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Effect of Nano-hydroxyapatite as an Alternative to Inorganic Dicalcium Phosphate on Growth Performance, Carcass Traits, and Calcium and Phosphorus Metabolism of Broiler Chickens

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ABSTRACT

The influence of nano-hydroxyapatite (NHA) as a source of calcium (Ca) and phosphorous (P) alternative to inorganic dicalcium phosphate (DCP) on the performance, carcass traits, and the Ca and P contents in serum, bone, and excreta was evaluated in broiler chickens. One-day-old un-sexed Arbor Acres Plus broiler chickens (n.= 160) were randomly assigned to four treatment groups of four replicates with 10 birds in each replicate. The birds received, for 35 days, one of four isocaloric isonitrogenous experimental diets differing in DCP and NHA inclusion levels as follows: diet 1 served as control and contained 2% DCP (100% DCP); diet 2 contained 1% DCP plus 0.1% NHA (50% DCP); diet 3 having 0.5% DCP plus 0.1% NHA (25% DCP); diet 4 having a 0.1% NHA only (0% DCP). The NHA was prepared by the wet chemical method and was shown to have Rod-like shape with 20-100 nm size, 98% purity, 30.8% Ca and 11% available P. Results indicated that the dietary supplementation of 0.5% DCP plus 0.1% of NHA (diet 3) or 0.1% of NHA (diet 4) decreased the excreted P ($P \le 0.05$) without negative impacts on growth performance parameters compared to control group. There was no difference ($P > 0.05$) in the carcass characteristics and serum Ca-P profile of broilers among the four dietary treatments. There was no difference ($P > 0.05$) in the tibia bone ash as well as the Ca and P levels among experimental diets. Thus, dietary supplementation of 0.1% of NHA can be safely used instead of inorganic DCP in broiler diets.

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Introduction

Calcium (Ca) and phosphorus (P) are essential major elements involved in various biological processes necessary for poultry welfare and performance, and both elements are required in large amounts in poultry diets. The poultry requirements of Ca and P are routinely provided using conventional inorganic sources (limestone, oyster shell, and mono- and- dicalcium phosphates), which are of low bioavailability that may contribute to environmental deterioration from discharged minerals, particularly P, which is mostly associated to phytate; one of the anti-nutritional factors poorly utilized by poultry (Viveros *et al*., 2000; Sharpley *et al*., 2007; Humer *et al*., 2015). Moreover, the inorganic P sources are relatively expensive, which encouraged the use of exogenous phytases to improve phytate P utilization in cereals and oilseed meals (Selle and Ravindran, 2007; Slominski, 2011; Romano and Kumar, 2018). However, supplemental inorganic Ca sources have been shown to interfere with P digestibility and phytases activity (Selle *et al*., 2009; Cowieson *et al*., 2016; Walk, 2016; Bedford and Rousseau, 2017). Some reports are claiming that phytases activity in poultry intestine can be enhanced upon feeding low levels of inorganic Ca, feeding Ca-free diets with Ca being fed separately or formulating diets based on digestible Ca rather than on total Ca (Wilkinson *et al*., 2014; Cowieson *et al*., 2016; Bedford and Rousseau, 2017). However, these feeding strategies have not been agreed upon yet in poultry production besides that marginal Ca deficiency has deleterious effects on poultry health and performance (Wilkinson *et al*., 2014; Cowieson *et al*., 2016).

Due to the continuous search for alternative sources of macro-minerals, which may be more efficiently used by birds due to, for example, their size, nanotechnology has aroused increasing interest. A general aim was to reduce the use of large amounts of selected sources of elements through the application of more highly bioavailable forms due to effective

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absorption (Gonzales-Eguia *et al*., 2009).

Based on the above context, current research on mineral nutrition is aimed at reducing the inclusion levels of inorganic sources and replacing them by nanoparticles (NPs) forms, which have the potential of high availability, minimal antagonisms and low environmental impact in addition to other health benefits for poultry (Gangadoo *et al*., 2016; Anwar *et al*., 2019; Patra and Lalhriatpuii, 2019).

Recent studies have addressed nanoparticles of Ca-P in feed, including Ca phosphate (Vijayakumar and Balakrishnan, 2014a; Samanta *et al*., 2019) and dicalcium phosphate (Hassan *et al*., 2016; Mohamed *et al*., 2016), Ca carbonate (Salary *et al*., 2017) and hydroxyapatite (Sohair *et al*., 2017). In laying hens, the application of Ca-P NPs has been investigated in a few studies and the findings are largely promising in terms of egg yield and quality (Ramesh, 2014; Ganjigohari *et al*., 2018).

