Revista Brasileira de Ciência Avícola
ISSN: 1516-635X
revista@facta.org.br
Fundação APINCO de Ciência e Tecnologia Avícolas
Brasil

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Revista Brasileira de Ciência Avícola, vol. 18, núm. 1, enero-marzo, 2016, pp. 73-85
Fundação APINCO de Ciência e Tecnologia Avícolas
Campinas, Brasil

Brazilian Journal of Poultry Science
Revista Brasileira de Ciência Avícola
ISSN 1516-635X Jan - Mar 2016 / v.18 / n.1 / 073-086
http://dx.doi.org/10.1590/18069061-2015-0016

# *Effects of 1.25-Dihydroxycholecalciferol and Hydroalcoholic Extract of Withania Coagulans Fruit on Bone Mineralization and Mechanical and Histological Properties of Male Broiler Chickens*

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■Keywords

1.25-dihydroxycholecalciferol, Withania coagulans, bone, broiler chickens.

Submitted: February/2015
Approved: March/2015

## ABSTRACT

An experiment was conducted to investigate the effects of hydroalcoholic extract of *Withania coagulans* (WC) fruit and 1.25-dihydroxycholecalciferol (1.25-$(OH)_2$ $D_3$) on bone mineralization, mechanical and histological properties of male broiler chickens at 21 and 42 d of age. A total of six hundred male day-old Ross 308 broiler chickens were randomly distributed according to a completely randomized experimental design in a 2×3×2 factorial arrangement with 12 treatments of five replicates of 10 birds each. Treatments consisted of two basal diets (positive control with adequate Ca level and negative control with 30% less Ca), three levels of WC (0, 100, or 200 mg/kg diet), and two levels of 1.25-$(OH)_2$ $D_3$ (0 or 0.5 µg/kg diet). Birds were housed in floor pens. The diets were fed *ad libitum* from one to 42 days of age. On day 21 and 42, one bird per replicate was sacrificed and its tibiae were removed. Both Ca and P retention increased when dietary Ca level was reduced ($p<0.001$). The addition of 200 mg WC/kg to positive control diet increased Ca retention ($p<0.01$). Except for tibia diameter, no significant main effects of experimental treatments were observed on tibia physical characteristics or on bone mineralization. The diet with 30% Ca reduction decreased tibia diameter at 42 days of age ($p<0.05$). The dietary addition of 1.25-$(OH)_2$ $D_3$ increased tibia fracture energy, width of tibia mineralized zone, and serum Ca at 42 days of age ($p<0.05$). At 21 days of age, supplementation of 100 mg WC/kg increased cortical thickness ($p<0.05$). At 42 days of age, supplementation of 100 mg WC/kg increased tibia shear force ($p<0.05$) and fracture energy ($p<0.01$). The results of this experiment showed that supplementation of 100 mg/kg hydroalcoholic extract of WC fruit increased tibia cortical thickness, shear force, and fracture energy.

## INTRODUCTION

Dietary and endogenous vitamin $D_3$ metabolism in the bird is a complex process. This process involves the hydroxylation of vitamin $D_3$ molecule at the position 25 in the liver to produce 25-OH-cholecalciferol (25-OH-$D_3$), the main circulating form of vitamin $D_3$ in the blood. The circulating 25-OH-cholecalciferol is then transported to the kidneys and hydroxylated at the position 1 to produce 1.25-dihydroxycholecalciferol [1.25-$(OH)_2$ $D_3$], the most biologically active hormonal metabolite of the vitamin $D_3$ (Norman, 2008).

The molecule 1.25-$(OH)_2$ $D_3$ has a well-recognized role in the regulation of mineral metabolism. It has been established that dietary supplementation with 1.25-$(OH)_2$ $D_3$ and other metabolites of vitamin $D_3$ improve bone mineralization in broiler chickens fed a diet with low Ca and high or adequate P levels (Edwards, 1989; Ledwaba & Roberson, 2003). Also, it has been reported that the dietary supplementation of

cholecalciferol derivatives improves bone mechanical properties of broilers, such as shear force and shear fracture energy in conventional diets with low cholecalciferol (Noff *et al.,* 1982; Atencio *et al.,* 2005). It is well known that the administration of estradiol hormones mediate renal 25-hydroxyvitamin $D_3$ 1α-hydroxylase ($VD_3$ 1α hydroxylase) (E.C.1.14.13.13), the enzyme responsible for the metabolic conversion of 25-OH-$D_3$ into 1α,25-$(OH)_2$ $D_3$ (Soares, 1984; Reddy & Tsering, 1989).

There are 23 known species of *Withania* (Solanaceae plants) and among these only two, *Withania somnifera* (WS) and *Withania coagulans* (WC), are economically important and widely cultivated (Panwar & Tarafdar, 2006). *Withania coagulans* L. Dunal is a small evergreen shrub that has been shown to possess several medicinal properties as a remedy for dyspepsia, flatulent colic, and other intestinal diseases (AbouZid *et al.,* 2010). These effects are attributed to a group of steroidal lactones called withanolides, comprising a group of C-22 and C-26 patterns (Dewir *et al.,* 2010). Previous chemical investigations of this plant identified several withanolides in WC fruits (Atta-ur-Rahman *et al.,* 2003; Prasad *et al.,* 2010). Tahmasbi *et al* (2012) reported that the dietary supplementation of 130 mg/kg of an alcoholic extract of the root of WS increased Ca and P retention in the tibiae of aged laying hens. They hypothesized that mechanism of these changes is driven by estrogen-like withanolides that may be stimulate the activity of renal 25-hydroxyvitamin $D_3$ 1α-hydroxylase, increasing the production of 1.25-$(OH)_2$ $D_3$. Also, Nagareddy & Lakshmana (2006) suggested a possible effect of WS on Ca and P metabolism. Because of the involvement of 1.25-$(OH)_2$ $D_3$ in bone mineralization and the possible effect of withanolides on Ca and P metabolism, it was therefore of interest to investigate the comparative and cumulative effects of 1.25-$(OH)_2$ $D_3$ and a hydroalcoholic extract of the WC fruit added to diets with low and adequate Ca levels on bone mineralization and bone mechanical and histological characteristics of male broilers.

## MATERIAL AND METHODS

### Production of *Withania coagulans* fruit extract

Dried WC fruits were purchased from the local market in Saravan, Sistan, and Baluchestan, Iran, and authenticated at the Herbarium of Botany Directorate in Ferdowsi University of Mashhad Iran. The fruits were coarsely ground and soaked in 50% ethanol with occasional shaking at room temperature. After three days, the ethanol soluble materials were filtered and concentrated using a rotary evaporator (Laborota 4000, Heidolph, Germany), and then freeze-dried for 24 h to produce a powder. The dried powder was stored at -20°C until use in the experiment.

### Birds, husbandry and treatments

Six hundred day-old male Ross 308 broiler chickens were purchased from a commercial hatchery, and randomly allocated to 12 experimental treatments with five replicates (10 birds per replicate). The experiment was conducted as a 2×3×2 factorial arrangement with two basal diets (negative and positive controls), three WC levels (0, 100, or 200 mg/kg diet), and two levels of 1.25-$(OH)_2$ $D_3$ (0 or 0.5 μg/kg diet).

