VETERINARSKI ARHIV 83 (6), 639-652, 2013

Amelioration of chicken infectious anaemia virus induced immunosuppression by immunomodulator and haematinic supplementation in chicks

Prakash Bhatt[1], Santosh K. Shukla[2], Mohd Yaqoob Wani[3], Ruchi Tiwari[4], and Kuldeep Dhama[5]*

[1]*Veterinary Clinics, College of Veterinary and Animal Sciences, Govind Ballabh Pant University of Agriculture and Technology, Pantnagar, Udham Singh Nagar, Uttarakhand, India*

[2]*Department of Clinical Medicine, College of Veterinary and Animal Sciences, Govind Ballabh Pant University of Agriculture and Technology, Pantnagar, Udham Singh Nagar, Uttarakhand India*

[3]*Immunology Section, Indian Veterinary Research Institute (IVRI), Izatnagar, India*

[4]*Department of Microbiology and Immunology, College of Veterinary Sciences, Pandit Deen Dayal Upadhyaya Veterinary University (DUVASU), Mathura, India*

[5]*Avian Diseases Section, Division of Pathology, Indian Veterinary Research Institute (IVRI), Izatnagar, India*

BHATT, P., S. K. SHUKLA, M. Y. WANI, R. TIWARI, K. DHAMA: Amelioration of chicken infectious anaemia virus induced immunosuppression by immunomodulator and haematinic supplementation in chicks. Vet. arhiv 83, 639-652, 2013.

ABSTRACT

Nutritional immunomodulation represents a new frontier area of research for achieving rational goals of efficient and healthy livestock production, as continuous genetic selection and intensive productions systems have made the animals highly susceptible to various pathogens. In this study, the amelioration of haematinic and immunomodulator on chicken infectious anemia virus (CIAV) induced immmunosupression was studied, with regard to haematological and immunological parameters. Vaccines against Newcastle disease (ND) and infectious bursal disease (IBD) were used as markers for immunological parameter studies. A total of 80 specific pathogen free (SPF) broiler chicks, negative for CIAV antibodies, were randomly divided into four groups (Group I-IV) and were vaccinated against ND and IBD on days 1 and 12, respectively. All chicks of groups II, II and IV were inoculated with CIAV intramuscularly on day 15 of the study, while group I served as control. The chicks of group III and IV were treated with a haematinic (haemocare) and haematinic (haemocare) + immunomodulator (Immuno care), respectively, in drinking water for the entire study period (28 days). Live body weights, haematological parameters, along with humoral immune response (HIR) were measured at regular intervals. The CIAV infected groups showed a significant decline in cell count of erythrocyte and most leukocyte lineages, reduction in live body weights, and antibody titres against ND and IBD viruses. The

*Corresponding author:

Dr. Kuldeep Dhama, Senior Scientist, Avian Diseases Section, Division of Pathology, Indian Veterinary Research Institute, Izatnagar, Uttar Pradesh - 243 122, India, Phone: +91 581 2310074 (Off.); +91 983 7654 996; Fax: +91 581 2303 284; +91 581 2302 179; E-mail: kdhama@rediffmail.com

ISSN 0372-5480
Printed in Croatia

intensity of body weight loss, anaemia, leukopenia and depressed humoral immune responses were less severe in CIAV infected chicks fed with haematenic and immunomodulator supplementation diets. Interestingly, dually treated chicks (group IV) had a significantly higher response than the group treated by haematinic alone . In conclusion, the use of immunomodulator plus haematinic supplementation is suggested for providing better protection compared to haematinic supplementation alone, and the combination can be used for prophylaxis and therapeutic purposes against CIA, an economically important and emerging disease of poultry.

Key words: chicken infectious anaemia, immunotherapeutic, humoral immune response, immunomodulators, haematinics

Introduction

Chicken infectious anaemia (CIA) is an emerging disease responsible for considerable health problems and economic losses to the poultry industry worldwide (McNULTY, 1991; HAGOOD et al., 2000; DHAMA et al., 2008, SCHAT, 2003; SCHAT, 2009; OLUWAYELU, 2010; BHATT et al., 2011). The disease is characterized by poor weight gain, severe anemia, aplasia of the bone marrow and generalized lymphoid atrophy (McNULTY, 1991; ADAIR, 2000; SCHAT, 2003; DHAMA et al., 2002; DHAMA et al., 2008). First reported in 1979 in Japan, the etiological agent of disease, chicken infectious anaemia virus (CIAV), is the smallest DNA virus classified within the genus *Gyrovirus* of family *Circoviridae*. The virus has gained much importance due to its notable characteristics, such as its hardy and highly contagious nature, ubiquitous distribution, vertical transmission, latent infections of layer flocks and infections of SPF eggs, and the potential for inducing marked immunosuppression (McILROY et al., 1992; ROSENBERGER and CLOUD, 1998; DE HERDT et al., 2001; SCHAT, 2003; ELTAHIR et al., 2011). In India CIAV has been placed on the list of emerging viruses which can cause a severe threat to the poultry industry, thereby warranting the need for ascertaining the epidemiological status of the disease in the country and to devise effective control measures (NATESAN et al., 2006; BHATT et al., 2011; DHAMA et al., 2008).

