《Research Note》

Use of 25-hydroxycholecalciferol in Diets of Broiler Chickens: Effects on Growth Performance, Immunity and Bone Calcification

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An experiment was conducted in mixed sexes broiler chickens 1-d-old (Ross 308) to investigate the effects of 25-hydroxycholecalciferol (25-OH-D₃) on growth performance parameters, immunity and bone calcification. A completely randomized design with 4 experimental diets was used: 1) diet with cholecalciferol (VIT-D3) at 200 IU/kg of feed (NRC-1994 level); 2) diet with 25-OH-D₃ at 69 µg/kg of feed; 3) diet with VIT-D3 at 2000 IU/kg of feed (commercial level); and 4) as diet 3+25-OH-D₃ at 69 µg/kg of feed. Experimental treatments consisted in 6 repetitions of 10 chickens each. On 21 d of trial, chickens fed diets with VIT-D3 at commercial level (with or without 25-OH-D₃) increased the BWG ($P<0.05$) than those supplemented with VIT-D3 at NRC-1994 level or single 25-OH-D₃. The feed conversion ratio of chickens was improved ($P<0.01$) by using diets supplemented with VIT-D3 at commercial level (with or without 25-OH-D₃) than that diet with VIT-D3 at NRC-1994 level. Calcium content in tibias of broiler (21 d old) was increased ($P<0.05$) with the use of diets added 25-OH-D₃+VIT-D3 (commercial level) respect to those diets with VIT-D3 at NRC-1994 or commercial level. At 14 d, chickens fed diets with 25-OH-D₃ increased ($P<0.05$) the delayed cutaneous hypersensitivity response than those fed only VIT-D3 (NRC-1994 or commercial levels). The antibody response against Newcastle disease vaccine was better ($P<0.05$) in chickens fed diets with commercial levels of VIT-D3 (with or without 25-OH-D₃) than those diets, VIT-D3 NRC-1994 level or single 25-OH-D₃ supplementation. It can be concluded that higher levels of VIT-D3 (2000 IU/kg of feed) than those recommended by NRC (1994) improved the growth performance and the antibody response; and the supplementation of 25-OH-D₃ at 69 µg/kg of feed increased the cellular immune response and the bone calcification of broiler chickens.

Key words: bone calcification, broilers, growth performance, 25 hydroxycholecalciferol, immunity

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Introduction

In poultry feeding, studies about the different groups of nutrients including vitamins have shown a close relationship between nutrition and health (Kidd, 2004; Chou *et al.*, 2009). Cholecalciferol (VIT-D3) is necessary for the intestinal absorption, blood transport and efficient metabolism of calcium and phosphorus. In addition, studies have shown an important role for the VIT-D3 in the development of skin and blood cells, as well it is involved in controlling cellular activities of host immune system (Bunce *et al.*, 1997; Capiati *et al.*, 2002; Lymboussaki *et al.*, 2009). Under intensive rearing conditions, VIT-D3 is added intentionally into the

feeds because birds are maintained in the absence of sunlight. Cholecalciferol coming from exogenous source is absorbed primarily in the duodenum and its absorption is depending of micelle formation. In the liver, VIT-D3 is hydroxylated to produce 25-OH-D₃, which is the main VIT-D3 metabolite in the blood. Further, 25-OH-D₃ is hydroxylated once again in the kidney to produce 1 α , 25-(OH)₂-D₃, which is the most biologically active, hormonal metabolite of the VIT-D3 (DeLuca and Zierold, 1998). Since, the efficiency of conversion for cholecalciferol to active metabolites [25-OH-D₃ and 1 α , 25-(OH)₂-D₃] in the biological systems is not 100%, serum 25-OH-D₃ is considered the best available biomarker of exposure to dietary sources of VIT-D3. These observation have stimulated the research towards the study of using VIT-D3 metabolites in poultry feed in other to improve bird productivity, bone calcification and egg sell quality (Garcia *et al.*, 2001; Keshavarz, 2003; Driver *et al.*, 2006; Shim *et al.*, 2008; Chou *et al.*, 2009; Han *et al.*, 2009; Valkonen *et al.*, 2010). Nowadays, animal welfare is a big concern in the livestock industry of developed countries. In last 50th years,

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genetic selection was focused in the improvement of faster growth, livability, and feed efficiency. Nevertheless, others aspects linked to animal welfare like metabolic disorders, mayor susceptibility to infectious diseases, and skeletal problems have also been increased. Since the influence of VIT-D₃ in bone mineralization and immune response have been well documented. We postulate that the use of active metabolites such as 25-OH-D₃ in broiler diets might be a nutritional alternative to improve not only growth performance but also the chickens health status or welfare. This study was carried out with the goal of evaluating the addition of 25-OH-D₃ as a source or complement of VIT-D₃ in diets of broiler chickens and its effects in productivity, bone mineralization and immunity.