The 1.25-$(OH)_2$ $D_3$ (Sigma Aldrich, St. Louis, MO, USA) was used in liquid form with corn oil as a carrier (10 μg/mL). Both control diets (Table 1) were based on corn and soybean meal and were formulated to meet the requirements suggested by the Ross 308 manual (Ross, 2007) for all nutrients, except for Ca, which was reduced by 30% (obtained by reducing limestone inclusion and adding fine sand) in the negative control diet. All birds had free access to feed and water throughout 42-day experimental period. All procedures were approved by the Ferdowsi University of Mashhad Animal Care Committee.

### General procedures

Feed intake (FI) and body weight gain (BWG) were recorded at 11, 24 and 42 days of age to assess growth performance. At 15 days of age, birds were fasted for 16 h, and experimental diets containing 0.3% chromium oxide were fed to measure mineral retention (ash, Ca and P). After 24 h of adaptation, the excreta from each pen was collected for 72 h to determine ash, Ca, and P retention. Feed and excreta samples were oven-dried at 105°C for 24 h.

At 21 and 42 days of age, one bird was removed from each pen and blood samples were taken from the brachial vein. The serum was separated by centrifugation at 1500 × g for 15 minutes, and stored at -20°C until Ca, P, and alkaline phosphatase (ALP) analyses.

After the birds were sacrificed by cervical dislocation (21 and 42 day), both tibiae were dissected from the carcass and the adherent tissues were removed. The left tibia was stored at -20°C for subsequent measurement of mechanical properties and mineral content. The proximal epiphysis and mid-diaphysis of right tibia were cut to collect 0.5-cm thickness

**Table 1.** Composition of the basal diets (g/kg).

| Ingredient | Starter (1-10 day) | | Grower (11-23 day) | | Finisher (24-43 day) | |
|---|---|---|---|---|---|---|
| | - | + | - | + | - | + |
| Corn | 520.0 | 520.0 | 532.0 | 532.0 | 530.0 | 530.0 |
| Soybean meal | 350.0 | 350.0 | 370.0 | 370.0 | 369.0 | 369.0 |
| Corn gluten meal | 50 | 50 | - | - | - | - |
| Vegetable oil | 32.7 | 32.7 | 58.0 | 58.0 | 65.6 | 65.6 |
| Limestone | 5.3 | 13.1 | 4.0 | 10.7 | 3.7 | 10.3 |
| Dicalcium phosphate | 17.5 | 17.5 | 15.5 | 15.5 | 14.0 | 14.0 |
| Salt | 3.5 | 3.5 | 4.7 | 4.7 | 4.1 | 4.1 |
| DL-Methionine | 3.2 | 3.2 | 2.8 | 2.8 | 2.0 | 2.0 |
| L-Lysine HCL | 4.0 | 4.0 | 1.3 | 1.3 | - | - |
| Threonine | 1.0 | 1.0 | - | - | - | - |
| Vitamin premix[1] | 2.5 | 2.5 | 2.5 | 2.5 | 2.5 | 2.5 |
| Mineral premix[2] | 2.5 | 2.5 | 2.5 | 2.5 | 2.5 | 2.5 |
| Sand | 7.8 | - | 6.7 | - | 6.6 | - |
| *Calculated nutrients and energy* | | | | | | |
| AME, (MJ/kg) | 12.6 | 12.6 | 13.1 | 13.1 | 13.3 | 13.3 |
| Crude protein (g/kg) | 235.2 | 235.2 | 211.5 | 211.5 | 209.1 | 209.1 |
| Lysine (g/kg) | 14.4 | 14.4 | 12.4 | 12.4 | 11.3 | 11.3 |
| Methionine (g/kg) | 7.0 | 7.0 | 6.1 | 6.1 | 5.2 | 5.2 |
| TSAA[3] (g/kg) | 10.7 | 10.7 | 9.5 | 9.5 | 8.6 | 8.6 |
| Calcium (g/kg) | 7.3 | 10.4 | 6.3 | 9.0 | 5.9 | 8.5 |
| Non-phytate P (g/kg) | 5.0 | 5.0 | 4.5 | 4.5 | 4.2 | 4.2 |
| Total P (g/kg) | 7.0 | 7.0 | 6.8 | 6.8 | 6.5 | 6.5 |
| Analyzed Ca and total P concentration | | | | | | |
| Calcium (g/kg) | 7.5 | 10.7 | 6.5 | 9.3 | 6.1 | 8.8 |
| Total P (g/kg) | 7.4 | 7.4 | 7.1 | 7.1 | 6.7 | 6.7 |

[1]Vitamin premix provided per kilogram of diet: vitamin A, 11,000 IU; vitamin D3, 1,800 IU; vitamin E, 11 mg; vitamin K3, 2 mg; vitamin B2, 5.7 mg; vitamin B6, 2 mg; vitamin B12, 0.024 mg; nicotinic acid, 28 mg; folic acid, 0.5 mg; pantothenic acid, 12 mg; choline chloride, 250 mg.

[2]Mineral premix provided per kilogram of diet: Mn, 100 mg; Zn, 65 mg; Cu, 5 mg; Se, 0.22 mg; I, 0.5 mg; and Co, 0.5 mg.

[3]Total sulfur amino acids

samples. Both longitudinal epiphyseal and latitudinal diaphyseal samples were fixed in 10% phosphate buffer formalin for 12 h at 4°C, decalcified in 10% formic acid, embedded in paraffin, and 5 μm sections were prepared using a microtome. The sections were stained with hematoxylin and eosin (HE).

The width of growth plate zones (proliferative zone, hypertrophic zone, and mineralized zone) and cortical thickness were measured using an optical microscope (Olympus BX41TF, Tokyo, Japan), photographed with a digital camera system (Olympus DP12 U-TV0.5 XC-2, Japan), and the images were analyzed using Soft Imaging System (Olysia Soft Imaging System, Germany).

The 3-point bending test was used to determine the mechanical properties of the left tibia. The frozen left tibia was thawed at room temperature for 4 h, oven-dried at 105°C for 24 h, and defatted with diethyl ether for 48 h. Bone length and diameter at the center of diaphysis were measured using digital calipers. The mechanical properties were determined using an Instron Universal Testing Machine (Model H5KS, Tinius Olsen Company). The bones were held in identical positions. The distance between the two supporting ends was 5 cm and the 10 mm diameter crosshead probe (50 kg) approached the bone at 5 mm/min until fracture occurred. During the experiment care was taken to ensure that the impact of crosshead was at the midpoint. Using the software (Q Mat), mechanical parameters such as shear force, shear fracture energy, maximal deflection before fracture, and stiffness were calculated. Broken tibia samples were collected, dried over night at 105 °C in an oven, and ashed at 600 °C for 16 h for mineral content measurement.

## Chemical analysis

The concentrations of Ca, inorganic P and ALP in the serum samples were determined using an automatic blood chemical analyzer (Random Access Analyser A15, Biosystem Corp, Spain). The concentrations of Ca and total P in the diets, excreta, and bones were determined by atomic absorption spectrophotometry (Varian SpectrAA 50B Atomic Absorption Spectrometer, Varian Ltd, USA) (AOAC, 2005 method 927.02) and total P was colorimetrically measured using the

molybdovanadate method (AOAC, 2005 method 965.17). The concentration of chromium in dried samples of feed and excreta was measured according to the procedure described by Williams *et al.* (1962) using an atomic absorption spectrophotometry (Varian SpectrAA 50B Atomic Absorption Spectrometer, Varian Ltd, USA).

### Statistical analysis

This experiment was conducted according to a completely randomized design with 2×3×2 factorial arrangement, totaling 12 treatments. All experimental data were subjected to analysis of variance using the GLM procedure of SAS (SAS Institute, 2003). Significant differences among the means were determined by Duncan's multiple-range test at $p<0.05$.