Being a potent immunosuppressive agent, CIAV increases the susceptibility of very young unprotected chicks to other secondary infections, *viz.* viral, bacterial and fungal agents, and has also been found to depress vaccinal immune responses and production performance in field conditions (ADAIR, 2000; TODD, 2000; DE HERDT et al., 2001; SCHAT, 2009; HOERR, 2010). The virus also seems to play a key role in the etiology of several other multifactorial diseases, *viz.* haemorrhagic syndrome, haemorrhagic anaemia syndrome, infectious/aplastic anaemia, anaemia-dermatitis syndrome, gangrenous dermatitis and blue wing disease (BULOW, 1991; POPE, 1991; TORO et al., 2000; HAGOOD et al., 2000; SCHAT, 2003; DHAMA et al., 2008). Reliable diagnostics, viz., enzyme linked immunosorbent assay (ELISA), virus neutralization (VN) and immunofluorescent assay (IFA), along with recent DNA detection techniques, such as polymerase chain reaction (PCR), restriction enzyme (RE) analysis and sequencing, have emerged as effective

diagnostic tools and characterizing the virus at genomic level (McNULTY, 1991; POPE, 1991; DHAMA et al., 2002; DHAMA et al., 2008; SCHAT, 2009; ANCI et al., 2012). Vaccination strategies inclusive of live-attenuated and inactivated vaccines are available, and recombinant (r)-DNA vaccine and immune complex vaccine have been recently reported to be protective (SCHAT, 2009; DHAMA et al., 2008).

As with other viral infections, there is no specific therapeutic approach for the treatment of CIA infected birds; however, broad spectrum antibiotics are generally used to control or avoid secondary bacterial infections. Birds in convalescent stages can be provided with immunostimulants and hematinics to boost their immune system and the process of blood formation, respectively (McNULTY, 1991; DHAMA et al., 2002; SCHAT, 2003). Therefore, effective therapeutic regimens need to be evaluated to counter the massive immunosuppression produced by the disease and to reduce economic losses. The present study was therefore undertaken to assess the ameliorative potential of immunomodulator and haematinic supplementation in chicks experimentally infected by the CIA virus, and to assess their prophylactic and therapeutic potentials by measuring the haematological effects and vaccinal responses as immune parameters.

Materials and methods

Experimental chicks. Specific pathogen free (SPF) chicks (n = 80) were obtained from Venkateshwara Hatcheries Private Limited, Pune, and maintained in standard management conditions and given normal basal ration and water *ad libitum*. Chicks were also given antibiotics for first six days in prophylactic doses to prevent secondary bacterial infections. All the experimental procedures on the chicks were carried out according to the recommendations and approval of the Institute Animal Ethics Committee (IAEC) under the guidelines set forth by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

Virus. Chicken infectious anaemia virus (Indian isolate, strain A; accession No. AY583755), maintained in the Avian Disease Section, Division of Pathology, IVRI, Izatnagar, UP (India) was propagated in MDCC-MSB1 cells and was used as the challenge virus for inducing infection (NATESAN et al., 2006).

Immunomodulator and hematinic. An immunomodulator (Immuno care), consisting of: Vitamin E and C, Amino acids, Omega fats, Sodium chloride, Potassium chloride, Selenium, Manganese sulphate and *Withania somnifera* (Ashvagandha), and a hematinic (haemocare) consisting of: elemental iron, Vitamin B_{12} and B_6, Folic acid, Potassium chloride, Sodium chloride, Vitamin K3, Copper sulphate and Copper chloride.

Experimental design. For the experimental study, a total of 80 specific pathogen free (SPF) broiler chicks were divided randomly into four groups (Group I-IV) with 20 chicks each. Chicks of all four groups were vaccinated against Newcastle disease (ND) by live

attenuated 'F' strain (NDV-F) and Georgia strain of infectious bursal disease (IBD) on day 1 and 12, respectively. Screening of SPF chicks for absence of CIAV antibodies was performed using a commercially available ELISA kit (IDEXX Laboratories, USA) on day 14.

Chicks of groups II, III and IV were inoculated with CIAV intramuscularly, at a dose of 0.5 mL ($10^{4.5}$ $TCID_{50}$/0.1mL), on day 15 of the study. The chicks of groups III and IV were treated with a haematinic (haemocare) and haematinic (haemocare) + immunomodulator (Immunocare), respectively. The haematinic and immunomodulator were given 5 mL and 10 mL each per 100 birds in drinking water, for 0-2 weeks and 2-4 weeks, respectively, for the entire study period of 28 days. The chicks of groups I and II served as the healthy (virus negative) control and infected virus control, respectively. Chicks of all the groups were monitored for live body weight, haematological parameters and immune responses. About 5 mL of pooled blood samples were collected from 5 chicks taken randomly from each group on days 14 (0 day post infection, DPI), 21 (7 DPI) and 28 (14 DPI). The blood was divided into two parts, one part was taken in heparinised vials (10-20 IU/mL) for haematological estimations and the second part was taken in sterilized vials for serum collection and immunological assay.

Haematological parameters. Haematological parameters, *viz.,* packed cell volume (PCV), haemoglobin (Hb), total erythrocyte count (TEC), total leukocyte count (TLC), differential leukocyte count (DLC) - percent lymphocyte count (PLC) and percent heterophil count (PHC), were measured in the experimental chicks at regular intervals (on the 0^{th}, 7^{th} and 14^{th} DPI) as per the standard protocols (CAMPBELL,1995)

Immunological studies. Serum was harvested from coagulated blood using the standard protocol, and stored at -20 ºC unti used for determining the humoral immune response. The antibody titer was assessed against NDV and IBD vaccines in CIAV inoculated chicks with the help of Haemeagglutination Inhibition (HI) and Enzyme linked immunosorbent assay (ELISA), respectively.

