THE AFFECT OF DIETARY LYSINE AND PHOSPHORUS DEFICIERNCY ON IMMUNE FUNCTION IN BROILER CHICKEN

by

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(Under the Direction of Jean E. Sander)

ABSTRACT

The influence of lysine and phosphorus deficiency on chicken immune function was conducted with broiler chickens fed lysine-deficient diets at 65% of the control diet and phosphorus-deficient diets at 67% of the control diet. The evaluation of humoral immune function was evaluated by measuring the antibody production to Newcastle disease vaccination using the hemagglutination inhibition (HI) test and enzyme-linked immunosorbent assay (ELISA). The cellular immune function was evaluated by cutaneous basophile hypersensitivity and lymphocyte proliferation test. Result of lysine deficiency indicates reduced humoral immune function measured by ELISA but not by HI and the cell-mediated immune function also was reduced by lysine deficiency. Result of phosphorus deficiency indicates that phosphorus deficiency had no great influence on chicken immune function under the parameters of this study.

INDEX WORDS: Lysine, phosphorus, immunity, broiler, HI, ELISA, hypersensitivity, lymphocyte.

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DEDICATION

To my parents and wife,

whose love, patience and support enabled me

to complete this work

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CHAPTER 1

INTRODUCTION

The nutritional requirement of essential amino acids for chicken has been studied in detail (5, 7, 9, 10, 19, 23). Lysine along with methionine, the most limiting amino acid in corn-soybean diet, which affects many factors such as heat stress, growth rate, feed efficiency etc. (8, 12), has been evaluated in great depth. The reason for such detail is because lysine is used as the reference amino acid to which all other essential amino acids, like metheonine and threonine (13, 14, 15), are added to poultry ration to provide the ideal amino acid ratio. Generally, the proper level of lysine in the diet can maximize chicken growth, especially in meat yield and feed efficiency (3,12,23).

According to National Research Council (NRC) (3), the lysine requirement in the diet for broiler chicks has three levels based on the age of the bird. They are 1.1% of the diet for 0 to 3 wk old chicks, 1.0% of the diet for 3 to 6 wk old chicks and 0.85% of the diet for 6 to 8 wk. chicks. However the level of lysine added to commercial broiler starter chicken diets in field usage is around 1.2% to 1.5%. The NCR's report suggests only the minimum requirement amount, but many factors, such as temperature and diseases, also affect the requirement for lysine (8). Lysine influences growth performance but the

increased performance offered by the addition of lysine will reach a plateau when lysine is over 1.2% of total starter diet (9). Feeding a level of lysine over that which is required by the chicken will result in smaller increases in growth and efficiency. Economically, this becomes an inefficient use of the amino acid. Metabolically, excess lysine cannot be used very efficiently because the receptors for lysine become saturated and the turn over of protein remains at steady state. Beside the effect of lysine on chick growth, different levels of dietary lysine may also affect the immune system (16, 18). In the poultry industry, clinical disease is usually prevented through the use of vaccination. A deficiency of lysine may reduce the immune function of the chicken and result in an economic detriment by the reduced effect of vaccination.

Phosphorus (P) is an important mineral in the chicken. It is involved in muscle and skeletal growth, amino acid and carbohydrate metabolism. Phosphorus is essential in the utilization of energy and is a component of nucleic acid, DNA and RNA. The high energy compounds, *adenosine di-* and *triphosphate*, and *creatine phosphate,* all are derived from phosphorus. Calcium (Ca) and vitamin D_3 are required for the phosphorus utilization. The adequate desired Ca and P ratio is about 2:1 (3, 17). An imbalance or deficiency of Ca or P will cause inefficient unitization of both elements. Vitamin D_3 is also required for the calcium and phosphorus utilization. Proper administrations of vitamin D_3 can optimize

the Ca and P utilization, and also prevent or cure mild calcium and phosphorus deficiency (1, 11, 17). The deficiency of phosphorus or imbalance in the Ca:P ratio of the diet will result in poor skeletal development (rickets) and birds failing to thrive to marketing (17, 20, 21).

According to National Research Council (NRC) report in 1994, the phosphorus requirement in the diet for broiler chicks has three levels based on the age of the bird (3). They are 0.45% of the diet for 0 to 3 wk old chicks, 0.35% of the diet for 3 to 6 wk old chicks and 0.3% of the diet for 6 to 8 wk. The phosphorus level within the blood is an important indicator of nutrition status. The blood contains 35 to 45 mg of phosphorus per 100 ml, most of which is in the blood cells and about 10 % in inorganic form (4, 17).

Studies have been done to determine the benefit of adequate nutrition for optimal poultry production, but there is little data about the interaction of phosphorus and immune function in chicken. Also, since lysine is so commonly limiting in poultry diets, the goal of this study was to determine if there would be an effect on immune function in broiler chickens fed lysine deficient and phosphorus deficient diets.

In both the experiments, the evaluation of humoral immune function was evaluated by measuring the antibody production to Newcastle disease virus (NDV) using the hemagglutination Inhibition (HI) test (22) and enzyme-linked immunosorbent assay

(ELISA). The cellular immune function was evaluated through the use of cutaneous basophile hypersensitivity test (2), and lymphocyte proliferation test (6).

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CHAPTER 2

REVIEW OF LITERATURE

Lysine. Lysine has been determined to be one of the most limiting amino acids in animal diets by many investigators, and the study of lysine requirements also supports those observations (18, 26, 28, 29, 36, 56). However, the terminology of "first limiting amino acid" is not clear in its meaning. Chickens can produce many but not all of the amino acids through the metabolic system. Lysine can not be produced by the system and it must be obtained in the diet. There are ten considered essential amino acids, because they can not be synthesized by the body and must be added in the diet to achieve the nutrient requirements of the chicken (40, 47). Lysine is an important element in the protein structures, like muscle and organs; hence the chicken will not grow well without adequate lysine. When lysine is deficient, many parts of metabolic system will not function and this is the reason lysine is referral to as the "first limiting amino acid".

Research has been conducted to determine the actual requirement of lysine in the chicken. According to National Research Council report in1994. (14), the lysine requirement for broiler chickens has three levels. They are 1.1% for 0 to 3 wk. old chicks, 1.0% for 3 to 6 wk. old chicks and 0.85% for 6 to 8 wk. old chicks. The actual

requirement in field usage is around 1.2%, because many elements, like temperature and diseases, will affect the utilization (27, 44).

Lysine has a significant influence on growth (5, 7, 9, 10, 11). If a relationship chart of chicken growth performance and lysine is drawn, the growth performance will reach a plateau at the ratio of around 1.2% lysine in the total diet (28, 56). Above this amount of dietary lysine, some improvements will still occur but the increase becomes less. Therefore, except in some special situations such as recovery after disease, adding excess lysine in the diet is not cost effective due to inefficient utilization. Excess lysine is not used efficiently because the receptors for lysine are saturated. Therefore, the turn over of protein remains at steady state and the requirement of lysine will decrease slowly (20, 26, 29).

Lysine is a very important element in the diet but other amino acids are also essential. Methionine, threonine and valine, are usually thought of as second, third and fourth limiting amino acids in corn soybean diets (26, 35, 39). Compared to lysine, they are not as important for growth because the requirement of lysine is mainly used for protein synthesis and its influence is very important in the chicken. Methionine and threonine perform complex and multiple roles in chicken growth, therefore specific changes on one system are less apparent (26, 35, 39). The balance of amino acids in the

diet becomes important because there is evidence that an imbalance of amino acids will cause poor growth and reduced diseases resistance (2, 11, 30, 45).

Digestible lysine in the diet. Not all lysine in the diet is digestible. Normally, the concentration of total lysine in diet ranges from 0.5% to 0.7% (25), but the digestible lysine within is around 80%. In the report of NRC in 1994 (14) recommended 1.1% for 0 to 3 week old broiler, 1.0% for 3 to 6 week old broiler and 0.85% for 6 to 8 week old broiler. The concentration of available lysine in the feed ingredients is usually not enough and this means additional lysine such like L-lysine HCl or another synthetic form of lysine must be added into the diet to meet the requirement. Extensive work has been done to estimate lysine digestibility. One common method to estimate the digestibility of lysine is by comparing intubated chickens fed a basal diet to a feed deprived control, and measures the excretion level of the amino acid in the feces (17, 25). However, a good base diet of corn and soybean meal for chicken growth is adequate, when fortified with essential levels of crystalline amino acids (2, 3, 17, 18).

The lysine requirement of male and female broiler chicken. Much work has been done to estimate an accurate amount of lysine required by broiler chickens, which supports the recommendation of NRC (14, 28, 36, 56). However, there is still not sufficient data about the specific requirements of male and female broiler chickens (28,

56). There is some suggestion that male broilers needs more lysine than female broilers due to their higher growth and body composition. In the research of Han and Baker (22), male broiler chickens required higher levels of lysine than females, because males had more protein and less fat in their weight gain. Male chicks eat more feed and gain weight faster than females by about 12% (25). However, the actual feed efficiency between sexes is similar (25, 56). Breast yield does not tend to increase along with the lysine level (25). The recommended dietary lysine content for male chickens is 1.01% and 0.99% for female chickens during 3-6 weeks of age (25), which is very close to the NRC recommendation. Vazquez (56) has done similar work on 0-3 weeks old chickens and their prediction of 1.209% of lysine for both male and female chicken is a little higher than the NRC standard**.**

The lysine requirement of fast and slow growing broiler chickens. Han and Baker (25) tried to determine whether fast and slow growing broilers at the same age required the same or different lysine contents in the diet. According to the experimental data, no difference was found. The efficiency of lysine utilization for these two strains was similar; fast-growing chicks do not require a higher concentration of dietary lysine than slow-growing chicks (28).

The influence of heat stress. Heat can be a problem during the rearing of commercial broiler chickens. Mendes and Watkins (44) indicated 37 C heat-stressed males do not appear to require a higher level of dietary lysine than those housed at 24 C**.** Compared to males, female may have a higher requirement for lysine at 37 C than at 24 C. Panting was observed in chicks maintained in the 37 C temp, but the chicks seemed to adapt the condition over a 14-day period (44). Heat stress also reduced feed intake by 22% and weight gain by 20% in both male and female chicks. The main effect of sex difference was significant on feed efficiency, with male chicks having higher gain:feed ratios than females (44). The metabolism of protein is one main heat producer, which means high protein containing diet will cause chicken to produce more heat than normal diet. Therefore, the amino acid balance in diet is very important for heat-stressed chicks (27, 44).

The influence of body weight within different strain. Regardless of sex, crossbred chicks selected for heavy body weight gain faster and eat more feed than those selected for light body weight (27). Both heavy and light body weight gain birds need the same level of lysine to achieve maximal growth. However, selective heavy weight gain chicks need higher lysine level to achieve the maximal feed efficiency (27, 44).

Phosphorus. Phosphorus (P) is an important mineral in the chicken for bone formation. It is also a major component involved in muscle, amino acid and carbohydrate metabolism. Phosphorus is essential in the utilization of energy and is a component of nucleic acid, DNA and RNA. The high energy compounds, *adenosine di-* and *triphosphate*, and *creatine phosphate,* all are derived from P. Calcium (Ca) and vitamin D_3 are required for the P utilization. The adequate desired Ca and P ratio is about 2:1 (14, 40). An imbalanced or deficiency of Ca or P will cause inefficient unitization of both elements. Vitamin D_3 is also required for proper absorption of Ca from the gut. Proper administrations of adequate vitamin D_3 can optimize the Ca and P utilization, and also prevent or cure mild calcium and P deficiency. The deficiency of P or imbalance in the Ca:P ratio of the diet will result in poor skeletal development (rickets) and birds fail to thrive (40, 50, 51). Under the deficiency of either Ca or P, the excess of either element will precipitate the other in the intestine and block absorption.

