Effect of 25-hydroxycholecalciferol Supplementation on Broiler, Layer and Turkey Birds Growth Performance and Immune System

DISSERTATION

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ABSTRACT

In vitro and in vivo studies were conducted to identify the effects of 25-hydroxycholecalciferol (25(OH)D) supplementation on poultry immune cells function, cytokine production, and production parameters following an inflammatory or coccidial challenge. Supplementing chicken macrophages with 25(OH)D in the presence of LPS increased nitrite production, mRNA amounts of IL-1β, IL-10, 1α-hydroxylase and 24-hydroxylase in HD11 cells. The 25(OH)D treatment increased heterophil nitrite production post-LPS treatment. Chicken macrophage HD11 cell line treated in vitro with 200 nM of 1,25-dihydroxycholecalciferol (1,25(OH)2D) had increased nitrite production (P < 0.01) compared to HD11 cells treated with 0 or 200 nM of 25(OH)D post-coccidial antigen treatment. Treating HD11 cells with 25(OH)D decreased IL-10 mRNA by 1.7-fold, but 1,25(OH)2D treatment increased the amount of IL-10 mRNA (P < 0.01) post-coccidial antigen treatment. 25(OH)D or 1,25(OH)2D treatment decreased (P <0.01) 1α-hydroxylase mRNA amounts in HD11 cells, post-coccidial antigen treatment. T cell line, ConA-B1-VICK, stimulated with 100 nM 1,25(OH)2D or with supernatants from HD11 cells treated with 25(OH)D plus LPS had 1.3-fold less (P < 0.01 ) IFN-γ mRNA compared to the group treated with 25(OH)D.

Broiler birds were fed a basal diet supplemented with either cholecalciferol (3000 IU/kg) or 25(OH)D (69 µg/kg). At 21 and 35 d of age birds supplemented with 25(OH)D gained
approximately 2.5% (P = 0.03) and 3.8% (P < 0.01) respectively, more body weight than the birds supplemented with cholecalciferol over the 24 h post-LPS injection. Birds fed 25 or 50 µg/kg -hydroxycholecalciferol had lower (P = 0.010) IL-1β mRNA in the liver compared with those birds fed 6.25 µg/kg of 25-hydroxycholecalciferol or 250 IU cholecalciferol post-LPs injection. Post-LPS injection, birds supplemented with 25-hydroxycholecalciferol had increased 1α-hydroxylase mRNA amounts in liver (P = 0.07). Layer birds were fed a basal diet supplemented with 25(OH)D at 6.25, 25, 50 or 100 µg/kg and at 21 d of age orally challenged with 1 x 10^5 live coccidia oocysts. Compared to the control birds fed similar levels of 25(OH)D and unchallenged with the coccidia oocyst, birds challenged with the coccidia oocyst had 15% reduced BW gain in the groups supplemented with either 6.25, 25, and 50 µg/kg of 25(OH)D but had only 4% reduced BW gain in birds fed 100 µg/kg of 25(OH)D (P < 0.01). Birds fed 100 µg/kg 25(OH)D had decreased (P = 0.01) CD8+ cell percentages in cecal tonsils in both coccidial oocyst challenged and unchallenged birds, compared to the control birds fed 6.25 µg/kg 25(OH) and unchallenged with coccidial oocysts. At 15 d post coccidia challenge, birds fed 100 µg/kg 25(OH)D and challenged with coccidial oocysts had 17% more CD4+CD25+ cells (P = 0.02) in the cecal tonsil compared to the birds fed 100 µg/kg 25(OH)D and unchallenged with coccidial oocysts. At d 6 post coccidia challenge, among birds challenged with coccidial oocysts, birds fed 100 µg/kg 25(OH)D had 3.4-fold (P = 0.14) decrease in IL-1β and 4.9-fold increase in IL-10 mRNA amounts in the cecal tonsils compared to birds fed 6.25 µg/kg 25(OH)D.

Turkey poults were fed a basal diet supplemented with 25(OH)D at 27.5, 55, 82.5 or 110 µg/kg and at 21 d of age orally challenged with 1 x 10^4 live coccidia oocysts. At 21 d of
age, there was a trend in increasing the mean BW of birds supplemented with 25(OH)D at 82.5 µg/kg (P = 0.09). At d 7 post coccidia challenge, birds fed above 55 µg/kg 25(OH)D had increased IL-1β mRNA amounts in the cecal tonsils compared to control birds.

In conclusion, higher doses of 25(OH)D supplementation improved BW gain in broiler birds post-LPS challenge and decreased the inflammatory cytokine IL-1β. In layer birds post-coccidial oocyst challenge, birds supplemented with the highest dose of 25(OH)D had the least decrease in BW gain along with an increase in the percentage of T-regulatory cells and anti-inflammatory cytokine IL-10 compared to the control group. In turkey poultys supplementing with high doses of 25(OH)D supplementation increased mean BW and post-coccidial oocyst challenge there was still an increased BW gain.
Dedication

Dedicated to my late father and mother, John Andrew Morris and Cicily Morris, who in the middle of their financial struggles, encouraged and provided us the best education to achieve the dreams in our life.
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Chapter 1: Introduction

Coccidiosis is the major disease affecting poultry and is caused by a protozoan parasite belonging to the genus *Eimeria*, resulting in impaired body weight (BW) gain and feed utilization (1). The economic loss to the poultry industry due to coccidiosis has been estimated at a staggering loss of three billion dollars globally and more than 800 million dollars annually in the United States (2, 3). The most pathogenic species responsible for causing coccidiosis are *Eimeria acervulina*, *Eimeria maxima* and *Eimeria tenella* in chicken and *Eimeria adenoeides* in turkeys (2, 4-6). However, increasing resistance to anti-coccidial drugs poses a serious challenge in combating coccidiosis and alternative nutritional interventional strategies have been attempted in controlling coccidiosis (7).

Birds combat the coccidial infection by mounting an inflammatory response mediated by increased production of nitric oxide and pro-inflammatory cytokines such as IL-1β and IFN-γ (8, 9). However, pro-inflammatory cytokines are responsible for mediating the systemic acute phase response by the liver, which releases acute phase proteins (APP) to cause anorexia, fever and decreased growth in animals (10-13). Studies conducted in broiler birds have demonstrated that, subsequent to a lipopolysaccharide (LPS) challenge, there is a dramatic increase in the production of APP by the liver. To
meet increased demands for the production of APP lysine requirements increase by six fold. Hence lysine, under normal physiological conditions used for growth is diverted for mounting immune responses (10). Because of these reasons, immune responses in birds have significant energetic cost and can decrease BW gain.

The 25-hydroxycholecalciferol 25(OH)D is the first hydroxylation product in the biosynthesis of active vitamin D and is converted into its active form the 1, 25-dihydroxycholecalciferol (1,25(OH)\textsuperscript{2}D) by the enzyme 1α-hydroxylase (14). Studies in birds have demonstrated 25(OH)D to increase the tibial ash and body weight gain and to decrease the incidence of tibial dyschondroplasia (15, 16). Though 1α-hydroxylase enzyme is mostly expressed in kidneys, extra renal expression of 1α-hydroxylase in cells of the immune system provides for a local production of active vitamin D (17, 18).

Studies conducted in other species have demonstrated immunomodulatory effects of vitamin D. Short term treatment of macrophages with 1,25(OH)\textsuperscript{2}D increased inflammatory responses by increasing macrophage NO production and IL-1β. However, long term exposure of monocytes with 1,25(OH)\textsuperscript{2}D increased the anti-inflammatory cytokine IL-10 and in cells of adaptive immunity favored the phenotypic differentiation of T-cells into T-regulatory cells that suppress the inflammation (19, 20). Because excess immune responses in birds comes with a cost, it would be beneficial to investigate if nutritional interventional strategies, such as 25(OH)D supplementation could negate some of the excess inflammatory response and ameliorate the loss of BW gain during an inflammation.

The broad objectives of this study are

2. To compare the effects of supplementing dietary 25-hydroxycholecalciferol and cholecalciferol on the immune system and growth performance of broiler chickens after an LPS challenge.

3. To study the effect of supplementation with different doses of 25-hydroxycholecalciferol on the immune system, growth performance and fecal oocyst output in layer chickens and turkey poults post-coccidial infection.

Our main hypotheses are

1. 25-hydroxycholecalciferol treatment will increase the inflammatory response in monocytes, macrophages and neutrophils.

2. Supplementing 25-hydroxycholecalciferol treatment in poultry diet will improve growth performance and suppress inflammation during an LPS challenge or coccidial infection.
Chapter 2: Review of Literature

**Vitamin D3 (Cholecalciferol), metabolism and mechanism of action**

Vitamin D is a collective term that encompasses both cholecalciferol (vitamin D3) derived from the metabolism of cholesterol and vitamin D2 (ergocalciferol), obtained from the plant steroid ergosterol (21). Vitamin D3, is generated endogenously in the skin of animals when exposed to ultra violet light and is the inactive form of Vitamin D. Due to modern indoor husbandry practices employed in poultry production, vitamin D is an essential nutrient in the diets of broiler chicken. The National Research Council (NRC) requirement for Vitamin D concentrations in poultry feed is 200 IU/kg of diet. In the poultry industry, vitamin D is fed in excess at concentrations up to 4000 IU/kg of diet (15). Vitamin D3 is converted into its active form following a two-step hydroxylation process mediated by two key enzymes: 25-hydroxylase and 1α-hydroxylase. The first step occurs mostly in the liver by the enzyme 25-hydroxylase, which hydroxylates cholecalciferol at the 25-C position, to form 25-hydroxycholecalciferol. Limited hydroxylation at the 25-C position also occurs in the skin and intestines (22) (Figure 1). Unlike the 1α-hydroxylase enzyme which converts 25-hydroxycholecalciferol to the active form in the kidney, the 25-hydroxylase enzyme is poorly regulated. Serum concentrations of 25-hydroxycholecalciferol increase in parallel with the amounts of
cholecalciferol (23). After hydroxylation in the liver, 25-hydroxycholecalciferol binds to a Vitamin D binding Protein (DBP) and is transported to the kidneys. Proteins megalin and cubilin are expressed in the proximal tubules of kidneys and are receptors for DBP. Through a process of endocytosis, 25-hydroxycholecalciferol bound to DBP is endocytosed into the cell. Inside the lysosome DBP is degraded, and the released 25-hydroxycholecalciferol is converted into 1,25-dihydroxycholecalciferol by the enzyme 1α-hydroxylase (24, 25). Transforming growth factor-β (TGF-β) and insulin upregulate megalin expression (26, 27). The subsequent hydroxylation of 25-hydroxycholecalciferol occurs at the 1-C position and is mediated by 1α-hydroxylase in the kidneys to produce 1,25-dihydroxycholecalciferol, the active form of vitamin D3 (14) (Figure:2). Metabolites of vitamin D, 25-hydroxycholecalciferol and 1,25-dihydroxycholecalciferol, are lipophilic and penetrate cell membranes with relative ease and translocate to nucleus (28, 29). Vitamin D3 is bound to DBP (22) and is transported to the liver for subsequent steps of hydroxylation and activation (30). Vitamin D binding protein is a carrier protein found in the plasma and responsible for transport of cholecalciferol from plasma to liver, 25-hydroxycholecalciferol to kidneys and 1,25-dihydroxycholecalciferol to the target organs (31). Plasma concentrations of DBP is in excess compared to its major binding ligand 25-hydroxycholecalciferol and is not affected by the plasma concentrations of 25-hydroxycholecalciferol (32, 33).

Inside the nucleus 1, 25-dihydroxycholecalciferol binds to a family of receptors called nuclear hormone receptors. The classical action of 1, 25-dihydroxycholecalciferol is elicited through its binding to the vitamin D receptor (VDR) which is located in the nucleus. Transcription of VDR genes is induced by 1,25-dihydroxycholecalciferol (34).
Following ligand binding, VDR are activated and act as transcription factors (21). Binding of 1, 25-dihydroxycholecalciferol to VDR induces further recruitment of retinoid X receptors (RXR). This heterodimer complex consisting of VDR, RXR, and 1, 25-dihydroxycholecalciferol stably binds to VDR response elements (VDREs) in the promoter regions of responsive genes and induces or represses transcription of those genes (22, 35) (Figure: 3).

**Central role of kidneys on vitamin D3 regulation**

Vitamin D forms are tightly regulated by the concentration of available extracellular calcium. The major endocrine organ responsible for regulating concentration of calcium is the parathyroid gland. Low concentrations of serum calcium are detected by the G-protein coupled calcium sensing receptors located in the parathyroid gland. Stimulation of the calcium sensing receptors leads to the release of parathyroid hormone (PTH). PTH mediates its actions through PTH receptors, belonging to the family of G-protein coupled receptors. PTH acts on bones to promote osteoclast activity, thereby increasing calcium release from bones. In the proximal cells of the kidneys, PTH exerts its effect on 1α-hydroxylase both at the transcriptional and post-transcriptional stages (36, 37). Promoter regions for 1α-hydroxylase gene contain specific regions for binding to PTH and stimulate transcription of 1α-hydroxylase (38). Activated 1, 25-dihydroxycholecalciferol stimulates intestinal absorption of calcium and phosphate and tubular reabsorption of calcium in the kidneys (39). An increased concentration of 1,25-dihydroxycholecalciferol facilitates its own inactivation by inhibiting subsequent 1α-hydroxylase activation in the kidneys (40). 1, 25-dihydroxycholecalciferol suppresses
PTH transcripts and increases the expression of 24-hydroxylases and the production of fibroblast growth factor (FGF-23) from bones (17). FGF-23 suppresses 1α-hydroxylase expression and induces the production of 24- hydroxylase in the kidney (30, 41).

Promoter regions of 24- hydroxylase genes have two VDREs, which makes them highly inducible by 1,25-dihydroxycholecalciferol (42). 24-hydroxylase can hydroxylate both 25-hydroxycholecalciferol and 1, 25-dihydroxycholecalciferol, with 1,25-dihydroxycholecalciferol being the preferred substrate. Hydroxylation products 24,25-dihydroxyvitamin D₃ or 1,24,25-trihydroxy vitamin D₃ are inactive forms of vitamin D₃ and are excreted as calcitroic acid (17) (Figure:4).

**Extra renal expression of Vitamin D3**

Expression of 25-hydroxylase is primarily limited to the liver and to a lesser extent in skin (43, 44). Although the kidney is the major organ that expresses 1α-hydroxylase, extra renal expression has been reported in several species (18), including chickens (45). 1α-hydroxylase is expressed in many tissues including bones, dendritic cells, macrophages, skin, parathyroid and brain (46-48). Extra renal expression of 1α-hydroxylase facilitates the conversion of 25-hydroxycholecalciferol to the active form of vitamin D3 in organs other than kidneys (17).

**25-hydroxycholecalciferol as a dietary supplement in birds**

In growing turkeys, calcium deficiency is associated with decreased growth rate, bone deformities and economic loss (49). The incidence of tibial dyschondroplasia is reduced in young chicks when they are fed diets high in calcium (50). Vitamin D plays a
major role in calcium absorption by increasing its intestinal absorption and thereby favoring proper mineralization of bones in growing birds (51). 25-hydroxycholecalciferol has been used as a substitute for cholecalciferol in the diets of chicken (15). A comparison of the net absorption rate of cholecalciferol and 25-hydroxycholecalciferol supplemented in the diet of 20-d-old broiler chickens showed that 25-hydroxycholecalciferol is absorbed better than cholecalciferol; thus, the higher biopotency for 25-hydroxycholecalciferol is due to this difference in net absorption (52). Supplementing 25-hydroxycholecalciferol in the diets of chickens deficient in vitamin D improved bone ash compared to equivalent amounts of cholecalciferol (16). In broilers, 25-hydroxycholecalciferol had increased potency compared to cholecalciferol in improving the bodyweight gain and decreasing the incidence of tibial dyschondroplasia (15). A greater potency was also observed in broilers when supplemented with 25-hydroxycholecalciferol at lower amounts compared to cholecalciferol (15). Diet supplemented with 25-hydroxycholecalciferol as the sole source of vitamin D improved small intestinal morphology and protective humoral immunity in broiler chickens compared to cholecalciferol during Salmonella Typhimurium infection (53).

In summary, overall performance in chicken can be improved by substituting cholecalciferol with 25-hydroxycholecalciferol as the sole source of vitamin D in their diets. However, no studies have been done so far investigating the effect of 25-hydroxycholecalciferol supplementation during an immune challenge in chickens.

**Immune system and associated components**

*a.) Innate and Adaptive immunity*
Innate and adaptive immunity are two major components of the immune system (167). Cells associated with both innate and adaptive immunity originate from pluripotent hematopoietic stem cells. Hematopoietic stem cells will differentiate into either a common myeloid or a lymphoid progenitor depending on the availability of the local factors in the microenvironment. Myeloid stem cells give rise to progenitor cells of red blood cells or white blood cells such as monocytes, macrophages, neutrophils, basophils, eosinophils, mast cells, dendritic cells and platelets. Lymphoid progenitor cells give rise to B, T, dendritic and NK (Natural killer) cells (167). The major cell types of innate immunity are monocytes/macrophages, neutrophils and dendritic cells. Cells of adaptive immunity include T and B lymphocytes. T cells are further classified into T-helper cells, cytotoxic T cells and suppressor or regulatory T cells (54).

b.) Monocytes

Monocytes develop from hematopoietic stem cells in the bone marrow (55). This development involves several intermediary precursors or progenitor stages. Myeloid progenitors pass through sequential stages: from a common myeloid progenitor to granulocyte/macrophage progenitor and the macrophage/dendritic cell (DC) progenitor (MDP). MDPs proliferate and differentiate in the bone marrow and give rise to monocytes and dendritic cell precursors (56). In vivo, MDP is a common precursor for monocytes, macrophages and two different subsets of DC: the conventional dendritic cells (cDCs) and the plasmacytoid dendritic cells (pDCs) (57).
Monocytes play an important role in scavenging toxic compounds and removing apoptotic cells (58, 59).

c.) Macrophages

During an infection, tissue injury, or inflammation, circulating monocytes are recruited to the affected site and differentiate into resident tissue macrophages (56). These macrophages have a pro-inflammatory phenotype and secrete a variety of inflammatory mediators and cytokines such as TNF-α, IL-1, and reactive oxygen and nitrogen species. Reactive oxygen and nitrogen species (nitric oxide) act as antimicrobial compounds responsible for killing pathogenic bacteria (60). Inflammatory macrophages also secrete cytokines IL-12 and IL-23, which drive the clonal expansion and proliferation of TH1 and TH17 subsets of antimicrobial T cells in the adaptive immune response. The TH1 subset of T cells produces cytokines like interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α). The TH17 subset of T cells produces IL-17 cytokines. These subsets of T cells are important in driving further inflammatory responses (60, 61).

