DIETARY FACTORS INFLUENCING CALCIUM AND PHOSPHORUS UTILIZATION BY BROILER CHICKS

by

ANASTASSIA LIEM

(Under the Direction of Gene M. Pesti)

ABSTRACT

Calcium and phosphorus are the most abundant minerals in the body of animals. They are macrominerals in animal nutrition, as they are required at relatively high levels. Calcium and phosphorus absorption and metabolism are influenced by many factors, such as the levels and ratio of inclusion in the diet, vitamin D_3 and its derivatives, phytase, and organic acids. The effects of the above factors are investigated in four separate battery studies.

Study one investigated the effects of phytase and 1α-OHD_3 on Ca, P and phytate P utilization. Supplementation of 1α-OHD_3 and phytase to P-deficient corn-soybean meal and corn-peanut meal based broiler diets increased P, and phytate P utilization, as indicated by an increase in bone ash, body weight gain, plasma P, phytate P and P retention, and also reduction in incidence of P-deficiency rickets. Study two investigated the effects of combinations of phytase, methionine source, and calcium or 1α-OHD_3 on phosphorus utilization in broilers. Phytase, 1α-OHD_3, and HMB (an organic acid) increased phytate P utilization, and the effect of each supplement often depended on the levels of other supplements and nutrients. Study three evaluated the efficacy of several 1α-OHD_3 compounds as a substitute for cholecalciferol. Slope ratio analysis of data from the measurements of 16-d BWG, plasma Ca, rickets and bone ash

indicated the bioavailability of the different 1α-OHD_3 (except for the 5, 6 trans 1α-OHD_3 which was inactive) to be 7 to 15 times more active as compared to D_3. There were differences between the 1α-OHD_3 compounds due to source, processing, and cis-trans isomerism. Study four investigated the effect of lithocholic acid, a bile acid which has been reported to have vitamin D activity in rats, on commercial broiler chicks. Lithocholic acid, at low levels, had some vitamin D_3 activity in chicks, as indicated by increased plasma Ca and calbindin expression. It also caused severe hepatotoxicity and growth depression to chicks.

INDEX WORDS: broilers, calcium, phosphorus, 1α-OHD_3, vitamin D_3, lithocholic acid, phytate P utilization

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ANASTASSIA LIEM

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ANASTASSIA LIEM

Major Professor:	Gene M. Pesti
Committee:	Michael Azain Samuel E. Aggrey Robert B. Beckstead Wan-I Oliver Li

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
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DEDICATION

I dedicate this dissertation to my parents for their love and support.

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CHAPTER 1

INTRODUCTION

Calcium and phosphorus are the most abundant minerals in the body of animals. They are also minerals required at the highest dietary levels by poultry. While the calcium requirement is easily satisfied with the supplementation of limestone, satisfying phosphorus requirement with inorganic phosphorus supplementation is relatively more costly. Plants store phosphorus in the form of myo-inositol 1,2,3,4,5,6-hexakisphosphate or phytate phosphorus (Nelson, 1968) which is mostly unavailable to monogastric animals such as birds. There are incentives to maximize the utilization of phytate P, such as possible reduction in feed inorganic phosphorus, which might result in reduction in feed cost and reduction in phosphorus content of the manure. Phytate P utilization is influenced by numerous factors, such as dietary calcium and phosphorus, phytase, vitamin D_3 and its derivatives, and organic acids (Ravindran et al., 1995; Rafacz-Livingston et al., 2005; Liem et al., 2008).

Calcium and phosphorus have an antagonistic relationship. Increasing dietary Ca reduced P absorption, and also reduced the utilization of phytate P (Edwards and Veltman, 1983; Davis, 1959; Waldroup et al., 1963; MacDonald and Solvyns, 1964; Kondos and McClymont, 1967). Vitamin D_3 increased calcium intestinal absorption, removing calcium from the gastrointestinal tract, and subsequently increased phytate P utilization (Edwards, 1993).

Vitamin D_3 is required by chicks for normal growth and bone development. Several derivatives, such as 1α-OHD_3, and 25-OHD_3, have been reported to have higher biological

potency compared to cholecalciferol (Boris et al, 1977, Edwards et al, 2002). Recent studies also reported that lithocholic acid, a bile acid, could have some vitamin D activity in rats (Nehring et al, 2007). Early research with lithocholic acid in chicks indicated that lithocholic acid caused severe growth depression and hepatotoxicity (Edwards, 1961; Leveille et al, 1962; Hunt et al, 1964).

In the current study, the effects of dietary calcium, phytase, vitamin D_3 and its derivatives, and organic acids on phytate P utilization and their interactions were investigated. An experiment was also conducted to evaluate the efficacy of different preparations, and isomers of 1α-OHD_3 in comparison to cholecalciferol. Lithocholic acid activity in chicks were also studied by measuring performance, plasma calcium, and expression of genes normally upregulated by vitamin D_3.

CHAPTER 2

LITERATURE REVIEW

Importance of Calcium and Phosphorus

Calcium and phosphorus are the most abundant minerals in the body of animals. They are often discussed together due to their interdependent functions in bone formation. Major constituent of bone is made of hydroxyapatite crystal with the molecular formula $Ca_{10}(PO_4)_6(OH)_2$ (Scott et al., 1976). Calcium is also found in plasma and the cytosol. It is critical for the activity of many enzymes, including those for nerve impulse transmission, and muscle contraction. It is also required in blood coagulation (McDonald et al.,2002). Phosphorus is found in bone, phosphoproteins, nucleic acids, and phospholipids. It is also part of ADP and ATP, making it essential in energy metabolism (McDonald et al., 2002).

Regulation of calcium and phosphorus in the body

The majority, 99%, of calcium in the body is found in bones. The remaining 1% is found in plasma in 3 fractions: ionized calcium (50%), protein-bound (primarily albumin) calcium (40%), and soluble complexes with citrate and phosphate (10%).

Plasma calcium is very tightly regulated by the actions of parathyroid hormone (PTH), vitamin D, and calcitonin. This regulation is critical for normal cell function, neural

transmission, membrane stability, bone structure, blood coagulation, and intracellular signaling. PTH stimulates bone resorption and increased Ca release into the circulation. It also increased renal reabsorption of Ca, and inorganic phosphate excretion. PTH also upregulates 25-hydroxyvitamin D-1α -hydroxylase (CYP1α) which consequently increased the levels of 1,25 dihydroxy cholecalciferol (1,25 $(OH)_2D_3$), the biologically active form of vitamin D. Vitamin D increases intestinal absorption of Ca and renal reabsoption . Vitamin D also increases bone resorption, increasing Ca in circulation. Calcitonin inhibits bone resorption and increase Ca renal excretion. The interactions among PTH, vitamin D, and calcitonin maintain normal plasma Ca levels (Molina, 2006).

Parathyroid hormone

Parathyroid gland chief cells synthesize PTH continuously. It is secreted continuously with about 6-7 superimposed pulses per hour. PTH is synthesized as a pre-propeptide, which is rapidly cleaved to pro-PTH and the mature form of PTH. Changes in plasma Ca are detected by the parathyroid Ca^{2+} sensing receptor, a G protein coupled receptor located on the plasma membrane of the parathyroid chief cells. It is also found in kidney tubule cells and thyroid C cells. PTH release is increased by hypocalcemia, hyperphosphatemia, and catecholamines; and decreased by hypercalcemia, vitamin D, and severe hypomagnesemia. While three types of PTH receptors have been identified, the important physiologic effects of PTH are mediated by PTHR1. It is expressed in bone osteoblasts and kidney. The transcellular reabsorption of Ca in nephron's distal tubule is regulated by PTH, 1,25$(OH)_2D_3$, and calcitonin. PTH increases the insertion of calcium channels (unidirectional) in the apical membrane and facilitates Ca entry. PTH decreases renal reabsorption of phophorus (Brown et al, 1993) by decreasing type II Na^+/Pi cotransporter expression, and increases its internalization via coated vesicles.

In bone, PTH binds to its receptors on osteoblasts and stimulates the expression of receptor activator of nuclear factor-κB ligand (RANKL) on the cell surface. RANKL binds to RANK (a cell surface protein on osteoclast precursor) and activates osteoclast precursor differentiation into mature osteoclast with ruffled membrane which facilitates bone resorption. PTH also decreases osteoprotegerin (which can bind to RANKL and inhibit RANKL/RANK interaction) release from osteoblast. The overall effect of PTH is to increase Ca level in circulation from bone resorption and renal reabsorption (Molina, 2006).

Calcitonin

Calcitonin is a peptide hormone secreted by parafollicular or C cells in thyroid gland. Its release is regulated by plasma Ca, detected by Ca receptor in C cells. The main functions of caltonin are to decrease plasma Ca and phosphate concentration. This is primarily mediated by decreasing bone resorption. Calcitonin inhibits osteoclast differentiation, inhibits its motility, secretory activity, and numbers in bone (long term). In kidney, calcitonin inhibits renal calcium reabsorption and increases Ca excretion (Molina, 2006).

Vitamin D and its molecular mechanism of action

Steps on metabolism of vitamin D_3 have been elucidated in the past few decades. Several researchers published detailed and inclusive reviews on metabolism steps, regulations, structures and dynamics of vitamin D receptor, and newly discovered roles of vitamin D (Jones et al, 1998, Christakos et al, 2007). There are 2 forms of vitamin D, cholecalciferol (D_3) and ergocalciferol (D_2) which comes from plant sources. Since vitamin D_2 has little or no activity in chicks, the following review focuses on cholecalciferol.

Vitamin D_3 is made from its precursor, 7-dehydrocholecalciferol in the skin in an ultraviolet dependent process. It is then activated by 25-hydroxylation in the liver and 1α-

hydroxylation in the kidney, forming the biologically active hormone, 1,25 dihydroxy cholecalciferol (1,25 $(OH)_2D_3$). The 25-hydroxylation in the liver is mediated by a P-450 enzyme in the mitochondria of hepatocyte, vitamin D_3-25-hydroxylase (CYP27). This enzyme is only loosely regulated. The 1α hydroxylation is mediated by 25-hydroxyvitamin D-1α -hydroxylase (CYP1α) in the mitochondria of renal proximal tubular cell. This enzyme is tightly regulated by the levels of plasma 1,25-$(OH)_2D_3$ and calcium. CYP1α is upregulated, and activated by parathyroid hormone. Parathyroid gland, which has been known to be the calcium sensing organ (Potts,1993; Rasmussen and DeLuca, 1963; Rubin et al, 1985), releases PTH within seconds of even slight hypocalcemia (Silver et al, 1996). PTH mediated by cAMP, increases the mRNA level of CYP1α (Horiuchi et al, 1977). The renal enzyme is strongly upregulated by the parathyroid hormone (PTH), which in the proximal convoluted tubule cells, activates the 25-hydroxyvitamin D-1α -hydroxylase (25-OH-D_3-1α -OHase) that converts 25-hydroxyvitamin D_3 to the active hormone, 1,25-$(OH)_2D_3$ (Garabedian et al, 1972; Shinki et al, 1998; Tanaka et al, 1975) . Another enzyme that is involved in vitamin D metabolism is 24(OH)ase, the enzyme involved in metabolic inactivation of 1,25$(OH)_2D_3$. The enzyme converts 1,25$(OH)_2D_3$ to 1,24,25-$(OH)_3D_3$, which is 10 less active biologically (Castillo et al, 1978; Tanaka et al, 1977). The expression of 24(OH)ase in kidney and small intestine is upregulated by 1,25$(OH)_2D_3$, protecting the body from hypercalcemia (Kleiner-Bossaller and DeLuca, 1974; Kumar et al, 1978).

The effects of vitamin D on gene expression are mediated by vitamin D receptor (VDR). VDR is a member of superfamily of nuclear receptor (Evans, 1988). In the nuclear receptor family, close structural similarities were found in the nuclear receptor group with metabolic functions, such as PXR, CAR, and FXR, all of which bile acid or xenobiotic receptors involved

in bile acid detoxification and homeostasis (Moore et al, 2006). VDR, like other members of nuclear receptor superfamily, consists of several domains (divided by function). The function of A/B domain at the NH_2 terminus has not been described. The next sections are the DNA binding domain (C), the hinge domain (D), followed by ligand binding domain (E) at the COOH-terminal. The ligand binding domain interacts with high affinity with ligand ($1,25(OH)_2D_3$), and is responsible for dimerization with Retinoid X Receptor (RXR) (Evans, 1988). The DNA binding domain consists of two zinc finger motifs (Lee et al, 1993). One of the fingers interacts with the major groove of DNA and binds to specific DNA sequence in the promoter region of target genes (Vitamin D Response Element / VDRE).

The proposed mechanism in which vitamin D increases target gene transcription is described below (Jones et al, 1998). Upregulations of target genes are mediated by binding of VDR to vitamin D response element located in the promoter region of target genes. Binding of $1,25(OH)_2D_3$ to the ligand binding domain of the receptor increases heterodimerization of VDR with retinoid X receptor /RXR (Kimmel-Jehan et al, 1997). The complex then binds to response elements, and induces a bend in the DNA (Jehan-Kimmel et al, 1996). Binding of ligand to VDR also appears to induce conformational change in the COOH terminal of the VDR, allowing a region (termed AF-2 domain) to interact with other transcription factors, such as coactivator protein SRC-1. More recent studies indicated that coactivator proteins have intrinsic histone acetylase activity (Chen et al, 1997; Torchia et al, 1997). Coactivator proteins also bind to other proteins such as calcium-binding protein and p300, which also possess histone acetylase activity (Kouzarides and Bannister, 1997). Recruitment of coactivators and the above proteins to the promoter site appear to cause conformational change in DNA structure, due to the action of histone acetylase, which results in histone release from DNA. This opens the promoter to the

transcriptional machinery. Therefore, the net result of binding of ligand/receptor complex to upregulated target promoter is an increase in the transcription of the gene, which (may) lead to an increase in the production of the particular protein.

Vitamin D also downregulates the expression of several genes, such as PTH, PTHrP, and CYP27B1 (Kim et al, 2007, Murayama et al, 1998, Murayama et al, 2004, Zehnder et al, 2001). Negative VDREs, have been mapped in the human and rat PTH promoter, and human PTHrP gene promoter. These negative VDRE's are bound to either VDR/VDR homodimers or VDR/RXR heterodimers (Demay et al, 1992, Russell et al, 1999). Several possibilities have been proposed. First, VDR may bind to a down regulatory response element, and disrupt the binding of upregulatory transcription factors, which in turn decrease rate of transcription. Another possibility is that binding of 1,25-$(OH)_2D_3$ to inhibitory response element may recruit repressor proteins which in turn decrease the rate of transcription (Jones et al, 1998).

VDR has been cloned from several species with considerable similarity in size and sequence (Jones et al, 1998). Two forms have been cloned from chicken intestine and kidney (Lu et al, 1997). The primary protein structures of avian and mammalian species are 80-90% similar (Lu et al, 1997). The DNA binding domains between avian and mammalian species are very similar, except for some conservative substitutions in the second zinc finger region. In the ligand binding domain, several amino acids changes were also found. The major differences between avian and mammalian receptors were found in the hinge region, and at the N-terminus.

Vitamin D endocrine system is not only central in bone and calcium homeostasis. In human, VDR expression has been detected in almost all human tissue. It is also been suggested that vitamin D pathways are also involved in the immune response, diabetes, cardiovascular diseases, and cancer. Since VDR mediates important effects of vitamin D, mutations in VDR

gene can lead to defects in gene activation, calcium homeostasis, immune response, disease susceptibility (Valdivielso and Fernandez, 2006). Polymorphism, or genetic variants, in VDR gene exists in human population. The associations between polymorphisms in VDR gene and human diseases (diabetes, renal disease, cancer, bone biology) were summarized in Valdivieso and Fernandez (2006).

Vitamin D research in chicken

The availability of vitamin D has made raising poultry indoors possible. Chicks acquire cholecalciferol either from the diet, or from irradiation of 7-dehydrocholesterol with ultraviolet light from the sun or artificial source. The skin of legs and feet of chicken contains about eight times as much 7-dehydrocholesterol as the body skin (Koch and Koch, 1941). In chicks, vitamin D deficiency leads to suppressed growth, and rickets, characterized by severe leg weakness, soft beaks, and difficulty to walk (Scott et al, 1976).

Vitamin D requirement for broiler chicks is 200 IU/kg of diet according to NRC (1994). However, numerous papers since the publication of NRC (1994) indicated that the requirement for vitamin D_3 was much higher. Depending upon the criteria evaluated, the requirement for vitamin D_3 were different (Edwards et al., 1994). The requirement for growth was the lowest, 275 ICU (6.9 μg)/kg. The requirement for bone ash was 503 ICU (10.1 μg)/kg, for blood plasma Ca : 552 ICU(13.8 μg)/kg, and for vitamin D rickets prevention : 904 ICU (22.6 μg)/kg. This requirement also varied based on the amount of calcium and phosphorus, and protein source in the diet. In Kasim et al. (1996), which fed purified animal protein as the sole protein source, cholecalciferol requirement for growth was 100 IU/kg, for bone ash 200 IU/kg, and for rickets

prevention was less than 400 IU/kg. When the diet was low in available phosphorus, much higher levels of cholecalciferol was needed to maximize bone ash and growth (Baker et al, 1998).

A few sources of vitamin D_3 are available commercially for poultry production, with cholecalciferol as the most widely used form. Another derivative, 1α-OHD_3 has been available, but not approved for use in the US. 1α-OHD_3 is a synthetic derivative of cholecalciferol. It is not naturally found in the body of animals. Haussler et al. (1972) reported that 1α-OHD_3 was not found after injection of radioactive cholecalciferol into a vitamin D-deficient animal. It was first reported to be active in inducing calcium absorption and bone mobilization in chickens by Haussler et al. (1973) in a study with White Leghorn cockerels. In the study, 1α-OHD_3 was found to be as effective (mole to mole basis) as 1, 25$(OH)_2D_3$ in mobilizing calcium from the bone and also inducing intestinal calcium absorption, and at least 10 times as active as cholecalciferol . Another early study (Boris et al, 1977) also reported that 1α-OHD_3 increased tibia ash more effectively than cholecalciferol and 25-OHD_3. Since then, numerous studies confirmed that 1α-OHD_3 has activity as an effective substitute for cholecalciferol in increasing Ca absorption, plasma Ca, body weight gain, tibia ash, and also increasing phytate P utilization (Edwards, 1994, 2002; Biehl et al., 1995, 1998; Biehl and Baker, 1997a, b, Edwards et al, 2002). The effects of vitamin D derivatives on phytate P utilization will be further discussed in the section Vitamin D and its derivatives increase phytate P utilization.

Leg abnormalities affected by dietary calcium, phosphorus, and vitamin D

Calcium deficiency rickets, phosphorus deficiency rickets, and tibial dyschondroplasia (TD) are directly related to dietary calcium, phosphorus, and vitamin D. The dietary calcium and phosphorus combination that results in high (over 90%) incidences in these leg problems is illustrated in the Figure 2.1.

