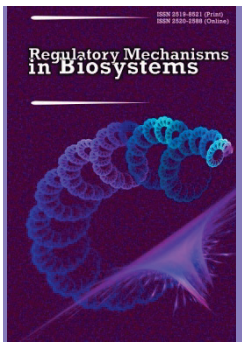

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Influence of nanosilver in hybrid carriers on morphological and biochemical blood parameters of laying hens

L. V. Shevchenko*, Y. Y. Dovbnia*, N. M. Permyakova**,
T. B. Zheltonozhskaya**, S. V. Shulyak***, D. O. Klymchuk****

**National University of Life and Environmental Sciences of Ukraine, Kyiv, Ukraine*
***Institute of Macromolecular Chemistry, National Academy of Sciences of Ukraine, Kyiv, Ukraine*
****State Scientific Research Institute of Laboratory Diagnostics and Veterinary and Sanitary Expertise, Kyiv, Ukraine*
*****M. G. Kholodny Institute of Botany, National Academy of Sciences of Ukraine, Kyiv, Ukraine*

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National University of Life and Environmental Sciences of Ukraine, Heroiv Oborony st., 15, Kyiv, 03041, Ukraine. Tel.: +38-050-193-10-29. E-mail: shevchenko_laris@ukr.net

Institute of Macromolecular Chemistry, National Academy of Sciences of Ukraine, Kharkivske Shosse, 48, Kyiv, 02160, Ukraine. Tel.: +38-050-782-55-40. E-mail: zheltonozhskaya@ukr.net

State Scientific Research Institute of Laboratory Diagnostics and Veterinary and Sanitary Expertise, Donetska st., 30, Kyiv, 02000, Ukraine. Tel.: +38-066-402-30-29. E-mail: dia_sveta_@ukr.net

M. G. Kholodny Institute of Botany, National Academy of Sciences of Ukraine, Tereschenkovska st., 2, Kyiv, 01601, Ukraine. Tel.: +38-050-708-01-36. E-mail: microscopy.botany@gmail.com

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The search for an alternative to antibiotics in poultry has led to a study of the effectiveness of using nanosilver preparations in the production of table eggs. The experiment determined the effect of the drug nanosilver in carriers based on polymer/inorganic hybrids (AgNPs/SPH) on morphological and biochemical parameters of the blood of laying hens. For this, 45 Hy-Line W36 hens were used at the age of 38 weeks, which were randomly divided into three groups. The AgNPs/SPH solution was administered 3 times a month with an interval of 10 days at concentrations of 0.0, 1.0, and 2.0 mg/L (0.0, 0.2 and 0.4 mg per hen per day). The introduction of AgNPs/SPH in doses of 0.2 and 0.4 mg per hen per day three times a month did not have a significant effect on the morphological parameters of the blood. A single dose of 0.2 mg AgNPs/SPH solution per hen per day increased the level of total protein, glucose, cholesterol, as well as the activity of alanine aminotransferase and alkaline phosphatase in the blood serum and decreased albumin, creatinine and gamma-glutamyl transpeptidase activity. Feeding laying hens a solution of nanosilver in a larger dose had a less pronounced effect on these indicators. Two-fold administration of AgNPs/SPH solution at a dose of 0.2 mg per laying hen per day increased only gamma-glutamyl transpeptidase activity in the blood serum, but decreased the level of total activity of protein, albumin, phosphorus, and alkaline phosphatase. At the same time, the drug nanosilver in double dose per day caused an increase in albumin content and alkaline phosphatase activity in the serum of hens. Triple feeding of laying hens with a solution of nanosilver at a dose of 0.2 mg per hen per day did not affect most of the biochemical parameters of serum, but in the double dose increased the content of total protein against the background of lowered cholesterol and gamma-glutamyl transpeptidase activity. With the increase in the frequency of feeding laying hens solutions of nanosilver in carriers based on polymer / inorganic hybrids, the level of severity of their impact on the metabolic profile of serum decreased. The results of research can be the basis for determining the optimal interval of application of nanosilver drugs in poultry, depending on the method of their synthesis and stabilization.

Keywords: nanoparticles; polymer / inorganic carrier; red blood cells; white blood cells; the body of a bird.

Introduction

The issue of reducing the use of antibiotics in animal husbandry sparked a search for alternative preparations that have germicidal, virucidal, and fungicidal effects in the animal body (Anwar et al., 2019). Such preparations include silver nanoparticles (AgNPs) and other metals 0.1–100 nm in size, which are widely used in the food industry, medicine, and animal husbandry (Nikalje, 2015; Cameron et al., 2018). A number of studies have proven the positive effect of silver nanoparticles used as disinfectants (Deshmukh et al., 2019), stimulants of the body's immune response (Kulak et al., 2018a), anticarcinogenic (Gomathi et al., 2020), preventive and therapeutic antiviral agents (Pangestika & Ernawati, 2017; Kiseleva et al., 2020). However, several studies point to the negative impact of silver nanoparticles on birds, which is characterized by a decrease in productivity and absence of germicidal effect against some infectious agents (Vadalasetty et al., 2018). In particular, AgNPs have been shown to cause dose-dependent toxicity, and their repeated administrations to rats have caused severe (acute) toxicity in form of congestions, hemorrhage, cell degeneration, apoptosis, and necrosis of liver and kidney tissues. In the blood serum of these animals, nanosilver caused an increase in the levels of malondialdehyde, activity of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and a decrease in the levels of glutathione, immunoglobulins G, M, and total protein (Hassanen et al., 2019).

The effect of nanosilver preparations on the morphological and biochemical parameters of animal blood depends on many factors, including the animal species and breed (Jafarzadeh et al., 2015; Abdelsalam et al., 2019), their physiological state and sex (Heydrnejad et al., 2015), dose of the preparation (Sulaiman et al., 2015), properties of the AgNPs stabilizer (Bélteky et al., 2019), nanoparticle size (Chen et al., 2015) and synthesis method (Xu et al., 2020). Despite the large number of studies on the effects of silver nanoparticles on the body of chickens, the question of their dosage, duration and interval of use of poultry remains unclear.