Macrophages are found in almost all tissue types, depending upon the status of the local environment, they exhibit different phenotypic characters. The microenvironment in which macrophages reside will dictate their further differentiation into different subsets (62). Macrophages are broadly classified into M1 macrophages (classically activated), M2 macrophages (alternatively activated), and also regulatory macrophages (Mreg). Macrophages that are activated by lipopolysaccharide (LPS) and type 1 inflammatory cytokines IFN-γ, IL-2, and IL-12
are associated with inflammatory conditions and are active against microorganisms and tumor cells. They differentiate into the classically activated M1 macrophages and express most of the toll-like receptors (TLR) and opsonic receptors. They secrete cytokines IL-12, TNF-α, IL-1β, IL-23, and IL-6 and also express inducible nitric oxide synthase (iNOS) (62, 63).

d.) Toll-like receptors

During the early stages of an infection, innate immunity serves as the first barrier against infection and sets the stage for the adaptive phase of the immune response. For this, the cells of the innate immunity rely on specialized receptors called toll-like receptors (TLRs) (64). Toll-like receptors are a family of germ line encoded pattern recognition receptors (PRRs) that recognize specific pathogen associated molecular patterns (PAMPs) found in bacteria, viruses and fungi (65). TLRs belong to type 1 transmembrane proteins consisting of three distinct domains: an extracellular leucine rich repeat section that recognizes PAMPs, a transmembrane domain and a downstream signaling intracellular Toll-interleukin 1 (IL-1) receptor (TIR) domain in cytoplasm (65). In humans, TLR1-10 and in mice, TLR1-13 has been identified. In chickens, 8 TLRs have been identified including a unique TLR15 which is not found in mammals (64, 66). Each toll-like receptor is specific to a unique pathogen associated molecular pattern (Table 1) (49, 66, 67). In general, TLRs can be divided into two subgroups depending on their cellular location and the PAMPs they recognize. TLR1, TLR2, TLR4, TLR5, TLR6, and TLR11 are localized on cell surfaces and recognize outer microbial cell wall components such as LPS,
lipoproteins, and proteins. The other group of TLRs (TLR3, TLR7, TLR21, TLR8, and TLR9) recognizes microbial components such as nucleic acids located in intracellular compartments like endosomes, lysosomes, and the endoplasmic reticulum (65).

e.) Lipopolysaccharide and Toll-like receptor signaling

LPS is found in the outer membrane of gram negative bacteria. It consists of three distinct regions: 1) lipid A- hydrophobic layer consisting of glucosamine molecules 2) layer of oligosaccharide molecules attached to lipid A and 3) O-antigen, containing repeating saccharide units of different sizes that vary with bacterial species and strains (68). During a bacterial infection, LPS is released into the host blood stream and binds to a lipid binding protein (LBP). Clusters of differentiation proteins (CD14) a leucine-rich repeat protein are anchored to the cell membrane by glycosylphosphatidylinositol. CD14 enhances the host response by binding to LPS and transferring it to its TLR4/MD-2 receptor complex. Myeloid differentiation protein (MD-2) is a soluble protein that associates with the extracellular ligand binding domain of TLR4 and is the primary molecule for recognition of LPS (68, 69).

Binding of LPS to the TLR4/MD-2 complex leads to its activation and further downstream signaling. Initial extra cellular binding of LPS to TLR4 is characterized by a refolding of the cytoplasmic signaling domain of TLR4, the Toll-interleukin-1 receptor (TIR). This leads to the recruitment of four adaptor molecules (TIRAP, MyD88, TRAM, and TRIF) which leads to activation of two distinct signaling pathways: MyD88 dependent pathways and TRIF-dependent pathways (66, 68).
MyD88 dependent pathway signaling, toll like receptors result in recruitment of MyD88 and TIRAP adaptor proteins. MyD88 recruits subsequent signaling molecules downstream of the pathway including IRAKs, TRAF6, and TAK1 complex. This leads to the final activation of IκB kinase (IKK) pathway. NF-κB is held in an inactive form within the cytosol by binding to the inhibitory protein IκB.

Phosphorylation of IκB, by IκB kinase, results in proteasomal degradation of IκB protein and the subsequent release of NF-κB leading to its translocation to the nucleus and activation of NF-κB signaling pathways (Figure:5) (66, 68). Most TLRs (TLR5, TLR2/TLR1, TLR2/TLR6, TLR7, and TLR9) signal through the MyD88 signaling pathway which leads to the production of inflammatory cytokines. TLR4 has MyD88, and TRIF-dependent signaling pathways leading to the production of both inflammatory cytokines and Type 1 IFN.

Activation of NF-κB signaling pathway leads to the production of inflammatory cytokines. In later stages of TLR4 signaling, the complex consisting of LPS/TLR4/MD-2 is internalized and localized into endosomes. This forms a complex with TRAM and TRIF which recruits TRAF3 and further recruitment of kinase proteins TBK1 and IKKi. This catalyzes the phosphorylation of the transcription factor, interferon regulatory factor (IRF3), leading to the subsequent transcription and expression of type 1 IFN-genes (66, 68, 70).

f.)  **T cells**

T and B cells are the major cell types of adaptive immunity which recognize specific epitopes in pathogens through B-cell receptors (BCR) or T-cell receptors
T cells are further classified as helper T cells (CD4+) and cytotoxic T cells (CD8+) based on the presence of co-receptors. Naïve T cells develop in the thymus and migrate into the peripheral secondary lymphoid organs. When naïve T cells encounter a foreign antigen derived peptide in the context of a major histocompatibility complex, it gets activated and proliferates into effector T cells specific to that particular pathogen (72, 73). CD4+ T cells recognize foreign antigens presented on MHC-class II molecules and differentiate into effector T cells and regulatory T cells. T-helper cells are further classified into different subsets as T-helper (Th1, Th2 and Th17), induced T-regulatory cells (iTreg) and natural T-regulatory cells (nTregs) (71, 73). Differentiation of naïve T cells into different subsets of T-helper cells depends on the cytokines secreted by antigen presenting cells (dendritic cells and macrophages) and activated T cells (73, 74). Th1 cells secrete cytokine IFN-γ, IL-2 and TNF-α and are important in the fight against intracellular pathogens. Th2 cells secrete mostly cytokine IL-4, IL-5, IL-6, IL-10 and are responsible for humoral (antibody) mediated immune responses against extracellular pathogens (53, 72). Th17 cells produce inflammatory cytokines, IL-17 and IL-22, and are implicated in conditions like autoimmunity and infection (75). T-regulatory cells are further classified into natural T regulatory cells and induced T regulatory cells.

Nitric oxide production, regulation and functions

Nitric oxide is produced in almost all cells of the innate immune system including macrophages, dendritic cells, neutrophils, eosinophils, natural killer (NK) cells and mast
Nitric oxide (NO) is produced from the amino acid L-arginine in the presence of NADPH and O2. These are converted to NO and citrulline in a two-step sequential reaction catalyzed by the enzyme nitric oxide synthase (NOS). In the first oxidation reaction in the presence of the enzyme NOS, NADPH and O2 convert one molecule of arginine into oxidized arginine. The second sequential oxidation reaction converts oxidized arginine into NO and citrulline (77, 78). Three different isoforms of nitric oxide synthase (NOS) are present in mammals and these are neuronal (n) NOS, endothelial (e) NOS and inducible (i) NOS (79). nNOS is expressed in neurons of the brain and peripheral nervous system and eNOS in endothelial cells. nNOS and eNOS are low output, calcium dependent enzymes producing nitric oxide constitutively in a pulsatile manner. These enzymes are constitutively expressed and are inactive in normal resting cells. A stimulus that increases the intracellular calcium ion concentration activates these enzymes and results in NO production (77, 79). iNOS is a high output calcium independent enzyme that is not expressed in resting cells, but is increased in response to LPS, double stranded RNA (dsRNA) and inflammatory cytokines such as tumor necrosis factor alpha (TNF-α), interferon-γ (IFN-γ) or interleukin-1 (IL-1). iNOS can induce the production of NO for an extended period of time until the enzyme is deactivated. Studies in mice and humans have found that cytokines and transcription factors, including NF-κB, activating protein (AP-1), signal transducer and activator of transcription (STAT) -1α and interferon regulatory factor-1 (IRF-1) bind to promoter regions in the iNOS gene and activate iNOS gene expression and further production of NO (76, 77). Activated macrophages up regulate iNOS expression (80). Although LPS and IFN-γ are the most potent inducers of iNOS, other cytokines have also been implicated in the up regulation
of iNOS. Cytokines IL-12 and IL-18 are potent inducers of iNOS expression in macrophages mediated through IFN-γ (76, 81). Inflammatory cytokines IL-1β, IL-17 and tumor necrosis factor (TNF-α) up regulate iNOS expression by inducing the NF-κB pathway. IFN-γ mediates the induction of iNOS through STAT -1α and IRF-1 transcription factors (82-84). Apart from cytokines and LPS, bacterial, viral or protozoan parasites that activate TLRs like TLR-2, TLR-5 or TLR-9 have been shown to activate NO production by macrophages (85-89). NO also affects the transcription of the iNOS gene. Low concentrations of NO during the initial stages of macrophage activation activate NF-κB and up regulate iNOS expression while high concentrations of NO inactivate the transcription of iNOS (90, 91).

iNOS amounts are negatively regulated in macrophages transcriptionally and post transcriptionally by the anti-inflammatory cytokine TGF-β. TGF-β destabilizes the iNOS mRNA, retards synthesis and accelerates degradation of NOS2 protein (92). The enzyme arginase controls the extracellular arginine concentration, a substrate for the enzyme iNOS, by degrading arginine into urea and ornithine (76, 93). T-helper (TH2) cytokines, IL-4, IL-10, IL-13, TGF-β, LPS, dexamethasone and cyclic AMP, strongly induce arginase expression in macrophages and bone-marrow derived dendritic cells (94, 95). IFN-γ, in the presence of either LPS or TNF-α, up regulates the enzyme arginase prior to induction of iNOS. This limits the availability of substrate arginine for NO production (94-96). Cytokines IL-4 and IL-13 inhibit iNOS gene transcription and protein expression. IL-4 inhibits IFN-γ induced expression of iNOS by decreasing the expression of IRF-1 mRNA (76, 97).
NO produced in infected hosts mediates a multitude of functions which include antiviral, proinflammatory, antibacterial and antiparasitic actions (98-102). Peroxynitrite (ONOO−) is a potent antibacterial compound produced from the reaction of host derived iNOS and pathogen derived O2- within the microbes. This is a cytotoxic molecule and is implicated in the killing of invading microbes (99, 103, 104). NO also enhances expression of IFN-γ, a potent inflammatory cytokine that fights against invading microbes (105). Indirectly iNOS mediates antiparasitic action by limiting the availability of arginine needed for synthesis of polyamines and cell proliferation in trypanosomes, schistosomes and giardia. Local depletion of arginine by the induction of iNOS, or arginase in macrophages, can lead to inhibition of growth and/or death of parasites (106-108). Non-activated rat peritoneal macrophages express high amounts of iNOS mRNA and NOS protein and are resistant to *Toxoplasma gondii* infection. Stimulation of mouse or rat macrophages with both LPS and IFN-γ resulted in upregulation of iNOS mRNA and increased NO production which inhibited proliferation of *Toxoplasma gondii*. Inhibition of arginase activity restricted the proliferation of *Toxoplasma gondii* in mouse macrophages (109).

**Coccidiosis**

*a.) Avian Coccidiosis, Common Species and Life Cycle*

Coccidiosis is a major parasitic disease affecting poultry and the economic loss to the poultry industry has been estimated at three billion dollars globally and more than 800 million dollars annually in the United States (2, 3). Avian coccidiosis is caused by a protozoan parasite that belongs to the genus *Eimeria*. *Eimeria* infects the
gastrointestinal tract of poultry producing characteristic lesions, destruction of
intestinal epithelia and subsequently decreasing absorption of nutrients accompanied
by diarrhea (110). There are seven prevalent species of *Eimeria* infecting chickens:
*Eimeria tenella*, *Eimeria acervulina*, *Eimeria brunetti*, *Eimeria maxima*, *Eimeria
cratrix*, *Eimeria praecox*, and *Eimeria mitis*. Among the seven species, *Eimeria
acervulina*, *Eimeria maxima*, and *Eimeria tenella* are the most pathogenic to chicken,
whereas *Eimeria praecox* and *Eimeria mitis* are considered benign as they do not
cause mortality or pathogenic lesions in chickens (2, 4, 6). Each species of *Eimeria*
have specific predilection sites in the gut. *Eimeria acervulina* infects the upper small
intestine (duodenum) and produces characteristic white lesions in the gut. *Eimeria
maxima* targets the midgut (jejunum) and results in petechiae while *Eimeria tenella*
targets the paired cecal pouches and results in bloody diarrhea (2, 110). *Eimeria
species infecting poultry have a homoxenous life cycle (4). *Eimeria* has both an
extracellular and intracellular life cycle and also exhibits both asexual and sexual
reproduction.

The life cycle of *Eimeria* begins with a process called sporulation which is
shedding of oocysts in the feces and undergoing sporogony (meiosis) in the presence
of atmospheric oxygen. This requires approximately 24 h for completion (4, 111).
Sporogony results in the formation of a sporulated oocyst that contains four
sporocysts with two sporozoites in each sporocyst. The infective cycle starts with the
ingestion of a sporulated oocyst by a susceptible host. They undergo a process called
excystation within the intestinal lumen of the birds aided by the local factors available
within the intestine like trypsin, bile and carbon-dioxide. Released sporozoites
penetrate the intestinal villus and develop locally at the penetration site for species like *Eimeria brunetti* and *Eimeria Praecox* (4). In the more pathogenic species of *Eimeria*, sporozoites are transported to different sites, like crypt epithelium, by different cell types, including lymphocytes, and undergo further development (156-158). Sporozoites of *Eimeria tenella* invade heterophils and merozoites invade goblet cells and mast cells (159). Macrophages also play a role in the transport of *Eimeria* parasites (160). Sporozoites invade the intestinal epithelium and develop into the next stage as trophozoites. Trophozoites undergo further rounds of asexual reproduction by nuclear division followed by cytoplasmic division to give rise to immature meronts (schizonts) (4, 111). Meronts undergo further rounds of asexual reproduction by multiple fission, giving rise to several merozoites. Host cells that contain merozoites subsequently rupture and the released merozoites penetrate other cells. They undergo further rounds of merogonic cycle culminating with a sexual reproduction or gametogeny at the last merogonic life cycle. Merozoites penetrate host cells and develop into male (microgamonts) and female (macrogamonts) forms. Microgamonts undergo several rounds of division and give rise to microgametes which are motile and flagellated. Each macrogamont gives rise to a single macrogamete. Microgametes enter cells containing macrogamonts, leading to subsequent penetration and fertilization within the cell. Following fertilization, there is formation of an oocyst wall and the oocyst matures within this oocyst wall. When the host cell ruptures, oocysts are released into the intestinal lumen and excreted out in feces. Sporulation occurs outside the host in the environment and the life cycle continues (4, 111).
There are four prevalent pathogenic species of *Eimeria* that infect turkey: *E. adenoeides*, *E. meleagrimitis*, *E. dispersa*, and *E. gallapovonis*. Among these, *E. adenoeides* is considered to be the most pathogenic infecting ceca. Infection with *E. adenoeides* causes significant reduction in feed intake, body weight gain, feed conversion and mortality if exposed to high doses of sporulated oocyst (5, 112).

b.) Coccidiosis and Immune Responses

*Eimeria* parasites are highly immunogenic and can elicit an immune response comprised of both the innate and adaptive arms of the immune system. Chickens develop protective immunity to reinfection by homologous species of parasites, but not to heterologous species of parasites (4,113,161). An efficient immune response to *Eimeria* infection in birds prevents sporozoite development, but not its invasion (162). Innate immunity constitutes the first line of defense against a coccidial infection. During coccidial infection macrophages play a critical role in the host defense mechanism. Macrophage inhibitory factor (MIF) is secreted by different cell types including monocytes and macrophages (163). MIF is an important mediator of the host defense against different microorganisms including protozoan parasites (164). MIF induces secretion of inflammatory cytokines IL-17, IL-1, TNF-α, IL-8, and IFN-γ in mammals (114). In poultry, infection with *Eimeria tenella* induces high amounts of MIF transcripts (165). In vitro treatment of chicken macrophages with recombinant chicken MIF increased transcription of inflammatory cytokines IL-6, IL-17, and TNF (161). Sporozoites of *Eimeria acervulina* are found to accumulate in chicken macrophages (111). Stimulation of HD11 cells (chicken macrophage cell
lines) or peripheral blood derived chicken macrophages with lipopolysaccharide or *Eimeria tenella* derived sporozoites or merozoites, and produced tumor-necrosis like factor (TNF) (115). Six days following a primary infection with *Eimeria acervulina*, *Eimeria tenella*, or *Eimeria maxima*, plasma nitric oxide concentrations are upregulated. The genotype of a chicken also plays a role in the NO production following infection with *Eimeria tenella* (168,169). Macrophages stimulated with cytokine IFN-γ produce nitric oxide and reactive oxygen species that inhibit the growth of pathogenic bacteria and inhibit the replication of *Eimeria tenella* (9,170,171). Following an oral challenge with live *Eimeria maxima* oocysts in chicken genotypes containing either M5.1 or M15.2B haplotypes, at their MHC locus, the former had a higher number of transcripts encoding IL-1β, IL-6, IL-12, IL-17A, IL-8, inducible nitric oxide synthase, and lipopolysaccharide induced tumor necrosis factor-alpha factor in intestinal intraepithelial lymphocytes. Chickens with a M5.1 haplotype also had higher amounts of the cytokines IFN-γ, IL-8, and IL-15 cytokines in the spleen, a decreased parasitic load in the feces, and reduced weight loss compared to those with a M15.2B haplotype (172). Chickens infected with *Eimeria tenella* have higher levels of cytokine IL-6, an important mediator of both a Th1 and Th2 response (173). Stimulation of chicken heterophils and blood derived macrophages with heat killed sporozoites of *Eimeria tenella* increased the transcripts of TLR4, TLR15, and MyD88, indicating the involvement of a TLR signaling pathway and innate immunity (3).

