Veterinary Microbiology 231 (2019) 24–32

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Immunological and pathological effects of vitamin E with Fetomune Plus® on chickens experimentally infected with avian influenza virus H9N2

Walaa F. Awadin[a], Abdelfattah H. Eladl[b,*], Reham A. El-Shafei[c], Mohamed A. El-Adl[d], Hanaa S. Ali[e]

[a] Department of Pathology, Faculty of Veterinary Medicine, Mansoura University, Mansoura, Egypt
[b] Department of Poultry Diseases, Faculty of Veterinary Medicine, Mansoura University, Mansoura, Egypt
[c] Department of Pharmacology, Faculty of Veterinary Medicine, Mansoura University, Mansoura, Egypt
[d] Department of Biochemistry and Chemistry of Nutrition, Faculty of Veterinary Medicine, Mansoura University, Egypt
[e] Department of Pathology, Animal Health Research Institute, Mansoura branch, Egypt

ARTICLE INFO

Keywords:
AIV H9N2
Vitamin E
Fetomune Plus®
Gene expression
Histopathology
Immunohistochemistry
Chickens

ABSTRACT

Avian influenza virus (AIV) H9N2 infection causes economic losses on poultry farms, and immunostimulants are essential for improving chicken immunity. This study evaluated the immunological and pathological effects of vitamin E with Fetomune Plus® (a commercial product based on a yeast extract and vitamins) on chickens experimentally infected with AIV H9N2. Three groups of white Hy-Line chicks were included. The G1 group was kept as an uninfected untreated control, the G2 group was intranasally infected with the AIV H9N2 strain (0.5 ml of 10^6 50% egg infectious dose (EID_{50})), and the G3 group was infected and treated with vitamin E (200 mg/kg of diet) and Fetomune Plus® (1 ml/liter of drinking water) for four weeks. The gene expression of interferon-gamma (IFN-γ), interleukin (IL)-6, and IL-2 was determined at 3, 5 and 7 days post-infection (PI). Virus shedding titers and rates and haemagglutination inhibition (HI) antibody titers were detected. Clinical signs, mortalities and post-mortem lesions were recorded. The birds were weighed, and relative organ weights were calculated. Tissue specimens were taken for histopathological examination and immunohistochemistry (IHC). The expression of IFN-γ in the duodenum revealed a significant increase in G2 compared to G3 at 3 days PI, while the duodenal and splenic expression of IL-6 was significantly increased in G2 compared to G3 at 5 days PI. IL-2 was overexpressed in the duodenum in G3 compared to G2 at 3 and 5 days PI. A significant decrease ($P \leq 0.05$) in the virus shedding titer and an increase in the HI titers were detected in G3 compared to G2. The clinical signs and the mortality rate were clearly appeared in G2 than in G3. By IHC, lower H9N2 staining intensity was observed in the examined organs from G3 than in those from G2. In conclusion, as a first report, vitamin E with Fetomune Plus® supplementation for four weeks could improve the immunological and pathological effects of H9N2 infection on chickens.

1. Introduction

Avian influenza virus (AIV) H9N2 circulates widely in domestic poultry around the world (Karimi-Madab et al., 2010). The pathogenicity of AIV H9N2 is variable and depends on the presence of other pathogens. Although AIV H9N2 is classified as a low pathogenic strain, it can cause serious disease and high mortality in broilers due to mixed infections (Seifi et al., 2010). The genome sequence of the first AIV H9N2 isolate from chickens in Egypt was reported in 2012 by Abdel-Moneim et al. (2012).

Commonly, the immune response in poultry can be enhanced by preventing deficiency syndromes via the administration of vitamins at

Abbreviations: AIV, avian influenza virus; LPAI, low pathogenic avian influenza; IHC, immunohistochemistry; IL, interleukin; IFN-γ, interferon-gamma; HI, haemagglutination inhibition; GMTs, geometric mean titers; BW, body weight; PI, post-infection; RNA, ribonucleic acid; PCR, polymerase chain reaction; cDNA, complementary deoxynucleic acid; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HPF, high power field; PBS, phosphate buffered saline; NDV, Newcastle disease virus; MEVAC, Middle East for veterinary vaccines; EID_{50}, 50% embryo infective dose; DAB, diaminobenzidine; Ct, threshold cycle; NLQP, National laboratory for veterinary quality control on poultry production; HA, haemagglutinin; ANOVA, analysis of variance; IKKγ, IκBα kinase gamma; NF-κB, nuclear factor-κappaB

* Corresponding author.
E-mail address: abdelfatahpo@yahoo.com (A.H. Eladl).

https://doi.org/10.1016/j.vetmic.2019.02.028
Received 2 January 2019; Received in revised form 19 February 2019; Accepted 19 February 2019

levels higher than the currently recommended levels (Rama-Rao et al., 2004). Vitamins act as co-factors in various physiological processes, such as growth, development, maintenance, reproduction and immunity. Vitamins deficiency in the diet might cause decreased immunity (Bourre and Galea, 2006). Vitamin E is a well-known antioxidant and is one of the most commonly studied nutrients that can enhance the immune response of birds by improving humoral and cell-mediated immunity (Niu et al., 2009). Vitamins and minerals have positive effects on live weight gain and haemagglutination inhibition (HI) antibody titers in Newcastle disease (ND)-vaccinated chickens (Monoura et al., 2008). Rehman et al. (2018) suggested that supplementation with vitamin E can ameliorate Newcastle disease virus (NDV) infection by improving oxidative stress and histopathological changes in the duodenum and jejunum of infected chickens. In addition, the effect of vitamin E on influenza virus infection was reviewed by Mileva and Galabov (2018).

Beta-glucan is extracted from yeast and fungi and is characterized by its immunomodulatory effects due to its ability to enhance the immune system without causing the deterioration of the functions of other organs (Jacob and Pescatore, 2017). It has been suggested that beta-glucan can contribute to immune regulation through its anti-inflammatory role via the down-regulation of interleukin (IL)-8 expression (Cox et al., 2010). Moreover, Akhtar et al. (2016) showed that the use of mannan-oligosaccharide may reduce the spread of AIV H9N2 infection by decreasing virus shedding in poultry.

AIV H9N2 infection is associated with massive mortality and loss of production on poultry farms (Nagarajan et al., 2009), and the application of immunostimulants in poultry production is becoming essential for improving chicken immunity (Abdukalykova and Ruiz-Feria, 2006). Moreover, dietary supplementation with vitamin E has productive, reproductive and immunological effects on chickens (Alm El-Dein et al., 2013; Alm El-Dein et al., 2013). Therefore, we hypothesized that dietary supplementation of AIV H9N2-experimentally infected chickens with vitamin E and Fetomune Plus® in the drinking water would improve the immune reaction of the chickens. Gene expression levels of immune genes, virus shedding titers and HI antibody titers were evaluated. The final body weight (BW), relative organ weights (bursa of Fabricius & spleen), clinical signs, mortality rate, histopathology and immunohistochemical staining for H9N2 were detected.