New ways to reduce the toxicity of nanosilver preparations in animals and humans are being actively sought today. It was found that toxicity of AgNPs in relation to human cells depends significantly on the nature (and size) of coating agents (Kennedy et al., 2014; Li et al., 2014). Therefore, the creation of effective safe carriers of AgNPs is an urgent task for their successful use in living organisms. It was shown that silica / polyacryla-

mide hybrids (SPH) based on biocompatible and biodegradable silicananoparticles and grafted polyacrylamide (PAAm) chains are effective matrices for the synthesis and stabilization of small AgNPs (<10 nm) in an aqueous medium (Zheltonozhskaya et al., 2019, 2021). The resulting composition AgNPs/SPH consisted of separate swollen hybrid particles filled with one or more silver nanoparticles, which had a small size (10–40 nm) and high antibacterial and antifungal activity. In this regard, these hybrid particles can be considered as promising candidates for AgNPs delivery to living organisms, including chickens. At the same time, detailed studies are needed to uncover their possible risks for the health of animals, as well as hens raised for laying table eggs (Samari et al., 2018; Ranoszek-Soliwoda et al., 2019). Therefore, the aim of our research was to study the effect of the nanosilver preparations in SPH carriers on morphological and biochemical blood parameters of laying hens, in order to assess their safety when used in egg farming.

Materials and methods

Synthesis and determination of the main parameters of the silica / polyacrylamide hybrid. Sample SPH was synthesized by radical grafting polymerization of acrylamide (AAm) from the surface of a silica sol according to the study (Zheltonozhskaya et al., 2021). Aerosil A-175 from "Orisil" (Ukraine) with a specific surface area of $1.82 \cdot 10^5$ m^2/kg, cerium (IV) ammonium nitrate (CAN) from "Aldrich" (USA) and AAm from "Merck" (Germany) were used in this synthesis. Weight ratios: $[CeIV]/[SiO_2] = 0.2$ and $[CeIV]/[AAm] = 7.72 \cdot 10^{-3}$ and the concentration of $CSiO_2 = 1.35$ g/L were chosen. The synthesis was carried out at 23 ºC in an inert (argon) atmosphere with stirring for 24 hours by adding CAN to 100 cm^3 of an aqueous dispersion of silica sol and then the required weight AAm. The gelled product was diluted with deionized water, re-precipitated with acetone, re-dissolved in water, and freeze-dried.

The main molecular parameters of the SPH sample were found by the general method developed earlier (Zheltonozhskaya et al., 2021). So, the average size of silica nanoparticles ($RSiO_2$) was found by static light scattering. Determination of the number (N) and molecular weight of the grafted PAAm chains (MvPAAm) was performed using elemental analysis, dynamic thermogravimetric analysis and viscometry. The obtained molecular characteristics of the SPH are shown in Table 1.

Table 1
Main parameters of the synthesized silica/polyacrylamide hybrid

Sample	R_{SiO2}, nm	M_{vPAAm}, kDa	N
SPH	7.7	1513	8

To obtain the AgNPs/SPH preparation, the in situ reduction of Ag+-ions with sodium borohydride in an aqueous solution of the hybrid was carried out in accordance with the previously developed technique (Zheltonozhskaya et al., 2021). An eight-fold molar excess of $NaBH_4$ was used for the complete conversion of Ag+-ions to AgNPs at the selected concentration of silver nitrate ($CAgNO_3 = 36.4$ mg/L). A hybrid solution with a concentration of CSPH = 1.0 g/L was mixed with $AgNO_3$ and stored for 30 min in a dark box; then a reducing agent was added. Three minutes after the addition of the reducing agent, a yellow colour appeared, corresponding to the colour of the diluted dispersion of AgNPs in water. The nanoparticle formation was monitored by changes in the integrated intensity of the surface plasmon resonance band (SPRB) at about 400 nm in the UV-Vis spectrum. Extinction spectra were recorded every 3 min for 90 min in the range 200–1000 nm using a Cary 50 Scan UV-Vis spectrometer from "Varian" (USA). The resulting nanosilver preparation was re-precipitated with ethanol, centrifuged at 6000 rpm, and re-dissolved in deionized water to remove byproducts of the reduction reaction. For biological experiments, this basic preparation with CAgNPs = 24 mg/L was diluted to the required concentration.

The morphology and size of the pure hybrid, as well as its preparation with AgNPs in aqueous solutions, were determined using transmission electron microscopy (TEM). Micrographs were obtained on a JEM-I230 device ("JEOL", Japan) operating at an accelerating voltage of 80 kV. Small drops (~$1 \cdot 10^{-4}$ mL) of dispersions of the hybrid and the nanosilver preparation in deionized water (CSPH = 1 g/L) were applied to copper grids coated with Formvar films and carbon. Then they were dried in air for ~1–2 min and in a vacuum desiccator for 24 hours. The sizes of individual structural elements in TEM images were determined at high magnification using an image and fax viewer.

The stability of the AgNPs/SPH preparation with respect to the release of silver nanoparticles at different pH values and the addition of salt to CNaCl = 9 g/L (in "physiological solution") was checked by dialysis. A preparation of nanosilver or a dispersion of pure SPH with a volume of 20 mL was poured into a standard dialysis vessel, the flat bottom of which was replaced by a semi-permeable cellulose membrane with a diameter of ~3.5 cm and a permeability of 20 kDa. This vessel was immersed to a certain depth in a beaker, placed on a magnetic stirrer and filled with 100 mL of various external media, such as deionized water with pH = 5.6 and 9.0, as well as NaCl solution. Samples of the external environment (dialysate) with a volume of 5 mL were taken from the beaker after a certain time for several days. They were studied by UV-Vis spectroscopy as described above.

It is important to note the presence of both individual diffuse hybrid particles with a shape close to spherical and predominantly smooth surface, and their fractal aggregates of various sizes and grape-like shapes (Fig. 1). Using TEM images in Figure 1, the sizes of individual SPH particles and their fractal aggregates were determined (Table 2).

