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Effect of silver nanoparticles on the immune, redox, and lipid status of chicken blood

K. Ognik[1], E. Cholewińska[1], A. Czech[1], K. Kozłowski[2], Ł. Wlazło[3], B. Nowakowicz-Dębek[3], R. Szlązak[4,5], K. Tutaj[5,6]

[1]Department of Biochemistry and Toxicology, Faculty of Biology, Animal Sciences and Bioeconomy, University of Life Sciences in Lublin, Lublin, Poland
[2]Department of Poultry Science, Faculty of Animal Bioengineering, University of Warmia and Mazury, Olsztyn, Poland
[3]Department of Animal Hygiene and Environment, Faculty of Biology and Animal Breeding, University of Life Sciences in Lublin, Lublin, Poland
[4]Department of Metrology and Modelling of Agrophysical Processes, Bohdan Dobrzański Institute of Agrophysics of the Polish Academy of Sciences, Lublin, Poland
[5]Department of Biophysics, Institute of Physics, Faculty of Mathematics, Physics and Computer Science, Maria Curie Skłodowska University, Lublin, Poland
[6]Chair of Soil Science, Environmental Chemistry and Hydrology, Faculty of Biology and Agriculture, University of Rzeszów, Rzeszów, Poland

ABSTRACT: The aim of the study was to analyze how *per os* application of hydrocolloids of silver nanoparticles (22 nm) and lipid-coated silver nanoparticles (5 nm) affect the immune, redox, and lipid status of the blood of broiler chickens. The experiment was conducted on 60 chickens. The first group was the control (Group C). The chickens in Group II received a silver nanoparticle hydrocolloid (Ag-nano) at a dose of 5 mg/kg body weight (BW) per day. The chickens in Group III received a lipid-coated silver nanoparticle hydrocolloid (AgL-nano) at a dose of 5 mg/kg BW per day. Blood for analysis was collected from 24- and 38-day-old chickens and several blood parameters were determined. The increase in phagocytosis and in the metabolic activity of leukocytes observed following *per os* administration of chemically synthesized silver nanoparticles differing in size and in the presence or absence of a lipid coating may indicate a developing inflammatory state in the organism. The decrease in haemoglobin content and antioxidant enzyme activity and the increase in the content of iron, bilirubin, and lipid peroxidation products are indicative of oxidative stress, although in the case of administration of AgL-nano the oxidation effect appears to be greater. Administration of silver nanoparticles to the chickens, particularly 5 nm lipid-coated nanoparticles, probably led to a disturbance in protein catabolism in the organism, which is evidenced by the decrease in the activity of the liver enzymes AST and ALT and the decreased concentration of the main protein metabolism products (creatinine and urea).

Keywords: chickens; nanosilver; immunology; antioxidant

INTRODUCTION

Recent years have seen a return to the use of silver as a microbicidal agent in the form of solutions, suspensions and/or nanoparticles. Owing to their confirmed antimicrobial properties, nanoparticles of noble metals (mainly silver) are currently used in animal production as disinfectants and

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to reduce emissions of ammonia and nitrogen oxides (Xu et al. 2013). A study by Dobrzanski et al. (2010) shows that the use of nanosilver as a microbicidal preparation applied to litter each time it is changed in the broiler house brings positive results (reduction in the number of harmful Salmonella, Escherichia coli, and Streptococcus bacteria and in the total number of mesophilic bacteria). Recently, intensive *in vitro*, *in vivo*, and *in ovo* research on the use of nanosilver and nanogold as prophylactic feed additives for animals has been undertaken as well (Sawosz et al. 2009; Ahmadi and Kurdestani 2010). The rationale for these studies lies in reports indicating a positive, often selective effect of nanosilver on the bacteria of the digestive tract in poultry – mainly the ability to inhibit the development of pathogenic bacteria.

Thus so far only a few studies have been conducted on poultry to evaluate the effect of nanosilver on the immune and redox response and the lipid status of chicken blood (Ahmadi 2012; Pineda et al. 2012; Bhanja et al. 2015). *In vitro* studies on stabilized cell lines and *in vivo* studies on laboratory animals have shown that the spectrum of action of nanoparticles of various metals on the immune or redox system can be quite broad (from stimulation to suppression), depending on the dosage, size of nanoparticles, medium, and the route and duration of application, as well as the production method (Javanovic and Palic 2012; Xu et al. 2013). Hence this is a question that has not been fully researched and much remains to be elucidated.

Therefore it was considered worth analyzing how *per os* application of hydrocolloids of silver nanoparticles (22 nm) and lipid-coated silver nanoparticles (5 nm) affect the immune, redox, and lipid status of the blood of broiler chickens.

MATERIAL AND METHODS

Nanoparticle synthesis. The silver nanoparticle hydrocolloid (Ag-nano) was synthesized according to the method given by Pyatenko et al. (2007). In this method silver ions ($AgNO_3$) were reduced with trisodium citrate at a temperature of about 100°C, which at the same time served as a stabilizer of the nanoparticles produced. Silver nitrate ($AgNO_3$) was purchased from Sigma-Aldrich, St. Louis, USA. The hydrocolloid of lipid-coated silver nanoparticles (AgL-nano) was obtained in two stages. First the nanoparticles were synthesized according to the method described by Oliveira et al. (2005). This method involves reduction of silver ions ($AgNO_3$) by sodium borohydride. Binding of dodecanethiol to the surface of the nanoparticles allows them to be suspended in the organic phase. In order to transfer them to water they were incorporated in the hydrophobic phase of single bilayer liposomes, prepared according to Batzri and Korn (1973). Injecting an ethanolic solution of phospholipid admixed with dodecanethiol-coated nanoparticles into water results in the formation of water-soluble lipid-silver hybrid nanoparticles (AgL-nano). In the second stage, 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) (Sigma-Aldrich) was added to nanoparticles (Ag at a concentration of 2 mg/ml) suspended in anhydrous ethanol. DPPC is a phospholipid also known as L-β,γ-dipalmitoyl-α-lecithin. It is a lipid component of pulmonary surfactant. Next, the nanoparticles with the lipid were injected into water heated to 50°C, constantly mixed on a magnetic stirrer. The final concentration of silver in the hydrocolloids was 0.1 mg/ml. The final concentration of silver in the hydrocolloids was 0.1 mg/ml for AgL-nano and 2 mg/ml for Ag-nano. Ag-nano hydrocolloids at a concentration of 2 mg/ml were diluted to a concentration 0.1 mg/ml. To test the stability of the hydrocolloids, their absorption was measured immediately after synthesis and 24, 48, and 72 h later (Figures 1 and 2). Spectra were measured on a Cary 50 UV-Vis spectrophotometer (Varian Australia Pty Ltd., Mulgrave, Australia). On the basis of photographs taken by a transmission electron microscopes Tecnai G2 T20 X-TWIN (FEI, Hillsboro, USA) and LEO 912AB (Carl Zeiss GmbH, Jena, Germany), the average size of the Ag-nano and AgL-nano silver nanoparticles was estimated at 22 nm (Figure 1) and at about 5 nm (Figure 2), respectively.

