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Dynamic of apoptosis of cells in duodenal villi infected with *Eimeria acervulina* in broiler chickens

Peter Major[1], Štefan Tóth[2], Mária Goldová[1]*, Viera Révajová[3], Ivona Kožárová[4], Mikuláš Levkut[3], Jana Mojžišová[1], Vladimír Hisira[5] & Tomáš Mihok[6]

[1]*Department of Epizootology and Parasitology, University of Veterinary Medicine and Pharmacy, Komenského 71,SK-04181 Košice, Slovakia; e-mail: goldova@uvm.sk*
[2]*Department of Histology and Embryology, Faculty of Medicine, P.J. Šafárik University, Šrobárova 2, SK-04001 Košice, Slovakia*
[3]*Department of Pathological Anatomy and Pathological Physiology, University of Veterinary Medicine and Pharmacy, Komenského 71, SK-04181 Košice, Slovakia*
[4]*Department of Food Hygiene and Technology, University of Veterinary Medicine and Pharmacy, Komenského 71, SK-04181 Košice, Slovakia*
[5]*Clinic of Ruminants, University of Veterinary Medicine and Pharmacy, Komenského 71, SK-04181 Košice, Slovakia*
[6]*Department of Nutrition, Dietetics and Animal Breeding, University of Veterinary Medicine and Pharmacy, Komenského 71, SK-04181 Košice, Slovakia*

Abstract: The aim of our study was to evaluate the parasite – host interactions at apoptosis level. We studied histopathological changes and time course of apoptosis in the duodenum during *Eimeria acervulina* infection. One-day-old broiler chicks were randomly allocated into two equal groups. At the age of two weeks the first group was experimentally infected with a pure suspension of sporulated *E. acervulina* oocysts. The second group served as a negative control. Tissue samples from the upper part of duodenum were obtained at 0.5, 1, 2, 3, 4, 5 and 6 days post infection. Biopsies of duodenum were studied immunohistochemically using DeadEnd^{TM}Colometric TUNEL System for apoptosis detection in duodenal mucosa. Number of parasites in duodenal epithelium was also investigated. Our experimental results demonstrate: (i) macroscopic and histopathological changes in epithelium detected mainly in proximal segment of duodenum in infected groups; (ii) the number of developmental stages of *E. acervulina* (DSEA) during our trial increased, reaching the maximum 5 days post infection (dpi) (332.2 ± 16.12) (mean ± SEM), whereas the amount of DSEA declined significantly as late as 6 dpi (124.6 ± 3.91); (iii) the highest apoptosis level was recorded in initiatory 0.5 dpi (13.2 ± 1.02) and on the end of parasite development cycle after 5 dpi (12.6 ± 1.36). Finally, results showed that there was a period of inhibition of apoptosis during infection by *E. acervulina*.

Key words: apoptosis; duodenum; *Eimeria acervulina*; parasite

Introduction

Poultry coccidiosis is an enteric disease caused by intracellular protozoan parasites *Eimeria* spp. (Protozoa: Apicomplexa: Eimeriidae). It is one of the most common diseases of poultry and has significant economical implication. High prevalence of the disease is found in any intensive poultry rearing industry. Seven species of *Eimeria* are known to be infective for chickens (Allen & Fetterer 2002).

Eimeria acervulina is one of the most widespread species of coccidia in the chickens. It initially invades the duodenal villi enterocytes. In massive infection it has been shown to invade also the posterior segments of the small intestine (Pellérdy 1974). The infection results in characteristic white petechial lesions in the intestinal lining associated with malabsorption of nutrients. *Eimeria acervulina* is generally considered to be moderately pathogenic, thus causing a chronic disease which leads to poor weight gains without a significant increasing mortality (Taylor et al. 2007). On the other hand, the damage in the gut epithelium caused by the parasite may increase the risk of secondary infection with bacteria such as *Clostridium perfringes*, the causative agent of necrotic enteritis (Al-Sheikhly & Al-Saieg 1980).