Limited studies are available on the response of mineral retention and excretion, skeletal system, and carcass traits to the application of Ca-P NPs in broiler diets. Based on the beneficial effects of nano-DCP, the present study evaluated the use of nano-hydroxyapatite (NHA) at a very low dose (0.1% of the diet, as air-dried) on broiler performance, carcass traits, serum, and bone mineral profile and excreted P in broiler diets.

Materials and methods

This experimental trial was carried out at the Poultry and Animal Research Center, Faculty of Veterinary Medicine, Cairo University (January- February 2020). All experimental procedures were approved by the Institutional Animal Care and Use Committee of Cairo University (Vet CU20022020126).

Preparation and composition of nano-hydroxyapatite

The NHA was prepared, as air-dried nanoparticles, by the NanoTech Egypt Center (NanoTech Egypt for Photo-Electronics, Al Giza, Egypt) adopting the wet chemical method (Sokolova *et al*., 2006) as previously described (Vijayakumar and Balakrishnan, 2014a; Hassan *et al*., 2016; Mohamed *et al*., 2016) using calcium nitrate (18 mM) and di-ammonium hydrogen phosphate (10.8 mM) in the presence of NaOH to keep the basic medium at pH 9-11. The morphological structure properties were investigated through using transmission electron microscopy, and X-ray diffraction (XRD) measurements (Bisht *et al*., 2005). The prepared NHA particles were shown to have a Rod-like shape and a size range from 100.0±20. nm to 20.0±10.0 nm (Fig. 1). The purity, Ca and available P levels of the NHA particles were 97.99%, 30.76%, and 11.06%, respectively. Furthermore, the crystallographic structure of the NHA particles was investigated via monitoring the XRD patterns, as shown in Fig. 2. The NHA particles show a crystalline structure with the monoclinic phase as a main crystallographic structure in the as-prepared samples with three strongest reflection planes 161, 142 and 060, as a reference to XRD card no. 01-082-1553. The infrared spectra of the synthesized powders are given in Fig. 3. As reported by Paz *et al.* (2012) the characteristic bands of internal phosphate $(PO_4)^{3-}$ mode were assignments in the spectrum: the band at 472 cm^{-1} was assigned to OPO bending mode; the presence of two characteristic bands around 568 and 601 cm^{-1} correspond to OPO bending mode; the doublet in the range 1100-1000 cm was assigned to PO anti-symmetric stretching mode. These bands indicate the characteristic molecular structures of the polyhedrons of the PO in the apatite lattice. Further, at 3572 cm^{-1} the main hydroxyl vibration was observed in the spectrum (Paz *et al*., 2012).

Birds and experimental design

A total of 160 one-day-old un-sexed Arbor Acres Plus broiler chickens were randomly divided into four treatment groups of 4 replicates in each (10 chicks/replicate). Replicates were randomly allocated in a floor pens equipped with wheat straw litter, with a bird density 10 birds/m^2. Four isocaloric isonitrogenous experimental diets were formulated, according to Arbor Acres Plus nutrient specification manual 2019, as fol-

Table 1. Ingredients and calculated nutrient composition of experimental diets (as fed, %)

Item	Starter				Grower				Finisher			
	Control	Diet 2	Diet 3	Diet 4	Control	Diet 2	Diet 3	Diet 4	Control	Diet 2	Diet 3	Diet 4
Ingredients												
Yellow corn	53.82	55.7	55.97	56.92	56.97	58.07	58.62	58.22	61.1	61.85	62.55	63.5
Corn gluten meal 60%	3	4	3.3	3.5	2	3	3.4	2	2.4	2.1	2	2.2
Soybean meal 47%	36.3	35	36.2	36	32.9	32.4	32.1	34	27.7	28.5	28.7	28.1
Soybean oil	2.4	2.1	2	1.7	4	3.6	3.5	4	4.8	4.8	4.5	4.5
Di-calcium phosphate	2	1	0.5	0	1.8	0.9	0.45	0	1.6	0.8	0.4	0
Limestone, ground	1.2	0.8	0.65	0.5	1.2	0.8	0.7	0.55	1.3	0.75	0.65	0.5
Nano-NHA	0	0.1	0.1	0.1	0	0.1	0.1	0.1	0	0.1	0.1	0.1
Common salt	0.4	0.4	0.4	0.4	0.35	0.35	0.35	0.35	0.35	0.35	0.35	0.35
DL-Methionine	0.18	0.18	0.18	0.18	0.2	0.2	0.2	0.2	0.15	0.2	0.2	0.2
L-Lysine	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.22	0.2	0.2	0.2
L-Threoinine	0.05	0.05	0.05	0.05	0.03	0.03	0.03	0.03	0	0	0	0
Betaine	0.1	0.1	0.1	0.1	0	0	0	0	0	0	0	0
Sodium butyrate	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Broiler premix[1]	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
Total	100	100	100	100	100	100	100	100	100	100	100	100
Nutrient composition												
ME (kcal/kg)	3000	3010	3020	3030	3110	3130	3150	3170	3200	3230	3245	3280
Crude protein%	22.75	22.9	23	23	21	21.3	21.5	21.6	19	19.3	19.4	19.5
Calcium%	0.98	0.62	0.47	0.3	0.95	0.61	0.47	0.31	0.93	0.56	0.43	0.29
Total phosphorus%	0.72	0.48	0.34	0.23	0.67	0.46	0.35	0.23	0.62	0.4	0.34	0.22
Ava. P	0.47	0.31	0.22	0.15	0.44	0.3	0.22	0.15	0.40	0.26	0.22	0.15