The adaptive immune response varies according to the species of *Eimeria* infecting the chicken. In an infection by *Eimeria acervulina*, CD4+ T cells do not
have any role in the immune response, as opposed to an *Eimeria tenella* infection where they play a significant role (Swinkels et al., 2007).

During a primary infection with *Eimeria tenella* or *Eimeria acervulina* depletion of CD8+ T cells decreases oocyst output. Decreased output is hypothesized to be due to a decrease in the CD8+ T cells that act as transporters for the sporozoites. However, CD8+ T cells are critical in subsequent infections because depletion of CD8+ T cells increases the oocyst output (111, 116). The major cytokine implicated in the control of an *Eimeria infection* is IFN-γ. Infection with *Eimeria tenella* and *Eimeria maxima* increases the number of transcripts of the inflammatory cytokine IFN-γ and IL-1β along with iNOS transcripts in the jejunum and cecum (8). Multiple infections with *Eimeria tenella* increased the number of T cells secreting IFN-γ and decreased oocyst shedding (117).

During a primary infection with *Eimeria adenoeides* there was an increase in the white blood cell (WBC) populations due to elevated levels of lymphocytes and monocytes. The lymphocytes population consisted of both CD4+ and CD8+ T cells (118). Further studies have demonstrated that during an *E. adenoeides* infection there is infiltration of CD4+ and CD8+ T cells into the infected area. Along with an increase in the lymphocyte infiltration there was also an increase in the expression of inflammatory cytokines such as IFN-γ, IL-2, IL-12 and IL-18 (119).

*Vitamin D3 and Immune responses*

Vitamin D3 plays a major role in modulating immune responses. 1,25-dihydroxycholecalciferol which is the active form of vitamin D3 can act both in an
autocrine and a paracrine manner on immune cells (22). Monocytes and macrophages constitutively express VDR (67). The presence of VDR and 1, 25-dihydroxycholecalciferol in monocytes help their maturation into macrophages (120). Vitamin D₃ production in macrophages is regulated differently compared to the kidney. In macrophages, production of Vitamin D₃ is independent of the overall calcium status or 1,25-dihydroxycholecalciferol concentration (17). Human monocyte derived macrophages and dendritic cells constitutively express low amounts of 1α-hydroxylase. 1α-hydroxylase expression in macrophages is regulated by immune stimuli (121). In monocytes, TLR4 stimulation by LPS increases 1α-hydroxylase expression and the biosynthesis of 1, 25-dihydroxycholecalciferol from 25-OH cholecalciferol. 1,25-dihydroxycholecalciferol acts in an autocrine manner and increases microbicidal activity in monocytes (122, 123). There is a bidirectional effect observed when monocytes are stimulated with LPS in the presence of 1,25-dihydroxycholecalciferol in human monocytes. During the first 8 h of LPS stimulation IL-10, the anti-inflammatory cytokine, was down regulated and this changed to upregulation 48 h after stimulation (20). Stimulation of human peripheral blood derived monocytes with both IFNγ and TLR4 agonist LPS showed a synergistic induction of 1α-hydroxylase mRNA transcripts. This was followed by a concomitant increase in the enzyme 1α-hydroxylase and 1, 25-dihydroxycholecalciferol production. Increased amounts of 1, 25-dihydroxycholecalciferol is not a negative regulator of further production of 1α-hydroxylase in monocytes, as it is in kidneys during an infection (124, 125). During an infection or inflammation, 24-hydroxylase, the negative regulator of 1,25-dihydroxycholecalciferol, is not induced, sustaining production of 1,25-
dihydroxycholecalciferol in monocytes and dendritic cells (22). The differentiation and maturation status of monocytes determine their ability to respond to 1,25-
dihydroxycholecalciferol and induction of 24-hydroxylase. Undifferentiated monocytes are susceptible to 1,25-dihydroxycholecalciferol mediated induction of 24-hydroxylase but fully differentiated or activated macrophages are insensitive. Cytokine IFN-γ signaling leads to increased STAT-1 accumulation in the nucleus. STAT-1 binds to the DNA binding domain of VDR and prevents the VDR/RXR/1,25-
dihydroxycholecalciferol complex from interacting with the VDREs for 24-hydroxylase and prevents its transcription (126).

TLR signaling pathways are involved in the induction of 1α-hydroxylase. Blocking the key mediators of the TLR signaling pathway using inhibitors for MAPK, JAK or NF-κB completely blocked the IFNγ and TLR4 mediated induction of 1α-hydroxylase enzyme expression (121). Promoter regions for 1α-hydroxylase in humans have several potential sites for binding of different transcription factors including NF-κB. Blocking NF-κB inhibited the induction of 1α-hydroxylase mRNA expression in human monocytes. JAK-STAT and p38 mitogen-activated protein kinase (MAPK) pathways also induce 1α-hydroxylase mRNA expression in monocytes (121). 1, 25-
dihydroxycholecalciferol, along with LPS, or the inflammatory cytokine TNF-α, increased the mRNA amounts of IL-1β in a dose dependent manner. This was mediated by the activation of Erk1/2 pathway and increased phosphorylation of C/EBPβ a key transcriptional factor for the IL-1β gene. Also 1, 25-dihydroxycholecalciferol in the presence of LPS increased the expression of TLR4 in human macrophages (7, 120).

Stimulation of HL-60 (human macrophage-like cell line) with LPS produces nitric oxide
and this production is mediated by increased iNOS transcripts. 1, 25-dihydroxycholecalciferol alone at nanomolar concentration induced NO production in an HL-60 cell line and inhibited the growth of *Mycobacterium tuberculosis* in a NO dependent manner (127).

In humans, 1, 25-dihydroxycholecalciferol converted dendritic cells into tolerogenic dendritic cells that had decreased inflammatory cytokine production (128). Tolerogenic dendritic cells induce naïve CD4+CD25- T cells into CD4+CD25+ T regulatory cells (129). 1,25-dihydroxycholecalciferol inhibited the production of inflammatory cytokine IFN-γ and IL-17 and upregulated the production of IL-10 the anti-inflammatory cytokine in CD4+ T cells. 1, 25-dihydroxycholecalciferol also upregulated the production of CTLA-4 and FoxP3 in CD4+ T cells a marker of T-regulatory cells (19). T-cells have limited expression of 1α-hydroxylase enzyme and rely on extrinsic sources of 1,25-dihydroxycholecalciferol. Production of 1,25-dihydroxycholecalciferol by activated monocytes or dendritic cells could act in a paracrine manner on CD4+ T cells and convert them to a regulatory phenotype (Figure:6) (130).

**Conclusions**

Supplying 25-hydroxycholecalciferol might increase the total pool of active vitamin D due to extra renal expression of 1α-hydroxylase by passing liver and increasing overall production of 1,25-dihydroxycholecalciferol from 25-hydroxycholecalciferol. Supplementing 25-hydroxycholecalciferol can be beneficial early in an infection. During infection, 25-hydroxycholecalciferol can increase the local availability of active vitamin D in cells of the innate immune system by local production
and release via increased expression of \(1\alpha\)-hydroxylase. Early in an infection, 1,25-dihydroxycholecalciferol helps to limit infection by acting through an autocrine mechanism in monocytes to increase an inflammatory immune response and downregulating an anti-inflammatory response. During later stages of infection, 1,25-dihydrocholecalciferol could act through an autocrine mechanism to upregulate anti-inflammatory genes in monocytes. In addition to this it could act on T-cells by inducing the formation of T-regulatory cells through a paracrine mechanism (Figure 7). Therefore, the objectives of this thesis is to


2. To compare the effects of supplementing dietary 25-hydroxycholecalciferol and cholecalciferol on the immune system and growth performance of broiler chickens after an LPS challenge.

3. To study the effect of supplementation with different doses of 25-hydroxycholecalciferol on the immune system, growth performance and fecal oocyst output in layer chickens and turkey poults post-coccidial infection.
Table 1. Summary of toll-like receptors and their corresponding ligands. TLR21 is a non-mammalian homologue of TLR9 found in birds, fishes and frogs. TLR15 is found only in chickens and the corresponding ligand is not identified.

<table>
<thead>
<tr>
<th>Toll-like receptor</th>
<th>PAMPs recognized</th>
<th>Species detected</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR2/1, TLR2/6</td>
<td>Peptidoglycans, lipoteichoic acids (LTA), Zymosan, lipoarabinomannan, lipoproteins</td>
<td>Gram-negative and Gram-positive bacteria, Fungi, viruses and parasites</td>
</tr>
<tr>
<td>TLR2</td>
<td>tGPI-mutin</td>
<td>Trypanosoma</td>
</tr>
<tr>
<td>TLR3</td>
<td>dsRNA</td>
<td>Viruses</td>
</tr>
<tr>
<td>TLR4</td>
<td>LPS, mannannan, glycosylphospholipids</td>
<td>Gram-negative bacteria, Fungus, Trypanosoma</td>
</tr>
<tr>
<td>TLR5</td>
<td>Flagellin</td>
<td>Gram-negative and Gram-positive bacteria</td>
</tr>
<tr>
<td>TLR7</td>
<td>Single-stranded RNA</td>
<td>Viruses, Bacteria</td>
</tr>
<tr>
<td>TLR8</td>
<td>Single-stranded RNA</td>
<td>Viruses</td>
</tr>
<tr>
<td>TLR9</td>
<td>Unmethylated C-G motifs, hemozoin</td>
<td>Bacteria, molds, Plasmodium and viruses</td>
</tr>
<tr>
<td>TLR11</td>
<td>Profilin-like molecules</td>
<td>Toxoplasma gondii</td>
</tr>
<tr>
<td>TLR21</td>
<td>CpG-ODN</td>
<td>Bacteria</td>
</tr>
</tbody>
</table>
Figure 1. General overview of vitamin D action
25-hydroxylation (CYP2R1)
- Mostly expressed in liver.
- Hydroxylate cholecalciferol at 25-C position.
- Low levels in skin and testis.
- Poorly regulated.
- Suppressed by 1,25-(OH)2 D3 in prostate cancer cells.

25-hydroxycholecalciferol/DBP-complex
Liver
(cytochrome P450 vitamin D 25 hydroxylase - CYP2R1, CYP2D11, and CYP2D25)

Kidney (Proximal tubule cells-PTC)
(1alpha,25-hydroxyase (CYP27B1), Megalin and Cubulin)

Binds to megalin and cubulin (endocytic receptors for DBP in PTC)

Internalized-Endosomal vesicle-acidification-Lysosomal vesicle-Megalin degradation-release of 25-OH cholecalciferol-cytosol

Megaline and cubulin
- Expressed in PTC.
- Glycoprotein - belongs to low density lipoprotein receptor family.
- Endocytic receptors for DBP.
- Mediate uptake of DBP from glomerular filtrate.
- Down regulated by TGF-β.
- Upregulated by insulin/high glucose.

Figure 2. Biosynthesis of active vitamin D
**Parathyroid hormone**
- Acts through PTH receptors in kidneys and bones.
- Specific to proximal cells of kidneys.
- Increase osteoclastic activity in bone.
- Demineralization and calcium release.
- Increases 1 alpha-hydroxylase production by kidneys.
- Regulation at transcriptional and post-transcriptional levels.
- Involves second messengers generated by protein kinases A and C.
- For 1 alpha-hydroxylase production.
- Acts on promoter regions of 1 alpha-hydroxylase (DNA methylation and demethylation).

**Vitamin D receptor**
- Nuclear hormone receptor.
- Located in nucleus.
- Expressed in tubular epithelial cells of kidneys, small and large intestine, monocytes, macrophages, dendritic cells and T cells, parathyroid gland, bones etc.
- Undetectable in muscle and liver.
- Binding of 1,25-dihydroxycholecalciferol to VDR induces homo or heterodimerization with Retinoid X receptor and promotes high affinity DNA binding.
- Complex binds to promoter regions of VDRE.
- Inhibitory VDRE and activating VDRE.

**1,25-dihydroxycholecalciferol**
- Active form of vitamin D.
- Increased serum levels inhibit PTH and 1 alpha-hydroxylase transcription.

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Figure 3. Regulation of vitamin D metabolizing enzymes
Figure 4. Regulation of vitamin D metabolizing enzymes by active vitamin D mediated signaling.
Figure 5. TLR activation and NF-κB signaling pathways
Figure 6. Vitamin D and immune system
Figure 7. Proposed mechanism of action of 25-hydroxycholecalciferol
Chapter 3: In vitro 25-hydroxycholecalciferol treatment of lipopolysaccharide-stimulated chicken macrophages increases nitric oxide production and mRNA of interleukin-1beta and 10

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Abstract

25-hydroxycholecalciferol (25(OH)D) is an intermediate metabolite during the biosynthesis of active vitamin D. In vitro studies were conducted in chicken monocytes and a chicken macrophage cell line (HD11) to study the effects of supplementing with 25(OH)D on nitrite production and mRNA amounts of interleukin (IL)-1β, IL-10, 1α-hydroxylase and 24-hydroxylase post-lipopolysaccharide (LPS) challenge. Supplementing HD11 cells and monocytes with 25(OH)D in the presence of LPS increased nitrite production by approximately 3-fold and 5-fold, respectively, compared to the LPS group. There was a linear increase in nitrite production by HD11 cells when treated with increasing doses of 25(OH)D. At 48 h post-LPS treatment, HD11 cells treated with either 25(OH)D or 1,25-dihydroxycholecalciferol(1,25(OH)2D) in the
presence of LPS had higher amounts of IL-1β mRNA compared to the LPS group or the group treated with LPS and cholecalciferol (Chol). At 48 h, HD11 cells treated with 25(OH)D and stimulated with LPS had higher amounts of IL-10 mRNA compared to the LPS group or the groups treated with LPS and Chol or 1,25(OH)_{2}D. At 48 h post-LPS treatment, HD11 cells treated with either 25(OH)D or 1,25(OH)_{2}D and stimulated with LPS had higher amounts of 1α-hydroxylase and 24-hydroxylase mRNA compared to the group treated with LPS and Chol. In summary, a 25(OH)D treatment increased nitrite production and mRNA amounts of IL-1β, IL-10, 1α-hydroxylase and 24-hydroxylase in HD11 cells following LPS stimulation.)

**Introduction**

Vitamin D3, also known as cholecalciferol (Chol), is generated endogenously in the skin of animals when exposed to ultraviolet light and is the inactive form of vitamin D. A two-step hydroxylation process mediated by two key enzymes, 25-hydroxylase and 1α-hydroxylase, converts Chol into its active form. The first step occurs mostly in the liver by the enzyme 25-hydroxylase, which hydroxylates Chol at the 25-C position to form 25-hydroxycholecalciferol (25(OH)D) (22). The second key enzyme, 1α-hydroxylase, located predominately in the kidneys, mediates the subsequent hydroxylation of 25(OH)D at the 1-C position to produce 1,25-dihydroxycholecalciferol (1,25(OH)_{2}D), the active form of vitamin D (14). Even though the kidney is the major organ that expresses 1α-hydroxylase, extra-renal expression of 1α-hydroxylase occurs in several organs of different species (18), including chickens (45). Extra-renal expression
of 1α-hydroxylase facilitates local synthesis of the active metabolite 1,25(OH)\textsuperscript{2}D from 25(OH)D in organs other than the kidneys (17).

Chol plays a major role in modulating immune responses, particularly in macrophages. The 1,25(OH)\textsuperscript{2}D acts in an autocrine manner and increases microbicidal activity of bovine monocytes (123). LPS stimulation increases 1α-hydroxylase expression and the biosynthesis of 1,25(OH)\textsuperscript{2}D from 25(OH)D in monocytes. The effect of 1,25(OH)\textsuperscript{2}D treatment on LPS-stimulated monocytes is dependent on the time point post-LPS stimulation. At 8 h post-LPS stimulation, 1,25(OH)\textsuperscript{2}D treatment downregulates IL-10 expression, but at 48 h post-LPS challenge, 1,25(OH)\textsuperscript{2}D treatment upregulates IL-10 expression in human monocytes (20). Treating human macrophages with 1,25(OH)\textsuperscript{2}D with either LPS or TNF-α increases the mRNA amounts of IL-1β in a dose-dependent manner (120, 131).

Nitric oxide (NO) is produced in cells of the innate immune system including macrophages, dendritic cells, neutrophils, eosinophils, natural killer (NK) cells and mast cells (76). The 1,25(OH)\textsuperscript{2}D alone at nanomolar concentrations induces NO production in an HL-60 cell line and inhibits the growth of Mycobacterium tuberculosis in an NO-dependent manner (127). Even though studies in other species have implicated a role for 1,25(OH)\textsuperscript{2}D in NO production in macrophages and although extra renal expression of vitamin D metabolizing enzymes 1α-hydroxylase and 24-hydroxylase has been reported in chickens (45), no studies have been conducted in chicken macrophages following a 25(OH)D treatment. Our previous in vivo study demonstrated that broiler birds supplemented with 25(OH)D had increased body weight gain and decreased expression of inflammatory cytokine IL-1β compared to the cholecalciferol-supplemented group.
post-LPS injection (132). In the present study, we investigated the effects of in vitro supplementation of 25(OH)D on chicken monocytes and HD11 cells following an LPS challenge.

**Materials and Methods**

All animal protocols were approved by the Institutional Animal Care and Use Committee at The Ohio State University.

*Effect of 25(OH)D treatment on chicken monocyte nitrite production*

Approximately 10 ml of blood was layered over 5 ml of Histopaque-1077 and centrifuged at 400 X g at 10°C for 15 min. The cells at the interface were washed and resuspended in complete RPMI-1640 medium. Cells, 1 X 106 cells/well were plated in 500 µl of RPMI-1640 medium supplemented with 10% FBS, 1% non-essential amino acids, L-glutamine and 2% penicillin and streptomycin and allowed to adhere overnight at 37°C and 5% CO2 in 48 well plates. Following overnight incubation, the medium was removed and fresh medium supplemented with 0 or 200 nM of 25(OH)D (Santa Cruz Biotechnology, USA) was added in six replications (n = 6). Monocytes were stimulated with 0 µg/well of LPS (control) or 0.5 µg/well of Salmonella typhimurium LPS (#L9516, Sigma, St. Louis, MO) for 24 h. Nitrite concentration of the cell culture supernatant was quantified using Greiss reagent (Ricca Chemical Company, Arlington, TX) following manufacturer’s instructions (133). Data presented are representative of two independent experiments.
**Effect of 25(OH)D treatment on HD11 cell nitrite production**

HD11 cells, a chicken macrophage cell line, were grown in 48 well plates (5 X 10^3 cells/well) in 500 μl of RPMI complete growth medium, as described above, and allowed to adhere overnight at 37°C and 5% CO₂. Following overnight incubation, the medium was removed, and fresh RPMI medium supplemented with 0 or 200 nM of 25(OH)D was added in six replications (n = 6). HD11 cells were then stimulated with 0 (control) or 0.5 μg/well of LPS for 24 h. Nitrite concentration of the cell culture supernatant was quantified as described above.

