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The Bioavailability and Effects of Nano-Vitamin D_3 and Micro-Vitamin D_3 on Performance and Bone Characteristics in Broiler Chickens

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Abstract

This study aimed to investigate the effects of nano-vitamin D_3 (NVD$_3$) and micro-vitamin D_3 (MVD$_3$) on performance and bone parameters in broiler chickens. A total of 792 one-d-old Cobb 500 male broiler chickens were randomly distributed in a completely randomized design according to a 2×6 factorial arrangement of 12 dietary treatments with six replicates of 11 chicks. The treatments were: basal diet (control); basal diet supplemented with 100, 200, 400, 800, and 1600 (IU/kg diet) commercial MVD$_3$; and a basal diet supplemented with the same levels of NVD$_3$. The basal diet consisted of 0.5% Ca and 0.25% non-phytate phosphorus and was free of vitamin D_3 supplement. There were significant differences ($P < 0.01$) in feed conversion ratio due to supplementation with NVD$_3$ from 4 to 10, 11 to 21, and 4 to 21 days of age compared to those receiving MVD$_3$. The chicks receiving NVD$_3$ had significantly ($P < 0.01$) higher live body weight (LBW) at 21-d of age and daily weight gain from 4 to 21-d of age compared to MVD$_3$. The chicks receiving NVD$_3$ had a significantly ($P < 0.01$) higher concentration of ash and Ca content in the tibia. Bone stiffness and strength, tibia weight per length index, tibia weight, tibia diameter, organic matter (OM), OM/ash, and OM/length at 21-d were significantly ($P < 0.01$) improved in birds fed diets supplemented with NVD$_3$. Relative bioavailability of the NVD$_3$ source to the MVD$_3$ was more efficient according to performance and bone mineralization measurements. There was better growth performance and bone parameters in the 800 and 1,600 IU/kg levels of both kind than at the lowest level. Assessing the data of all experimental indexes showed that 800 IU/kg exhibited significant linear effects, suggesting that it can be the ideal level of supplementation of NVD$_3$, and, on the other side, the rate of 1600 IU/kg could be the maximum supplementation of NVD$_3$ in broiler chickens.

Introduction

The main objective of modern poultry nutrition is to fully utilize the genetic potential of birds by providing complete diets covering all known nutrient requirements. Because of the genetic improvement, rapid-growing and heavy commercial broilers, , are prone to leg problems and skeletal disorders such as lameness, rickets, and tibia dyschondroplasia (TD). Nutritional factors such as VD$_{3,}$ due to its essential role in calcium and phosphorus metabolism and increasing bone ash density, are known to improve skeletal disorders (Lohakare *et al.*, 2005; Garcia *et al.*, 2013). In addition, VD$_3$ plays an important role in calcium homeostasis in the bones, intestines, kidneys, and oviduct (de Matos, 2008).

The NRC (1994) recommends that broiler diets must be fortified with a minimum of 200 IU/kg of VD$_3$. However, commercial broiler diets are commonly fortified with this vitamin 20 to 25 times (Ross Broiler Nutrition Specification, 2019) above the NRC recommendations to prevent production losses and skeletal disorders (Fritts and Waldroup, 2003). The very low content of VD$_3$ in feedstuffs is generally ignored in diet formulation, and the complete requirement is supplied by using vitamin supplements (NRC, 1994).

Studies have shown that vitamins with smaller particles as vitamin premixes, were more effective and showed higher bioavailability (Yanjing and Huang, 2010). Nanoparticles with smaller size have better

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properties than larger ones such as site-specific actions, lower degradation, and higher bioavailability (Pedersen *et al*., 2009). Nano form of supplementation increases the surface area and surface activity which possibly could increase absorption and thereby utilization of vitamins. (Ahn, 2010; Cai *et al*., 2012). Nanotechnology entities help to improve the bioavailability, solubility, absorbability, and half-life of conventional natural products (Fesseha *et al*., 2020). Sheikhsamani *et al.* (2016) demonstrated that nano-multivitamin at 100 and 150% of the recommended levels based on Ross Nutrition Specification resulted in a significant increase in the average weight and daily weight gain of broiler chicks during the starter period compared to conventional multivitamin supplementation. Zhang *et al*. (2010) studied the effect of composite vitamin nano-emulsion on growth performance and immunity of broilers and concluded that composite vitamin nano-emulsion could be used to increase the growth performance and immunity in the broiler. Hassan *et al*. (2016) concluded that using nano di-calcium phosphate in broilers diets allows successfully reduce the dietary di-calcium phosphate by 75%, and using of dicalcium phosphate in nanoparticle size allows to reduce the excreted Ca and P by about 50%, which reduces the impact of poultry on environmental pollution.

