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INFLUENCE OF THE ENZYME PHYTASE ON THE CLINICAL STATUS, SOME PLASMA MACROELEMENTS AND THE HISTOSTRUCTURE OF FEMUR AND TIBIA IN CHICKENS

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Summary

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Two experiments with 90 growing chickens at the age of 30 days: 50 broilers (4 experimental groups and 1 control group) (experiment I), and 40 commercial chickens (3 experimental groups and 1 control group) (experiment II), divided into groups of ten were carried out. The aim of the experiments was to establish the optimal levels of the enzyme phytase in the daily rations of chickens, without disturbing their mineral homeostasis. Different amounts of the enzyme (as the product Natuphos) were added to the chickens' diet for 60 days. The plasma concentrations of calcium, inorganic phosphate, magnesium, and alkaline phosphatase were studied; also, histological investigations of femoral and tibial specimens on the 30^{th} and 60^{th} day from the start of the treatment were done. We found that, after treatment, the chickens of the two experiments exhibited increased levels of calcium and inorganic phosphate, while the activity of the alkaline phosphatase was significantly lowered. The histological study of femur and tibia showed a thinned compacta and enlargement of the central (Haversian) canals in the broiler chickens at the end of the trials. The results showed that in broiler chickens, phytase supplementation was favourable at 750–1500 FTU/kg food up to the age of 60 days, while for commercial chickens the respective dosage was 1250 FTU/kg food up to the age of 90 days, without addition of dicalcium phosphate.

Key words: bone histology, growing chickens, minerals, phytase

INTRODUCTION

The addition of phytase to the chickens' food is economically justified (Qian *et al.* 1996a), because it increases the live body weight and the organs' weight (Mitchel *et al.*, 1997; Vetesi *et al.*, 1998; Yan *et al.*, 2000; Viveros *et al.* 2002; Tsokova *et al.*, 2004), proportionally to phytase amount in food.

The insufficient absorption of phytin phosphorus is the reason for supplementation of chicken ration with inorganic phosphorus. This causes significant environmental problems since the amounts of excreted phosphorus (P) and other minerals in the soil increase (Van der Klis *et al.*, 1997; Zanini & Sazzad, 1999; Paik, 2003), while the enzyme phytase can decrease the quantities of excreted minerals (Gordon & Roland, 1997; Farrell & Martin, 1998; Ferguson *et al.*, 1998; Rutherfurd *et al.*, 2002) and increases the plasma levels of P and Ca. In a study of ours (Tzokova, 2004) we also found significant reduction in the levels of calcium, phosphorus, magnesium, and the trace ele-

ments copper, iron, zinc, and manganese in the ileal chyme of broiler chickens, under the influence of the enzyme phytase.

According to Keshavarz (2000), low levels of phytase (300 FTU/kg food as Natuphos) and high levels of non-phytin phosphorus (NPP), do not influence growth and mineral homeostasis in commercial chickens at the age of 6 to 18 weeks, while higher phytase levels (600 FTU/kg food) and 0.34% NPP used by Qian *et al.* (1996b) or phytase levels ranging from 750 to 1500 FTU/kg food for broiler chickens after the age of 12 weeks (Tsokova *et al.*, 2005), caused the birds to spend more time sitting on the litter, increased weight and strength of femur and tibia, as well as bending and thickening of the proximal tibia. Other authors (Zhang *et al.* 2000) claim that 2500 FTU/kg food in broilers did not have any negative effects on the histological findings from the tibia after a 5-week administration, while in turkeys receiving phytase (600 FTU/kg food), numerous hypertrophies in the area of the trabecules, for high levels of NPP were observed (Qian *et al.*, 1996c).

Because of the different positions of some authors on the influence of various doses of the enzyme phytase and NPP in the chickens' diet on mineral homeostasis and because of the fact that in scientific literature, there is no information on the mentioned substances' influence on growing broilers after the 45th day, we were motivated to perform respective experiments.

MATERIALS AND METHODS

Animals

For the experiments, we used 90 chickens at the age of 30 days, all of them bought from the poultry-breeding farm at the town of Chirpan. A part of 50 birds were Hybro-PN broiler chickens, weighing 828–953 g, randomly divided into 5 groups of 10 chickens each, and another part of 40 birds were Hyssex Brown chickens weighing 529–556 g, randomly divided into 4 groups of 10 chickens each.

The chickens were reared in the stationary facility of the Clinic of Internal Non-Infections Diseases, using a floor-rearing system, with all control and test groups kept under the same zoohygienic conditions. They were fed with a forage mixture in accordance to their age, containing raw protein – 19%, Ca – 1%, P – 0.70%, of which 0.18% NPP and exogenous phytase with activity of 2500 FTU/g (as the product Ronosin, DSM, Switzerland) – 500 FTU/kg food. The chickens were given free access to food and water.

