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Mucosal- Submucosal Changes in Rabbit Duodenum during Development

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***Abstract*—**The sequential morphologic changes of rabbit duodenal mucosa-submucosa were studied from primodial stage to birth in 15 fetuses and during the early days of life in 21 rabbit newborns till maturity using light, scanning and transmission electron microscopy. Fetal rabbit duodenum develops from a simple tube of stratified epithelium to a tube containing villus and intervillus regions of simple columnar epithelium. By day 21 of gestation, the first rudimentary villi were appeared and by day 24 the first true villi were appeared. The Crypts of Lieberkuhn did not appear until birth. By the first day of postnatal life the duodenal glands appeared. The histological maturity of the rabbit small intestine occurred one month after birth. In conclusion, at all stages, the sequential morphologic changes of the rabbit small intestine developed to meet the structural and physiological demands during the fetal stage to be prepared to extra uterine life.

***Keywords*—**Duodenum, mucosa, submucosa, morphogenesis, rabbit.

I. Introduction

THE demands to the small intestine are minimal in the fetus, on exposure of the newborn to the external environment, the small intestine is presented with virtually all the nutrients required by the rapidly growing newborn. So that, immediately after birth, the small intestinal mucosa must be capable of regulating intestinal absorption not only to maintain nutrition and homeostasis, but also to facilitate the rapid growth of the developing organism. This requires that by birth the intestinal mucosa be highly differentiated and capable of many of the physiological and biochemical functions which characterize the small intestinal mucosa of the adult [1]. The mammalian fetal small intestine undergoes extensive morphologic development during gestation to be prepared for extra uterine life [2]. Associated with marked alterations in the structure of the small intestine during development, there are profound changes in its biological capabilities [3], [4]. Although the duodenum is a tiny fraction of the small intestine, it is the site of most of the breakdown of the food passing through it [5].

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The duodenal wall has submucosal glands which contain neutral or acidic mucin glycoproteins or the combination of both type of mucins [6], [7], [8], [9], [10], [11].

As an economic animal, especially for meat and fur production, the natural development of rabbit duodenum is more important specially the mucosa (villi) for absorption, submucosa (glands) for enzymatic digestion. We aim to re-evaluate the morphogenesis of duodenal mucosa and submucosa using light and electron microscope from early prenatal life till maturity. Since, to our knowledge, studies published on the morphological aspect of the rabbit duodenum during the pre- and postnatal life were very little, the current study was conducted to add further information in this issue.

II. Materials and Methods

A. Animals

Embryos, fetuses, newborn and adult V-line white rabbits were obtained from the farm of Faculty of Agriculture, Alexandria University. The rabbits were mated and the dates were recorded considering the day of mating to be the day 0. The female were then segregated for getting the prenatal samples. Postnatally, the day of delivery was considered the day 0 of postnatal life. The rabbits were housed in separate cages with 12 hour light/12 hour dark cycles and allowed free access to water and rations.

B. Histological Technique

1. Samples

The pregnant rabbits, at different stages of pregnancy, starting from the embryonic day 15 (E15), E18, E21, E24, E27, three embryo in each age (n=15) and full term, were slaughtered and the embryos and fetuses were removed. In case of fetuses under E22, the whole abdominal cavity was fixed in 10% neutral buffered formalin, Bouin's solution. In case of older fetuses the whole gastrointestinal tract (GIT) was dissected out after incision of the abdominal wall and preserved in the same fixatives. After the initial period of preservation, the small intestine was dissected and samples were taken from duodenum. Postnatally, samples were taken from apparently healthy animals aged 1, 4, 7 days old, 2 weeks, 1 month, 2 and 6 months old rabbits, three animals of each age (n=21). The animals were slaughtered and the duodenum was dissected out and fixed in the same fixative. Procedures involving animals and their care were conducted in conformity with the standards for animal experiments and are in compliance with the NIH Guide for the Care and Use of Laboratory Animals (1996).

2. Processing and Staining

The samples were then, dehydrated in ascending grade of ethyl alcohol, cleared using xylene, embedded in paraffin and blocked. Thin sections (3-7 µm thick) were prepared and mounted on egg albumin-glycerin coated glass slides, dried and stained with the following stains; Hematoxylin and eosin (H and E) for general inspection of the organs. Combination of Periodic Acid Schiff reaction (PAS) and Alcian blue (AB) for the demonstration of the neutral mucins and acid mucins [12].