Animals. The material for the study consisted of one-day-old ROS 308 chickens (♂) raised until the 6th week of age. The study was carried out with the consent of the Local Ethics Commission (2014). The birds were kept in pens on straw litter and reared in standard hygiene conditions in a building with regulated temperature and humidity. The birds had permanent access to drinking water and received *ad libitum* complete feed mixtures appropriate for the rearing period according to the nutrient requirements of broiler chickens (Smulikowska and Rutkowski 2005). The experiment was carried out on 60 chickens assigned to three experimental

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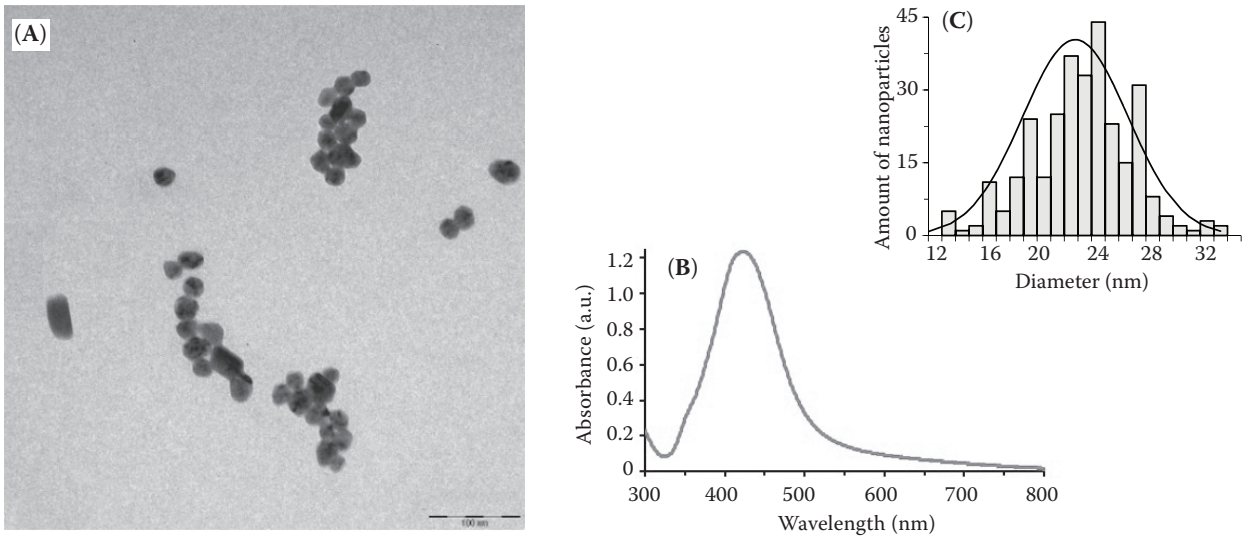

Figure 1. Hydrocolloid of silver nanoparticles (Ag-nano). Transmission electron microscopy (TEM) image (**A**), absorption spectrum (**B**), size distribution (**C**)

groups of 20 each (5 × 4 repetitions). The first group was the control (Group C), which did not receive silver nanoparticles. The chickens from Group C received distilled water via a tube into the crop. The chickens belonging to Group II received an aqueous solution of silver nanoparticles (Ag-nano) at a dose of 5 mg/kg body weight (BW) via a tube into the crop. The chickens in Group III received an aqueous solution of lipid-coated silver nanoparticles (AgL-nano) at a dose of 5 mg/kg BW via a tube into the crop. The silver nanoparticles (Ag-nano and AgL-nano) were administered to the chickens on the first three days of the 2nd, 4th, and 6th week of life, i.e. on days 8–10, 22–24, and 36–38 of age. The final concentration of silver in the hydrocolloids was 0.1 mg/ml for AgL-nano and 2 mg/ml for Ag-nano. Ag-nano hydrocolloids at a concentration of 2 mg/ml were diluted to a concentration 0.1 mg/ml. So that the chicks in the Ag-nano and AgL-nano group would receive a dose of 5 mg/kg BW in the 2nd week of life (on days 8, 9, and 10), every day 20 ml of hydrocolloid was administered in 5 doses of 4 ml each. So that the chicks would receive a dose of 5 mg/kg BW in the 4th week of life (on days 22, 23, and 24), every day 65 ml of hydrocolloid was administered in 5 doses

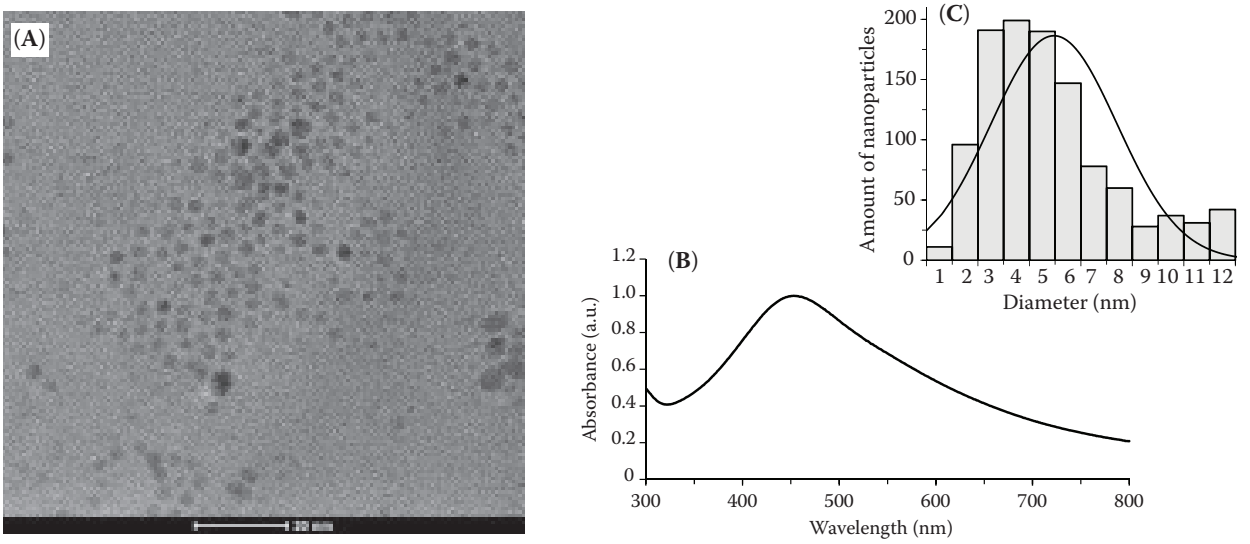

Figure 2. Hydrocolloid of lipid-coated silver nanoparticles (AgL-nano). Transmission electron microscopy (TEM) image (**A**), absorption spectrum (**B**), size distribution (**C**)

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of 13 ml each. So that the chicks would receive a dose of 5 mg/kg BW in the 6th week of life (on days 36, 37, and 38), every day 120 ml of hydrocolloid was administered in 5 doses of 24 ml each.