Haemagglutination inhibition assay (HI). The HI test was carried out to determine the specific humoral antibody response to ND vaccination in the experimental chicks. The test was conducted using the β-procedure as described by ALLAN and GOUGH (1974). Two fold serial dilutions of the test sera were made in normal saline solution (NSS), pH 7.2, in round bottomed microtitre plates, in a volume of 50 µL. Four HA units of ND virus in equal volume (50 µL) was added to each serum dilution, mixed by gentle shaking and the plates were kept at room temperature for 10 minutes. Freshly prepared 1% RBC suspension was added to each well in 50 µL volume. The negative controls were set up by taking NSS and 1% RBC alone, whereas the virus control was set up by making serial two-fold dilution of 4 HA virus and adding 1% chicken RBC. The plates were incubated at room temperature for 30 minutes or until the RBCs in

the control wells formed a clear button at the bottom. The HI-titre of the serum was calculated as the reciprocal of the highest dilution of the test sera, showing complete inhibition of haemagglutination of RBCs. The mean HI titre (log_2) for different groups was calculated for comparison.

Enzyme linked immunosorbent assay (ELISA). ELISA was carried out to determine the specific humoral antibody response to IBD vaccination in the experimental chicks. ELISA was performed by single dilution method as per the procedure described by CHANDRASEKHAR (1994). A local Indian isolate of IBD virus adapted in chicken embryo fibroblast culture (CEF) (titer log_{10} 4.3 $TCID_{50}$/mL) was used as antigen for ELISA. The concentration of viral antigen was determined by checker board titration. Briefly, the antigen was diluted at 1: 50 in carbonate-bicarbonate buffer (pH 9.5) and 50 µL of this diluted virus was added to each well of a 96 well ELISA plate (NUNC). The plate was incubated overnight at 4 ºC. The next day, antigen coated plates were washed three time with PBS-T (phosphate-buffered saline containing 0.05% Tween-20) for 5 minutes each and tapped thoroughly. The unreacted sites were blocked by adding 100 µL of 5 % skimmed milk powder to each well of the plate for 1 hour at 37 ºC. The plates were again washed as mentioned above. 1: 100 single dilutions of the test serum samples were made in another 96 well microtitre plate, by adding 200 µL of dilution buffer and 2 µL of test serum. The positive control serum (raised in the chicken against purified and confirmed local Indian isolates of the IBD virus, maintained in the Avian disease section, IVRI) and the negative serum were also diluted to 1:100 in PBS-T. The diluted negative serum was added in 100 µL to the first three wells of the first row (A1, A2, A3) and 100 µL of diluted positive serum was added to the first three wells of the last row (H1, H2, H3) of the ELISA plate, while the last three wells of the last row (H10, H11, H12) were kept blank. In the remaining wells of the antigen-coated plate, 100 µL of test serum from the dilution plate was transferred to each of the corresponding three wells. The plate was again incubated at 37 ºC for 1 hour and washed three times with PBS-T. Then 100 µL of rabbit anti-chicken horse radish peroxidase conjugate (Sigma), diluted to 1: 5000 in PBS-T, was added to each well and the plates were incubated at 37 ºC for 1 hour. To each well of the plate, 100 µL of freshly prepared substrate solution of ortho-diphenylenediamine dihydrochloride (OPD) in citrate buffer was added, followed by H_2O_2 and then incubated at 37 ºC for 30 minutes in the dark. The reaction was stopped by adding 100 µL of stop solution (1N H_2SO_4) to each well of the plate. The plate was read at 492 nm in an ELISA reader (ECIL Microscan MS 5605).

The average absorbance of positive and negative controls was calculated from the absorbance value of the ELISA plate and the corrected positive control (CPC) value was determined, by subtracting the average negative absorbance from average positive absorbance.

The specific value (Sp. Value) was calculated using the following formula:

$$\text{Sp. Value} = \frac{\text{Average absorbance of test sample - Average absorbance of negative control}}{\text{Corrected positive control}}$$

The titre was then calculated by

Log_{10} titre = [1.464 x $\log_{10}$ Sp.] + 3.197

Titre = Antilog of $\log_{10}$ titre.

Statistical analysis. The results were analyzed by the standard method (SNEDECOR and COCHRAN, 1994).

Results

Live body weights. The mean live body weights of different groups on day 14 (0DPI) were in the range of 350.5 ± 16.06 and 357 ± 21.24 g. Groups challenged with CIAV showed a significant ($P<0.05$) decline in their mean live body weight as compared to the control group (I) both on days 21 (7DPI) and 28 (14DPI) of the study. However group IV chicks supplemented with immunomodulator and haematinic had significant ($P<0.05$) mean live body weight gain, as compared to group II and III on day 28 (14DPI) (Table 1).

Table 1. Mean live body weight of different treatment groups challenged with CIAV infection

	Mean live body weight (g)		
Groups	14th day (0 DPI)	21st day (7 DPI)	28th day (14 DPI)
I	350.5 ± 16.06^{Aa}	599 ± 34.22^{Cb}	803 ± 39.74^{Cc}
II	353 ± 17.51^{Aa}	496 ± 48.06^{Ab}	588.5 ± 54.32^{Ac}
III	353 ± 23.83^{Aa}	503.5 ± 53.23^{ABb}	593 ± 48.77^{Ac}
IV	357 ± 21.24^{Aa}	538 ± 32.08^{Bb}	678 ± 40.84^{Bc}

The values (Mean ± SD) having at least one common superscript (Capital letters in columns and small letters in rows) do not differ significantly ($P<0.05$) for any parameter

Hematological parameters. The hematological parameters studied during the present study include PCV, Hb, TEC, TLC, DLC in terms of PHC and PHC. The effect of haematinic and haematinic + immunomodulator on these haematological parameters in CIAV infected chicks are given in Table 2.

