The effect of humic acid on zinc accumulation in chicken broiler tissues

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ABSTRACT: Thirty-six selected male chickens were allocated into four groups C, HA, ZN, ZN + HA, and the experiment was initiated after 4 days of an adaptation period. Group C was fed a complete feed mixture without supplements. Group HA was fed the same diet with 500 mg of humic acid per chicken and day. Group ZN was loaded with 240 mg Zn (as 600 mg $ZnSO_4$) per chicken and day, and birds of the group ZN + HA were loaded as those of the latter group, with additional 500 mg of humic acid per chicken and day. The treatments were carried out for 10 days. Subsequently, the chickens were slaughtered and samples of liver, kidneys, leg muscles and blood were collected for Zn level assessment. The Zn levels detected in group C can be considered as the background value, found commonly in broiler chickens fed the complete feed mixtures enriched with trace elements. The following concentrations were detected (mg Zn/kg): 40.1 ± 13.4 in muscles, 81.0 ± 6.6 in kidneys, 72.2 ± 15.0 in liver, and in blood serum 1.04 ± 0.45 mg Zn/l. Increased Zn levels by 11 to 30% ($P > 0.05$) compared to group C were found in group HA in all tissues studied. The ten-day treatment with 600 mg $ZnSO_4$ (240 mg Zn/day, i.e. 2 400 mg Zn per 10 days) resulted in increased Zn levels in all investigated tissues, significantly in kidneys ($P < 0.01$), liver ($P < 0.01$) and blood serum ($P < 0.01$). The mean levels 430.5 ± 159.0, 149.8 ± 41.9 and 57.9 ± 22.7 mg Zn/kg, and 4.14 ± 0.9 mg/l were found in liver, kidneys, leg muscles, and blood serum, respectively. After the same treatment with zinc sulphate together with humic acid (group ZN + HA), no significant changes of Zn levels in the investigated tissues were reported. Concentrations of the selected parameters of metabolism in chickens ranged within the reference limits; significant differences between experimental and control groups (cholesterol $P < 0.05$; lactate $P < 0.05$; calcium $P < 0.01$) were found sporadically.

Keywords: zinc sulphate; liver; kidney; leg muscle; biochemical profile

Zinc has been used as a growth stimulator of farm animals for several decades, and is important as an activator of significant enzymes and hormones. Interactions exist among zinc and other elements, and zinc and iron, copper, manganese, cobalt and molybdenum metabolism. If copper is used as a

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growth stimulator, signs of zinc deficiency may appear in case that the zinc supply is insufficient. On the other hand, feed mixtures rich in zinc but low in copper may result in the development of anaemia. Antagonistic relations between zinc and copper lie in their competition during intestinal absorption (Van Campen and Scaife, 1967; Southern and Baker, 1983). As reported by Galyean et al. (1999), Zn, Cu, Se and Cr supplements can play a positive role in the immune response of poultry.

In veterinary practice and animal husbandry, the amounts of zinc in the rations must be monitored, particularly in pigs and poultry, where both primary and secondary deficiencies of zinc may occur. Zinc deficiency in poultry results in growth retardation, poor plumage and deficient development of leg and wing bones (ODell and Savage, 1957). Suchý et al. (1998) reported a positive effect of zinc on the testis development in male chickens.

Recently, supplementation with zinc oxide and copper sulphate has increased in some countries to promote performance and improve health status as a consequence of the ban on the use of nutritional antibiotics (Anonymous, 1997; Hansen, 2000; Pluske et al., 2002). It is worrying that the use of zinc compounds at therapeutic levels elevates Zn excretion into the environment.

Humic acids (HA) can markedly influence various chemical and biochemical processes owing to their structure. Their structure, chemical and physical properties and biological activity have been the objectives of study in different spheres (Yoruk et al., 2004; Rath et al., 2006; Van Rensburg et al., 2006; Ipek et al., 2008). HA are organic compounds which can bind metal cations. Some of the functional groups present in humic acids can act as ligands. With metals, an almost infinite number of humic-metallic complexes can be formed. Not only Zn, Cu and other trace elements, but also heavy metals such as Cd and Pb can enter these bonds (Boyd et al., 1981; Senesi et al., 1985; König et al., 1986; Kang et al., 1991; Livens, 1991).