Although many studies have focused on the importance of nanotechnologies in poultry production (Hu *et al*., 2012; Sahoo *et al*., 2014; Vijayakumar and Balakrishnan, 2014; Elkloub *et al.*, 2015; Nguyen *et al*., 2015), there is no evidence regarding the bioavailability and effect of vitamin D_3 nanoparticles in broilers. Thus, the aim of the current investigation was to evaluate the impacts of supplementing nano-vitamin D_3 (NVD_3) and micro-vitamin (MVD_3) at different levels on performance and bone parameters in broiler chickens.

Materials and Methods

Bird husbandry and experimental design

This study was conducted according to the protocols of the Animal Care Committee at the Ferdowsi University of Mashhad, Iran. A completely randomized design arranged as a 2×6 factorial arrangement was used to determine the effects of two sources of vitamin D_3 (NVD_3 and MVD_3), each with six dietary levels. Seven hundred ninety-two one-day-old male chicks of the Cobb 500 broiler strain were obtained from a commercial hatchery and were allocated to 12 treatments (72 pens; 11 birds per pen), Birds were reared in floor pens (110 cm×100 cm) from 1 to 21 days. Each pen was equipped with one tube feeder and a nipple drinker. Feed and water were provided *ad-libitum* and with a photoperiod of 21 hours of daylight per day.

Table 1. Composition and nutrient content of basal diets

Ingredients (%)	Starter (1-3 days)	Starter (4-10 days)	Grower (11-21 days)
Corn	53.72	53.60	55.33
Soybean meal	38.89	38.89	37.46
Soybean oil	3.11	3.11	3.62
Salt	0.37	0.37	0.34
Dicalcium phosphate	1.90	0.54	0.59
Limestone	0.85	0.40	0.39
DL-Methionine	0.39	0.39	0.29
L-Lysine	0.27	0.27	0.10
Threonine	0.00	0.00	0.05
Sand	0	1.93	1.33
Vitamin permix[1]	0.25*	0.25	0.25
Trace Mineral permix[2]	0.25	0.25	0.25
Calculated chemical composition			
Crude protein (%)	23.00	23.00	20.80
Metabolizable Energy (Kcal/Kg)	3000	3000	3000
Calcium (%)	0.96	0.5	0.5
Available phosphorus (%)	0.48	0.25	0.25
Sodium (%)	0.16	0.16	0.15
Arginine (%)	1.53	1.53	1.41
Lysine (%)	1.44	1.44	1.24
Methionine (%)	0.71	0.71	0.60
Methionine + cystine (%)	1.08	1.08	0.95
Threonine (%)	0.97	0.97	0.85
Tryptophan (%)	0.28	0.28	0.26

*It consists of 9790 IU vitamin D_3 per kilogram of diet in addition to other compositions of vitamin premix.

[1] Supplied in milligrams per kilogram of diet (except as noted): vitamin A (as all trans-retinyl acetate), 5,500 IU; vitamin E (all-rac-α-tocopherol acetate), 11 IU; riboflavin, 4.4; Ca pantothenate, 12; nicotinic acid, 44; choline Cl, 220; vitamin B12,9 µg; vitamin B6, 3.0; menadione (as menadiol- one sodium bisulfite), 1.1; thiamin (as thiamin mononitrate), 2.2; folic acid, 3.0; and biotin, 0.3.

[2] Supplied in milligrams per kilogram of diet: Mn (MnSO4), 36; Zn (ZnSO4), 33; Fe (FeSO4), 20; Cu (CuSO4ↄ5H2O), 0.76; I [Ca (IO3)2], 0.75; Mg (MgO), 12; and Se (Na2SeO3), 1.14.

The temperature was set at 32°C±1 on the first day and decreased linearly by 0.5°C per day until the final temperature reached 21°C±1. Ultraviolet radiation was kept out of the room by opaque plastic sheeting on the windows and using standard incandescent bulbs.

Diets

The corn-soybean meal basal diet (Table 1) in mash form was formulated to meet or exceed the nutritional requirements of the chicks from hatch to three days as recommended by the Cobb 500 Nutrition Supplement. Then, from day 3 to 21, chicks received the diet recommended by Cobb 500 Nutrition Supplement, for all nutrients except calcium, phosphorus, and VD_3. Birds were fed a starter diet until day 10, followed by a grower diet until day 21.

Pure crystalline cholecalciferol (D_3) designated by Sigma Chemical Co. as the Sigma Reference Standard that pure crystalline cholecalciferol meets or exceeds all U.S. Pharmacopeia specifications was used in this study. The crystalline D_3 was diluted with propylene glycol to obtain dilutions of 10 µg/mL of vitamin D_3, which was then used to prepare the experimental diets. Nano-VD_3 with the standard particle size of 823 nm was provided by Nanoceutical Solutions® (San Antonio, TX, USA).

