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A Study of Programmed Cell Death in Cecal Tonsil of Infected Chickens by Infectious Bursal Disease Virus with Using Electronic Microscope

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Abstract: Infectious Bursal disease is one of the important diseases of poultry. This disease caused severe economical losses such as mortality, retardation of growth and immunosuppressant, especially at 3-6 weeks chickens. Histopathologic lesions were appeared in Cecal tonsil and other lymphoid tissues. Destruction of infected lymphocytes with virus and peripheral cells and depletion of lymphocytes in Cecal tonsil were caused by necrosis and apoptosis. In this study, 50 SPF 28 days olds chickens were divided in to tow groups (control and experimental) with 25 chickens in each group. The experimental group was infected orally by 106EID50 in 1 mL of IR499 (IRAN499) virus (vvIBDV) in control group physiological saline solution was used. At 4th days post infection, all birds were sacrificed and their Cecal tonsil were taken out and prepared for EM (Electronic Microscope) assay and light microscopic study. By light microscopic study about numeration of apoptotic cells, statistic difference were appeared ($p = 0.000$) between control and experimental groups. With EM, apoptotic cells were appeared by submargination of chromatin of nuclear membrane concurrent chromatin condensation in experimental group but there were not any apoptotic cells in control group. Apoptosis was appeared by attachment of virus to IgM+ receptors of LB surface and to enforce of cells to secretion of some cytokines. VP2 and 17KD were major viral proteins induced apoptosis in bursa and spleen in infected chickens. In this study and previous studies were demonstrated that IBDV (Infectious Bursal Disease Virus) affected chickens with both of necrosis and apoptosis.

Key words: Infectious bursal disease, cell programmed death, cecal tonsil, EM, cytokines, Iran

INTRODUCTION

Infectious bursa disease is an acute contagious viral disease, especially in 3-6 weeks aged young chicks. In this disease lymphoid tissue especially, bursa of fabricius is the target tissue. However in other lymphatic organs such as spleen, thymus, harderian glands, cecal tonsils and in non lymphatic organs such as breast and femur muscles, mucous gland between preventriculus and gizzard, kidney and bone marrow, create macroscopic and microscopic spoilage (Bozorgmehri-Fard *et al*., 1998; Tanimura and Sharma, 1977; Vegad, 2004). The creator agent of disease is a delink RNA virus of Birnaviridae (family) of Avibirna virus genus. Serotype 1 that infects poultry, from the virulence concern is variable from very virulent strains to a pathogenic strain (Baxendale, 2002). Apoptosis or programmed cell death is the organized cellular death that occur according exact genetically planning. Infected chicks with Gumboro virus, amount of apoptotic cells in bursa of fabricius has a direct relation with tense and amount of RNA virus. Namely, RNA virus multiplication the increase, act as an apoptosis inducer. In early status of infection, the amount of virus RNA is low, whereas the amount and rate of programmed cell death in spleen and bursa is high. It seems that, the increase of cytotoxines production such as Interlockin3 TNF likeness agent and interferon has an important role in apoptosis occurrence this infection. According to the studies, 2-6 days after infection by Gumboro virus, lyses and number decline of heterophil myelocyte is visible, specially in 2-3 days after infection. According to the Tunel staining, determinate that high cellular classes decline occurred by apoptosis and necrosis. It is known the studies indicate that chicks have receptors (Notch-1) on their lymphocyte B cells surface. The stimulation these receptors decrease cellular growth and apoptosis. Stimulation biochemical products of these receptors are Hairy-1 that induces apoptosis but as like as Notch-1 does not cause any pause in G1stage of cellular growth (Jungmann *et al*., 2001).

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MATERIALS AND METHODS

In this research, 50 SPF leghorn chicks (28 days old) selected and devised in two groups (experimental and control groups) and each 25 chicks placed in a separate room. Then experimental group, infected with Gumboro virus strain IRAN499 (IR499) that is a high acute strain of Gumboro disease virus. They infected way was oral and its amount was 1 mL with 10^6EID_{50} titer. Whereas for control group physiological serum. At 4th day after infection when mortality, all of dead chicks, test group chicks and control group chicks microscopically studied and cecal tonsils were removed and get used transported to pathologic laboratory to provide samples for hematoxilin-eosin staining to study by light microscope. Immediately after necropsy, samples was put in 10% formalin buffer and to then samples provided by routine paraffin form and hematoxilin-eosin staining.

Then in each slides 4 areas selected and studied with number 100 magnifications and apoptotic cells in each group (control and experimental) were counted and determined their mean. Data statistically analyzed according to Mann-Whitney and t-test methods. Some part of mentioned samples (with 1 cm scantling) were put in fixing fluid that was made of 86 mL physiological serum, 10 mL of 37% formaldehyde, 1.16 g $NaH_2PO_4.H_2O$, 0.27 g NaOH, 4 mL of 25% gluteraldehyde.

