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Comparative immune response and pathogenicity of the H9N2 avian influenza virus after administration of Immulant®, based on *Echinacea* and *Nigella sativa,* in stressed chickens

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ABSTRACT

Avian influenza vaccines are commonly used in the poultry industry, and some medicinal plants can increase the efficacy of such vaccines. The objective of this study was to evaluate the effect of Immulant® (IMU) (a commercial product based on *Echinacea* and *Nigella sativa*) on stress induced by dexamethasone (DEX) in chickens vaccinated (VAC) against the H9N2 avian influenza virus (AIV-H9N2). Seven experimental groups were included: the negative control, VAC, DEX, VAC + DEX, VAC + DEX + IMU, VAC + IMU and IMU groups. The vaccinated chickens (at 10 days of age) were injected daily with DEX for three days pre-vaccination and for three days pre-challenge and orally administered 1% IMU for 6 weeks post-vaccination (PV). The chickens were then challenged intranasally with AIV-H9N2 at 28 days PV. Serum, blood, tracheal and cloacal swabs and tissue samples were collected in the 1st and 4th weeks PV and at different time points post-challenge. The results showed significant changes ($P \leq 0.05$) in oxidative stress and antioxidant biomarkers (malondialdehyde, nitric oxide and reduced glutathione), haematological and immunological parameters, final live weights, relative organ weights and histopathological lesions between the VAC + DEX group and the VAC group. Moreover, IMU significantly increased protection rates post-challenge, HI antibody titers and heterophil phagocytic activity and decreased DEX-induced stress and virus shedding titers. In conclusion, oral administration of 1% IMU for six weeks can enhance the immune response after AI-H9N2 vaccination and reduce the pathogenicity of infection in stressed chickens.

1. Introduction

Avian influenza (AI) is an important disease that is responsible for severe economic losses on affected poultry farms worldwide [1]. The first H9 isolate in Egypt was identified in chickens in 2012 [2]. H9N2 avian influenza virus (AIV) infection in poultry farms results in several clinical signs, depending on the viral strain and the presence of immunosuppressive diseases [1]. Inactivated vaccines derived from field strains of AIV-H9N2 have been recently used in Egyptian poultry farms (breeders, layers and broilers) [3].

Different methods are urgently needed to enhance the vaccination responses of the available field vaccines to prevent severe economic losses within the poultry industry [4]. Many immunostimulatory substances have been used in poultry with success, such as herbal extracts, which significantly increase the HI antibody titers to Newcastle disease and AI vaccines [5].

Abbreviations: AIV, avian influenza virus; DPC, days post-challenge; EID_{50}, 50% embryo infective dose; HI, haemagglutination inhibition; LPAI, low pathogenic avian influenza; MEVAC, Middle East company for veterinary vaccine; NLQP, national laboratory for veterinary quality control on poultry production; PV, post-vaccination; PC, post-challenge; IMU, Immulant®; DEX, dexamethasone; VAC, vaccinated; BW, body weight; GMTs, geometric mean titers; TLC, Total leukocyte count; H/L, Heterophil/ lymphocyte; sMDA, serum malondialdehyde; rMDA, liver homogenate malondialdehyde; NO, nitric oxide; GSH, reduced glutathione; IHC, immunohistochemistry

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Dexamethasone (DEX) is an immunosuppressant that is commonly used to study and mimic stress factors in poultry diseases [6]. The DEX immunosuppression model is intended to mimic excessive stressors. The low and medium level of stress can be useful for the immune system, while the combination of excessive transport, heat and humidity represent severe stressors lead to immunosuppression that can be induced by DEX stress model [6]. Moreover, the two best criteria of the DEX stress model are the exact daily treatment and the predictable stress responses in a known time [7]. There may be a cumulative effect of several stressors throughout poultry production that results in physiological changes and immunosuppression. Additionally, the DEX stress model can provide justification for management measures intended to reduce stress [8].

Stress is considered one of the major predisposing factors for poultry diseases, and stress due to intensive poultry rearing, increased ammonia, malnutrition, and immunosuppressive diseases leads to immunosuppression [9]. AI may occur after vaccination failure on poultry farms due to immunosuppression or other causes [4].

In recent years, research has focused on finding new additives to improve performance and stimulate the immune response in birds. The best methods should be used to study such extracts and investigate the effects of mixing different medicinal plants [10]. Improvements in growth rate and the immune responses have been reported in vaccinated chickens treated with *Echinacea* and *Nigella sativa* [11,12]. Moreover, feeding *Echinacea purpurea*, particularly for six weeks, could improve the feed conversion rate, the number of blood cells and the enhancement of immune response in chickens [11]. Infection with AIV isolated from waterfowl can suppress T-cell function and impact macrophage phagocytic activity [13]. Protection against infection has mainly been achieved through leukocytes and antibody production, and heterophils play an essential role in host defence in birds through phagocytosis [14]. The selection of healthy chicks may be associated with hens that have effective phagocytosis [15].

We hypothesize that the Immulant® (IMU), a commercial product containing *Echinacea* (coneflower) extract and *Nigella sativa* (black seed) extract as medicinal plants, may improve heterophil phagocytic activity in vaccinated and immunosuppressed chickens. The different plant extracts of the immune stimulant may have different actions and the effects of mixture should not be attributed to a single plant because the effects of each plant may be enhanced or reduced by the other plant extract. Therefore, more research is needed on the interactions between combinations of plant extracts [16]. Furthermore, we assume that the dual plant extracts of the tested immune stimulant may reduce the pathogenicity of AIV-H9N2 infection, mitigate the induced oxidative stress as well as improve the immune responses and the feed conversion by their potential synergism rather than individual effects [11,12].

Therefore, the present study was designed to determine the effects of using the tested immune stimulant in vaccinated chickens by measuring clinical, haematological, biochemical, and histopathological effects. The specific aims of this study included determining (i) the effect of DEX-induced stress on the health status and immune responses of AI-H9N2 vaccinated chickens and (ii) the effect of the tested immune stimulant on DEX-stressed chickens post-vaccination (PV) and post-challenge (PC) with AIV-H9N2.

2. Materials and methods

2.1. Experimental chickens

Two hundred ten one-day-old white Hy-Line chicks (the same hatching batch) were purchased from a commercial poultry farm (Dakahlia, Egypt) in which there was no history of AIV-H9N2 infection. The birds were kept in cages in a well-ventilated and strictly isolated room and a commercial balanced starter ration, free from any medication, was provided. All measures of biosecurity were considered. Food and water were provided to the birds *ad libitum* throughout the experiment (52 days).

All bird experiments were performed in accordance with the recommendations of the "Guide for the Care and Use of Laboratory Animals" and approved by the Ethics Committee of the Faculty of Veterinary Medicine, Mansoura University. The birds were confirmed to have no H9 or H5 viruses or antibodies by isolation in embryonated eggs and HI testing [17].

