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THE EFFECT OF ADMINISTRATION OF SILVER NANOPARTICLES ON THE IMMUNE STATUS OF CHICKENS*

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Abstract
The purpose of the study was to verify the hypothesis that there are doses of silver nanoparticles (Ag-NPs) that improve immune defence in chickens without compromising their health. To verify this hypothesis, an attempt was made to determine which doses of Ag-NPs (5 nm) consumed by chickens as a result of administration of hydrocolloids during varying time periods at a concentration of 5 or 10 mg Ag/l stimulate immune defence. The experiment was performed on 296 chickens assigned to 3 treatments. Chickens from the control treatment received drinking water without experimental additives. Chickens from the T-5 and T-10 treatments received a hydrocolloid of Ag-NPs at a concentration of 5 mg/l (treatment T-5) or 10 mg/l (treatment T-10) from their second week of life. Blood for analysis was collected at the age of 42 days from 8 birds per treatment. Ceruloplasmin (Cp), leukocyte count (WBC), erythrocyte sedimentation rate (ESR), interleukin IL-6, immunoglobulins IgA, IgY, phagocytic cells (% PC), phagocytic index (PI), nitroblue tetrazolium reduction (NBT), and lysozyme content in the blood was determined. Application of Ag-NPs at a concentration of 5 mg/l or 10 mg/l in the dose range of 2.87–12.25 mg/bird (administration of Ag-NPs in concentration 5 mg/l in weeks: 2; 2 and 3; 2 and 4; 2 and 5; 2 and 6 or concentration 10 mg/l in week 2) resulted in an immunostimulatory effect expressed as an increase in heterophil respiratory burst and an increased concentration of lysozyme. Higher doses of Ag-NPs exerted a pro-inflammatory effect, as indicated by elevated levels of IL-6 and ceruloplasmin, as well as a high ESR. They also stimulated B lymphocytes to produce IgA and IgA immunoglobulins.

Key words: silver nanoparticles, chicken, blood, immune parameters, haematological indices

Nanoparticles, due to their small size (less than 100 nm) and large specific surface area, have different properties from those of 'macro' forms. They are characterized by high chemical reactivity, a tendency to agglomerate, porosity and considerable affinity for a number of biological structures (Shahbazi et al., 2013; Zhao and Riediger, 2014; Ognik et al., 2017). Owing to the unique optical, magnetic, mechanical and electronic properties of nanomaterials, they have broad biomedical, industrial and agricultural applications (Hu et al., 2006; Sekhon, 2014). Recently scientists have

also taken up new challenges involving intensive *in vitro*, *in vivo* and *in ovo* research to evaluate the potential use of silver nanoparticles as prophylactic feed additives for animals (Shahbazi et al., 2013; Ognik et al., 2016 a). Silver nanoparticles are used in livestock facilities for deodorizing and to reduce emissions of ammonia and nitrogen oxides (Dobrzański et al., 2010). Nanoparticles can easily penetrate biological membranes, block ion channels, inhibit enzymatic proteins, and interact with DNA. These processes can cause damage to cells and tissues and generate oxidative stress with all its consequences (Jia et al., 2009; Pan et al., 2009).

Metal nanoparticles can react with immune system components to stimulate or inhibit it. Many studies indicate the stimulatory effect of silver nanoparticles on the activity of phagocytic cells – macrophages, dendritic cells and peripheral blood phagocytes (Małaczewska, 2015; Dykman et al., 2004; Ognik et al., 2016 a). These cells easily ingest nanoparticles, which can lead to the stimulation and expression of pro-inflammatory cytokines such as TNF-alpha, IL-1, or IL-6 (Yen et al., 2009; Lee et al., 2012). Some authors, as mentioned above, have observed detrimental effects in the form of inflammation or immunotoxicity. Others authors report anti-inflammatory and antioxidant properties of silver nanoparticles, manifested as a decrease in the level of pro-inflammatory cytokines and indicators of oxidative stress (Pedersen et al., 2009; Victor et al., 2012; Sumbayev et al., 2013). Most studies on the biological effects of Ag-NPs have been conducted on experimental animals (mice and rats) (Kiruba et al., 2010), while information is lacking on the impact of their application in poultry.

The purpose of the study was to verify the hypothesis that there are doses of Ag-NPs that improve immune defence in chickens without compromising their health. To verify this hypothesis, an attempt was made to determine which doses of Ag-NPs (5 nm) consumed by chickens as a result of administration of hydrocolloids during varying time periods at a concentration of 5 or 10 mg Ag/l stimulate immune defence.

Material and methods

Nanoparticles

The subject of the study was an aqueous solution of a silver nanocolloid at a concentration of 50 mg/l. Concentrations of 5 mg/l and 10 mg/l were prepared from this solution for the purposes of the experiment. The silver nanoparticles were non-ionic, nanocrystalline, chemically pure particles 5 nm in size, produced in a physical process (a non-explosive, high-current method for degradation of metals) by a patented technology licensed by Nano Technologies Group, Inc. (USA).

