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THROMBOCYTE RESPONSE TO LIPOPOLYSACCHARIDE IN STRESS INDUCED BROILER CHICKS

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THROMBOCYTE RESPONSE TO LIPOPOLYSACCHARIDE IN STRESS INDUCED
BROILER CHICKS

A Thesis
Presented to
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In Partial Fulfillment
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Master of Science
Animal and Veterinary Science

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by
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August 2007

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ABSTRACT

The role of thrombocytes, nucleated blood leukocytes, on immune responses in chickens fed diets supplemented with vitamin C has not been studied in the past. This study investigates the effects of three types of diets (control, control with corticosterone, and control with corticosterone and vitamin C) in 14-day old broiler chicks on the expression of pro-inflammatory cytokines IL-1β, IL-6, and IL-12 in thrombocytes. In addition, the effect of LPS, a microbial component to which broiler chicks can be exposed, on the expression of these pro-inflammatory cytokines was investigated. After two weeks of feeding, blood samples from the chicks were collected and the thrombocytes isolated were incubated with LPS for an hour. Then, RNA was extracted from the samples to study the expression of pro-inflammatory cytokines using real-time RT-PCR. The study found that the diets fed to broiler chicks do not affect the expression of pro-inflammatory cytokines IL-1β, IL-6, and IL-12 in thrombocytes. However, LPS does increase the expression of pro-inflammatory cytokines IL-1β, IL-6, and IL-12. The fact that thrombocytes are so abundant, and can be stimulated by LPS makes them the primary effector cell in innate host defenses against bacterial infections in poultry.

DEDICATION

To my loving parents, Dr. Md. Ferdous Alam and Sayeeda Banu. Without your love, encouragement, and dedication, the completion of this thesis and the degree would not have been possible. I love you both with all my heart.

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Especially, I want to thank my mother-in-law for taking care of my baby while I was in class and in the lab, and for her encouragement as I pursued my Master's degree. Although my parents could not be here with me, their love and support have always been my inspiration. I also want to thank my sister, Sadia, who persuaded me to pursue further studies abroad and my brother, Raquib, for his encouragement. Finally, I would like to thank my husband, Taufique, for all his love, care, and support, and for calming and reassuring me when I was stressed. Also, I thank my son Irfan for bringing so much joy and happiness into my life.

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REVIEW OF LITERATURE

Mammalian Thrombocytes

Mammalian thrombocytes are more commonly known as platelets. Platelets are derived from megakaryocyte of the bone marrow (Wright, 1910) and lungs (Martin and Levine, 1991) as small-enucleated cells. They are the smallest of blood cells. They are the most sensitive cellular elements in the blood because of their ability to respond to a variety of chemical and physical stimuli with the intention to minimize a possible blood loss when the vascular endothelium is damaged (Klinger, 1997). The dominant hormone controlling the development of megakaryocytes is thrombopoietin. However, many cytokines and hormones, including interleukins (ILs) 3, 6 and 11, also take part in this process (Kaushansky, 1995). Platelets are a key element linking the process of homeostasis, and the initiation of wound repair (George, 2000). Besides their role in homeostasis and thrombus formation after endothelial injury, thrombocytes also take part in the processes of inflammation and tissue repair that follow (Klinger, 1997). Platelets are the first cellular corpuscles that accumulate at a site of damage of the vascular wall within the surrounding tissue.

Avian thrombocytes

Avian thrombocytes are homologus in function to mammalian platelets (Lucas and Jamroz, 1961). Avian bone marrow lacks megakaryotes so there are conflicting views on the differentiation of thrombocytes (Sugiyama, 1926). One group believes that thrombocytes are descendants of lymphocytes or primitive lymphoid cells, while a second group believes in their endothelial origin. According to Lucas and Jamroz (1961), thrombocytes arise from the antecedent mononucleated cells. A third group considers

thrombocytes as damaged or degenerated red cells, and a fourth group thinks of thrombocytes as transitory forms of lymphocytes. There are also researchers that believe thrombocytes have a hemoblastic nature or are capable of developing into red blood cells, while others regard them as a type of giant cell. According to Archer (1971), large cells in chicken bone marrow appear to be multinucleated and could be the precursors of thrombocytes.

Ultrastructurally thrombocytes in peripheral blood of different avian species such as chicken, turkey and Japanese quail are very similar (Sweeny and Carlson, 1968; Simpson, 1968; and Nirmalan et al., 1972). Although mammalian thrombocytes are not nucleated, most avian thrombocytes are nucleated blood leukocytes and represent the most abundant white blood cell types in chicken blood (Chang and Hamilton, 1979a). In circulating blood, the concentration of thrombocytes is 30,000 cells/mm^3 (Lucas and Jamroz, 1961) compared to only 5,000 cells/mm^3 for concentration of heterophils (Glick, 1958). The ultrastructure of thrombocytes can be described as round, oval or somewhat elongated cell with an irregular outline extending into occasional fine pseudopodial processes (Lucaz and Jamroz, 1961; and Hodges, 1979). Thrombocytes are characterized mainly by the large, round to oval nucleus which is frequently irregular and indented in outline, sharply divided into blocks of heavily condensed, granular heterochromatin with intervening areas of pale euchromatin, and the presence of several cytoplasmic vacuoles (Maxwell and Trejo, 1970; Hodges, 1979; and Swayne et al., 1986). The cytoplasm also contains a few mitochondria, smooth and rough endoplasmic reticulum, microtubules, and often a well developed Golgi apparatus (Maxwell and Trejo, 1970; and Hodges, 1979). Maxwell (1973) compared thrombocytes in six species of domestic bird (duck, geese, turkey, pigeon, quail and guinea-fowl) and found little difference in ultrastructural

features, confirming that thrombocytes are ultrastructually very similar in different avian species.

Although thrombocytes and erythrocytes bear a superficial resemblance in both shape and general appearance, thrombocytes are usually smaller in size and possess a larger but more rounded nucleus (Hodges, 1979) as observed in Figure 1. Thrombocytes have cytoplasm with reticulated appearances and normally stain pale, dull blue with traces of purplish shades (Lucaz and Jamroz, 1961). Morphologically they can be distinguished from monocytes and lymphocytes by electron microscopy and in fresh preparations by phase microscopy. Since they are very similar in specific gravity and size to small lymphocytes, when chicken blood is centrifuged under conventional separation procedures, the thrombocytes separate out with the leukocytes into the buffy coat (Horiuchi et al., 2004).
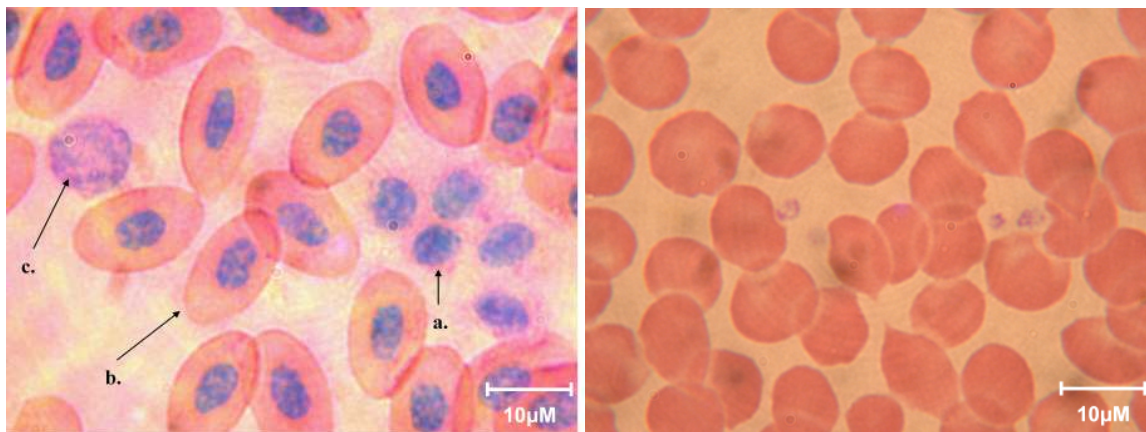
Figure 1: Photomicrographs of Wright's stained smears of chicken (left panel, a. nucleated thrombocyte, b. nucleated red blood cell, c. lymphocyte) and human (right panel, platelets appear among enucleated red blood cells) blood.

Avian Thrombocytes in Blood Coagulation

Thrombocytes play a primary role in homeostasis similar to that of mammalian platelets by aggregating to form a homeostatic plug (Stalsberg and Prydz, 1963); however, the rate at which clumping takes place is much slower than that of platelets (Hodges, 1979). Stalsberg and Prydz (1963) studied formation of the homeostatic plug in the chick embryo through in vivo microscopy, in sections of homeostatic plugs, and by measurement of primary bleeding time. They concluded that homeostatic plugs consisted mainly of thrombocytes. They also found that the ability to form a homeostatic plug is connected with the development of different stages of thrombocyte precursors. Venkatayan and Nambiar (1968) demonstrated that in domestic fowl, with increase in the concentration of thrombocytes in blood, there is a marked decrease in the clotting time. The avian intrinsic blood-clotting pathway has been shown to be relatively weak compared to the extrinsic clotting pathway (Stopforth, 1970; and Archer, 1971). Stopforth (1970) suggested that Factors V and VII might be low or absent in domestic chicken as compared with a typical mammalian system, but Factor X activity was present. Also, thrombocytes contain very little intrinsic thromboplastin so they are not very efficient in the initiation of the clotting process (Archer, 1971). The extrinsic clotting system is the most important coagulation pathway in domestic fowl and is initiated by the release of tissue thromboplastin (factor III or tissue factor) from aged tissue and thrombocytes (Archer, 1971; Hodges, 1979; and Morrisey et al., 2003). It has been established that although platelets have been shown to aggregate in the presence of adenosine diphosphate (ADP), avian thrombocytes do not aggregate by ADP or adenosine triphosphate (ATP), while micromolar concentrations of serotonin (5-hydroxytryptamine, 5-HT) are enough to cause aggregation of avian thrombocytes

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(Belamarich and Simoneit, 1973). Stiller et al. (1975) showed that platelet aggregation is related to the release of 5-HT from the intracellular granules of thrombocytes, where serotonin acts as a functional counterpart of ADP. Avian thrombocytes have also been demonstrated to secrete antiheparin proteins (Wachowicz et al., 1981).

Avian Thrombocytes in Phagocytosis

Glick et al., (1964) first demonstrated that circulating thrombocytes were phagocytic in nature. Later, Clarson et al. (1968) demonstrated phagocytic and trephocytic activities of chicken thrombocytes by microscopy and vital staining techniques. Kuruma et al. (1970) examined living thrombocytes by phase contrast microscopy and confirmed the incessantly moving and extending pseudopodia. Chang and Hamilton (1976) suggested that thrombocytes are the primary circulating phagocytes in chicken. Thrombocytes phagocytized about 3 times as rapidly as heterophils and monocytes (Chang and Hamilton, 1979a). Chang and Hamilton (1979a) also calculated that the circulating thrombocytes engulfed 1.7 as many bacteria as the heterophil and monocyte together. Avian thrombocytes are capable of phagocytosing bacteria (Glick et al., 1964; and Carlson et al., 1968), dye particles (Carlson et al., 1968), and viruses (Hodges, 1979). Carlson et al. (1968) also demonstrated presence of large, acid phophatase-positive granules histochemically in the cytoplasm of avian thrombocytes, which may be lysosomal structures, associated with phagocytic activity. Carlson and Allen (1969) showed that although avian thrombocytes had phagocytic ability, they did not play a major phagocytic role in inflammatory reactions. Chang and Hamilton (1979b) examined the percentage phagocytosis of chicken thrombocytes and found it to be insensitive to dietary aflatoxin. All other phagocytes such as monocytes, heterophils, and macrophages in this study were significantly inhibited because thrombocytes were

dependent upon a heat sensitive factor in the plasma for phagocytosis, whereas the other phagocytic cells were shown to be Complement dependent that was adversely affected by aflatoxin (Chang and Hamilton, 1979b).

