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STRUCTURAL AND ULTRASTRUCTURAL ASPECTS OF PIGS INTESTINAL ADENOMATOSIS

ASPECTE STRUCTURALE ȘI ULTRASTRUCTURALE ÎN ADENOMATOZA INTESTINALĂ SUINĂ

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ABSTRACT | REZUMAT

To highlight the structural and ultrastructural aspects of porcine intestinal adenomatosis, the study was carried out in an open-circuit slaughterhouse, where during the cutting,through the macroscopic examination of the small intestine, lesions characteristic of *Lawsonia intracellularis* infection were observed. Ileum samples were taken for histopathological examination, immunohistochemistry, and double-contrast transmission electron microscopy. The histopathological examination revealed the atrophy of the intestinal villi with the proliferation of immature enterocytes and the absence of goblet cells. In the lamina propria, the inflammatory infiltrate was moderate and only in a few cases became abundant, reaching the muscular tunic of the intestinal mucosa. Immunohistochemistry allowed the confirmation of the aetiology, with the presence of the bacteria both on the surface of the intestinal villi and in the macrophages in the lamina propria. Moreover, using alcian blue immunohistochemistry, the absence of goblet cells on the surface of the intestinal villi was more visible compared to the examination of the samples by Diff-Quick staining. Electron microscopy identified mitochondrial vacuolations in enterocytes without affecting the smooth endoplasmic reticulum (SER), rough endoplasmic reticulum (RER), Golgi apparatus, and lysosomes. In some cases, the enterocytes had a karyopycnotic nucleus, a sign of necrosis, and a lack of microvilli, implying epithelial desquamation. In areas where goblet cells persisted, they showed no structural changes.

Keywords: swine proliferative enteropathy, intestinal adenomatosis, *Lawsonia intracellularis*

Pentru a evidenția aspectele structurale și ultrastructurale în adenomatoza intestinală suină, studiul a fost realizat pe probe recoltate dintr-un abator cu circuit deschis, unde pe parcursul tăierii, prin examinarea macroscopică a intestinului subțire au fost observate leziuni caracteristice infecției cu *Lawsonia intracellularis*. Au fost prelevate probe de ileon pentru a fi supuse examenului histopatologic, imunohistochimic și microscopiei electronice de transmisie cu dublă contrastare. Examenul histopatologic a pus în evidență atrofia vililor intestinali cu proliferarea enterocitelor imature și absența celulelor caliciforme. În lamina propria, infiltratul inflamator prezenta un caracter moderat și doar în puține cazuri devenea abundent, ajungând până la nivelul tunicii musculare a mucoasei intestinale. Imunohostochimia a permis confirmarea etiologiei, cu prezența bacteriei atât la suprafața vililor intestinali cât și în macrofagele din lamina propria. Mai mult, prin utilizarea imunohistochimiei cu albastru de alcian, absența celulelor caliciforme la suprafața vililor intestinali a fost mai vizibilă, comparativ cu examinarea probelor prin colorația Diff-Quick. Microscopia electronică a permis identificarea vacuolizărilor mitocondriale ale enterocitelor, fără a fi afectate REN, RER, aparatul Golgi și lizozomii. În unele cazuri, enterocitele prezentau nucleu kariopicnotic, fenomen asociat cu necroză și lipsa microvililor, implicit descuamări epiteliale. În zonele în care persistau încă celulele caliciforme, acestea nu prezentau modificări structurale.

Cuvinte cheie: enterita proliferativă suină, adenomatoza intestinală, *Lawsonia intracellularis*

The enteric diseases of pigs caused by different bacteria, viruses, and parasites are the most important pathological processes that cause significant economic losses. Among enteric diseases with bacterial aetiologies, infection with *Lawsonia intracellularis* seems to be a global problem, affecting farmed pigs' health and product efficacy. *Lawsonia intracellularis* is a Gram-negative, anaerobic obligate intracellular bacterium infecting the small intestine and infrequently also the large intestine of pigs, causing two syndromes: proliferative haemorrhagic enteritis (PHE), or the acute form, and intestinal adenomatosis (PE), or the chronic form of the diseases. The acute form is characterised by haemorrhagic diarrhoea and death. In the chronic form, the clinical signs are summed up as the reduced average daily weight gain, increased feed conversion ratio, and uneven growth, with or without diarrhoea (8, 11). Four forms of disease evolution were recognised based on the morphopathological lesions: porcine intestinal adenomatosis, necrotic enteritis,regional ileitis,and haemorrhagic proliferative enteritis. Necrotic enteritis results from secondary bacterial infection leading to

