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Effect of Vitamin D_3 and/or Zeolite Supplementation to Laying Hen Rations Added Microbial Phytase on Some Blood Indices. 1. Calcium and Inorganic Phosphorus Levels and Alkaline Phosphatase Activity

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Abstract: The aim of this study was to examine the effect of vitamin D_3 and/or zeolite supplementation in presence of phytase enzyme on serum total Calcium (Ca), inorganic Phosphorus (Pi) levels and Alkaline Phosphatase (ALP) activity in laying hens. A total of 120, 28 weeks old laying hens were used for the study. The laying hens were separated to 4 equal groups (5 replicates). The treatment groups were as follows: control diet (300 FTU phytase per kilogram), trial 1 diet (300 FTU phytase + 400 IU vitamin D_3), trial 2 diet (300 FTU phytase + 400 IU vitamin D_3 + 2% zeolite) and trial 3 diet (300 FTU phytase + 2% zeolite). The experimental period was 16 week. Blood samples were taken on weeks 4, 8, 12 and 16. There were no significant differences between groups for serum ALP activity and Pi levels on weeks 4, 8, 12 and 16. During the study, serum Ca levels were significantly higher in the trial 2 than those of other groups ($p<0.05$). In conclusion, a significant increase or decrease was not seen in the effect of phytase as a result of the addition of vitamin D_3 or zeolite, respectively. There was a significant phytase, vitamin D_3 and zeolite interaction for serum Ca level.

Key words: Laying hen, phytase, serum indices, vitamin D_3, zeolite, calevel

INTRODUCTION

Major ingredients used in poultry feeds are of plant origin. About two-thirds of the Phosphorus (P) in feedstuffs is present as phytate P which is poorly utilized by poultry (Carlos and Edwards, 1998). The inability of poultry to utilize phytate P due to lack of endogenous phytase, results in the addition of inorganic phosphates to poultry diets in order to meet the P requirements. This practice results in substantial excretion of phytate P, leading to the accumulation of P in soils and its entry into surface and ground waters and sparking off major environmental concerns (Kornegay and Harper, 1997). The process of producing inorganic phosphates for animal usage requires expensive capitalization and manufacturing inputs that make it expensive feed ingredient.

So, any attempt to improve utilization of phytate P in laying hens could reduce feed costs and P pollution (Carlos and Edwards, 1998). Studies with broiler chickens fed corn-soybean diets indicate phytate P utilization of between 10 and 53% (Ballam *et al*., 1984; Edwards and Veltmann, 1983). Simons *et al*. (1990) reported that addition of phytase increased dietary P availability to 65% and reduced P excretion by 50% in broilers.

Phytase is an enzyme which hydrolyzes phytate to inositol and inorganic phosphate. Enzyme is at low level in the chicken gastrointestinal tract but is present in some cereals and at high concentration in microbial sources. The need to supplement the diets of monogastric animals with inorganic P (Pi) can be decreased if phytate P can be made available to animals by treating the grains with phytase (Huff *et al*., 1998). Nelson and Ferrara (1968) reported that the addition of phytase to grains and feeds was an effective way to increase P availability in poultry. The supplementing diets with phytase reduced the need to supplement diets with Pi and decreased P excretion (Biehl and Baker, 1997). Phytate P utilization of broiler chicks was enhanced with the addition of a combination of phytase and 1, 25-dihydroxycholecalciferol (1, 25-$(OH)_2D_3$) (Carlos and Edwards, 1998). Vitamin D can affect the utilization of phytate P but the interaction of vitamin D and phytase has not been elucidated yet (Lei *et al*., 1994). Calcium (Ca) and P are the most significant minerals participating in metabolic interaction with vitamin D

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(Mrljak *et al*., 1999). Ca has a key role in many biological processes being an essential component of bone and egg shell (Bogin *et al*., 1996). Serum Pi concentration is greatest during the period of egg shell calcification as a result of bone mobilization (Mongin and Sauveur, 1979).

Primary response is improved egg shell quality for laying hens. Sodium Aluminosilicate (SAS) may improve Ca absorption and utilization because SAS has a high affinity for Ca (Moshtaghian *et al*., 1991).

The aim of this study was to examine the effect of vitamin D_3 and/or zeolite supplementation in presence of phytase enzyme on serum Ca and Pi levels and Alkaline Phosphatase (ALP) activity in laying hens.

MATERIALS AND METHODS

Animals and treatments: A total of 120, 28 weeks old laying hens were used for the study. The hens were reared in a enclosed house with ventilation fans. Lighting schedule followed a 16 h light: 8 h dark cycle. All hens were housed in individual cages. The laying hens were separated to 4 equal groups (5 replicates).