The small size and smooth surface of individual SPH particles (Fig. 1, Table 2) confirmed the earlier conclusion about strong interaction of PAAm chains with the silica surface in aqueous medium.

Table 2
Dimensions of structural elements of SPH in aqueous solutions

Sample	d [a], nm	l [b], nm
SPH	20 – 45	61 – 590

Note: [a] – diameter of spherical particles; [b] – size (length) of fractal aggregates.

The reduction of silver nitrate with borohydride was carried out in an aqueous solution of SPH carriers in two separate stages to achieve the maximum yield of AgNPs. At the first stage, silver ions were bound to amide groups of PAAm chains and to a weakly negatively charged silica surface due to coordination and electrostatic interactions, respectively.

This led to the accumulation of Ag+-ions in the polymer layer around the central inorganic particles. At the second stage, the added reducing agent penetrated into the grafted layer saturated with silver ions and initiated the reduction process there with a high rate and yield of AgNPs. The silver nanoparticles grown in this way were stabilized directly in the grafted PAAm layer.

The results of these studies made it possible to predict the sufficient stability of the nanosilver preparation in the body of laying hens.

The final step was the characterization of the morphology and size of SPH carriers and AgNPs included in them. In these TEM researchers, the base dispersion of the preparation, obtained after synthesis and purification from unwanted by-products, was used (Fig. 2a, b). In both TEM images, numerous spherical silver nanoparticles can be observed embedded in swollen individual or aggregated SPH carriers. Due to the strong swelling of the initial hybrid particles during the formation of AgNPs (this effect is clearly seen from a comparison of Fig. 1 and 2), it was difficult to determine clear boundaries and exact sizes of SPH carriers together with bound metal nanoparticles. Their approximate dimensions can be estimated as ~31–47 nm. But the exact sizes of silver nanoparticles were easily calculated using Figure 2b.

Thus, the diameter of the spherical AgNPs in the base preparation was 1.2–9.6 nm. It should also be noted that the strong swelling of SPH particles and the disappearance of their boundaries after the formation of AgNPs testified to the "detachment" of PAAm chains from the silica surface. To prepare drinking water for the laying hens, the base preparation was diluted 12 and 24 times to achieve CAgNPs = 1 and 2 mg/L. Obviously, under these conditions, the process of disaggregation took place, and the final drinking water contained mainly individual swollen particles of the SPH carrier filled with one or more AgNPs.

Animals, experimental design and housing. All experiments were conducted in compliance with the requirements of the European Convention for the Protection of Vertebrate Animals used for Experimental and

Other Scientific Purposes as of 1986, as well as the law of Ukraine "On the protection of animals against ill-treatment" No. 3447-IV as of 21/02/2006 last amended on 04/08/2017. The study was approved by the Commission on Bioethics of the National University of Life and Environmental Science of Ukraine as of 11/2018.

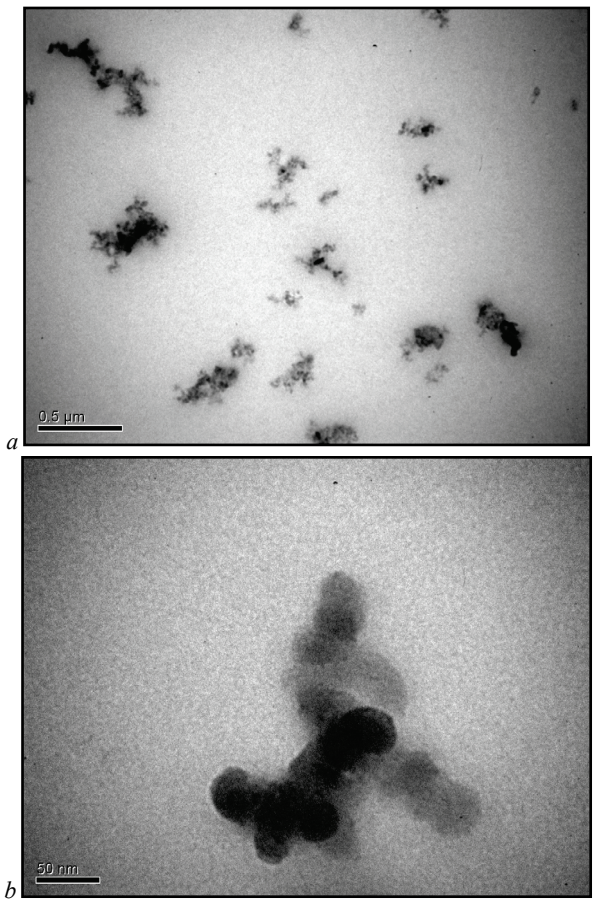

Fig. 1. TEM images of SPH aggregates existing in aqueous solutions with lower (*a*) and higher (*b*) magnification: CSPH = 1 g/L; T = 20 ºC

45 Hy-Line W36 laying hens aged 38 weeks were used for the experiment. Laying hens were randomly assigned into three groups (n = 15); after adaptation, they were watered with a solution of nanosilver preparation in hybrid carriers with a concentration of CAgNPs = 0.0, 1.0 and 2.0 mg/L, which corresponded to a dose of 0.0, 0.2 and 0.4 mg nanosilver per hen, three times a month with 10-day intervals. All hens were fed with commercial compound feed, the composition of which met the poultry's requirements in terms of nutrients and bioactive substances.

The hens were watered ad libitum with the help of drinking cups, equipped with graduated cylinders for tracking the consumption of the solution of nanosilver preparation and water. The hens were kept in premises with adjustable ventilation in cages with 5 animal units in each. The hens received 16 hours of light per day (lighting intensity – 30 Lux), the nighttime comprised 8 hours. The room temperature was maintained at 21–22 ºC, relative humidity – 60–62%.

Throughout the experiment, the feed and water consumption, as well as safety of animal units in each group were under control. On the 10th day after the nanosilver solution was administered, in the morning before feeding, blood from the axillary veins of hens in each group was collected in two test tubes. Blood with EDTA anticoagulant was collected in the first test tube to determine the blood corpuscles, hematocrit, and hemoglobin levels in blood. Blood without anticoagulant was collected in the second test tube to obtain blood serum. Blood serum for the studies was obtained by gentle centrifugation at 2000 rpm for 10 minutes and stored at –20 ºC.