To analyze the effect of different doses of 25(OH)D on nitrite production, HD11 cells (2 X 10^5 cells/well) were grown in 48 well plates in 500 μl of RPMI medium supplemented with either 0, 10, 100, 200 or 500 nM of 25(OH)D and stimulated with 0.5 μg/well of LPS for 12 h in six replications (n = 6). Nitrite concentration of the cell culture supernatant was measured using Greiss reagent as described above.

**Effect of 25(OH)D treatment on IL-1β, IL-10, 1α-hydroxylase and 24-hydroxylase mRNA content of HD11 cells**

HD11 cells (2 X 10^5 cells/well) were grown in 48 well plates in 500 μl of RPMI medium and allowed to adhere overnight at 37°C and 5% CO₂. Following overnight incubation, the medium was removed and fresh RPMI medium supplemented with 200 nM of Chol, 25(OH)D, or 1,25(OH)²D was added in eight replications (n = 8). HD11 cells were stimulated with 0 (control) or 0.5 μg/well (1 μg/mL) of LPS for 12 or 48 h after which total RNA was extracted from HD11 cells using Tri-reagent (Molecular Research Center, Inc., USA) reverse-transcribed into cDNA. Because insufficient RNA was isolated from
some of the wells, the replication size ranged from 5 to 8 in different treatment groups. cDNA were analyzed for relative amounts of IL-1β (5′-tcctccagccagaagta-3′ and 5′-caggcggtagagaattga-3′), IL-10 (5′-caatccagggcgtagacta-3′ and 5′-ggcaggacctaagtgtgtag-3′), 1α-hydroxylase (5′-tcgtgctgctagagatagac-3′ and 5′-actgccacatgtgtggt-3′) and 24-hydroxylase (5′-aaaccctgtaacccctctg-3′ and 5′-ccagttcaccacctgct-3′) mRNA amounts by quantitative real-time PCR (iCycler, BioRad) using SyBr green after normalizing for β-actin (5′-accggactttaccaagct-3′ and 5′-gactgctgctgacaccttc-3′) mRNA amounts as described previously (133,142). Annealing temperatures for IL-1β, 1α-hydroxylase, IL-10 and 24-hydroxylase primers were 57.5°C, 55°C, 58°C and 55°C, respectively. The threshold (Ct) values were determined by iQ5 software (BioRad, Hercules, CA).

Statistical analysis

Data were analyzed using the Generalized Linear model of ANOVA (Minitab Inc., PA, USA). When the effects were significant (P < 0.05), differences between means were analyzed by Tukey’s least square means comparison. The effect of different doses of 25(OH)D on nitrite production by HD11 cells was analyzed by SAS (SAS 9.3, NC, USA). Orthogonal polynomial contrasts were used to test for linear and quadratic responses to different doses of 25(OH)D; due to unequal treatment spacing, PROC IML of SAS was used to generate orthogonal polynomial contrast coefficients.
Results

Effect of 25(OH)D treatment on nitrite production from chicken monocytes and HD11 cells

At 24 h post- LPS treatment, chicken monocytes treated with 25(OH)D and LPS had approximately 5-fold (P < 0.01) more nitrite in the supernatant compared to the group with 0.5 μg/well of LPS and 0 nM of 25(OH)D (Figure 1 A). Similarly, HD11 cells treated with 200 nM of 25(OH)D and stimulated with 0.5 μg/well of LPS had approximately 3-fold (P < 0.01) more nitrite in the supernatant compared to the group with 0.5 μg LPS and 0 nM of 25(OH)D (Figure 1 A).

Effect of different doses of 25(OH)D treatment on HD11 cell nitrite production

Treating HD11 cells with 200 and 500 nM of 25(OH)D in the presence of 0.5 μg/well of LPS increased the nitrite content of the cell culture supernatant (P < 0.01) (Figure 1 B) compared to that of the group with 0 nM 25(OH)D.

Effect of 25(OH)D treatment on HD11 cell IL-1β mRNA content

At 12 h post-LPS treatment, HD11 cells treated with LPS had higher IL-1β mRNA content than HD11 cells treated with 0 μg/well of LPS and 0 nM of Chol, and treating LPS-stimulated HD11 cells with 200 nM Chol, 25(OH)D or 1,25(OH)₂D further increased IL-1β mRNA content (P = 0.18). At 48 h post-LPS treatment, HD11 cells treated with either 25(OH)D or 1,25(OH)₂D in the presence of LPS had higher expression (P < 0.01) of IL-1β mRNA compared to the HD11 cells treated with 0.5 μg/well of LPS and 0 nM of Chol (Figure 2 A). At 48 h post-LPS treatment, treating HD11 cells with 200
nM Chol and 0.5 μg/well LPS did not increase IL-1β mRNA expression compared to the group with 0.5 μg/mL LPS and 0 nM Chol.

*Effect of 25(OH)D treatment on HD11 cell IL-10 mRNA content*

At 12 h post-LPS treatment, HD11 cells treated with either 25(OH)D or 1,25(OH)\(^2\)D in the presence of LPS had numerically higher expression (P = 0.25) of IL-10 mRNA compared to the group treated with 0 μg/well of LPS and 0 nM of Chol. At 48 h, HD11 cells treated with 25(OH)D and stimulated with LPS had higher expression (P < 0.01) of IL-10 mRNA compared to the group with 0.5 μg/mL LPS and 0 nM of Chol (Figure 2 B).

*Effect of 25(OH)D treatment on HD11 cell 1α-hydroxylase and 24-hydroxylase mRNA content of HD11 cells*

At 48 h post-LPS treatment, LPS stimulation of HD11 cells treated with either 25(OH)D or 1,25(OH)\(^2\)D increased 1α-hydroxylase (P < 0.01) or 24-hydroxylase mRNA (P < 0.01) amounts compared to the group treated with 0.5 μg/well of LPS or 200 nM of Chol (Figure 3 and 4 ) respectively. At 48 h post-LPS treatment, LPS stimulation of HD11 cells treated with Chol did not increase 1α-hydroxylase (P < 0.01) and 24-hydroxylase mRNA amounts compared to the group treated with 0.5 μg/well of LPS and 0 nM of Chol. In HD11 cells stimulated with 0 μg/well of LPS, treatment with 200 nM of 25(OH)D decreased both 1α-hydroxylase (P < 0.01) and 24-hydroxylase mRNA amounts compared to the group treated with 0 μg/well of LPS and 0 nM of Chol.
Discussion

The effect of 25(OH)D treatment on nitrite production in cells of innate immunity such as monocytes and macrophages during an LPS treatment was studied. During inflammation, circulating monocytes are recruited to the site of inflammation and could be differentiated into inflammatory macrophages (56). These macrophages secrete a variety of inflammatory mediators and cytokines like TNF-α, IL-1β, and reactive oxygen and NO. Reactive oxygen and NO act as antimicrobial compounds responsible for killing pathogenic bacteria (60).

The 1,25(OH)²D alone at nanomolar concentrations induced NO production in an HL-60 cell line and inhibited the growth of *Mycobacterium tuberculosis* in a NO-dependent manner (127). In our studies, in vitro 25(OH)D treatment increased NO production from HD11 cells and chicken monocytes post-LPS treatment. LPS stimulation increased 1α-hydroxylase mRNA amounts in cells treated with 25(OH)D and thereby facilitated the synthesis of 1,25(OH)₂D from 25(OH)D. Increased production of 1,25(OH)₂D might have been responsible for the increased NO production observed in HD11 cells and chicken monocytes treated with 25(OH)D post-LPS treatment.

In vitro studies conducted in human dendritic cells used 25(OH)D at doses ranging from 100 to 200 nM (128). In this study, in vitro 25(OH)D treatment increased HD11 cell NO production linearly (P < 0.01) in a dose-dependent pattern at doses above 200 nM post-LPS treatment. The 1,25(OH)²D, along with LPS or the inflammatory cytokine TNF-α, increased mRNA amounts of IL-1β in a dose-dependent manner (120, 131).
Studies conducted elsewhere in human monocytes showed a bidirectional effect. When monocytes were stimulated with LPS in the presence of 1,25(OH)\(_2\)D during the first 8 h of LPS stimulation, the anti-inflammatory cytokine IL-10 was down-regulated, and this changed to up-regulation 48 h after stimulation (20). IL-1\(\beta\) was suppressed in broiler chickens supplemented with 25(OH)D (132). In this study, increased expression of IL-1\(\beta\) mRNA was observed in HD11 cells treated with 25(OH)D or 1,25(OH)\(_2\)D at both 12 and 48 h post-LPS stimulation. However, the magnitude of response at 48 h decreased at least 8- to 10-fold compared to the magnitude at 12 h. Consistent with the several fold decrease in expression of IL-1\(\beta\) mRNA at 48 h, there was an increased expression of IL-10 mRNA in the group treated with 25(OH)D and stimulated with LPS. Increased expression of IL-10 mRNA in 25(OH)D treated macrophages might be a compensatory feedback mechanism to suppress further expression of inflammatory cytokine IL-1\(\beta\) in HD11 cells.

Macrophages and dendritic cells derived from human monocytes constitutively express low amounts of 1\(\alpha\)-hydroxylase. The 1\(\alpha\)-hydroxylase expression in macrophages is regulated by immune stimuli (121). In monocytes, TLR4 stimulation by LPS increases 1\(\alpha\)-hydroxylase expression and the biosynthesis of 1,25(OH)\(_2\)D from 25(OH)D (127). At 48 h, 1\(\alpha\)-hydroxylase mRNA amounts were increased only in groups stimulated with LPS and treated with 25(OH)D or 1,25(OH)\(_2\)D. Increased amounts of 1\(\alpha\)-hydroxylase might facilitate the increased production of 1,25(OH)\(_2\)D from 25(OH)D. The 1,25(OH)\(_2\)D facilitates its own inactivation by increasing the expression of 24-hydroxylase, the negative regulator of active vitamin D synthesis (17). Undifferentiated monocytes are susceptible to 1,25(OH)\(_2\)D mediated induction of 24-hydroxylase, but fully differentiated
or activated macrophages are insensitive (126). In this study, even though 24-hydroxylase mRNA expression was down regulated at 12 h, groups treated with either 25(OH)D or 1,25(OH)\(^2\)D in HD11 cells were subsequently upregulated at 48 h. A plausible reason for increased expression of 24-hydroxylase mRNA could be induction by 1,25(OH)\(^2\)D.

In summary, we demonstrated that 25(OH)D increased the production of NO and mRNA expression of IL-1\(\beta\) and IL-10 in HD11 cells. There was a biphasic, time-dependent induction of cytokines IL-1\(\beta\) and IL-10 in HD11 cells treated with 25(OH)D. Further, we demonstrated a capability of HD11 cells for local production of active vitamin D by the induction of the 1\(\alpha\)-hydroxylase enzyme subsequent to an LPS challenge.

**Fundings**

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Figure 8. Effect of 25(OH)D treatment on nitrite production from (A) chicken monocytes and HD11 cells. Peripheral blood monocytes or HD11 cells were treated with 0 or 200 nM of 25(OH)D and stimulated with 0 or 0.5 μg/well LPS for 24 h. Nitrite concentration in the supernatant was determined. Bars (± SEM) without a common superscript differ significantly within a cell type. P values for monocytes: 25(OH)D, \( P < 0.01 \); LPS, \( P < 0.01 \); 25(OH)D*LPS, \( P < 0.01 \); HD11 cells, 25(OH)D, \( P < 0.01 \); LPS, \( P < 0.01 \); 25(OH)D *LPS, \( P < 0.01 \). \( n=6 \). Data presented are representative of two independent experiments (B) Different doses of 25(OH)D on nitrite production by HD11 cells. HD11 cells were grown in a medium supplemented with 0, 10, 100, 200 or 500 nM of 25(OH)D and stimulated with 0.5 μg/well of LPS for 12 h. P values: \( P < 0.01 \) (linear), \( P = 0.20 \) (Quadratic). \( n=6 \) for all treatment groups except for 200 nM 25(OH)D where \( n=12 \). Data presented is representative of one experiment.
Figure 9. Effect of 25(OH)D treatment on HD11 cell (A) IL-1β and (B) IL-10 mRNA content. HD11 cells were grown in a medium supplemented with Chol, 25(OH)D or 1,25(OH)₂D and stimulated with 0 or 0.5 µg/well of LPS for 12 and 48 h. At 12 and 48 h post-LPS challenge, mRNA amount was analyzed by real time PCR and normalized to β-actin and compared to the group not stimulated with LPS or treated with Chol, 25(OH)D or 1,25(OH)₂D, so that all bars represent fold change compared to the no LPS group. Bars (± SEM) without a common superscript differ significantly within a time point. n=8 except for treatment groups discussed in materials and methods. P values for IL-1β: 12 h: Diet, \( P = 0.18 \); LPS, \( P < 0.01 \); Diet *LPS, \( P = 0.18 \); 48 h: Diet, \( P < 0.01 \); LPS, \( P < 0.01 \); Diet *LPS, \( P < 0.01 \). P values for IL-10: 12 h: Diet, \( P = 0.17 \); LPS, \( P < 0.01 \); Diet *LPS, \( P = 0.25 \); 48 h: Diet, \( P < 0.01 \); LPS, \( P < 0.01 \); Diet *LPS, \( P < 0.01 \). Data presented are the average of two independent experiments.
Figure 10. Effect of 25(OH)D treatment on HD11 cell 1α-hydroxylase mRNA content. HD11 cells were grown in a medium supplemented with Chol, 25(OH)D or 1,25(OH)\(_2\)D and stimulated with 0 or 0.5 μg/well of LPS for 12 and 48 h. At 12 and 48 h post-LPS challenge, mRNA amount was analyzed by real time PCR and normalized to β-actin and compared to the group not stimulated with LPS or treated with Chol, 25(OH)D or 1,25(OH)\(_2\)D, so that all bars represent fold change compared to the no LPS group. Bars (± SEM) without a common superscript differ significantly within a time point. n=8 except for treatment groups discussed in materials and methods. P values for 1α-hydroxylase mRNA: 12 h: Diet, P = 0.12; LPS, P = 0.04; Diet *LPS, P = 0.56; 48 h: Diet, P = 0.06; LPS, P = 0.33; Diet *LPS, P < 0.01.
Figure 11. Effect of 25(OH)D treatment on HD11 cell 24-hydroxylase mRNA content. HD11 cells were grown in a medium supplemented with Chol, 25(OH)D or 1,25(OH)$_2$D and stimulated with 0 or 0.5 μg/well of LPS for 12 and 48 h. At 12 and 48 h post-LPS challenge, mRNA amount was analyzed by real time PCR and normalized to β- actin and compared to the group not stimulated with LPS or treated with Chol, 25(OH)D or 1,25(OH)$_2$D, so that all bars represent fold change compared to the no LPS group. Bars (± SEM) without a common superscript differ significantly within a time point. n=8 except for treatment groups discussed in materials and methods. P values for 24-hydroxylase mRNA: 12 h: Diet, $P = 0.15$; LPS, $P < 0.01$; Diet *LPS, $P < 0.01$; 48 h: Diet, $P < 0.01$; LPS, $P < 0.01$; Diet *LPS, $P < 0.01$. 
Chapter 4: 25-hydroxycholecalciferol supplementation improves growth performance and decreases inflammation during an experimental lipopolysaccharide injection

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Abstract

Three experiments were conducted to study the effects of 25-hydroxycholecalciferol supplementation on body weight gain, IL-1β and 1α-hydroxylase mRNA expression in different organs of broiler chickens following a lipopolysaccharide (LPS) injection. In experiment I, birds were fed a basal diet supplemented with either cholecalciferol (3000 IU/kg) or 25-hydroxycholecalciferol (69 µg/kg). At 21 and 35 d of age, birds were injected with LPS. Post-LPS injection, birds supplemented with 25-hydroxycholecalciferol gained approximately 2.5 % (P = 0.03) and 3.8% (P < 0.01), respectively, more body weight than the birds supplemented with cholecalciferol over the 24 h period. In experiment II, birds were fed basal diets supplemented with 25-hydroxycholecalciferol at 6.25, 25 and 50 µg/kg feed or cholecalciferol at 250 IU/kg
At 35 d of age, birds were injected with LPS. Birds fed 25-hydroxycholecalciferol at 25 and 50 µg/kg and injected with LPS had approximately 7-fold and 3-fold less (P = 0.010) IL-1β mRNA in the liver compared with those birds fed 6.25 µg/kg of 25-hydroxycholecalciferol and the cholecalciferol (250 IU/kg) group. In experiment III, birds were fed a basal diet supplemented with either cholecalciferol (3000 IU/kg) or 25-hydroxycholecalciferol (69 µg/kg). At 28 d of age, birds were injected with LPS and those fed 25-hydroxycholecalciferol and injected with LPS had 1.1-fold less (P < 0.01) IL-1β mRNA in the liver than the other groups. After an LPS injection, birds supplemented with 25-hydroxycholecalciferol had increased 1α-hydroxylase mRNA amounts in liver (P = 0.07). In conclusion, 25-hydroxycholecalciferol supplementation at higher doses improved growth performance and decreased inflammatory gene IL-1β mRNA amounts in the liver post-LPS injection.

**Key words:** 25-hydroxycholecalciferol, lipopolysaccharide, inflammation

**Introduction**

Vitamin D₃, also known as cholecalciferol, is the inactive form of vitamin D that can be generated endogenously in the skin of animals exposed to ultraviolet light. Vitamin D₃ is converted into its active form following a two-step hydroxylation process mediated by two key enzymes, 25-hydroxylase, and 1α-hydroxylase. The first hydroxylation step occurs in the liver by the enzyme 25-hydroxylase, which hydroxylates cholecalciferol at the 25-C position to form 25-hydroxycholecalciferol. The subsequent hydroxylation of 25-hydroxycholecalciferol occurs at the 1-C position and is mediated by 1α-hydroxylase in the kidneys to produce 1, 25-dihydroxycholecalciferol, the active form
of vitamin D (14). Increased levels of 1,25-dihydroxycholecalciferol downregulates further conversion by inhibiting 1α-hydroxylase (40).