They were fed a corn and soybean meal basal diet (NRC, 1994). The treatment groups were as follows: control diet (300 phytase units (FTU) phytase (from *Aspergillus niger*) (Natuphos 600, BASF Corp., Mt. Olive, NJ 07828 USA. kg^{-1}), trial 1 diet (300 FTU phytase + 400 IU vitamin D_3 (AMP Medizintechnik GmbH Statteggerstrasse 31b 8045 Graz, Austria.)), trial 2 diet (300 FTU phytase + 400 IU vitamin D_3 + 2% zeolite (a natural zeolite, clinoptilolite) (Zeotech Corp., Albuquerque, NM 87107 USA)) and trial 3 diet (300 FTU phytase + 2% zeolite). The experimental period was 16 week. Feed and water were consumed *ad libitum* by the laying hens. Composition and calculated nutrients in diets are shown in Table 1.

Table 1: Composition and calculated nutrients in diets (%)

Composition of nutrients	Control (P)	Trial 1 (P+D_3)	Trial 2 (P+D_3+ZE)	Trial 3 (P+ZE)
Corn	63.00	63.00	63.00	63.00
Soybean meal, dehulled	24.00	24.00	24.00	24.00
Vegetable oil	1.20	1.20	1.20	1.20
Limestone	7.58	7.58	7.58	7.58
Dicalcium phosphate	1.06	1.06	1.06	1.06
Vitamin premix[1]	0.25	0.25	0.25	0.25
Mineral premix[2]	0.25	0.25	0.25	0.25
DL-Methionine	0.16	0.16	0.16	0.16
Iodized salt	0.50	0.50	0.50	0.50
Sand	2.00	2.00	-	-
Zeolite	-	-	2.00	2.00
Phytase (FTU)	300.00	300.00	300.00	300.00
Vitamin D_3 (IU)	-	400.00	400.00	-
Calculation of nutrients				
Crude protein	16.00	16.00	16.00	16.00
Metabolizable energy (kcal kg^{-1})	2750.00	2750.00	2750.00	2750.00
Calcium	3.50	3.50	3.50	3.50
Phosphorus, total	0.50	0.50	0.50	0.50

[1]Provided per kilogram of diet: vitamin A, 4400 IU; vitamin D_3, 1000 IU; vitamin E, 11 IU; riboflavin, 4.4 mg; d-pantothenic acid, 12 mg; nicotinic acid, 44 mg; choline chloride, 220 mg; vitamin B_{12}, 9 μg; vitamin B_6, 3 mg; menadione sodium bisulfite complex, 2.33 mg; folic acid, 3 mg; biotin, 0.3 mg; thiamin, 2.2 mg; ethoxyquin, 125 mg; [2]Provided per kilogram of diet: manganese, 75 mg; zinc, 75 mg; iron, 75 mg; copper, 5 mg; iodine, 0.75 mg; selenium, 0.1 mg

Analytical analysis: Blood samples were taken on weeks 4, 8, 12 and 16. They were collected from vena brachialis of hens with no anticoagulanted vacutainer tubes. After sampling, tubes were centrifugated at 5000 g for 10 min after they were left at 37°C for 30 min. Serum samples were transferred to 2 mL volume Eppendorf microcentrifuge tubes. Samples were stored at -20°C prior to analysis. Serum Ca and Pi levels and ALP activities were analysed by using commercial kits[3] and a Technicon RA-1000 autoanalyser (DSG UK Limited, Unit 1B, 13-4 King's Gardens Hove, BN3 2PG, UK).

Statistical analysis: Data were compared by using analysis of variance (ANOVA, Duncan's multiple range test) between groups within each blood sampling week for all blood indices. Results are presented as mean±SE. All statistical analysis was performed using software package program (SPSS for windows, Standard version 10.0, 1999; SPSS Inc., Headquarters, Chicago, IL, USA). A significance level of $p<0.05$ was employed in the analysis of data from groups (Snedecor and Cochran, 1980).

RESULTS AND DISCUSSION

The effects of the different dietary treatments on serum Ca levels are shown in Table 2. During the study, serum Ca levels were higher in the phytase, zeolite and vitamin D_3 added group than in the other groups ($p<0.05$). The statistical differences were not found between the only phytase added group, the phytase and vitamin D_3 added group and the phytase and zeolite added group. Table 3 presents the effects of phytase and vitamin D_3 and/or zeolite on serum Pi levels. There were no significant differences between groups for serum Pi levels in all weeks. However, the levels were tended to be higher in the phytase, zeolite and vitamin D_3 added group and the phytase and zeolite added group.