C. Scanning Electron Microscopical Examination

For scanning electron microscope (SEM), 6 samples from duodenum of rabbit fetus at E27 and from 2 months old rabbit, three of each age was used. The samples were immediately immersed in 4F1G fixative (2% formaldehyde, 1.25% glutaraldehyde in 0.1 M sodium cacodylate) at pH 7.2 and stored at 4 ºC. The fixed samples were washed in 0.1 M cacodylate containing 5% sucrose processed through tannic acid, dehydrated in graded ethanol series. The critical point dried samples (with carbon dioxide) were then attached to stubs with colloidal carbon and coated with gold palladium in a sputtering device. The samples were examined and photographed with Jeol SEM operating 15Kv in the Faculty of Science, Alexandria University.

D. Transmission Electron Microscopical Examination

For transmission electron microscopic examination (TEM), samples from duodenum of fetuses (at E27) and from 2 months old rabbits were used. Pieces of 1 mm3 were cut then fixed immediately for 2 hours at 40C in 6% solution of phosphate buffered glutaraldehyde at pH 7.4 [13]. After initial fixation, the tissue were washed in several changes of cold (40C) 0.1 M. phosphate buffer every 15 minutes for 2 hours. The tissue was post fixed in 1% solution of osmium tetroxide in cold (40C) 0.1 M phosphate buffer (pH 7.2) for 2 hours. Then they were rapidly dehydrated through ascending grades of ethyl alcohol then transferred to propylene oxide and placed overnight in a 1:1 mixture of propylene oxide and epoxy araldite. Then they were embedded in epoxy araldite [14]. Semithin sections (1µm) were cut firstly and stained with toludine blue and viewed with light microscope to select a suitable area for electron microscope examination. Then ultrathin sections (60-100 nm) were cut by a glass knife with LKB ultramicrotome then they were stained with uranyl acetate followed by lead citrate [14]. The sections were examined with Jeol 100 CX electron microscope in the Faculty of Medicine, Tanta University.

III. Results

Fig. 1 shows the dramatic growth which occurred in fetal rabbit duodenum between the E15 and E27 of gestation. The duodenum of fetal rabbits on the fifteenth and eighteen day of gestation was a simple tube with a tiny elliptical lumen surrounded by stratified epithelial cells two to three layers in thickness. The basal aspect of the deepest layer of the epithelium was surrounded by concentrically arranged mesenchymal cells (Fig. 1A). The lumen gradually became triangular in shape with epithelial ridges. The mesenchymal tissue interfered between the ridges forming the lamina propria. At E21, the epithelial ridges increased in number with mesenchymal cores of the lamina propria. These epithelial outgrowths were considered to be the first rudimentary villi where the lining epithelium gradually became a single layer (Fig. 1B). Few smooth muscle layers were first seen at this stage following the lamina propria. At E24, the duodenal epithelial outgrowths were elongated, and the number of sectioned villi increased. The villi were covered by single layer of epithelial cells with centrally located nuclei however some of the intervillus epithelium remained stratified (Fig. 1C). At E27, the duodenal villi increased in length and the whole duodenal epithelium became single layer of columnar cells. Few scattered goblet cells in the villus epithelium stained PAS positive could be seen (Fig. 1D).

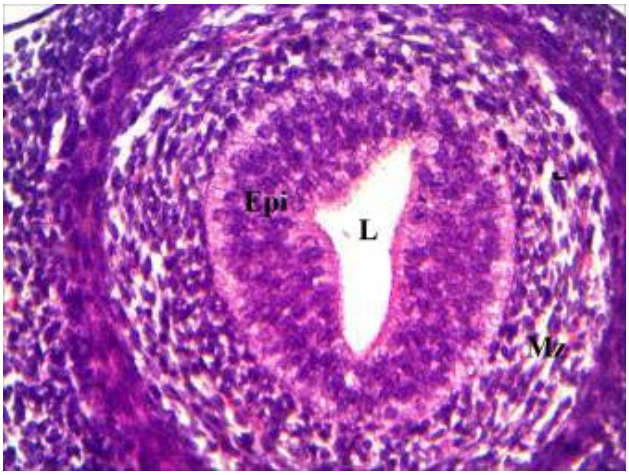

Fig. 1A The duodenal wall at its earliest stage of development consisted of elliptical lumen (L) surrounded by stratified epithelium (Epi) and mesenchymal tissue (Mz). X40

The scanning electron microscope showed the duodenal villi irregular, finger-like with globular surface. The villus wall had well-ordered hexagonal epithelial cells and goblet cells in between, and the surface of the epithelial cell was adorned with microvilli (Fig. 2A and B). Under transmission electron microscope, the columnar epithelial cells (surface enterocyte) of the duodenal villi had short irregular microvilli with a distinct glycocalyx and long core of actin filament. There were numerous round mitochondria filled the apical cytoplasm and around the nucleus together with rough endoplasmic reticulum. The basally located nuclei were large, ovoid, with condensed and diffuse chromatin. The cytoplasm possessed small vesicles near the junctional areas and under the microvilli and evenly distributed glycogen granules. The intercellular borders below the junctional complex appeared interdigitated (Fig. 3A and B). The presence of goblet cells and enteroendocrine cells was confirmed at the stage of duodenal epithelium differentiation. The goblet cells at this stage of development began to differentiate and filled with supranuclear accumulation of electron dense granules which

differed in size. Some of these cells reached the surface with small dispersed microvilli in the apical part, and the others did not reach the surface. The enteroendocrine cells at this stage were located in between the enterocytes and characterized by a low cell to nucleus ratio and diffuse nuclear chromatin. We noticed the presence of basal small heterogeneous, electron dense granules in these cells (Fig. 3C and D).

