Annals of Warsaw University of Life Sciences – SGGW
Animal Science No 52, 2013: 141–149
(Ann. Warsaw Univ. of Life Sci. – SGGW, Anim. Sci. 52, 2013)

Effect of nanoparticles of copper and copper sulfate administered *in ovo* on hematological and biochemical blood markers of broiler chickens

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Abstract: *Effect of nanoparticles of copper and copper sulfate administered in ovo on hematological and biochemical blood markers of broiler chickens.* At the first stage of the study the experimental material included 300 clutching eggs of Hubbard Flex chickens. The eggs were divided into three groups: control (without injection *in ovo*), Nano50 (*in ovo* injection of colloidal copper nanoparticles) and Nano$CuSO_4$ (*in ovo* injection of colloidal nanoparticles of copper sulphate). Experimental solutions were administered by *in ovo* injection using a sterile needle 0.3 mm as follows: Nano50 group – colloid of copper nanoparticles (concentration: 50 ppm), and Nano$CuSO_4$ group – colloid of copper sulfate (concentration: 50 ppm), to the air cell of the egg. The eggs were incubated under standard conditions. After hatching, 50 chicks were selected from each group for 42-day rearing. Birds were fed standard complete feed mixtures for broilers. On the last day of rearing (day 42), 12 females and 12 males were selected from each group and their blood was sampled for assays of hematological and biochemical markers. Hematological analyses included determinations of: WBC, RBC, Hb, heterophils, lymphocytes, monocytes, eosinophils and basophils, whereas biochemical analyses included assays of the following markers in blood serum: glucose, cholesterol, triglycerides, HDL-cholesterol, urea, calcium, magnesium, phosphorus, iron, ASPAT, and ALAT. The use of copper nanoparticles evoked an increase in blood levels of RBC, HGB, HTC, heterophils, monocytes and basophils. In addition, in blood serum in contributed to reduced concentrations of glucose and cholesterol and increased levels of selected microelements: calcium, phosphorus and iron.

Key words: *in ovo*, nanoparticles, colloid, hematological and biochemical markers in chicken blood

INTRODUCTION

Copper is an element commonly occurring in organisms of plants and animals. As a microelement, it is a constituent of active centers of many enzymes being of key significance to metabolic processes. In addition, it is a component and activator of enzymes in many redox reactions, however its major roles are seen in the synthesis of red blood cells and assistance in connective tissue formation. Also, this element plays an important role in iron metabolism – it facilitates its absorption and binding to transferrin, which transfers iron to erythrocytes, where hemoglobin is being synthesized. Copper present in ceruloplasmin (serum protein) is one of the most active forms of this element in organisms and in this form it regulates iron metabolism and

transport (Witkiewicz 2008). By activating the enzyme indispensable of erythrocytes synthesis, it influences the proper functioning of the erythropoietic system. Deficiency of copper and iron leads to anemia and low utilization of vitamin C. Significant is also its impact on the regeneration of connective tissue by, e.g., synthesis of collagen and elastin and on the development of the nervous system by synthesis of dopamine. Furthermore, copper and zinc prevent damages induced by free oxygen radicals. This element plays also a significant role in the regulation of glucose and cholesterol metabolism. It is a constituent and an activator of a numerous group of enzymes, particularly of oxidases: cytochrome, lysyl, ascorbic, ketocholic superoxide dismutase and tyrosinase (Makarski and Zadura 2006). Copper deficiency induces osteoporosis, reduced body immunity, increases blood level of cholesterol and anemia. In chickens it mainly contributes to: growth retardation, disorders of the nervous and cardiovascular systems, anemia, disorders in plumage pigmentation, disorders in ossification, and myocardial fibrosis. When copper deficiency is also a reduced production of eggs, but the larger mass. Moreover, the eggs are often without the shell (in the membrane of the egg) or with a thin shell, or distorted. Deficiency revealed disturbances in reproduction and development of sperm and high mortality of embryos during hatching. In addition, results in poor pigmentation colorful feathers feathered breeds, slow growth, reducing body weight, anemia and ultimately death. Symptoms of copper deficiency include poor production results in dairy cattle, loss of hair color and hair loss as well as reproduction disorders. Copper deficit deteriorates keratin synthesis and, as a result, causes more frequent incidence of lameness and increased incidence of mastitis. Poultry demand for copper is not that big, but necessary for the proper development of the organism. In poultry, is from 5 to 25 mg in 1 kg of the mixture (Smulikowska 1996), depending on the species, age and the production direction. Why or copper in 1924 was included in the essential micronutrients. Furthermore, it is one of two major transition metals present in the body.