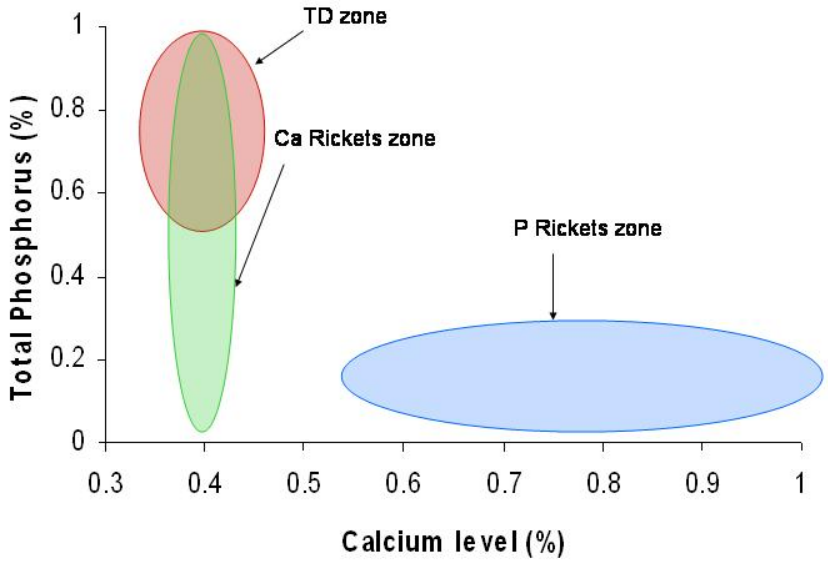

Figure 2.1. Combinations of dietary calcium and phosphorus that produce high (over 90%) incidence of tibial dyschondroplasia, calcium deficiency rickets, and phosphorus deficiency rickets (Pesti et al., 2005).

The incidence of TD in many broiler and turkey flocks is about 30% (Riddell, 1992). It is characterized by a lesion of abnormal mass of cartilage in the metaphysis below the growth plate. In TD, there is failure in the following processes that are required for normal bone development: hypertrophy, mineralization, vascular invasion and removal of cartilage of the growth plate. The

incidence of TD was increased with high phosphorus, low Ca diet. Its incidence can be ameliorated by vitamin D_3 and its derivatives (Edwards, 1990).

Calcium deficiency rickets occurs when chicks are fed low calcium diet (especially under 0.4%), and or diets deficient in vitamin D_3. The lesion is characterized by lengthened, striated appearance of the growth plate, soft, weak bone and beak. The thickening of the epiphyseal plate is due to an accumulation of proliferation chondrocytes (Jande and Dickson, 1980), and a variable increase in the length of the perforating epiphyseal vessels (Lacey and Huffer, 1982). The incidence and severity of calcium rickets in chicks can be reduced by supplementation of vitamin D and its derivatives (Edwards et al, 2002).

Phosphorus rickets incidence are highest when chicks are fed diets low in phosphorus, regardless of calcium level. The birds are often small, with ruffled feathering, and soft bones and beak. It is characterized by lengthened primary spongiosa but with normal proliferating zones. The accumulation of hypertrophic chondrocytes is not accompanied by any change in perforating epiphyseal vessels (Lacey and Huffer, 1982). The incidence of phosphorus rickets in chicks fed phosphorus deficient diets can be reduced by supplementation of cholecalciferol, 1α-OHD_3, 25-OHD_3, and 1,25$(OH)_2D_3$ (Edwards, 2002).

Phytate Phosphorus

Plants such as cereal grains store most of their phosphorus in the form of phytate phosphorus. The phytate phosphorus content of cereal grains increases on a percentage basis during maturation. The term “phytic acid” is used for several chemical compounds a 6 member carbon ring with 1, 2, 3, 4, 5 or 6 phosphates attached. It occurs in nature in the phytate form

with mixtures of Na, K, Mg, Ca, etc. making up the ionic salt. Phytate is found in cereal grains, legumes, and oil-bearing plants. The phytate P content of some feed ingredients was summarized in previous reviews (Boland et al., 1975; Jongbloed and Kemme, 1990; Kirby and Nelson, 1988; Lolas et al., 1976; Nelson et al., 1968a; Ravindran et al., 1994; Ravindran et al., 1995).

In cereal grains, phytate is found in the seed, with very little in leaves and stems. Corn has about 88% of its phytate P in the germ, and about 3% in the endosperm (Odell et al., 1972). In recent releases of genetically modified corn varieties, the phytate P content was changed in the germ, but not much in the endosperm (Raboy et al., 1990). A typical broiler diet, which is predominantly corn and soybean meal, will contain approximately 0.25 % phytate phosphorus. However, there have been some variations in the phytate P value of feed, corn and soybean meal over the years. The variations may be due to changes in analytical techniques, cultivars, and soil P levels. An early study (Common, 1940) reported yellow maize (2 samples) phytate content to be 0.149 and 0.276%. Nelson et al. (1968a) reported the phytate P value for corn and SBM to be 0.17 and 0.38%, respectively. Another study (Odell et al., 1972) reported the phytate P content of corn to be 0.25%. Kasim and Edwards (1998) reported the phytate P content of corn grain and 44% protein soybean meal samples to be 0.27 and 0.50%, respectively. Further studies (Kasim and Edwards, 2000) tested the biological availability of phytate from 4 different corn samples whose phytate P value ranged from 0.18 to 0.25%. In a recent study, Kwanyuen and Burton (2005) found low phytate soybean meal to contain 0.0957% phytate P, while standard and 48% protein soybean meal contained 0.4083 and 0.6392% phytate phosphorus, respectively. The NRC values for the phytate P content of corn and soybean meal are 0.20 and 0.40 % respectively. Because of the variation in phytate P content of corn and soybean meal, phytate P content of feed

can be variable and difficult to predict. The analyzed value of feed is expected to differ from the formulated value.

Bioavailability of Phytate Phosphorus

The most common consensus among previous research indicated that about one third of phytate phosphorus in feed is available to poultry. Many of the studies on the subject measure the utilization of phytate phophorus by comparing performance and bone ash of chicks fed diet containing phytate P to chicks fed mainly or solely inorganic phosphorus. A few studies actually determined the retention or hydrolysis or disappearance of phytate P as a direct measure of its utilization. The retention or hydrolysis or disappearance of phytate P was determined by calculating the the percentage of phytate P in the diet retained in the birds' body by calculating the phytate P content of the diet and feces. One of the early studies (Lowe et al., 1939) showed that phytic acid was poorly utilized by chicks, especially for bone mineralization. And while White Leghorn hens generally utilized phytate phosphorus better than broilers, they utilized phytate P half as effectively as dicalcium phosphate (Gillis et al., 1953). Utilization of phytate P by turkey was reported to be much lower, as low as 2 % according to Gillis et al. (1957). Another study (Ashton et al., 1960) fed P^{32} labeled calcium phytate and reported phytate P retention of 20% by 4 week old birds, and 36-49% by 6 week old birds. When purified phytic acid salts are fed, the availability is dependent on the cations in the salt. Calcium phytate phosphorus utilization is 75-80% of sodium phytate's (Maddaiah et al.,1963). Free phytic acid is more available than sodium phytate or calcium phytate (Waldroup et al., 1964). In a short abstract, Nott at al. (1967) reported that at optimum calcium levels, laying hens of unspecified genetics utilized very little phytate P. Leske and Coon (1999) reported the phytate P hydrolysis of corn

and SBM respectively to be 27 % and 35 % without phytase, and 58 % and 41 % with 600 units phytase / kg feed.

Phytase

Four years after Posternak (1903) first described phytic acid, Suzuki et al. (1906) discovered that rice bran extract contains an enzyme (phytase) that will split P off the myoinositol hexaphospho-molecule. Phytases include a group of enzymes that catalyze the stepwise cleavage of inorganic phosphorus from phytate, resulting in myo-inositol and mono to penta inositol phosphate as intermediates. There are two phytase categories recognized; 3-phytases start cleaving phosphate from position 3 of myo-inositol, while 6-phytases start at position 6 (IUPAC-IUB, 1976). Phytase activity has been found in plants (feed ingredients), the intestinal mucosa of animals, including chickens, and in fungi. Eeckhout and De Paepe (1994) tested 285 feed samples for phytase activity, and reported a wide range of phytase activity. Wheat, wheat bran, and rye have very high phytase activity, while corn and soybean meal have low activity. Plant phytases have optimum pH in the range of 4.0 to 7.5, as reviewed by Wodzinski and Ullah (1996). Intestinal phytase activity was discovered in the intestinal mucosa of albino rats (Patwardhan, 1937), and then in chicken, pig, and cow (Spitzer and Phillips, 1945). Shieh and Ware (1968) at International Minerals and Chemicals Corporation screened over 2000 samples for phytase activity, and found that *Aspergillus niger* NRRL 3135 syn *A. ficuum* produces 2 phytases, which they named A and B at the highest yield. The phytase from International Minerals was Chemicals was later used by Nelson et al. (1968b, 1971) and Rojas and Scott (1969) to conduct studies on the effect of adding phytase to the diet on the availability of natural phytate phosphorus in the diet to young chickens. Nelson et al (1968b) fed soybean

meal treated with culture filtrate from *Aspergillus ficuum* and reported that birds fed the treated soybean meal had increased percentages of bone ash, comparable to birds fed 0.23% more total P. Nelson et al.(1971) later fed phytase produced by the same fungi, and based on bone ash data, suggested that total hydrolysis of phytate P can occur when the diet was supplemented with phytase. Rojas and Scott (1969) reported that treating cottonseed meal with culture filtrate containing phytase increased the availability of phytate P, protein, zinc and metabolizable energy when added to the diet of young chickens. Using one of the first available commercial phytase preparations, Simons et al. (1990) studied increasing phytase supplementation of 0, 250, 500, 750, 1000, and 1500 units /kg in a diet containing 0.6% Ca and 0.45% P and found the availability of P in 21-24 day old broilers to be 49.8, 56.5, 59.6, 59.5, 62.5, and 64.5 % respectively. They suggested that the apparent optimum P availabilities were obtained when 800 units of phytase /kg of diet were fed. Other studies (Kornegay et al., 1996; Yi et al., 1996) showed that phytase addition increased apparent retention of P, Ca, and decreased P excretion. The studies also predicted that 1 g P from defluorinated phosphate was equivalent to 939 (Kornegay et al., 1996) and 785 (Yi et al., 1996) U of phytase. Mitchell and Edwards (1996a; 1996b) reported that phytase, together with 1,25- $(OH)_2D_3$ had additive effects on increasing phytate P retention, and also bone ash in male broilers 1 – 21 and 0 – 35 days of age. In a further study, Shirley and Edwards (2003) supplemented a log dose of phytase to a corn and soybean meal based diet, 0 to12,000 U/kg, and reported that retention of Ca, P and phytate increased linearly and quadratically to the log dosing of phytase. The study also reported that nitrogen retention and apparent metabolizable energy increased linearly to log dose of phytase. Shirley and Edwards (2003) also observed that an additional response in phytate P utilization was produced by supplementation of phytase up to 12000 U/kg.

Vitamin D and its derivatives increase phytate P utilization

Vitamin D has been shown to increase Ca absorption from the GI tract (Coates and Holdsworth, 1961). The study showed that vitamin D, given since hatching or as a single dose (at least 8 hours prior to measuring), increased Ca absorption from washed duodenal loops in vivo. The effect was (partly) mediated by vitamin D dependent - calcium binding protein production in kidney and intestine (Christakos and Norman, 1980; Wasserman and Taylor, 1966). This resulted in the lower Ca concentration in the GI tract, lowering its chelation with phytic acid, which in turn increased phytate P utilization. Also, vitamin D has been shown to be involved in phosphate transport across the intestine (Harrison and Harrison, 1961; Wasserman and Taylor, 1973). Steenbock, et al. (1953) first reported that vitamin D supplementation increased phytase activity in the duodedum of leghorn birds. Another study (Davies et al., 1970) reported that increasing levels of vitamin D_3 increased intestinal phytase and phosphatase activities to a great extent when the diet was deficient in P, and slightly when the diet was adequate in P. An early study (Matterson et al., 1945) reported that addition of increasing levels of vitamin D from cod liver oil and irradiated animal sterols to phosphorus deficient diet increased percent bone ash. Increasing cholecalciferol inclusion into a low P, normal Ca diet, increased phytate P digestibility from 51 to 59%, and the effect was also seen in a low P, low Ca diet, where phytate P digestibility increased from 65 to 77% (Mohammed et al., 1991).

Edwards (1993) has shown that adding a vitamin D derivative (5 µg/kg of 1,25-dihydroxycholecalciferol) to the diet increased phytate P retention from 31.4% to 68.4% in broilers fed a phosphorus deficient diet. The study also reported the additive effect of vitamin D on top of phytase. The phytate P retention of birds supplemented with phytase of 75, 150, 300, 600 units/kg were 56.4, 59.1, 52.7, 64.9 % respectively. When 10 µg of 1, 25

dihydroxycholecalciferol was added, the retention increased to 80.6, 87.4, 83.4, and 82.2 % respectively. This finding was confirmed by other studies (Mitchell and Edwards, 1996a). Edwards (1993) also reported that addition of 10 μg of 1, 25-$(OH)_2D_3$ even with low level of phytase (75 U/kg) decreased the amount of inositol hexaphosphate and inositol pentaphosphate in excreta from 4.23, and 0.490 mg/g to 1.80 and 0.105 mg/g, respectively.

Biehl et al. (1995) fed graded levels of 1αOHD3 from 0 to 40 μg/kg in a corn soy diet deficient in Ca and P. They reported a linear response in tibia ash from 33.0 to 40.5% when 1αOHD3 was supplemented from 0 to 20μg/kg. The addition of 20μg/kg of 1αOHD3 and 1200 U/kg of phytase to a broiler diet deficient in Ca and P produced the same or better responses for weight gain, and percentage and weight of tibia ash compared to supplementation of 0.1% P. In a further study Baker et al. (1998) reported that the addition of cholecalciferol at 0 to 1250 μg/kg to broiler diet limiting in available phosphorus increased weight gain, and percent and weight of tibia ash linearly, although there was no marked improvement in weight gain when cholecalciferol was added above 37.5 μg/kg. Biehl et al. (1998) also tested the efficacy of 2 other D_3 derivatives relative to 1αOHD3 in improving phytate P bioavailability in a P-deficient corn and soybean meal-based diet. The percent tibia ash data suggested that 20-epi-19-nor-1, 25-$(OH)_2D_3$ had the same activity as 1αOHD3 in improving phytate P availability, whereas 20-epi-19-nor-1αOHD3 was less effective compared to 1αOHD3. A study (Edwards, 2002) compared the efficacies of 1,25-$(OH)_2D_3$, 1αOHD3 , and 25-OHD3 for stimulating phytate utilization. The study reported that 1,25-$(OH)_2D_3$ resulted in the highest increase in phytate P retention, followed by 1αOHD3 supplementation. Supplementation of 25-OHD3 to the diet produced inconsistent increases in phytate P retention. The study agrees with previous works on vitamin D metabolism in poultry (Myrtle and Norman, 1971; Norman et al., 1971; Soares et al., 1995).

Effects of Dietary Ca and P levels on phytate P utilization

As calcium and inorganic phosphorus levels in the diet increase, phytate P utilization decreases. Calcium in the diet reduces phytate P hydrolysis due to the formation of insoluble Ca-phytate complexes. An earlier study (Davies et al, 1970) also reported that increasing level of vitamin D_3 increased intestinal phytase and phosphatase activity, especially when the diet is deficient in P. A more recent study indicated that increasing dietary Ca level decreased intestinal phytase activity (Applegate et al., 2003).

A study with laying hens (Nott et al., 1967) reported that at 50 and 72 weeks, increasing Ca from 3.0 to 3.5 and 4.0% decreased phytate P retention. Edwards and Veltmann (1983) found that phytate P retentions by young broiler chicks were highest when chicks were fed about 0.7%Ca and 0.8%P (37%) and as Ca and P increase to 1.65 and 1.09% respectively, the retention decreased to 6%. Ballam et al. (1984) observed that chicks fed 1.0% Ca and 0.5% available phoshorus had retained 9.5% of non phytin phosphorus, while chicks fed 0.85%Ca and 0.42% nonphytin phosphorus retained 22.6%. In further studies, Ballam et al. (1985) showed that increasing Ca from 0.09 to 1.0% Ca decreased phytate P hydrolysis regardless of P level. However, increasing inorganic P in the diet decreased phytate P hydrolysis when Ca level is at 1.0% but not at 0.09%. In another study with laying hens, increasing dietary phosphorus altered phytate P retention in an inconsistent manner (Scheideler and Sell, 1987). Mohammed et al. (1991) reported that decreasing Ca level from 1 to 0.5% increased phytate P retention. However, decreasing inorganic P level from 0.45 to 0.26% did not. Another study (Sooncharernying and Edwards, 1993) showed that decreasing nonphytate phosphorus from 0.42 to 0.27% increased retention of inositol hexaphosphate from gizzard, small intestine and cloaca. Qian et al. (1997)

reported that changing the Ca: total P ratio from 1.1:1 to 2.0:1 decreased P retention (and phytase efficacy) at different levels of D_3 and phytase supplementation.

Effects of organic acids on phytate P utilization

Organic acids in poultry industry refer to a large group of acidic organic compounds (that often have little in common among each other). Some organic acids have been reported to increase phytate P utilization by poultry, mostly indicated by performance and bone ash as the parameters measured. Boling et al. (2000b) reported that increasing level of citric acid up to 6% linearly increased percent tibia ash from 26.9% (P-deficient basal diet) to 38.6%. They suggested that citric acid chelated Ca in the diet and prevent the formation of Ca phytate complexes and therefore increased phytate P utilization. There seems to be a difference in the response of broilers vs. laying hens to citric acid. Further work (Boling et al., 2000a) reported that citric acid supplementation to laying hens from 22 to 40 weeks of age fed a P deficient diet did not improve phosphorus utilization as indicated by the performance of the hens. The reason why citric acid did not improve phytate P utilization by laying hens was unknown, but they hypothesized that it might be associated with the high Ca level in the diet (3.8%). Boling-Frankenbach et al. (2001) showed that citric acid supplementation of broilers decreased the requirement for inorganic phosphate supplementation by about 0.10% (of the diet). In an abstract, Shellem and Angel (2002) reported that citric acid supplementation increased percent bone ash, but the improvement was mediated by reduction in feed consumption and growth. Snow et al. (2004) showed that citric acid had additive effects on phytate P utilization (tibia ash) when fed with phytase and1αOHD_3. Rafacz-Livingston et al.(2005) expand the research with more organic acids, and reported that sodium gluconate, calcium gluconate, glucono-δ-lactone, 2-hydroxy-4methylthio

butanoic acid (Alimet), and citric acid improve phytate P utilization, but fumaric acid and EDTA did not. Rafacz-Livingston et al. (2005) suggested that citric acid improved apparent P digestibility of broiler chicks by lowering the pH of digesta in small intestine which inhibits formation of mineral phytate complexes that are resistant to hydrolysis endogenous phytase. One of the compounds tested in in Rafacz-Livingston et al. (2005), 2-hydroxy-4methylthio butanoic acid (Alimet), is a liquid methionine source commonly used in the poultry industry. Liem et al. (2008) studied the effect of substituting 2-hydroxy-4methylthio butanoic acid for DL-Methionine in combination with phytase, and reported that even at a very low level (0.2%), 2-hydroxy-4methylthio butanoic acid can increase phytate P utilization, as indicated in increased bone ash and phytate P hydrolysis. In addition, the paper also reported that citric and malic acid can improve phytate P utilization, while fumaric acid and EDTA did not. The effect of organic acids on phytate P utilization could not be explained by their Ca chelating abilities. EDTA, which has tremendous chelating activity, did not improve phytate P utilization (Rafacz-Livingston et al, 2005; Liem et al, 2008).

Lithocholic acid – toxicity and vitamin D activity

Bile acids are mostly known for their functions in aiding lipid digestion and their role in cholesterol metabolism. However, recent studies showed that certain bile acids also play a role in signaling pathways. Lithocholic acid is one of the main bile acids found in mammals and birds. It is a secondary bile acid, formed from primary bile acids chenodeoxycholic acid and ursodeoxycholic acid which are produced in liver from cholesterol. The primary bile acids above

are conjugated with taurine or gylcine, and enter enterohepatic circulation. In intestine, the acids are deconjugated (by bacterial peptidases) and converted to lithocholic acid by 7α - dehydroxylation by bacteria (Hofmann, 2004).

