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THE EFFECT OF *Neem* LEAVES EXTRACT AS IMMUNSTIMULANT BEFORE CHICKEN INFECTIOUS ANEMIA VACCINATION IN BROILERS

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Abstract: Chicken infectious anemia (CIA) has lately arisen as a major problem in poultry production due to poor growth, high mortalities. We successfully used PCR on seven flocks of chickens - (three breeder and four broiler) in the Egyptian governorates of Dakahlia and Damietta to detect CIAV in tissues of probable CIAV-infected birds. Numerous substances, including triterpenoids and glycosides, are found in *Neem* and are assumed to be the source of its antiviral effects. All birds were randomly allocated into eight groups (n=20 each) for different treatments, including vaccination, feeding *Neem* 8%, feeding infected birds 8%, vaccination and infected birds, feeding infected birds 8%, and finally feeding infected birds 8% and vaccination. One-hundred and sixty birds were randomly divided into eight groups (n=20 each). The groups were differentially-treated as the followings; healthy birds, birds infected with CIAV, birds fed *Neem* 8%, infected birds fed *Neem* 8%, vaccinated birds, vaccinated and infected birds, birds fed *Neem* 8% and vaccinated, and finally the infected ones fed *Neem* 8% and vaccinated. The study is aimed to detect the immunostimulant effect of *Neem* leaves extract 8% on immune response post vaccination against CIAV in broilers. The group taken immunstimulant (*Neem* leaves extract 8%) along with vaccination increased immune response of birds since titers of the enzyme-linked immunosorbent assay were 15200 ($P \leq 0.05$) than the group valued 14732 of vaccination only. However, in groups (*Neem* vaccinated infected, infected and vaccinated infected) were 14663, 12600 and 12091 ($P<0.05$, $P<0.05$ and $P<0.01$, respectively). Infected group exhibited indications of CIAV as despondency, while group vaccinated and treated with *Neem* leaves extract 8% appeared normal after challenging with CIAV. The hematocrit values of infected group and vaccinated treated infected group were 21 and 30, respectively. Histopathological changes in *Neem* vaccinated group after challenging with CIAV showed increasing in the thickness of both cortex and medulla of thymic lobules beside over population. In the latest group, bone marrow showed activation and proliferated hemopoitic elements with regenerative centers. We conclude that the combination of *Neem* leaves extracts 8% and CIAV vaccination is a potent antiviral and has immunstimulant properties during the production cycle of broilers.

Key words: CIAV; PCR; histopathology; enzyme-linked immunosorbent assay; broilers

Introduction

One of the most prevalent viral diseases in the poultry industry is the chicken infectious anemia (CIA), resulting in significant mortality, lower productivity, and expensive preventive medicine costs. Chicken infectious anemia virus (CIAV) infection can show up as clinical or subclinical symptoms of birds such as anemia, thymus degeneration, bone marrow depletion, and immunosuppression characterize the disease's outbreak (1,2). The immunosuppressive effect, whether direct or indirect, induces significant lymphocyte depletion in main and inferior lymphoid organs (3). Moreover, erythroblastoid cells of the bone

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marrow and immune cells in the thymus cortex of one-day old chick are destroyed (4). CIAV detection via molecular technology, like as polymerase chain reaction (PCR) is a rapid, sensitive and simple way for detecting viral nucleic acid (5). blood and tissue samples were used from diseased broiler breeder chicks and detected CIAV-specific 418-base-pair by PCR (6). However, there are limited investigations on the CIAVs that are prevalent in Egypt (7, 8). the efficacy and safety of live CIAV vaccines was assessed and it was found that live vaccines give the birds measurable titers to CIAV by the first week reaching the peak titers by the eighth week (9). Non-vaccinated hens took the virus shed by the vaccinated ones and had lower antibody titers. Medicinal plants (Azadirachta indica) are used by humans to fight ailments. It known as "*Neem*", has gotten a lot of attention from the medical community throughout the world because of its vast range of medicinal characteristics (10). *Neem* contains numerous components, including triterpenoids and glycosides, which are thought to be responsible for the herb's antiviral properties (11). The current study is aimed to determine the prevalence of CIAV infection in some flocks in two Egyptian governorates (Dakahlia and Damietta) by using PCR in broiler and breeder chicken flocks, and then to examine the hematological, pathological, and value of live CIAV vaccine in developing immunity against the illness with or without Neem leaves extract 8% treatment in experimental chicks against CIAV infection with estimation of immune response.

