RESEARCH ARTICLE

Sokoto Journal of Veterinary Sciences

(P-ISSN 1595-093X: E-ISSN 2315-6201)

http://dx.doi.org/10.4314/sokjvs.v16i2.9

Igwe & Eze /*Sokoto Journal of Veterinary Sciences*, **16**(2): 63 - 74.

Leucogram, serum protein parameters and histopathology of broiler chickens (*Gallus gallus domesticus*) treated with cyclophosphamide and infected with velogenic Newcastle disease virus

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Publication History:
Received: 05-10- 2017
Accepted: 06-02-2018

Abstract

The present study evaluated the changes in leucogram, serum protein parameters and histology of lymphoid organs associated with cyclophosphamide–induced immunosuppression in broiler chickens infected with velogenic Newcastle disease virus. At four-week-old, one hundred broiler chickens were randomly assigned into four groups of 25 each viz: A/CYTI – cyclophosphamide treated and velogenic Newcastle disease virus infected, B/CYTU – cyclophosphamide– treated and uninfected, C/CYNTI – cyclophosphamide non-treated, infected and D/CYNTU– untreated uninfected. Groups A/CYTI and B/CYTU were injected with cyclophosphamide at the dose of 75mg/kg body weight daily for 3 days while groups A/CYTI and C/CYTU were infected with velogenic Newcastle disease virus at six-week-old. Blood samples were collected from randomly selected chickens in each group for leucogram and serum protein assays, while tissue samples were collected for histopathology. Cyclophosphamide induced significantly ($P < 0.05$) lower levels of circulating total leucocytes, lymphocytes and heterophils on days 7 and 14 post-treatment. On day 14 post-treatment, the total serum protein values of groups A/CYTI and B/CYTU were significantly ($P < 0.05$) lower than those of groups C/CYNTI and D/CYNTU. Examination of the tissue sections showed severe diffused lymphocytic necrosis and depletion in the bursa, spleen and thymus. Newcastle disease virus infection induced significantly ($P < 0.05$) higher levels of circulating total leucocytes, lymphocytes and heterophils and significantly ($P< 0.05$) decreased serum proteins in cyclophosphamide treated chickens on day 3 PC. The results showed that severe decreased leucogram and serum proteins and lymphoid cell depletion in cyclophosphamide treated broiler chickens, which together with the increased leukogram observed following velogenic Newcastle disease virus infection could be used as indicators of exposure to immunosuppressants.

Keywords: Broiler chickens, Cyclophosphamide, Leucogram, Serum proteins, Newcastle disease

Introduction

Avian clinical pathological assays are relevant tools in evaluating the physiological and pathological statuses of birds that aid veterinary clinicians to arrive at proper diagnosis of diseases, guide prognosis and assess of the efficacy of therapeutic interventions, and toxicity of drugs and chemical substances (Campbell & Coles, 1986; Samour, 2009). Haematology is one of the cornerstones of medical diagnosis and is currently considered an integral part of clinical laboratory diagnostics in avian species (Samour, 2009). Serum biochemical assessment, along with haematology and physical examination, helps to predict pathological processes in the vital internal organs of the body such as the liver, muscle, heart, pancreas and kidney (Harr, 2009; Cerón *et al*., 2010). It also helps to establish the presence or absence of disease of an organ, and determines the nature and extent of a disease process (static, progressive or regressive) by serial performance of laboratory tests for the internal organs where little or no clinical signs of disease are observed even when seriously ill (Harr, 2009; Cerón *et al*., 2010). Evaluation of total and differential white blood cell counts is commonly used as clinical indicator of haematological, immunological and infection status (Harr, 2009).

White blood cells make up about 1 percent of blood, but their small number belies their immense importance. They play a vital role in the body's immune system—the primary defense mechanism against invading bacteria, viruses, fungi, and parasites. They often accomplish this goal through phagocytosis (Coles, 1986; Teske, 2010). White blood cells also produce antibodies, which are released into the circulating blood to target and attach to foreign organisms. After attachment, the antibody may neutralize the organism, or it may elicit help from other immune system cells to destroy the foreign substance (Teske, 2010). There are several varieties of white blood cells, including neutrophils (heterophils in birds), monocytes, and lymphocytes, all of which interact with one another and with plasma proteins and other cell types to form the complex and highly effective immune system (Coles, 1986). Therefore, white blood cells are the effector cells of immune responses (Ono *et al*., 2003; Latimer & Bienzle, 2010; Teske, 2010). Avian white blood cells are functionally equivalent to mammalian white blood cells (Latimer & Bienzle, 2010; Wakenell, 2010). Heterophils provide first line of defence against invading microorganisms, tissue trauma, or any inciting inflammatory signal and play an important role in phagocytosis, elimination of microorganisms and accomplish effective non-oxidative bacterial killing within both the blood stream and tissues by relying more heavily on oxygen-independent (non-oxidative) mechanisms by myeloperoxidase -independent methods that involve lysozyme, acid phosphatase, cathepsin, and β -glucuronidase (hydrolase) activities, and cationic proteins (Evans *et al*.. 1995; Latimer & Bienzle, 2010; Wakenell, 2010). Monocytes are the precursors of the mononuclear phagocytic system, that is, connective tissue macrophages, osteoclasts, alveolar macrophages, perisinusoidal macrophages in the liver (Kupfer cells), and macrophages of lymphoid organs, and bone marrow (Campbell, 1994; Teske, 2010). Eosinophils are associated with allergic reactions, chronic inflammation and play a major role in host defense against helminthic parasites (Latimer & Bienzle, 2010). The lymphoid cells are key components of the immune system (Day, 2010). Lymphocytes are important for cell mediated and antibody-mediated responses as well as nonspecific natural killer cell cytotoxicity (Ono *et al*., 2003; Latimer & Bienzle, 2010).