For second fixation to create suitable slices of samples, they were put in osmium tetra oxide and then samples were put in 30, 30, 60, 70, 80, 85 and 95° ethanol for about 10 min and in 100, 100 and 100° ethanol for about 15 min. After these periods, samples were put in propylene oxide for 3 times and each period last 10 min. Finally the samples entered in Epon solution, get formed, cut and staining (uranyl acetate-lead citrate staining) and they were studied with electronic microscope at demandable magnification (Sharma *et al*., 2002; Vasconcelos and Lam, 1994).

RESULTS AND DISCUSSION

Results of the Gumboro virus effect on necropsy finding are shown in Table 1. The results of light microscopic by computation of apoptotic cells, number with ×100 in 4 field and to get their average (Table 2). After statistic analysis (with t-test and Mann-Whitney Method), the mean of experienced apoptotic cells determined in control group (0.68±0.852) and in test group (25.56±12.007) with attention to both of test, means had signification difference ($p = 0.000$). With attention to light and electronic microscopes studies in control group in appreciable number of apoptotic cells that in normal condition exist in cecal tonsils were observed. In cecal tonsils of control group chicks, cell's nucleus observed at centre and they were not fragmental or not marginal. Also, cells cytoplasm's observed without alteration and fragmentation or various cytoplasmic bubbles (Fig. 1). Within cecal tonsil of infected chicks, apoptotic cells observed with definite chromatin fragmentation and concentration in hematoxilen-eosin staining (Fig. 2).

Within electron microscopic study, apoptotic cells of cecal tonsils with cell cytoplasm destroyed, nucleus fragmentation, chromatin concentration and lunette constriction were observed. Sting A shown the chromatin of nucleus apoptotic cells in situation fragmentation and marginal. Sting B show a view of apoptotic cell with lunette constriction, marginal and fragmentation of chromatin under nucleus membrane (Fig. 3). Generally, electronic microscopic studies on the cecal tonsils

Table 1: Necropsy finding in experimental group

Necropsy findings	Frequency of necropsy finding
Petechiae in leg muscles	20
Petechiae in breast muscles	4
Petechiae in Isthmus (between proventiculus and gizzard)	9
Kidney changes	5
Bursa edema without bloodshed (gelatin with yellow exudates)	21
Bursa edema with bloodshed veins	10
Atrophic bursa	0
Spleen changes (edema and bloodshed)	8
Hemoraghia in cecal tonsils	10
Mortality	13

Table 2: The apoptotic computational cells number in cecal tonsils in the control and experimental groups

No. of chickens	Control group	Experimental group
The number of apoptotic cells		
1	0	28
2	0	11
3	2	12
4	0	20
5	1	33
6	1	12
7	2	43
8	1	28
9	1	21
10	0	11
11	3	18
12	1	26
13	2	44
14	1	19
15	0	21
16	0	32
17	0	54
18	0	11
19	0	21
20	1	34
21	0	25
22	0	47
23	0	13
24	0	26
25	1	29

Values are defined as $p = 0.001$

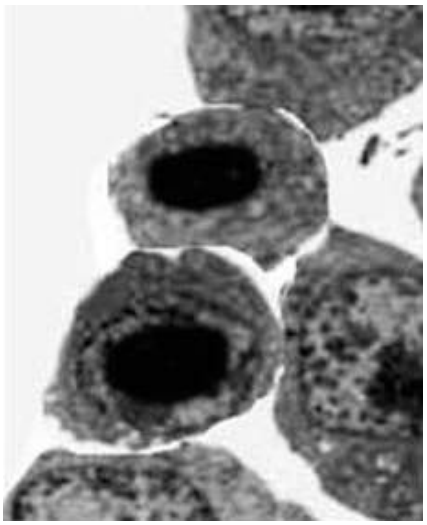

Fig. 1: Lymphocytes cells of cecal tonsil. As it shown lymphocyte cells are absolutely natural, cells nucleus are in center without fragmentation or chromatin marginalization. Cell cytoplasm has no change and fragmentation or different bubbles. uranyl acetate-lead citrate staining ×8000