Role of Platelets in Innate Immunity

Platelets play an important role in innate immunity such as inflammatory response, expression of molecules that modulate many different cell types. The mammalian thrombocytes contain an array of potent pro-inflammatory substances, and, therefore, are regarded as mediators and effector cells in inflammation. Activated platelets express receptors for adhesive proteins, different cytokines and other mediators that make direct contact with endothelial cells, granulocytes, monocytes, and lymphocytes to either stimulate or inhibit functions of other cellular partners in inflammation (Klinger and Jelkmann, 2002). They function as effector cells by directly interacting with bacteria, viruses, fungi, protozoans, and releasing anti-microbial proteins to limit infections. Their capability to interact with these pathogens and other foreign material is possibily a phylogenetic vestige and may explain existence of IgE dependent killing mechanism of the platelet (Klinger, 1997). Platelets enhance antigen presentation, improve CD8 T-cell responses and play a critical role in T-cell dependent humoral immunity (Elzey et al., 2005). The expression of CD154 (CD40 ligand) has been shown to directly stimulate endothelial cells to initiate inflammation at the vessel wall (Hen et al., 1998). Also, activation of the platelets may be responsible along with other inflammatory mechanisms in sepsis which is a serious medical condition resulting from immune response to severe infection (Andonegui et al., 2005).

Mammalian thrombocytes like avian thrombocytes are phagocytic cells with cytoplasm that is predominantly composed of granules containing bioreactive molecules

(Weksler et al, 1992). Activated platelets like other phagocytic cells such as neutrophils and monocytes express surface antigens such as P-selectin (Weksler et al, 1992; and Klinger, 2002). They also express chemotactic factors for one or more classes of leukocytes such as MIP-1α, 12-HETE, PAF, PDGF, PF-4, β-TG, TGF-β and RANTES, while cationic proteins, histamine, serotonin, PGE2, and PGD2 are vasoactive (Klinger, 1997; Klinger et al. 2002; and Elzey et al., 2005).

Besides releasing molecules that attract and modulate effector cells of the innate immunity, platelets perform direct anti-microbial functions. Platelets are capable of binding, aggregating, and internalizing microorganisms that enhance the clearance of pathogens from the bloodstream. Platelets and microorganisms have been found to interact through specific ligands, suggesting pathogens may exploit platelets as adhesive sites via molecular mimicry (Sullam et al., 1987; and Robet et al., 1992). Platelets facilitate microbial adhesion to fibrin matrices or endothelial cells in vitro (Scheld et al., 1978; and Klotz et al., 1989). Platelets are suggested as facilitating infection by aggregation and internalization of microorganisms, which may protect pathogens from exposure to antibiotics or clearance by neutrophils or other leukocytes (Clawson and White, 1971). These studies indicate platelets facilitate pathogen survival, endothelial cell penetration, or dissemination of microorganisms into deeper tissue parenchyma by which they inhibit immune response. But there is a compelling body of evidence that indicates platelets to have a key role in host defense against infection (Yeaman, 1997). Platelets release anti-bacterial/anti-fungal proteins after internalizing bacteria and viruses (Yeaman, 1997) and release reactive oxygen species upon activation (Chakrabarti et al., 2005).

Toll-like Receptors in Thrombocytes

Toll-like receptors (TLRs) are central to innate immunity for early host response to microbial components (innate immunity) because of their role in pathogen recognition (Medzhitov, 2001; Takeda et al, 2003; Takeda and Akira, 2005; and Underhill and Ozinsky, 2002). Recently mammalian thrombocytes have been shown to express specific TLRs (TLR2, TLR4, and TLR9) in human and murine tissues (Cognasse et al., 2005; Andonegui et al., 2005; and Aslam et al., 2006). Stimulation of most TLRs triggers a classical signaling cascade involving association of Toll/IL-1 receptor (TIR) domain containing adaptor molecules such as myeloid differentiation primary response protein 88 (My88) and MyD88 adaptor like, that leads to the activation of nuclear factor-κB (NF-κB) and the expression of pro-inflammatory cytokine genes. Also, TLR4 and TLR3 activation trigger an alternative-signaling pathway involving adaptors such as TIR domain containing adaptor protein inducing INFβ (TRIF) and TIRF-related adaptor protein, which lead to the induction of the INF-inducible genes and a late NF-κB response (Takeda and Akira, 2005). Common TLR signaling pathways are shown in Figure 2. Activation of these pathways via respective TLRs leads to cascading events resulting in gene expression of various inflammatory cytokines and INF-β, which potentiate innate immunity. Since activation of the innate immune system is a prerequisite for the induction of the adaptive immune system, TLRs are essential elements of host defense against pathogens (Elzey et al., 2003).
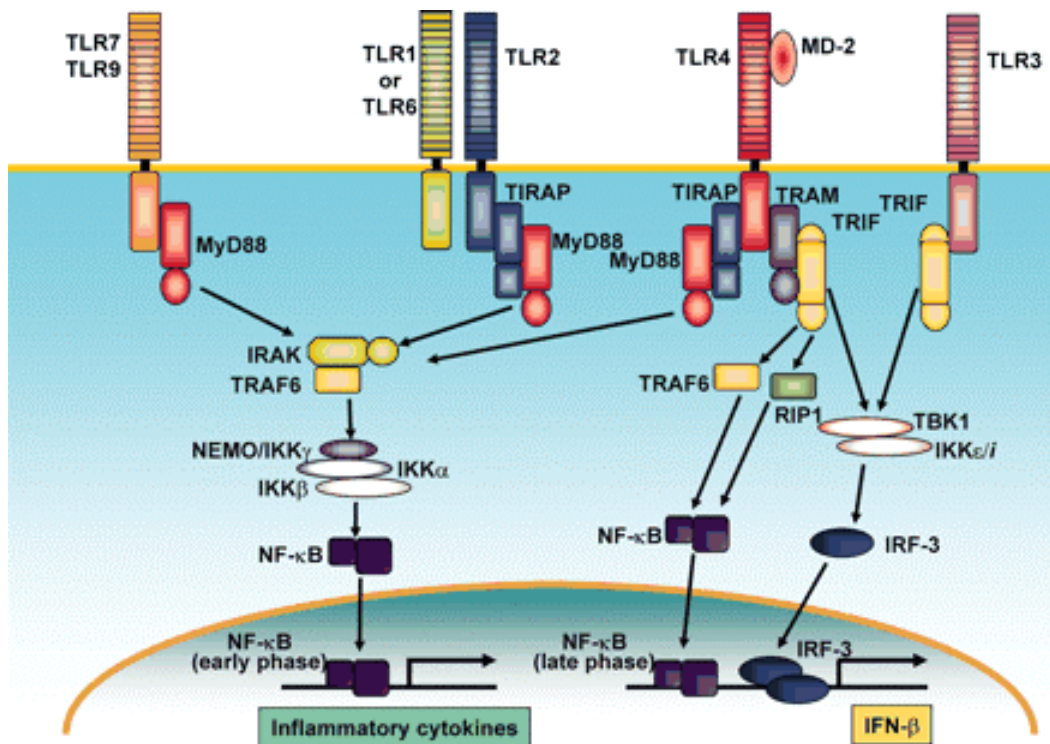
Figure 2: TLR signaling pathway. Most TLRs triggers a classical signaling cascade involving association of Toll/IL-1 receptor (TIR) domain containing adaptor molecules. MyD88, a TIR domain-containing adaptor, associates with the cytoplasmic TIR domain of TLRs, and recruits IRAK to the receptor upon ligand binding. IRAK then activates TRAF6, which leads to the activation of the IκB kinase (IKK) complex consisting of IKKα, IKKß and NEMO/IKKγ. The IKK complex phosphorylates IκB that results in nuclear translocation of NF-κB. A second adaptor protein, TIRAP is involved in the MyD88-dependent signaling pathway via TLR2 and TLR4. In the case of TLR3- and TLR4-mediated signaling pathways, activation of IRF-3 and induction of IFN-β are observed in a MyD88-independent manner. A third adaptor protein, TRIF is essential for the MyD88-independent pathway. A fourth adaptor, TRAM is specific to the TLR4-mediated MyD88-independent/TRIF-dependent pathway (Takeda and Akira, 2005).

Effect of Lipopolysaccharide on Thrombocytes

Lipopolysaccharide (LPS) also known as endotoxin, a major component found on the outer layer surface of gram-negative bacteria is known to activate innate immune response (Raetz, 1990). Commercial broilers are constantly exposed to airborne microorganisms and, therefore, LPS and other microbial components. LPS stimulation affects the cell function and gene expression in chicken monocytes and macrophages (Dil and Qureshi, 2002). LPS recognition in mammals occurs through a multi-protein interaction (TLR4 and other molecules). LPS binds to LPS-binding protein (LBP) in the blood serum and this complex is subsequently recognized by CD14, a protein that exists both in soluble form and as glycosylphosphatidylinositol-anchored molecule preferentially expressed in monocytes, macrophages and neutrophils (Aderem and Ulevitch, 2000; and Takeda et al., 2003). CD14 is a high affinity receptor for LPS in both forms and CD14$^{-/-}$ mice are at least 100X more resistant to LPS-induced mortality (Haziot et al., 1996 and Andonegui et al., 2005). Stimulation of TLR4 by LPS activates a signaling cascade, which is characterized by the transcription and translation of pro-inflammatory cytokines such as IL-1, tumor necrosis factor-α (TNF-α), and IL-8 that trigger innate immune defenses such as inflammation, fever, and phagocytosis in order to provide an immediate response against the invading microorganism. This process also stimulates production of cytokines such as IL-6 that promotes B-lymphocyte activity and IL-12 that promotes T-lymphocyte activity. The signaling process for TLR4 due to LPS stimulation is shown in Figure 3. TLRs also play an important role in adaptive immunity by triggering various secondary signals needed for the production of antibodies (humoral immunity) and the production of cytotoxic T-lymphocytes and additional cytokines (cell-mediated immunity).
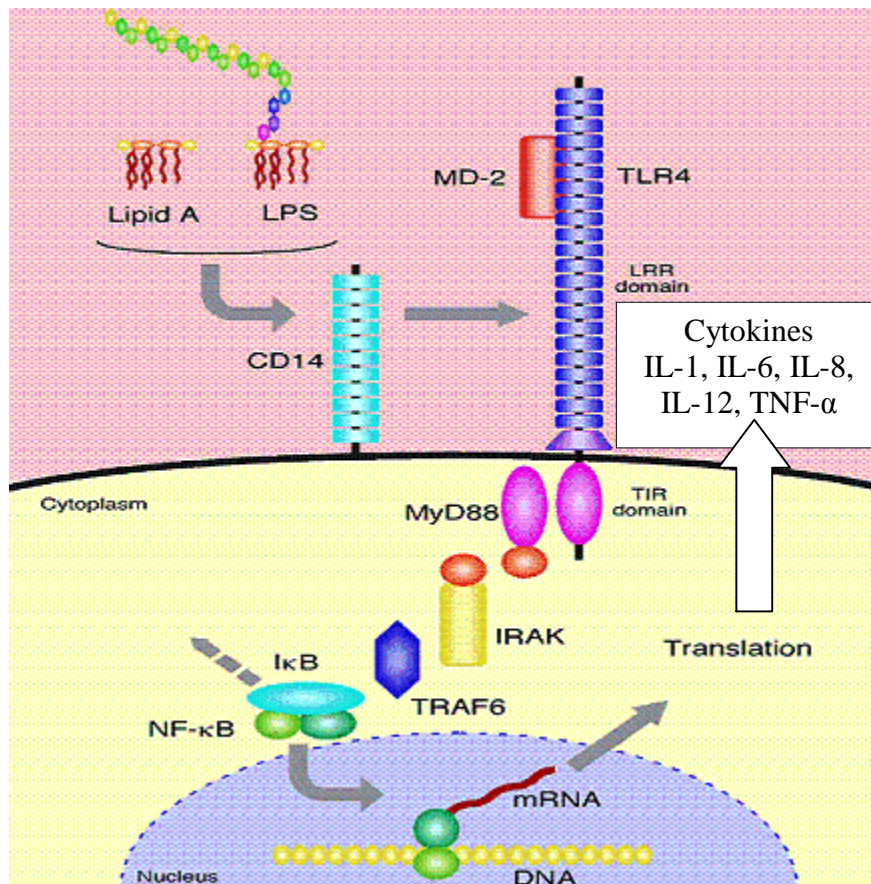
Figure 3: Toll-Like Receptors (TLRs) responding to lipopolysaccharide (LPS) from the gram-negative bacterial cell wall. (Adapted from http://www.gak.co.jp/FCCA/glycoword/ISA01/fig03.gif).