2.2. Drugs, medicinal plants and toxicity test

- **Dexamethasone sodium phosphate®**: Injectable solution, each 2 ml ampoule contains 8 mg of DEX in the sodium phosphate form (Almriya Pharmaceutical Industries, Alexandria, Egypt, Batch No. 2615037A). The chickens were injected with DEX (2 mg/kg BW, I/M) as a stress model [18].
- **Immulant®**: This is a commercial product; each 5 ml contains 83.5 mg of *Echinacea* dry extract and 0.008 ml of *Nigella sativa* oil. It is produced by Mepaco, Medifood, Enshas El-Raml, Sharkia, Egypt. Immulant® (IMU) (1%) was used in the drinking water (10 ml/liter) for six weeks [11].
- **Determination of acute toxicity of Immulant®**: The LD_{50} of the Immulant® product was determined using the OECD guideline on animal acute toxicity testing [19]. Twenty-four white Hy-Line chicks at 10 days of age were used. The chicks were given food and water *ad libitum* and the appetence of the tested immune stimulant was observed, including the smell and taste of the plant extract. The chicks were divided into four groups of six chicks each and were treated with the Immulant® at different doses of 1, 10 and 100 ml/L orally with drinking water while the last group was used as a control. They were observed for 24 h for signs of toxicity such as incoordination, reluctance to move, depression, diarrhea and mortality.

2.3. Vaccine, antigen and challenge strain

- **ME Fluvac H9N2 vaccine (A/Chicken/Egypt/11490v/NLQP/2011**, related to G1-like lineage group B viruses) is an inactivated water-in-oil emulsion vaccine produced by Middle East for Veterinary Vaccines (ME VAC) in Egypt.
- **AI H9N2 standard diagnostic antigen** is a low-pathogenic AI (LPAI) antigen derived from A/Chicken/Egypt/11490v/NLQP/2011 (H9N2) related to G1-like lineage group B viruses. This antigen was obtained from ME VAC and used for HI test.
- **The LPAI AIV-H9N2 strain** is a challenge strain (KX663332, A/chicken/Egypt/Mansora-36/2015) that was kindly obtained [20]. This AIV-H9N2 strain is related to group B viruses (G1-like lineage viruses). This H9N2 virus was titrated in 10-day-old specific pathogen-free (SPF) embryonated chicken eggs and calculated as previously described [21]. At 38 days of age, the challenged birds were intranasally inoculated with H9N2 virus (0.5 ml of 10^6 EID_{50}/bird) [22].

2.4. Grouping and experimental design (Table 1)

Two hundred ten 1-day-old, white Hy-Line chicks were allocated into 7 groups. Each group consisted of 30 chicks (15 × 2 replicates). The first group (G1) was kept as an unvaccinated control, G2 was the VAC only group, G3 was the DEX-treated group, G4 was the VAC + DEX group, G5 was the VAC + DEX + IMU group, G6 was the VAC + IMU group and G7 was the IMU group. The chicks were subcutaneously injected with the inactivated H9N2 vaccine at 10 days of age as a common age of AI vaccination in the field [23]. The chicks were injected with DEX (2 mg/kg BW, I/M) as a stress model to induce the stress and immunosuppression, with daily injections for three successive days pre-vaccination and for additional three days pre-challenge. The chicks were administered 1% IMU in the drinking water

Table 1
Grouping and experimental design.

Group			G1 Negative control	G2 VAC	G3 EX	G4 VAC + DEX	G5 VAC + DEX + IMU	G6 VAC + IMU	G7 IMU
Number of birds			30 (15 × 2)	30 (15 × 2)	30 (15 × 2)	30 (15 × 2)	30 (15 × 2)	30 (15 × 2)	30 (15 × 2)
Vaccination (AIV-H9N2)		10 days of age	–	+	–	+	+	+	–
Dexamethasone (2 mg/kg BW, I/M)		daily for 3 days pre-vaccination and 3 days pre-challenge	–	–	+	+	+	–	–
1% IMU D.W. (six weeks PV)		10–52 days of age	–	–	–	–	+	+	+
Sampling	1st week PV	17 days of age	• Blood with anticoagulant (n = 8) for TLC, H/L ratio and heterophil phagocytic activity. • Serum (n = 8) for HI test and antioxidant test (sMDA). • Liver homogenate (n = 4) for antioxidant tests (rMDA, NO, and GSH) • Organs of the same birds (n = 4) (bursa and spleen) for relative organ/ BW.						
	4th week PV	38 days of age	• Blood with anticoagulant (n = 8) for TLC, H/L ratio and heterophil phagocytic activity, • Serum (n = 8) for HI test • Organs (n = 4) (bursa and spleen) for relative organ/ BW • Organs of the same birds (n = 4) (liver, spleen and jejunum) for histopathology						
AIV-H9N2 challenge (n = 20)		38 days of age	–	+	+	+	+	+	+
Sampling	1st week PC	45 days of age	• Blood with anticoagulant (n = 8) for TLC, H/L ratio and heterophil phagocytic activity. • Serum (n = 8) for HI test and antioxidant test (sMDA) • Swabs (n = 6) (Tracheal and cloacal) for shedding titers at 2, 5, 8 and 10 days PC • Liver homogenate (n = 4) for antioxidant tests (rMDA, NO, and GSH) • Organs (n = 4) (bursa and spleen) for relative organ/ BW • Organs of the same birds (n = 4) (lung, liver, spleen and jejunum) for histopathology and lungs for immunohistochemistry						
	2nd week PC	52 days of age	• Serum (n = 8) for HI test						

(10 ml/liter) for six weeks post-vaccination (PV). The choice of the use dosage was according to the toxicity test performed before the experiment. At 38 days of age (28 days PV), twenty birds from each group were intranasally inoculated with AIV-H9N2 (0.5 ml of 10^6 EID_{50}).

Clinical signs, mortality, and post-mortem lesions were recorded daily for 14 days PC. The liver homogenate of the euthanized birds (n = 4, each group) was used to estimate the chosen antioxidant (GSH) and oxidative stress markers (rMDA and NO) known in chickens [10,24–26] in the 1st week PV and the 1st week PC. Random blood (n = 8) and serum (n = 8) samples were collected in the 1st and 4th weeks PV and the 1st and 2nd weeks PC for total leukocyte counts, H/L ratios, phagocytic activity tests, HI, and antioxidant tests (sMDA). At 2, 5, 8 and 10 days PC, tracheal and cloacal swabs (n = 6, each group) were collected to assess virus shedding. BW and relative organ weights (bursa and spleen) were calculated from euthanized birds (n = 4) in the 1st and 4th weeks PV and the 1st week PC. The organs of the same euthanized birds (n = 4) were collected for histopathological examination in the 4th week PV (liver, spleen and jejunum) and the 1st week PC (lung, liver, spleen and jejunum). The lungs were also collected for detection of AIV-H9N2 by immunohistochemistry (Table 1).

2.5. Laboratory methods

2.5.1. Antioxidant and oxidative stress biomarkers

Liver homogenate (n = 4) was used to estimate antioxidant and oxidative stress biomarkers. A lipid peroxidation marker (MDA), nitric oxide (NO) and reduced glutathione (GSH) were estimated by the enzymatic colorimetric method (using ready-made kits obtained from Biodiagnostic, Egypt) as previously described [24]. The separated serum samples (n = 8) were stored at −20 °C until the use in the antioxidant test (sMDA).

