Histological and Histochemical investigation of the development of the New -Zealand rabbit’s gastric glands

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ABSTRACT
The present study aimed to provide a detailed description of the normal development of rabbit stomach and focusing on the histogenesis of gastric glands. In a total, 24 New Zealand White rabbit fetuses were collected at gestational days 21, 25, and 29. The stomachs of the collected fetuses were fixed in 10% neutral buffered formalin and prepared by paraffin technique then stained with Harris's Haematoxylin and Eosin, Masson's Trichrome stain, Orcein, Periodic acid-Schiff, Alcian blue, and Bromophenol blue stains. The results revealed that, at 21st gestational day, the different parts of the stomach including, cardia, fundus and pylorus could be easily distinguished. On 25th developmental day, the gastric mucosal folds were more prominent in the cardia than fundus and pylorus. At 29th developmental day, tunica mucosa and tunica submucosa of the fetal stomach were laid in longitudinally oriented folds known as rugae. The gastric gland in this age became well developed containing well-demarcated oxyntic and peptic cells. In conclusion, the rabbit stomach is completely differentiated during the embryonic life and the gastric glands were functionally active.

Keywords: Gastric glands, Histological, Parietal cells, Peptic cells, Rabbit, Stomach
1. Introduction
The rabbit is a widely distributed animal species used for economical and laboratory purposes (Hristov et al., 2006). It is used in laboratory investigations because it is an ideal experimental model (Ghoshal and Bal, 1989).

The stomach is an important organ that has numerous functions including digestion of food, regulation of metabolic homeostasis and immune defense. Depending on their unique dietary needs and habits, vertebrates have adapted variations in their structural and histological organization of the stomach (McCracken and Wells, 2017). The stomach of rabbits is of the monolocular type. It is divided into three regions: cardiac, fundic and pyloric regions each containing different types of glands (Madge, 1975).

Embryologically, the stomach is derived from the foregut. At early stage of embryonic life, the primitive stomach appears as a fusiform dilatation at the caudal part of the foregut (Sadler, 2011). With the advancement of age and development of the esophagus, the fetal stomach migrates caudally and became enlarged to occupy a larger area in the fetal abdominal cavity (Kumar, 2015). At the histological level, many authors reported that the development of fetal gastric mucosa occurs very early during fetal life (Kammeraad, 1942), (Sommer and Kressin, 2001), (Rodrigues et al., 2014) and (Nivedita et al., 2016). It is well known that the epithelial components of the stomach are endodermal in origin, whereas the connective tissue and muscle components are mesodermal in origin (Esrefoglu et al., 2017).

The morphological and functional characteristics of the adult mammalian stomach have been expansively studied by many authors in many experimental animal models (Madge, 1975), (Ghoshal and Bal, 1989), (Hristovet al., 2006), (Khalel, 2012) and (Asmaa, 2010). However, there is a clear limitation on the available data concerning the normal stomach development in the fetal rabbits generally and the histogenesis of gastric glands particularly.

The gastric glands were firstly recorded in rabbit fetuses on 23rd gestational day, the mucosal epithelia was stratified and undulating with primary glands (Yaman and Girgin, 2001). The same authors added that the folds possess cellular cords projecting downwards into the underlying connective tissue. The majority of these cords appear solid with no lumen. In mice fetuses, the gastric glands are primarily formed on 15.5th gestational day (Hiroshi Fukamachi, 1979). Esrefoglu et al., 2017 and Kammeraad, 1942, in rat fetuses recorded that, the gastric pits are firstly noted on the 15th day of gestation. In cat fetuses, the glands begin to evaginate into primitive pits and tubules on the 42nd day of gestation (Knospe, 1996). On the 24th gestational day of rabbit fetuses, the gastric epithelium has a simple columnar structure and the parietal cells occur between the bases of the epithelial cells of the gastric glands (Yaman & Girgin, 2001; Hayward, 1967and Menzies, 1958). Yeomans et al., 1976 stated that, in rat fetuses on 21st gestational day, the surface cells mostly contain mucous granules and, in fundus, some parietal cells are usually identifiable. Just before birth, the gastric epithelium contains well-defined glands containing parietal and mucus-secreting cells (Hayward, 1967, in rabbit and Yeomans et al., 1976 and Helander, 1969a in rat).