Control, having 2% inorganic Di-calcium phosphate (DCP) (100% DCP); Diet 2, containing 1% DCP plus 0.1% nano-hydroxyapatite (NHA) (50% DCP); Diet 3, having 0.5% DCP plus 0.1% NHA (25% DCP); Diet 4, containing 0.1% NHA only (0% DCP).

[1]Contains per Kg premix: 1200000 IU vit. A, 350000 IU vit. D, 3,4000 mg vit. E, 250mg vit. B1, 800 mg vit. B2, 600 mg vit. B6, 3.2 mg vit.B12, 450 mg vit. K3,4.5g nicotinic acid,1.5g Ca pantothenate,120 mg folic acid, 5mg biotin, 55 mg choline chloride,3g Fe,2 g Cu, 10 g Mn,8 g Zn oxide, 0.15 mg sodium selenite,120 mg I, 40 mg Co.

lows: diet 1 served as control and containing 2% DCP (100% DCP); diet 2 having 1% DCP plus 0.1% NHA (50% DCP); diet 3 containing 0.5% DCP plus a 0.1% NHA (25% DCP); diet 4 having a 0.1% NHA only (0% DCP). The Ca:P ratio was maintained at 2:1 in all diets. The formulation and nutrient composition of these diets are shown in Table 1. The whole feeding period was 35 days. Birds were subjected to 1 h darkness and 23 h light during the first week of the experiment followed by 18 h light and 6 h darkness for the rest of experimental period (35 days) (Olanrewaju *et al*., 2006). The body weight (BW) changes and actual feed intake were recorded weekly to calculate the body weight development, weight gain, and feed conversion ratio (FCR) along the whole experimental period for all experimental groups. All birds were vaccinated throughout the experimental period against avian flu, New Castle, IB, and IBD according to the routine prophylactic vaccination program.

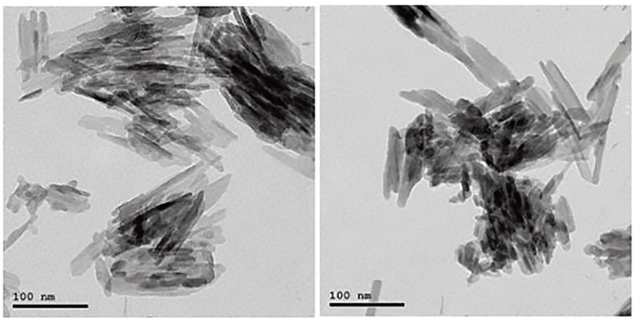

Fig. 1. Transmission Electron Microscopy micrographs of the as-prepared nano-hydroxyapatite.

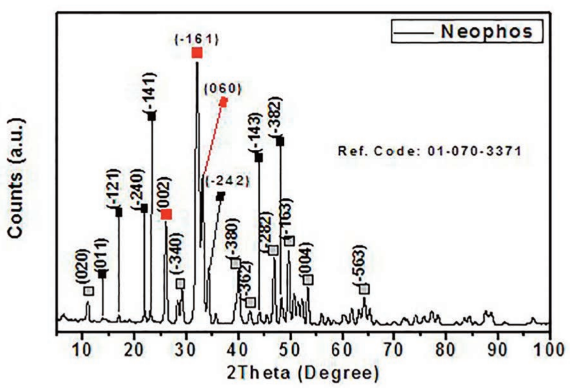

Fig. 2. XRD Patterns of nano-hydroxyapatite Nanoparticles.