Experimental design

The microbial enzyme phytase with activity of 5000 FTU/g (as the product Natuphos, BASF, Ludwigshafen, Germany) was added to the chickens' food for 60 days, as followed: for experiment I (broilers) – 1st group with 250 FTU/kg food, 2nd group with 500 FTU/kg food, 3rd group with 750 FTU/kg food, and 4th group with 1000 FTU + 0.75 g dicalcium phosphate/kg food. For experiment II (commercial chickens) – 1st group with 250 FTU/kg food, 2nd group with 500 FTU/kg food, 3rd group with 750 FTU + 0.75 g dicalcium phosphate/kg food. The food of control groups was not supplemented with Natuphos.

Clinical status and biochemical studies of blood plasma

Clinically, the chickens were examined following the routine methods of locomotor activity assessment and visible changes in the extremities.

One mL heparinized blood from all

groups of growing chickens was sampled from *v. cutanea ulnaris superficialis* between 10 and 11 AM on the 30th and 60th day from the start of the experiment. In separated plasma, we studied the levels of Ca, P, and Mg, colorimetrically with Ciba Corning kits, while the alkaline phosphatase (AP) was assayed by a kit of Roche Diagnostics, Germany, on an automated analyzer "Reflotron", Germany.

Histological studies of femur and tibia

To conduct the histological studies, 5 chickens from the control and test groups were euthanized, on the 30th and 60th day from the experiment's start, respectively by intraperitoneal injection of pentobarbital sodium at doses of 150 mg/kg body weight. Post-mortem, bone material was obtained from the large tubular bones (femur and tibia). With the aid of bone scissors and a small saw, samples were taken from bone epiphysis and diaphysis. The histological processing was performed on material fixed in 10% aqueous solution of neutral formalin by the method of Volkova & Eletskii (1976), and the staining of samples of the materials was performed according to Vitanov *et al.* (1995). With the aid of a light microscope, we studied the histological samples at different magnifications.

RESULTS

The locomotor activity of commercial chickens from all groups did not exhibit any changes. In the 60-day-old broilers there were no visible changes in the hindlegs, yet they spent more time sitting on the litter than commercial chickens did, and at the age of 67–70 days, bone deformations of the one or both tibias were noticed in individuals from the experimental broiler groups, accompanied by enlarged proximal end of the tibia, lameness, and difficult walking that aggravated with age.

Data on the changes in plasma levels of Ca, P, Mg and AP in growing broiler chickens are presented in Table 1. The results for Ca in the plasma of broiler chickens from both performed tests showed significantly higher levels for all experimental groups throughout the first analysis (for groups 1 and 4 – $P<0.05$; for groups 2 and 3 – $P<0.01$), while during the second sampling, only the first experimental group did not exhibit any significant difference vs the control group (for group 3: $P<0.05$; for groups 2 and 4: $P<0.01$).

The inorganic phosphate on the 30th and 60th day of the experiment, had significantly higher values in the chickens of groups 1, 2 and 3, compared to the control group ($P<0.01$). The differences in plasma Mg, for both testings, were insignificant in comparison with the control values.

The data for blood plasma AP activity showed that it was significantly lower for groups 2 and 4 ($P<0.01$) for the first testing, while for the second testing, it was lower for experimental groups 2, 3 and 4 ($P<0.01$) compared to controls.

The results from the changes in the plasma levels of Ca, P, Mg, and AP in the growing commercial chickens are presented in Table 2. The values of Ca in commercial chickens during the first blood sampling were significantly higher than control values - $P<0.05$ in the 1st and 3rd group, and $P<0.01$ for 2nd group. By the 60th day, only the 2nd group showed statistically significant difference vs the control group. Higher levels of inorganic phosphate were determined for all experimental groups, yet the difference with the control group values were significant only

Table 1. Changes in plasma Ca, P, Mg and alkaline phosphatase (AP) in growing broiler chickens (10 birds in each group) after supplementation of the forage with the enzyme phytase with or without dicalcium phosphate (mean ± SEM)