2. Materials and Methods
I- Source of specimens
Specimens of the present study were collected from 24 New-Zealand White apparently healthy rabbit fetuses on gestational days 21, 25, and 29. The experimental animals were obtained from the laboratory animal house at the Faculty of Medicine, Assiut University.

II- Sampling procedures
Each pregnant normal and apparently healthy rabbit doe was sacrificed and the uterus was extracted. The fetuses were quickly removed from the uterus, washed by normal saline and immediately embedded in 10% neutral buffered
formalin. Macerated fetuses were recorded as non-survivors and excluded from further analysis.

III- Light microscopy

Eight fetuses for each developmental age were prepared for light microscopical investigation. The prepared specimens included: The abdominal region of fetuses collected on gestational days 21, 25 and 29.

All these specimens were immediately fixed in 10% neutral buffered formalin for 7-22 hours. The fixed materials were dehydrated, cleared and embedded in paraffin wax. Step serial sagittal and transverse sections were obtained at 5-7 μm and stained with the following histological stains: Harris's Haematoxylin and Eosin, Masson's Trichrome, Orcein, Periodic acid-Schiff, Alcian blue (AB), Bromophenol blue. All stains were cited by (Bancroft and Gamble, 2008).

3. Results

On 21st gestational day, the different parts of the stomach including, cardia, fundus, body, and pylorus could be easily distinguished. Mucosal foldness was highly distinct in the cardiac region and became less distinct in the fundus, body and pyloric regions (Fig. 1 A-D-G). Moreover, it was observed that the thickness of the gastric mucosa was greater in the cardiac region and became lesser in the fundus, body and pyloric regions (Fig. 1 A-D-G). The gastric mucosa consisted of lamina epithelialis, lamina propria, and muscularis mucosa. The gastric epithelium was variable from stratified to simple columnar. However, the majority of the epithelial cells contained oval apically located nuclei and showed basophilic cytoplasm (Fig. 1 B-E-H). The cytoplasm of the epithelial cells contained PAS-positive granules (Fig. 1 C-F-I). It was observed that gastric pits were first recognized during this stage of development and led to shallow primitive tubular or alveolar gastric glands (Fig. 1 A).

On 25th developmental day, the gastric mucosal folds were more prominent in the cardia than fundus, body, and pylorus (Fig. 2 A, Fig. 3 A and Fig. 4 A). The gastric surface epithelial cells appeared columnar with basally located nuclei and acidophilic cytoplasm. The surface epithelium was interrupted by the openings of the gastric pits (Fig. 2 A, Fig. 3 A and Fig. 4 A). PAS and alcian blue positive intracellular granules were demonstrated at the apical portions of the gastric epithelial cells. This mucoid coat gave a strong PAS and alcian blue positive reactions in the all gastric regions (Fig. 2 C-D, Fig. 3 E-F-G and Fig. 4 D-E). At the pyloric region, some gastric epithelial cells showed a bromophenol blue positive reaction (Fig. 4 G). At cardia, gastric glands appeared as branched tubular glands, the terminal portions of the glands were frequently coiled with relatively large lumens (Fig. 2 A-F). At fundus and body, gastric glands appeared as simple and branched tubular glands, the depth of these glands was significantly lesser than that in cardiac region (Fig. 3 A-B). At the pyloric region, the glands appeared simple tubular shallow glands (Fig. 4 A-B). At this stage of development, parietal and chief cells were firstly recognized within the lining epithelium of the gastric glands at all gastric regions. Parietal cells occupied a peripheral position at the body and base of the gland and appeared as sporadic, large cells bearing deeply acidophilic cytoplasm and relatively small and rounded nuclei (Fig. 3 B-C-D). While chief cells were located near the bottom of the gland and appeared rounded or polyhedral cells with centrally located nuclei. They stained deep blue with bromophenol blue stain (Fig. 2 E, Fig. 3 H-I and Fig. 4 F). The lamina propria and tunica submucosa consisted mainly of elastic and collagen fibers which were more prominent than the previous age (Fig. 2 F-G, Fig 3. J and Fig. 4 H-I). The muscularis layer appeared thicker and more developed than the previous age. At cardia, it was differentiated into the inner longitudinally oriented layer and outer circularly oriented layer (Fig. 2 A-F). At fundus, body and pyloric regions, the muscularis layer appeared thinner than that at the cardiac region and appeared as interrupted bands of
circularly arranged smooth muscle fibers (Fig. 3 A and Fig. 4 A-I). The serosa consisted of highly vascular loose connective tissue covered by mesothelium (Fig. 2 A-F, Fig. 3 A and Fig. 4 A-I).