Hematocrit (Ht) was determined by centrifuging whole blood in capillary tubes with the use of CM-3 MicroMed centrifuge (Ukraine) and identifying the blood plasma/blood corpuscles ratio (in %). All blood cells were counted in blood smears manually. The sum of red blood cells (RBC) was expressed in terms of 10^{12}/L. To determine the number of white blood cells, whole blood smears were stained with the use of LDF 200 reagent kit (Erba Lachema, Czech Republic). The sum of white blood cells (WBC) was expressed in terms of 10^{9}/L. A white blood cell differential was performed in whole blood smears stained according to Pappenheim. For this purpose, 100 cells were counted and their percentage ratio to the total leukocyte count was determined.

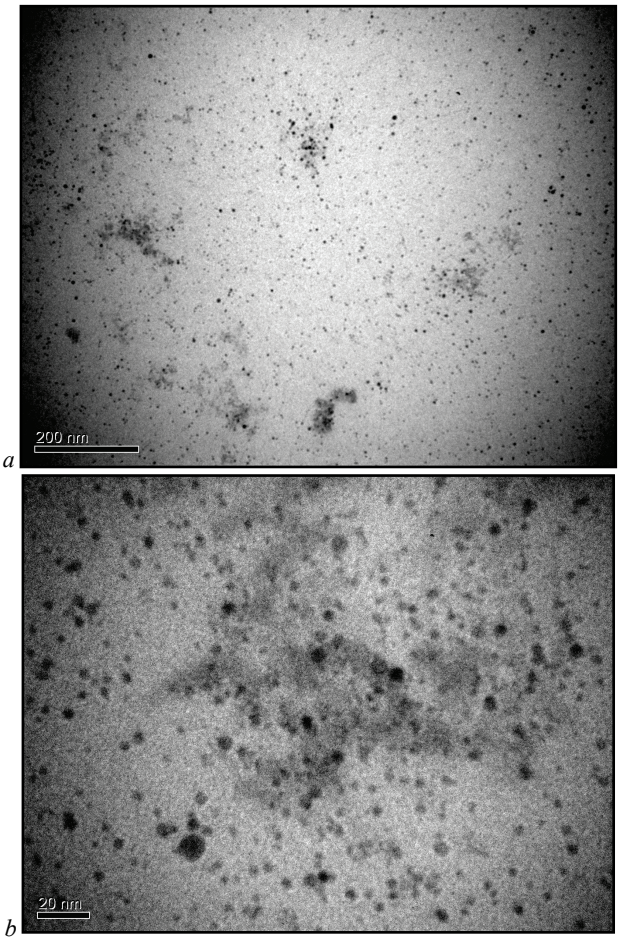

Fig. 2. TEM images of the AgNPs/SPH composition with lower (*a*) and higher (*b*) magnification: CSPH = 1 g/L; CAgNPs = 24 mg/L; T = 20 ºC

In the blood of hens, hemoglobin content (Hb) was determined, and in the blood serum – the content of total protein, albumin, creatinine, glucose, cholesterol, total calcium, inorganic phosphorus, potassium, magnesium, as well as the activity of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), gamma-glutamyl transpeptidase (GGT) with the use of Pointe Scientific Inc. reagent kits (USA) and Pointe 180 semi-automatic analyzer (Poland).

Statistical processing of the obtained results was performed using the program ANOVA, the data in the tables are presented in the form of x ± SD (standard deviation). The difference between the groups was considered probable using the Tukey test at $P < 0.05$ (taking into account the Bonferroni correction).

Results

Effect of nanosilver preparation on hematological parameters of hens. The use of nanosilver preparation in polymer/inorganic carriers did not affect the hens' behaviour, their consumption of feed and water. In the course of the experiment, there were also no deaths or signs of diseases in the hens from the experimental groups. Single, two-time and three-time administration of the nanosilver preparation to the laying hens at doses of 0.2 and 0.4 mg per animal unit per day did not change the number of RBC, WBC and their subpopulations in the blood of hens; though hematocrit was reduced ($P < 0.05$) only at a single use of the preparation as compared to the control (Table 3).

Table 3
Hematological parameters of laying hens after application of the drug nanosilver (x ± SD, n = 15)

Indicator	Frequency of treatment with AgNPs	The dose of AgNPs, mg/hen		
		0.0	0.2	0.4
Red blood cells, RBS, 10^{12}/L	single use	2.85 ± 0.28	2.77 ± 0.30	2.67 ± 0.14
	double use	3.12 ± 0.22	3.09 ± 0.26	3.31 ± 0.22
	triple use	2.89 ± 0.17	3.04 ± 0.31	3.00 ± 0.30
Hemoglobin, g/L	single use	102.24 ± 8.33	94.38 ± 4.78	103.02 ± 5.36
	double use	95.93 ± 4.26	104.87 ± 8.42	100.18 ± 11.01
	triple use	102.06 ± 11.30	109.33 ± 10.57	109.33 ± 17.14
Hematocrit, %	single use	28.14 ± 1.90^{a}	24.14 ± 1.42^{b}	27.03 ± 2.08^{ab}
	double use	27.14 ± 1.24^{ab}	25.93 ± 1.50^{ab}	27.56 ± 1.69^{ab}
	triple use	25.42 ± 1.34^{ab}	25.57 ± 1.79^{ab}	25.57 ± 2.56^{ab}
White blood cells, WBC, 10^{9}/L	single use	24.96 ± 2.92	23.78 ± 0.94	24.02 ± 1.39
	double use	25.86 ± 2.91	26.42 ± 2.57	25.64 ± 3.92
	triple use	25.77 ± 1.85	23.45 ± 2.61	23.45 ± 1.60
Heterophils, %	single use	41.01 ± 5.33	47.84 ± 6.81	45.21 ± 6.76
	double use	39.44 ± 5.41	40.65 ± 4.39	39.40 ± 6.07
	triple use	42.01 ± 6.89	43.02 ± 9.03	43.03 ± 5.52
Eosinophils, %	single use	4.82 ± 2.16	4.01 ± 1.58	4.83 ± 0.84
	double use	5.61 ± 2.07	5.25 ± 3.70	2.04 ± 0.44
	triple use	3.81 ± 3.27	5.41 ± 4.10	5.42 ± 2.04
Basophils, %	single use	2.02 ± 1.58	1.81 ± 1.64	2.64 ± 0.84
	double use	1.81 ± 1.64	3.21 ± 2.17	1.64 ± 0.57
	triple use	1.22 ± 0.83	3.03 ± 1.87	3.04 ± 0.94
Monocytes, %	single use	5.81 ± 2.17	6.24 ± 1.48	4.41 ± 0.57
	double use	4.84 ± 3.11	7.84 ± 2.17	7.82 ± 1.79
	triple use	11.41 ± 3.51	8.21 ± 5.21	8.24 ± 2.70
Lymphocytes, %	single use	46.42 ± 7.16	40.22 ± 4.09	43.03 ± 7.84
	double use	48.43 ± 3.36	43.22 ± 5.89	49.21 ± 6.53
	triple use	41.62 ± 4.72	40.44 ± 9.02	40.41 ± 8.06