Egg laying represents a major challenge for Ca metabolism in poultry. Laying hens must maintain a high blood calcium concentration because of rapid transfer of Ca to the egg shell (Simkiss, 1961). Increased Ca requirement is evident primarily in increased intestinal absorption of Ca (Hurwitz *et al*., 1973). Body regulates Ca and P homeostasis via the actions of vitamin D_3, parathyroid hormone and calcitonin on the small intestine, kidneys and bone (Li *et al*., 1998).

Table 2: Serum calcium levels in laying hens fed rations added microbial phytase and supplemented vitamin D_3 and/or zeolite (mg dL^{-1})

	Groups			
	Control (P)	Trial 1 (P+D_3)	Trial 2 (P+D_3+ZE)	Trial 3 (P+ZE)
Weeks	(x±SE)			
4	20.81±0.71^b	20.82±1.11^b	22.84±0.62^a	21.45±1.02^b
8	20.75±0.70^b	20.72±0.91^b	22.92±1.36^a	21.01±0.88^b
12	20.63±0.57^b	20.69±0.95^b	22.81±0.65^a	21.00±1.08^b
16	20.67±0.62^b	20.92±0.99^b	22.75±0.74^a	21.37±1.02^b

x±SE: Mean±Standard Error; ^{a,b}different superscrips indicate significant differences between treatment groups ($p<0.05$); P: Phytase, D_3: vitamin D_3: ZE zeolite

Table 3: Serum inorganic phosphorus levels in laying hens fed rations added microbial phytase and supplemented vitamin D_3 and/or zeolite (mg dL^{-1})

	Groups			
	Control (P)	Trial 1 (P+D_3)	Trial 2 (P+D_3+ZE)	Trial 3 (P+ZE)
Weeks	(x±SE)			
4	7.56±0.65	7.69±0.56	7.97±0.55	7.98±0.62
8	7.66±0.68	7.63±0.69	7.93±0.59	7.75±0.70
12	7.67±0.69	7.57±0.85	8.04±0.58	7.95±0.46
16	7.81±0.51	7.94±0.53	7.94±0.42	7.95±0.52

x±SE: Mean±Standard Error; P: Phytase, D_3: vitamin D_3: ZE zeolite

In Ca-or vitamin D-deficient hens, control hens had significantly higher total plasma Ca and Pi concentrations than the deficient hens (Ruschkowski and Hart, 1992). Norman (1987) reported that elevated blood 1,25-$(OH)_2D_3$ concentrations induce increased absorption and retention of Ca and raise serum Ca level. Capdevielle *et al.* (1998) stated that absorption of P is increased by vitamin D_3 or 1,25-$(OH)_2D_3$ and by 1,25-$(OH)_2D_3$ when the phosphate is complexed with phytate. Moreover, P retention and absorption have been shown to be two times higher in vitamin D-supplemented pigs compared with pigs fed vitamin D-deficient diets (Fontaine *et al.*, 1985), serum Pi concentration is greatest during the period of egg shell calcification as a result of bone mobilization (Mongin and Sauveur, 1979).

Huff *et al.* (1998) showed that supplemental dietary phytase has no significant effect on serum Ca and P concentrations in broilers. However, Carlos and Edwards (1998) reported that addition of phytase significantly improved plasma Ca levels at 9th week in laying hens. Laying hens beyond 1 year of age lose ability to modulate cholecalciferol metabolism to compensate for the inadequate Ca intake (Bar and Hurwitz, 1987). Addition of 1,25-$(OH)_2D_3$ may cause the increased utilization of Ca and stimulate the natural intestinal phytase activity in the gut (Carlos and Edwards, 1998). Li *et al.* (1998) reported that addition of 2000 IU vitamin D to the phytase supplemented diet resulted in numerical improvements in P and Ca digestibility but the differences were significant for only Ca digestibility ($p<0.05$).

Furthermore, same researchers determined that there were no additional benefits from feeding vitamin D in combination with phytase over those seen with phytase alone. Fontaine *et al.* (1985) suggested that elevated dietary vitamin D_3 levels can alleviate some unfavorable effects of dietary Ca although phytase activity of intestine and phosphatase activity of mucous membranes are not affected.

Ca absorption can be promoted by vitamin D_3, so the negative effect on phytate utilization can be alleviated and the formation of phytic Ca can be reduced (Pointillart *et al.*, 1987). Addition of microbial phytase to a corn-soybean diet containing no Pi supplementation, liberated enough P from phytate to allow pig performance similar to the performance achieved with diet containing supplemental Pi.

