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RESEARCH ARTICLE

Effect of Aflatoxin Induced Immunosuppression on Pathogenesis of H9N2 Avian Influenza Virus

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A B S T R A C T

The study was performed to investigate the immunosuppressive effect of aflatoxin on the pathogenesis of H9N2 AI virus in SPF chickens. The experiment was carried out on 110 unvaccinated day old SPF chicks. They were divided into four groups of 25 birds each. Group I was kept as a non-treated and non-infected control; group II was intranasally infected with H9N2 AI virus at the 4th week of age; group III was fed on a diet containing 0.75 ppm aflatoxin from day one through the entire experiment period and group IV was fed on diet containing 0.75 ppm aflatoxin as group III and infected intranasally with H9N2 AI virus at the 4th week of age. Five chicks were kept as contact control (without infection) to group II and group IV. Five chickens from each group were slaughtered at 4th, 9th, 14th, 20th and 27th DPI Serum was collected from all slaughtered birds (5 serum samples/group/slaughter time) for serology (HI). Specimens from nasal conchae, trachea, lungs, liver, kidneys, bursa of Fabricius, thymus, spleen, pancreas and brain were collected from slaughtered birds for histopathology and immunohistochemistry. The histopathological lesions were more severe and persist till the end of the experiment in group IV. Using immunoperoxidase technique viral antigens were detected in the nasal conchae, trachea, lungs, thymus, kidneys and brain in group II while in group IV it extended further to the pancreas and bursa of Fabricius. In conclusion, the immunosuppression caused by aflatoxin increased the severity of lesions and allowed the virus to be disseminated to more organs.

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INTRODUCTION

The H9N2 AIV has been isolated from chicken and reported many times from various countries (Alexander, 2003; Ahad *et al*., 2013a; 2013b). Although the H9N2 avian influenza virus (AIV) was reported to be of low pathogenicity in chickens (Bano *et al*., 2003), it has been incriminated in several out breaks since its first isolation (Mo *et al*., 1998). More recently it was isolated in association with widespread and serious disease problems in commercial chickens in Iran and Pakistan (Alexander, 2000). In Egypt, it seems that H9N2 has been circulating in an undetectable manner since a serological evidence of H9 spread throughout Egypt has been recorded on 2009-2010 (Afifi *et al*., 2013). Successively, H9N2 AIV has been isolated from a broiler-breeder farm found in northern part of Egypt as well as from commercial bobwhite quail (*Colinus virginianus*) (Abdel-Moneim *et al*., 2012; El Zoghby *et al*., 2012).

In the last few years, the H9N2 AIV seems to regain importance as novel genotypes continue to arise. The recently isolated genotypes from poultry in Pakistan were found to contain NS genes similar to highly pathogenic H7N3 and H5N1 viruses (Iqbal *et al*., 2009). Moreover, the Egyptian H9N2 strain was found to have a genetic constitution that suggest the ability of the virus to acquire basic amino acids in the HA connecting the peptide sequence needed to become highly pathogenic (Abdel-Moneim *et al*., 2012).

Since it is presumed that only highly pathogenic avian influenza (HPAI) viruses cause systemic infection and death in chickens, it is unclear why H9N2 LPAI viruses induce relatively high mortality in the field populations of chickens. Previous available data on the pathogenesis of LPAI viruses in chickens suggest that the H9N2 LPAI viruses in domestic poultry farms result in diverse clinical syndromes of varying severity depending on the viral strain as well as co-infection with

immunosuppressive diseases (Bano *et al*., 2003; Kishida *et al*., 2004).

Natural toxins produced by molds or fungus have threatened the quality and safety of food and have caused severe losses to poultry industry in recent times (Saeed *et al.*, 2009). In warm and moist climates, feed is vulnerable to invasion by aflatoxigenic Aspergillus spp. with the subsequent production of aflatoxin during harvesting, processing, transportation and storage (Park *et al*., 2001). Aflatoxicosis caused severe depression in growth rate and immune suppression in broilers. Feeding levels lower than 20 $\mu g.Kg^{-1}$ aflatoxin may still reduce their resistance to disease, decrease their ability to withstand stress and bruising, and generally make them unthrifty (Coelho, 1990). Consequently the concept of our study was based on demonstrating the immunosuppressive effect of low aflatoxin consumption on the pathogenesis and viral antigen distribution of H9N2 AIV in SPF chickens.