2. Materials and methods

2.1. Birds and the experimental procedure

Ninety, 1-day-old white Hy-Line chicks from the same hatch were obtained from a commercial poultry farm (Dakahlia governorate, Egypt) that did not have a history of AIV H9N2 infection. All birds were reared in cages in a strictly isolated room and were provided with a basal balanced diet (NRC, 1994; Table 1). The birds were not given any medications. All biosecurity features and hygienic measures were followed. The birds were given feed and water *ad-libitum*. The birds were shown to be free from influenza virus by virus isolation in embryonated eggs and HI test (Iqbal et al., 2013). None of the birds were vaccinated with the AIV vaccine. At the age of 28 days, the birds were allocated into three groups (10 birds X3 replicates per group). The G1 group was kept as an uninfected and untreated negative control, the G2 group was infected with the AIV H9N2 strain, and the G3 group was infected with the AIV H9N2 strain and treated with 200 mg/kg of diet vitamin E. Dietary supplementation with vitamin E was administered from the 14th to 42nd day of the chicken life (the 42nd day was the end of the experimental period), and Fetomune Plus® was administered at a dose of 1 ml/liter of drinking water during the same period. The chicks in G1 and G2 were fed a basal balanced diet. The infected birds were intranasally inoculated with AIV H9N2 (0.5 ml of 10^6 EID_{50}/bird) (Arafat et al., 2018) at 28 days old. At 3, 5 and 7 days post-infection (PI), tissue specimens from the spleen and duodenum were collected from six slaughtered birds per group for the detection of the gene expression levels of interferon-gamma (IFN-γ), IL-6, and IL-2, and tracheal and cloacal swabs (n = 9 per group) were performed to measure the virus shedding titers and rates. The haemagglutination inhibition geometric mean titers (HI-GMTs) were estimated from collected serum (n = 9 per group) on days 0, 7 and 14 PI. The clinical signs, mortalities and post-mortem lesions in all groups were recorded. At 7 days PI, other tissue specimens were collected from the trachea, lungs, liver, spleen and duodenum of the six slaughtered birds in all groups for histopathological examination and immunohistochemical staining. Finally, six birds per group were weighed at 0 day PI (initial live BW) and 14 days PI (final live BW), and the relative organ weights (spleen and bursa) (n = 6) were calculated at 14 days PI (at the end of the experiment; 42 days of age).

Table 1
Ingredients (%) and proximate composition of the corn-soybean meal basal diet.

Ingredients %	Grower Control	Finisher Control
Corn grain (8.5%)	60.1	66.2
Soybean meal (44%)	27.6	22
Corn gluten (62%)	5.5	5.5
oil	3	3
Lime stone	1.5	1.5
Dicalcium phosphate	1.7	1.3
Premix*	0.25	0.25
salt	0.3	0.3
DL- Methionine	0.12	0.12
kDL-Lysine	0.1	0.1
Proximate composition (calculated)		
CP %	21.17	19.01
ME kcal/kg	3129	3197
Ca %	1.02	0.9
Available P %	0.45	0.35

Control represents corn-soybean meal basal diet. Vitamins and minerals premix used to cover the required vitamins and minerals per each kilogram diet (Vit. A, 10,000 I.U.; Vit. D3, 1500 I.U.; Vit. E, 10 mg; Vit. K3, 2 mg; Vit. B1, 2 mg; Vit. B2, 5 mg; Vit. B6, 3 mg; Vit. B12, 0.01 mg; Niacin, 27 mg; Folic acid, 1 mg; Biotin, 0.05 mg; Pantothenic acid, 10 mg; Mn, 60 mg; Zn, 50 mg; Cu, 10 mg; I, 0.1 mg; Se, 0.1 mg; Co, 0.1 mg; Fe, 50 mg).

All birds in this study were managed under the "Guide for the Care and Use of Laboratory Animals", and this study was approved by the Ethics Committee of the Faculty of Veterinary Medicine, Mansoura University.

2.2. Supplements and the challenge strain

-Vitamin E: Vitamin E was provided by Multivita Company, The 6th of October Governorate, Egypt. It was added at a dose of 200 mg/kg of diet (Niu et al., 2009) for 4 weeks.

- **Fetomune Plus®**: Fetomune Plus® is a commercial product composed of a yeast extract (mannan and beta-glucan), vitamin C, vitamin E, selenium, methionine, lysine, choline, zinc, copper, and manganese in addition to a plant extract and Lactobacillus. It was provided by Perfect Pharm Company (Kafr El-Zayat, Egypt). It was added at a dose of 1 ml/liter of drinking water for 4 weeks.

-**Challenge strain:** The low pathogenic AIV H9N2 strain (KX663332, A/chicken/Egypt/Mansoura-36/2015) used as the challenge strain was kindly provided by a researcher at the Department of Poultry Diseases, Animal Health Research Institute, Mansoura branch (Asmaa, 2016). This H9N2 strain was titrated in 10-day-old specific pathogen-free embryonated chicken eggs. The titration was calculated as previously indicated (Reed and Muench, 1938).

2.3. Tissue sampling

Tissue specimens from the spleen and duodenum were collected from six slaughtered birds per group on the third, fifth and seventh day PI and then stored at −80 °C until use for ribonucleic acid (RNA) isolation and complementary deoxynucleic acid (cDNA) synthesis to detect the gene expression levels of IFN-γ, IL-6, and IL-2. At 7 days PI, other tissue specimens were collected from the trachea, lungs, liver, spleen and duodenum of the birds in all groups and fixed in 10% neutral buffered formalin for histopathological examination and immunohistochemical staining.

2.4. Gene expression analysis of immune genes

2.4.1. RNA isolation and cDNA synthesis

Approximately 100 mg of tissue from the duodenum and spleen was homogenized in Trizol™ reagent (Invitrogen, UK) according to the manufacturer's instructions (Simms et al., 1993). The concentration of RNA was detected using a nano-spectrophotometer (Q5000 UV–vis spectrophotometer, San Jose, USA) to check the RNA purity and concentration. A total of 1 µg of RNA was reverse transcribed into cDNA with a high capacity cDNA reverse transcription kit (Applied Biosystems) using random hexamers in a 20 µl reaction volume that was diluted 1:20 for further downstream analysis.

2.4.2. Quantification of immune gene levels using real-time polymerase chain reaction (PCR)

In the current study, real-time PCR for the amplification and relative quantification of immune gene expression was conducted with a Pikoreal real-time PCR system (Thermo Fisher Scientific). Real-time PCR was conducted to assess the immune genes listed in Table 2 with their primer sequences and accession numbers in GenBank. Real-time PCR was performed using TOP real qPCR 2× premix (Enzynomics, South Korea) with the following cycling conditions: initial denaturation at 95 °C for 8 min, followed by 40 cycles of 95 °C for 40 s, 55 °C for 30 s and 72 °C for 40 s. Then, the reaction was terminated by a final elongation cycle at 72 °C for 7 min. The expression analysis was performed using the $2-\Delta\Delta CT$ method adopted by Livak and Schmittgen (2001). The fold expression was normalized against the expression of the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and this approach was suitable to use in chickens due to the minimal change in the expression pattern of GAPDH (Hong et al., 2006).

2.5. Virus shedding titers and rates in tracheal and cloacal swabs

Tracheal and cloacal swabs were collected from nine birds per group at 3, 5 and 7 days PI to evaluate virus shedding titers and rates. The swabs were vortexed with sterile phosphate-buffered saline (PBS, 2 ml) containing 1% gentamicin. Viral RNA was extracted using a QIAamp viral RNA Mini kit (Qiagen, Germany, GmbH), and PCR amplifications were performed in a final volume of 25 µl containing RNA template (7 µl), 2× QuantiTect Probe RT-PCR Master Mix (12.5 µl), PCR-grade water (4.125 µl), 0.5 µl of each primer (50 pmol conc.), 0.125 µl of probe (30 pmol conc.) and 0.25 µl of QuantiTect RT Mix. The real-time RT-PCR primers and probe sequences were previously described by **Ben** Shabat et al. (2010) and were as follows: H9F: 5′-GGAAGAATTAATT ATTATTGGTCGGTAC-3′; H9R: 5′−CCACCTTTTTCAGTCTGACATT-3′; and H9Probe: [FAM] AACCAGGCCAGACATTGCGAGTAAGATCC [TAMRA]. Reverse transcription was performed (52 °C for 30 min), followed by 40 cycles of denaturation (94 °C for 15 s), annealing, and extension (60 °C for 45 s). A standard curve was calculated, and the Ct values were converted into EID_{50}/ml (Lee and Suarez, 2004).

Table 2
Primers sequences of immune genes.