Expression of 25-hydroxylase is limited primarily to the liver and to a smaller extent, to the skin (43, 44). Though the kidney is the major organ that expresses 1α-hydroxylase, extra renal expression of the enzyme has been reported in several species (18), including chickens (45). Extra renal expression of 1α-hydroxylase facilitates the local conversion of 25-hydroxycholecalciferol to the active form of Vitamin D₃ (17) in organs other than the kidney. Supplementing 25-hydroxycholecalciferol, rather than cholecalciferol, can thus be expected to increase the local availability of active vitamin D. Studies have shown that 25-hydroxycholecalciferol is more potent than cholecalciferol as measured by increased tibial ash in broiler chickens (16). In broilers, 25-hydroxycholecalciferol has been reported to improve bodyweight gain and decreasing the incidence of tibial dyschondroplasia when compared with cholecalciferol (15).

Lipopolysaccharide is a potent immunogen in stimulating an inflammatory response in both invivo and invitro experiments (134). LPS stimulation leads to release of inflammatory cytokines IL-1β and TNF-α (134, 135). LPS-mediated TLR4 signaling in bovine monocytes induces the expression of 1α-hydroxylase which leads to increased production of 1, 25-dihydroxycholecalciferol from 25-hydroxycholecalciferol and increased microbicidal activity (123). The active form of Vitamin D₃ plays a major role in modulating immune responses in an autocrine manner in cells of the innate immune system and in a paracrine manner in cells of the adaptive immune system (22). Supplementing 25-hydroxycholecalciferol can be beneficial during an immune challenge
because it can bypass the 25-hydroxylase step in the liver and will be converted to active vitamin D locally.

Our hypothesis is that 25-hydroxycholecalciferol supplementation will suppress the inflammatory response and improve body weight gain in broiler chickens post-LPS challenge.

**Materials and Methods**

Three experiments were conducted to study the effect of 25-hydroxycholecalciferol supplementation after an LPS injection. All animal protocols were approved by the Institutional Animal Care and Use Committee at The Ohio State University. All in vivo experiments were repeated at least once.

**Experiment I**

Experiment I was conducted to study the effects of 25-hydroxycholecalciferol supplementation on broiler body weight gain following an LPS challenge. A total of 195 one-day-old chicks (Aviagen 708; Orrville chick hatchery, Orrville, OH) were randomly distributed to one of three experimental groups. Each treatment was replicated in five floor pens of thirteen chicks per replication (n = 5). The first two experimental groups were fed a basal diet supplemented with either cholecalciferol (3000 IU/kg of diet) or 25-hydroxycholecalciferol (HyD, DSM nutritional products, Netherlands) at 69 µg/kg of diet (equivalent to 2760 IU of cholecalciferol) as the supplemental source of vitamin D. The third experimental group was fed a basal diet supplemented with 25-hydroxycholecalciferol (69 µg/kg) for the first 14 d and cholecalciferol (3000 IU/kg)
from 14 to 35 days of age. The basal diet was based on corn and soybean meal (Table 1). Average body weight of birds in each pen was measured weekly. At 21 and 35 d of age, one bird per pen was randomly selected and injected with Salmonella typhimurium LPS (#L9516, Sigma Chemicals, St Louis, MO) at 500 μg/kg body weight. Body weight gain was measured at 24 h post-LPS injection. Similar results were obtained in a second independent experiment; hence the data presented is representative of both independent experiments.

**Experiment II**

Experiment II was conducted to study the effects of different levels of 25-hydroxycholecalciferol supplementation on the expression of IL-1β mRNA in the liver post-LPS injection. A total of 144 one-day-old chicks (Aviagen 708 X Ross; Orrville chick hatchery, Orrville, OH) were randomly distributed to one of four treatment groups. Each treatment was replicated in six pens of six chicks per replication (n = 6). The four experimental groups were fed a basal diet supplemented with either cholecalciferol (250 IU/kg) or 25-hydroxycholecalciferol (6.25, 25 or 50 μg/kg) which is equivalent to 250, 1000 or 2000 IU of cholecalciferol. At 35 d of age, one bird per pen was injected with 250 μg/kg body weight LPS. As a control, one bird per pen not injected with LPS or phosphate buffered saline (PBS) was sacrificed. At 6 h post-LPS challenge, samples of the cecal tonsils, breast muscle, kidney, liver, and spleen were collected in RNAlater (Quiagen, Valencia). At 24 h, RNAlater was discarded, and the organs were stored at -80°C until further analysis.
Experiment III

Experiment III was conducted to study the effects of 25-hydroxycholecalciferol supplementation on the expression of IL-1β mRNA in the liver and 1α-hydroxylase mRNA in different organs post-LPS injection. A total of 80 one-day-old chicks (Aviagen 708 X Ross; Orrville chick hatchery, Orrville, OH) were randomly distributed to one of two treatment groups. Each treatment was replicated in four pens of 10 chicks per replication (n = 4). The two experimental groups were fed a basal diet supplemented with either cholecalciferol (3000 IU/kg) or 25-hydroxycholecalciferol (69 µg/kg). At 28 d of age, one bird per pen was injected with 500 µg/kg body weight LPS. As a control, one bird per pen was not injected with LPS or PBS was sacrificed. At 24 h post-LPS challenge, samples of kidney and liver were collected in RNaLater (Quiagen, Valencia). At 24 h, RNaLater was discarded, and the organs were stored at -80°C until further analysis.

mRNA analysis

Total RNA from the in vivo studies was extracted from tissue samples collected from the different organs and analyzed for relative amounts of IL-1β (5'-tctccagccagaagtga-3' and 5'-caggcgtagaaatgag-3') and 1α hydroxylase (5'-tcgtggcaggaatacagaga-3' and 5'-actgccacatctttggt-3') after normalizing for β-actin (5'-acggcactgttaccaacc-3' and 5'-gactgtgctgacaccttca-3') mRNA levels as described previously (Shanmugasundaram and Selvaraj, 2010). The annealing temperatures were as follows: IL-1β 57.5 °C 1α hydroxylase 60 °C and β-actin 57 °C. The fold change from the reference was calculated as 2(Ct Sample)/2(Ct Reference), where Ct is the threshold
cycle (Selvaraj et al., 2010). The reference group was the control group fed cholecalciferol with no LPS injection.

Results

Experiment I

The mean body weights of birds in the different experimental groups at 21 and 35 d of age were 684.5 g and 1690 g, respectively. At both 21 (P = 0.03) and 35 (P < 0.01) days of age and at 24 h post-LPS challenge, birds supplemented with 25-hydroxycholecalciferol had greater body weight gains than those fed diets with cholecalciferol (Fig.1). At 21 and 35 d of age and at 24 h post-LPS injection, birds supplemented with 25-hydroxycholecalciferol gained 2.5% (P = 0.03) and 3.8% (P < 0.01), respectively, more body weight than the challenged birds supplemented with cholecalciferol. The 24 h post-LPS body weight gain did not differ significantly between birds supplemented with 25-hydroxycholecalciferol for the first 14 d and the birds supplemented with cholecalciferol from day 0 at both 21 and 35 d of age.

Experiment II

IL-1β mRNA relative abundance in the liver

Birds fed 25-hydroxycholecalciferol at 25 and 50 µg/kg and injected with LPS had approximately 7-fold and 3-fold less (P = 0.01) IL-1β mRNA in the liver compared to those birds fed 6.25 µg/kg 25-hydroxycholecalciferol and the low cholecalciferol group (Fig.2).
Experiment III

IL-1β mRNA relative abundance in the liver

Birds fed 25-hydroxycholecalciferol and injected with LPS had 1.1-fold less (P < 0.01) IL-1β mRNA than the other groups (Fig.3).

1α-hydroxylase mRNA relative abundance in different organs

In the presence of a LPS injection, birds supplemented with 25-hydroxycholecalciferol had increased 1α-hydroxylase mRNA amounts in the liver (P = 0.07) (Fig.4). The relative abundance of 1α-hydroxylase mRNA in the kidney were not different among different treatment groups (P = 0.60).

Discussion

This research note studied the effects of dietary supplementation of 25-OH cholecalciferol on short term body weight gain and vitamin D metabolizing enzyme following an inflammatory stimulus.

Birds supplemented with 25-hydroxycholecalciferol had increased body weight gain at 24 h post-LPS injection compared to birds fed cholecalciferol. LPS decreased body weight gain through production of an inflammatory cytokine like IL-1β (136). Liver cells have specialized macrophages, Kupffer cells that secrete IL-1β following an immune stimulation (137). In rodents, cytokine IL-1β increases the mRNA amounts and expression of leptin which increases anorexia (135). Birds fed higher amounts of 25-hydroxycholecalciferol had decreased expression of IL-1β in the liver post-LPS injection and increased bodyweight gain. Human monocyte derived macrophages and dendritic cells constitutively express low levels of 1α-hydroxylase and 1α-hydroxylase expression
in macrophages is regulated by immune stimuli (121). In this study the amount of 1α-hydroxylase mRNA was increased in liver post-LPS injection in birds supplemented with 25-hydroxycholecalciferol. Supplementing 25-hydroxycholecalciferol during inflammatory stimuli most likely increased the local production of active vitamin D3 in extra-renal organs like liver and the induction of tolerance. This may be a plausible explanation for the decreased production of inflammatory cytokines in the liver. Because increased levels of inflammatory cytokines will lead to a more systemic effect in birds, manifested by anorexia, suppression of inflammatory cytokines in these birds might contribute to normal appetite and body weight gain. However, more studies needed to be conducted to see if the there is any difference in feed intake between the different treatment groups during an LPS challenge.

Extra-renal expression of 1α hydroxylase mRNA is present in day-old chicks, with breast and thigh muscles having the greatest amount of 1α hydroxylase mRNA (45). In this study, extra renal expression of 1α hydroxylase mRNA in the kidneys and liver is present in birds supplemented with cholecalciferol or 25-hydroxycholecalciferol. Extra renal expression of 1α-hydroxylase enzyme suppresses the immune response by increasing the production of active vitamin D (17). Increased amounts of 1α hydroxylase mRNA observed in birds fed 25-hydroxycholecalciferol and subjected to an immune challenge might be the result of a compensatory mechanism to suppress the inflammation by increasing active vitamin D production in liver.

In conclusion, higher doses of 25-hydroxycholecalciferol supplementation improved growth performance and decreased expression of the inflammatory gene IL-1β mRNA
post-LPS injection; thus, it might be beneficial to supplement 25-hydroxycholecalciferol rather than cholecalciferol during an immune response.

**Acknowledgements**

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Table 2. Composition of experimental diets

<table>
<thead>
<tr>
<th>Ingredient (g/kg of diet)</th>
<th>Cholecalciferol/vitamin D₃</th>
<th>25(OH)D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn</td>
<td>59.79</td>
<td>59.74</td>
</tr>
<tr>
<td>Soybean meal</td>
<td>33.30</td>
<td>33.30</td>
</tr>
<tr>
<td>Blended fat</td>
<td>3.00</td>
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</tr>
<tr>
<td>Dicalcium Phosphate</td>
<td>1.80</td>
<td>1.80</td>
</tr>
<tr>
<td>Limestone</td>
<td>1.20</td>
<td>1.20</td>
</tr>
<tr>
<td>Vitamin and Mineral mix¹</td>
<td>0.11</td>
<td>0.16</td>
</tr>
<tr>
<td>Salt</td>
<td>0.40</td>
<td>0.40</td>
</tr>
<tr>
<td>DL-methionine</td>
<td>0.30</td>
<td>0.30</td>
</tr>
<tr>
<td>L-Lysine</td>
<td>0.10</td>
<td>0.10</td>
</tr>
</tbody>
</table>

¹ Vitamins and minerals were provided in the form and amount described in the NRC Standard Reference Diet for chickens (NRC, 1994). Cholecalciferol diets had cholecalciferol at 3,000 IU/kg of feed. The 25-hydroxycholecalciferol (25(OH)D) diet had 25-hydroxycholecalciferol (HyD, DSM Nutritional Products, Heerlen, the Netherlands) at 69 µg/kg feed.
Figure 12. 24 h body weight gain of birds in different experimental groups. Day-old birds were fed diets supplemented with either cholecalciferol (Vit D3) or 25-hydroxycholecalciferol (25-OH Vit D3) or 25-hydroxycholecalceferol for first 14 d and replaced with Vit D3 after 14 d. At 21 and 35 d of age, birds were injected or not injected with lipopolysaccharide (LPS). At 24 h post-LPS challenge, body weights were measured and body weight gain was expressed as pre-LPS body weight. Means (± SD). P values. 21 d: P = 0.03; 35 d: P = 0.01. Data presented are representative of two independent experiments.
Figure 13. IL-1β mRNA relative abundance in the liver of birds in different experimental groups. Day-old birds were fed diets supplemented with either cholecalciferol at 250 IU/kg of feed or 25-hydroxycholecalciferol (25-OH Vit D3) at 6.25, 25 or 50 μg/kg of feed for 28 d. At 28 d of age, birds were injected or not injected with lipopolysaccharide (LPS). At 24 h post-LPS challenge, IL-1β mRNA amount was analyzed by real time PCR and normalized to β-actin and compared with group fed 250 IU of Vit D3 and not injected with LPS, so all bars represent fold change compared to that group. Means (± SD). P values of liver: Diet, \( P = 0.82 \); LPS, \( P = 0.01 \); Diet*LPS, \( P = 0.16 \). Data presented is representative of one experiment.
Figure 14. IL-1β mRNA relative abundance in the liver of birds in different experimental groups. Day-old birds were fed diets supplemented with either cholecalciferol (Vit D3) or 25-hydroxycholecalciferol (25-OH Vit D3) for 28 d. At 28 d of age, birds were injected or not injected with lipopolysaccharide (LPS). At 24 h post-LPS challenge, IL-1β mRNA amount was analyzed by real time PCR and normalized to β-actin and compared with group fed 3000 IU of Vit D3 and not injected with LPS, so that all bars represent fold change compared to that group. Means (± SD). P values of liver: Diet, $P = 0.01$; LPS, $P = 0.87$; Diet*LPS, $P < 0.01$. Data presented is representative of one experiment.
Figure 15. 1α-Hydroxylase mRNA relative abundance in the kidney and liver of birds in different experimental groups. Day-old birds were fed diets supplemented with either cholecalciferol (Vit D3) or 25-hydroxycholecalciferol (25-OH Vit D3) for 28 d. At 28 d of age, birds were injected or not injected with lipopolysaccharide (LPS). At 24 h post-LPS challenge, 1α-hydroxylase mRNA amounts amount was analyzed by real time PCR and normalized to β-actin and compared with group fed 3000 IU of Vit D3 and not injected with LPS, so that all bars represent fold change compared to that group. Means (±SD). P values: Kidney, Diet, $P = 0.40$; LPS, $P = 0.27$; Diet*LPS, $P = 0.60$; Liver, Diet, $P < 0.01$; LPS, $P = 0.66$; Diet*LPS, $P = 0.07$. Data presented is representative of one experiment.
Chapter 5: Effect of in vitro and in vivo 25-hydroxyvitamin D treatment on macrophages, T cells and layer chickens during a coccidia challenge

This chapter has been accepted in the journal, Journal of Animal Sciences and has been reformatted for thesis.

Abstract

This article describes the in vitro and in vivo effects of a 25-hydroxycholecalciferol (25(OH)D) treatment in layer hens during a mixed coccidia challenge. HD11 cells (chicken macrophage cell line) were treated in vitro with a coccidia antigen or in a medium supplemented with either 1,25-dihydroxycholecalciferol (1,25(OH)\(^2\)D) or 25(OH)D. HD11 cells treated in vitro with 200 nM of 1,25(OH)\(^2\)D had increased nitrite production (P < 0.01) compared with HD11 cells treated with 0 or 200 nM of 25(OH)D. Treating HD11 cells with 25(OH)D decreased IL-10 mRNA by 1.7-fold, but 1,25(OH)\(^2\)D treatment increased the amount of IL-10 mRNA by 2.7-fold (P < 0.01) compared with the group treated with 0 nM of 25(OH)D. Post-coccidial antigen stimulation, 25(OH)D or 1,25(OH)\(^2\)D treatment decreased (P < 0.01) 1α-hydroxylase mRNA amounts in HD11 cells. Stimulating primary T cells in vitro with Con-A
decreased (P = 0.020) the 1α-hydroxylase mRNA amounts by three fold. ConA-B1-VICK cells stimulated with 100 nM 1,25(OH)₂D or with supernatants from HD11 cells treated with 25(OH)D plus LPS had 1.3-fold less (P < 0.01) IFN-γ mRNA compared with the group treated with 25(OH)D. Layer birds were fed a basal diet supplemented with 25(OH)D at 6.25, 25, 50 or 100 µg/kg and at 21 d of age orally challenged with 1 x 105 live coccidia oocysts. Compared with the control birds fed similar levels of 25(OH)D and unchallenged with the coccidia oocyst, birds challenged with the coccidia oocyst had 15 % reduced BW gain in the groups supplemented with either 6.25, 25, and 50 µg/kg of 25(OH)D but there was only a 4 % reduced BW gain in birds fed 100 µg/kg of 25(OH)D (P < 0.01). Birds fed 100 µg/kg 25(OH)D had decreased (P = 0.012) CD8+ cell percentages in cecal tonsils in both coccidial oocyst challenged and unchallenged birds, compared with the control birds fed 6.25 µg/kg 25(OH) and unchallenged with coccidial oocysts. At 15 d post coccidia challenge, birds fed 100 µg/kg 25(OH)D and challenged with coccidial oocysts had 17% more CD4+CD25+ cells (P = 0.018) in the cecal tonsil compared with the birds fed 100 µg/kg 25(OH)D and unchallenged with coccidial oocysts. At d 6 post coccidia challenge, among birds challenged with coccidial oocysts, birds fed 100 µg/kg 25(OH)D had 3.5-fold increase (P < 0.01) in IL-10 and numerically decreased IL-1β mRNA amounts in the cecal tonsils compared with birds fed 6.25 µg/kg 25(OH)D. In conclusion, supplementing birds with 100 µg/kg 25(OH)D could be a nutritional strategy to reduce the production losses post coccidia challenge.