Animals

The material for the study consisted of day-old Ross 308 chickens (male) raised until the age of 42 days. The experimental procedure was approved by the Second Local Ethics Committee for Experiments with Animals in Lublin (approval no. 30/2014). The birds were kept in pens on straw litter and reared in standard condi-

tions in a building with regulated temperature and humidity. They had permanent access to drinking water and received complete compound feeds *ad libitum* (Table 1). The nutritional value of the feeds was adjusted to meet the requirements of practical feeding of broiler chickens in Poland. The nutritional value of the basal diets was calculated according to the Polish Feedstuff Analysis Tables (Smulikowska and Rutkowski, 2005). The experiment was performed on 296 (128 × 2 + 40) chickens, with day-old chicks assigned to three treatments. The experimental design and doses of Ag-NPs administered to the chickens are presented in Figure 1 and Table 2. The control treatment (C) consisted of 40 chickens (10 chickens in each of 4 pens). Chickens from the control treatment received drinking water without experimental additives. Chickens from the T-5 and T-10 treatments, initially 128 in each treatment, received a hydrocolloid of Ag-NPs at a concentration of 5 mg/l (treatment T-5) or 10 mg/l (treatment T-10) from their second week of life. The chickens in the T-5 and T-10 treatments received Ag-NPs as follows: 1 cycle × 7 days (week 2); 2 cycles × 7 days (weeks 2 and 3; weeks 2 and 4; weeks 2 and 5; or weeks 2 and 6); 3 cycles × 7 days (weeks 2, 3 and 4; weeks 2, 3 and 5; weeks 2, 3 and 6; weeks 2, 4 and 5; weeks 2, 4 and 6; or weeks 2, 5 and 6); 4 cycles × 7 days (weeks 2, 3, 4 and 5; weeks 2, 3, 4 and 6; weeks 2, 3, 5 and 6 or weeks 2, 4, 5 and 6); and 5 cycles × 7 days (weeks 2, 3, 4, 5, and 6). At the end of the experiment, the body weight of chickens was monitored. At 42 days of age, blood samples were collected from 8 birds per treatment. After slaughter (at 42 days of age), the spleen, thymus, and bursa of Fabricius were collected from 8 birds per treatment for organosomatic evaluation. The organosomatic index was calculated by the following formula: [Weight of organ (g)/Body weight (g)] × 100.

Table 1. Composition of diets for broiler chickens

Ingredients (g/kg)	Starter weeks 1–3	Grower weeks 4–5	Finisher week 6
1	2	3	4
Wheat	452.8	367.6	330.7
Maize	150.0	250.0	300.0
Soybean meal	272.2	227.9	178.1
Rapeseed meal	20.0	40.0	60.0
Soybean oil	20.0	40.0	60.0
DDGS[1]	40.1	43.6	46.9
Monocalcium phosphate	11.0	5.4	2.1
$CaCO_3$[2]	16.1	10.9	8.5
NaCl	3.6	3.2	2.8
DL-Met (99%)	3.6	2.4	2.0
L-Lys HCl (78%)	4.3	2.9	3.1
L-Thr (99%)	1.3	0.9	0.8
Premix[3, 4]	5.0	5.0	5.0
Calculated composition (g/kg)			
ME (kcal/kg)	3070	3140	3190
Crude protein	210.0	198.5	187.5
Crude fibre	27.2	29.8	32.2

Table 1 – contd.

	1	2	3	4
Crude fat		65.9	74.5	81.4
Lys		13.5	11.7	10.9
Met		6.7	5.5	5.0
Met + Cys		10.1	8.8	8.3
Trp		2.5	2.3	2.1
Arg		13.1	12.1	11.1
Ca		9.8	7.3	6.0
P available		3.9	2.8	2.1
Na		1.6	1.5	1.4

[1] DDGS – maize distillers dried grains with solubles.

[2] Calcium carbonate.

[3] Vitamin provided per kilogram of diet: wks 1–3: vitamin A – 15,000 IU; vitamin D_3 – 5,000 IU; vitamin E – 112 IU; vitamin K_3 – 4 mg; vitamin B_1 – 3 mg; vitamin B_2 – 8 mg; vitamin B_6 – mg; vitamin B_{12} – 16 mg; folic acid – 2 mg; biotin – 0.2 mg; nicotinic amide – 60 mg; calcium pantothenicum – 18 mg; choline – 1.8 g; wks 4–5: vitamin A – 12,000 IU; vitamin D_3 – 5,000 IU; vitamin E – 75 IU; vitamin K_3 – 2 mg; vitamin B_1 – 2 mg; vitamin B_2 – 6 mg; vitamin B_6 – 4 mg; vitamin B_{12} – 16 mg; folic acid – 1.75 mg; biotin – 0.05 mg; nicotinic amide – 60 mg; calcium pantothenicum – 18 mg; choline – 1.6 g; wk 6: vitamin A – 12,000 IU; vitamin D_3 – 5,000 IU; vitamin E – 75 IU; vitamin K_3 – 2 mg; vitamin B_1 – 2 mg; vitamin B_2 – 5 mg; vitamin B_6 – 3 mg; vitamin B_{12} – 11 mg; folic acid – 1.5 mg; biotin – 0.05 mg; nicotinic amide – 35 mg; calcium pantothenicum – 18 mg; choline – 1.6 g.