Beryllium in the diet also causes rickets because of the formation of insoluble beryllium phosphate which blocked the absorption of P (47).

According to NRC report in 1994 (14), the P requirement in the diet for broiler chicks has three levels based on the age of the bird. They are 0.45% of the diet for 0 to 3 wk old chicks, 0.35% of the diet for 3 to 6 wk old chicks and 0.3% of the diet for 6 to 8

wk. The P level within the blood is important indicator of nutrition status. The blood contains 35 to 45 mg of P per 100 ml, most of which is in the blood cells and about 10 % in inorganic form (16, 40).

The availability of phosphorus. Inorganic P existing in soil and rock is unavailable to chicken and other animals. Rock P must be heat treated to convert it into other forms, such as α-, or β-tricalcium phosphorus (47). The calcined or heated P causes a structural change making it available to the chicken. Also, the γ-calcium phosphorus, β-calcium phosphorus, γ-calcium metaphosphate and calcium phosphate are available to chicken. Potassium metaphosphate is unavailable to chicken (47).

Phytin P is unavailable to chicken as with other simple-stomach animals unless supplemented with phytase enzyme (40). Only the ruminant can naturally utilize phytin P due to the production of phytase by the rumen microflora. About one third of P in plants is present as non-phytin P and available to chickens (47). Therefore, the supplement of phytase in the diet has resulted in improvements in chicken growth and P utilization which lowers the dietary requirement of non-phytate P (33, 34, 47, 59). In cottonseed meal, the phytase treated diet has almost complete hydrolysis of the phytin and releases available P for chicken utilization. This also frees some protein from protein-phytate complex in the meal and reduces the gossypol toxicity of the glanded cottonseed meals

(47). However, according to Boling (8) the phytase does not improve protein utilization. There is additional improvement, however in metabolizable energy by the phytase hydrolysis (47). Another way to improve the P utilization within corn-soybean meal diets is with citric acid (9, 10). However, the mechanism of how citric acid improves the P utilization from the diet is not clear (10). One possible hypothesis is the binding of citric acid with Ca reducing the inhibitory effects of Ca on intestinal phytic acid hydrolysis, which increased the release of available P in diet (9, 10).

Avian Immunity. The immune system is a mechanism used by animals to protect themselves from foreign components by eliminating the foreign materials or destroying these components before or while they cause damage inside the animal`s body (1). Therefore, the immune system is equipped with the ability to recognize which components belong inside the body and what is foreign material. Basically, the foreign material is called the antigen (Ag) and one major responding immune component is the antibody (Ab). A wide variety of high molecular weight compounds can act as Ag`s (1, 46). However, the portion, which is recognized by an Ab or T-cell receptor (TCR), is referred to as an epitope. The relationship between Ag and Ab is like a key and lock in

that they must match exactly (46). Therefore the immune system must have the ability to respond to the enormous diversity that exists in the nature.

The immune system of chickens has been studied for a long time beginning with the discovery of the bursa of Fabricius**,** which produces the B cell responsible for the humoral part of the chicken immune system (48, 54). The immune system of the chicken is remarkably sophisticated and shares many similarities with the equivalent mammalian systems. However, there are some important differences, such as the ontogeny of the B cell and the different isotypes of Ab. The avian immune system is divided into specific and non-specific immune mechanisms (48). Specific immune mechanisms, on one hand, are characterized by specificity, heterogeneity, and memory (46). Specificity indicates the immune responses are specific for distinct Ag`s. Heterogeneity expresses the diversity of immune system and the memory function enables the immune system to be capable of remembering the foreign material for future response. These mechanisms are divided into cellular and humoral components. Non-specific immune mechanisms include the innate or inherent mechanisms through the action of cytotoxic and natural killer (NK) cells which help the chicken resist disease (1, 46).

Chickens generate Ab`s in response to antigenic stimulation and this is called the humoral response. The dominant Ab-producing cell is B-lymphocytes. The Ab is

responsible for recognition and neutralization of a specific Ag (1, 46). The combination of Ab and Ag will neutralize or lyses the Ag and then the Ag will be eliminated by the other cell mechanisms. There are three major classes of Ab`s in chickens, IgM, IgG (or called IgY) and IgA (48). Because of the specific character of Ab, every individual has more than $1X10^7$ different Ab molecules ready for all kinds of Ag`s (1, 58). In order to generate all the complicated diversity of Ab`s to respond to the wide variety of foreign materials, chickens achieve this goal by gene conversion just like mammals (1).

Another important immune cell is T lymphocytes. It is the principal cell of the cellular immune response, also called the cell-mediated response (1, 48). As in mammals, chicken T cells engage in helper and cytotoxic functions to achieve the immune mechanism. The chicken T cell differentiates into two pathways, α/β and γ/δ, which are different from mammals (12). Like the enormous number of Ab, the T cell generates its diversity through gene combinatorial and junctional mechanisms similar to the mechanisms that operate in mammalian TCR (1).

For non-specific immune mechanisms, the innate effecter mechanism includes those mediated by NK cells and Ag dependent cellular cytotoxcity (ADCC) (1, 46). Also, protein hormones, called cytokines, secreted by activated T lymphocytes can enhance the

ability of whole mechanism. Recently, genes of several avian cytokines have been cloned and expressed that allow detailed research to be done. (58)

However, many naturally occurring viruses and environmental factors cause immunosupression in chickens. Mechanisms and strategies have been developed, such as immunomodulation, to enhance immune responsiveness in commercial poultry (46, 54).

The Ontogeny of the immune response. The immune response consists of two fundamental divisions; the humoral or cellular mediated reactions (1, 46). These two components closely collaborate to eliminate infectious organisms and carry out immune surveillance for tumors. The ability to differentiate between self and non-self is a fundamental function of all immune reactions. Exposure to foreign Ag`s not only triggers an immune response, it also results in the development of immune memory, which enables an enhanced response that will deal with further exposure. (1, 46, 58)

The primary lymphoid organs**,** such as bone marrow, thymus and bursa of Fabricius**,** are the locations where lymphocytes develop and the T cell and B cell receptor genes rearrange (48). The lymphocytes then migrate to secondary lymphoid organs such as spleen, intestinal lymphoid tissue and the avian lymph nodes. Avian lymph nodes are functionally similar to mammalian lymph nodes, however, they lack an external capsule and the sheaths of lymphoid tissue containing germinal centres surround lymphatic

vessels (48, 49). The bursa of Fabricius can act as both as primary and secondary lymphoid organ. Naive T-cells and B-cells can be stimulated to replicate in the secondary organs (54). The resultant cells are memory cells and effector cells that in the case of B-cells are plasma cells responsible for the production of immunoglobulins (55).

The cells of the immune system are extremely mobile. Their targeting of foreign material is assisted by adhesion molecules on blood vessel endothelium and corresponding molecules exist on the lymphocytes and other circulating cells (1). These mechanism greatly enhance the response ability of immune cells.

Cells of innate immunity. In addition to macrophages, NK cells and the cells of ADCC also contribute to the innate immunity in chickens (1, 46). Avian NK cells are large granular lymphocytes and are thus morphologically similar to mammalian NK cells (46). They lack surface markers expressed by T cells but do contain cytoplasmic CD3 γ, δ and ε chains, indicating they may be derived from T cell lineage (46). The NK cells also express certain cytokine receptors and can be maintained *in vitro* as long-term cultures in the presence of cytokine-rich conditioned media (1, 12, 46).

The NK cells selectively identify and lyse virus-infected and tumor target cells (1, 46, 48, 58). These cells are present naturally in chickens and do not need prior antigenic exposure for target recognition. Unlike cytotoxic T cells, the NK cells are not Major

Histocompatability Complex (MHC) -restricted; in fact down-regulation of MHC-I surface expression by virus infection or tumor transformation may enhance the susceptibility of the cells to NK lysis (48, 58). Early immune responses especially those involving viruses and tumors rely on NK cells which are cytotoxic, produce interferon **(**IFN**)** and carry out Ag dependant cell mediated cytotoxicity (15, 58).

B cell development (humoral response). The predominant circulating immunoglobulin in birds is IgY (15), the avian equivalent of mammalian IgG. This is a relatively rigid molecule without a hinge region and one additional domain. Chicken IgY is the isotype responsible for maternal immunity as it is transferred to the chick via the yolk sac (15, 48).

Chickens also have pentameric IgM similar to mammalian IgM, which is expressed on the surface of most B cells as B cell receptor (15, 49). Chicken IgA, which has at times been referred to as IgB, is different from mammalian IgA. Immunoglobulin A is the major secretory immunoglobulin and plays a major role in local immunity. It forms a dimer, which binds to a poly-immunoglobulin receptor on mucosal epithelial cells (15, 48, 58). It is transported to the luminal surface of the cells where it is discharged still attached to a fragment of the receptor, which is known as a transport piece. Diversity of immunoglobulin variable regions is generated in a manner that is quite different from

their mammalian counterparts (55). Germline genes contribute very little to the diversity, which relies heavily on somatic mutation (55).

Avian B-cells do not appear to produce IgD and its function in mammals is also unclear (48). Allergic responses as in mammals are initiated by degranulation of mast cells, which are likely to have IgE receptors (48).

T cell development (Cell-mediated immunity). T lymphocytes are the principal cells of the cellular immune response. Many studies show a number of monoclonal antibodies can be used to identify the TCR and differentiation of the Ag`s have been used to categorize T cells into subsets in chickens (48, 49). The T-cell precursors migrate from locations such as bone marrow to the thymus, which is the location where intense selection pressure is applied (15). Cells are selected for their ability to recognize the host cells through MHC mechanism, and their ability not to recognize self-peptides (1, 48, 49). Similar to mammals, the avian TCR is a multi chain complex and consists of two parts, which are formed by glycoprotein chains designated aṣ αβ or γδ̣ units (12, 48). They are used for the signal transudations of the CD3 complex and the antigenic binding site of the complex. The γδ̣ T cells can be identified by TCR 1 monoclonal antibodies and αβ̣ T cells by TCR 2 and TCR 3 monoclonal antibodies. Thus, the avian T cells differentiate into two different pathways based on the expression of the two TCR chains (12, 48, 49).

The study of T cell development in birds is facilitated by the relative simplicity of the avian αβ TCR gene loci, especially with regard to variable (V) gene diversity (12). There are only two avian Vα and two Vβ gene families, Vβ1 and Vβ2 (12). In birds and mammals, TCR diversity is generated largely by variation of the nucleotide sequence in the third complementarity-determining region formed by the joins between rearranged V, D (diversity), and J (joining) genes (12, 48). Since the avian Vβ1 and Vβ2 gene segments combine with a single D and the same four Jβ gene segments, the Vβ1 and Vβ2 subpopulations of T cells may recognize a similar spectrum of antigenic peptides (12, 48). Different physiological roles for the $V\beta1^+$ and $V\beta2^+$ subsets of T cells are nevertheless implied by the preferential homing of $V\beta1^+$ T cells to mucosal surfaces of the body, where they help B cells to produce secretory IgA. The limited sequence homology between the Vβ1 and Vβ2 genes (~30 percent) suggests their receptor chain products may recognize different types of peptide-presenting MHC molecules (42, 49). The biological rules for pairing of the two Vβ families with the two Vα families to form the αβ TCR are being explored (42, 49). Studies of avian ontogeny indicate the influx of thymocyte progenitors in waves. Each progenitor wave gives rise to γδ T cells three days earlier than αβ T cells and to alternating waves of departure of these subpopulations en route to the periphery (12, 49).