## RESULTS AND DISCUSSION

### Live performance and mineral retention

No effects ($p>0.05$) were observed on growth performance or ash retention (Table 2). Feed intake (FI), body weight gain (BWG) and feed conversion ratio (FCR) were significantly influenced by the two-way interaction between the control and WC treatments. There were significant interactions between control × 1.25-$(OH)_2$ $D_3$ and WC × 1.25-$(OH)_2$ $D_3$ for FI. The addition of 200 mg WC/kg to the positive control diet resulted in FI depression (3699 *vs*. 4168 g; $p<0.05$), whereas no FI difference was observed between the supplementation of 0.5 µg/kg 1.25-$(OH)_2$ $D_3$ and 200 mg/kg WC (4072 *vs*. 4168 g). The birds fed the negative control diet showed lower FI in comparison with those

**Table 2 –** Effect of *Withania coagulans* (WC), 1.25 $(OH)_2$ $D_3$ and type of control diet on body weight gain (BWG), feed intake (FI),and feed conversion ratio (FCR) determined at 42 days of age and mineral retention of broilers with 19-21 days of age.

| Treatment | | | | | | Mineral retention | | |
|---|---|---|---|---|---|---|---|---|
| Control | WC (mg/kg) | 1.25 $(OH)_2$ $D_3$ (µg/kg) | FI (g) | BWG (g) | FCR (g/g) | Ash (%) | Ca (%) | P (%) |
| - | 0 | 0 | 3905$^{abcd}$ | 2225$^{c}$ | 1.75$^{a}$ | 62.3 | 82.8$^{ab}$ | 75.8 |
| - | 0 | 0.5 | 3787$^{cd}$ | 2215$^{c}$ | 1.71$^{ab}$ | 62.8 | 82.2$^{ab}$ | 76.7 |
| - | 100 | 0 | 4019$^{abc}$ | 2357$^{abc}$ | 1.70$^{ab}$ | 64.4 | 82.6$^{ab}$ | 78.1 |
| - | 100 | 0.5 | 3812$^{bcd}$ | 2291$^{bc}$ | 1.66$^{ab}$ | 57.9 | 84.6$^{a}$ | 76.1 |
| - | 200 | 0 | 3828$^{bcd}$ | 2284$^{bc}$ | 1.67$^{ab}$ | 62.4 | 81.3$^{ab}$ | 76.8 |
| - | 200 | 0.5 | 3923$^{abcd}$ | 2428$^{ab}$ | 1.61$^{b}$ | 63.0 | 82.3$^{ab}$ | 77.6 |
| + | 0 | 0 | 4012$^{abc}$ | 2407$^{abc}$ | 1.66$^{ab}$ | 59.0 | 70.0$^{c}$ | 71.9 |
| + | 0 | 0.5 | 4168$^{a}$ | 2511$^{a}$ | 1.66$^{ab}$ | 61.5 | 68.5$^{c}$ | 73.7 |
| + | 100 | 0 | 3820$^{bcd}$ | 2240$^{bc}$ | 1.70$^{ab}$ | 66.3 | 71.3$^{c}$ | 74.6 |
| + | 100 | 0.5 | 3894$^{bcd}$ | 2268$^{bc}$ | 1.72$^{ab}$ | 66.6 | 71.0$^{c}$ | 74.7 |
| + | 200 | 0 | 3699$^{d}$ | 2237$^{bc}$ | 1.65$^{ab}$ | 63.4 | 75.5$^{bc}$ | 74.9 |
| + | 200 | 0.5 | 4072$^{ab}$ | 2348$^{abc}$ | 1.73$^{a}$ | 65.7 | 81.6$^{ab}$ | 73.6 |
| SEM | | | 82.36 | 60.71 | 0.03 | 2.07 | 2.31 | 1.72 |
| Main effect | | | | | | | | |
| Control | | - | 3879 | 2300 | 1.68 | 62.0 | 82.7$^{a}$ | 76.8$^{a}$ |
| | | + | 3944 | 2335 | 1.69 | 63.7 | 72.9$^{b}$ | 73.9$^{b}$ |
| WC | | 0 | 3968 | 2339 | 1.70 | 61.4 | 75.9 | 75.9 |
| | | 100 | 3886 | 2289 | 1.69 | 63.6 | 77.4 | 75.9 |
| | | 200 | 3881 | 2324 | 1.67 | 63.8 | 80.3 | 75.7 |
| 1.25 $(OH)_2$ $D_3$ | | 0 | 3881 | 2292 | 1.69 | 62.8 | 77.3 | 75.4 |
| | | 0.5 | 3942 | 2344 | 1.68 | 62.9 | 78.2 | 75.3 |
| | | | | | | p | | |
| Control | | | 0.174 | 0.321 | 0.883 | 0.200 | <0.001 | <0.001 |
| WC | | | 0.254 | 0.494 | 0.369 | 0.232 | 0.135 | 0.594 |
| 1.25 $(OH)_2$ $D_3$ | | | 0.199 | 0.146 | 0.625 | 0.964 | 0.620 | 0.980 |
| Control × WC | | | 0.031 | <0.001 | 0.041 | 0.053 | 0.007 | 0.683 |
| Control × 1.25 $(OH)_2$ $D_3$ | | | 0.005 | 0.410 | 0.044 | 0.172 | 0.684 | 0.616 |
| WC × 1.25 $(OH)_2$ $D_3$ | | | 0.036 | 0.243 | 0.738 | 0.249 | 0.249 | 0.807 |
| Control × WC × 1.25 $(OH)_2$ $D_3$ | | | 0.999 | 0.653 | 0.516 | 0.664 | 0.664 | 0.581 |

$^{a-d}$Means within each column with no common superscript differ significantly ($p<0.05$).

fed the positive control diet at similar levels of 1.25-$(OH)_2$ $D_3$ (0.5 μg/kg) and WC (0 mg/kg) (3787 *vs*. 4168 g; p<0.01). The effect of the interaction between the control and WC on BWG showed that the birds fed the negative control diet with no dietary supplementation of WC had significantly lower BWG in comparison with those fed the positive control diet (2215 *vs*. 2511 g; p<0.001). The birds fed the negative control diet supplemented with 200 mg/kg WC presented lower FCR than those fed the positive control diet and similar WC concentration (1.61 *vs*. 1.73 g/g; p<0.05). Data showed that supplementation of 1.25-$(OH)_2$ $D_3$ improved FCR when added to the negative control diet compared with the positive control diet, regardless dietary WC level (1.61 *vs*. 1.73 g/g).

Both Ca and P retention increased when dietary Ca level was reduced (p<0.001). Our findings are in accordance with those of Mirakzehi *et al.* (2013), who reported that Ca and P retention increased when dietary Ca decreased to 70% of the recommended concentration. Previous published research revealed that birds fed low-Ca diets presented greater Ca retention compared with those offered adequate Ca level (Mitchell & Edwards, 1996; Plumstead *et al.,* 2008). It was shown that phytate P utilization in corn-soybean diets is influenced by dietary Ca and P levels (Scheideler & Sell, 1987). Earlier research also reported higher phytate P utilization in laying hens fed low dietary Ca level (Van der Klis *et al.,* 1994). A two-way interaction among controls and WC was detected on Ca retention. This interaction clearly showed that supplementation of WC at level of 100 mg/kg improved dietary Ca retention in broilers fed the negative control diet compared with those fed the positive control diet (84.67% *vs*. 71.03%; p<0.01). These observations confirm the previous report of Mirakzehi *et al.* (2013), who found greater Ca retention at a supplementary WS level of 150 mg/kg in broilers fed a negative control diet.