The normal value of PCV was found to be 0.33-0.34 (v/v) in normal chicks. Inoculation of CIAV was found to decrease normal PCV values on both days 7 and 14 post inoculation in a temporal fashion. Both, haematinic and haematinic + immunomodulator treatments were found to significantly reduce the effect of CIAV induced PCV reduction; however, amelioration was significantly higher (0.26 v/v) in the haematinic + immunomodulator

treated group than with haematinic (0.23 v/v) alone on the 14th day PI. Regarding Hb concentrations, the groups treated with haematinic and haematinic + immunomodulator showed significantly lower reduction compared to the infected control group on both the 7th and 14th DPI. The normal value of Hb concentration (117-119 g/L) was reduced to 67.66 g/L in CIAV infected chicks, and the highest improvement in Hb concentration was recorded in the haematinic + immunomodulator treated (82.06 ± 2.01 g/L) group on the 14th day PI. Similar to the values of Hb and PCV, TEC also showed a significant reduction in CIAV infected compared to normal healthy control chicks on both the 7th and 14th

Table 2. Effect of haematinic and haematinic + immunomodulator supplementation on hematological profile in CIAV infected chicks.

Parameters	Group No.	CIAV Post inoculation day		
		0	7	14
Hemoglobin (g/L)	I	119.24 ± 1.00	121.96 ± 2.20^{C}	123.58 ± 2.01^{C}
	II	119.14 ± 1.67^{a}	101.30 ± 1.10^{Ab}	67.66 ± 1.72^{Ac}
	III	117.15 ± 2.55^{a}	107.38 ± 2.67^{Bb}	76.09 ± 1.53^{Bc}
	IV	118.90 ± 1.27^{a}	116.24 ± 2.09^{BCa}	82.08 ± 2.01^{Bb}
PCV (L/L)	I	0.33 ± 0.01	0.34 ± 0.01^{C}	0.34 ± 0.00^{C}
	II	0.32 ± 0.01^{a}	0.28 ± 0.00^{Ab}	0.20 ± 0.01^{Ac}
	III	0.32 ± 0.01^{a}	0.29 ± 0.00^{Bb}	0.23 ± 0.00^{Bc}
	IV	0.32 ± 0.01^{a}	0.29 ± 0.00^{Bb}	0.26 ± 0.00^{Dc}
TEC ($x10^{12}$/L)	I	3.36 ± 0.05	3.43 ± 0.06^{B}	3.43 ± 0.09^{C}
	II	3.37 ± 0.06^{a}	2.67 ± 0.05^{Ab}	1.99 ± 0.011^{Ac}
	III	3.27 ± 0.16^{a}	2.72 ± 0.02^{ABb}	2.20 ± 0.01^{Bc}
	IV	3.20 ± 0.01^{a}	3.27 ± 0.05^{Ba}	2.83 ± 0.01^{Bb}
TLC ($x10^{9}$/L)	I	22.17 ± 0.76	23.16 ± 0.31^{C}	22.68 ± 0.60^{C}
	II	22.33 ± 1.04^{a}	18.90 ± 0.28^{Ab}	15.88 ± 0.15^{Ac}
	III	22.17 ± 1.04^{a}	19.21 ± 0.50^{Ab}	16.27 ± 0.51^{Ac}
	IV	22.17 ± 0.76^{a}	21.17 ± 0.71^{Ba}	17.95 ± 0.14^{Bb}
PLC (%)	I	60.17 ± 0.76	60.76 ± 1.55^{B}	59.71 ± 0.67^{C}
	II	60.83 ± 1.61^{a}	52.66 ± 0.69^{Ab}	48.50 ± 1.36^{Ac}
	III	59.83 ± 1.89^{a}	52.50 ± 0.59^{Ab}	49.67 ± 1.21^{ABc}
	IV	60.50 ± 2.18^{a}	54.05 ± 1.08^{ABb}	51.35 ± 1.51^{BCb}
PHC (%)	I	29.13 ± 1.31	30.08 ± 1.22^{A}	30.83 ± 0.45^{A}
	II	29.90 ± 1.15^{a}	39.88 ± 2.26^{Bb}	45.22 ± 1.10^{Cc}
	III	29.17 ± 0.76^{a}	39.85 ± 2.82^{Bb}	44.33 ± 0.58^{Cc}
	IV	29.17 ± 1.26^{a}	37.98 ± 0.98^{Bb}	40.32 ± 0.59^{Bc}

The values (Mean ± SD) having at least one common superscript (Capital letters in columns and small letters in rows) does not differ significantly ($P < 0.05$) for any parameter
PCV - packed cell volume, TEC - total erythrocyte count, TLC - total leukocyte count, PLC - percent lymphocyte count, PHC - percent heterophil count

days PI. The normal TLC value was found to be 22-23×10^9/L, and CIAV infection was found to reduce it on both days 7 (18.9 ± 0.28) and 14 (15.88 ± 0.15) PI, respectively. Treatment with haematinic alone was unable to improve the TLC reduction, but the combination of haematinic + immunomodulator significantly improved the TLC values on both the 7th (21.17 ± 0.71) and 14th days (17.95 ± 0.14) PI, compared to the respective CIAV infected control group. The mean PLC values were significantly decreased while the PHC % showed an increasing trend in all the CIAV inoculated chicks in a temporal fashion. The group treated with haematinic + immunomodulator showed significant improvement in both in PLC (51.35 ± 1.51) and PHC (40.31 ± 0.59) on the 14th DPI.