Many chemicals are immunosuppressive (Halloran, 2004; Wiseman, 2016) and may alter the resistance or increase the susceptibility of the host to various infectious diseases (Okoye *et al*. 1992; Wiseman, 2016). Cyclophosphamide (CY), has received a significant amount of attention as an anti-tumor as well as an immune suppressing drug in animals and humans for clinical use in immune diseases and transplantation (Wiseman, 2016). CY acts as an alkylating agent, causing damage to nucleic acids, including breaks in DNA strands, cross-links between two DNA strands and cross-linking between DNA and RNA or protein (Hengstler *et al*., 1997). Linking results in a major disruption of the DNA function and subsequent interference with normal cell mitosis and kills cells undergoing multiplication (Colvin, 1982; Hengstler *et al*., 1997; Chinnaswamy *et al*., 2011). CY has its greatest effect on the B lymphocytes of laboratory animals, humans and birds and depending on the dose, is relatively T-lymphocyte sparing, with the capability of inhibiting both humoral and cell-mediated immune responses (Winkelstein, 1973; Ficken & Barnes, 1988; Wiseman, 2016). Humoral immunity is affected by the toxic effect on B cells, and cell-mediated immunity is affected by the toxic effect on cyclophosphamide-sensitive suppressor T cells (Winkelstein, 1973; Wiseman, 2016). CY causes

profound changes in certain haematologic parameters in chickens such as thrombocytopaenia and also hypocelluar bone marrow resulting in severe hypoplasia of granulopoiesis predisposing birds to infectious agents (Fulton *et al*., 1996).

Newcastle disease (ND), a highly contagious and devastating viral disease of many avian species worldwide, is in the Office International des Epizooties (OIE) list of notifiable diseases (OIE, 2017). ND is caused by virulent strains of avian paramyxovirus-1 (APMV-1), of the genus *Avulavirus*, subfamily *Paramyxovirinae*, family *Paramyxoviridae* and order *Mononegavirales* (Miller & Koch, 2013; OIE, 2012). The disease results in significant economic losses in poultry sector and for countries that export poultry or poultry products, due to losses resulting from trade restrictions and embargoes (Alexander *et al*., 2012; OIE, 2012; OIE, 2017). Velogenic Newcastle disease virus (vNDV) infection causes increased circulating white blood cells (lymphocytes, heterophils, monocytes and eosionphils) in laying chickens (Igwe *et al*., 2017), severe atrophy of the lymphoid organs due to necrosis and depletion of the lymphocytes resulting in immunosuppression (Kapczynski *et al*., 2013; Ezema *et al*. 2016; Igwe & Eze, 2016).

Due to a lack of biosecurity and management practice, birds are constantly exposed to the risk of immunosuppressants, which might lead to increased susceptibility to diseases and vaccination failures (Hoerr, 2010). A common occurrence in nature is that a flock could have been infected or exposed to an immunosuppressive condition and then followed by vNDV infection. Knowing that ND and CY affect the haemopoietic and lymphoid tissues which are directly involved in immunological functions, there is the need to investigate the changes that occur in haematology and biochemistry levels of an immune suppressed flock. The cyclophosphamide used in this study is only an agent to induce immunosuppression. Therefore, the present study evaluated the changes in leucogram, serum protein parameters and histology associated with CY (an immunosuppressive agent) in broiler chickens infected with vNDV.

Materials and Methods

The experimental protocols were reviewed and approved by the University Ethics Committee on Medical and Scientific Research. This study was scrutinized and approved by the Michael Okpara University of Agriculture, Umudike, Committee on Medical and Scientific Research Ethics and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and later amendments.

Broiler chickens

One hundred day-old White Marshall broiler chicks (*Gallus gallus domesticus*) procured from a reputable local commercial hatchery were used for the study. The birds were randomly separated into four groups of 25 broiler chicks each. The groupings and their treatments were:

Group A consisted of CY-treated and vNDV challenged chickens (CYTI).

Group B consisted of CY-treated and unchallenged chickens (CYTU).

Group C consisted of CY-non-treated and vNDV challenged chickens (CYNTI).

Group D consisted of CY-non-treated and unchallenged chickens (CYNTU).

Brooding was done separately for each of the groups on deep litter under the same environmental conditions and they were not vaccinated against any disease. Feed and water were provided *ad libitum*. The chicks were kept in isolation in a Poultry Experimental Unit under strict biosecurity measures. General care of the birds was provided in accordance with the Institutional Animal Care and Use Committee as outlined in the 1998 Code of Federal Regulations, Animals and Animal Products (USDA, 1998).

Cyclophosphamide treatment

Cyclophosphamide (Paucocyclo®, Keally Pharmaceutical Pty, Ltd, Amber- India) was obtained in a dry form containing active ingredients. The fresh daily aqueous solution was prepared as needed.

At four-week-old, broiler chickens of Groups A/CYTI and B/CYTU were each weighed and administered 75 mg/kg of CY each day intramuscularly (IM) in the breast muscle on days 28, 29 and 30 of age. Groups C/CYNTI and D/CYNTU chickens were similarly weighed and injected with the equal volume of distilled water**,** IM as placebo. Chemical ablation was achieved according to the methods of Reynolds & Maraqa (1999) and Kim *et al*. (2003). The four experimental groups were housed separately.

All the groups exposed to CY were observed twice daily for effects of CY treatment (clinical signs) from day 0 through to day 14 (day 0 vNDV PI) post-CY-treatment.