Role of Vitamin C

Vitamin C, also referred to as L-ascorbic acid, is synthesized from available precursors by most mammals but not by human and nonhuman primates, guinea pigs, the Indian fruit bat, several birds, and some fish (Rumsey and Levine, 1998). The major function of vitamin C is its role in many enzymatic reactions where it serves to maintain prosthetic metal ions in their reduced state (Fe^{2+}, Cu^+) and as a scavenger of harmful oxidative products including free radicals or reactive oxygen species (ROS) in order to protect tissues from oxidative damage and maintain the appropriate redox state to

facilitate cellular signal transduction (Padh, 1991; and Rose and Bode, 1993). According to Sies (1991), redox homeostasis is maintained by pro-oxidant/antioxidant balance, and the imbalance in favor of the pro-oxidant system will result in oxidative stress. Antioxidant defense mechanisms protect the cell against cellular oxidants and repair systems that prevent the accumulation of oxidatively damaged molecules. In order to minimize oxidative damage, antioxidants (vitamin C and E, glutathione, uric acid) and antioxidant enzymes (catalase, superoxide dismutase, and glutathione peroxidase) play vital roles in protecting cells from harmful effects of ROS (Altan et al., 2003).

The level of protection from vitamin C depends on the amount of uptake by a particular type of cell. For example, in human, vitamin C content per cell has been shown to be higher in mononuclear leukocytes and granulocytes than in platelets and erythrocytes (Evans et al., 1982). The concentration of intracellular vitamin C in turn influences inflammatory, neoplastic, and apoptotic processes via inhibition of NF-κB activation (Carcamo et al., 2002).

Effect of Vitamin C on Stress Induced Chicken

Chickens are exposed to a variety of stressors during their lifetime. Stress is known to affect the redox balance in poultry. When subjected to acute stress or chronic stress, broiler chickens show different biochemical responses that are related to the nature of stressor and the adaptation process to the stressor (Lin et al., 2004). In broiler chickens, plasma corticosterone concentrations can be used to assess physiological effects of stress (Post et al., 2003). The concentration of corticosterone in plasma is a reliable indicator of stress in birds. Corticosterone is a steroid hormone produced in the cortex of the adrenal glands. In many species, corticosterone is the principal glucocorticoid, involved in regulation of fuel metabolism, immune reactions, and stress

responses. Oxidative stress induced in chickens by dietary corticosterone causes marked regression of lymphoid tissues and reduction in tissue ascorbic acid without impairment in biosynthesis of Vitamin C as measured by L-gulonolactone oxidase activity (Maurice et al., 2006). Neither the effect of these changes nor the effect of dietary ascorbic acid intervention on cellular inflammatory processes in chickens is known.

Vitamin C or ascorbic acid is recommended as a nutritional intervention for chickens to alleviate stress due to high temperature. This is because during stress it is assumed that the requirement for vitamin C exceeds synthesizing ability (Gous and Morris, 2005). Supplemental ascorbic acid in poultry diets at 1000 mg/kg alleviates clinical symptoms of infectious bursal disease (IBD) and reduces morbidity and mortality in non-vaccinated chicks challenged with IBD (Amakye-Amin et al, 2000). Satterlee et al. (1989) demonstrated that ascorbic acid supplementation reduces the stress response evident in broiler chickens during cooping/starvation before being taken to an abattoir for processing. Even though the ameliorative effect of vitamin C was not observed in an experiment conducted in West Africa (Okoye et al., 1998), vitamin C increased resistance to Newcastle disease, mycoplasma, and *Escherichia coli* when administered at a tenth of the concentration ascorbic acid (Gross, 1992). In recent years, interest in studying the effect of ascorbic acid on cellular signal transduction modulated by the redox state of the cell has grown.

Currently, there is no information on the effects of ascorbic acid on the inflammatory process in an avian model like poultry. Vitamin C has been shown to inhibit activation of NF-κB by tumor necrosis factor (Bowie and O'Neill, 2000; and Zhang and Chen, 2004) in cell culture models and in mice and to interrupt cardiac inflammatory cytokine secretion (Horton et al., 2001). Pro-inflammatory cytokines are

produced via activation of NF-κB, and there are two main signaling pathways that activate NF-κB (Figure 4). The classical pathway is triggred by pro-inflammatory stimuli and genotoxic stress, including the cytokines, such as TNF and IL-1; bacterial cell-wall components, such as LPS; viruses; and DNA-damaging agents that lead to IKKβ- and IKKγ-dependent phosphorylation of IκB (Karin, 2005). This results in proteosomal degradation and subsequent release of the NF-κB dimmers (discussed previously in Toll-like receptors in Thrombocytes; Karin, 2005). The alternative pathway is triggered by certain TNF-family members that lead to the phosphorylation of p100 by IKKα and the degradation of its carboxy-terminal half by the proteasome, which works independently of IKKβ and IKKγ (Karin, 2005).
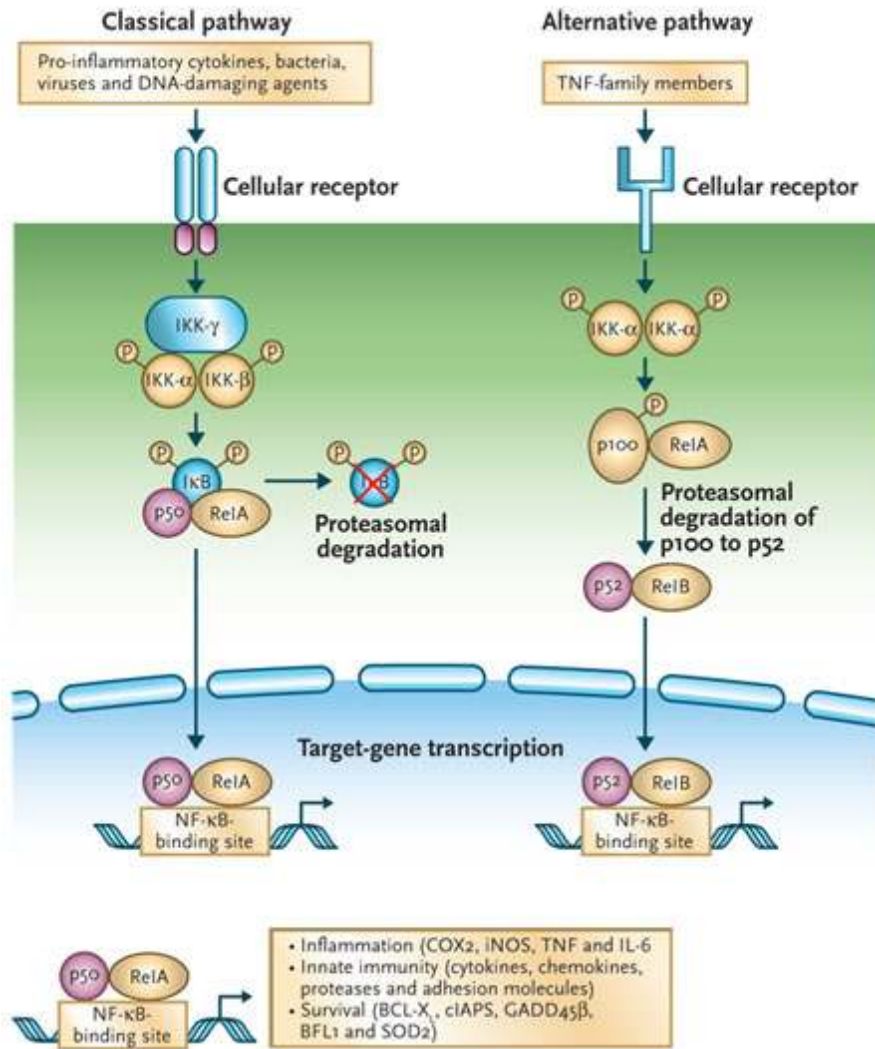
Figure 4: Activation of NF-κB through classical and alternative pathway (Karin, 2005).

Indicators of Stress in Chicken

Blood parameters such as corticosteroid concentration (Edens and Siegel, 1975; Edens and Siegel, 1976; Siegel, 1980; and Gross and Siegel, 1983), heterophil to lymphocyte (H/L) ratio (Beuving et al., 1989; Gross and Chickering, 1987; and Jones, 1989), concentration of lymphocytes in plasma (Ben at al., 1976; and Gross and Siegel, 1983), and changes in basophil count or granulation (Mitchell et al., 1992; and Maxwell et al., 1992) have been previously used to detect stress in chicken. The secretion of

corticoids is most characteristic of the stress response. Therefore, environmental stress increases the level of corticosteroid in blood plasma. It has also been shown that H/L ratio increases with physiological and physical stressors such as fasting, frustration, water deprivation, and crowding (Beuving et al., 1989; Gross and Chickering, 1987; and Jones, 1989). However, chickens can adapt to these stressors. For example, the H/L ratio increases significantly due to initial fasting but only increases a small amount due to repeated fasting (Gross and Siegel, 1986), and H/L ratio increases within half an hour of an exposure to cold stress but returns to pre-exposure level one day after exposure (Gross, 1989).

Since vitamin C inhibits activation of NF-κB by tumor necrosis factor (Bowie and O'Neill, 2000; and Zhang and Chen, 2004), it may affect the inflammatory cytokine production in chicken. In this study, we have investigated the effect of purified LPS in pro-inflammatory cytokine (IL-1β, IL-6 and IL-12) mRNA expression by thrombocytes in stressed broiler chicks with and without supplemental vitamin C.