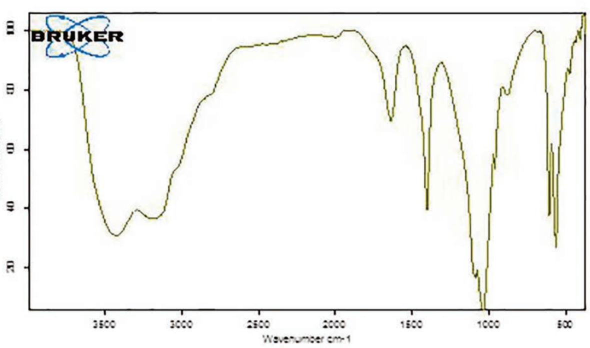

Fig. 3. FT-IR Spectrum of nano-hydroxyapatite Nanoparticles.

Sampling and measurements

Fecal samples from each replicate of the four treatment groups were collected at day 33 through day 35 of age, to measure Ca and P excretion. The collected feces were dried in a hot air oven at 60°C for 24 h then the dried excreta collected throughout the three days were pooled, finely ground, thoroughly mixed and placed in a plastic jar for the measurement of Ca and P contents by the Inductively coupled plasma atomic emission spectroscopy (ICP-AES; Thermo Sci, Model: iCAP6000 series) (Hassan *et al*., 2016).

Five birds from each replicate (20 birds per treatment) were randomly selected at the 35th day of age for performing various measurements. The birds were slaughtered to determine the dressing yield, breast muscle yield, thigh yield, tibia weight, tibia index (% of live body weight) according to El-Banna *et al*. (2008). During slaughter, serum samples were collected and used for serum Ca and P analysis using Atomic Absorption Spectrophotometer Sunostk, SBA733 Plus. Calorimetric methods were used to determine calcium (Gindler and king, 1972) and phosphorus (El-Merzabani *et al*., 1977) using commercial kits (Biodiagnostics, Egypt) following the manufacturer's instructions; absorbance was measured at 585 nm and 460 nm for Ca and P, respectively. Samples of thigh muscles were collected to determine Ca and P retention by ICP-AES (Thermo Sci, Model: iCAP6000 series). The left and right tibial bones were dissected out and their adhered muscles together with the connective tissue were thoroughly removed manually, then the weight of each tibial bone was recorded and expressed in percentage relative to live body weight (tibia index). Medio-lateral view, M/L radiographs of the bones were obtained at 50-52 kv/32mAs. The radiographs were examined for length, width, and densitometry according to Harash *et al*. (2020). Tibia length was measured from the proximal end to the distal end and the width at the medial diaphysis. This observation was recorded with the aid of computer software program (Digimizer Image Analysis Tool (MedCalc Software, version 4.2.5.0). The tibial bones were boiled in water for 5 min, dried in a hot air oven for overnight, then de-fatting of dried bones was carried out using diethyl ether, followed by petroleum spirit for 16 h each. Estimation of bone ash percentage of the dried defatted bone was carried out according to AOAC (2000). The soluble ash was assayed for Ca and P contents by ICP-AES (Thermo Sci, Model: iCAP6000 series). The Ca and P contents of the bone were expressed in % per gram of tibia ash.

Statistical analysis

Data were analyzed with the One-Way ANOVA of the Statistical Package for the Social Sciences (SPSS, version 18.0 for Windows). When a significant difference ($P \leq 0.05$) was detected, the Duncan's Multiple Range Test was used to separate the treatment means (Nie *et al*., 1975). Data are presented as Mean±S.E.

Results

Growth performance

The growth performance and feed consumption of broiler chickens fed diets supplemented with DCP and/or NHA are shown in Table 2. The data showed that the final body weight and BWG were lower ($P \leq 0.05$) in chickens fed diet 2 (1% DCP plus 0.1 %NHA) or diet 3 (0.5% DCP plus 0.1 %NHA) but were similar ($P > 0.05$) to those fed the diet 4 (0% DCP) compared to those fed the control diet. The feed consumption was similar ($P > 0.05$) among all treatments, but the FCR was higher ($P \leq 0.05$) in chickens fed diet 2 than those fed the other treatments. The mortality rate was numerically higher in chickens fed diet 2 or diet 3 than those fed the control or diet 4.

Carcass traits

The carcass traits of birds fed diets supplemented with DCP and/or NHA are presented in Table 3. The dressing percentage, breast yield, thigh weight as well as the Maryland yield were similar among treatments (P>0.05). The carcass, breast, and drumstick weights were lower in birds fed diet 2 than in birds fed the other three treatments (P≤0.05).