Gene	Sequence (5'-3')	Amplicon size (bp)	Accession number
GAPDH	F:GGTGGTGCTAAGCGTGTTAT R:ACCTCTGTCATCTCTCCACA	264	K01458
Il-2	F:TCTGGGACCACTGTATGCTCT R:ACACCAGTGGGAAACAGTATCA	256	AF000631
Il-6	F:CAAGGTGACGGAGGAGGAC R:TGGCGAGGAGGGATTTCT	254	AJ309540
IFN-γ	F:AGCTGACGGTGGACCTATTATT R:GGCTTTGCGCTGGATTC	259	Y07922

2.6. HI test and the H9N2 antigen

HI (beta-procedure) test was performed for the detection of AIV H9N2 antibody titers, and the HI-GMTs were estimated according to the method described by Beard (1989). The AIV H9N2 standard diagnostic antigen is a low pathogenic avian influenza (LPAI) antigen, A/Chicken/Egypt/11490v/NLQP/2011 (H9N2). This antigen was kindly provided by the Middle East for Veterinary Vaccines (ME VAC), Egypt.

2.7. BW and organ/BW ratios

Six birds per group were weighed at 0 day PI (for the mean initial live BW) and 14 days PI (for the mean final live BW) and then the relative organ weight (spleen and bursa) (n = 6) was calculated at 14 days PI (42 days of age). The weights of the bursa and spleen were each divided by the chicken's total body weight and then multiplied by 100 to obtain the organ/BW ratios (Mazariegos et al., 1990).

2.8. Histopathology and immunohistochemistry (IHC)

All tissue specimens were routinely processed for paraffin embedding. Two sets of paraffin sections were cut and prepared. After deparaffinization and dehydration, one set was stained with haematoxylin and eosin according to the protocol described by Bancroft and Gamble (2007). The other set of paraffin-embedded sections was deparaffinized with xylene and rehydrated through a graded series of ethanol. Blocking steps in the procedure included the incubation of the slides with 3% H2O2. Antigen retrieval was performed by treating the tissue sections with a quenching solution for 5 min, followed by rinsing the slides in a bath of phosphate buffered saline (PBS), pH 7.2. Primary antibodies were prepared at the Animal Health Research Institute, Cairo, Egypt and applied to the slides for 60 min. Two drops of a horseradish peroxidase polymer conjugate were applied and incubated for 10 min. For staining, a 3,3′-diaminobenzidine (DAB) chromogen solution (1 drop) was poured onto the tissue sections. Finally, the tissue sections were counterstained with Mayer's haematoxylin for 1 min. The entire procedure was performed as previously described by Aslam et al. (2015). Negative control slides were prepared by incubating sections with a saline solution. The staining intensity was observed by light microscopy and scored as described by Abdel-Moneim et al. (2009): 0 (no positive cells per high-power field (HPF)), 1 (infrequent = small number of positive cells per HPF), 2 (common = moderate number of positive cells per HPF), and 3 (widespread = high numbers of positive cells per HPF).

2.9. Statistical analysis

Statistical analysis of the data was performed using one-way analysis of variance (ANOVA) followed by the least significant difference test using a confidence level of 0.05 to assess the differences among the three groups. Statistical analysis was performed using SPSS 19 software (SPSS Inc., Chicago, Illinois) (Brosius, 2011). The gene expression levels of pro-inflammatory mediators at the three time points were tested using two-way ANOVA employing Tukey's test as a post-hoc test to

compare the differences among the means. Data were expressed as the mean ± standard error, and the results of the three replicates in each group were merged. Differences between means were considered significant when $P \leq 0.05$. Since scores for immunohistochemical staining intensity did not fit a normal distribution, non-parametric comparisons were performed with the Kruskal-Wallis test, and then each group was compared pairwise with the other groups using Dunn's test.

3. Results

3.1. Gene expression analysis of immune genes

In the duodenum, the IFN gamma level showed a significant ($P \leq 0.05$) decrease at all time points in the G3 group compared with the other study groups. Moreover, significant IFN gamma overexpression was observed in the G2 group (11-fold) compared with the other groups, and this overexpression started to significantly decrease on day 5. A significant increase in the expression of IL-6 was detected in the G2 group compared with the other groups on the fifth day ($P \leq 0.05$), and the G2 group IL-6 expression started to decline significantly on the seventh day ($P \leq 0.05$). Vitamin E supplementation as well as the mannan polysaccharide and glucan levels showed significant declines at all time points and in all groups compared with group G2 on the fifth day and the control group at all time points ($P \leq 0.05$). The expression of IL-2 was higher in group G3, especially on the 5th day, than in the other groups and started to significantly decrease on the seventh day ($P \leq 0.05$) (Fig. 1 A–C).

In the spleen, IFN gamma revealed an overall increase in expression in G3 on the fifth day ($P \leq 0.05$). On the 3rd day, a remarkable overexpression of IFN gamma was detected in group G2, but this expression also declined significantly on the fifth and seventh days of infection ($P \leq 0.05$). Similarly, the expression of IFN gamma in group G2 showed a significant decrease on the fifth day and started to decrease significantly on the seventh day ($P \leq 0.05$). A significant increase in IL-6 expression was observed in group G2 on the fifth day compared with group G3 at all time points ($P \leq 0.05$). The expression of IL-2 exhibited significant increases in both the fifth and seventh days PI in group G2 compared with group G3 ($P \leq 0.05$). In general, the expression of IL-2 was significantly downregulated in all groups compared with the control group ($P \leq 0.05$) (Fig. 2A–C).

3.2. Clinical signs, the HI-GMTs, virus shedding titers and rates, mortality, the mean BW, relative organ weights and gross lesions

No maternal HI antibody titers for AIV H9N2 were detected before infection in the experimental chicks at 1, 10 and 28 days of age. The HI-GMTs were significantly higher ($P \leq 0.05$) in G3 (72.1 & 128.3) than G2 (14.6 & 27.9) at 7 and 14 days PI, respectively. Clinical signs were absent in G1 and G3, while mild respiratory signs were observed in G2. The infected birds showed depression with low feed and water intakes, respiratory sounds, nasal discharge, and diarrhea. The signs appeared between 2 and 7 days PI. Virus shedding titers (tracheal and cloacal swabs) were significantly higher ($P \leq 0.05$) in G2 than G3 at 3, 5 and 7 days PI. Viral shedding titers were positively detected in the swabs from G2 at 3, 5 and 7 days PI, while the titers were detected in the swabs from G3 at 3 and 5 days PI, showing that G3 had a short period of shedding. In the present study, there was no viral shedding from the negative control group at any time point. The higher number of shedders and shedding rates during the 3rd, 5th and 7th day PI were recorded in G2 than those in G3 in both tracheal and cloacal swabs. During the whole experimental period, the mortality rate was approximately 10% in G2 (3 dead out of 30 chicks), while it was 0.0% in G3 (0 dead out of 30 chicks) and G1. There was a non-significant change in the mean initial live BW at 0 day PI among the three experimental groups (Table 3). The calculated mean final live BW was significantly higher ($P \leq 0.05$) in G3 (693 ± 76.3) than in G2 (534 ± 23.6). In addition,

