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Bone Characteristics of 16 Wk-Old-Turkeys Subjected to Different Dietary Supplements and Simulated Stress

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Abstract: The effect of two nutritional supplements, a Yeast Extract (YE) and a vitamin D₃ formulation (VD) on growth and structural properties of bones from turkeys, transiently subjected to a simulated stress using Dexamethasone (Dex) administration, was determined. The birds were fed diets with or without YE or VD supplements during wks of 6, 11 and 15 post hatch. At weeks 6 and 15 of age, half of the birds in each treatment group received 3 intramuscular injections of Dex at concentrations of 2 mg/kg BW on 3 alternating days to induce simulated stress. At 16 wk of age, the birds were weighed, bled prior to euthanasia and the tibia were harvested at necropsy to determine their mineral content, density and biomechanical properties. Bone Mineral Density (BMD) and Bone Mineral Content (BMC) of whole tibia were determined by Dual Energy X ray Absorptiometry (DEXA) and the biomechanical properties using Instron material testing machine. The ash yield and bone densities were determined using bone marrow free mid diaphyseal segments manually by ashing and Archimedes principle. Serum Ca, P, protein and alkaline phosphatase measured using a clinical chemistry analyzer. Neither YE nor VD had any effect on body or bone weights by itself or in combination. Dex reduced both BW and bone weights. DEXA estimated BMD and BMC of whole tibia were reduced in Dex-stressed birds but it was not evident measuring the diaphyseal bone and ash densities. Dex treatment lowered the breaking strength and the plasticity of bone but had no significant effect on its stiffness. Dex treated turkeys showed higher relative bone weights indicating faster recovery of bones from Dex induced growth suppression. Overall, these results suggest that decreased bone mass due to Dex-induced growth suppression reduces bone strength and can alter some structural properties. Intermittent treatment with either VD or YE individually or in combinations do not provide much protection against the negative effects of stress.

Key words: Turkeys, tibia, yeast extract, vitamin D, dexamethasone

INTRODUCTION

Leg problems associated with bone weakness are prevalent in young fast growing commercial poultry which render them lame leading to the deterioration of their gait scores, performance and welfare (Bradshaw *et al.*, 2002; Julian, 2005; Knowles *et al.*, 2008; Talaty *et al.*, 2010). Stress is an important factor in poultry production which can affect skeletal health (Rath *et al.*, 2000). Stress of non infectious origins in commercial poultry production can stem from various physical and environmental constraints such as crowding, transportation, extended light period, wet litter and the lack of physical exercise (Dawkins *et al.*, 2004; Knowles *et al.*, 2008). In commercial turkey production, the birds are usually transferred to separate housing conditions several times between their brooding and growth period (www.ansci.umn.edu/poultry/resources/turkeymgmt.htm) which can cause additional stress. To mitigate different production associated health problems and improve their resistance to stress, the commercial poultry are

often supplemented with different micro nutrients such as vitamins, minerals, prebiotics and probiotics (Patterson and Burkholder, 2003; Huff *et al.*, 2002; 2007; Cetin *et al.*, 2005). YE byproducts such as glucan and mannan oligosaccharides are widely used as prebiotic feed supplements in livestock and poultry diets to promote growth, improve feed conversion, immunity and overall performance (Westendorf and Wohlt, 2002; Ferket *et al.*, 2002; Huff *et al.*, 2007; 2011). However, their effect on bone health is not known. Vitamin D is an osteotropic factor with many physiological benefits (DeLuca, 2008) but its protective effect on stress induced changes in poultry is not known. Hence, the objective of this study was to evaluate the effects of dietary supplements, a Yeast Cell Extract (YE) and a vitamin D₃ (cholecalciferol) preparation (VD) on growth and structural properties of bones of control and the turkeys that were temporarily subjected to a simulated stress using Dexamethasone (Dex) administration. This was designed to mimic the transfer stress of

commercial turkeys to different housing conditions during production (Huff *et al.*, 2000; 2002). The effects of the treatments were evaluated using tibia bones harvested at 16 wk of age.