## Bone physical characteristics and mineralization

At 21 days of age, the experimental treatments did not affect tibial physical characteristics (weight, length, or diameter; Table 3). Also, no effects were observed on tibia mineralization (p>0.05). The non-significant effects of the negative control diet on bone mineralization suggest that the lowest amount of calcium fed in this experiment (70% of Ca requirement) was not low enough to cause any negative effect on bone mineral retention. In the other words, broilers are able to adapt to moderate Ca deficiencies by increasing absorption rates and utilization efficiency and reducing the excretion of the restricted nutrients (Yan *et al.,* 2005).

In the present experiment, the non-significant differences in tibial mineral retention between the negative and the positive control groups are consistent with the mineral retention results. This may be probably due to the higher Ca and P retention observed with the negative control compared with the positive control diet.

At 42 days of age, birds fed the positive control diet had higher tibia diameter compared with those fed the negative control diet (p<0.05; Table 4). Rousseau *et al.* (2012) reported that broilers fed the adequate dietary Ca presented normal tibia diameter, and emphasized the need for a balanced and adequate supply of Ca and P to optimize certain bone parameters. However, when Ca was decreased by 30%, a greater increase in Ca and P retention was observed in broilers fed the negative control diets. Considering that poultry cope with Ca and P deficiencies through an active regulatory phenomenon that leads to increased mineral utilization efficiency, bone criteria cannot be optimized if the mineral supply is not well balanced (Rousseau *et al.,* 2012). Also, a three-way interaction of control, WC and 1.25-$(OH)_2$ $D_3$ treatments was observed on tibia bone diameter (p<0.05). The maximum bone diameter was detected in birds fed the positive control diet supplemented with 0.5 μg/kg 1.25-$(OH)_2$ $D_3$. The interaction showed that birds fed the low Ca diet with no WC and supplemented with 0.5 μg/kg 1.25-$(OH)_2$ $D_3$ had the lowest tibia diameter compared to those fed the positive control diet with both supplements (7.54 *vs*. 8.65 mm; p<0.05). Also, the interaction suggests that the supplementation of 100 mg/kg WC to the positive control diet containing 0.5 μg/kg 1.25-$(OH)_2$ $D_3$ decreased tibia bone diameter (7.49 *vs*. 8.65 mm; p<0.05).

## Bone histology

The results indicated that birds supplied with 100 mg/kg of WC had a higher bone cortical thickness in comparison with those fed 0 and 200 mg/kg WC at 21 days of age (p<0.05; Table 5). Mineralized zone width was also increased in response to the addition of 1.25-$(OH)_2$ $D_3$ at 42 d of age (p<0.05). There was a two-way significant interaction between the controls and 1.25-$(OH)_2$ $D_3$ on cortical thickness and mineralized zone width at 21 and 42 days of age, respectively. Also, an interaction between dietary WC and 1.25-$(OH)_2$ $D_3$ was observed for mineralized zone width and cortical

thickness at 21 and 42 days of age, respectively. The interaction between dietary WC and 1.25-$(OH)_2$ $D_3$ on mineralized zone showed that, regardless of dietary Ca concentration, the birds given only 100 mg/kg WC had greater mineralized zone width compared with those fed diets containing 100 mg/kg WC and 0.5 μg/kg 1.25-$(OH)_2$ $D_3$ (2448 *vs*. 1768 μm; p<0.01). The interaction between the control and the 1.25-$(OH)_2$ $D_3$ treatments showed that, regardless of dietary WC level, the supplementation of 0.5 μg/kg 1.25-$(OH)_2$ $D_3$ increased cortical thickness in birds receiving the positive control compared with those that consumed the negative control diet (1577 *vs*. 1190 μm; p<0.05). The interaction between the control and the 1.25-$(OH)_2$ $D_3$ treatments suggests that, regardless of dietary level of WC, the addition of 1.25-$(OH)_2$ $D_3$ to the negative control diet increased mineralized zone width (2322 *vs*. 1595 μm; p<0.05). The interaction of WC with 1.25-$(OH)_2$ $D_3$ indicates that cortical thickness was increased in 42-d-old birds fed 0.5 μg/kg 1.25-$(OH)_2$ $D_3$ and 100 mg/kg WC, regardless of dietary Ca level (1782 *vs*. 1425 μm; p<0.05). This result leads us to suggest that the synergistic effects of 1.25-$(OH)_2$ $D_3$ and WC can beneficially influence bone cortical thickness. At 42 days of age, the main effects of the control diets and WC on proliferative zone width and cortical thickness approached significance (p=0.054 and p=0.065, respectively). In spite of no significant effects of dietary WC on tibia mineral retention, the tibial cortical thickness of broilers fed the WC supplemented

**Table 3 –** Effect of *Withania coagulans* (WC), 1.25 $(OH)_2$ $D_3$ and type of control diet on the bone characteristics of 21-d-old broilers.

| Treatment | | | Tibia physical properties | | | Tibia mineral retention | | |
|---|---|---|---|---|---|---|---|---|
| Control | WC (mg/kg) | 1.25 $(OH)_2$ $D_3$ (μg/kg) | Weight (g) | Length (mm) | Diameter (mm) | Ash (%) | Ca (%) | P (%) |
| - | 0 | 0 | 1.63 | 65.7 | 5.31 | 51.0 | 35.6 | 24.3 |
| - | 0 | 0.5 | 1.52 | 65.8 | 5.16 | 51.6 | 36.9 | 24.1 |
| - | 100 | 0 | 1.53 | 64.7 | 5.19 | 52.6 | 34.9 | 24.0 |
| - | 100 | 0.5 | 1.63 | 66.2 | 4.98 | 51.5 | 36.6 | 24.1 |
| - | 200 | 0 | 1.48 | 64.3 | 5.20 | 52.0 | 37.6 | 23.9 |
| - | 200 | 0.5 | 1.74 | 65.7 | 5.36 | 49.6 | 36.6 | 24.3 |
| + | 0 | 0 | 1.66 | 65.9 | 5.35 | 55.1 | 34.7 | 24.3 |
| + | 0 | 0.5 | 1.70 | 65.9 | 5.08 | 52.4 | 35.9 | 24.1 |
| + | 100 | 0 | 1.84 | 66.0 | 5.75 | 49.3 | 35.2 | 23.8 |
| + | 100 | 0.5 | 1.55 | 64.8 | 5.40 | 57.1 | 37.2 | 24.1 |
| + | 200 | 0 | 1.58 | 63.8 | 5.02 | 53.1 | 36.0 | 24.3 |
| + | 200 | 0.5 | 1.73 | 66.4 | 5.31 | 52.5 | 35.9 | 24.4 |
| SEM | | | 0.11 | 0.88 | 0.18 | 1.37 | 1.27 | 0.17 |
| Main effect | | | | | | | | |
| Control | | - | 1.59 | 65.4 | 5.20 | 51.4 | 36.3 | 24.1 |
| | | + | 1. 68 | 65.5 | 5.32 | 53.3 | 35.8 | 24.2 |
| WC | | 0 | 1.63 | 65.8 | 5.22 | 52.5 | 35.8 | 24.2 |
| | | 100 | 1.64 | 65.4 | 5.33 | 52.6 | 36.03 | 25.05 |
| | | 200 | 1.63 | 65.1 | 5.22 | 51.8 | 36.4 | 24.2 |
| 1.25 $(OH)_2$ $D_3$ | | 0 | 1.62 | 65.1 | 5.30 | 52.24 | 35.6 | 24.1 |
| | | 0.5 | 1.65 | 65.8 | 5.21 | 52.5 | 36.5 | 24.2 |
| | | | p | | | | | |
| Control | | | 0.166 | 0.936 | 0.252 | 0.310 | 0.593 | 0.836 |
| WC | | | 0.986 | 0.464 | 0.650 | 0.491 | 0.270 | 0.123 |
| 1.25 $(OH)_2$ $D_3$ | | | 0.447 | 0.150 | 0.417 | 0.528 | 0.197 | 0.344 |
| Control × WC | | | 0.894 | 0.980 | 0.052 | 0.658 | 0.544 | 0.235 |
| Control × 1.25 $(OH)_2$ $D_3$ | | | 0.596 | 0.625 | 0.853 | 0.679 | 0.632 | 0.902 |
| WC × 1.25 $(OH)_2$ $D_3$ | | | 0.275 | 0.219 | 0.122 | 0.757 | 0.113 | 0.134 |
| Control × WC × 1.25 $(OH)_2$ $D_3$ | | | 0.240 | 0.298 | 0.841 | 0.333 | 0.814 | 0.605 |

