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Effect of copper nanoparticles and copper sulfate administered *in ovo* on copper content in breast muscle, liver and spleen of broiler chickens

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Abstract: *Effect of copper nanoparticles and copper sulfate administered in ovo on copper content in breast muscle, liver and spleen of broiler chickens*. The initial experimental material included 300 hatching eggs of Hubbard Flex chickens. The eggs were divided into three groups: control, NanoCu and $CuSO_4$. Eggs from groups NanoCu and $CuSO_4$ were subjected to *in ovo* injection to the air cell of egg. Experimental solutions were administered by *in ovo* injection using a sterile needle and a 0.3 mL syringe as follows: NanoCu – colloid of copper nanoparticles, concentration 50 ppm; and $CuSO_4$ – colloid of copper sulfate, concentration 50 ppm. The eggs were incubated under standard conditions. After hatching, 50 chicks were selected from each group for 42-day rearing. The birds were fed standard feed concentrates for broilers. In that last day of rearing (42 day), 12 females and 12 males were selected from each group. The right part of their breast muscle, their liver and spleen were collected for copper content determinations. Results of this experiment confirm previous scientific reports which demonstrate that the greatest accumulation of copper is observed in soft organs like liver or spleen.

Key words: nanoparticles, in ovo, copper, copper sulfate, egg

INTRODUCTION

Researches concerning content of mineral elements in tissues of farm animals are interesting both from the point of view of the good of animals and people's health as people use animal products. These researches include not only exposition to heavy metals, such as cadmium and lead, but also concentration and transferring in the food chain of indispensable elements, such as zinc, copper and magnesium. Metals which are the components of animal tissues are consumed by people and therefore they directly affect the condition of public health (Nriagu et al. 2009).

Particular ability to accumulate some of the elements has animal liver which is commonly used in cooking. Liver is widely considered among numerous societies as the source of macro- and microelements and vitamins soluble in fats. Kunachowicz et al. (1998), report that the highest content of copper in chicken meat and edible products is in liver (0.3 mg/100 g). Due to this fact, liver is one of the best indicators showing demand for particular elements in organism (Dorton et al. 2003).

Although meat is a significant component of human diet, it can also accumulate heavy metals which in concentrations exceeding reference values may pose toxic risk. Level of trace elements in meat and in meat products depends on different factors, such as condition of environment, type of grazing land and genetic features of organisms (Demirezen and Aksoy 2004). Rays et al. (1994) underline that toxicity can be harmful even in small intensity upon long-term consumption, owing to its capability to accumulate in human and animal bodies. Although copper has significant role in proper functioning of the organism, its high intake can cause health problems and lead to damage of liver and kidneys (Bendeddouche et al. 2014).

Currently, copper is added as copper sulphate to pre-mixes of blends for broiler chicken due to its anti-bacterial properties and to promote the effect of growth (Pesti and Bakalii 1996). Owing to the fact that copper has anti-bacterial properties and it can become an alternative to antibiotics, growing interest has been observed in this element used in production of poultry. Different sources and forms of copper have also diverse bio-availability and influence on animals. Du et al. (1996) claim that the most assimilable copper is organic one and nanoparticles of copper. However, still the most widely used form of copper is copper sulphate due to its lower price and easy access.

The aim of this experiment was to determine the effect of copper nanoparticles and copper sulfate administered *in ovo* on the content of copper in breast muscle, liver and spleen of broiler chickens.

MATERIAL AND METHODS

In ovo injection and incubation of hatching eggs

300 hatching eggs of Hubbard Flex broiler chickens (average weight 62.25 ±2.2 g) were the experimental material in the first stage of the study. The eggs were stored for 4 days at a temperature of 12°C and humidity of 73%. The eggs were weighed and divided into three experimental groups: C, NanoCu, $CuSO_4$, 100 eggs each.

Experimental solutions were administered by *in ovo* injection to the air cell of egg using a 0.3-mL sterile syringe with a needle in the following doses for particular groups: NanoCu (colloid of copper nanoparticles, concentration 50 ppm) and $CuSO_4$ (copper sulfate colloid, concentration 50 ppm). Holes after injection were sterile-tightened and the eggs were placed in an incubator and hatched under standard conditions (temperature 37.8°C, humidity 60%, eggs rotation per hour by 90° angle for 18 days). The eggs were hatched in an incubator by a German company Heka equipped in temperature, air humidity and egg rotation controlling module. During incubation, the eggs were two-fold light-exposed on 6^{th} and 18^{th} day, and weighed in order to determine egg weight loss. On 19th day of incubation, the eggs were transferred to a hatching compartment with a temperature of 37.0–37.5°C and relative humidity of 75–80%. After hatching, one-day chicks were evaluated and healthy chicks with healed navels were selected for further rearing experiment.