MATERIALS AND METHODS

Experimental birds, virus and aflatoxin procurement: One hundred and ten unvaccinated day old SPF chicks were purchased from El Fayoum egg production project, Agricultural Research Center, Ministry of Agriculture (Fayoum, Egypt) and were used to carry out this experiment.

Avian influenza A virus, A/turkey/Wisconsin/1/1966 (H9N2) was kindly obtained from viruses collection of department of virology, faculty of veterinary medicine, Cairo University. The virus was propagated in 11 day old SPF hatching eggs via allantoic sac inoculation. The virus titration to estimate EID_{50}value was performed according to Reed and Muench (1938).

Concentrates containing an estimated amount of aflatoxin were obtained from biochemistry department, Animal Health Institute, El Dokki and were used to prepare diet containing 0.75 ppm aflatoxin as a final concentration.

Experimental design: One hundred and ten day old chicks were divided into four groups. Group I non-treated non-infected, group II Infected, Group III non-infected and fed with feed containing 0.75 ppm aflatoxin from day one and through the entire experimental period, group IV infected and fed as group III (Table 1). The birds of group II and IV were infected with 10^6 EID_{50}/100µl/bird H9N2 at 28th day of age. After infection, the birds were observed for 27 days. Five chicks were kept as non-infected contact control in group II and group IV. Five chickens from each group were slaughtered on the 4th, 9th, 14th, 20th and 27th day post infection. The chickens of the contact control in group II and group IV were slaughtered at the end of the experiment.

Serum was collected from the slaughtered birds (5 serum samples/group/slaughter time) for determination of antibody titer against avian influenza virus using HI tests (OIE, 2012)**.** Four hemagglutination units of homologous antigen which was prepared from the same strain used for infection, was used. The birds were daily observed throughout the experimental period to record the signs and deaths. At the times of slaughter, the birds were weighed and subjected to postmortem examination.

Table 1: Experimental design

Groups	N	Feed with aflatoxin	Infection*	Samples
I	25	-	-	**
	25			**
II	5 contact	-	+	Tissue specimens only 5 birds slaughtered at 27th DPI
III	25	+	-	**
	25			**
IV	5 contact	+	+	Tissue specimens only 5 birds slaughtered at 27th DPI

* Intranasal infection with H9N2 at day 28 (10^6 EID_{50}/100µl/bird); ** Tissue specimens and serum samples /chicken at slaughter (5 birds/4th, 9th, 14th, 20th & 27th DPI).

Histopathology and immunohistochemistry: Tissue specimens from nasal chonchae, trachea, lung, liver, kidneys, spleen, pancreas, bursa of Fabricius, thymus and brain were collected from the five slaughtered birds from each group at 4th, 9th, 14th, 21st, 27th DPI. Tissue specimens were fixed in 10% buffered formalin and then processed by the routine paraffin embedding technique, sectioned at 5 micron thickness and stained with Hematoxylin and Eosin (Bancroft *et al*., 1996).

Serum containing high antibody titer against H9 antigen was used as primary antibody in the immunohisto-chemical technique. The serum prepared by intranasal inoculation of ten chickens with H9 N2 virus at dose level 10^6 EID_{50}/ 100 µL/bird at 4th week of age and repeated at 8th weeks. The geometric HI mean titer of the collected serum was Log_2 7. This serum was used for the detection of H9 N2 antigen in the unstained paraffin sections prepared from different organs in the experiment as described by Colvin *et al*. (1988). Peroxidase conjugated anti-chicken antibodies were obtained from (Abcam, Cambridge, UK). Diaminobenzoic acid (DAB) was used for development of the color.

RESULTS AND DISCUSSION

Clinical signs: No mortalities were observed in all groups throughout the entire observation period. The clinical signs were restricted to depression, decreased food consumption and sneezing. They were observed in the two infected groups (Group II and IV) but were more prominent in the chickens fed on aflatoxin and inoculated with H9N2 AIV (Group IV). A matter which agreed with previous studies that showed H9N2 LPAIV cause only transient mild clinical signs and seldom cause mortality in experimentally infected SPF chickens (Mo *et al*., 1998; Lee *et al*., 2007). However, it was reported that the clinical signs were more severe in SPF chickens receiving cyclosporine (immunosuppressant agent) and infected with H9N2 than those observed in chickens which was only infected with H9N2 (Kwon *et al*., 2008). It was believed that high mortalities in the field were due to environmental stress and concurrent secondary infections (Bano *et al*., 2003). Therefore it can be assumed that immunosuppression has an effect on the severity of clinical signs but it doesn't equal the influence of secondary bacterial infection.