There were numerous studies published in the 1960's reporting the effect of dietary LCA in chicks and hens. Feeding LCA at 0.2% severely depressed growth and increase serum cholesterol in chicks (Edwards, 1961). Another research group published a series of paper reporting that dietary LCA increased plasma phospholipids levels, and liver size (by 3.5 times normal) which was partially attributed to bile duct proliferation (Leveille et al, 1962; Hunt et al, 1964; Hunt et al, 1963).

The effect of LCA varies when fed to other species. In rabbit (which naturally has less chenodeoxycholic acid compared to other species), LCA at 0.25 % decreased body weight, increased cholesterol and plasma phospholipids, similar to the effects in chicken. In guinea pig, hamster, and mouse, feeding LCA at the same level did not reduce body weight, or increase cholesterol. The increase liver weights were also not observed in guinea pig, hamster, and mouse (Hunt et al, 1964).

Lithocholic acid is not toxic or only mildly toxic in species capable of one or more mechanism to detoxify LCA (Hofmann, 2004). Chronic feeding of LCA in rats only caused a mild hepatotoxicity, due to rat's liver's ability to hydroxylate LCA at C-6. LCA is also relatively non toxic to human. In human, conjugated LCA is sulfated at C-3, secreted in bile, and eventually excreted in the feces (Hofmann, 2004).

More recent studies suggested that bile acids are involved in signaling pathways, generally acting as activators of transcription factors (nuclear receptors) whose target genes

appear to play a role in bile acids synthesis, conjugation, transport, and also members of the cytochrome P450 system.

Vitamin D receptor (VDR) is a member of nuclear receptor family. Upon binding of ligand, VDR heterodimerizes with the retinoid X receptor (RXR), and binds to specific DNA sequences (response elements) in the promoter regions of the target genes. Vitamin D target genes are involved in a wide array of physiological functions. Calbindin , and ATP2B1 are among the genes influenced by vitamin D (and or VDR) which are involved in calcium transport, and calcium absorption. A recent study also reported that vitamin D, through VDR, increased transcription of CYP3A4 (Makishima et al, 2002), a member of the P450 family which is involved in cellular detoxification in human colon cancer cell line HT-29 (Jurutka, et al, 2005). VDR is also reported to regulate transcription of sulfotransferase (SULT2A1) in human, rat and mouse (Chatterjee et al., 2005; Seo et al., 2007). As mentioned before, sulfation is involved in LCA detoxification in human.

Vitamin D receptor is not only activated by 1,25 dihydroxy D_3. Makishima, et al (2002) reported that LCA and its 3-keto metabolite activate VDR, as determined in "a ligand-screening assay based on the ligand-induced interaction of a nuclear receptor with its coactivator". Nehring, et al (2007) went further to demonstrate that LCA has vitamin D activity in vivo in rats. They reported that dietary LCA increases plasma Ca, increases the expression of TRPV6, calbindin, and Ca^{2+}ATPase (ATP2B1) mRNA in the intestine. TRPV6 is a calcium channel located on the apical side of duodenum mucosal cells. It allows unidirectional calcium entry into the cell. Inside the cell, calcium binds to calbindin, which facilitates cytosolic calcium diffusion from the apical influx to the basolateral efflux sites. Calcium is transported out of the cell by Ca^{2+}ATPase located on the basolateral membrane (Molina, 2006).

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CHAPTER 3

THE EFFECT OF PHYTASE IN COMBINATIONS WITH 1α-OHD_3 ADDED TO P-DEFICIENT CORN-SOYBEAN MEAL, AND CORN-PEANUT MEAL BASED BROILER DIETS[1]

[1]Liem, A., H.M. Edwards Jr., G.M. Pesti, A. Atencio. To be submitted to Poultry Science

ABSTRACT

Supplementation of 1α-OH D_3 and phytase have been shown to influence phosphorus, including phytate phosphorus utilization in broiler chicks. Two battery studies were conducted from 1 to 16d to investigate the effects of supplementing increasing phytase (log) dose, with the combination of 1α-OHD_3 in the diet on broilers performance and mineral utilization. In Experiment 1, the birds were randomly allocated to 6 treatments: 5 treatments with increasing phytase levels from 0 to 12000 U/kg, and 1 treatment with of 5 μg/kg of 1α-OHD_3. In Experiment 2, the birds were subjected to 8 treatments: 0, 433, 1333, and 4000 U/kg phytase, with and without of 5 μg/kg of 1α-OHD_3.

Supplementation of 1α-OHD_3 and phytase to P-deficient corn-soybean meal and corn-peanut meal based broiler diets increased P, and phytate P utilization, as indicated by an increase in bone ash, body weight gain, plasma P, phytate P and P retention, and also reduction in incidence of P-deficiency rickets. Retention numbers are often calculated by using indigestible markers such as chromic oxide and celite (for acid insoluble ash determination). In Experiment 1, both markers were utilized. While there were differences between the 2 data sets, there were moderately high correlations, leading to similar general conclusions.

Keywords: 1α-OHD_3, phytase, phytate P utilization

INTRODUCTION

Phytate phosphorus is poorly utilized by non ruminants such as chickens, as they do not have the microflora that might significantly hydrolyze phytate phosphorus. Many factors have been reported to influence the birds' ability in utilizing phytate P, such as phytase, both endogenous and exogenous, dietary Ca and P, fiber, metal cations, fiber, organic acids, vitamin D and its derivatives (as reviewed in Ravindan et al, 1995). As energy price increase, the cost of inorganic phosphorus supplementation increases due to the high energy required for its production. The cost factor, coupled with increasing environmental pressure to reduce phosphorus in poultry manure, give incentive for poultry research and industry to evaluate methods to increase the availability of phytate P.

Phytase was shown to increase phytate P utilization by broiler chicks as early as 1970's. Since then different kinds of fungal and bacterial phytases have been available commercially; and have been used to improve phytate P utilization. Shirley and Edwards (2003) demonstrated that chickens continued to respond to increasing dose of phytase up to very high levels (12000 units/kg), much higher than the recommended dose of 600 U/kg.

Increasing level of cholecalciferol in the diet increases phytate phosphorus utilization in broiler chicks (Mohammed et al., 1991). Vitamin D_3 derivatives also have been shown to increase the ability of broiler chicks in utilizing phytate phosphorus: 1, 25 dihydroxycholecalciferol (Edwards, 1993, 1994; Mitchell and Edwards, 1996a,b), 1α-cholecalciferol (Edwards, 1994; Biehl et al., 1995, Biehl and Baker, 1997a,b). When the efficacy of some of the vitamin D derivatives on stimulating phytate P utilization were compared, high levels of D_3 increased phytate P utilization, although the increase was not as great as the effect

from 1,25-$(OH)_2D_3$ followed by 1α-OHD_3, as measured by plasma Ca and P, incidence of P rickets, bone ash, and retention of Ca, P, and phytate P (Edwards 1994, 2002). Supplementation of 25-OH D_3 in general, produced smaller and more inconsistent responses to these criteria, indicating some inconsistency in its ability to increase phytate P utilization.

Phytase, in combination with vitamin D and its derivatives, have been shown to increase phytate P utilization. Some studies have shown that interactions are present between the effects of phytase and 1α-OHD_3. In this study, the effects of increasing phytase (log) dose, with the combination of 1α-OHD_3 in the diet were evaluated. Regression equations for phytase effect on the criteria measured, and use it to approximate the relative equivalency of 5 μg/kg of 1α-OHD_3.

MATERIALS AND METHODS

General Procedure

All procedures involving research animals were approved by the University of Georgia IACUC. Two experiments were conducted for 16 d with 1-d old straight run Cobb x Cobb broiler chicks. All experiments were conducted in electrically heated wire mesh-floored battery brooder. Mash feed and water are provided at all time. Ultra violet irradiation was eliminated by fitting Arm-a0Lite sleeves to all the fluorescent lights in the room and battery brooders (Edwards et al., 1994). The lights were on 24 h. Room temperature was maintained at 22 C at all times. Feed samples were randomly sampled at mixing, excreta samples were pooled for last 72 hrs of the experiment. Feed and excreta samples were analyzed for Ca (Hill, 1965), total P(Latta and Eskin, 1980). Ca, P, and phytate P retentions were calculated by using the method of Edwards and Gillis (1959).

At the end of the experiments, one bird from each pen was selected randomly and blood sample was obtained by heart puncture for plasma Ca (Experiment 1) and dialyzable P content (Experiment 2). The birds were weighed by pen, and their feed consumption recorded. They were then terminated by carbon dioxide asphyxiation. P-deficiency rickets incidences were determined by making a longitudinal cut across the right tibia. The left tibias were then collected for bone ash determination.

Experiment 1

Experiment 1 was conducted to evaluate the effects of increasing log dose of phytase, and 5 µg/kg of 1α-OHD_3 on the performance of young broiler chicks fed phosphorus deficient corn-soybean meal based diet.The corn-soybean meal basal diet is shown in Table 3.1. The phytase obtained from BASF (Natuphos®) had 10000 U/g. The 1α-OHD_3 was obtained from Vitamin Derivatives Inc. Both chromic oxide and celite were added to the diet as indigestible marker. The retention data obtained from both chromic oxide and acid insoluble ash (AIA) are then compared. The treatments in the experiment were: 0, 444, 1333, 4000, 12000 U/kg phytase, and 5 µg/kg of 1α-OHD_3. The supplements were added on top of the basal diet shown in Table 3.1.

Experiment 2

Experiment 2 was conducted to evaluate the effect of increasing dose of phytase with and without 5 µg/kg of 1α-OHD_3. The corn-peanut meal basal diet is shown in Table 3.1. Lysine, threonine, and tryptophan were added to fulfill the amino acids requirements. The experiment was designed as 2 x 4 factorial, with 2 levels of 1α-OHD_3, 0 and 5 µg/kg; and 4 levels of phytase, 0, 444, 1333, and 4000 U/kg diet.

Statistical Analyses

Experiment 1 was analyzed as one-way analysis of variance. In a separate analysis, regression equation were obtained and used to approximate the relative equivalency of 5 µg/kg of 1α-OHD_3. Experiment 2 was analyzed as a 2 x 4 factorial design. Analyses of variance were performed on all data from both experiments using the GLM procedure of SAS appropriate for the respective designs.

RESULTS

Experiment 1

Increasing level of phytase (linearly and quadratically, $p<0.05$) increased body weight gain (BWG), feed intake. (Table 3.2). Using the regression analysis (linear equation) that were performed for the first 5 treatments, the increase in BWG that were obtained by adding 5 µg/kg of 1α-OHD_3 was equivalent to 648 U/kg of phytase. There was no significant effect on feed efficiency (gain/feed) due to treatment.

Increasing phytase levels linearly increased bone ash (percent and mg/ tibia). While using the regression equation did not give a probable estimate, the effect of 5 µg/kg of 1α-OHD_3 on bone ash were comparable to 444 U/kg of phytase (Table 3). Phytase also linearly reduced the incidence and severity of P-deficiency rickets. The birds in basal treatment had 80% incidence of rickets. Additions of 12000 units of phytase to this very P-deficient diet almost eliminated P-rickets (3% incidence). The effect of 5 µg/kg of 1α-OHD_3 on P-rickets incidence was approximately equivalent to the effect of 458 units of phytase (from the regression equation). Plasma Ca was not significantly affected by treatments.

The analyzed phytate P and AIA content of the feed were different from the formulated (predicted) value. The feed was formulated to contain 0.25 % phytate P, and 0.65% AIA. The

analyzed value for phytate P and AIA were 0.39% and 0.55%, respectively. Retention data calculated from both analyzed value and formulated are shown in Tables 3.3 and 3.4, respectively.

The results below refer to the retention data obtained from real lab value (Table 3.3). While retention data obtained from using Cr_2O_3 and AIA as indigestible marker were not identical, there were moderate correlations between the numbers (Figure 3.1). In general retention numbers obtained with AIA were higher than numbers obtained with Cr_2O_3. However, when both data sets were statistically analyzed, they lead to the same general conclusion.

Phytase did not increase retention of calcium. It linearly increased total P and phytate P retention. The effect of of 5 µg/kg of 1α-OHD_3 on total P retention was comparable to the effect from 444 U/kg of phytase. The effect of 5 µg/kg of 1α-OHD_3 on phytate P retention was comparable to dosage of phytase lower than 444 U/kg. Fitting regression equation for both criteria would give inappropriate estimate (the intercepts were higher than the number corresponding to 0 U/kg of phytase).

Experiment 2

The results from Experiment 2 are shown in Tables 3.5, 3.6, and 3.7. BWG were increased by addition of phytase, but not 1α-OHD_3. Feed intake was not significantly affected by treatment. Therefore, feed conversion ratio was improved by phytase, but not 1α-OHD_3.

There was an interaction between phytase and 1α-OHD_3 on plasma P taken on day 8. On treatments without 1α-OHD_3, adding 4000 U/kg of phytase to the basal diet increased plasma P from 3.13 to 4.34 mg/dl. When 1α-OHD_3 was present in the diet, adding phytase decreased plasma P from 4.82 to 2.51 mg/dl. This interaction was not present in plasma P taken at 16 day as both phytase and 1α-OHD_3 significantly increased 16 d plasma P. Tibia ash was also

increased by the addition of phytase and 1α-OHD_3. The effect of phytase and 1α-OHD_3 on percent tibia ash were additive.

Both phytase and 1α-OHD_3 also significantly reduced the incidence and severity of P-deficiency rickets, and tibial dyschondroplasia (TD). Neither phytase nor 1α-OHD_3 affected nitrogen retention in this experiment. Addition of 1α-OHD_3 increased retention of Ca, P, and phytate P. Phytase increased P and phytate P retention.

DISCUSSION

The results show that microbial phytase and 1α-OHD_3 increased P utilization in broiler chicks. The responses were similar to those reported from the laboratory in other studies (Shirley and Edwards, 2004, Edwards, 2002). In both experiments, adding phytase to a diet deficient in P produced an increase in BWG, bone ash, and reduced incidence and severity of P-deficiency rickets. Phytase liberates phosphate from phytate phosphorus, allowing the birds to utilize phytate more efficiently (Nelson et al, 1968). The size of the responses (on BWG, tibias ash, P-rickets incidence) from high levels of phytase was comparable to those reported in Shirley and Edwards, 2004. In contrary, other studies (Schoner et al, 1991; Simons et al, 1990) have suggested that total P utilization begins to plateau with supplementation of around 500 U/kg of phytase. However, the increase in Ca and nitrogen retention that were reported in the previous paper was not seen in the current study (both experiments).

Adding vitamin D and its derivatives increases Ca and P transport across the intestinal mucosa (Coates and Holdsworth, 1961; Harrison and Harisson, 1961; Wasserman and Taylor, 1973). Calcium in the diet reduces phytate P hydrolysis due to the formation of insoluble Ca-

phytate complexes. An earlier study (Davies et al, 1970) also reported that increasing level of vitamin D_3 increased intestinal phytase and phosphatase activity, especially when the diet is deficient in P. In our study, adding 5 μg/kg of 1α-OHD_3 increased plasma P, bone ash, and reduced leg abnormalities, indicating an increase in phosphorus utilization, mostly coming from the phytate P in the diet (as P retention increased 5 %, phytate P retention increased 13 %, Experiment 2). The effect of 5 μg/kg of 1α-OHD_3 in Experiment 1 was comparable to approximately 400 to 600 U/kg of phytase, depending on the criteria measured. The comparisons were mostly by observation rather than fitting the response obtained from 5 μg/kg of 1α-OHD_3 to the linear equation, as the number generated from the equation often gives estimates below 0 U/kg phytase.

The performance of the birds in Experiment 2 was comparable to Experiment 1. The differences seen in BWG were not unusual to occur between experiments, although in this study, the difference in metabolizable energy contents of the basal diets might also contribute to the lower overall BWG in Experiment 2 (389 vs 430 grams). The use of peanut meal, with added lysine, threonine, and tryptophan, as a protein source in Experiment 2 did not produce a marked difference in the performance, and P utilization in chicks.

There were some unexplained trends in plasma P data from Experiment 2. On day 8, increasing levels of phytase resulted in an increase in plasma P when 1α-OHD_3 was not present in the diet. The opposite trend was seen when 1α-OHD_3 was present. This interaction was statistically significant ($p<0.05$). However, this interaction was not seen in plasma P taken on day 16, where both 1α-OHD_3 and phytase increased plasma P.

There were interactions between the effect of 1α-OHD_3 and phytase on P rickets and TD. When 1α-OHD_3 was present in the diet the incidence of leg abnormalities was very low, hence

the phytase supplementation produced little or no effect. When 1α-OHD_3 was not present in the diet, phytase reduced incidence and severity of P-rickets and TD.

In Experiment 2, the use of both chromic oxide and celite allowed us to make side by side comparison of the digestibility data obtained by each marker. However, there were discrepancy in the calculated (formulated) and analyzed chromic oxide and AIA value of the feed, which made markers comparison more complicated. While both data sets in general gave the same general conclusion in terms of the effects of the supplements, there were notable differences between the data sets. When the analyzed feed values for phytate P and AIA were used the retention values were consistently higher when AIA level were used in calculations. These differences were much smaller when formulated values for the feed were used in the calculation. This raised the concern if the feed sample taken in this experiment was a good representative sample of the basal diet. Laboratory analytical error might also contribute to the discrepancy between analytical and formulated values for the feed. Scott and Boldaji (1997) conducted a study on determining apparent metabolizable energy of wheat or barley based diet with and without enzyme, using chromic oxide and 3 levels of celite. They found that in barley based diet (and not wheat based diet) using chromic oxide resulted in lower AME determinations in one treatment, and higher AME in the other treatment relative to the AME levels determined using AIA. They also reported that the levels of celite added in the diet influenced the AME determinations, implying that not only type of marker but also inclusion level can influence digestibility data.

From the data in both experiments, 1α-OHD_3 and phytase increased P and phytate P utilization. While the broilers continue to response to levels of phytase much higher than the recommended dose, commercial application should depend on the price of inorganic phosphorus

and phytase itself. The effects of 1-OHD_3 and phytase were mostly additive, as adding both supplements produced better results than the addition of each supplement by itself.

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TABLE 3.1. Composition of basal diets, Experiments 1 and 2

Ingredient	Experiment 1	Experiment 2
	----------%----------	
Ground yellow corn	52.75	50.73
Soybean meal (dehulled)	38.09	-
Peanut meal	-	39.82
Vegetable oil	5.00	5.00
Iodized sodium chloride	0.45	0.45
DL-Methionine	0.19	0.19
Vitamin premix[1]	0.25	0.25
Trace mineral premix[2]	0.08	0.08
Dicalcium Phosphate	0.60	0.60
Limestone	1.99	1.99
Cr_2O_3	0.10	0.10
Celite	0.50	-
Lysine	-	0.592
Threonine	-	0.195
Tryptophan	-	0.002
Calculated composition[3]		
ME, kcal	3.14	3.05
CP, %	23.23	24.05
Calcium, %	1.00	0.96
Phosphorus-total, %	0.50	0.47
Phytate phosphorus, %	0.24	0.24
Analyzed composition		
Calcium, %	1.05	1.10
Nitrogen, %	3.61	3.86
Total phosphorus, %	0.53	0.53
Phytate phosphorus, %	0.39	0.29

[1]Vitamin mix provided the following (per kilogram of diet): Thiamin-mononitrate, 2.4 mg; nicotinic acid, 44 mg; riboflavin, 4.4 mg; D-Ca pantothenate, 12 mg; vitamin B_{12} (*cobalamin*), 12.0 ug; pyridoxine-HCl, 2.7 mg; D-biotin, 0.11 mg; folic acid, 0.55 mg; menadione sodium bisulfate complex, 3.34 mg; choline chloride, 220 mg; cholecalciferol, 1,100 IU; trans-retinyl acetate, 5,500 IU; all-rac-tocopherol acetate, 11 IU; ethoxyquin, 150 mg.