**Table 4 –** Effect of *Withania coagulans* (WC), 1.25 $(OH)_2$ $D_3$ and type of control diet on the bone characteristics of 42-d-old broilers.

| Treatment | | | Tibia physical properties | | | Tibia mineral retention | | |
|---|---|---|---|---|---|---|---|---|
| Control | WC (mg/kg) | 1.25 $(OH)_2$ $D_3$ (µg/kg) | Weight (g) | Length (mm) | Diameter (mm) | Ash (%) | Ca (%) | P (%) |
| - | 0 | 0 | 5.56 | 95.46 | 7.86$^{ab}$ | 48.9 | 38.8 | 24.3 |
| - | 0 | 0.5 | 5.43 | 98.04 | 7.54$^{b}$ | 49.9 | 38.4 | 24.2 |
| - | 100 | 0 | 5.91 | 98.07 | 7.74$^{ab}$ | 47.7 | 37.9 | 24.0 |
| - | 100 | 0.5 | 5.50 | 96.73 | 7.65$^{ab}$ | 48.5 | 38.6 | 24.1 |
| - | 200 | 0 | 5.45 | 90.95 | 7.67$^{ab}$ | 49.2 | 37.9 | 24.2 |
| - | 200 | 0.5 | 5.12 | 93.96 | 7.76$^{ab}$ | 49.3 | 38.0 | 24.5 |
| + | 0 | 0 | 5.64 | 97.12 | 8.01$^{ab}$ | 48.7 | 38.00 | 24.5 |
| + | 0 | 0.5 | 6.28 | 98.25 | 8.65$^{a}$ | 47.4 | 38.3 | 24.1 |
| + | 100 | 0 | 5.68 | 95.22 | 8.41$^{ab}$ | 47.5 | 38.8 | 24.3 |
| + | 100 | 0.5 | 5.45 | 93.30 | 7.49$^{b}$ | 48.5 | 38.0 | 24.02 |
| + | 200 | 0 | 5.72 | 97.32 | 8.12$^{ab}$ | 49.4 | 38.0 | 24.4 |
| + | 200 | 0.5 | 6.09 | 97.89 | 8.25$^{ab}$ | 48.4 | 38.9 | 24.1 |
| SEM | | | 0.30 | 2.36 | 0.30 | 0.74 | 0.53 | 0.20 |
| Main effect | | | | | | | | |
| Control | | - | 5.49 | 95.5 | 7.70$^{b}$ | 49.0 | 38.3 | 24.2 |
| | | + | 5.81 | 96.52 | 8.15$^{a}$ | 48.3 | 38.3 | 24.2 |
| WC | | 0 | 5.73 | 97.2 | 8.01 | 48.0 | 38.3 | 24.1 |
| | | 100 | 5.64 | 95.8 | 7.82 | 48.0 | 38.3 | 24.1 |
| | | 200 | 5.59 | 95.0 | 7.95 | 49.1 | 38.2 | 24.3 |
| 1.25 $(OH)_2$ $D_3$ | | 0 | 5.66 | 95.6 | 7.97 | 48.63 | 38.2 | 24.3 |
| | | 0.5 | 5.64 | 96.3 | 7.89 | 48.7 | 38.4 | 24.2 |
| | | | p | | | | | |
| Control | | | 0.096 | 0.519 | 0.044 | 0.136 | 0.551 | 0.746 |
| WC | | | 0.566 | 0.189 | 0.934 | 0.138 | 0.754 | 0.848 |
| 1.25 $(OH)_2$ $D_3$ | | | 0.965 | 0.674 | 0.754 | 0.834 | 0.838 | 0.580 |
| Control × WC | | | 0.311 | 0.088 | 0.789 | 0.407 | 0.419 | 0.996 |
| Control × 1.25 $(OH)_2$ $D_3$ | | | 0.315 | 0.412 | 0.567 | 0.222 | 0.782 | 0.051 |
| WC × 1.25 $(OH)_2$ $D_3$ | | | 0.792 | 0.772 | 0.395 | 0.407 | 0.769 | 0.368 |
| Control × WC × 1.25 $(OH)_2$ $D_3$ | | | 0.349 | 0.538 | 0.024 | 0.491 | 0.324 | 0.548 |

$^{a-b}$Means within each column with no common superscript differ significantly ($p<0.05$).

diets increased ($p<0.05$). Although there is no direct evidence to explain the precise mechanism by which the tibial cortical thickness increased in broilers consuming 100 mg/kg WC, it can be speculated that the positive effect of withanolides on endocrine systems (Mishra *et al.*, 2000) may have improved collagen stabilization on diaphysis regions and consequently cortical thickness increased. It has been demonstrated that collagen stability against collagenase could be enhanced through various plant secondary metabolites, such as flavonoids, withanolides, and tannins containing steroidal lactone unit. Withanolides and flavonoids were found to be the preferred chelator for the preparation of collagen inhibitors (Krishnamoorthy *et al.,* 2011). The results of the present study are consistent with the report of Mirakzehi *et al.* (2013), who found that dietary supplementation of 1.25-$(OH)_2$ $D_3$ improved mineralized zone width. An *in-vitro* study reported that 1.25-$(OH)_2$ $D_3$ regulates c-myc protein level (Minghetti & Norman, 1988). Dietary supplementation of 1.25-$(OH)_2$ $D_3$, 3 days prior to killing the chickens increased the level of the c-myc protein in growth plate compared with that of the birds fed the non-supplemented diet (Loveridge *et al.,* 1993). It has previously been demonstrated that c-myc protein is a potent inducer of apoptosis (Farquharson & Jefferies, 2000) to form the matrix vesicles that mediate the mineralization of the growth plate matrix.

## Bone biomechanical properties

At 21 days of age, only the control diets affected fracture deflection ($p<0.01$), as shown in Table 6. Tibia fracture deflection was reduced in birds that received low dietary Ca. At 42 days of age, the main effects of the control diets and WC on tibia shear force were significant ($p<0.05$). Feeding diets containing low dietary Ca level resulted in the reduction of tibia shear force compared with the positive control diet.