The avian TCR γ locus contains three Vγ subfamilies, three Jγ gene segments, and a single constant-region Cγ gene (12, 49). The members of each Vγ subfamily undergo random V-Jγ rearrangement during each embryonic wave to generate a diverse TCR γ gene repertoire early in ontogeny (12). There is evidence that suggests a relationship between αβ and γδ T cells (48, 49, 58). The γδ T cells require growth factors produced by αβ T cells, and the activated γδ T cells in turn may present the helper αβ T cells with antigenic peptides via their class II MHC molecules (12, 49, 58).

T-cells bearing the CD8 accessory molecule mainly have a cytotoxic function while the T-cells with the CD4 accessory molecules develop to be helper T-cells (1, 12). In mammals two major groups are recognized, Th1 and Th2. Th1 cells predominantly produce IFN and stimulate cell mediated responses while the Th2 cells produce a quite different array of cytokines and stimulate Ab production (1, 12).

In chickens the heterophils, which are the equivalent of the mammalian neutrophils, destroy foreign organisms by phagocytosis (15). Antigens may also be phagocytosed by the mononuclear phagocytic system, which consists of circulating macrophages and a range of similar cells throughout the tissues. Macrophages may be activated to destroy intracellular organism by the action of cytokines or chemical messengers produced by T-cells, which recognize the foreign Ag`s (15). The macrophages are also able to destroy

tumor cells and are important sources of secreted proteins such as the complement components. Antigens, which are partly degraded to peptides, may be presented on the surface of the phagocytic cell in association with the MHC II molecules (23, 42). This is the form that is recognized by helper T-cells. Foreign peptides are also presented by B-cells and by the Ag presenting cells known as dendritic cells (23, 42).

A second group of MHC proteins, the MHC I molecules also present peptides, which are derived from the cytoplasm of cells (23, 42). These MHC I molecules sample the cytosol of cells allowing cells that are changed by intracellular organisms or neoplastic events to be recognized. These altered cells are recognized by the cytotoxic T-cells which discharge granules which containing perforins responsible for producing pores in the cell membrane and granzymes which initiate the destruction of the cell (23, 42). In addition, lymphotoxins, which are in the family of tumor necrosis factor, destroy target cells by initiating apoptosis. Apoptosis is a form of regulated, physiologic cell death in which the nucleus undergoes condensation and fragmentation (15). The dead cell will be rapidly phagocytoesed without its inside content released (1).

Immune response assay. For the humoral immune response, there are several detection and measurement methods. Basically, they are divided into three categories, primary binding, secondary binding and tertiary (*in vivo*) binding tests (53). The

sensitivity of tests is also in the same order. Primary binding tests, like Enzyme Linked Immunosorbent Assay (ELISA) and competitive radioimmunoassay, are the most sensitive test to directly measure interaction between Ag and Ab. The secondary binding tests, like ring test, gel precipitation, hemagglutination-inhibition (HI) and virus neutralization, measure the formation of immune complexes *in vitro* (52, 53). The tertiary binding tests, like passive cutaneous anaphylaxis, measures the development of humoral immune response *in vivo*. The secondary and tertiary binding tests are usually less sensitive than primary binding test because they do not directly measure the interaction between Ag and Ab (52, 53). However, they are thought to be easier to perform and can provide more practical information for the immune response.

Cellular immune response tests are more difficult to perform than humoral response because live whole animals are needed for testing (52, 53). Among the cellular immune response tests, the skin test and cutaneous basophil hypersensitivity test are very popular (13). The skin test is based on the positive delayed hypersensitivity reaction that may occur with infectious disease where cell-mediated immunity plays a significant role. The immune response is measured by the extent of the lesion in the inflammatory area. The cutaneous basophil hypersensitivity is caused by Ag`s triggering a similar inflammatory response in the skin (13). The lesion is infiltrated with large number of basophils as well

as mononuclear cells in response to the presence of Ag. This test can be performed in the chicken interdigital skin and detects the immune response by measurement of the thickness of the skin (13).

Enzyme Linked Immunosorbent Assay (ELISA), Enzyme Immunoassay (EIA) or Solid-Phase Immuosorbent Assay (SPIA), are a very sensitive laboratory methods used to detect the presence of Ag**'**s or Ab's of interest in a wide variety biological samples (19, 52, 58). Many variations in the methodology of the ELISA have evolved since its development in the 1960s but the basic concept is still the imunological detection and quantitation of single or multiple Ag or Ab in one sample (usually serum) (19, 52). It became very popular with the advanced computer and equipment support. Many automatic systems have been introduced into the laboratory, making the routine assay of Ab much easier. Also, many inexpensive commercial test kits for ELISA are available.

Hemagglutination-inhibition (HI) Test. Test is commonly used to quantitate a specific Ag response in chickens. The inhibition of hemagglutinating (HA) Ag by specific antibodies is the basis of the HI test, so the basic components of the HI test are the HA**-**Ag and serum (52, 57). The principal of HI is to use the red blood cell as a visual marker to measure the ratio of Ab against HA**-**Ag in serum. The constant-serum (β technique) is used much more than is the constant-antigen (α technique) (52, 57).

Because of the obvious color, red blood cells in the assay serve as indicator particles, allowing easy verification of hemagglutination. If a high titer Ab exists in the serum, a negative hemagglutination tests will result. This is because the high titer Ab binds to the Ag first and the Ag cannot bind to red blood cell to hemagglutinate. However, if serum containing low titer antibody is used, a positive test will result because the Ab cannot bind to all of the Ag and Ag will bind to the red blood cell resulting in hemagglutination. However, there is an exception to this; when a serum containing very high titer Ab is used, a positive hemagglutination can result. This is caused by the excess Ab binding to the red blood cell, causing the red blood cells to form a network similar to agglutination (52, 57). This assay is easy to perform but aseptic technique is essential.

Hemagglutination inhibition determines the minimum dilution of serum required to completely inhibit agglutination of red blood cells in the presence of an agglutinating Ag. Typically, sera are diluted 2-fold and added to the Ag followed by the addition of red cells (52, 57). Three or five fold dilution may also be used for more sensitive results. The highest dilution that inhibits agglutination is the "titer" of that serum (i.e. A titer of 640 means that the last dilution to completely inhibit agglutination of red cells was 1:640). Usually, eyesight is used to judge the test titer, so evaluatior bias may occur. Therefore it

takes time and experience to establish a self-standard in order to ensure the repeatable result.

Immunosuppression. Stress, viruses, mycotoxins and imbalanced nutrition can induce immunosuppression that contributes to the pathogenesis of a variety of infectious diseases in poultry. Immunosuppressed birds respond poorly to commonly used vaccines and experience an increased incidence of opportunistic infections (7, 52). For example, environmentally or socially stressed poultry are more susceptible to *Mycoplasma gallisepticum* and to viral diseases such a Newcastle disease, hemorrhagic enteritis and Marek`s disease (15). Although the mechanisms of immunosuppression are not fully understood and seem to be different for each virus or environmental element, certain common features still exist. Immunosuppressed, birds usually show a reduced function of humoral and cellular immunity (7, 15, 52). Interestingly, immunosuppression also decreases the nutritional requirement (37). Reduced immune function lowers the nutrition ingredient demand of chicken and also inhibits growth. The impact of immunosuppression in poultry can be reduced by improving the environment. This is done by routine cleaning of facility and maintaining the quality of the feed.

Immunomodulation. To enhance the immune responsiveness, there are many strategies currently being developed. Immunomodulation refers to the pysiological or

pharmacological manipulation of the immune system to affect a beneficial outcome for the poultry flock in a particular situation (46, 52). In poultry production, the goal of immunomodulation is often to minimize the incidence or severity of infectious disease. Thus beneficial manipulations are often presumed to enhance the immune response or decrease the threshold required for initiating a response. Synonyms for positive immunomodulation include immunopotentiation, immunostimulation, immunotherapy and biological response modification (46, 52). Immunomodulation differs from vaccination in that it is non-specific, and does not require prior knowledge of challenging pathogens. However, immunomodulation can improve the vaccinal immunity and promote responses for increasing the protection (46, 52). Immunomodulation could also reduce the negative impact when immunosuppression is caused by extraneous agents or by certain vaccine viruses.

Amino Acids, Lysine, Phosphorous and Immunity. In the study of Lotan on rats (41), supplementation of lysine not only supports rate of growth but also has some effect on the thymus weight and other parameters of immune system, including increasing the ability of the body to reject skin allografts. However, he also noted the effect of lysine on body weight also correlates with liver, spleen and thymus weights. The studies of Jose in

mice (31) and Klasing in chickens (37) have reported similar results showing the limitation of lysine in diet had a slight depressive effect on the immune response.

In the study of Konashi (38), several essential amino acid deficiencies in broiler chickens were evaluated and the effect on the immune response of a specific amino acid deficiency could not be generalized. But the combination of isoleucine, leucine and valine deficiency in diet has the great potential to affect the immune response. They concluded that amino acid deficiencies may modify cell-mediated immune response in chickens, which related to the development of the lymphoid organs and antibody production. Glick et.al. (21, 22) has done similar work on amino acid deficiencies and immune response in chickens. They found the serum protein of chickens fed amino acid deficient diets was significantly reduced, and this was related to the humoral immune response. For cell-mediated immune response, the delayed hypersensitivity response to human gamma globulin was also significantly reduced. These findings are in accordance with Bounous` study in mice (11), where restricted amino acids in the diet suppressed the humoral and cell-mediated immune response.

In the study by Kegley (32), the results suggest that increasing supplemental phosphorus increased average daily gain and enhanced cell-mediated immune response but decreased humoral immune response in pigs. The phytohemogglutinin (PHA)

response to lymphocyte blastogenesis assay was increased by increasing supplemental P. The antibody level to injection of sheep blood cells and ovalbumin decreased by increasing supplemental P. However, this is in contrast to the study of Aslam in broiler chickens (5), in which the antibody titer against sheep blood cells increased with the increased available P concentration.

Newcastle Disease. Newcastle disease (ND) is avian viral disease that occurs around the world. It can cause up to 100% mortality in susceptible chickens. While many avian species may become infected, dramatic losses are seen most often in the domestic fowl and turkeys, and to a lesser extent in pheasants (4). Because it has the potential to cause great impact in poultry industry, it is one of the most important diseases of 20th century.

Newcastle disease was named by Dolye in 1926 because he reported the first outbreak on a farm near Newcastle-upon-Tyne, England (4). The name was coined by Dolye as a temporary measure because he wished to avoid a descriptive name that might be confused with other diseases. Nevertheless the name of ND is considered to be better than names which attempted to describe the other diseases and is probably the reason the name has lasted more than 60 years (4, 24).

After the first reported outbreak in Newcastle-upon-Tyne, many cases began to emerge worldwide, including Java, Ranikhet in India, Colombo in Sri Lanka (Ceylon), Korea and Manila. All the cases appeared to cause severe disease during the period from 1926 to 1940 (4). Also, several of the outbreaks were seen near seaports, so disease transmission was thought to be transported from the tropic forests of south–east Asia to other countries by transportation of refrigerated meat (4). At the time, this disease was considered a highly lethal and capable of killing all ages of birds. In the United States of America, this highly virulent disease did not appear during that time, but a relatively mild poultry disease called avian penumoencephalitis was reported in California by Beach in 1944 (4). Its symptoms were similar to ND and the bird's response was indistinguishable from ND virus on immunological tests. Although this disease was mildly virulent, it also presented a major threat to the poultry industry during that time. How long this disease existed in USA before it was identified is not known but there is evidence showing this disease was already present on the east coat in 1938. Subsequently, many cases of ND were reported all over the world. By 1966, the situation stabilized and a highly virulent virus was endemic in the tropics, a milder disease was occurring in North America and western Europe and an intermediate type was present in Iran and Arab countries to the west. It had been spread to most of the countries of Middle east by the end of 1960s. By

the middle of 1970s, the vaccination and slaughter of infected farms helped control the spread of this disease, but the cost was high (4, 24).