Material and methods

PCR for detection of CIAV

DNA was taken out by QIA amp DNA mini kit according to producer's directions. Amplification of fractional VP1 gene was done according to the previous protocol (13). The definite intensified PCR product was illustrious using agarose gel electrophoresis according to previ-ous methodology (14).

Titration (Dose)

The virus was intramuscularly injected at day 21 by 0.1ml contained 102CID50 (18). The 50 SPF chicks.

To determine the Chicken Infectious Dose (CID), two groups of specified pathogens free (SPF) chicks received injections at one day old. Tenfold sequential dilution (10-1-10-5) with five chicks each dilution was carried out using the Reed and Meunch technique (17).

CID 50: It means half of the inoculated an-imals shows the positive reaction. Reed and Muench method computing a 50% end point of a virus titration. Calculate the proportionate distance between dilutions which infect above and below 50% of the chicks.

Equation

$$PD: \frac{\text{No of mortality above 50\% - 50\%}}{\text{(No of mortality above 50\% - No of mortality belowe 50\%)}}$$

Table 1: The grouping of birds in the study.

Groups no.	Birds no./group	Treatment of *Neem* 8%	Period of *Neem* treated (days)	Vaccination against CIAV at day 5	CIAV challenge day
1	20	-	-	-	-
2	20	-	-	-	21
3	20	+	1-30	-	-
4	20	+	1-30	-	21
5	20	-	-	+	-
6	20	-	-	+	21
7	20	+	1-30	+	-
8	20	+	1-30	+	21

Vaccine used

CIAV vaccine Nobilis® CAVP4 is a live attenuated vaccine against CIAV strain with active components per dose 26P4 ≥ 3.0log10TCID50 with dose 0.2 mL per bird (Patch no. A043AJ01) at the 5th days of age.

Bird management

The birds were housed under standard environmental and hygienic conditions for 30 days and fed organic feed (2 weeks' starter feed 21% protein and 2950 k.cal. /kg and complete the experiment with grower feed 19% and 2800 k.cal. / kg feed), with continuous lighting throughout the experiment and water ad-libitum. The eight groups were differentially treated as showed in Table 1.

The birds reared for thirty days in rigorous biosafety procedures and standard conditions (level 3). Moreover, CIAV strains were obtained from tissue homogenate from flock no.1 at age of 6 weeks (Ross breeder species), which more pronounced postmortem lesions. The homogenized tissues with PBS have been treated with antibiotics. After repeating the freezing and thawing process three times, the sample was centrifuged at 3000 rpm for 20 minutes. The supernatant was PCR-tested for Avian Influenza (H5 or H9), Infectious Bursal Disease, and Newcastle Disease to confirm that the sample were devoid of these viruses. The breeder hens and the flock we choose the chicks from were free from Adeno virus. The virus was taken from the samples of the infected flocks that we performed a PCR (the first flock in the field infected flocks). The dose of the virus measured according to Reed and Meunch technique.

Tissue specimens

The samples were taken from seven chicken flocks (4 commercial broilers, aged 2 ~ 4 weeks old) and 3 breeder chicken, aged 6 ~ 8 weeks old) located in two Egyptian governorates (Dakahlia and Damietta) during 2021 (shown in Table 2) - All the birds were not got vaccine against CIAV. Moreover, the tissue samples taken from freshly dead birds (postmortem) suffered from suspected CIAV infection with anemia, stunting, depression, pale comb & wattles, poor growth, weakness, and droopy appearance in chicken (Figure 1). Various tissues were used for sampling such as (thymus, bone marrow, bursa of Fabricious, and spleen), following the previous protocol (12).