INTRODUCTION

Mammalian thrombocytes, more commonly known as platelets, are the smallest blood cell ebing only fragments of the megakaryocyte cytoplasm (Klinger, 1997). Platelets have become intriguing subjects for immunological research in recent years because of their role in natural host defense mechanisms (Meseguer et al., 2002). Platelets are mostly known for the process of homeostasis and the initiation of wound repair (George, 2000). Besides their role in homeostasis and thrombus formation after endothelial injury, platelets also take part in the processes of inflammation and tissue repair that follows (Klinger, 1997). Platelets release a vast array of bio-reactive molecules such as chemotactic factors for other cell types, cationic proteins, histamine, serotonin, PGE2, and PGD2 (Klinger 1997; Klinger et al., 2002; and Elzey et al., 2005). The role of thrombocytes in adaptive immunity has been recently reviewed since the discovery of their CD154 (CD40L) expression that directly stimulates endothelial cells to initiate inflammation at the vessel wall (Henn et al., 1998; Elzey et al., 2003; and Elzey et al., 2005).

Chicken thrombocytes are nucleated blood leukocytes and represent the most abundant white blood cell types in chicken blood (Chang and Hamilton, 1979a). Since the avian bone marrow lacks megakaryocytes, thrombocytes arise from the antecedent mononucleated cells (Lucas and Jamroz, 1961). Thrombocytes are homologus in function to mammalian platelets. Mammalian platelets have been shown to play an important role in innate immunity and a potential to serve as a link between innate and adaptive immunity. Chicken thrombocytes have been used in this study to analyze gene

expression of certain pro-inflammatory cytokines that will enable us to examine a unique side of immune response in chickens.

Commercial broilers are exposed to a variety of stressors during their lifecycle. They are raised in an environment where they can be exposed to airborne microorganisms and microbial components such as lipopolysaccharide (LPS). Stress is known to affect the redox balance in poultry. Oxidative stress induced in chickens by dietary corticosterone causes marked regression of lymphoid tissues and reduction in tissue ascorbic acid without impairment in biosynthesis of Vitamin C (Maurice et al., 2006). Dietary supplementation of vitamin C has been shown to have beneficial effects on stressed broiler chickens due to high temperature (Gous and Morris, 2005). In a study done by Amakye-Amin and collegues (2000), supplemental vitamin C alleviated clinical symptoms of infectious bursal disease (IBD) and reduced morbidity and mortality in non-vaccinated chicks challenged with IBD.

Although vitamin C has been shown to be advantageous in some case, there is no information on the effects of vitamin C or ascorbic acid on the inflammatory process in an avian model like poultry. Cytokines are vital constituents in the regulation of immunity and inflammation, and thrombocytes are known to have an important role in innate immunity. In this study, we will investigate the effect of purified LPS on pro-inflammatory cytokine (IL-1β, IL-6 and IL-12) mRNA expression by thrombocytes in stressed broiler chicks with and without supplemental vitamin C.

MATERIALS AND METHODS

Chickens and Diets

The 54 broiler chicks used for this study were obtained from a local hatchery and received standard commercial starter diet until two weeks of age. For the first experiment, 18 chicks were randomly assigned into three groups that received different diets from 2-4 weeks of age and were housed at the Morgan Poultry Center, Clemson, SC. The dietary groups were a control group fed the control diet (standard corn-soy diet), a stressed group fed the control diet with 30 mg/kg corticosterone to stimulate oxidative stress, and a vitamin C group fed the control diet with 30 mg/kg corticosterone plus 500 mg/kg vitamin C. The second experiment was a repeat of the first experiment but included 36 chicks (12 per dietary groups).

Thrombocyte isolation

Blood samples were collected into syringes from the wing vein with 10% EDTA as anticoagulant for thrombocyte isolation. The collected blood was stored on ice until brought back to the laboratory. The thrombocyte isolation protocol used here was modified from the protocol used by Horiuchi et al. (2004). The blood samples were diluted (1:1) with calcium and magnesium free Hanks balanced salt solution (HBSS) (Cambrex Bio Sciences Walkersville Inc., Walkersville, MD). Diluted blood samples were then layered on a lymphocyte separation medium (Density 1.077-1.080g/ml, Mediatech. Inc., Herdon, VA) and centrifuged at 1700xg for 30 minutes at 23ºC. The band containing the thrombocytes were then collected and washed twice and resuspended in calcium and magnesium free HBSS. Smears were prepared with the purified

thrombocytes in order to assess purity. The smear was made by putting a drop of the cell suspension on a microscope slide and spreading it with a beveled edged microscope slide. The slide was then covered in Wright's Stain, rinsed, air dried and finally cover-slipped. The cell suspensions contained an average of 91% thrombocytes. Trypan blue solution (0.4% w/v in normal saline) was an exclusion stain required for the quantification of viable cell numbers using a SPolite® Hemacytometer (Baxter Healthcare, McGaw Park, IL). The cell concentrations were adjusted to 1 x 10^7/ml for the following experiments.

Thrombocyte Stimulation

The isolated thrombocytes (1 x 10^7 in 1 ml) were incubated with 10μg Ultra pure *S. Minnesota* LPS (InvivoGen, San Diego, CA) on a rocking platform at 40ºC, and 5% CO_2 for an hour.

RNA Isolation and Quantification

After thrombocyte stimulation, cells were centrifuged at 5000xg for 2 minutes to pellet. The pellets were stored in 100 μl of RNA*later* (Qiagen Inc., Valencia, CA), a RNA stabilizing solution overnight at 4ºC. After 24 hours in RNA*later*, the cells were centrifuged again to remove the supernatant and stored at -20ºC for use later.   The RNeasy Kit (Qiagen Inc., Valencia, CA) was used and the manufacturer's protocol was followed to isolate the total RNA from these samples. First the cells were disrupted by addition of buffer RLT (included in the RNeasy Kit), a lysis buffer. Then to homogenize the samples, the cell lysates were pipetted onto QIAshredder spin columns (Qiagen Inc., Valencia, CA) placed in 2 mL collection tubes and centrifuged at maximum speed. Before being centrifuged on the RNeasy mini columns (Qiagen Inc., Valencia, CA), 70% ethanol was added to the homogenized cell lysates. The RNA samples were treated with an on-column DNase, RNase-free DNase Set (Qiagen Inc., Valencia, CA) to remove any

possible contamination from chicken genomic DNA. Then, the RNeasy columns were
centrifuged several times after addition of several buffers according to the manufacturer's
protocol to finally isolate RNA from the samples. Isolated RNA was quantified using an
Eppendorf BioPhotometer (Eppendorf, Hamburg, Germany).

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

RT-PCR was performed using a QuantiTech SYBR Green RT-PCR Kit (Qiagen
Inc., Valencia, CA) using an Eppendorf Mastercycler ep *realplex* (Eppendorf North
America, Westbury, NY).   Primers used for GAPDH, IL-1β, IL-6 and IL-12
amplification were designed by and purchased from Integrated DNA Technologies Inc.
(Coralville, IA). The primers used for RT-PCR are listed in Table 1. The RT-PCR
mixture consisted of 0.25 μl QuantitTect RT Mix, 12.5 μl 2×QuantitTect SYBR Green
RT-PCR Master Mix, 0.5 μL of 0.5 μM of each specific primer listed in Table 1, 10 μL of
1 ng/μL template RNA, and 1.25 μL of RNase-free water to make the final reaction
volume of 25 μL. The cycling profile used for all the reactions is 1 cycle of 50°C for
30.min, 95°C for 15 min, and 40 cycles of 94°C for 15 s, 57°C for 20 s, and 72°C for 20s.

Table 1: List of primers used for RT-PCR.

Target RNA	Accession No.	Primer Sequence (5'-3')
GAPDH	NM_204305	F: ATGCCATCACAGCCACACAGAAGA
		R: ATGCCATCACAGCCACACAGAAGA
IL-1β	Y15006	F: GCTCTACATGTCGTGTGTGATGAG
		R: TGTCGATGTCCCGCATGA
IL-6	AJ309540	F: ATGTGCAAGAAGTTCACCGTGTGC
		R: TTCCAGGTAGGTCTGAAAGGCGAA
IL-12	NM_213571	F: TGTCTCACCTGCTATTTGCCTTAC
		R: CATACACATTCTCTCTAAGTTTCCACTGT

Data Collection and Analysis

Fluorescence data collection was performed during the extension step of the cycling protocol. The data obtained from the RT-PCR, was first analyzed using information on the cycle number (C_t values). Software recorded cycle number for each sample at which the fluorescence crosses the arbitrary threshold; this crossing point is referred as the C_t value. Samples that express more of a given gene product will have lower C_t value, while lower expressing samples will have higher C_t values.

Relative quantification of mRNA levels was determined through the use of the relative fold change calculation according to Pfaffl (2001b). This method of relative quantification is usually used when amplification efficiencies (E) between target and housekeeping genes are approximately equal to one another, which was the case for these reactions. Amplification efficiencies used here were calculated by using Equation 1, where E represents amplification efficiency and s signifies slope.

$$E(s) = 10^{-(1/s)} - 1 \qquad (1)$$

The efficiencies for each gene (GAPDH, IL-1β, IL-6, and IL-12) were calculated by plotting the log of the template dilution versus Ct values. Five-fold dilutions of total cellular RNA from broiler chickens were used to generate standard curves for analysis. The specifics of the standard curve are summarized in Table 2. Total cellular RNA used ranged from 3.13 to 50 ng. No-template controls were used in order to confirm target specific amplification, and no-reverse transcriptase controls were used to detect primer dimer formation.

Table 2: Details of the standard curves used for data analysis.

Gene of Interest	Efficiency	Slope	R^2
GAPDH	0.98	-3.405	0.989
IL-1β	0.99	-3.339	0.99
IL-6	0.91	-3.526	0.994
IL-12	0.94	-3.507	0.99

Relative change in IL-1β, IL-6, and IL-12 mRNA expressions following stimulation by LPS were expressed in terms of fold change in expression levels. Fold expression values were determined using Equation 2.

$$FoldExpression = \frac{\left(1 + E_{Target}\right)^{\Delta C_{t,Target}}}{\left(1 + E_{GADPH,Control}\right)^{\Delta C_{t,GAPDH}}} \tag{2}$$

where,

$$\Delta C_{t,Target} = (C_{t,\text{Target, Control}} - C_{t,\text{Target, LPS}})$$

and

$$\Delta C_{t,GAPDH} = (C_{t,\text{GAPDH, Control}} - C_{t,\text{GAPDH, LPS}})$$

Statistical Analysis

The factorial experiments were conducted using split-plot design with 18 chicks (whole plots) randomly assigned to provide 6 replications for each of the three diets in experiment one and 36 chicks (whole plots) randomly assigned to provide 12 replications for each of the three diets in experiment two. The blood sample collected from each of the 16 (2 out of 18 chicks died) and 36 chicks was split into two parts (sub-plots) with one part serving as a control (no LPS) and the other part stimulated with LPS. The linear model (Equation 3) for this study was:

$$Y_{ijk} = \mu + \alpha_i + d_{ik} + \beta_j + (\alpha\beta)_{ij} + e_{ijk} \tag{3}$$

where $i = 1,..,3$, $j = 1,2$, $k = 1,..,6$ for 1st study and $k=1,..,12$ for the 2nd study, Y_{ijk} denotes observed response of jth level of LPS from k^{th} chick that received i^{th} diet, μ denotes grand mean, α_i denotes effect of ith diet, d_{ik} denotes whole plot error, β_j denotes effect of jth level of LPS, $(\alpha\beta)_{ij}$ denotes interaction effect between i^{th} diet and j^{th} level of LPS, and e_{ijk} denotes sub-plot error. Analysis of variance was performed with the General Linear Model procedure of SAS 9.1 (SAS Manual 2000), and hypothesis testing was conducted using $\alpha = 0.05$. In order to test for homogeneity of the obtained data, Levene's test for homogeneity was performed. Hartley's Test was performed in order to ensure that there was equality of variance and the equal variance assumption was applicable in our model.