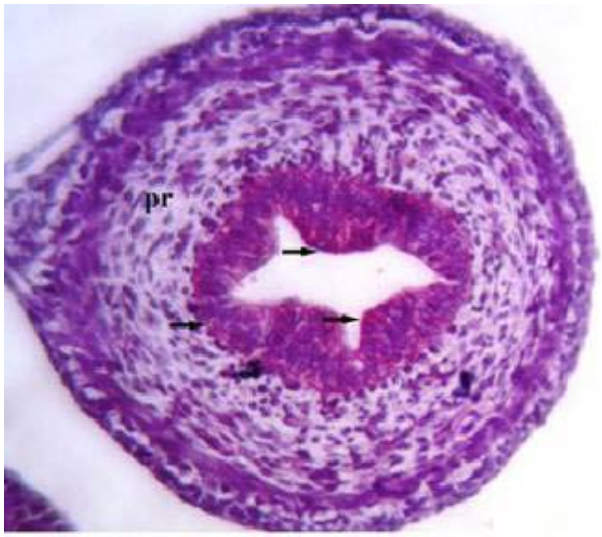

Fig. 1B At E21 the duodenal wall showed folded mucosa (arrows) surrounded by CT of lamina propria submucosa (pr). PAS X40

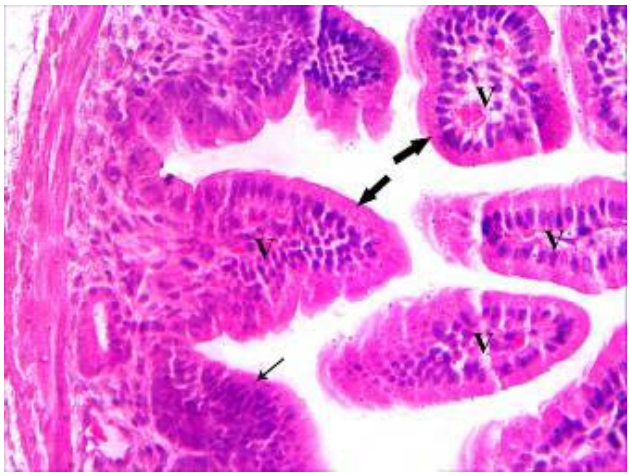

Fig. 1C At E24 the duodenal wall showed primitive villi (V) covered by simple columnar epithelium (broad arrow). H and E X40

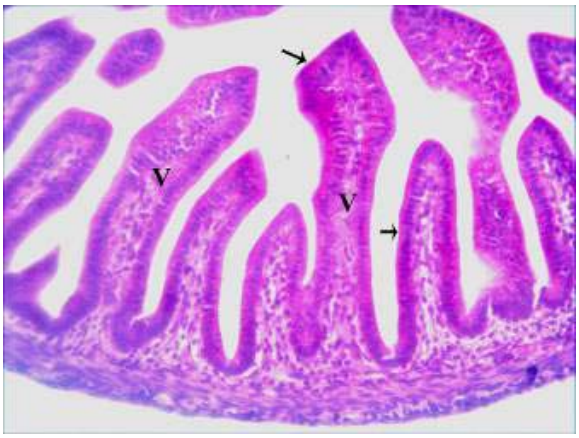

Fig. 1D At E27, The duodenal villi increased in length and covered by single layer of columnar cells (arrow). Few scattered goblet cells stained PAS positive could be seen. PAS X40