The results and conclusions from *in vitro* experiments cannot be simply applied to *in vivo* experiments. The presence of several ions of one metal in body fluids and inside the cells, toxic metal accumulation in extracellular spaces and interactions of toxic metals with biological ligands are factors that may change the biological effect of humic acids.

The ability of humic acids to bind heavy metals was confirmed in several studies by different authors. However, studies investigating a possible effect of HA on microelements which are essential for animals and are included in mineral supplements of feed mixtures or are applied as a replacement of nutritional antibiotics appear sporadically (Islam et al., 2005). Regarding the fact that humic acids are used in horses, ruminants, pigs and poultry at the doses from 500 to 2 000 mg per kg of live weight to treat or prevent diarrhoea, dyspepsia and acute intoxication (Anonymous, 1999), the study on a relationship between humic acids and microelements is highly desirable.

The objective of our study was to investigate the effect of oral application of permitted preventive dose of HA on zinc levels in blood serum and its deposition in organs and muscles of broiler chickens.

MATERIAL AND METHODS

The experiment was carried out at the experimental animal facility of the Veterinary and Pharmaceutical University, Brno, in the period March 7–22, 2007. Thirty-six selected hybrid Ross 308 male chickens with a body weight 2 500 ± 306 g, aged 45 days, were allocated into 4 groups of 9 birds each:

Group C – negative control – chickens were fed the complete feed mixture BR 2 without supplements.

Group HA – positive control – chickens were fed the complete feed mixture BR 2 plus 500 mg humic acid per chicken and day.

Experimental group ZN – chickens were fed the feed mixture plus 240 mg Zn (600 mg $ZnSO_4$) per chicken and day.

Experimental group ZN + HA – chickens were fed the feed mixture plus 240 mg Zn per chicken and day, and concurrently 500 mg of humic acid per chicken and day were administered orally.

The experiment started after a 4-day adaptation period. The capsules containing Zn or HA were wetted with oil and administered on the tongue root every morning. The treatment with the substances under study was performed for 10 days; after slaughter, the liver, kidney and leg muscle samples from the right hind limb (*mm. flexores perforantus et perforati*) were collected for Zn level assessment. At the same time, blood was collected for a biochemical analysis of selected parameters (total protein, albumin, cholesterol, triacylglycerols, glucose, lactate, ALP, AST, LDH, calcium, phosphorus, magnesium, zinc). The chickens were fed the

Table 1. The composition of complete feed mixture (%) and the content of basic nutrients (g/kg)

Wheat	24.80
Maize 9% crude protein	35.00
Soya extracted meal 46% crude protein	31.60
Soya oil	4.40
Biolysin 65[1]	0.40
DL-methionine 100%	0.25
L-threonine 100%	0.06
Monocalcium phosphate MCP-F	1.08
Salt	0.35
Ground limestone	1.59
Vitamin/mineral suplement[2]	0.23
Sodium monensinate	0.24
Total	100.00
Dry matter	886.40
Crude protein	210.00
Fat	48.20
Fibre	25.70
Ash	52.00
ME (MJ/kg)	13.12

[1]contains 50.7% of L-lysine sulphate (Evonik Degussa GmbH)

[2]contents of additives per 1 kg of diet: vitamin A 14 000 IU; vitamin D_3 5 000 IU; vitamin K 2.45 (mg); vitamin E 52.5 (mg); vitamin B_1 2.45 (mg); vitamin B_2 6.75 (mg); vitamin B_6 3.75 (mg); vitamin B_{12} 0.022 (mg); niacin 30 (mg); folic acid 0.8 (mg); calcium pantothenate 9.75 (mg); choline chloride 750 (mg); biotin 0.088 (mg); L-lysine HCl 2.12 (g); D,L-methionine 1.85 (g); L-threonine 1.25 (g); cobalt 0.262 (mg); iodine 0.375 (mg); selenium 0.13 (mg); copper 7.00 (mg); manganese 82.5 (mg); zinc 45 (mg); iron 80 (mg); sodium 0.5 (g); phosphorus 0.15 (g); calcium 2.5 (g); avilamycin 10 (mg)

same diet and drinking water was available *ad libitum* prior to and in the experimental period. Composition of the feed mixture is shown in Table 1.

