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Assessment of Possible Antiviral and immune stimulating Effects of Aloe Vera Water Extract (AVWE) on Very virulent Infectious Bursal Disease Virus Infection.

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ORIGINAL ARTICLE

Assessment of Possible Antiviral and Immunostimulating Effects of Aloe Vera Water Extract on Very Virulent Infectious Bursal Disease Virus Infection

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Abstract

OBJECTIVE: Evaluation of the antiviral and immunostimulatory effects of aloe vera.
DESIGN: Experimental study.
PROCEDURES: A 10 % [aloe vera water extract (AVWE)] was prepared, and its antiviral effect against vvIBDV was tested in embryonated chicken eggs (ECEs). Five groups were created (Ge1−Ge5); Ge1 was a negative control, Ge2 was a positive control, and Ge3, Ge4, and Ge5 were prophylactic, antiviral, and treatment groups, respectively. While the immunostimulatory effects of 10 % AVWE were tested in chicks, five groups were created (Gc1−Gc5); Gc1, a negative control, Gc2 a positive control; and Gc3, Gc4, and Gc5, the protected, protected-treated, and treated groups, respectively.
RESULTS: In ECEs, Ge2, Ge3, and Ge5 embryos showed typical vvIBDV lesions with a high vvIBDV load in real-time RT-PCR. However, the Ge1 and Ge4 embryos were normal and showed negative results on RT-PCR. In challenged birds, Gc1 did not show any clinical signs or PM changes. Gc2 and Gc5 showed the typical signs of vvIBDV infection. Gc3 and Gc4 showed mild symptoms. There was a statistically significantly lower body weight in the Gc2 group than in the other groups. Gc4 had the highest total leukocyte and lymphocyte counts. Gc4 also showed the highest antibody titer against vvIBDV with mild histopathological lesions.
CONCLUSION AND CLINICAL RELEVANCE: AVWE (10 %) has a potent antiviral effect against vvIBDV, with no prophylactic or treatment effects on ECEs. In contrast, the use of 10 % AVWE before and after vvIBDV challenge in chicks showed a promising protective effect.

Keywords: Aloe vera, Antiviral, Histopathology, Immunostimulant, Very virulent infectious bursal disease virus

1. Introduction

The poultry industry faces many difficulties due to common viral infections, such as infectious bursal disease virus (IBDV), Newcastle disease virus, infectious bronchitis, and Fowl pox virus [1,2]. IBDV belonging to the Birnaviridae family, causes Gumboro disease in poultry [3]. IBDV is a nonenveloped double-stranded bisegmented RNA virus that infects chicks at 3−6 weeks [4,5]. Viral diseases are considered the most prominent diseases in the poultry industry because viruses not only cause damage to bird health but also open the gates for other infectious agents, such as bacteria, fungi, and mycoplasmas. The lymphoid organs, including the bursa of Fabricius, are the main targets of IBDV, which can damage the avian immune system and cause immunosuppressive diseases [6]. IBDV virulence can be classified into three types (mild, virulent, and very virulent strain). The mild and virulent strains induced up to 30 and 60 % mortality, respectively, whereas the

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very virulent infectious bursal disease virus (vvIBDV) strains caused more than 70 % mortality. Therefore, the poultry industry is economically affected by this disease [7]. Nowadays, the focus is on medicinal plants (phytotherapy), which are used for the prevention and treatment of some diseases in both animals and humans. Many researchers have used medicinal plant extracts as antivirals and immunostimulating agents. Moreover medicinal plants may reduce drug resistance and modify the action of the immune system against viral-related diseases [8]. Aloe vera is a phytogenic plant with great medicinal potential. Aloe vera has antiviral, antioxidant, antidiabetic, anti-inflammatory, antifungal, and antitumor effects, which are attributed to many ingredients in aloe vera extract, such as vitamins, anthraquinones, enzymes, and saccharides [9]. The aim of this study was to measure the antiviral effects of aloe vera against vvIBDV in embryonated chicken eggs (ECEs) by assessing its immune-stimulating effect on susceptible vvIBDV-challenged chickens.

2. Materials and methods

2.1. Ethical considerations

The experimental procedures were performed in accordance with the guidelines for international animal welfare and the relevant Egyptian legislation. Ethical approval was obtained from the Research, Publication, and Ethics Committee of the Faculty of Veterinary Medicine, Mansoura University, Egypt (04/2018).

2.2. Standard infectious bursal disease virus

A standard vvIBDV virus strain (IBDV/Dakahlia, Egypt/2016) from a previous study [10] was used. This strain has a GenBank accession number MK988513.

2.3. Aloe vera water extract

Fresh, mature, and healthy aloe vera leaves were purchased from a commercial source and thoroughly washed with water. A cut was then made in the leaves, the latex was removed, and the gel was collected. The gel was then diluted to a 10 % (w/v) concentration by mixing 100 g of fresh aloe vera gel with 1 l of boiling water and mixed thoroughly by shaking for 7 min. The 10 % aloe vera water extract (AVWE) was stored at room temperature for 6−8 h before use [11].

2.4. Assessment of aloe vera water extract antiviral effect against very virulent infectious bursal disease virus in embryonated chicken eggs

2.4.1. Emberyonated chicken eggs

One-day-old specific pathogen-free (SPF-ECEs) were obtained from SPF chicken farms (Koum Oshiem, Fayoumi, Egypt). They were kept in an egg incubator at 37 °C with humidity 60 % for 11 days and then used for the toxicity assay, preparation of seed viruses, virus titration, and assessment of the antiviral effect of AVWE against vvIBDV.

2.4.2. Toxicity assay

This was performed according to Mosad *et al.* [10] to determine the maximum nontoxic concentration of AVWE in ECEs. About 0.1 ml of 10-fold dilution (10^{-1}—10^{-5}) of AVWE was inoculated into the allantoic cavity of 11-day-old ECEs in triplicate. All inoculated ECEs were incubated at 37 °C until hatching and were candled daily to check viability.

2.4.3. Preparation and titration of seed virus stock

The 11-day-old SPF-ECEs ($n = 10$) were inoculated with 0.2 ml of standard vvIBDV strain on the CAM, then incubated for 72 h at 37 °C; then the CAMs were harvested, homogenized, centrifuged, and the supernatant fluid containing the seed virus was stored at −80 °C. The seed virus was titrated by preparation of a 10-fold serial dilution (10^{-1}—10^{-9}), and then inoculated on the CAM of 11-day-old SPF-ECEs (5 ECEs/dilution), and the virus titer was estimated as the egg infective dose 50 % per ml (EID50/ml) as previously described by Reed and Menuch [12]. The viral titer was $10^{4.5}$ EID50/ml.

2.4.4. Testing of the aloe vera water extract antiviral effect against very virulent infectious bursal disease virus

This was done according to Ghoke *et al.* [13] and Taechowisa *et al.* [14]. The 10 % AVWE (100 μl) was inoculated into the allantoic sac, while 100 μl of vvIBDV ($10^{4.5}$ EID50/ml) was inoculated via the CAM of 11-day-old ECEs. Eleven-day-old ECEs ($n = 50$) were divided into five groups (Ge1−Ge5) and 10 ECEs/group. Ge1 a negative control group, in which SPE-ECEs were inoculated with 100 μl of PBS in the allantoic sac, Ge2 was a positive control group, in which SPE-ECEs were inoculated with 100 μl of vvIBDV via CAM. Ge3 was a prophylactic group in which SPE-ECEs were inoculated with 100 μl of 10 % AVWE in the allantoic sac and incubated at 37 °C for 2 h. Then, the ECEs were inoculated with 100 μl of vvIBDV via CAM. Ge4 was

an antiviral group, in which a mixture of 10 % AVWE with an equal volume of vvIBDV was incubated for 2 h at 37 °C, and then 100 μl of the previous mixture was inoculated on the CAM of the ECEs. Ge5 was a treatment group, in which 100 μl of vvIBDV was inoculated on the CAM of ECEs and incubated at 37 °C for 2 h, then 100 μl of 10 % AVWE was inoculated into the allantoic sac. The inoculated ECEs were incubated at 37 °C for 72 h and then examined daily, and any deaths during the first 24 h were discarded. After 72 h, ECEs were examined for embryonic lesions, and CAMs were harvested for determination of viral load by RT-PCR.