RESULTS

Appearance

The three groups of chicks had clear differences in appearance in both studies. The control group was the healthiest looking among the three groups by being largest in size, best feathered, and comparatively easiest to bleed having larger wing veins. The stressed group receiving control diet with added 30 mg/kg corticosterone was the smallest in size, worst feathered, most difficult to bleed having very small veins and hardest to handle as they appeared very stressed. The vitamin C group that was fed control diet with 30 mg/kg corticosterone plus 500 mg/kg vitamin C had intermediate features in body size, feathering, and vein size.

Thrombocyte Purity Heterophil to Lymphocyte Ratio

The isolated thrombocyte preparation contained on average 91% thrombocytes, 6.4% lymphocytes and 2.6% heterophils. These values were obtained only for the first study.

Heterophil to Lymphocyte Ratio

Heterophil to lymphocyte ratio was also recorded for only the first study. The heterophil to lymphocyte ratio was 0.419 for the control group, 0.29 for the stressed group and 0.320 for the vitamin C group.

Comparison of C_t values

No significant interaction was observed between the two factors, diet and LPS stimulation in Study 1 and 2 (p= 0.8067 and p= 0.2080). Statistical analysis of C_t values showed no significant difference (p>0.05) in the 3 diet groups in the level of the

housekeeping gene, GAPDH and the cytokine gene expression of IL-1β, IL-6 and IL-12 due to LPS stimulation in Study 1 and 2 (Table 3). The C_t values for both studies are provided in Figures 5 and 6. In Figures 5 and 6, the horizontal axis represents the types of gene expression (housekeeping gene GAPDH, IL-1β, IL-6, IL-12). LPS stimulation affected the C_t values for housekeeping gene, GAPDH in Study 1 but it did not affect C_t values in Study 2 (Table 4). However it is evident from these figures that the LPS stimulation increases the expression of inflammatory cytokines IL-1β, IL-6, and IL-12. Note that increase in gene expression decreases C_t value. Table 4 demonstrates that diet did not significantly affect the expression of pro-inflammatory cytokines, while Table 3 shows a significant increase in expression of pro-inflammatory cytokines IL-1β, IL-6 and IL-12 due to LPS stimulation in both studies. Although housekeeping gene (GAPDH) was not supposed to be affected by LPS stimulation, a significant change in GAPDH was observed in the first study due to LPS stimulation.

Table 3: Effect of diet on expression of certain genes when stimulated with LPS.

Study 1		Study 2	
Gene	p-value[3]	Gene	p-value
GAPDH	0.2080	GAPDH	0.8067
IL-1β	0.4743	IL-1β	0.7353
IL-6	0.2609	IL-6	0.9806
IL-12	0.9525	IL-12	0.92.11

[3] The p-values observed by performing analysis of variance (ANAOVA) to detect the interaction between the two factors diet and expressions of certain genes when stimulated with LPS revealed that there is insufficient evidence of interaction between these two factors 0.05 level of significance.

Table 4: Effect of LPS on expression of housekeeping and pro-inflammatory cytokine genes.

Study 1		Study 2	
Gene	p-value[4]	Gene	p-value
GAPDH	0.0313	GAPDH	0.7614
IL-1β	<0.0001	IL-1β	<0.0001
IL-6	<0.0001	IL-6	<0.0001
IL-12	<0.0001	IL-12	<0.0001

[4] The p-values observed by performing analysis of variance (ANAOVA) to test the effect of LPS on the expression of house-keeping and different inflammatory cytokine genes at the 0.05 level of significance
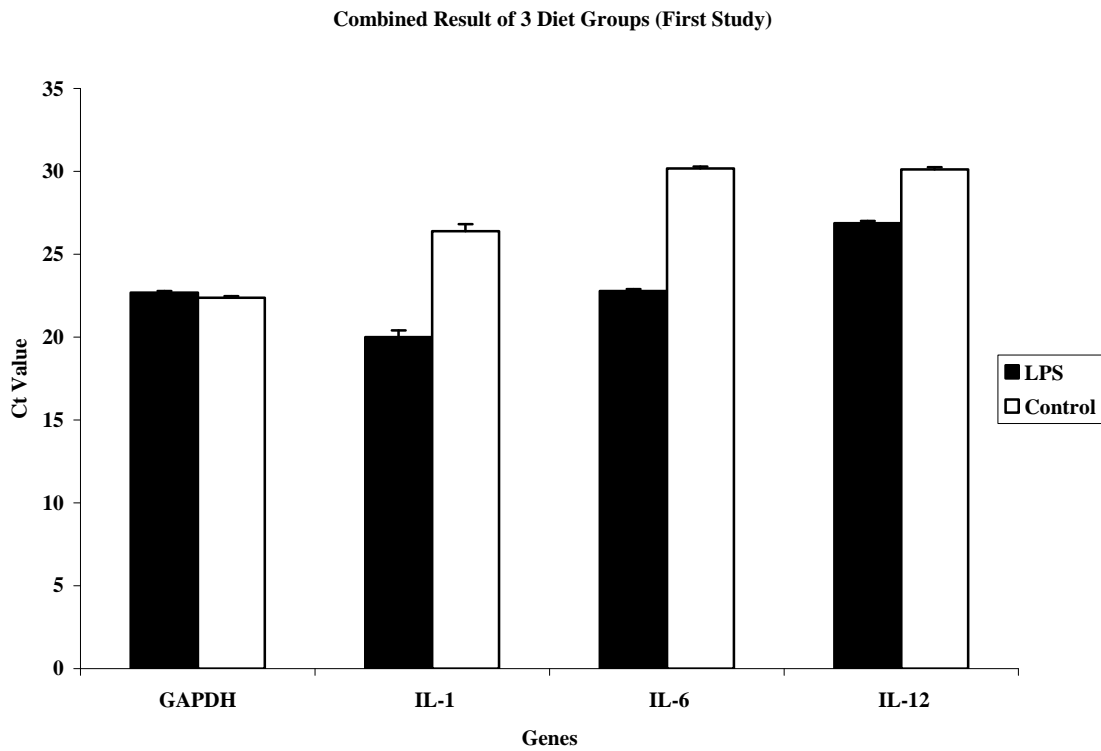
Combined Result of 3 Diet Groups (First Study)

Figure 5: The mean C_t values of GAPDH, IL-1β, IL-6 and IL-12 for LPS treated or control without LPS for Study 1. Error bars show ±SEM with 14 samples.

Combined Results of 3 Diet Groups (Second Study)
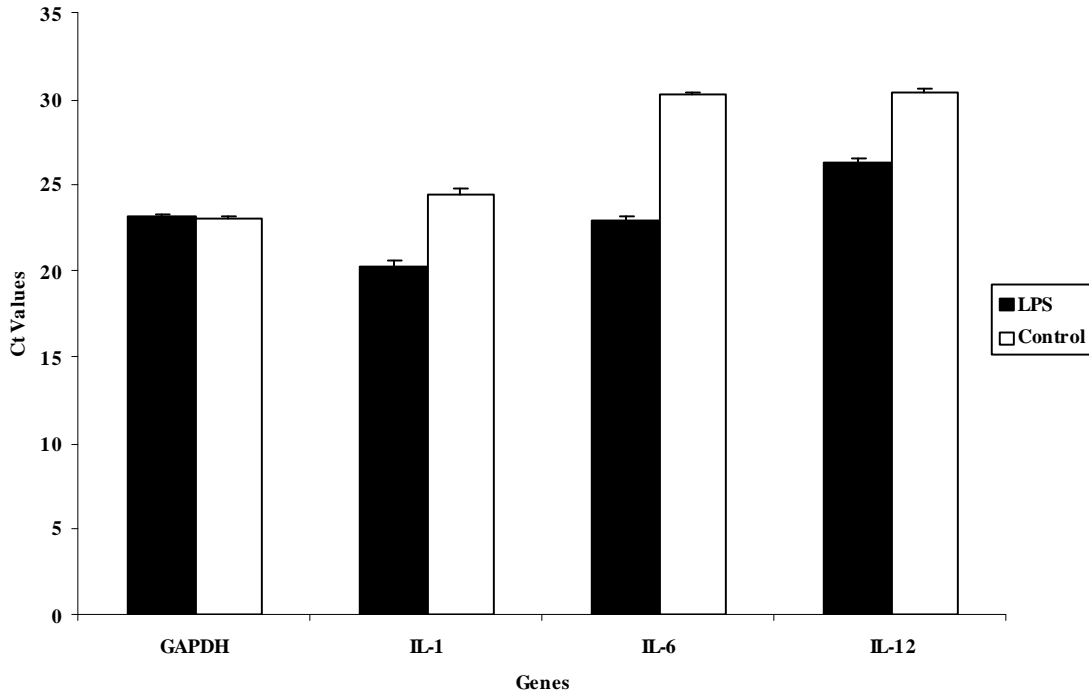
Figure 6: The mean C_t values of GAPDH, IL-1β, IL-6, and IL-12 for LPS treated or control without LPS for Study 2. Error bars show ± SEM with 36 samples.

Further Analysis of C_t values

Each sample of extracted RNA had a different A_{260}/A_{280} value. These values are obtained from Eppendorf BioPhotometer (Eppendorf, Hamburg, Germany) during quantification. The statistical analysis discussed above was repeated using A_{260}/A_{280} values of the samples as a covariate to determine if there was a relationship between C_t and A_{260}/A_{280} values. Plots generated with C_t values on the Y-axis and A_{260}/A_{280} values on the X-axis showed random distribution rather than any specific slope or pattern (Data not shown).

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Comparison of Fold Expression

Equation (2) was used to compute the fold change in expression levels using the C_t value for both studies. Statistical analysis using the fold expression values support the results from Analysis of C_t values that diet do not affect the expression of pro-inflammatory cytokines and LPS stimulation increased the level of expression compared to control group (Table V). The values of the fold expression for study 1 and study 2 are shown in Figures 7, 8, and 9 respectively. It is evident from these figures that the relative fold expression increased for all three pro-inflammatory cytokine IL-1β, IL-6, and IL-12. In both studies, the fold change in expression level for IL-6 was the highest ranging from 220 to 480.

In the first study that was done with only 16 chicks, fold expression values ranged from 0.736 to 709.511, while for the second study with 36 chicks the values ranged from 0.27 to 1157.91. The highest variation was specially observed in the relative fold expression of IL-6 in both studies. In order to test for homogeneity of the obtained data since there was a large variation, Levene's test for homogeneity was performed. Levene's test for homogeneity indicated that the data was homogeneous enough to perform analysis of variance (p-values ranged from 0.0229 to 0.8993 for the first study and 0.0139 to 0.181 for the second). Hartley's Test was performed in order to further validate our results. Hartley's test indicated that there was equality of variance and the equal variance assumption was applicable in our model.

Table V: Effect of dietary groups on LPS induced fold expression.

Study 1		Study 2	
Gene	p-value	Gene	p-value
IL-1β	0.9974	IL-1β	0.3016
IL-6	0.5500	IL-6	0.6453
IL-12	0.3282	IL-12	0.6474

Fold Expression of the Pro-inflammatory Cytokine lL-1 in the Three Groups of Chicks

Figure 7: Relative quantification in terms of fold expression of pro-inflammatory cytokine, IL-1β in response to LPS stimulation in the 3 groups of broiler chicks in the first and second study. Diet 1, 2 and 3 were fed to the Control Group, the Stressed Group and the Vitamin C Group respectively. Although LPS stimulation increased level of IL-1β mRNA expression in all three groups of chick, the level of expression was not statistically significant in the three dietary groups.