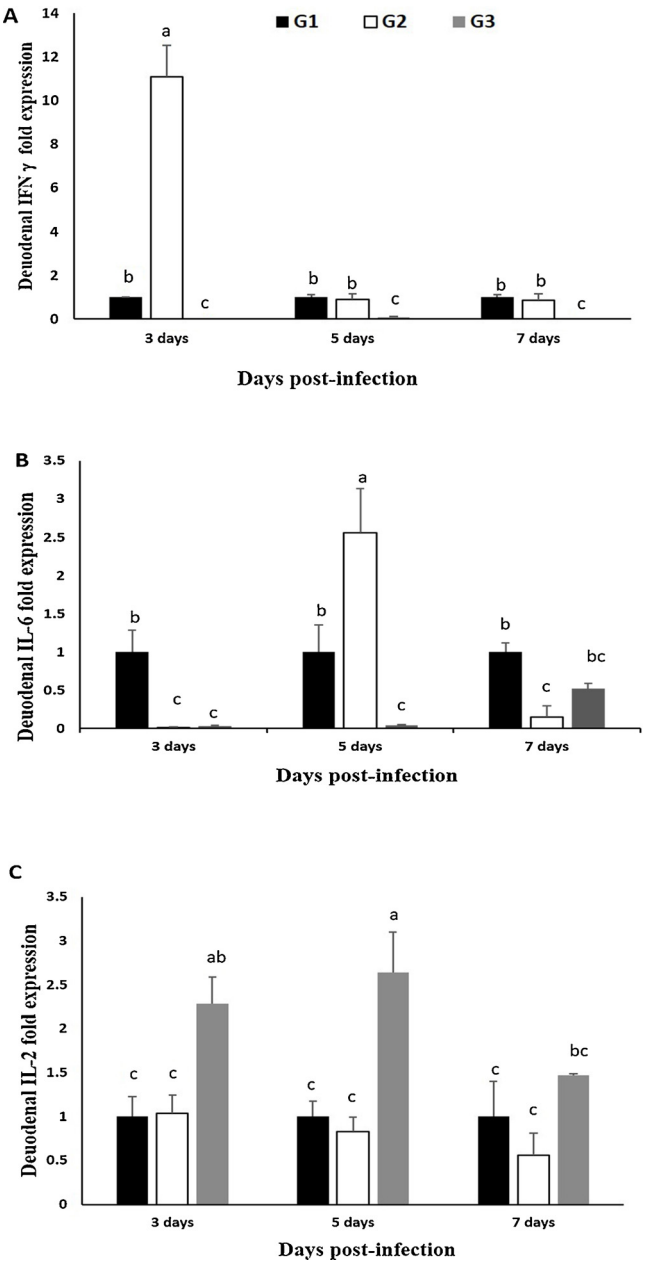

Fig. 1. Expression of IFN-gamma (A), IL-6 (B) and IL-2 (C) in the duodenum of chickens infected with avian influenza virus H9N2 and treated with vitamin E and Fetomune® plus. G1, negative control group; G2, infected group; G3, infected + treated group. Columns contain different letter indicated significant change where significant level at 0.05.

the relative organ weights (spleen & bursa) were significantly increased ($P \leq 0.05$) in G3 compared with G2 (Table 3). The post-mortem lesions during the necropsy of the three dead birds in G2 revealed petechial haemorrhage in the proventriculus, excess mucous in trachea and severe congestion in both trachea and lungs. In contrast, there were no observed gross lesions in G1 and G3 slaughtered birds. The challenge virus H9N2 in the mortalities of G2 was re-isolated from different organs (trachea and lung) and swabs (tracheal and cloacal) in embryonated eggs and the positive result was given when tested by the haemagglutination test and the H9N2 virus detection was confirmed by real-time RT-PCR.

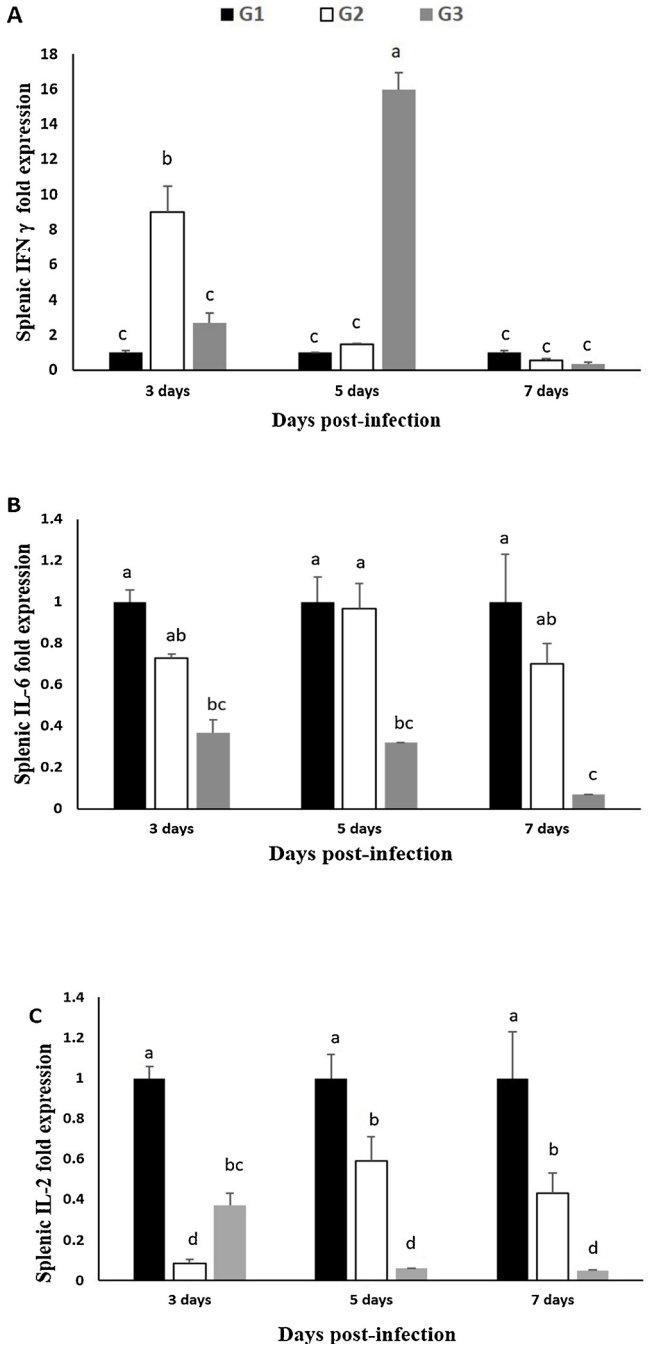

Fig. 2. Expression of IFN-gamma (A), IL-6 (B) and IL-2 (C) in the spleen of infected chickens with avian influenza virus H9N2 and treated with vitamin E and Fetomune® plus. G1, negative control group; G2, infected group; G3, infected + treated group. Columns contain different letter indicated significant change where significant level at 0.05.

3.3. Histopathology and IHC

The trachea exhibited normal organization in G1; epithelial necrosis, oedema and leukocytic cell infiltration in G2; and mild epithelial hyperplasia with deciliation in G3 (Fig. 3**A–C)**. By IHC, a stronger positive signal for the H9N2 viral antigen was detected in the trachea from G2 than in that from G3 (Fig. 3**D–F). The lung** appeared normal in G1; congestion, perivascular and interstitial oedema, and leukocytic cell infiltration were observed in the lungs in G2; and congestion with interstitial and perivascular oedema were observed in the lungs in G3 (Fig. 4**A–C)**. By IHC, a stronger positive signal for the H9N2 viral

Table 3
Mean Live body weight (BW), relative organ weight (spleen & bursa), HI-GMTs, virus shedding titers (log_{10} EID_{50}/ml), shedding rate and surviving chicks' percentage post-infection (PI) with AIV-H9N2 in the experimental groups.

Groups		BW (n = 6)		Relative organ weight (14 days PI) (n = 6)		HI-GMTs (days PI) (n = 9)			Virus shedding titers (Shedders %)* (days PI) (n = 9)						Surviving Chicks** (%)
									3		5		7		
		Initial BW 0 day PI	Final BW 14 days PI	Spleen index	Bursa index	0	7	14	T	C	T	C	T	C	
G1	Control	433.6 ± 18.2^{a}	732 ± 48.4^{a}	0.23 ± 0.03^{a}	0.26 ± 0.12^{a}	0.0^{a}	0.0^{c}	0.0^{c}	0.0 ± 0.0^{c} (0/9)	0.0 ± 0.0^{c} (0/9)	0.0 ± 0.0^{c} (0/9)	0.0 ± 0.0^{c} (0/9)	0.0 ± 0.0^{c} (0/9)	0.0 ± 0.0^{c} (0/9)	30/30 (100)^{a}
G2	AIV-H9N2	459.8 ± 33.5^{a}	534 ± 23.6^{c}	0.18 ± 0.02^{b}	0.21 ± 0.09^{b}	0.0^{a}	14.6^{b}	27.9^{b}	4.8 ± 0.28^{a} (9/9)	3.2 ± 0.92^{a} (8/9)	3.1 ± 0.16^{a} (9/9)	1.6 ± 0.34^{a} (7/9)	0.9 ± 0.11^{a} (5/9)	0.6 ± 0.21^{a} (4/9)	27/30 (90)^{c}
G3	AIV-H9N2 + Vit E and Fetomune Plus®	441.2 ± 12.7^{a}	693 ± 76.3^{b}	0.21 ± 0.04^{a}	0.25 ± 0.12^{a}	0.0^{a}	72.1^{a}	128.3^{a}	1.3 ± 0.61^{b} (5/9)	0.9 ± 0.22^{b} (4/9)	0.8 ± 0.25^{b} (4/9)	0.5 ± 0.10^{b} (2/9)	0.0 ± 0.00^{b} (0/9)	0.0 ± 0.00^{b} (0/9)	30/30 (100)^{b}

The different letters within the same column were significantly different at $P \leq 0.05$.

