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Toxicity of nanosilver in intragastric studies: Biodistribution and metabolic effects

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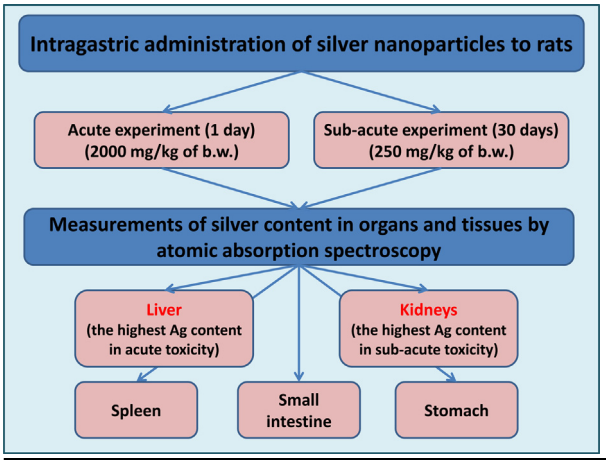
HIGHLIGHTS

- Acute and sub-acute intragastric administrations of AgNPs do not result in rats' lethality or pronounced toxic effects.
- Hematological and biochemical parameters do not change after rats' exposure to AgNPs.
- Silver absorbs from the gastrointestinal tract and distributes to various secondary organs.
- The liver and kidneys are the major target organs for AgNPs.
- Silver is efficiently excreted from rats.

GRAPHICAL ABSTRACT



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ABSTRACT

The unique physicochemical properties of silver nanoparticles explain their extensive application in consumer goods, food, and medicinal products. However, the biological effects of nanosilver after peroral exposure of mammals are still debatable. This study describes the biodistribution and biological action of 12 nm non-coated silver nanoparticles intragastrically administered to male rats after acute (single exposure) and sub-acute (multiple exposures over 30 days) toxicity experiments. The daily doses were 2000 and 250 mg/kg of body weight for single and multiple administrations, respectively. Silver tissue detection was conducted by elemental analysis with the help of atomic absorption spectroscopy. An estimation of the state of exposed animals was made and the dynamics of hematological and biochemical parameters of rats was studied. It was demonstrated that single and multiple administrations resulted in silver accumulation in the liver, kidneys, spleen, stomach, and small intestine. After both one- and repeated-dose exposures, the highest Ag contents were detected in the liver (0.87 ± 0.37 μg/g of organ) and kidneys (0.24 ± 0.02 μg/g of organ). The concentrations of silver detected in tissues were far smaller

Abbreviations: AAS, atomic absorption spectroscopy; AgNPs, silver nanoparticles; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GIT, gastrointestinal tract; HPLC, high performance liquid chromatography; ICP-MS, inductively coupled plasma mass spectrometry; PBS, 50 mM potassium phosphate buffer, pH 7.4, containing 0.1 M NaCl; ROS, radical oxygen species.

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than the administered doses (<99%), indicating its efficient excretion from the organism. Acute and subacute exposures caused no animal mortality or signs of toxicity, manifested as changes in outward appearance or notable deviations in behavior or locomotor activity. Postmortem study revealed no visible pathomorphological abnormalities of internal organs. Hematological indices and biochemical parameters of the treated rats did not differ from those of the vehicle control animals. Overall, it can be concluded that nanosilver is able to be absorbed from the gastrointestinal tract into the bloodstream and accumulate in the secondary organs of rats. It showed no distinct toxicity under the experimental conditions of this study.