Copper is an important microelement, indispensible for vital processes and for the proper development of living organisms as it plays significant metabolic functions. For instance, it facilitates iron absorption from the gut, takes part in hemoglobin synthesis and therefore has a great impact on red blood cells production (erythropoiesis), it is also an indispensable element of many enzymes (Brzozowski 2007). Both its deficiency and excess may evoke adverse and toxic effects. However, information is lacking on the boundary between its necessary and toxic concentrations in a body. It is common knowledge that intracellular copper occurs mainly in mitochondria and in the nucleus. It is capable of establishing links with nucleic acids wherein it may induce permanent structural changes. It forms links especially easily with various sulfur-containing proteins, particularly with low-molecular metallothionein.

Nanotechnology – which is one of the most intensively developing sciences – has entered into many research disciplines including: chemistry, physics, bioengineering and biomedicine.

Nanotechnological processes have enabled structural modifications of many simple and complex substances, owing to which they can be transformed into submicroscopic objects. What is more, it has been quite recently discovered that the submicroscopic fragments of the matter are characterized by exceptional biochemical traits. Examples of these substances include nanoparticles constituted by atoms of silver, chromium or copper. Nanoparticles are microscopic molecules the size of which is measured in nanometers (nm). They are defined as particles sized less than 100 nm. A nanometer equals one millionth of a millimeter or the width of three or four atoms. By comparison, a human hair is ca. 10–50 nm in width, whereas red blood cells are 2–6 nm and DNA is 2–12 nm in diameter.

The objective of this study was to determine the effect of copper and copper sulfate nanoparticles injected *in ovo* on hematological and biochemical blood markers of broiler chickens.

MATERIAL AND METHODS

Characterization of copper nanoparticles

The shape and size of the copper nanoparticles were inspected (Fig. 1) using a JEM-1220 (JEOL, Tokyo, Japan) transmission electron microscope (TEM) at 80 KeV, with a Morada eleven-megapixel camera (Olympus Soft Imaging Solutions, Münster, Germany). Samples for the TEM were prepared by placing droplets of hydrocolloids onto Formvar-coated copper grids (Agar Scientific, Stansted, UK). Immediately after drying

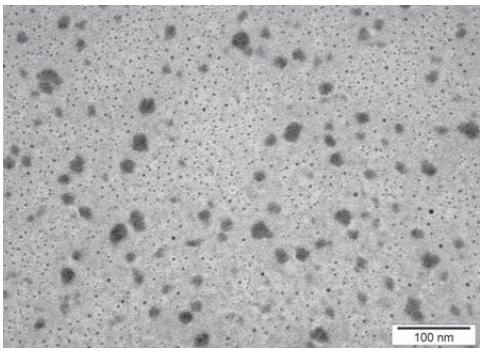

FIGURE 1. Copper nanoparticles visible under electron microscope

of the droplets in dry air, the grids were inserted into the TEM. The test was performed in triplicate. The zeta potential in water was measured using a Zetasizer Nano ZS model ZEN3500 (Malvern Instruments, Malvern, UK).

In ovo injection and incubation conditions of clutching eggs

At the first stage of the study, the experimental material included 300 clutching eggs of Hubbard Flex chickens (average weight 62.25 ±2.2g), that were stored for 4 days at a temperature of 12°C and humidity of 73%. The eggs were weighed and divided into three groups: C (control), Nano$CuSO_4$, and Nano50 (each of 100 eggs). Experimental solutions were administered by *in ovo* injection using a sterile needle 0.3 ml as follows: Nano50 group – colloid of copper nanoparticles (concentration: 50 ppm), and Nano$CuSO_4$ group – colloid of copper sulfate (concentration: 50 ppm), to the air cell of the egg. Injection orifices were tight-sealed and eggs were fixed in an incubator under standard conditions (temperature 37.8°C, humidity 60%, egg revolution by 90° once a day

for 18 days). The eggs were incubated in a hatching apparatus by Heka company equipped in a temperature, air humidity and egg rotation control module. During incubation, the eggs were light-exposed twice (in day 6 and 18 of incubation) and weighed in order to determine egg weight loss. On day 19 of incubation, the eggs were transferred to a hatcher with a temperature of 37.0–37.5°C and relative air humidity of 75–80%. After hatching, one-day chicks were evaluated and healthy birds with healed navels were selected for further rearing.