Table 3. Effect of haematinic and haematinic + immunomodulator supplementation on NDV and IBV vaccine induced humoral immune response in CIAV infected chicks.

Parameters	Group No.	CIAV Post inoculation day		
		0	7	14
HI titre (log_2) against NDV	I	10.32 ± 0.00	9.99 ± 0.58^{C}	9.32 ± 1.00^{C}
	II	10.65 ± 0.58^{a}	7.65 ± 0.58^{Ab}	4.99 ± 0.58^{Ac}
	III	10.32 ± 0.00^{a}	7.99 ± 0.58^{Ab}	5.32 ± 1.00^{Ac}
	IV	10.65 ± 0.58^{a}	8.99 ± 0.58^{Bb}	7.32 ± 0.00^{Bc}
ELISA antibody titre against IBD	I	1.49 ± 0.05^{a}	549.69 ± 24.97^{Cb}	963.28 ± 31.69^{Cc}
	II	1.66 ± 0.10^{a}	22.04 ± 1.59^{Ac}	16.72 ± 1.84^{Ab}
	III	1.51 ± 0.03^{a}	22.17 ± 1.71^{Ab}	20.71 ± 2.21^{Ab}
	IV	1.55 ± 0.05^{a}	57.71 ± 2.61^{Bb}	67.07 ± 4.23^{Bc}

The values (Mean ± SD) having at least one common superscript (Capital letters in columns and small letters in rows) does not differ significantly ($P<0.05$) for any parameter

Immunological studies. The antibody titres were assessed against NDV and IBD vaccines as markers to determine the effect of haematinic and haematinic + immunomodulator treatment on CIAV inoculated chicks. HI and ELISA tests were carried out to determine the specific humoral antibody response against the NDV and IBD vaccines administered to the experimental chicks, respectively. All the groups inoculated with CIAV showed a marked reduction in antibody titres against the respective vaccines; however significantly higher antibodies were recorded for both NDV and IBD in the group treated with haematinic + immunomodulator combination on both the 7th and 14th DPI, as compared to the CIAV infected control group. Haematinic treatment alone was not found to improve antibody titres significantly (Table 3).

Discussion

Infection with CIAV is of global level significance as it adversely affects poultry health and production systems, especially the broiler industry and the production of SPF eggs (McILROY et al., 1992, ADAIR, 2000; TODD, 2000; DHAMA et al., 2002; DHAMA et al., 2008; SCHAT, 2009; HOERR, 2010). Specific clinical signs of the disease are generally observed during the first 3-4 weeks of age, when susceptible chicks (without maternally derived antibodies) are infected horizontally (McNULTY, 1991; SMYTH et al., 1993; HAGOOD et al., 2000; SCHAT, 2003). Although mortality from the disease is not generally beyond 5-10% (within 2-4 wks), the associated secondary infections cause more severe clinical complications and higher mortality, which are frequently observed in field conditions (HAGOOD et al., 2000; BALAMURUGAN and KATARIA, 2006). Keeping in view the high metabolic stress, immature immune status and the crucial stage for most vaccination programs, the present study was designed to study the amelioration of CIAV induced immunosuppression by nutritional supplementation with an immunomodulator and haematinic, by determining their effects on hematological and immunological parameters.

Chicken infectious anemia virus causes suppression in the differentiation and proliferation of haematopoietic precursor cells, thereby leading to transient destruction of erythroblastoid and granuloblastoid cell lineages in the bone marrow which is characterized by a drastic reduction in the production of mature red blood cells (erythropoiesis) and myelopoiesis, leading to hypoplasia, anaemia and panleukopenia (McNULTY, 1991; DHAMA et al., 2008). In the present study, all such changes were clinically observed in the CIAV infected chicks. A significant ($P<0.05$) decline in Hb, PCV and TEC was observed in all the CIAV infected groups as compared to the healthy control chicks. The leukocyte lineages also showed a significant ($P<0.05$) decline in TLC, percent lymphocyte count and percent basophil and eosinophil counts in the virus challenged groups, as compared to the control group.

Intensive genetic selection for certain specific traits, such as meat and egg production, in poultry has altered their immune system, and commercial poultry rearing practices have made them at increased risk for exposure to pathogenic organisms (KORVER, 2012). Also, due to the continuous and indiscriminate use of antibiotics, many pathogens have now acquired multiple drug resistance and their presence aggravates the stress conditions and mortality patterns in birds, especially where immune functions are already affected, such as immuno-suppressive CIAV co-infections. In such situations nutritional immunomodulation represents a rational goal to achieve efficient production of healthy birds and their economic benefits. In this study, a combination of various vitamins, minerals, amino acids, fatty acids and inorganic substances, along with the herb *Withania somnifera* (Ashvagandha) was used as an immunomodulating agent. The role of iron, copper, and

manganese has been well established in hematopoiesis. Omega-3 polyunsaturated fatty acids play their role by reducing the growth-suppressive effects of inflammation in poultry (KORVER, 2012). Vitamin E acts as free radical scavenger and prevents highly susceptible n-3 PUFA from oxidation, and is also said to enhance macrophages phagocytic activity and antibody response (FRIEDMAN et al., 1998; KONJUFCA et al., 2004). In the present study it was found that nutritional immunomodulation indeed has positive and beneficial effects on weekly body weight gain and the compromised haematological parameters in the infected chicks, as indicated by the lower severity and intensity of anaemia and leukopenia in CIAV infected groups, which were supplemented with haematinic and immunomodualtor. Interestingly, the dually treated (haematinic + immunomodulator) group IV chicks showed significantly greater improvements, in both body weight gain and haematological parameters, as compared to all other infected groups.

