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Effect of Vitamin D₃ and/or Zeolite Supplementation to Laying Hen Rations Added Microbial Phytase on Some Blood Indices 2. Total Cholesterol, 1,25-Dihydroxycholecalciferol and Oestradiol-17 β Levels ^[1]

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Summary

The aim of this study was to examine the effect of vitamin D₃ and/or zeolite supplementation in the presence of phytase enzyme on serum total cholesterol, 1,25-dihydroxycholecalciferol (1,25-(OH)₂D₃) and oestradiol-17 β 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 D₃), experimental 2 diet (300 FTU phytase + 400 IU vitamin D₃ + 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 D₃ added group (P<0.05). Serum 1,25-(OH)₂D₃ 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-17 β 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 D₃ 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)₂D₃ levels, and phytase enzyme and vitamin D₃ supplementation increased serum oestradiol-17 β levels.

Keywords: Hen, Vitamin D₃, Zeolite, Phytase, Cholesterol, 1,25-Dihydroxycholecalciferol, Oestradiol-17 β

Mikrobiyal Fitaz İlaveli Yumurta Tavuğu Rasyonlarına D₃ Vitamini ve/veya Zeolit Eklenmesinin Bazı Kan Parametreleri Üzerine Etkisi 2. Total kolesterol, 1,25- Dihidroksikolekalsiferol ve Östradiol-17 β Düzeyleri

Özet

Çalışmanın amacı yumurta tavuklarında fitaz enzimi varlığında yeme vitamin D₃ ve/veya zeolit ilavesinin serum total kolesterol, 1,25-dihidroksikolekalsiferol (1,25-(OH)₂D₃) ve östradiol-17 β 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 D₃ vitamini), deneme 2 rasyonu (300 FTU fitaz + 400 IU D₃ 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 D₃ vitamini ilave edilen gruptan anlamlı ölçüde daha yüksek bulunmuştur (P<0.05). Serum 1,25-(OH)₂D₃ 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-17 β düzeyleri 4 ve 12. haftalarda fitaz ve zeolit ilave edilen grupta diğer gruplardan daha yüksek, 12. haftada fitaz, D₃ 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)₂D₃ düzeylerini artırmış, fitaz enzimi ile D₃ vitamini ilavesi ise serum östradiol-17 β düzeylerini yükseltmiştir.

Anahtar sözcükler: Yumurta tavuğu, Vitamin D₃, Zeolit, Fitaz, Kolesterol, 1,25-Dihidroksikolekalsiferol, Östradiol-17 β



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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 system¹. 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 chickens⁴. 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 diet⁶⁻⁸. Many studies have indicated that dietary inclusion of sodium aluminosilicate had beneficial effects on the performance of poultry⁹⁻¹¹.

Dietary and endogenous vitamin D₃ is hydroxylated at position 25 of the vitamin D₃ molecule in the liver to produce 25-hydroxycholecalciferol (25-OHD₃), which is the main circulating vitamin D₃ metabolite in the blood. The circulating 25-OHD₃ is hydroxylated in position 1 of the molecule in the kidney to produce 1,25-dihydroxycholecalciferol (1,25-(OH)₂D₃)¹². This active form of vitamin D₃ is involved in the biosynthesis of Ca-binding protein, which is involved in active transport of Ca across the intestinal wall¹³. Parfitt et al.¹⁴ postulated that 1,25-(OH)₂D₃ 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)₂D₃ level when the female birds approached sexual maturity, under the influence of oestradiol. The increase of 1,25-(OH)₂D₃ production is required for the supply of Ca, the mineralization of egg shell and the medullary bone¹⁵. The known strongest stimulation of Ca absorption is 1,25-(OH)₂D₃, which is regulated according to Ca needs¹⁶.

In the previous studies, dietary feed additives were separately examined in different experimental groups of hens. The present study was designed to investigate the synergical effects of two or three dietary feed

additives in the same experimental group. The effect of vitamin D₃ and/or zeolite supplementation in presence of phytase enzyme on serum total cholesterol, 1,25-(OH)₂D₃ 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 diet¹⁷. 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 D₃], experimental 2 diet [300 FTU phytase + 400 IU vitamin D₃ + 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)₂D₃ was extracted as described by Stampfer & Zucker¹⁸. Serum 1,25-(OH)₂D₃ (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+D ₃)	Experimental 2 (P+D ₃ +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 D₃, 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 B₁₂, 9 µg; vitamin B₆, 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, **D₃:** vitamin D₃, **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 groups¹⁹.