Note: ^{a}, ^{b}, ^{c} – different superscript letters indicate values that probably differed in the table ($P < 0.05$), if superscripts are absent, the probable difference between the values is not detected by comparison with Tukey test taking into account the Bonferroni correction.

Table 4
Biochemical parameters of serum of laying hens after application of the drug nanosilver (x ± SD, n = 15)

Indicator	Frequency of treatment with AgNPs	The dose of AgNPs, mg/hen		
		0.0	0.2	0.4
Total protein, g/L	single use	42.30 ± 1.93^{a}	59.14 ± 3.44^{b}	44.48 ± 2.60^{a}
	double use	66.04 ± 4.62^{b}	43.15 ± 2.83^{a}	64.58 ± 3.48^{b}
	triple use	44.16 ± 2.78^{a}	45.96 ± 3.81^{a}	59.10 ± 2.26^{b}
Albumin, g/L	single use	6.98 ± 0.31^{ab}	2.20 ± 0.21^{c}	6.96 ± 0.72^{ab}
	double use	6.59 ± 0.56^{b}	3.22 ± 0.31^{c}	8.19 ± 0.63^{a}
	triple use	7.24 ± 0.50^{ab}	7.11 ± 0.44^{ab}	6.80 ± 0.56^{ab}
Creatinine, μmol/L	single use	91.12 ± 2.93^{a}	45.09 ± 5.41^{c}	56.56 ± 16.53^{bc}
	double use	69.80 ± 4.47^{b}	73.92 ± 4.35^{ab}	66.82 ± 4.71^{b}
	triple use	69.84 ± 5.99^{b}	76.00 ± 3.38^{ab}	64.40 ± 4.97^{bc}
Glucose, mmol/L	single use	4.32 ± 0.22^{a}	10.57 ± 0.77^{b}	7.05 ± 2.66^{ab}
	double use	10.64 ± 1.35^{b}	10.04 ± 0.59^{b}	10.33 ± 0.85^{b}
	triple use	9.92 ± 1.18^{b}	11.88 ± 0.50^{b}	8.74 ± 0.72^{b}
Cholesterol, mg/dL	single use	143.16 ± 14.11^{a}	591.91 ± 17.74^{b}	189.98 ± 41.25^{a}
	double use	171.18 ± 72.52^{a}	139.40 ± 11.49^{a}	211.63 ± 9.24^{b}
	triple use	186.04 ± 31.43^{a}	218.24 ± 9.03^{a}	125.46 ± 5.87^{b}
Alanine aminotransferase (ALT), U/L	single use	11.36 ± 1.45^{a}	19.18 ± 2.80^{b}	12.28 ± 0.82^{a}
	double use	11.21 ± 1.19^{a}	12.65 ± 1.12^{a}	13.35 ± 1.61^{a}
	triple use	11.72 ± 1.66^{a}	13.64 ± 0.89^{a}	11.10 ± 1.14^{a}
Aspartate aminotransferase (AST), U/L	single use	92.44 ± 4.36^{a}	89.44 ± 6.12^{ab}	64.82 ± 12.41^{b}
	double use	105.32 ± 8.48^{a}	102.64 ± 5.27^{a}	113.63 ± 4.81^{a}
	triple use	126.40 ± 16.52^{a}	109.01 ± 8.47^{a}	137.86 ± 10.67^{a}
Alkaline phosphatase (ALP), U/L	single use	330.72 ± 10.81^{a}	607.51 ± 58.94^{b}	302.92 ± 14.87^{a}
	double use	724.42 ± 150.20^{b}	175.77 ± 6.12^{a}	1033.66 ± 48.53^{c}
	triple use	186.36 ± 23.34^{a}	204.88 ± 5.00^{a}	184.74 ± 31.56^{a}
Gamma-glutamyl transpeptidase (GGT), U/L	single use	7.08 ± 0.59^{a}	1.12 ± 0.18^{b}	8.85 ± 1.35^{a}
	double use	1.72 ± 0.13^{b}	7.25 ± 0.32^{a}	2.08 ± 0.23^{b}
	triple use	5.76 ± 0.86^{a}	6.52 ± 0.46^{a}	3.72 ± 0.55^{ab}
Ca, mmol/L	single use	4.29 ± 0.31^{a}	3.96 ± 0.60^{a}	4.38 ± 0.78^{a}
	double use	2.36 ± 0.48^{b}	3.63 ± 0.44^{ab}	2.65 ± 0.22^{b}
	triple use	2.81 ± 0.46^{b}	3.17 ± 0.38^{ab}	3.11 ± 0.16^{ab}
P, mmol/L	single use	1.53 ± 0.41^{ab}	2.34 ± 0.19^{ab}	1.74 ± 0.22^{ab}
	double use	2.51 ± 0.47^{b}	1.18 ± 0.11^{a}	2.53 ± 0.27^{b}
	triple use	1.78 ± 0.49^{ab}	1.15 ± 0.11^{a}	2.29 ± 0.32^{ab}
K, mmol/L	single use	5.01 ± 0.26^{ab}	6.30 ± 0.34^{ab}	7.48 ± 2.21^{b}
	double use	4.51 ± 0.38^{a}	4.41 ± 0.40^{a}	4.72 ± 0.25^{a}
	triple use	4.38 ± 0.31^{a}	5.25 ± 0.16^{ab}	4.32 ± 0.41^{a}
Mg, mmol/L	single use	0.95 ± 0.11	0.91 ± 0.16	1.22 ± 0.18
	double use	1.11 ± 0.32	1.09 ± 0.18	0.95 ± 0.14
	triple use	1.06 ± 0.26	0.99 ± 0.10	1.12 ± 0.24