[2]Trace mineral mix provides the following (per kilogram of diet): manganese ($MnSO_4.H_2O$), 60 mg; iron ($FeSO_4.7H_2O$), 30 mg; zinc (ZnO), 50 mg; copper ($CuSO_4.5H_2O$), 5 mg; iodine (ethylene diamine dihydroiodide), 1.5 mg.

[3]Calculated from NRC (1994).

TABLE 3.2. The effect of phytase and 1α-OHD_3 supplementation to a P-deficient corn-soybean meal diets on broiler performance, bone ash, leg abnormalities and plasma Ca, Experiment 1.

Diet and Levels									
Phytase	1α-OHD_3	BWG	FI	FE	%Bone ash	mg bone ash	P rickets score	P rickets incidence	Plasma Ca
U/kg	µg/kg	g	g					%	mg/dl
0	-	359±14	425±24	0.85±0.02	31.7±0.5	314±8	1.99±0.28	79.8±4.0	9.43±1.27
444	-	446±24	545±22	0.82±0.01	34.0±0.2	404±20	1.96±0.10	74.2±6.7	9.37±0.87
1333	-	462±14	557±4	0.83±0.02	36.6±0.4	481±19	1.31±0.12	37.5±10.3	8.55±0.62
4000	-	461±11	579±22	0.80±0.01	39.3±0.2	545±16	0.75±0.25	12.5±4.8	9.43±0.30
12000	-	480±24	575±29	0.84±0.01	41.6±0.3	608±29	0.25±0.25	2.8±2.8	10.18±0.31
	5	426±10	516±16	0.83±0.01	34.8±0.5	407±7	1.79±0.19	59.2±5.4	10.78±0.24
Probability									
Linear		0.0005	0.0002	0.4154	<.0001	<.0001	<.0001	<.0001	0.5304
Quadratic		0.0404	0.0104	0.1579	0.9380	0.5743	0.2952	0.8789	0.2660
Equation									
Linear		$\hat{y} = 421.4 + 0.00057x$	$\hat{y} = 512.2 + 0.0067x$	$(\hat{y} = 0.82 + 0.000000077x)$	$\hat{y} = 34.4 + 0.00063x$	$\hat{y} = 412.4 + 0.01756x$	$\hat{y} = 1.742 - 0.00014x$	$\hat{y} = 61.84 - 0.00576x$	$(\hat{y} = 9.08 + 0.0000855x)$
Quadratic		$\hat{y} = 403.3 + 0.025x - 0.0000016x^2$	$\hat{y} = 481.1 + 0.040x - 0.0000027x^2$	$(\hat{y} = 0.84 - 0.000015x + 0.0000000012x^2)$	$(\hat{y} = 32.9 + 0.0024x - 0.00000014x^2)$	$(\hat{y} = 364.5 + 0.0688x - 0.0000041x^2)$	$(\hat{y} = 2.001 - 0.000417x + 0.000000023x^2)$	$(\hat{y} = 77.95 - 0.02308x + 0.0000014x^2)$	$(\hat{y} = 9.2161 - 0.000052x + 0.000000011x_2)$

TABLE 3.3. The effect of phytase and 1α-OHD_3 supplementation to a P-deficient corn-soybean meal diets on Ca, P, and phytate P retention using Cr_2O_3 or acid insoluble ash as markers(calculated with real lab value= pp 0.39%,AIA=0.55%), Experiment 1.

Diet and Levels		Chromic oxide				Acid Insoluble Ash			
Phytase	1α-OHD_3	Ca retention	P retention	Phytate P retention	N retention	Ca retention	P retention	Phytate P retention	N retention
U/kg	µg/kg	%	%	%	%	%	%	%	%
0	-	52.37±6.00	57.26±6.33	57.34±4.12	60.06±8.43	61.24±5.54	66.17±3.02	65.14±3.94	69.09±3.56
444	-	50.33±6.34	59.17±4.08	61.97±4.44	68.08±3.57	58.71±6.10	66.62±1.01	68.67±2.76	73.93±1.68
1333	-	57.20±6.81	63.68±5.39	67.94±6.12	64.28±1.77	64.46±3.91	69.83±1.74	73.90±2.19	69.05±3.27
4000	-	49.00±4.78	67.58±2.72	78.39±2.90	69.95±1.76	56.44±4.74	72.60±1.75	81.79±2.45	74.62±0.57
12000	-	56.22±6.04	70.64±2.96	90.64±2.66	69.12±1.98	61.85±2.52	74.14±1.37	92.12±1.46	72.29±2.67
	5	52.91±5.51	60.15±4.40	59.66±6.19	63.49±4.99	61.89±3.22	67.90±1.18	67.85±2.91	69.59±5.43
Probability									
Linear		0.7426	0.0264	<.0001	0.1656	0.9451	0.0024	<.0001	0.4005
Quadratic		0.8802	0.9219	0.2328	0.6198	0.9064	0.8098	0.1263	0.6945
Equation									
Linear		(ŷ = 52.06457628 + 0.00026991x)	ŷ = 62.16691238+ 0.00255692x	ŷ = 40.98038331 + 0.00398880 x	(ŷ = 64.62161544 + 0.00047154 x)	(ŷ = 60.40603308 + 0.00003789 x)	ŷ = 67.69140372 + 0.00061348 x	ŷ = 68.95311834+ 0.00207296 x	(ŷ = 71.27405781 + 0.00014721 x)
Quadratic		(ŷ = 53.25632859 – 0.00101197x =0.00000010 x^2)	(ŷ = 58.65057275+ 0.00633920 x - 0.00000031 x^2)	(ŷ = 35.49489348 + 0.00988916 x - 0.00000048 x^2)	(ŷ = 62.80663417 + 0.00242378 x - 0.00000016 x^2)	(ŷ = 61.74874977 -0.00140637 x + 0.00000012 x^2)	(ŷ = 66.23789719 + 0.00217692 x - 0.00000013 x^2)	(ŷ = 66.21677584+ 0.00501625 x -0.00000024 x^2)	(ŷ = 70.19374778 + 0.00130923 x - 0.00000009 x^2)

TABLE 3.4. The effect of phytase and 1α-OHD_3 supplementation to a P-deficient corn-soybean meal diets on Ca, P, and phytate P retention using Cr_2O_3 or acid insoluble ash as markers (calculated with feed's formulated value), Experiment 1.

Diet and Levels		Chromic oxide				Acid Insoluble Ash			
Phytase	1α-OHD_3	Ca retention	P retention	Phytate P retention	N retention	Ca retention	P retention	Phytate P retention	N retention
U/kg	μg/kg	%	%	%	%	%	%	%	%
0	-	52.37±6.00	57.26±6.33	33.45±6.43	60.06±8.43	54.20±6.55	60.02±3.57	35.73±7.25	63.47±4.21
444	-	50.33±6.34	59.17±4.08	40.68±6.93	68.08±3.57	51.20±7.21	60.55±1.19	42.25±5.08	69.19±1.98
1333	-	57.20±6.81	63.68±5.39	49.99±9.55	64.28±1.77	58.00±4.62	64.34±2.06	51.87±4.03	63.43±3.87
4000	-	49.00±4.78	67.58±2.72	66.30±4.52	69.95±1.76	48.52±5.61	67.62±2.07	66.43±4.52	70.01±0.67
12000	-	56.22±6.04	70.64±2.96	85.39±4.14	69.12±1.98	54.92±2.98	69.44±1.62	85.46±2.68	67.26±3.16
	5	52.91±5.51	60.15±4.40	37.07±9.66	63.49±4.99	54.96±3.80	62.07±1.39	40.72±5.36	64.06±6.42
Probability									
Linear		0.7426	0.0264	<.0001	0.1656	0.9451	0.0024	<.0001	0.4005
Quadratic		0.8802	0.9219	0.2328	0.6198	0.9064	0.8098	0.1263	0.6945
Equation									
Linear		(ŷ = 52.06457628 + 0.00026991x)	ŷ = 60.16483322 + 0.00098528 x	ŷ = 40.98038331 + 0.00398880 x	(ŷ = 64.62161544 + 0.00047154 x)	(ŷ = 53.20713000+ 0.00004478 x)	ŷ = 61.81711349+ 0.00072503 x	ŷ = 42.76083999 + 0.00382178 x	(ŷ = 66.05115923+ 0.00017398 x)
Quadratic		(ŷ = 53.25632859 – 0.00101197x =0.00000010 x^2)	(ŷ = 57.99253103 + 0.00332187x - 0.00000019 x^2)	(ŷ = 35.49489348 + 0.00988916 x - 0.00000048 x^2)	(ŷ = 62.80663417 + 0.00242378 x - 0.00000016 x^2)	(ŷ = 54.79397700- 0.00166208 x + 0.00000014 x^2)	(ŷ = 60.09933304+ 0.00257272 x -0.00000015 x^2)	(ŷ = 37.71601946 0.00924814 x -0.00000044 x^2)	(ŷ = 64.77442919+ 0.00154727 x -0.00000011 x^2)

TABLE 3.5. The effect of phytase and 1α-OHD_3 supplementation to a P-deficient corn-peanut meal diets on broiler performance. Experiment 2.

Diet and levels						
Phytase	1α-OHD_3	n	BWG	FI	FE	F / G
0	0	3	366 ± 9	481 ± 16	0.76 ± 0.01	1.32 ± 0.01
444	0	3	399 ± 8	518 ± 11	0.77 ± 0.00	1.30 ± 0.00
1333	0	3	393 ± 18	502 ± 26	0.78 ± 0.01	1.28 ± 0.01
4000	0	3	423 ± 12	528 ± 10	0.80 ± 0.01	1.25 ± 0.01
0	5	3	357 ± 6	470 ± 9	0.76 ± 0.00	1.31 ± 0.00
444	5	3	372 ± 9	494 ± 5	0.77 ± 0.01	1.30 ± 0.02
1333	5	3	400 ± 4	508 ± 12	0.79 ± 0.01	1.27 ± 0.02
4000	5	3	390 ± 7	499 ± 12	0.78 ± 0.01	1.28 ± 0.01
0		6	362 ± 5[b]	476 ± 9	0.76 ± 0.00[b]	1.32 ± 0.01[a]
444		6	390 ± 7[a]	506 ± 7	0.77 ± 0.01[ab]	1.30 ± 0.01[ab]
1333		6	396 ± 8[a]	505 ± 13	0.79 ± 0.01[a]	1.27 ± 0.01[b]
4000		6	407 ± 9[a]	514 ± 9	0.79 ± 0.01[a]	1.26 ± 0.01[b]
	0	12	395 ± 8[a]	507 ± 9	0.78 ± 0.01	1.28 ± 0.01
	5	12	382 ± 6[a]	493 ± 6	0.78 ± 0.01	1.29 ± 0.01
ANOVA (probabilities)		df				
Phytase		3	0.0021	0.0634	0.0090	0.0083
1α-OHD_3		1	0.0805	0.1549	0.5818	0.5819
Phytase x 1α-OHD_3		3	0.2812	0.6199	0.4964	0.5183

TABLE 3.6. The effect of phytase and 1α-OHD_3 supplementation to a P-deficient corn-peanut meal diets on plasma P and bone ash. Experiment 2.

Diet and levels			Plasma P	Plasma P	mg ash	% bone ash
Phytase	1α-OHD_3	n	8day mg/dl	16day mg/dl	/ tibia	
0	0	3	3.13 ± 0.22[a]	2.44 ± 0.17	341 ± 12	34.29 ± 0.53
444	0	3	3.83 ± 0.32[a]	2.06 ± 0.13	367 ± 21	35.65 ± 0.30
1333	0	3	3.49 ± 0.75[a]	2.84 ± 0.20	410 ± 18	37.85 ± 0.09
4000	0	3	4.34 ± 0.67[a]	3.21 ± 0.38	480 ± 25	39.25 ± 0.41
0	5	3	4.82 ± 0.69 [a]	2.62 ± 0.18	352 ± 7	35.83 ± 0.55
444	5	3	3.54 ± 0.55[ab]	2.76 ± 0.37	421 ± 3	38.20 ± 0.15
1333	5	3	2.89 ± 0.22[b]	2.93 ± 0.21	447 ± 8	39.19 ± 0.32
4000	5	3	2.51 ± 0.15[b]	4.03 ± 0.28	473 ± 14	40.99 ± 0.57
0		6	3.98 ± 0.50	2.53 ± 0.12[b]	346 ± 7[c]	35.06 ±0.48[d]
444		6	3.68 ± 0.29	2.41 ± 0.24[b]	394 ± 15[b]	36.93 ± 0.59[c]
1333		6	3.19 ± 0.37	2.89 ± 0.13[b]	429 ± 12[b]	38.52 ± 0.33[b]
4000		6	3.42 ± 0.51	3.62 ± 0.28[a]	477 ± 13[a]	40.12 ± 0.50[a]
	0	12	3.70 ± 0.27	2.64 ± 0.17[b]	400 ± 18[b]	36.76 ± 0.60[b]
	5	12	3.44 ± 0.33	3.08 ± 0.20[a]	423 ± 14[a]	38.56 ± 0.59[a]
ANOVA (probabilities)		df				
Phytase		3	0.4574	0.0010	<.0001	<.0001
1α-OHD_3		1	0.4816	0.0248	0.0443	<.0001
Phytase x 1α-OHD_3		3	0.0223	0.4063	0.2382	0.4768

TABLE 3.7. The effect of phytase and 1α-OHD_3 supplementation to a P-deficient corn-peanut meal diets on leg abnormalities. Experiment 2.

Diet and levels			P-Rickets	P-Rickets	TD	TD
Phytase	1α-OHD_3	n	incidence %	score	incidence %	score
0	0	3	23.33 ± 6.67[a]	1.44 ± 0.29[a]	10.00 ± 0.00[a]	1.67 ± 0.33[a]
444	0	3	20.00 ± 5.77[a]	1.33 ± 0.33[a]	0.00 ± 0.00[b]	0.00 ± 0.00[b]
1333	0	3	3.33 ± 3.33[b]	0.33 ± 0.33[ab]	3.33 ± 3.33[b]	0.33 ± 0.33[b]
4000	0	3	0.00 ± 0.00[b]	0.00 ± 0.00[b]	0.00 ± 0.00[b]	0.00 ± 0.00[b]
0	5	3	10.00 ± 5.77[a]	0.67 ± 0.33[a]	0.00 ± 0.00[a]	0.00 ± 0.00[a]
444	5	3	0.00 ± 0.00[a]	0.00 ± 0.00[a]	0.00 ± 0.00[a]	0.00 ± 0.00[a]
1333	5	3	0.00 ± 0.00[a]	0.00 ± 0.00[a]	0.00 ± 0.00[a]	0.00 ± 0.00[a]
4000	5	3	3.33 ± 3.33[a]	0.33 ± 0.33[a]	0.00 ± 0.00[a]	0.00 ± 0.00[a]
0		6	16.67 ± 4.94	1.06 ± 0.26	5.00 ± 2.24	0.83 ± 0.40
444		6	10.00 ± 5.16	0.67 ± 0.33	0.00 ± 0.00	0.00 ± 0.00
1333		6	1.67 ± 1.67	0.17 ± 0.17	1.67 ± 1.67	0.17 ± 0.17
4000		6	1.67 ± 1.67	0.17 ± 0.17	0.00 ± 0.00	0.00 ± 0.00
	0	12	11.67 ± 3.66	0.78 ± 0.22	3.33 ± 1.42	0.50 ± 0.23
	5	12	3.33 ± 1.88	0.25 ± 0.13	0.00 ± 0.00	0.00 ± 0.00
ANOVA (probabilities)		df				
Phytase		3	0.0049	0.0081	0.0018	0.0003
1α-OHD_3		1	0.0107	0.0105	0.0010	0.0006
Phytase x 1α-OHD_3		3	0.0507	0.0328	0.0018	0.0003

TABLE 3.8. The effect of phytase and 1α-OHD_3 supplementation to a P-deficient corn-peanut meal diets on Ca, P, phytate P, and N retention. Experiment 2.

Diet and levels			Ca retention	P retention	Phytate P	N retention
Phytase	1α-OHD_3	n			retention	
			%	%	%	%
0	0	3	53.25±2.15	57.07±0.99	36.08±1.69	69.70±4.12
444	0	3	41.99±6.90	56.82±1.03	37.77±7.29	72.66±2.11
1333	0	3	44.64±5.01	59.84±0.38	39.38±5.38	71.97±0.78
4000	0	3	46.44±1.60	65.50±1.00	54.16±1.77	74.06±1.76
0	5	3	52.51±3.34	61.49±2.20	44.26±7.31	72.57±1.25
444	5	3	52.19±2.29	63.13±1.74	50.33±2.25	72.54±3.04
1333	5	3	54.31±2.67	66.10±1.22	54.47±2.36	71.99±3.11
4000	5	3	59.03±2.74	68.38±0.81	67.69±2.79	72.69±2.84
0		6	52.88± 1.78	59.28± 1.46	40.17± 3.82	71.13± 2.03
444		6	47.09± 3.97	59.98± 1.68	44.05± 4.42	72.60± 1.66
1333		6	49.48± 3.33	62.97± 1.51	46.92± 4.28	71.98± 1.44
4000		6	52.73± 3.15	66.94± 0.86	60.92± 3.37	73.38± 1.52
	0	12	46.58 ± 2.28	59.81± 1.12	41.84±2.95	72.10± 1.17
	5	12	54.51 ± 1.45	64.78± 1.05	54.19±3.16	72.45± 1.14
ANOVA (probabilities)		df				
Phytase		3	0.3700	<.0001	0.0016	0.8464
1α-OHD_3		1	0.0082	<.0001	0.0013	0.8500
Phytase x 1α-OHD_3		3	0.3200	0.5018	0.8815	0.8676

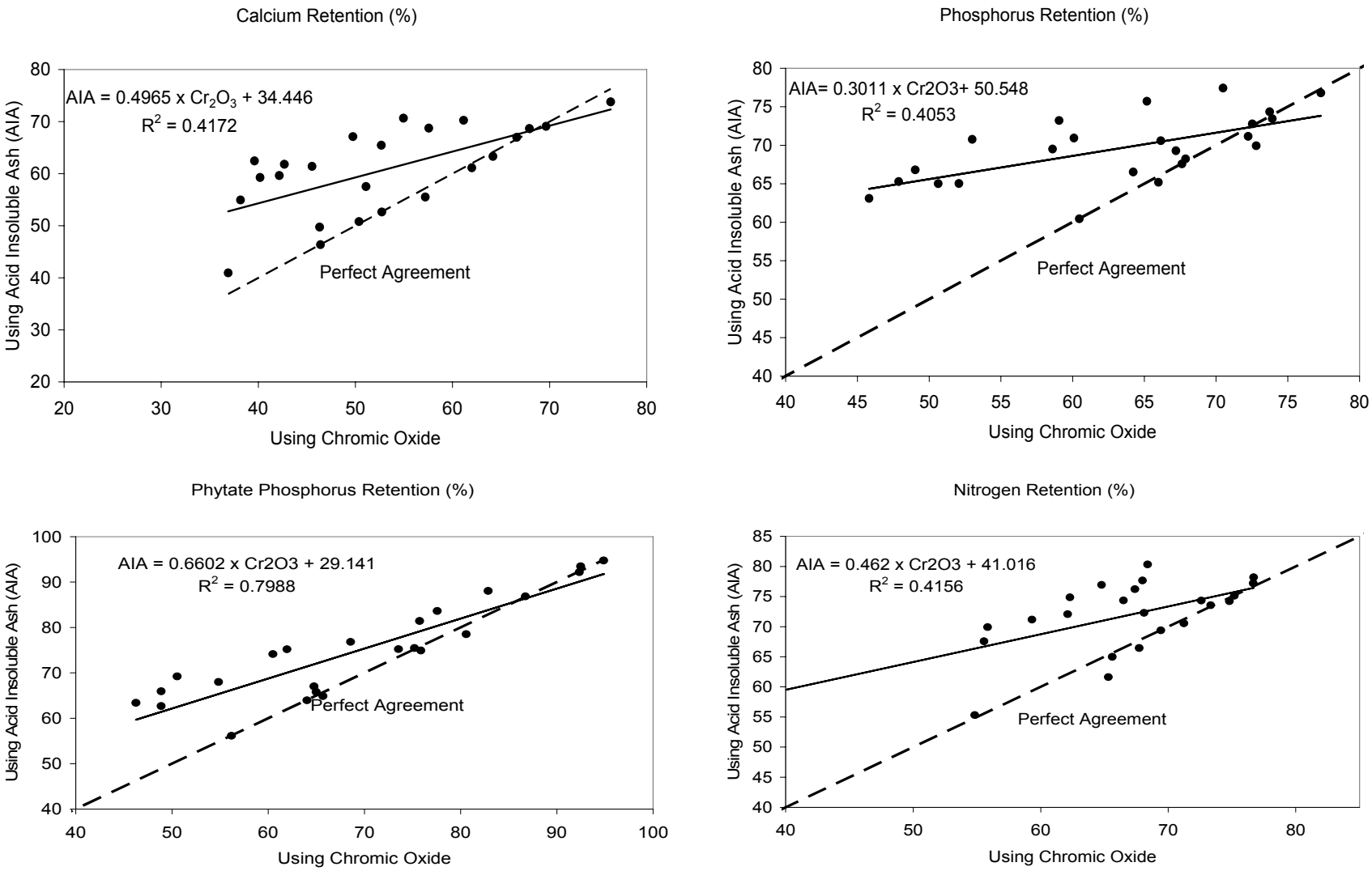

Figure 3.1. The correlation between retention values calculated using chromic oxide and acid insoluble ash as indigestible markers.