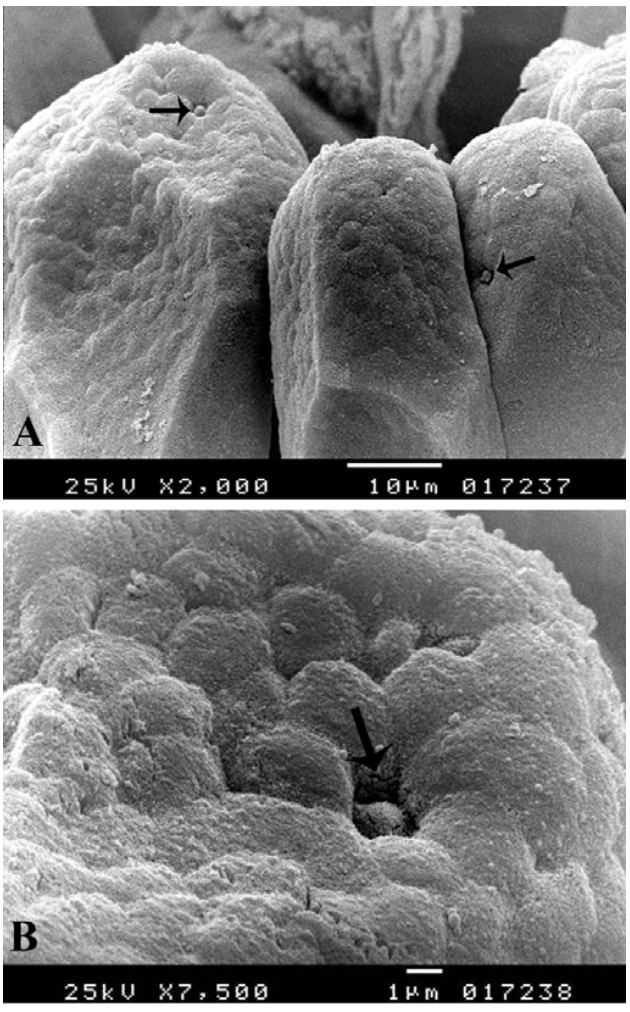

Fig. 2 At E27 the duodenal villi were irregular, finger-like with globular surface and goblet cells (arrow) in between