* Positive shedders % = number of positive shedders/ total number of swabs X 100.

** Mortality percentage was 10% in G2 AIV-H9N2 infected group (3 dead out of 30 chicks), while it was 0.0% in G3 AIV-H9N2 + Vit E group (0 dead out of 30 chicks). HI-GMTs, haemagglutination inhibition geometric mean titers; T, Tracheal; C, Cloacal.

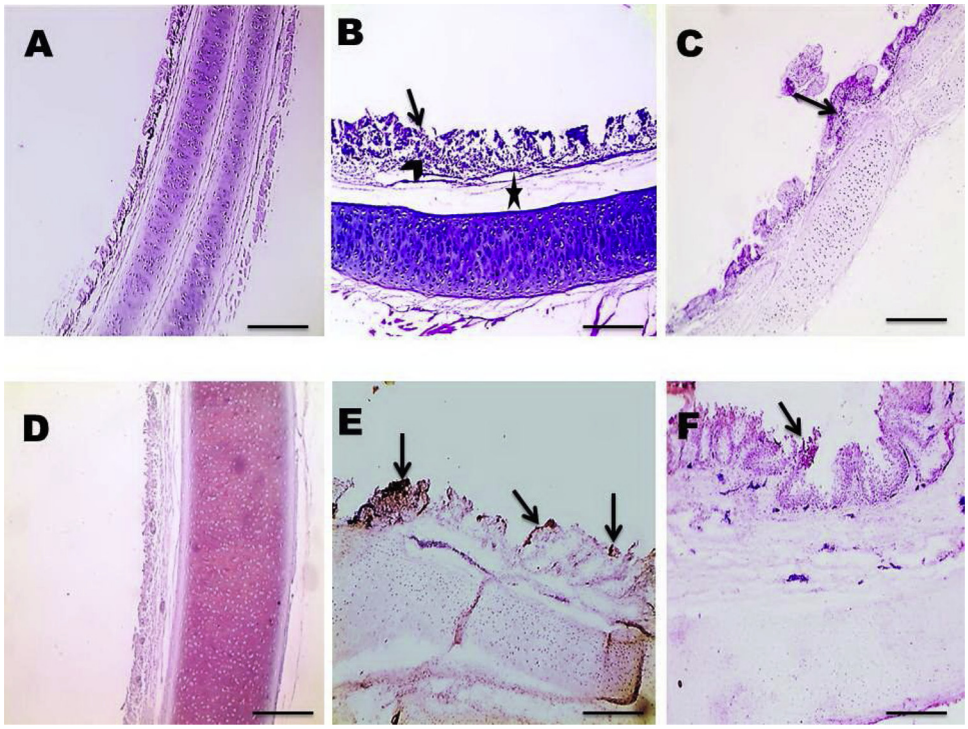

Fig. 3. Histological pictures of trachea show normal picture in G1 (**A**), necrotic epithelium (arrow) with leukocytic infiltration (arrowhead) and edema (asterisk) in lamina propria in G2 (**B**) and mild epithelial hyperplasia with deciliation (arrow) in G3 (**C**) H&E × 100 bar 100. IHC staining of H9 antigen shows negative in G1 (**D**), strong in G2 (**E**) and weak in G3 (**F**) immunolabelling of antigen in mucosa (IHC counterstained with Mayer's hematoxylin, X: 100 bar 100).

antigen was detected in the lungs from G2 than in those from G3 (Fig. 4**D-F**).

The liver showed few small follicular lymphocytic aggregations in G1; perivascular leukocytic cell infiltration, multifocal large follicular aggregation of lymphocytes in the hepatic parenchyma, vacuolar degeneration of the hepatocytes, perivascular focal parenchymal haemorrhage and dilation of the bile ducts with hyperplastic degenerated biliary epithelium in G2; and perivascular leukocytic cell infiltration, a few small follicular aggregations of lymphocytes in the hepatic parenchyma and vacuolar degeneration of the hepatocytes in G3 (**Fig. S1 A–C**). A stronger positive signal for the H9N2 viral antigen was detected by IHC in the liver from G2 than in that from G3 (**Fig. S1 D–F**).

The spleen was normal in G1 and consisted of a thick splenic capsule, white pulp that was composed of reticular cells and fibers with small-, medium- and large-sized lymphocytes and distributed plasma cells, and less distinct red pulp composed of venous sinuses, reticular cells, macrophages, lymphocytes and red blood cells. The spleen in G2 showed severe depletion of the lymphocytes from lymphoid follicles with follicular shrinkage, congestion, haemorrhage and fibrin deposition in the red pulp. In G3, the spleen revealed mild depletion of the

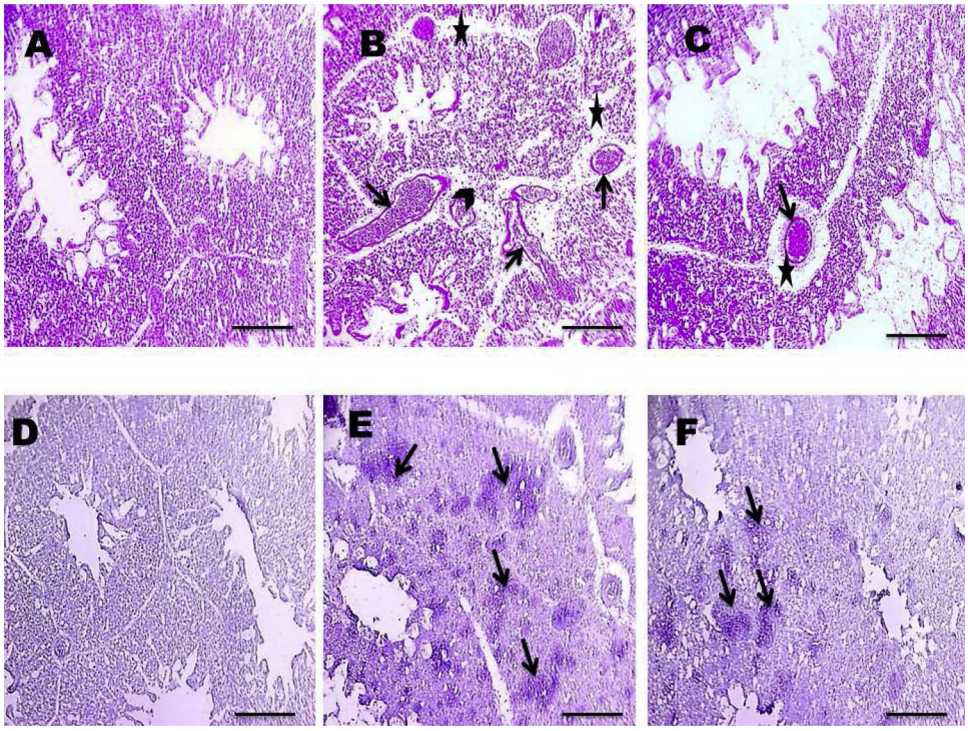

Fig. 4. Histological pictures of lung show normal picture in G1 (**A**), severe congestion (arrows), interstitial and perivascular edema (asterisks) with leukocytic cells infiltration (arrowhead) in G2 (**B**), mild congestion (arrow) and perivascular edema (asterisk)) in G3 (**C**) H& E × 100 bar 100. IHC staining of H9 antigen shows negative (**D**), strong (arrows) (**E**) and weak (arrows) (**F**) immunolabelling in mucosa of G1, G2 and G3, respectively (IHC counterstained with Mayer's hematoxylin, X: 100 bar 100).