[4] Trace minerals provided per kilogram of diet: Mn, 100 mg; Zn, 80 mg; Fe, 80 mg; Cu, 8 mg; I, 1 mg; Se, 0.15 mg; coccidiostat – salinomycin (except wk 6).
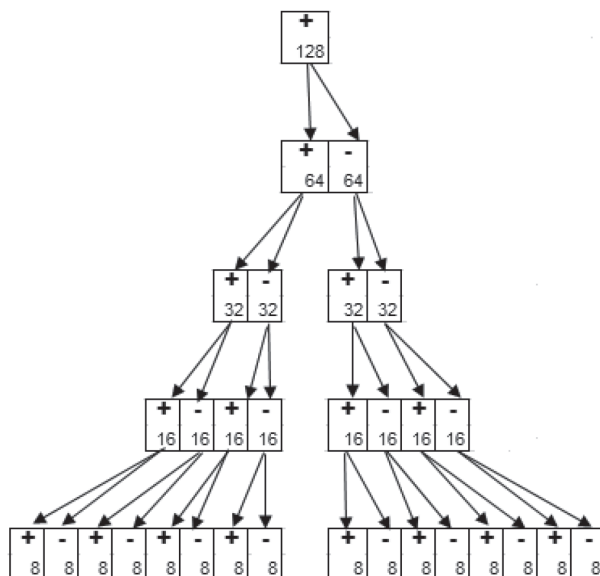

(+) – application for 7 days of a hydrocolloid of Ag-NPs at a dose of 5 mg/l – treatment T-5 or 10 mg/l – treatment T-10.

(–) – no application of a hydrocolloid of Ag-NPs.

Figure 1. Experimental design of Ag-NPs administered to chickens in treatments T-5 and T-10. Numbers on diagram represents numbers of chickens in treatments

Laboratory analysis

Blood for analysis was collected into test tubes with an anticoagulant (heparin) from the wing vein of chickens at the age of 42 days (from 8 birds per treatment). For analysis in plasma, the blood samples were centrifuged at 3,000 *g* for 10 min. Leukocyte count (WBC) in the blood was determined in an Abacus Junior Vet haematology analyser (Diatron, Hungary). The Wintrobe method was used to determine the erythrocyte sedimentation rate (ESR), i.e. the rate at which erythrocytes settle out of unclotted blood in one hour (Bomski, 1995). Ceruloplasmin in the blood plasma (Cp) was determined by the p-phenylenediamine colorimetric method according to Sunderman and Nomoto (1970). 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 phagocytic index (PI) (Siwicki et al., 1994). The respiratory burst activity of the heterophils was quantified by nitroblue tetrazolium reduction (NBT) to formazan as a measurement of production of oxygen radicals (Park et al., 1968). Immunoglobulins IgA and IgY and interleukin IL-6 in the blood were determined in an ELISA reader using assays from Elabscience Biotechnology Co., Ltd. Lysozyme activity was determined by the turbidimetric method (Siwicki and Anderson, 1993).

Statistical analysis

To compare the control treatment with the experimental treatments (Ag-NPs), the data were subjected to a studentized t-test procedure (one-way analysis of variance). In a model without the control (C), two-way ANOVA was performed to examine the main effects: C – effect of Ag-NP concentration (5 and 10 mg/l), T – effect of time (five variants of cyclical administration as described above), and the interaction between these two factors (C×T). If the analysis revealed a significant interaction (P≤0.05), the differences between treatments were then determined by the Newman-Keuls post hoc test at P≤0.05. The statistical analysis was performed according to the GLM procedure in Statistica 8.0 PL software (StatSoft Corp., Kraków, Poland). Treatment effects were considered to be significant at P≤0.05. All data were expressed as mean values with pooled SE. Student's t-test was used to compare the slopes of the regression equations.

<div align="center">

Results

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The experimental treatments, i.e. periodic *per os* administration of a hydrocolloid of Ag nanoparticles, and the mean total intake of Ag-NPs by chickens are presented in Table 2. In the T-5 chickens, the doses ranged from 2.87 to 31.87 mg/bird depending on the time of administration of Ag-NPs, while the T-10 chickens received Ag-NPs in a range of 5.75–63.74 mg/bird.