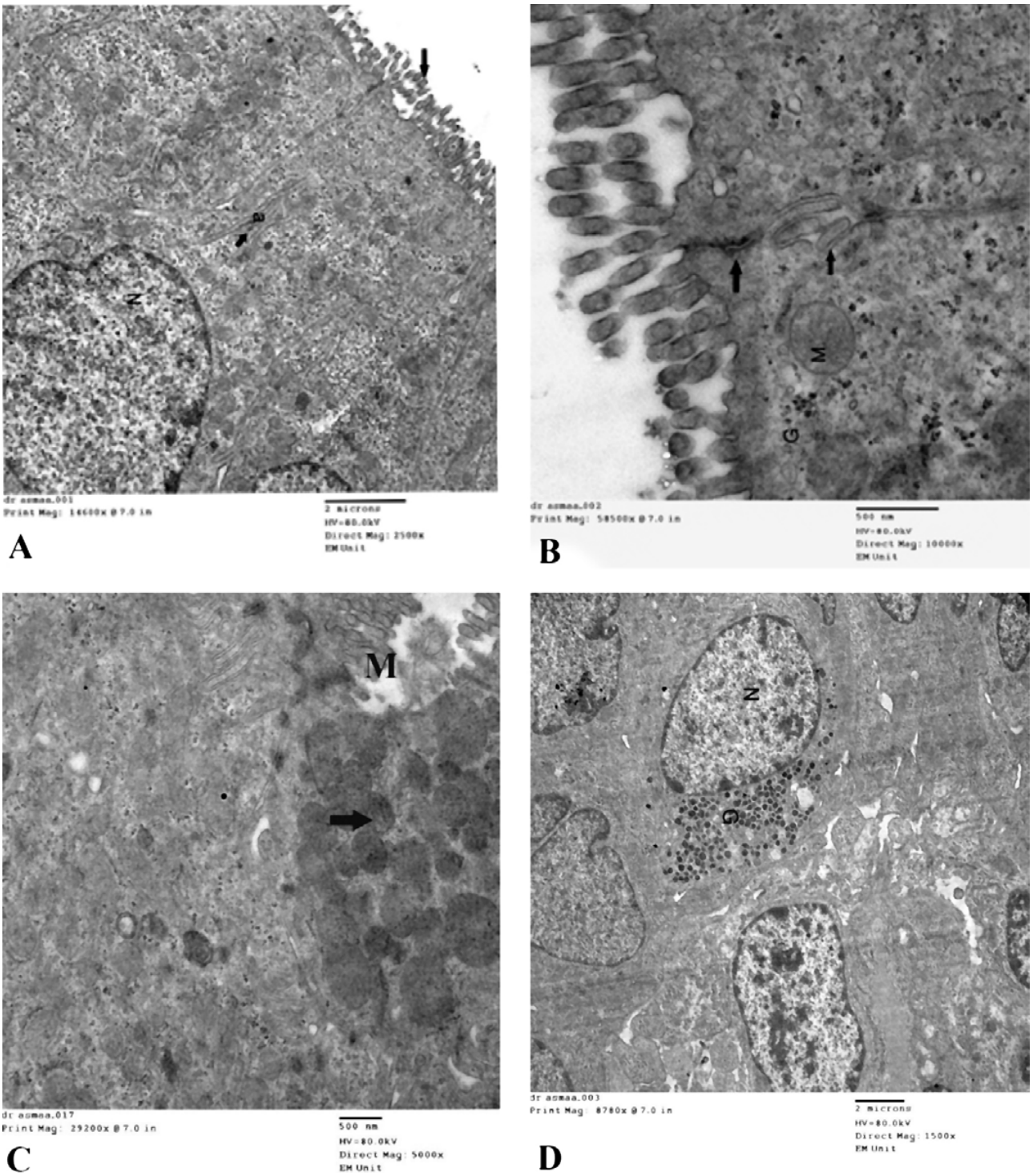

Fig. 3 Showing the ultrastructure of duodenal epithelium at E27 of rabbit's fetal life. Notice the microvilli on the surface, the junctions between enterocytes in A and B, the goblet cell with its secretory granules in C and the enteroendocrine cells in D

Primitive crypts of Lieberkuhn appeared in the duodenal mucosa just after birth, at one day old, in the form of invaginations at the base of villi. We noticed the submucosal duodenal glands (of Brunner) for the first time at 4 days-old.

The glandular epithelium was of columnar type and appeared lighter than that of the crypts (Fig. 4).

At one month-old rabbit, the duodenal wall became histologically mature with broader villi those had goblet cells positive for PAS-AB at pH 2.5. The Brunner's glands increased in number and composed serous and mucous cells.

The mucous cells in the acini of Brunner's glands stained dark with alcian blue at pH2.5 while serous cells gave a positive reaction with PAS (Fig. 5).

Under SEM, the duodenal villi of two months old rabbit became broader and tongue-like. The surfaces of the tongue-shaped villi showed many irregular folds that branched and anastomosed subdivided the surfaces into many polygonal areas. The goblet cells were evenly distributed along the surface of the villi and were active in mucus secretion (Fig. 6A and B). Under the transmission electron microscope, surface enterocyte of the duodenal villi showed numerous, closely packed, tall, uniform and parallel microvilli. There was a decrease in the glycogen particles or totally absent. The crypt cells showed fewer microvilli than that of the epithelial cells and junctional complex on their lateral surfaces. The goblet cells appeared mature, with a typical goblet shape, extensive mucin droplets with moderate electron dense components, and basally located nucleus with small dispersed microvilli in the apex (Fig. 7A and B). Some secretory units of the duodenal glands were mucous with serous demilunes (mixed in type) and the others were serous. The lumen had scattered, short microvilli on the surface of the lining cells. In the mucus cells, the nucleus and the majority of organelles were situated in the basal cytoplasm while the apical cytoplasm was occupied by the secretory granules.

The basal cytoplasm contains rough endoplasmic reticulum and nucleus, with condensation of chromatin material (Fig. 7C and D). From six months-old and later, the small intestine did not show any further developmental changes.

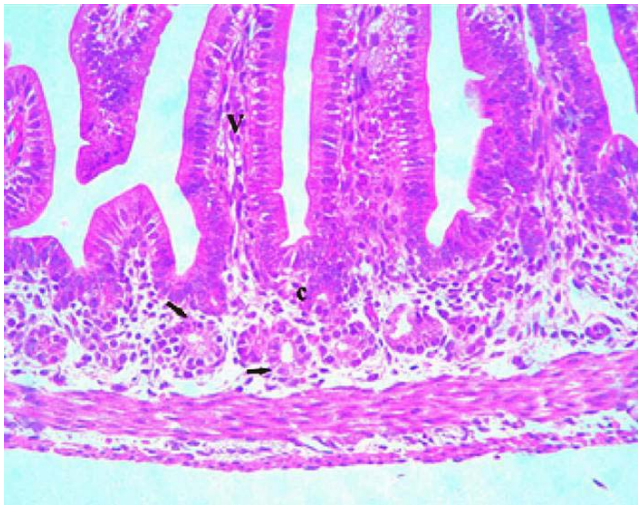

Fig. 4 The duodenal mucosa and submucosa showed the primitive crypts of Lieberkuhn (c), long villi (V). The submucosal duodenal glands (of Brunner) recorded for the first time at 4 days. H&E.X40