Calcium and phosphorus concentration in serum, thigh muscle and excreta

The Ca and P concentrations in serum, thigh muscle and excreta of broiler chickens fed diets supplemented with DCP and/or NHA are presented in Table 4. The Ca and P levels in serum and thigh muscle were similar among treatments (P>0.05). There was no significant difference in Ca excretion among dietary treatments with decreasing DCP levels, while there was a significant reduction in P excretion in groups fed NHA only compared with the control group.

Bone mineral assay

The tibial bone morphometry and mineral content of broiler chickens fed diets supplemented with inorganic DCP and/or NHA are presented in Table 5. Except for the tibial width (cm), there were no significant differences (p>0.05) in tibial weight (g), tibial index (% of Live BW), tibial length (cm), tibial cortex (cm), tibial ash and tibial Ca and P content among treatments; the tibial width increased with the NHA supplementation compared to the control.

Discussion

The final body weight and BWG of broilers in the control group appeared similar to birds fed diets with a very low dose of NHA i.e. 0.1% NHA (diet 4) implying that the intervention made in feed did not negatively influence the metabolism and that supplementation of a very low level of NHA (0.1%) was sufficient to meet Ca and P requirements of the broilers. This

Table 2. Effect of nano-hydroxyapatite on the performance parameters of broiler chickens

Item	Experimental diets			
	Control	Diet 2	Diet 3	Diet 4
Initial body Weight (g)	42±1.15[a]	42±0.44[a]	43.5±0.28[a]	43±0.58[a]
Final body weight (g)	2467±60.3[a]	2235±44.5[b]	2322±44.7[b]	2340±10.4[ab]
Feed intake (g)	3406.7±12.6[a]	3245.5±18.3[a]	3233±128.7[a]	3329±3.51[a]
Weight gain (g)	2425.5±59.2[a]	2195±44.5[b]	2279±44.5[b]	2299±11.0[ab]
Feed conversion ratio	1.41±0.03[b]	1.48±0.03[a]	1.42±0.03[b]	1.44±0.01[b]
Mortality%	2.5	7.5	5	2.5

Control, having 2% inorganic Di-calcium phosphate (DCP) (100% DCP); Diet 2, containing 1% DCP plus 0.1% nano-hydroxyapatite (NHA) (50% DCP); Diet 3, containing 0.5% DCP plus 0.1% NHA (25% DCP); Diet 4, having 0.1% NHA only (0% DCP).
Values are expressed as Mean±SE
Values of different superscripts in the same row differ significantly at P ≤0.05.

Table 3. Effect of nano-hydroxyapatite on the carcass traits of broiler chickens

Item	Experimental diets			
	Control	Diet 2	Diet 3	Diet 4
Live weight (g)	2368 ± 112[a]	2120 ± 115[a]	2280 ± 142.9[a]	2329 ± 93.9[a]
Carcass weight (g)	1801± 69.5[a]	1557 ± 88.2[b]	1701 ± 99.2[a]	1709 ± 73.7[a]
Dressing%	76.3 ± 2.06[a]	73.4 ± 0.54[a]	74.9 ± 0.91[a]	73.4 ± 0.65[a]
Breast weight (g)	640.8 ± 19.9[a]	545 ± 36.07[b]	605.8 ± 40.5[ab]	595.8 ± 23.9[ab]
Breast yield%	35.7 ± 0.88[a]	34.9 ± 0 .51[a]	35.4 ± 0.43[a]	34.9 ± 0.63[a]
Thigh weight (g)	483 ± 22.9[a]	435 ± 26.8[a]	458.3 ± 21.9[a]	476.6 ± 25.7[a]
Drumstick weight (g)	225 ± 13.4[a]	180 ± 9.3[b]	215 ± 15.86[a]	211.6 ± 11.2[a]
Maryland yield%	39.3 ± 1.06[a]	39.5 ± 0.52[a]	39.5 ± 0.58[a]	40.2 ± 0.51[a]

Control, having 2% inorganic Di-calcium phosphate (DCP) (100% DCP); Diet 2, having 1% DCP plus 0.1% nano-hydroxyapatite (NHA) (50% DCP); Diet 3, having 0.5% DCP plus 0.1% NHA (25% DCP); Diet 4, having 0.1% NHA only (0% DCP).
Values are expressed as Mean±SE
Values of different superscripts in the same row differ at P ≤0.05

Table 4. Effect of nano-hydroxyapatite on calcium and phosphorus levels in serum, thigh muscle and excreta of broiler chickens