Table 1: Turkey feed composition

Ingredient	g/1,000 g
Yellow corn	439.0
Soybean meal	495.0
Rendered poultry oil	7.4
Di calcium phosphate	32.0
Calcium carbonate	9.5
Salt	3.7
Vitamin premix ¹	5.0
Choline chloride 60%	2.5
Trace minerals ²	4.0
DL Methionine 99.5%	1.7
Lysine 98%	0.2

¹[Vitamin A (vitamin A acetate), 7,778 IU; cholecalciferol, 5,556 IU; vitamin E (DL- α -tocopheryl acetate), 16.67 IU; vitamin B₁₂, 0.013 mg; riboflavin, 6.7 mg; niacin, 39 mg; pantothenic acid, 10 mg; menadione (menadione dimethylpyrimidinol), 1.5 mg; folic acid, 0.9 mg; choline, 895 mg; thiamine (thiamine mononitrate), 1.56 mg; pyridoxine (pyridoxine hydrochloride), 2.78 mg; D-biotin, 0.067 mg; ethoxyquin, 125 mg].

²[(Ca calcium carbonate) 62 mg; Mn (manganese sulfate), 100 mg; Mg (magnesium oxide) 27 mg; Zn (zinc sulfate) 100 mg; Fe (ferrous sulfate) 50 mg; Cu (copper sulfate) 10 mg; I (calcium iodate) 1 mg]

MATERIALS AND METHODS

Diet and experimental stress: All experiments were conducted following the University of Arkansas, Institutional Animal Care and Use Committee guidelines. Day-old male turkey poults were wing-banded and brooded for the first 6 weeks, at a density of 30 birds/4 m² pen under an alternate light and dark period of 23 h and 1 h respectively and provided *ad libitum* access to water and feed composed per NRC recommended dietary guidelines (National Research Council, 1994) (Table 1). Each nutritional treatment consisted of replicate pens of birds. Dietary supplements consisted of YE (Alphamune™, Alpharma Animal Health) added to the feed at the concentration of 1,008 g/ton and VD (High-D 2X dispersible liquid, Alpharma Animal Health) provided in drinking water at approximately 4,000 IU/bird/day. The treatments were given as single supplement or in combination. The VD solution was prepared fresh and half of the daily dosage was added to bell waterers in assigned pen daily at 8:00 am and 4:00 pm respectively. The control fed turkeys received regular diets without supplements. Birds were provided these treatments for the first week after placement and then at wks 6, 11 and 15 for a period of 7 days each. At wk 6, half of each treatment group was given an intramuscular injection of dexamethasone (Sigma, St. Louis, MO) in saline, at the concentration of 2 mg/ kg BW, in the thigh muscle on 3 alternate days to simulate conditions of stress (Huff *et al.*, 2007) and

placed in separate pens. Based on preliminary studies which showed no differences between saline injected and non injected birds, the nonstressed and control-diet fed turkeys were used as controls. At 16 wk of age, 4 birds/pen (8 birds/treatment group) were bled by venipuncture, killed by CO₂ asphyxiation and weighed. Blood samples were collected in BD serum collector tubes (VWR.com), centrifuged and the serum was stored at -20°C. Tibia from both legs of each bird was removed, cleaned of extraneous tissue such as muscle and periosteum, weighed and stored at -20°C for subsequent measurements.

Bone weight, mineral content and density: After thawing the bones to room temperature, each tibia was weighed to determine relative bone weights with respect to BW. The Bone Area, Mineral Content (BMC) and Mineral Density (BMD) of whole bones inclusive of epiphyseal cartilage were determined by Dual Energy X-ray Absorptiometry (DEXA) (www.gehealthcare.com/DEXA). DEXA has been used to measure bone qualities of poultry for genetic selections (Zotti *et al.*, 2003; Hester *et al.*, 2004; Schreiweis *et al.*, 2005). The BMC represents the total mineral content (g) of the bone and the BMD calculated as the ratio of BMC to the measured area of the bone expressed as g/cm². These parameters are not only correlative of bone strength but also predictive of overall skeletal health and fracture risk (Keenan *et al.*, 1997; Watts, 2002).