Table 2. Experimental design and doses of Ag-NP administered to chickens

Treatment	Cyclical administration of Ag-NPs	Week of administration of Ag-NPs	Total Ag-NPs applied, mg/bird
C	0	0	0
T-5$_{(2)}$	1×7	2	2.87
T-5$_{(2,3)}$	2×7	2,3	7.80
T-5$_{(2,4)}$	2×7	2,4	9.47
T-5$_{(2,5)}$	2×7	2,5	10.97
T-5$_{(2,6)}$	2×7	2,6	12.25
T-5$_{(2,3,4)}$	3×7	2,3,4	14.40
T-5$_{(2,3,5)}$	3×7	2,3,5	15.90
T-5$_{(2,3,6)}$	3×7	2,3,6	17.18
T-5$_{(2,4,5)}$	3×7	2,4,5	17.56
T-5$_{(2,4,6)}$	3×7	2,4,6	18.84
T-5$_{(2,5,6)}$	3×7	2,5,6	20.34
T-5$_{(2,3,4,5)}$	4×7	2,3,4,5	22.50
T-5$_{(2,3,4,6)}$	4×7	2,3,4,6	23.77
T-5$_{(2,3,5,6)}$	4×7	2,3,5,6	25.27
T-5$_{(2,4,5,6)}$	4×7	2,4,5,6	26.94
T-5$_{(2,3,4,5,6)}$	5×7	2,3,4,5,6	31.87
T-10$_{(2)}$	1×7	2	5.75
T-10$_{(2,3)}$	2×7	2,3	15.61
T-10$_{(2,4)}$	2×7	2,4	18.94
T-10$_{(2,5)}$	2×7	2,5	21.94
T-10$_{(2,6)}$	2×7	2,6	24.50
T-10$_{(2,3,4)}$	3×7	2,3,4	28.80
T-10$_{(2,3,5)}$	3×7	2,3,5	31.80
T-10$_{(2,3,6)}$	3×7	2,3,6	34.36
T-10$_{(2,4,5)}$	3×7	2,4,5	35.12
T-10$_{(2,4,6)}$	3×7	2,4,6	37.68
T-10$_{(2,5,6)}$	3×7	2,5,6	40.68
T-10$_{(2,3,4,5)}$	4×7	2,3,4,5	44.98
T-10$_{(2,3,4,6)}$	4×7	2,3,4,6	47.54
T-10$_{(2,3,5,6)}$	4×7	2,3,5,6	50.54
T-10$_{(2,4,5,6)}$	4×7	2,4,5,6	53.88
T-10$_{(2,3,4,5,6)}$	5×7	2,3,4,5,6	63.74

Effect of concentration of silver nanoparticles

As the concentration of Ag in the hydrocolloid of silver nanoparticles increased, the levels of IgA and IgY increased in the plasma of the chickens (P=0.032 and P=0.003, respectively) (Table 5).

Table 3. Body weight of chickens (42nd day of life)

	Treatment		Number of chickens	Body weight (kg)
Effect of additive (Ag-NPs)		C	n=40	2.24
		Ag-NPs	n=256	2.25
P-value				0.78
Effect of concentration (C)		5	n=128	2.25
		10	n=128	2.24
Effect of time of application (T)	1×7	(2)	n=16	2.25 ab
	2×7	(2,3)	n=16	2.20 b
		(2,4)	n=16	2.30 a
		(2,5)	n=16	2.29 a
		(2,6)	n=16	2.28 ab
	3×7	(2,3,4)	n=16	2.25 abc
		(2,3,5)	n=16	2.26 ab
		(2,3,6)	n=16	2.25 abc
		(2,4,5)	n=16	2.23 abc
		(2,4,6)	n=16	2.23 abc
		(2,5,6)	n=16	2.29 a
	4×7	(2,3,4,5)	n=16	2.27 ab
		(2,3,4,6)	n=16	2.26 ab
		(2,3,5,6)	n=16	2.27 ab
		(2,4,5,6)	n=16	2.21 b
	5×7	(2,3,4,5,6)	n=16	2.16 c
P-value				
C effect				0.462
T effect				0.044
C × T interaction				0.301

a, b, c, … – means within a column with no common letter differ significantly at P≤0.05.

Effect of application time of silver nanoparticles

The chickens from the 1×7, 2×7, 3×7 and 4×7 treatments attained similar body weights at 42 days of age; only the 5×7 treatment led to a lower body weight in the chickens in this treatment as compared to the most of treatments (Table 3).

