P.O.Box 2345, Beijing 100023,China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

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• BASIC RESEARCH •

Morphological changes of cell proliferation and apoptosis in rat jejunal mucosa at different ages

Li Wang, Jian Li, Qing Li, Jian Zhang, Xiang-Lin Duan

Li Wang, Jian Li, Qing Li, Jian Zhang, Xiang-Lin Duan, Life Science College, Hebei Normal University, Shijiazhuang 050016, Hebei Province, China
Supported by the Natural Science Foundation of Hebei Province, No.303158; Education Department Foundation of Hebei Province, No.2002136
Correspondence to: Xiang-Lin Duan, Life Science College, Hebei Normal University, Shijiazhuang 050016, Hebei Province, China. duanxianglin@mail.hebtu.edu.cn
Telephone: +86-311-6269480 **Fax:** +86-311-5828784
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Abstract

AIM: To study the changes of cell proliferation and apoptosis in rat jejunal epithelium at different ages.

METHODS: Cell proliferation and apoptosis of the jejunal mucosal and glandulous epithelia from birth to postnatal 12th month were observed using immunocytochemistry (ICC), and TUNEL method. The height of villus, the thickness of muscle layer and the number of goblet cells in jejunal mucosal and glandulous epithelia were measured by BeiHang analytic software and analyzed by STAT.

RESULTS: (1) Proliferating cell nuclear antigen (PCNA) positive cells of jejunal glandulous recess were found and increased in number from birth to the postnatal 3rd month. The number of PCNA positive cells peaked in the postnatal 3rd month, and decreased from then on. (2) The number of apoptotic cells also peaked in the postnatal 3rd month, showing a similar trend to that of the PCNA positive cells. (3) The height of jejunal villus increased after birth, peaked in the postnatal 3rd month and decreased from then on. The jejunal muscle layer became thicker in the postnatal 3rd week and the postnatal 12th month. The number of goblet cells of the jejunal mucosal and glandulous epithelia had a linear correlation with age.

CONCLUSION: (1) PCNA positive cells are distributed in the jejunal glandulous recess. (2) Apoptotic cell number peaks in the postnatal 3rd month, indicating that cell proliferation and apoptosis are developed with the formation of digestive metabolism as rat grows to maturity. (3) The thickness of jejunal muscle layer increases to a maximum in the postnatal 3rd week, which may be related to the change in diet from milk to solid food. (4) The number of goblet cells increases rapidly in the postnatal 3rd week, probably due to ingestion of solid food.

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INTRODUCTION

The small intestine is the primary digestive apparatus of mammals, and nutrient absorption is ongoing mostly via intestinal epithelium. Mathan *et al* in 1976[1-4] observed the embryogenesis and postnatal change of rat duodenal villi using transmission electron microscope and scanning electron microscope respectively. Weinstein *et al*[5-7] described the ultrastructure and function of intestinal epithelial lining in 1981. In 1995, Gao *et al*[8-10] studied the epithelialization, expression pattern, and transformation of some enzymes of duodenum with histological methods. However, the status of proliferation and apoptotic changes in developing jejunal epithelial lining has not yet been elucidated. The present study was to provide a digestive physiological proof more directly in proliferation and apoptotic changes at four representative developmental stages in rats: birth, postnatal 3rd week, postnatal 3rd month, and postnatal 12th month.