Biomechanical properties: Structural properties of the bones was determined using left tibia of each bird by 3 point flexural bending method with an Instron 4502 material testing machine. The mid-diaphyseal diameter of the bone at the site of impact was measured using a dial caliper; the bones were held in identical positions and the test was done at the impact rate of 30 mm/min as described previously (Rath *et al.*, 1999). Breaking strength (load at failure), stress at yield (force per area at deformation), strain (relative change in length, plasticity) and the elastic modulus (stiffness) were determined to assess bone mechanical properties (Huff *et al.*, 1980; Turner and Burr, 1993; Zioupos and Currey, 1998).

Diaphyseal bone and ash density: The bone density and bone mineral concentrations of diaphyseal bone segments were determined using manual methods. Approximately 1 cm length of a tibia was cut from the midsection of right tibia using a scroll saw and cleaned of bone marrow with a jet stream of water and dried and the volumes of the hollow tibia sections were determined using a Mettler AT200 density kit (Memphis Scale Works Inc., TN) by Archimedes' principle (Keenan *et al.*, 1997). The densities of bone pieces were calculated by dividing the mass (initial weight) by the volume of bone corrected with the density of water at

room temperature. Following density measurements the bone sections were further dried at 95°C for 18 h to determine their dry weights and ashed at 750°C for 17 h in porcelain crucibles. Ash density (ash weight g /volume, cm³) was determined according to Gafni *et al.* (2002).

Blood biochemistry: Serum was used to determine protein, calcium, phosphate and Alkaline Phosphatase (ALP) concentrations using a Corning clinical chemistry analyzer (Chiron Corporation, San Jose, CA) according to the manufacturer's instructions.

Statistical analysis: The group means and Standard Error of Means (SEM) were calculated and the data

analyzed as 2 x 2 x 2 factorial arrangements using SAS software (SAS Institute, 2002). All ratio and percentage data were arcsine transformed and others presented as raw data. A p-value of ≤ 0.05 was considered significant.

RESULTS

The results are presented in Table 2 and 3. Neither of the supplements by itself nor in combination affected either BW or relative bone weights of non stressed birds. Table 2 shows administration of Dex significantly suppressed BW (7.66 vs 12.54, $p < 0.0001$), tibia weights (83.53 vs 97.61, $p < 0.0001$) but resulted in an increase in relative weights of tibia (5.98 vs 5.07, $p < 0.0001$). The interaction between VD supplement and administration of Dex only affected tibia weights (87.26 vs 100.52,

Table 2: Effect of supplemental Yeast Extract (YE) and Vitamin D₃ (VD) on body weight and bone parameters of turkeys with or without simulated stress induced by Dexamethasone (Dex) treatment (n = 8)

Treatments			BW (kg)	Tibia weight (g)	Relative bone wt	DEXA-BMD (g/cm ²)	DEXA-BMC (g)	Bone density (AP) (g/cm ³)	Ash yield (g/cm ³)
YE	VD	Dex							
-	-	-	12.58±0.49	99.56±2.39	5.12±0.07	0.32±0.01	13.10±0.60	1.59±0.02	1.01±0.02
-	-	+	7.64±0.21	78.77±4.59	5.82±0.21	0.32±0.01	10.73±0.85	1.56±0.01	0.99±0.01
+	-	-	12.49±0.37	101.47±2.40	5.18±0.09	0.39±0.02	16.06±0.92	1.55±0.05	0.97±0.05
+	-	+	7.54±0.38	81.34±4.77	5.95±0.13	0.33±0.02	10.70±0.94	1.53±0.03	1.03±0.02
-	+	-	12.90±0.51	97.59±3.38	5.00±0.09	0.35±0.02	14.05±0.88	1.60±0.02	1.09±0.02
-	+	+	7.98±0.33	85.29±2.48	5.96±0.17	0.32±0.01	11.08±0.58	1.59±0.03	1.02±0.02
+	+	-	12.18±0.46	91.82±2.35	4.99±0.09	0.33±0.01	12.58±0.54	1.62±0.01	1.02±0.01
+	+	+	7.66±0.31	88.72±1.40	6.20±0.11	0.30±0.01	10.44±0.60	1.57±0.02	1.08±0.01
Source of variation						(p-value)			
YE			0.274	0.812	0.240	0.380	0.664	0.315	0.904
VD			0.676	0.800	0.818	0.055	0.273	0.051	0.004
YE*VD			0.446	0.449	0.910	0.011	0.019	0.381	0.970
Dex			<0.0001	<0.0001	<0.0001	0.005	<0.0001	0.208	0.739
YE*Dex			0.719	0.275	0.394	0.326	0.243	0.003	0.871
VD*Dex			0.689	<0.006	0.059	0.215	0.905	0.364	0.922
YE*VD*Dex			0.702	0.344	0.631	0.080	0.186	0.600	0.581

