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RESEARCH ARTICLE

Effect of Vitamin D_3 and/or Zeolite Supplementation to Laying Hen Rations Added Microbial Phytase on Some Blood Indices *2. Total Cholesterol, 1,25-Dihydroxycholecalciferol and Oestradiol-17β Levels* [1]

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Summary

The aim of this study was to examine the effect of vitamin D_3 and/or zeolite supplementation in the presence of phytase enzyme on serum total cholesterol, 1,25-dihydroxycholecalciferol (1,25-$(OH)_2D_3$) and oestradiol-17β levels in laying hens. A total of 60 laying hens, 28-wk-old were separated to 4 equal groups. The hens were fed control diet (300 phytase units (FTU) phytase per kilogram), experimental 1 diet (300 FTU phytase + 400 IU vitamin D_3), experimental 2 diet (300 FTU phytase + 400 IU vitamin D_3 + 2% zeolite) and experimental 3 diet (300 FTU phytase + 2% zeolite). Serum total cholesterol levels were not statistically different between groups except for week 12. On week 12, these levels were significantly higher in the phytase and zeolite added group than in the phytase and vitamin D_3 added group ($P<0.05$). Serum 1,25-$(OH)_2D_3$ levels were higher in the only phytase added group than in the other groups on week 16 and lower in the phytase and zeolite added group than in the other groups on week 8 ($P<0.05$). Serum oestradiol-17β levels were higher in the phytase and zeolite added group than in the other groups on weeks 4 and 12, and lower in the phytase, vitamin D_3 and zeolite added group than in the other groups on week 12 ($P<0.05$). Consequently, serum total cholesterol levels were not affected by different feeding regimes, phytase enzyme added to ration increased serum 1,25-$(OH)_2D_3$ levels, and phytase enzyme and vitamin D_3 supplementation increased serum oestradiol-17β levels.

Keywords: *Hen, Vitamin D_3, Zeolite, Phytase, Cholesterol, 1,25-Dihydroxycholecalciferol, Oestradiol-17β*

Mikrobiyal Fitaz İlaveli Yumurta Tavuğu Rasyonlarına D_3 Vitamini ve/veya Zeolit Eklenmesinin Bazı Kan Parametreleri Üzerine Etkisi *2. Total kolesterol, 1,25- Dihidroksikolekalsiferol ve Östradiol-17β Düzeyleri*

Özet

Çalışmanın amacı yumurta tavuklarında fitaz enzimi varlığında yeme vitamin D_3 ve/veya zeolit ilavesinin serum total kolesterol, 1,25-dihidroksikolekalsiferol (1,25-$(OH)_2D_3$) ve östradiol-17β düzeyleri üzerine etkisini incelemektir. 28 haftalık 60 adet yumurta tavuğu 4 eşit gruba ayrılmıştır. Tavuklar kontrol rasyonu (300 fitaz ünitesi (FTU) fitaz/kg), deneme 1 rasyonu (300 FTU fitaz + 400 IU D_3 vitamini), deneme 2 rasyonu (300 FTU fitaz + 400 IU D_3 vitamini + %2 zeolit) ve deneme 3 rasyonu (300 FTU fitaz + %2 zeolit) ile beslenmişlerdir. Serum total kolesterol düzeyleri 12. hafta hariç, gruplar arasında anlamlı ölçüde farklı bulunmamıştır. 12. haftada düzeyler fitaz ve zeolit ilave edilen grupta fitaz ve D_3 vitamini ilave edilen gruptan anlamlı ölçüde daha yüksek bulunmuştur ($P<0.05$). Serum 1,25-$(OH)_2D_3$ düzeyleri 16. haftada sadece fitaz eklenen grupta diğer gruplardan daha yüksek, 8. haftada fitaz ve zeolit ilave edilen grupta diğer gruplardan daha düşük saptanmıştır ($P<0.05$). Serum östradiol-17β düzeyleri 4 ve 12. haftalarda fitaz ve zeolit ilave edilen grupta diğer gruplardan daha yüksek, 12. haftada fitaz, D_3 vitamini ve zeolit eklenen grupta diğer gruplardan daha düşük bulunmuştur ($P<0.05$). Sonuç olarak, serum total kolesterol düzeyleri farklı besleme rejimlerinden etkilenmemiş, rasyona ilave edilen fitaz enzimi serum 1,25-$(OH)_2D_3$ düzeylerini artırmış, fitaz enzimi ile D_3 vitamini ilavesi ise serum östradiol-17β düzeylerini yükseltmiştir.