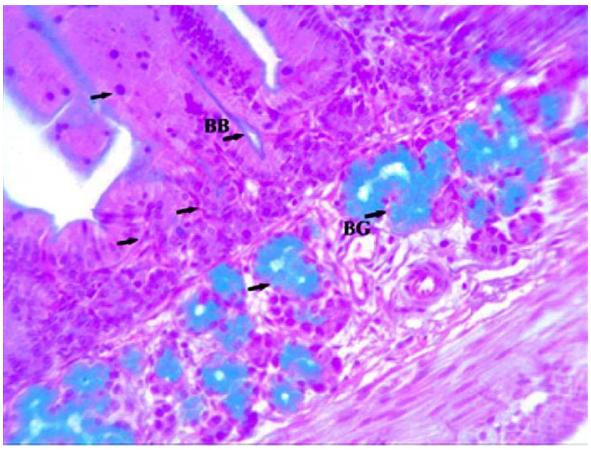

Fig. 5 The duodenum at one month showed the goblet cells with AB positive (arrow), Brush border (BB), Brunner's glands (BG), luminal edge. PAS-AB combination X40

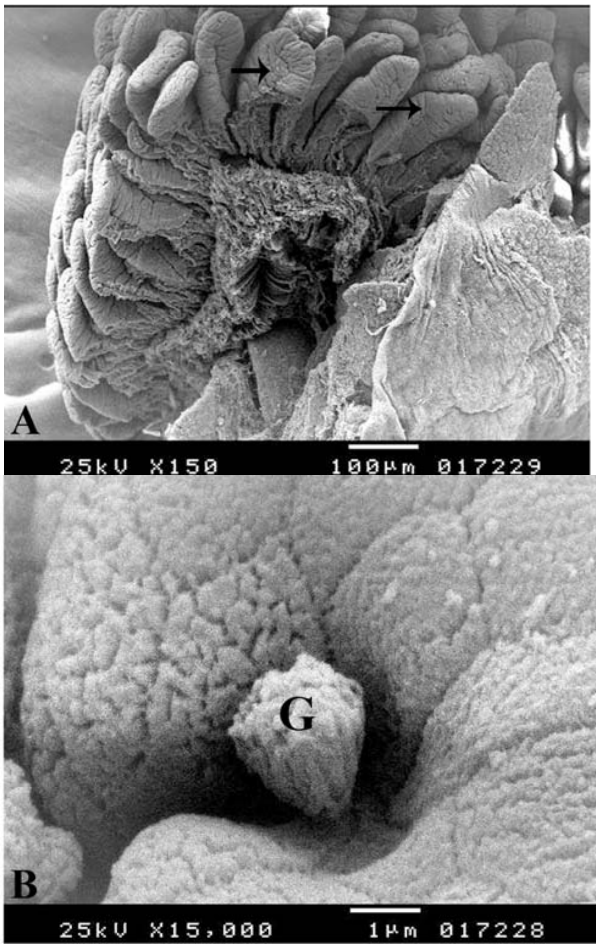

Fig. 6 The duodenal wall at two months old rabbit showed broader and tongue-like. The surfaces of the tongue-shaped villi show many irregular folds in A and goblet cells on the top of the villi in B containing mucous (G)

IV. Discussion

In present study the duodenal mucosa of rabbit showed rapid developmental changes only in the late gestation and in the newly born animals; although the intestinal glands were differentiated and still in the primitive stage. A similar developmental picture has been described in the mouse where the complete mucosal components were not present until the neonatal period [15]. Moreover in the human [16], bovine and ovine fetuses [17], [18] the development of mucosal structures started as early as the first third of gestation, but develops less rapidly where the different mucosal structures are present and fully mature by late gestation and at birth. This was dissimilar to our findings in rabbit as the villi, the crypt as well as the glands began in the late gestation or just after birth and became mature at one month old. This is may be attributed to that, the rabbit are gradually deprived of their maternal supply of nutrients after birth till one month and henceforth depend on their own alimentary tract digestion and absorption. So the development of the crypt and the gland become more rapidly after birth depending on the degree of tissue and cellular differentiation which has taken place. This development is correlated to the length of the gestation period.

The present study revealed that the rabbit duodenal mucosa did not form villi directly, but first formed multilayered epithelial ridges at E18, which in turn transformed into primitive villi with a stratified epithelium and a central core of mesenchyme at E21. A similar developmental pattern was reported by [19] in rabbits. In an accord, in mouse [20], rat [21], [22], [27] in chick intestine [21], [23], [24], [25] the morphogenesis of intestinal mucosa does not start by forming pre-villus ridges which then segment, but immediately forms epithelial elevations which project into the lumen and later acquire a core of mesenchyme. [20] in mouse suggested that the dynamic interaction between two layers growing at different rates plays an important role in the morphogenesis of the intestinal wall. The outer layer might exert a restraining action on the rapid growth of the inner layer, thus contributing to the folding of the mucosa.