Figure 1: Clinical examination of naturally infected bird with CIAV

Table 2: Data of the birds suspected to be infected with CIAV within Dakahlia and Damietta governorates in 2021

Flock no.	Locality	Breed	Age (week)	Total no.	Mortality % (daily)
1	Dakahlia	Ross Breeder	6	5500	0.3
2	Dakahlia	Ross Breeder	8	4500	0.3
3	Damietta	Avian 48 Breeder	7	7500	0.4
4	Dakahlia	IR Broiler	4	10000	0.35
5	Damietta	Cobb Broiler	3	2500	0.4
6	Dakahlia	Cobb Broiler	4	4000	0.3
7	Damietta	Cobb Broiler	3	4500	0.3

Herbal antiviral and immunstimulant (Neem leaves extract 8%)

Aqueous *Neem* leaves extract was prepared according to the protocol of (15). Eighty mL from this aqueous solution was then collected and mixed with one liter of drinking water, also 8% *Neem* leaves extract supplemented as ad-libidum (16).

Hematocrit values

Blood samples were collected weekly from day-1 old till day-28 from jugular vein of chicks on 3.5% sodium citrate solution. PCVs were determined (19).

ELISA test

Serum samples from non-heparinized blood samples were obtained individually at 7, 14, 21, and 28 days. Commercial CIAV antibody ELISA kit of IDEXX (ref. no: 99-08702 – LOT: FU728) was used and performed based on the manufacturer instructions.

Histopathological examination

For microscopic inspection, small sections of the thymus, bursa of Fabricious, bone marrow, and spleen were fixed in buffered neutral formalin solution at a 10% concentration (20).

Statistical analysis

Data from ELIZA titers were utilized to analyze the variance using SPSS program through One way-ANOVA.

Results

Clinical symptoms and postmortem lesions of infected flocks

The examined infected flocks were suffered from anemia, stunting, depression, pale comb & wattles, poor growth, weakness, and droopy appearance with high daily mortality rates ranging from 0.3-0.4%. Although necropsy results revealed paleness in the bone marrow, hemorrhage under the skin and bursa of Fabricious and thymus atrophy can range from mild to severe cases. Additionally, many cases of subcutaneous and muscle bleeding were reported in Table 1.

Using PCR for detection of CIAV-DNA in tissues

Liver, spleen, thymus and bone marrow of seven commercial broiler and breeder chicken flocks were examined. The primer set was designed to amplify a 418 bp fragment of the vp1 gene (2,4,8) - At the proper size band of 418 bp, five flocks tested positive with an incidence rate of 71.42%, as shown in Table 3. The findings revealed that all flocks of various ages except for flocks 5 and 6 were positive for CIAV.

Clinical and postmortem findings of the experimental birds

The CIAV-infected group displayed anorexia, despondency, general weakness, a droopy appearance, pale comb and wattles, stunting, and growth retardation (Group. 2). Thymus glands that have atrophied, subcutaneous bleeding, fatty yellow bone marrow, regressed bursa of Fabricious, liver and spleen showing and enlargement and paleness, and slowing of keel bone ossification were among the pathological findings. The groups given *Neem* leaves extract (8%) had normal immune organs, while the control group did not. The parameters used in the experiment were exclusive for histopathological changes, hematocrite values and ELIZA because the aim of the experiment is to assess the immunstimulant effect and the mortality rates. The occurrence of other factors rather than infection (like blood sampling or faulty injection for example) may give a faulty figure to the *Neem* effect. Also, the clinical finding was not a main parameter in assessing the immunstimulant effect. The chicks in all groups appeared normal may be due to strictly hygienic conditions unlike the field situation in low biosecurity farms.