2.5. Assessment of aloe vera water extract immune stimulating effect against very virulent infectious bursal disease virus in chicken

2.5.1. Experimental animals

One-day-old Cobb broiler chicks ($n = 100$), with an average weight of 46 g, were purchased from a commercial source. They were fed a commercial diet (Dakahlia Poultry Company, Dakahlia, Egypt). A starter diet was fed from the first to 21st day of age and then a grower diet from 22nd to 42nd day of age. Food and water were provided *ad libitum* throughout the experiments. The chicks were not administered any medication or vaccinations.

2.5.2. Testing of aloe vera water extract immune stimulating effect in chicks

The vvIBDV ($10^{4.5}$ EID50/ml) was inoculated in challenged birds by the eye drop method (1 ml/bird) at 21st day of age, while the AVWE was added to the drinking water at a final concentration of 10 %. One-day-old Commercial Cobb broiler chicks ($n = 100$) were divided into five groups (Gc1−Gc5) of 20 chicks each. Gc1 was used as the negative control group, in which birds were kept without virus inoculation or AVWE administration. Gc2 was a positive control group, in which the chicks were challenged with vvIBDV ($10^{4.5}$ EID50/ml) by the eye drop method on the 21st day of age without AVWE administration. Gc3 was a protected group, in which the chicks received 10 % AVWE from the 7th day till 21st day of age and then challenged with vvIBDV ($10^{4.5}$ EID50/ml) by the eye drop method at 21st day of age. Gc4 was a protected-treated group, in which the chicks received 10 % AVWE from the 7th day to 21st day. On day 21st of age they were challenged with vvIBDV ($10^{4.5}$ EID50/ml) and treated with 10 % AVWE from day 21st to 42nd day of age. Gc5 was a treated group, in which chicks were challenged with vvIBDV ($10^{4.5}$ EID50/ml) by the eye drop method at day 21st of age and then treated with 10 % AVWE from day 21st to 42nd day of age. The chicks were examined daily to record the clinical signs and mortality. On the 5th day postchallenge (26th day of age), three birds were randomly selected from each group and euthanized, and their organs (bursa, spleen, thymus, and kidney) were collected and fixed in 10 % neutral buffered formalin for histopathological examination. Three birds were randomly selected from each group on 28th, 35th, and 42nd day of age, and their body weights were recorded. Whole blood samples were collected from the patients with anticoagulant (heparin 20 IU/ml) for total leukocyte count (TLC) and lymphocytic count. Serum samples were also collected, inactivated at 56 °C for 30 min, and stored at −20 °C until used for evaluation of IBDV humeral immune response by enzyme-linked immunosorbent assay (ELISA). These birds were then euthanized, and their tissues (bursa and spleen) were collected, weighed, pooled from each bird, homogenized, and used for vvIBDV detection by RT-PCR.

2.5.3. Evaluation of total leucocytic count and lymphocytic count

TLC and lymphocytic count were performed according to Chubb and Rowell [15] as follows: the blood samples were diluted with Natt and Herrick [16] solution using an improved Neubauer hemocytometer red blood cells pipette. The number of leukocytes in the central red blood cells squares was counted, and the number of leukocytes per ml blood was calculated according to the following equation: white blood cells/ml blood $= N \times 10 \times 100$, where N is the number of leukocytes or lymphocytes in the central square.

2.5.4. Evaluation of humeral immune response in collected serum samples using enzyme-linked immunosorbent assay

The tested samples were diluted to 1 : 500 with the sample diluent and mixed thoroughly. ELISA test procedure, calculation, and interpretation were performed according to Müller and Becht [17].

2.5.5. Real-time RT-PCR detection of very virulent infectious bursal disease virus in experimental chicks

Viral RNA extraction from tissue samples: tissue samples (bursa and spleen) were collected from each bird, pooled, and homogenized, and a 10 % concentration (w/v) was prepared. The samples were centrifuged at 2000 rpm for 15 min, and the supernatant was used for viral RNA extraction using the QIAamp MinElut Virus spin kit (QIAGEN, GmbH, Hilden, Germany).

Real-time RT-PCR assay: a one-step RT-PCR assay was carried out using the QuantiNova Probe RT-PCR kit according to Peters *et al.* [18]. The forward primer was 5′-GTCGAGTGGATATTGGCCCC-3′ and reverse primer 5′-GGCTCCTGCGTTA TTCTTGC-3′ (Metabion, Germany) was used to amplify an IBDV segment A 136 bp amplicon within the VP4 gene. FAM − 5′-CAACGCCTATGGCGA-GATTGAGAACGTGAG-3′ − BHQ1 (Metabion, Ger many) was designed with a sequence specific to highly virulent IBDV strains. The reaction mixture was composed of 10 μl of 2 × QuantiNova Probe RT-PCR Master Mix, 0.8 μl forward primer, 0.8 μl reverse primer, 0.2 μM TaqMan Probe, 5 μl template RNA and RNase-free water up to 20 μl then the reactions were mixed thoroughly. The real-time cycler was programmed based on the program outlined in Table 1. Data acquisition was performed during the combined annealing and extension step.

2.5.6. Histopathological examination

Bursa, spleen, thymus, and kidney tissue samples were fixed in 10 % neutral buffered formalin and then used to prepare paraffin tissue section at 3−4 μm thickness. Tissue sections were stained with hematoxylin and eosin for pathological examination [19].

2.6. Statistical analysis

2.6.1. Software and data expression

Data were entered and analyzed using the IBM SPSS software (IBM Corp. Released 2019. IBM SPSS Statistics for Windows, Version 26.0.; IBM Corp., Armonk, New York, USA). Quantitative data were initially tested for normality using the Shapiro−Wilk test, with data being normally distributed if the *P* value less than or equal to 0.05. Quantitative data are expressed as mean ± SD.

2.6.2. Data comparison

Effects of one factor (group) on a quantitative data: one-way analysis of variance was used for normally distributed data with no significant outliers in all groups, whereas the Kruskal−Wallis *H* test was used for non-normally distributed data and/or the presence of significant outliers in at least one group.

Effects of two factors (group and time) on a quantitative data: two-way analysis of variance was used to ascertain the group × time interaction effect on the quantitative data.

Table 1. Detailed real-time RT-PCR cycling condition.

Steps	Time	Temperature	Number of cycle
Reverse transcription	10 min	45 °C	1 cycle
PCR initial activation	5 min	95 °C	1 cycle
Two-step cycling			
Denaturation	20 s	95 °C	40 cycle
Combined annealing/ extension	60 s	60 °C	

2.6.3. Significance level

For any of the tests, the results were considered statistically significant if the *P* value was less than or equal to 0.05.

2.6.4. Charts

Appropriate charts were used to graphically present the results whenever needed including profile plots.

3. Results

3.1. Assessment of aloe vera water extract antiviral effect against very virulent infectious bursal disease virus in embryonated chicken eggs

3.1.1. Toxicity assay

All dilutions of AVWE were nontoxic, as all inoculated ECEs successfully hatched without any embryonic death. Hatched chicks were monitored for 5 days after hatching and were healthy; therefore, the MNTD was 10^{-1}, so 10 % AVWE was used.