As the length of administration of Ag-NPs increased (thereby increasing the intake of Ag-NPs), the ESR, IL-6 and Cp activity in the blood increased as well (P=0.002, P=0.004 and P=0.003, respectively) (Table 4). In the treatments in which the intake of Ag-NPs was higher than 12.25 mg/bird, the increases in ESR and IL-6 in the blood were expressed by linear regression equations with very high or high coefficients of determination: $R^2= 0.951$ and $R^2= 0.726$, respectively (Table 7, Figure 2). Furthermore, a statistical interaction of C×T was observed for ESR, IL-6 and Cp, as the higher concentration of silver (T-10) applied in doses higher than 12.25 mg/bird increased the value of these parameters (Table 4). As the length of administration of Ag-NPs increased, the content of IgA and IgY increased in the blood (P<0.0001 and P=0.37, respectively), while NBT and lysozyme activity decreased (P=0.004 and P=0.001, respectively) (Table 5). In the treatments in which the intake of Ag-NPs was higher than 12.25 mg/bird, the increase in the content of IgA and IgY was also expressed by regression equations with high coefficients of determination ($R^2= 0.603$ and $R^2= 0.545$, respectively) (Table 7, Figure 2). As the time of administration of Ag-NPs increased, the weight of the lymphoid organs, i.e. the thymus and the bursa of Fabricius, decreased (P=0.047 and P=0.019, respectively) (Table 6).

Table 4. Indicators of inflammation in chicken blood

Treatment			ESR (mm h⁻¹)	WBC (10^9 l⁻¹)	Cp (U l⁻¹)	IL-6 (pg ml⁻¹)
Effect of additive (Ag-NPs)		C	2.53	23.40	0.269	0.08
		Ag-NPs	5.00	23.90	0.510	0.18
P-value			0.001	0.269	<0.0001	0.042
Effect of concentration (C)		5	4.93	24.01	0.500	0.173 b
		10	5.08	23.80	0.521	0.201 a
Effect of time of application (T)	1×7	(2)	2.52 d	22.85	0.264 d	0.085 d
	2×7	(2,3)	3.30 c	22.89	0.272 d	0.108 c
		(2,4)	2.29 d	23.95	0.299 d	0.060 d
		(2,5)	2.24 d	21.50	0.279 d	0.080 d
		(2,6)	2.28 d	23.70	0.245 d	0.090 c
	3×7	(2,3,4)	4.09 c	22.87	0.657 b	0.210 b
		(2,3,5)	4.15 c	22.81	0.586 b	0.220 b
		(2,3,6)	3.48 c	24.65	0.722 ab	0.210 b
		(2,4,5)	6.42 b	23.05	0.478 bc	0.090 c
		(2,4,6)	7.15 a	23.40	0.356 c	0.090 c
		(2,5,6)	3.15 c	22.74	0.312 c	0.104 c
	4×7	(2,3,4,5)	7.31 ab	24.65	0.684 b	0.320 a
		(2,3,4,6)	7.28 ab	24.00	0.786 ab	0.350 a
		(2,3,5,6)	7.31 ab	25.15	0.638 b	0.320 a
		(2,4,5,6)	7.04 ab	24.6	0.817 a	0.260 b
	5×7	(2,3,4,5,6)	9.28 a	25.26	0.803 a	0.370 a
P-value						
C effect			0.628	0.167	0.547	0.024
T effect			0.002	0.062	0.003	0.004
C × T interaction			0.035	0.359	0.044	0.002

ESR – erythrocyte sedimentation rate, WBC – leukocytes, Cp – ceruloplasmin, IL-6 – interleukin 6.
a, b, c – means within a column with no common letter differ significantly at P≤0.05.

Table 5. Immune parameters of chicken blood

Treatment		IgA (ng ml⁻¹)	IgY (ng ml⁻¹)	%PC	PI	NBT	LYSOZYME (mg l⁻¹)
Effect							
of additive (Ag-NPs)	C	0.190	0.635	36.26	5.26	32.60	4.25
	Ag-NPs	0.372	0.874	36.33	4.98	30.01	4.15
P-value		<0.0001	0.001	0.682	0.079	0.071	0.063
Effect of concentration (C)	5	0.344	0.797	36.71	5.01	30.37	4.21
	10	0.400	0.951	35.94	4.95	29.66	4.11
Effect of time of application (T)	1×7 (2)	0.195 d	0.634 c	41.77	4.97	35.45 ab	6.15 a
	2×7 (2,3)	0.180 d	0.615 c	40.63	4.93	39.71 a	6.33 a
	(2,4)	0.180 d	0.596 d	35.57	4.62	36.12 a	5.78 a
	(2,5)	0.181 d	0.646 c	39.20	5.13	37.81 a	4.93 b
	(2,6)	0.190 d	0.665 c	37.31	4.79	38.35 a	5.77 a
	3×7 (2,3,4)	0.430 b	0.845 b	35.81	5.13	28.91 b	4.30 b
	(2,3,5)	0.410 b	0.747 bc	35.09	5.16	29.60 b	3.74 c
	(2,3,6)	0.320 b	0.884 b	35.25	5.11	30.82 b	3.96 c
	(2,4,5)	0.280 c	0.729 bc	34.59	5.14	28.86 b	3.79 c
	(2,4,6)	0.380 bc	0.852 b	34.50	5.07	26.17 c	3.15 c
	(2,5,6)	0.320 bc	1.311 a	36.73	5.04	26.31 c	4.34 b
	4×7 (2,3,4,5)	0.630 a	1.230 a	34.99	5.08	24.33 c	3.02 d
	(2,3,4,6)	0.610 a	1.340 a	35.73	4.73	24.90 c	2.90 d
	(2,3,5,6)	0.460 b	0.839 b	35.20	4.79	23.55 c	2.69 d
	(2,4,5,6)	0.500 ab	0.933 ab	34.12	4.79	25.16 c	2.76 d
	5×7 (2,3,4,5,6)	0.660 a	1.240 a	34.79	5.21	25.82 c	2.86 d
P-value							
C effect		0.032	0.003	0.223	0.066	0.351	0.664
T effect		<0.0001	0.037	0.071	0.284	0.004	0.001
C × T interaction		0.063	0.081	0.092	0.588	0.082	0.124