CIAV is believed to be a potent immunosuppressive agent, as it causes marked damage of haematopoietic and lymphopoietic tissue, *viz.* stem cells in bone marrow and precursor T-lymphocytes in thymus (GORYO et al., 1989a,b; SMYTH et al., 1993; TODD, 2000; DHAMA et al., 2008). Immunosuppression so induced is caused at least in part by apoptosis induced by VP3 protein (apoptin), not only on the primary lymphoid organs, but also on secondary lymphoid tissues, thereby suppressing the helper (CD4+) and cytotoxic (CD8+) T-lymphocytes populations (HU et al., 1993; POPE, 1991; PASCUCCI 1997; ADAIR, 2000; SCHAT, 2003). Poor antibody responses are thus generally observed after CIAV infection as a consequence of depressed T_h responses (OTAKI et al., 1988a and b). This immunodepression dramatically leads to susceptibility of birds to secondary infections (viral, bacterial or fungal origin); depressed vaccinal immunity against other poultry pathogens; enhanced vaccination reactions; aggravation of the residual pathogenicity of attenuated vaccine viruses and the emergence of a variant virus; and vaccine failure / outbreaks (ADAIR et al., 1993; DHAMA et al., 2002; PASCUCCI, 1997; TODD, 2000; DHAMA et al., 2008). In the field, CIAV infection seems to cause few signs of disease, however, dual infections are more serious. Sub-clinical CIAV infections in older chickens have also been reported to cause substantial economic losses, by affecting the growth and health of birds.

In the present study, humoral immune responses (HIR) against NDV and IBD vaccines were assayed as indicators to determine the effects of CIAV induced immune dysfunctions at an early age. HIR against NDV and IBD were assessed by determining the specific antibody vaccine titres by employing HI and ELISA, respectively. Previous studies by ZHENG and LIU (1996 a,b) indicated that ND-HI vaccine titres were low in CIAV infected birds vaccinated at one day old. While studying the immune response to the ND vaccine, RAGLAND et al. (1998) also reported that an increase in CIAV detection correlated inversely with ND-HI titres. To overcome this immunosuppressive condition

induced by CIAV, an immunomodulator containing Ashwagandha was selected for the present study. Several studies have indicated various roles played by Ashwagandha, such as: immunoprotective, myeloprotective and anti-inflammatory effects, along with potentiation of NK cells, T cell proliferation, and humoral and cellular immune response (DAVIS and KUTTAN, 2000 and 2002; PADMAVATHI et al., 2005). In the present study, a significant decline in the antibody HI titre against NDV and ELISA titres against IBD was observed in all the CIAV infected chicks compared to the healthy control group. Nutritional supplementation with haematinic plus immunomodulator was found to ameliorate the CIAV induced immune suppression, as indicated by the induction of higher antibody titres for NDV and IBD vaccines. Similar studies using dietary ascorbic acid supplementation found reduced IBDV induced immunosuppression and improved both humoral and cellular immune responses (WU et al., 2000).

On the basis of the above findings, it may be concluded that nutritional immunomodulation may act as a supplementary prophylactic approach for ameliorating CIAV induced immune suppression in poultry birds, as indicated by improvements in erythrocyte and leukocyte cell lineages. Also the present data highlighted the beneficial effects of dietary supplementation on the humoral branch of the immune system, which is necessary for prevention of many important viral pathogens and thus for vaccination programs. It is therefore suggested that immunomodulators along with haematinics should be supplemented to chicks during their early susceptible age (3-4 weeks) to minimize the immunosuppression caused by ubiquitous CIAV. Such types of prophylactic regimes will thus not only help alleviate the huge economic losses caused by this important virus, but will also improve the general immune status of birds against various other pathogens and thereby help to reduce antibiotic usage and the development of drug resistance.

Acknowledgements

The authors are very thankful to the Dean, College of Veterinary and Animal Sciences, Dean, Post Graduate Studies and Director, Experiment Station for providing the necessary facilities to carry out this research work.

References

ADAIR, B. M. (2000): Immunopathogenesis of chicken anemia virus infection. Dev. Comp. Immunol. 24**,** 247-255.

ADAIR, B. M., C. D. McCONNELL, F. MCNEILLY, M. S. McNULTY, F. COUDERT (1993): Effect of chicken anaemia virus on macrophage and lymphocyte functions in chickens. Avian Immunol. Progress 53, 219-223.

ALLAN, W. H., R. E. GOUGH (1974): A standard haemagglutination inhibition test for Newcastle disease. 1. A comparison of macro and micro methods. Vet. Rec. 95, 120-123.

ANCI, S. MANOHARAN, P. RAMADASS, K. KUMANAN (2012): Assessment of polymerase chain reaction sensitivity for the detection of chicken anaemia virus using different primers for three genes. Vet. Arhiv 82, 89-102.

BALAMURUGAN, V., J. M. KATARIA (2006): Economically important non-oncogenic immunosuppressive viral diseases of chicken - current status. Vet. Res. Commun. 30, 541-566.