3.1.2. Lesions of very virulent infectious bursal disease virus in inoculated embryos

The embryos of Ge1 (negative control group) and Ge4 (antiviral group) were normal and showed no lesions in any of the inoculated ECEs. Ge2 (positive control group), embryos Ge3 (prophylactic group), and Ge5 (treatment group) showed edema in the head and abdomen, dwarfism, subcutaneous hemorrhage with a pale greenish liver, and some embryos showed deformities (Fig. 1).

3.1.3. Real time RT-PCR

Embryos of Ge1 (negative control group) and Ge4 (antiviral group) showed negative results on RT-PCR. Ge2 (positive control group), embryos Ge3 (prophylactic group), and Ge5 (treatment group) yielded positive results in RT-PCR with low ct values, indicating high vvIBDV titers in the examined CAMs (Table 2 and Fig. 2). Real-time RT-PCR (RRT-PCR) revealed a statistically significant difference among the five groups ($P = 0.027$). There was no statistically significant difference in RRT-PCR between Ge1 and Ge4, which revealed that AVWE has a potent antiviral effect with no prophylactic or treatment effect against vvIBDV.

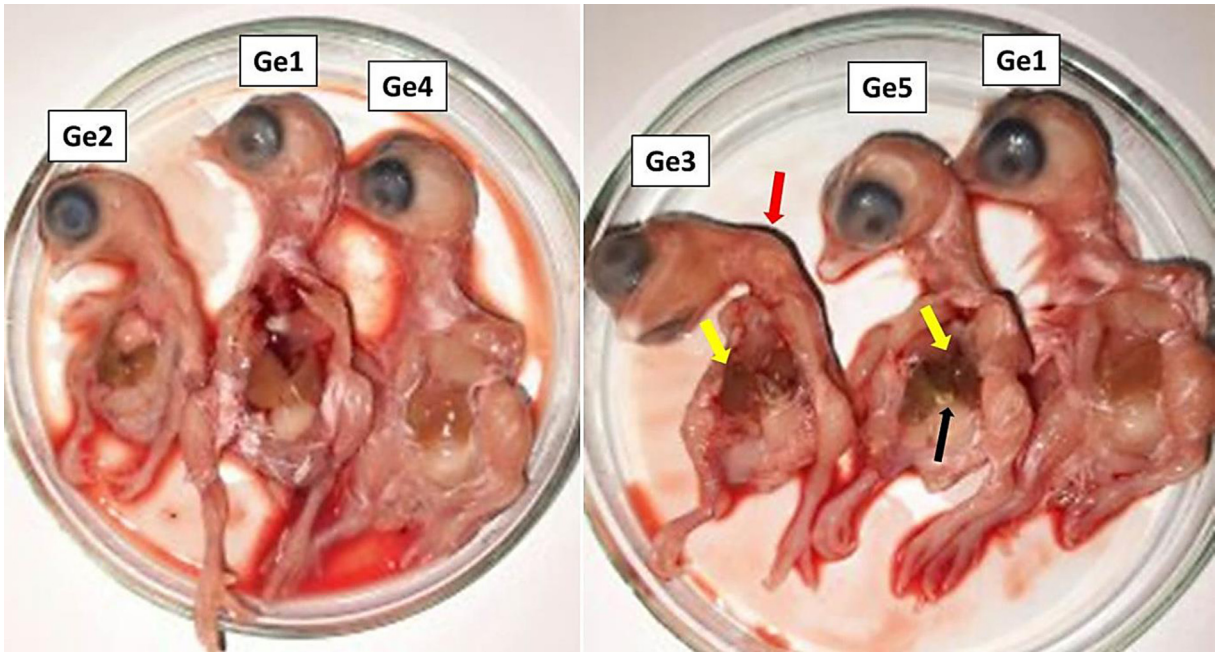

Fig. 1. Embryos of Ge1 (negative control) and Ge4 (antiviral group) were normal. Ge2 (positive control), embryos Ge3 (prophylactic) showing dwarfism, greenish liver, and deformities in neck (red arrow), Ge5 (treatment) showed edema in head and abdomen, dwarfism, with pale greenish liver (yellow arrow) and liver necrosis (black arrow).

3.2. Immune stimulating effect of 10 % aloe vera water extract in very virulent infectious bursal disease virus challenged chicks

3.2.1. Clinical signs and postmortem changes in very virulent infectious bursal disease virus experimentally challenged chicks

None of the experimental groups (Gc1−Gc5) did not showed any clinical signs or mortality until the time of challenge (21st day of age). Gc1 (negative control group) did not develop any clinical signs or post mortem changes throughout the experiment. Gc2 (positive control group) showed typical vvIBDV clinical signs including depression, trembling, anorexia, vent pecking, ruffled feathers, poor appetite, whitish diarrhea, and dehydration from the third day postchallenge. Gc3 (protected group) and Gc4 (protected group) developed mild signs of depression and ruffled feathers, respectively. Gc5 (treatment group) showed clinical signs of depression, ruffled feathers, anorexia, vent pecking, whitish diarrhea, and dehydration. No mortality was recorded for Gc1 during the experiment. Gc2, Gc3, Gc4, and Gc5 recorded mortality rates of 25 % (520/birds), 15 % (3/20 birds), 5 % (1/20 birds), and 15 % (3/20 birds), respectively.

3.2.2. Effect of 10 % aloe vera water extract on the body weight of very virulent infectious bursal disease virus challenged chicks

The effect of oral administration of 10 % AVWE on the body weight of chicks at different time points in the five test groups showed statistically significant differences in body weight between five groups. On days 28, 35, and 42 of age, there was a significantly lower body weight in Gc2 (positive control) than in all other groups, which indicates that the use of 10 % AVWE can improve the body weight of vvIBDV-infected chickens (Table 3 and Fig. 3).

Table 2. Statistical analysis real-time RT-PCR Ct values of five test groups.

Statistic	Ge1	Ge2	Ge3	Ge4	Ge5	H [4]	*P* value
Median	0	12.10	12.40	0	11.60		
Minimum	0	11.70	10.20	0	10.60	10.990	0.027
Maximum	0	12.90	12.55	0	12.90		
Pairwise comparisons	A	B	B	A	B		

Notes: The Kruskal−Wallis *H* test was used to test for significance. Pairwise comparisons are presented as letters; similar letters indicate statistically insignificant results, and different letters indicate statistically significant results. Ge1, negative control; Ge2, positive control; Ge3, prophylactic; Ge4, antiviral; and Ge5 = treated.