Rearing, housing conditions and feeding

A further stage of the study included 150 Hubbard Flex chickens (50 birds from each group) that were kept on litter till 42 days of age under typical zoohygienic conditions, in a room without daylight. One-day chickens after weighing and tagging were divided into three groups (control – C, Nano50, and Nano$CuSO_4$) in two replications, 25 birds each. Stock density in a henhouse reached 11 birds per 1 m^2. Immediately after moving to a production hall, the chickens from all groups were vaccinated against Marek's disease, infectious bronchitis and coccidiosis.

The mean air temperature in the room accounted for 24°C, and under radiators – for 34.5°C. The following parameters of the microclimate were measured since the 1st week to the end of rearing: humidity, temperature and contents of toxic gases: ammonia, hydrogen sulfide and carbon dioxide. A three-stage feeding program was applied in the rearing period: starter (crumb), grower and finisher (granulate) – Table 1. The birds were fed *ad libitum*. Body weight of the birds (on day: 1, 14, 35, 42 day), their mortality and feed intake were monitored in the rearing period.

Determinations of hematological and biochemical blood markers

In week 6 of rearing, 12 hens and 12 cocks with body weight similar to the average body weight in a group were selected from each group for slaughter. Blood was sampled from their brachial vein to 3-ml-heparinized test tubes. Hematological analyses were conducted in full blood for: WBC (white blood cells), RBC (red blood cells), Hb (hemoglobin), HCT (hematocrit), heterophils, lymphocytes, monocytes, eosinophils, and basophils. The following biochemical markers were assayed in blood serum (30 min after sampling 3 ml of blood were centrifuged at 3,000 rpm for 10 min): glucose, cholesterol, triglycerides, HDL-cholesterol, urea, calcium, magnesium, phosphorus, iron, ASPAT, and ALAT using a CORMAY kit.

Analytical results were subjected to a statistical analysis by computing mean values and standard deviation using the analysis of variance calculated with the least squares method in a statistical software SPSS 19.0 PL (SPSS Inc., Chicago, II, USA).

RESULTS AND DISCUSSION

Right after mercury, cadmium and copper represent the most toxic heavy metals (Dojlido 1995). Even their minute quantities affect the course of biological processes (Swędrzyńska and Sawicka 2010). Results of assays of hematological blood

TABLE 1. Composition and nutritive value of feed mixtures

Specification	Starter (1–14 day)	Grower (15–35 day)	Finisher (36–42 day)
Content (%)			
Corn	10.0	11.4	10.0
Wheat	53.0	55.0	59.6
Soybean meal	30.6	27.4	23.2
Feeding chalk	1.19	1.20	1.11
Acidic sodium carbonate	0.20	0.14	0.14
NaCl	0.24	0.28	0.28
Stimulant	0.01	0.01	0.01
FOSF 2-Ca	1.18	0.78	0.70
Soybean oil	2.10	2.40	3.60
Methionine	0.48	0.42	0.36
Lysine	0.36	0.34	0.36
Threonine	0.14	0.13	0.14
Premix	0.50	0.50	0.50
Nutritive value			
Metabolizable energy (ME) (MJ/kg)	12.52	12.76	13.20
Fat (%)	3.67	4.00	5.14
CP (%)	21.99	20.78	19.26
Methionine (%)	0.70	0.63	0.57
Methionine + Cysteine (%)	1.08	1.01	0.92
Lysine (%)	1.38	1.28	1.19
Ash (%)	5.83	5.35	4.96

markers are presented in Table 2. The *in ovo* injection of a copper sulfate colloid increased counts of WBC, lymphocytes and eosinophils compared to the other groups. In turn, the Nano50 group was characterized by a decreased number of leucocytes compared to the remaining groups. A similar effect was observed by Dmoch and Polonis (2007), who in their study with turkeys were adding copper chelate with lysine to drinking water for birds.