Apoptosis (programmed cell death) is normal part of development and tissue homeostasis (Vaux & Strasser 1996). Apoptosis is present in the intestinal crypts and regulates the total amount of progenitor stem cells (Potten 1997). It also influences the desquamation or exfoliation process of epithelial cells on the villous tips by anoikis as a phenomenon of apoptosis (Thompson et al. 2001). Anoikis is the subset of apoptosis triggered by inadequate or inappropriate cell-matrix

* Corresponding author

contacts, and it maintains the correct cell number in high-turnover intestinal epithelium. Under physiological conditions in the mammalian small intestine it is present mainly in the intestinal crypts (less than 1%) as spontaneous apoptosis (Potten 1997). The apoptosis is significant under physiological and various pathological conditions. Furthermore, apoptosis is an important regulator of the host's response during various intracellular protozoan infections, and helps eliminate damaged or infected cells (Williams 1994; Lüder et al. 2001). However, some pathogens have evolved distinct strategies to induce or inhibit the host cell apoptosis in order to ensure their intracellular survival. Among parasites it is well documented in some apicomplexan parasites, e.g., *Cryptosporidium parvum* (Chen et al. 2001), *Toxoplasma gondii* (Keller et al. 2006), *Theileria parva* (Heussler et al. 2001), *Eimeria tenella, Eimeria necatrix* and *Eimeria bovis* (Cacho et al. 2004; Lang et al. 2009). Direct effects of parasites and their metabolites, as well as indirect mechanisms via the induction of antigen-specific T cells, participate in the modulation of host cell death (Lüder et al. 2001).

The aim of our study was to investigate the dynamic of apoptosis of cells in duodenal villi during parasite – host interactions in relation to number of developmental stages of *Eimeria acervulina* in cells of duodenal villi in chickens.

Material and methods

Parasites
Pure culture of *Eimeria acervulina* was obtained by single oocysts isolation on agar (Tsutsumi 1972). The sporulated *E. acervulina* oocysts were stored in 2.5% potassium dichromate according to Raether et al. (1995).

Experimental animals and experimental design
A total of 50 one-day-old broiler chicks (hybrid Ross 308, Agrocass s.r.o.) were randomly allocated into two equal groups. Each group was housed in a separate wire suspended cage, equipped with an infrared lamp. At the age of two weeks one group was experimentally infected with a pure suspension of sporulated *E. acervulina* oocysts. The infectious dose represented 1×10^4 oocysts/bird. The second group served as a negative control.

Histological examination
Intestinal tissue samples for histological examination were obtained at 0.5, 1, 2, 3, 4, 5 and 6 days post infection (dpi), fixed in 10% buffered formol and subjected to routine paraffin embedding. Histological sections (5 μm thick) were stained with haematoxylin-eosin and examined for morphological determination of the developmental stages of *E. acervulina* (DSEA).

Detection of apoptotic cells
In the present study, the apoptotic cell death was detected by DeadEnd® Colometric TUNEL System (Promega, Germany) technique to assess DNA fragmentation in the intermediate and late stages of apoptosis cascade. In the duodenum positive apoptotic cells were sought in the intestinal epithelium – *lamina epitelialis mucosae* and in the mucosal connective tissue – *lamina propria mucosae*. The resulting value is the number of apoptosis positive cells in the *tunica musoca (lamina propria + lamina epithelialis mucosae)* of the small intestine.

TUNEL immunohistochemistry
Detection of apoptosis was achieved using terminal deoxynucleotidyl-transferase-mediated deoxyuridine triphosphate *in situ* nick end-labeling (TUNEL) in paraffin-embedded 4-μm-thick sections, according to the manufacturer's application instructions (DeadEnd™ Colometric TUNEL System Promega, Germany). The DeadEnd™ Colometric TUNEL System is a modified TUNEL Assay designed to provide simple, accurate and rapid detection of apoptotic cells in situ at the single-cell level. The DeadEnd™ Colometric TUNEL System measures nuclear DNA fragmentation, which is an important biochemical indicator of apoptosis. Using this procedure, positive nuclei of apoptotic cells are stained dark brown. Sections were counterstained with Mayer's hematoxyline (Merck, Germany).

Counting of DSEA and apoptotic cells
The investigated histological sections were divided into 10 randomly selected fields of duodenal mucosa (histotopography: *lamina epithelialis + lamina propria mucosae* of correctly formed intestinal villi with appropriate mucosa) per section. Each section was studied under 400× magnifications by light microscope (Olympus BX 500) and QuickPHOTO Industrial 2.3 software was used. The mean number of apoptotic cells and DSEA per each field was calculated for further statistical analysis.