Anahtar sözcükler: *Yumurta tavuğu, Vitamin D_3, Zeolit, Fitaz, Kolesterol, 1,25-Dihidroksikolekalsiferol, Östradiol-17β*

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INTRODUCTION

Phosphorus (P) is a critical and expensive mineral in poultry nutrition. The major portion of P present in cereals, cereal byproducts and vegetable protein supplements is in the form of phytic acid and phytate. P from phytate is poorly available to the chicken due to lack of phytase in the digestive system [1]. Cromwell et al.[2] have indicated that the addition of microbial phytase to diets can release inorganic phosphate from phytate, improving P availability. Phytase releases other nutrients bound by phytic P, improving the digestibility and retention of protein, some amino acids and calcium (Ca) [3]. A purified preparation of phytase made from Aspergillus ficuum fermentation was shown to be effective in hydrolyzing phytate P when added to a corn-soybean diet for chickens [4]. Simons et al.[5] reported that the addition of phytase increased dietary P availability to 65% and reduced P excretion by 50% in 3-wk-old broilers.

Studies with broiler chickens fed corn-soybean diets indicated that phytate P utilization were between 10 and 53%. Phytate P utilization from corn-soybean diets has been shown to be influenced by Ca and P levels in the diet, synthetic zeolite and the aluminum content of the diet [6-8]. Many studies have indicated that dietary inclusion of sodium aluminosilicate had beneficial effects on the performance of poultry [9-11].

Dietary and endogenous vitamin D_3 is hydroxylated at position 25 of the vitamin D_3 molecule in the liver to produce 25-hydroxycholecalciferol (25-OHD_3), which is the main circulating vitamin D_3 metabolite in the blood. The circulating 25-OHD_3 is hydroxylated in position 1 of the molecule in the kidney to produce 1,25-dihydroxy-cholecalciferol (1,25-$(OH)_2D_3$) [12]. This active form of vitamin D_3 is involved in the biosynthesis of Ca-binding protein, which is involved in active transport of Ca across the intestinal wall [13]. Parfitt et al.[14] postulated that 1,25-$(OH)_2D_3$ is the only vitamin D metabolite essential for normal bone growth and bone development.

There was a dramatic increase in the serum 1,25-$(OH)_2D_3$ level when the female birds approached sexual maturity, under the influence of oestradiol. The increase of 1,25-$(OH)_2D_3$ production is required for the supply of Ca, the mineralization of egg shell and the medullary bone [15]. The known strongest stimulation of Ca absorption is 1,25-$(OH)_2D_3$, which is regulated according to Ca needs [16].

In the previous studies, dietary feed additives were seperately examined in different experimental groups of hens. The present study was designed to inverstigate the synergical effects of two or three dietary feed additives in the same experimental group. The effect of vitamin D_3 and/or zeolite supplementation in presence of phytase enzyme on serum total cholesterol, 1,25-$(OH)_2D_3$ and oestradiol-17β levels in laying hens were studied.

MATERIAL and METHODS

Animals, Diets and Feeding

A total of 60 laying hens 28-wk-old were used in the study. The hens were reared in a pen with ventilation fans. All hens were housed in the individual cages with 16:8 h light and dark cycle . The laying hens were separated to 4 equal groups (5 replicates). They were fed a corn and soybean meal basal diet [17]. 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)) per kilogram], experimental 1 diet [300 FTU phytase + 400 IU vitamin D_3], experimental 2 diet [300 FTU phytase + 400 IU vitamin D_3 + 2% zeolite (a natural zeolite, clinoptilolite (Zeotech Corp., Albuquerque, NM 87107 USA))] and experimental 3 diet [300 FTU phytase + 2% zeolite]. The experimental period was 16 weeks. Feed and water were consumed ad libitum by the laying hens. Composition and calculation of nutrients in diets are shown in *Table 1*.