Materials and Methods

Animal Housing

An experiment was conducted with two-hundred and forty 1-d-old mixed sexes (50% males and 50% females) broilers chickens (Ross 308). All experimental conditions and animal protocols were accepted by the Ethical Commission of the Faculty of Veterinary Medicine and Zootecnia / University National Autonomous of Mexico Chickens were placed in two brooders batteries cages (PetersimeTM) into an experimental house (FMVZ-UNAM, facilities). Feed and water were provided for *ad-libitum* consumption throughout 21 d of experimentation. Twice daily observations were recorded for general flock condition, temperature, lighting, water, feed, and mortality.

Experimental Design and Diets

A completely randomized design with four experimental treatments was used. Sorghum+soybean meal (Table 1) based diets in mash form isoproteinic and isoenergetic with a variation regarding the vitamin premix (Table 2) or VIT-D₃ (level and source) were used: diet with VIT-D₃ at 200 IU/kg of feed or NRC (1994) level; 2) diet without VIT-D₃ but with 25-OH-D₃ at 69 µg/kg of feed (according to the producer recommendations, this dose is equivalent to 3000 IU/kg; Hy-D, DSM, Jalisco, Mexico); 3) diet with VIT-D₃ at 2000 IU/kg of feed or commercial level; and 4) as diet 3+ 25-OH-D₃ at 69 µg/kg of feed. Each treatment included 6 repetitions of 10 chickens each.

Animal Performance

Chickens were weighed by pen group at 0 and 21 d of age. As growth performance parameters average of body weight

gain (BWG), feed intake, and feed conversion ratio were calculated from 1–21 d of age.

Bone Ash, Ca and P Content

To determine bone ash, calcium and phosphorus content, 6 chickens per treatment were sacrificed on day 21 of trial. The corresponding tibia from the left leg was excised; tibias bones were oven-dried at 105°C for 24 h and ashed in a muffle furnace at 600°C for 6 h. The percentage ash, calcium and phosphorus were determined according (AOAC; 1990) the samples were taken and slightly modified by methodologies described by Brenes *et al.*, 2003; Potter 1988; Potter *et*

Table 1. **Ingredient composition (g/kg) and calculated nutrient analysis of experimental diets**

Item	1–21 d of trial
Ingredient	
Sorghum	563.0
Soybean-meal (460 g CP/kg)	368.0
Calcium carbonate (380 g ca/kg)	19.0
Soybean oil	29.0
Dicalcium phosphate	8.7
Sodium chloride	4.0
Vitamin & mineral premix ¹	4.2
DL- Methionine (99% purity)	2.2
L-Lysine HCl (78.5% purity)	0.9
L-Threonine (98.5% purity)	0.2
Choline chloride (60% purity)	0.8
Calculated analysis	
CP (%)	22.00
ME (kcal/kg)	2950
Total Lys (%)	1.25
Total Met+Cys (%)	0.90
Total Thr (%)	0.84
Ca (%)	1.00
Total P (%)	0.70
Available P (%)	0.45

¹ Included per kg of feed: vitamin A, 12 000 IU; vitamin E, 25 mg; vitamin K₃, 2.5 mg; thiamine, 2.0 mg; riboflavin, 5.0 mg; pyridoxine, 3.0 mg; cyanocobalamin, 20 µg; biotin, 75 µg; folic acid, 0.8 mg; nicotinic acid, 30 mg; calcium pantothenate, 10 mg; Fe (ferrous sulfate), 50 mg; Zn (zinc oxide) 50 mg; Mn (manganese sulfate), 110 mg; Cu (copper sulfate), 12 mg; I (potassium iodate), 0.30 mg; Se (sodium selenite), 200 µg; Co (cobalt sulfate), 0.20 mg; Zn bacitracin, 50 mg; ethoxyquin, 200 mg; acid propionic, 500 mg.

Table 2. **Effect of dietary VIT-D₃ and 25-OH-D₃ on growth performance parameters of broiler chickens from 1 to 21 days old**

Per kg basal diet	Weight gain, g	Feed intake, g	Feed conversion, g/g
VIT-D ₃ , 200 IU	495.0 ^B ±14.63	675.7±19.89	1.37 ^a ±0.05
25-OH-D ₃ , 69 µg	498.8 ^B ±18.45	646.0±21.08	1.30 ^{ab} ±0.05
VIT-D ₃ , 2000 IU	560.9 ^A ±21.47	670.7±11.54	1.20 ^b ±0.04
VIT-D ₃ , 2000 IU + 25-OH-D ₃ , 69 µg	596.4 ^A ±25.36	713.4±20.38	1.25 ^b ±0.03
Probability	NS	0.024	0.008

^{A,B} Values within a column not sharing a common superscript are statistically different ($P < 0.05$).

al., 1995.