On the 29th developmental day, both tunica mucosa and submucosa of the fetal stomach were laid in longitudinally oriented folds known as rugae (Fig. 5 A-B and Fig. 8 A). The gastric mucosal folds were more prominent than the previous gestational age and more prominent in the cardiac region than in fundus, body and pyloric regions (Figs. 5 D, Fig. 14 A, and Fig. 8 B). The gastric epithelial cells appeared columnar with oval basally located nuclei and acidophilic vacuolated cytoplasm (Fig. 5 C and Fig. 7 A). PAS and alcian blue positive intracellular granules were demonstrated at the apical portions of these cells (Fig. 6 A-B, Fig. 7 B-C and Fig. 8 B-C). Mucous neck cells were recognized in groups or clusters in the neck of the gastric gland and appeared cuboidal with spherical nuclei (Fig. 5 C-E and Fig. 7 A). The surface epithelium was interrupted by the openings of the gastric pits which were relatively wider than previous age, and was invaginated to various extends in the lamina propria, leading to the branched tubular gastric glands (Fig. 5 C-E, Fig. 7 B and Fig. 8 D). At cardiac region, gastric glands appeared as highly coiled, highly branched tubular glands (Fig. 5 D-E), while in fundus and body, the gastric glands appeared as branched tubular glands (Fig. 7 B), however, in pylorus, the gastric glands still appeared as simple tubular glands (Fig. 8 D). Parietal cells occupied a peripheral position and appeared as large triangular cells with rounded nuclei and deeply acidophilic cytoplasm (Fig. 5 D-E and Fig. 7 A). Chief cells were located near the bottom of the gland and gave a strong bromphenol blue positive reaction (Fig. 6 C-D, Fig. 7 D and Fig. 8 D). It was obvious that the parietal and chief cells in the fetal rabbit gastric glands were more numerous than that observed than that in the previous age. The lamina propria and tunica submucosa consisted of a well-developed network of elastic and collagen fibers demonstrated by orcein and Masson's trichrome stains (Fig. 6 E-F, Fig. 8.E). The muscularis layer appeared well developed and the serosa consisted of highly vascular loose connective tissue containing blood capillaries and nerve fibers and covered by mesothelium (Fig. 6 E, Fig. 7 A).

**Legends**

Fig. 1 Transverse section in fetal rabbit stomach at 21st gestational day showing cardiac region (A-C), fundus and body (D-F) and pyloric region (G-I)

(A) Gastric mucosa at cardiac region showing, gastric pit (*) and primitive gastric gland (black arrow heads). Note the interrupted bands of ill developed smooth muscle fibers representing the first appearance of muscularis mucosa (Mm), interrupted bands of immature smooth muscle fibers of tunica muscularis (Ms). (Haematoxylin and Eosin)

(B) Cardiac region, (E) fundus, body and (H) pyloric region, the lining epithelium (Ep) varied from stratified and simple columnar with apically situated nuclei (white arrow head). (Haematoxylin and Eosin)