Histopathological lesions: The respiratory lesions were varied in severity between the infected groups (group II and group IV) according to the duration of infection and

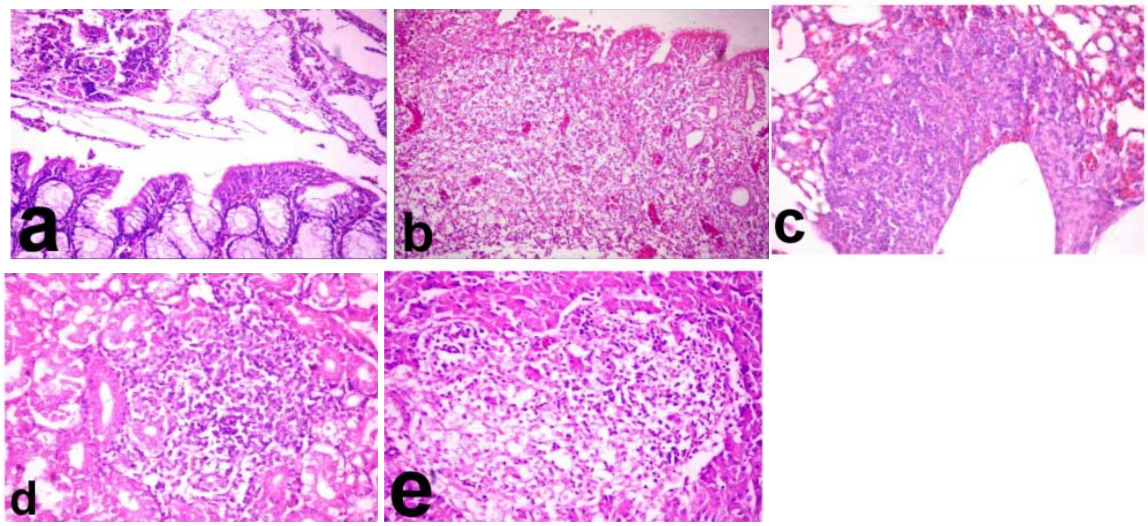

Fig. 1: Histopathological observations. a-Nasal chonchae of chicken in group II (4 days PI) showing mucus exudates, inflammatory cells and erythrocytes in the lumen, b-Nasal chonchae of chicken in group IV(20 days PI) showing necrosis of the lining epithelium, congestion of blood vessels, mononuclear inflammatory cells infiltration and edema of submucosa; c-Lung of chicken in group II (9 days PI) showing congestion and focal aggregation of lymphocytes adjacent to the parabronchus; d-Kidney of chicken in group II (9 days PI) showing focal interstitial mononuclear inflammatory cells aggregation and e-Pancreas of chicken in group IV (20 days PI) showing focal area of necrosis with inflammatory cell infiltration. H & E; a and b: X 200; c-e: X 400.