During the experiment, significant ($P \leq 0.05$) changes were determined in the number of erythrocytes (RBC) in the peripheral blood of the birds exposed to copper. An increased percentage of RBC was noted in the group Nano50. The copper-exposed chickens were characterized by an increased frequency of changed erythrocytes in the peripheral blood that were effectively compensated for by the increasing number of juvenile erythrocytes in blood, owing to which the number of red blood cells remained unchanged (C, Nano$CuSO_4$) or even increased (Nano50). Analyses showed also increased concentration of hemoglobin

TABLE 2. Hematological blood markers of chickens (♀ + ♂)

Specification	Group			
	C	Nano50	$NanoCuSO_4$	SE
WBC – white blood cells (g/l)	13.77	12.00	14.52	1.62
RBC – red blood cells (t/l)	2.84^{b}	3.44^{a}	2.82^{b}	0.12
Hb – hemoglobin (g/l)	134.66^{b}	154.75^{a}	148.0^{b}	7.74
HCT – hematocrit (l/l)	0.36	0.43	0.40	0.02
Heterophils (%)	6.33^{B}	13.75^{A}	7.75^{B}	2.32
Lymphocytes (%)	84.00	75.25	86.50	4.90
Monocytes (%)	7.33^{A}	8.00^{A}	2.00^{B}	2.12
Eosinophils (%)	1.00	1.50	2.50	1.09
Basophils (%)	1.33	1.50	1.25	0.37

a, b – statistically significant differences at $P \leq 0.05$, A,B – statistically significant differences at $P \leq 0.01$. Conversion factor: g/dl×10 = g/l, g/l×0.1 = g/dl, g/dl×0.6206 = mmol, mmol×1.611 = g/dl; l/l×100 = %, %×0.01 = l/l; 10^9/l = thou./mm^3, thou./mm^3×1 = 10^9/l, 10^9/l = G/l, thou./mm^3 = 10^3/mm^3 = = 10^3/μl; t/l = 10^{-15}; Tl = 10^{12}; p/g = 10^{-12}g.

which allowed the birds to keep oxygen transport at an appropriate level. This group was additionally characterized by increased numbers of heterophils, monocytes and basophils.

Changes in erythropoietic parameters in the chickens from experimental groups referred mainly to an increased concentration of hemoglobin in peripheral blood, which was usually accompanied by an increased number of RBC (Table 2). The increased level of hemoglobin could be due to its continued synthesis by erythrocytes already circulating in the peripheral blood. Speckner et al. (1989) emphasized the capability of mature erythrocytes of fish for hemoglobin synthesis that may continue even for some time after cells release from the erythropoietic tissue. It is also likely that the increased level of hemoglobin in the group Nano50 could be linked with the homeopoetic function of copper. This element was shown to directly stimulate erythrocytes synthesis, as it determines iron absorption into the body and its incorporation to hemoglobin (Fox 2003, Mullally et al. 2004). Results reported by Dmoch and Polonis (2007), demonstrated a reducing tendency in the levels of hematocrit and hemoglobin after the application of copper chelate.

The analysis of other studies on the impact of metals on erythropoietic parameters of fish demonstrated a decreasing tendency in the number of erythrocytes, hematocrit value and hemoglobin concentration in blood. Reduced values of these parameters upon the influence of cadmium were reported by Vincent et al. (1996) and upon the influence of copper – by Ates et al. (2008). Decreased values of these parameters were consequently leading to anemia development in fish (Ruperelia et al. 1990).

Furthermore, analyses demonstrated a reduced percentage of monocytes in the $NanoCuSO_4$ group, which was accompanied by a suppressed metabolic activity of these cells. Reduction was also observed

in the phagocytic activity in the group of chickens characterized by WBC number increase. This indicates that $NanoCuSO_4$ could contribute to accelerated damage of phagocytes and to inhibited metabolic activity of these cells.