By E24, we found that the primitive villi elongated, acquired the shape of the true villi and covered with one layer of epithelial cells together with the appearance of smooth muscles following the lamina propria. These finding was similar to those described by [3] in the fetal rabbit. It was suggested that the developing smooth muscle layer (musculature) caused this transformation of ridges into true villi by forcing the epithelium to buckle and this is the same as suggested in chicken by [23] but substantial evidence was presented later that the mesenchyme was essential for this development and that the mechanism was intrinsic to the epithelium [24], [28].

The present study revealed that under SEM, the surface of the duodenal villi shows a globular appearance at E21. This was similar to that found in rat by [29] who suggested that this globular appearance probably reflects a dynamic turnover of epithelial cells during the remodeling of the finger-like villi.

The shape of the rabbit duodenal villi at birth (one day-old) was regular and finger-like and their density increased. These findings were similar to that described in the early postnatal period in piglets [30], [31]. The last author suggested that these seem to be associated with mucosal stretching, matching the dramatic increase in length and width of the entire intestine. With increasing age, the duodenal villi become tongue-shape with a hexagonal arrangement of the epithelial cells. At the tips of the villi the epithelial cells often take a round or convex form. Similar observations were reported in mouse [32], [33], [34], [35]. These authors suggested that, these features probably reflect the fact that the epithelial cells are continuously shedding from the tips of the villi in the cycle of epithelial replacement.

In the present study the development of the Brunner's glands was independently of the crypts of Lieberkuhn. Similar observations were reported by [19] in rabbit. In contrast [27] in rat and [36] in human mentioned that the duodenal glands (Brunner's gland) sprout off from the crypt of Lieberkuhn as double outgrowths. In classic carbohydrate histochemistry, a positive PAS reaction indicate the presence of neutral carbohydrate, while positive alcian blue reactions at pH 2.5 indicate the presence of acidic sulphated and acidic carboxylated residues [37]. In the present study the mucous cells and the excretory ducts of Brunner's gland did not react with PAS; they reacted positively with alcian blue at pH2.5 and the opposite for the serous cells. These findings were similar to that observed in the Angora rabbit [38], domestic rabbit and American (Cottontail) rabbit [7]. This may refer to that the mucous cells in the acini of Brunner's glands contained neutral, carboxylic and sulpho acidic mucin, while serous cells contain neutral mucopolysaccharides [38], [39]. By transmission electron microscope, our results revealed that, at the late stage of gestation, the enterocyte of the duodenal villi had short irregular microvilli, with a distinct glycocalyx and long actin filament in their core, and there were small vesicle in the apical cytoplasm. Small apical granules in the intestinal crypt cell were noted in mouse [40], [41], in rat [42]. Two contradictory views were proposed as to nature of these granules; [41] regarded them as secretory granules, while [42] suggested an absorptive function. Additionally, [43] reported that apical vesicles engaged in the transport of the IgA-secretory component complex. Whereas [44] suggested that surface-forming vesicles supply the plasmalemmal component and glycocalyx for the formation of microvilli. The increase in the number and length of the microvilli postnatally more than prenatally and from the crypt to the villus suggest a relationship between the position of the epithelial cells and the functional capacities of these cells. A similar relationship has been noted by [45] and by [46] for cells of the jejunum. The mucosal development was always more advanced in the cranial parts of the rabbit fetal small intestine than in the more caudal regions.