Velogenic Newcastle disease virus challenge

The challenge vNDV used, duck/Nigeria/Plateau/Kuru/113/1992, was obtained

from National Veterinary Research Institute (NVRI), Vom. It was isolated from an apparently healthy duck and characterized as viscerotropic velogenic NDV (vvNDV) by Echeonwu *et al*. (1993) and Igwe *et al.* (2014). The inoculum had a median embryo infective dose (EID_{50}) of $10^{6.46}$ per ml.

At six-week-old (day 14 post-CY-treatment), the chickens in each group were found to be serologically negative for NDV antibodies by haemagglutination inhibition (HI) test using the method described by OIE (2012). Each broiler chicken in groups A/CYTI and C/CYNTI was inoculated intranasally (I/N) with 0.2 ml of the viral inoculum, while each broiler chicken in groups B/CYTU and D/CYNTU (corresponding non-infected chickens) received 0.2 ml of phosphate buffered saline (PBS) through the same route as placebo.

Blood sample collection

Ten birds from each group were randomly selected and three millilitres of blood were collected from each bird through the jugular vein for leucogram and serum protein analyisis. The samples were collected at weeks 4 (before the CY treatment), 5 and 6 of age, immediately before vNDV infection (day 0 of challenge), and on days 3 and 21 post-challenge (PC).

Leucogram analysis - One millilitre of the blood samples from each bird was collected into a labeled clean sample bottle containing 1 mg of ethylenediaminetetraacetic acid (EDTA) powder as an anticoagulant and used immediately for haematologic analysis using standard procedures. Total white blood cell (Total WBC) counts were done by the haemocytometer method using Natt and Herrick's solution as the diluting fluid (Campbell, 1994), while the smears for differential leucocyte count for each blood sample were prepared and stained by the Leishman technique and enumerated by the battlement counting method (Thrall & Weiser, 2002).

Serum protein determination - Two millilitres of the blood samples from each bird were collected into sterile test tubes and allowed for 30 minutes to clot. The clotted samples were centrifuged at 3000 rpm for 10 minutes. Serum samples were harvested and used immediately for determination of serum protein levels while the remaining portion was stored in the freezer at −20 °C and used later for quantification of NDV antibody titres using the standard technique described by OIE (2012). Total serum protein was determined using direct Biuret method as described by Lubran (1978). The serum albumin level was determined using bromocresol green method (Doumas & Peters, 1997). Serum globulin was calculated as follows:

Globulin = total serum proteins – serum albumin (Coles, 1986).

Histopathology

Trachea, lung, liver, kidney, bursa of Fabricius, spleen and thymus were collected from each group on days 7 and 14 post-CY-treatment and on days 4 and 5 post-ND infection, fixed in 10% buffered formalin, and routinely processed for microscopic examination by standard techniques.

Statistical analyses

Data generated for the study were subjected to one way analysis of variance (ANOVA). Variant means were separated post hoc using the least significant difference (LSD) method (Okafor, 1992). Probabilities less than 0.05 were accepted as significant.

Results

Leucocyte value

The total WBC values of the CY treated, A/CYTI and B/CYTU, groups were significantly ($P < 0.05$) lower than those of non- CY treated, C/CYNTI and D/CYNTU, groups on days 7 and 14 post-CY treatment (Figure 1). Following vNDV infection, the total WBCs of the vNDV infected groups A/CYTI and C/CYNTI were significantly ($P < 0.05$) higher than those of non-infected groups, B/CYTU and D/CYNTU, while the total WBCs of group C/CYNTI were significantly ($P < 0.05$) higher than those of A/CYTI on day 3 PC (day 17 post-CY treatment) (Figure 1).

The lymphocyte counts of groups A/CYTI and B/CYTU were significantly ($P < 0.05$) lower than those of C/CYNTI and D/CYNTU on days 7 and 14 post-CY treatment (Table 1). The lymphocyte counts of the vNDV infected groups A/CYTI and C/CYNTI were significantly ($P < 0.05$) higher than those of their controls, B/CYTU and D/CYNTU, respectively, while those of C/CYNTI were significantly ($P < 0.05$) higher than those of A/CYTI on day 3 PC (day 17 post-CY treatment) (Table 1). The heterophil counts of groups A/CYTI and B/CYTU were significantly ($P < 0.05$) lower than those of C/CYNTI and D/CYNTU on days 7 and 14 post-treatment. On day 3 PC, the heterophil counts of groups A/CYTI and C/CYNTI were significantly ($P < 0.05$) higher than those of B/CYTU and D/CYNTU (Table 1). Eosinophil,

monocyte and basophil counts of CY-treated and non-treated groups and vNDV infected groups compared with their controls were erratic throughout the study.

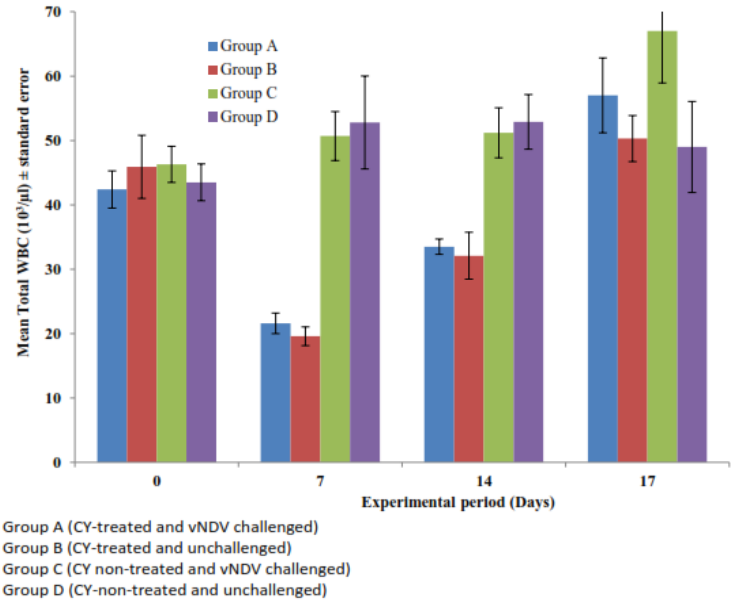

Figure 1: The total white blood cell counts (Total WBC) of broilers treated with cyclophosphamide and infected with velogenic Newcastle disease virus