Groups	Ca	P	Mg	AP
After 30 days of treatment				
Control group	2.28±0.22	2.18±0.19	0.99±0.07	2910±130
Experimental group 1 – phytase 250 (750) FTU/kg	2.77±0.00*	2.60±0.11*	1.17±0.10	2675±575
Experimental group 2 – phytase 500 (1000) FTU/kg	3.41±0.36**	2.63±0.22**	1.16±0.14	2020±280**
Experimental group 3 – phytase 750 (1250) FTU/kg	3.19±0.47**	2.24±0.28	0.92±0.06	2400±340
Experimental group 4 – phytase 1000 (1500) FTU/kg + 0.75 g $CaHPO_4.2H_2O$/kg	2.82±0.14*	2.15±0.21	0.94±0.05	1450±330**
After 60 days of treatment				
Control group	2.35±0.18	2.45±0.14	1.12±0.18	2838±206.94
Experimental group 1 – phytase 250 (750) FTU/kg	2.92±0.08	2.9±0.12**	1.18±0.09	2712±217.75
Experimental group 2 – phytase 500 (1000) FTU/kg	3.17±0.00**	3.18±0.17**	1.10±0.03	1881±210.60**
Experimental group 3 – phytase 750 (1250) FTU/kg	2.97±0.21*	2.93±0.13**	1.02±0.08	1954±219.17**
Experimental group 4 – phytase 1000 (1500) FTU/kg + 0.75 g $CaHPO_4.2H_2O$/kg	2.73±0.13**	2.39±0.15	1.17±0.17	1204±147.25**

The phytase doses put into brackets, indicate the total amount of enzyme supplemented to the forage, representing a sum of a background (500 FTU/kg) and additional amounts (indicated by the digits before brackets); * $P< 0.05$, ** $P <0.01$ vs the control group.

Table 2. Changes in plasma Ca, P, Mg and AP in growing commercial chickens (10 birds in each group) after supplementation of the forage with the enzyme phytase with or without dicalcium phosphate (mean ± SEM)

Groups	Ca	P	Mg	AP
After 30 days of treatment				
Control group	2.18±0.13	2.12±0.14	0.92±0.07	3060±180.36
Experimental group 1– phytase 250 (750) FTU/kg	2.54±0.22*	2.36±0.18	0.95±0.08	3146±161.14
Experimental group 2 – phytase 500 (1000) FTU/kg	2.74±0.18**	2.48±0.12**	0.90±0.09	2374±213.30**
Experimental group 3 – phytase 750 (1250) FTU/kg + 0.75 g $CaHPO_4.2H_2O$/kg	2.46±0.13*	2.1±0.17	0.92±0.06	1834±115.22**
After 60 days of treatment				
Control group	2.12±0.14	2.16±0.12	0.94±0.07	2819±323.09
Experimental group 1 – phytase 250 (750) FTU/kg	2.36±0.18	2.64±0.14**	1.04±0.09	1737±173.37**
Experimental group 2 – phytase 500 (1000) FTU/kg	2.48±0.12**	2.66±0.09**	1.03±0.07	1233±162.45**
Experimental group 3 – phytase 750 (1250) FTU/kg + 0.75 g $CaHPO_4.2H_2O$/kg	2.1±0.17	2.32±0.22	0.92±0.03	1229.8±208.87**

The phytase doses put into brackets, indicate the total amount of enzyme supplemented to the forage, representing a sum of a background (500 FTU/kg) and additional amounts (indicated by the digits before brackets); * $P< 0.05$, ** $P <0.01$ vs the control group.

for the 2nd group by day 30 ($P<0.01$), while by day 60, significant differences were established for the 1st and 2nd groups ($P<0.01$). The plasma Mg from both measurements exhibited only insignificant variations, just as it was for broiler chickens. The activity of plasma AP from both measurements was lower in the groups of chickens that received additional phytase. The differences towards the control groups were significant at the first testing for experimental groups 2 and 3 ($P<0.01$) and at the second testing for all experimental groups ($P<0.01$).

Histological analysis of femur and tibia

The first analysis of femur and tibia specimens from broiler and commercial chickens (on the 30th day of the experiment) did not exhibit any histostructural changes in the control groups, and only insignificant changes in the experimental groups were observed. In several samples from the 3rd group of broilers, the bone trabeculae in the epiphyseal spongiosa were thinned at places, and the spaces between them were filled with traces of bone marrow (Fig. 1).

On the 60th day of the experiment's start (2nd analysis), the histological study on chicken femur and tibia revealed that the general histological structure was intact for control and experimental commercial chickens and for control broiler chickens, while for the experimental broilers, there were changes, such as thinned compact bone at places, appearance of resorption surfaces (on the account of spongiosa of the diaphyses and the belonging part of the compacta), increases in the central (Haversian) canals and loss of bone trabeculae within the epiphyseal spongiosa (Fig. 2 and Fig. 3).