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intestinal adenomatosis, and regional ileitis represents the healing stage (5, 13). Also, granulomatous enteritis has been associated with *L. intracellularis* infection (7, 19, 23). The increased prevalence of the infection in pig farms worldwide (1, 2, 3, 16, 17) has led to the need to apply some prophylaxis methods to reduce economic losses. To date, prophylaxis against *L. intracellularis* infection has been achieved by either the administration of subtherapeutic levels of in-feed antibiotic growth promoters or vaccination (6, 10, 12, 15). However, the use of antibiotics as a prevention method in pig breeding has begun to be limited or even prohibited due to the risk of bacterial antibiotic resistance, so vaccination remains a basic method that seems to have proven its effectiveness (6, 10, 15). Despite decades of research on this pathogen, there are still unknown aspects, such as the organism's pathogenesis and virulence factors (21). This study aims to highlight the ultrastructural and structural aspects of intestinal adenomatosis, as a first step in unravelling the mechanisms of disease pathogenesis.

MATERIALS AND METHODS

The samples

The study was conducted in one pig slaughterhouse with an open circuit from Caraș-Severin County, where proliferative enteritis lesions were noticed during the cutting. Samples of the ileum were collected to highlight intestinal adenomatosis's structural and ultrastructural aspects.

The histological technique

Permanent slides of collected tissues were prepared according to Sadeghipour and Babaheidarian (19). The paraffin embedding technique was used to create a formalin-fixed, paraffin-embedded tissue block. Briefly, the samples were fixed in 10% formalin, dehydrated using alcohol in increasing concentrations (80°, 90°, and absolute), clarified through three successive baths of benzene, and then embedded in paraffin. The paraffin blocks were cut at 5 µm using a microtome to generate thin sections of tissue contained in paraffin. The sections displayed on the slide were deparaffinised using three successive baths of benzene and decreasing alcohol concentrations (absolute, 70°, 50°), rehydrated with distilled water, and coloured according to the Diff-Quick (Medion®Diagnostics, Germany) manufacturer's instructions.

The immunohistochemical technique

According to the immunohistochemical technique (IHC), the paraffin blocks obtained by the protocol described previously (18), were used. The chosen method was a combination of the immunochemical technique and Alcian blue staining (9). In histopathological diagnosis, Alcian blue staining highlights acidic mucopolysaccharides and, respectively, proteoglycans. In other words, according to the manufacturer's instructions, it allows the examiner to distinguish goblet cells, respectively, by their depletion or hyperplasia.

The IHC involves two important steps, as described by Magaki et al. (2019), namely antigenic retrieval and immunolabeling (14).

Antigenic retrieval was achieved by exposing the deparaffinised and rehydrated sections to heat in a sodium citrate solution (pH 6) for 30 minutes. Hydrogen peroxide was used at a concentration of 3% to block the endogenous peroxidase for 5 minutes.

The immunolabeling involved using the NovoLink Max Polymer Detection System (Novocastra, Newcastle Upon Tyne, UK), with the stages being performed by the automated staining system DakoCytomation Autostainer. The chromogen used consisted of 3,3-diaminobenzidine, and modified Lille haematoxylin was applied for counterstaining.

Electron microscopy technique

The transmission electron microscopy (TEM) method was used, according to Winey et al. (22), with slight modifications to describe the ultrastructural aspects of intestinal adenomatosis. The technique protocol involved seven stages: fixation, dehydration, infiltration and inclusion, encapsulation, block modelling, sectioning and contrast.

Fixation involved two distinct steps: the prefixation stage, performed with a 2.7% glutaraldehyde solution in 0.1M phosphate buffer (pH 7.2) for 180 minutes, and the post-fixation stage, carried out with a 2% osmium tetroxide solution in 0.15M phosphate buffer (pH 7.4) for 90 minutes. Between the two stages, the samples were exposed to four successive baths, one hour each, using 0.1M phosphate buffer (pH 7.4).