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 D₃ added group.

Serum 1,25-(OH)₂D₃ Levels

Table 3 presents the effects of phytase and vitamin D₃ and/or zeolite on serum 1,25-(OH)₂D₃ levels. Serum 1,25-(OH)₂D₃ 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 D₃ and/or zeolite**Tablo 2.** Mikrobiyal Fitaz ilaveli yumurta tavuğu rasyonlarına D₃ vitamini ve/veya Zeolit eklenmesi sonucundaki serum total kolesterol düzeyleri (mg/dl)

Groups Weeks	Control (P)		Exp. 1 (P+D ₃)		Exp. 2 (P+D ₃ +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, **D₃:** Vitamin D₃, **ZE:** zeolite

Serum Oestradiol-17β Levels

Table 4 shows the effects of phytase and vitamin D₃ 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 D₃ 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 D₃ added group and the phytase, vitamin D₃ 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+D ₃)		Experimental 2 (P+D ₃ +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+D ₃)		Experimental 2 (P+D ₃ +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 gastrointestinal tract²⁰. Adding exogenous microbial phytase to poultry diets results in less supplementation of inorganic phosphates to feed and less excretion of phytate P into the environment²¹. 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 availability²⁴. Edwards⁷ 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 availability²⁴.

The 1,25-(OH)₂D₃ is considered to be the most active form of D₃ derivatives in stimulating Ca and P absorption

and in Ca mobilization from the bone²⁵. Endo et al.²⁶ reported that 1,25-(OH)₂D₃ stimulates calcification of bone synergistically with parathormone. Kato et al.²⁷ provided that 24,25-(OH)₂D₃ together with 1,25(OH)₂D₃ improved bone mechanical strength parameters in chickens. Ruschkowski and Hart²⁸ stated that, in their study, plasma 1,25-(OH)₂D₃ concentrations were significantly higher in the calcium-deficient hens than the control or vitamin D-deficient hens. 1,25-(OH)₂D₃ 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)₂D₃ levels.

Absorption of P is increased by vitamin D₃ or by 1,25-(OH)₂D₃ even when phosphate make complexes with phytate³⁰. Elevated plasma concentrations of P would be expected to have an inhibitory effect on the renal 1-hydroxylase, which converts 25-(OH)D₃ to 1,25-(OH)₂D₃. Contrarily, lower blood P concentrations would be expected to enhance the activity of the renal 1-hydroxylase³¹. Therefore, it was expected that dietary phytase and vitamin D₃ combination increased serum P levels and so did not increase serum 1,25-(OH)₂D₃ concentration.

Indeed, in the present study, serum 1,25-(OH)₂D₃ levels were not higher in the phytase and vitamin D₃ added group than in the only phytase added group.

Plasma 1,25-(OH)₂D₃ level may be a beneficial indicator of vitamin D₃ status in laying hens³². Parathyroid hormone, Ca and P are unlikely to play roles in the adaptive increase in the level of 1,25-(OH)₂D₃ in the blood of chicks given a minimal amount of D₃¹⁵. Sedrani¹⁵ demonstrated a significant increase in the serum level of 1,25-(OH)₂D₃ associated with a diet low in vitamin D₃ as compared with a normal diet. Similarly, in the current study, vitamin D₃ added groups (experimentals 1 and 2) generally had the lower serum 1,25-(OH)₂D₃ levels than other groups.

Frost et al.³² studied the effect of dietary supplementation of zeolite and/or vitamin D₃ on plasma 1,25-(OH)₂D₃ levels in laying hens. They reported that there were no significant interactions for plasma 1,25-(OH)₂D₃ between zeolite (0.75%) and/or vitamin D₃ (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 D₃ system, namely the increased production of 1,25-(OH)₂D₃. 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)₂D₃ 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 D₃ added group and the phytase and zeolite added group. The reason of difference between these two groups is unknown.

Ruschkowski and Hart²⁸ 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 D₃ added group and the phytase, vitamin D₃ 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 Hart²⁸. The reasons of this may be due to the same precursor (cholesterol) and the steroid structure of vitamin D₃ and oestradiol-17β.

Consequently, phytase added to the diet increased serum 1,25-(OH)₂D₃ levels and phytase plus vitamin D₃ 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 D₃ 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)₂D₃ and oestradiol-17β levels in the present study, zeolite supplementation along with phytase and/or vitamin D₃ supplementation is not advised.

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