Blood Sampling and Analysis

Blood samples were taken on weeks 4, 8, 12, 16. They were collected from vena brachialis of hens to the vacutainer tubes with no anticoagulant. After sampling, tubes were centrifuged at 3000 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 total cholesterol levels were analysed by using commercial kit (AMP Medizintechnik GmbH Statteggerstrasse 31b 8045 Graz, Austria) and a Technicon RA-1000 autoanalyser (DSG UK Limited, Unit 1B, 13-4 King's Gardens Hove, BN3 2PG, UK). 1,25-$(OH)_2D_3$ was extracted as described by Stampfer & Zucker [18]. Serum 1,25-$(OH)_2D_3$ (DRG Instruments GmbH, Germany Division of DRG International Inc. Frauenbergstraße 18, D-35039 Marburg, Germany) and oestradiol 17β (DIMA Gesellschaft für Diagnostika mbH, Robert-Bosch-Breite 23, 37079 Goettingen, Germany) levels were analysed by using commercial ELISA kits and a microplate reader (Bio-Tek Instruments, Inc., P.O. Box 998, Highland Park, Winooski, Vermont 05404, USA).

Statistical Analysis

Data were compared by using analysis of variance

Table 1. *Composition and calculation of nutrients in diets*
Tablo 1. *Yemin içeriği ve kimyasal bileşimi*

Nutrients	Control (P)	Experimental 1 (P+D3)	Experimental 2 (P+D3+ZE)	Experimental 3 (P+ZE)
Composition of nutrients, %				
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 [a]	0.25	0.25	0.25	0.25
Mineral premix [b]	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	300	300	300
Vitamin D_3, IU	-	400	400	-
Calculation of nutrients, %				
Crude protein	16.00	16.00	16.00	16.00
Metabolizable energy, kcal/kg	2750	2750	2750	2750
Calcium	3.50	3.50	3.50	3.50
Phosphorus, total	0.50	0.50	0.50	0.50

[a] Provided per kilogram of diet: vitamin A, 4.400 IU; vitamin D_3, 1.000 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. [b] 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. **P:** Phytase, **D_3:** vitamin D_3, **ZE:** zeolite

(ANOVA, Duncan's multiple range test) between groups within each blood sampling week for all blood indices. Results are presented as mean±SD. All statistical analysis were 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 [19].

RESULTS

Serum Total Cholesterol Levels

The effects of the different dietary treatments on serum total cholesterol levels are presented in *Table 2*. Serum total cholesterol levels were insignificantly different between groups except for week 12. On week 12, they were significantly higher in the phytase and zeolite added group than in the phytase and vitamin D_3 added group.

Serum 1,25-$(OH)_2D_3$ Levels

Table 3 presents the effects of phytase and vitamin D3 and/or zeolite on serum 1,25-$(OH)_2D_3$ levels. Serum 1,25-$(OH)_2D_3$ levels were higher in the only phytase added group than in the other groups on week 16 and lower in the phytase and zeolite added group than in the other groups on week 8 ($P<0.05$).

Table 2. *Serum total cholesterol levels (mg/dl) in laying hens fed rations added microbial phytase and supplemented vitamin D_3 and/or zeolite*

Tablo 2. *Mikrobiyal Fitaz ilaveli yumurta tavuğu rasyonlarına D_3 vitamini ve/veya Zeolit eklenmesi sonucundaki serum total kolesterol düzeyleri (mg/dl)*

Groups Weeks	Control (P)		Exp. 1 P+D3)		Exp. 2 (P+D3+ZE)		Exp. 3 (P+ZE)	
	n	x±SD	n	x±SD	n	x±SD	n	x±SD
4	14	167±23 [a]	14	171±17 [a]	14	161±25 [a]	15	156±24 [a]
8	15	163±29 [a]	13	164±21 [a]	13	170±20 [a]	13	157±13 [a]
12	15	166±14 [ab]	15	158±25 [b]	14	167±24 [ab]	13	178±12 [a]
16	11	169±09 [a]	14	160±23 [a]	15	159±27 [a]	12	171±10 [a]

n: number of animal **x±SD:** mean±standard deviation
[a,b:] Different superscripts indicate significant differences between treatment groups ($P<0.05$) **P:** Phytase, **D_3:** Vitamin **D_3, ZE:** zeolite

Serum Oestradiol-17β Levels

Table 4 shows the effects of phytase and vitamin D_3 and/or zeolite on serum oestradiol-17β levels. Serum oestradiol-17β levels were higher in the phytase and zeolite added group than in the other groups on weeks 4 and 12, and lower in the phytase, vitamin D_3 and zeolite added group than in the other groups on week 12 ($P<0.05$). Also, the levels were significantly different between the only phytase added group, the phytase and vitamin D_3 added group and the phytase, vitamin D_3 and zeolite added group on week 16.