Statistical analysis
The statistical analysis was performed using GraphPad InStat version 3.01 (GraphPad Software, San Diego, CA). The quantitative results were determined by one-way ANOVA with a multiple comparison Tukey-Kramer *post-hoc* test. The results were expressed as mean ± SEM. P values less than 0.05 were considered significant.

Results

Macroscopic lesions
Pathological changes were observed in the upper part of the duodenum of the infected animals during the experimental period. The wall of the duodenum was slightly thickened, and the mucosal layer presented whitish, elongated lesions. The gut content was of hydrous nature.

Histopathological findings
In negative control group the morphology of duodenal wall was found to be intact without pathological changes. In experimental group the histological picture varied: in intestinal lining progressive alterations in epithelial morphology were in relation with development cycle of *Eimeria acervulina*. First DSEA were found on 0.5 dpi (8.2 ± 1.28). Significant changes were not found in number of DSEA on 1, 2, 3 dpi in comparison to 0.5 dpi (Fig. 1). On 4 dpi number of DSEA was higher ($P < 0.01$) than in 0.5 dpi. The number of DSEA on 5 and 6 dpi was enormously higher ($P < 0.001$) than on other followed days (Fig. 2), on the other hand, density of DSEA was higher ($P < 0.001$) on 5 dpi than on 6 dpi (Table 1).

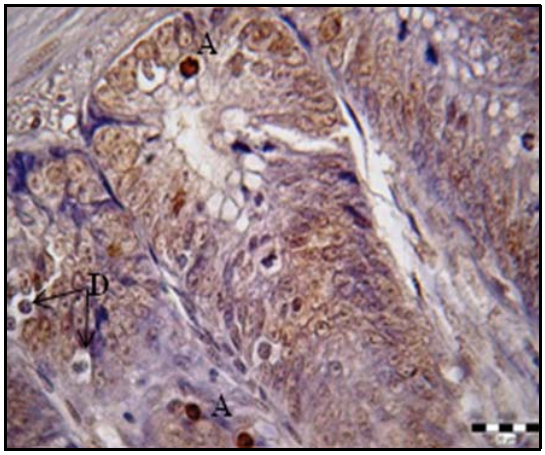

Fig. 1. Low number of DSEA (D) and apoptotic cells (A) on the base of duodenal villi and crypts 1 dpi. Scale 20 μm.

Table 1. The total number of DSEA in duodenal epithelium in particular time intervals post infection and statistical comparison.

dpi	DSEA
Negative control	0.0
0.5	8.2 ± 1.28^{ac}
1	31.0 ± 2.39^{c}
2	31.6 ± 0.51^{c}
3	31.2 ± 2.13^{c}
4	42.4 ± 2.54^{bc}
5	332.2 ± 16.12^{cd}
6	124.6 ± 3.91^{d}

Explanations: Specific superscripts in column indicate significant differences: ${}^{ab}P < 0.01$, ${}^{cd}P < 0.001$.

Imunohistochemical evaluation of apoptosis

In negative control (NC) group the level of apoptosis in intestinal epithelium was in average 3.4 ± 0.93. Apoptotic cells were detected mostly on the tip of duodenal villi, no cells affected by apoptosis were found in crypts. Number of apoptotic cells was higher ($P < 0.001$) in 0.5 dpi in comparison to NC. Similarly, density of apoptotic cells was higher ($P < 0.05$) on 1 and 2 dpi than in NC (Fig. 1). On the other hand, number of apoptotic cells was lower ($P < 0.05$) on 1 and 2 dpi than in 0.5 dpi. Density of apoptotic cells in 3 dpi was similar to 2 dpi. Number of apoptotic cells increased ($P < 0.001$) on days 4, 5, 6 dpi in comparison to the NC (Fig. 2). The quantity of apoptotic cells was higher ($P < 0.001$) on 4 and 6 dpi than on 2 and 3 dpi (Table 2).