Hematocrit values

At 28 days of age, the CIAV-infected group (Group. 2) had symptoms of normocytic normochromic anaemia which represented a significant decrease in PCV to less than 22% (21.1%). When compared to the normal group (27.5%), the PCV

Table 3: Frequency of CIAV detection by PCR among examined flocks in Dakahlia and Damietta governorates

Flock No.	Locality	Breed	Age (week)	No of examined birds	Vaccination of CAV	PCR results
1	Dakahlia	Ross Breeder	6	5	-	+
2	Dakahlia	Ross Breeder	8	5	-	+
3	Damietta	Avian 48 Breeder	7	5	-	+
4	Dakahlia	IR Broiler	4	5	-	+
5	Damietta	Cobb Broiler	3	5	-	-
6	Dakahlia	Cobb Broiler	4	5	-	-
7	Damietta	Cobb Broiler	3	5	-	+
Total	**35** samples				**25** (+ve) samples, **(71.4%)**	

values of the CIAV-infected *Neem*-treated group (Group 3) and the infected vaccine group (Group 4) decreased by 50% and 75%, respectively. In infected treated and vaccinated group Interestingly, group 5 showed increase in PCV values (30%) compared to vaccinated infected and/or treated infected groups as shown in (Figure 2).

ELISA results

The immune response increased in *Neem* vaccinated and vaccinated groups as the ELISA titers were 15200 and 14732, respectively while ELISA titers in groups (*Neem* vaccinated infected, infected and vaccinated infected) were 14663,12600 and 12091. The negative control group had maternal antibodies as it is broiler chicks not SPF as shown in Table 4.

On day 21, the results showed that supplementation of *Neem* 8% improved significantly the immune status of birds infected with CIAV, vaccinated, and vaccinated then infected with the CIAV infection (P=0.02, P=0.011, and P=0.034, respectively) compared with those only vaccinated birds followed by the viral infection. Further, on day 28, Moreover, *Neem* 8% plus vaccination significantly (P=0.048, P=0.036, and P=0.025) the birds' immunity compared to infected, infected and supplemented with *Neem* 8%, and vaccinated ones, respectively. Supplementation of *Neem* 8% plus vaccination optimized the immunity compared with vaccinated birds only. It was clear that the birds' immunity was shaped on day 28, and therefore the birds adapted to the viral load through the compensatory mechanism and targeted the homeostatic immune balance - It comes from the fact that our results showed that supplementation of Neem 8% plus infection improved the negative feedback mechanism of birds to slightly represented as the control birds Immune response was significantly increased in groups treated with 8% neem.

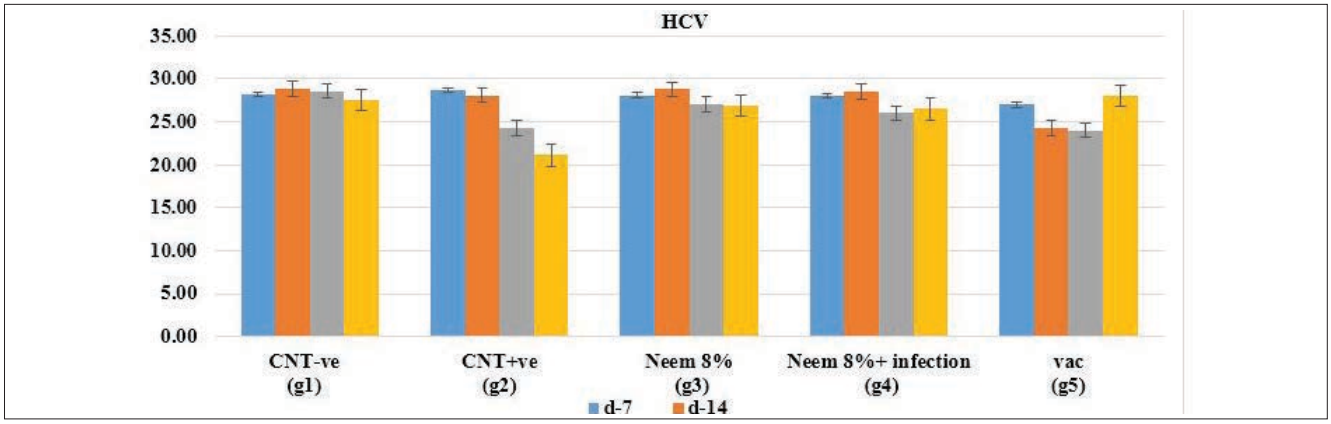

Figure 2: Hematocrit value at 7, 14, 21, and 28 days of age showing increased PCV values in "infected treated and vaccinated group" compared to vaccinated infected and treated infected groups.