Classification and Epidemiology. Newcastle disease virus is a paramyxovirus with a diameter about 100-200 nm. Its origin is paramyxoviridae family. This family includes two subfamilies and four genera. The subfamily paramyxovirinae includes rubulavirus, paramyxovirus and morbillivirus. The other subfamily pneumovirinae, has a single genus pneumovirus. There are nine serogroups of avian paramyxoviruses, PMV-1 to PMV-9. Newcastle disease is classified as type 1 avian paramyxovirus (PMV-1) (24). All of the PMV type viruses have the identical morphology and helical symmetry structure in RNA with the envelopes containing the neuraminadase spikes and hemagglutinin. The enzyme neuraminadase and hemagglutinin serve as surface Ag`s. Dimer formation of hemagglutinin with avian red blood cells results in hemagglutination, allowing the use of the hemagglutination inhibition test to identify the antigenic variation between strains (24). Based on this, there were twelve different strains developed by cross serum neutralization, but there is no significant difference between strains after advanced study (4, 24). However, the pathotypes still remain distinct. Beard and Hanson, grouped NDV strains and isolates into five pathotypes according to their disease signs**:** 1) viscerotropic velogenic (VVND), or Doyle`s form, is an acute, lethal infection of all ages of chickens

and the sign for it is prominent hemorrhagic lesions in the guts**,** 2) neurotropic velogenic (NVND), or Beach`s form, causes high mortality with respiratory and neurological signs, but no gut pathology**,** 3) mesogenic NDV, Beaudette`s form, has low mortality but some respiratory and nervous signs**,** 4) lentogenic NDV, Hitchner`s form, causes mild or inapparent respiratory infections**,** and 5) asymptomatic enteric NDV presents light gut lesion and no obvious disease (4, 24). However, these five groups still do not cover all types, because cross reaction between strains sometimes makes the identification so difficult.

Pathogenicity Tests. In order to identify the different pathotypes, several test methods were devised (52). The most common tests used in the laboratory are the intracerebral pathogenicity index (ICPI) and the intravenous pathogenicity index (IVPI). The ICPI is determined by using the day old specific-pathogen-free (SPF**)** antibody free chick test. It uses the chick's brain as virus host and mortality is the indicator of pathogenicity. The indicator uses numbers from 0 and 2**;** "0" means normal, "1" is sick and "2" if dead, so the more pathogenic strains give higher scores. The IVPI test is conducted in six-weeks-old SPF chicks. The score system is similar with the ICPC but is

scored between 0-3 to determine the severity. Beside these two tests, there are mean death time in eggs (MDT) and intracloacal inoculation pathogenicity test in chickens (4, 24, 52).

Isolation and culture. In order to obtain successful virus samples of avian paramyxovirues from birds, the most common material is feces or tracheal swabs. In addition to those, clinical lesions should be collected because the different strains will infect different organ**s**. For VVND, the common collection sites are lung, spleen, liver, heart and brain. Because the infectivity of virus will be reduced by putrefaction, the collected sample must be chilling for further transportation and storage (4, 24, 52).

For proper culture of avian paramyxoviruses, there are many available cell-culture systems and most of them need the addition of trypsin (0.01 mg/ml) to accelerate growth (52). However, the most sensitive method is the inoculation of embryonated chicken eggs. For this method, nine to ten days old embryonated chicken eggs should be obtained from SPF or NDV Ab free flocks and prepared for inoculation into the allantoic cavity (52). The suspension of virus containing medium needs to be washed and centrifuged to make a concentrated suspension. After inoculation, the eggs should be placed at 37 C and incubated for five to seven days. Egg yolk inoculation appears an alternative way to culture the virus and has been successful in some cases (4, 52).

Hemagglutination inhibition (HI) test and enzyme-linked immunosorbent assay (ELISA) for NDV. Hemagglutination inhibition test is commonly used to detect the immune response to NDV or the other avian paramyxoviruses. Usually, the ND virus will be treated with formalin to inactivate it. Different strains of NDV will have different titer response, and there are some strains which are resistant to the formalin treatment (52). Therefore, viral preparation of Ag for HI test must be carefully tested. When conducting an HI test after vaccination, the time for detection is dependent on the type of vaccination. Some vaccines will result in a detectable level of Ab after seven to eight days, but some just need two or three days (52, 57).

Another test used is ELISA, which has become very popular with the development of advanced computer and analysis equipment. The introduction of these automatic systems into the laboratory, makes the routine assay of Ab much easier than before. The preparation of ELISA test is similar to the HI and they both need Ab. The basic difference is ELISA needs a culture plate with specific pre-coated Ab on it and some response indicators (52).

The comparison of HI and ELISA is difficult due to differences in sensitivity and specificity, but there is some degree of agreement between them when analyzed statistically (6, 19, 43).

Studies have been done to determine the benefit of adequate nutrition for optimal poultry production, but there is little data about the interaction of lysine and phosphorus with immune function in chicken. The goal of this study was to determine if there would be an effect on immune function in broiler chickens fed lysine deficient and phosphorus deficient diets.

In both the experiments, the evaluation of humoral immune function was evaluated by measuring the antibody production to NDV using the HI test (52, 57) and ELISA. The cellular immune function was evaluated through the use of cutaneous basophile hypersensitivity test (13), and lymphocyte proliferation test (23).

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CHAPTER 3

THE INFLUENCE OF LYSINE IN CHICKEN IMMUNE FUNCTION[1]

[1]Chen, C., J. E. Sander and N. M. Dale. To be submitted to Avian Disease.

SUMMARY. The influence of lysine deficiency in chicken immune function was conducted with broiler chickens fed lysine-deficient diets with lysine at 65% of the control diet. The evaluation of humoral immune function was evaluated by measuring the antibody production to Newcastle disease vaccination (NDV) using the hemagglutination inhibition (HI) test and enzyme-linked immunosorbent assay (ELISA). The cellular immune function was evaluated through the use of cutaneous basophil hypersensitivity test and lymphocyte proliferation test. Result indicates that humoral immunity assays show a reduction in antibody response to NDV vaccination in broiler chickens fed a lysine deficient diet, when measured by ELISA but not when measured by HI. The cell-mediated immune function also was also reduced by lysine deficiency.

INTRODUCTION

The nutritional requirement of essential amino acids for chicken has been studied in detail (4, 10, 12, 14, 24, 29). Lysine, one of the most limiting amino acid, which affects many factors such as heat stress, growth rate, feed efficiency (12, 15) etc., has been evaluated in great depth. The reason for such detail is because lysine is used as the reference amino acid to which all other essential amino acids, like metheonine and threonine (16, 17, 18), are added to poultry ration to provide the ideal protein ratio. Generally, the proper level of lysine in the diet can maximize chicken growth, especially in meat yield (3, 15) and feed efficiency (29).

According to National Research Council (NRC) report in 1994 (3), the lysine requirement in the diet for broiler chicks has three levels based on the age of the bird. They are 1.1% of the diet for 0 to 3 wk old chicks, 1.0% of the diet for 3 to 6 wk old chicks and 0.85% of the diet for 6 to 8 wk. chicks. However the level of lysine added to commercial broiler chicken diets in field usage is around 1.2% to 1.5%. The NCR's report suggests only the minimum requirement amount, but many factors, such as temperature and disease, also affect the utilization of lysine (12). Lysine influences growth performance but the increased performance offered by the addition of lysine will reach a plateau when lysine is over 1.2% of total diet (10). Feeding a level of lysine over

that which is required by the chicken will result in smaller increases in growth and efficiency. Economically, this becomes an inefficient use of the dietary costs. Metabolically, excess lysine cannot be used very efficiently because the receptors for lysine become saturated and the turn over of protein remains at steady state. Beside the effect of lysine on chick growth, different levels of dietary lysine may also affect the immune system (22, 23). In the poultry industry, clinical disease is commonly prevented through the use of vaccination. A deficiency of lysine may reduce the immune function of the chicken and result the reduced effect of vaccination.

Many studies have been done to determine the benefit of adequate nutrition for the optimal poultry production, but there is little data about the interaction of lysine and immune function in poultry. The goal of this study was to determine if there would be an effect on productivity and immune function in broiler chickens fed a lysine deficient diet. The evaluation of humoral immune function was evaluated by measuring the antibody production to Newcastle disease vaccination (NDV) using the hemagglutination inhibition (HI) test (28, 30)and enzyme-linked immunosorbent assay (ELISA). The cellular immune function was evaluated through the use of cutaneous basophil hypersensitivity test (2), and lymphocyte proliferation test (9).

MATERIAL AND METHODS

Animals: The experiment was conducted utilizing four hundred and eighty unvaccinated one-day-old Ross X Ross broiler chickens, which were purchased from a nearby commercial hatchery. All chickens were wing banded and housed in electrically heated pens on floor and fed *ad libitum* for the first two weeks with the standard starter diet, which consisted 23% of crude protein and 3147 Kcal/Kg metabolizable energy, provided from University of Georgia feed mill. When they were twelve days old, all chickens were individually weighed and bled. Enzyme-linked immunosorbent assay was performed on all blood samples in order to select out two hundred and forty chickens, which would carry the most uniform maternal antibody level against NDV. Then, at fourteen days old, these two hundred and forty chickens were randomly assigned into floor pens located in environmentally regulated houses. Light was provided 23 hours daily and nipple-drinking water system provided consistent water supply. The birds also began to receive control (nutritionally balanced) and experiment diets (lysine deficient) at this point. The control and experiment diets would be weighed before being fed to the chickens. Feed and water were provided *ad libitum*. Also, the mortality was counted and the feed efficiency calculated at the end of the experiment.

Diets: The control diet was formulated to meet the normal nutrient requirement that would meet the NRC (3) recommended levels as listed in Table 3.1. The lysine deficient diet was formulated to include all the same amino acids as in the control diet with lysine at 65% of the control diet as in Table 3.2. Both the diets were based on cotton seed meal and similar ingredients were added to provide recommended nutrient requirements, resulting in 22% crude protein and 2950 Kcal/Kg metabolizable energy.

Experimental design: The effect of lysine deficiency was evaluated on feed conversion, body weight, lymphoid organ weights (thymus, spleen and bursa of Fabricius), lymphocyte proliferation, cutaneous basophil hypersensitivity responses and antibody titer against NDV by ELISA and HI tests.

The two hundred and forty selected birds were randomly divided into sixteen groups. At fourteen days of age, one half of the sixteen groups were fed a lysine deficient diet and the other half groups were fed a control diet. At nineteen days of age, after five days of diet adaptation, half of the groups on each diet were vaccinated for NDV by eye drop. The NDV vaccine (B1 type, B1 strain, Select Laboratories, Inc., Gainesville, Georgia (ND-0620)) was used for the vaccination after rehydration as described in the manufactures instructions. Treatment groups consisted of control diet with NDV vaccination, lysine deficient diet with NDV vaccination, control diet with no-NDV

vaccination and lysine deficiency diet with no-NDV vaccination. There were four replicates per treatment group and fifteen birds in each replicate. Vaccinated groups were housed in separate but identical housing to avoid potential vaccine spread. At forty two days of age, birds were individually weighed, bled and euthanized by cervical disarticulation at the end of the experiment, which was at forty two days of age. The lymphoid organs (thymus, spleen and bursa of Fabricius) were carefully removed and weighed individually.

Hemagglutination inhibition (HI) test and enzyme-linked immunosorbent assay (ELISA): Besides the original baseline bleeding before grouping birds, blood samples were collected twice again at twenty-eight days and forty-two days of age from all the experiment chickens. The collected blood samples were allowed to sit overnight at room temperature to separate blood cells and serum, which were used for ELISA and HI tests.