BMC = Bone Mineral Content; BMD = Bone Mineral Density, Dex = Dexamethasone; DEXA = Dual Energy X-ray Absorptiometry. AP = Archimedes Principle

Table 3: Effect of Yeast Extract (YE) and Vitamin D₃ (VD) supplements on bone area, diameter and biomechanical properties of turkeys with or without simulated stress induced by Dexamethasone (Dex) treatment (n = 8)

Treatments			DEXA-Bone area (cm ²)	Bone diameter (mm)	Breaking strength (kg)	Strain (mm/mm)	Stress at yield (kg/mm ²)	Elastic modulus (kg/mm ²)
YE	VD	Dex						
-	-	-	40.57±1.66	13.80±0.46	68.68±3.60	0.21±0.03	2.15±0.32	26.25±5.82
-	-	+	33.00±1.51	12.30±0.51	59.14±3.77	0.13±0.02	2.57±0.31	36.90±5.89
+	-	-	41.63±1.03	13.49±0.33	93.80±10.09	0.30±0.08	2.92±0.26	32.40±3.54
+	-	+	31.88±1.52	12.44±0.34	55.88±3.61	0.12±0.01	2.27±0.22	38.57±7.02
-	+	-	40.38±0.89	14.11±0.28	71.09±4.62	0.23±0.01	1.98±0.19	23.13±2.95
-	+	+	35.00±0.80	12.96±0.32	60.99±4.42	0.15±0.03	2.19±0.21	30.99±2.56
+	+	-	38.50±0.76	13.62±0.40	74.44±4.16	0.23±0.04	2.38±0.30	26.88±3.30
+	+	+	34.88±0.81	13.12±0.18	52.32±3.50	0.16±0.03	1.78±0.14	24.60±2.86
Source of variation						(p-value)		
YE			0.619	0.631	0.261	0.379	0.499	0.688
VD			0.516	0.089	0.206	0.972	0.028	0.030
YE*VD			0.461	0.887	0.068	0.459	0.505	0.419
Dex			<0.0001	0.0002	<0.0001	0.0004	0.383	0.086
YE*Dex			0.878	0.291	0.0075	0.431	0.010	0.259
VD*Dex			0.013	0.385	0.300	0.276	0.820	0.385
YE*VD*Dex			0.236	0.839	0.267	0.330	0.713	0.661

Dex = Dexamethasone; DEXA = Dual Energy X-ray Absorptiometry

$p < 0.006$). DEXA estimated BMC and BMD showed increases in turkeys fed both YE and VD (BMC: 12.49 vs 11.92, $p = 0.019$; BMD: 0.34 vs 0.32, $p = 0.011$). Dex-stressed birds showed decreased DEXA-BMC (10.74 vs 13.95, $p < 0.0001$) and BMD (0.32 vs 0.35, $p = 0.005$). None of the feed supplements was able to modulate stress induced changes in these parameters. Dry bone density and ash yield of diaphyseal bones increased in turkeys fed VD. YE supplementation in Dex treated birds increased ash yield (YE*Dex 1.05 vs 1.02, $p = 0.003$). Both tibial diameters and DEXA estimated bone areas were decreased in Dex-stressed birds (Table 3). Dex treatment decreased bone breaking strength (57.08 vs 77.00, $p < 0.0001$) but had no significant effect on stress at yield or modulus of elasticity (Table 3). YE supplement interacting with stress significantly weakened the breaking strength (66.10 vs 69.89, $p = 0.0075$). VD decreased stress at yield in control, non stressed turkeys (2.09 vs 2.48, $p = 0.028$) and YE interacting with stress also decreased it significantly (2.07 vs 2.35, $p = 0.01$). The strain values of Dex treated turkey bones decreased (0.14 vs 0.24, $p = 0.0004$). VD decreased the bone's modulus of elasticity (26.40 vs 33.53, $p = 0.03$). None of the treatments had any effect on serum protein, Ca, P, or ALP values (data not shown).