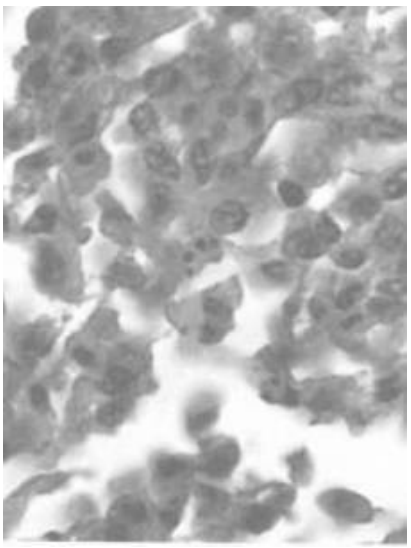

Fig. 2: The cross micrograph of cecal tonsil in septic chicken to Gumboro virus (experimental group): A few number of apoptotic cells are visible with fragmentation and certain density of chromatin. The hematoxilen-eosin staining ×100

(control and experimental group), determined that control group had received physiological serum, inappreciable number of apoptotic cells that normally experience were observed. When as test group chicks that received infectious virus, besides necrosis, apoptotic lymphocytes

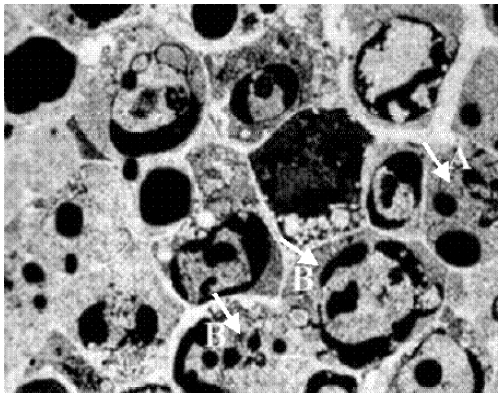

Fig. 3: The existence of apoptotic cells in cecal tonsil are observed together chromatin density and their piecemeal and with aggregation under the nucleus membrane. In the chromatin themes crescent aggregation are observed. The uranyl acetate-lead citrate staining ×8000

cells were observed as concentrated and fragmentated chromatin and in some cases lunette chromatin under nucleus membrane of apoptotic cells that determine apoptosis occur in lymphocytic cells and cecal tonsils peripheral cells. According to the other researchers studies, it is assignable that beside occurring necrosis in lymphocytic cells specially, lymphocytes exist in bursa, spleen and other lymphatic organs also, apoptosis is one of important processes that occur in these organs, during infecting with Gumboro virus. Virus after infecting macrophages and lymphatic cells of duodenum, jejunum, cecum, exited to liver and was infected liver coopfer cells and cause viremia and then exited to bursa and start its main reproduction and create histopathological alterations as follow (Baxendale, 2002):

- Inhibits stemcell substitution to mature B lymphocyte
- B lymphocyte cytolysis by necrosis
- Lymphatic cells apoptosis

Therefore, B lymphocytes decline and humeral immune system weakness and flock will be sensitive to all kinds of infection such as bacterial infections and vaccination programs defeating that caused by decreasing production of antibodies (Genova, 2000). Also in these modification, central region of bursa afflicted to cystic cavitations that it cause is lymphatic cells of bursa lymphatic follicles necrosis and the number of plical fold were decreased (Hiari and Funakoshi, 1987).

B and T lymphatic cells, in Gumboro disease are target cells for cellular death. As apoptosis in these cells are occurred in three processes. At first stage, apoptosis

starter signals affected by Fas (CD95) receptors were began and at second stage, cells were afflicted to morphologic modification and chromatin concentration, cytoplasmic vacuolation and activate internal endonoclease and at third stage, apoptotic bodies digested by macrophages (Fernandez *et al*., 1997). Immatase B lymphocyte cells that have surfaced IgM^+ receptors are major target of Gumboro's virus (Kim and Sharma, 2000).

According to the researchers studies, Gumboro's virus after contact with IgM^+ (on the surface of LB) with a content of a VP_2 and 17KD (NS) proteins and with BCL2 proceeds inhibiting cause apoptosis in lymphatic cells as they compel to produce some of cytokines such as ALFA TNF, interlokin 6, 8 alike agents and NOIF and secretion of NO by macrophages, at first excite the apoptosis in peripheral cells and then with infectious progress apoptosis excite infected cells. This process is visible in illustration of electron micrograph with chromatin fragmentation and concentration and chromatin accumulation under nucleus membrane surface (Inoue *et al*., 1999; Domanska *et al*., 2002).

CONCLUSION

According to the researcher's studies on Gumboro virus's effects on bursa and spleen and disease progress with use up necrosis, apoptosis excites and cytokine producers realized statistical significant results of cecal tonsil apoptotic cells count in this study. It is mean that destruction effects and immune system weakness were not only by necrosis effects but apoptosis and some of cytokines products had important role in disease progress (Kun and Vikram, 2001; Saif and Phil, 2003; Tanimura and Sharma, 1997, 1998).

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