Humoral Immune Response

The effect of different treatments on humoral immune response was examined by means of antibody production after a simultaneous vaccination against Newcastle disease virus (NDV). At 10 d of chicken age, all chickens were vaccinated against NDV, with an inactivated vaccine in emulsion (newca-mex, Avimex Animal health; Mexico D.F., Mexico) by subcutaneous injection (1 ml, 2/3 from the neck) and attenuated live vaccine "LaSota" strain by ocular via (eye drop 0.03 ml, lasota; Avimex Animal health). Serum samples were taken at 11 days post-vaccination (18 samples per treatment), frozen at -20°C , and specific antibody titers against NDV were determined by hemagglutination inhibition assay (Gomez *et al.*, 2009).

Cell-mediated Immune Response

The effect of different treatments on cell-mediated immune response was examined by cutaneous basophilic hypersensitivity test (Edelman *et al.*, 1985; Corrier and DeLoach, 1990). Analysis of cell-mediated immune response at 14 days was performed as described by Corrier and DeLoach (1990). Briefly, an intradermic inoculation of phytohemagglutinin-P (150 $\mu\text{g}/0.1\text{ ml}$ of PHA-P, Sigma Chemical; St. Louis, Mo) was carried out in the inter-digital membrane of 3rd and 4th phalanges of the right inferior extremity and sterile saline solution (0.1 ml) of the left foot (6 chickens per treatment). Twenty-four hours post-inoculation the thickness of the inter-digital membrane was measured in millimeters using a micrometer.

Statistical Analysis

Experiment was analyzed by one-way ANOVA as a randomized complete design with 4 treatments and 6 repetitions (SAS Institute, 1985). Differences between treatments were established by Tukey's test at $P \leq 0.05$ probability. The data expressed as percentages were transformed to arcsine values and the data of antibody titers were transformed to Log^2 prior to statistical analysis.

Results

Animal Performance

On 21 d of experimentation, chickens fed diets contained VIT-D3 at a commercial level with and without an addition of 25-OH-D₃, presented greater BWG ($P < 0.024$) compared to those chickens fed diets with a level of VIT-D3 recommended by the NRC (1994) and diets with the only sup-

plementation of 25-OH-D₃ (Table 2). The feed conversion ratio was better ($P < 0.008$) in treatments which included VIT-D3 at a commercial level (with and without an addition of 25-OH-D₃) than treatments according to the requirements of VIT-D3 set by NRC (1994) level but, it was similar to that treatment with the simple supplementation of 25-OH-D₃ (Table 2). The general mortality was not affected by any experimental diets ($P > 0.05$, data not included).

Bone Ash, Ca and P Content

On 21 d of trial, the percentages of ashes and phosphorus measured in tibias of chickens were not affected statistically ($P > 0.05$) by the use of any experimental diet (Table 3). The percentage of calcium was increased ($P < 0.038$) in chickens fed diets including 25-OH-D₃ + VIT-D3 at commercial level respect to those not supplemented 25-OH-D₃, VIT-D3 in diets at NRC (1994) or at commercial level (Table 3).

Humoral Immune Response and Cell-mediated Immune Response

At 21 d of chicken age, the antibody response of NDV was better ($P < 0.05$) in treatments with an inclusion of VIT-D3 at level similar to that used in a commercial premix of vitamins (with and without 25-OH-D₃ supplementation) respect to those treatments with VIT-D3 at NRC (1994) and with the simple use of 25-OH-D₃ (Table 4). Regarding the cell-mediated immune response measured at 14 d of trial, an increasing of cutaneous basophilic hypersensitivity response ($P < 0.05$) was observed in chickens fed diets supplemented 25-OH-D₃ than those supplemented with only VIT-D3 (NRC-1994 and commercial levels) (Table 4).