IgA – immunoglobulin A, IgY – immunoglobulin Y, %PC – phagocytic cells, PI – phagocytic index, NBT – test of reduction of nitroblue tetrazolium by heterophils.
a, b, c – means within a column with no common letter differ significantly at P≤0.05.

Table 6. Organosomatic index of immunocompetent organs

Treatment			Organosomatic index		
			Spleen	Thymus	Bursa of Fabricius
Effect of additive (Ag-NPs)		C	0.093	0.311	0.164a
		Ag-NPs	0.095	0.303	0.123b
P-value			0.723	0.584	<0.001
Effect of concentration (C)		5	0.093	0.305	0.123
		10	0.097	0.300	0.124
Effect of time of application	1×7	(2)	0.094	0.318 a	0.135 a
(T)	2×7	(2,3)	0.097	0.296 b	0.128 abc
		(2,4)	0.092	0.310 ab	0.130 ab
		(2,5)	0.096	0.305 b	0.124 b
		(2,6)	0.106	0.304 b	0.124 b
	3×7	(2,3,4)	0.092	0.288 bc	0.125 b
		(2,3,5)	0.096	0.287 bc	0.127 b
		(2,3,6)	0.089	0.304 b	0.123 b
		(2,4,5)	0.095	0.320 a	0.125 bc
		(2,4,6)	0.090	0.315 a	0.122 b
		(2,5,6)	0.094	0.301 b	0.118 c
	4×7	(2,3,4,5)	0.089	0.285 bc	0.121 b
		(2,3,4,6)	0.100	0.300 b	0.118 c
		(2,3,5,6)	0.104	0.311 a	0.112 c
		(2,4,5,6)	0.098	0.299 b	0.121 b
	5×7	(2,3,4,5,6)	0.089	0.279 c	0.118 c
P value					
C effect			0.114	0.345	0.621
T effect			0.117	0.047	0.019
C × T interaction			0.108	0.035	0.058

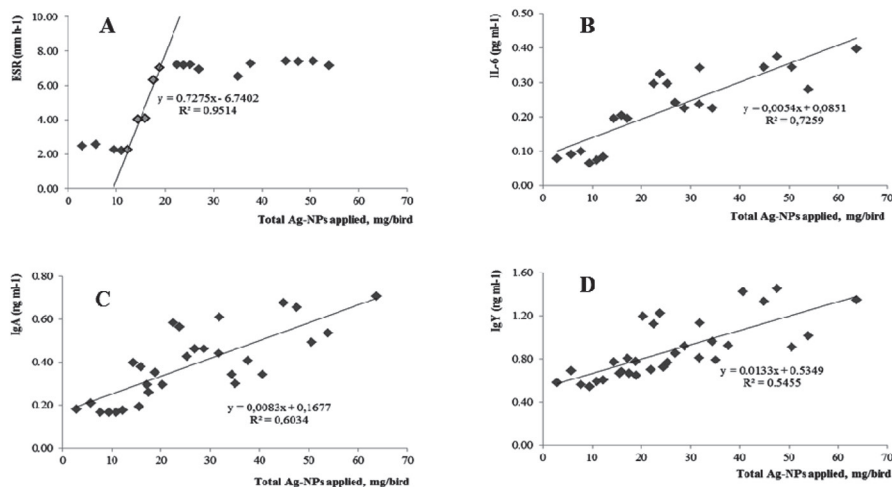a, b, c – means within a column with no common letter differ significantly at P≤0.05.