Fold Expression of Pro-inflammatory Cytokine IL-6 in the Three Groups of Chicks
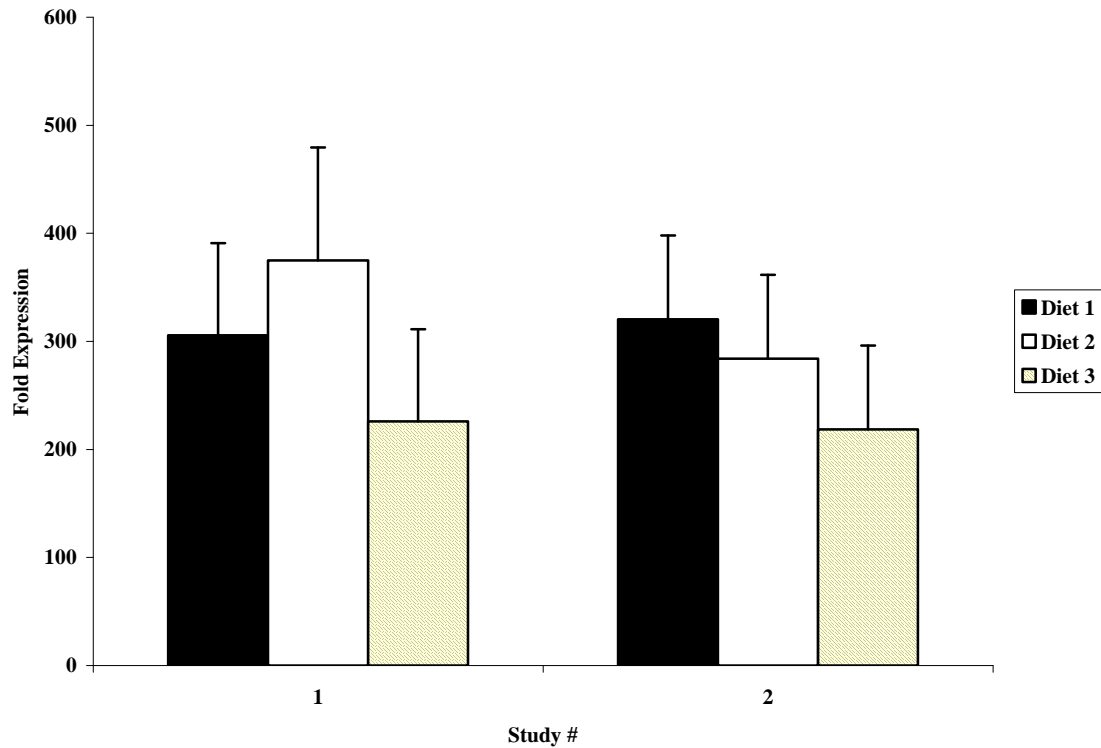
Figure 8: Relative quantification in terms of fold expression of pro-inflammatory cytokine, IL-6 in response to LPS stimulation in the 3 groups of broiler chicks in the first and second study. Diet 1, 2 and 3 were fed to the Control Group, the Stressed Group and the Vitamin C Group respectively. Although LPS stimulation increased level of IL-6 mRNA expression in all three groups of chick, the level of expression was not statistically significant in the three dietary groups.

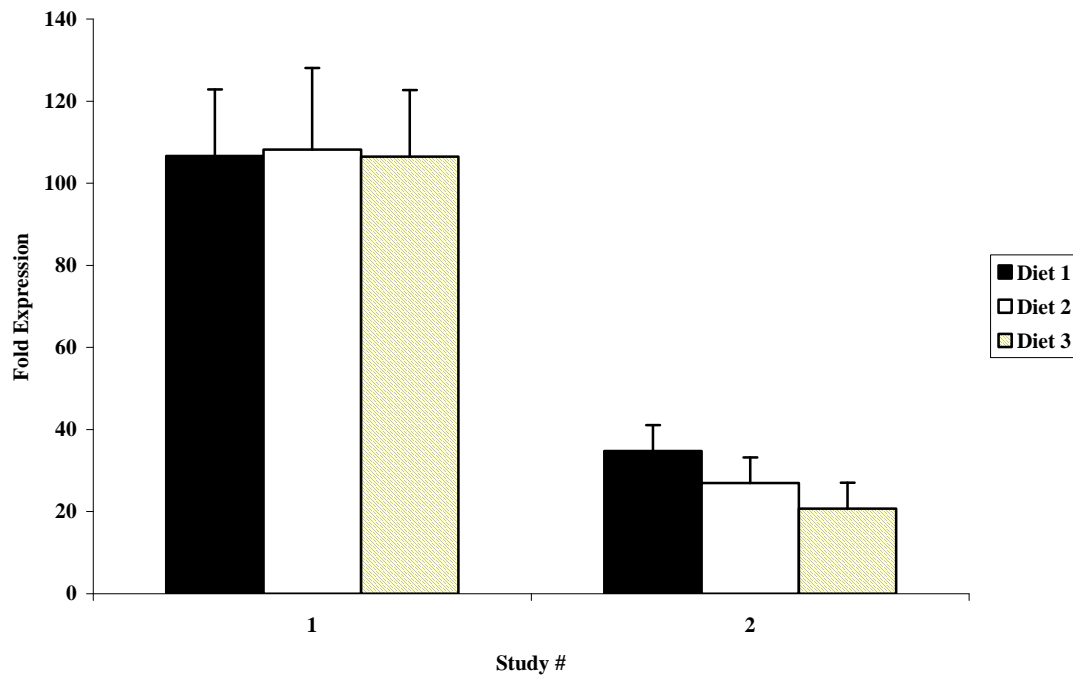**Fold Expression of Pro-inflammatory Cytokine IL-12 in the Three Groups of Chicks**
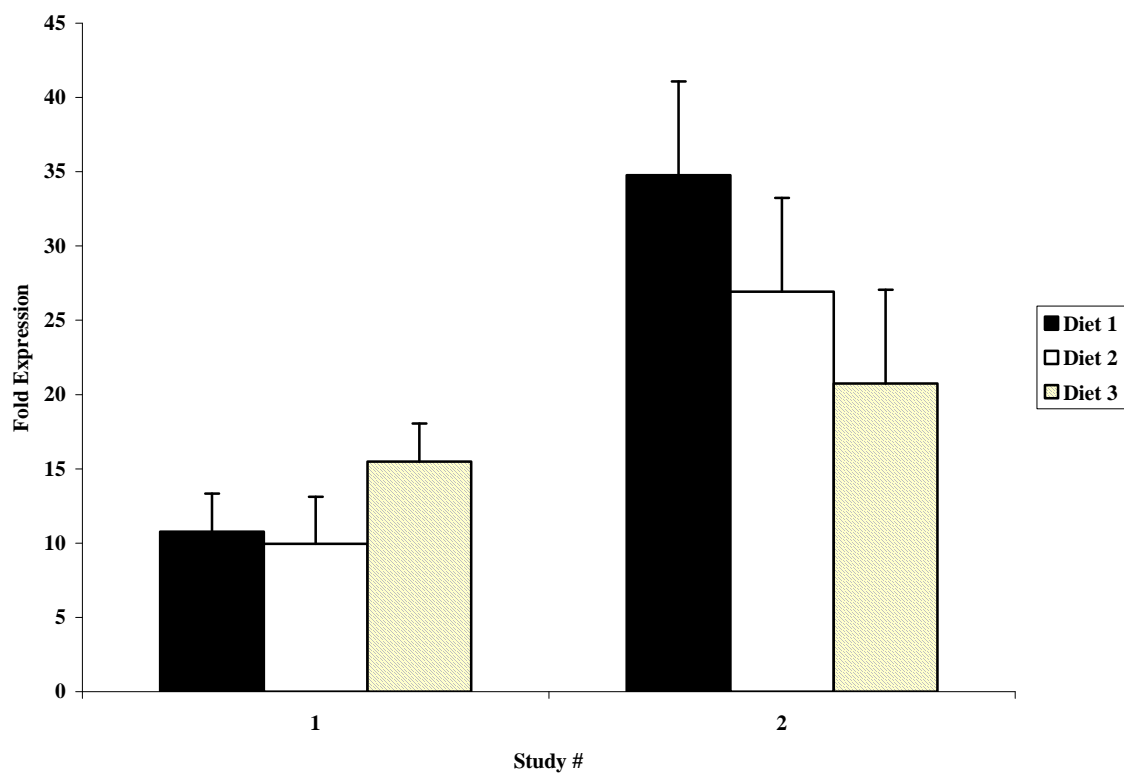
Figure 9: Relative quantification in terms of fold expression of pro-inflammatory cytokine, IL-12 in response to LPS stimulation in the 3 groups of broiler chicks in the first and second study. Diet 1, 2 and 3 were fed to the Control Group, the Stressed Group and the Vitamin C Group respectively. Although LPS stimulation increased level of IL-12 mRNA expression in all three groups of chick, the level of expression was not statistically significant in the three dietary groups.

DISCUSSION

The motivation for this study is based on (i) recent interest in the literature on the role of thrombocytes (i.e., mammalian platelets) as a link between innate and adaptive immune response; and (ii) role of dietary vitamin C supplementation on immune response in chickens. In this study, we investigated the effect of types of diet in broiler chicks on the expression of pro-inflammatory cytokines IL-1β, IL-6, and IL-12 in thrombocytes that play an important role in innate immune response with potential effects on adaptive immune response. We also investigated the effect of LPS, a microbial component to which broiler chicks can be exposed, on the expression of these pro-inflammatory cytokines.

It has been previously reported that supplementing vitamin C in the diet may alleviate stress in broiler chickens (Gous and Morris, 2005), improve clinical symptoms of IBD, and reduce morbidity and mortalility in non-vaccinated chicks challenged with IBD (Amakye-Amin et al., 2000), and reduce stress response observed in broiler chicken being prepared for processing prior to slaughter (Satterlee et al., 1989). In this study, the overall appearance of the group of chickens that received supplemental vitamin C was better than the stressed broiler chicks which supports the previous reports mentioned above.

In this study, any increase in heterophil to lymphocyte (H/L) ratio in stressed chicks was not observed. In fact, the H/L ratio decreased compared to control group. However, note that only 18 chicks were used for the first study, and the blood was

collected two weeks after initial exposure of stress induced by 30 mg of corticosterone per kg feed. The decrease in H/L ratio may be due to chickens adapting to stress during an intermediate stage of resistance.

However the results from analysis of RT-PCR data did not show any significant differences in cytokine expressions due to addition of vitamin C. This may be due to low vitamin C content in chicken thrombocytes compared to platelets in human (Evans et al., 1982). Another possible explanation may be that the thrombocytes remain fairly unaffected during stress even with supplemental vitamin C. According to Gross (1989), the addition of corticosterone (200 mg/kg) to the diet did not affect the thrombocytes morphology score (TMS) but increased H/L ratio, and injection of an adrenal blocker, 1,1-dichloro-2, 2 bis p-choro-phenyl ethane (ABC), decreased H/L values but again did not alter TMS. Since corticosterone and ABC failed to alter TMS, Gross (1989) concluded that thrombocytes are not sensitive to circulating corticosterone concentrations. This feature of the thrombocytes might be a way for the immune system to protect the body during stress and challenge to bacterial infection. We have already discussed how thrombocytes play an important role in protecting innate immunity by being able to bind, aggregate, and internalize microorganisms, and express a vast array of potent pro-inflammatory substances. Thrombocytes are exceptional because stress induced increases in levels of corticosterone do not affect their activity but at the same time pathogenic microorganisms easily stimulate their pro-inflammatory effect.