Dietary treatments

Based on the AOAC (932.16; 2000) chick bioassay method to determine the bioavailability of VD_3, the basal diet supplemented with 0.5% Ca and 0.25% NPP and was lack of VD_3. According to the HPLC test, each gram of fed corn and soybean meal in the diet contained 60.49 and 36.93 IU of VD_3, respectively. In addition, both NVD_3 and MVD_3 tested contained 492649 and 494132 IU of VD_3 per gram, respectively. The experimental treatments were as follows: basal diet (control); basal diet supplemented with 2.5, 5, 10, 20, and 40 (µg/kg diet) commercial MVD_3; and a basal diet supplemented with 2.5, 5, 10, 20, and 40 (µg/kg diet) NVD_3. Both VD_3 sources were sprayed into the experimental diets to meet the above levels of VD_3 in each treatment.

Performance

The average body weight (BW), feed intake (FI), and body weight gain (BWG) were determined by measuring the weight of all chicks and the amount of consumed feed per pen at 3, 10, and 21 days of age. Feed conversion ratio (FCR) was calculated at the same ages. Mortality of birds was recorded daily to calculate survival rate and adjust FI and FCR; (total feed intake divided by the weight of live birds plus dead birds).

Bone sampling and analytical methods

On day 21, after random selection of two chickens from each replicate, the birds were first weighed and then killed by cervical dislocation for measuring tibia characteristics. Each bird's left and right tibia were removed, transferred to sealed plastic bags, and maintained at -20 °C for further analysis. Based on the method proposed by AOAC (2000), the left tibias were first subjected to 5 min boiling to loosen the muscle tissue, and then the meat, connective tissue, and fibula bone were completely removed using scissors and forceps. After cleaning the tibiae, they were placed in an ethanol container (to remove water and polar lipids) for 48 hours. The bones were then extracted in anhydrous ether for 24 hours (to remove nonpolar lipids). The tibias were dried at 105°C for 24 hours and then weighed. The weight of the tibia was recorded. To determine Tibia ash content, the ash of the bone was used in a muffle furnace for 18 hours at 600 °C. The left tibia was employed to measure ash, in addition to Ca, P, Fe, Zn, Mn, and Mg content, using HPLC. The right tibia was utilized for the analysis of the breaking strength. A fully digital electronic universal testing machine was employed to determine the breaking strength of the tibia. The tibias were cradled on two supporting points at a distance of 4 cm apart. A 50 kg load cell and a crosshead speed of 10 mm/min were used to apply the force at the center of the same side of each tibia (Han *et al*., 2016b). Seedor index, an indicator of bone density, is the value that can be calculated by dividing the weight of the bone by its length, as suggested by Seedor *et al.* (1991). There is a positive relation between Seedor index and bone density, so that the higher the value of the index, the denser the bone (Klein *et al*., 1991).

Statistical Analysis

Replicate means were subjected to an analysis of variance (ANOVA) using the general linear model (GLM) procedures of SAS software (SAS, 2002). According to ANOVA, differences among treatment means were compared using Duncan's test, and the values obtained were considered different at a statistical level of 5%. Statistical Analysis of variance and regression analysis and all possible interactions among main effects were completed using the GLM procedure of SAS software. The relative potencies of the tested products were determined using a slope ratio method (Littell *et al*., 1997). The linear and quadratic effects of nano-VD_3 and micro-VD_3 were evaluated by using orthogonal polynomials.

Table 2. The effect of using nano vitamin D_3 and micro vitamin D_3 on the performance in 10, 21 days old chicks (g/chick/day)[1]