Serum proteins

On days 14 and 17 post- CY treatment (day 3 PC), the total serum protein values of groups A/CYTI and B/CYTU were significantly ($P < 0.05$) lower than those of groups C/CYNTI and D/CYNTU, while the values of group C/CYNTI were significantly ($P < 0.05$) higher than those of A/CYTI, B/CYTU and D/CYNTU on day 3 PC (Figure 2). The serum albumin levels of group C/CYNTI were significantly ($P < 0.05$) higher than those of groups A/CYTI, B**/**CYTU and D/CYNTU on day 3 PC (Figure 3). The serum globulin levels of group A/CYTI and B**/**CYTU showed significant ($P < 0.05$) decrease when compared with those of groups C/CYNTI and D/CYNTU on days 14 and 17 pos- CY treatment (day 3 PC), while the levels of group CYNTI were significantly ($P < 0.05$) higher than those of A/CYTI, B/CYTU and D/CYNTU on day 3 PC (Figure 4).

Histopathology

Trachea, lung, liver, and kidney of the sacrificed broilers in all the groups showed no microscopic lesions. Sections of the bursa showed folded but intact plica epithelium, hyperaemia, marked interfollicular fibroplasias, follicular

Table 1: The absolute differential leucocyte counts of broilers treated with cyclophosphamide and infected with velogenic Newcastle disease virus, (Mean ± SD)

Exptl. period (days)	Groups	Lymphocyte counts (10^3/µl)	Heterophil counts (10^3/µl)	Eosinophil counts (10^3/µl)	Monocyte counts (10^3/µl)	Basophil counts (10^3/µl)
0	CYTI	25.25 ± 2.13	14.52 ± 1.13	1.28 ± 0.38 ^{ab}	1.22 ± 0.44	1.24 ± 0.32
	CYTU	27.94 ± 2.36	16.19 ± 2.46	0.78 ± 0.27 ^{b}	0.91 ± 0.28	0.68 ± 0.25
	CYNTI	21.97 ± 1.26	19.25 ± 4.72	2.27 ± 0.71 ^{a}	1.07 ± 0.31	1.34 ± 0.43
	CYNTU	26.27 ± 2.86	13.99 ± 0.75	0.78 ± 0.20 ^{ab}	0.61 ± 0.31	0.76 ± 0.23
7	CYTI	12.74 ± 0.95 ^{a}	6.02 ± 0.77 ^{a}	1.69 ± 0.80 ^{a}	0.56 ± 0.27 ^{ab}	0.60 ± 0.28
	CYTU	9.56 ± 1.63 ^{a}	9.11 ± 1.29 ^{a}	0.70 ± 0.12 ^{ab}	0.10 ± 0.10 ^{a}	0.14 ± 0.10
	CYNTI	31.65 ± 4.07 ^{b}	16.69 ± 1.43 ^{b}	1.43 ± 0.36 ^{a}	0.52 ± 0.33 ^{ab}	0.42 ± 0.11
	CYNTU	32.28 ± 5.28 ^{b}	19.10 ± 3.89 ^{b}	0.07 ± 0.07 ^{ab}	1.20 ± 0.44 ^{b}	0.14 ± 0.14
14	CYTI	20.97 ± 3.90 ^{a}	12.07 ± 1.54 ^{a}	0.20 ± 0.08	0.00 ± 0.00	0.26 ± 0.11
	CYTU	12.68 ± 1.84 ^{b}	15.22 ± 7.48 ^{b}	0.68 ± 0.18	1.98 ± 0.16	1.54 ± 0.13
	CYNTI	33.41 ± 3.83 ^{c}	17.01 ± 7.21 ^{c}	0.58 ± 0.36	0.09 ± 0.04	0.09 ± 0.09
	CYNTU	34.17 ± 5.14 ^{c}	18.39 ± 4.56 ^{c}	0.13 ± 0.12	0.12 ± 0.09	0.11 ± 0.11
17	CYTI	25.73 ± 0.87 ^{a}	31.17 ± 7.12 ^{c}	0.02 ± 0.02 ^{ab}	0.08 ± 0.08	0.00 ± 0.00
	CYTU	22.70 ± 4.16 ^{b}	26.97 ± 4.93 ^{a}	0.35 ± 0.13 ^{b}	0.28 ± 0.11	0.00 ± 0.00
	CYNTI	36.57 ± 4.30 ^{c}	29.55 ± 9.90 ^{c}	0.30 ± 0.53 ^{a}	0.58 ± 0.44	0.00 ± 0.00
	CYNTU	28.78 ± 4.60 ^{d}	19.43 ± 5.38 ^{b}	0.38 ± 0.21 ^{b}	0.28± 0.12	0.13 ± 0.00

$^{a, b, c,d}$ Different superscripts in a column indicate significant differences between the groups, $p < 0.05$

atrophy with severe lymphocytic depletion in all the follicles and hyperplasia of the follicular epithelium in CY treated group when compared with non-CY treated groups (Plate I) on days 7 to 14 post-CY treatment. Increased numbers of macrophages and few plasma cells were present in the plicae of the bursa. At day 19 post-CY treatments, evidence of lymphocytic regeneration was observed in few follicles in group B/CYTU broiler chickens (Plate II); while marked depletion of lymphocytes and lymphocytic necrosis and depletion was observed in infected groups A/CYTI and C/CYNTI, respectively. Lymphocytic necrosis and depletion with only the reticular cell network remaining were observed in the thymus, caecal tonsils, and spleen of A/CYTI and B/CYTU broilers on days 7 and 14 post-CY treatment (Plates III & IV); while evidence of lymphocytic regeneration was observed in few follicles in group B/CYTU on day 19 post- CY treatment (day 5 PC) which was not observed in vNDV infected group A/CYTI.