Dehydration was performed by increasing acetone concentrations in aqueous solutions, starting from 30% up to three pure acetone baths. Then, the samples were exposed to propylene oxide baths which act as a dehydrator and fixative, being very fluid and miscible with Epon 812, ensuring an excellent infiltration of the tissues at the molecular level. Infiltration and inclusion consisted of maintaining the samples for one hour in mixtures of Epon 812 with anhydrous acetone in proportions of 1/3, 1/1, 2/1, 3/1, and pure Epon 812. Afterwards, the samples were encapsulated in gelatin capsules and kept at 60 °C for 72 hours. Finally, the blocks were modelled and sectioned to contrast.

To determine the region of interest, semi-thin slices (250–600 nm) were stained with Epoxy dye (Epoxy tissue stain) and inspected using an optical microscope. To double contrast the ultrafine sections (60–70 nm), uranyl acetate and lead citrate were used. Double contrasted sections were examined under the transmi-

ssion electron microscope, and selected images were captured sequentially and stored in the database using the Megaview III camera programme, Soft Imaging Analysis. The obtained images were processed using the Wiever Imaging Analysis programme.

RESULTS AND DISCUSSIONS

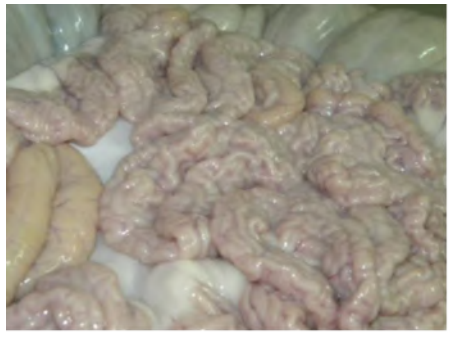

Fig. 1. Porcine intestinal adenomatosis - cerebriform aspects of the small intestine

The macroscopic examination of the gastrointestinal masses of pigs allowed the identification of the characteristic lesions described in the specialised literature (5, 13) on *L. intracellularis* infection. The cerebriform aspect of the small intestine attracted attention (Fig. 1). The oedema of the serosa was accompanied by the increased proliferation of the mucosa, with the presence of folds and, respectively, the narrowing of the intestinal lumen. No hemorrhagic, ulcerative, or necrotic areas were observed.

The overall image of the studied ileum fragments allowed the identification of the atrophy of the intestinal villi, which appears as a consequence of the increased rate of enterocyte loss. Following epithelial losses, villi contract adaptively, acquiring a flattened appearance (Fig. 2A). Atrophied villi were covered by poorly differentiated cells. The lamina propria, the compound interposed between the surface epithelium and the muscular mucosae, containing intestinal glands, vascular formations, and nerve threads emitted by the submucous plexus, as well as lymphatic follicles (4), presented a moderate leukocyte infiltrate (Fig. 2B). Still, in some cases, the leukocyte infiltrate took on an abundant character, extending to the muscular tunic of the mucosa of the small intestine (Fig. 2C). From a histological point of view, the intestinal epithelium of healthy organisms is simple, single-layered, cylindrical, and composed of enterocytes, goblet cells, and argentaffin cells (4). However, in the case of the samples studied, the intestinal epithelium appears multi-layered, with numerous immature enterocytes (Fig. 2D). If the strains of *L. intracellularis* stimulate the proliferation of immature enterocytes, delay the maturation of enterocytes, or act through both pathogenetic mechanisms, it remains an enigma, but the result of the infection consists in epithelial hyperplasia as a consequence of the mobilisation, on the surface of the villi or in the intestinal glands, of imma-