Specification of the active substance

Humic acid (HA) was prepared in the Research Institute of Inorganic Chemistry (Ústí nad Labem, Czech Republic) by sedimentation and centrifugation of potassium humate after coagulation with sulphuric acid at pH 1.5–1.7. HA was separated by filtration, washed with distilled water and dried in the air at room temperature. The composition of HA (batch No. B03A1) is presented in Table 2.

Zn assessment

Zn content in the collected samples of liver, kidneys and muscles was detected as follows: approximately 0.8–1.0 g of fresh sample was lyophilized and then about 0.1–0.2 g of dry matter was digested in an MLS-1200 MEGA microwave system (Milestone, Italy). The content of Zn was determined by GF-AAS method using a Solaar 939 atomic absorption spectrometer (ATI Unicam, United Kingdom). Blood serum was analyzed without previous lyophilization. Two analyses were performed for each sample. The content of zinc in feed mixture, humic acid, placebo and capsule are shown in Table 3. Reliability of the analytical method used was proved by a certified reference material for trace elements DORM-2 (dogfish muscle; National Research Council, Canada).

Assessment of selected metabolic parameters

Total protein, albumin, cholesterol, triacylglycerols (TAG), glucose, lactate, aspartate aminotransferase (AST), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), calcium, phosphorus and magnesium blood serum levels were determined photometrically using a Cobas EMIRA biochemical analyzer (Roche Co.) with commercial kits (Biovendor – Laboratorni medicina a.s., Brno, Czech Republic).

Table 2. Humic acid analysis (mass %)

Sample	Water	Dry matter	Ash		HA	
			in sample	in dry matter	in sample	in dry matter
B03A1	9.37	90.63	3.74	4.13	86.89	95.87

Table 3. Zinc contents in feed mixture, humic acid, placebo and capsule

Sample	Zn (mg/kg)
Feed mixture	132
Humic acid	1.31
Placebo	4.47
Capsule	< detection limit

Chicken body weight assessment

The chickens were weighed at the beginning and termination of the experimental period to the nearest 0.1 g always at the same time and at the same sequence of groups. Relative growth rate (RGR), which takes into account the initial body weight, was calculated. The following formula was used for the calculation:

$$q = ((Yt - Yo)/Yo) \times 100$$

where:

Yt, *Yo* = the final and initial body weight, respectively

Health status

The health status of chickens was monitored daily. No clinical symptoms of any disease were observed. One chicken from the group ZN + HA died due to ascites diagnosed by necropsy. The patho-anatomic finding had no association with the treatment.

Statistical evaluation

The obtained data were evaluated using the programme Unistat 5.1. ANOVA was used for data processing, followed by Tukey-HSD test (Zar, 1999) in order to find pairs of groups with significant differences.

RESULTS AND DISCUSSION

In the negative control (group C), which was fed a complete feed mixture without supplement, the highest zinc levels were found in kidneys (81.0 ± 6.6 mg/kg) (Table 4), followed by liver (72.2 ± 15.0 mg/kg), and the lowest levels (40.1 ± 13.4 mg

Table 4. Zinc concentrations in muscles, liver, kidneys (mg/kg) and blood serum (mg/l) of chicks