Item		4-10 (10) days				11-21 (21) days				4-21 (Total) day		
Nano-VD_3 IU(μg) /kg	Micro-VD_3 IU(μg) /kg	DBWG(g)	DFI(g)	FCR(g/g)	BW(g)	DBWG(g)	DFI(g)	FCR(g/g)	BW(g)	DBWG(g)	DFI(g)	FCR(g/g)
0	0	16.08	20.80	1.29	217.9	28.57	46.18	1.62	333.2	22.32	33.53	1.50
100 (2.5)		16.00	19.87	1.24	213.1	39.66^{c}	57.55	1.45^{a}	473.3^{c}	28.74	40.15^{a}	1.40^{a}
200 (5)		16.26	20.16	1.24	215.5	40.50^{c}	58.45	1.44^{a}	487.1^{c}	29.44	40.98^{a}	1.39^{a}
400 (10)		16.13	19.63	1.22	216.8	43.87^{ab}	59.47	1.35^{bc}	513.4^{ab}	31.14	41.19^{a}	1.32^{b}
800 (20)		16.39	19.17	1.20	215.6	44.60^{a}	59.41	1.33^{c}	526.3^{a}	31.78	41.12^{a}	1.29^{b}
1600 (40)		16.52	19.37	1.17	224.2	44.05^{ab}	59.56	1.35^{c}	520.1^{a}	31.53	41.29^{a}	1.31^{b}
	100 (2.5)	15.53	19.67	1.27	209.2	38.06^{d}	54.97	1.44^{a}	432.6^{d}	26.93	37.52^{b}	1.39^{a}
	200 (5)	16.31	20.67	1.27	219.7	40.79^{c}	58.33	1.43^{a}	481.7^{c}	29.32	40.70^{a}	1.39^{a}
	400 (10)	16.24	20.14	1.24	214.8	40.80^{c}	57.85	1.42^{a}	485.0^{c}	29.34	40.24^{a}	1.37^{a}
	800 (20)	16.07	19.93	1.24	217.2	41.80^{bc}	58.52	1.40^{ab}	494.8^{bc}	30.06	40.93^{a}	1.36^{ab}
	1600 (40)	16.42	19.63	1.19	215.2	44.37^{ab}	60.99	1.38^{bc}	521.0^{a}	31.52	41.95^{a}	1.33^{bc}
Pooled *SEM*		0.669	0.740	0.028	8.560	1.390	1.820	0.024	11.540	1.000	1.310	0.019
P-value												
VD_3 source		0.416	0.068	<.0001	0.414	0.0004	0.104	0.0001	<.0001	0.0001	0.053	<.0001
Levels		0.168	0.038	<.0001	0.207	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
VD_3 source × levels		0.826	0.613	0.237	0.415	0.005	0.08	0.0001	<.0001	0.054	0.047	<.0001
Linear (Nano-VD_3)		0.152	0.011	<.0001	0.050	<.0001	0.038	<.0001	<.0001	<.0001	0.1062	<.0001
Quadratic (Nano- VD_3)		0.685	0.954	0.439	0.655	0.009	0.308	0.002	0.004	0.049	0.408	0.008
Linear (Micro- VD_3)		0.132	0.488	0.0004	0.432	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
Quadratic (Micro- VD_3)		0.493	0.298	0.582	0.578	0.535	0.880	0.410	0.083	0.835	0.510	0.256
Means of sources												
Nano-particles		16.26	19.64	1.21^{b}	217.10	42.53^{a}	58.89	1.39^{b}	504.1^{a}	30.53^{a}	40.95	1.34^{b}
Micro-particles		16.11	20.01	1.24^{a}	215.23	41.16^{b}	58.13	1.41^{a}	483.1^{b}	29.44^{b}	40.27	1.37^{a}
Means of levels												
100 IU/kg		15.76^{b}	19.77^{ab}	1.25^{a}	211.2^{b}	38.86^{d}	56.26^{c}	1.45^{a}	453^{e}	27.83^{d}	38.83^{b}	1.39^{a}
200 IU/kg		16.29^{ab}	20.41^{a}	1.25^{a}	217.6^{ab}	40.65^{c}	58.39^{b}	1.44^{a}	484.4^{d}	29.38^{c}	40.84^{a}	1.39^{a}
400 IU/kg		16.19^{ab}	19.89^{ab}	1.23^{b}	215.8^{ab}	42.33^{b}	58.66^{b}	1.39^{b}	499.2^{c}	30.24^{b}	40.71^{a}	1.35^{b}
800 IU/kg		16.23^{ab}	19.55^{b}	1.20^{bc}	216.4^{ab}	43.2^{ab}	58.97^{ab}	1.37^{c}	510.6^{b}	30.92^{ab}	41.03^{a}	1.33^{c}
1600 IU/kg		16.47^{a}	19.50^{b}	1.18^{c}	219.7^{a}	44.21^{a}	60.27^{a}	1.36^{c}	520.6^{a}	31.53^{a}	41.62^{a}	1.32^{c}

[a,b,c,d] Means with different superscript letters within a column are significantly different ($P < 0.05$).

[1] Daily body weight gain (DBWG), Daily feed Intake (DFI), Feed conversion ratio (FCR), Body weight (BW).

Results

Growth performance

The effects of different sources and levels of VD$_3$ in broiler diets on growth performance at 4-21 days of age are shown in Table 2. The results indicated that FCR was linearly affected by the sources and levels of VD$_3$ and the chicks receiving NVD$_3$ had significantly ($P < 0.01$) lower FCR (1.208) from 4 to 10 days of age compared to those receiving MVD$_3$ (1.242). Daily BWG and FCR of chicks from 11 to 21 days of age and their BW at day 21 were linearly changed by different sources and levels of VD$_3$. There was also an interaction effect between sources and levels of VD$_3$.