Results of analyses of biochemical blood markers are provided in Table 3. The *in ovo* injection of copper nanoparticles resulted in decreased blood levels of glucose and cholesterol. The reduced concentration of glucose confirms findings of Makarski and Zadura (2006) who were administering copper chelate to turkeys with drinking water. The applied element had some effect on lipid and cholesterol metabolism as well as on properties of myelin sheaths of nervous fibers. Turnlund et al. (1988) and Bakalii et al. (1995) claim that copper addition contributes to a decreased level of triglycerides and reduced cholesterol synthesis in blood plasma and tissues of animals. In our experiment, the lowest level of triglycerides was determined in the $NanoCuSO_4$ group, whereas the highest concentration of HDL was assayed in the control group. The administration of copper nanoparticles caused an increase in the blood level of uric acid. A similar effect on increased levels of glucose and uric acid was observed by Dmoch and Polonis (2007), applying a copper-lysine chelate to chickens. The addition of copper contributed also to increased serum levels of calcium, phosphorus and iron compared to the remaining groups. The increased levels of these elements may be reflected in production effectiveness as they contribute to improved bone mineralization. Upon the use of Cu-Lys chelate Dmoch and Polonis (2007) noted an increase in the level of calcium and to, a lesser extent, in the level of phosphorus. In the case of hepatic enzymes, a reduced level of ALAT was observed in the groups subjected to *in ovo* injection with copper and copper sulfate colloids. An opposite effect was reported for ASPAT concentration which in the $NanoCuSO_4$

TABLE 3. Biochemical blood markers of chickens (♀ + ♂)

Specification	Group			
	C	Nano50	$NanoCuSO_4$	SE
Glucose (mmol/l)	13.29	12.85	12.47	1.03
Cholesterol (mmol/l)	3.82	3.43	3.50	0.39
Triglycerides (mmol/l)	0.59	0.62	0.51	0.55
HDL (mmol/l)	2.39	1.95	2.11	0.44
Urea (mmol/l)	2.70	3.10	2.80	0.12
Calcium (mmol/l)	2.27	2.72	2.29	0.33
Magnesium (mmol/l)	0.80	0.85	0.98	0.14
Phosphorus (mmol/l)	1.93	2.27	1.87	0.23
Iron (mmol/l)	24.40	30.50	20.50	3.17
ALAT (mmol/l)	16.00	12.00	12.00	4.96
ASPAT (mmol/l)	368.50^{B}	498.50^{B}	911.00^{A}	100.78

A,B – statistically significant differences at $P \leq 0.01$.

group was almost threefold higher than in the control group. Such a result may be indicative of liver lesions, however histopathological examination of liver excluded the pathological effect of copper on this organ.

CONCLUSION

Results obtained in this study demonstrate that the erythropoietic system of chickens is susceptible to effects of metals, including copper (nanoparticles), which shows that changes in blood may be used as an indicator of the impact of toxic substances on chickens. Simultaneously, the analysis of changes in the peripheral blood enables more precise evaluation and interpretation of the effect a given factor has on bird body.

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Streszczenie: *Wpływ nanocząstek miedzi i siarczanu miedzi podawanych in ovo na wskaźniki hematologiczne i biochemiczne krwi kurcząt brojlerów.* Materiał doświadczalny w pierwszym etapie stanowiło 300 jaj lęgowych kurcząt Hubbard Flex. Jaja podzielono na trzy grupy: kontrola, Nano50 i $NanoCuSO_4$, z czego jaja z grupy Nano50 i $NanoCuSO_4$ poddane zostały zabiegowi iniekcji *in ovo*. Eksperymentalne roztwory podano poprzez wstrzyknięcie *in ovo*, przy użyciu sterylnej igły 0,3 mm kolejno do grup: Nano50 (koloid nanocząstek miedzi, stężenie 50 ppm), $NanoCuSO_4$ (koloid siarczanu miedzi, stężenie 50 ppm) do komory powietrznej jaja. 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 krew celem określenia wskaźników hematologicznych i biochemicznych. Wykonano oznaczenia hematologiczne, tj.: WBC, RBC, Hb, heterofile, limfocyty, monocyty, eozynofile, bazofile. Do oznaczenia wskaźników biochemicznych pobrano krew na surowicę, w której oznaczono wskaźniki biochemiczne: glukoza, cholesterol, triglicerydy, HDL-cholesterol, mocznik, wapń, magnez, fosfor, żelazo, ASPAT, ALAT. Zastosowanie nanocząstek miedzi wpłynęło na wzrost poziomu RBC, HGB, HTC, heterofili, monocytów i bazofili. Dodatkowo w surowicy stwierdzono obniżenie stężenia glukozy i cholesterolu przy jednoczesnym wzroście poziomu wybranych mikroelementów: wapnia, fosforu i żelaza.

MS. received in November 2013

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