(C) Cardiac region, (F) fundus& body and (I) pyloric region, the cytoplasm of the lining epithelial cells contains PAS positive granules (black arrow heads). (Periodic acid–Schiff)

(D) Gastric mucosa at Fundic region, primitive gastric glands (black arrow head), lamina muscularis mucosa (Mm), tunica muscularis (Ms). (Haematoxylin and Eosin)

(G) Gastric mucosa at pyloric region, lamina muscularis mucosa (Mm), tunica muscularis (Ms). (Haematoxylin and Eosin)

Fig. 2 Transverse section in cardiac region of fetal rabbit stomach at 25th gestational day showing:-
(A) Simple columnar surface epithelial cells (black arrow heads), openings of gastric pits (star), simple branched gastric glands (white arrow head), lamina muscularis mucosa (Mm), tunica muscularis arranged inner longitudinal and outer circular (Ms) and serosa contains well developed blood vessels (se). (Haematoxylin and Eosin)

(B) Simple columnar surface epithelial cells (black arrow heads), parietal cells at the body and basal parts of the gland (white arrow heads). (Haematoxylin and Eosin)

(C) Positive PAS intra cellular granules at the apices of gastric epithelial cells (black arrow head) and positive thick mucus layer covers the mucosa (white arrow head). (Periodic acid–Schiff)

(D) Positive alcian blue intra cellular granules at the apices of gastric epithelial cells (black arrow head) and positive thick mucus layer covers the mucosa (white arrow head). (Alcian blue)

(E) Chief cells are stained deep blue (black arrow heads). (Bromophenol blue)

(F) Fine collagen fibers in the lamina propria and tunica sub mucosa (black arrow heads). (Masson’s trichrome)

(G) Fine elastic fibers in the lamina propria and tunica sub mucosa (black arrow heads). (Orcein)

Fig. 3 Transverse section in fundic region of fetal rabbit stomach at 25th gestational day showing:-

(A) Openings of gastric pits (black arrow heads), branched gastric glands (white arrow heads), lamina muscularis mucosa (Mm) and interrupted bands of circular smooth muscle fibers of tunica muscularis layer (Ms) (Haematoxylin and Eosin)

(B-C-D) Numerous parietal cells appear highly acidophilic (black arrow heads). (Haematoxylin and Eosin)

(E-F) Positive PAS intra cellular granules at the apices of surface epithelial cells (black arrow heads) and positive thick mucus layer covers the mucosa (white arrow head) (Periodic acid–Schiff)

(G) Positive alcian blue intra cellular granules at the apices of surface epithelial cells (black arrow heads) and positive thick mucus layer covers the mucosa (white arrow head) (Alcian blue)

(H-I) Chief cells are deep blue (black arrow heads). (Bromophenol blue)

(J) Fine elastic fibers in the lamina propria and tunica sub mucosa (black arrow head). (Orcein)

Fig. 4 Transverse section in pyloric region of fetal rabbit stomach at 25th gestational day showing:-

(A) Openings of gastric pits (black arrow heads), gastric glands (white arrow heads), lamina muscularis mucosa (Mm), and interrupted bands of circular smooth muscle fibers of tunica muscularis layer (Ms). (Haematoxylin and Eosin)

(B-C) Higher magnification of the pyloric glands. Note the gastric epithelial cells appear columnar with acidophilic cytoplasm and basal basophilic nuclei. (Haematoxylin and Eosin)

(D) Positive PAS intra cellular granules at the apices of surface epithelial cells (black arrow heads) and positive thick mucus layer covers the mucosa (white arrow head). (Periodic acid–Schiff)

(E) Positive alcian blue intra cellular granules at the apices of surface epithelial cells (black arrow heads) and positive thick mucus layer covers the mucosa (white arrow head). (Alcian blue)
(F-G) Chief cells (black arrow heads), and some of surface epithelial cells appear deep blue (white arrow heads). (Bromophenol blue)