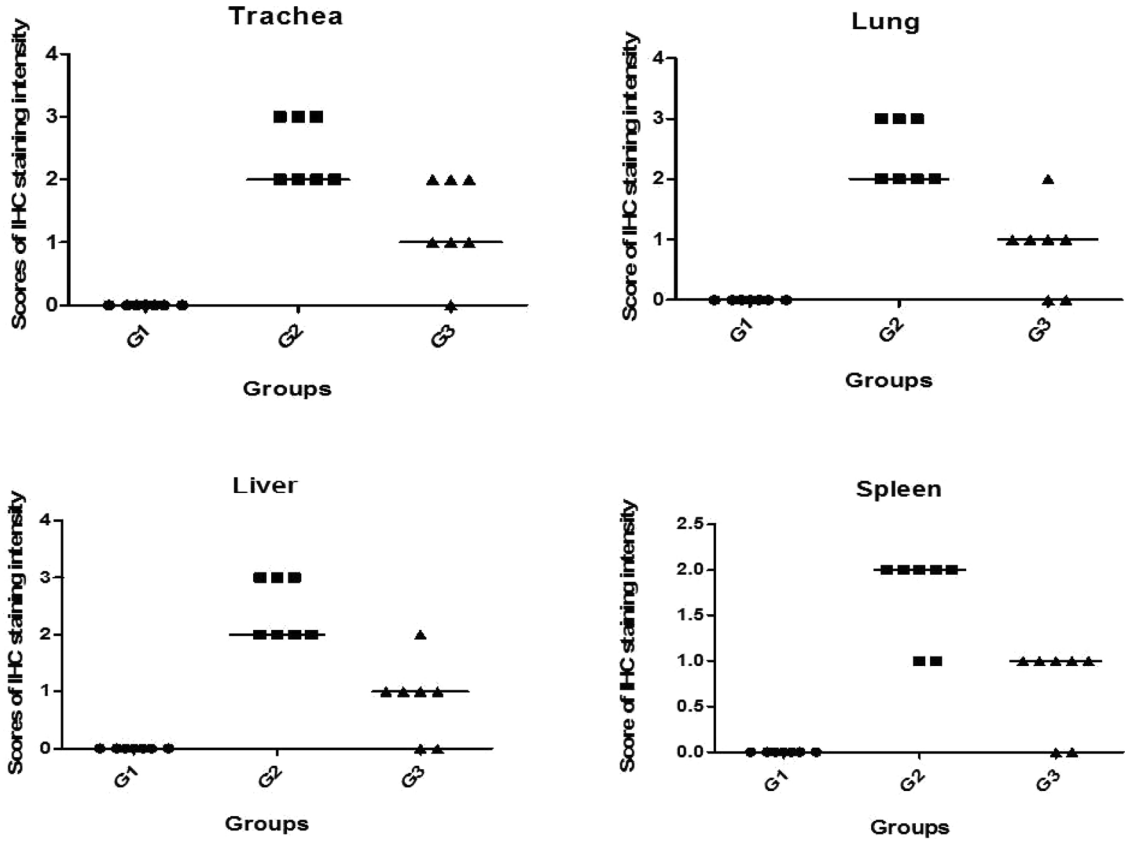

Fig. 5. Statistical analysis of IHC staining intensity in trachea, lung, liver and spleen. The significance level was set at $P \leq 0.05$.

lymphocytes from lymphoid follicles (**Fig. S2 A–F**). A stronger positive signal for the H9N2 viral antigen was detected by IHC in the spleen from G2 than in that from G3 (**Fig. S2 G–I**). **The duodenum** appeared normal in G1 but showed loss or necrosis of the intestinal villi and focal enteritis characterized by focal leukocytic cell infiltration in G2. In contrast, the duodenum showed only loss of the tips of the intestinal villi in G3 (**Fig. S3**).

Statistical analysis of the immunohistochemical staining scores in these organs showed significant differences only between G1 and G2 (Fig. 5).

4. Discussion

The present study evaluated the immunological and pathological effects of vitamin E with Fetomune Plus® (a commercial product based on a yeast extract, vitamins and minerals) on chickens experimentally infected with AIV H9N2. In this study, the challenge strain was a low pathogenic AIV H9N2 strain belonged to the G1 lineage circulating in the Middle East.

In the current study, chickens infected with AIV H9N2 exhibited a significant increase in IFN-gamma expression, with an 11-fold increase at 3 days PI that subsided within 5 days to the basal levels in the duodenum and spleen. In a study mentioned by Li et al. (2018), H9N2 infection could spread to the intestinal mucosa and significantly increase the expression of IFN-gamma due to the disturbance of the intestinal microbiota and inflammatory episodes in the intestinal epithelium on the fifth day PI, which suggested that natural killer cells in the enterocyte population were activated (Volmer et al., 2011). In our research, a significant decrease in the expression of IFN-gamma was detected after 5 days of infection and remained until the seventh day, and this expression pattern showed an inverse relationship with the virus shedding titer. Wang et al. (2012) found that AIV was capable of controlling the expression of interferons by suppressing the IκBα kinase gamma (IKK©) pathways, as these pathways control the activity of nuclear factor-κappaB (NF-κB) which regulates the expression of interferons. Furthermore, a highly pathogenic AIV strain caused the upregulation of the expression of different pro-inflammatory cytokines that later started to exhibit downregulated expression as a consequence of the induction of apoptosis in the affected organ (Suzuki et al., 2009).

In contrast, vitamin E and Fetomune Plus® were capable of reducing the expression of IFN-gamma at all time points. Vitamin E has been reported to essentially produce a non-significant change in IFN-gamma expression. Furthermore, it has been suggested that vitamin E is also implicated in the suppression of NF-κB, which has a potential role in the expression of IFN-gamma (Li-Weber et al., 2002). Beta-glucan is known to enhance the immune status of un-challenged birds, but it has also been shown to alter the expression of certain pro-inflammatory cytokines, such as IL-8 and IFN-gamma, in the duodenum and ileum (Cox et al., 2010). In the spleen, supplementation with Fetomune Plus® (composed of beta-glucan and mannan-oligosaccharides) and vitamin E induced the overexpression of IFN-gamma after 5 days PI, and this effect was also investigated by work conducted by Estrada et al. (1997) using oat-extracted glucan applied to murine splenic cell lines, which showed that the glucan induced significant overexpression of IFN-gamma.

The IL-6 level is thought to correspond with the magnitude of AIV infection and the severity of disease (Svitek et al., 2008). In our results, IL-6 in the duodenum started to be overexpressed on the fifth day, but the IL-6 level dropped significantly lower than the basal level on the seventh day. AIV is capable of overexpressing IL-6 successively, as shown in a research work focused on influenza A virus by Wang et al. (2012). Several studies have suggested that the expression of IL-6 peaks on the 3rd to 5th days PI, as IL-6 is secreted during the early stages of infection (Teijaro et al., 2011). Tocopherols can initiate a series of anti-inflammatory reactions, which are marked by a decrease in circulating cytokine expression that induces the expression of inflammatory

mediators such as IL-6 and IL-beta (Singh and Jialal, 2004).

There was a non-significant change in the IL-2 gene expression between G1 and G2. It was previously shown that AIV in chickens and ducks might be associated with feeble expression of IL-2 in the late stage of infection. As the expression of IL-2 in AIV infection is restricted in the first few hours (up to 36 h) of infection, IL-2 expression begins to decrease gradually (Adams et al., 2009). In NDV infection, beta-glucan extracted from mushrooms is capable of increasing the expression of IL-2 as a defensive mechanism to alleviate the damaging effect of the virus on the splenocytes of chicken embryos (Jacob and Pescatore, 2017).

Vitamin E is characterized by its potential role in stimulating the immune system in chickens. In chickens exposed to an intravenous injection of lipopolysaccharides, a non-significant change in IL-2 expression is observed when α-tocopherol acetate is injected into the spleen of the exposed chicks (Leshchinsky and Klasing, 2003). Dalia et al. (2018) also detected a non-significant change in splenic IL-2 expression in broiler females that were supplemented with 100 mg/kg of diet for 3 weeks. It has also been suggested that higher doses of tocopherols can downregulate the expression of several cytokines such as IL-2, IL-5 and IL-10, which could explain the significant decrease in IL-2 expression on the seventh day (McCary et al., 2011).