2.5.2. TLC and heterophil/lymphocyte ratio

Eight blood samples with EDTA were randomly collected from the wing vein and used to determine the total leukocyte count (TLC) and differential leukocyte count [27]. All types of heterophils and lymphocytes were considered for calculation of the H/L ratio [28].

2.5.3. Phagocytic activity

Heparinized blood (n = 8) was used for the phagocytic activity assay. Phagocytic activity was estimated as previously described [29]. A killed *Candida* suspension was used, and the smears were stained with Giemsa. Phagocytic activity was calculated [30] as the ratio of the number of heterophils with phagocytized *Candida* cells to the total number of inspected heterophils. The phagocytic index was the average number of ingested *Candida* cells per phagocytizing heterophil. The suspension of *Candida* was prepared as previously indicated [31].

2.5.4. BW and organ-to-BW ratio

BW and the organ-to-BW ratio were calculated for each bird. Bursae and spleens of different euthanized birds (n = 4) were collected from each experimental group at 1 and 4 weeks PV and at one week PC. The organ-to-BW ratio was calculated by dividing the organ weight by the chicken's total BW and then multiplying by 100 [32].

2.5.5. Histopathology and immunohistochemistry (IHC)

The organs of the euthanized birds (n = 4) were collected for histopathological examination in the 4th week PV (liver, spleen and jejunum) and the 1st week PC (lung, liver, spleen and jejunum). All samples were fixed in 10% neutral buffered formalin for processing. Paraffin sections were prepared and stained with haematoxylin and eosin (H&E) as previously indicated [33]. The scoring system for lesions in the studied fields was described previously [34]: (−) no lesions, (+) mild lesions of less than 20%, (++) moderate lesions of 20–60% and (+++) severe lesions of greater than 60%.

The lungs were also collected for detection of AIV-H9N2 by immunohistochemistry in the 1st week PC. The avidin-biotin complex method was applied to formalin-fixed, paraffin-embedded tissue sections of the lungs. After dewaxing and dehydration, avidin and biotin-labelled peroxidase antibodies specific for the H9N2 virus were applied to the slides. After washing with PBS, the slides were incubated with an anti-chicken secondary antibody (1:200). The staining intensity observed by light microscopy was scored based on the number of positive cells per high-power field, as follows: − (negative), + (small number), ++ (moderate number), and +++ (large number), as previously

described [35].

2.5.6. AIV-H9N2 PCR monitoring

At 2, 5, 8 and 10 days PC, tracheal and cloacal swabs (n = 6) were collected from each experimental group to estimate virus shedding. The viral RNA was extracted using the QIAamp viral RNA Mini kit (Qiagen, Germany) as indicated in the manufacturer's instructions. PCR was 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 used were described previously [36]: **H9F**: 5′-GGAAGAATTAATTATTATTGGTCGGTAC-3′; H9R: 5′-CCACCT TTTTCAGTCTGACATT-3′; **H9Probe**: [FAM] AACCAGGCCAGACATTG CGAGTAAGATCC [TAMRA]. Reverse transcription was performed (at 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 generated with RNA extracted from the AIV-H9N2 virus; the Ct values of samples were converted into EID_{50}/ml as previously described [37] for the determination of shedding titers.

2.5.7. Serological test

The HI (beta procedure) test was carried out for the detection of AIV-H9N2 antibody titers, and geometric mean titers were estimated as previously described [38]. A standard antigen AIV-H9N2 was used for the HI test.

2.6. Statistical analysis

Statistical analysis of the data was performed using one-way ANOVA followed by the least significant difference test to determine significant differences among the seven tested groups using a confidence level of 0.05. The analyses were performed with SAS statistical software [39].

3. Results

3.1. Mortality, clinical signs, and post-mortem lesions

Toxicity studies of Immulant® revealed that the product exhibited no signs of toxicity such as incoordination, reluctance to move, depression, diarrhea or mortality with normal water drinking, indicating palatable smell and taste of the product in chicks even at the highest dose of 100 ml/L compared to the control group. In the experimental study, no mortality was recorded in the negative control group. The protection rates obtained PC with the AIV-H9N2 strain correlated with the results of the HI test. The VAC group did not show any mortality (0%), in contrast to the VAC + DEX group (10%). In addition, no mortality was detected in the VAC groups after administration of 1% IMU (VAC + DEX + IMU and VAC + IMU) (0%), which correlated with the highest HI titers in both treated groups (Table 2). There was a significant ($P \leq 0.05$) lower mortality in the non-vaccinated IMU group (5%) than in the DEX group (15%). In this study, no clear respiratory signs were recorded in the groups treated with 1% IMU (VAC + DEX + IMU, VAC + IMU and IMU), while mild respiratory signs were recorded in the VAC group, followed by moderate signs in the VAC + DEX group and severe signs in the DEX group. The challenged birds showed respiratory signs, such as sneezing, coughing, rales, swelling of the periorbital sinuses with conjunctivitis, and white diarrhea. The post-mortem lesions included petechial haemorrhage on the proventriculus and congested trachea and lungs.

3.2. Antioxidant activity and oxidative stress

The antioxidant and oxidative stress results are shown in Fig. 1. Serum MDA and liver NO levels were significantly ($P \leq 0.05$) higher in all groups than in the control group at 1st week PV and 1st week PC. Hence, the DEX administration at a dose of 2 mg/kg of BW and/or vaccination in chicks significantly ($P \leq 0.05$) increased serum MDA and liver oxidative stress markers (rMDA and rNO). However, compared to those in the VAC, DEX and VAC + DEX groups, significant decreases ($P \leq 0.05$) in MDA and NO were observed after administration of 1% IMU (VAC + DEX + IMU, VAC + IMU and IMU). Conversely, all groups had significantly lower levels of GSH ($P \leq 0.05$) than the control group, but the groups treated with 1% IMU showed a significantly higher level ($P \leq 0.05$) than the VAC, DEX, and VAC + DEX groups at the two determined time points. Thus, DEX treatment and/or vaccination significantly ($P \leq 0.05$) reduced liver antioxidant capacity, as indicated by the detection of a lower level of rGSH in the tested groups than in the control group (Fig. 1).

3.3. TLC and heterophil/lymphocyte (H/L) ratio

The groups treated with 1% IMU (VAC + DEX + IMU, VAC + IMU and IMU) showed significantly ($P \leq 0.05$) higher TLCs than the VAC + DEX group in the 1st and 4th weeks PV and the 1st week PC (Table 3). The H/L ratio reflected the stress differences between groups (Fig. 2). The VAC + DEX group recorded a significantly higher H/L ratio at $P \leq 0.05$, followed by the DEX group, when compared to the control group and the groups treated with 1% IMU (VAC + DEX + IMU, VAC + IMU and IMU) at the three time points. However, the H/L ratio of the VAC group was significantly ($P \leq 0.05$) higher than that of the control and 1% IMU-treated groups in the 1st week PC.