Laboratory analysis. At the end of the 24th and 38th day of age, blood was collected from the wing vein of 12 birds from each group.

Blood samples were taken after an 8-hour fasting with free access to drinking water. An aqueous solution of heparin sodium salt at 0.01 ml per 1 ml blood was used to stabilize the blood samples. Haematocrit value (Ht), haemoglobin level (Hb), and total white blood cell (WBC) and red blood cell (RBC) counts were determined by standard methods according to Feldman et al. (2000). The immunological analyses involved determination of phagocytic activity of leukocytes against the *Staphylococcus aureus* 209P strain, expressed as the percentage of phagocytic cells (%PC) and the phagocytic index (PI) (Siwicki and Anderson 1993). The respiratory burst activity of the neutrophils was quantified by reduction of nitroblue tetrazolium (NBT) to formazan as a measurement of production of oxygen radicals (Park et al. 1968). Serum lysozyme activity was determined by the turbidimetric method (Siwicki and Anderson 1993).

Kits developed by Cormay (PZ CORMAY S.A., Lomianki, Poland) were used to determine the

Table 1. Haematological and immunological indices in the blood plasma of chicken receiving silver nanoparticles

Parameter	Day of life[1]	Group C[2] (control)	Group II[3] (Ag-nano)	Group III[4] (AgL-nano)	SEM	*P*-value
Ht (l/l)	24 (n = 12)	33.8	33.3	31.8	0.486	0.236
	38 (n = 12)	31.3	29.5	31.0	1.006	0.782
	$\bar{x}$ (n = 24)	32.5	31.4	31.4	0.615	0.696
Hb (g/l)	24 (n = 12)	4.68	4.61	4.32	0.096	0.947
	38 (n = 12)	6.51^{a}	6.46^{a}	5.22^{b}	0.408	0.045
	$\bar{x}$ (n = 24)	5.59^{a}	5.53^{a}	4.77^{b}	0.275	0.037
RBC (10^{12}/l)	24 (n = 12)	1.80	1.75	1.60	0.043	0.104
	38 (n = 12)	1.65	1.66	1.60	0.020	0.399
	$\bar{x}$ (n = 24)	1.72	1.71	1.60	0.025	0.055
WBC (10^{9}/l)	24 (n = 12)	15.59	15.99	16.11	0.165	0.460
	38 (n = 12)	19.33	19.29	19.22	0.175	0.974
	$\bar{x}$ (n = 24)	17.46	17.64	17.66	0.426	0.979
Lysozymes (mg/l)	24 (n = 12)	1.89^{b}	2.14^{a}	2.24^{a}	0.055	0.007
	38 (n = 12)	2.30^{a}	2.09^{ab}	2.01^{b}	0.049	0.015
	$\bar{x}$ (n = 24)	2.09	2.11	2.12	0.036	0.933
PI	24 (n = 12)	5.69^{b}	6.81^{a}	6.93^{a}	0.673	0.0002
	38 (n = 12)	6.53	6.41	6.74	0.106	0.296
	$\bar{x}$ (n = 24)	6.11^{b}	6.61^{a}	6.83^{a}	0.113	0.015
%PC	24 (n = 12)	42.51^{b}	46.88^{a}	47.87^{a}	0.758	0.014
	38 (n = 12)	44.44	43.99	41.36	0.875	0.343
	$\bar{x}$ (n = 24)	43.47	45.44	44.61	0.604	0.640
NBT (%)	24 (n = 12)	20.04^{b}	23.28^{a}	23.63^{a}	0.232	0.045
	38 (n = 12)	20.09	21.16	21.59	0.322	0.143
	$\bar{x}$ (n = 24)	20.06^{b}	22.22^{a}	22.61^{a}	0.197	0.042

Ht = haematocrit, Hb = haemoglobin, RBC = red blood cell, WBC = white blood cell, PI = phagocytic index, %PC = percentage of phagocytic cells, NBT = test reduction of nitroblue tetrazolium

[1]blood taken on days 24 and 38 of life, [2]group receiving distilled water, [3]aqueous solution of silver nanoparticles at a dose of 5 mg/kg body weight, [4]aqueous solution of lipid-coated silver nanoparticles at a dose of 5 mg/kg body weight

[a,b]values in rows marked with different letters differ significantly at $P \leq 0.05$, $P \leq 0.01$

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following biochemical indices in the blood plasma samples: uric acid (UA), urea (UREA), bilirubin (BIL), creatinine (CREAT), cholesterol (CHOL) and its fractions – high-density (HDL) and low-density (LDL), triacylglycerol (TG), and activity of the enzymes alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), and lactate dehydrogenase (LDH). Spectrophotometric assays were also used to test the blood plasma samples for activity of antioxidant enzymes: superoxide dismutase (SOD), by the adrenaline method with a modification of the wavelength to 320 nm to increase the selectivity of transient reaction products (Bartosz 2004), and catalase (CAT), according to Bartosz (2004). Total antioxidant potential (ferric reducing ability of plasma – FRAP) and plasma concentrations of vitamin C and glutathione (GSH + GSSG) were determined according to Bartosz (2004). In addition, the biological material was analyzed for levels of lipid peroxidation products: peroxides (LOOH) according to Gay and Gebicki (2002) and malondialdehyde (MDA) as an end product of tissue lipid oxidation according to Salih et al. (1987). The concentration of phosphorus (P), calcium (Ca), magnesium (Mg), sodium (Na), potassium (K), copper (Cu), zinc (Zn), and iron (Fe) in the blood plasma was determined in the Central Apparatus Laboratory, University of Life Sciences in Lublin, Poland by the flame AAS technique on a UNICAM 939 spectrometer (Cecil Instruments Ltd., Milton Technical Centre, Cambridge, UK).

Statistical analysis. Numerical data were processed by the analysis of variance (ANOVA) and the results were presented as mean values for groups and standard deviations (StatSoft Inc. 2009).