	C (n = 9)	HA (n = 9)	ZN (n = 9)	ZN + HA (n = 8)
	x ± SD	x ± SD	x ± SD	x ± SD
Muscle	40.1 ± 13.4	52.2 ± 15.6	57.9 ± 22.7	62.1 ± 23.5
CV (%)	33.5	29.9	39.2	37.9
Index (%)	100	130.2	100	107.2
Kidney	81.0 ± 6.6^{A}	91.6 ± 36.1^{A}	149.8 ± 41.9^{B}	143.8 ± 22.7^{B}
CV (%)	8.2	39.4	28.0	15.8
Index (%)	100	113.1	100	96.0
Liver	72.2 ± 15.0^{A}	88.1 ± 18.5^{A}	430.5 ± 159^{B}	416.0 ± 183.4^{B}
CV (%)	20.7	72.7	36.9	44.1
Index (%)	100	122.0	100	96.6
Blood serum	1.04 ± 0.45^{A}	1.16 ± 0.26^{A}	4.14 ± 0.9^{B}	4.37 ± 0.87^{B}
CV (%)	43.5	22.5	21.7	20.0
Index	100	111.5	100	105.6

^{A,B}significant differences ($P \leq 0.01$); CV = coefficient of variation (%)

per kg) were detected in muscle tissues. The preferential Zn deposition in kidneys and liver is in accordance with the data of Kottferová et al. (2002), who found Zn levels 12.83, 18.44 and 13.37 mg/kg in the wet samples of hind leg muscle, liver and kidneys, respectively. These values recalculated to dry matter basis correspond to our results. Concentrations reported for humans are similar to those found in our experiment: 54 mg/kg, 55 mg/kg and 60 mg/kg Zn in muscles, kidneys and liver, respectively (Lentner, 1981). The Zn levels detected in the negative control (group C) can be considered as a background commonly found in chicken broilers fed the complete feed mixtures supplemented with trace elements. The above-mentioned Zn concentrations affected the levels detected in all experimental groups.

After humic acid treatment (group HA), the Zn level in muscle tissue, kidneys and liver increased by about 22% (Table 4). This finding is in contrast to the traditional general concept. Humic acids are weak organic acids that can bind metal cations. Many of the functional groups present in humic acids can act as ligands producing thus humic-metallic complexes. These bonds can be entered by Zn, Cu, Mn, Au, Fe, Al, Se, and also heavy metals such as Cd and Pb (Boyd et al., 1981; Senesi et al., 1985; König et al., 1986; Kang et al., 1991; Livens, 1991). These complexes can be then released from the organisms into the environment. Ipek et al. (2008) found a significant increase in Fe plasma levels when HA was administered. Fe and Zn are known to be able to participate actively in the ligand formation with organic compounds and therefore, the ability of HA to act in a ligand former may explain their facilitatory action in inorganic ion transport through biological membranes (Addington and Schauss, 1999; Islam et al., 2005).

Treatment of the experimental groups of chickens (ZN and ZN + HA) with 240 mg Zn (600 mg $ZnSO_4$) per chicken and day caused an increase in Zn levels in all tissues under investigation. The mean Zn level in the group ZN was 430.5 ± 159 mg per kg in liver, which is six times more compared to group C; the zinc level in kidneys was 149.8 ± 41.9, and 57.9 ± 22.7 mg/kg in leg muscle.

The application of zinc sulphate and humic acid (group ZN + HA) did not have any significant effect on Zn levels in all the investigated tissues. The mean zinc levels were 416.0 ± 183.4, 143.8 ± 22.7 and 62.1 ± 23.5 mg/kg in liver, kidneys and leg muscle, respectively.

Of all the investigated groups, the highest Zn levels were found in liver in both experimental groups; lower levels were found in kidneys, and the lowest in leg muscle (Table 3). Zinc, similarly like copper,

Table 5. Levels of selected biochemical parameters in the blood serum of chickens