CHAPTER 4

THE EFFECT OF PHYTASE, METHIONINE SOURCE, CALCIUM, AND 1α-OHD_3 ON PHYTATE PHOSPHORUS UTILIZATION IN BROILER CHICKS.[1]

[1]Liem, A., H.M. Edwards Jr., G.M. Pesti. To be submitted to Poultry Science

ABSTRACT

Phytase, 1-alpha cholecalciferol (1α-OHD_3), Ca, and organic acids such as 2-hydroxy-4 methythio butanoic acid have been shown to influence phytate P utilization. Two experiments were conducted from 0 to 16 d in battery pens to determine the effects of different combinations phytase, methionine source, 1α-OHD_3 (in Experiment 1), and Ca (in Experiment 2) and their interactions on phytate P utilization in young broiler chicks. In both experiments, birds were fed P deficient diet, corn and soybean meal based diets. In Experiment 1, a 2 x 2 x 2 factorial design with 2 methionine sources (DL-Methionine / DLM and 4-methylthio butanoic acid / HMB), 2 levels of 1α-OHD_3(0 and 5μg/kg), with and without 500 U/kg of phytase (Natuphos®, BASF) was implemented. . The design of Experiment 2 is a 2 x 2 x 2 factorial design with 2 methionine sources, 2 levels of dietary Ca (0.6 and 0.9%), and 2 levels of 1α-OHD_3(0 and 5μg/kg).

Birds fed phytase had higher bone ash, phytate P disappearance, and lower P-deficiency rickets incidence. The effect of HMB often depends on other supplements. In Experiment 1, HMB reduced incidence of P-rickets and increased percent bone ash, especially when 1α-OHD_3 was not present in the diet. In Experiment 2, HMB increased percent bone ash and incidence of tibial dyschondroplasia (overall) and reduced P-rickets incidence in 2 dietary combinations: high calcium no phytase, and low calcium with phytase (significant 3 way interactions for P-rickets incidence). The addition of 1α-OHD_3 increased feed efficiency, percent bone ash and reduced P-rickets incidence.

While phytase, 1α-OHD_3, and HMB in general increased phytate P utilization, the effect of each supplement often depends on the levels of other supplements and nutrients (Ca). This interaction should be considered when adjusting feed formulation for adding the supplements.

Keywords: phytase, 1α-OHD_3, calcium, methionine hydroxy analog.

INTRODUCTION

Phosphorus is one of the major minerals required by chicks. The cost of inorganic phosphorus supplementation is the third most expensive cost in feed. Optimizing phytate phosphorus utilization might reduce feed cost in addition to reducing phosphorus excretion into the environment.

Numerous factors influence phytate phosphorus utilization by broiler chicks, such as Ca, P, phytase, fiber, organic acids, vitamin D_3 and its derivatives (Ravindran et al., 1995).

Phytase was shown to increase phytate P utilization by broiler chicks since the 1960's (Nelson et al, 1968). Since then different kinds of fungal and bacterial phytases have been available commercially; and have been used to improve phytate P utilization.
Edwards and Veltmann (1983) demonstrated that increasing dietary Ca and inorganic P reduces utilization of phytate P. Calcium, added even at normal levels to P-deficient diets, have been shown to dramatically reduced P utilization, as indicated by poor bone ash, impaired growth, and increased incidence and severity of P rickets in young broilers (Davis, 1959; Waldroup et al., 1963; MacDonald and Solvyns, 1964; Kondos and McClymont, 1967). Edwards and Veltmann (1983) demonstrated that phytate P retention was highest when the diet contained low levels of calcium and phosphorus. However, maximum bone ash and body weight gain was not obtained by feeding low levels of Ca and P, hence balancing objectives is important.

Vitamin D increases Ca absorption from the GI tract (Coates and Holdsworth, 1961). This results in lower Ca concentration in GI tract, lowering its chelation with phytic acid, which in turn increased phytate P utilization. Increasing level of cholecalciferol in the diet increases phytate phosphorus utilization in broiler chicks (Mohammed et al., 1991). Vitamin D_3 derivatives also have been shown to increase the ability of broiler chicks in utilizing phytate phosphorus

such as 1, 25 dihydroxycholecalciferol (Edwards, 1993, 1994; Mitchell and Edwards, 1996a,b), 1α-cholecalciferol (Edwards, 1994; Biehl et al., 1995, Biehl and Baker, 1997a,b).

Some organic acids, such as citric acid is also thought to increase phytate P utilization by chelating Ca in the diet. Increasing levels of citric acid has been shown to increase bone ash, and body weight when added to a P deficient diet (Boling et al, 2000). However, other organic acids such as EDTA, which has tremendous chelating ability, reduced bone ash and body weight (Rafacz-Livingston et al, 2005; Liem et al, 2008). The previous studies also reported that the following organic acids can increase phytate P utilization, either indicated by increased performance, bone ash, or phytate P retention: sodium gluconate, calcium gluconate, glucono-δ-lactone, 2-hydroxy-4 methythio butanoic acid, and citric acid. Substituting 2-hydroxy-4 methythio butanoic acid, a common methionine source often referred as methionine hydroxy analog, for DL-methionine have been shown to increase phytate P utilization (Liem et al, 2008).

The objective of the current study is to evaluate the effects of phytase, methionine source, 1α-OHD_3 (in Experiment 1), and Ca (in Experiment 2) and their interactions on phytate P utilization in young broiler chicks.

MATERIALS AND METHODS

General Procedure

Two experiments were conducted for 15d with 1-d old broiler Cobb x Cobb 500 mixed sex chickens from a commercial hatchery. All procedures were approved by the University of Georgia IACUC. The mash corn-soybean meal basal diets for both experiments are shown in Table 4.1. The experiments were conducted in electrically heated wire mesh-floor Petersime®

battery brooders. Ultraviolet irradiation was eliminated from the chick room by fitting Arm-a-Lite® sleeves to all fluorescent fixtures in the room and battery brooders (Edwards et al., 1994). The fluorescent lights were on 24 h each day. The temperature of the room was maintained at 22 C. The chicks were given access to water and mash-feed ad libitum.

At termination of the experiments, the birds were weighed by pen and their feed consumption recorded. They were killed by carbon dioxide asphyxiation and examined at random for phosphorus deficiency rickets by making a longitudinal cut across the right tibia. Chickens with P-deficiency rickets have normal proliferating prehypertrophied zone and lengthened metaphyseal primary spongiosa (Edwards, 1993). Left tibias were removed for dry fat-free bone ash determination according to AOAC International (1995).

Feces samples were collected for the last 72 hours of the experiments (d13 morning-d16 morning). Chromic oxide was added to the diets at 0.1% (Table 1).Feed and excreta samples were analyzed for phytate P (Latta and Eskin, 1980) and chromic oxide (Brisson, 1956). Phytate P retention was calculated using the methods of Edwards and Gillis (1959). Percent phytate P retained = 100 – ((% Cr_2O_3 in feed / % Cr_2O_3 in feces) x (% phytate P in feces / % phytate P in feed) x 100).

Experiment 1

In Experiment 1, 240 chicks were randomly allocated to 24 pens, which were assigned to 8 treatments. The design of Experiment 1 is a 2 x 2 x 2 factorial design with 2 methionine sources (DL-Methionine / DLM and 4-methylthio butanoic acid / HMB), 2 levels of 1α-OHD_3(0 and 5µg/kg), with and without 500 U/kg of phytase (Natuphos®, BASF). The DL-methionine was substituted for the hydroxyl analog of the same amount, taking into account that the HMB is 88% pure. DL-methionine was added at 0.2%, HMB was added at 0.227%.

Experiment 2

In Experiment 2, 240 chicks were randomly allocated to 24 pens, which were assigned to 8 treatments. The design of Experiment 2 is a 2 x 2 x 2 factorial design with 2 methionine sources (DLM and HMB), 2 levels of dietary Ca (0.6 and 0.9%), and 2 levels of 1α-OHD_3 (0 and 5μg/kg). Limestone was added at the expense of corn in diets containing 0.9% Ca.

RESULTS

Experiment 1

The results from Experiment 1 are shown in Tables 4.2, and 4.3. None of the supplements influenced body weight gain (BWG) of the birds; however, both phytase and 1α-OHD_3 increased feed efficiency (gain/feed). HMB, phytase and 1α-OHD_3 reduced the incidence of P-deficiency rickets. There was an interaction between methionine source and 1α-OHD_3 effects. When HMB was present in the diet, adding 1α-OHD_3 did not produce P-rickets reduction as much as when DLM was in the diet, and vice versa. There was an interaction between phytase and methionine source on tibial dyshondroplasia (TD) incidence. When DLM is in the diet, adding phytase had little or no effect on TD incidence, when HMB is in the diet, adding phytase increased the incidence of TD, and vice versa.

Phytase and 1α-OHD_3 increased percent bone ash on average by 2.07 and 1.02% respectively. There was an interaction between the effect of methionine source and 1α-OHD_3on bone ash. When DLM was in the diet, adding 1α-OHD_3 increased bone ash, whereas when HMB was in the diet, 1α-OHD_3had little or no effect, and vice versa.

Only phytase contributed to the variations in mg/tibia ash, and phytate P disappearance from GI tract.

Experiment 2

Methionine source did not influence BWG. Phytase increased BWG on average by 46 grams, while increasing Ca level decreased BWG by 100 grams on average. The feed efficiency (gain/feed) was significantly lower for birds fed HMB, while not affected by phytase and Ca levels.

The incidence of P-deficiency rickets was reduced by addition of phytase and increased by the increasing level of Ca. A three way interaction (between phytase, Ca, and methionine source) was observed for P-deficiency rickets. In this experiment, substituting HMB for DLM increased TD incidence from 15 to 26 % on average. Increasing Ca level from 0.6 to 0.9 level reduced TD incidence from 34 to 7% on average.

HMB and phytase significantly increases percent tibia ash. There was a three way interaction on percent tibia ash. Phytase increased mg/tibia ash, while Ca decreased mg/tibia ash. The only factor that significantly contributed to the phytate P disappearance was Ca. Neither phytase nor methionine source significantly affect phytate P disappearance.

DISCUSSION

Phytase cleaves phosphate from myoinositol ring, liberating phosphate that otherwise is mostly unavailable to nonruminants. Phytase and other phosphatase' activities are influenced by many factors, such as pH and cations (Ravindran et al, 1995). Adding organic acids are thought to influence both these factors. Citric acid is thought to increase phytate utilization by chelating

Ca in the diet (Boling et al, 2000). Organic acids also might decrease the pH of the digesta, making it closer to the optimum pH for phytase to work. However, not all organic acids that decrease pH or chelate Ca can improve phytate P utilization. EDTA, which has more chelating ability than HMB did not improve phytate P utilization (Rafacz-Livingston et al, 2005; Liem et al, 2008).

In our study, substituting HMB in place of DL-methionine resulted in a subtle improvement in phytate P utilization, which in general was very similar to what is reported in Liem et al (2008). The effect was often obscured when 1α-OHD_3 was added to the diet, such as seen for P-rickets incidence. For percent bone ash, HMB actually numerically reduced bone ash when 1α-OHD_3 was present in the diet. This observation, which is yet to be explained, suggests that adding both 1α-OHD_3 and HMB simultaneously to a P-deficient diet might impair P utilization in birds. In the second experiment, the effects of HMB depended on phytase and Ca levels, as significant 3 way interactions were present for percent bone ash, and P-rickets incidence. Birds fed HMB also had lower feed efficiency compared to birds fed DLM. This was not expected, nor observed in our previous studies, as methionine was not the limiting nutrient in these diets.

Phytase has been shown since late 1960's to increase phytate P utilization (Nelson, 1968; Nelson et al, 1971). Adding phytase increases BWG (only in Experiment 2), feed efficiency (Experiment 2), tibia ash (%), phytate P disappearance (Experiment 1), consistent with previous studies (Shirley and Edwards, 2003). In our current study, not all the effects typically associated with phytase were seen. This could be due to the differences in birds' abilities to use phytate P which might be influenced by breeders' vitamin D status (Atencio et al, 2005). The birds'

performance in Experiment 1 was noticeably better than the performance of birds fed similar diets in our previous studies (Liem et al, 2008).

Adding phytase to these birds might not produce an improvement as dramatic as in lesser quality batch of birds.

Adding vitamin D and its derivatives increases Ca and P transport across the intestinal mucosa (Coates and Holdsworth, 1961; Harrison and Harisson, 1961; Wasserman and Taylor, 1973). Calcium in the diet reduces phytate P hydrolysis due to the formation of insoluble Ca-phytate complexes. An earlier study (Davies et al, 1970) also reported that increasing level of vitamin D_3 increased intestinal phytase and phosphatase activity, especially when the diet is deficient in P. Previous studies demonstrated that addition of 1α-OHD_3 increase phytate P utilization (Biehl et al, 1995, 1998; Edwards, 2002). In our study, adding 1α-OHD_3 produced similar results. The additional information from our study is the interaction between methionine source (organic acid), and 1α-OHD_3. The effect of 1α-OHD_3 was less prominent when HMB was present in the diet.

The effect of increasing Ca levels in a P deficient diet severely reduced birds' performance and ability to utilize phytate P (Davis, 1959; Waldroup et al., 1963; MacDonald and Solvyns, 1964; Kondos and McClymont, 1967). This same trend was seen in our study. Calcium levels also influence the effects of phytase and HMB.

Phytase, methionine source, 1α-OHD_3, and calcium have been demonstrated before to influence birds' performance and phytate P utilization. However, when adding or changing the levels of one supplement or nutrient, the interactions between the factors should be considered.

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TABLE 4.1. Composition of basal diets, Experiments 1 and 2

Ingredient	Experiment 1	Experiment 2
	----------%----------	
Ground yellow corn	53.89	54.40
Soybean meal (dehulled)	38.00	38.00
Vegetable oil	5.00	5.00
Iodized sodium chloride	0.45	0.45
Methionine source	0.20	0.20
Vitamin premix[1]	0.25	0.25
Trace mineral premix[2]	0.08	0.08
Dicalcium Phosphate	0.56	0.56
Limestone	1.47	0.96
Cr_2O_3	0.10	0.10
Calculated composition[3]		
ME, kcal	3.18	3.19
CP, %	23.29	23.33
Calcium, %	0.79	0.60
Phosphorus-total, %	0.49	0.49
Phytate phosphorus, %	0.25	0.25

[1]Vitamin mix provided the following (per kilogram of diet): Thiamin-mononitrate, 2.4 mg; nicotinic acid, 44 mg; riboflavin, 4.4 mg; D-Ca pantothenate, 12 mg; vitamin B_{12} (*cobalamin*), 12.0 ug; pyridoxine-HCl, 2.7 mg; D-biotin, 0.11 mg; folic acid, 0.55 mg; menadione sodium bisulfate complex, 3.34 mg; choline chloride, 220 mg; cholecalciferol, 1,100 IU; trans-retinyl acetate, 5,500 IU; all-rac-tocopherol acetate, 11 IU; ethoxyquin, 150 mg.

[2]Trace mineral mix provides the following (per kilogram of diet): manganese ($MnSO_4.H_2O$), 101 mg; iron ($FeSO_4.7H_2O$), 20 mg; zinc (ZnO), 80 mg; copper ($CuSO_4.5H_2O$), 3 mg; iodine (ethylene diamine dihydroiodide), 0.75 mg, magnesium (MgO), 20 mg; selenium (sodium selenite), 0.3 mg.

[3]Calculated from NRC (1994).

[4] In Experiment 2, DL-Methionine was added at 0.2%, HMB was added at 0.227% (taking into account that HMB is 12% water)

TABLE 4.2. The effects of methionine source, phytase, and 1-alpha D_3 on performance, P-deficiency rickets, and TD incidence, Experiment 1.

Treatment	DL-Met	HMB	phytase	1-alpha D_3	BWG	Feed effi.	P rickets incidence	TD incidence
	%	%	U/kg	µg/kg	g	g/g	%	%
1	0.2	0	0	0	431±20	0.82±0.04	64±11	4±4
2	0.2	0	500	0	466±20	0.86±0.02	14±8	0±0
3	0.2	0	0	5	484±14	0.86±0.02	22±8	5±5
4	0.2	0	500	5	474±16	0.98±0.08	5±5	5±5
5	0	0.227273	0	0	491±13	0.81±0.02	21±8	0±0
6	0	0.227273	500	0	475±17	0.85±0.01	8±8	18±5
7	0	0.227273	0	5	471±23	0.85±0.02	22±3	4±4
8	0	0.227273	500	5	481±18	0.88±0.02	4±4	15±8
Parameters				df				
Methionine source				1	0.2335	0.2306	0.0315	0.1066
Phytase				1	0.7069	0.0338	0.0002	0.0918
1-alpha D_3				1	0.3613	0.0429	0.0176	0.6598
Met. Source x phytase				1	0.5757	0.4588	0.0940	0.0300
Met. Source x 1-alpha D_3				1	0.1539	0.4714	0.0339	0.7618
Phytase x 1-alpha D_3				1	0.7094	0.5191	0.1877	0.8254
Met. Source x phytase x1-alpha D_3				1	0.1872	0.3866	0.0818	0.4283

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Main effect means				
Met. Source				
DL-Met	464±10	0.88±0.03	26±8	3±2
HMB	479±8	0.85±0.01	14±4	9±3
Phytase				
0 U/kg	469±10	0.83±0.01	32±6	3±2
500 U/ kg	474±8	0.89±0.02	8±3	10±3
1-alpha D_3				
0 µg/kg	466±10	0.84±0.01	27±8	6±3
5 µg/kg	478±8	0.89±0.02	13±3	7±3

TABLE 4.3. The effects of methionine source, phytase, and 1-alpha D_3 on bone ash and phytate P disappearance, Experiment 1.