3.4. Phagocytic activity and phagocytic index

The phagocytic activity ratio is illustrated in Fig. 3. The 1% IMU-treated groups (VAC + DEX + IMU, VAC + IMU and IMU) achieved

Table 2
Effect of Immulant® on HI geometric mean titers (GMTs) in vaccinated and dexamethasone-treated chickens and mortalities % post-challenge with AIV-H9N2.

Parameters	G1 Control	G2 VAC	G3 DEX	G4 VAC + DEX	G5 VAC + DEX + IMU	G6 VAC + IMU	G7 IMU	*P* value
W1 PV (17 days of age)								
HI	0.0^{d}	13.0^{b}	0.0^{d}	6.1^{c}	21.1^{a}	27.9^{a}	0.0^{d}	0.013
W4 PV (38 days of age)								
HI	0.0^{d}	55.7^{b}	0.0^{d}	28.6^{c}	94.4^{a}	119.4^{a}	0.0^{d}	0.017
W1 PC (45 days of age)								
HI	0.0^{c}	48.5^{a}	19.1^{b}	22.1^{b}	64.0^{a}	68.7^{a}	35.3^{b}	0.013
W2 PC (52 days of age)								
HI	0.0^{c}	66.3^{a}	28.1^{b}	33.2^{b}	76.1^{a}	86.4^{a}	52.8^{b}	0.041
Mortalities % post-challenge								
AIV-H9N2 challenge (n = 20)	$0/20\ (0)^{d}$	$0/20\ (0)^{d}$	$3/20\ (15)^{a}$	$2/20\ (10)^{b}$	$0/20\ (0)^{d}$	$0/20\ (0)^{d}$	$1/20\ (5)^{c}$	0.022

W1 PV& W4 PV: one week& four weeks post-vaccination. W1 PC & W2 PC, one and two weeks post-H9-challenge.
The different letters within the same row were significantly different at $P \leq 0.05$.

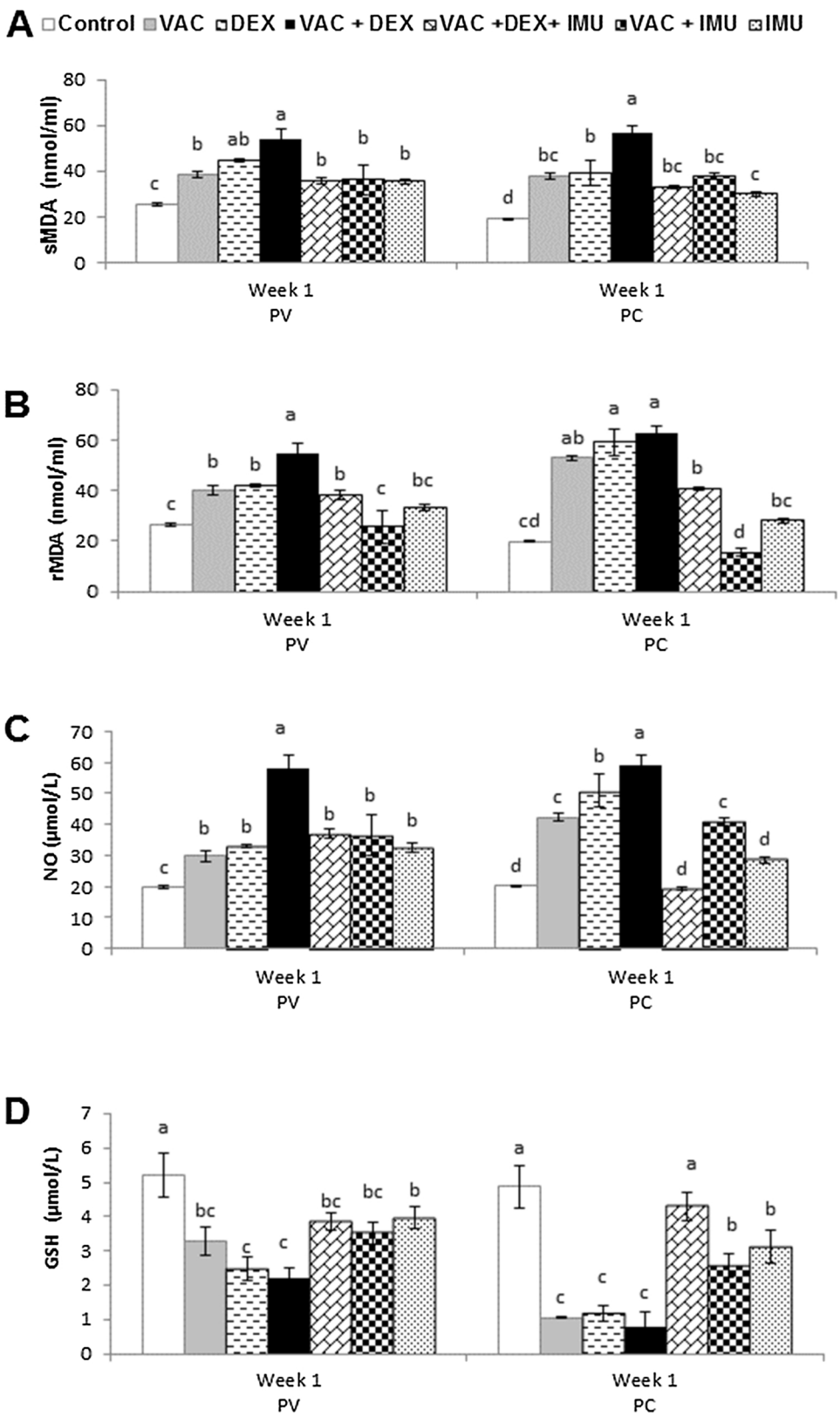

Fig. 1. Antioxidant and oxidative stress parameters of the experimental chickens at 1st week post-vaccination (PV) and 1st week post-challenge (PC). sMDA, serum malondialdehyde; rMDA, liver homogenate malondialdehyde; NO, nitric oxide; GSH, reduced glutathione. [a–d]Bars (mean ± SE) with different letters in the same period were significantly different at $P \leq 0.05$.

higher significance ($P \leq 0.05$) in terms of phagocytic activity ratios than the VAC, DEX and VAC+DEX groups in the 1st week PC. In comparison, this ratio was significantly lower in the DEX and VAC + DEX groups than in all other experimental groups PV and PC. The VAC, DEX and VAC + DEX groups had significantly lower ratios than the control group in the 1st week PC. The phagocytic index showed non-significant ($P > 0.05$) differences between all groups and the control group in the 1st week PC (data not shown).

Table 3
Total leukocyte count (x10^3/μl) in the experimental groups of chickens (mean ± SE).