Item	Experimental diets			
	Control	Diet 2	Diet 3	Diet 4
Serum calcium (mg/dl)	10.3 ± 0.16[a]	10.2 ± 0.03[a]	10.1 ± 0.10[a]	10.1 ± 0.05[a]
Serum phosphorus (mg/dl)	5.02 ± 0.08[a]	4.62 ± 0.05[a]	4.57 ± 0.05[a]	4.59 ± 0.04[a]
Thigh muscle Ca%	0.12 ± 0.01[a]	0.11 ± 0.05[a]	0.12 ± 0.02[a]	0.13 ± 0.08[a]
Thigh muscle P%	0.54 ± 0.01[a]	0.42 ± 0.05[a]	0.45 ± 0.02[a]	0.47 ± 0.08[a]
Excreta Ca%	2.60 ± 0.12[a]	2.58 ± 0.20[a]	2.56 ± 0.03[a]	2.32 ± 0.16[a]
Excreta P%	1.10 ± 0.03[b]	0.75 ± 0.06[b]	0.69 ± 0.02[b]	0.65 ± 0.09[b]

Control, having 2% inorganic Di-calcium phosphate (DCP) (100% DCP); Diet 2, containing 1% DCP plus 0.1% nano-hydroxyapatite (NHA) (50% DCP); Diet 3, containing 0.5% DCP plus 0.1% NHA (25% DCP); Diet 4, having 0.1% NHA only (0% DCP).
Values are expressed as Mean±SE
Values of different superscripts in the same row differ significantly at P ≤0.05

Table 5. Effect of nano-hydroxyapatite on tibial bone morphometry and tibial bone calcium and phosphorus contents of broiler chicks

Item	Experimental diets			
	Control	Diet 2	Diet 3	Diet 4
Tibia weight (g)	45.0 ± 2.20[a]	46.6 ± 2.10[a]	43.2 ± 1.53[a]	45.0 ± 1.83[a]
Tibia index%	1.93 ± 0.15[a]	1.98 ± 0.14[a]	1.80 ± 0.09[a]	1.90 ± 0.04[a]
Tibia total ash (%)	28.4 ± 1.76[a]	31.4 ± 2.60[a]	26.9 ± 2.55[a]	30.1 ± 0.80[a]
Tibia length (cm)	7.31 ± 0.14[a]	7.57 ± 0.12[a]	7.30 ± 0.05[a]	7.42 ± 0.09[a]
Tibia width (cm)	1.63 ± 0.11[b]	1.86 ± 0.11[a]	1.90 ± 0.08[a]	2.10 ± 0.06[a]
Tibia cortex (cm)	0.13 ± 0.005[a]	0.14± 0.01[a]	0.18 ± 0.01[a]	0.15 ± 0.005[a]
Tibia Ca (% of tibia ash)	24.9 ± 3.23[a]	19.5 ± 1.38[a]	21.2 ± 2.25[a]	19.8 ± 2.01[a]
Tibia P (% of tibia ash)	16.6 ± 1.83[a]	13.9 ± 0.93[a]	14.6 ± 1.53[a]	13.5 ± 1.22[a]

Control, having 2% inorganic Di-calcium phosphate (DCP) (100% DCP); Diet 2, containing 1% DCP plus 0.1% nano-hydroxyapatite (NHA) (50% DCP); Diet 3, containing 0.5% DCP plus 0.1% NHA (25% DCP); Diet 4, having 0.1% NHA only (0% DCP).
Values are expressed as Mean±SE
Values of different superscripts in the same row differ significantly at P ≤0.05