Treatment	DL-Met	HMB	phytase	1-alpha D_3	Bone ash	Bone ash	Phytate P disappearance
	%	%	U/kg	µg/kg	%	mg/tibia	%
1	0.2	0	0	0	32.80±0.77	426±10	59.07±1.53
2	0.2	0	500	0	35.29±0.38	476±24	66.23±4.52
3	0.2	0	0	5	35.06±0.49	472±8	59.97±1.01
4	0.2	0	500	5	36.97±0.15	499±30	67.64±2.51
5	0	0.227273	0	0	34.28±0.83	485±13	58.70±3.96
6	0	0.227273	500	0	36.20±0.38	489±18	65.27±2.91
7	0	0.227273	0	5	34.31±0.40	463±19	64.43±3.81
8	0	0.227273	500	5	36.31±0.48	498±14	68.39±3.04
Parameters				df			
Methionine source				1	0.5203	0.2443	0.6661
Phytase				1	<.0001	0.0375	0.0111
1-alpha D_3				1	0.0145	0.2923	0.2245
Met. Source x phytase				1	0.7526	0.4703	0.6334
Met. Source x 1-alpha D_3				1	0.0214	0.1279	0.4698
Phytase x 1-alpha D_3				1	0.7386	0.8802	0.8144
Met. Source x phytase x1-alpha D_3				1	0.6594	0.3213	0.7289

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Main effect means			
Met. Source			
DL-Met	35.03±0.49	468±12	63.22±1.63
HMB	35.27±0.38	484±8	64.20±1.81
Phytase			
0 U/kg	34.11±0.37	461±9	60.54±1.42
500 U/ kg	36.19±0.24	490±10	66.88±1.47
1-alpha D_3			
0 µg/kg	34.64±0.46	469±11	62.32±1.79
5 µg/kg	35.66±0.36	483±10	65.11±1.55

TABLE 4.4. The effects of methionine source, phytase and calcium on performance, P-deficiency rickets and TD incidence, Experiment 2.

Treatment	DL-Met	HMB	phytase	Ca	BWG	Feed eff.	P rickets incidence	P-rickets 3 incidence	TD incidence
	%	%	U/kg	%	g	g/g	%	%	%
1	0.2		0	0.6	465±14	0.86±0.02	29±2	12±7	26±4
2	0.2		0	0.9	317±17	0.83±0.03	97±3	75±14	0±0
3	0.2		500	0.6	465±13	0.82±0.02	23±3	13±7	30±6
4	0.2		500	0.9	404±6	0.83±0.02	63±9	33±3	3±3
5		0.227	0	0.6	432±9	0.81±0.01	40±10	20±6	40±10
6		0.227	0	0.9	426±15	0.78±0.02	79±6	61±6	14±3
7		0.227	500	0.6	450±4	0.82±0.01	7±3	0±0	40±10
8		0.227	500	0.9	407±12	0.79±0.01	73±8	54±7	11±6
Parameter				df					
Metsource				1	0.3013	0.0159	0.4136	0.9405	0.0188
Phytase				1	<.0001	0.6920	0.0005	0.0051	0.8218
Ca				1	<.0001	0.1679	<.0001	<.0001	<.0001
Ca x Metsource				1	0.0997	0.3725	0.8752	0.5614	0.8958
Phytase x Metsource				1	0.7329	0.2120	0.9960	0.5270	0.5558
Phytase x Ca				1	0.0004	0.3475	0.9960	0.1690	0.8299
Phytase x Ca x Metsource				1	0.4802	0.4333	0.0079	0.0158	0.8792

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Main effect means					
Met. Source					
DL-Met.	413±19	0.84±0.01	53±9	33±9	15±4
HMB	403±15	0.80±0.01	49±9	34±8	26±5
Phytase					
0 U/kg	385±20	0.82±0.01	61±9	42±9	20±5
500 U/kg	431±9	0.82±0.01	41±9	25±7	21±5
Calcium					
0.6 %	453±6	0.83±0.01	25±4	11±3	34±4
0.9 %	363±14	0.81±0.01	78±5	56±6	7±2

TABLE 4.5. The effects of methionine source, phytase and calcium on bone ash and phytate p disappearance, Experiment 2.

Treatment	DL-Met	HMB	phytase	Ca	Bone ash	Bone ash	Phytate P disappearance
	%	%	U/kg	%	%	mg/tibia	%
1	0.2		0	0.6	31.75±0.13	420±11	62.37±3.09
2	0.2		0	0.9	27.46±0.15	274±4	45.95±6.34
3	0.2		500	0.6	32.84±0.34	447±15	67.71±3.58
4	0.2		500	0.9	30.93±0.42	378±13	59.49±3.91
5		0.227	0	0.6	31.66±0.27	402±10	65.53±6.16
6		0.227	0	0.9	28.47±0.38	284±18	51.22±8.88
7		0.227	500	0.6	33.94±0.26	448±6	69.51±1.23
8		0.227	500	0.9	30.66±0.19	372±14	54.48±3.67
Parameter				df			
Metsource				1	0.0435	0.7293	0.7238
Phytase				1	<.0001	<.0001	0.0900
Ca				1	<.0001	<.0001	0.0018
Ca x Metsource				1	0.7476	0.5469	0.7490
Phytase x Metsource				1	0.9213	0.9319	0.4335
Phytase x Ca				1	0.0113	0.0037	0.6125
Phytase x Ca x Metsource				1	0.0073	0.3014	0.5465

Continued on the next page

Main effect means			
Met. Source			
DL-Met.	30.74±0.62	380±21	58.88±3.07
HMB	31.18±0.60	377±19	60.18±3.35
Phytase			
0 U/kg	29.83±0.58	345±21	56.27±3.66
500 U/kg	32.09±0.43	411±12	62.80±2.31
Calcium			
0.6 %	32.55±0.30	429±8	66.28±1.86
0.9 %	29.38±0.46	327±16	52.78±2.99

CHAPTER 5

THE EFFICACY OF DIFFERENT PREPARATIONS OF 1α-OHD_3 DERIVATIVES ON PERFORMANCE AND CALCIUM UTILIZATION IN YOUNG BROILER CHICKS

[1]Liem, A., H.M. Edwards Jr., G.M. Pesti, A. Atencio. To be submitted to Poultry Science

ABSTRACT

Vitamin D_3 is required by chicks for growth, bone health, and normal physiological functions. There are a few commercially available derivatives of vitamin D_3, such as (5,6 cis)1α-OHD_3. In this study, three experiments were conducted using a corn-soybean meal diets that meets or exceeds the NRC (1994) requirements for all nutrients except cholecalciferol to evaluate the efficacy of several 1α-OHD_3 compounds as a substitute for cholecalciferol. Cobb x Cobb straight-run day-old chicks were raised in Petersime battery brooders from 1 to 16d. In Experiment 1, D_3 at 0, 2.5, and 5 μg/kg, two levels of spray dried 1α-OHD_3 (0.625, and 1.25 μg/kg), and one level of crystalline 1α-OHD_3 at 1.25 μg/kg of feed were fed. In Experiment 2, D_3 at 0, 2.5, 5 and 10 μg/kg and two levels (0.625, and 1.25 μg/kg) of spray dried 1α-OHD_3 processed at 100°C and at 200°C were fed. In Experiment 3, D_3 at 0, 2.5, 5 and 10 μg/kg and two levels (0.625, and 1.25 μg/kg) of 5, 6 cis 1α-OHD_3 or 5, 6 trans 1α-OHD_3 were fed. Slope ratio analysis of data from the measurement of 16-d BWG, plasma Ca, rickets and bone ash indicated the bioavailability of the different 1α-OHD_3 (except for the 5, 6 trans 1α-OHD_3 which was inactive) to be 7 to 15 times more active as compared to D_3.

There were differences between the 1α-OHD_3 compounds due to source, processing, and cis-trans isomerism. The effects of spray dried and crystalline 1α-OHD_3 supplied at the same level were comparable for all the parameters measured, except for percent tibia ash, and Ca rickets incidence, for which the effects of crystalline 1α-OHD_3 were better. The effects of 1α-OHD_3 processed at different temperatures were similar for tibia ash and plasma calcium, but differences were observed for BWG and feed intake. 1α-OHD_3 processed at 100°C increased BWG and feed intake while 1α-OHD_3 processed at 200°C did not. The 5, 6 cis 1α-OHD_3 was found to be much more active compared to D_3, while the 5,6 trans 1α-OHD_3 was not.

Keywords : 5,6 cis1α-OHD_3, 5,6 trans 1α-OHD_3, cholecalciferol, calcium, spray dry temperature

INTRODUCTION

Vitamin D_3 is required by animals for various functions, mainly related to calcium metabolism. In the body vitamin D_3 undergo modifications resulting in the active hormonal form 1, 25$(OH)_2D_3$. There are a few forms of vitamin D_3 derivatives that are available commercially, such as 1-alpha hydroxyl cholecalciferol (1α-OHD_3). It was first reported to be active in inducing calcium absorption and bone mobilization in chickens by Haussler et al. (1973) in study with White Leghorn cockerels. In the study, 1α-OHD_3 was found to as effective (mole to mole basis) as 1, 25$(OH)_2D_3$ in mobilizing calcium from the bone and also inducing intestinal calcium absorption, and at least 10 times as active as cholecalciferol . Another early study (Boris et al, 1977) also reported that 1α-OHD_3 increased tibia ash more effectively than cholecalciferol and 25-OHD_3. Since then, numerous studies confirmed that 1α-OHD_3 has activity as an effective substitute for cholecalciferol in increasing Ca absorption, plasma Ca, body weight gain, tibia ash, and also increasing phytate P utilization (Edwards, 1994, 2002; Biehl et al., 1995,1998; Biehl and Baker, 1997a,b, Edwards et al, 2002).

Commercial applications of 1α-OHD_3 are made possible by dilution and further processing of the compound, such as spray drying. A spray dryer mixes a heated gas with an atomized (sprayed) liquid stream within a vessel (drying chamber) to accomplish evaporation and produce a free flowing dry powder with a controlled average particle size.

The objective of this study was to evaluate the efficacy of different processing conditions and isomers of 1α-OHD_3. The synthesis of 1α-OHD_3 starts with 5, 6 cis cholecalciferol as the starting material. The Barton-Hesse synthesis consists of 6 steps which yield 5, 6 trans 1α-OHD_3 (as the product of step 5) before conversion to the final product 5, 6 cis 1α-OHD_3. In this study 2 different isomers of 1α-OHD_3, 5, 6 cis 1α-OHD_3 and 5, 6 trans 1α-OHD_3, were tested. Spray

dried products of 1α-OHD_3 were also compared to crystalline 1α-OHD_3, and D_3. There have been little data (from both chemical and animal research) on the heat stability of 1α-OHD_3. Higher temperatures facilitate faster drying process, but have the potential of destroying the compound. In this study, the effects of processing temperatures on two spray dried products were also evaluated and compared to crystalline D_3.

MATERIALS AND METHODS

General Procedures

Three experiments were conducted for a period of 16d using 240 mixed-sex 1-d-old Ross × Ross chicks (each) from a commercial hatchery. All procedures that involve research animals have been approved by the University of Georgia IACUC. The corn-soybean meal basal diet used is shown in Table 5.1. All experiments were conducted in electrically heated wire mesh-floor battery brooders with feed and water always available. Ultraviolet irradiation was eliminated from the chick room by completely covering glass windows with opaque plastic sheets and by fitting Arm-a-Lite® sleeves to all the fluorescent fixtures in the room and battery brooders (Edwards et al., 1994). The fluorescent lights were on 24 h each day. The temperature of the room was maintained at 22 C.

At the termination of the experiments, one bird from each pen was selected randomly and a blood sample was obtained by heart puncture for plasma Ca content. The birds were weighed by pen and their feed consumption was recorded. They were then killed by carbon dioxide asphyxiation and examined at random for rickets and tibial dyschondroplasia (TD). A longitudinal cut was made across the right tibia and diagnosis for rickets was made based on the

width of the growth plate band and its appearance (Edwards et al., 1994). Bone ash on a dry fat-free basis (AOAC International, 1995) was determined on the left tibia.

Pure crystalline cholecalciferol (D_3) designated by Sigma Chemical Co. as Sigma Reference Standard meets or exceeds all U.S. pharmacopoeia specifications and was used in both of these studies. All 1α-OHD_3 compounds were provided by Vitamin Derivatives, Inc. (Winterville, GA). Crystalline D_3 and 1α-OHD_3 compounds were diluted with propylene glycol to produce dilutions of 10 μg/mL and 50 μg/mL of the D_3 and dilutions of 2 μg/mL and 10 μg/mL of the 1α-OHD_3 compounds, which were used in the preparation of the experimental diets.

Experiment 1

The basal diet (Table 5.1) and two levels of D_3 were fed (at 2.5, and 5μg/kg) of feed to establish the responses to D_3. Two levels of spray dried 1α-OHD_3 (0.625, and 1.25 μg/kg) were fed. One level of crystalline 1α-OHD_3 was fed at 1.25 μg/kg of feed. Four pens of 10 day-old, mixed-sex Cobb x Cobb chicks were fed each diet for 16 d.

Experiment 2

The basal diet (Table 5.1) and three levels of D_3 (at 2.5, 5, and 10 μg/kg of feed) were fed to establish the responses to D_3. Two levels of 1α-OHD_3 processed at 100°C (1α-OHD_3 A) and at 200°C (1α-OHD_3 B) were fed (0.625, and 1.25 μg/kg). Three pens of 10 day-old, mixed-sex Cobb x Cobb chickens were fed each diet for 16 d.

Experiment 3

The basal diet (Table 5.1) and three levels of D_3 (at 2.5, 5, and 10 μg/kg of feed) were fed to establish the responses to D_3. Two levels of 5, 6 cis 1α-OHD_3 and 5, 6 trans 1α-OHD_3 were

fed (0.625, and 1.25 μg/kg). Three pens of 10 day-old, mixed-sex Cobb x Cobb chickens were fed each diet for 16 d.

Statistical analyses

Analysis of variance and regression analyses were completed using the general linear models procedure of SAS software (SAS Institute,2008). In Experiment 1, treatment means were compared using the Student Newman Keul's multiple range test. Relative potencies of the tested products were determined by a slope ratio method (Littell et al., 1995).

RESULTS

Experiment 1

Addition of D_3 increased BWG and tibia ash, while having no effect on calcium or TD incidences (Tables 2, 3). Feed intake, feed efficiency and TD incidence and severity were not affected by treatments. Both the spray dried and crystalline 1α-OHD_3 increased BWG, tibia ash, and reduced Ca rickets incidence and severity more efficiently than higher levels of D_3. The effect of 0.625 μg/kg of 1α-OHD_3 on body weight gain was comparable to the effect of 5 μg/kg of D_3, and higher than that for tibia ash. The effects of spray dried and crystalline 1α-OHD_3 supplied at the same level were comparable for all the parameters measured, except for percent tibia ash and Ca rickets incidence, for which the effect of crystalline 1α-OHD_3 were better.

Experiment 2

Chicks responded to D_3 levels with increased BWG, feed intake, tibia ash, plasma Ca, and reduced TD incidence (Table 5.4, 5.5, and 5.6). Feed efficiency was not affected by treatment. 1α-OHD_3 processed at 100°C (1α-OHD_3 A) increased BWG and feed intake while 1α-OHD_3 processed at 200°C (1α-OHD_3 B) did not. 1α-OHD_3 B increased BWG and feed intake

when added at 0.625 µg/kg, but not at 1.250 µg/kg. 1α-OHD_3 processed at either temperature was 8 to 15 times more effective (weight basis) than D_3 in increasing plasma Ca, tibia ash, and reducing TD incidence. The differences between 1α-OHD_3 processed at the two temperatures that were observed for body weight and feed intake were not observed for plasma Ca and tibia ash. Both 1α-OHD_3 reduced the severity of TD (score), incidence and severity of Ca rickets, whereas D_3 fed at the level fed in this experiment did not. The confidence interval for relative bioavailability analysis for some of the criteria measured (TD score, Ca-deficiency rickets incidence and severity) were larger than others due to the lack of response (slope) from levels of D_3.

Experiment 3

Increasing levels of vitamin D_3 increased body weight gain, feed intake, but not feed efficiency (Table 5.7). The 5, 6 trans 1α-OHD_3 did not have activity on the criteria measured, and subsequently excluded from the slope ratio analysis. The basal diet served as the first point in both the D_3 and 5,6 cis 1α-OHD_3 analysis. From the analysis, 5,6 cis 1α-OHD_3 was 7 and 6 more active in increasing BWG and feed intake, respectively. Feed efficiency was not affected by treatments.

Vitamin D_3 increased plasma Ca, and percent and mg ash / tibia (Table 5.8). The 5,6 cis 1α-OHD_3 were 5 to 6 times more effective in increasing the above criteria, while the trans isomer was not active.

TD incidence was increased by the addition of both D_3 and 5,6 cis 1α-OHD_3; however, observed differences were probably due to chance ($p<0.05$). The incidence, but not the severity of calcium rickets deficiency was reduced by increasing levels of D_3 and 5,6 cis 1α-OHD_3.

DISCUSSION

The results from experiment 1, 2 and 3 indicate that 1α-OHD_3 is an effective alternative to cholecalciferol as the vitamin D source for broiler chicks, consistent with previous research (Haussler et al, 1973; Edwards et al, 2002). However, the birds in Experiment 1 and 3 were still deficient in vitamin D_3. While the tibia ash data implied that the birds have acceptable (if not adequate) bone mineralization, optimal body weight gain was not achieved compared to previous experiments with adequate or excess vitamin D (Edwards et al, 2002). A portion of birds which received the highest levels of D_3 and 1α-OHD_3 also still displayed Ca rickets and TD, which was also observed in previous studies (Edwards, 1990; Edwards et al, 2002). According to NRC (1994), there should have been plenty of Ca, available phosphorus and vitamin D (in the treatment with 5 μg/kg of D_3). However, it is clear that NRC recommendation for vitamin D was not enough to prevent rickets in young broiler chicks. The birds in Experiment 2 which received either one of the 1α-OHD_3 tested had observed higher body weight gain, bone ash, and much lower TD and calcium rickets incidence compared to in Experiment 1 and 3. The compounds that were used in each experiment were processed slightly differently (crystalline vs. spray dried, processing temperature), and might have different activities in chicks. The differences between experiments might also due to differences in chick quality.

Slope ratio analysis had been used before to estimate the relative bioavailability of 1α-OHD_3 as compared to D_3 (Edwards et al, 2002). The standard errors and confidence intervals reported in this study were larger than those in Edwards et al (2002). The previous study reported standard error of 0.03 for RBV of 4.48 (from measurements of percent bone ash). In comparison, in Experiment 3, 1α-OHD_3 was 5.7 ± 0.14 more active than D_3 in increasing percent bone ash; in Experiment 2, the RBV was about 10 ± 7, which might be due to the fact that percent bone ash

peaked at middle level of the 1α-OHD_3 supplied. In the previous paper, when several of the criteria peaked at intermediate levels of supplementation, the levels on the plateau were not used to obtain regression equation for the slopes, which was done in this paper (which often only had 3 levels in the regression).

The processing temperature on spray dried 1α-OHD_3 was indicated in Experiment 2. While not much has been reported for the stability of 1α-OHD_3, increasing temperature has been shown to increase loss during pelleting (Riaz et al, 2009). The 1α-OHD_3 processed at higher temperature increased percent tibia ash and plasma Ca, but not body weight gain. The birds which received 1.25 μg/kg seemed to exhibit some of the Ca toxicity, as indicated by high plasma Ca level and lowered body weight gain. Toxicity due to 1α-OHD_3 was not expected at this level, as higher levels of 1α-OHD_3 have been fed with no adverse effects on body weight gain (Edwards, 1994, 2002; Biehl et al., 1995,1998; Biehl and Baker, 1997a,b, Edwards et al, 2002).