Note: see Table 3.

Effect of nanosilver preparation on the biochemical parameters of the hens' blood serum. A single administration of the solution of nanosilver preparation to laying hens at a dose of 0.2 mg per hen per day contributed to an increase ($P < 0.05$) in the content of total protein, glucose, cholesterol, as well as ALT and ALP activity in the blood serum following the decrease ($P < 0.05$) in albumin, creatinine and GGT activity compared to the control (Table 4). At a dose of 0.4 mg per day, the nanosilver preparation had a less pronounced effect on tissue metabolism characterized by a decrease ($P < 0.05$) in creatinine, as well as AST activity in blood serum compared to the control. Single administration of both doses of nanosilver did not affect the level of calcium in blood serum of laying hens.

After two-time administration of the solution of nanosilver preparation to the laying hens at the dose of 0.2 mg per hen per day, the GGT activity in the blood serum increased ($P < 0.05$), while the level of total protein, albumin, phosphorus, as well as ALT activity, decreased ($P < 0.05$) compared to the control and AgNPs/SPH 0.4 group (Table 4). Two-time administration of the nanosilver preparation to laying hens at the dose of 0.4 mg per animal unit per day increased only the albumin level and ALP activity in the blood serum compared to the control. Two-time oral administration of both doses of AgNPs/SPH did not affect the level of creatinine, glucose, potassium, magnesium, as well as ALT and AST activity in the blood serum compared to the control (Table 4).

Three-time administration of the solution of nanosilver preparation in polymer / inorganic hybrid carriers at the dose of 0.2 mg per animal unit per day did not affect the abovementioned biochemical parameters (Table 4). The 0.4 mg of the nanosilver preparation per day resulted in the increase ($P < 0.05$) of the total protein following the decrease ($P < 0.05$) of cholesterol and GGT activity. Such dose of the preparation did not change other parameters of the blood serum of hens compared to the control. During this period, the blood serum of hens receiving AgNPs/SPH at the dose of 0.2 mg per animal unit per day showed the lower level ($P < 0.05$) of total protein, while the levels of creatinine, cholesterol, as well as GGT activity, were higher ($P < 0.05$) than in hens receiving AgNPs/SPH at the dose of 0.4 mg per animal unit per day (Table 4).

Discussion

Effect of nanosilver preparation on hematological parameters of hens. The effect of nanosilver on the body of animals depends on a significant number of factors, in particular on the method of their introduction into the body (Sarhan & Hussein, 2014). One of the most effective and acceptable ways of administering nanosilver preparations to hens is oral, which involves their passing through the mucous membrane of the digestive system, blood and liver and providing a systemic effect on the body. Watering the laying hens with the solution of nanosilver preparation with a particle size of 1.2–9.6 nm in SPH carriers reduced the hematocrit content in the blood only at the dose of 0.2 mg per hen per day (~0.13 mg/kg body mass) after a single administration. Chen et al. (2015) explained such AgNPs-induced cytotoxicity by the nanoparticles directly interacting with RBC, which resulted in oxidative stress, membrane damage and subsequent hemolysis. Moreover, AgNPs cytotoxicity towards blood cells depended on their size. Taking fish RBC, the nucleus of which is similar to poultry RBC nucleus, it was proven that the use of AgNPs of different diameters (15, 50, and 100 nm) and 50 nm particles stabilized with citrate anions showed the highest level of adsorption and absorption by RBC, while 15 nm particles showed a greater ability to cause damage to cell membranes and hemolysis.

We did not register a significant effect of the nanosilver preparation in SPH carriers on hemoglobin content and count of RBC, WBC and their subpopulations in the hens' blood, therefore we can consider this preparation as biocompatible and safe for blood cells. This is obviously driven by the good transport properties of hydrophilic hybrid carriers used to produce and deliver AgNPs. Data presented in the study by Ognik et al. (2016) confirm that the effect of nanosilver on the hematological parameters of hens depends on the AgNPs size and stabilizer. In particular, this research showed that administration of 22 nm hydrocolloid silver nanoparticles to broiler chickens at the dose of 5 mg/kg body mass did not affect the levels of hemoglobin and hematocrit, WBC and RBC count, while administration of 5 nm lipid-coated silver nanoparticles resulted in a significant decrease in the hemoglobin level in the blood. The study by Dosoky et al. (2021) showed that 20 nm silicon dioxide nanoparticles, doped with nanosilver (SiO_2@AgNP) and coated with starch, did not negatively affect the growth performance, hematological and biochemical parameters when used in poultry feed at doses of 2, 4, 8 ppm/kg feed. According to the data provided by Ahmadi (2009), the use of commercial colloidal nanosilver at significantly higher doses (300, 600, and 900 ppm) did not negatively affect the hematological parameters of broiler chickens as well. Gholami-Ahangaran & Zia-Jahromi (2014) suggest that colloidal nanosilver (Nanocid) at the dose of 2500 ppm stimulated erythropoiesis in broiler chickens, when feeds contained aflatoxin, and thus reduced its negative effect on the body. Another study (Kulak et al., 2018b) revealed that oral administration of 5 nm nanosilver colloidal dispersion to broiler chickens in the dose range 2.87–12.25 mg/animal unit produced a positive immunostimulatory effect manifested through an increase in heterophil respiratory burst and higher lysozyme concentration in the bloodstream. Higher doses of AgNPs showed an anti-inflammatory effect in broiler chickens due to an increase in interleukin 6 and ceruloplasmin levels, as well as high erythrocyte sedimentation rate and stimulation of IgA and IgY synthesis by B-cells.