Hemagglutination inhibition test was performed according to the method described (28, 30). Briefly, 25 ul each serum sample diluted in 100ul of NDV antigen was placed in the first column of ninety-six-well plates. The rest of the wells in the plate contained 50 ul of NDV antigen. Then two fold serial dilutions followed by passing 50 ul from one column to the next column to the end of the plate. The antigen (NVD-EP-2 LaSota, Poultry Disease Research Center, Athens, GA) used to dilute the serum contained 10

Hemagglutination activity units (HA). Then, 50 ul of prepared 0.5% chicken red blood was added into each well and forty-five minutes of incubation at room temperature was requirement for the hemagglutination action before results were observed. The HI titer was determined by the well of the highest serum dilution where a clear button was seen. In this study, triplicate tests were made to assure the accuracy of data reading.

Enzyme-linked immunosorbent assay was done by commercial Newcastle disease antibody test kit test that purchased from IDEXX (IDEXX Laboratories Inc., Westbrook, MW). Titers were obtained and calculated as described by IDEXX. Briefly, 5 ul of each serum sample was used to perform the test and an optical density of 650 nm wavelength was used for reading the color change. Triplicate tests were also made in this assay.

Evaluation of the cutaneous basophil hypersensitivity responses: Three days after the NDV vaccination, at twenty two days of age, five birds per pen from each replicate including vaccinated and non-vaccinated groups were randomly selected to perform the cutaneous basophil hypersensitivity assay as described by Corrier (2). All the birds were intradermally injected in the toe web between 3^{rd} and 4^{th} digits of right foot with 100 ug of Phytohemagglutinin (PHA-P) (SIGMA Chemical CO., St. Louis, Missouri), in 0.1 ml of phosphate buffered saline (PBS) solution. The left foot of each

chicken was also injected with 0.1ml of PBS in the corresponding toe web to serve as a control. The toe web thickness was measured before and after the injection.

The evaluation of the cutaneous basophil hypersensitivity response was determined by measuring the toe web thickness, which was measure by a constant-tension, dial micrometer (Mitutoyo Ltd., Aurora, IL). Two methods were used, which were PHA1, which measured the difference in thickness of inter-digital skin before and after the PHA-P/PBS solution injection, and PHA2, PHA-P post-injection skin thickness minus the PBS post injection skin thickness (2).

Lymphocyte proliferation assay: At twenty two days of age, five birds from each pen, which were not included in the cutaneous basophil hypersensitivity assay, including all the replicates from both NDV vaccinated and non-vaccinated group, were randomly selected to perform lymphocyte proliferation assay. At twenty six days of age, this assay was repeated again to compare the result. As described by Gogal (9), 1.5 ml blood from each bird was collected into a heparinized (SIGMA Chemical CO., St. Louis, Missouri) syringe. Blood from five birds per pen would be pooled in one tube, mixed well to prevent clotting, and left at room temperature during the blood collecting process. After blood collection, the pooled blood was centrifuged for 10 min at 50 x g. The lymphocyte-rich buffy coat layer, which formed on top of the blood was gently swirled to

collect and was held on ice. This centrifugation and swirl technique was done twice to maximize lymphocyte collection. After collection of the suspension, it was washed three times with cold RPMI-1640 (SIGMA Chemical CO., St. Louis, Missouri) and centrifuge at 250xg at 4C. Then, the centrifuged pellet was suspended into 2 ml complete media, which contained cold RPMI-1640 containing 10% fetal bovine serum (SIGMA Chemical CO., St. Louis, Missouri), 2mM glutamine (ICN, Costa, CA), 50 IU Penicillin (ICN), and 50 mg/ml streptomycin (ICN). One hundred ul of the cell suspension was measured and incubated for 5 min with 900 ul Natt-Herrick stain, then lymphocytes were counted on a hemacytometer. Lymphocytes stained dark blue, and thrombocytes had a dull stain. The lymphocytes were then resuspended into complete media and adjusted to $5x10^6$ lymphocytes/ml. The lymphocyte proliferation assay was performed in 96-well round bottom tissue culture plate. One hundred ul of adjusted lymphocyte suspension was added to triplicate wells containing 100 ul of either complete media alone, which served as negative control for baseline proliferation, 100 ul of media containing 50 ug/ml concanavalin A (ConA, SIGMA), or 100 ul of media containing 2.5 ug/ml phytohemagglutin (PHA-P, SIGMA). The plates with lymphocytes would be incubated at 37 C with 5% CO_2 in a tissue culture incubator for 24 hr. After 16-18 hr of culture, 20ul of the Alamar blue (Accumed International Inc.,Westlake, Ohio) was added to each well

and returned to the incubator. Then the plate was read on ELISA plate reader at 595 nm wavelength after full 24 hrs incubation.

The evaluation of read out optical density data was calculated as stimulation index (SI) as describe by Frome (6).

Statistical analysis: Data collected from all the experiments were subjected to ANOVA procedures by using General Linear Model's procedure of SAS software (SAS Institute, 2001). The difference level $P \leq 0.05$ will be considered as significant.

RESULTS

Body weight and the weight of thymus, spleen and bursa of Fabricius: Table 3.3 shows the final body weight, the weight of thymus, spleen and bursa of Fabricius as well as feed efficiency, which were obtained at the end of the experiment. The table also presents the ratio of these immune organs to the body weight. The body weight of the lysine deficient diet groups was significantly lower than the control diet groups. There was no significant difference between NDV and non-NDV vaccination group on the lysine deficient diet. However, there was a significant difference based on vaccination status in birds fed the control diet. The non-NDV vaccinated groups had lower body weights than the NDV vaccinated groups.

For the weight of spleen, thymus and bursa of Fabricius, there is significant ifference between the control and lysine deficiency diet groups regardless of vaccination status. From the view of correlation percentage of body weight and immune organs, there are some differences. Lysine deficiency does not affect the correlation rate except for the thymus, where a lysine deficient diet resulted in a relative decrease in the thymus weight. Here, the vaccination with NDV did not show a significant effect on thymus weight. The spleen to body weight ratio was significantly larger for chickens not vaccinated when compared to NDV vaccinated birds regardless of the diets. This was also seen with the bursa of Fabricius.

Mortality rate for all treatment groups was low (data not shown). The NDV vaccinated control diet group had one dead bird and two birds died among lysine deficient group. For non-NDV vaccination group, there was one dead bird in both control and lysine deficient group.

The chickens fed with lysine deficiency diet also had decreased feed efficiency on both NDV and non-NDV vaccination groups. The non-NDV vaccination groups have better feed efficiency in general.

Enzyme-linked immunosorbent assay (ELISA): Table 3.4 shows the relative ELISA titers, which has three time frames. Before the NDV vaccination, all the groups

show no significant difference except for the control diet group for non-NDV vaccination, which was significantly lower than the others. The ELISA titers are low and would be considered negative in the non-NDV vaccinated group despite the increase over time. Within the NDV vaccinated groups, the control and lysine deficient diet groups do have significant difference. The lysine deficiency groups show much lower ELISA titers at both 10 days and 25 days after vaccination. The ELISA titer climbs through the experiment as expected after NDV vaccination for both control diet groups and lysine deficiency groups, but birds fed adequate lysine produced significantly more antibody than the lysine deficiency groups. There is significant difference in ELISA titer between non-NDV and NDV vaccination groups as expected.

Hemagglutination inhibition (HI) test: Table 3.5 shows the hemagglutination inhibition (HI) test titers at three time frames, before NDV vaccination, and 10 and 25 days after NDV vaccination. Before the NDV vaccination, all the groups show no significant difference. At 10 and 25 days after NDV vaccination, there is no significant difference between control and lysine deficiency diet group for both NDV and non-NDV vaccination groups. However, there is significant difference between NDV and non-NDV vaccination group. Also the HI titer climbs through the experiment for the NDV vaccination groups on both control and lysine deficient diet groups as expected.

Cutaneous basophil hypersensitivity responses: Table 3.6 shows the cutaneous basophil hypersensitivity responses to PHA-P. There were two different methods to interpret the response (2). Using the PHA1 method of calculation, there is significant difference between control diet group and lysine deficient diet group on both NDV and non-NDV vaccination groups. Using the PHA2 method of calculation again, there is again a significant difference between control and lysine deficient diet group for both non-NDV vaccination groups and NDV vaccination groups.

Lymphocyte proliferation assay: Table 3.7 shows the result of Lymphocyte proliferation assay. The value is presented as stimulation index (SI). For ConA, there is no significant difference between control and lysine deficient diet groups regardless of the NDV vaccination status at day 4 and day 8 post NDV vaccination. There is increase in stimulation index (SI) from day 4 to day 8 post NDV vaccination. With PHA-P, there was a significant reduction in SI in non-NDV vaccinated chickens fed lysine deficient diet.

DISCUSSION

It is clear from the results reported that overall body weight and the weight of thymus, spleen and bursa of Fabricius were affected by the lysine deficiency diet. The reduced body weight has been already reported to be significant features of lysine

deficiency diet on broiler chickens (14, 18, 29). The smaller size of all immune organs was most likely related to the overall body weight reduction. This finding is similar to both Lotan et al. and Okumura and Mori`s studies (23, 27), where lysine deficiency severely reduced the body and organ weights. All the data suggest lysine is a key amino acid for chicken growth, which agree with the studies of Jensen et al. and Okumura and Mori (15, 27). Comparing the feed efficiency, the control diet group had better feed efficiency rate than lysine deficiency group on both NDV and non-NDV vaccination groups.

In the immune organs weights relative to body weight percentage, only the thymus had significant difference between control and lysine deficient diet groups, with chickens fed a lysine deficient diet having smaller thymus. This finding is similar to Lotan`s study on rat growth (23), suggesting that lysine may have effect on thymus growth.

Feed efficiency of both control diet groups and lysine deficiency diet groups of NDV vaccinated and non vaccinated was worse than normal average, which is close to 2.0 at seven weeks of age (26). The reduced feed efficiency occurring in control diet groups was not expected. The lower than expected feed efficiency may be caused by chickens digging out the feed from the feeding pan. Some researches indicated that

immunosuppression caused a reduction of the amino acid requirement, which resulted lower feed efficiency (19, 20, 21).

The ELISA data before diet treatment shows most of the chickens had antibody titers in the low but expected range except for chicken which were placed in the control diet groups with non-NDV vaccination. Although the birds were randomly placed, this group has a standard deviation larger than mean, which indicates the individual variance is large within this group. The HI data at day 0 post-vaccination shows the corresponding groups have no significant difference from the other groups. Viewing the ELISA data at 10 days and 25 days of NDV vaccination, there is significant difference between the control and lysine deficient diet groups which were vaccinated for NDV but there is no significant difference within non-NDV vaccinated groups. The former result indicates a reduced response to vaccine by a lower production of antibodies to NDV in the lysine deficient groups. The later result could be simply explained as no NDV stimulation in non-NDV vaccinated groups. The HI data shows there is no significant difference between control and lysine deficient diet groups within NDV or non-NDV vaccination groups. In other studies of HI and ELISA relationship (1, 25), ELISA and HI have a highly significant agreement between these two tests. However, the dose or route of inoculation of NDV vaccine may influence the correlation between them. Both ELISA

and HI titer shows shows a response to NDV vaccination throughout the experiment. ELISA test suggests the lysine deficient diet reduced the antibody production, but the HI test only shows numerical decrease. These observations were similar with the studies of Glick et al., Hill, and Klasing (7, 13, 20), where the deficiency of lysine may have had a slight effect on the formation of antibody.

Generally speaking, the HI test is more specific and ELISA is quantitative or sensitive. Hemagglutination inhibition test is based on the binding of a specific hemogglutinin antigen of NDV. Because of the nature of NDV, it has several surface antigens which could be bound by non specific antibody within chicken serum, as a result the ELISA has greater sensitivity but less specific and accurate (5).