RESULTS

Silver nanoparticles administered *per os*, differing in size and in the presence or absence of a lipid coating, had no effect on the growth performance

Table 2. Activity of antioxidant and liver enzymes in the blood plasma of chicken receiving silver nanoparticles

Parameter	Day of life[1]	Group C[2] (control)	Group II[3] (Ag-nano)	Group III[4] (AgL-nano)	SEM	*P*-value
SOD (U/ml)	24 (n = 12)	35.01^{a}	25.18^{b}	33.45^{a}	1.545	< 0.001
	38 (n = 12)	31.06	31.96	31.60	0.328	0.595
	$\bar{x}$ (n = 24)	33.03^{a}	28.57^{b}	32.52^{ab}	0.767	0.022
CAT (U/ml)	24 (n = 12)	0.881^{a}	0.763^{b}	0.884^{a}	0.024	0.038
	38 (n = 12)	0.887^{a}	0.775^{b}	0.885^{a}	0.023	0.045
	$\bar{x}$ (n = 24)	0.884^{a}	0.769^{b}	0.884^{a}	0.016	0.003
ALP (U/l)	24 (n = 12)	1168.2^{ab}	1384.6^{a}	972.4^{b}	70.71	0.024
	38 (n = 12)	1708.9^{a}	348.9^{b}	437.2^{b}	26.80	0.0002
	$\bar{x}$ (n = 24)	1438.5^{a}	866.7^{b}	704.8^{b}	122.5	0.025
ALT (U/l)	24 (n = 12)	3.03	2.81	3.60	0.185	0.208
	38 (n = 12)	5.67	4.50	5.19	0.796	0.734
	$\bar{x}$ (n = 24)	4.35	3.65	4.39	0.655	0.765
AST (U/l)	24 (n = 12)	152.1^{b}	160.9^{ab}	194.1^{a}	10.75	0.027
	38 (n = 12)	157.6^{a}	100.7^{b}	109.3^{b}	10.21	0.015
	$\bar{x}$ (n = 24)	154.8	130.8	151.7	9.136	0.533
LDH (U/l)	24 (n = 12)	457.5	457.9	463.3	6.127	0.930
	38 (n = 12)	803.7^{a}	431.7^{b}	514.7^{b}	62.41	0.006
	$\bar{x}$ (n = 24)	630.6^{a}	444.8^{b}	489.0^{b}	33.92	0.050

SOD = superoxide dismutase, CAT = catalase, ALP = alkaline phosphatase, ALT = alanine aminotransferase, AST = aspartate aminotransferase, LDH = lactate dehydrogenase

[1]blood taken on days 24 and 38 of life, [2]group receiving distilled water, [3]aqueous solution of silver nanoparticles at a dose of 5 mg/kg body weight, [4]aqueous solution of lipid-coated silver nanoparticles at a dose of 5 mg/kg body weight

[a,b]values in rows marked with different letters differ significantly at $P \leq 0.05$, $P \leq 0.01$

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Table 3. Content of redox status indicators in the blood plasma of chicken receiving silver nanoparticles

Parameter	Day of life[1]	Group C[2] (control)	Group II[3] (Ag-nano)	Group III[4] (AgL-nano)	SEM	*P*-value
FRAP (μmol/l)	24 (*n* = 12)	113.1	108.1	110.7	3.604	0.881
	38 (*n* = 12)	83.86	28.19	39.04	13.36	0.209
	$\bar{x}$ (*n* = 24)	98.48	68.14	74.87	9.925	0.448
GSH + GSSG (μmol/l)	24 (*n* = 12)	0.064	0.068	0.067	0.001	0.551
	38 (*n* = 12)	0.062	0.051	0.057	0.002	0.264
	$\bar{x}$ (*n* = 24)	0.063	0.059	0.062	0.001	0.720
VIT C (mg/l)	24 (*n* = 12)	0.153	0.132	0.092	0.018	0.440
	38 (*n* = 12)	0.119	0.104	0.118	0.017	0.938
	$\bar{x}$ (*n* = 24)	0.135	0.118	0.105	0.012	0.617
BIL (μmol/l)	24 (*n* = 12)	2.87	2.99	3.05	0.089	0.784
	38 (*n* = 12)	5.46	6.41	6.83	0.467	0.591
	$\bar{x}$ (*n* = 24)	4.16	4.70	4.94	0.540	0.643
UA (μmol/l)	24 (*n* = 12)	106.6	94.6	104.0	4.06	0.510
	38 (*n* = 12)	220.0	184.0	197.3	23.36	0.330
	$\bar{x}$ (*n* = 24)	163.3	139.3	150.7	19.71	0.740
UREA (mmol/l)	24 (*n* = 12)	0.071	0.069	0.056	0.005	0.489
	38 (*n* = 12)	1.15[a]	1.15[a]	0.65[b]	0.097	0.018
	$\bar{x}$ (*n* = 24)	0.60	0.61	0.35	0.121	0.655
CREAT (μmol/l)	24 (*n* = 12)	21.89	23.26	28.73	2.162	0.450
	38 (*n* = 12)	27.36[a]	26.01[a]	9.57[b]	3.017	0.001
	$\bar{x}$ (*n* = 24)	24.62	24.63	19.15	1.854	0.403
LOOH (μmol/l)	24 (*n* = 12)	4.12	4.46	4.11	0.128	0.513
	38 (*n* = 12)	1.62[b]	2.81[a]	2.78[a]	0.219	0.008
	$\bar{x}$ (*n* = 24)	2.87 [b]	3.63[a]	3.44[a]	0.253	0.046
MDA (μmol/l)	24 (*n* = 12)	0.346[b]	0.470[a]	0.627[a]	0.046	0.001
	38 (*n* = 12)	0.612[b]	0.699[a]	0.711[a]	0.049	0.050
	$\bar{x}$ (*n* = 24)	0.479[b]	0.584[a]	0.669 [a]	0.042	0.015

FRAP = total antioxidant potential of plasma, GSH + GSSG = glutathione, VIT C = vitamin C, BIL = bilirubin, UA = uric acid, UREA = urea, CREAT = creatinine, LOOH = peroxides, MDA = malondialdehyde

[1]blood taken on days 24 and 38 of life, [2]group receiving distilled water, [3]aqueous solution of silver nanoparticles at a dose of 5 mg/kg body weight, [4]aqueous solution of lipid-coated silver nanoparticles at a dose of 5 mg/kg body weight

[a,b]values in rows marked with different letters differ significantly at $P \leq 0.05$, $P \leq 0.01$

parameters of the chickens (body weight gain in 1–42 days of age was 2.32–2.37 kg/bird, feed conversion in 1–42 days of age was 1.74–1.76 kg/kg weight gain) (Ognik et al. 2016).