MATERIALS AND METHODS

Materials

Sprague-Dawly rats (male, Grade II, Certificate *No* 04036, obtained from the Experimental Animal Center of Hebei Province) were divided into four groups: newborn (n=6), postnatal 3rd week (n=6), postnatal 3rd month (n=6) and postnatal 12th month (n=6). The rats were held for 12 hours without food. The mean weight was 5.6±0.3 g for newborn, 53.5±1.2 g for postnatal 3rd week, 390±0.9 g for postnatal 3rd month and 601.7±3.4 g for postnatal 12th month. The abdominal cavity was opened immediately after the rat was killed with ether. Several segments of jejunum (1-2 cm) were removed and placed immediately in a fixative consisting of 4 % paraformaldehyde and 0.01 mol/L phosphate buffered saline (PBS, pH7.4) (4 ℃, 12 h). The segments were used for TdT-mediated X-dUTP nick end labeling (TUNEL) examination and HE staining, and other segments were placed in a fixative consisting of Bouin's solution. Fixed jejunum segments were embedded in paraffin and continuously sliced up at 6 μm thickness and mounted onto glass slides covered with 3-aminopropyl-triethoxysilane (APES). They were dried at 37 ℃, and used for immunostaining of proliferating cell nuclear antigen (PCNA).

Reagents

Rabbit anti-rat PCNA antibody and SP kit were purchased from Beijing Zhongshan Biotechnology Company. TUNEL examination kit was purchased from Wuhan Boster Biological Technology Company.

HE staining

Several segments of jejunum were fixed in formalin and embedded in paraffin. Slides were cut and stained with hematoxylin and eosin according to routine methods.

Immunohistochemistry

Immunohistochemical staining for PCNA was performed using SP technique with following procedures. Mounted specimens were washed in 0.01 mol/L phosphate-buffered saline (PBS). Endogenous peroxidase was blocked by 0.3 % H_2O_2 in methanol for 25 min. Slides were washed with PBS followed by incubation in normal goat serum for 30 min at room temperature. The primary rabbit anti-rat PCNA antibody was

diluted 1:75 and applied to sections for 12 hours at 4 ℃. Slides were washed with PBS again and incubated with biotinylated secondary antibody for 60 min at 37 ℃. After rinsed in PBS, the slides were incubated with peroxidase-conjugated streptavidin for 60 min at 37 ℃. Colour reaction was performed by incubating the sections with diaminobenzidine-H_2O_2 for 5 min. The sections were counterstained with hematoxylin, dehydrated in gradient alcohol, and hyalinized in dimethylbenzene. Finally, the sections were mounted and observed under a microscope. PBS was used as a substitute for primary antibody as negative control.

TdT-mediated X-dUTP nick end labeling (TUNEL) examination technique

Mounted specimens were dewaxed and endogenous peroxidase was blocked by 3 % H_2O_2 in methanol for 10 min. After washed with water, 20 μl labeling buffer (1 μl TdT, 1 μl DIG-UTP, and 18 μl Labeling Buffer) was added to each section and incubated for 12 hours at 4 ℃. The sections were washed with 0.01 mol/L TBS and 50 μl blocking solution was added to each slide, followed by incubation for 30 min at room temperature. Biotinylated antidigoxin antibody was added to the slides for 30 min at 37 ℃. SABC was added for 40 min at 37 ℃ followed by washing with TBS. Then the specimens were incubated with diaminobenzidine-H_2O_2 for 5 min. After rinsed in tap water, the sections were counterstained with hematoxylin. Then they were dehydrated in gradient alcohol and hyalinized in dimethylbenzene. The sections were mounted and observed under a microscope. PBS was used as a substitute for primary antibody as negative control.

Data processing

The Northern image analyzing software was employed to analyze the stained area (the percentage of immunoreaction positive cells in total number of cells within a vision field). Three sections were selected from each experimental animal, and ten fields of vision from each section were inspected by statistical random sampling. The villus height was measured, the apoptotic cells under 16×40 double OLYMPUS microscope with testing resection and the number of goblet cells in unit area were counted[11]. The proliferation index was calculated. Under 15×10 double microscope, the thickness of muscle layer was measured according to the statistical random sampling with a micrometer, then the mean value was calculated. The values were expressed as $\bar{x}\pm s$. Statistical analysis was performed using the STAT software and analysis of variance was used as appropriate. $P<0.05$ was considered significant.