Table 4: The ELISA results

Group no.	Group name	Total no.	Serum samples no.	Time of samples collected							
				7 days		14 day		21 day		28 days	
				GMT	CV (%)	GMT	CV (%)	GMT	CV (%)	GMT	CV (%)
1	CNT-ve	20	5	15250	2.2	14952	3.3	14752	2.5	14644	7.3
2	CNT+ve	20	5	15300	3.5	12525	7.7	12600	5.7	15489 c	2.5
3	*Neem* 8%	20	5	14995	3	13884	3.5	15200	3.6	15500	1.5
4	*Neem* 8%+infec.	20	5	12694	23	13584	7.2	14472a	2.1	15601 c	1
5	vac.	20	5	14824	1.4	13554	12.5	14732b	13	15695 c	1.7
6	vac+infec.	20	5	13226	8.9	14356	3.3	12091	11	15.35	7
7	*Neem* 8%+vac.	20	5	13781	8.4	13830	2.4	14197a	2.3	15183	6.8
8	*Neem* 8%+vac+ infec.	20	5	13028	15	13418	12	14663a	7.3	13606	17

Vac; vaccinated, inf; infection, GMT: Geometric mean titer, CV%: Coefficient variance.
Statistical analysis represented by SPSS-One ANOVA.
[a]$P \leq 0.05$ compared with vac+infec group.
[b]$P \leq 0.01$ compared with vac+infec group.
[c]$P \leq 0.05$ compared with *Neem* 8% + vac group.

Histopathological changes

Histopathological pictures of lymphoid organs of negative control (Group. 1) and treated group (Group. 3) showed a typical normality of histological pictures, thymic lobules' cortex & medulla and lymphoid follicles of the bursa. Splenic elements, capsule & hemopoitic centers and matrix of the bone marrow are within the histo-morphological picture (Figure 3: A-D). Infected group (Group. 2) (Figure 3: E-H) showed hemorrhages and edema in medulla of thymic lobules (arrow) beside intense lymphoid depletion from cortex. The bursal lymphoid follicles showed atrophy besides intra and inter follicular heterophilic infiltration. Moreover, the spleen picture showed severe lymphoid depletion while treated challenged group (Group. 4) showed activated hemopoitic centers with reduction of stromal cells in thymus with bursal regenerative attempts in some bursal lymphoid follicles. Besides, the spleen showed hyperplastic white pulps, and the bone marrow showed activated hemopoitic centers with regenerative attempts and numerous stem cells (Fig. 3I, Figure 3L). In (Group. 8); treated vaccinated group and challenged revealed an increase in the thickness of both cortex and medulla of thymic lobules and hyperplasia with proliferated hemopoitic elements. In addition, bone marrow showed numerous regenerative hemopoitic centers within fatty marrow (Figure 3Q, T) compared with vaccinated and challenged group (Group. 6) which showed moderate atrophy with moderate necrotic changes in all lymphoid organs (Figure 3M, P). On the other hand, the treated vaccinated group (Group. 7) displayed nearly normal thymic, Fabricious bursa, and splenic architecture. Additionally, the bone marrow region had few fat globules and nearly normal architecture, including all the hematological series (Figure 3A).