Data pertaining to the haematological and immune indicators in the chicken blood are presented in Table 1. Application of the hydrocolloid of 22 nm silver nanoparticles (Ag-nano) was not found to affect haemoglobin content, haematocrit or white and red blood cell counts in the blood of the experimental chickens. Application of lipid-coated 5 nm silver nanoparticles (AgL-nano) caused a significant decrease in blood haemoglobin content in the chickens on day 38 of age. The results of the immunological analyses performed on day 24 of age of the chickens showed an increase in lysozyme activity, PI, %PC, and NBT in the blood of the chickens that received per os nanosilver (Ag-nano) and nanosilver (AgL-nano). On day 38 no influence of the administration of nanosilver increasing these immune system indices was confirmed.

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Table 4. Content of lipid indicators in the blood plasma of chicken receiving silver nanoparticles

Parameter (mmol/l)	Day of life[1]	Group C[2] (control)	Group II[3] (Ag-nano)	Group III[4] (AgL-nano)	SEM	*P*-value
CHOL	24 (n = 12)	2.17[b]	3.54[a]	3.52[a]	0.091	0.0198
	38 (n = 12)	1.83[b]	3.58[a]	2.92[a]	0.452	< 0,001
	$\bar{x}$ (n = 24)	2.00[b]	3.56[a]	3.22[a]	0.237	0.005
HDL	24 (n = 12)	1.66	2.54	2.68	0.086	0.827
	38 (n = 12)	1.31	2.42	1.70	0.057	0.444
	$\bar{x}$ (n = 24)	1.49	2.48	2.19	0.144	0.913
LDL	24 (n = 12)	0.378[b]	0.847[a]	0.680[ab]	0.077	0.510
	38 (n = 12)	0.381[b]	0.906[a]	1.030[a]	0.414	0.001
	$\bar{x}$ (n = 24)	0.380[b]	0.876[a]	0.855[a]	0.211	0.005
TG	24 (n = 12)	0.291	0.337	0.353	0.018	0.431
	38 (n = 12)	0.306	0.559	0.419	0.053	0.149
	$\bar{x}$ (n = 24)	0.298	0.448	0.386	0.030	0.121

CHOL = cholesterol, HDL = high-density cholesterol, LDL = low-density cholesterol, TG = triacylglycerol

[1]blood taken on days 24 and 38 of life, [2]group receiving distilled water, [3]aqueous solution of silver nanoparticles at a dose of 5 mg/kg body weight, [4]aqueous solution of lipid-coated silver nanoparticles at a dose of 5 mg/kg body weight

[a,b]values in rows marked with different letters differ significantly at $P \leq 0.05$, $P \leq 0.01$

The results pertaining to the activity of selected antioxidant enzymes (Table 2) show that administration of Ag-nano significantly reduced the activity of the antioxidant enzymes SOD and CAT, which was most visible on day 24 of age of the chickens. Administration of the lipid-coated 5 nm silver hydrocolloid (AgL-nano) had no effect on SOD and CAT activity.

Administration of Ag-nano and AgL-nano nanosilver to the chickens led to a reduction in the activity of the liver enzymes ALP and LDH, mainly visible on day 38 of age of the chickens. However, the significant decrease in AST activity on day 38 of age of the chickens in groups AG-nano and AgL-nano had no significant effect on differences in the mean values.

The indicators illustrating the intensity of oxidation processes and low-molecular-weight antioxidants are presented in Table 3. Administration of the hydrocolloid of nanosilver (Ag-nano) and lipid-coated nanosilver (AgL-nano) significantly increased the level of LOOH and MDA. In the blood serum of the 38-day-old chickens receiving AgL-nano *per os* a significant decrease in UREA and CREAT was observed.

The results presented in Table 4 showed that administration of Ag-nano and AgL-nano to the chickens significantly increased the content of total and LDL cholesterol.

Administration of nanosilver (Ag-nano and AgL-nano) to the chickens had no effect on the content of most of the macro- and micronutrients tested in the blood. Administration of Ag-nano and AgL-nano was only found to significantly increase the content of iron in the blood plasma of the chickens (Table 5).

DISCUSSION

Both the 22 nm Ag-nano and the 5 nm AgL-nano administered *per os* to chickens stimulated the process of phagocytosis and increased the metabolic activity of leukocytes. Increased phagocytosis is one of the defence mechanisms observed during worsening inflammatory states. Silver nanoparticles administered to mice and rats have been found to induce inflammatory reactions, which were not limited to local changes but included systemic changes as well (Liu et al. 2013). Upon contact of metal nanoparticles with body fluids a structure is formed (mainly of fibrinogen, immunoglobulin, albumin, and complement system proteins) that stimulates the complement system. At a later stage the development of an inflammatory state is observed, during which phagocytic reactions intensify (Javanovic and Palic 2012). Studies conducted *in vitro* or on laboratory animals have also found that metal nanocolloids influence proliferation

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Table 5. Content of minerals in the blood plasma of the chicken receiving silver nanoparticles

Parameter	Day of life[1]	Group C[2] (control)	Group II[3] (Ag-nano)	Group III[4] (AgL-nano)	SEM	*P*-value
P (mmol/l)	24 (n = 12)	1.82	1.81	2.02	0.074	0.489
	38 (n = 12)	3.03	2.70	2.90	0.1323	0.659
	$\bar{x}$ (n = 24)	2.42	2.25	2.46	0.371	0.976
Ca (mmol/l)	24 (n = 12)	3.06	3.58	2.90	0.1689	0.252
	38 (n = 12)	2.36	2.70	3.01	0.162	0.297
	$\bar{x}$ (n = 24)	2.71	3.14	2.95	0.128	0.415
Mg (mmol/l)	24 (n = 12)	0.43	0.37	0.37	0.022	0.453
	38 (n = 12)	0.32	0.29	0.28	0.013	0.387
	$\bar{x}$ (n = 24)	0.37	0.33	0.32	0.016	0.361
Na (mmol/l)	24 (n = 12)	137.2	136.5	135.3	1.30	0.872
	38 (n = 12)	135.3	133.4	137.3	0.889	0.220
	$\bar{x}$ (n = 24)	136.2	134.9	136.3	0.774	0.749
K (mmol/l)	24 (n = 12)	3.66	4.02	3.96	0.072	0.055
	38 (n = 12)	3.75	4.05	4.01	0.067	0.129
	$\bar{x}$ (n = 24)	3.70	4.03	3.98	0.048	0.403
Cu (µmol/l)	24 (n = 12)	2.22	2.33	2.89	0.202	0.401
	38 (n = 12)	5.45	4.11	4.67	0.277	0.134
	$\bar{x}$ (n = 24)	3.83	3.22	3.78	0.321	0.715
Zn (µmol/l)	24 (n = 12)	20.71	20.66	21.49	0.234	0.299
	38 (n = 12)	20.60	20.76	21.44	0.187	0.150
	$\bar{x}$ (n = 24)	20.65	20.71	21.46	0.145	0.068
Fe (µmol/l)	24 (n = 12)	21.28[b]	33.45[a]	38.75[a]	2.10	0.002
	38 (n = 12)	26.01[b]	45.52[a]	39.08[a]	4.59	0.163
	$\bar{x}$ (n = 24)	23.64[b]	39.48[a]	38.91[a]	6.49	0.006