RESULTS

The number of jejunal mucosal goblet cells increased from birth to postnatal 3rd week and decreased from postnatal 3rd week to postnatal 3rd month, and then increased again after the postnatal 3rd month (Figure 1, Table 1). The number of

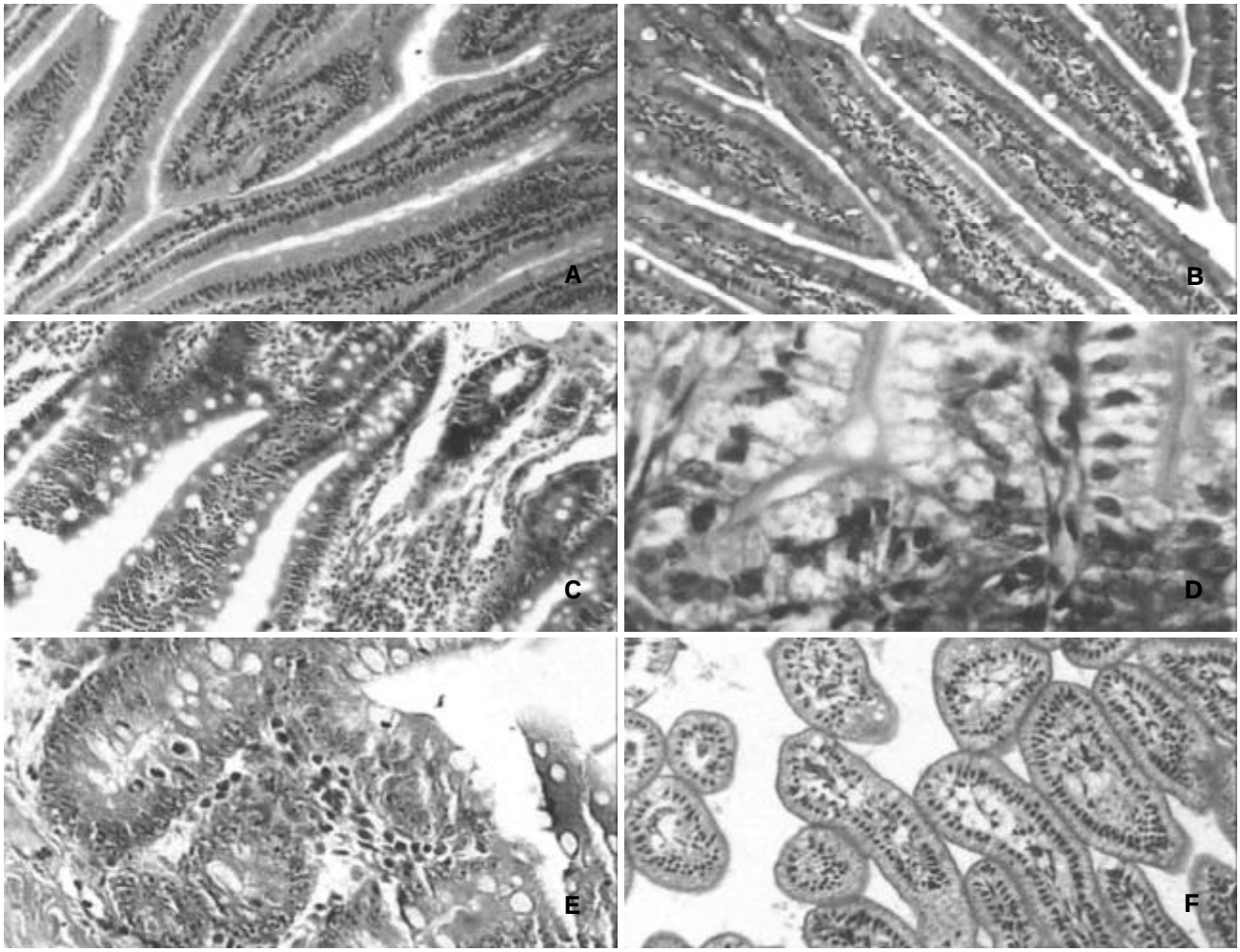

Figure 1 A: The number of goblet cells and the morphology of jejunal villi in the postnatal 3rd month ×100. B: The number of goblet cells in rat jejunal villi in the postnatal 3rd month ×100. C: The number of goblet cells and the morphology of jejunal villi in the postnatal 12th month ×100. D: There were few or no goblet cells in the jejunal glands of newborn rats ×400; E: The number of goblet cells in rat jejunal gland was much higher in the postnatal 12th month than in any other month ×400. F: The jejunal villi of newborn rats displayed ateliosis and were very small ×200.

glandulous epithelial goblet cells had a linear correlation with age (Figure 1), and there were significant differences between groups ($P<0.01$, Table 1). The height of jejunal villus increased from birth, peaked in the postnatal 3rd month and decreased from then on, and the villi were swollen in postnatal 12th month (Figure 1, Table 1). The thickness of jejunal muscle layer appeared crest on postnatal 3rd week and postnatal 12th month, the muscle fibers were quite dense in the postnatal 3rd month but very loose in the postnatal 3rd week (Table 1).

The immunostaining of PCNA positive cells was shown as light brown deposited in the nuclei. The PCNA positive cells in rat jejunum were mainly distributed in jejunal glandulous recess and proper lamina (Figure 2). The PCNA positive cells were shown to increase from birth to postnatal 3rd month, and decrease from then on (Figure 2, Table 2).

Table 2 Surface densities of PCNA-positive cells and the number of apoptotic cells at different stages ($\bar{x}\pm s$, $n=6$)

Age	Surface densities of PCNA-positive cells	The number of apoptotic cells
Newborn group	0±0	0±0
Postnatal 3rd week	0.022±0.012	9.71±2.43
Postnatal 3rd month	0.449±0.063[b]	38.83±7.41[b]
Postnatal 1 year	0.096±0.045[d]	11.36±3.14[d]