As the above data show, there is no unanimity on the way how nanosilver particle size, coating type, and dosing affect the hematological parameters of various animal species. However, it is believed that one mechanism of WBC response to non-self stimuli, including nanosilver particles, is degranulation and subsequent release of enzymes (i.e., proteinases such as elastase) and other biologically active substances (i.e., cytokines such as interleukins and chemotactic proteins of monocytes) (de la Harpe et al., 2019). In addition, respiratory burst is another mechanism applied by WBC to eliminate unwanted and exogenous agents, such as microbes and nanoparticles (Lin et al., 2018). Excessive production of reactive oxygen intermediates and other free radicals can be toxic to both phagocytes and surrounding cells, can cause apoptosis and inflammation (Laridan et al., 2019), and affect the metabolic state of the body and the functional state of vital organs, including the liver.

Effect of nanosilver preparation on the biochemical parameters of hen blood serum. One indicator reflecting the protein-synthesizing function of the hen's liver is the level of serum total protein and albumin. Watering the laying hens with the solution of nanosilver preparation in SPH carriers had a contradictory effect on the levels of serum total protein and albumin, depending on the dose and frequency of administration. The 0.2 mg AgNPs/SPH dose produced more dynamic fluctuations of these parameters than 0.4 mg AgNPs/SPH per hen per day. In our experiment, the effect of both doses of nanosilver on the levels of serum albumin decreased with increasing frequency of administration. Changes in the activity of ALT, AST, ALP were also detected by Ahmadi (2012) in the serum of broiler chickens fed supplements of commercial AgNPs throughout the rearing period. Whereas the main function of albumin is to provide oncotic pressure and ion transport, it can be assumed that silver nanoparticles can bind to chicken serum albumin, which has been proved by the example of bovine serum albumin (Gnanadhas et al., 2013; Gessmann et al., 2018). As a result, conformational changes or even protein damage could occur (Chen et al., 2012), which is probable reason for significant fluctuations in its content in the blood serum of chickens in our experiment. Consequently, the hens develop adaptive mechanisms to nanosilver preparations, the direction of which depends on the dose, use duration, and type of AgNPs stabilizer.

The level of serum creatinine is considered to be one of the parameters characterizing the functional state of the liver. As the byproduct of protein metabolism, creatinine is formed in the muscles and, after entering the bloodstream, is excreted by the kidneys. In our experiment, serum creatinine of hens receiving AgNPs/SPH with drinking water at doses of 0.2 and 0.4 mg per hen per day (~0.13 and ~0.27 mg/kg of poultry body weight) decreased only after a single administration, later this indicator was kept at the control level. The decrease in serum creatinine of broiler chickens was also observed in the study by Ognik et al. (2016), where the broiler chickens received lipid-coated nanosilver preparation, while the hydrocolloid nanosilver without a coating did not cause such an effect. The authors emphasize that along with a decrease in the activity of the AST and ALT enzymes responsible for the catabolic pathway of amino-

acids, a decrease in the plasma concentration of the main products of protein metabolism (creatinine and urea), may indicate a violation of protein catabolism in hens receiving 5 nm lipid-coated nanosilver preparation.

Potassium is one of the main intracellular elements in the animal body. In the course of our experiment, its level in the blood serum of hens increased with administration of AgNPs/SPH at the dose of 0.4 mg. Nevertheless, Ognik et al. (2017) obtained opposite results indicating that 5 nm colloidal nanosilver blocked potassium absorption into the blood of broiler chickens. The authors suggest that K and Ag compete for ion channels involved in intestinal absorption of both elements since they both have similar atomic radii and the same oxidation number. In such case, the difference in the results obtained may be due to the different response of metabolism of broiler chickens and laying hens to different nanosilver preparations, as well as the presence of polymer/inorganic hybrid stabilizer in our experiment, which could have significantly reduced the rate of release of silver ions and their oxidation.

Our experiment showed that the nanosilver preparation in SPH carriers caused an increase in serum glucose after its single administration at the dose of 0.2 mg per hen per day. Nanosilver preparation at the dose of 0.4 mg per hen generated a much less marked effect comparable with the tendency towards increasing serum glucose. Such effect may be due to the temporary blocking by small-sized (5 nm) nanosilver of glucose consumption by the cells as a result of oxidative stress, which was observed on HepG2 cell culture by Lee et al. (2015).

The increase in serum cholesterol after the single administration of 0.2 mg nanosilver preparation per hen per day, which was revealed in our study, may be due to the effect of the nanosilver on the hen's endocrine profile. The study by Katarzyńska-Banasik et al. (2021) proved the effect of hydrocolloid nanosilver on ovarian steroidogenesis and thyroid hormone metabolism in domestic hens. The 100-ppm dose of 50 nm hydrocolloid nanosilver in feed caused the increase in triiodothyronine, which can stimulate cholesterol synthesis in the liver of hens (Lopez et al., 1984).

In our experiment, a higher dose of the nanosilver preparation (0.4 mg per hen per day) reduced serum cholesterol only after three-time administration. This is consistent with the results of the studies on broiler chickens that received AgNPs at doses of 2, 4, 6, 8 and 10 ppm/kg of diet (Elkloub et al., 2015). In the study by Saleh & El-Magd (2018), the use of nanosilver preparation at the dose of 50 ppm/kg of feed for 12 days caused a decrease in not only cholesterol levels but also in triglycerides and serum glucose in broiler chickens.