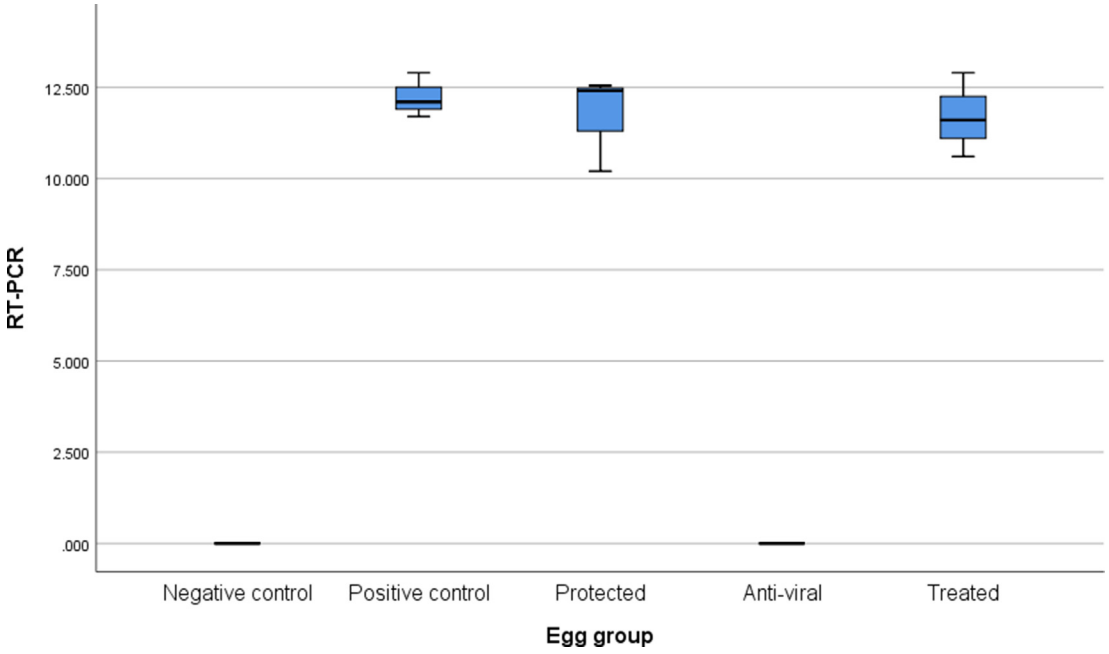

Fig. 2. Boxplot for RRT-PCR in the 5 test groups. RRT-PCR, real-time RT-PCR.

Table 3. Two-way analysis of variance study of the body weight over time in the five groups.

Time	Gc1	Gc2	Gc3	Gc4	Gc5	Total	*F*	*P*	Partial η^2
Day 28	792 ± 125.5	604 ± 72.96	802 ± 128.64	810 ± 131.22	817 ± 133.50	765 ± 132			
Day 35	1342 ± 36.01	1102 ± 24.29	1346 ± 36.23	1359 ± 36.94	1362 ± 37.10	1302 ± 108			
Day 42	1898 ± 72.04	1592 ± 50.69	1920 ± 73.73	1936 ± 74.96	1948 ± 75.89	1858.80 ± 151	3.354	<0.001	0.535
Total	697.42 ± 666	586 ± 547.42	707.29 ± 670.3	712.78 ± 676.4	717.29 ± 679.7	684.18 ± 639			

3.2.3. Effect of 10 % aloe vera water extract on the bursa and spleen weights of very virulent infectious bursal disease virus challenged chicks

The effect of oral administration of 10 % AVWE on the bursa and spleen weights of vvIBDV-challenged chicks at different time points was assessed. There was no statistically significant difference in the bursa and spleen weights among the five groups on days 28, 35, and 42 of age (Tables 4 and 5 and Fig. 3).

3.2.4. Effect of 10 % aloe vera water extract on the total leucocytic count of very virulent infectious bursal disease virus challenged chicks

The effects of 10 % AVWE on the TLC of vvIBDV-challenged chicks at different time points are shown in Table 6 and Fig. 4. At 28, 35, and 42 days of age, there was a statistically significant difference in Gc4>Gc3>Gc5>Gc2. From these results, we concluded that the use of AVWE for both protection

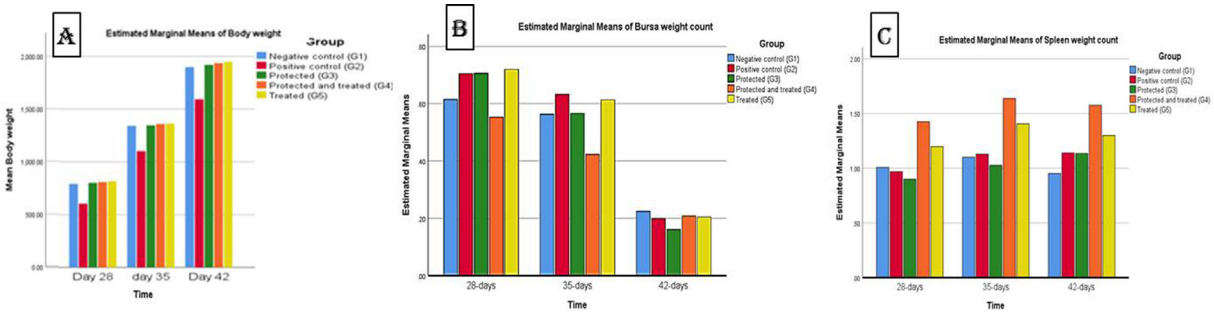

Fig. 3. Body weight, bursa, and spleen weight over time in the five groups. (A) In days 28, 35, and 42, there was a statistically significantly lower body weight in G2 (positive control) versus all other groups. (B) No statistically significant difference in bursal weight between five groups in days 28, 35, and 42. (C) No statistically significant difference in spleen weight between five groups in days 28, 35, and 42.

Table 4. Two-way analysis of variance study of the bursal weight over time in the five groups.

Time	Gc1	Gc2	Gc3	Gc4	Gc5	*F*	*P*	Partial η^2
Day 28	0.50 ± 0.14	0.40 ± 0.14	0.80 ± 0.14	0.90 ± 0.14	0.70 ± 0.14			
Day 35	0.50 ± 0.14	0.40 ± 0.14	0.70 ± 0.14	0.70 ± 0.14	0.50 ± 0.14	1.455	0.115	0.333
Day 42	0.20 ± 0.14	0.10 ± 0.14	0.20 ± 0.14	0.20 ± 0.14	0.10 ± 0.14			
Total	1.20 ± 0.42	0.9 ± 0.42	1.7 ± 0.42	1.8 ± 0.42	1.3 ± 0.42			

Table 5. Two-way analysis of variance study of the spleen weight over time in the five groups.

Time	Gc1	Gc2	Gc3	Gc4	Gc5	*F*	*P*	Partial η^2
Day 28	1.10 ± 0.60	1.60 ± 1.28	0.90 ± 0.405	1.0 ± 0.50	0.90 ± 0.41			
Day 35	1.20 ± 0.72	1.80 ± 1.62	1.1 ± 0.605	1.20 ± 0.72	1.0 ± 0.50	0.302	0.999	0.094
Day 42	1.00 ± 0.50	1.70 ± 1.45	1.2 ± 0.72	1.1 ± 0.605	1.1 ± 0.61			
Total	3.30 ± 1.82	4.92 ± 4.35	3.2 ± 1.73	3.3 ± 1.825	3 ± 1.52			

Table 6. Two-way analysis of variance study of the total leukocyte count over time between five groups.

Time	Gc1	Gc2	Gc3	Gc4	Gc5	Total	*F*	*P*	Partial η^2
Day 28	12.20 ± 0.03	13.73 ± 0.64	15.40 ± 0.05	16.03 ± 0.13	14.30 ± 1.7	14.33 ± 1.5			
Day 35	9.80 ± 0.04	10.30 ± 0.01	13.07 ± 0.16	15.07 ± 0.09	13.06 ± 0.09	12.26 ± 2.0	20.44	<0.001	0.875
Day 42	10.67 ± 0.57	12.10 ± 0.18	12.90 ± 0.05	13.07 ± 0.09	12.40 ± 0.02	12.22 ± 0.91			
Total	11.82 ± 1.64	12.54 ± 1.65	13.29 ± 1.78	13.79 ± 1.7	12.96 ± 1.64	12.88 ± 1.79			

Data expressed as mean ± SE.
P value is for time × group interaction.