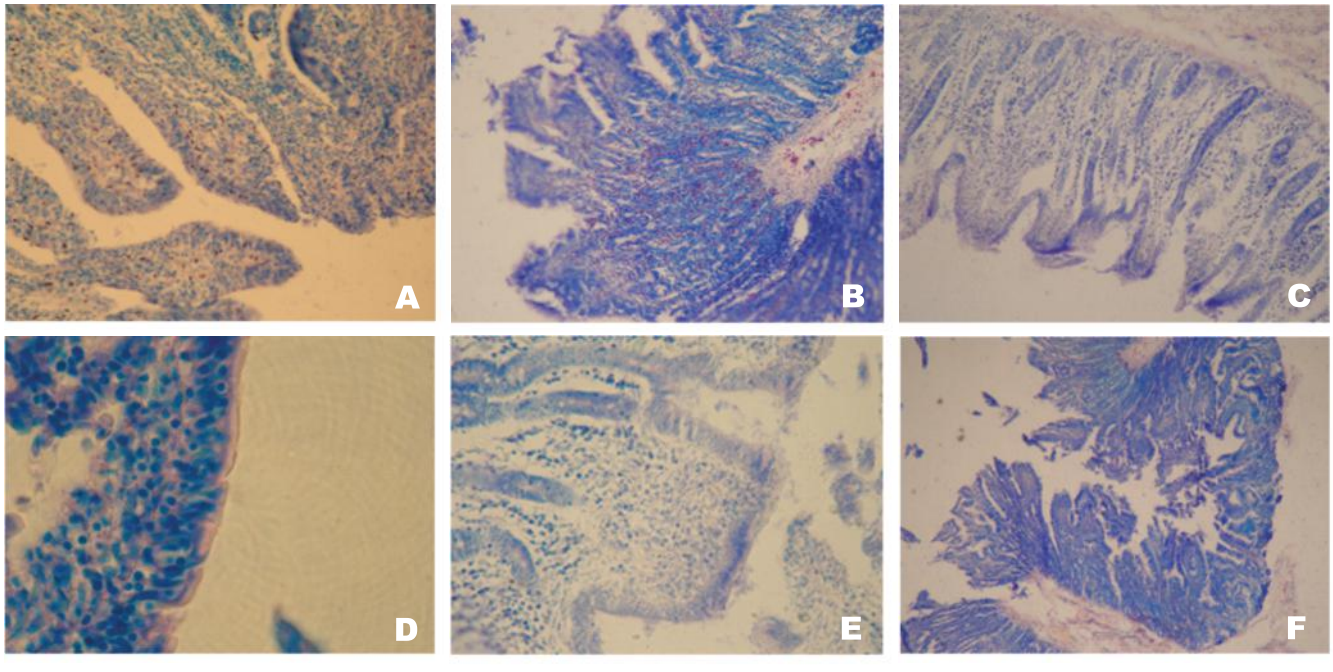

Fig. 2. A. Epithelial losses, contracted villi with a flattened appearance (Diff-quick, x100); B. Moderate leukocyte infiltrate (Diff-quick, x400); C. Leukocyte infiltrate reaching to the muscular tunic of the mucosa (Diff-quick, x200); D. The absence of the goblet cells on the surface of the villi (Diff-quick, x1000); E. (Diff-quick, x400); F. General aspects of the intestinal adenomatosis lesions with immature enterocyte proliferation and depletion of the goblet cell (Diff-quick, x100)