(H) Well recognized elastic fibers in the lamina propria and sub mucosa (black arrow heads). (Orcein)

(I) Well recognized collagen fibers in tunica sub mucosa (black arrow head). (Masson’s trichrome)

Fig. 5 Transverse section in cardiac region of fetal rabbit stomach at 29th gestational day showing:

(A) Connecting area of the esophagus (E) and cardiac region (C) of stomach (cardiac opening). Note the clear gastric rugae (black arrow head). (Haematoxylin and Eosin)

(B) Higher magnification of gastric rugae (GR). (Haematoxylin and Eosin)

(C) Wide gastric pits of the glands (star), surface mucous cells (white arrow head), and mucous neck cells (black arrow head). (Haematoxylin and Eosin)

(D-E) Branched tubular glands (white arrow heads) and parietal cells (black arrow heads). (Haematoxylin and Eosin)

Fig. 6 Transverse section in cardiac region of fetal rabbit stomach at 29th gestational day showing:

(A) Positive PAS intra cellular granules at the apices of surface epithelial cells and mucous neck cells in the lumen of gastric pits (black arrow heads). (Periodic acid–Schiff)

(B) Positive alcian blue intra cellular granules at the apices of surface epithelial cells (black arrow heads). (Alcian blue)

(C-D) Chief cells are deep blue (black arrow heads). (Bromophenol blue)

(E) Well recognized collagen fibers in tunica sub mucosa (black arrow head), and thick muscularis layer (Ms). (Masson’s trichrome)

(F) Well recognized elastic fibers in the lamina propria and tunica sub mucosa (black arrow heads). (Orcein)

Fig. 7 Transverse section in fundic region of fetal rabbit stomach at 29th gestational day showing:

(A) Mucous neck cells (white arrow head), parietal cells (black arrow heads), lamina muscularis mucosa (Mm) and longitudinally arranged muscularis layer (Ms) and serosa (Se). (Haematoxylin and Eosin)

(B) Positive PAS intra cellular granules at the apices of surface epithelial cells and mucous neck cells in the lumen of gastric pits (black arrow heads). (Periodic acid–Schiff)

(C) Positive alcian blue intra cellular granules at the apices of surface epithelial cells and mucous neck cells in the lumen of gastric pits (black arrow heads). (Alcian blue)

(D) Chief cells are deep blue (black arrow heads). (Bromophenol blue)

Fig. 8 Transverse section in pyloric region of fetal rabbit stomach at 29th gestational day showing:

(A) Connecting area of the pyloric region of the stomach (P) and duodenum (D) (pyloric sphincter). (Haematoxylin and Eosin)

(B) Positive intracellular granules at the apices of gastric epithelial cells and mucous neck cells (black arrow heads). (Periodic acid–Schiff)

(C) Positive intracellular granules at the apices of gastric epithelial cells (black arrow heads). (Alcian blue)

(D) Chief cells are deep blue (black arrow heads). (Bromophenol blue)
(E) Well recognized elastic fibers in the lamina propria and tunica sub mucosa (black arrow head) (Orcein)

Fig. 1
Fig. 2

Fig. 3
Fig. 6

Fig. 7
4. Discussion
The present study has been achieved as an attempt to investigate the normal development of rabbit gastric glands from the early embryonic stage to the late fetal stage. The present investigation showed that on the 21st gestational day, the different parts of the stomach including, cardia, fundus, body, and pylorus could be easily distinguished. The gastric mucosa was thrown into distinct folds and consisted of lamina epithelialis, lamina propria, and muscularis mucosa. Similar findings were observed by (Hayward, 1967) and (Yaman and Girgin, 2001).

In the present study, the gastric epithelium at 21st gestational day was variable from stratified to simple columnar and their cytoplasm contained PAS-positive granules. Similar findings were observed by (Yaman and Girgin, 2001) and (Menzies, 1958) who found PAS-positive few granules and rings in the supranuclear region of a few cells.

