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COMPARATIVE EVALUATION OF APOPTOSIS INDUCED CHANGES IN THYMOCYTES OF SPF CHICKS CO-INFECTED WITH CHICKEN INFECTIOUS ANAEMIA VIRUS (CIAV) AND MAREK'S DISEASE VIRUS (MDV)

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KEYWORDS

Chicken infectious anaemia virus

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Co-infection

Apoptosis

Thymocytes

ABSTRACT

Chicken infectious anaemia (CIA) and marek's disease (MD) are highly prevalent immunosuppressive viral infections in India and so natural co-infection is also likely to occur. The present study evaluated the extend of apoptosis induced in the thymocytes of SPF chicks challenged with CIAV and MDV both alone and in combination. Seventy two (72) SPF chicks were randomly allocated into 4 groups viz., CIAV (challenged with CIAV on day1), MD (challenged with vMDV on day14), CIAV + MD (challenged with CIAV on day1 and vMDV on day14) and CONTROL (without virus challenge). Three birds from each group were sacrificed on day14, 28 and 42, followed by collection of thymus and thymocytes were separated and suspended in PBS as single cell suspension. Further treatment of cells to release caspase3 and its colorimetric evaluation were done using caspACE assay kit (Promega). Results of present study indicated a significantly higher ($p<0.05$) level of caspase3 released in CIAV and CIAV + MD groups from day 14 onwards, reached peak on day 28 but by day 42 CIAV alone group showed a decline while in co-infected group the level remained significantly higher ($p<0.05$). So results of this study point to a greater extend of apoptosis in co-infected group, aggravating the immunosuppression.

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1 Introduction

Co-infections with different etiological agents are much frequent in Indian poultry industry, among which infections associated with immunosuppressive pathogens inflicts wide spread impact on the economy (Li et al., 2016; Zhang et al., 2017). Since global requirement for poultry products is increasing, poultry industry is forced to enforce intensive production facilities which ultimately predisposes to stress and immunosuppression (Padhy et al., 2015). This necessitates wider/broader assessments and diagnosis regarding the prevalence of immunosuppression in chicken caused by both noninfectious and infectious causes (Dhama et al., 2008; Wani et al., 2015). Immunosuppressive infections predispose the birds to vaccine failure, loss of natural immunity, growth retardation, low feed conversion efficiency and adversely affects the breeding policies and national disease control measures (Bhatt et al., 2011). Both Chicken Infectious Anaemia (CIA) and Marek's Disease (MD) are highly prevalent viral immunosuppressive diseases of Indian poultry (Gupta & Deka, 2016; Ganar et al., 2017). So it is highly warranted to have clear cut idea over the extend of pathogenesis like apoptosis induced in the target tissues of susceptible birds which are coinfectd with CIAV and MDV so as to rethink the vaccination and management practices to curtail the economic losses.

The genetically controlled process of apoptosis or programmed cell death is sequel to several infectious and non infectious pathological events. Though it is necessary to have balanced cellular apoptosis for normal physiology, uncontrolled or unregulated decrease in apoptosis of cells leads to tumour conditions while increase will end in fatal outcomes (Elmore, 2007). Primarily there exists an intrinsic and an extrinsic pathway, the former being initiated with caspase8 activation while the latter depends on activation of caspase9 (Cohen, 1997). Both the pathways are finally terminated with the executioner factor caspase3 for final apoptotic events (Kvansakul, 2017). It is the apoptosis induced by CIAV in the lymphoblastoid cells like thymocytes, which is the main pathological mechanism exerted in chicken and clinical manifestations and secondary complications are attributed to this mechanism only (Crowley et al., 2011). Hence the present study has been executed to elucidate the extend of apoptosis in thymocytes in chicks coinfectd with CIAV and MDV for the better understanding of the coinfection pathology.

2 Materials and Methods

2.1 Experimental birds

The present study was conducted in total 72 specific pathogen free (SPF) chicks, hatched out aseptically in the hatchery unit of ICAR-Central Avian Research Institute, Izatnagar, from SPF eggs procured from the M/S Venkateshwara Hatcheries Group Limited (VHL), Pune, Maharashtra, India.

2.2 Challenge viruses

Indian field isolates of CIAV strain A and virulent MDV maintained in the Avian Diseases sections, ICAR-IVRI was used for the study after following standard virus titration methods for each virus.

2.3 Study site

Chicks were housed in the experimental sheds of Avian disease section within IVRI campus with proper biosecurity measures for challenge study. Control group was kept separately in the control shed at considerable distance. Birds were provided with adlibitum feed and water after autoclaving.

2.4 Experimental design

Chicks were randomly divided into 4 groups (CIAV, MD, CIAV + MD AND CONTROL) with 18 birds per group. CIAV and CIAV + MD groups were challenged with CIA virus at a dose of 50CID₅₀ intramuscularly at day old age as per Kaffashi et al. (2006). MD group was challenged with 1000PFU of MDV intraperitoneally at 14th day of age. Control group was maintained uninfected. For assessing the caspase3 secretion from thymocytes, 3 birds from each group were sacrificed on 14th, 28th and 42nd days post CIAV infection, from 4 groups, followed by aseptic separation of thymocytes and cells were treated for caspase3 release and colorimetric estimation using commercial assay kit as per manufactures protocol. For the separation of thymocytes, thymus lobes were collected from sacrificed chicks aseptically and placed in sterile petri dishes. After washing with sterile PBS (pH 7.4), tissue was transferred to another dish, minced into 3-4mm pieces with cold PBS and washed to remove RBCs. Again minced for individual thymocytes and centrifuged at 250g for 5 minutes, discarded supernatant, cells suspended in 5ml PBS. Individual cell suspension was filtered through cell strainer, collected in sterile tubes suspended in PBS and cell count was assessed.