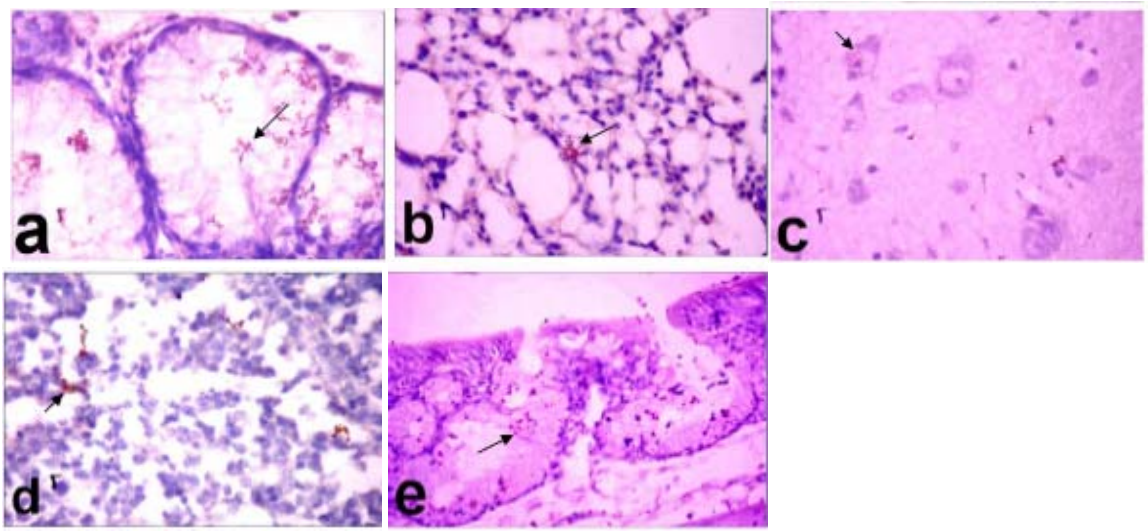

Fig. 2: Immunoperoxidase findings. a-Nasal chonchae of chicken in group II (4 days PI) showing localization of AIV antigen (arrow) in the mucus gland epithelium detected (indirect immunoperoxidase technique); b-Lung of chicken in group II (4 days PI) showing AIV antigen (arrow) in the pneumocytes and lumen of the air capillaries detected (indirect immunoperoxidase technique; c-Brain of chicken in group IV (9 days PI) showing brown granules of viral antigen (arrow); d-Bursa of chicken in group IV (9days PI) showing brown granules of viral antigen (arrow); and e-Nasal chonchae of contact chicken to group II showing brown granules of viral antigen in the epithelium of the mucus glands. a-e: X 1000.

the organs affected. However, the inflammatory reaction was most prominent in the nasal conchae than in the trachea and lungs. The nasal conchal lesions in group II appeared in the form of activation of the mucous secreting glands with inflammatory exudates in the lumen (Fig. 1a) as well as severe congestion and hemorrhages in the submucosa at 4th and 9th DPI. The severity of the lesions reached the peak by the 14th day where severe necrosis with denudation of mucosa appeared. Severity of the lesions decreased by the 20th day and subsided by 27th day. In group IV, the intensity of the lesions was more severe and persistent till the end of experiment (Fig. 1b). The tracheal lesions in group II were most prominent on the 9th day and subsided by 14th day of experiment whereas it persisted in group IV till the 27th day post infection. Concerning the lungs, the examined sections revealed severe congestion of all pulmonary blood vessels with multiple focal areas of hemorrhages. Focal aggregations of lymphocytes were commonly observed perivascular and close to the parabronchi (Fig. 1c). Inflammation of the wall of secondary bronchi with exudation in the air way was a common finding in group IV.

In our study, the infected chickens which were fed on aflatoxins showed more severe histopathological lesions than the group infected with the virus only. Therefore it

can be assumed that immunosuppression which was induced by dietary aflatoxicosis had an effect on the severity of the histopathological lesions recorded. This agrees with what was mentioned earlier about the effect of immunosuppression on the pathogenicity of the virus (Toroghi and Momayez, 2006; Aamir *et al*., 2007).

The renal lesions observed in both infected groups were in the form of tubulointerstitial nephritis (Fig. 1d). These results were close to the results of Pazani *et al*. (2008) who said that severe congestion, urate deposition and non-suppurative focal interstitial nephritis were the predominant histological lesions. The pancreas was affected only in group IV and the pancreatic lesions were manifested by focal acinar necrosis associated with inflammatory cells infiltration (Fig. 1e). The pancreatic lesions of the infected group with H9N2 recorded in the current study appeared non-specific although few cases at 20 days post-infection showed pancreatitis. The lesions in the infected chickens fed on aflatoxins were remarkably more severe and extended till the end of experiment. Pancreatitis was recorded in intratracheally inoculated chickens with H9N2 as a non-specific lesion (Hablovarid *et al*., 2004). Thus, the debate aroused from whether the pancreatic lesions are specific or non-specific and whether the incriminated route of infection had an effect on the involvement of the pancreas can be conclusively settled by direct antigen detection.

The lesions in the lymphoid organs (bursa of Fabricius, thymus and spleen) varied between the infected groups from lymphocytic necrosis, lymphocytic depletion with reticular cells -hyperplasia but the changes were more severe and prominent in group IV. The examined sections of brain in group II revealed neuronal degeneration and perivascular lymphocytic cuffing. Moreover, the brain in group IV exhibited areas of malacia, focal gliosis and marked demyelination.