Table 2. The total number of apoptotic cells in duodenal epithelium in particular time intervals post infection and statistical comparison.

dpi	Apoptotic cells
Negative control	3.4 ± 0.93^{ae}
0.5	13.2 ± 1.02^{af}
1	7.8 ± 0.58^{bc}
2	4.6 ± 0.40^{bg}
3	4.6 ± 0.40^{bg}
4	11.8 ± 0.49^{fh}
5	10.0 ± 1.05^{f}
6	12.6 ± 1.36^{fdh}

Explanations: Specific superscripts in column indicate significant differences: ${}^{ab}P < 0.05$; ${}^{cd}P < 0.01$, ${}^{ef,gh}P < 0.001$.

Discussion

In a healthy organism apoptosis plays an important role in displacing old, damaged and redundant cells and hence is a key factor in holding tissue homeostasis (Vaux & Strasser 1996; Ramachandran et al. 2000). Particularly essential is the apoptosis in gastrointestinal system, where constant and rapid renewal of intestinal cells is in progress providing normal function. In intestinal crypts proliferation of immature stem cells occurs. These differentiate gradually and migrate to the top of villi. The immature epithelial cells after certain time undergo apoptosis and are replaced by new cells from intestinal crypts. Similar compensation and cell replacement with presence of apoptotic cell death is present also in the villus connective tissue. In control group number of apoptotic cells found in our trial can correspond with normal renewal of intestinal cells. According to Ramachandran et al. (2000) the apoptosis

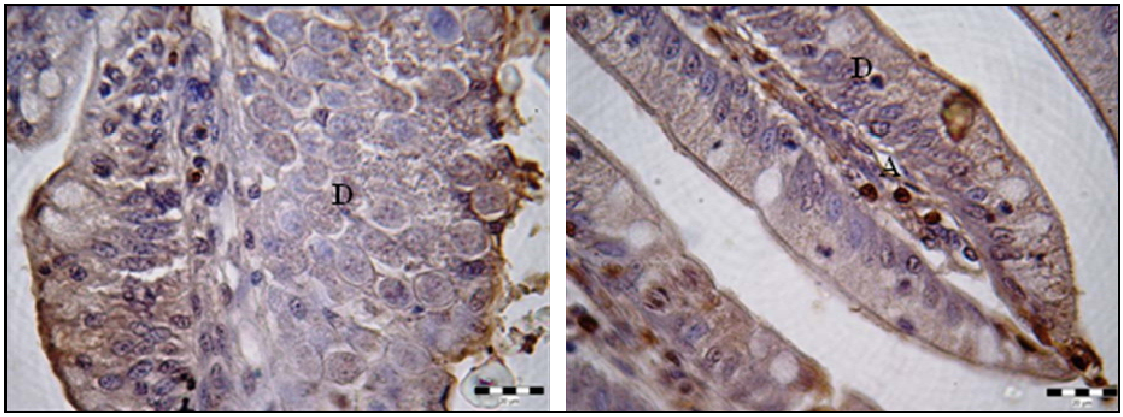

Fig. 2. Numerous DSEA (D) and apoptotic cells (A) on the apical part of duodenal villi 5 dpi. Scales 20 μm.

predominantly occurs at the top of intestinal villi and only rarely in intestinal crypts, what is in conformity with our findings.

The current study demonstrates the qualitative and quantitative changes in dynamics of apoptosis of enterocytes and connective tissue cells in the course of *E. acervulina* infection. We confirmed that apoptosis is a vital part of complex host parasite interactions in the course of disease. Our results revealed the increase of *E. acervulina* developmental stages numbers during first days post infection with maximum at 5 dpi (332.2 ± 16.12) topographically localised mainly in apical part of duodenal villi. In infected chicken significant enhancement in apoptosis level appeared at 0.5 dpi when compared with uninfected birds, whereas the apoptotic cells were found at top of duodenal villi, corresponding to the expected parasite location.