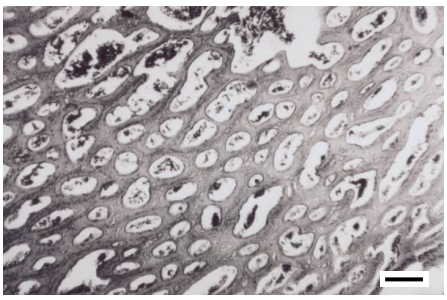

Fig. 1. Spongiosa from the tibia in a broiler chicken after 30-day treatment with phytase at 1250 FTU/kg forage. Bar= 10.5 μm.

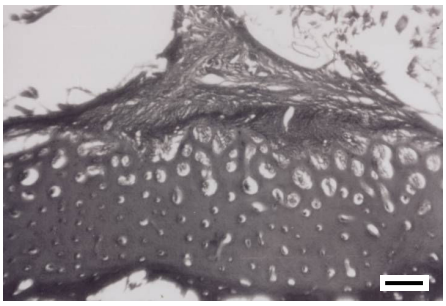

Fig. 2. Tibial area in a broiler chicken with all structural elements with various-degree structural alterations after 60-day treatment with phytase at 1500 FTU/kg forage. Bar= 5 μm.

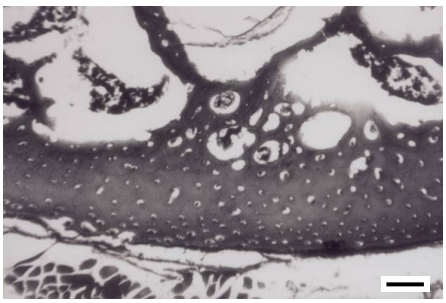

Fig. 3. Tibial epiphyse of a broiler chicken, after 60-day treatment with phytase at 1250 FTU/kg forage. The compact bone and the visible part of the spongiosa exhibit histological alterations. Bar= 5 μm.

DISCUSSION

The decreased locomotor activity and the more time spent sitting in the broilers at the age of 67–70 days (37–40 days after the experiment's start) could have been caused by the fast accumulation of biomass for a short time (Quamane, 1985; Tsokova *et al.*, 2004) and insufficient mineralization of the skeleton, exhibited by mild or severe deformations, mostly of the tibia, accompanied by increased width of its proximal end (Tsokova *et al.*, 2005). Commercial chickens from the experimental groups did not exhibit any changes in their activity or any visible deformations in the tubular bones for the whole duration of the study, which showed that probably, these changes were partially caused by genetic errors.

The obtained results from blood plasma tests after the addition of the enzyme phytase and the nutrient mineral dicalcium phosphate, showed better absorption of P from food and an enhanced general metabolism. This was confirmed by the significantly higher levels of Ca and P in the experimental groups, with better manifested signs in the broiler groups. The concentration of these elements was equalized for most experimental groups, and for some of them it was higher than for P, compared to the concentration of Ca (Edwards & Veltmann, 1984; Riddell & Pass, 1987; Roberson *et al.*, 1993), which causes metabolic acidosis. It could also cause conversion of 25-hydroxycholecalciferol to 1,25-dihydroxycholecalciferol in the kidneys and increased number of broilers affected by dyschondroplasia. AP decreased proportionally to the increase of the phytase doses in the diet for all experimental groups, which contradicts to the findings by Crespo *et al.* (2002). Our results comply with the results of other authors (Bar *et al.*,2002), which point out that the AP levels are negatively correlated to the levels of Ca.

The results of the histological analysis of femur and tibia samples showed that the experimental groups of stock chickens have preserved (unchanged) histostructure within the test period (60 days). We assume that the observed structural changes in the bones of broilers at the second testing were caused by a process of disturbed mineral metabolism in birds. We also assume that the unnatural increase of osteons in the bones was a "compensation" reaction towards the disturbed Ca:P balance in blood. It could be hypothesized that the impaired Ca:P ratio would cause destructive processes in the birds' bones in the future, which would render their movements even more difficult, because of the lameness and fractures.

In conclusion, the addition of the enzyme phytase at doses of 750 or 1500 FTU/kg food (1st and 2nd experimental groups) was favourable up to the age of 60 days for broilers, without additions of NPP, but not for chickens bred to be future breeders, because of occurring fast growth and harmful histological and macroscopic changes in the tubular bones of the legs.

The exogenous phytase added to the food of commercial chickens, non-supplemented with NPP, at dose of 1250 FTU/kg food was economically advantageous within the test period (90 days of age for the chickens).

The addition of microbial phytase to the feed of the experimental groups of growing chickens from experiments I and II caused increased plasma levels of Ca and P, and reduced activity of AP.

Within the experimental period, treatment caused broiler chickens to exhibit various-degree macroscopic and histological symptoms of dyschondroplasia.

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