Figure 2. Influence of total Ag-NPs applied on erythrocyte sedimentation rate (ESR) (A), interleukin 6 (IL-6) (B), immunoglobulin A (IgA) (C), and immunoglobulin Y (IgY) (D)

Table 7. Effect of Ag-NP dose on linearity of changes in selected parameters

Item	Regression equation	Comment
ESR (mm h^{-1})	y = 0.7275x − 6.7402; R² = 0.9514	Plateau in the range of 2.87–12.25 mg/bird; linear increase in the range of 12.25–18.84 mg/bird; plateau above 18.84 mg/bird
IL-6 (pg ml^{-1})	y = 0.0054x + 0.0851; R² = 0.7259	Plateau in the range of 2.87–12.25 mg/bird; linear increase in the range of 12.25–63.74 mg/bird
IgA (ng ml^{-1})	y = 0.0083x + 0.1677; R² = 0.6034	Plateau in the range of 2.87–12.25 mg/bird; linear increase in the range of 12.25–63.74 mg/bird
IgY (ng ml^{-1})	y = 0.0133x + 0.5349; R² = 0.5455	Plateau in the range of 2.87–12.25 mg/bird; linear increase in the range of 12.25–63.74 mg/bird
%PC	y = -0.1073x + 39.196; R² = 0.4707	Plateau in the range of 2.87–12.25 mg/bird; inear decrease in the range of 12.25–63.74 mg/bird

Discussion

When Ag-NPs enter the body they encounter lymphoid cells, especially tissue macrophages, and dendritic cells, with which they may interact. Due to such interactions, it is likely that the Ag-NPs will exert an immunotropic effect during their application (Luo et al., 2015). The results of the study showed that the two concentrations of Ag-NPs (5 and 10 mg/l) administered periodically influenced humoral immunity (increased levels of immunoglobulins and interleukin 6). Due to their quantity and functions, heterophils are the most important peripheral blood phagocytes in chickens (called blood microphages). However, unlike neutrophils (the equivalent of heterophils in mammals), their antimicrobial activity is associated more with oxygen-independent mechanisms than with oxygen-dependent processes (Ognik and Sembratowicz, 2012; Sembratowicz et al., 2004; Harmon, 1998). The literature does not provide the results of studies on the effect of oral administration of Ag-NPs to chickens on non-specific immune mechanisms. The results of the analysis of indicators of the phagocytic activity of peripheral blood leukocytes in the chickens indicated that they were not affected by the experimental treatments. On the other hand, the study found that administration of Ag-NPs at doses from 2.87 to 12.25 mg/bird resulted in an increase in lysozyme activity and the bactericidal activity of heterophils measured in the NBT reduction assay. The increase in the number of cells reducing NBT may be indicative of the stimulatory effect of small doses of Ag-NPs on heterophils and may suggest the increased capacity of heterophils for respiratory burst and production of superoxide radicals. Once ingested by macrophages, the nanoparticles are enclosed in phagosomes, which chronically stimulates them to respiratory burst, leading to their NETosis. As a result, the nanoparticles are released and become available for other phagocytes, thus enabling their long-term recirculation in the body. It is likely that many mature forms of heterophils die as a result of NP-induced NETosis, and the new immature cells may not be fully competent (Javanovic and Palic, 2012). During the formation of a heterophil extracellular trap (HET), consisting of nuclear chromatin and a number of biocidal proteins (Bartneck

et al., 2010; Chuammitri, 2009), degranulation of granulocytes and monocytes and the release of enzymes contained in them, such as lysozyme, may occur as well. This may also explain the increased level of this enzyme in the blood of the chickens. Our study also showed that doses greater than 12.25 mg/bird reduced the activity of lysozyme and the NBT value. *In vitro* studies performed on murine macrophage lines and *in vivo* studies on laboratory animals have shown that heterophils, which are phagocytic macrophages, ingest metal nanoparticles, which may induce a suppressive effect of these nanoparticles. Nanoparticles ingested by heterophils may also cause a decrease in the quantity of reactive oxygen species produced by macrophages and in the production of pro-inflammatory cytokines. Bancos et al. (2014) report strong (over 50%) inhibition of the phagocytic ability of macrophages by gold nanoparticles that have accumulated in these cells.