The chicks receiving NVD$_3$ had significantly ($P<0.01$) higher daily BWG (42.53) and BW (504.09) and lower FCR (1.387) from 11 to 21 days of age compared to those receiving MVD$_3$. In addition, there was better growth performance in the 800 and 1,600 IU/kg levels than in the other levels. From 4 to 21 days of age, daily BWG and FCR were linearly dependent on the source and level of VD$_3$, and feeding NVD$_3$ resulted in higher daily BWG (30.53) and better FCR (1.343) ($P < 0.01$). Daily FI also showed a significant increase ($P < 0.05$) as VD$_3$ levels increased in the diet in all periods.

Bone composition

The influence of dietary sources and levels of VD$_3$ and their interaction on the tibia composition of broiler chickens at 21 of age is shown in Table 3. The results showed that the ash and Ca content of the tibia were linearly influenced by the sources and levels of VD$_3$ supplied ($P < 0.01$). The chicks receiving NVD$_3$ had significantly ($P < 0.01$) higher concentrations of ash (0.376) and Ca (37.62) compared to those receiving MVD$_3$. Overall, chicks fed diets with 800 or 1600 IU/kg had a higher concentration of ash and Ca than those fed lower levels. There was no significant difference between the concentrations of other tibia minerals.

Table 3. The effect of using nano vitamin D$_3$ and micro vitamin D$_3$ on the bone composition of 21 days old chicks

Item		Bone minerals						
Nano-VD$_3$ IU(μg) /kg	Micro-VD$_3$ IU(μg) /kg	Ca (%/ash)	P (%/ash)	Fe (ppm/ash)	Zn (ppm/ash)	Mn (ppm/ash)	Mg (%/ash)	Ash (%)
0	0	33.69	15.68	81.22	351.1	4.66	0.95	0.309
100 (2.5)		34.88	15.9	81.09	354.1	7.89	0.94	0.364
200 (5)		36.84	16.56	81.55	351.9	7.72	0.92	0.369
400 (10)		37.77	16.08	81.31	350.5	7.86	0.9	0.374
800 (20)		39.81	16.91	81.13	355.9	7.78	0.88	0.387
1600 (40)		38.8	16.88	80.71	350.9	7.45	0.86	0.388
	100 (2.5)	34.4	16.53	81.25	355.3	7.76	1.00	0.36
	200 (5)	35.66	16.44	81.40	355.7	7.89	0.94	0.362
	400 (10)	36.23	16.42	81.79	354.7	7.58	0.92	0.373
	800 (20)	38.5	16.79	81.01	352.4	7.69	0.89	0.377
	1600 (40)	38.29	16.78	81.17	357.0	7.79	0.86	0.377
Pooled *SEM*		0.535	10.140	4.090	18.230	1.290	0.110	0.009
P-value								
VD$_3$ source		<.0001	0.650	0.860	0.626	0.980	0.450	0.003
Levels		<.0001	0.412	0.994	0.999	0.996	0.171	<.0001
VD$_3$ source ×levels		0.070	0.864	0.999	0.974	0.979	0.983	0.504
Linear (Nano-VD$_3$)		<.0001	0.091	0.833	0.919	0.644	0.128	<.0001
Quadratic (Nano-VD$_3$)		0.0018	0.542	0.850	0.832	0.779	0.944	0.613
Linear (Micro- VD$_3$)		<.0001	0.549	0.900	0.999	0.934	0.053	0.0001
Quadratic (Micro-VD$_3$)		0.6922	0.664	0.763	0.875	0.770	0.824	0.293
Means of sources								
Nano-particles		37.62^{a}	16.47	81.16	352.7	7.74	0.90	0.376^{a}
Micro-particles		36.62^{b}	16.59	81.34	355	7.74	0.92	0.37^{b}
Means of levels								
100 IU/kg		34.64^{e}	16.22	81.1	354.7	7.82	0.97^{a}	0.362^{c}
200 IU/kg		36.25^{d}	16.50	81.5	353.8	7.80	0.93^{ab}	0.365^{c}
400 IU/kg		37.00^{c}	16.25	81.5	352.6	7.72	0.91^{ab}	0.373^{b}
800 IU/kg		39.16^{a}	16.857	81.00	354.2	7.73	0.88^{ab}	0.382^{a}
1600 IU/kg		38.55^{b}	16.834	80.9	353.9	7.62	0.86^{b}	0.382^{a}

^{a,b,c,d,e} Means with a different superscript letter within a column are significantly different ($P < 0.05$).

