylorus part. We also found no expression of ghrelin

in the pylorus as reported in the previous study[12].There was also diffuse expression of ghrelin seen in remaining layers of the stomach and blood vessels. The location of ghrelin cells is towards the basal part of the gland indicates its secretions to be poured to the blood circulation to operate its activities. Initially, ghrelin is identified in embryonic day 18 in stomach of neonatal rats in the gastric gland. It showed that ghrelin-immunopositive cells appeared in the glandular base of the fundic gland at 1 week of age and they were found in the glandular base and the glandular neck at 3 weeks of age. Then the distribution of ghrelin cells was extended from the glandular base to the glandular neck during the post neonatal developmental period.

VI. CONCLUSION

There is a sequential pattern of development and differentiation of gastric glands and neuroendocrine cells in the lining of stomach of human fetuses. Ghrelin expression is increased in stomach with advancing age and in corresponding ages of fetus. The very early appearance of ghrelin cells in the stomach during the entire period of period of prenatal development suggest that ghrelin is involved in local control of growth, differentiation, functioning of cell tissue in which they are localized.

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