BHATT, P., S. K. SHUKLA, M. MAHENDRAN, K. DHAMA, M. M. CHAWAK, J. M. KATARIA (2011): Prevalence of chicken infectious anaemia virus (CIAV) in commercial poultry flocks of Northern India: a serological survey. Transbound. Emerg. Dis. 58, 458-460.

BULOW, V. V. (1991): Avian infectious anemia and related syndromes caused by chicken anaemia virus. Crit. Rev. Poult. Biol. 3, 1-17.

CAMPBELL, T. W. (1995): Avian Haemtology and Cytology (2nd ed), Iowa State University Press, Ames, USA.

CHANDRASEKHAR, P. (1994): Studies on immunomodulatory effect of MDP, Poly I:C and Interferon gamma in chicks immunized with IBD vaccine. M.V.Sc Thesis submitted to IVRI, Izatnagar (U.P.), India.

DAVIS, L., G. KUTTAN (2000): Immunomodulatory activity of *Withania somnifera*. J. Ethnopharmacol. 71, 193-200.

DAVIS, L., G. KUTTAN (2002): Effect of *Withania somnifera* on cell mediated immune responses in mice. J. Exp. Clin. Canc. Res. 21, 585-590.

DE HERDT, P., G. VAN DEN BOSCH, R. DUCATELLE, E. UYTTEBROEK, C. SCHRIER (2001): Epidemiology and significance of chicken infectious anemia virus infections in broilers and broiler parents under nonvaccinated European circumstances. Avian Dis. 45, 706-708.

DHAMA, K., J. M. KATARIA, B. B. DASH, N. SENTHILKUMAR, S. TOMAR (2002): Chicken infectious anemia (CIA): a review. Ind. J. Comp. Microbiol. Immunol. Infect. Dis. 23, 1-15.

DHAMA, K., M. MAHENDRAN, R. SOMVANSHI, M. M. CHAWAK (2008): Chicken infectious anaemia virus: an immunosuppressive pathogen of poultry - a review. Indian J. Vet. Pathol. 32, 158-167.

ELTAHIR, Y. M., K. QIAN , W. JIN, P. WANG, A. QIN (2011): Molecular epidemiology of chicken anemia virus in commercial farms in China. Virol. J. 8, 145. doi: 10.1186/1743-422X-8-145.

FRIEDMAN, A., I. BARTOV, D. SKLAN (1998): Humoral immune response impairment following excess vitamin E nutrition in the chick and turkey. Poult. Sci. 77, 956-962.

GORYO, M., S. HAYASHI, K. YOSHIZAWA, T. UMEMURA, C. ITAKURA, S. YAMASHIRO (1989a): Ultrastructure of the thymus in chicks inoculated with chicken anaemia agent (MSB1-TK5803 strain). Avian Pathol. 18, 605-617.

GORYO, M., T. SUWA, T. UMEURA, C. ITAKURA, S. YAMASHIRO (1989b). Ultrastructure of bone marrow in chicks inoculated with chicken anemia agent (MSB1-TK5803 strain). Avian Pathol. 18, 329-343.

HAGOOD, L. T., T. F. KELLY, J. C. WRIGHT, F. J. HOERR (2000): Evaluation of chicken infectious anemia virus and associated risk factors with disease and production losses in broilers. Avian Dis. 44, 803-808.

HOERR, F. J. (2010): Clinical aspects of immunosuppression in poultry. Avian Dis. 54, 2-15.

HU, L. B., B. LUCIO, K. A. SCHAT (1993). Depletion of $CD4^+$ and $CD8^+$ T lymphocyte subpopulation by CIA-1, a chicken infectious anemia virus. Avian Dis. 37, 492-500.

KONJUFCA, V. K., W. G. BOTTJE, T. K. BERSI, G. F. ERF (2004): Influence of dietary vitamin E on phagocytic functions of macrophages in broilers. Poult. Sci. 83, 1530-1534.

KORVER, D. R. (2012): Implications of changing immune function through nutrition in Poultry. Animal Feed Sci. Technol. 173, 54-64.

McILROY, S. G., M. S. McNULTY, D. W. BRUCE, J. A. SMYTH, E. A. GOODALL, M. J. ALCORN (1992): Economic effects of clinical chicken anemia agent infection on profitable broiler production. Avian Dis. 36, 566-574.

McNULTY, M. S. (1991): Chicken anemia agent: a review. Avian Pathol. 20, 187-203.

NATESAN, S., J. M. KATARIA, K. DHAMA, S. RAHUL, N. BARADHWAJ (2006): Biological and molecular characterization of chicken anemia virus isolates of Indian origin. Virus Res. 118, 78-86.

OLUWAYELU, D. O. (2010): Diagnosis and epidemiology of chicken infectious anemia in Africa. Afr. J. Biotechnol. 9, 2043-2049.

OTAKI, Y., T. NUNOYA, M. TAJIMA, A. KATO, Y. NOMURA (1988a): Depression of vaccinal immunity to Marek's disease by infection with chicken anemia agent. Avian Pathol. 17, 333-347.

OTAKI, Y., M. TAJIMA, K. SAITO, Y. NOMURA (1988b): Immune response of chicks inoculated with chicken anemia agent alone or in combination with Marek's disease or turkey herpes virus. Jap. J. Vet. Sci. 50, 1040-1047.

PADMAVATHI, B., P. C. RATH, A. R. RAO, R. P. SINGH (2005): Roots of *Withania somnifera* inhibit forestomach and skin carcinogenesis in mice. Evidence based complement. Altern. Med. 2, 99-105.