These results are consistent with those of O'Connor-Dennie & Southern (2005), who demonstrated that Ca-deficient diets, containing 8 g/kg Ca, resulted in poor bone strength in broilers. The poor bone strength seen in the birds fed low dietary Ca in the present

**Table 5 –** Effect of *Withania coagulans* (WC), 1.25 $(OH)_2$ $D_3$ and type of control diet on tibia histological measurements of broilers at 21 and 42 days of age .

| Treatment | | | 21 days | | | | 42 days | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Control | WC (mg/kg) | 1.25 $(OH)_2$ $D_3$ (µg/kg) | PZ[1] (µm) | HZ[2] (µm) | MZ[3] (µm) | CT[4] (µm) | PZ (µm) | HZ (µm) | MZ (µm) | CT (µm) |
| - | 0 | 0 | 806 | 306 | 1788$^{c}$ | 1260$^{ab}$ | 986 | 266 | 1919$^{abc}$ | 1625$^{ab}$ |
| - | 0 | 0.5 | 743 | 307 | 2092$^{abc}$ | 1269$^{ab}$ | 1056 | 266 | 2322$^{a}$ | 1425$^{b}$ |
| - | 100 | 0 | 893 | 322 | 2448$^{a}$ | 1570$^{a}$ | 1011 | 276 | 1762$^{bc}$ | 1703$^{ab}$ |
| - | 100 | 0.5 | 753 | 299 | 1952$^{abc}$ | 1401$^{ab}$ | 947 | 289 | 2032$^{ab}$ | 1657$^{ab}$ |
| - | 200 | 0 | 725 | 279 | 1880$^{bc}$ | 1323$^{ab}$ | 1007 | 257 | 1595$^{c}$ | 1515$^{ab}$ |
| - | 200 | 0.5 | 740 | 320 | 2094$^{abc}$ | 1190$^{b}$ | 891 | 261 | 1963$^{abc}$ | 1632$^{ab}$ |
| + | 0 | 0 | 799 | 344 | 2031$^{abc}$ | 1329$^{ab}$ | 948 | 295 | 1935$^{abc}$ | 1634$^{ab}$ |
| + | 0 | 0.5 | 775 | 255 | 2226$^{abc}$ | 1433$^{ab}$ | 911 | 264 | 1934$^{abc}$ | 1560$^{ab}$ |
| + | 100 | 0 | 760 | 327 | 2311$^{ab}$ | 1369$^{ab}$ | 871 | 285 | 1897$^{abc}$ | 1648$^{ab}$ |
| + | 100 | 0.5 | 721 | 273 | 1768$^{c}$ | 1577$^{a}$ | 911 | 224 | 1748$^{bc}$ | 1782$^{a}$ |
| + | 200 | 0 | 724 | 259 | 2090$^{abc}$ | 1268$^{ab}$ | 924 | 235 | 1959$^{abc}$ | 1431$^{b}$ |
| + | 200 | 0.5 | 828 | 278 | 1927$^{bc}$ | 1380$^{ab}$ | 910 | 262 | 1969$^{abc}$ | 1752$^{a}$ |
| SEM | | | 47.8 | 31.6 | 145.2 | 106.0 | 61.2 | 21.9 | 128.5 | 84.50 |
| Main effect | | | | | | | | | | |
| Control | | - | 774 | 307 | 2042 | 1336 | 983 | 269 | 1932 | 1591 |
| | | + | 769 | 287 | 2051 | 1401 | 912 | 261 | 1907 | 1634 |
| WC | | 0 | 780 | 303 | 2034 | 1322$^{b}$ | 975 | 273 | 2028 | 1561 |
| | | 100 | 781 | 305 | 2118 | 1485$^{a}$ | 936 | 268 | 1860 | 1699 |
| | | 200 | 756 | 284 | 1998 | 1291$^{b}$ | 933 | 254 | 1871 | 1582 |
| 1.25 $(OH)_2$ $D_3$ | | 0 | 784 | 306 | 2076 | 1357 | 958 | 269 | 1845$^{b}$ | 1593 |
| | | 0.5 | 761 | 289 | 2018 | 1375 | 939 | 261 | 1995$^{a}$ | 1634 |
| | | | p | | | | | | | |
| Control | | | 0.768 | 0.064 | 0.848 | 0.225 | 0.054 | 0.493 | 0.734 | 0.402 |
| WC | | | 0.684 | 0.373 | 0.525 | 0.043 | 0.553 | 0.444 | 0.130 | 0.065 |
| 1.25 $(OH)_2$ $D_3$ | | | 0.408 | 0.462 | 0.355 | 0.519 | 0.574 | 0.531 | 0.049 | 0.400 |
| Control × WC | | | 0.221 | 0.778 | 0.285 | 0.481 | 0.740 | 0.412 | 0.123 | 0.901 |
| Control × 1.25 $(OH)_2$ $D_3$ | | | 0.203 | 0.472 | 0.312 | 0.030 | 0.647 | 0.290 | 0.010 | 0.091 |
| WC × 1.25 $(OH)_2$ $D_3$ | | | 0.116 | 0.099 | 0.003 | 0.707 | 0.638 | 0.414 | 0.693 | 0.017 |
| Control × WC × 1.25 $(OH)_2$ $D_3$ | | | 0.898 | 0.937 | 0.712 | 0.832 | 0.389 | 0.311 | 0.985 | 0.944 |

[a-c]Means within each column with no common superscript differ significantly ($p<0.05$).
[1]PZ= Proliferative zone
[2]HZ= Hypertrophic zone
[3]MZ= Mineralized zone
[4]CT=Cortical thickness

**Table 6 –** Effect of *Withania coagulans* (WC), 1.25 $(OH)_2$ $D_3$ and type of control diet (negative control with 30% lower Ca level and positive with the recommended Ca level) on bone mechanical properties of broilers at 21 and 42 days of age.