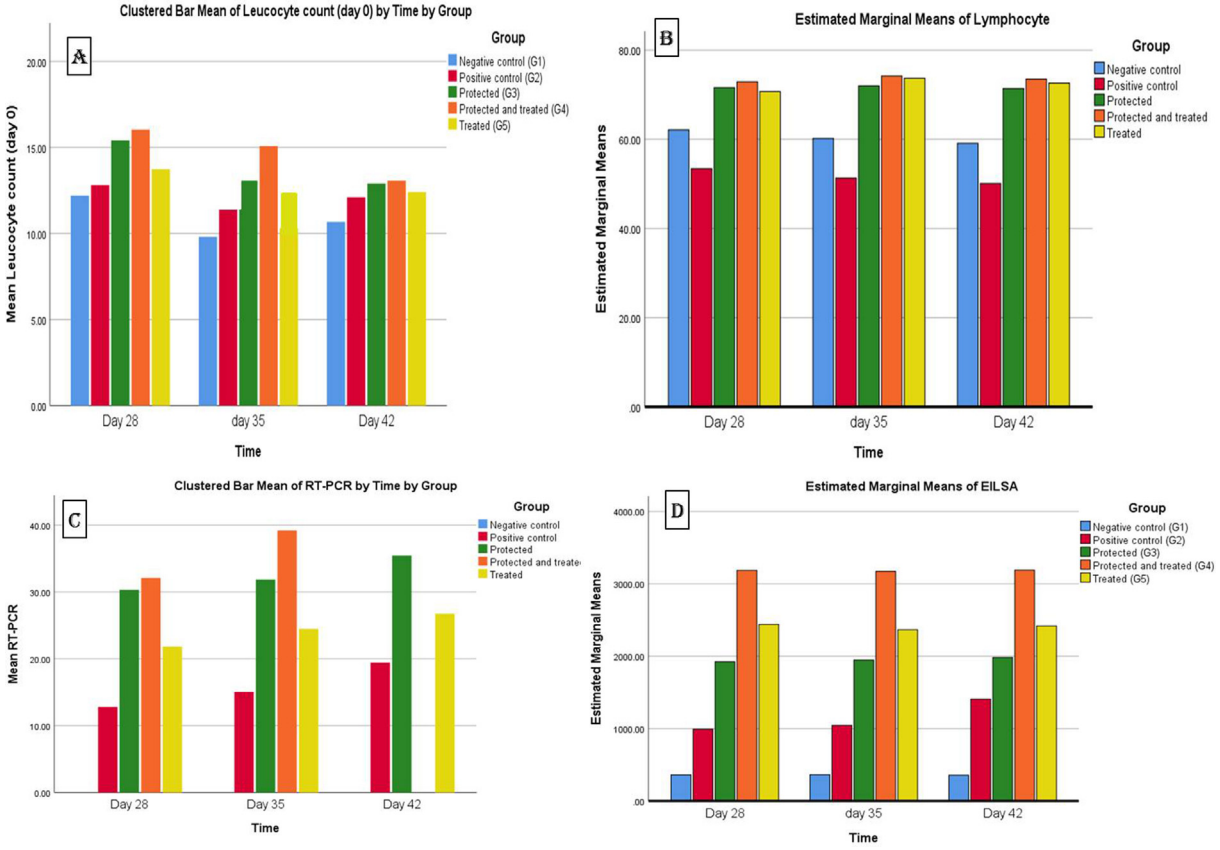

Fig. 4. TLC, lymphocytic count, RRT-PCR, and ELISA over time between five groups: (A) TLC in which there was a statistically significantly higher TLC in G4> G3>G5>G2 at the days 28, 35, and 42. (B) Lymphocytic count in which there was a statistically significantly higher lymphocytic count in G4>G5>G3 at the days 28, 35, and 42. (C) Real-time PCR in which there was a statistically significantly higher C_t in G4>G3>G5>G2 at the days 28 and 35 while the day 42, there was no statistically significant difference between G1 and G4. (D) ELISA antibody titer has a statistically significantly higher in G4>G5>G3>G2 at the days 28, 35, and 42. ELISA, enzyme-linked immunosorbent assay; RRT-PCR, real-time RT-PCR; TLC, total leukocyte count.

and treatment of vvIBDV showed promising results in improving the TLC against the virus.

3.2.5. Effect of 10 % aloe vera water extract on the lymphocytic count of very virulent infectious bursal disease virus challenged chicks

The effect of 10 % AVWE on the lymphocyte count of vvIBDV-challenged chicks at different time points is shown in Table 7 and Fig. 4. On days 28, 35, and 42, there was a significant difference in G4>G5>G3. From these results, we concluded that the use of AVWE for both protection and treatment of vvIBDV provided promising results in improving the cellular immune response against the virus.

3.2.6. Effect of 10 % aloe vera water extract on very virulent infectious bursal disease virus load in challenged chicks

The effect of 10 % AVWE on vvIBDV load in challenged chicks at different time points is shown in Table 8 and Fig. 4. In the RT-PCR results, a lower virus C_t indicates a higher virus titer. On days 28 and 35, there was a statistically significant increase in G4>G3>G5>G2. On day 42, there was no statistically difference between G1 and G4 indicating no virus load in challenged chickens in G4 at 42 days of age (21-day postchallenge).

3.2.7. Effect of 10 % aloe vera water extract on enzyme-linked immunosorbent assay antibody titer

The effect of oral administration of 10 % AVWE on the ELISA antibody titer of chicks at different time points is illustrated in Table 9 and Fig. 4. At three time points 28, 35, and 42, ELISA antibody titers were significantly higher in G4>G5>G3>G2. G4 showed the highest antibody titer against vvIBDV, which recommended using 10 % AVWE for both protection and treatment against vvIBDV, as it gives promising results of higher antibody titers.

3.2.8. Histopathological examination of very virulent infectious bursal disease virus challenged chicks

Bursa, spleen, thymus, and kidney tissue samples were collected on the fifth day post-vvIBDV challenge for pathological examination. These lesions are summarized in Figs. 5–8.

4. Discussion

Infectious bursal disease is a highly contiguous immunosuppressive disease affecting young chickens. The severity of the disease is attributed to its high mortality rate, which can reach up to 30 and 60 % in layers and broilers, respectively, leading to severe economic losses. The immunosuppressive action induced by IBDV makes chickens more susceptible to

Table 7. Two-way analysis of variance study of the lymphocytic count over time between five groups.

Time	Gc1	Gc2	Gc3	Gc4	Gc5	Total	*F*	*P*	Partial η^2
Day 28	62.20 ± 0.62	53.40 ± 0,53	71.60 ± 0.72	72.9 ± 0.73	70.70 ± 0.71	66.14 ± 7.7			
Day 35	60.20 ± 0.60	51.30 ± 0.51	72.00 ± 0.72	74.20 ± 0.74	73.70 ± 0.73	66.28 ± 9.4	119.75	<0.001	0.976
Day 42	59.10 ± 0.59	50.10 ± 0.50	71.40 ± 0.71	73.50 ± 0.74	72.60 ± 0.73	65.34 ± 9.6			
Total	61.35 ± 1.7	57.11 ± 5.7	69.64 ± 3.5	70.68 ± 3.5	69.52 ± 2.8	65.67 ± 6.7			

Data expressed as mean ± SE.
P value is for time × group interaction.

Table 8. Two-way analysis of variance study real-time RT-PCR over time between five groups.

Group	G1	G2	G3	G4	G5	Total	*F*	*P*	Partial η^2
Day 28	0 ± 0	12.8 ± 0.36	30.30 ± 2.51	32.09 ± 1.68	21.81 ± 0.21	19.34 ± 12.35			
Day 35	0 ± 0	15.05 ± 0.17	31.82 ± 1.25	39.18 ± 0.33	24.46 ± 0.456	22.11 ± 14.13	286.532	<0.001	0.987
Day 42	0 ± 0	19.42 ± 1.62	35.42 ± 0.84	0 ± 0	26.74 ± 1.38	16.32 ± 14.78			
Total	0 ± 0	15.76 ± 3.03	19.40 ± 12.35	23.76 ± 18.10	24.34 ± 2.26	19.27 ± 13.68			

Data expressed as mean ± SE.
P value is for time × group interaction.