Discussion

Because of the increased susceptibility to subsequent infections, viral infections that impair the immune system have a huge financial impact on the poultry industry. One of the most common immunosuppressive diseases in poultry is chicken infectious anemia virus (CIAV). CIAV has emerged as a major poultry virus, causing serious financial harm to the global poultry industry (8,21-24). Furthermore, earlier research has revealed that CIAV is widely distributed in Egypt's commercial poultry sectors (8). The pathophysiological profile is characterized by atrophy of the thymus, generalized hemorrhages in the skin muscles, bone marrow aplasia, immunological suppression, and anemia in chickens; which are the only known hosts afflicted by this virus (22,25). In our study, three breeder farms and four broiler

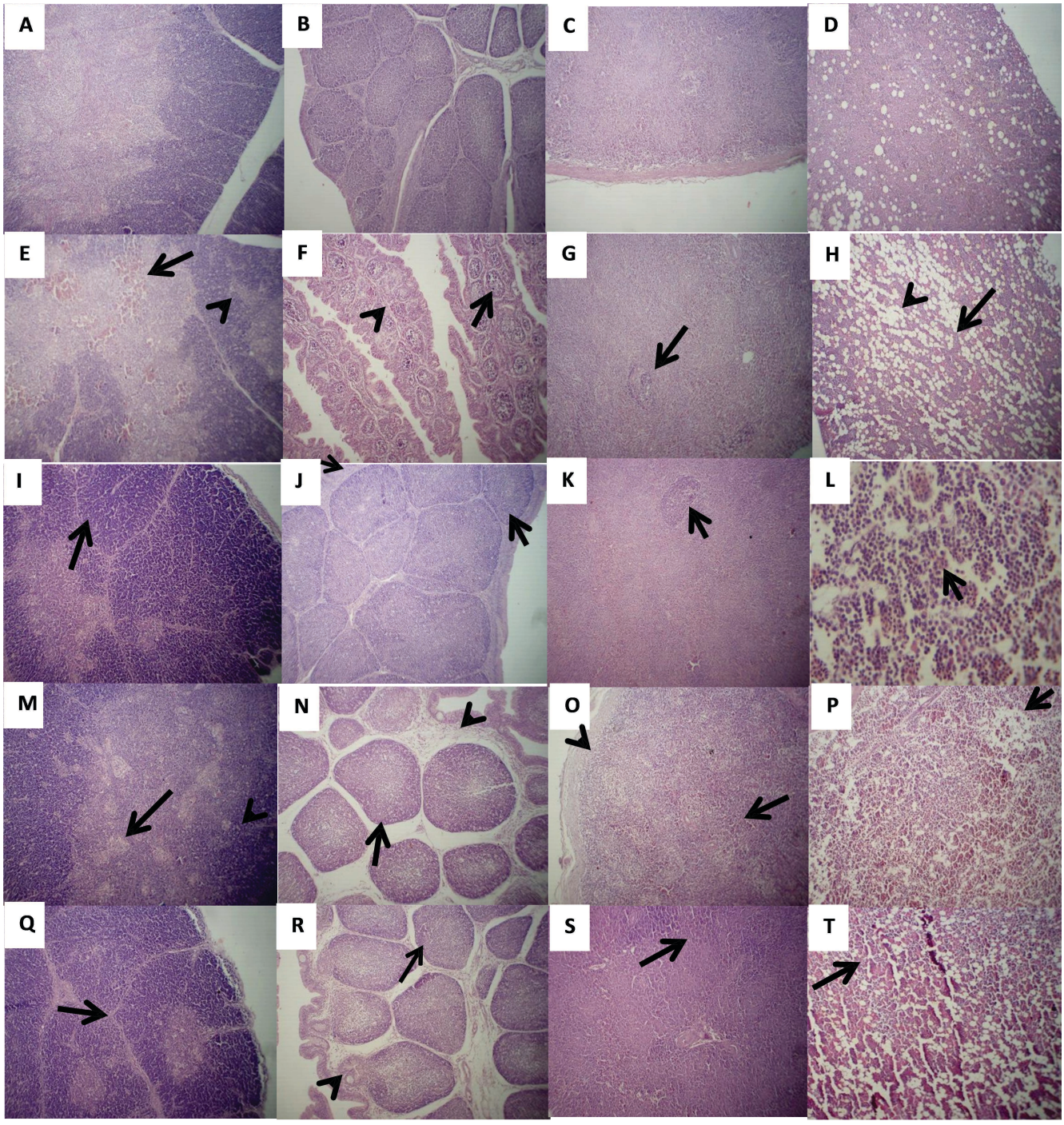

Figure 3: Histopathological findings of experimentally infected chicks with CIA virus thymus, bursa of Fabricious, spleen and bone marrow: (A-D) negative control group; (E-H): positive control group; (I-L): The group treated with *Neem* 8% and challenged; (M-P): vaccinated and challenged group; (Q-T): group treated with *Neem* 8%, vaccinated and challenged.

farms are used as sources for the thymic loops, bone marrow, bursa of Fabricious, and spleen in Dakahlia & Damietta governorates. The suspected flocks giving suspected signs and lesions of CIAV such as drooping aspect, pale combs and wattles, overall weakness, depression, high mortalities, stunting, and growth retardation. Necropsy findings include watery blood, yellow, fatty bone marrow, particularly atrophied thymus glands, atrophied bursa of Fabricious, and enlarged livers and spleens. Clinical symptoms and postmortem lesions were consistent with the findings of (3,26-

30). In flocks of broiler and breeder chickens, polymerase chain reaction (PCR) was employed to identify the presence of the chicken infectious anemia virus (CIAV). In Damietta and Dakahlia provinces of Egypt, out of 7 tested commercial flocks, 5 flocks were positive as reported in the study. For the recognition of CIAV-DNA in birds' tissues, the PCR test was the method of choice. Compared to virus isolation through cell culture, it was more precise and sensitive; especially because the same tissues used to isolate viruses can also be utilized to extract DNA (8,31,32). CIA has no specific therapy because it is a viral disease, and its significant immunosuppressive effect raises its economic danger to poultry producers. Herbal immunomodulation is a new field in research for establishing effective and healthy chicken production due to the birds' increased sensitivity to several diseases as a result of constant genetic selection and intensive production practices (33-35). Therefore, in our study, the immunostimulant effects of *Neem* were evaluated with concentration 8% compared with vaccination against CIAV. Infected group displayed CIAV symptoms such anaemia, stunting, sadness, and a pale comb and wattles, poor growth, weakness and droopy appearance with high daily mortality rates ranging from 0.3-0.4% hence the mentioned mortality rate is for the infected flocks in the survey not in the experiment. However, chicken's group vaccinated and treated with *Neem* leaves extract 8% appeared normal after challenging with CIAV. The hematocrit values were 21.10 (in infected