[1]blood taken on days 24 and 38 of life, [2]group receiving distilled water, [3]aqueous solution of silver nanoparticles at a dose of 5 mg/kg body weight, [4]aqueous solution of lipid-coated silver nanoparticles at a dose of 5 mg/kg body weight

[a,b]values in rows marked with different letters differ significantly at $P \leq 0.05$, $P \leq 0.01$

of B and T cells (Javanovic and Palic 2012); depending on the dose and duration of application this effect can be stimulatory or inhibitory. There have also been studies in which intraperitoneal administration of silver nanoparticles to mice induced no immune response in the organism, despite the fact that they were confirmed to have accumulated in all of the organs, including the spleen (Kiruba et al. 2010). Another study, in which chickens received nanosilver in concentrations of 20, 40, and 60 ppm/kg of feed, showed that this nanometal could accumulate in the lymphatic organs, having an immunosuppressive effect on the organism (Ahmadi 2012).

The present study showed no effect of the application of Ag-nano and AgL-nano on most of the haematological indices in the blood of the chickens. It should be emphasized, however, that administration of AgL-nano to the chickens caused a decrease in haemoglobin content in the blood. There have been reports indicating that nanosilver can affect haematological indices in the blood of birds. Ahmadi and Kurdestani (2010) found that broiler chickens receiving silver nanoparticles had lower WBC and RBC counts and a lower level of Hb in the blood. Gholami-Ahangaran and Zia-Jahromi (2014) suggest that colloidal nanosilver stimulates erythropoiesis. The authors found that colloidal nanosilver administered with aflatoxin, which decreases haemoglobin content and lowers the haematocrit in the blood of chickens, reduced the negative effects of the toxin on these blood indices. The decreased haemoglobin content in the blood may be the effect of intensification of

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oxidation and phagocytic processes in the chickens receiving nanosilver. Independently of its other physiological functions, haemoglobin is involved in redox reactions, because the erythrocyte transports molecular oxygen, which is a potential source of reactive oxygen species. Moreover, erythrocyte membranes contain large quantities of polyunsaturated fatty acids, which in an environment rich in oxygen and oxygen radicals can be oxidized by these radicals. Haemoglobin is also responsible for generation of reactive oxygen species as a consequence of enzymatic and non-enzymatic haem degradation (Bylund et al. 2010). The reduced haemoglobin content, increased lipid peroxide content, increased iron content, and insignificantly elevated bilirubin content observed in the study may indicate an effect of nanosilver on oxidation of haemoglobin, and in consequence on haem degradation. As noted above, administration of nanosilver stimulated the phagocytosis mechanism in the birds. Superoxide anion radical and hydrogen peroxide generated in phagocytosis take part in haem degradation (Bylund et al. 2010). The mechanism of interaction of hydrogen peroxide with haem is based on the reaction of oxidation of iron (II) to iron (III) by hydrogen peroxide anion generated from hydrogen peroxide. The reaction of hydrogen peroxide anion with haemoglobin generates a peroxide–iron (III) complex as well as biliverdin and bilirubin (Hersleth et al. 2008). Products generated by the decomposition of haem, such as iron and biliverdin, are toxic in high concentrations because they initiate oxidation processes in the erythrocyte (Pradines et al. 2005). An elevated level of iron, which is a catalyst of the Fenton reaction, contributes to the production of hydroxyl radicals and to lipid peroxidation. The biliverdin resulting from haem degradation is immediately transformed into bilirubin, which in its free form (not conjugated with glucuronic acid) is toxic in high concentrations because it damages nerve cells. Bilirubin and biliverdin in low concentrations have been shown to have an anti-inflammatory (antioxidant) effect, because they capture reactive oxygen and nitrogen species (Hersleth et al. 2008). Moreover, the elevated iron level noted in the present study may result from disruptions in the functioning of certain enzymes regulating systemic homeostasis of iron. As silver exhibits high affinity for sulfhydryl and disulfide groups, it may cause suppression of delta-aminolevulinic acid dehydratase and ferrochelatase, resulting in haem decomposition and accumulation of zinc protoporphyrin and delta-aminolevulinic acid, which in turn initiates the process of lipid peroxidation (Sharma et al. 2014).

Additional evidence that the Ag-nano and AgL-nano applied in the experiment initiated oxidative stress in the organism of the chickens is the increase noted in the plasma content of LOOH and MDA. Ahmadi (2012) also noted an increase in the plasma content of MDA in chickens resulting from the administration of nanosilver in dosages of 20, 40, and 60 ppm/kg of feed. An elevated level of MDA (an end product of lipid peroxidation) can modify the physical properties of cell membranes, disturbing the hydrophobicity of the lipid interior and the two-layer structure of the membranes, which can lead to a loss of cell membrane integrity. Oxidative stress and lipid peroxidation are important processes that can cause damage to liver cells. Functional damage to hepatocytes is associated with energy depletion and intensive production of reactive oxygen species, which can lead to a vicious circle of cellular stress and metabolic disturbances (Ognik and Wertelecki 2012). The slight decrease observed in the present study in activity of the enzymes AST and ALT, which are responsible for directing amino acids onto catabolic pathways and reducing the plasma concentration of the main protein metabolism products (creatinine and urea), may indicate disturbed protein catabolism in the chickens receiving 5 nm AgL-nano.

After penetration of cells, most nanoparticles enter the mitochondria, which leads to lipid peroxidation and damage to proteins, enzymes, and nucleic acids of the mitochondria. The oxidative stress and increased production of reactive oxygen species in the mitochondrion lead to activation of cellular antioxidant defence: superoxide dismutase (SOD), glutathione peroxidase (PGx), catalase (CAT), and cellular glutathione (GSH) (Ognik and Wertelecki 2012). Administration of Ag-nano probably induced severe oxidative stress in the chickens, which was manifested as a decrease in the activity of antioxidant enzymes (SOD and CAT) in their blood plasma. After administering nanosilver to chickens in concentrations of 20, 40, and 60 ppm/kg of feed, Ahmadi (2012) also observed a decrease in the activity of antioxidant enzymes (SOD, PGx, and CAT) in the blood plasma, which was in direct proportion to the dose of nanosilver in the feed.