| Treatment | | | 21 days | | | | 42 days | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Control | WC (mg/kg) | 1.25 $(OH)_2$ $D_3$ (µg/kg) | SF[2] (N) | FD[3] (mm) | FE[4] (N-mm) | Stiffness (N/mm) | SF (N) | FD (mm) | FE (N-mm) | Stiffness (N/mm) |
| - | 0 | 0 | 89.5 | 0.55 | 31.4 | 193 | 188$^{bc}$ | 0.63 | 59.4 | 304 |
| - | 0 | 0.5 | 93.6 | 0.51 | 30.2 | 188 | 232$^{abc}$ | 0.73 | 123 | 211 |
| - | 100 | 0 | 113 | 0.57 | 31.2 | 205 | 208$^{abc}$ | 0.70 | 71.5 | 296 |
| - | 100 | 0.5 | 107 | 0.54 | 28.7 | 200 | 199$^{bc}$ | 0.70 | 84.3 | 286 |
| - | 200 | 0 | 92.0 | 0.52 | 26.1 | 174 | 173$^{c}$ | 0.56 | 47.5 | 314 |
| - | 200 | 0.5 | 98.5 | 0.49 | 23.4 | 184 | 180$^{bc}$ | 0.60 | 64.7 | 290 |
| + | 0 | 0 | 99.3 | 0.59 | 29.7 | 169 | 200$^{bc}$ | 0.69 | 62.5 | 310 |
| + | 0 | 0.5 | 112 | 0.80 | 38.2 | 166 | 184$^{bc}$ | 0.61 | 67.9 | 319 |
| + | 100 | 0 | 110 | 0.53 | 21.1 | 190 | 246$^{ab}$ | 0.72 | 104 | 341 |
| + | 100 | 0.5 | 107 | 0.58 | 27.0 | 184 | 271$^{a}$ | 0.84 | 127 | 316 |
| + | 200 | 0 | 121 | 0.69 | 41.0 | 180 | 200$^{bc}$ | 0.68 | 65.9 | 308 |
| + | 200 | 0.5 | 110 | 0.61 | 32.1 | 209 | 224$^{abc}$ | 0.66 | 75.4 | 328 |
| SEM | | | 10.7 | 0.04 | 5.31 | 17.4 | 20.0 | 0.04 | 15.2 | 31.6 |
| Main effect | | | | | | | | | | |
| Control | | - | 99.1 | 0.53$^{b}$ | 28.3 | 191 | 193$^{b}$ | 0.65 | 74.8$^{b}$ | 288 |
| | | + | 110.3 | 0.62$^{a}$ | 32.0 | 182 | 221$^{a}$ | 0.70 | 82.3$^{a}$ | 320 |
| WC | | 0 | 98.6 | 0.59 | 32.5 | 178 | 195$^{b}$ | 0.65 | 77.1$^{b}$ | 293 |
| | | 100 | 109 | 0.56 | 27.3 | 195 | 232$^{a}$ | 0.74 | 96.46$^{a}$ | 308 |
| | | 200 | 106 | 0.58 | 30.6 | 186 | 194$^{b}$ | 0.63 | 63.4$^{b}$ | 311 |
| 1.25 $(OH)_2$ $D_3$ | | 0 | 104 | 0.58 | 30.3 | 185 | 202 | 0.66 | 66.6$^{b}$ | 311 |
| | | 0.5 | 105 | 0.57 | 30.0 | 188 | 214 | 0.69 | 91.6$^{a}$ | 298 |
| | | | | | | p | | | | |
| Control | | | 0.084 | 0.001 | 0.265 | 0.455 | 0.045 | 0.150 | 0.002 | 0.173 |
| WC | | | 0.353 | 0.327 | 0.615 | 0.438 | 0.025 | 0.053 | 0.001 | 0.659 |
| 1.25 $(OH)_2$ $D_3$ | | | 0.924 | 0.621 | 0.756 | 0.743 | 0.272 | 0.9060 | 0.009 | 0.998 |
| Control × WC | | | 0.361 | 0.043 | 0. 068 | 0.295 | 0.047 | 0.744 | 0.088 | 0.883 |
| Control × 1.25 $(OH)_2$ $D_3$ | | | 0.876 | 0.118 | 0.619 | 0.747 | 0.905 | 0.756 | 0.730 | 0.422 |
| WC × 1.25 $(OH)_2$ $D_3$ | | | 0.674 | 0.176 | 0.616 | 0.740 | 0.958 | 0.212 | 0.762 | 0.821 |
| Control × WC × 1.25 $(OH)_2$ $D_3$ | | | 0.681 | 0.138 | 0.575 | 0.907 | 0.244 | 0.415 | 0.816 | 0.793 |

[a-c]Means within each column with no common superscript differ significantly ($p<0.05$).
[1]SF= Shear force
[2]FD= Fracture deflection
[3]FE= Fracture energy

study may be attributed to the small bone diameter (Schwartz & Biewener, 1992) observed in these birds. The supplementation of WC at 100 mg/kg of diet significantly increased the shear force compared with 0 and 200 mg/kg.

In spite of the significant effects of type of control diet and dietary level of WC on shear force, no significant effect of the dietary supplementation of 100 mg/kg WC or of the positive control diets was found on bone ash. Wilson (1991) demonstrated that shear force increases with increasing bone ash content. He also showed that 58.7% of the variation in shear force can be attributed to body weight and ash content. Bonser & Casinos (2003) concluded that ash content may be

an efficient predictor of differences in the mechanical competence in between-bone comparisons. On the other hand, Onyango *et al.* (2003) reported that shear force did not increase linearly or quadratically when dietary Ca and P were increased. Noff *et al.* (1982) also observed similar improvements in bone mechanical properties in broilers fed diets containing vitamin D metabolites, but these results were not reflected on bone ash content. They hypothesized that bone ash values are the average for the whole bone and do not actually represent the mid shaft region, the component loaded in bending.

At 42 days of age, the effects of dietary WC supplementation on fracture deflection only approached significance (p=0.053). Also, a two-way interaction between the control and the WC treatments was observed for shear force at that age. The maximum value was recorded in the broilers fed the positive control diet supplemented with 100 mg/kg WC. However, the birds fed the negative control diet supplemented with 200 mg/kg WC showed the lowest shear force value. Regardless of dietary level of 1.25-$(OH)_2$ $D_3$, the two-way interaction showed that the effects of the dietary supplementation of WC on shear force were more pronounced when added the positive control diet at the level of 100 compared with 200 mg/kg (271 *vs*. 200 N; p<0.05).

The main effects of the control, WC and 1.25-$(OH)_2$ $D_3$ treatments on fracture energy were significant (p<0.01). The results clearly indicated that birds fed the diet with adequate Ca or the diet containing 1.25-$(OH)_2$ $D_3$ presented higher tibia fracture energy (p<0.01). Also, the maximum value of tibia fracture energy was observed with the supplementation of 100 mg/kg WC (p=0.001). Frost & Roland (1991) reported that supplementing the diet with 0.5 or 1 µg/kg 1.25-$(OH)_2$ $D_3$ significantly and linearly increased tibia bone density and breaking strength in 75-week-old hens. On the other hand, Rennie *et al.* (1997) observed no significant difference in the bone strength of hens fed 5µg/kg 1.25-$(OH)_2$ $D_3$. The beneficial effects of 1.25-$(OH)_2$ $D_3$ on tibia fracture energy are reflected in bone histological findings, as evidenced by increased bone mineralized zone width. Liu *et al.* (2003) concluded that bone strength is related with its material (minerals) and structural properties (matrix chemistry).

The structural properties of bones depend in part on bone geometry (Zernicke *et al.,* 1995). The current study indicated that the bone mineral content of birds fed the two control diets and different WC levels presented no significant differences, suggesting that the deleterious effects of the negative control diets and the positive effect of 100 mg/kg WC are mainly related to structural properties of the tibia. Further, geometrical and histological observations demonstrated that cortical thickness were markedly improved in 21-d-old birds fed 100 mg/kg WC and tibia diameter was reduced those fed the negative control diets. These results are in agreement with those of Fleming *et al.* (1994), who observed that bone strength increased with increasing cortical thickness. *Whitania coagulans* is known to contain estrogen-like withanolides (Atta-ur-Rahman *et al.,* 2003; Prasad *et al.,* 2010). As previously mentioned, plant secondary metabolites had beneficial effects on bone strength as a result of the prevention of connective tissue breakdown (Rasool & Varalakshmi, 2007). There were no differences in tibia stiffness among treatments as measured at 21 and 42 days of age. Data showed that WC dietary supplementation had no effect on the mineral matrix. Rath *et al.* (1999) reported that tibia stiffness is closely related with the mineral matrix.