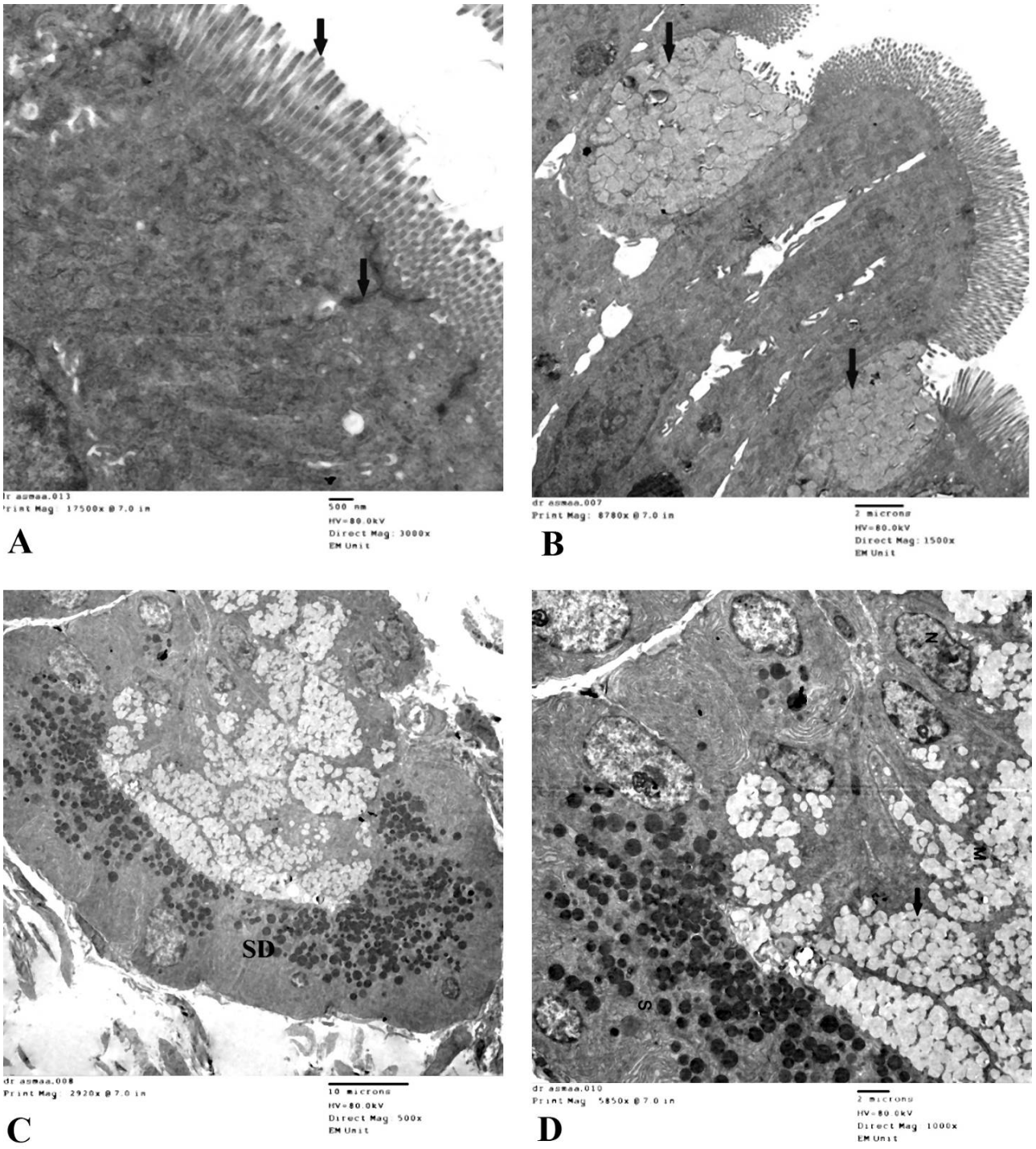

Fig. 7 TEM of the duodenal epithelium and Brunner's glands showing the enterocytes with microvilli and goblet cells in A and B, the Brunner's glands of seromucoid type in C and D

A similar cranio-caudal gradient development was found also in ovine and bovine intestine [17], [18]. This may be attributed to that in the late gestation to early postnatal the newborn rabbit need the enzymatic secretions of the glands to digest the milk in the duodenum at first.

Electron microscopic studies of the rabbit Brunner's glands

have revealed fine structural characteristics of mucous and serous type. A similar result was found by [8], [47] in the rabbit, [48] in mouse [49] in cat and [8], [47], [50], [51] in horse. While, [52], reported that the glands in the Guinea pig are typically mucous in type. Also, [53], [7] in the studies of eight wild ungulate species native to North America these glands were consisting of only mucous cells. That may be correlated with the histochemical and physiological differences among species.

The luminal surface of the mucous cells was irregular due to the presence of short microvilli and the apical cytoplasm is occupied by the secretory products, with pale interior, arranged into discrete granules. Serous cells were present as complex of pyramidal cells at the blind ending to the mucous tubules giving the appearance of demilune. Their basal cytoplasm contains the nucleus, granular endoplasmic reticulum and the apical cytoplasm was occupied by secretory products. Similar results were found by [54] in rabbit, who found that mucous cells appear to possess features intermediate between those of rat and mouse and the apical cytoplasm, is occupied by secretory droplets which appear pale within their interior and after glutaraldehyde fixation the droplet usually are discrete but they show a tendency to fuse into complexes after direct fixation in osmium tetraoxide. These authors suggested that some of the morphological differences noted in the secretory product of glands of various species may be a result of different methods of preparation. The general morphogenesis pattern of the small intestinal mucosa of the rabbit in the present study during fetal and neonatal periods was similar to those described in other vertebrate species [26], although the chronologic sequences were different.

V. Conclusion

Finally we can conclude that the general pattern of morphogenesis of the duodenal mucosa of the rabbit in the present study during fetal and neonatal periods was similar to those described in other vertebrate species, although the chronologic sequences were different. The rapid developmental pattern of mucosal structures of small intestine of rabbits could be obviously recognized in the late gestation and in the new born animal. So the maturity of the rabbit small intestine became completed at one month age thus, the young rabbit can change the feed behavior from milk suckling to ration eating.

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