1. Introduction

Nanosilver is nowadays one of the most widely known nanomaterials, with a number of production and practical applications. In ionic and colloidal form, silver has been utilized since the nineteenth century mainly as an antimicrobial agent for medical treatment (Chen and Schluesener, 2008; Ahamed et al., 2010; Prabhu and Poulose, 2012; Wei et al., 2015). Today, silver nanoparticles (AgNPs) are used in a huge number of consumer goods, including textiles, kitchenware, food storage bags and containers, clothes, sanitary and cosmetic products, baby nursing bottles, toys, and many others (Seltenrich, 2013; Yang and Westerhoff, 2014; Gaillet and Rouanet, 2015). As a food additive, nanosilver is used as an anti-caking agent, as well as to clarify beverages (Gaillet and Rouanet, 2015). Nanosilver's antibacterial properties also make it suitable for use in water purifying systems, bedding, paints, surface coatings in washing machines, and refrigerators (You et al., 2012). Due to their well-known bacteriostatic, antiviral, and antifungal action, AgNPs are extensively used as dietary supplements, for wound dressings and dental hygiene, and in implants and other medical devices (Mijnendonckx et al., 2013). AgNP applications in the treatment of breast cancer, leukemia, and different carcinomas have been proposed in several investigations (Wei et al., 2015). Beside this, AgNPs are involved in industrial processes as catalysts, and are exploited in electronics and optics (Wijnhoven et al., 2009; Zeng et al., 2010).

In a number of investigations, an appreciable release of nanosilver from functionalized materials – including clothes, textiles, and paints – into the environment has been demonstrated (Reidy et al., 2013; Yang and Westerhoff, 2014). Close day-to-day contact with goods containing nanosilver, as well as regular consumption of food products and medications containing AgNPs, is causing significant concern about the potential adverse effects of human exposure to nanoparticulate silver. The mechanism of toxicity induced by nanoparticulate silver is still unresolved, but is believed to be related to the generation of radical oxygen species (ROS) and oxidative stress resulting in apoptosis, lipid peroxidation, DNA and protein damage, membrane leakage, and other dysfunctions (Volker et al., 2013; McShan et al., 2013).

The main problem in the assessment of silver biological activity is to distinguish the effects promoted by silver in nanoparticulate form from those of dissolved Ag^+ ions released from nanoparticle surface in aqueous media. For this reason, many *in vivo* toxicological studies include simultaneous experiments on administration of soluble silver salts ($AgNO_3$, CH_3COOAg, etc.). The adverse effects of silver nanoparticles arise due to their large surface area and high reactivity. Ionic silver that is also highly reactive can diffuse across biological barriers and penetrate into cells to achieve equilibrium concentrations (Reidy et al., 2013), whereas nanoparticles absorbed through endocytosis accumulate in cells, followed by possible ion release and subsequent destructive action (this effect is known as the "Trojan horse"). Additionally, changes that can occur in the digestive tract – aggregation, interaction with biological environment molecules, or enzyme transformation – and affect the physicochemical characteristics of nanomaterial and, consequently, its bioavailability should be taken into consideration (Bohmert et al., 2014; Gaillet and Rouanet, 2015). However, the biological response to both silver forms was shown to be quite similar in many studies (Ahamed et al., 2015).

Toxic effects of ingested nano-dispersed silver and the underlying mechanisms of these effects, as well as the biodistribution of silver nanoparticles through organs and tissues, are discussed in a number of studies and reviews (Johnston et al., 2010; Reidy et al., 2013; Hadrup and Lam, 2014; Gaillet and Rouanet, 2015). A dose-dependent bioaccumulation of silver nanoparticles in different organs was demonstrated in studies of Kim et al. (2008) and Kim et al. (2010). A comparison of toxic effects and biodistribution of silver in nano- and microform was carried out by Park et al. (2010). Tissue clearance of silver nanoparticles was investigated by Lee et al. (2013). Lethality and general toxicity signs were estimated in studies of Maneewattanapinyo et al. (2011) and Kim et al. (2013).