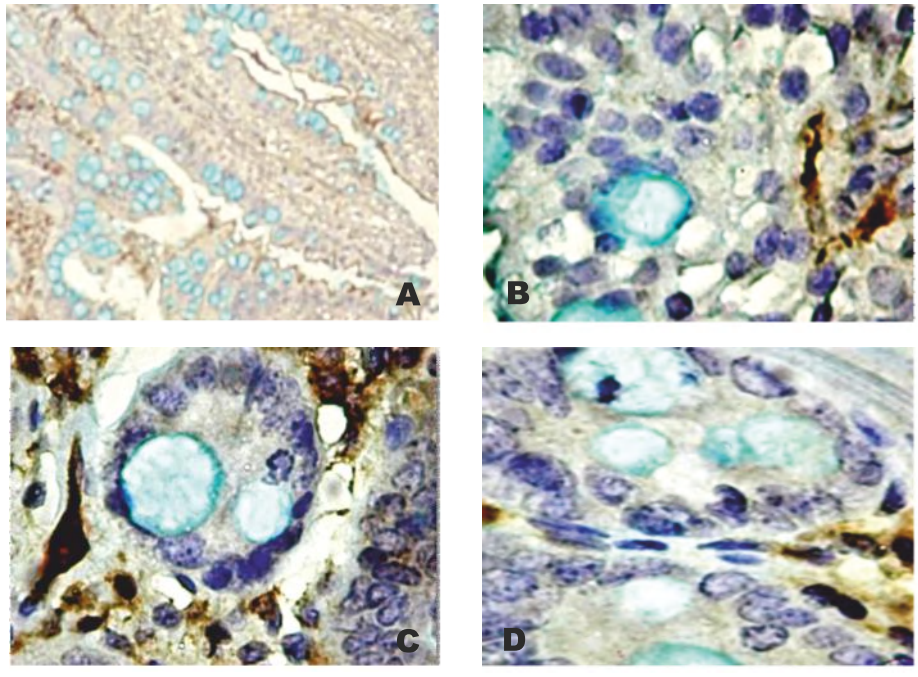

Fig. 3. A. Overview of the ileum, the presence of bacterial antigen on the surface of intestinal villi and lamina propria between the intestinal glands (IHC – double staining with Alcian blue, x100); B. Mucosa epithelium of the ileum - enterocyte proliferation in the intestinal villi; C. The presence of *L. intracellularis* in macrophages (IHC double staining - with Alcian blue, x1000); D. Cross-section through the intestinal glands, the presence of antigen both in macrophages of intestinal glands and in enterocytes (IHC – double staining with Alcian blue, x1000)

ture enterocytes (11). In the hyperplastic areas, goblet cells are absent (Figs. 2D and 2E). The epithelial hyperplasia areas alternate with epithelial desquamation (Fig. 2C). Desquamated cells and cellular detritus are entrained into the intestinal lumen. Consequently, the histopathological lesions observed in this study broadly coincided with the literature data (7, 23).

The immunohistochemical technique allowed the highlighting of histopathological lesions and the identification of bacterial antigens (Fig. 3A). Epithelial desquamation was joined by epithelial proliferations of the ileum mucosa (Fig. 3B). Bacterial antigens were identified both in enterocytes (Fig. 3C) and in macrophages in the lamina propria (Fig. 3D).

The electron microscopy technique revealed the changes, especially in the enterocytes. The karyopyknotic nucleus (condensation of chromatin with the appearance of a smaller, tachychromatic nucleus) and the absence of the cell membrane observed in many enterocytes from the ileum samples studied signify a process of cellular necrosis (Figs. 4A and 4B). This process, extended over certain areas, causes a lack of microvilli and, respectively, the inability of the small intestine to absorb, resulting in the weakening of the body in the presence of appetite. Cellular debris was entrained into the intestinal lumen (Fig. 4C). The cytoplasm shows numerous mitochondria with a rarefied matrix and altered cristae. Mitochondria represent the cell's energy centres, generating most of the ATP. Under physiological conditions, the functioning of the mitochondria requires the integrity of all its component structures and a functional balance that admits minor variations without jeopardising its normality. The rarefaction of the mitochondrial matrix implies secondary dysfunction, which can also occur due to the action of some extrinsic factors (toxins, viral diseases, drugs, etc.). This ultrastructural change can be attributed to the NTTLi gene of *L. intracellularis* strains, which encodes the ATP/ADP translocase. This enzyme catalyses specific exchanges between bacterial ADP and host ATP, allowing the bacterium to use the energy of the infected cell (8). As ATP levels are depleted, reversible changes in mitochondrial conformation and function are observed, and the cell rapidly cannot maintain homeostasis. Beyond a certain level, the process becomes irreversible. The cell membrane can become the major site of alterations, thus losing its ability to regulate osmotic pressure. Membrane damage results from releasing cellular enzymes into the tissue space and producing a non-specific inflammatory response (17). Moreover, it has been demonstrated that one of the pathogenic factors of the bacterium is the type III secretion system (11), which, among its multiple roles, also has a cytotoxicity function. No changes