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Ahmadi (2012) explains the results obtained as the effect of oxidative stress.

In the blood plasma of the chickens receiving 22 nm Ag-nano or 5 nm AgL-nano, changes were also noted in the activity of the liver enzyme ALP. The activity of liver enzymes usually increases when they are released from hepatocytes into the blood due to cardiac infarction or liver damage caused by toxins or viruses. Reduced AST activity is observed in kidney disorders and reduced ALP activity in bone growth disorders and vitamin C deficiencies (Aragon and Younossi 2010). Reduced activity of AST and ALP in the blood plasma of chickens receiving nanosilver in concentrations of 20, 40, and 60 ppm/kg of feed was noted by Ahmadi (2012). Other studies in which chickens were given nanosilver found no effect on the activity of the liver enzymes AST and ALT (Andi et al. 2011). Similarly, Sawosz et al. (2009) observed no effect of administration of nanosilver to chickens on the activity of AST, ALT or ALP. The present study found a decrease in LDH activity in the plasma of chickens receiving Ag-nano and AgL-nano. On the basis of LDH activity it is possible to determine the degree of cell membrane integrity under the influence of oxidative stress. Oxidative stress induced by administration of nanosilver usually causes a decrease in mitochondrial function, manifested as a decrease in LDH activity (Carr et al. 2000).

The results of the experiment showed that administration of an aqueous solution of silver nanoparticles (Ag-nano and AgL-nano) to chickens had a negative effect on the lipid profile of the blood, causing an increase in CHOL and in LDL cholesterol. Similarly, when Ahmadi (2012) gave chickens nanosilver in dosages of 20, 40, and 60 ppm/kg of feed, they found that it had a negative effect on the blood lipid profile, causing an increase in CHOL, LDL, and TG. Another study by Ahmadi et al. (2013) found that application of lower doses, i.e. 4, 8, and 12 ppm/kg of feed, had no effect on CHOL content, but doses of 8 ppm/kg and 12 ppm/kg increased LDL cholesterol and reduced HDL cholesterol. Modification of the LDL cholesterol fraction is usually initiated by lipid peroxidation, during which polyunsaturated fatty acids (PUFA) are degraded, followed by an increase in the formation of lipid, triglyceride, and phospholipid peroxides from cholesterol esters, which are further decomposed to aldehyde products such as malondialdehyde. Moreover, LDL oxidation taking place during an inflammatory reaction can also occur in enzymatic reactions, e.g. by myeloperoxidase. However, the entire LDL oxidation process, whether initiated by lipid peroxidation or resulting from inflammatory reactions, is determined by the presence of a high plasma concentration of LDL (Carr et al. 2000).

The analysis of available literature and the results of the present study lead to the conclusion that a substantial number of the studies carried out thus far on the effect of nanosilver on poultry indicate that it has a negative influence on the organism (suppressing the immune response and inducing oxidative stress). It should be emphasized, however, that there have also been studies showing no negative effect of nanosilver on the health of poultry. The discrepancies in the results obtained could be due to the use of nanosilver produced by different methods. In the available publications the authors do not provide information on the method used to produce the nanoparticles administered to the animals, which makes it difficult to compare the results obtained with the results of studies by other authors. During chemical synthesis of nanoparticles a certain quantity of active silver ions may be adsorbed on their surface, whereas nanosilver produced by physical methods has no (or very few) active silver ions on its surface. The antibacterial effectiveness, for example, of silver and its compounds is known to be directly proportional to the amount of biologically active Ag^+ ions released. Moreover, ionic silver nanoparticles can react with hydrochloric acid in the digestive tract to form salts. Biologically active Ag^+ ions have been shown to have high affinity for a number of functional groups, including phosphate, carboxyl, amine, sulfhydryl, and imidazole groups, which are components of cellular proteins and nucleic acids (Liu et al. 2013; Ognik et al. 2016). For this reason, as many researchers emphasize, the biological activity of nanosilver may differ to some extent depending on the production method, medium, particle size, dosage, and duration and means of administration.

CONCLUSION

The increased phagocytosis and metabolic activity of leukocytes noted in the present study following *per os* administration of chemically synthesized silver nanoparticles differing in size and

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in the presence or absence of a lipid coating (Ag-nano and AgL-nano) may indicate a developing inflammatory state in the organism. The decrease noted in haemoglobin content and antioxidant enzyme activity and the increased content of iron, bilirubin, and lipid peroxidation products indicate the occurrence of oxidative stress, although in the case of administration of AgL-nano the oxidation effect appears to be stronger. Administration of nanosilver to the chickens, especially AgL-nano, probably led to a disturbance in protein catabolism in the organism, as indicated by the reduction in activity of the liver enzymes AST and ALT and the reduced concentration of the main products of protein metabolism (creatinine and urea).

To sum up, administration of chemically synthesized nanosilver to chickens, particularly 5 nm nanoparticles with a lipid coating (which facilitates penetration of the cell through the cell membrane), may have a negative effect on the health of the birds. The present findings are only relevant for the nanoparticles used in this study and cannot be generalized.

REFERENCES

Ahmadi F. (2012): Impact of different levels of silver nanoparticles (Ag-NPs) on performance, oxidative enzymes and blood parameters in broiler chicks. Pakistan Veterinary Journal, 32, 325–328.

Ahmadi F., Kurdestani A.H. (2010): The impact of silver nanoparticles on growth performance, lymphoid organs and oxidative stress indicators in broiler chicks. Global Veterinaria, 5, 366–370.

Ahmadi F., Khah M.M., Javid S., Zarneshan A., Akradi L., Salehifar P. (2013): The effect of dietary silver nanoparticles on performance, immune organs, and lipid serum of broiler chickens during starter period. International Journal of Biosciences, 3, 95–100.

Andi M.A., Mohsen H., Farhad A. (2011): Effects of feed type with/without nanosil on cumulative performance, relative organ weight and some blood parameters of broilers. Global Veterinaria, 7, 605–609.

Aragon G., Younossi Z.M. (2010): When and how to evaluate mildly elevated liver enzymes in apparently healthy patients. Cleveland Clinic Journal of Medicine, 77, 195–204.

Bartosz G. (eds) (2004): Second Face of Oxygen. PWN, Warsaw, Poland.

Batzri S., Korn E.D. (1973): Single bilayer liposomes prepared without sonication. Biochimica et Biophysica Acta, 298, 1015–1019.