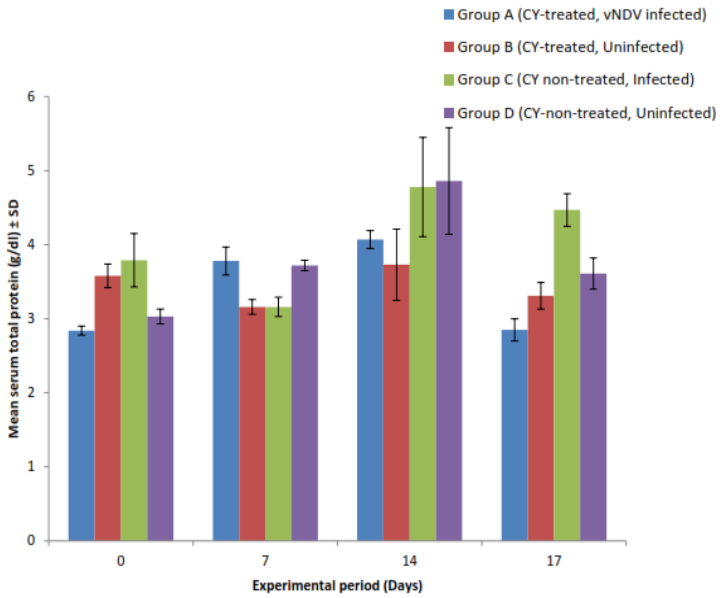

Figure 2: The serum total protein levels of broilers treated with cyclophosphamide and infected with velogenic Newcastle disease virus, Mean **±** SD (g/dl)

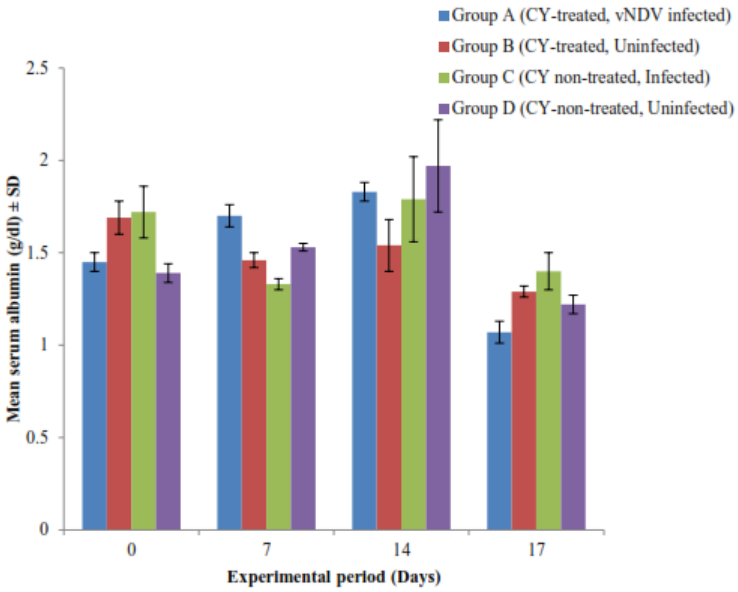

Figure 3: The serum albumin levels of broilers treated with cyclophosphamide and infected with velogenic Newcastle disease virus, (g/dl) ± SD

Discussion

Total white blood cell counts (Total WBCs) and differential leucocyte counts reflect the systemic status of an animal in relation to its response and adjustment to injurious agents, stress, and/or deprivation; the indices are of value in confirming or eliminating a tentative diagnosis, in making a prognosis and guiding therapy (Coles 1986; Wakenelll, 2010). The total WBCs and differential leucocyte counts could further provide information on the severity of an injurious agent, the virulence of an infecting organism, the susceptibility of a host, and the nature, severity and duration of a disease process (Campbell, 1994; Harr, 2009; Latimer & Bienzle, 2010). The findings in the present study of significantly lower total WBC levels in the CY treated groups at days 7 and 14 post-CY treatment showed significant direct cytotoxic effects of CY on circulating white blood cells, and the severity and duration of the cytotoxic process. The decreased total WBCs observed in the present study is of great significance to immunologist, biomedicals and veterinary clinicians as an aid in determination of physiological and pathological status of birds exposed to immunosuppressive agents. The decreased values observed in the present study are also of clinical implication for poultry farmers that ignorantly expose their birds to environmental chemical agents like pesticides or herbicides and environmental immunosuppressant due to lack of biosecurity and

management practise. Considering that white blood cells play a vital role in the body's immune system (phagocytosis) and are involved in production of antibodies (Coles, 1986; Teske, 2010), continued exposure to immunosuppressive agents will increase susceptibility of the host to various infectious diseases. In addition, their decreased values on days 7 & 14 post-CY treatment could be used as indicators of stress response and sensitive markers crucial to immune function in birds exposed to immunosuppressive agents, After a sharp decrease at days 7 & 14 post-CY treatment in group B/CYTU, there was a gradual rise from day 17 post-CY treatment that most likely represented haematopoietic tissue stem cell sparing effect of CY. Post-CY treatment, haematopoietic system expansion above normal levels is observed in some studies and is suggested to be caused by expansion of the stem cell population (Fulton *et al*., 1996; Lohmann & Schreml, 1982).