is consistent with the findings of Mishra *et al.* (2019), who observed a non-significant difference in the body weight of broiler fed either 0.42% or 0.85% of Ca-P NPS of the diet (51 nm to 200 nm) compared to the control (100% DCP). Moreover, the results of broiler performance in the present study are in agreement with those obtained by Hassan *et al.* (2016) who observed that using Ca-P NPs at lowest levels of 0.88% and 0.44% instead of the conventional DCP in broiler chickens' diets did perform as well as control one. Also, Sohair *et al.* (2017) recorded no difference in BWG and FCR during the finishing period of broilers fed 0.08%, 0.12%, and 0.16% NHA of the diet, compared with the control group (100% DCP). Contrary to the results of the present study, Vijayakumar and Balakrishnan (2014a) reported that the cumulative gain in body weight of broiler chickens fed with rations containing 50% and 60% Ca-P NPs had significantly higher body weight gain than the rest of the treated groups. Also, Hassan *et al.* (2016) reported that the body weight gain of broilers was proportionally increased as the level of nano calcium phosphate increased from 0.44 to 1.75%. The non-significant difference in this study in body weight and BWG of broilers in group 4 might be due to using a very low level of NHA i.e. 0.1% against the above-mentioned trials. Matuszewski *et al.* (2020) stated that in the case of broiler chickens, the concentration, source or particle size of Ca have a lesser effect on their production results. Also, it could be explained that the conventional/inorganic DCP had a low bioavailability. On the other hand, the small size (ranging from 20 to 100 nm) and the larger surface area of NHA, probably increased bioavailability and give the same results as the control group. The variations in the results obtained from the different feeding trials might be related to many factors such as the size and solubility of the nanoparticle, experimental design, i.e. inclusion rate of nano-feed supplement, feed types, and management factors (Li *et al.*, 2016). Among the NHA supplemented groups, significantly lower body weight and BWG of broilers were recorded in broilers fed DCP mixed with NHA (diets 2 and 3) than that of the control group. This might be due to supplementation of conventional DCP at a high level i.e. 50 or 25 % with NHA lowered the body weights of broilers. This might be one of the causes of lower body weight in those groups as high Ca in poultry feed has been also resulted in decreased bird performance (Sebastian *et al.*, 1996) and macro mineral utilization and bioavailability (Simpson and Wise, 1990). A study conducted by (Long *et al.* (1984) demonstrated that Ca administered in excess to broiler chickens caused the formation of insoluble Ca phosphates ($Ca_3(PO_4)_2$) in their intestines, which led to P deficiency. The average cumulative feed consumption and FCR of all the treated groups did not differ significantly. The current observations are consistent with Mishra *et al.* (2019), who stated that an increase or a decrease in P content of the diet did not significantly influence the feed intake of broiler and FCR. (Jiang *et al.*, 2013; Thacker *et al.*, 2013) also stated that feed efficiency in broiler chicks was similar despite differences in the dietary available P content. In contradiction to this finding, significantly higher feed consumption and better FCR were reported in broilers fed Ca-P NPs supplemented diets (Hassan *et al.*, 2016; Vijayakumar and Balakrishnan, 2014a). The difference between the present findings with those recorded by Hassan *et al.* (2016) was that they fed three levels of either conventional DCP or Ca-P NPs at 1.75, 1.31 and 0.88% and compared the feed consumption and FCR but in the present study, the conventional DCP was replaced with only 0.1% NHA. Also, Vijayakumar and Balakrishnan (2014a) replaced DCP with Ca-P NPs keeping P level constant, but in the present study low inclusion level of NHA i.e. (0.1%) with low P levels were found in different treatments and in addition to diet 4 in which the whole amount of conventional DCP was replaced by only 0.1% of NHA. Even if non-significant, BW, BWG and FCR was observed in broiler birds in NHA supplemented groups was higher than the breed catalogue of broilers 2226 g, 2183 g, 1.48 respectively. It seems that the broiler chickens tolerate a relatively wide range of Ca and P concentrations as far as the Ca to P ratio in their diet generally assumed at 1:1–2:1 (Matuszewski *et al.*, 2020).

The non-significant difference in dressing percentage among treatments indicates that the intervention made in feed did not negatively influence the metabolism. Similarly, Vijayakumar and Balakrishnan (2014b) reported no significant difference in dressing percentage, heart weight, and liver weight by Ca-P NPs supplementation. Also, Mishra *et al.* (2019) observed that no significant difference in dressing percentage, breast, thigh, and drumstick yield with nano dicalcium phosphate supplementation. On the contrary, Sohair *et al.* (2017) stated that the highest carcass weight values were recorded for birds fed 6 and 8% NHA at 6 weeks of age, compared to the control group. This variation among these studies might be due to the difference in dietary treatment and the duration of the experiments.

The serum mineral profile did not show any significant difference due to the interventions made in the diet, which again reinforces the recommendation of replacing the conventional DCP supplementation with Ca-P NPs as the birds were healthy. The results are in line with (Mishra *et al.* (2019), who observed that the serum Ca and P levels were similar for both the control and Ca-P NPs- supplemented groups. Vijayakumar and Balakrishnan (2014b) also reported no significant difference in the serum Ca and P with supplementation of Ca-P NPs to broiler birds.

The results regarding Ca and P content in the thigh muscle reinforce the recommendation of replacing the conventional DCP supplementation by the NHA as Ca and P content in thigh muscle in experimental groups was comparable to the control one. Sohair *et al.* (2017) reported that the addition of nano dicalcium phosphate improved the bioavailability of Ca and P. In this regard, Gross *et al.* (2014) found that nanoparticle-sized ingredients of calcium phosphate have a larger specific surface area and surface roughness compared to the traditional form. Also, Rajendran (2013) reported that a nano-sized calcium phosphate materials have greater bioactivity compared to traditional materials since dietary fortification with mineral in a nano form increases functionality and subsequent bioavailability by increasing the surface area.