Table 3. *Serum 1,25-dihydroxycholecalciferol levels (pg/ml) in laying hens fed rations added microbial phytase and supplemented vitamin D3 and/or zeolite*

Tablo 3. *Mikrobiyal fitaz ilaveli yumurta tavuğu rasyonlarına D3 vitamini ve/veya Zeolit eklenmesi sonucundaki serum 1,25-dihidroksikolekalsiferol düzeyleri (pg/ml)*

Groups / Weeks	Control (P)		Experimental 1 P+D3)		Experimental 2 (P+D3+ZE)		Experimental 3 (P+ZE)	
	n	x±SD	n	x±SD	n	x±SD	n	x±SD
4	10	206±109 [a]	10	200±153 [a]	14	232±136 [a]	14	267±166 [a]
8	12	195±085 [a]	08	220±118 [a]	11	206±110 [a]	12	103±041 [a]
12	10	110±042 [a]	14	119±049 [a]	11	115±038 [a]	12	142±075 [a]
16	13	266±129 [a]	10	162±125 [b]	09	172±073 [b]	15	116±046 [a]

n: number of animal **x±SD:** mean±standard deviation
[a,b:] Different superscripts indicate significant differences between treatment groups (P<0.05) **P:** Phytase, **D3:** Vitamin **D3, ZE:** zeolite

Table 4. *Serum oestradiol-17β levels (pg/ml) in laying hens fed rations added microbial phytase and supplemented vitamin D3 and/or zeolite*

Tablo 4. *Mikrobiyal fitaz ilaveli yumurta tavuğu rasyonlarına D3 vitamini ve/veya Zeolit eklenmesi sonucundaki serum östradiol-17β düzeyleri (pg/ml)*

Groups / Weeks	Control (P)		Experimental 1 P+D3)		Experimental 2 (P+D3+ZE)		Experimental 3 (P+ZE)	
	n	x±SD	n	x±SD	n	x±SD	n	x±SD
4	15	615±242 [b]	12	480±228 [b]	13	361±626 [b]	16	987±441 [a]
8	13	480±116 [a]	13	462±118 [a]	12	464±107 [a]	14	440±227 [a]
12	11	280±034 [ab]	14	271±035 [b]	13	217±046 [c]	14	327±111 [a]
16	15	344±062 [b]	12	432±096 [a]	13	206±146 [c]	15	195±089 [c]

n: number of animal **x±SD:** mean±standard deviation
[a,b:] Different superscripts indicate significant differences between treatment groups (P<0.05) **P:** Phytase, **D3:** Vitamin **D3, ZE:** zeolite

DISCUSSION

Poultry are unable to utilize phytate P. This is due to the low endogenous phytase activity in the gastro-intestinal tract [20]. Adding exogenous microbial phytase to poultry diets results in less supplementation of inorganic phosphates to feed and less excretion of phytate P into the environment [21]. In some studies, the effectiveness of phytase was negatively related to the amount of inorganic P in the diet [22,23].

Zeolite contains 14.6% aluminum, which may form a complex with P in the digestive tract and reduce P availability [24]. Edwards [7] showed that P utilization may be impaired by zeolite supplementation of chick diets and that the effects of zeolite were due to increased excretion of phytate P. Zeolite decreases utilization of dietary P by laying hens. The negative influence of zeolite on P may be due to the aluminum in zeolite forming complexes with P and reducing P availability [24].

The 1,25-$(OH)_2D_3$ is considered to be the most active form of D_3 derivatives in stimulating Ca and P absorption and in Ca mobilization from the bone [25]. Endo et al.[26] reported that 1,25-$(OH)_2D_3$ stimulates calcification of bone synergistically with parathormone. Kato et al.[27] provided that 24,25-$(OH)_2D_3$ together with 1,25$(OH)_2D_3$ improved bone mechanical strength parameters in chickens. Ruschkowski and Hart [28] stated that, in their study, plasma 1,25-$(OH)_2D_3$ concentrations were significantly higher in the calcium-deficient hens than the control or vitamin D-deficient hens. 1,25-$(OH)_2D_3$ functions to resorb additional Ca from the bones. Frost et al.[29] showed that marginal dietary P levels altered the circadian rhythm of plasma 1,25-$(OH)_2D_3$ levels.