Key words: coccidiosis, HD11 cells, 25-hydroxyvitamin D, layer chicken, T-cells
**Introduction**

Coccidiosis is the major parasitic disease affecting poultry and results in severe economic loss due to impaired body weight gain and feed utilization (1). Increasing resistance to anti-coccidial drugs poses a serious challenge in combating coccidiosis, and alternative strategies such as nutritional intervention have been attempted (7).

Vitamin D is an immunomodulatory nutrient, and the active form of vitamin D, 1, 25-dihydroxycholecalciferol (1,25(OH)₂D), improves host defenses against intracellular pathogens like *Mycobacterium tuberculosis* by increasing macrophage antimicrobial peptide cathelicidin (138). Earlier we identified that in vitro supplementation of 25-hydroxyvitamin D (25(OH)D), an inactive form of vitamin D, increases macrophage nitric oxide (NO) production during an inflammatory challenge (139). Replication of *Eimeria tenella* is inhibited by NO (7, 140). Active Vitamin D is produced from 25(OH)D by the enzyme 1α-hydroxylase, expressed mostly in kidneys (24). However, extra-renal presence of 1α-hydroxylase in macrophages facilitates local production of active Vitamin D from 25(OH)D (141). Of interest is identifying whether or not supplementing 25(OH)D improves host defense against coccidiosis.

Because mammalian T cells have limited expression of the 1α-hydroxylase enzyme, they rely on extrinsic sources of 1,25(OH)₂D (130) for their conversion into a regulatory phenotype that suppresses excess inflammatory responses (19). Identifying that chicken T cells can convert 25(OH)D into 1,25(OH)₂D during a coccidial infection would promote application of 25(OH)D to prevent excess inflammatory response associated with coccidiosis. This study investigated the in vitro and in vivo effects of 25(OH)D supplementation during a coccidia infection in chickens.
**Materials and Methods**

All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at The Ohio State University. Specific pathogen-free layer birds (White Leghorn) were housed in individual battery cages and provided feed and water ad libitum.

**Preparation of Coccidial Antigens for In Vitro Studies**

Coccidial antigens for in vitro studies were prepared by slight modifications to the established procedures (Annamalai and Selvaraj, 2012; You, 2014). Briefly, $1 \times 10^7$ coccidial oocysts containing a mixture of *E. acervulina*, *E. maxima* and *E. tenella* (Inovocox, Zoetis, Florham Park, NJ) were resuspended in 6 mL of 1x PBS and placed in a -70°C freezer for 30 min to begin freeze-thaw cycles. Following freezing, coccidial oocysts were subsequently thawed at room temperature for 45 min. The freeze-thaw cycle was repeated for a total of three times. At the end of the final freeze-thaw cycle, 500 µL of oocysts were mixed with approximately an equal volume of 0.4-to 0.6-mm glass beads and subjected to a 4-min oscillation cycle of 50 oscillations/s in a TissueLyser LT (Qiagen, Valencia, CA). The oscillation cycles were repeated four times with a resting period of 10-15 s between each cycle. The lysed solution was centrifuged at 9000 x g for 6 min to remove intact cells and cell debris. The protein content of the supernatant was determined using the Nanodrop Spectrophotometer (ND-1000, NanoDrop Technologies Inc, Wilmington, DE).

*Effect of 25(OH)D Treatment on HD11 Cell Nitrite Production*
HD11 cells were obtained from Dr. Mike Kogut, Southern Plains Agricultural Research Center, College Station, TX. HD11 cells, a chicken macrophage cell line, were grown in 48 well plates (4 x 10^5 cells/well) in 500 μL of RPMI-1640 growth medium supplemented with 10% fetal bovine serum (FBS), 1% non-essential AA, L-glutamine and 2% penicillin and streptomycin and allowed to adhere overnight at 37°C and 5% CO_2. Following overnight incubation, the medium was removed. Fresh RPMI medium, supplemented with 200 nM of 25(OH)D (Santa Cruz Biotechnology, Dallas, TX) or 1,25(OH)_2D (Cayman Chemical, Seattle, WA), was added in eight replications (n = 8) and stimulated with 15 μg/well coccidial antigens for 12 h. Nitrite content in the cell culture supernatant was quantified using Griess reagent (Ricca Chemical Company, Arlington, TX) following the manufacturer’s instructions (133).

Effect of 25(OH)D Treatment on IL-1β, IL-10 and 1a-hydroxylase mRNA Content of HD11 Cells

HD11 cells (4 X 10^5 cells/well) were grown in 48-well plates in 500 μL of RPMI medium and allowed to adhere overnight at 37°C and 5% CO_2. Following overnight incubation, medium was removed and fresh RPMI medium supplemented with 200 nM of 25(OH)D or 1,25(OH)_2D was added in eight replications (n = 8). HD11 cells were stimulated with 15 μg/well of coccidial antigen (control) or with coccidial antigen in the presence of 200 nM of 25(OH)D or 1,25(OH)_2D for 12 or 48 h respectively. At both 12 and 48 h, total RNA was extracted from HD11 cells using Tri-reagent (Molecular Research Center, Cincinnati, OH., USA) and reverse transcribed into cDNA. cDNA was analyzed for relative amounts of IL-1β (5'-tcttcagccagaagtga-3’and 5’-
caggggtagaagatgaagc-3'), IL-10 (5'-caatccagggagatgaact-3' and 5'-
gcaggcacctctgtgta-3'), and 1α-hydroxylase (5'-tcgtggcaggatacaga-3' and 5'-
actgccatactttggttt-3') mRNA amounts by quantitative real-time PCR (iCycler, BioRad)
using SyBr green after normalizing for β-actin (5'-accggactgttacacca-3' and 5'-
gactgctgctgacacctca-3') mRNA amounts as described previously (45, 142). Annealing
temperatures for IL-1β, 1α-hydroxylase and IL-10 primers were 57.5°C, 55°C and 58°C,
respectively (139). The fold change from the reference was calculated as 2(Ct
Sample)/2(Ct Reference), where Ct is the threshold cycle (142). The reference group was
the control group stimulated with 15 µg/well of coccidial antigen. The threshold (Ct)
values were determined by iQ5 software (BioRad, Hercules, CA). Because insufficient
RNA was obtained for some samples at 48 h, the replication size was 6 (n=6) for RT-
PCR analysis.

Isolation of Primary T cells and Estimating 1α-hydroxylase mRNA Content

The thymus was isolated from 8-to 10-wk-old White Leghorn chickens, and mononuclear
cells were obtained using Ficoll Density Gradient Separation (Histopaque 1077, Sigma,
St. Louis, MO) by methods described earlier (143). CD4+ T cells were isolated using
MACS Microbeads technology (Miltenyi Biotec, Auburn, CA, USA) by positive
selection using mouse-anti-chicken CD4-IgG-coated with magnetic beads (133). Purity of
isolated CD4+ cells was determined by a flow cytometer (Gauva Easycyte, Millipore,
Billerica, MA) (> 90%). Isolated CD4+ cells (1 x 106) were grown in an RPMI medium
supplemented with 2.5% FBS, 2% chicken serum (CS), and 2% penicillin plus
streptomycin and stimulated with 1 µg/mL of Concanavalin A (Con-A) (Sigma Aldrich,
St. Louis, MO) for 48 h at 37°C. At the end of stimulation, RNA was extracted from CD4+ cells as described earlier, reverse transcribed into cDNA and analyzed for relative amounts of 1α-hydroxylase mRNA relative abundance by quantitative real-time PCR after normalizing for β-actin.

**Effect of 25(OH)D Treatment on a Chicken T Cell Line (ConA-B1-VICK) on IFN-γ mRNA Relative Abundance.**

RPMI medium supplemented with 2.5% fetal bovine serum, 2% chicken serum and 2% penicillin plus streptomycin was used for growing ConA-B1-VICK cells (ATCC, Manassas, VA). ConA-B1-VICK cells (2 x 10^7 cells/well) were stimulated with 7.5 µg/mL of Con-A for 120 h at 37°C and 5% CO2 in 48 well plates in a RPMI medium containing either 200 nM of 25(OH)D or 100 nM 1,25(OH)_2D or with 1:5 dilution of supernatants from HD11 cells treated with 25(OH)D in the presence or absence of an LPS stimulation. At the end of stimulation, RNA was extracted from ConA-B1-VICK cells as described earlier, reverse transcribed to cDNA, and analyzed for relative abundance of 1α-hydroxylase and IFN-γ mRNA (5'-gtgaagaaggtgaaagatatcatgga-3' and 5'-gctttgcgctggattctca-3') relative abundance by quantitative real-time PCR after normalizing for β-actin as described above (141, 143). Annealing temperature for IFN-γ was 55°C. The reference group was the group stimulated with 7.5 µg/mL of Con-A in a RPMI media containing 200 nM of 25(OH)D.
Animals, Housing and Coccidial Infection

A total of 200 1-d-old White Leghorn chicks hatched at the Ohio Agricultural Research and Development Center (OARDC, Wooster, Ohio) hatchery were randomly distributed to 1 of 4 treatment groups. Each treatment was replicated in 10 individual battery cages (n=10) with a total of 5 chicks in each individual battery cage. All birds were wing-tagged and weighed individually. The 4 experimental groups were fed a basal diet (Table 3) supplemented with 25-hydroxycholecalciferol (HyD, DSM nutritional products, Netherlands) at doses of 6.25, 25, 50 or 100 µg/kg, which are equivalent to 250, 1000, 2000 or 4000 IU/kg of diet of cholecalciferol. The mean body weights of chicks at d-1 in the different experimental groups supplemented with 25-hydroxycholecalciferol at doses of 6.25, 25, 50, or 100 µg/kg of diet were 41.5, 42, 42.1, and 41 g, respectively. At 21 d of age, birds were weighed. Five individual battery cages per treatment (n=5) were chosen, and all birds in the pen were orally challenged with 1 x 10^5 live coccidial oocysts (Inovocox, Zoetis, Florham Park, NJ) in 100 µL of PBS, as described previously, to induce a coccidial infection (133). The birds in the remaining 5 battery cages/treatment were left unchallenged. The experimental design was a 4 x 2 factorial arrangement of treatments, and the unchallenged birds in each treatment group served as the control.

Effect of Different Doses of 25(OH)D on Body Weight Gain and Fecal Coccidia Oocyst Load Post Coccidia Challenge

Birds were weighed individually on the day of coccidial challenge and on the 6-day post coccidia challenge. The gain in body weight over a 6-d period was calculated as the difference in the body weight compared with the initial body weight and expressed as a
percentage. On d-6 and 15 of post-coccidia challenge fecal samples were collected from individual battery cages in air tight plastic bags and stored at 4°C until further analysis. On the day of analysis, samples were homogenized, and coccidial oocysts were enriched using a salt flotation technique described previously (144). Coccidial oocysts were diluted and counted using the McMaster Counting Chamber (Chalex Corporation, Ketchum, ID, USA).

Effect of Different Doses of 25(OH)D on CD4+, CD8+ and CD4+ CD25+ Cell Percentage in Cecal Tonsils Post Coccidia Challenge

One bird was selected randomly from each individual battery cage (n=5) on d-14 and 15 post coccidia challenge. Cecal tonsils were collected to determine the percentage of CD4+ and CD8+ cells on d-14 and of Tregs (CD4+ CD25+) on d-15. Single cell suspensions of the cecal tonsils were enriched for the mononuclear cells by Density Gradient Separation as described previously (145). The percentages of CD4+, CD8+ and Tregs (CD4+ CD25+) in the mononuclear cell populations were determined in a Flow Cytometer as described previously (146). CD4+CD25+ cell percentages were expressed as a percentage of total CD4+ cells to facilitate comparison between samples.

Effect of Different Doses of 25(OH)D Treatment on IL-1β, IL-10 and 1α-hydroxylase mRNA Amounts in Cecal Tonsils and Liver

At 6 d post coccidia challenge, 1 bird was selected randomly from each individual battery cage (n=5/treatment) and sacrificed. Samples of the cecal tonsils and liver were collected in RNALater (Qiagen, Valencia, CA). After 24 h, RNALater was discarded, and the organs
were stored at -70°C until further analysis. Total RNA was extracted from the different organs and analyzed for relative amounts of IL-1β, IL-10, and 1α hydroxylase after normalizing for β-actin mRNA levels as described above. The reference group was the group fed 6.25 µg/kg of 25(OH)D and not challenged with coccidia parasite.

Statistical Analysis

For the in vitro study, data were analyzed using a one-way ANOVA (JMP, SAS Institute Inc., Cary, NC, USA) to determine the effects of 25(OH)D treatment on dependent variables. For the in vivo study, data were analyzed using a factorial arrangement with factors in the model being 25(OH)D and coccidial parasite injection and their interaction. Differences between means were analyzed by Tukey’s least square means comparison.

Results

Effect of 25(OH)D Treatment on HD11 Cell Nitrite Production and IL-1β mRNA Relative Abundance

At 12 h post coccidial antigen stimulation, HD11 cells treated with 200 nM of 1,25(OH)2D had increased nitrite production (P < 0.01) compared with HD11 cells treated with 0 or 200 nM of 25(OH)D (Fig. 16A). At 12 h post coccidial antigen stimulation, HD11 cells treated with 200 nM of 25(OH)D or 1,25(OH)2D had 28-and 14-fold lower (P<0.01) IL-1β mRNA abundance compared with the control group treated with 0 nM of 25(OH)D or 1,25(OH)2D (Fig. 16B). At 48 h post coccidial antigen stimulation, HD11 cells treated with 200 nM of 25(OH)D or 1,25(OH)2D had no
significant differences in IL-1β mRNA abundance (P=0.93) compared with the control group (Fig. 16B).

*Effect of 25(OH)D Treatment on HD11 cell, IL-10 and 1α-hydroxylase mRNA Relative Abundance*

At 12 h post coccidial antigen stimulation, treatment of HD11 cells with 25(OH)D decreased IL-10 mRNA by 1.7-fold, but 1,25(OH)₂D treatment increased the abundance of IL-10 mRNA by 2.7-fold (P < 0.01) compared with the group treated with 0 nM of 25(OH)D or 1,25(OH)₂D (Fig. 17A). At 48 h post coccidial antigen stimulation, treatment of HD11 cells with 1,25(OH)₂D increased the amounts of IL-10 mRNA by approximately 17-fold (P < 0.01) compared with the group with 0 nM of 25(OH)D or 1,25(OH)₂D (Fig. 17A). HD11 cells treated with 200 nM of 25(OH)D or 1,25(OH)₂D had 1.5-fold lower (P <0.01) and 1.2-to 2.7-fold lower (P <0.01) 1α-hydroxylase mRNA abundance at 12 and 48 h respectively compared with the group treated with 0 nM 25(OH)D or 1,25(OH)₂D (Fig. 17B).

*Effect of Proliferation on T cell 1α-hydroxylase and Supernatants from HD11 cells*

*Treated with 25(OH)D on ConA-B1-VICK IFN-γ mRNA Relative Abundance*

Stimulating primary T cells for 48 h with Con-A decreased (P = 0.020) the 1α-hydroxylase mRNA abundance by threefold (Fig. 18A). ConA-B1-VICK cells stimulated with100 nM 1,25(OH)₂D or with supernatants from HD11 cells treated with 25(OH)D plus LPS had approximately 1.3-fold lower (P < 0.01) IFN-γ mRNA compared with the group treated with 25(OH)D (Fig. 18B).
Effect of 25(OH)D Supplementation on Body Weight Gain and Fecal Oocyst Shedding

Post Coccidia Infection in Layer Chickens

There was a main effect of coccidial oocyst challenge on the body weight gain over a 6-d period (P<0.01) in layer chickens (Fig.19A). Compared with the control birds fed similar levels of 25(OH)D and unchallenged with the coccidia pathogen, birds challenged with the coccidia pathogen had approximately 15 % reduced body weight gain in the groups supplemented with either 6.25, 25, and 50 μg/kg of 25(OH)D but had only 4 % reduced body weight gain in birds fed 100 μg/kg of 25(OH)D. There was no significant effect of 25(OH)D treatment on the fecal oocyst shedding at d 6 post coccidial challenge (Fig. 19B).

Effect of 25(OH)D Supplementation on Cecal Tonsil CD4+, CD8+ and CD4+CD25+

Percentages Post Coccidial Oocyst Infection in Layer Birds

Birds fed 100 μg/kg 25(OH)D had decreased (P = 0.013) CD8+ cell percentages in cecal tonsils in both coccidial-oocyst-challenged and unchallenged birds, compared with the control birds fed 6.25 μg/kg of 25(OH)D and not challenged with coccidial oocysts (Fig. 20A). At 15 d post coccidial oocyst challenge, birds fed 100 μg/kg 25(OH)D and challenged with coccidial oocysts had 17% more CD4+CD25+ cells (P = 0.018) in the cecal tonsil compared to the birds fed 100 μg/kg 25(OH)D and not challenged with coccidial oocysts (Fig. 20B). At 14 d post coccidial oocyst challenge, CD4+ cell percentages in cecal tonsils of birds fed 25, 50, and 100 μg/kg 25(OH)D and challenged with coccidia oocysts did not differ significantly from that in the control group fed 6.25 μg/kg 25(OH)D.
Effect of 25(OH)D Supplementation on Cecal Tonsil IL-1β, IL-10 and 1α-hydroxylase mRNA Amounts and Liver 1α-hydroxylase mRNA Relative Abundance Post Coccidia Oocyst Infection in Layer Birds

At d 6 post coccidial oocyst challenge, among birds challenged with coccidial oocysts, birds fed 25, 50 and 100 µg/kg 25(OH)D had 1.7, 4.2, and 3.4-fold numerical decrease in the IL-1β mRNA relative abundance in the cecal tonsils compared with the birds fed 6.25 µg/kg 25(OH)D (Fig.21A). The group fed 6.25 µg/kg of 25(OH)D and challenged with coccidial oocysts had no significant difference in the IL-1β mRNA relative abundance in the cecal tonsils compared with the control birds fed 6.25 µg/kg 25(OH)D and unchallenged with coccidial oocysts (Fig.21A).

In groups fed 25(OH)D at 6.25, 25, 50 and 100 µg/kg of feed and challenged with coccidial oocysts, IL-10 mRNA relative abundance increased by approximately 3.3-, 4.5-, 4.9- and 3.5-fold (P < 0.01) compared with the control group (Fig.21B).

There were no significant differences in the cecal tonsils and liver 1α-hydroxylase mRNA amounts among different treatment groups (P > 0.10).