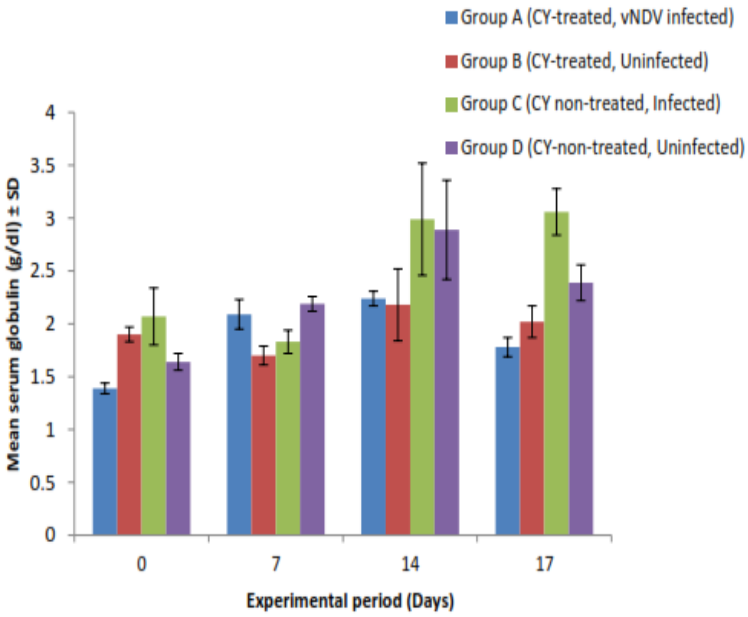

Figure 4: The serum globulin levels of broilers treated with cyclophosphamide and infected with velogenic Newcastle disease virus, Mean ± SD (g/dl)

Results of the differential leucocyte counts showed that heterophils and lymphocytes were significantly decreased. This corresponds to the findings in 3-weeks-old CY-treated chickens (Fulton *et al*., 1996), with heterophils and lymphocytes being the most sensitive cell types.. The heteropaenia observed in this study is suggestive that CY, an immunosuppressive agent, affects production or release of heterophils from the bone marrow. Heteropaenia in CY treatment studies has been reported to be induced by myelosuppression due to prevention of replication of granulocytic precursors in the bone marrow, that is, the mechanism responsible for the myelosuppression occurs as a response to blockade of actively dividing cells within the bone marrow (Fulton *et al*., 1996). Since heterophils have phagocytic function similar to their mammalian

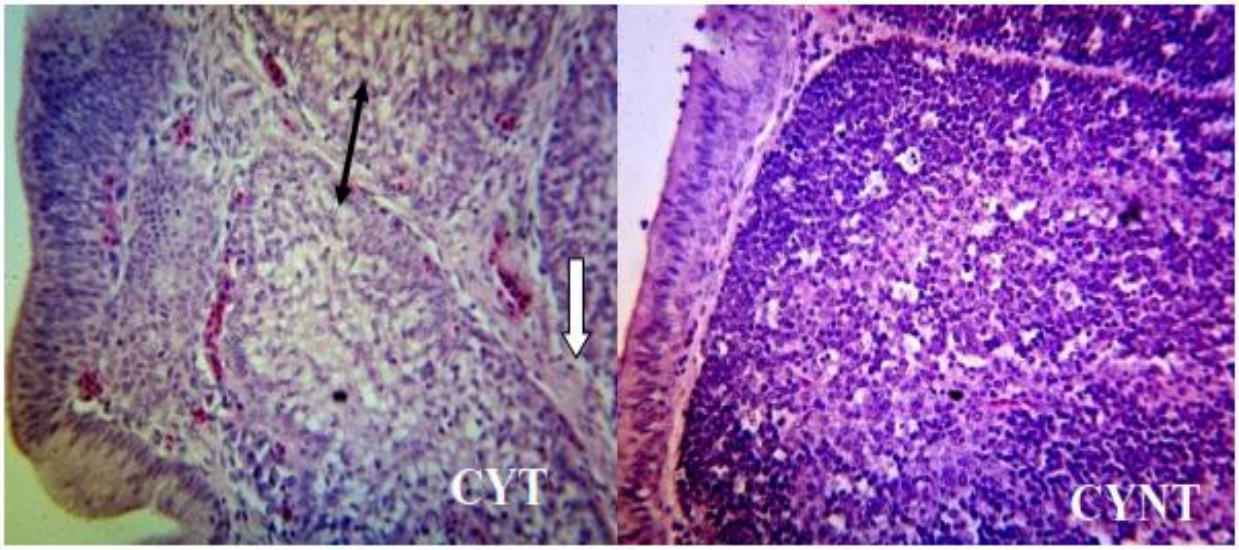

Plate I: Photomicrograph of bursa of Fabricius from cyclophosphamide treated (CYT) and nontreated (CYNT) broiler chickens. A marked depletion of lymphocytes in all the follicles (double arrow), and interfollicular fibroplasia (down arrow) were observed on day 7 post-cyclophosphamide treatment. H&E. X 400

counterparts (Coles, 1986; Teske, 2010), another implication of their decreased values is their inability to perform their vital roles.