Table 4. The effect of using nano vitamin D_3 and micro vitamin D_3 on bone strength parameters of 21 days old chicks[1]

Level in the diet		Parameters													
Nano-VD_3 IU(μg) /kg	Micro-VD_3 IU(μg) /kg	Stiff	BS	W/L Index (RT)	W/L Index (LT)	RTW	LTW	LRT	LLT	T D	DRTW	om	om/A	A/L	om/L
0	0	27.24	55.6	58.8	57.7	3.48	3.57	59.1	61.9	4.75	1.28	0.69	2.23	0.523	1.16^{a}
100 (2.5)		67.12	117.9^{c}	70.4	67.0	4.53	4.41	64.2	65.8	6.17	1.69	0.63	1.74	0.567^{c}	0.98^{ab}
200 (5)		69.04	123.4^{bc}	70.4	75.6	4.55	4.91	63.4	64.8	5.70	1.71	0.63	1.70	0.582^{bc}	0.99^{ab}
400 (10)		74.95	124.2^{bc}	74.0	72.6	4.64	4.66	62.6	64.2	5.65	1.74	0.62	1.67	0.597^{abc}	0.84^{c}
800 (20)		77.99	133.8^{a}	76.25	70.9	4.80	4.6	63.1	64.9	5.81	1.79	0.61	1.58	0.614^{ab}	0.97^{b}
1600 (40)		75.85	129.3^{ab}	76.0	74.2	4.66	4.68	61.2	62.9	5.88	1.75	0.61	1.57	0.634^{a}	1.00^{ab}
	100 (2.5)	61.02	104.2^{c}	67.9	65	4.13	4.10	60.8	63.0	5.83	1.56	0.63	1.77	0.593^{bc}	1.05^{a}
	200 (5)	61.53	118.1^{b}	65.8	63.5	4.07	4.04	61.8	63.5	5.36	1.52	0.63	1.75	0.586^{bc}	1.03^{ab}
	400 (10)	69.56	121.2^{ab}	68	66.5	4.23	4.24	62.2	63.8	5.58	1.57	0.62	1.67	0.60^{abc}	1.00^{ab}
	800 (20)	74.40	128.0^{a}	68.8	64.7	4.26	4.08	61.9	63.1	5.33	1.59	0.62	1.65	0.60^{ab}	1.00^{ab}
	1600 (40)	72.20	128.8^{a}	67.6	65.6	4.29	4.28	63.3	65.1	5.6	1.61b	0.62	1.65	0.596^{abc}	0.98^{b}
Pooled *SEM*		4.092	4.073	5.162	5.540	0.371	0.418	1.880	2.064	0.461	0.090	0.008	0.070	0.022	0.033
P-value															
VD_3 source		<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.067	0.132	0.013	<.0001	0.003	0.004	0.711	<.0001
Levels		<.0001	<.0001	0.350	0.410	0.593	0.649	0.988	0.983	0.104	0.195	<.0001	<.0001	0.0002	<.0001
VD_3 source ×levels		0.761	0.006	0.722	0.289	0.982	0.545	0.057	0.067	0.874	0.846	0.504	0.556	0.011	<.0001
Linear (Nano- VD_3)		0.0002	<.0001	0.034	0.2411	0.323	0.719	0.024	0.021	0.393	0.067	<.0001	<.0001	<.0001	0.9773
Quadratic (Nano-VD_3)		0.149	0.733	0.761	0.532	0.803	0.618	0.960	0.867	0.085	0.589	0.613	0.674	0.695	<.0001
Linear (Micro- VD_3)		<.0001	<.0001	0.710	0.718	0.320	0.443	0.034	0.241	0.464	0.209	0.0001	0.0001	0.306	0.0003
Quadratic (Micro-VD_3)		0.143	0.054	0.927	0.655	0.913	0.790	0.853	0.854	0.589	0.842	0.293	0.266	0.643	0.468
Means of sources															
Nano-particles		72.9^{a}	125.7^{a}	73.72^{a}	72.09^{a}	4.64^{a}	4.65^{a}	62.96	64.57	5.84^{a}	1.741^{a}	0.623^{b}	1.656^{b}	0.599	0.959^{b}
Micro-particles		67.7^{b}	120.1^{b}	67.6^{b}	65.1^{b}	4.2^{b}	4.15^{b}	62.03	63.74	5.54^{b}	1.574^{b}	0.629^{a}	1.703^{a}	0.597	1.016^{a}
Means of levels															
100 IU/kg		64.0^{c}	111.0^{c}	69.18	66.0	4.33	4.26	62.5	64.4	6.00^{a}	1.631	0.637^{a}	1.759^{a}	0.580^{c}	1.020^{a}
200 IU/kg		65.2^{c}	120.8^{b}	68.8	69.6	4.31	4.48	62.6	64.1	5.53^{b}	1.618	0.634^{a}	1.733^{a}	0.584^{bc}	1.012^{ab}
400 IU/kg		72.2^{b}	122.7^{b}	71.06	69.5	4.43	4.45	62.4	64.0	5.61^{ab}	1.663	0.626^{b}	1.676^{b}	0.598^{ab}	0.925^{c}
800 IU/kg		76.1^{a}	130.9^{a}	72.56	67.8	4.53	4.34	62.5	64.0	5.57^{b}	1.694	0.617^{c}	1.616^{c}	0.612^{a}	0.988^{b}
1600 IU/kg		74.0^{ab}	129.1^{a}	71.86	69.9	4.47	4.48	62.2	64.0	5.74^{ab}	1.683	0.617^{c}	1.614^{c}	0.615^{a}	0.992^{ab}