[b]$P<0.01$, *vs* Postnatal 3rd week. [d]$P<0.01$, *vs* Postnatal 3rd month.

The apoptotic cells were stained by TUNEL and positive cells were shown as dark brown in the nuclei. The apoptotic positive cells were mainly distributed in lamina propria (Figure 2). The number of apoptotic positive cells increased from newborn to postnatal 3rd month, and then decreased with aging. The pattern was similar to that of the PCNA positive cells (Figure 2, Table 2).

DISCUSSION

The goblet cell is a typical cell capable of excreting glycoprotein, containing a lot of spumous grume and granules of inhomogeneous electronic density. The jejunal glandulae of newborn rats could not develop but goblet cells can be seen in the villi. The number of goblet cells increased in the postnatal 3rd month, which might be related to the change in diet from milk to solid food. In the postnatal 12th month, the number of goblet cells increased again, and in this phase the increase of goblet cells led to the increase of mucus, which has the functions of lubricating mucosa and defending intestinal wall. The increasing change in the number of gerontic goblet cells might be a kind of protective mechanism against the weakening of excreting function of digestive gland in gerontic rat.

Cheng *et al*[12] considered that rat intestinal villi had perfectly developed on the 21st day of pregnancy, but there were only a few intestinal glands and few goblet cells. Our result was coincident with that of Cheng *et al*. We found that along with

Table 1 Developmental changes in cell type and morphology of rat villi after staining ($\bar{x}\pm s$, $n=6$)

Group	Number of villus goblet cells (entries/μm²)	Number of crypt goblet cells (entries/μm²)	Thickness of muscle layer (μm)	Villus height (μm)
Newborn group	4.89±2.67	0±0	32.0±15.71	315.20±43.74
Postnatal 3rd week	7.14±1.54[b]	4.47±1.10[b]	64.86±14.91[b]	532.75±53.36[b]
Postnatal 3rd month	4.44±1.11	5.27±0.93	56.34±15.41	629.64±48.22
Postnatal 1 year	9.31±1.50[d]	6.49±1.98	60.48±11.93	510.95±82.93[d]

[b]$P<0.01$, *vs* Newborn group. [d]$P<0.01$, *vs* Postnatal 3rd month.