The cellular immune response detected by cutaneous basophil hypersensitivity responses shows lysine deficient diet groups have a significantly reduced response to the mitogen compared to control diet groups. Two different methods to compare the result were used in order to eliminate the influence from different individual toe web thickness. Different chickens have different body weights and inflammatory reactions. PHA1 only shows the inflammatory degree that each chickens response to the PHA-P injection. PHA2 eliminate the different toe web thickness within each individual chicken. When both results are considered, a more specific result is obtained. Our observation indicate

the lysine deficiency has reduced the function of the T cell against this mitogen to some degree, which agrees with report of Jose and Good in mice (16) and Glick et al. in chicken (8). The result is also in agreement with the observation of reduced thymus weight in this study.

For the lymphocyte proliferation assay, there is no significant difference between control and lysine deficiency diet groups regardless NDV vaccination status. Basically, the result suggests the lysine deficiency has no statistically significant effect on cellular immune function. The lymphocyte proliferation assay appears to have a high standard deviation within all of the groups, which maybe due to the experimental skill or individual difference.

CONCLUSION

Dietary lysine deficiency causes a major reduction in the body and thymus weight of chickens. Lysine also has some degree of influence in chicken immune function. Humoral immunity assays show a reduction in antibody response to NDV vaccination in broiler chickens fed a lysine deficient diet, when measured by ELISA but not when measured by HI. The cell-mediated immunity function also was also reduced by lysine deficiency.

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Table 3.1 Composition of the control diet fed to broiler chickens for 42 days.

Ingredients	Percentage
Yellow corn	48.030
Cottonseed ML	42.000
Fat, Poultry	5.667
Limestone	1.127
Phos. Defluor.	1.531
Lysine-L 78.4	0.596
Salt	0.308
Meth DL-98	0.253
Br. Vitamins	0.250
Soy Meal 48	0.163
Tr. Min. / Br.	0.075
Calculated composition	
ME. (Kcal/Kg)	2950.000
Crude protein	22.000
Arginine	2.180
Lysine	1.250
Methionine	0.550
Meth & Cystine	0.920
Tryptophane	0.250
Crude Fat	7.490
Crude Fiber	1.210
Calcium	1.000
Phosphorus-AV	0.450
Phosphorus-TO	0.840
Sodium	0.210
Xanthophyll (mg/Kg)	10.560
Choline (mg/Kg)	1141.010

Table 3.2 Composition of the lysine deficient diet.

Ingredients	Percentage
Yellow corn	47.835
Cottonseed ML	42.000
Fat, Poultry	5.448
Limestone	1.136
Phos. Defluor.	1.520
Salt	0.308
Meth DL-98	0.239
Br. Vitamins	0.250
Soy Meal 48	1.204
Tr. Min. / Br.	0.075
Calculated composition	
ME. (Kcal/Kg)	2950.000
Crude protein	22.000
Arginine	2.220
Lysine	0.810
Methionine	0.540
Meth & Cystine	0.920
Tryptophane	0.260
Crude Fat	7.270
Crude Fiber	1.230
Calcium	1.000
Phosphorus-AV	0.450
Phosphorus-TO	0.840
Sodium	0.210
Xanthophyll (mg/Kg)	10.560
Choline (mg/Kg)	1169.630

Table 3.3 Relative weights of thymus, spleen and bursa of Fabricius and feed efficiency of NDV vaccinated and non-NDV vaccinated broiler chickens fed control and lysine deficiency diets.

	NVD vaccination				Non-NDV vaccination			
	Control		Lysine Deficiency		Control		Lysine Deficiency	
	Mean[C]	SD	Mean	SD	Mean	SD	Mean	SD
BW[A] (gm)	1910.3[a]	285.09	1401.91[c]	215.39	1786.71[b]	244.04	1347.57[c]	190.36
SW[A] (gm)	2.66[a]	0.67	2.02[b]	0.50	2.84[a]	0.78	2.15[b]	0.54
SW/BW[B] (%)	0.14[a]	0.035	0.144[a]	0.032	0.16[b]	0.043	0.16[b]	0.034
TW[A] (gm)	6.05[a]	2.04	3.90[b]	1.50	6.21[a]	2.14	4.00[b]	1.41
TW/BW[B](%)	0.316[ab]	0.095	0.274[c]	0.085	0.350[a]	0.123	0.294[bc]	0.087
BFW[A] (gm)	1.10[a]	0.37	0.77[b]	0.20	1.19[a]	0.40	0.83[b]	0.27
BFW/BW[B](%)	.057[b]	0.018	0.057[b]	0.014	.068[a]	0.022	0.062[ab]	0.022
FE[A]	2.575413		3.125962		2.280576		2.687604	

[A]BW, body weight; SW, spleen weight; TW, thymus weight; BFW, bursa of Fabricius weight; FE, feed efficiency

[B]Organ label followed by /BW presented as body weight relative percentage

[C]Mean values within a row followed by different lower case superscripts are significantly different: $P \leq 0.05$.

Table 3.4 Enzyme-linked immunosorbent assay titer of broiler chickens fed control or lysine deficient diets at the time of and after Newcastle disease virus vaccination.

	NVD vaccination				Non-NDV vaccination			
	Control		Lysine Deficiency		Control		Lysine Deficiency	
Days Post Vaccination	Mean[A]	SD	Mean	SD	Mean	SD	Mean	SD
0 days	77.32[a]	112.04	76.4[a]	99.76	50.79[b]	105.97	98.89[a]	125.82
10 days	2258.47[a]	3538.7	766.19[b]	1516.9	104.69[b]	282.13	111.63[b]	213.14
25 days	8213.53[a]	5553.9	5331.15[b]	4455.1	131.62[c]	315.77	231.18[c]	427.69

[A]Mean values within a row followed by different lower case superscripts are significantly different: $P \leq 0.05$.

Table 3.5 Hemagglutination inhibition test titer of broiler chickens fed control or lysine deficient diets at the time of and after Newcastle disease virus vaccination.

	NVD vaccination				Non-NDV vaccination			
	Control		Lysine Deficiency		Control		Lysine Deficiency	
Days Post Vaccination	Mean[A]	SD	Mean	SD	Mean	SD	Mean	SD
0 day of age	14.58[a]	10.39	15.69[a]	10.79	14.92[a]	10.72	13.9[a]	12.18
10 days	90.68[a]	92.64	89.83[a]	79.28	5.76[b]	6.49	3.56[b]	6.09
25 days	254.41[a]	673.15	200.69[a]	256.97	7.29[b]	7.84	8.22[b]	11.62

[A]Mean values within a row followed by different lower case superscripts are significantly different:$P \leq 0.05$.

Table 3.6 Cutaneous basophil hypersensitivity responses, measured in mm, elicited by an injection of phytohemagglutinin-P, using a phosphate buffer saline as the control.

	NVD vaccination				Non-NDV vaccination			
	Control		Lysine Deficiency		Control		Lysine Deficiency	
	Mean[A]	SD	Mean	SD	Mean	SD	Mean	SD
PHA1[B]$_{(mm)}$	1.11^{a}	0.13	0.90^{b}	0.19	1.08^{a}	0.25	0.91^{b}	0.14
PHA2[C]$_{(mm)}$	0.63^{a}	0.15	0.47^{bc}	0.17	0.58^{ab}	0.24	0.41^{c}	0.15

[A] Mean values within a row followed by different lower case superscripts are significantly different:$P \leq 0.05$.

[B]PHA1 was calculated by subtracting pre-injection skin thickness from post-injection skin thickness of the right toe web injected by PHA-P/PBS solution.

[C]PHA2 was calculated by post-injection of right toe web skin thickness, PHA-P/PBS solution injected, minus left toe skin thickness, PBS solution injected.

Table 3.7 Lymphocyte proliferation assay stimulation index from broiler chickens fed control or lysine deficient diets after Newcastle disease virus vaccination.

	NVD vaccination				Non-NDV vaccination			
	Control		Lysine Deficiency		Control		Lysine Deficiency	
Days Post Vaccination	SI[A]	SD	SI	SD	SI	SD	SI	SD
ConA								
4 days	0.87[a]	0.43	0.89[a]	0.35	0.68[a]	0.33	0.89[a]	0.10
8 days	1.51[a]	0.54	1.14[a]	0.26	1.47[a]	0.53	1.70[a]	0.20
PHA-P								
4 days	0.80[ab]	0.50	1.23[a]	0.33	0.74[ab]	0.40	0.50[b]	0.13
8 days	1.56[a]	0.53	1.17[a]	0.60	1.28[a]	0.55	1.86[a]	0.34

[A]Stimulation index (SI) values within a row followed by different lower case superscripts are significantly different: $P \leq 0.05$.

CHAPTER 4

THE INFLUENCE OF PHOSPHORUS IN CHICKEN IMMUNE FUNCTION[1]

[1]Chen, C., J. E. Sander and N. M. Dale. To be submitted to Avian Disease.

SUMMARY. The influence of phosphorus deficiency on chicken immune function was studied with broiler chickens fed phosphorus-deficient diets with phosphorus at 67% of the control diet. The evaluation of humoral immune function was evaluated by measuring the antibody production to Newcastle disease vaccination (NDV) using the hemagglutination inhibition (HI) test and enzyme-linked immunosorbent assay (ELISA). The cellular immune function was evaluated through the use of cutaneous basophil hypersensitivity test and lymphocyte proliferation test. Result indicates that 67% phosphorus deficiency has no influence in chicken immune function within the parameters of this study.

INTRODUCTION

Phosphorus (P) is an important mineral in the chicken. It is involved in muscle and skeletal growth, amino acid and carbohydrate metabolism. Phosphorus is essential in the utilization of energy and is a component of nucleic acid, DNA and RNA. The high energy compounds, *adenosine di-* and *triphosphate*, and *creatine phosphate,* all are derived from phosphorus. Calcium (Ca) and vitamin D_3 are required for the phosphorus utilization. The adequate Ca and P ratio is about 2:1 (5, 12). An imbalanced or deficiency of Ca or P will cause inefficient unitization of both elements. Vitamin D_3 is also required the calcium and phosphorus utilization. Proper administrations of vitamin D_3 can optimize the Ca and P utilization, and also prevent or cure mild calcium and phosphorus deficiency (2, 10, 12). The deficiency of phosphorus or imbalance in the Ca:P ratio of the diet will result in poor skeletal development (rickets) and birds fail to thrive (12, 15, 16).

According to National Research Council (NRC) report in 1994 (5), the phosphorus requirement in the diet for broiler chicks has three levels based on the age of the bird. They are 0.45% of the diet for 0 to 3 wk old chicks, 0.35% of the diet for 3 to 6 wk old chicks and 0.3% of the diet for 6 to 8 wk. The phosphorus level within the blood is an important indicator of nutrition status. The blood contains 35 to 45 mg of phosphorus per 100 ml, most of which is in the blood cells and about 10 % in inorganic form (6, 12).

Studies have been done to determine the benefit of adequate nutrition for optimal poultry production, but there is little data about the interaction of phosphorus and immune function in chicken. The goal of this study was to determine if there would be an effect on immune function in broiler chickens fed a phosphorus deficient diet. The evaluation of humoral immune function was evaluated by measuring the antibody production to Newcastle disease virus (NDV) using the hemagglutination inhibition (HI) (17, 18) test and enzyme-linked immunosorbent assay (ELISA). The cellular immune function was evaluated through the use of cutaneous basophil hypersensitivity test (4), and lymphocyte proliferation test (9).