LPS affects cell function and induces gene expression in chicken monocytes, macrophages, and heterophils (Dil et al., 2002a; Dil et al, 2002b; Hussain and Qureshi, 1997; and Hussain and Qureshi, 1998). In fact, we have demonstrated in this study that LPS also induces expression of the pro-inflammatory cytokines IL-1β, IL-6 and IL-12 in

thrombocytes similar to heterophils, the assumed primary effector cells of the innate host defenses to bacterial infections in poultry. Heterophils are the avian equivalent of the neutrophils and function as professional phagocytes to aid in regulation of innate host defenses (Kogut et al, 2002). Chicken heterophils constitutively express TLR1/6/10, TLR2 type 1, TLR2 type 2, TLR3, TLR4, TLR5, and TLR7 mRNA (Kogut et al., 2005a). Our laboratory has recently determined that thrombocytes express TLR2, TLR3 and TLR4 (Scott and Dimmick Owens, unpublished). Therefore, the observed LPS effects on broiler chick thrombocytes are mediated through TLR4 with presumed activation of NF-κB.

Since the first demonstration of avian thrombocytes as phagocytes by Glick et al. (1964), there has been extensive work done in the area of phagocytic ability of thrombocytes (Clarson et al., 1968; Carlson and Allen, 1969; Strez and Weiss, 1973; Chang and Hamilton, 1976; Chang and Hamilton, 1979a, and Chang and Hamilton, 1979b). There are also some reports available in which avian thrombocytes have been shown to play a major role in homeostasis like mammalian platelets by aggregating to form a homeostatic plug (Stalsberg and Prydz, 1963; and Hodges, 1979). Thrombocytes have also been shown to secrete antiheparin proteins (Wachowicz et al., 1981). But to date, there is no study done with thrombocytes that shows their ability to be major effector immune cells by being stimulated by LPS or other pathogenic components. Although heterophils have been studied so far as primary effector cells in the innate immune response, the finding of this study along with the unpublished data of Scott and Dimmick Owens that demonstrated that thrombocytes express TLR2, TLR3 and TLR4 potentially places thrombocytes as the primary effector cells. Thrombocytes are the most abundant white blood cells in the avian blood (Chang and Hamilton, 1979a). There

are almost 6 times more thrombocytes than heterophils in avian ciculating blood (Lucas and Jamroz, 1961; and Glick 1958). This demonstrates that although thrombocytes and heterophils both have similar effector cells functions in the innate host defenses to bacterial infections, thrombocytes by shear number can claim the role of being the primary effector cell in poultry.

CONCLUSION AND FUTURE WORK

In summary, it has been found that the types of diet in broiler chicks did not effect the expression of pro-inflammatory cytokines IL-1β, IL-6, and IL-12 in thrombocytes. Also, LPS does increase the expression of pro-inflammatory cytokines IL-1β, IL-6, and IL-12. In the future, as a follow up experiment one could (i) investigate the expression of other genes in addition to IL-1β, IL-6, and IL-12 in similar groups of chicks; (ii) investigate the effect of vitamin C on gene expression by collecting other cells for example from spleen, liver, etc.; and (iii) investigate the pro-inflammatory cytokine response of thrombocytes in similar groups of chicks challenged by injection of a microbial component.

LITERATURE CITED

Aderem, A.; and Ulevitch, R.J. 2000. Toll-like receptors intheinduction of the innate immune response. Nature 406: 782-787.

Altan, Ö; Pabuçcuoğlu, A.; Altan, A.; Konyalioğlu, S.; and Bayraktar H. 2003. Effect of heat stress on oxidative stress, lipid peroxidation and some stress parameters in broilers. British Poultry Science 44**:** 545–550.

Amakye-Anim J.; Lin, T.L.; Hester, P.Y.; Thiagarajan, D.; Watkins, B.A.; and Wu, C.C. 2000. Ascorbic acid supplementation improved antibody response to infectious bursal disease vaccination in chickens. Poultry Science 79(5): 680-8.

Andonegui, G; Kerfoot, SM; McNagny, K; Ebbert, KV; Patel, KD; and Kubes, P. 2005. Platelets express functional Toll-like receptor-4. Blood 106(7): 2417-23.

Archer, R. K. 1971. Blood coagulation. Physiology and Biochemistry of Domestic Fowl, 2nd Edition (Bell, D.J. and Freeman, B.M. editors), Academic Press, London: 898-910.

Aslam, R.; Speck, E.R.; Kim, M.; Crow, A.R.; Bang, K.A.; Nestel, F.P.; Ni, H.; Lazarus, A.H.; Freedman, J.; and Semple, J.W. 2006. Platelet toll-like receptor expression modulates lipopolysaccharide-induced thrombocytopenia and tumor necrosis factor production in vivo. Blood 107(2): 637-641.

Belamarich, F.A.; and Simoneit, L.W. 1973. Aggregation of duck thrombocytes by 5-hydroxytryptamine. Microvascular Research 6: 229-234.

Ben Nathan, D.; Heller, E.D.; and Perek, M. 1976. The effect of short heat stress upon leucocyte count, plasma corticosterone level, plasma and leucocyte ascorbic acid content. British Poultry Science 17: 481-485.

Beuving, G.; Jones, R.B.; and Blokhuis, H.J. 1989. Adrenocortical and heterophil/lymphocyte responses to challenge in hens showing short or long tonic immobility reactions. British Poultry Science 30: 175-184.

Bowie, A.; and O'Neill, L.A. 2000. Oxidative stress and nuclear factor-kappaB activation: a reassessment of the evidence in the light of recent discoveries. Biochemical Pharmacology 59(1): 13-23.

Bowie, A.; and O'Neill, L.A. 2000. Vitamin C inhibits NF-kappa B activation by TNF via the activation of p38 mitogen-activated protein kinase. The Journal of Immunology 165(12): 7180-7188.

Carcamo, J.M.; Pedraza, A.; Borquez-Ojeda, O.; and Golde, D.W. 2002. Vitamin C suppresses TNF alpha-induced NF kappa B activation by inhibiting I kappa B alpha phosphorylation. Biochemistry 41(43): 12995-13002.

Carlson, H.C.; Sweeny, P.R.; and Tokaryk, J.M. 1968. Demonstration of phagocytic and trephocytic activities of chicken thrombocytes by microscopy and vital staining techniques. Avian Diseases 12: 700-715.

Carlson, H.C.; and Allen, J.R. 1969. The acute inflammatory reaction in chicken skin:blood cellular response. Avian Diseases 13: 817-833.

Chang, C. F.; and Hamilton, P. B. 1976. Phagocytic properties of chicken thrombocytes. Poultry Science 55: 2018.

Chang, C. F.; and Hamilton, P. B. 1979a. The thrombocyte as the primary circulating phagocyte in chickens. Journal of Reticuloendothel Society 25: 585-590.

Chang, C. F.; and Hamilton, P. B. 1979b. Refractory phagocytosis by chicken thrombocytes during aflatoxicosis. Poultry Science 58: 559-561.

Chakrabarti, S;Varghese, S.;Vitseva, O.;Tanriverdi, K.; and Freedman, J.E. 2005. CD40 Ligand Influences Platelet Release of Reactive Oxygen Intermediates. Arteriosclerosis, Thrombosis, and Vascular Biology 25: 2448-2434.

Clawson, CC.; and White, JG. 1971. Platelet interaction with bacteria. II. Fate of bacteria. American Journal of Pathology 65: 381–98.

Cognasse, F.;Hamzeh, H.; Chavarin, P.; Acquart, S.; Genin, C.; and Garraud, O. 2005. Evidence of toll-like receptor molecules on human platelets. Immunology & Cell Biology 83: 196-198.

Dil, N.; and Qureshi, M.A.; 2002a. Differential expression of inducible nitric oxide synthase is associated with differential Toll-like receptor-4 expression in chicken macrophages from different genetic backgrounds. Veterinary Immunology and Immunopathology 84 (3–4): 191–207.

Dil, N; and Qureshi, MA. 2002b. Involvement of lipopolysaccharide related receptors and nuclear factor κB in differential expression of inducible nitric oxide synthase in chicken macrophages from different genetic backgrounds. Veterinary Immunology and Immunopathology 88 (3–4): 149–161.

Edens, F.W.; and Siegel, H.S. 1975. Adrenal responses in high and low ACTH response lines of chickens during acute heat stress. General Comparative Endocrinology 25: 64-73.

Edens, F.W.; and Siegel, H.S. 1976. Modification of corticosterone and glucose responses by sympatholytic agents in young chickens during acute heat exposure. Poultry Science 55: 1704-1712.

Elzey, B.D.; Sprague, D.L.; and Ratliff, T.L. 2005. The emerging role of platelets in adaptive immunity. Cellular Immunology 238: 1-9.

Elzey, B.D.; Tian, J.; Jensen, R.J.; Swanson, A.K.; Lees, J.R.; Lentz, S.R.; Stein C.S.; Nieswandt, B.; Wang, Y.; Davidson, B.L.; and Ratliff, T.L. 2003. Platelet-mediated modulation of adaptive immunity. A communication link between innate and adaptive immune compartments. Immunity 19: 9-19.

Evans, R.M.; Currie, L.; and Campbell, A. 1982. The distribution of ascorbic acid between various cellular components of blood, in normal individuals, and its relation to the plasma concentration. British Journal of Nutrition 47(3): 473-82.

George, J.N. 2000. Platelets. Lancet 355: 1531-1539.

Glick, B. 1958. The Effect of cortisone acetate on the leukocytes of young chickens. Poultry Science 37: 1446-1452.

Glick, B.; Sato, K.; and Cohenour, F.; 1964. Comparison of phagocytic ability of normal and bursectomized birds, Journal of Reticuloendothel Society 1: 442-449.

Gous, R. M.; and Morris, T. R. 2005. Nutritional interventions in alleviating the effects of high temperatures in broiler production. Worlds Poultry Science 61: 463–475.

Gross, W.B. 1989. Factors affecting chicken thrombocyte morphology and the relationship with heterophil:lymphocyte ratios. British Poultry Science 30(4): 919-925.

Gross, W.B. 1992. Effects of ascorbic acid on stress and disease in chickens. Avian Diseases 36: 688-692.

Gross, W.B.; and Chickering, W. 1987. Effects of fasting, water deprivation and adrenal-blocking chemical on resistance to Escherichia coli challenge. Poultry Science 66: 270-272.

Gross, W.B.; and Siegel, P.B. 1986. Effects of initial and second periods of fasting on heterophil/lymphocyte ratios and body weight. Avian Diseases 30: 345-346.

Haziot, A.; Ferrero, E.; Köntgen, F.; Hijiya, A.; Yamamoto, S.; Silver, J, Stewart, C.L.; and Goyert, S.M. 1996. Resistance to endotoxin shock and reduced dissemination of Gram-negative bacteria in CD14-deficient mice. Immunity 4: 407-414.

Henn, V.; Slupsky, J. R.; Grafe, M.; Anagnostopoulos, I.; Forster, R.; Muller-Berghaus, G.; and Kroczek, R. A. 1998. CD40 ligand on activated platelets triggers an inflammatory reaction of endothelial cells. Nature 391: 591-594.