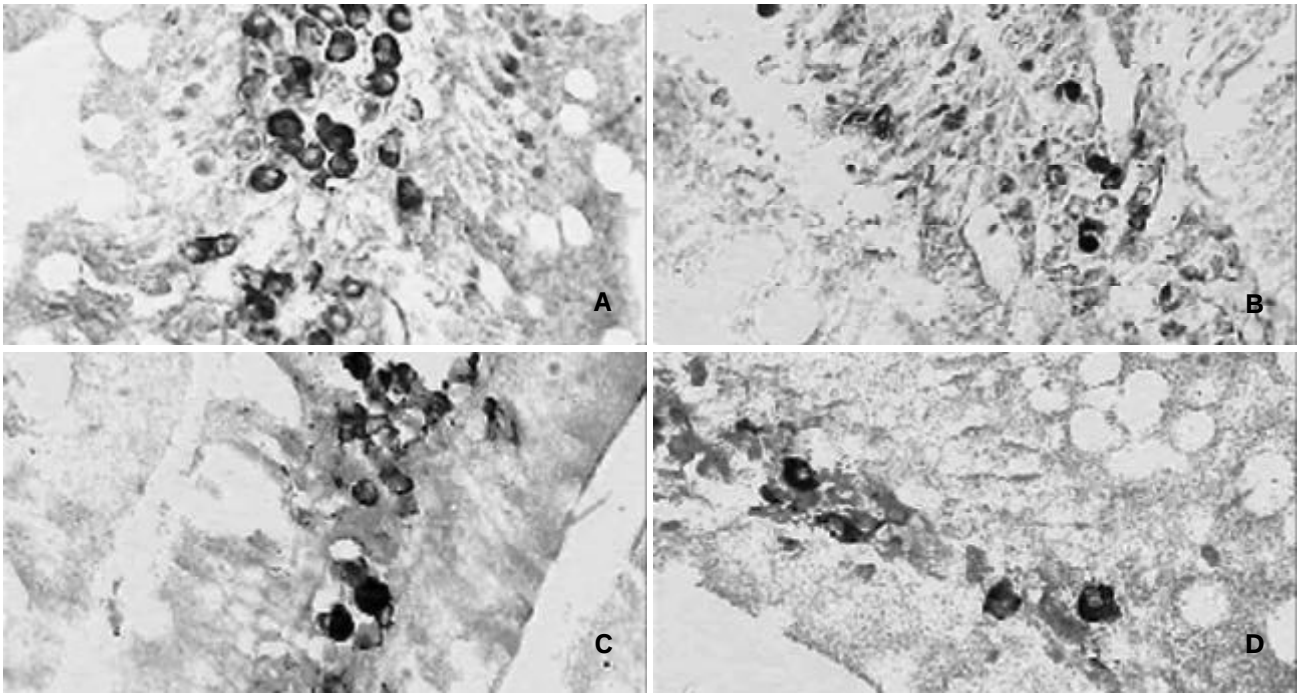

Figure 2 A: The expression of PCNA positive cells of the rat jejunum in the postnatal 3rd month ×400. B: The number of jejunal PCNA positive cells in the postnatal 12th month ×400. C: The expression of apoptotic positive cells of rat jejunum in the postnatal 3rd month ×400. D: The number of rat jejunal apoptotic positive cells in the postnatal 12th month ×400.

the development of intestinal glands the number of goblet cells in the intestinal gland gradually increased in the rat of postnatal 3rd week, postnatal 3rd month and postnatal 12th month, and this phenomenon might be corresponding to hearty digestive function in mature rats.