MATERIAL AND METHODS

Animals: The experiment was conducted utilizing four hundred and eighty unvaccinated one-day-old Ross X Ross broiler chickens, which were purchased from nearby commercial hatchery. All chickens were wing banded and housed in electrically heated pens on the floor and fed *ad libitum* for the first two weeks with the standard starter diet, which consisted 23% of crude protein and 3147 Kcal/Kg metabolizable, provided from University of Georgia feed mill. When they were twelve days old, all chickens were individually weighed and bled. Enzyme-linked immunosorbent assay was performed on all blood samples in order to select out two hundred and forty chickens,

which would carry uniform maternal antibody level against Newcastle Disease. Then, at fourteen days old, these two hundred and forty chickens were randomly assigned to floor pens located in environmentally regulated houses. Light was provided 23 hours daily and nipple-drinking water system provided consistent water supply. The birds also received control (nutritionally balanced) and experiment diets (phosphorus deficient) at this point. The control and experiment diets would be weighed before being fed to the chickens. Feed and water were provided *ad libitum*. Also, the mortality was counted and the feed efficiency calculated at the end of the experiment.

Diets: The control diet was formulated to meet the normal nutrient requirement that would meet the NRC (5) recommended levels as listed in Table 4.1 The phosphorus deficient diet was formulated to include all the same amino acids as in the control diet with phosphorus at 67% of the control diet as listed in table 4.2. Both the diets were based on cotton seed meal and similar ingredients were added to provide recommended nutrient requirements, resulting in 22% crude protein and 2950 Kcal/Kg metabolizable energy.

Experimental design: The effect of phosphorus deficiency was evaluated on feed conversion, body weight, lymphoid organ weights (thymus, spleen and bursa of

Fabricius), lymphocyte proliferation, cutaneous basophil hypersensitivity responses and antibody titer against NDV by ELISA and HI tests.

The two hundred and forty selected chickens were divided into sixteen groups. At fourteen days of age, half of the sixteen groups were fed a phosphorus deficient diet and the other eight groups were fed the control diet. At nineteen days of age, after five days of diet adaptation, half of the groups on each diet were vaccinated for NDV by eye drop. The NDV vaccine (B1 type, B1 strain, Select Laboratories, Inc., Gainesville, Georgia (ND-0620)) was used for the vaccination after rehydration as described in the manufactures instructions. Treatment groups consisted of control diet with NDV vaccination, phosphorus deficient diet with NDV vaccination, control diet with no-NDV vaccination and phosphorus deficient diet with no-NDV vaccination. There were four replicates per treatment and fifteen birds in each replicate. Vaccinated groups were housed in separate but identical housing to avoid potential vaccine spread. At forty two days of age, birds were individually weighed, bled and euthanized by cervical disarticulation at the end of the experiment. The lymphoid organs (thymus, spleen and bursa of Fabricius) were carefully removed and weighed individually.

Hemagglutination inhibition (HI) test and enzyme-linked immunosorbent assay (ELISA): In addition to the original baseline bleeding before grouping birds, blood

samples were collected twice again at twenty-eight days and forty-two days of age from all the experiment chickens. The collected blood samples were allowed to sit overnight at room temperature to separate blood cells and serum, which was used for ELISA and HI tests.

Hemagglutination inhibition test was performed according to the method described (17, 18). Briefly, 25 ul each serum sample diluted in 100ul of NDV antigen was placed in the first column of ninety-six-well plates. The rest of the wells in the plate contained 50 ul of NDV antigen. Then two fold serial dilutions followed by passing 50 ul to the next column to the end of the plate. The antigen (NVD-EP-2 LaSota, Poultry Disease Research Center, Athens, GA) used to dilute the serum contained 10 hemagglutination activity (HA) units. Then, 50 ul of prepared 0.5% chicken red blood was added into each well and forty-five minutes of incubation at room temperature was requirement for the hemagglutination action before results were observed. The HI titer was determined by the well of the highest serum dilution where a clear button was seen. In this study, triplicate tests were made to assure the accuracy of data reading.

Enzyme-linked immunosorbent assay was done by commercial Newcastle disease antibody test kit test that purchased from IDEXX (IDEXX Laboratories Inc., Westbrook, MW). Titers were obtained and calculated as described by IDEXX. Briefly, 5 ul of each

serum sample was used to perform the test and an optical density of 650 nm wavelength was used for reading the color change. Triplicate tests were also made in this assay.

Evaluation of the cutaneous basophil hypersensitivity responses: Three days after the NDV vaccination, at twenty two days of age, five birds per pen from each replicate including vaccinated and non-vaccinated groups were randomly selected to perform the cutaneous basophil hypersensitivity assay as described by Corrier (4). All the birds were intradermally injected in the toe web between 3^{rd} and 4^{th} digits of right foot with 100 ug of Phytohemagglutinin (PHA-P) (SIGMA Chemical CO., St. Louis, Missouri), in 0.1 ml of phosphate buffered saline (PBS) solution. The left foot of each chicken was also injected with 0.1ml of PBS in the corresponding toe web to serve as a control. The toe web thickness was measured before and after the injection.

The evaluation of the cutaneous basophil hypersensitivity response was determined by measuring the toe web thickness, which was measure by a constant-tension, dial micrometer (Mitutoyo Ltd., Aurora, IL). Two methods were used, which were PHA1, the subtracting of thickness of inter-digital skin before and after the PHA-P/PBS solution injection, and PHA2, post-injection skin thickness of PHA-P/PBS minus the PBS post injection skin thickness (4).

Lymphocyte proliferation assay: At twenty two days of age, five birds from each pen, which were not included in the cutaneous basophil hypersensitivity assay, including all the replicates from both NDV vaccinated and non-vaccinated groups**,** were randomly selected to perform the lymphocyte proliferation assay. At twenty six days of age, this assay was repeated to compare the results. As described by Gogal (9), 1.5 ml blood from each bird was collected into a heparinized (SIGMA Chemical CO., St. Louis, Missouri) syringe. Blood from five birds per pen would be pooled in one tube, mixed well to prevent clotting, and left at room temperature during the blood collecting process. After finishing blood collection, the pooled blood was centrifuged for 10 min at 50 x g. The lymphocyte-rich buffy coat layer, which formed on top of the blood was gently swirled to collect and was held on ice. This centrifugation and swirl technique was done twice to maximize lymphocyte collection. After collection of the suspension, it was washed three times with cold RPMI-1640 (SIGMA Chemical CO., St. Louis, Missouri) and centrifuge at 250xg at 4C. Then, the centrifuged pellet was suspended into 2 ml complete media, which contained cold RPMI-1640 containing 10% fetal bovine serum (SIGMA Chemical CO., St. Louis, Missouri), 2mM glutamine (ICN, Costa, CA), 50 IU Penicillin (ICN), and 50 mg/ml streptomycin (ICN). One hundred ul of the cell suspension was measured and incubated for 5 min with 900 ul Natt-Herrick stain, then lymphocytes were counted on a

hemacytometer. Lymphocytes stained dark blue, and thrombocytes had a dull stain. The lymphocytes were then resuspened into complete media and adjusted to $5x10^6$ lymphocytes/ml. The lymphocyte proliferation assay was performed in 96-well round bottom tissue culture plate. One hundred ul of adjusted lymphocytes suspension was added to triplicate wells containing 100 ul of either complete media alone, which served as negative control for baseline proliferation, 100 ul of media containing 50 ug/ml concanavalin A (ConA, SIGMA) or 100 ul of media containing 2.5 ug/ml PHA-P (SIGMA). The plates with lymphocytes would be incubated at 37 C with 5% CO_2 in a tissue culture incubator for 24 hr. After 16-18 hr of culture, 20ul of the Alamar blue (Accumed International Inc.,Westlake, Ohio) was added to each well and returned to the incubator for 24 hours. Then the plate was read on an ELISA plate reader at 595 nm wavelength.

The evaluation of the read out of the optical density data was calculated as stimulation index (SI) as describe by Frome (8). It is a method to evaluate the increased proliferation of lymphocyte after they are stimulated.

Statistical Analysis: Data collected from all the experiments were subjected to ANOVA procedures by using General Linear Model's procedure of SAS software (SAS Institute, 2001). The difference level $P \leq 0.05$ will be considered as significant.

RESULTS

Body weight and the weight of thymus, spleen and bursa of Fabricius: Table 4.3 shows the final body weight, the weight of thymus, spleen and bursa of Fabricius as well as feed efficiency, which were obtained at the end of the experiment. The table also presents the ratio of these immune organs to the body weight. The body weight of the P deficient diet groups was not significantly different from the control diet groups. There was also no significant difference in body weight between NDV and non-NDV vaccination groups for birds fed a P deficient diet. However, there was a significant difference based on vaccination status in birds fed the control diet. The non-NDV vaccinated groups had lower body weights than the NDV vaccinated groups.

For the actual weight of spleen, there is no significant different between control and P deficient diet groups regardless of the vaccination status. Thymus weight appears significantly heavier in non-NDV vaccinated groups fed a P deficient diet, when comparing to vaccinated birds fed control diet. For the weight of bursa of Fabriucius, there is a significant reduction in NDV vaccinated chickens fed a P deficient diet compared to all other groups.

A P deficient diet does not affect immune organ to body weight ratio within birds of similar vaccination status. The vaccination status does affect the result of the bursa of

Fabriucius to body weight ratio where the bursa was significantly reduced in NDV vaccinated groups when compared to non-NDV vaccinated groups.

Mortality rate for all treatment groups was low (data not shown). The NDV vaccinated control diet groups had one dead bird and one bird died among P deficient groups. For non-NDV vaccination groups, there was only one dead bird in control diet groups.

The P deficient groups also had a slightly increased feed efficiency in both NDV and non-NDV vaccination groups. The non-NDV vaccination groups have better feed efficiency in general.

Enzyme-linked immunosorbent assay (ELISA): Table 4.4 shows the relative ELISA titers, which has three time frames. Before the NDV vaccination, all the groups show no significant difference except for the control diet groups for Non-NDV vaccination, which was significantly lower than the others. The ELISA titers are low and would be considered negative within the non-NDV vaccination groups despite the increase over time. Within the NDV vaccination groups, the control diet groups have a significant increase in NDV titer only at 25 days after vaccination when compared to the chickens fed a P deficient diet. The ELISA titer climbs through the experiment as expected after NDV vaccination for both control diet groups and P deficient groups.

There is significant difference between non-NDV and NDV vaccination groups as expected.

Hemagglutination inhibition (HI) test: Table 4.5 shows the Hemagglutination Inhibition (HI) test titers at three time frames, before NDV vaccination, and 10 and 25 days after NDV vaccination. Before the NDV vaccination, all the groups show no significant difference. At 10 and 25 days after NDV vaccination, there is no significant difference between control and P deficient diet groups regardless of vaccination status. However, there is significant difference between NDV and non-NDV vaccination groups regardless of treatment diet. Also the HI titer climbs through the experiment for the NDV vaccination groups on both control and P deficient diet groups as expected.

Cutaneous basophil hypersensitivity responses: Table 4.6 shows the cutaneous basophil hypersensitivity responses to PHA-P. There were two different methods to interpret the response (4). Neither PHA1 nor PHA2 calculation methods showed a significant difference between control diet groups and P deficient diet groups in both NDV and non-NDV vaccination groups. NDV vaccination status did not affect the cutaneous basophil hypersensitivity response.

Lymphocyte proliferation assay: Table 4.7 shows the result of Lymphocyte proliferation assay. The value is presented as stimulation index (SI). For both ConA and

PHA-P assays, there is no significant different between control and P deficient diet groups regardless of the NDV vaccination status at day 4 and day 8 of post NDV vaccination. There is an increase in SI values from day 4 to day 8 post NDV vaccinations on all groups.