Groups								
Periods	G1 Control	G2 VAC	G3 DEX	G4 VAC + DEX	G5 VAC + DEX + IMU	G6 VAC + IMU	G7 IMU	*P* value
W1 PV	11.33 ± 0.67^{b}	15.93 ± 1.11^{a}	11.17 ± 0.73^{b}	11.50 ± 1.26^{b}	16.5 ± 0.29^{a}	17.50 ± 0.76^{a}	14.36 ± 1.85^{a}	0.026
W4 PV	10.73 ± 0.98^{ab}	11.00 ± 0.58^{ab}	11.00 ± 0.64^{ab}	9.67 ± 0.55^{b}	11.13 ± 0.59^{ab}	13.73 ± 0.18^{a}	12.31 ± 0.25^{a}	0.050
W1 PC	10.50 ± 0.29b^{b}	14.34 ± 0.71^{a}	10.71 ± 1.0^{b}	12.67 ± 0.54^{ab}	13.29 ± 0.99^{a}	14.50 ± 0.58^{a}	13.51 ± 0.71^{a}	0.014

G: group; W: week; PV: post-vaccination; PC: post-challenge.
The different letters within the same row were significantly different at $P \leq 0.05$.

3.5. BW and relative organ weights

The mean BW was significantly lower ($P \leq 0.05$) in birds from the DEX-treated groups (DEX and VAC+DEX) than in non-treated groups (control and VAC) (Table 4). However, the mean BW was not significantly changed ($P > 0.05$) between the birds administered 1% IMU (VAC+DEX+IMU, VAC+IMU and IMU) and the control group in the 1st and 4th weeks PV and the 1st week PC (Table 4). In addition, we noticed that H9N2-AI vaccination at 10 days of age may not affect the chickens' final live weight, and a non-significant change ($P > 0.05$) was detected between the control (G1) group and the VAC (G2) group at 38 days of age.

A study of relative organ weight showed that organ weights were comparable among the VAC, DEX-treated and non-treated groups. The mean bursa weight-to-BW ratios and spleen weight-to-BW ratios were significantly lower ($P \leq 0.05$) in the DEX-treated groups (DEX and VAC+DEX) than in the non-treated groups (control and VAC) at the three time points. Moreover, non-significant changes ($P > 0.05$) were detected in the relative organ weights (spleen and bursa) between the control (G1) and 1% IMU-treated groups at the three time points (Table 4).

3.6. Histopathology and immunohistochemistry

The histopathological examination revealed that the VAC + DEX group showed higher lesion scores in the examined organs than the VAC group before and after the AIV-H9N2 challenge. A clear reduction in lesion scores occurred in all examined organs from the 1% IMU-treated groups (VAC + DEX + IMU, VAC + IMU and IMU) either before or after AIV-H9N2 challenge. However, lesion scores were much lower in the VAC + IMU group than in the VAC + DEX + IMU and IMU groups (Table S1 & Fig. S2). By IHC, H9 viral antigen showed variable staining intensities in the lung, particularly in the alveolar walls (Fig. 4A–G), with negative staining in the negative control and VAC + IMU groups, mild staining in the VAC, VAC + DEX + IMU and IMU groups, moderate staining in the VAC + DEX group and strong staining in the DEX group.

3.7. Virus shedding titers and period

The viral shedding titers and period of AIV-H9N2 were tested in tracheal and cloacal swabs from different groups at 2, 5, 8 and 10 days PC (Fig. 5). Viral shedding titers were positively detected in swabs from the DEX and VAC + DEX groups at 2, 5, and 8 days PC, while titers were detected in swabs from the VAC group and the 1% IMU-treated groups (VAC + DEX + IMU, VAC + IMU and IMU) at 2 and 5 days PC, indicating a short period of shedding. Moreover, tracheal and cloacal shedding titers from chickens in the DEX and VAC + DEX groups were significantly ($P \leq 0.05$) higher than those from chickens in the VAC group or the 1% IMU-treated groups at the same time points. The lowest viral shedding titers were detected in the VAC+IMU group, followed by the VAC+DEX+IMU group and the IMU group at 2 and 5 days PC in tracheal swabs ($P = 0.022$ and $P = 0.017$, respectively) and in cloacal swabs ($P = 0.023$ and $P = 0.035$, respectively), and these differences were significant. In this study, there was no viral shedding from the negative control group on any day or from any group at 10 days PC (Fig. 5).

3.8. HI titers of AIV-H9N2

No maternal HI antibody titers of AIV-H9N2 were detected in the experimental chicks either on the 1st day of age or on the 10th day of

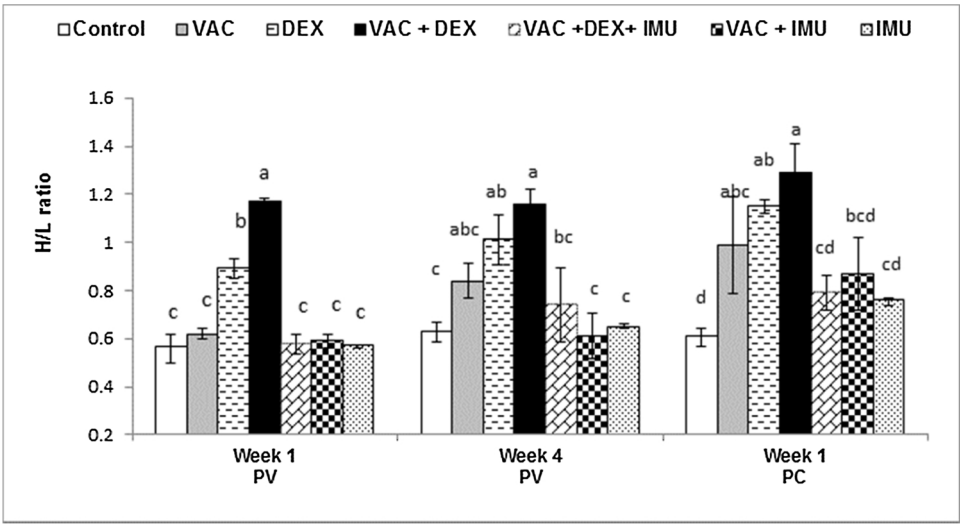

Fig. 2. Heterophil lymphocyte (H/L) ratio (mean ± SE) in the blood of the experimental chickens. PV, post-vaccination; PC, post-challenge. $^{a–d}$ Bars with different letters in the same period were significantly different at $P \leq 0.05$.

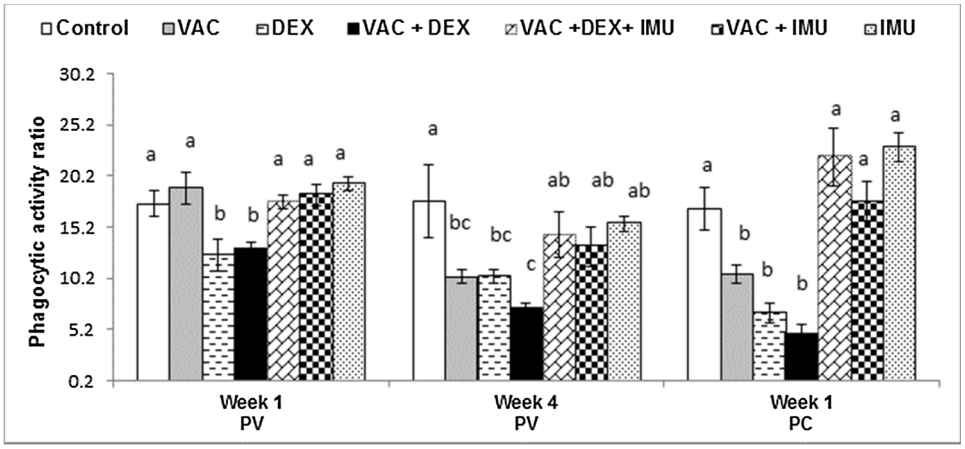

Fig. 3. Heterophils phagocytic activity ratio (mean ± SE) of the experimental chickens PV, post-vaccination; PC, post-challenge. [a–c] Bars with different letters in the same period were significantly different at $P \leq 0.05$.