Bhanja S.K., Hotowy A., Mehra M., Sawosz E., Pineda L., Vadalasetty K.P., Kurantowicz N., Chwalibog A. (2015): In ovo administration of silver nanoparticles and/or amino acids influence metabolism and immune gene expression in chicken embryos. International Journal of Molecular Sciences, 16, 9484–9503.

Bylund J., Brown K.L., Movitz C., Dahlgren C., Karlsson A. (2010): Intracellular generation of superoxide by the phagocyte NADPH oxidase: how, where, and what for? Free Radical Biology and Medicine, 49, 1834–1845.

Carr A.C., McCall M.R., Frei B. (2000): Oxidation of LDL by myeloperoxidase and reactive nitrogen species: reaction pathways and antioxidant protection. Arteriosclerosis, Thrombosis and Vascular Biology, 20, 1716–1723.

Dobrzanski Z., Zygadlik K., Patkowska-Sokola B., Nowakowski P., Janczak M., Sobczak A., Bodkowski R. (2010): The effectiveness of nanosilver and mineral sorbents in the reduction of ammonia emissions from livestock manure. Przemysł Chemiczny, 4, 348–351. (in Polish)

Feldman B., Zinki J., Jain N. (2000): Schalm's Veterinary Haematology. Lippincott Williams & Wilkins, Philadelphia, USA.

Gay C.A., Gebicki J.M. (2002): Perchloric acid enhances sensitivity and reproducibility of the ferric–xylenol orange peroxide assay. Analytical Biochemistry, 304, 42–46.

Gholami-Ahangaran M., Zia-Jahromi N. (2014): Effect of nanosilver on blood parameters in chickens having aflatoxicosis. Toxicology and Industrial Health, 30, 192–196.

Hersleth H.P., Hsiao Y.W., Ryde U., Gorbitz C.H., Andersson K.K. (2008): The crystal structure of peroxymyoglobin generated through cryoradiolytic reduction of myoglobin compound III during data collection. Biochemical Journal, 412, 257–264.

Javanovic B., Palic D. (2012): Immunotoxicology of non-functionalized engineered nanoparticles in aquatic organism with special emphasis on fish-review of current knowledge, gap identification, and call for further research. Aquatic Toxicology, 118–119, 141–151.

Kiruba D., Tharmaraj V., Sironmani A.T., Pitchumani K. (2010): Toxicity and immunological activity of silver nanoparticles. Applied Clay Science, 48, 547–551.

Liu H.L., Yang D.F., Yang H.L., Zhang H.S., Zhang W., Fang Y.J., Lin Z.Q., Tian L., Lin B.C., Yan J., Xi Z.G. (2013): Comparative study of respiratory tract immune toxicity induced by three sterilization nanoparticles: silver, zinc oxide and titanium dioxide. Journal of Hazardous Materials, 248–249, 478–486.

Ognik K., Wertelecki T. (2012): Effect of different vitamin E sources and levels on selected oxidative status indices in blood and tissues as well as on rearing performance of

doi: 10.17221/80/2015-CJAS

slaughter turkey hens. The Journal of Applied Poultry Research, 2, 259–271.

Ognik K., Sembratowicz I., Cholewinska E., Wlazlo L., Nowakowicz-Debek B., Szlazak R., Tutaj K. (2016): The effect of chemically-synthesized silver nanoparticles on performance and the histology and microbiological profile of the jejunum in chickens. Annals of Animal Science, 16, 1–12.

Oliveira M.M., Ugarte D., Zanchet D., Zarbin A.J. (2005): Influence of synthetic parameters on the size, structure, and stability of dodecanethiol-stabilized silver nanoparticles. Journal of Colloid and Interface Science, 292, 2429–435.

Park B.H., Fikrig S.M., Smithwick E.M. (1968): Infection and nitroblue tetrazolium reduction by neutrophils. The Lancet, 7, 532–534.

Pineda L., Chwalibog A., Sawosz E., Lauridsen C., Engberg R., Elnif J., Hotowy A., Sawosz F. (2012): Influence of in ovo injection and subsequent provision of silver nanoparticles on growth performance, microbial profile, and immune status of broiler chickens. Open Access Animal Physiology, 4, 1–8.

Pradines B., Ramiandrasoa F., Hammadi A., Henry M., Briolant S., Orlandi-Pradines E., Bogreau H., Mosnier J., Baret E., Kunesch G., Le Bras J., Rogier C. (2005): Generation of free radicals and enhancement of hemin-induced membrane damage by a catechol iron chelator in Plasmodium falciparum. International Journal of Biological Sciences, 5, 463–471.

Pyatenko A., Yamaguchi M., Suzuki M. (2007): Synthesis of spherical silver nanoparticles with controllable sizes in aqueous solutions. The Journal of Physical Chemistry C, 111, 7910–7917.

Salih M., Smith D.M., Price J.F., Dawson L.E. (1987): Mosified extraction 2-thiobarbituric acid method for measuring lipid oxidation in poultry. Poultry Science, 66, 1483–1488.

Sawosz E., Grodzik M., Zielinska M., Niemiec T., Olszanka B., Chwalibog A. (2009): Nanoparticles of silver do not affect growth, development and DNA oxidative damage in chicken embryos. Archiv für Geflügelkunde, 73, 208–213.

Sharma B., Singh S., Siddigi N.J. (2014): Biomedical implications of heavy metals induced imbalances in redox systems. BioMed Research International, 2014: 640754.

Siwicki A.K., Anderson D.P. (1993): Nonspecific defense mechanisms assay in fish: II. Potential killing activity of neutrophils and macrophages, lysozyme activity in serum and organs and total immunoglobulin (Ig) level in serum. In: Siwicki A.K, Anderson D.P., Waluga J. (eds): Fish Diseases Diagnosis and Prevention Methods. FAO-Project GCP/INT/526/JPN. Inland Fisheries Institute, Olsztyn, Poland, 105–112.

Smulikowska S., Rutkowski A. (2005): Nutrient Requirements of Poultry. Feeding Recommendations and Nutritive Value of Feed. The Kielanowski Institute of Animal Physiology and Nutrition of the Polish Academy of Sciences, Jabłonna, Poland. (in Polish)

Xu Y., Tang H., Liu J.H., Wang H., Liu Y. (2013): Evaluation of the adjuvant effect of silver nanoparticles both in vitro and in vivo. Toxicology Letters, 12, 42–48.

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Corresponding Author

dr hab. Katarzyna Ognik, University of Life Sciences in Lublin, Faculty of Biology, Animal Sciences and Bioeconomy, Department of Biochemistry and Toxicology, Akademicka 13, 20-950 Lublin, Poland
Phone: +48 81 445 69 31, e-mail: kasiaognik@poczta.fm