Table 9. Two-way analysis of variance study of enzyme-linked immunosorbent assay antibody titer over time between five groups.

Time	Gc1	Gc2	Gc3	Gc4	Gc5	Total	*F*	*P*	Partial η^2
Day 28	360 ± 2.65	992.33 ± 11.23	1923.33 ± 55.08	3186.7 ± 32.2	2440 ± 52.9	1780.7 ± 1042.7			
Day 35	365 ± 5.57	1044.7 ± 22.47	1946.7 ± 64.29	3171.7 ± 20.21	2366.7 ± 40.4	1778.9 ± 1020.09	23.061	<0.001	0.860
Day 42	357.33 ± 6.42	1408 ± 36.17	1983.33 ± 55.08	3189 ± 6.56	2418.67 ± 35.85	3182.44 ± 20.92			
Total	361.11 ± 5.6	1148.4 ± 197.5	1951.11 ± 56.9	1871.33 ± 988.4	2408.44 ± 50	1810.31 ± 994.9			

Data expressed as mean ± SE.
P value is for time × group interaction.

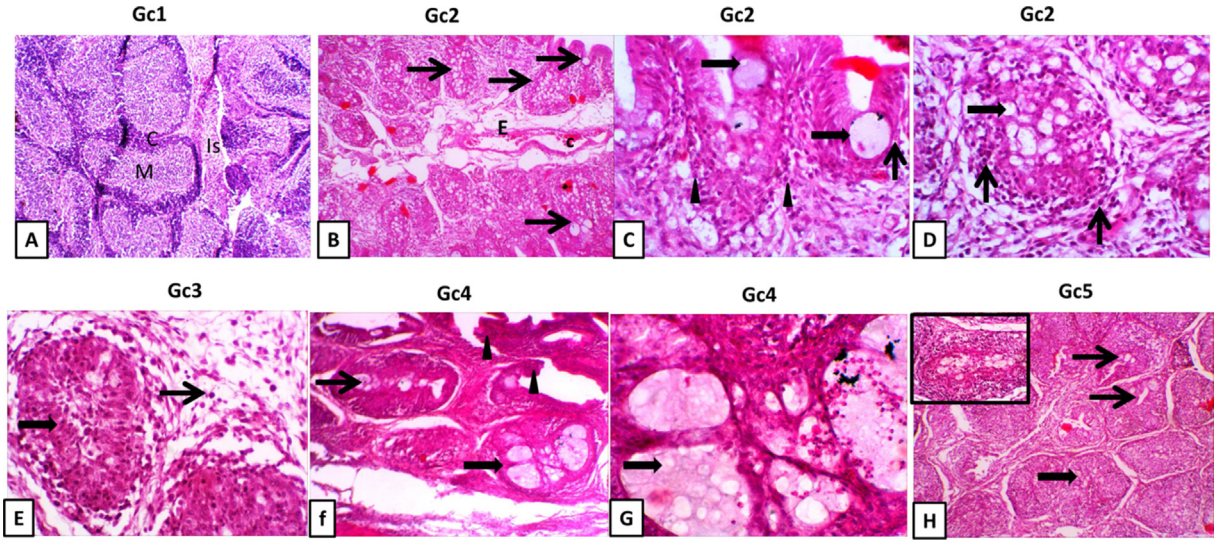

Fig. 5. Representative photomicrograph of bursa of Fabricius: (A) Gc1 bursa had normal arrangement of the follicular cortex (C) and medulla (M) with interfollicular septa (Is) in between, H&E, × 400. (B) Gc2 exposed group showing diffuse cystic vacuolation in epithelial lining and bursal follicles (thin arrows) with lymphoid depletion and marked interfollicular edema (E) and congestion (c), H&E, × 100. (C) High power of Gc2 epithelial lining of bursa showing large vacuolation containing faint eosinophilic fluid (thick arrows) and necrotic epithelial cells (thin arrow) with subepithelial leukocytic infiltrations (arrowheads), H&E, × 400. (D) High power on bursal follicles showing marked lymphoid depletion replaced by cystic cavities in follicular medulla with heterophilic infiltrations in cortex (thin arrows), H&E, × 400. (E) Gc3 bursa showing hypercellularity of follicular bursa (thick arrow) with few interfollicular inflammatory infiltrates (thin arrow), H&E, × 400. (F,G) Gc4 bursa showing papillary proliferation of bursal epithelium (arrowheads) with few, large cystic cavities filled with eosinophilic proteinaceous materials and infiltrated with eosinophils (thick arrows) (high power in F), some follicles showing epithelized cyst (thin arrow), H&E, × 100 (g) and × 400. (H) Gc5 bursa showing mild cystic cavities in follicular medulla (thick arrow), others showing epithelized cysts (thin arrows), H&E, × 100. Inset, enlarged follicles with tightly packed cortical lymphocytes with numerous activated reticulocytes around a limited medullary cavity, H&E, × 400. Gc1 = control negative, Gc2 = control positive, Gc3 = protected group, Gc4 = protected and treated group, Gc5 = treated group. H&E, hematoxylin and eosin.

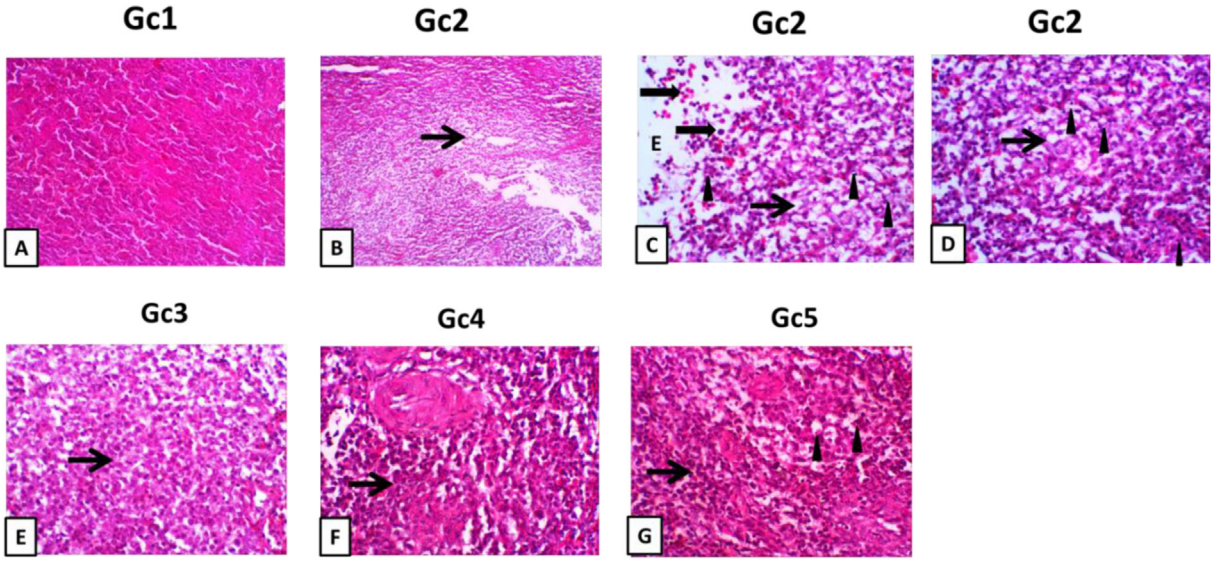

Fig. 6. Representative photomicrograph of spleen showing (A) Gc1 spleen with normal histological architecture of red and white pulp, H&E, × 100. (B) Spleen of Gc1 positive exposed section showing massive lymphoid depletion (thin arrow), H&E, × 100. (C) Gc2 spleen showing diffuse lymphoid depletion with necrotic cells (arrowheads) and edema (E), hemorrhage admixed with numerous inflammatory cells (thick arrows), H&E, × 400. (D) Gc2 spleen showing necrosis of lymphocytes (arrowheads) and lymphoid depletion (thin arrow), H&E, × 400. (E) Gc3 spleen showing splenic hypercelluarity (thin arrow), H&E, × 400. (F) Gc4 spleen showing marked lymphocytic hyperplasia (thin arrow), H&E, × 400. (G) Gc5 spleen showing few lymphocyte necrosis (arrowheads) with moderate lymphoid proliferations (thin arrow), H&E, × 400.