DISCUSSION

From the results it is evident that neither YE nor VD by itself or in combination had any effect on BW of turkeys and Dex treatment suppressed growth that could not be prevented by these supplements. The studies on the effects of prebiotic or probiotic feed supplements on bone are limited although some studies have reported positive effects of these supplements on bone growth in mammalian species (Scholz-Ahrens *et al.*, 2007; Kim *et al.*, 2009). The structural properties of bone is influenced by its mass, shape and material content (Nigg and Grimston, 1994; Frost, 1997; Rath *et al.*, 2000). YE supplement did not change bone weight or its mineral content and had no effect on its biomechanical properties. Although VD did not affect bone mass, it increased BMC, ash yield and had only a moderate effect on bone density. VD though decreased stiffness it did not change bone breaking strength. The effect of VD on different parameters of poultry bone has been studied by many investigators and its beneficial effects on poultry bone health in general, is established (Frost and Roland, 1991; Roberson, 1999; Bar *et al.*, 2003; Fritts and Waldroup, 2003; Whitehead *et al.*, 2004). However, Vitamin D has not been shown to increase bone weight although it affects its mineral content. Ferket *et al.* (2009) observed very marginal effect of HyD (25 hydroxy cholecalciferol) on bone strength of turkeys but they found it to increase bone strength when given together with organic mineral supplements. YE and VD interaction showed a very limited effect on bone mass or its mechanical properties.

Dex treatment suppressed body growth and reduced total tibia weights. Neither YE nor VD by itself or in combinations modulated Dex induced stunting of bone growth or changed their structural properties to any significant extent. VD interacting with Dex increased tibia weights although it did not reverse the trend. The negative effect of glucocorticoids on body weight is presumed to be due to its inhibitory effect on feed intake, feed conversion efficiency and increased energy expenditure that influence the growth hormone-insulin-like growth factor axis (Allen, 1996; Leili and Scanes, 1998; Lin *et al.*, 2006; Virden *et al.*, 2007). Glucocorticoids impair longitudinal growth of bone by retarding growth plate development (Baron *et al.*, 1992). Although the density and ash content of diaphyseal segments of Dex treated turkeys remained unaffected, the DEXA estimated decreases in BMC and BMD may be due to the changes estimated from whole bone data including growth plate. Dex treatment resulted in the reduction of ultimate breaking strengths of bones possibly due to their reduced mass but it had no significant effect on stress and stiffness values. The plasticity of bones was lowered by Dex as indicated by their decreased strain values. The plastic property of the bone can be related to their collagenous matrix components (Ritchie *et al.*, 2009). Glucocorticoids down regulate extracellular matrix production including collagen synthesis (Delany *et al.*, 2004) and retard growth plate development in young animals (Smink *et al.*, 2002; Lui and Baron, 2011). It is possible that increased spatial content of inorganic matrix relative to organic collagenous matrix decreased the plastic properties of bone in Dex-stressed turkeys. The increase in relative tibia weights in Dex-stressed turkeys is intriguing but it may be explained if it is assumed that bone growth recuperates faster than general growth from the suppressive effects of glucocorticoids. In mammalian studies it has been shown that temporary growth suppression of bones by glucocorticoid is reversed by a certain mechanism called 'catch-up growth' (van der Eerden *et al.*, 2003; Lui and Baron, 2011). Rabbits showing reduced bone weights and densities due to massive glucocorticoid treatment at 5 wk of age would recover their bone weights following discontinuation of treatment (Gafni *et al.*, 2002). Assuming that similar compensatory growth mechanisms operate in avian species, the increase in relative tibia weights in Dex treated turkeys is possible. In conclusion, our study shows that transient but massive stress such as induced by Dex can suppress growth of young birds affecting their bone. The bones appear to be more resilient than BW with respect to recovery from stress induced growth suppression. Short term supplementation of YE does not affect physical changes in bone. These results also suggest that severe stress during early growth periods though transient can negatively affect growth and persist through the production period.

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