Several authors demonstrate that pathological processes initiate the increase of apoptosis in enterocytes and adjacent parts of intestinal epithelium. In humans, enhanced intestinal apoptotic activity was observed during the infection with pathogens *Salmomella* spp., *Shigella* spp., entheropathogenic *E. coli*, HIV virus and *Helicobacter pylori* (Kim et al. 1998; Jones et al. 1999; Crane et al. 1999; Clayton et al. 1992). When infecting the cell cultures with *Cryptosporidium parvum* during first 12 hours, significant increase of cells undergoing apoptosis was observed by McCole et al. (2000). The induction of apoptosis appears to be the result of organism reaction on penetrating pathogen, through destruction of potential living niche and exposing of pathogens to different cells of the immune system. Intestinal lymphocytes play an important role, taking part both in cellular and humoral response against coccidian infection. Intraepithelial lymphocytes are in particular the T lymphocytes (Major et al. 2011) whereas in *lamina propria mucosae* especially plasma cells occur – effectors B lymfocytes producing immunoglobulines (Guy-Grand et al. 1991). Cytoplasmatic lymphocytar granula contain besides other substances also perforin that is able to initialize apoptosis (Iwanaga 1995). It has been demonstrated that CD3+, CD8+ and TCR2+ intraepithelial lymphocytes take part in apoptosis induction in epithelial cells in chicken ceca (Takeuchi et al. 1999). Iwanaga (1995) found considerable numbers of sporozoites of *E. acervulina* inside of macrophages. These cells, when stimulated by sporozoites and merozoites, produce significant amounts of TNF (tumor necrosis factor) which is a pro-apoptotic cytokine playing also an important role in apoptosis induction (Zhang et al. 1995).

In the present study on days 1, 2 and 3 post infection the frequency of apoptosis decreased. This effect caused more likely parasites developing in host cells and inhibition of the apoptotic activity. Similarly to other intracellular parasites *E. acervulina* needs for completing its life cycle an adequate environment within the host cell, where particular reproduction phases are in progress. The cell death would interrupt the development cycle causing consequently the death of parasite. It is therefore necessary for the parasite to avoid the host cell death and ensure its own intracellular survival (Moss et al. 1999). Parasites interfere in complex apoptotic mechanisms by many means. In course of infection with *E. tenella* and *E. necatrix* in assaulted cells the transcription factor NF-$_{K}$B is activated which after passage into cell nucleus initializes genes responsible for cell growth and apoptosis inhibition (Karin & Lin 2002). During infection of endothelial cells with *E. bovis* other anti-apoptotic factors are expressed – c-IAP1 and c-FLIP (Lang et al. 2009). Anti-apoptotic mechanisms employed by *E. acervulina* are still not explained completely, we suggest, that similar factors are involved as in other *Eimeria* species.

Our current trial demonstrated a higher prevalence of cell death on 4, 5, 6 dpi with maximum number of apoptotic cells on 6 dpi. The amount of developmental stages of *E. acervulina* also increased, with the highest frequency on 5 dpi, what is related to parasite life cycle. Gametogony in *E. acervulina* begins five days post infection producing massive amount of immature oocysts. Releasing of oocysts from enterocytes is followed by cell destruction (Taylor et al. 2007). In this stage the parasite does not depend on host cell and cell death and its disintegration allows the dissemination of parasite in environment. Cacho et al. (2004) observed reduced secretion of anti-apoptotic factors during maturation of the second generation of *Eimeria tenella* and *E. necatrix* schizonts, followed by initiation of enterocytes apoptosis.

Results of current experiment suggest that intracellular parasite *E. acervulina* is able to modify flexible the apoptotic cell death in host tissue to complete its life-cycle. The significant increase number of apoptotic cells in intestinal epithelium in our trial can be the result of decreased anti-apoptotic activity of *E. acervulina*. We suppose that during individual phases of asexual reproduction as well as during oocysts release from host cells the parasite developed the anti-apoptotic activity. Due to lower amount of developing parasites in 1st and 3rd schizont generation, the number of apoptotic cell death in duodenal epithelium did not change markedly. It can be concluded that overall presence of apoptotic cells in infected animals was generally above 10, with significant reduction during the 2 and 3 dpi allowing the parasite to remain within the cells and fulfil the life cycle. Massive enterocytes destruction led to the damage of entire duodenal epithelium. Our finding of the shortening of intestinal villi, deletion of striated border and changes in modality and form of epithelial cells are in conformity with the suggestion of Ramachandran et al. (2000). The authors consider that the shortening of the villi being the concomitant of increased enterocytal apoptosis in course of pathological conditions. On the other hand, increased level of apoptosis may facilitate the regeneration of damaged mucosa by displacing damaged and functionless cells, contiguous with physiological processes in tissue.

Further studies for determination of apoptosis dy-

namic during infection of another *Eimeria* spp. are needed.

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