### Serum Ca, P and alkaline phosphatase levels

As shown in Table 7, serum P concentration increased in response a 30% reduction in dietary Ca at 21 days of age, whereas an opposite result was observed at 42 days of age when the two control diets were compared (p<0.05). Dietary treatments did not affect ALP activity at none of the ages evaluated (p>0.05). Our study confirms the findings of Mirakzehi *et al.* (2013), who reported that 1.25-$(OH)_2$ $D_3$ increased serum P concentration in birds fed a diet with 30% less Ca. The significantly higher serum P concentration in the birds fed the negative control diet might have been related to elevated levels of P retention in this group. At 42 days of age, birds fed 0.5 µg/kg 1.25-$(OH)_2$ $D_3$ had higher concentration of serum Ca than those fed the diet with no supplementation of 1.25-$(OH)_2$ $D_3$ (10.2 *vs*. 8.65 mg/dL) (p<0.01). Tanaka *et al.* (1973) indicated that 1.25-$(OH)_2$ $D_3$ increases gut absorption of calcium and phosphorus. Some studies reported no differences in serum Ca concentrations in broilers fed 5 or 10µg/kg 1.25-$(OH)_2$ $D_3$ (Edwards, 1993; Roberson & Edwards, 1994). However, a linear increase in plasma Ca was also noted in birds fed 5µg/kg 1.25-$(OH)_2$ $D_3$ (Mitchell *et al.,* 1997). It seems that the effects of 1.25-$(OH)_2$ $D_3$ supplementation on serum Ca resulted in a larger mineralized zone width at 42 days of age. The effects of WC on serum P concentration was only approached significance (p=0.053) at 42 days of age. At 21 days of age, a two-way interactions between 1.25-$(OH)_2$ $D_3$

**Table 7 –** Effect of *Withania coagulans* (WC), 1.25 $(OH)_2$ $D_3$ and type of control diet (negative control with 30% lower Ca level and positive with the recommended Ca level) on blood characteristics of broilers at 21 and 42 days of age.

| Treatment | | | 21 day | | | 42 day | | |
|---|---|---|---|---|---|---|---|---|
| Control | WC (mg/kg) | 1.25 $(OH)_2$ $D_3$ (µg/kg) | Ca (mg/dL) | P (mg/dL) | ALP (U/L) | Ca (mg/dL) | P (mg/dL) | ALP (U/L) |
| - | 0 | 0 | 8.94$^{ab}$ | 6.60$^{a}$ | 9772 | 8.83 | 6.60 | 3047 |
| - | 0 | 0.5 | 9.12$^{ab}$ | 5.18$^{ab}$ | 9901 | 9.23 | 5.17 | 2522 |
| - | 100 | 0 | 8.79$^{ab}$ | 6.21$^{ab}$ | 13481 | 8.19 | 6.81 | 2476 |
| - | 100 | 0.5 | 9.61$^{ab}$ | 5.94$^{ab}$ | 13726 | 9.96 | 6.26 | 2929 |
| - | 200 | 0 | 8.19$^{b}$ | 5.60$^{ab}$ | 12037 | 9.56 | 5.32 | 2260 |
| - | 200 | 0.5 | 9.89$^{ab}$ | 7.04$^{a}$ | 10199 | 10.33 | 6.59 | 2045 |
| + | 0 | 0 | 9.74$^{ab}$ | 5.95$^{ab}$ | 86.27 | 9.28 | 7.91 | 2686 |
| + | 0 | 0.5 | 9.24$^{ab}$ | 4.86$^{b}$ | 9244 | 10.06 | 6.56 | 2651 |
| + | 100 | 0 | 9.83$^{ab}$ | 5.04$^{ab}$ | 8624 | 9.21 | 8.06 | 2706 |
| + | 100 | 0.5 | 9.95$^{ab}$ | 5.38$^{ab}$ | 8335 | 9.69 | 8.39 | 2366 |
| + | 200 | 0 | 10. 2$^{a}$ | 5.42$^{ab}$ | 12075 | 6.83 | 6.51 | 2434 |
| + | 200 | 0.5 | 8.61$^{ab}$ | 5.65$^{ab}$ | 10703 | 12.19 | 6.90 | 2391 |
| SEM | | | 0.65 | 0.63 | 1713.5 | 0.859 | 0.826 | 246.1 |
| Main effect | | | | | | | | |
| Control | | - | 9.06 | 6.07$^{a}$ | 11575 | 9.35 | 6.15$^{b}$ | 2511 |
| | | + | 9.60 | 5.40$^{b}$ | 9563 | 9.53 | 7.36$^{a}$ | 2576 |
| WC | | 0 | 9.26 | 5.69 | 9386 | 9.35 | 6.56 | 2671 |
| | | 75 | 9.54 | 5.62 | 10582 | 9.22 | 7.29 | 2695 |
| | | 150 | 9.19 | 5.87 | 11253 | 9.73 | 6.38 | 2245 |
| 1.25 $(OH)_2$ $D_3$ | | 0 | 9.28 | 5.80 | 10812 | 8.65$^{b}$ | 6.89 | 2589 |
| | | 0.5 | 9.39 | 5.64 | 10177 | 10.28$^{a}$ | 6.58 | 2500 |
| | | | p | | | | | |
| Control | | | 0.188 | 0.024 | 0. 074 | 0.708 | 0.025 | 0.967 |
| WC | | | 0.756 | 0.416 | 0.322 | 0.730 | 0.053 | 0.122 |
| 1.25 $(OH)_2$ $D_3$ | | | 0.754 | 0.778 | 0.690 | 0.003 | 0.622 | 0.537 |
| Control × WC | | | 0.942 | 0.935 | 0.080 | 0.660 | 0.328 | 0.595 |
| Control × 1.25 $(OH)_2$ $D_3$ | | | 0.045 | 0.816 | 0.946 | 0.234 | 0.364 | 0.908 |
| WC × 1.25 $(OH)_2$ $D_3$ | | | 0.785 | 0.043 | 0.711 | 0. 113 | 0.107 | 0.776 |
| Control × WC × 1.25 $(OH)_2$ $D_3$ | | | 0.286 | 0.660 | 0.972 | 0. 062 | 0.414 | 0.396 |

$^{a,b}$Means within each column with no common superscript differ significantly ($p<0.05$).

and control or WC on serum Ca and P was observed ($p<0.05$). Serum Ca concentration was higher in birds fed the positive compared with the negative control diet with no supplementation of 1.25-$(OH)_2$ $D_3$ (10.2 *vs*. 8.19 mg/dL) ($p<0.05$). Regardless the dietary Ca concentration, the birds given 200 mg/kg WC and 0.5 µg/kg 1.25-$(OH)_2$ $D_3$ had higher serum P concentration compared with those receiving only 0.5 µg/kg 1.25-$(OH)_2$ $D_3$ (7.04 *vs*. 4.86 mg/dL; $p<0.05$).

The current experiment demonstrated that Ca and P retention are significantly modified by the level of dietary Ca. The negative control diets resulted in poor bone strength, whereas the supplementation of 100 mg/kg WC improved bone mechanical properties. Our results suggest that the deleterious effects of the negative control diets and positive effect of WC on bone strength are mainly related to the structural properties of the tibia. In addition, the geometrical and

histological results showed that cortical thickness was markedly increased in birds fed 100 mg/kg WC. The administration of 1.25 $(OH)_2$ $D_3$ had beneficial effects on bone strength of 42-d-old broilers, which was accompanied by a thicker mineralized zone.

## ACKNOWLEDGMENTS

The authors wish to express appreciation to Ferdowsi University of Mashhad, Iran. Appreciation is also expressed to the staff at the farm of Ferdowsi University of Mashhad for their support during the experiment.

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