[a,b,c,d] Means with a different superscript letter within a column are significantly different ($P < 0.05$).

[1] Stiffness, Bone Strength (BS), Weight/Length Index (right tibia), Weight/Length Index (left tibia), Weight right tibia (RTW), Weight left tibia (LTW), Length right tibia (LRT), Length left tibia (LLT), Tibia Diameter (TD), Weight of Dried Right Tibia (DRTW), om (1-%ash), om/ash, ash/length, om/length

Table 5. The relative bioavailability of using nano vitamin D_3 and micro vitamin D_3 in 21 days old chicks

	performance[1]								bone			
	FCR 4-10 d	BWG 11-21 d	FI 11-21 d	FCR 11-21 d	BW 21 d	BWG 4-21 d	FI 4-21 d	FCR 4-21 d	Ash	Ca	Bone Strength	Bone Stiffness
Source of variance (probabilities)												
Slope difference	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
Curvature												
Slope Micro-VD3	-0.0012	0.31	0.07	-0.006	1.82	0.066	0.051	-0.0049	0.0008	0.19	0.78	0.54
Slope Nano-VD3	-0.0046	0.57	0.15	-0.01	3.78	0.094	0.049	-0.0084	0.0013	0.28	1.17	0.88
R^2	0.56(4)	0.9(3)	0.78(3)	0.88(3)	0.89(4)	0.78(5)	0.67(5)	0.84(3)	0.86(4)	0.88(4)	0.94(3)	0.92(3)
RBV	3.71	1.79	1.91	1.63	2.07	1.4	0.95	1.69	1.56	1.48	1.5	1.61
Standard error	0.0007	0.09	0.05	0.001	0.45	0.014	0.017	0.001	0.0002	0.016	0.141	0.107
Lower 95% fiducial limit	3.52	9.87	2.55	0.99	1.17	0.79	0.22	1.01	0.89	1.24	1.01	1.05
Upper 95% fiducial limit	3.91	2.60	3.83	2.27	2.98	2.00	1.67	2.37	2.23	1.71	1.98	2.16
intercepts	1.26	38.02	56.9	1.47	460.5	28.7	39.8	1.41	0.36	34.5	112.2	62.7

[1] Body weight gain (BWG), Feed Intake (FI), Feed conversion ratio (FCR), Body weight (BW).

Bone strength parameters

The results in Table 4 compared the effect of dietary sources and levels of VD_3 and their interaction on tibia characteristics of broiler chickens at 21 of age. The results showed that stiffness, bone strength, om (1% of ash), om/ash, and om/length were linearly influenced by the sources and levels of VD_3 supplied ($P < 0.01$). The chicks fed diets with 800 or 1600 IU/kg improved these parameters more than those fed lower levels. Stiffness, bone strength (BS), tibia weight per length index (WLI), tibia weight (TW), tibia diameter (TD), om (1-%ash), om/ash, and om/length at 21 d were significantly ($P < 0.01$) improved in birds fed diets supplemented with NVD_3 as compared to those fed diets supplemented with MVD_3 (Table 4). There was no interaction between dietary VD_3 levels and VD_3 sources for tibia stiffness and weight/length index, but breaking strength, ash/length, and om/length at 21 days of age were significantly influenced by interactions between VD_3 sources and levels ($P < 0.01$).

Relative bioavailability

The slope ratio method for the variables of performance and bone mineralization response was employed to measure the relative bioavailability of NVD_3 to MVD_3 in broiler chickens (Table 5). RBV of the NVD_3 source to the MVD_3 was estimated to be around 150% more efficient according to measured parameters except for FCR in 4-10 days which bioavailability of NVD_3 was more than 370%. Biological activity was calculated by using performance and bone mineralization measurements. However, BWG (11-21 d), bone strength, and bone stiffness with the values of 0.9, 0.94, and 0.92 had the best coefficients of determination (R^2), respectively.