Table 4
Effect of Immulant® on mean body weight (BW), and mean organ to BW ratios of vaccinated and dexamethasone-treated chickens challenged with AIV-H9N2 (mean ± SE).

Parameters	G1 Control	G2 VAC	G3 DEX	G4 VAC + DEX	G5 VAC + DEX + IMU	G6 VAC + IMU	G7 IMU	*P* value
W1 PV (17 days of age)								
BW	287.5 ± 9.2^{a}	296 ± 15.5^{a}	236 ± 33.9^{d}	249.3 ± 14.7^{c}	295 ± 4.9^{a}	298.7 ± 16.3^{a}	297.3 ± 11.8^{a}	0.014
Spleen/BW ratio	0.24 ± 0.01^{a}	0.21 ± 0.04^{ab}	0.18 ± 0.04^{b}	0.19 ± 0.03^{b}	0.25 ± 0.06^{a}	0.23 ± 0.02^{a}	0.24 ± 0.03^{a}	0.012
Bursa/BW ratio	0.25 ± 0.04^{a}	0.20 ± 0.09^{b}	0.17 ± 0.03^{c}	0.18 ± 0.08^{c}	0.27 ± 0.06^{a}	0.28 ± 0.05^{a}	0.26 ± 0.05^{a}	0.015
W4 PV (38 days of age)								
BW	660.0 ± 8.0^{a}	655.3 ± 23.0^{a}	595.0 ± 18.7^{b}	608.3 ± 18.1^{b}	651.7 ± 20.5 ^{a}	670.0 ± 6.1^{a}	662.5 ± 9.2^{a}	0.024
Spleen/BW	0.26 ± 0.04^{a}	0.22 ± 0.01^{b}	0.19 ± 0.02^{c}	0.18 ± 0.004^{c}	0.21 ± 0.02^{b}	0.26 ± 0.01^{a}	0.25 ± 0.06^{a}	0.010
Bursa/BW	0.22 ± 0.01^{a}	0.24 ± 0.008 ^{a}	0.13 ± 0.007^{c}	0.13 ± 0.004^{c}	0.19 ± 0.005^{b}	0.23 ± 0.02^{a}	0.24 ± 0.03^{a}	0.012
W1 PC (45 days of age)								
BW	822 ± 25.2^{a}	795 ± 27.4^{a}	617 ± 29.8^{b}	656 ± 29.8^{b}	793 ± 25.2^{a}	816 ± 21.7^{a}	802 ± 21.7^{a}	0.050
Spleen/BW	0.19 ± 0.04^{a}	0.16 ± 0.02^{b}	0.15 ± 0.07^{c}	0.15 ± 0.07^{c}	0.21 ± 0.03^{a}	0.20 ± 0.06^{a}	0.22 ± 0.05^{a}	0.031
Bursa/BW	0.23 ± 0.12^{a}	0.19 ± 0.09^{b}	0.17 ± 0.14^{c}	0.17 ± 0.14^{c}	0.23 ± 0.12^{a}	0.24 ± 0.12^{a}	0.25 ± 0.11^{a}	0.024

W1 PV & W4 PV: one week& four weeks post-vaccination. W1 PC, one week post-H9-challenge.
The different letters within the same row were significantly different at $P \leq 0.05$.

age immediately before vaccination. The VAC group showed significantly elevated ($P \leq 0.05$) HI antibody titers against the H9N2 compared to those of the VAC+DEX group. Moreover, there was a significant increase ($P \leq 0.05$) in antibody titers against the H9N2 vaccine after administration of 1% IMU (VAC+DEX+IMU and VAC+IMU) in the 1st and 4th weeks PV and the 1st and 2nd weeks PC (Table 2). These results show that administration of the H9N2 vaccine on day 10 of age can be considered protective, as indicated by the high HI antibody titers in the 4th week PV.

4. Discussion

The present study was planned to evaluate the effects of medicinal plants in a commercial product (Immulant®) based on *Echinacea* and *Nigella sativa* on immune response of vaccination and pathogenicity of the H9N2 AIV in chickens stressed by DEX.

In the present study, the obtained protection rates PC with the AIV-H9N2 strain were observed to correlate with the results of the HI test. In addition, the birds challenged with AIV-H9N2 showed respiratory signs, white diarrhea and congested trachea and lungs. Similar clinical signs and post-mortem lesions have been described previously [40]. In this study, mild to severe respiratory signs in birds PC after vaccination and/or DEX treatment were observed because the chickens were stressed at the time of vaccination, similar to typical field conditions. No clear respiratory signs were recorded in the vaccinated and 1% IMU-treated groups, establishing that IMU could rescue the immune response, even with DEX treatment. Similar results were obtained by Arafat et al. [22], who explained that severe clinical signs and high mortality associated with AIV-H9N2 could be due to environmental stress or co-infection with the ILT virus vaccine. On the other hand, Kilany et al. [41] found that no challenged H9N2 vaccinated birds displayed any clinical signs, while the non-vaccinated challenged birds displayed mild respiratory signs.

Reactive oxygen and nitrogen species (ROS and RNS) are vital by-products that contain free radicals and can damage cell biomolecules such as lipids. When immunological defences fail to control these species, damage occurs and is called "oxidative stress". Nitric oxide (NO) and malondialdehyde (MDA) are vital biochemical markers of oxidative stress. Reduced glutathione (GSH) is an antioxidant biomarker that is synthesized in the liver [25].

In the present study, the DEX-treated and/or vaccinated groups showed that stress elevated MDA and NO levels and reduced GSH levels. However, there was a significant decrease in MDA and NO levels and an increase in GSH levels after administration of IMU. NO plays a beneficial role in many physiological functions; however, its over-production can induce cytotoxic effects [24]. The GSH, MDA and NO results obtained in this study are similar to the results of a previous study [36]. NO values were significantly elevated after administration of *E. purpurea* in rabbits. Furthermore, in pasteurellosis-vaccinated rabbits, GSH levels were increased by administration of *E. purpurea* for 2 weeks [10].