Rearing, housing conditions and feeding

Further stage of the study included 150 Hubbard Flex broiler chickens (50 birds in each group), that were kept on litter until 42 days of age under standard animal husbandry conditions, in a room without the access of daylight. One-day chicks after weighing and tagging with individual tags were divided into three groups (C – control, NanoCu, $CuSO_4$), in two replications, 25 birds each. Stock density in a poultry house was 11 birds per 1 m^2. Immediately after introduction to the poultry house, the chickens from all groups were vaccinated against Marek's disease, infectious bronchitis and coccidiosis.

A three-stage feeding program was applied during rearing: starter (crumb), grower and finisher (granulate) – Table 1; the birds were fed *ad libitum*. Body weight of the birds (1st, 14th, 35th and 42th), mortality rate and feed intake were controlled throughout the rearing period.

TABLE 1. Feed mixture composition and nutritional value according to producer's

Specification	Starter (1–14 days)	Grower (15–35 days)	Finisher (36–42 days)
Diet component [%]			
Corn	10.00	11.40	10.00
Wheat	53.00	55.00	60.80
Soybean meal	30.60	27.40	21.60
Feeding limestone	1.19	1.20	0.97
Sodium bicarbonate	0.20	0.14	0.16
NaCl	0.24	0.28	0.26
Stimulant	0.01	0.01	0.01
Dicalcium phosphate	1.18	0.78	0.64
Soybean oil	2.10	2.40	4.40
Methionine 84% calcium salt	0.48	0.42	0.28
Lysine	0.36	0.34	0.28
Threonine	0.14	0.13	0.10
Premix C196 PX05802 0.5%	0.50	0.50	0.50
Nutritional value			
ME [kcal]	2 990.20	3 047.19	3 217.10
Fat [%]	3.67	4.00	5.92
Protein [%]	21.99	20.78	18.51
Fiber [%]	3.60	2.55	2.41
Ash [%]	5.83	5.35	4.67
Lysine [%]	1.38	1.28	0.97
Methionine + cystine [%]	1.08	1.01	0.76
Available phosphorus [%]	0.45	0.38	0.35

Provided per kilogram of diet: STARTER: vitamin A 11.00 K UL; organic phosphorus 0.59%; calcium 0.98%; phosphorus available 0.45%; calcium chloride 0.24%; sodium 0.15%; chlorine 0.27%; potassium 0.90%; magnesium 0.17%; manganese 142.32 mg; copper 31.59 mg; selenium 0.41 mg; iron 191.51 mg; sulfur 0.34%; zinc 116.80 mg; lysine 1.36%; methionine 0.31%; GROWER: vitamin A 11.00 K UL; organic phosphorus 0.51%; calcium 0.87%; phosphorus available 0.38%; calcium chloride 0.28%; sodium 0.15%; chlorine 0.29%; potassium 0.85%; magnesium 0.16%; manganese 141.84 mg; copper 30.82 mg; selenium 0.41 mg; iron 174.55 mg; sulfur 0.32%; zinc 115.03 mg; lysine 1.26%; methionine 0.30%; FINISHER: vitamin A 11.00 K UL; vitamin D3 3.00 K UL; vitamin E; organic phosphorus 0.73%; calcium 0.35%; calcium chloride 0.26%; sodium 0.15%; chlorine 0.27%; potassium 0.74%; magnesium 0.15%; manganese 140.80 mg; copper 29.92 mg; selenium 0.40 mg; iron 159.92 mg; sulfur 0.28%; zinc 113.14 mg; lysine 1.06%; methionine 0.27%.

Slaughter and collection of material for analyses

In the 6th week of rearing, 12 cocks and 12 hens, with body weights similar to the average body weight in a group, were selected from each group. After 12-hour fasting, the chickens were transported to a poultry slaughter house. After 24 h of air-chilling of the carcasses at 4°C, slaughter traits were analyzed and contents of breast muscles, leg muscles, adipose fat and giblets in the carcass were calculated. For determinations of copper content, the right part of breast muscle, liver and spleen were collected from each bird.

Determination of copper content in breast muscles, liver and spleen

Samples to be analyzed were subjected to microwave mineralization in a Milestone 1200 mineralizer equipped in high-pressure mineralization vessels. The size of the weighed portion depended on the character of the samples and ranged from ca. 0.5 to 1.0 g. The samples were mineralized with 7 mL of concentrated (69%) nitric acid V of spectral purity and 1.1 mL of concentrated (30%) hydrogen peroxide also of spectral purity. The optimized controlling programme of the mineralization process was presented in Table 2.