With the commence of sucking activity of newborn rat, the digestive tract starts its digestive activities. The newborn rat villi are very fine, and immature. In this period the intestinal digestive function is quite feeble, great molecule materials are assimilated via endocytosis with the help of lysosomes, thus the nutrients are absorbed. This absorptive manner only suits to the materials in milk[13]. Milk has balanced and all-sided nutrition, and contains many growth factors which are necessary to the development of the rat, so it can promote intestinal cells to develop[14]. To absorb various kinds of nutrients, gastrointestinal tract of rat develops rapidly and matures gradually to adapt to the digestive function[15]. In the postnatal 3rd week, villi become primarily matured. The muscle layer is quite thick, and the enteric digestive function of rat is boosted up. In this phase, endocytosis has weakened, and the rat can gradually digest food, and many absorptive mechanisms like glucide simple diffusing, solvent dragging, active ion transport and co-transport appear. In the postnatal 3rd month, villi heighten and peak, which increase the intestinal digestive areas. In this period, microvilli on the top of intestinal villus can be seen under an electron microscope, and they gather together to be known as brush border. Villi and microvilli increase the intestinal digestive area to 200 m^2 or more. There are capillary, lymphatic capillary, smooth muscle fiber and nerve net in intestinal villi, and one or more central small arteries and a central chyle vessel in each intestinal villus. The villus height increases with advancing age. The structures of capillary, lymphatic capillary, smooth muscle fiber and nerve net in intestinal villi are perfectly developed, in favor of substance countercurrent exchange in ascending and descending vessels of villi. At the same time, the muscle fiber relaxation and contraction and the pump function of central chyle vessels in villi help digestion. Besides, increase of muscle fibers which enhance the whip and rhythmic condensing movement is also helpful to digestion, and this period becomes the strongest phase of the digestive function. In the postnatal 12th month, there are effete changes like swelling and shortening of some villi, and the enteric digestive function slacks up, thus food is insufficiently digested in the enterocoelic cavity. The phenomenon of exocytosis manner assimilating large molecule substances appear again, maybe this manner is a kind of compensation for the digestion.

PCNA has a molecular weight of 36 kDa and is an assistant protein of DNA polymerase δ in terms of its physiological function. It can objectively reflect the proliferation degree of cells, and is a better proliferating marker of cells. PCNA immunostaining signal is almost completely disappeared in the nuclei. Positive substance takes on the form of dispersion or granule, or a combination of the two forms. The cell nuclei of strong positive cells are brown-yellow, nuclei of positive cells are yellow, the cell nuclei of feeble positive cells are buff. The proliferating bloom region of PCNA positive cells is distributed in jejunal glandulous recess, and the differentiation of positive cells on the top of villi is a sort of "ladder-like" movement[16-19]. The experiment showed that the proliferative index of PCNA positive cells in the recess increased from birth to postnatal 3rd month, and reached the crest in postnatal 3rd month, which made the cells in recess to be continuously hyperplastic. The proliferation index decreased at postnatal 12th month, indicating that the proliferation of cells becomes weak as the organism ages.

As the fastest renewing cells in the body, the cell cycle of small intestinal epithelia only exists for 12 h, and they also have apoptosis[20-23]. As early as the end of 19th century, the granules of epithelial cells in embryonic digestive tube under optical microscope were named "meconium corpuscles". Harmon *et al*[24-27] confirmed under the electron microscope that in fact meconium corpuscles were the apoptotic globules in digestive tract epithelia. Iwanaga *et al*[28-34] considered that macrophages in small intestinal proper lamina of adult rats could induce apoptosis of epithelia. It was observed in this experiment that the apoptotic cell number of intact jejunal villi of SD rats increased from birth to postnatal 3rd month and reached its peak in postnatal 3rd month, which may be indicated that when the rat grows up, cell proliferation and apoptosis activity became abundant with the buildup of body metabolism function. The metabolic level decreased in postnatal 12th month with the senescence of body, the number of apoptotic cells began to decline compared with that in the postnatal 3rd month.

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