A similar pattern of decline and gradual rise was observed in the blood lymphocyte levels compared with those of controls in the present study from day 7 post-CY treatment. Because lymphocytes are the predominant circulating white blood cells in chickens (Fulton *et al*., 1996; Campo *et al*., 2008; Latimer & Bienzle, 2010), lymphocytopaenia recorded in the present study is suggestive of decreased numbers of lymphoid cells due to direct destruction and blockade of actively dividing B and T cells and immune, differentiated lymphocytes by CY toxicity (Colvin, 1982; Hengstler *et al*., 1997). This was supported by histopathology where all the bursa follicles consisted only of macrophages due to generalized depletion of lymphocytes. Also microscopic lesions comprising of severe diffused lymphocytic necrosis and depletion in the thymus, caecal tonsils and periellipsoid lymphoid aggregates in the spleen were observed in the CY treated chickens in this experiment. This is contrary to the results of Fulton *et al*. (1996), in which CY had no effect on thymus at 75mg/kg body weight, but in agreement with the findings of Misra & Bloom (1991) in which bursae accumulated twice the amount of active CY compounds inhibiting mitosis with resultant lymphocytolysis. Also this result agrees with the findings of Winklestein (1973) and Okoye *et al*. (1992) where CY treatment resulted in severe necrosis and depletion of lymphocytes in the thymus and spleen. Cyclophosphamide is an alkylating agent that is toxic to all cells to differing degrees, with lymphoid and haematopoetic cells forming a particularly sensitive target (Hengstler *et al*., 1997), resulting in B-cell depression and depletion, and T-cell suppression and necrosis (Okoye *et al*., 1992; Winklestein (1973). The gradual return to normal peripheral blood lymphocyte counts in group B/CYTU on day 17 post-CY treatment supported by histopathology where multifocal regeneration occurred in few follicles in bursa of Fabricius, and diffused regeneration in thymus and spleen by days 19 to 20 post-CY treatment in this study could be due to the stem cell sparing effect of cyclophosphamide and the continuous replication of lymphocyte precursors in the lymphoid organs (Ahmed & Hombal, 1984).

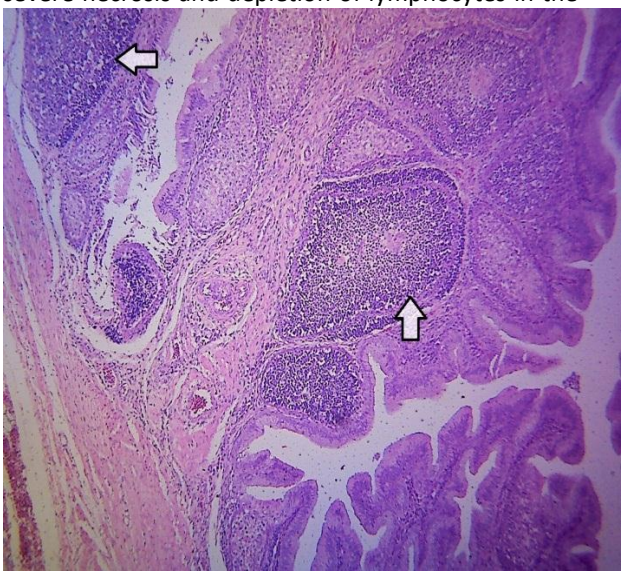

Plate II: Photomicrograph of bursa of Fabricius of group B/CYTU showing regeneration of lymphocyte in some follicles (arrow) on day 19 post-cyclophosphamide treatment. H&E. X100

Heteropaenia was induced from day 7 post-CY treatment, while heterophilia was induced on day 3 PI following vNDV infection in the present study**.** The findings of significantly lower heterophil counts are similar to the findings of Fulton *et al*. (1996) in CY-treated in 3-weeks-old specific pathogen free (SPF) chickens on day 10, and Ficken & Barnes (1988) in turkeys on day 3 post-CY treatment. Although transient heterophilia were induced in chickens and in these turkeys, 24 to 48 hours after CY administration in their studies, which resulted from tissue damage caused by intramuscular injection of CY. In the present study, if heterophilia was earlier induced, probably it would have been negated by the toxic effects of CY.

The reversibility of the leucopaenia in this study on day 17 post-CY treatment also supports the mechanism of myelosuppression as the cause of leucopaenia which coincided with a return of blood heterophil levels that were equal to those of control group.

The subsequent leucocytosis and heterophilia observed in this study following vNDV infection in the infected group C/CYNTI compared with other three groups correspond with the findings of Igwe *et al*. (2017), who reported heterophilia at day 3 post- vNDV infection. This indicates the acute systemic nature of ND and correlates with acute inflammatory changes observed in the present study in the lymphoid organs and in other visceral organs of birds infected with vNDV (Igwe & Eze, 2016; Igwe *et al*., 2018). They are also good indicators of stress in chickens observed in conjunction with tissue damage induced by inflammation or viral infections (Latimer & Bienzle, 2010). Eosinophils, basophils, and monocytes, as