TABLE 2. Optimized controlling programme of the mineralization process

Stage	Time [min]	Power [W]
1	1	250
2	1	0
3	5	250
4	5	400
5	5	650

The content of copper was determined with the method of atomic emission spectrometry with inductively-coupled plasma (ICP-AES) using an iCAP 6500 spectrometer by Thermo Scientific company. Optimized parameters of spectrometer work were presented in Table 3. The spectrometer was calibrated with multi-element standard solutions prepared from an ICP Multi element standard solution IV CertiPUR by Merck company. A few analytical lines (presented in Table 3) were collected for copper determinations. The final result of the analysis represented the arithmetic mean of partial results obtained for selected analytical lines devoid of interference. The quality of study results was confirmed with the use of reference material, i.e. lyophilized chicken meat NCS ZC73016 with a certified content of copper.

TABLE 3. Optimized parameters of spectrometer work

Parameters	Conditions of iCAP 6500 spectrometer work
Power generator [W]	1 150
Atomizer	type Meinhard
Mist chamber	cyclone
Plasma gas [l/min]	12
Supporting gas [l/min]	0.5
Gas flow rate in atomizer [l/min]	0.5
Length of copper line [nm]	324.7
	327.3
	224.7
	219.9

Statistical analysis

The data obtained were analyzed statistically using a multi-factor analysis of variance (least squares) using SPSS 21.0 software (SPSS, Chicago, IL, USA). Only significant interactions between factors ($P \leq 0.01$ or $P \leq 0.05$) were considered in the study. The level of significance was determined after performing preliminary statistical analyses

RESULTS AND DISCUSSION

Akan et al. (2010), who determined concentration of heavy metals (Cu, Zn, Co, Mn, Mg, Fe, Cr, Cd, As, Ni and Pb) in internal organs and meat of selected species of farm animals, conclude that the highest concentration of these elements is in liver and kidneys, whereas the lowest in meat. It also confirms (Tekin-Özan 2008) that metals accumulate in the biggest amount in tissues and soft organs. Similar effect were confirmed in our research (Table 4) in which content of copper was the highest in liver and next in spleen, whereas the lowest in breast muscle. Niedziółka et al. (2007) in their researches aiming to determine the rate of selected elements in meat, liver of kid and ram fed full portions of blend with 10% of linseed observed that liver was the organ of concentration of copper, zinc and manganese. Although the amount of copper in muscular tissue of analysed animals was small, the authors concluded statistically vital differences ($P < 0.01$) (kid 0.08 mg/100 g; ram 0.07 mg/100 g).

The *in ovo* injection of copper colloids applied in the study caused a significant ($P < 0.05$) decrease in copper content in breast muscles of the experimental chickens only in male species NanoCu (0.31 mg/100 g) and CuSO4 (0.30 mg/100 g) compared to control group (0.39 mg/100 g). The concentration of copper in muscular tissue in female species was on the same level and no significant differences were observed.

TABLE 4. Content of copper in breast muscle, liver and spleen of broiler chickens (mg/100 g product)

Sex	Group	Breast muscle	Liver	Spleen
♂♂	C	0.39 ± 0.04^{a}	3.20 ± 0.46^{Bb}	0.65 ±0.07
	NanoCu	0.31 ± 0.03^{b}	4.34 ± 0.37^{A}	0.75 ±0.08
	CuSO4	0.30 ± 0.03^{b}	4.14 ± 0.46^{a}	0.68 ±0.07
♀♀	C	0.27 ±0.03	4.12 ±0.45	0.64 ±0.07
	NanoCu	0.26 ±0.03	3.77 ±0.41	0.73 ±0.08
	CuSO4	0.26 ±0.03	3.98 ±0.44	0.72 ±0.08

^{A,B} Mean values within a sex group differ significantly at $P < 0.01$;
^{a,b} mean values within a sex group differ significantly at $P < 0.05$.

Didanto and Sarkar (1997) claim that absorbing of copper occurs mostly in stomach and small intestine of most of animal species. Thornburg (2000) in his research on dogs demonstrated that absorption of copper occured mostly in the upper part of the small intestine, and that liver was the main organ of copper capture. After absorption in intestine and penetration to blood serum, copper ions bind with albumins. Within 2 h, they are inbuilt to liver cells. Herein copper is either stored (in liver liposome) or is bound with apoceruloplasmin forming ceruloplasmin which then is secreted to blood plasma. Ceruloplasmin is the main factor of copper transport from liver to other tissues and acts as a donor of this metal in production of copper-dependant enzyme (Kochanowska et al. 2008).