Discussion

The present study document a consistent effect of NVD_3 to increase performance and bone parameters as an alternative source of VD_3 with higher bioavailability when fed to broiler chickens from days 3 to 21. Previous studies have shown that feeding broilers with Ca and NPP-deficient diets along with adding VD3 can linearly increase the growth performance and bone quality (Aburto *et al*., 1998; Baker *et al*., 1998; Rama Rao *et al*., 2009). Thus, to have a Ca and NPP-deficient diet the Ca and NPP levels were chosen at 0.50% and 0.25%, respectively. The optimal dietary Ca to NPP ratio is 2.0 to increase bone mineralization and growth performance in broiler chickens (Han *et al*., 2016a).

NVD_3 significantly improved DFCR over 4 to 10, 11 to 21, and 4 to 21 days, and it was found that increasing the amount of VD_3 to 800 and 1600 IU/kg in the diet increased the amount of dailyFI, as a result, the best FCR was observed by adding 800 IU/kg nano vitamin D_3 to the diet. In addition to DFI and DFCR, NVD_3 treatments increased DWG and BW compared to equal amounts of MVD_3, and the best result was observed in chicks fed an 800 IU/kg diet for 11 to 21 days. No significant differences were observed even at higher levels of nano and micro VD_3. The DFI increased linearly when more VD_3 was supplied, and these two factors resulted in the best DFCR being assigned at 800 and 1600 IU/kg NVD_3 and 1600 IU/kg MVD_3. Furthermore, the same results have been observed in some studies, reporting that increasing the rate of VD_3 addition improves WG, FI, and FCR of broilers fed with various concentrations of VD_3 (Yarger *et al.*, 1995; Fritts and Waldroup, 2003; Driver *et al*., 2006). These results contrast with those of Lohakare *et al*. (2005) and Vieites *et al*. (2014), who reported that the inclusion of different levels of VD_3, with fixed calcium and phosphorus levels did not affect broiler performance parameters.

The VD_3 levels and sources in the present study did not affect the phosphorus, iron, zinc, manganese, and magnesium contents of the tibia bone. The calcium and ash content in the tibia bone was increased after supplementation with different sources and levels of VD_3. The highest calcium content was found in the group receiving 800 IU/kg NVD_3. Lohakare *et al*. (2005) found that increasing the level of VD_3 from 200 to 1800 IU/kg in broilers diet had no significant effect on bone Ca and P at 21 d of age. Han *et al*. (2013) did not find significant differences in tibia ash, breaking strength, and bone Ca and P among the chickens fed with 25 to 1,000 µg/kg VD_3. Different researchers pointed out the positive effects of VD_3 and its derivatives on growth performance and tibia weight, tibia strength, and the percentage of ash and Ca in broiler chickens (Fritts and Waldroup, 2003; Han *et al*., 2016a). Atencio *et al*. (2005) reported that as the level of VD_3 increased in the diet, BWG, tibia ash, plasma Ca increased and TD incidence and Ca rickets decreased.

In this study, NVD_3 significantly improved the bone parameters, stiffness, bone strength, Seedor index, tibia weight, and diameter and the highest value was found in the group receiving 800 IU/kg NVD_3. Previous studies have reported that increasing levels of VD_3 increased plasma Ca concentrations, tibial mineral deposition, and bone strength (Shirley *et al*., 2003; Kim *et al*., 2011; Sun *et al*., 2013). The result suggests that the addition of NVD_3 at the level of 800 IU/kg facilitates calcium absorption and enhances tibial development.

RBV is the ratio between the standard amount and testing source that is required to generate equivalent responses, in which a nutrient source at different levels is compared to a reference standard based on a biological response such as growth, bone mineralization and, etc. (Littell *et al*., 1995). The possible mechanism by which dietary NVD_3 may affect performance and bone parameters is its high bioavailability by reducing the need for the presence of lipids for absorption, especially at the starter period due to low lipase activity, and improving transmucosal transport. Nanoparticles can also improve the therapeutic effect, biodegradation, and photostability of VD_3 (Ahn, 2010). Bone density responses had high R^2 in comparison with performance; this is in line with results reported by the previous studies (Han *et al*., 2016a; Leyva-Jimenez *et al*., 2018). Lotfi *et al.* (2015) evaluated the effect and bioavailability of nano and micro vitamin K_3 in broilers' diets and showed that nanoparticle size affected positively bone weight and bone strength more than microparticle size and also reported an increased bioavailability in nano vitamin K_3 within 145 % compared to micro vitamin K_3.

Conclusion

The findings of the current study showed that the addition of NVD_3 to broiler chicks' diets could improve the performance and bone quality than supplementation of MVD_3. Relative bioavailability of VD_3 increased with dietary NVD_3. Considering all experiment indexes, 800 IU/kg exhibited significant linear effects and thus can be recommended as the optimum supplementation level of NVD_3. More studies are required to measure the bioavailability of NVD_3 in commercial flocks with standard levels of P and Ca.

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