Discussion

Results of this study confirmed that VIT-D3 added in diets of broiler chickens at 10 times (2000 IU/kg of feed) the recommendations of NRC (1994), or those levels commonly included in commercial feed formulations improved the body weight gain and the feed conversion ratio. This result is in agreement with others studies conducted previously in broiler chicken (Xu *et al.*, 1997; Kasim and Edwards, 2000; Fritts and Waldroup, 2003; Khan *et al.*, 2010). It was confirmed that the recommendation of NRC (1994) for VIT-D3 in broiler diets, is mainly to avoid deficiency signs more than to reach higher production index. In agreement with Rath *et al.* (2007) and Chou *et al.* (2009) data of this study did not show negative effects in chicken productivity by adding 25-OH-D₃ as a source of VIT-D3, or in combination

Table 3. Effect of dietary VIT-D₃ and 25-OH-D₃ on the concentration of ash, calcium and phosphorus in tibias

Per kg basal diet	Ash, g/100 tibia	Calcium, g/100 tibia	Phosphorus, g/100 tibia
VIT-D ₃ , 200 IU	36.6 $\pm$ 2.13	10.7 $\pm$ 0.88 ^b	5.9 $\pm$ 0.41
25-OH-D ₃ , 69 μg	38.0 $\pm$ 2.80	11.6 $\pm$ 0.83 ^{ab}	6.5 $\pm$ 0.52
VIT-D ₃ , 2000 IU	38.9 $\pm$ 1.24	11.1 $\pm$ 0.54 ^{ab}	6.7 $\pm$ 0.24
VIT-D ₃ , 2000 IU + 25-OH-D ₃ , 69 μg	39.6 $\pm$ 1.58	12.6 $\pm$ 0.43 ^a	6.5 $\pm$ 0.23
Probability	NS	0.05	NS

^{a,b} Values within a column not sharing a common superscript are statistically different ($P < 0.05$).

Table 4. Effect of dietary VIT-D₃ and 25-OH-D₃ on immune response of broiler chickens

<i>Per kg basal diet</i>	Cutaneous basophilic hypersensitivity response, mm	NDV antibody titers, Log ²
VIT-D ₃ , 200 IU	0.034±0.0010 ^b	47.2±13.01 ^a
25-OH-D ₃ , 69 µg	0.064±0.0019 ^a	45.2±13.23 ^a
VIT-D ₃ , 2000 IU	0.037±0.0011 ^b	58.1±13.25 ^b
VIT-D ₃ , 2000 IU + 25-OH-D ₃ , 69 µg	0.065±0.0002 ^a	61.0±12.12 ^b
<i>Probability</i>	<i>0.05</i>	<i>0.05</i>

^{a,b} Values within a column not sharing a common superscript are statistically different ($P < 0.05$).

* Response to coetaneous hypersensitivity.

** Obtained antibodies by the hemoagglutination inhibition test for the Newcastle disease.

with commercial levels of VIT-D₃ in the diet. On the contrary, Fritts and Waldroup (2003) concluded that including 25-OH-D₃ in broiler chicken diets can represent an increment in the body weight of broiler chickens more than that observed using VIT-D₃. The active form of VIT-D₃ (1,25 dihydroxycholecalciferol) is mainly produced in the kidney due to the action of 1 α -hydrolase from the 25-OH-D₃, it is attributable to the latter different functions, and the maintenance of calcium levels in serum as an outstanding one. Furthermore, 25-OH-D₃ participates more actively than VIT-D₃ in the calcium absorption at intestinal level, the mobilization of calcium to bones and calcium fixation (Applegate *et al.*, 2003; Ledwaba and Roberson, 2003; Fritts and Waldroup, 2005; Driver *et al.*, 2006;) which was also observed in this study with treatments including 25-OH-D₃ which demonstrated an increase in the calcium deposition in the tibias of sacrificed birds.

Some studies (Etten *et al.*, 2008; Chou *et al.*, 2009; Khan *et al.*, 2010) reported that VIT-D₃ can modulate the immune response and productive parameters in broilers chickens. Similarly to these studies, our results confirmed that some immune parameters of chickens were affected by both, source and level of VIT-D₃ supplemented in the diet of broilers. It has been described that 1,25-OH-D₃ molecule plays an important function regarding the immune response, as it can stimulate hematopoiesis when linking to VIT-D₃ receptor (VDR), which are located intracellular in the nucleus membranes of several hematopoietic cells such as lymphocytes B and T (Capiati *et al.*, 2002; Enioutina *et al.*, 2009). In fact, it was postulated that 25-OH-D₃ presents a series of advantages like a higher persistence in the blood and is able to compete with VDR in a 2 or 3 to 1 proportion with respect to 1, 25-OH-D₃ (Capiati *et al.*, 2002). In our study, 25-OH-D₃ favored the cutaneous basophilic hypersensitivity response (CBH) more than VIT-D₃ this observation implies that there was a higher response of T cells in the birds fed with 25-OH-D₃. Since, elicited CBH response in birds by inoculation with PHA-P is a Thymus dependent response mediated by T cells (Corrier and De Loach, 1990). Moreover, our data were similar with Khan *et al.* (2010), it showed that commercial level of VIT-D₃ were more effective to induce better antibody response versus NDV vacci-

nation.

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