Immunohistochemistry: The viral antigen was demonstrated in the nasal chonchae, trachea, lungs, kidneys, thymus and brain in both infected groups (Fig. 2 a, b & c). Substantially, the viral antigen was also recorded in the pancreas and bursa in the immunosuppressed infected chickens only (Fig. 2d). The viral antigen was also demonstrated in the respiratory organs of contact chickens found in company of the two infected groups (Fig. 2e). The reaction was severe in contact birds.

Similarly, previous studies were capable of demonstrating the viral nucleoprotein and antigen (H9N2) in trachea and lungs using immunoflorescence technique and immunoperoxidase technique following intranasal and intratracheal infection with H9N2 virus, respectively (Hablovarid *et al*., 2004; Hadipour, 2009; Hadipour *et al*., 2009). In our study the viral antigen was also demonstrated in the kidney. This agrees with Hadipour (2009) and Hadipour *et al*. (2009) who were also capable of demonstrating the viral antigen in kidneys following intranasal inoculation using immunoflorescence technique.

Viral antigen was detected in bursa of Fabricius in the immunosuppressed group and not detected in the group II a matter agreed with Kwon *et al*. (2008). The viral antigen was demonstrated by immunoperoxidase in the pancreatic acini in the group IV only which suggest the enhanced distribution of the viral antigen in the group IV. Consequently, viral antigen detection in pancreas can be attributed to the immunosuppressive effect of the aflatoxin which allowed the virus dissemination to different organs. In the current study, the brain lesions were noticed in both groups (group II and group IV). The viral antigen detected in the brain of both groups confirms that the histo-pathological lesions were due to the direct effect of the virus.

Serological studies: The HI test was performed on the serum obtained from the five chickens slaughtered from each group at each time of slaughter. The serum was collected at the 4th, 9th, 14th, 21st and 27th DPI. The HI test revealed a gradual increase in the HI titer and number of birds developing antibodies. Collectively, the geometric mean titer was higher and developed faster in group II (Fig. 3).

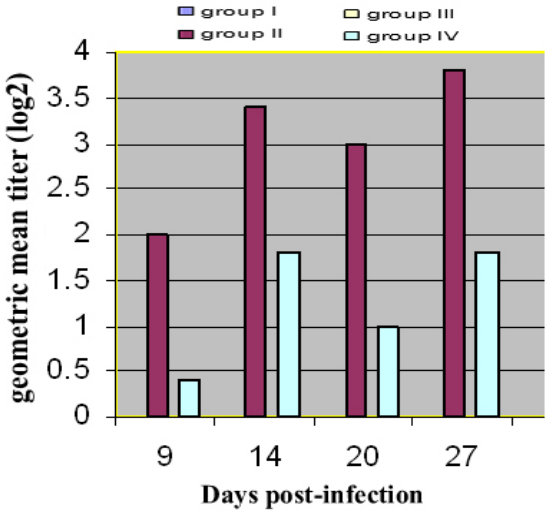

Fig 3: Chart showing the geometric mean titer for the four groups using hemagglutination inhibition test

In our study, the geometric mean titer of group II ranged from Log_2 2 at 9th DPI to Log_2 3.8 at 27th DPI whereas of group IV ranged from Log_2 0.4 to Log_2 1.8. These results agreed with Gharaibeh (2008) who stated that both broiler and SPF-infected groups with H9N2 virus were seroconverted on the hemagglutination inhibition test at 16 DPI giving a geometric mean titer of Log_2 8.2 and Log_2 9.3, respectively. Similarly, the Indian virus isolates of H9N2 gave a positive HI test with H9 reference antisera with titers ranging from - Log_2 4 to Log_2 9 (Nagarajan *et al*., 2009)

Conclusion: The present study has provided an insight into the pathogenesis of the virus in tissue. It showed that the virus (H9N2 AI) alone produced mild histopathological lesions that were confined to the respiratory organs, brain, kidneys and lymphoid organs. These lesions alleviated at 27 DPI. On the other hand, the lesions were more severe and persist in infected chickens fed on aflatoxins. These data can help explain the high mortalities found in field. It was presumed that immunosuppression caused by aflatoxin together with the

lymphocytic depletion induced by the virus facilitates the invasion with secondary bacterial infection.

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