In this study, the VAC + DEX group recorded the highest and most significant H/L ratio in comparison to the control group and the groups treated with 1% IMU. The H/L ratio is not the only valid parameter for

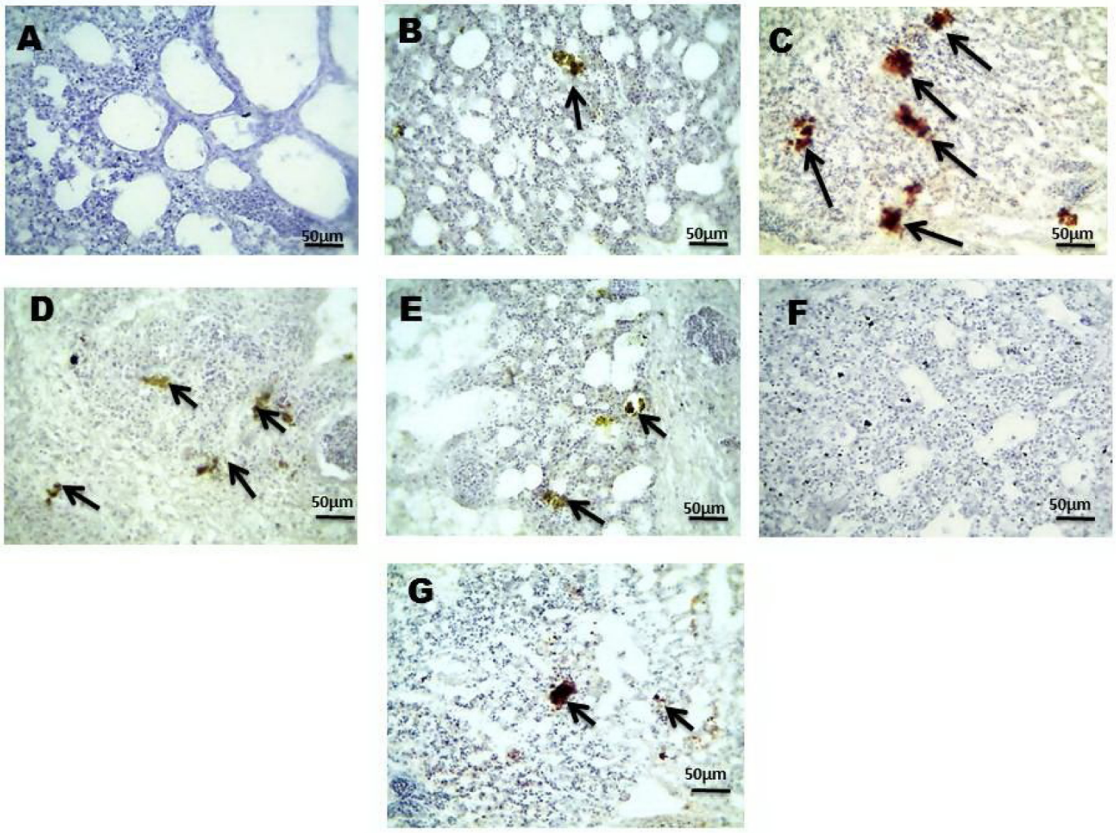

Fig. 4. Microscopical pictures of immunostaind lung of chickens at the 1st week post- challenge with AIV H9N2: **(A)** G1 (negative control group) shows negative staining; (B) G2 (VAC group) shows mild positive staining; (C) G3 (DEX group) shows strong positive staining; (D) G4 (VAC + DEX group) shows moderate positive staining; (E) G5 (VAC + DEX + IMU group) shows mild positive staining; (F) G6 (VAC + IMU group) shows negative staining; (G) G7 (IMU group) shows mild positive staining. Arrows point to brown positive signal in alveolar wall. IHC counterstained with Mayer's haematoxylin, X: 200.

evaluating stress status in birds; in particular, in turkeys [38], the leukocyte profile is also required [28]. In another study, a decrease in lymphocytes was observed in DEX-treated turkeys, which might have occurred because CD8+ T-lymphocytes play a role in the clearance or absence of AIV infection [42]. Glucocorticoids and stress shift the H/L ratio and several other parameters [43]. In our findings, the 1% IMU-treated groups showed significantly higher TLCs at $P \leq 0.05$ than the VAC + DEX group. Similarly, a significant degree of leukocytosis was observed after administering a specific dose (49 mg/kg of BW) of *E. purpurea* to rabbits [10].

The results of this study show a decrease in the heterophil phagocytic activity of immunosuppressed chickens. Heterophils have selective and sensitive phagocytic activity and play an important role in host defence [44]. In addition, the development of healthy chicks may be related to the selection of hens with more efficient immune systems, including more effective phagocytosis [14]. Our results suggest that IMU may improve heterophil phagocytic activity in vaccinated and immunosuppressed chickens. To the best of our knowledge, this is the first study to report on heterophil phagocytic activity against *Candida albicans* and the phagocytic index through a simple method in chickens. In the current study, medicinal plant-treated groups showed clear positive results in terms of heterophil phagocytic activity. These results were partially in harmony with those of a previous study showing that the phagocytic activities of blood mononuclear cells were enhanced in chickens treated with plant essential oils and vaccinated with the AI-H5N2 vaccine [45]. In our study, the non-significant changes in the phagocytic index might be due to the large particle size of *Candida albicans* in relation to the size of the heterophils; the phagocytic index depends on the size of the engulfed particle, which changes according to the surface features of the phagocytic cell [30].

The findings of this study showed that DEX has a depressive effect on the humoral and cellular immune responses of vaccinated birds against the AIV-H9N2 subtype, as shown by a significant decrease in the HI antibody titers PV and PC with AIV-H9N2, reductions in TLCs and heterophil phagocytic activity and significant increases in the H/L ratio and induced oxidative stress biomarkers. In this study, we suggest that stress factors leading to immunosuppression could be a factor contributing to AI vaccination failure on poultry farms. Similarly, Yu et al. [4] reported that AI vaccination failure on poultry farms may be due to immunosuppression induced by stress. Types of stress models are environmental stressors (heat or cold stress), injection preparations, social groupings, and toxic substances [7]. The two best criteria of the DEX stress model are the exact and possible daily treatment and the predictable stress responses in a known time [7]. The DEX stress model can also provide a justification for management approaches intended to reduce stress [8]. Virden and Kidd [46] showed that the DEX stress model has been validated and may be the best method of the physiological stress because specific effects of specific stressors can be avoided. They confirmed that DEX injection decreased body weight, immune responses and lymphoid organs, and increased H/L ratio. DEX is commonly used stress model to study and mimic severe stressors in poultry diseases. The combination of excessive transport, heat and humidity represent severe stressors that can be induced by DEX stress model [6]. Conversely, the heat stress model may not cause changes in markers of stress, such as corticosterone and H/L ratio due to the adaptability to high temperatures because of high metabolic rate of broiler chickens [47].