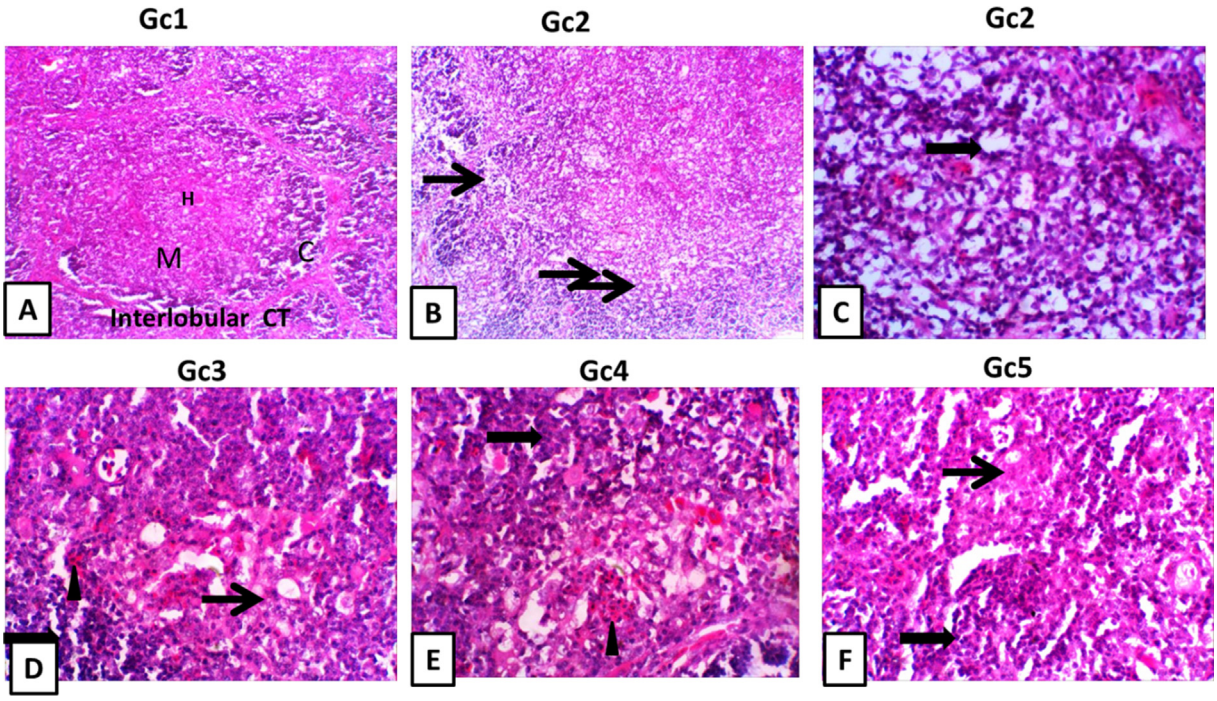

Fig. 7. Representative photomicrograph of thymus showing (A) Gc1 thymus with normal arrangement of connective tissue septa (IC) around a darkly basophilic cortex (c) and lightly stained medulla (M) with Hassel corpuscles (H), H&E, × 100. (B) Gc2 thymus group showing marked cortical and medullary lymphoid depletion (thin arrows), H&E, × 100. (C) High power of Gc2 thymus showing medullary lymphoid depletion (thick arrow) and necrotic cell (thin arrow), H&E, × 400. (D) Gc3 thymus exposed group showing cystic epithelial (thin arrow) with few necrotic cells, hemorrhage (arrowhead). (E) Gc4 thymus had few hemorrhage (arrowhead) and cortical lymphoid proliferations (thick arrow), H&E, × 400. (F) Gc5 group showed focal area of medullary necrosis (thin arrow) with cortical lymphoid proliferation (thick arrow), H&E, × 400.

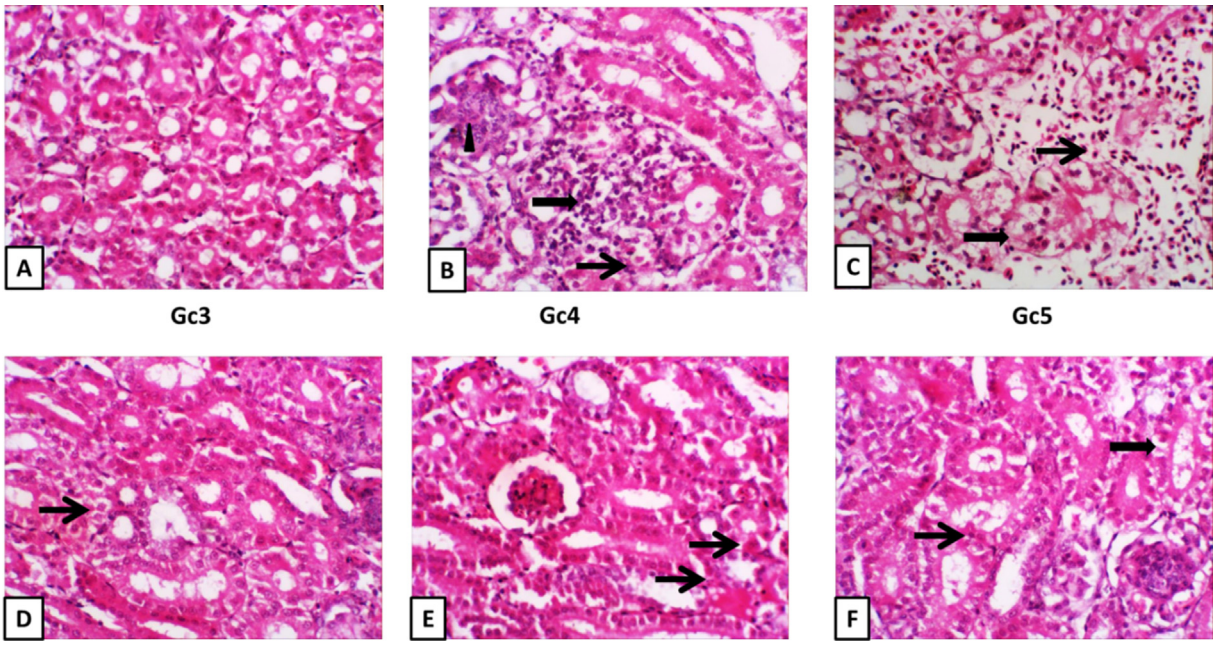

Fig. 8. Representative photomicrograph of kidney showing (A) Gc1 kidney with normal renal tubular arrangement, H&E, × 400. (B) Gc2 kidney had diffuse tubular necrosis (thin arrow) with marked leucocytic infiltrations (thick arrow) and atrophied glomerulus (arrowhead), H&E, × 400. (C) Gc2 kidney showing diffuse hemorrhage (thin arrow), widely separated massively damaged renal tubules (thick arrow), H&E, × 400. (D) Gc3 kidney showing focal area of tubular epithelial cell individualization (thin arrow), H&E, × 400. (E) Gc4 kidney had few tubular cells necrosis (thin arrows), H&E, × 400. (F) Gc5 kidney showing normal tubular arrangement with individual tubular epithelial cell degeneration (thick arrow) and necrosis (thin arrow), H&E, × 400.

secondary bacterial infection and leads to vaccination failure; therefore, researchers have attempted to find new drugs using medicinal plants [20,21].