Hodges, R.D. 1979. The blood cells. In: Form and Function in Birds, Vol. 1(King, A.S.; and McLelland, J., Eds), Academic Press, New York: 361-379.

Horiuchci, H.; Tanka, K.; Shigeta, A.; Yoshida, K.; Kushima, K.; Ohta, H.; Furusawa, S; and Matsuda, H. 2004. A monoclonal antibody against chicken thrombocytes reacts with the cells of thrombocyte lineage. The Journal of Veterinary Medical Science 66(3): 243-250.

Horton, J.H.; White, D.J.; Maass, D.L.; Hybki, D.P.; Haudek, S.; and Giroir, B. 2001. Antioxidant vitamin therapy alters burn trauma-mediated cardiac NF-☐B activation and cardiomyocyte cytokine secretion. The Journal of Trauma Injury, Infection, and Critical 50(3): 397-408.

Hussain, I.; and Qureshi, M.A. 1997. Nitric oxide synthase activity and mRNA expression in chicken macrophages. Poultry Science 76(11): 1524–1530.

Hussain, I.; and Qureshi, M.A. 1998. The expression and regulation of inducible nitric oxide synthase gene differ in macrophages from chickens of different backgrounds. Veterinary Immunology and Immunopathology 61(2–4): 317–329.

Jones, R.B. 1989. Chronic stressors, tonic immobility and leucocytic responses in the domestic fowl. Physiology and Behavior 46: 439-442.

Karin,M. 2005. Cancer Research in Flames Tracking inflammation's role in promoting malignancy could lead to better treatments. The Scientist 19(23)**:** 24-25.

Kaushansky, K. 1995. Thrombopoietin: the primary regulator of platelet production. Blood 86: 419–431.

Klinger, M.H. 1997. Platelets and inflammation. Anatomy and Embryology 196: 1-11.

Klinger, M.H.; and Jelkmann, W. 2002. Role of blood platelets in infection and inflammation. Journal of Interferon & Cytokine Research 22: 913-922.

Klotz, S.A.; Harrison, J.L.; and Misra, R.P. 1989. Aggregated platelets enhance adherence of Candida yeasts to endothelium. The Journal of Infectious Diseases160: 669–677.

Kogut, M.; Rothwell, L; and Kaiser, P. 2002. Differential effects of age on chicken heterophil functional activation by recombinant chicken interleukin-2. Developmental and Comparative Immunology 26(9): 817-30.

Kogut, M.H.; Iqbal, M.; He, H.; Philbin, V.; Kaiser, P.; and Smith A. 2005a. Expression and function of Toll-like receptors in chicken heterophils. Developmental and Comparative Immunology 29(9): 791-807.

Kogut, M.H.; He, H.; and Kaiser, P. 2005b. Lipopolysaccharide binding protein/CD14/ TLR4-dependent recognition of salmonella LPS induces the functional activation of chicken heterophils and up-regulation of pro-inflammatory cytokine and chemokine gene expression in these cells. Animal Biotechnology 16(2): 165-81.

Kuruma, I.; Okada, T.; Kataoka, K.; and Sorimachi, M.; 1970. Ultrasctructural observation of 5-hydroxytryptamine-storing granules in the domestic fowl thrombocytes. Zeitschrift Fuer Zellforsch 108: 268-281.

Lin, H.; Decuypere, E.; and Buyse, J. 2004. Oxidative stress induced by corticosterone administration in broiler chickens (Gallus gallus domesticus) 1. Chronic exposure. Comparative Biochemistry and Physiology - Part B: Biochemistry & Molecular Biology 139: 737-744.

Lucas, A. M.; and Jamroz, C. 1961. Atlas of Avian Hematology. Department of Agriculture Monograph 25, U.S**.**

Martin, J.F.; and Levine, R.F. 1991. Evidence in favour of the lungs as the site of platelet production. The platelet in health and disease. Oxford: Blackwell Scientific (Page, C.P., Editor): 1–9.

Maurice, D.; Lightsey, S. F.; Toler, J.; and Canty, S. 2006. Effect of chronic stress on ascorbic acid synthesis, tissue ascorbic acid and total antioxidant capacity in chickens (Gallus gallus domesticus). Journal of Animal Physiology and Animal Nutrition. In Review.

Maxwell, M.H. 1973. Comparison of heterophil and basophil ultrastructure in six species of somestic birds. Journal of Anatomy 115: 187-202.

Maxwell, M.H.; and Trejo, F. 1970. The ultrastructure of white blood cells and thrombocytes of the domestic fowl. British Veterinary Journal 126: 583-592.

Maxwell, M.H.; Robertson, G.M.; Mitchell, M.A.; and Carlisle, A.J. 1992a. The fine structure of broiler chicken blood cells, with particular reference to basophils, after severe heat stress. Comparative Haemotology International 2: 190-200.

Medzhitov, R. 2001. Toll-like receptors and innate immunity. Nature Reviews Immunology 1: 135-145.

Meseguer, J.; Ngeles Estban, M. A.; and Guez, A. R. 2002. Are Thrombocytes and Platelets True Phagocytes? Microscopy Research and Technique 57: 491–497.

Mitchell, M.A.; Kettlewell, P.J.; and Maxwell, M.H. 1992. Indicators of physiological stress in broiler chickens during road transportation. Animal Welfare. 1: 91-103.

Morrisey, J.K.; Paul-Murphy, J.; Fialkowski, J.P.; Hart, A.; and Darien, B.J. 2003. Estimation of Prothrombin Times of Hispaniolan Amazon Parrots (*Amazona ventralis*) and Umbrella Cockatoos (*Cacatua alba*). Journal of Avian Medicine and Surgery 17(2): 72–77.

Nirmalan, G.P.; Atwal, O.S.; and Carlson, H.C. 1972. Ultrastructural studies on the leucocytes and thrombocytes in the circulating blood of Japanese quail. Poultry Science 51: 2050-2055.

Okoye, J.O.A; Okoshi, L.I.; Ezema, A.R.S.; Chinwuba, A.R.S.; Oburnwe, K.G.; Orajaka, L.J.E.; and Orwor, R.C. 1998. Effect of dietary ascorbic acid supplementation on resistance to infectious bursal disease of chickens., in: Samberg Y (Ed) Proceedings of 10th European Poultry Conference, Jerusalem, Israel, pp 555-558 (World Poultry Science Assocoation).

Padh, H. 1991. Vitamin C: newer insights into its biochemical functions. Nutrition Reviews 49(3): 65-70.

Pfaffl, M.W. 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Research 29: 2002–2007.

Post, J.; Rebel, J.;and Ter Huurne, A. 2003. Physiological effects of elevated plasma corticosterone concentrations in broiler chickens. An alternative means by which to assess the physiologicaleffects of stress. Poultry science 82: 1313-1318.

Raetz, C.R.H.; Ulevitch, R.J.; Wright, S.D.; Sibley, C.H.; Ding, A.; and Nathan, C.F. 1991. Gram-negative endotoxin: an extraordinary lipid with profound effects on eukaryotic signal transduction. The Journal of the Federation of American Societies for Experimental Biology 5: 2652-2660.

Ries, S.; Kaufer, I.; Reinacher, M.; and Weiss, E. 1984. Immunomorphologic characterization of chicken thrombocytes. Cell Tissue Research 236(1): 1-3.

Robert, R.; Senet, C.; and Mahaza, V. 1992. Molecular basis of the interaction between Candida albicans, fibrinogen, and platelets. Journal of Mycologic Medicine 2: 19–25.

Rose, R.C.; and Bode, A.M. 1993. Biology of free radical scavengers: an evaluation of ascorbate. The Journal of the Federation of American Societies for Experimental Biology 7(12): 1135-42.

Rumsey, M.L. 1998. Absorption, transport, and disposition of ascorbic acid in humans. The Journal of Nutritional Biochemistry 9 (3): 116-130 S.

Saterlee, D.G.; Aguilera-Quintana, I.; Munn, B.J.; and Kautmann, B.A. 1989. Vitamin C amelioration of the adrenal stress response in broiler chickens being prepared for slaughter. Comparative Biochemistry And Physiology A Vol. 94 (4): 569-74.

Scott, T.R.; and Dimmick-Owens, M. unpublished data.

Scheld, W.M.;Valone, J.A.; and Sande, M.A. 1978. Bacterial adherence in the pathogenesis of endocarditis: interaction of bacterial dextran, platelets, and fibrin. The Journal of Clinical Investigation 61: 1394–404.

Siegel, H.S. 1980. Physiological stress in birds. BioScience. 301:529-533.

Sies, H. 1991. Oxidative stress: from basic research to clinical application, American Journal of Medicine 91: 31S–38S.

Simpson, C.F. 1968. Ultrastructural features of the turkey thrombocyte and lymphocyte. Poultry Science 47: 848-850.

Stalsberg, H.; and Prydz, H. 1963. Studies on chick embryo thrombocytes II. Function in primary homeostatsis. Thrombosis et Diathesis Haemorrhagia. (Stuttg.) 9: 291-299.

Stiller, R.A.; Belamarich, F.A.; and Shepro, D. 1975. Aggregation and release in thrombocytes of duck. American Journal of Physiology 229: 206-210.

Stopforth, A. 1970. A Study of coagulation mechanisms in domestic chickens. Journal of Comparative Pathology 80: 525–533.

Sugiyama, S. 1926. Origin of thrombocytes and of different types of blood-cells as seen in the living chick blastoderm. Carnegie Institute Washington Pub. No. 363. Contributions to Embryology 18: 121-147.

Sullam, P.M.;Valone, F.H.; and Mills, J. 1987. Mechanisms of platelet aggregation by viridans group streptococci. Infection and Immunity 55: 1743–50.

Swayne, D.E.; Stockham, S.L.; and Johnson, G.S. 1986. Cytochemical properties of chicken blood cells resembling both thrombocytes and lymphocytes. Veterinary Clinical Pathology15(2): 17-24.

Sweeny, P.R.; and Carlson, H.C. 1968. Electron microscopy and histochemical demonstration of lysosomal structures in chicken thrombocytes. Avian Diseases 12: 636-644

Takeda, K.; and Akira, S. 2005. Toll-like receptors in innate immunity. International Immunology 17: 1-14.

Takeda, K.; Kaisho, T.; and Akira, S. 2003. Toll-like receptors. Annual Review of Immunology 21: 335-376.

Underhill, D. M.; and Ozinsky, A. 2002. Toll-like receptors: key mediators of microbe detection. Current Opinion in Immunology 14: 103-110.

Venkatayan, S.; and Nambiar, K.T.K.; 1968. The role of thrombocytes on coagulation of blood in the domestic fowl. Indian Veterinary Journal 45: 499-504.

Wachowicz, B.; Krajewski, T.; and Stefanczyk, B. 1981. Antiheparin proteins secreted by avian thrombocytes. Journal of Thrombosis and Haemostasis 45(1): 98.

Weksler, B.B. Platelets. 1992. Inflammation: basic principles and clinical correlates. 2nd Edition (Gallin, J.I., Goldstein. I.M., and Snyderman, R. Eds), New York, Raven.

Wright, J.H. 1910. The histogenesis of blood platelets. Journal of Morphology 21: 263–268.

Yeaman, M.R. 1997. The role of platelets in antimicrobial host defense. Clinical Infectious Diseases 25: 951-970.

Zhang, Y.; and Chen, F. 2004. Reactive oxygen species (ROS), troublemakers between nuclear factor-kappaB (NF-kappaB) and c-Jun NH(2)-terminal kinase (JNK). Cancer Research 64(6): 1902-1905.