Discussion

Stimulating HD11 cells that were pre-treated with either 25(OH)D or 1,25(OH)\textsuperscript{2}D with coccidial antigen decreased 1α-hydroxylase mRNA amounts. We earlier reported that HD11 cells treated in vitro with the bacterial LPS have increased 1α-hydroxylase mRNA (141). This suggests that coccidial antigens are a poor in vitro inducer of 1α-hydroxylase mRNA in macrophages. In line with prior findings in our lab (141) and by other researchers (127), 1,25(OH)\textsuperscript{2}D treatment increased nitrite production in HD11 cells,
whereas cells treated with 25(OH)D did not increase nitrite production. Decreased 1α-hydroxylase mRNA relative abundance post in vitro coccidial antigen stimulation in 25(OH)D treated cells can explain the inability of 25(OH)D to induce NO production as decreased 1α-hydroxylase will decrease the conversion and subsequent availability of active Vitamin D in those cells.

1,25(OH)²D converts naïve T cells into T regulatory cells (19). T regulatory cells secrete anti-inflammatory cytokines IL-10 and TGF-β that inhibit the expression of pro-inflammatory cytokines such as IFN-γ, IL-17, and IL-21 (19, 145, 147). In this study, 1,25(OH)²D treatment decreased the IFN-γ mRNA content of a T cell line. Prior studies have demonstrated that T cells have limited 1α-hydroxylase activity (130), and that they rely on neighboring bystander macrophages or dendritic cells for 1,25(OH)²D. In this study, proliferating T cells decreased the 1α-hydroxylase mRNA relative abundance, suggesting that proliferating T cells may not be able to efficiently utilize 25(OH)D. Because our previous in vitro studies identified that treating HD11 cells with an inflammatory molecule, LPS, upregulated the 1α-hydroxylase amounts, we hypothesized that treating ConA-B1-VICK cells with supernatants from HD11 cells treated with 25(OH)D and stimulated with LPS suppress the production of pro-inflammatory cytokines. In this study, T cell lines treated with supernatants from LPS-stimulated macrophage cells and treated with 25(OH)D suppressed the IFN-γ mRNA amounts. T cells treated with supernatants from macrophages that were not stimulated with LPS did not suppress IFN-γ mRNA amounts, showing that chicken T cells, similar to their mammalian counterparts (130) may not utilize 25(OH)D efficiently as a source of 1,25(OH)²D.
Both in vitro and in vivo, the 25(OH)D treatment decreased IL-1β mRNA amounts post coccidial treatment. Parallel to the decreased IL-1β mRNA amounts, there was an increase in the IL-10 mRNA amounts both in vivo and in vitro, post coccidial treatment. Increased cecal tonsil T regulatory cell percentages in birds supplemented with 100 μg/kg of 25(OH)D post coccidial infection challenge most likely contributed to the increase in the IL-10 in vivo because T regs are a natural source of IL-10 (146, 148). In addition to the anti-inflammatory effects of vitamin D mediated through Tregs, vitamin D curtails the inflammatory pathway by upregulating IL-10 in macrophages (149). Decreased IL-1β mRNA amounts observed both in vitro and in vivo might be due to the anti-inflammatory effects of cytokine IL-10. Although supplementing 25(OH)D increased the mRNA amounts of IL10, an anti-inflammatory cytokine, feeding 25(OH)D increased CD8+ cell percentage in the cecal tonsils. This is interesting in the context of a coccidial infection because early studies have demonstrated a role for CD8+ T cells in acting as transporters for sporozoite and increasing the fecal oocyst shedding (150). Since our data from fecal oocyst counts are inconclusive to support this argument, more studies need to be done using 25(OH)D to investigate the effects of 25(OH)D on CD8+ T cells and its relative contribution in fecal oocyst shedding. In our earlier study, we observed that supplementing with higher doses of 25(OH)D in broiler diets increased body weight gain and decreased expression of the IL-1β mRNA post LPS injection (132). In this study, the highest BW gain over a 6 d period and decreased IL-1β mRNA amounts were observed only in the group supplemented with a dose of 25(OH)D above 25 μg/kg. Here we propose high doses of 25(OH)D supplementation in layer birds as a strategy to direct the immune response towards an anti-inflammatory nature. The decreased expression of
IL-1β and IFN-γ is due to a synergistic action by cells from both innate and adaptive arms of the immune system involving macrophages and T regs, and the cytokine IL-10. Even though an initial inflammatory response is needed to combat pathogens, an excess inflammatory response in birds comes with a decrease in BW gain (10).

In conclusion, our current findings suggest a unique nutritional intervention strategy of supplementing with a high dose of 25(OH)D to decrease the production losses associated with a coccidial infection.

Acknowledgements

We acknowledge Keith Patterson, Jack Sidle, Jordan Welsh, Jarrod Snell (Ohio Agricultural Research and Development Center) for help with Animal Husbandry, Matthew Walston and Ashley Markazi for help with sample collection and analysis, Michelle Hendrick (The Ohio State University) for proof reading the manuscript and Orrville Hatcheries (Orrville, Ohio) for donating chicks for this study.
Table 3. Ingredients and Calculated Nutrient Composition of the Basal Diets

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¹ Vitamins and minerals were provided in the form and amount described in the NRC Standard Reference Diet for chickens (NRC, 1994). Experimental diets had 25-hydroxycholecalciferol (HyD, DSM nutritional products, Netherlands) at doses of 6.25, 25, 50, and 100 µg/kg of feed.
Figure 16. Effect of 25(OH)D treatment on HD11 cell (A) nitrite production and (B) IL-1β mRNA relative abundance. (A) HD11 cells were grown in a medium supplemented with 200 nM of 25(OH)D or 1,25(OH)$_2$D and stimulated with 15 μg/mL of coccidial antigens. At 12 h post coccidial antigen stimulation, nitrite concentration in the supernatant was quantified using Griess reagent. Means (± SEM) without a common superscript differ significantly within a treatment group. P values for nitrite: P < 0.01. n = 8. (B) For mRNA analysis, at 12 and 48 h post coccidial antigen stimulation, mRNA relative abundance was analyzed by real time PCR and normalized to β-actin and compared to the group stimulated with coccidial antigen and treated with 0 nM of 25(OH)D or 1,25(OH)$_2$D, so that all bars represent fold change compared to this group. Means (± SEM) without a common superscript differ significantly within a treatment group. P values: IL-1β: 12 h: P < 0.01; 48 h: P = 0.929. n=6. Data presented are the average of two independent experiments.
Figure 17. Effect of 25(OH)D treatment on HD11 cell (A) IL-10 and (B) 1α-hydroxylase mRNA relative abundance. HD11 cells were grown in a medium supplemented with 200 nM of 25(OH)D or 1,25(OH)_2D and stimulated with 15 μg/mL of coccidial antigens for 12 and 48 h. At 12 and 48 h post coccidial antigen stimulation, mRNA relative abundance was analyzed by real time PCR and normalized to β-actin and compared to the group stimulated with coccidial antigen and treated with 0 nM of 25(OH)D or 1,25(OH)_2D, so that all bars represent fold change compared to this group. Means (± SEM) without a common superscript differ significantly within a treatment group. P values for IL-10: 12 and 48 h: P < 0.01. P values: 1α-hydroxylase: 12 and 48 h: P < 0.01. n=6. Data presented are the average of two independent experiments.
Figure 18. Expression of 1α-hydroxylase (A) in primary T cells and (B) IFN-γ mRNA relative abundance in ConA-B1-VICK cells treated with 100 nM 1,25(OH)₂D₃ or with supernatant from HD11 cells treated with 25(OH)D plus LPS. (A) Isolated thymic CD4+ cells (1 x 10⁶) were stimulated with 1 µg/mL of Con-A for 48 h. At 48 h post-Con-A stimulation, mRNA relative abundance was analyzed by real time PCR and normalized to β-actin and compared to the 0 h group not stimulated with Con-A, so that all bars represent fold change compared to the 0 h group. P values: 1α-hydroxylase: P = 0.020. n=3 (B) ConA-B1-VICK cells (2 x 10⁷), were grown in a medium containing either 200 nM of 25(OH)D, 100 nM of 1,25(OH)₂D₃ or with 1:5 dilution of supernatants from HD11 cells treated with 25(OH)D in the presence or absence of an LPS stimulation and stimulated with 7.5 µg/mL of Con-A for 120 h. At 120 h post Con-A stimulation, mRNA relative abundance was analyzed by real time PCR and normalized to β-actin and compared to the group treated with 25(OH)D, so that all bars represent fold change compared to this group. Means (± SEM) without a common superscript differ significantly within a time point. P values: IFN-γ: P < 0.01. n=4 except for the treatment group discussed in materials and methods.
Figure 19. Effect of different doses of 25(OH)D on (A) body weight gain and (B) fecal coccidia oocysts. Day-old birds were fed diets supplemented with 25(OH)D at doses of 6.25, 25, 50 or 100 µg/kg of feed. At 21 d of age, birds were weighed individually and orally challenged with 1 x 10^5 live coccidial oocysts. (A) At 6 d post coccidia challenge, body weights were measured and % body weight gain over a 6 d period was expressed as pre-coccidia body weight. Means (± SEM) without a common superscript differ significantly within a group. P values: % body weight gain over a 6 d period: Diet, P = 0.421; coccidia, P < 0.01; Diet* coccidia, P = 0.511. n=5. (B) At d 6 post coccidia challenge, fecal samples were collected and coccidial oocysts enriched using the salt flotation technique and subsequently diluted and counted using the McMaster Counting Chamber. Means (± SEM) without a common superscript differ significantly within a group. P values: d 6 fecal coccidial oocyst: Diet, P = 0.659; coccidia, P < 0.01; Diet* coccidia, P = 0.660. n=5. Data presented are from one experiment.
Figure 20. Effect of different doses of 25(OH)D on (A) CD4+, CD8+ (B) and double positive CD4+ CD25+ cell percentage in cecal tonsils post coccidia challenge. At d 14 and 15 post coccidia challenge, cecal tonsils were collected and enriched for lymphocytes by Ficoll density gradient separation. The percentages of CD4+ and CD8+ cells and Tregs (CD4+ CD25+) were calculated by Flow cytometry on d 14 and 15, respectively. Means (± SEM) without a common superscript differ significantly within a group. P values: d 14: CD4+ cells: Diet, P = 0.042; coccidia, P < 0.01; Diet* coccidia, P = 0.129. CD8+ cells: Diet, P < 0.01; coccidia, P = 0.553; Diet* coccidia, P < 0.01. n=5. P values: d 15: CD4+ CD25+ cells: Diet, P = 0.864; coccidia, P = 0.046; Diet* coccidia, P = 0.018. n=5. Data presented are from one experiment.
Figure 21. Effect of different doses of 25(OH)D treatment on (A) IL-1β and (B) IL-10 mRNA relative abundance in cecal tonsils. At d 6 post coccidia challenge, cecal tonsils were collected and IL-1β and IL-10 mRNA amounts were analyzed by real time PCR and normalized to β-actin and compared with the group fed 6.25 µg/kg of 25(OH)D and not infected with coccidia, so that all bars represent fold change compared to that group. Means (± SEM) without a common superscript differ significantly within a group. P values: cecal tonsils: IL-1β: Diet, P = 0.041; coccidia, P = 0.182; Diet* coccidia, P = 0.137. IL-10: Diet, P = 0.062; coccidia, P < 0.01; Diet* coccidia, P < 0.01. n=5. Data presented are from one experiment.
Chapter 6: Effect of in vitro 25-hydroxycholecalciferol treatment of lipopolysaccharide on heterophil nitrite production and dietary supplementation on body weight gain and mRNA expression of IL-1β and 1α-hydroxylase and T-cell percentages in turkey poults post coccidial challenge

Abstract

Two experiments were conducted to study the effects of 25-hydroxycholecalciferol (25(OH)D) in vitro treatment on heterophil nitrite production post-lipopolysaccharide (LPS) challenge and the effects of 25(OH)D in vivo supplementation in turkey birds post-coccidial challenge. Heterophils were cultured in the presence of 0 or 200 nM of 25(OH)D and stimulated with 1 μg/mL of LPS for 24 h. 25(OH)D treatment increased heterophil nitrite production by approximately 3-fold change (P = 0.02) compared to the control group with 0 nM of 25(OH)D. Turkey poults were fed a basal diet supplemented with 25(OH)D at 27.5, 55, 82.5, or 110 µg/kg and at 21-d of age orally challenged with 1 x 10^4 live coccidia oocysts. At 21-d of age, there was a trend in increasing the mean BW of birds supplemented with 25(OH)D at 82.5 µg/kg (P = 0.09). Compared to the control birds fed similar levels of 25(OH)D and unchallenged with the coccidia oocyst, birds challenged with the coccidia oocyst had 20, 13, and 24 % increased (P = 0.01) BW gain in the groups supplemented with either 27.5,
55, and 82.5 μg/kg of 25(OH)D and a 3 % reduced BW gain in birds fed 110 μg/kg of 25(OH)D (P = 0.01). At d 7 post coccidia challenge, in birds challenged with coccidia oocysts, birds fed 55, 82.5 and 110 μg/kg 25(OH)D had 1.71, 1.21 and 1.90 fold (P < 0.01) increase and those fed 27.5 μg/kg 25(OH)D had 1.34 fold decrease in IL-1β mRNA relative abundance in the cecal tonsils compared to control birds fed 27.5 μg/kg 25(OH)D and not challenged with coccidial oocyst. In conclusion, 25(OH)D treatment increased heterophils nitrite production post-LPS challenge and increased BW gain and cecal tonsil IL-1β mRNA relative amounts post-coccidial challenge.

**Introduction**

Coccidiosis is a major parasitic disease affecting turkeys and is caused by the protozoan parasite belonging to genus *Eimeria*. Among different species of *Eimeria* that infect turkeys such as *E. adenoeides* is considered the most pathogenic and prevalent species (119, 151). *E. adenoeides* colonizes in the ceca and causes economic loss due to reduced feed intake, impaired body weight gain, poor feed conversion, and, in severe infection, mortality (5). Turkeys infected with oocysts from *E. adenoeides* have decreased body weight gain, increased oocyst shedding, increased CD4+ and CD8+ cells and IFN-γ mRNA *relative abundance* (118, 119). Nutritional interventional strategies, such as supplementing with dietary putrescine in turkey poultams ameliorated the loss of bodyweight gain post coccidial infection in the recovery period (151).

The active form of vitamin D, 1, 25-dihydroxycholecalciferol (1,25(OH)₂D), is produced from 25(OH)D by the enzyme 1α-hydroxylase, expressed mostly in the kidneys. However, extra-renal presence of 1α-hydroxylase in macrophages (141) and
cecal tonsils facilitates local production of active Vitamin D from 25(OH)D. Our prior studies have demonstrated, during a mixed coccidial challenge in layer birds, that supplementing with high doses of 25(OH)D increased the BW gain over a 6-d period and decreased IL-1β mRNA relative abundance and increased T-regulatory cell percentages in cecal tonsils.

Earlier, we identified that in vitro supplementation of 25-hydroxyvitamin D (25(OH)D), an inactive form of vitamin D, increases chicken macrophage nitric oxide (NO) production during an inflammatory challenge (141). Replication of *E. tenella* is inhibited by NO (7, 140).

The aim of this study is to investigate the in vitro effects of 25(OH)D supplementation on chicken heterophils nitrite production following an LPS challenge. Further, this study investigates the in vivo effects of supplementing with 25(OH)D in turkey birds during a coccidial challenge.

**Materials and Methods**

All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at The Ohio State University. Day old healthy Hybrid Converter Large White turkeys (Cooper Farms, Ohio) were housed in individual battery cages and provided feed and water ad libitum.

*Effect of 25(OH)D treatment on heterophil nitrite production*

Isolation of heterophils was done by already established procedures with slight modifications (Redmond et al., 2009). Briefly, 10 ml of blood containing EDTA (0.5 M)
was collected from layer birds by terminal cardiac puncture and layered over a discontinuous density gradient (Histopaque with specific gravity 1.077 over 1.119 g/mL Sigma Aldrich) and centrifuged at 400 x g at 10°C for 15 min. After centrifugation, the interface and the Histopaque-1.119 fraction were washed and resuspended in complete RPMI-1640 medium (Hahn-Dantona et al., 2000). Cells 3 X 10^6 cells/well were plated in 500 µl of RPMI-1640 medium supplemented with 10% FBS, 1% non-essential amino acids, L-glutamine and 2% penicillin and incubated at 37°C and 5% CO2 in 48-well plates. The medium was supplemented with 0 or 200 nM of 25(OH)D (Santa Cruz Biotechnology, USA), and the experiment was conducted in six replications. Heterophils were stimulated with 1 µg/well of Salmonella typhimurium LPS (#L9516, Sigma, St. Louis, MO) for 24 h. Nitrite concentration of the cell culture supernatant was determined using Greiss reagent (Ricca Chemical Company, Arlington, TX) following manufacturer’s instructions (Annamalai and Selvaraj, 2012).

**Animals, Housing and Coccidia challenge**

A total of 200 one-d-old Hybrid Converter Large White turkeys (Cooper Farms, Ohio) were randomly distributed to one of four treatment groups. Each treatment was replicated in ten individual battery cages with a total of 5 poults in each individual battery cage. All poults were wing tagged and weighed individually. The 4 experimental groups were fed a basal diet (Table 1) supplemented with 25-hydroxycholecalciferol (HyD, DSM nutritional products, Netherlands) at doses of 27.5, 55, 82.5, or 110 µg/kg of diet, which are equivalent to 1100, 2200, 3300 or 4400 IU of cholecalciferol. At 21 d of age birds were weighed. Five individual battery cages per treatment were chosen, and all poults in
the pen were orally challenged with $1 \times 10^4$ live coccidial oocysts (Immucox, Vetech, Ontario, Canada) in 100 µL of PBS to induce a coccidial infection. The poults in the remaining five battery cages/treatment were left unchallenged. The experimental was analyzed as a completely randomized design with a 4 x 2 factorial arrangement of treatments, and the unchallenged poults in each treatment group served as the control.

*Effect of different dose of 25(OH)D on body weight gain and fecal coccidia oocyst load post coccidia challenge*

Poults were weighed individually on the day of coccidial challenge and on the sixth day post coccidia challenge. The gain in body weight over a 6-d period is calculated as the difference in the body weight compared to the initial body weight and expressed as a percentage. On d 6, 9, and 12, post-coccidia challenge fecal samples were collected from individual battery cages in air tight plastic bags and stored at 4°C until further analysis. On the day of analysis, samples were homogenized, and coccidial oocysts were enriched using a salt flotation technique described previously (Levine et al., 1960). Coccidial oocysts were diluted and counted using the McMaster counting chamber (Chalex Corporation, Ketchum, ID, USA).