PASCUCCI, S. (1997): Immunosuppressive effect of chicken anaemia virus. Selezione Veterinaria 8-9, 599-609.

POPE, C. R. (1991): Chicken anemia agent. Vet. Immunol. Immunopathol. 30, 51-65.

RAGLAND, W. L., H. MAZIJA, V. CVELIC-CABRILO, V. SAVIC, R. NOVAK, M. POGACNIK (1998): Immune suppression of commercial broilers in Croatia, Slovenia and Bosnia and Herzegovina from 1981 to 1991. Avian Pathol. 27, 200-204.

ROSENBERGER, J. K., S. S. CLOUD (1998): Chicken anemia virus. Poult. Sci. 77, 1190-1192.

SCHAT, K. A. (2003): Chicken infectious anemia. In: Diseases of Poultry, 11th ed. (Saif, Y. M., H. J. Barnes, J. R. Glisson, A. M. Fadly, L. R. Mcdougald, D. E. Swayne, Eds.). Iowa State University Press. Ames, USA. pp. 182-202.

SCHAT, K. A. (2009): Chicken anemia virus. Current Topics Microbiol. Immunol. 331, 151-183.

SMYTH, J. A., D. A. MOFFETT, M. S. McNULTY, D. TODD, D. P. MACKIE (1993): A sequential histopathologic and immunocytochemical study of chicken anemia virus infection at one day of age. Avian Dis. 37, 324-338.

SNEDECOR, G. W., W. G. COCHRAN (1994): Statistical Methods. 8th ed., Iowa State University Press, Iowa.

TODD, D. (2000): Circoviruses: immunosuppressive threats to avian species: a review. Avian Pathol. 29, 373-394.

TORO, H., C. GONZALEZ, L. CREDA, M. HESS, E. REYES, C. GEISSE (2000): Chicken anemia virus and fowl adenoviruses: association to induce the inclusion body hepatitis/ hydropericardium syndrome. Avian Dis. 44, 51-58.

WU, C. C., T. DORAIRAJAN, T. L. LIN (2000): Effect of ascorbic acid supplementation on the immune response of chickens vaccinated and challenged with infectious bursal disease virus. Vet. Immunol. Immunopathol. 74, 145-152.

ZHENG, S. M., Z. G. LIU (1996a): Changes in immunoglobulins associated with humoral immunity in chicks infected with chicken anaemia virus. Chinese J. Vet. Sci. 16, 269-272.

ZHENG, S. M., Z. G. LIU (1996b): Changes in the immunoglobulin content and the HI titre after Newcastle disease vaccination of chicks infected with chicken anaemia virus. Acta Veterinaria et Zootechnica Sinica 27, 242-246.

Received: 5 September 2012
Accepted: 10 July 2013

BHATT, P., S. K. SHUKLA, M. Y. WANI, R. TIWARI, K. DHAMA: Poboljšanje potisnute imunoreaktivnosti prouzročene virusom zarazne anemije pilića upotrebom imunomodulatora i hematika. Vet. arhiv 83, 639-652, 2013.

SAŽETAK

Imunomodulacija putem hrane predstavlja novo područje istraživanja s ciljem da se postigne učinkovita proizvodnja zdrave stoke, jer su trajni genetski odabir i zahtjevni proizvodni sustavi učinili životinje visoko prijemljivima na različite patogene. U ovom je radu, na osnovi hematoloških i imunoloških pokazatelja, istražen učinak hematika i imunomodulatora na imunosupresiju izazvanu virusom zarazne anemije pilića. Cjepiva protiv newcastleske bolesti i zaraznog burzitisa kokoši rabljena su kao osnova za istraživanje imunoloških pokazatelja. Ukupno je 80 tovnih pilića, slobodnih od specifičnih patogenih uzročnika, negativnih na protutijela za virus zarazne anemije pilića, bilo nasumce podijeljeno u četiri skupine (I-IV) te prvog dana cijepljeno protiv newcastleske bolesti, a 12. dana protiv zaraznog burzitisa kokoši. Svi pilići skupine II, III i IV bili su intramuskularno zaraženi virusom zarazne anemije pilića 15. dana pokusa, dok je skupina I bila kontrolna. Pilići skupine III dobivali su hematik, a skupine IV hematik i imunomodulator u pitkoj vodi tijekom čitavog razdoblja istraživanja (28 dana). Tjelesna masa, hematološki pokazatelji i humoralni imunosni odgovor bili su mjereni u pravilnim razmacima. Skupina zaražena virusom zarazne anemije pokazivala je značajni pad broja eritrocita i leukocita te smanjenje tjelesne mase i titra protutijela za virus newcastleske bolesti i virus zaraznog burzitisa. Gubitak tjelesne mase, anemija, leukopenija i humoralni imunosni odgovor bili su slabiji u pilića zaraženih virusom zarazne anemije koji su dobivali hematik i imunomodulator u hrani. Zanimljivo je da je u pilića koji su dobivali dvostruki dodatak (skupina IV) zabilježen značajno bolji odgovor nego u pilića koji su dobivali samo hematik. Može se zaključiti da je upotreba imunomodulatora i hematika pružila bolju zaštitu u odnosu na upotrebu samog hematika. Kombinacija se može upotrijebiti za profilaksu i terapiju protiv zarazne anemije pilića, gospodarski važne i emergentne zarazne bolesti.

Ključne riječi: zarazna anemija pilića, imunoterapeutik, humoralni imunosni odgovor, imunomodulatori, hematici