Our findings showed lower virus shedding rate and titers with a shorter shedding period and improved gene expression levels (IFN-γ, IL-2 and IL-6) in the treated infected group compared with the untreated infected group. The administration of vitamins A, C, and E and selenium to chickens in excess of the currently recommended levels enhances disease resistance due to the significant stimulation of humoral and cellular immunity and phagocytosis (Abdukalykova and Ruiz-Feria, 2006). Dietary vitamin E may modulate pro-inflammatory cytokine expression through the antioxidant defence system in birds, as previously mentioned by Jang et al. (2014). Similarly, Akhtar et al. (2016) showed that the use of mannan-oligosaccharide may reduce the spread of disease by decreasing virus shedding during AIV H9N2 infection in poultry. In this study, the higher shedding titers and rates were recorded in G2 than G3. Similar results of virus shedding in the infected group were recorded by Kilany et al. (2016) who showed that the shedding rates were 100% on the 2nd and 4th day PI with AIV H9N2 till reached 0.0% on the 10th day PI.

The significantly increased HI-GMTs and relative organ weights (spleen & bursa) in G3 suggested that vitamin E increased the first defence barrier against infectious agents in broiler chicks (Safarizadeh and Zakeri, 2013). The mild clinical signs and post-mortem lesions produced by AIV H9N2 infection in this study were similar to those previously reported (Seifi et al., 2010; Arafat et al., 2018).

Our results showed that the dietary vitamin E with Fetomune Plus® supplementation produced higher mean final live BWs and reduced the mortality rate. Similarly, Monoura et al. (2008) showed that compared with the normal basal diet, dietary vitamins improved live weight gain and HI antibody titers in ND-vaccinated chickens. Similarly, compared with chicks in a control group (15 mg vitamin E/kg of diet), chicks administered a high level of vitamin E (150 mg/kg diet) show significant increases ($P \leq 0.05$) in their live BW and BW gain (Alm El-Dein et al., 2013; Alm El-Dein et al., 2013).

In addition, histopathological examination revealed milder lesions in the trachea, lungs, liver, spleen and duodenum from G3 than in those from G2. These findings may be due to the antioxidant property of vitamin E that protects phospholipids in the cell membrane. Mild lymphocyte depletion was observed in the spleen from G3. Previous studies have clarified the important role of vitamin E in protecting the lymphoid organs from the negative impacts of different stressors (Sodhi et al., 2006). The activities of the spleen, bursa and thymus are enhanced by dietary supplementation with vitamin E, leading to the production of numerous lymphocytes, which help improve the immunity of birds. The increased activities are associated with the presence of numerous large lymphocytes in the cortex areas of the bursa and thymus or in the red pulp area of the spleen (El-Gogary et al., 2015). In this study, the histopathological findings were compatible with the IHC results. The AIV H9N2 antigen staining intensity in the trachea, lungs, liver and spleen was significantly decreased in the treated group compared with the untreated infected group.

5. Conclusion

Our results may conclude that supplementation with vitamin E (200 mg/kg of diet) and Fetomune Plus® (1 ml/liter of drinking water) for four weeks improved the immunological and pathological effects of AIV H9N2 infection on chickens, as indicated by improved immune gene expression levels, virus shedding titers, HI-GMTs, and final BWs as well as decreases in the mortality rate, histopathological lesions and intensity of the staining for the AIV H9N2 antigen in the examined organs by IHC.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgement

The authors wish to acknowledge the departments of **Pathology, Poultry diseases, Pharmacology and Biochemistry** for their help, facilities and encouragement during the course of this study. Moreover, the authors would like to thank **Asmaa A. Al-Zayat**, Animal Health research institute, Mansoura branch for providing us the AIV-H9N2 strain. Egyptian knowledge bank is thanked for the proof editing of the manuscript from the nature research editing service.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2019.02.028.

References

Abdel-Moneim, A.S., Zlotowski, P., Veits, J., Keil, G.M., Teifke, J.P., 2009. Immunohistochemistry for detection of avian infectious bronchitis virus strain M41 in the proventriculus and nervous system of experimentally infected chicken embryos. Virol. J. 6, 15.

Abdel-Moneim, A., Afifi, M., El-Kady, M., 2012. Isolation and mutation trend analysis of influenza A virus subtype H9N2 in Egypt. Virol. J. 9, 173.

Abdukalykova, S.T., Ruiz-Feria, C.A., 2006. Arginine and vitamin E improve the cellular and humoral immune response of broiler chickens. Int. J. Poult. Sci. 5, 121–127.

Adams, S.C., Xing, Z., Li, J., Cardona, C.J., 2009. Immune-related gene expression in response to H11N9 low pathogenic avian influenza virusinfection in chicken and Pekin duck peripheral blood mononuclear cells. Mol. Immunol. 46, 1744–1749.

Akhtar, T., Ara, G., Ali, N., ud Din Mufti, F., Imran Khan, M., 2016. Effects of dietary supplementation of mannan-oligosaccharide on virus shedding in avian influenza (H9N2) challenged broilers. IJVR 17, 268–272.

Alm El-Dein, A.K., Soliman, M.M., Abd El-Aziz, S., 2013. Effect of dietary vitamin E supplementation on some productive, reproductive and immunological traits of Inshas chickens strain. Egypt. Poult. Sci. J. 33, 939–955.

Arafat, N., Eladl, A.H., Marghani, B.H., Saif, M.A., El-Shafei, R.A., 2018. Enhanced infection of avian influenza virus H9N2 with infectious laryngeotracheitis vaccination chickens. Vet. Microbiol. 219, 8–16.

Aslam, R., Aslam, A., Tipu, Y., Nazir, J., Ghafoor, A., Fatima, S., 2015. Histopathological and immunohistochemical studies for the pathogenesis of a low pathogenicity H9 avian influenza virus in experimentally infected commercial broilers. Anim. Plant Sci. 25, 45–52.

Asmaa, A., 2016. Studies on Prevalence of H9 Avian Influenza Virus. Master Thesis, Department of Poultry Diseases, Faculty of Veterinary Medicine. Mansoura University, Mansoura, Egypt.

Bancroft, J.D., Gamble, M., 2007. Theory and Practice of Histological Techniques, 5th ed. Churchill Livingstone, London, UK, pp. 125–138.

Beard, C.W., 1989. Serologic procedures, in a laboratory manual for the isolation and identification of avian pathogens. In: Purchase, H.G., Arab, L.H., Domerumuth, C.H., Pearson, J.E. (Eds.), Am. Assoc. Avian Pathologist, Kennett Square., 3rd edn. pp. 192–200.

Bourre, J.M., Galea, I.F., 2006. An important source of Omega 3 fatty acids, vitamin D and E, Carotenoids, Iodine, Selenium; a new nayural multi enriched egg. J. Nut. Health Aging 10, 371–376.

Brosius, F., 2011. SPSS 19. MITP-Verlags GmbH & Co. KG.