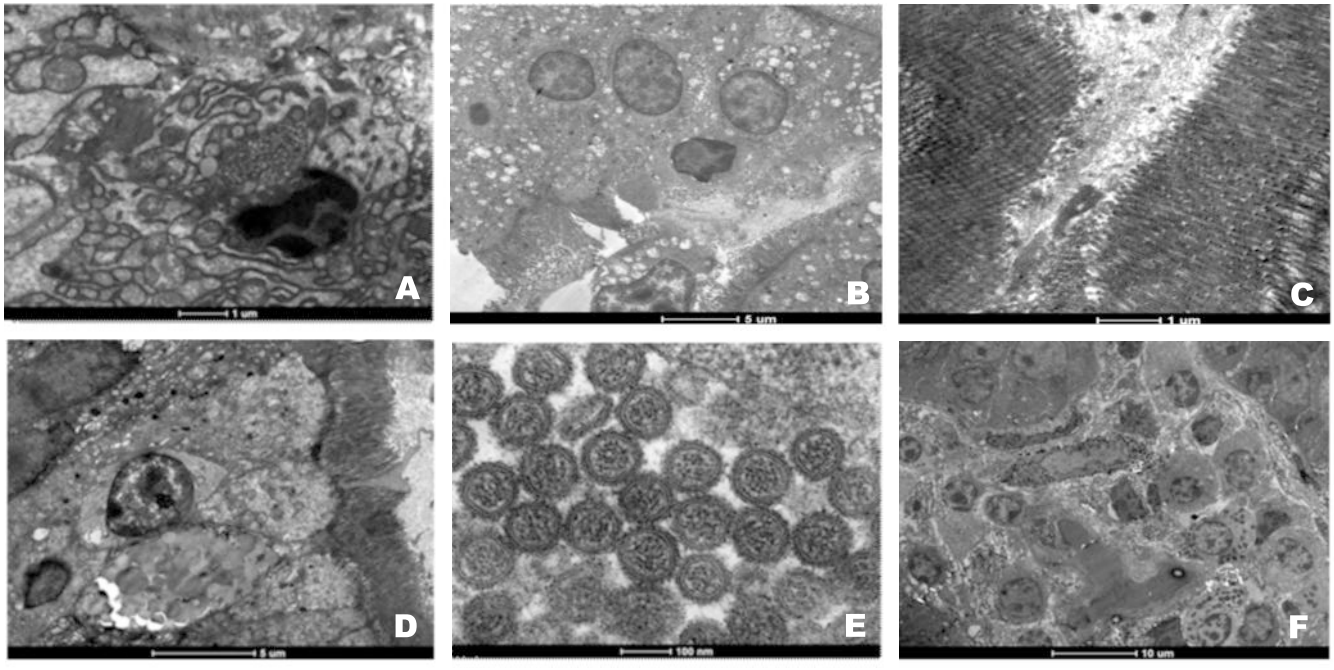

Fig. 4. A. Degenerative processes of the enterocyte (TEM); B. Cell necrosis and the absence of villi in affected areas (TEM); C. Typical brush border, cellular debris into the intestinal lumen (TEM); D. Goblet cell with glycoproteic mucus, enterocytes with mitochondrial vacuolisation (TEM); E. Normal aspect of microvilli, with actin filaments in the centre and the glycocalyx outside (TEM); F. Inflammatory infiltrate in lamina propria (TEM)

were noted in the smooth endoplasmic reticulum (SER), rough endoplasmic reticulum (RER), Golgi apparatus, or lysosomes of affected enterocytes.

In most cases, the absence of goblet cells was observed in areas of enterocyte proliferation. However, they could be observed in some areas of the intestinal villi (Fig. 4D) and at the level of the crypts without any structural changes, exercising their function even when the adjacent enterocytes suffered mitochondrial vacuolation.

Transverse sections through microvilli do not reveal changes in their structure, noting in the centre the presence of a bundle of actin filaments wrapped on the outside by the glycocalyx (Fig. 4E).

A leukocyte infiltrate was found in the lamina propria (Fig. 4F) The cellular infiltrate in the lamina propria of the intestinal mucosa is most often moderate, and consists of lymphoplasmocytes, mast cells, eosinophils and, to a lesser extent, macrophages.

As other authors have described, the macroscopic, histopathological, and immunohistochemistry examination at the slaughter age of pigs plays an essential role in diagnosing intestinal adenomatosis, a chronic disease with economic losses. The lesions described in the present study are similar to the data from the specialised literature (7, 20, 23, 24), except for the one observed by electron microscopy examination. As we know, this is the first time in the literature that the ultrastructural aspects of intestinal adenomatosis are described.

CONCLUSIONS

The present study highlights the histopathological lesions of the intestinal adenomatosis at the slaughter age of pigs, with desquamation and epithelial proliferations. Ultrastructural images demonstrated the existence of necrotic processes in enterocytes, with the presence of karyopyknotic nuclei, rarefied mitochondria, altered cristae, and lysed cell membranes. The cellular infiltrate in the lamina propria of the intestinal mucosa is moderate and consists of lymphoplasmocytes, mast cells, eosinophils, and macrophages.

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