	C*x* ± SD (*n* = 9)	HA*x* ± SD (*n* = 9)	ZN*x* ± SD (*n* = 9)	ZN + HA*x* ± SD (*n* = 8)
Total protein (g/l)	38.21 ± 2.05	38.25 ± 10.91	36.62 ± 3.17	38.73 ± 4.85
Albumin (g/l)	21.41 ± 2.14	22.0 ± 5.51	20.75 ± 2.47	23.93 ± 2.37
ALP (μkat/l)	15.87 ± 4.62	16.82 ± 6.56	25.18 ± 11.20	23.00 ± 6.37
LDH (μkat/l)	9.49 ± 5.14	9.47 ± 5.33	10.60 ± 4.00	11.41 ± 3.62
AST (μkat/l)	8.0 ± 2.41	9.07 ± 3.77	7.12 ± 6.19	4.99 ± 1.55
Cholesterol (mmol/l)	3.55 ± 0.49^b	3.63 ± 0.54^b	2.87 ± 0.46^a	3.33 ± 0.69^b
TAG (mmol/l)	1.01 ± 0.39	0.87 ± 0.38	1.19 ± 0.85	1.01 ± 0.26
Glucose (mmol/l)	14.42 ± 0.58^a	14.73 ± 1.77	14.56 ± 0.94	16.06 ± 1.47^b
Lactate (mmol/l)	9.16 ± 2.08^a	6.76 ± 1.39^b	7.43 ± 1.90	7.47 ± 1.62
Calcium (mmol/l)	2.96 ± 0.13	3.0 ± 0.14	2.83 ± 0.20^A	3.11 ± 0.14^B
Phosphorus (mmol/l)	2.30 ± 0.14	2.12 ± 0.11	2.11 ± 0.26	2.75 ± 1.47
Magnesium (mmol/l)	1.19 ± 0.08	1.17 ± 0.08	1.16 ± 0.17	1.22 ± 0.08

^{A,B}significant differences ($P < 0.01$); ^{a,b}significant differences ($P < 0.05$)

Table 6. Body weight of chickens and the weight gain (g)

	C (*n* =9)	HA (*n* =9)	ZN (*n* =9)	ZN + HA (*n* =8)
Live body weight 9.3.	2 433 ± 264.5	2 521 ± 266.1	2 352 ± 384.3	2 651 ± 309
CV (%)	10.9	10.6	16.28	11.7
Live body weight 22.3.	3 259 ± 381.6	3 414 ± 343.2	2 716 ± 581.6	3 196 ± 494.2
CV (%)	11.7	10.1	21.41	15.46
RGR (%)	33.9	35.4	15.5	20.6
Live body weight gain	825.8 ± 171.7	892.9 ± 136.9	346.1 ± 247.5	544.4 ± 311.5
CV (%)	20.8	15.3	71.51	57.2

RGR (%) = relative growth rate; CV (%) = coefficient of variation

accumulates predominantly in liver and kidneys, forming a resource that can be mobilized (Falandysz et al., 1991; Miles et al., 1998; Sadoval et al., 1999), which is in accordance with our results.

Blood serum levels of Zn were similar in the group C and HA (1.04 ± 0.45 and 1.16 ± 0.26 mg/l); significantly higher levels ($P \leq 0.01$) were found in the group ZN (4.14 ± 0.9) as well as in ZN + HA (4.37 ± 0.87 mg/l). Absorption is based on a rapid Zn penetration into the cells of the intestinal mucus and relatively slow transport into the blood. In blood, zinc is bound to plasma (especially to proteins) in 75%, to erythrocytes in 22%, and to leucocytes in 3% (Prasad, 1982).

The selected parameters of metabolism in chickens (Table 5) ranged within the reference limits (Meluzzi et al., 1992) and are in accordance with the data found by Demeterova and Mariscakova (2006) after the application of humic substances. The cholesterol level was significantly lower ($P < 0.05$) in the group ZN compared to the groups C, HA and ZN + HA, which agrees with the results of Rashtchizadeh et al. (2008), who found the antiatherogenic effect of zinc in the presence of copper.

There were significant differences in Ca levels between group ZN and ZN + HA ($P < 0.05$) with higher Ca levels in the group supplemented with HA. Contrary to our results, Rath et al. (2006) found that, besides other parameters, Ca had a tendency to decrease in broiler chickens fed diets supplemented with humic acid.

Mean initial body weight was taken into account by the calculation of relative growth rate over the experimental period. The RGR values in groups C and HA differed only slightly (33.9 vs. 35.4); they were lower in groups ZN and ZN + HA (15.5 vs. 20.6) (Table 6). With respect to the duration of the experimental period and no significant differences, the effect of Zn and HA treatment cannot be evaluated unambiguously.

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