Addition of microbial phytase improved Ca and P digestibility and reduced fecal P excretion (Li *et al.*, 1998). Li *et al.* (1998) reported that when both vitamin D_3 and phytase were added to diet, there was numerical improvement in serum P compared with diet containing phytase alone ($p>0.05$) in pigs. Also, they determined that there was not a significant difference between the vitamin D_3 and phytase added group and the only phytase added group for serum Ca level. Similarly in the current study, there were no significant differences between the phytase and vitamin D_3 added group and the only phytase added group for serum Ca and Pi levels.

SAS contains 14.6% aluminum, which may form a complex with P in digestive tract and reduce P availability (Lipstein and Hurwitz, 1982). Edwards (1988) showed that P utilization may be impaired by SAS supplementation of chick diets and that effects of SAS were due to increased excretion of phytate P. Beneficial effect seen in egg shell quality and increased Ca utilization from feeding sodium zeolite A (a synthetic SAS) is not accomplished through vitamin D_3 system, namely increased production of 1,25-$(OH)_2D_3$ (Frost *et al.*, 1992). Frost *et al.* (1992) reported that feeding sodium zeolite A at 0.75% did not alter normal bone resorption rhythms in laying hens. Watkins and Southern (1992) experienced reduced plasma P levels when broiler chicks were fed sodium zeolite A in low P diet but percentage of Ca or P in the bone ash was unaffected.

Oguz *et al.* (2000) noted that serum Ca and Pi values were negatively affected by the addition of clinoptilolite (zeolite) (both 1.5 and 2.5%) to basal diet in broilers. However, Dwyer *et al.* (1997) reported that the clinoptilolite treated group in broiler chicks was no significantly different from controls for P (6.14 vs. 6.15 mg dL^{-1}). Similarly, Frost *et al.* (1992) showed that feeding sodium zeolite A for a period of 8 week had no effect ($p>0.05$) on plasma total Ca and P levels. They also noted that there was significant sodium zeolite A and vitamin D_3 interaction for plasma P but not for plasma total Ca. Researchers stated that plasma P level decreased with supplemental sodium zeolite A (0.75%) when 0 ICU

Table 4: Serum alkaline phosphatase activities in laying hens fed rations added microbial phytase and supplemented vitamin D_3 and/or zeolite (U L^{-1})

	Groups			
Weeks	Control (P)	Trial 1 (P+ D_3)	Trial 2 (P+D_3+ZE)	Trial 3 (P+ZE)
	(x±SE)			
4	510.47±41.83	502.47±39.22	501.93±49.71	500.47±56.82
8	511.87±39.83	508.93±44.94	517.13±34.13	497.80±45.79
12	492.27±38.79	500.73±49.52	471.00±77.78	497.40±45.39
16	498.40±41.15	499.46±36.78	500.86±47.32	501.33±51.08

x±SE: Mean±Standard Error; P: Phytase, D_3: vitamin D_3: ZE zeolite

vitamin D_3 was fed whereas it increased when 175 ICU vitamin D_3 was fed. In the present study, the diet supplemented clinoptilolite (2%) beside of phytase did not significantly change serum Ca and Pi levels. Thus, P binding effect of zeolite (Moshtaghian *et al*., 1991) was not observed. The addition of vitamin D_3 to the phytase and zeolite supplemented diet significantly increased serum Ca but not Pi concentrations. So, it may be suggested that the phytase and zeolite supplemented to the diet neutralized each other in which the additional vitamin D_3 increased serum Ca levels.

Table 4 indicates the effects of phytase and vitamin D_3 and/or zeolite on serum ALP activities. Serum ALP activities were not significantly different between all groups.

ALP plays an important role in bone mineralization. The osteoblasts in bone increase ALP activity in response to poor bone mineralization which leads to elevation in bone and serum ALP levels. Since dietary P levels decrease, serum ALP level has been shown to increase (Koch *et al*., 1984). Pigs fed phytase had lower serum ALP level than control group indicating improved P status (Li *et al*., 1998). Similarly, Huff *et al*. (1998) reported that diet supplemented with phytase significantly decreased serum ALP activity.

Increasing cholecalciferol levels have been shown to increase intestinal phytase and ALP activity in chicks (Davies *et al*., 1970). Li *et al*. (1998) reported that when both vitamin D_3 and phytase were added to diet in pigs, there was lower serum ALP levels compared with diet containing phytase alone ($p>0.05$). Dwyer *et al*. (1997) stated that the clinoptilolite treated group in broiler chicks was no significantly different than controls for ALP levels. In the present study in similar to the findings of above researchers, there were no significant differences between all groups for serum ALP activity.

CONCLUSION

In this study, a significant increase or decrease was not seen in the effect of phytase as a result of the addition of vitamin D_3 or zeolite, respectively. There was a significant phytase, vitamin D_3 and zeolite interaction for serum Ca level.

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