Absorption of P is increased by vitamin D_3 or by 1,25-$(OH)_2D_3$ even when phosphate make complexes with phytate [30]. Elevated plasma concentrations of P would be expected to have an inhibitory effect on the renal 1-hydroxylase, which converts 25-$(OH)D_3$ to 1,25-$(OH)_2D_3$. Contrarily, lower blood P concentrations would be expected to enhance the activity of the renal 1-hydroxylase [31]. Therefore, it was expected that dietary phytase and vitamin D_3 combination increased serum P levels and so did not increase serum 1,25-$(OH)_2D_3$ concentration.

Indeed, in the present study, serum 1,25-$(OH)_2D_3$ levels were not higher in the phytase and vitamin D_3 added group than in the only phytase added group.

Plasma 1,25-$(OH)_2D_3$ level may be a beneficial indicator of vitamin D_3 status in laying hens [32]. Parathyroid hormone, Ca and P are unlikely to play roles in the adaptive increase in the level of 1,25-$(OH)_2D_3$ in the blood of chicks given a minimal amount of D_3 [15]. Sedrani [15] demonstrated a significant increase in the serum level of 1,25-$(OH)_2D_3$ associated with a diet low in vitamin D_3 as compared with a normal diet. Similarly, in the current study, vitamin D_3 added groups (experimentals 1 and 2) generally had the lower serum 1,25-$(OH)_2D_3$ levels than other groups.

Frost et al.[32] studied the effect of dietary supplementation of zeolite and/or vitamin D_3 on plasma 1,25-$(OH)_2D_3$ levels in laying hens. They reported that there were no significant interactions for plasma 1,25-$(OH)_2D_3$ between zeolite (0.75%) and/or vitamin D_3 (175 ICU/kg) added groups. They concluded that the beneficial effect usually seen in eggshell quality and increased Ca utilization from feeding zeolite is not accomplished through the vitamin D_3 system, namely the increased production of 1,25-$(OH)_2D_3$. In the present study, when the control, the experimental 2 and the experimental 3 groups were compared with each other, the effect of zeolite supplementation may be only seen in week 8. In this week, lower 1,25-$(OH)_2D_3$ concentration was determined in the experimental 3 group than in the control and the experimental 2 groups.

Oguz et al.[33] reported that serum cholesterol levels were negatively affected by the addition of clinoptilolite (zeolite) to the aflatoxin-free diet. Whereas Dwyer et al.[34] noted that the clinoptilolite treated group in broiler chicks was not significantly different from the controls for serum cholesterol values (177 vs 161 mg/dl). Peebles et al.[35] suggested that diet had no effect on serum cholesterol concentrations. In the present study, for serum cholesterol concentration, there were insignificant differences between all groups except the phytase and vitamin D_3 added group and the phytase and zeolite added group. The reason of difference between these two groups is unknown.

Ruschkowski and Hart [28] reported that mean plasma oestradiol-17β concentrations were higher in the control hens than the vitamin D3-deficient hens. In the current study, serum oestradiol-17β levels were variable between all groups. The levels were significantly different ($P<0.05$) between the only phytase added group, the phytase and vitamin D_3 added group and the phytase, vitamin D_3 and zeolite added group on week 16. On week 16, significantly higher serum oestradiol-17β level in the phytase and vitamin D3 added group (experimental 1) was an expected situation in similar to the study of Ruschkowski and Hart [28]. The reasons of this may be due to the same precursor (cholesterol) and the steroid structure of vitamin D_3 and oestradiol-17β.

Consequently, phytase added to the diet increased serum 1,25-$(OH)_2D_3$ levels and phytase plus vitamin D_3 supplementation increased serum oestradiol-17β levels although serum total cholesterol levels were not affected by different feeding regimes. Hence, it may be suggested that the supplementation of phytase and/or vitamin D_3 to the layer diets could improve the absorption of Ca from gut, the egg production and the eggshell quality. Because zeolite supplementation had exactly opposite effect on serum 1,25-$(OH)_2D_3$ and oestradiol-17β levels in the present study, zeolite supplementation along with phytase and/or vitamin D_3 supplementation is not advised.

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