Cox, C.M., Stuard, L.H., Kim, S., McElroy, A.P., Bedford, M.R., Dalloul, R.A., 2010. Performance and immune responses to dietary beta-glucan in broiler chicks. Poult. Sci. 89, 1924–1933.
Dalia, A.M., Loh, T.C., Sazili, A.Q., Jahromi, M.F., Samsudin, A.A., 2018. Effects of vitamin E, inorganic selenium, bacterial organic selenium, and their combinations on immunity response in broiler chickens. BMC Vet. Res. 14, 249.
El-Gogary, M.R., Ismail, F.S.A., El- Nadi, M.I., 2015. Effect of vitamin E supplementation and stocking density on broiler performance, carcass traits and histological responses of lymphoid organs. Asian J. Poult.Sci. 9, 70–84.
Estrada, A., Yun, C.H., Van Kessel, A., Li, B., Hauta, S., Laarveld, B., 1997. Immunomodulatory activities of oat beta-glucan in vitro and in vivo. Microbiol. Immunol. 41, 991–998.
Hong, Y.H., Lillehoj, H.S., Lee, S.H., Dalloul, R.A., Lillehoj, E.P., 2006. Analysis of chicken cytokine and chemokine gene expression following Eimeria acervulina and Eimeria tenella infections. Vet. Immunol. Immunopathol. 114, 209–222.
Iqbal, M., Yaqub, T., Mukhtar, N., Shabbir, M.Z., McCauley, J.W., 2013. Infectivity and transmissibility of H9N2 avian influenza virus in chickens and wild terrestrial birds. Vet. Res. 44, 100.
Jacob, J., Pescatore, A.J., 2017. Glucans and the poultry immune system. Am. J. Immunol. 13, 45.
Jang, I.-S., Ko, Y.-H., Moon, Y.-S., Sohn, S.-H., 2014. Effects of vitamin C or E on the pro-inflammatory cytokines, heat shock protein 70 and antioxidant status in broiler chicks under summer conditions. Asian-australas. J. Anim. Sci. 27, 749–756.
Karimi-Madab, M., Ansari-Lari, M., Asasi, K., Nili, H., 2010. Risk factors for detection of bronchial casts, most frequently seen in endemic H9N2 avian influenza infection, in poultry flocks in Iran. Prev. Vet. Med. 95, 275–280.
Kilany, W.H., Ali, A., Bazid, A.H., El-Deeb, A.H., El-Abideen, M.A., Sayed, M.E., El-Kady, M.F., 2016. Dose-Response Study of inactivated low pathogenic avian influenza H9N2 Virus in specific-pathogen-free and commercial broiler chickens. Avian Dis. 60, 256–261.
Lee, C.W., Suarez, D.L., 2004. Application of real-time RT-PCR for the quantitation and competitive replication study of H5 and H7 subtype avian influenza virus. J. Virol. Methods 119, 151–158.
Leshchinsky, T.V., Klasing, K.C., 2003. Profile of chicken cytokines induced by lipopolysaccharide is modulated by dietary alpha-tocopheryl acetate. Poult. Sci. 82, 1266–1273.
Li, H., Liu, X., Chen, F., Zuo, K., Wu, C., Yan, Y., Chen, W., Lin, W., Xie, Q., 2018. Avian influenza virus subtype H9N2 affects intestinal microbiota, barrier structure injury, and inflammatory intestinal disease in the chicken ileum. Viruses 10, E270.
Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2-\Delta\Delta CT$ method. Methods 25, 402–408.
Li-Weber, M., Weigand, M.A., Giaisi, M., Süss, D., Treiber, M.K., Baumann, S., Ritsou, E., Breitkreutz, R., Krammer, P.H., 2002. Vitamin E inhibits CD95 ligand expression and protects T cells from activation-induced cell death. J. Clin. Invest. 10, 681–690.
Mazariegos, L.A., Lukert, P.D., John, B., 1990. Pathogenicity and immunosuppressive prosperities of infectious bursal disease “intermediate” stains. Avian Dis. 34, 203–208.
McCary, C.A., Abdala-Valencia, H., Berdnikovs, S., Cook-Mills, J.M., 2011. Supplemental and highly elevated tocopherol doses differentially regulate allergic inflammation: reversibility of α-tocopherol and γ-tocopherol’s effects. J. Immunol. 186, 3674–3685.
Mileva, M., Galabov, A.S., 2018. Vitamin E and Influenza Virus Infection. In book: Vitamin E in Health and Disease. Chapter 4.. https://doi.org/10.5772/intechopen.80954.
Monoura, P., Rahman, M., Khan, M.F.R., Rahman, M.B., Rahman, M.M., 2008. Effect of vitamins, minerals and probiotics on production of antibody and live weight gain following vaccination with BCRDV in broiler birds. Bangl. J. Vet. Med. 6, 31–36.
Nagarajan, S., Rajukumar, K., Tosh, C., Ramaswamy, V., Purohit, K., Saxena, G., Behera, P., Pattnaik, B., Pradhan, H.K., Dubey, S.C., 2009. Isolation and pathotyping of H9N2 avian influenza viruses in Indian poultry. Vet. Microbiol. 133, 154–163.
Niu, Z.Y., Liu, F.Z., Yan, Q.L., Li, W.C., 2009. Effects of different levels of vitamin E on growth performance and immune responses of broilers under heat stress. Poult. Sci. 88, 2101–2107.
NRC (National Research Council), 1994. Nutrient Requirements of Poultry, 9th rev. ed. National Academy Press, Washington, DC.
Rama-Rao, S.V., Raju, M.V.L.N., Nagalakshmi, D., 2004. Nutritional Modulation to Enhance Immunity in Chickens. Poultry International April 2004, Watt Publishing Co, pp. 24–29.
Reed, L.J., Muench, H., 1938. Simple method of estimating fifty percent end point. Am. J. Hyg. 27, 493–497.
Rehman, Z.U., Che, L., Ren, S., Liao, Y., Qiu, X., Yu, S., Sun, Y., Tan, L., Song, C., Liu, W., Ding, Z., Munir, M., Nair, V., Meng, C., Ding, C., 2018. Supplementation of Vitamin E protects chickens from newcastle disease virus-mediated exacerbation of intestinal oxidative stress and tissue damage. Cell. Physiol. Biochem. 47, 1655–1666.
Safarizadeh, A., Zakeri, A., 2013. The effect of vitamin A and complex of vitamin E and selenium on growth factors and Humoral immunity in broiler chickens. Eur. J. Exp. Biol. 3, 99–102.
Seifi, S., Asasi, K., Mohammadi, A., 2010. Natural co-infection caused by avian influenza H9 subtype and infectious bronchitis viruses in broiler chicken farms. Vet. Arh. 80, 269–281.
Ben Shabat, M., Meir, R., Haddas, R., Lapin, I., Shkoda, E., Raibstein, I., Perk, S., Davidson, I., 2010. Development of a real-time TaqMan RT-PCR assay for the detection of H9N2 avian influenza viruses. J. Virol. Methods 168, 72–77.
Simms, D., Cizdziel, P.E., Chomczynski, P., 1993. TRIzol: A new reagent for optimal single-step isolation of RNA. Focus 15, 532–535.
Singh, U., Jialal, I., 2004. Anti-inflammatory effects of α-Tocopherol. Ann. N.Y. Acad. Sci. 1031, 195–203.
Sodhi, S., Sharma, A., Brar, R.S., 2006. A protective effect of vitamin E and Selenium in ameliorating the immunotoxicity of malathion in chicks. Vet. Res. Commun. 30, 935–942.
Suzuki, K., Okada, H., Itoh, T., Tada, T., Mase, M., Nakamura, K., Kubo, M., Tsukamoto, K., 2009. Association of increased pathogenicity of Asian H5N1highly pathogenic avian influenza virusesin chickens with highly efficient viral replication accompanied by early destruction of innate immune responses. J. Virol. 83, 7475–7486.
Svitek, N., Rudd, P.A., Obojes, K., Pillet, S., von Messling, V., 2008. Severe seasonal influenza in ferrets correlates with reduced interferon and increased IL-6induction. Virology 376, 53–59.
Teijaro, J.R., Walsh, K.B., Cahalan, S., Fremgen, D.M., Roberts, E., Scott, F., Martinborough, E., Peach, R., Oldstone, M.B., Rosen, H., 2011. Endothelial cells are central orchestrators of cytokine amplification during influenza virus infection. Cell 146, 980–991.
Volmer, C., Soubies, S.M., Grenier, B., Guérin, J.L., Volmer, R., 2011. Immune response inthe duck intestine following infection with low-pathogenic avian influenza viruses or stimulation with a Toll-like receptor 7 agonist administered orally. J. Gen. Virol. 92, 534–543.
Wang, Y., Zhou, J., Ruan, C., Du, Y., 2012. Inhibition of type I interferon production via suppressing IKK-gamma expression:a new strategyfor counteracting host antiviral defense by influenza A viruses? J. Proteome Res. 11, 217–223.