Colorimetric estimation was done using caspACE assay kit (Promega, USA) following manufactures protocol. Initially, a standard curve was prepared (Fig. 1) using different known concentrations of paranitroaniline (pNA) plotted against corresponding absorbance at 405nm for quantifying the caspase3 released from the cells. Thymocytes collected from each bird was centrifuged at 2000rpm for 5 minutes to obtain cell pellet, suspended with lysis buffer (provided in the kit) and added onto each well of 24-well culture plate (@ 1x10⁸ cells / ml). Cells were lysed by repeated freeze-thaw, then incubated on ice for 15 minutes, followed by centrifugation at 15,000 × g for 20 minutes at 4°C, and collected the supernatant fraction which was used as the enzyme source. The assay was performed in a total volume of

100µl in 96-well plates and reaction set up for each well is given in table 1. Then, 2µl of DEVD-pNA Substrate (10mM stock) was added to all wells and incubated at 37°C for 4 hours followed by measuring absorbance in the each wells at 405nm.

Quantitative analysis of caspase3 released by cells in each group was extrapolated from the standard curve (Figure 1). Values were analysed statistically by 2-way repeated measures ANOVA using Graphpad prism software version 7.

3 Results and Discussion

The caspase3 released by thymocytes of chicks in different groups under study is given in table 2 and figure 2. Caspase3 released is expressed in terms of and proportional to the paranitroaniline (pNA) released from the culture due to the action of the enzyme. Our results showed that the amount of caspase3 released on 14th day was significantly higher in CIAV and CIAV + MD groups, indicating the apoptosis in thymocytes induced by CIA virus. By 28th day level of apoptosis reached its peak in these two groups while a decline was indicated by 42nd day, but the level was significantly higher in co-infected group than CIAV alone. Caspase3 from MD group remained comparable to that of control group up to 28th day, whereas on 42nd day the level in MD group was significantly elevated than control but remained comparable with that of CIAV and at a significantly lower level with that of co-infected group.

Apoptosis induced by CIAV in the lymphoblastoid cells, thymocytes in particular, is the main pathological mechanism exerted in the host and clinical manifestations and secondary complications are attributed to this mechanism only (Smyth et al., 2006). Previous studies found out that CIAV induce cytopathogenic effects in vivo in chicken thymocytes and transformed cells of chicken origin in vitro via apoptosis (Noteborn, 2004; Kucharski et al., 2016). It has been reported that apoptin or VP3 protein of CIAV induces apoptosis by using host cellular components especially of intrinsic / mitochondrial apoptotic pathway and this involve the downstream caspases particularly caspase3 as the ultimate executioner of apoptosis (Danen-van Oorschot, 1999). Our results also run in accordance with the above reports since a significant increase in the release of caspase3 could be seen in the initial 4 weeks of CIAV infection. Reduction in apoptosis by day 42 indicates potential restriction over CIAV pathogenesis and apoptin induced apoptosis which can be attributed to the neutralizing antibodies developed by that time (Schat, 2009; Dhama et al., 2011). It has been reported that though MDV exerts lymphoproliferative

Table 1 Reaction set up for caspase colorimetric assay

Caspase assay buffer	32 µl
DMSO	2 µl
DTT, 100mM	10 µl
Sample cell extract	20µl (25–100µg total protein)
Deionised water	upto 98 µl

Figure 1 Standard curve generated with various concentrations of pNA and corresponding absorbance at 405nm for colorimetric estimation of caspase3 released by cells.

Table 2 Results showing the release of caspase3 by thymocytes in different groups

DAYS	EXPERIMENTAL GROUPS			
	CONTROL	CIAV	MD	CIAV + MD
14	39.20±7.03 ^a	379.58±35.30 ^b	56.92±3.83 ^a	327.95±33.67 ^b
28	46.04±6.91 ^a	823.93±75.78 ^b	166.67±31.65 ^a	753.27±94.60 ^b
42	42.31±2.96 ^a	198.62±43.70 ^b	214.27±16.16 ^b	505.38±2.91 ^c

Value given as mean ± SEM of picomols of pNA liberated due to caspase3 from the cells. Values bearing different superscripts differ significantly ($p < 0.05$) between groups within a day.

Figure 2 Results showing the release of caspase3 by thymocytes in different groups expressed as mean ± SEM of picomols of pNA liberated due to caspase3 from the cells. Values bearing different superscripts differ significantly ($p < 0.05$) between groups within a day.

changes in birds (Lee et al., 2000; Gimeno, 2008) an early cytolytic phase of MDV pathogenesis has been reported (Gimeno, 2008; Boodhoo et al., 2016) where it induces substantial apoptosis in immune cells especially B cells. This is evident in the substantial increase in the level of caspase3 released by MDV infected thymocytes during initial 4 weeks of MDV challenge. Results in CIAV + MD group showed a greater extend of apoptosis in this group at all time points than other two groups. This indicates that the restriction of CIAV induced apoptosis by day 42 (as evidenced in CIAV group) was violated in this group which may be due to decline in protective humoral responses and neutralizing antibody production, since coinfection with MDV specifically lyse the B cells. This indicates an aggravated tissue damage in the combined infection which is fatal and can pave for persistence in infection with sever degree of clinical manifestation at the cost of production loss.

Conclusion

The study indicates that the extend of apoptosis in chicken thymocytes induced by CIAV in co-infection with MDV is extensive and persisting in comparison with the CIAV alone infection.

Conflict of Interest

Authors would hereby like to declare that there is no conflict of interests that could possibly arise.

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