In our experiment, the 0.2 mg nanosilver preparation in SPH carriers per hen per day caused rather contradictory changes of serum calcium and phosphorus, though such changes were not observed at the dose of 0.4 mg per hen per day. These results are consistent with data obtained by Ognik et al. (2016) stating that oral administration of hydrocolloid silver nanoparticles (22 nm) and lipid-coated silver nanoparticles (5 nm) to broiler chickens at the dose of 5 mg/kg of body weight per day did not significantly affect the blood levels of most macro and microelements, except for iron.

ALT, AST, ALP and GGT are important enzymes that reflect the functional state of the liver and other vital organs. In our experiment, their activity in the blood serum of laying hens depended on both the dose and the frequency of administration of the nanosilver preparation. However, the pattern of such changes has not been stated. Ahmadi (2012) also detected the changes in ALT, AST, and ALP activity in the blood serum of broiler chickens fed with commercial AgNPs supplements (14.0 ± 0.8 nm) during the entire growth period (1–42 days) at doses of 20, 40, and 60 ppm AgNPs/kg of feed. However, feeding broiler chickens with the commercial nanosilver preparation at doses of 300, 600 and 900 ppm did not produce such an effect (Ahmadi, 2009). Elkloub et al. (2015) registered a decrease in AST activity in the blood serum of broiler chickens fed with AgNPs at doses of 2, 4, 8, and 10 ppm/kg of the diet, except for the dose of 6 ppm AgNPs.

The activity of liver enzymes usually increases when they are released from hepatocytes into the bloodstream due to cell damage. Such liver damage may also include damage caused by nanosilver preparations (Parang & Moghadamnia, 2018; Elalfy et al., 2020). The decrease in AST and ALP activity may be due to inactivation resulting from the affinity of AgNPs to thiol (–SH) groups, thereby causing a change in the functional state of proteins, as well as inactivation of amino transaminases (Sulaiman et al., 2015). Nevertheless, we believe that such changes could have happened during the development of adaptive responses in the bodies of laying hens, which is consistent with the data of the enzymatic activity in their blood after a three-time administration of nanosilver preparation at the end of the experiment. Wang et al. (2015) believe that high concentrations of nanosilver can cause obvious cytotoxicity due to the production of reactive oxygen intermediates. This can result in the oxidative stress in the bird's body (Ahmadi, 2012). In contrast, low levels of nanosilver tend to interrupt biological processing and signaling and disrupt normal organelle functions at the cellular level, which is not associated with reduced viability or cell death (Wang et al., 2015). Therefore, oral administration of AgNPs is less dangerous compared to parenteral administration, since in this case only 4–18% of silver nanoparticles are absorbed into the blood of animals (Sulaiman et al., 2015).

Important factors determining the bioavailability of silver nanoparticles to animals are their size and ability to aggregate (Sarhan & Hussein, 2014). The large size and aggregation of nanoparticles significantly limit their oral bioavailability. This method involves exposing nanosilver to different pH in the digestive system of hens, namely: pH ~ 4.8 in the crop content, pH ~ 3.5 in the gizzard stomach and pH > 6 in the intestines (Svihus, 2014), as well as electrolytes present in gastric acid and intestinal gland. The study of the aggregation capability of nanosilver particles about 10 nm in size, which were stabilized with citrate or tea extract, showed that while maintaining physiological pH and concentrations of sodium chloride, glucose and glutamine, the aggregation will develop at pH and C_{NaCl} = 10 mm. This largely determines the toxicity of such nanoparticles, which is reduced with increasing aggregation (Bélteky et al., 2019; Chen et al., 2020).

At the same time, large silver nanoparticles or developed aggregates of nanoparticles cannot penetrate into the circulatory system of the body through the intestinal epithelium. Due to this, the bioavailability of many silver preparations is reduced. Individual particles of our preparation have a relatively small size (~31–47 nm), which is the key to their penetration through the intestinal epithelium into the circulatory system of hens and ensuring high bioavailability of the preparation. Finally, biocompatible and biodegradable SPH carriers contribute to a more effective and prolonged action of the nanosilver preparation and largely protect the hen's body from the toxic effects of metal nanoparticles.

Studies have shown that a higher dose of AgNPs/SPH preparation (0.4 mg compared to 0.2 mg per hen per day) had a significantly lower effect on the metabolic state of poultry. This result cannot be yet clearly explained, but it is consistent with the data provided in the research with laboratory rats by Adeyemi & Adewumi (2014), which indicate that oral administration of nanosilver at the dose of 100 mg/kg of body weight led to more significant changes in blood biochemical parameters than doses of 1000 and 5000 mg/kg, respectively.

Conclusions

A nanosilver preparation containing biocompatible and biodegradable carriers filled with small silver nanoparticles was obtained by synthesizing a grafted silica / polyacrylamide hybrid and using it as hydrophilic matrix for the in situ formation of AgNPs. A pure hybrid formed compact, almost spherical particles with d = 20–45 nm and their fractal aggregates similar to grapes in an aqueous medium. The reaction of borohydride reduction of silver ions in hybrid solutions proceeded at a high rate and yield and was completed within 20 minutes. This led to the appearance in the hybrid polymer "corona" of small spherical AgNPs 1.2–9.6 nm in size. The formation of AgNPs was accompanied by the swelling of hybrid particles (up to 31–47 nm) and the "detachment" of polymer grafts from the silica surface. In the resulting preparation, silver nanoparticles remained stable to aggregation and were not released from hybrid carriers under normal physiological conditions.

The nanosilver preparation in polymer / inorganic hybrid carriers did not significantly affect the morphological blood parameters of hens when administered at doses of 0.2 and 0.4 mg per hen per day three times a month at an interval of 10 days. Administration of the nanosilver preparation to laying hens at the dose of 0.2 mg per animal unit per day at a 10-day interval had a more significant effect on the biochemical parameters

of the blood than the dose of 0.4 mg. The more frequently the nanosilver preparation was administered to the laying hens, the less marked was their influence on the metabolic profile of the blood serum. The results of the study proved the absence of toxicity of nanosilver preparation in polymer / inorganic carriers for laying hens and, thus, can be used as the basis for determining its optimal doses and mode of use in the event of infectious disease as an alternative to antibiotics in poultry farming.

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