The gastric pits, in the present study, were firstly recognized on the 21st day of gestation and they led to shallow primitive tubular or alveolar gastric glands. These primitive glands were lined by undifferentiated columnar cells. The mucosal epithelium was stratified and developing primary glands were first observed at this period. Similar observations were recognized by (Yaman and Girgin, 2001) on rabbits, (Esrefoglu et al., 2017) in rat on the 20th day of development and by (Hiroshi Fukamachi, 1979) in mice fetuses on the 15.5th gestational day. (Helander, 1969b) found that, at the 19th day old rat fetuses, the glandular lumens extended a short distance into the mucosa, but the basal half of the epithelium is still compact. Nalnophakoon, 1984, in pig fetuses, added that the gastric pits were formed as a result of depression in gastric mucosa toward the basement membrane and the expanded portion of the pit formed the gastric glands. The present study revealed that on the 25th developmental day, the gastric mucosal folds
were more prominent. The gastric surface epithelial cells appeared columnar with basally located nuclei and acidophilic cytoplasm. The surface epithelium was interrupted by the openings of the gastric pits which were more prominent and numerous than previous age. PAS and alcian blue positive intracellular apical granules and thick mucous layer covering the mucosa were demonstrated in all gastric regions. Similar observations were found by (Esrefoglu et al., 2017) in rat on the 20th day of development. (Madge, 1975) interpret that, the gastric mucus contains several mucoproteins and mucopolysaccharides. The mucus prevents the stomach wall from digesting itself by forming two layers, one over the surface of the gastric mucosa, and the other in-between the mucosal cells.

As revealed by the present study, parietal and chief cells were firstly recognized on the 25th developmental day within the lining epithelium of the gastric glands at all gastric regions. Parietal cells occupied peripheric position at the body and base of the gland and appeared as sporadic, large cells bearing deeply acidophilic cytoplasm and relatively small and rounded nuclei. While chief cells were located near the bottom of the gland and appeared rounded or polyhedral cells with centrally located nuclei. These findings supported by (Hayward, 1967) who observed the parietal cells on the 24-day old rabbit fetuses, and by (Menzies, 1958) who noted the first parietal cells appeared on the 23rd day of the fetal life of the rabbit and the first peptic cells appeared a few hours after birth, whereas some of the others report that they do not have that activity until birth (Salenius, 1962). Chimmalgi & Sant, 2005 and Keene & Hewer, 1929 support our result that the peptic cell became active before birth. They also added that eosinophilic cells appeared at the bottom of the gastric glands while in the upper portion of the glands mucous secreting columnar cells with basal vesicular nucleus were seen. A few cuboidal cells with rounded nuclei and granular cytoplasm, stained purplish with haematoxylin and eosin were recognized in the bottom of the gastric glands. These mark the first appearance of chief cells. (Helander, 1969b) mentioned that, carbonic anhydrase activity was first demonstrated in rat embryos on the 20th day of gestation. The present study revealed that at 29th gestational day, the gastric pits were relatively wider than previous age, and were invaginated to various extends in the lamina propria and leading to the branched tubular gastric glands.

In the cardiac region, gastric glands appeared as highly coiled, highly branched tubular glands, while in fundus and body, the gastric glands appeared as branched tubular glands, however, in pylorus the gastric glands still appeared as simple tubular glands. The same results were reported by (Leeson et al., 1985).

The present study revealed that parietal cells occupied a peripheral position and appeared as large triangular cells with rounded nuclei and deeply acidophilic cytoplasm. (Madge, 1975) interpret that, the hydrochloric acid can penetrate the mucus barriers. The HCL prevented from damaging the cell cytoplasm as a result of the buffering action of alkaline electrolytes trapped within the mucus layer. Mucus also can deactivate the pepsin. The chief cells were located near the bottom of the gland and gave a strong bromophenol blue positive reaction Chimmalgi & Sant, 2005 recorded same result.

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