*Effect of different dose of 25(OH)D on CD4+ and CD8+ cell percentage in cecal tonsils post coccidia challenge*

One poult was selected randomly from each individual battery cage (n=5) on d 6 and 9 post coccidia challenge. Cecal tonsils were collected to determine the percentage of CD4+ and CD8+ cells on d 6 and 9. Single cell suspensions of the cecal tonsils were
enriched for the mononuclear cells by density gradient separation as described previously (146). The percentages of CD4+ and CD8+ in the mononuclear cell populations were determined in a flow cytometer as described previously (152).

**Effect of different doses of 25(OH)D treatment on IL-1β and 1α-hydroxylase mRNA relative amounts in cecal tonsils**

At 7 d post coccidia challenge, one poult was selected randomly from each individual battery cage (n=5/treatment) and sacrificed. Samples of the cecal tonsils were collected in RNAlater (Quiagen, Valencia). After 24 h, RNAlater was discarded, and the organs were stored at -70°C until further analysis. Total RNA was extracted from the cecal tonsils using Tri-reagent (Molecular Research Center, Inc., Cincinnati, USA) and reverse transcribed to cDNA. cDNA was analyzed for relative abundance of IL-1β (5'-tctccagccagaatga-3'and 5'-caggcgtagaagatgaagc-3') and 1α-hydroxylase (5'-tcgtggcaggaatacagaga-3' and 5'-actgccacatctttgggttt-3') mRNA relative abundance by quantitative real-time PCR (iCycler, BioRad) using SyBr green after normalizing for β-actin (5'-accggactgtacctcat-3' and 5'-gactgctgctgacaccttca-3') mRNA relative abundance as described previously (45, 142). Annealing temperatures for IL-1β and 1α-hydroxylase primers were 57.5°C and 55°C, respectively. The fold change from the reference was calculated as 2(Ct Sample)/2(Ct Reference), where Ct is the threshold cycle (142). The reference group was the group fed 27.5 µg/kg of 25(OH)D and not challenged with coccidia parasite. The threshold (Ct) values were determined by iQ5 software (BioRad, Hercules, CA).
Statistical analysis

For the in vitro study, data were analyzed using a one-way ANOVA (Minitab Inc, PA, USA) to determine the effects of 25(OH)D treatment on dependent variables. For the in vivo study, data were analyzed using a 2-way ANOVA to test the interaction effects of 25(OH)D and coccidial parasite injection on dependent variables. When the effects were significant (P < 0.05), differences between means were analyzed by Tukey’s least square means comparison (JMP, SAS Institute Inc., Cary, NC, USA).

Results

Effect of 25(OH)D treatment on nitrite production from chicken heterophils

At 24 h post- LPS treatment, chicken heterophils treated with 25(OH)D had approximately 3.3-fold (P = 0.02) more nitrite in the supernatant compared to the group with treated with 1 μg/well of LPS and 0 nM of 25(OH)D (Figure 22).

Effect of 25(OH)D supplementation on mean BW, body weight gain and fecal oocyst shedding post coccidia infection in poults

At 21 d, poults supplemented with 25(OH)D at doses above 82.5 µg/kg of feed showed trend for an increased mean BW (P = 0.09). The mean BW of poults in different experimental groups fed 25(OH)D at doses of 27.5, 55, 82.5 or 110 µg/kg were 913, 896, 968 and 936 g respectively (Fig.23 A). There was a main effect of coccidial oocyst challenge on the body weight gain over a 6-d period in poults (P=0.01). Compared to the control birds fed similar levels of 25(OH)D and unchallenged with the coccidia pathogen, birds challenged with the coccidia pathogen had approximately 20, 13 and 24%
increased body weight gain in the groups supplemented with either 27.5, 55, and 82.5 μg/kg of 25(OH)D, but had 4% reduced body weight gain in birds fed 110 μg/kg of 25(OH)D (Fig. 23 B). There was no fecal oocyst shedding across any treatment groups on d 6, 9 or 12.

**Effect of 25(OH)D supplementation on cecal tonsil IL-1β and 1α-hydroxylase mRNA relative abundance post coccidia oocyst infection in turkey pouls**

At d 7 post coccidial oocyst challenge, among birds challenged with coccidial oocysts, birds fed 55, 82.5 and 110 μg/kg 25(OH)D had 1.7-, 1.2- and 1.9-fold (P < 0.01) increase and in birds fed 27.5 μg/kg 1.9-fold decrease (P < 0.01) in the IL-1β mRNA relative abundance in the cecal tonsils compared to the birds fed 27.5 μg/kg 25(OH)D (Fig. 24 A). There was no significant differences in the cecal tonsils (P = 0.77) 1α-hydroxylase mRNA relative abundance among different treatment groups (Fig. 24 B).

**Effect of 25(OH)D supplementation on cecal tonsil CD4+ and CD8+ percentages post coccidial oocyst infection in turkey pouls**

There was a main effect of coccidial oocyst challenge on the CD8+ cell percentages in cecal tonsils on both d 6 (P < 0.01) and 9 (P = 0.03) post coccidial oocyst challenge (Fig. 4A and B). However, there was no significant effect on the CD4+ cell percentages on d 6 (P = 0.77) or 9 (P = 0.68) across any treatment groups.
**Discussion**

This article considers the in vitro effects of 25(OH)D treatment on heterophils nitrite production post-LPS challenge and also the in vivo effects of 25(OH)D treatment in turkey poults during a mixed coccidial challenge.

Our prior studies have demonstrated treatment with 25(OH)D to increase the nitrite production in monocytes and chicken macrophage cell line (HD11) cells post-LPS challenge (139). Compared to monocytes and macrophages, heterophils produce less NO in response to LPS (153). The NO produced in infected hosts mediates a multitude of functions which includes antiviral, proinflammatory, antibacterial and antiparasitic actions (98, 99, 154). Even though our studies demonstrated decreased NO production by heterophils, we further demonstrated a capability for 25(OH)D treatment to increase the NO production in heterophils.

Coccidiosis is a major parasitic disease affecting both Turkey and poultry species and is caused by the parasite belonging to the genus *Eimeria*. Among different species of *Eimeria* that infect turkey, infection with *E. adenoeides* results in severe pathogenicity (155). Our prior studies have demonstrated during a mixed coccidial infection in layers birds, supplementing with higher doses of 25(OH)D, mitigated some of the production losses associated with coccidial infection in the BW gain. Birds supplemented with high doses of 25(OH)D had increased BW gain over a 6-d period and decreased expression of inflammatory cytokine IL-1β mRNA. In this study, birds challenged with coccidial pathogen had increased BW gain over a 6-d period compared to the control poults across all treatment groups, except the group supplemented with highest dose of 25(OH)D at 110 µg/kg from which a decrease in BW gain was observed. Further, there was also an
increased expression of IL-1β mRNA relative abundance in the cecal tonsils across all treatment groups, except the group treated with the lowest dose of 25(OH)D at 27.5 µg/kg. Since cytokines such as IFN-γ and IL-1β play a critical role in controlling Eimeria infections in poultry (8), more studies need to be conducted in turkeys to demonstrate the role of these cytokines in controlling coccidiosis in turkeys.

In this study, even though poults were infected with a high dose of live coccidial oocysts containing both E. adenoeides and E. meleagrititis, they failed to create any fecal oocyst shedding. Since the relative amounts of fecal oocyst shedding is a reliable indicator of infection, more studies needed to be conducted to determine the threshold of oocyst number needed to create clinical coccidiosis in turkey poults.

Our prior study in layer birds has demonstrated that in birds supplemented with 25(OH)D and challenged with coccidial oocysts had numerically increased CD8+ cell percentages in the cecal tonsils, and in the current study there was a main effect of coccidial treatment on the CD8+ cell percentages in the cecal tonsils. Earlier studies have also demonstrated a role for CD8+ T cells in acting as transporters for E.tenella and E.acervulina sporozoites in poultry and increasing the fecal oocyst shedding (150). In light of our initial findings, more studies need to be conducted to determine the relative contribution of 25(OH)D supplementation and CD8+ cells during an established clinical infection in turkey poults.

In summary, our current study has demonstrated the capability of 25(OH)D to increase in vitro nitrite production in heterophils post-LPS challenge and increase the BW gain and IL-1β mRNA amounts in cecal tonsils post-coccidia challenge in turkey poults.
Acknowledgements

We acknowledge Keith Patterson, Jack Sidle, Jordan Welsh, Jarod Snell (Ohio Agricultural Research and Development Center) for help with Animal Husbandry and my lab mates Matthew Walston and Ashley Markazi for help with sample collection and analysis and Cooper Farms in Ohio for donating turkey poults and Vetech, Canada for supplying the vaccine.
Table 4. Ingredients and Calculated Nutrient Composition of the Basal Diets

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<td>Dicalcium phosphate, 18.5%</td>
<td>2.90</td>
</tr>
<tr>
<td>Selenium, 90.8 mg/lb</td>
<td>0.10</td>
</tr>
<tr>
<td>DL-methionine</td>
<td>0.25</td>
</tr>
<tr>
<td>Choline Chloride</td>
<td>0.15</td>
</tr>
<tr>
<td>Premix</td>
<td>0.25</td>
</tr>
<tr>
<td>L-lysine</td>
<td>0.15</td>
</tr>
</tbody>
</table>

1 Vitamins and minerals were provided in the form and amount described in the NRC Standard Reference Diet for chickens (NRC, 1994). Experimental diets had 25-hydroxycholecalciferol (HyD, DSM nutritional products, Netherlands) at doses of 27.5, 55, 82.5 and 110 µg/kg of feed.
Figure 22. Effect of 25(OH)D treatment on nitrite production from chicken heterophils. Peripheral blood heterophil were treated with 0 or 200 nM of 25(OH)D and stimulated with 1 μg/well LPS for 24 h. Nitrite concentration in the supernatant was determined. Bars (+ SEM) without a common superscript differ significantly within a cell type. P values: $P = 0.02$. n=6. Data presented are from one experiment.
Figure 23. Effect of different doses of 25(OH)D on (A) Mean body weight and (B) % body weight gain. Day-old birds were fed diets supplemented with 25(OH)D at doses of 27.5, 55, 82.5 or 110 µg/kg of feed. At 21 d of age, birds were weighed individually and orally challenged with $1 \times 10^4$ live coccidial oocysts. (A) At 21 d body weights were measured and mean body weight was expressed. Bars (+ SEM) without a common superscript differ significantly within a group. P values: $P = 0.09$. n=5. (B) At 6 d post coccidia challenge, body weights were measured and % body weight gain over a 6 d period was expressed as pre-coccidia body weight. Bars (+ SEM) without a common superscript differ significantly within a group. P values: % body weight gain over a 6 d period: Diet, $P = 0.11$; coccidia, $P = 0.01$; Diet* coccidia, $P = 0.32$. n=5. Data presented are from one experiment.
Figure 24. Effect of different doses of 25(OH)D treatment on (A) IL-1β and (B) 1α-hydroxylase mRNA amounts in cecal tonsils. At d 7 post coccidia challenge, cecal tonsils were collected and IL-1β and 1α-hydroxylase mRNA amounts were analyzed by real time PCR and normalized to β-actin and compared with the group fed 27.5 µg/kg of 25(OH)D and not infected with coccidia, so that all bars represent fold change compared to that group. Bars (+ SEM) without a common superscript differ significantly within a group. P values: cecal tonsils: IL-1β: Diet, $P < 0.01$; coccidia, $P = 0.21$; Diet* coccidia, $P < 0.01$. 1α-hydroxylase: Diet, $P < 0.01$; coccidia, $P = 0.13$; Diet* coccidia, $P = 0.77$. n=5. Data presented are from one experiment.
Figure 25. Effect of different doses of 25(OH)D treatment on (A) d-6 and (B) d-9, CD8+ cell percentage in cecal tonsils post coccidia challenge. At d 6 and 9 post coccidia challenge, cecal tonsils were collected and enriched for lymphocytes by Ficoll density gradient separation. The percentages of CD8+ cells were calculated by Flow cytometry on d 6 and 9, respectively. Bars (+ SEM) without a common superscript differ significantly within a group. P values: d 6: CD8+ cells: Diet, P = 0.31; coccidia, P < 0.01; Diet*coccidia, P = 0.27. n=5. P values: d 9: CD8+ cells: Diet, P = 0.05; coccidia, P = 0.03; Diet*coccidia, P = 0.50. n=5. Data presented are from one experiment.
Chapter 7: Conclusions

Vitamin D3 is converted into its active form, 1,25-dihydroxycholecalciferol (1,25(OH)\textsubscript{2}D), following a two-step hydroxylation process mediated by two key enzymes; 25-hydroxylase and 1\textalpha-hydroxylase. However, extra renal expression of 1\textalpha-hydroxylase has been reported in several species, including chickens, and in many tissues, including immune cells. Even though immunomodulatory properties of vitamin D3 have been reported in different species and positive effects of 25-hydroxycholecalciferol (25(OH)D) supplementation on body weight gain has been observed in broiler chickens, there are no studies till date to our knowledge that investigated the effects of 25(OH)D supplementation on immune parameters of poultry following a LPS and or coccidial challenge.

Studies conducted in chapter 3, 5 and 6 investigated the in vitro effects of 25(OH)D treatment on immune cells stimulated with an LPS or coccidial antigen. Treating HD11 cells with 25(OH)D and stimulating with LPS antigen increased nitrite production but stimulating with coccidial antigen had no effect. Monocytes and heterophils treated with 25(OH)D following LPS stimulation had increased nitrite production. Our studies demonstrated that an increased production of IL-10 mRNA in HD11 cells requires the active form of vitamin D, 1,25(OH)\textsubscript{2}D. Because LPS stimulation increased the 1\textalpha-
hydroxylase mRNA amounts in HD11 cells, cells treated with 25(OH)D produce 
1,25(OH)_2D that increased the expression of IL-10 and IL-1β mRNA. Because the 
outcome of an immune response depend on antigenic properties, we investigated the in 
vitro effects of coccidial antigen stimulation in HD11 cells treated with 25(OH)D. In 
contrast to LPS challenge, stimulating HD11 cells with coccidial antigens decreased 1α-
hydroxylase mRNA amounts. Hence, HD11 cells supplemented with 25(OH)D lacked the 
intrinsic capability to produce 1,25(OH)_2D. Because expression of IL-10 mRNA amounts 
depends on the availability of 1,25(OH)2D, stimulating HD11 cells with coccidial 
antigens in a medium supplemented with 1,25(OH)2D had increased and those 
supplemented with 25(OH)D had decreased IL-10 mRNA relative abundance. The 
immune response is a concerted play involving not only members of innate immunity 
such as macrophages but also cells of adaptive immunity such as T-cells. The T-cells 
play an important role in mounting antigen specific immune response and dictating the 
balance of immune response to pro or anti-inflammatory phenotype. In our current study 
we identified, Con A-stimulation decreased 1α-hydroxylase mRNA amounts in chicken 
T-cells. Hence chicken T-cells, needed an extrinsic supply of 1,25(OH)_2D to convert it 
into a phenotype with decreased expression of the inflammatory cytokine IFN-γ mRNA. 
In chapter 4, 5 and 6 we addressed the in vivo effects of 25(OH)D supplementation in 
birds following an LPS or coccidial antigen challenge on its BW gain, coccidial oocyst 
output, expression of pro and anti-inflammatory cytokines, vitamin D metabolizing 
enzymes and T-cell characteristics. Post-LPS challenge, broiler birds supplemented with 
high doses of 25(OH)D, but not with cholecalciferol had increased BW gain and 1α-
hydroxylase mRNA amounts over a 24-h period. Supplementing with a higher dose of
25(OH)D decreased, whereas cholecalciferol increased, the mRNA amounts of inflammatory cytokine IL-1β. This effect was observed only in birds supplemented with 25(OH)D at a higher dose. Layer birds supplemented with the highest dose of 25(OH)D following a coccidial oocyst challenge could better tolerate the decrease in BW gain along with an increased percentage of T-regulatory cells. These birds also had decreased IL-1β and increased IL-10 mRNA expression.

In turkey poults 25(OH)D at a dose of 82.5 µg/kg had a trend in increasing in increasing the mean BW and BW gain following coccidial oocyst challenge. These birds also exhibited the greatest increase in IL-1β mRNA expression.

In conclusion in vitro 25(OH)D treatment modulated the immune responses in different cell types. In macrophages, it depends on the type of antigen and duration of antigenic stimulation. Treating with LPS for a short time increased the inflammatory response, whereas long time treatment increased the anti-inflammatory response in macrophages. However, when stimulated with a coccidial antigen, macrophages treated with 25(OH)D had no effect on the inflammatory or anti-inflammatory immune response. This is probably due to the inability of the coccidial antigens to stimulate the expression of 1α-hydroxylase mRNA in macrophages. Hence the synthesis of the 1α-hydroxylase enzyme needed for the biosynthesis of active vitamin D might be limiting. Since chicken T-cells had limited capability to synthesize 1α-hydroxylase mRNA, an extrinsic supply of active vitamin D was needed to convert it into an anti-inflammatory phenotype. In an in vivo condition the neighboring bystander macrophages could act as a source active vitamin D for the T-cells.
In our in vivo trials we investigated the effects of 25(OH)D supplementation in diets of broiler and layers chickens and turkey poults in the context of an acute inflammation induced by LPS or during a coccidial parasitic challenge. During the LPS challenge, broiler birds supplemented with 25(OH)D had increased BW gain and decreased inflammatory response compared to birds fed on a diet supplemented with cholecalciferol. Since inflammation decreases BW gain in broiler birds, our research provides an alternative to improve BW gain in broiler chickens during inflammation by supplementing with 25(OH)D. Coccidiosis is the major parasitic disease affecting poultry species causing severe economic losses due to decrease in feed intake and BW gain. However, increasing incidence of drug resistance to existing anti-coccidial drugs poses a serious challenge in combating coccidiosis. Hence alternative nutritional intervention strategies aimed at increasing the BW gain during a coccidial infection is warranted. In our current study we demonstrated the beneficial effects of supplementing with high doses of 25(OH)D in layer chicken and turkey poults in increasing BW gain. Our research thus identified 25(OH)D supplementation as a plausible nutritional strategy to improve BW gain during an acute inflammation in broiler chickens and in layer chicken and turkey poults during a coccidial infection.
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