DISCUSSION

Data shows the P deficient diet did not make any significant difference from the control diet on overall body and spleen weight. The thymus weight within non-NDV vaccinated groups was larger in chickens fed a P deficient diet and bursa of Fabricius in the NDV vaccinated groups was smaller than chickens fed a control diet. However, both of the organ to body weight ratio of thymus and bursa of Fabricius has no significant difference between control and phosphorus deficient diet groups. This suggests the significant difference of thymus and bursa of Fabricius weight related to individual body size and vaccine status and not affected by the dietary treatment specifically. Some studies (1, 6) indicate the deficiency of P in the diet results in a lowered appetite of the birds and causes reduced weight gain, poor feed efficiency, and high mortality. Our results disagree with their finding. Newcastle disease virus vaccination affected immune organ weight for the bursa of Fabricius, where NDV vaccinated groups has smaller bursa

in relative to body weight. This finding is similar with study of Li et al. in turkey (13) and this suggests the NDV vaccination has an effect on the bursa of Fabricius.

The ELISA data before diet treatment shows most of the chickens had low antibody titers. As stated before, birds which carried the most uniform maternal antibody level against Newcastle Disease were selected and randomly grouped for treatment. Although, the non-NDV vaccination group fed control diet has a significant lower value than other groups, it has a standard deviation larger than mean indicating the individual variance is large within this group and could be responsible for the unexplained difference in value. The HI data shows that corresponding groups had no significant difference from each other. In other studies of HI and ELISA relationship (3, 14), ELISA and HI have a high degree of agreement between these two tests. However, the dose or route of inoculation of NDV vaccine may influence the correlation between them (14). Viewing the ELISA data, at 25 days after NDV vaccination the P deficient diet group shows a significantly lower titer when compared to NDV vaccinated chickens fed control diets. There is no significant difference within non-NDV vaccination groups. The HI data shows there is no significant difference between control and P deficient diet groups within NDV or non-NDV vaccination groups. Both ELISA and HI titer shows the boost titer of NDV vaccination through the experiment. Enzyme linked immunosorbent assay suggests that P

deficient diet reduced the antibody production, but the HI test only shows numerical decrease. In the study by Kegley et. al., the result suggest that increasing supplemental phosphorus increased average daily gain and enhanced cell-mediated immune response but decreased humoral immune response in pigs (11). Our results do not agree with that.

Generally speaking, HI is more specific and ELISA is more quantitative or sensitive. Hemagglutination inhibition test is based on the binding of a specific hemogglutinin antigen of NDV. Because of the nature of NDV, it has several surface antigens which could be bound by non specific antibody within chicken serum, as a result the ELISA has higher sensitivity but less specific and accurate (7).

The cellular immune response detected by cutaneous basophil hypersensitivity responses shows P deficient diet groups have no significant difference from the control diet groups within both NDV and non NDV vaccination groups. The reason we use two different methods to compare the result is in order to eliminate the influence from different individual toe web thickness. Different chickens have different body weights and inflammatory reactions. PHA1 only shows the inflammatory degree of the chickens response to the PHA-P injection. PHA2 eliminate the different toe web thickness within each individual chicken. When both results are considered, a more specific result is

obtained. Our observation indicates the P deficiency has no effect of the function of T cell against mitogen.

In the lymphocyte proliferation assay, the data was presented as stimulation index (SI), which was ratio of stimulated lymphocyte (from treatment groups) verse lymphocytes without stimulation (from control groups). There was no significant difference in the SI between treatment groups. There was an increase in SI as the birds aged. The increase of the SI from day 4 to day 8 was consistent over all treatment groups and may have been simply a function of increased age or due to some other factor not measured in this study, which stimulated the cellular immune system equally across all groups. The lymphocyte proliferation assay suggests that P deficiency has no effect of cellular immune function in chickens.

CONCLUSION

In our study, a P deficiency of 67% does not have a great influence on either humoral or cellular immune function in the chicken under the parameters of this study. Also, no influence was seen on body and immune organs weight.

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Table 4.1 Composition of the control diet fed to broiler chickens for 42 days.

Ingredients	Percentage
Yellow corn	48.030
Cottonseed ML	42.000
Fat, Poultry	5.667
Limestone	1.127
Phos. Defluor.	1.531
Lysine-L 78.4	0.596
Salt	0.308
Meth DL-98	0.253
Br. Vitamins	0.250
Soy Meal 48	0.163
Tr. Min. / Br.	0.075
Calculated composition	
ME. (Kcal/Kg)	2950.000
Crude protein	22.000
Arginine	2.180
Lysine	1.250
Methionine	0.550
Meth & Cystine	0.920
Tryptophane	0.250
Crude Fat	7.490
Crude Fiber	1.210
Calcium	1.000
Phosphorus-AV	0.450
Phosphorus-TO	0.840
Sodium	0.210
Xanthophyll (mg/Kg)	10.56
Choline (mg/Kg)	1141.008

Table 4.2 Composition of the phosphorus deficient diet.

Ingredients	Percentage
Yellow corn	48.108
Cottonseed ML	42.000
Fat, Poultry	5.640
Limestone	1.827
Phos. Defluor.	0.698
Lysine-L 78.4	0.597
Salt	0.404
Meth DL-98	0.253
Br. Vitamins	0.250
Soy Meal 48	0.150
Tr. Min. / Br.	0.075
Calculated composition	
ME. (Kcal/Kg)	2950.000
Crude protein	22.000
Arginine	2.180
Lysine	1.250
Methionine	0.550
Meth & Cystine	0.920
Tryptophane	0.250
Crude Fat	7.470
Crude Fiber	1.210
Calcium	1.000
Phosphorus-AV	0.300
Phosphorus-TO	0.690
Sodium	0.210
Xanthophyll (mg/Kg)	10.560
Choline (mg/Kg)	1140.634

Table 4.3 Relative weights of thymus, spleen and bursa of Fabricius and feed efficiency of NDV vaccinated and non-NDV vaccinated broiler chickens fed on control and phosphorus deficiency diets.

	NVD vaccination				Non-NDV vaccination			
	Control		Phosphorus Deficiency		Control		Phosphorus Deficiency	
	Mean[C]	SD	Mean	SD	Mean	SD	Mean	SD
BW[A] (gm)	1910.3[a]	285.09	1871.65[ab]	289.50	1786.71[b]	244.04	1820.81[ab]	251.83
SW[A] (gm)	2.66[a]	0.67	2.64[a]	0.63	2.84[a]	0.78	2.76[a]	0.81
SW/BW[B] (%)	0.14[b]	0.035	0.142[b]	0.037	0.16[a]	0.043	0.149[ab]	0.039
TW[A] (gm)	6.05[a]	2.04	6.51[ab]	2.08	6.21[a]	2.14	7.12[b]	2.47
TW/BW[B](%)	0.316[a]	0.095	0.348[ab]	0.098	0.350[ab]	0.123	0.388[b]	0.114
BFW[A] (gm)	1.10[a]	0.37	1.06[b]	0.36	1.19[a]	0.40	1.21[a]	0.31
BFW/BW[B](%)	.057[b]	0.018	0.057[b]	0.019	.068[a]	0.022	0.067[a]	0.018
FE[A]	2.575413		2.304691		2.280576		2.207168	

[A]BW, body weight; SW, spleen weight; TW, thymus weight; BFW, bursa of Fabricius weight; FE, feed efficiency

[B]Weight lable followed by /BW as body weight relative percentage

[C]Mean values within a row followed by different lower case superscripts are significantly different: $P \leq 0.05$.

Table 4.4 Enzyme-linked immunosorbent assay titer of broiler chickens fed control or phosphorus deficient diets at the time of and after Newcastle disease virus vaccination.

	NVD vaccination				Non-NDV vaccination			
	Control		Phosphorus Deficiency		Control		Phosphorus Deficiency	
Days Post Vaccination	Mean[A]	SD	Mean	SD	Mean	SD	Mean	SD
0 days	77.32[a]	112.04	76.09[a]	107.36	50.79[b]	105.97	55.75[a]	84.07
10 days	2258.47[a]	3538.7	1575.23[a]	2023.4	104.69[b]	282.13	40.91[b]	148.07
25 days	8213.53[a]	5553.9	6702.14[b]	4906.8	131.62[c]	315.77	212.65[c]	375.67

[A]Mean values within a row followed by different lower case superscripts are significantly different: $P \leq 0.05$.

Table 4.5 Hemagglutination inhibition test titer of broiler chickens fed control or phosphorus deficient diets at the time of and after Newcastle disease virus vaccination.

	NVD vaccination				Non-NDV vaccination			
	Control		Phosphorus Deficiency		Control		Phosphorus Deficiency	
Days Post Vaccination	Mean[A]	SD	Mean	SD	Mean	SD	Mean	SD
0 day of age	14.58[a]	10.39	17.29[a]	10.64	14.92[a]	10.72	15.58[a]	13.56
10 days	90.68[a]	92.64	102.37[a]	89.99	5.76[b]	6.49	3.75[b]	5.49
25 days	254.41[a]	673.15	171.19[a]	184.92	7.29[b]	7.84	5.67[b]	6.47

[A]Mean values within a row followed by different lower case superscripts are significantly different:$P \leq 0.05$.

Table 4.6 Cutaneous basophil hypersensitivity responses, measured in mm, elicited by an injection of phytohemagglutinin-P, using a phosphate buffer saline as the control.

	NVD vaccination				Non-NDV vaccination			
	Control		Phosphorus Deficiency		Control		Phosphorus Deficiency	
	Mean[A]	SD	Mean	SD	Mean	SD	Mean	SD
PHA1[B]$_{(mm)}$	1.11[a]	0.13	1.13[a]	0.18	1.08[a]	0.25	1.13[a]	0.22
PHA2[C]$_{(mm)}$	0.63[a]	0.15	0.62[a]	0.18	0.58[a]	0.24	0.69[a]	0.22

[A]Mean values within a row followed by different lower case superscripts are significantly different:$P \leq 0.05$.

[B]PHA1 was calculated by subtracting pre-injection skin thickness from post-injection skin thickness of the right toe web injected by PHA-P/PBS solution.

[C]PHA2 was calculated by post-injection of right toe web skin thickness, PHA-P/PBS solution injected, minus left toe skin thickness, PBS solution injected.

Table 4.7 Lymphocyte proliferation assay stimulation index from broiler chickens fed control or phosphorus deficient diets after Newcastle disease virus vaccination.

	NVD vaccination				Non-NDV vaccination			
	Control		Phosphorus Deficiency		Control		Phosphorus Deficiency	
Days Post Vaccination	SI[A]	SD	SI	SD	SI	SD	SI	SD
ConA								
4 days	0.87[a]	0.43	0.95[a]	0.40	0.68[a]	0.33	0.85[a]	0.23
8 days	1.51[a]	0.54	1.17[a]	0.63	1.47[a]	0.53	1.59[a]	0.30
PHA-P								
4 days	0.80[a]	0.50	0.81[a]	0.38	0.74[a]	0.40	0.76[a]	0.50
8 days	1.56[a]	0.53	1.48[a]	0.54	1.28[a]	0.55	1.70[a]	0.39

[A]Stimulation index (SI) values within a row followed by different lower case superscripts are significantly different: $P \leq 0.05$.

CHAPTER 5

CONCLUSIONS

In the lysine deficiency study, it was shows that lysine has a major effect on thymus and body weight. For immune function, this study found that lysine deficiency may have some degree of influence in chicken immune function. Humoral immunity assay has numerical or statistic change depending on the test used to indicate a reduction in antibody production from the lysine deficiency. However, this experiment only was conducted with NDV and the conclusion affecting antibody performance can only be limited on the antibody to NDV. The cell-mediated immunity function also was reduced in the effect of a lysine deficiency, but not both of the assays can fully approve the effect of lysine deficiency. Further study should try to seek a way to eliminate the individual difference to get more detailed results to reveal the effect of lysine deficiency on the immune function.

The phosphorus (P) deficiency study does not show a great influence on either humoral or cellular immune function in the chicken. Also, no influence was seen on body and immune organs weight. However, P is an element that influences lots of metabolic and energy transfer functions within the chicken. Further study would benefit from

investigating different levels of P deficiency to see what if any level would have an influence on chicken immune function.