group) and 30.00 (in vaccinated and treated with *Neem* 8%). Hematocrit value of less than 27%, along with yellowish changes in the bone marrow and thymic atrophy, may be symptoms of CIA (3,29,30). The groups treated with *Neem* 8% showed faster recovery to the hematocrit values than positive control, and therefore these findings supported by (36). The latest author referred that the ameliorative effects of Azadirachta indica on CIAV-induced hematological alterations in chicks treated with Azadirachta indica (*Neem*). It indicates a significant improvement in hematological profiles and recovered from anemia within two weeks after infection. These findings emphasize the influence of *Neem* on the hematological parameters in birds by its hepatostimulatory and hepatoprotective effects – It indicates the release of erythropoietic factors by liver cells, which causes the production of more hemoglobin in the bone marrow. the effectiveness and safety of live CAV vaccines was assessed and we found measurable antibody titers by the first week after receiving a live CAV vaccination and the author evidenced that specific CAV antibodies were detectable in the birds, reaching maximal titers by the eighth week (9). When non-vaccinated birds encountered vaccinated chickens, the chickens shed virus and the non-vaccinated birds' antibody titers were lower. Concerning to the ELISA readings, the immune status of the birds was compared after different treatments. It was found that the immune response increased in *Neem*-treated and CAIV vaccinated, and vaccinated group as the ELISA titers were 15200 and 14732 respectively. However, birds treated with *Neem* 8% and infected gave higher ($P<0.05$) antibody response than which vaccinated and infected with virus. It means that using of *Neem* is better than vaccination while birds treated with *Neem* 8% and vaccinated after 5 days then infected at day 21 give higher antibody response ($P<0.05$) than vaccinated after 5 days and infected at day 21 without *Neem* which indicates immunstimulant effect of *Neem*. Treated group with *Neem* 8% and vaccinated at day 5 gives higher antibody response ($P=0.01$) than vaccinated group at day 5 and infected at day 14, indicating neutralization of antibodies by field strain and immunstimulant effect of *Neem*. It was clear that our results showed that supplementation of *Neem* 8% plus vaccination improved the negative feed-back mechanism of birds to slightly represented as the control birds. Histopathological changes in *Neem* vaccinated group post-challenge showed a wideness with increase in the thickness of both cortex and medulla of thymic lobules beside over population of cells (hyperplasia). Also, bone marrow showed activation and proliferated hemopoitic elements with regenerative centers. The bursal lymphoid follicles appeared broad and larger in diameter beside their overpopulation by lymphocytes. Herein, spleen showed intense hyperplastic changes in lymphocytes of white and red pulps than other groups. However, in vaccinated group challenged with virus showed atrophy and necrotic changes in all lymphoid organs. In group treated with *Neem* 8%, vaccinated and challenged revealed an increase in the thickness of both cortex and medulla of thymic lobules and hyperplasia with proliferated hemopoitic elements. In the same group, the bone marrow showed numerous regenerative