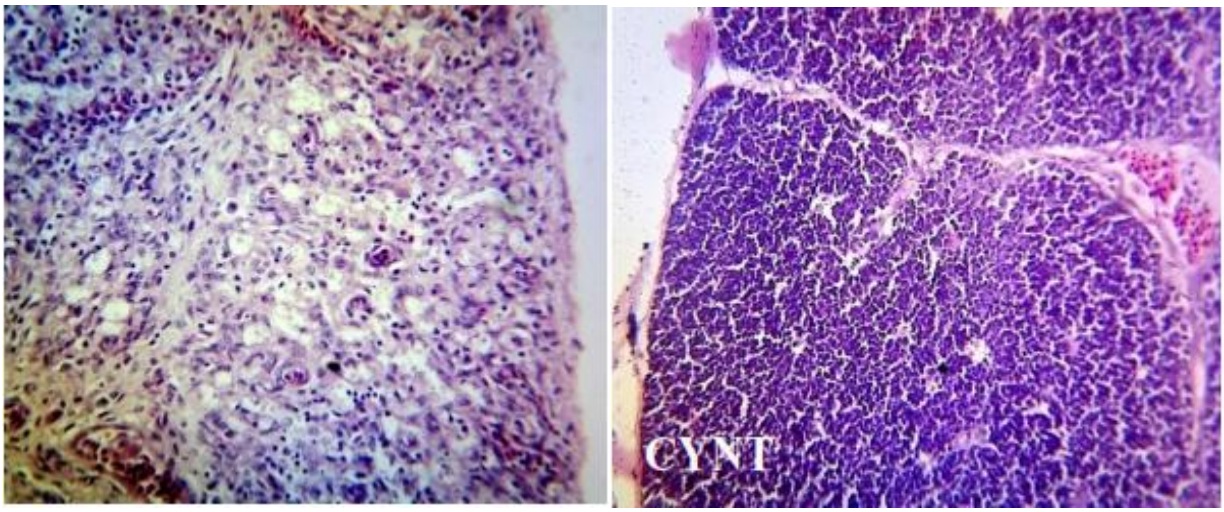

Plate III. Photomicrograph of thymus showing necrosis of lymphocytes following cyclophosphamide treatment in group CYT on day 7 post-treatment. For comparison, a section from normal thymus in untreated group CYNT is shown. H&E. X 400

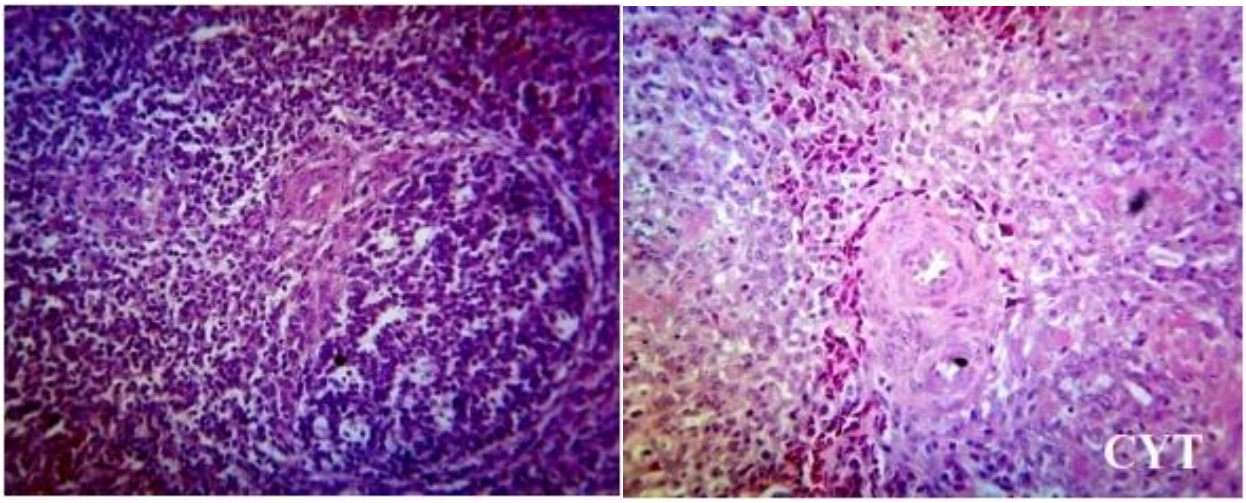

Plate IV. Photomicrograph of spleen showing necrosis of lymphocytes following cyclophosphamide treatment in group CYT (right) on day 7 post-treatment. For comparison, a section from normal spleen (left) in untreated group CYNT is shown. H&E. X 400

represented by a component of the differential blood count, were erratic and represented a small number of cells, which could be due to fluctuations of environmental temperature

Most proteins are produced by the liver, the exception being the immunoglobulins which are produced by the lymphocytes and plasma cells (Harr, 2009). In addition, the liver plays an important role in the uptake, storage, distribution of both nutrients and vitamins from the blood stream and many important metabolic pathways. It should be noted that serum biochemical assessment, along with haematology and physical examination, helps to predict pathological processes in the vital internal organs of the body such as the liver, muscle, heart, pancreas and kidney (Harr, 2009; Cerón *et al*., 2010). Though at the histologic level, no lesion was observed in the hepatocytes, the significantly lower levels of total serum proteins recorded in groups A/CYTI and B/CYTU on days 14 and 17 post-CY treatment is suggestive of decreased protein synthesis by the liver due to toxic effect of CY on the liver affecting its physiologic functional role (hepatic insufficiency). It is also suggestive of protein-losing enteropathies (diarrhoea) and dermatopathies (emaciation, feather loss, and bleeding skin) earlier observed in CY-treated groups coupled with vNDV infection. However, significantly higher total serum proteins recorded in the present study in non-CY, NDV infected group, C/CYNTI is suggestive of dehydration as a result of enteropathies caused by vNDV infection. ND has been reported to cause

haemorrhagic ulcers of mucosa of gastrointestinal tract, and degeneration and necrosis of the associated lymphoid organs (Igwe *et al*. 2014).

The albumin:globulin ratio is a good parameter to indicate health and disease responses (Harr, 2009). The significantly higher albumin and globulin levels in group C/CYNTI is an indication of dehydration and a complex homeostatic response to vNDV infection, respectively. In the present study, globulins increased in response to injury to the liver caused by antigenic challenge from a virulent NDV, which corresponds to the report of Harr (2009). In conclusion, the changes in leucogram, serum proteins and lymphoid organs in broilers could be used as indicators of immunosuppression.

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