Aloe vera is a popular medicinal plant mostly grown in India, China, and Egypt. Aloe vera has received increased attention, as many studies have shown its antibacterial, antiviral, and immunomodulatory effects [9].

The basal level of immune response can be increased using immunostimulating agents that enhance body resistance against various infections [22].

This study was performed to evaluate the antiviral and immunostimulatory effects of aloe vera against IBDV.

By testing the antiviral effect of 10 % AVWE on ECEs, typical IBDV lesions were detected in the positive control, prophylactic, and treatment groups with high virus load detection by RT-PCR. On the other hand, the negative control and antiviral group embryos showed no IBDV detection in RRT-PCR (negative results). From these results, we conclude that 10 % AVWE has a potent antiviral effect with no prophylactic or treatment effect against vvIBDV. These results match those of Shokraneh *et al.* [11], and Mawgod *et al.* [23], who postulated that aloe vera has antiviral effects through the presence of antiviral components.

Our findings on the mortality rate in the five tested groups revealed that the protected-treated group (Gc4) had the lowest mortality rate, followed by the protected (Gc3) and treated (Gc5) groups. In contrast, the highest mortality rate was recorded in the positive control (Gc2). From these findings, we concluded that prolonged use of the AVWE (before and after virus infection) cannot prevent viral infection but can improve the bird's resistance to viral infection and lower the mortality rate in the infected birds. These findings are in accordance with those of Sedeik *et al.* [24].

Our results showed a significantly higher body weight in the Gc4>Gc3>Gc5 group than in the positive control group, which showed a significantly lower body weight. These results were in accordance with Shokraneh *et al.* [11], Zayed *et al.* [25], who postulated that the addition of 1 % aloe vera in drinking water, as an additive to broiler chicken feed, has great potential for improving growth performance, intestinal health, and immune system response and can be used as an alternative to antibiotic growth promoters.

Spleen and bursa weights showed no statistically significant differences between the five groups ($P > 0.5$) on the 28th, 35th, and 42nd day of age, and these results were in agreement with those of Sharma *et al.* [26], who reported that aloe vera supplementation had no effect on the weight of lymphoid organs. However, Sunu *et al.* [27] reported that 2 % of aloe vera in feed increased the percentage of bursa of fabricius better than the control nontreated group.

The beneficial effects of herbal medicines are usually estimated using the TLC and lymphocyte count. White blood cells are the best guide for disease.

In this study, we found that administration of 10 % AVWE before and after vvIBDV challenge significantly improved TLC and lymphocytic counts, which significantly increased over time. TLC was highest in the protected-treated group (Gc4) followed by the protected group (Gc3), followed by the treated group (Gc5), while the positive control group (Gc2) showed a significant decrease in TLC and lymphocytic count, which is in agreement with Shokraneh *et al.* [11], Zayed *et al.* [25], Dziewulska *et al.* [28], who concluded that aleo vera has potent antiviral effects that are attributed to the stimulation of the immune system. Zayed *et al.* [25] also reported an increase in lymphocyte cell number in broilers supplemented with aloe vera. The improvement of TLC in Gc4, Gc3, and Gc5 compared to Gc2 was in accordance with Yadav *et al.* [29] and Singh *et al.* [30], who reported that the TLC increased in birds supplemented with 0.2 % aloe vera juice in drinking water. The findings of our study showed significant lymphopenia in the positive control group (Gc2), which was in accordance with Oladele *et al.* [31], who concluded that IBDV causes destruction of B lymphocytes in the bursa of Fabricius before their migration to the bloodstream, leading to a decrease in the number of lymphocytes; therefore, viral infections are usually associated with lymphopenia.

In RRT-PCR, at days 28 and 35, there was a lower viral load in G4>G3>G5>G2. No virus was detected in Gc4 chicks at the 42nd day of age, which revealed that the use of AVWE before and after vvIBDV challenge led to more rapid virus elimination than in the other groups.

Our present study showed that administration of 10 % AVWE increased the antibody titer against vvIBDV, and the highest antibody titer was found in the protected-treated group (Gc4); these findings are in agreement with those of Anunciado *et al.* [32] who confirmed that antibody production against IBDV was improved by administration of aloe vera to broiler chickens and indicated that this increase is attributed to aloe vera content such as acemannan.

Our results showed the beneficial effects of using 10 % AVWE on the health of tissues such as the bursa, spleen, thumus, and kidney in the protected-treated group versus all other groups, which were detected by histopathological examination; these

results were in agreement with Zayed *et al.* [25] and Sodani [33], who postulated the beneficial effects of aloe vera on tissue repair.

Regarding the histological examination, in our study, the bursa of the control positive group without aloe vera supplementation revealed lymphoid depletion and marked interfollicular edema and congestion, while the Gc3 and treated group Gc5 showed moderate lesions. The protected-treated group revealed papillary proliferation of the bursal epithelium with a few large cystic cavities filled with eosinophilic proteinaceous materials and infiltrated with eosinophils. The typical histological bursal lesions were similarly reported by Singh *et al.* [34] and Murmu *et al.* [35], which included moderate to severe lymphoid depletion in bursal follicles, cyst formation, follicular necrosis, and hemorrhage formation in the follicles of IBDV-infected chickens.

Our results in the spleen of the positive control group without aloe vera supplementation showed diffuse lymphoid depletion with necrotic cells and edema, hemorrhage, and mixed with numerous inflammatory cells, necrosis of lymphocytes, and lymphoid depletion with moderate lesions in the Gc3 and treated group Gc5. The protected-treated group exhibited marked lymphocytic hyperplasia in the spleen. Our results were in accordance with those of Sedeik *et al.* [24] and Cheville [36], who reported that the addition of medicinal plants was beneficial for repairing bursa and spleen tissues after infection.

In this study, the thymus of the positive control group without aloe vera supplementation showed marked cortical and medullary lymphoid depletion, while the protected-treated group revealed few hemorrhages and cortical lymphoid proliferations of the thymus, consistent with the results of Al Zubeedy [37] and Al-Jubori [38], who mentioned that destroying both B lymphocytes of bursa of Fabricius and T lymphocytes of thymus of IBDV-infected chickens. In contrast, the kidneys of the positive control group without aloe vera supplementation showed diffuse tubular necrosis with marked leukocytic infiltration, atrophied glomeruli, diffuse hemorrhage, and widely separated massively damaged renal tubules. While the protected-treated group revealed kidney had few tubular cells necrosis, kidney showing normal tubular arrangement with individual tubular epithelial cell degeneration matches with Zayed *et al.* [25], Sodani [33], Cheville [36], Ahmad *et al.* [39].

5. Conclusion

Aloe vera has potent antiviral effects on ECEs. We recommend the administration of 10 % AVWE in the drinking water of chickens before and after viral infection, as it can increase body weight gain and improve the immune response against vvIBDV with more rapid virus elimination from the bird's body.

Declarations

Ethics approval

The study followed the guidelines of the Institutional Animal Care and Use Committee, Mansoura University, Egypt.

Data access statement

All data are provided within this study.

Conflicts of interest

There are no conflicts of interest.

Funding statement

This study receives no fund.

Authors' contributions

This study was conducted with the cooperation of all authors.

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