In general, 1α-OHD_3 is a suitable alternative to cholecalciferol as the sole source of vitamin D in the diet. Except for the 5,6 trans 1α-OHD_3, all 1α-OHD_3 regardless of preparation were approximately 5 to 12 times more active than D_3, depending on the parameter measured

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TABLE 5.1. Composition of basal diets, Experiments 1, 2, and 3

Ingredient	Amount
	----------%----------
Ground yellow corn	53.065
Soybean meal (dehulled)	38.00
Vegetable oil	5.00
Iodized sodium chloride	0.45
DL-Methionine	0.22
Vitamin premix[1]	0.05
Trace mineral premix[2]	0.075
Dicalcium Phosphate	1.86
Limestone	1.28
Calculated composition[3]	
ME, kcal/g	3.15
CP, %	23.23
Calcium, %	1.00
Phosphorus-total, %	0.73
Available phosphorus, %	0.49

[1]Supplied in milligrams per kilogram of diet : vitamin A (as all trans-retinyl acetate), 5,500 IU; vitamin E (all-rac-α-tocopheryl acetate), 11 IU; riboflavin, 4.4; Ca panthotenate, 12; nicotinic acid, 44; choline Cl, 220; vitamin B_{12} , 9 μg; vitamin B_6, 3.0; menadione (as menadione sodium bisulfite), 1.1; thiamin (as thiamin mononitrate), 2.2; folic acid, 3.0; biotin, 0.3; and ethoxyquin, 125.

[2]Trace mineral mix provides the following (per kilogram of diet): manganese (MnSO4.H2O), 101 mg; iron (FeSO4.7H2O), 20 mg; zinc (ZnO), 80 mg; copper (CuSO4.5H2O), 3 mg; iodine (ethylene diamine dihydroiodide), 0.75 mg, magnesium (MgO), 20 mg; selenium (sodium selenite), 0.3 mg.

[3]Calculated from NRC (1994).

TABLE 5.2. Effect of vitamin D_3 and 1-alpha hydroxycholecalciferol [spray dry (SD) and crystalline (C)] on body weight gain, feed intake and feed conversion. Experiment 1

Treatment	D-source	Level	n	BWG	Feed intake	FCR
		µg/kg		g	g	g/g
1	D_3	0	4	313±14 [d]	421±8	1.35±0.04
2	D_3	2.5	4	322±5 [d]	439±6	1.36±0.01
3	D_3	5.0	4	365±10 [bc]	457±15	1.25±0.03
4	1α-OHD_3 SD	0.625	4	341±11 [cd]	459±11	1.35±0.02
5	1α-OHD_3 SD	1.250	4	400±3 [ab]	503±19	1.26±0.04
6	1α-OHD_3 C	1.250	4	386±10 [a]	474±38	1.23±0.08
ANOVA	p-value			0.0001	0.1151	0.1204

[a–d]Means within a column with no common superscript are significantly different ($P < 0.05$), SNK.

TABLE 5.3. Effect of vitamin D_3 and 1-alpha hydroxycholecalciferol [spray dry (SD) and crystalline (C)] on percentage tibia ash, milligrams of tibia ash, Ca rickets incidence, Ca rickets score 3 and TD incidence and TD score 3. Experiment 1.

Treatment	D-Source	Levels	n	Tibia ash	Tibia ash	Ca rickets incidence	Ca rickets score 3	TD incidence	TD score 3
		µg/kg		%	mg	%	%	%	%
1	D_3	0	4	24.08±0.20 [f]	224±7 [d]	82±9 [ab]	50±2 [a]	18±5	10.5±4.5
2	D_3	2.5	4	25.78±0.25 [e]	258±6 [c]	90±0 [a]	55±9 [a]	23±6	7.5±2.5
3	D_3	5.0	4	29.22±0.27 [c]	323±11 [b]	67±10 [ab]	48±14 [a]	21±5	8.0±5.3
4	1α-OHD_3 SD	0.625	4	26.81±0.52 [d]	275±10 [c]	80±8 [ab]	60±8 [a]	20±9	12.5±7.5
5	1α-OHD_3 SD	1.250	4	31.43±0.28 [b]	382±11 [a]	54±11 [b]	16±6 [b]	10±7	5.0±2.9
6	1α-OHD_3 C	1.250	4	32.95±0.27 [a]	384±12 [a]	27±2 [c]	20±4 [b]	7±5	5.0±2.9
ANOVA	p-value			0.0001	0.0001	0.0002	0.0030	0.4531	0.8276

[a–f]Means within a column with no common superscript are significantly different ($P < 0.05$), Student Neuman Keul.

TABLE 5.4. Slope ratio analysis for the effects of cholecalciferol, 1α-OHD_3 A and B on 16 d body weight gain, feed intake and feed efficiency. Experiment 2.

D_3	1α-OHD_3 A	1α-OHD_3 B	BWG	Feed intake	Feed efficiency
µg/kg	µg/kg	µg/kg	g	g	g/g
0	-	-	315±11	431±17	1.36±0.01
2.5	-	-	394±5	490±18	1.24±0.03
5	-	-	433±26	562±5	1.31±0.09
10	-	-	454±9	584±7	1.29±0.01
-	0.625	-	441±17	580±5	1.32±0.06
-	1.250	-	449±7	560±13	1.25±0.02
-	-	0.625	456±1	583±7	1.28±0.01
-	-	1.250	361±13	465±8	1.29±0.03

	1α-OHD_3 A	1α-OHD_3 B	1α-OHD_3 A	1α-OHD_3 B	1α-OHD_3 A	1α-OHD_3 B
Parameters			p-value			
D_3	0.0001	0.0173	.0005	.0132	.3924	.5107
1α-OHD_3	0.0004	0.3746	.0007	.4143	.2817	.5800
Slope difference	< 0.0001	-	< 0.0001	-	-	-
Curvature	0.3105	-	.0179	-	-	-
Intercept	352.706	-	466.170	-	-	-
Slope D_3	11.593	-	13.039	-	-	-
1α-OHD_3	89.914	-	96.277	-	-	-
Relative bioavailability	7.756	-	7.384	-	-	-
Standard error	1.714	-	1.735	-	-	-
Lower 95% fiducial limit	4.104	-	3.685	-	-	-
Upper 95% fiducial limit	11.408	-	11.082	-	-	-

TABLE 5.5. Slope ratio analysis for the effects of cholecalciferol, 1α-OHD_3 A and B on bone ash and plasma calcium. Experiment 2.

D_3	1α-OHD_3 A	1α-OHD_3 B	Bone ash	Bone ash	Plasma Ca
µg/kg	µg/kg	µg/kg	mg/tibia	%	mg/dl
0	-	-	257±13	25.2±2.1	7.29±0.46
2.5	-	-	353±9	29.2±1.3	8.20±0.41
5	-	-	529±31	34.5±1.3	9.26±0.38
10	-	-	609±17	38.4±0.5	9.74±0.16
-	0.625	-	639±36	39.6±0.3	10.32±0.27
-	1.250	-	604±19	39.7±0.2	10.28±0.29
-	-	0.625	660±25	39.8±0.1	10.11±0.63
-	-	1.250	499±30	39.1±0.5	11.76±0.38

Parameters	1α-OHD_3 A	1α-OHD_3 B	1α-OHD_3 A	1α-OHD_3 B	1α-OHD_3 A	1α-OHD_3 B
			p-value			
D_3	.0005	.0045	.0008	.0013	.0042	.0009
1α-OHD_3	<.0001	.0055	<.0001	.0002	.0001	<. 0001
Slope difference	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
Curvature	0.0082	0.0054	.0858	.1011	0.2296	0.3286
Intercept	321.432	332.373	27.947	27.899	7.861	7.656
Slope D_3	30.394	28.935	1.067	1.073	0.203	0.231
1α-OHD_3	282.656	239.949	11.256	11.681	2.335	3.410
Relative bioavailability	9.309	8.293	10.549	10.881	11.488	14.794
Standard error	1.708	2.510	7.603	7.442	2.261	1.839
Lower 95% fiducial limit	5.668	2.910	-5.184	-5.195	6.668	10.874
Upper 95% fiducial limit	12.950	13.675	27.057	26.957	16.308	18.715

TABLE 5.6. Slope ratio analysis for the effects of cholecalciferol, 1α-OHD_3 A and B on bone ash and plasma calcium. Experiment 2.

D_3	1α-OHD_3 A	1α-OHD_3 B	TD incidence	TD score	Ca-deficiency rickets incidence	Ca-rickets score
µg/kg	µg/kg	µg/kg	%		%	
0	-	-	92.6±7.4	2.74±0.26	24.8±2.6	2.83±0.17
2.5	-	-	96.7±3.3	2.72±0.12	22.57±8.9	2.33±0.67
5	-	-	58.0±10.3	2.78±0.22	29.4±2.4	2.78±0.22
10	-	-	20.0±15.3	1.80±0.92	16.7±6.7	2.67±0.33
-	0.625	-	0±0	0.00±0.00	0.00±0.00	0.00±0.00
-	1.250	-	7.4±7.4	0.67±0.67	0.00±0.00	0.00±0.00
-	-	0.625	7.0±3.5	2.00±1.00	3.70±3.70	1.00±1.00
-	-	1.250	3.7±3.7	0.67±0.67	0.00±0.00	0.00±0.00

Parameters	1α-OHD_3 A	1α-OHD_3 B	1α-OHD_3 A	1α-OHD_3 B	1α-OHD_3 A	1α-OHD_3 B	1α-OHD_3 A	1α-OHD_3 B
					p-value			
D_3	.0051	.0017	.5785	.2012	.6540	.5399	.5104	.8921
1α-OHD_3	<.0001	<.0001	.0042	.0077	.0009	.0008	<. 0001	.0005
Slope difference	< 0.0001	< 0.0001	.0010	.0155	.0003	.0006	< 0.0001	.0008
Curvature	.2546	.2403	.5598	.6543	.1620	.1810	.4282	.6189
Intercept	90.112	92.015	2.542	2.971	23.625	24.419	2.371	2.585
Slope D_3	-6.439	-6.693	-.044	-.101	-.329	-.435	.037	.009
1α-OHD_3	-81.771	-83.716	-2.014	-1.785	-22.680	-22.257	-2.276	-2.162
Relative bioavailability	12.699	12.508	45.524	17.610	68.926	51.194	-61.042	-248.066
Standard error	2.324	1.993	13.467	5.708	16.735	12.195	11.372	55.551
Lower 95% fiducial limit	7.746	8.261	16.820	5.445	33.257	25.201	-85.282	-366.471
Upper 95% fiducial limit	17.652	16.755	74.229	29.776	104.595	77.186	-36.803	-129.660

TABLE 5.7. Mean body weight gain (BWG), plasma Ca, tibia ash, slope ratio analysis and relative biological value (RBV) of 1α-OHD_3 as compared to D_3. Experiment 3.

D_3	5,6 cis 1α-OHD_3	5,6 trans 1α-OHD_3[1]	BWG [2]	Feed intake	Feed efficiency
µg/kg	µg/kg	µg/kg	g	g	g/g
0			259 ± 6	429 ± 18.5	1.66 ± 0.07
2.5			304 ± 12	507 ± 14.9	1.67 ± 0.02
5			335 ± 10	522 ± 24.7	1.56 ± 0.01
10			335 ± 20	549 ± 43.2	1.64 ± 0.04
	0.625		309 ± 13	494 ± 17.6	1.60 ± 0.01
	1.250		338 ± 9	535 ± 18.9	1.58 ± 0.02
		0.625	223 ± 16	407 ± 15.4	1.84 ± 0.08
		1.250	247 ± 6	427 ± 7.3	1.73 ± 0.02
Slope ratio analysis					
Source of variation					
Level			0.0083	0.0234	0.9288
Linear differences			0.0036	0.0408	0.2087
Quadratic			0.2018	0.9578	0.0685
Intercept			277.2166 ± 10.2358	454.6006 ± 18.0240	-
Slope D_3			7.1152 ± 1.8611	10.7887 ± 3.2242	-
Slope 1α-OHD_3			49.2533 ± 14.0248	64.3833 ± 24.6957	-
RBV (1α-OHD_3)			6.92	5.97	-
Standard error			0.26	0.34	-
Lower 95% fiducial limit			6.355	5.240	-
Upper 95% fiducial limit			7.489	6.696	-
R^2			0.55	0.45	-

[1]Source not included in the slope ratio analysis
[2]Mean ± SE

TABLE 5.8. Mean body weight gain (BWG), plasma Ca, tibia ash, slope ratio analysis and relative biological value (RBV) of 1α-OHD_3 as compared to D_3. Experiment 3.

D_3	5,6 cis 1α-OHD_3	5,6 trans 1α-OHD_3[1]	Plasma Ca [2]	Tibia ash	Tibia ash
µg/kg	µg/kg	µg/kg	mg/dl	%	mg
0			6.23 ± 0.84	24.80 ± 0.44	2253 ± 106
2.5			8.23 ± 0.38	27.57 ± 0.53	3561 ± 212
5			9.23 ± 0.67	33.40 ± 0.78	4241 ± 242
10			10.10 ± 0.20	36.93 ± 0.23	4967 ± 469
	0.625		7.70 ± 0.42	28.07 ± 2.09	3632 ± 293
	1.250		9.00 ± 0.26	34.73 ± 0.54	4701 ± 290
		0.625	8.47 ± 0.65	25.03 ± 0.42	2206 ± 154
		1.250	6.50 ± 0.15	23.79 ± 0.76	2321 ± 154
Slope ratio analysis					
Source of variation					
Level			0.0004	0.0001	0.0003
Linear differences			0.0186	0.0001	0.0003
Quadratic			0.5664	0.0471	0.5699
Intercept			6.817142 ± 0.3908	24.7928 ± 0.8301	2623.821 ± 223.463
Slope D_3			0.3700 ± 0.0699	1.3057 ± 1.1484	258.024 ± 39.974
Slope 1α-OHD_3			1.6857 ± 0.5355	7.4095 ± 1.1373	1651.905 ± 306.180
RBV (1α-OHD_3)			4.556	5.675	6.402
Standard error			0.577	0.135	0.168
Lower 95% fiducial limit			3.325	5.387	6.043
Upper 95% fiducial limit			5.787	5.963	6.761
R^2			0.65	0.85	0.76

[1]Source not included in the slope ratio analysis
[2]Mean ± SE

TABLE 5.9. Mean TD incidence, TD score, Ca rickets incidence, Ca rickets score and slope ration analysis and relative biological value (RBV) of 1α-OHD_3 as compared to D_3. Experiment 3.

D_3	5,6 cis 1α-OHD_3	5,6 trans 1α-OHD_3[1]	TD incidence	TD score	Ca rickets incidence	Ca rickets score
µg/kg	µg/kg	µg/kg	%		%	
0			0.08 ± 0.04	2.00	100 ± 0.00	2.83 ± 0.17
2.5			0.07 ± 0.03	1.67	97 ± 3.33	2.61 ± 0.14
5			11.10 ± 6.41	1.33	61 ± 3.21	2.50 ± 0.10
10			16.67 ± 6.67	2.90	30 ± 5.77	2.50 ± 0.29
	0.625		3.33 ± 3.33	0.33	97 ± 3.33	2.63 ± 0.12
	1.250		3.33 ± 3.33	1.00	30 ± 5.77	2.73 ± 0.15
		0.625	10.83 ± 0.83	2.67	100 ± 0.00	2.77 ± 0.03
		1.250	6.67 ± 3.33	1.67	100 ± 0.00	2.70 ± 0.10
Slope ratio analysis						
Source of variation						
Level			0.0065	0.1066	0.0001	0.1984
Linear differences			0.6287	0.3784	0.0001	0.9669
Quadratic			0.3185	0.4605	0.0211	0.7381
Intercept			-	-	111.131 ± 5.929	-
Slope D_3			-	-	-8.3666 ± 1.0606	-
Slope 1α-OHD_3			-	-	-56.5523 ± 8.1236	-
RBV (1α-OHD_3)			-	-	6.759	-
Standard error			-	-	0.133	-
Lower 95% fiducial limit			-	-	6.476	-
Upper 95% fiducial limit			-	-	7.043	-
R^2			-	-	0.567	-

[1]Source not included in the slope ratio analysis
[2]Mean ± SE

CHAPTER 6

EFFECTS OF LITHOCHOLIC ACID AND CHOLECALCIFEROL ON PLASMA CALCIUM AND CALBINDIN EXPRESSION IN COMMERCIAL BROILER CHICKS[1]

[1]Liem, A., G.M. Pesti, R. B. Beckstead, and H.M. Edwards Jr. To be submitted to Poultry Science

ABSTRACT

Lithocholic acid (LCA) is one of the main bile acids found in mammals and birds. While bile acids are predominantly known for their function in digestion, LCA has been reported to act as a ligand for vitamin D receptor. It has also been reported to carry out some functions of vitamin D in mice by increasing expression of calbindin, TRPV6 (a calcium channel), and ATP2B1 (CaATPase) in intestinal mucosa. Since there are similarities between mammalian and avian vitamin D receptors, we tested the hypothesis that LCA would have similar D_3 signaling capacity in chicken. LCA has also been shown to cause growth depression and growth suppression in chicks. This study also investigated if vitamin D_3 can alleviate the hepatotoxicity due to dietary LCA.

To investigate if LCA has vitamin D_3 activity in chickens, 240 Heritage broilers were randomly assigned to 6 treatments and raised from d 1-15 in a battery brooder. The first 3 experimental diets were fed from d1 to 15: basal diet deficient in vitamin D_3 (B), B+ 0.2% LCA, B + 400 ICU/kg vitamin D_3. The next 3 experimental diets were fed from d 8-15 (birds in these treatments received B diet from day1-7): B + 0.1% LCA, B+0.2% LCA, B + 400 ICU/kg vitamin D_3. To investigate if vitamin D_3 can alleviate the hepatotoxicity due to dietary LCA, 240 chicks were randomly assigned to 8 treatments of a 2 x 4 factorial design with 0 or 0.1% LCA, in combination with 4 levels of vitamin D_3 from 0 to 400 U/kg of diet.

Vitamin D_3, added at d 1 or d 8 increased body weight gain (BWG), increased plasma Ca, reduced the incidence of Ca rickets, and increased expression of calbindin. At the levels fed, LCA linearly increased plasma Ca and liver weight, and reduced the incidence of Ca rickets. At 0.1%, LCA increased expression of calbindin, but did not significantly affect expression of

ATP2B1. At 0.2%, LCA had no effect on the genes tested. Vitamin D_3 did not have any effect on relative liver weight, and did not alleviate the hepatotoxicity due to dietary LCA.

In conclusion, LCA increased plasma Ca and expression of calbindin in chicken intestinal mucosa ($p<0.05$). At 0.1%, LCA appears to have some vitamin D_3 activity in chickens. While at 0.2%, LCA is toxic to the birds and might cause an overall disruption of metabolic processes.

Keywords: cholecalciferol, lithocholic acid, vitamin D receptor

INTRODUCTION

Bile acids are mostly known for their function in aiding lipid digestion their role in cholesterol metabolism. However, recent studies showed that certain bile acids also play a role in signaling pathways. Lithocholic acid is one of the main bile acids found in mammals and birds. It is a secondary bile acid, formed from primary bile acids chenodeoxycholic acid and ursodeoxycholic acid which are produced in liver from cholesterol. The primary bile acids above are conjugated with taurine or gylcine, and enter enterohepatic circulation. In the intestinal lumen, the acids are deconjugated (by bacterial peptidases) and converted to lithocholic acid by 7α-dehydroxylation by bacteria (Hofmann, 2004).

There were numerous studies published in the 1960's reporting the effect of dietary LCA in chicks and hens. Feeding LCA at 0.2% severely depressed growth and increased serum cholesterol in chicks (Edwards, 1961). Another research group published a series of paper reporting that dietary LCA increased plasma lipid phosphorus levels, liver size (by 3.5 times normal) which was partially attributed to bile duct proliferation (Leveille et al, 1962; Hunt et al, 1964; Hunt et al, 1963).