hemopoitic centers within fatty marrow which are compatible with the findings of former studies (37,38). The previous authors showed that the immunosuppressive outcome is due to the virus destructive effect of hematological and lymphopoietic tissues, which results in a weakened immune response. CIAV infection in splenic T cells is characterized by decreased phytohemagglutinin reactivity, concanavalin A production, and a reduction in interleukin output (39). In addition, it was discovered that 8% *Neem* resulted in 100% recovery of leukocytes distributed in the hepatic parenchyma following bacterial infection (16). Macrophage concentration, interleukin-1 (IL-1) creation, Fc-receptor expression, phagocytosis, and bactericidal action are significantly reduced. Immunological response was altered due to the harmful effects of virus on lymphocyte and macrophage activities. It leads to increasing the risk of concomitant infection with other antigens and makes immunization ineffective (2). In this work, mild lymphoid follicle exhaustion was visible in the bursa. The finding is consistent with other authors who discovered substantial hypoplasia in hematopoietic cells in the bone marrow as well as significant lymphocyte depletion in the thymus and bursa of Fabricious (40). In addition, bursal alterations in lymphoid follicles with varying degrees of atrophy and dispersed necrotic foci was identified which attributed to secondary infections (6). The thymus and bone marrow samples were also positive because CIAV targets erythroid and lymphoid progenitor cells in these tissues (2).

Conclusions

It is concluded that CIAV damages the spleen, thymus and bone marrow, leading to high level of aplastic anemia, immunosuppression and a significant drop in PCV. The obtained results revealed that the use of *Neem* leaves extracts 8% in addition to vaccination rather than vaccination only with CIAV vaccine is a potential combination during the rearing period of chicken due to its high antiviral and immunstimulant effect. The finding could also help in preventing secondary infections of chicken and large financial losses for the chicken producers.

Author Contributions: AMEH and HMNT jointly developed the hypothesis and concept of the study and contributed to the chemicals and materials preparations, as well as the techniques performed. For this research and scientific paper, AMK and IFR are involved in the experimental procedures and analyses for this study and scientific paper. All the authors participated in the experimental analysis and helped with the manuscript's rewriting. The final manuscript was read and approved by all the authors.

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Institutional Review Board Statement: Animal Experimental Guidelines were followed, and the Animal Care and Use Committee of the Animal Health Research Institute, Zagazig University, Egypt.

The data that supports their conclusions is available from the authors of this manuscript upon request.

There are no conflicts of interest in the current work, according to the authors.

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