Stimulation of non-specific immune mechanisms may also be manifested as an enhanced pro-inflammatory response. This is a local or systemic reaction to a harmful agent, not necessarily an infectious agent. We analysed the inflammatory action of Ag-NPs by measuring ESR, ceruloplasmin (Cp) activity, and the interleukin 6 (IL-6) level. The results of the experiment indicate that administration of Ag-NPs at doses not exceeding 12.25 mg/bird did not induce an inflammatory response and therefore was well tolerated by the chickens. However, the application of Ag-NPs in longer cycles that resulted in the intake of higher doses than 12.25 mg/bird increased ESR, IL-6 and Cp activity in the blood of the chickens. Our study also showed that Ag-NPs at a concentration of 10 mg/L increased the IL-6 level more than the 5 mg/l concentration. These results indicate that, irrespective of the concentration of Ag-NPs, doses higher than 12.25 mg/bird may have a pro-inflammatory effect. In the acute phase of the inflammatory response, there is an increase in the concentration of pro-inflammatory cytokines (TNFα, IL-6, IL stimulating synthesis of acute phase proteins, i.e. C-reactive protein, ceruloplasmin, plasminogen, and haptoglobin, whose function is to restore homeostasis) (Polińska et al., 2009). Changes in the proportions of individual serum proteins during inflammation (an increase in globulins and fibrinogen and a decrease in albumin) result in an increase in the sedimentation rate. The available literature contains no reports on the effect of metal nanoparticles on the concentration of acute phase proteins, but Ag-NPs have been shown to stimulate the production of pro-inflammatory cytokines and reactive oxygen species (Lee et al., 2012; Yen et al., 2009; Ognik et al., 2018). The increased level of pro-inflammatory cytokines may be a measure of the immunomodulatory effect of nanoparticles, but also a measure of their immunotoxicity.

The literature provides few studies on the effect of silver nanoparticles on B lymphocyte proliferation and immunoglobulin production (Luo et al., 2015). Non-phagocytic cells were once thought not to be susceptible to the effects of nanoparticles. However, this is refuted by research by Joseph et al. (2013), who found that Au-NPs increased proliferation of B lymphocytes. Our study showed higher IgA and IgY content in the blood of chickens receiving Ag-NPs at a concentration of 10 mg/l than in chickens receiving 5 mg/l during an identical period. In addition, irrespective of the concentration used, extension of the application time of Ag-NPs (resulting in doses higher than 12.25 mg/bird) increased the IgA and IgY content in the blood

of the chickens. Sharma et al. (2017), in a study using a murine B-lymphocyte cell line, demonstrated that nanometals can affect these cells by stimulating the signalling pathway associated with transcription factor NF-κB, resulting in increased production of antibodies. The results of our experiment indicate the stimulatory effect of longer application (higher doses of silver nanoparticles) on IgA and IgY concentrations. It is worth noting that B-cell stimulation may be directly or indirectly influenced by cytokines released from macrophages or other phagocytes. The most important cytokine responsible for B cell activation is interleukin IL-6, also known as B-cell stimulatory factor-2 (Dinant and Dijkmans, 1999). As changes in immunoglobulin concentrations were correlated with increased IL-6 levels, the increase in immunoglobulins could be due to stimulation of phagocytes.

In the present study, extending the administration time (thereby increasing the silver intake) of Ag-NPs at concentrations of both 5 mg/l and 10 mg/l decreased the weight of lymphoid organs. After intravenous administration, nanoparticles get distributed to the colon, lungs, bone marrow, liver, spleen, and the lymphatics (Hagens et al., 2007). Accumulation of silver nanoparticles in organs affects their weight and biological functions. The liver is the main site of distribution and metabolism of gold nanoparticles, irrespective of the means of administration, and thus this organ is highly susceptible to their harmful effects (Balasubramanian et al., 2010; Cho et al., 2009). The results of a study by Ognik et al. (2016 b) found no negative effect of silver nanoparticles on the size and macroscopic appearance of the liver and other internal organs of chickens. The available literature contains few data on the effect of silver nanoparticles on carcass parameters of birds, but in a study by Ahmadi et al. (2013) a significant increase in the weight of the liver and the small intestine was observed in broilers that had received feed enriched with silver nanoparticles, while the weight of the heart, stomach and pancreas did not differ significantly from the control. According to the authors, the increase in the size of the liver may have been linked to the accumulation of silver nanoparticles in this organ; following absorption into the bloodstream it is known to penetrate various organs, particularly the liver and kidneys (Savolainen et al., 2010). Ahmadi and Rahimi (2011) found silver levels of about 0.1 mg/kg in the edible parts of broilers, such as the breast, thigh and liver.

To sum up, the results of our research provide useful information on the potential use of silver nanoparticles in chicken diets, but given the possible adverse effects of silver nanoparticles, it is crucial to choose the correct dose and duration of its administration. Oral application of Ag-NPs to chickens modified their biochemical and immunological blood parameters, but the effect depended mainly on the dose, which was a function of the time of administration of the silver nanoparticles.

Conclusions

Application of Ag-NPs at a concentration of 5 mg/l or 10 mg/l in the dose range of 2.87–12.25 mg/bird (administration of Ag-NPs in concentration 5 mg/l in weeks: 2; 2 and 3; 2 and 4; 2 and 5; 2 and 6 or concentration 10 mg/l in week 2) resulted in an immunostimulatory effect expressed as an increase in heterophil respiratory burst and an increased concentration of lysozyme. Higher doses of Ag-NPs exerted a pro-inflammatory effect, as indicated by elevated levels of IL-6 and ceruloplasmin,

as well as a high ESR. They also stimulated B lymphocytes to produce IgA and IgA immunoglobulins.

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