There was a significant reduction in P excretion in groups fed NHA compared with the control group. Birds fed 50% of conventional DCP plus 0.1% NHA excreted 31.8% less P while that fed 25% of CDCP plus 0.1% NHA excreted 37.2% less P than those fed 2% conventional DCP diet. Feeding a low level of 0.1% NHA decreased the excreted P by 40.9% compared to the control diet. In this regard, Hassan *et al.* (2016) found that feeding broiler chicks on diets containing 0.44% conventional DCP reduced the Ca and P excretion by 51 and 46%, respectively compared to the control diet. These results indicated that the addition of DCP in nano form enhances the absorption, therefore reduces the excretion of calcium and phosphorus and resulted in minimizing the concentrations needed for this feed supplement (Desai *et al.*, 1997; Sohair *et al.*, 2017). It has been hypothesized that different Ca nanoparticles, because of their high physical reactivity, could be an alternative to conventional forms as they can be supplied in much smaller doses in chicken diets. This would have the added advantage of significantly reducing the excretion of these minerals into the environment (Matuszewski *et al.*, 2020).

A non-significant difference in weight of the tibia and mean tibial bone width and cortex among treatments may indicate that the intervention made in feed did not negatively influence the bone. Little work has addressed the use of nanoparticles of Ca and P on the skeletal system. 99% of the Ca in the body is derived from the skeleton where, together with P, it forms hydroxyapatite (Bello *et al.*, 2014). Considering the nutritional factors, it is reasonable to pay attention to the two major macro-elements involved, the intake and common ratio of Ca and P which is necessary to ensure bone strength. These elements are the main contributors to bone mineral structure, where they occur in the form of hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) (Scott *et al.*, 1982; Turek, 1984). Ca and P in the form of the hydroxyapatite account for over 90% of the mineral fraction (Scott *et al.*, 1982). Also, it could be indicated that the bioavailability of hydroxyapatite in nano form is higher than conventional dicalcium phosphate. Gorbunoff (1984) and Spenlehauer *et al.* (1989) recorded that calcium phosphate nanoparticles have a chemical structure similar to that of the bone and hence has high biocompatibility and bioactivity. The obtained results are consistent with Vijayakumar and Balakrishnan (2014b), who found that tibial bone ash, Ca, and P contents are unison for both the control, which received 100% dicalcium phosphate and 50% calcium phosphate nanoparticles supplemented groups. Also, Mohamed *et al*. (2016) found that birds fed 0.44% of the nano DCP showed comparable values of tibia weight, length, width, and breaking strength as that fed 1.75% of the CDCP. The mean tibial weight, width, cortex and total ash in our control broilers were similar to those reported in previous studies (Applegate and Lilburn 2002; Viveros *et al.* 2002). Supplementation of nano-hydroxyapatite as nanoparticles did not affect the tibial bone total ash content. The data on mean Ca and P content as influenced by nano-hydroxyapatite nanoparticles revealed that there was no significant difference among the various treatments in tibial bone mineral contents. The mean Ca and P in broilers in the control group were comparable to the data recorded by Ahmad *et al.* (2000). Barreiro *et al.* (2009) reported that ash, Ca, and P in the bone of broilers reach their maximum at 22 days of age and older birds were more efficiently able to use organic P. Bone development was affected by in ovo application of Ca carbonate nanoparticles, resulting in significantly higher Ca and Cu, but not P, concentrations in tibia bones in broiler hatchlings (Salary *et al.*, 2017).

Conclusion

This study indicated that dietary supplementation of 0.5% DCP plus 0.1% NHA or only 0.1% NHA as a source of Ca and P has nearly the same effects on productive performance compared to diets containing 2% DCP. Dietary fortification with only 25% of the required DCP plus 0.1% NHA could be used instead of using 100% of the required DCP. Moreover, using 0.1% NHA allows effectively replacing 100% of the dietary DCP. Using NHA also allows the P excretion to be reduced by about 40.9%, which minimizes environmental pollution. Further, the tibial bone ash, Ca, and P contents are unison for the control group (which received 100% DCP) and other NHA-supplemented groups (that received only 50%, 25%, and 0% of the required DCP). It could be concluded that a very low dose of synthetic NHA could be used as an alternative source of Ca and P in broiler diets.

Conflict of interest

The authors declare that they have no conflict.

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