The effect of LCA varies when fed to other species. In rabbit (which naturally has less chenodeoxycholic acid compared to other species), LCA at 0.25 % decreased body weight, increased cholesterol and plasma phospholipids, similar to effects in chickens. In guinea pig, hamster, and mouse, feeding LCA at the same level did not reduce body weight, or increase cholesterol. The increased liver weights were also not observed in guinea pig, hamster, and mouse (Hunt et al, 1964).

Lithocholic acid is not toxic or only mildly toxic in species capable of one or more mechanism to detoxify LCA. Chronic feeding of LCA in rats only caused a mild hepatotoxicity,

due to rat's liver's ability to hydroxylate LCA at C-6. LCA is also relatively non toxic to human. In human, conjugated LCA is sulfated at C-3, secreted in bile, and eventually excreted in the feces. More recent studies suggested that bile acids are involved in signaling pathways, generally acting as activators of transcription factors (nuclear receptors) whose target genes appear to play a role in bile acids synthesis, conjugation, transport, and also members of the cytochrome P450 system (Hoffman, 2004).

Vitamin D receptor (VDR) is a member of nuclear receptor family. Upon binding of the ligand, VDR heterodimerizes with the retinoid X receptor (RXR), and binds to specific DNA sequences (response elements) in the promoter regions of the target genes. Vitamin D target genes are involved in a wide array of physiological functions. Calbindin , and ATP2B1 are among the genes influenced by vitamin D (and or VDR) which are involved in calcium transport, and calcium absorption. A recent study also reported that vitamin D, through VDR, increased transcription of CYP3A4, a member of the P450 family which is involved in cellular detoxification in human colon cancer cell line HT-29 (Jurutka, et al, 2005). VDR was also reported to regulate transcription of sulfotransferase (SULT2A1), which is involved in LCA detoxification, in humans, rats and mice (Chatterjee et al., 2005; Seo et al., 2007).

Vitamin D receptor is not only activated by 1,25 dihydroxy D_3. Makishima, et al (2001) reported that LCA and its 3-keto metabolite activate VDR, as determined in "a ligand-screening assay based on the ligand-induced interaction of a nuclear receptor with its coactivator". Nehring et al. (2007) went further to demonstrate that LCA has vitamin D activity in vivo in rats. They reported that dietary LCA increases plasma Ca, increases the expression of TRPV6, calbindin, and Ca^{2+}ATPase mRNA in the intestine.

Previous research has shown that the effect of LCA differs in chickens and other species. It also known that chicken and other species differ in their response to different vitamin D; that is vitamin D_2 is not active in birds, but is active in other species. This study investigated the involvement of lithocholic acid (LCA) in pathways normally regulated by vitamin D_3 in chickens, especially Ca homeostasis. The objectives of this study are to investigate if LCA has vitamin D_3 activity in chicks; and if vitamin D_3 can alleviate the hepatotoxicity due to LCA in chicks.

MATERIALS AND METHODS

Animal Study

Two experiments were conducted for a period of 15 d each using mixed-sex 1-d-old Heritage chicks from a commercial hatchery. All procedures were approved by the University of Georgia IACUC. The corn-soybean meal basal diet used is shown in Table 6.1. Both experiments were conducted in electrically heated wire mesh-floor battery brooders with feed and water always available. Ultraviolet irradiation was eliminated from the chick room by completely covering glass windows with opaque plastic sheets and by fitting Arm-a-Lite® sleeves to all the fluorescent fixtures in the room and battery brooders (Edwards et al., 1994). The fluorescent lights were on 24 h each day. The temperature of the room was maintained at 22 C.

In Experiment 1, 240 day ld chicks were randomly assigned to 6 dietary treatments, with 4 replicate pens of 10 chicks. The first 3 treatments (Table 6.2) were fed from day 1 to 15: Basal, Basal + 0.2% LCA, and Basal + 400 IU/kg of D_3. On day 7, blood samples were taken from 2 birds from each pen in these treatments for plasma Ca determination. Upon observation of

differences in plasma Ca among these treatments, the next 3 treatments were introduced to the rest of the birds. Treatments 4, 5, and 6 were: Basal + 0.1% LCA, Basal + 0.2% LCA, and Basal + 400 IU/kg of D_3. The birds in these treatments received basal diet from day 1-7.

In Experiment 2, chicks were randomly assigned to 8 dietary treatments with 3 replicate pens of 10 chicks each. The same basal diet was employed in a 2 x 4 factorial design. LCA was added at 0 or 0.1%, in combinations with 0, 100, 200 or 400 IU/kg of D_3.

At the termination of the experiments and on day 7 in Experiment 1, two birds from each pen were selected randomly and blood samples obtained by heart puncture for plasma Ca content. The birds were weighed by pen and their feed consumption recorded. They were then killed by carbon dioxide asphyxiation and examined at random for rickets and tibial dyschondroplasia (TD). A longitudinal cut was made across the right tibia and diagnosis for rickets was made based on the width of the growth plate band and its appearance (Edwards et al., 1994). Bone ash on a dry fat-free basis (AOAC International, 1995) was determined on the left tibia. Liver weights were obtained for two birds from each pen.

RNA Extraction and RT-PCR

Duodenal mucosa samples were collected from 2 birds from each pen in Exp. 1. The sample was taken from a $_3$ cm section on the ascending portion of the duodenal loop. The section was cleaned from digesta by spraying sterile saline solution. The mucosa is then scraped with a glass slide, placed in cryogenic vials and immediately immersed in liquid nitrogen prior tostorage in -80 ^{o}C freezer. Total RNA was extracted from tissue samples using the Tripure Isolation Reagent (monophasic solution of phenol and guanidine thiocyanate). RNA samples were stored at -80^{o}C.

Primers and TaqMan probes specific for chicken ATP2B1 (GeneID: 374244), calbindin 28kD (GeneID: 396519), and GAPDH (GenBank accession # M11213) were generated using Primer Express software version 2.0 (Applied Biosystems, Table 5.1) and were synthesized by Applied Biosystems. The probes for GHSR and GAPDH were labeled at the 5' end with FAM (6-carboxyfluorescein) as the reporter dye and at the 3' end with TAMRA (6-carboxy-*N,N,N'*,*N'*-tetramethylrhodamine) as the quencher dye. The forward, reverse and probe for all assays are as below:

Chicken GAPDH Real Time PCR Assay

Forward Primer: TTG GCA TTG TGG AGG GTC TT (20 BASES)

Reverse Primer: GGG CCA TCC ACC GTC TTC (18 BASES)

Probe: TGA CCA CTG TCC ATG CCA T (19 BASES)

Calbindin

Forward primer: CCGCCCAGTTCTTCGAGAT (19)

Reverse Primer: TGTAGCTCCTTCCCATCCATGT (22)

Probe: ACCACTACGACTCCGACG (18)

ATP2B1

Forward primer: TCGTGGTCAGATCTTGTGGTTT (22)

Reverse Primer: CTCATACAAAGAACTACGAAATGCATT (27)

Probe: CAGAATACAAACTCAGATACGAG (23)

CYP24

Forward primer: GGGCTACGGGCTGCTGAT (18)

Reverse Primer: TCTTCTGAAAGGCGCTTCTCA (21)

Probe: CTGGAAGGAAAGGACT (16)

Two step real time PCR was used. The cDNA was generated using the TaqMan Reverse Transcription kit (Applied Biosystems) following the manufacturer's protocol. The samples were stored at -20^{o}C or directly used in the next step. The real time reaction uses 25 uL sample reaction containing 100 ng cDNA, 1x TaqMan Universal PCR Master Mix (Applied Biosystems), 900 nM of the appropriate primer pair and 25 nM of the appropriate probe. The reactions were completed in an ABI 7500 Thermocycler (Applied Biosystems) with the following conditions: 10 minutes at 95 ^{o}C and 40 cycles each of 15 seconds at 95 ^{o}C and 1 minute at 60 ^{o}C. The reactions for each sample were performed in duplicate for all assays.

Statistical Analyses

In Experiment 1, PROC ANOVA was applied to detect differences between the means. The first, fourth and fifth treatments were also subjected to regression analysis to evaluate if there were linear or quadratic trends between those treatments. In Experiment 2, analysis of variance appropriate for factorial design was applied. For both experiments, while relative quantification ± SE was reported in the tables, all statistical analysis were performed on the data of dCt, as one of the options described in Yuan et al.(2006). The relative quantification (RQ/fold induction) was calculated with the $2^{-\Delta\Delta Ct}$ method. The amount of target, normalized to an endogenous reference and relative to a calibrator, is given by the formula $2^{-\Delta\Delta Ct}$. Hence, small variation in the dCt resulted in much larger variation in the fold induction. Employing dCt in the statistical

analyses also allowed each treatment to have variance, as opposes to using RQ data (in which the control/calibrator treatment will have no variance).

RESULTS

Experiment 1

Vitamin D_3 deficiency was established in birds fed the basal vitamin D_3 deficient diet by day 7, as indicated by the difference in plasma Ca (Table 6.4). The administration of LCA since day 1 reduced BWG, compared to the positive control (Basal + vitamin D_3) diet, and the basal diet (Table 6.2). However, when added from day 8, chicks fed 0.1 % LCA had better BWG at 15 d compared to the chicks fed basal diet, while chicks fed 0.2% LCA had the same BWG as the chicks fed basal diets.

LCA fed since d 1 increased plasma Ca at d 7, but the increase was not as large as the effect of vitamin D_3 (Table 6.4). Plasma Ca measured at d 15 was increased by the addition of vitamin D_3 and 0.2% LCA since d 1 or d 8, but not by 0.1% LCA added since d 8. When regression analysis was performed, increasing levels of LCA linearly increased plasma Ca. There was no significant difference in d 15 plasma Ca between the vitamin D_3 groups and the 0.2% LCA groups. Only vitamin D_3 added since d 1 or d 8 increased percent bone ash and milligrams ash / tibia.

TD incidence was not significantly affected by LCA, or vitamin D_3 added since day 1 (Table 6.3). The birds that received diet with vitamin D_3 from d 8 had significantly higher incidences of TD compared to other treatments. The incidence of Ca rickets was linearly decreased by increasing levels of LCA. However, the incidence of P rickets was increased linearly by LCA. Vitamin D_3 decreased the incidence of both Ca rickets and P rickets.

The expression of ATP2B1 was increased by the addition of vitamin D_3, but not LCA. The expression of calbindin was increased 800-900 fold by the addition of vitamin D_3. Calbindin expression was also increased by 0.1% LCA to a lesser extent, but not 0.2%LCA. Means separations by Duncan's Multiple Range Test, indicated that the expression of CYP24 was reduced by the addition of 0.2% LCA beginning on d8.

Experiment 2

In Experiment 2, birds fed 0.1% LCA had significantly lower BWG measured both on day 9 and day 15. Increasing levels of vitamin D_3 increased BWG measured on d 15, but not on d 9. There was a significant interaction between LCA and vitamin D_3 on 15d BWG. When there was no vitamin D_3 in the diet, reduction in BWG due to LCA was small, and as higher levels of vitamin D_3 were present in the diet, the difference between groups fed with and without LCA became larger. Relative liver weight was increased by 0.1% LCA. Vitamin D_3 did not affect relative liver weight, and no interactions between LCA and vitamin D_3 on relative liver weight were detected.

Birds fed 0.1% LCA had a lower incidence of TD, higher incidence of both Ca rickets and P rickets. Vitamin D_3 reduced the incidence of all leg abnormalities measured: TD, Ca and P rickets. There was no interaction between the effect of vitamin D_3 and LCA on leg abnormalities.

Plasma Ca was not increased by addition of 0.1% LCA, but was increased by vitamin D_3. Vitamin D_3 also increased bone ash percentage and mg ash/tibia, while both criteria were decreased by LCA. The interaction between the effects of LCA and vitamin D_3 on bone e ash indicated that negative effects of LCA was less prominent when no vitamin D_3 was present in the diet, and more prominent when higher levels of vitamin D_3 were present. And conversely, the

effects of vitamin D_3 were larger when no LCA was fed compared to when 0.1 % LCA was added.

Calbindin expression was increased by the addition of vitamin D_3. On average, LCA decreased the expression of calbindin, especially when higher levels of vitamin D_3 were fed, as indicated by the interaction. There was also a quadratic trend in the effect of vitamin D_3 when no LCA was added. The expression of ATP2B1 was not significantly affected by vitamin D_3, but reduced by LCA.

DISCUSSION

Vitamin D is an important hormone in regulating Ca homeostasis in the body of animals. Its active form, 1, 25 dihydroxy cholecalciferol binds to vitamin D receptor (VDR) to mediate its effects on expression of genes that play a role in Ca metabolism, such as calbindin, a calcium binding protein, ATP2B1, a Ca ATPase, among others. In Experiment 1, LCA was found to have some vitamin D activity in chicks, as indicated by the increase in plasma Ca and expression of calbindin. This was similar to the findings of Nehring et al. (2007), which reported that LCA had vitamin D activity in rats. However, the increase in ATP2B1 was not found in our study. The genetics of commercial broiler chicks are different from pure line rats, and more variation is expected with these crossbred chicks. The chicks in Experiments 1 and 2 also displayed symptoms of toxicity from dietary LCA, as it caused severe liver enlargement, accompanied with lowered body weight gain, consistent with older studies on feeding LCA to chicks (Edwards, 1961; Leveille et al, 1962; Hunt et al, 1964; Hunt et al, 1963). The severe toxicity might also interfere with overall metabolic functions, and might cause an overall decrease in gene expression.

While 0.1% LCA gave similar or better BWG compared to the vitamin D_3 deficient basal diet, when added to a diet sufficient in vitamin D_3, LCA caused markedly lower BWG. Adding vitamin D_3, did not alleviate any the hepatotoxicity due to LCA. Chicks seem to have no pathway (or at least none that is influenced by vitamin D_3 and VDR) to detoxify LCA, unlike humans and rodents.

Leg abnormalities were also influenced by dietary LCA. The reduction in incidence of TD, a leg problem often associated with rapid growth in broilers, might be caused by the reduction in growth. LCA decreased incidence of Ca rickets (and increased plasma Ca) in Experiment 1, but not in Experiment 2. This could be due to the difference in duration of LCA feeding (1 week in Experiment 1 vs. 15 days in Experiment 2). Prolonged feeding of LCA caused more severe reduction in BWG, and might cause a severe toxicity that disrupts many metabolic processes. In both experiments, LCA caused a bone abnormality characteristic of P-deficiency rickets in many of the birds. The bones were soft, poorly mineralized, which agreed with the low bone ash measurements, and had similar coloring and shape with P-deficiency rickets.

Lithocholic acid, at low levels, has some vitamin D_3 activity in chicks, as indicated by increased plasma Ca and calbindin expression. It also caused severe hepatotoxicity and growth depression to chicks. Vitamin D, and vitamin D receptor, that in some species could mediate detoxification of LCA, did not alleviate LCA toxicity in chicks.

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TABLE 6.1. Composition of basal diets, Experiments 1 and 2.

Ingredient	Amount
	----------%----------
Ground yellow corn	52.948
Soybean meal (dehulled)	38.00
Vegetable oil	5.00
Iodized sodium chloride	0.45
DL-Methionine	0.22
Vitamin premix[1]	0.067
Trace mineral premix[2]	0.075
Dicalcium Phosphate	1.86
Limestone	1.28
Cr_2O_3	0.10
Calculated composition[3]	
ME, kcal/g	3.15
CP, %	23.23
Calcium, %	1.00
Phosphorus-total, %	0.73
Available phosphorus, %	0.49

[1]Supplied in milligrams per kilogram of diet: vitamin A (as all trans-retinyl acetate), 5,500 IU; vitamin E (all-rac-α-tocopheryl acetate), 11 IU; riboflavin, 4.4; Ca panthotenate, 12; nicotinic acid, 44; choline Cl, 220; vitamin B_{12} , 9 μg; vitamin B_6, 3.0; menadione (as menadione sodium bisulfite), 1.1; thiamin (as thiamin mononitrate), 2.2; folic acid, 3.0; biotin, 0.3; and ethoxyquin, 125.

[2]Trace mineral mix provides the following (per kilogram of diet): manganese ($MnSO4 \cdot H_2O$), 101 mg; iron ($FeSO4 \cdot 7H_2O$), 20 mg; zinc (ZnO), 80 mg; copper ($CuSO4 \cdot 5H_2O$), 3 mg; iodine (ethylene diamine dihydroiodide), 0.75 mg, magnesium (MgO), 20 mg; selenium (sodium selenite), 0.3 mg.

[3]Calculated from NRC (1994).

TABLE 6.2. The effects of lithocholic acid and vitamin D_3 on 7 d body weight (BW), 15 d body weight gain (BWG), gain/feed, and relative liver weight. Experiment 1.

	Treatment Day 1-7		Day 8-15					
	LCA	D_3	LCA	D_3	d7 BW[1]	d15 BWG[1,2]	Gain/Feed	Relative liver weight[2,3]
	%	IU/kg	%	IU/kg	g	g	g/g	%
1	0	0	0	0	116 ± 3	234 ± 15[d]	1.03 ± 0.00	2.79 ±0.12[c]
2	0.2	0	0.2	0	100 ± 3	180 ± 5[e]	1.03 ± 0.00	9.90 ±0.87[a]
3	0	400	0	400	125 ± 2	380 ± 11[a]	1.02 ± 0.00	2.83 ±0.12[c]
4	0	0	0.1	0		283 ± 20[c]	1.25 ± 0.02	4.61 ±0.61[b]
5	0	0	0.2	0		246 ± 1[d]	1.34 ± 0.02	8.57 ±0.59[a]
6	0	0	0	400		335 ± 10[b]	1.16 ± 0.01	2.79 ±0.11[a]

Regression of treatments 1, 4, and 5

Parameter	df	p-value	
LCA	1	0.6459	<.0001

[1] Mean of 4 replicate pens ± SE. All 10 chicks in the pen were weighed collectively.
[2] Values within variables with no common superscripts differ significantly ($P < 0.05$) when tested with Duncan's Multiple Range Test following analysis of variance.
[3] Relative liver weight: liver weight / individual body weight x 100%. Body weight and liver weight were measured from 2 birds from each pen.

TABLE 6.3. The effects of lithocholic acid and vitamin D_3 on the incidence of tibial dyschondroplasia, Ca deficiency rickets, and P deficiency rickets[1,2]. Experiment 1.

	Treatment Day 1-7		Day 8-15				
	LCA	D_3	LCA	D_3	TD Incidence	Ca Rickets Incidence	P Rickets Incidence
	%	IU/kg	%	IU/kg	%		%
1	0	0	0	0	6.70 ± 3.88[b]	79.32 ± 3.70 [a]	44.64 ± 5.36 [bc]
2	0.2	0	0.2	0	3.57 ± 3.57[b]	9.82 ± 6.08[cd]	63.39 ± 12.68[b]
3	0	400	0	400	0.00 ± 0.00 [b]	0.00 ± 0.00 [d]	0.00 ± 0.00[d]
4	0	0	0.1	0	10.00 ± 4.08 [b]	57.50±19.74[ab]	30.00 ± 12.25[c]
5	0	0	0.2	0	0.00 ± 0.00 [b]	36.11 ± 6.83[bc]	87.22 ± 4.75[a]
6	0	0	0	400	35.00 ± 12.58[a]	7.50 ± 4.79[cd]	2.50 ± 2.50[d]
Regression of treatments 1, 4, and 5							
Parameter							
LCA					0.2097	0.0252	0.0321

[1] Mean leg problem incidences of 4 replicate pens ± SE. All 10 birds in each replicate pens were examined.
[2] Values within variables with no common superscripts differ significantly ($P < 0.05$) when tested with Duncan's Multiple Range Test following analysis of variance.