Our research conducted on three types of tissues proves that liver has the biggest tendency to accumulate copper. The level of copper in birds liver in research conducted after the injection of colloids both with nanoparticles of copper and with copper sulphate had a significant effect on increasing of this element content in both experimental groups of male species NanoCu (4.34 mg/100 g) and $CuSO_4$ (4.14 mg/100 g) compared to control group (3.20 mg/100 g). Accumulation of copper in liver of females has had a reverse tendency. In spite of lack of significant differences, liver of female group of experimented animals characterizes itself by smaller content of copper compared to females in control group.

Doudi and Setorki (2014) took liver and lungs to histological observation aiming to evaluate toxicity of nanoparticles of copper in vivo conditions. The histology of the hepatic tissues showed vasculature in central veins and portal triad vessels in all three treatment groups. Histology of lungs showed air sac wall thickening and increased fibrous tissue in all three groups with nano-copper. The results of research conducted by Chen et al. (2006) demonstrated that nanoparticles of copper cause severe toxicological effects and serious damage of kidneys, liver and spleen in experimental group of mice.

Content of copper in spleen of birds in our research did not differ between the groups of two sexes of birds. Marcinkowska and Dobicki (2014) confirmed higher capability to accumulate copper in liver and spleen than in gonads, gills, muscles and gastrointestinal track of perches from Barycz river, what is beneficial due to these fish use for consumption purposes. Nanoparticles of copper, as well as other nanoparticles of metals, get to the environment and human body in different ways, i.e. through sewerage or leak during transportation. However, research concerning activeness of nanoparticles of copper administrated subcutaneously showed that operating of nanoparticles in organisms depends mostly on applied dosage (Glushchenko et al. 1989). Lei et al. 2008 in histological image of liver and kidneys of rats treated with nanoparticles of copper on different level (50, 100 and 200 mg/kg daily through 5 days) showed that the effects caused by nanoparticles of copper in dosage of 100 or 50 mg/kg daily were slighter than when bigger dosage was applied. Sizova et al. (2012) underline that nanoparticles of copper are located in organs and tissues of the whole organism causing particular structural changes. According to the research, increase of nanoparticles of copper in organism up to the verge of toxicity (maximum dosage tolerable by organism) will cause necrosis of tissues and dystrophy.

CONCLUSIONS

Results of this experiment confirm previous scientific reports which demonstrate that the greatest accumulation of copper is observed in soft organs like liver or spleen. Moreover, the research conducted showed that male species accumulate copper in liver better. The differences which appeared during research between groups show that different forms of copper have different bioavailability, assimilability and ability to accumulate in organism. Simultaneously, it can be claimed that application of copper sulphate or nanoparticles of copper at the stage of developing chicken embryo do not pose threats of extensive accumulating of this element in organs and muscles of birds in the age at slaughter. As a result, meat and internal organs do not pose a threat to consumers' health.

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Streszczenie: *Wpływ nanocząstek miedzi i siarczanu miedzi podawanych in ovo na zawartość miedzi w mięśniu piersiowym, wątrobie i śledzionie kurcząt brojlerów*. Materiał doświadczalny początkowo stanowiło 300 jaj wylęgowych kurcząt Hubbard Flex. Jaja podzielono na trzy grupy: kontrola, NanoCu i $CuSO_4$, z czego jaja z grupy NanoCu i $CuSO_4$ poddano zabiegowi iniekcji *in ovo* do komory powietrznej. Eksperymentalne roztwory podano poprzez wstrzyknięcie *in ovo* przy użyciu sterylnej igły i strzykawki 0,3 ml kolejno do grup: NanoCu (koloid nanocząstek miedzi, stężenie 50 ppm), $CuSO_4$ (koloid siarczanu miedzi, stężenie 50 ppm). Jaja inkubowano w standardowych warunkach. Po wykluciu z każdej grupy wybrano po 50 piskląt do odchowu trwającego 42 dni. Ptaki żywiono standardowymi mieszankami pełnoporcjowymi dla brojlerów. W ostatnim 42. dniu odchowu z każdej grupy wybrano po 12 samic i 12 samców, od których pobrano prawą część mięśnia piersiowego, wątrobę i śledzionę celem określenia zawartości miedzi. Wyniki doświadczenia potwierdzają wcześniejsze doniesienia naukowe, że największą kumulację miedzi obserwuje się w narządach miękkich, takich jak wątroba czy śledziona.

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