In the current work, the chickens injected with DEX showed a significant reduction in mean BW, which may be attributed to anorexia and depression. These results are consistent with those of previous

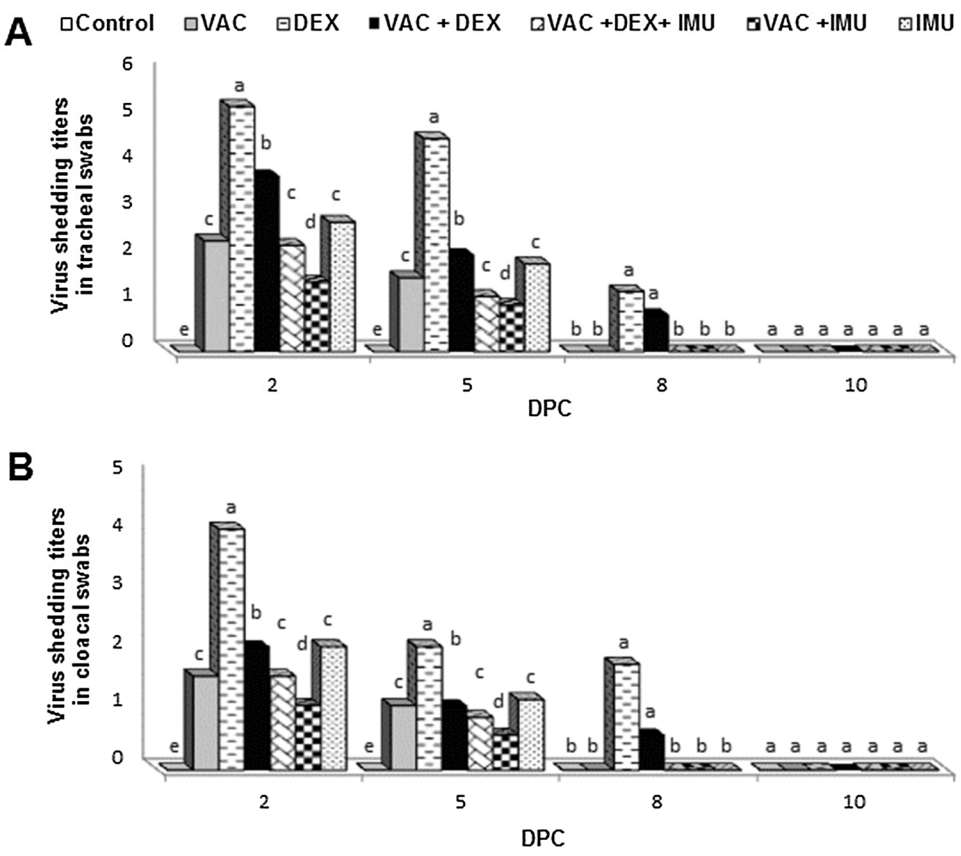

Fig. 5. Effect of Immulant® on mean AIV-H9N2 shedding titers (log_{10} EID_{50}/ml). Tracheal viral shedding (A) and cloacal viral shedding (B) of vaccinated and/or dexamethasone-treated chickens challenged with AIV-H9N2. DPC, days post-challenge. [a–d] Bars with different letters in the same period were significantly different at $P \leq 0.05$.

reports [48] that revealed that chickens treated with DEX had a decrease in BW gain compared with that of untreated chickens. Moreover, Vicuna et al. [49] showed that the administration of DEX decreased the weights of the immune organs (spleen and bursa of Fabricius). In the present study, the possible mechanism by which 1% IMU improves growth is due to its composition containing *Echinacea* and *Nigella sativa*. In a previous work, significant improvements in the final weights and feed conversion rates of vaccinated chickens treated with *Echinacea* treatment were shown [11]. Moreover, this difference may be due to the effect of black cumin seeds (*Nigella sativa*) on nutrient utilization, immunity, gut health and nitrogen excretion [12].

Low-pathogenic AIV primarily affects the respiratory and gastrointestinal tracts, but it less commonly affects the liver, spleen and brain [50]. Our histopathological findings showed the presence of different lesions in the liver, spleen and jejunum before challenge, mainly in the VAC and/or DEX-treated groups, due to stress stimulated by vaccination and/or DEX injection. Similar findings have been reported in rats due to DEX injection [51]. In the current study, more severe lesions were detected in the lungs, liver, spleen and jejunum post-H9 challenge, mainly in the VAC and/or DEX-treated groups. On the other hand, a previous study reported that the more cell injury was detected in tissues of the respiratory system and GIT than in other organs after AIV-H9N2 infection in birds [52].

Administration of 1% IMU could ameliorate the side effects of stress stimulated by vaccination and/or DEX injection either before or after challenge with AIV-H9N2. Moreover, IHC staining was absent from the lungs of the VAC + IMU group. In addition, mild staining was detected in the lungs of the IMU-treated groups, in contrast to the lungs of the VAC + DEX group (moderate staining) and the DEX group (strong staining), correlating with the results obtained for viral shedding titers. Therefore, 1% IMU as a commercial product (based on *Echinacea* and *Nigella sativa*) could reduce the pathogenicity of AIV-H9N2 infection and eliminate or decrease virus distribution *via* possible mechanisms related to enhancement of bird immune responses, changes in blood cell numbers, the reduction of induced oxidative stress, and the improvement of feed conversion [11,12].

In this work, the shedding titers of AIV-H9N2 from chicken groups treated with either DEX alone or VAC + DEX were significantly higher than the titers of those in the VAC group. Similarly, another study showed that DEX treatment may enhance the ability of LPAI virus to infect and replicate in immunosuppressed turkeys [18]. In this study, the VAC only group had no mortality, lower virus shedding titers than the non-vaccinated groups, and measurable HI responses, indicating that the vaccine is a protective vaccine. In addition, under stress, we observed mortality, high virus shedding titers, and weak HI antibody responses. There was a significant benefit of IMU treatment in "healthy" VAC birds in terms of prevention of shedding after challenge, as indicated by a short period of shedding (2 and 5 days PC) and the lowest virus shedding titers.

In this study, the IMU-treated groups had a significant increase in HI antibody titers PV and PC with AIV-H9N2, an increase in TLCs and heterophil phagocytic activity, a significant decrease in the H/L ratio and reduced DEX-stress biomarkers. Statistically significant seroconversion after vaccination was obtained by *Salmonella* Enteritidis bacterin with daily oral intake of *Echinacea purpurea* [53]. Furthermore, Dehkordi et al. [11] suggested that the feeding of *E. purpurea*, particularly for a long time (six weeks), could improve the feed conversion rate and blood cell counts and enhance the immune response in broilers. Therefore, based on our results, IMU could be used to enhance immune responses after H9N2 vaccination in immunosuppressed chickens.

5. Conclusion

In conclusion, the use of DEX as a stress model revealed that stress might be a contributing factor to AI-H9N2 vaccination failure on poultry farms because stress leads to immunosuppression and increased pathogenicity of infection; hence, vaccination alone is not a guarantee

of protection. Therefore, stress reduction during bird management is highly recommended. Interestingly, this is the first report that oral administration of 1% IMU for six weeks could improve final live weights, enhance the immune response after AIV-H9N2 vaccination and reduce the pathogenicity of viral infection in stressed chickens.

Conflict of interest

The authors declare that they have no conflict of interest.

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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.cimid.2019.05.017.

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