Yu et al. (2014), studied the effects of silver nanoparticles on pregnant dams and embryo-fetal development in rats. After peroral exposure to AgNPs (~6 nm) at concentrations of 100, 300, and 1000 mg/kg/day, animals were examined for teratogenic and embryotoxic effects. No treatment-related maternal or fetal lethality or toxicity signs were observed in the exposed animals. However, alterations in glutathione reductase and catalase activities and a decrease in glutathione content in maternal liver tissues after exposure to AgNPs indicated oxidative stress. The effects of nanosilver on oxidative stress and inflammation were also investigated by Ebabe Elle et al. (2013). Rats that perorally received silver nanoparticles at a daily dose of 500 mg/kg of body weight for 81 days displayed an increase in the production of liver and cardiac oxygen radicals (30% and 41%, respectively) and raised levels of inflammatory cytokines. Exposure to AgNPs resulted in liver damage and abnormalities in lipid metabolism, with the liver and heart being the most sensitive organs to the revealed effects.

Lee et al. (2012), studied the translocation of silver nanoparticles (~8 nm with 250 mg/kg dosing) to offspring after subchronic oral exposure of pregnant dams. Silver content was found in the brain, lungs, liver, and kidneys of pups by inductively coupled plasma mass spectrometry (ICP-MS) and electron microscopy on the 4th day after parturition. High accumulation of AgNPs in the tissues of the pups was demonstrated confirming that AgNPs can overcome the blood-placental barrier.

Besides studies of nanosilver toxicity on animal models, there is a toxicological experiment on human volunteers in the literature. Munger et al. (2014) studied *in vivo* human biodistribution, bioprocessing, and toxicity of AgNPs (~5–10 and ~33 nm) at a daily dose of 100 and 480 μg/day, respectively. Fourteen-day oral dosing of nanoparticulate colloidal silver caused no evident metabolic or hematologic changes, and no alterations in urine profile, physical state, or imaging morphology. No clinically important toxicity markers were detected, and no significant changes in pulmonary reactive oxygen species or pro-inflammatory cytokine generation

were found. Therefore, the authors concluded that there was no detectable toxicity caused by AgNPs in the conducted tests.

Loeschner et al. (2011), Hadrup et al. (2012), van der Zande et al. (2012), and Park (2013) conducted studies comparing the biodistribution and toxic effects of ionic and nanoparticulate silver. The main conclusions were that in its particulate form, silver was not bioavailable and was shown to rapidly eliminate with feces. Besides, the authors concluded that there was toxicity of ionic silver, but not of AgNPs.

It is worth noticing that according to estimations of Hadrup and Lam (2014) the extent of nanosilver adsorption varies from 0.4 to 18% for mammals and is about 18% for humans, which is not a particularly low figure.

Overall, the examples of *in vivo* studies discussed above demonstrate that silver nanoparticles undesirably interact with mammals and induce different degrees of various toxic effects with pronounced size – and dose – dependencies. Such factors as exposure time, surface charge, and coating also determine aspects of their biological action. Peroral toxicity of silver nanoparticles is most often manifested as changes in blood biochemistry and hematological indices, altered enzyme activities, and deviations in the state of the experimental animals. The principal mechanisms of toxicity can be attributed to ROS formation and oxidative stress mediated by AgNPs. Silver ions released from nanoparticles are considered to contribute to the discovered adverse effects in exposed animals. In some cases, ionic silver was found to cause similar but more pronounced effects and higher tissue biodistribution compared with nanoparticles after peroral exposure. AgNPs accumulated in all organs examined, with GIT organs (stomach, small intestine, and liver) and kidneys typically being the main targets.

All the observed effects indicate that there should be cause for serious concern about human health problems related to peroral exposure to nanosilver contained in numerous products, medications, and consumer goods. There is an unquestionable need for more investigations of silver-related biological effects. A clear understanding of the *in vivo* behavior of AgNPs administered perorally (or intragastrically) is critically important. To contribute to the understanding of nanosilver toxicity, we studied the biokinetics and biological effects of non-coated 12 nm silver nanoparticles in acute and sub-acute experiments in rats.

2. Materials and methods

2.1. Materials

We used 10 nm silver nanoparticles purchased from Nanostructured and Amorphous Materials Inc., USA, with the characteristics presented in Table 1. Food starch, Tween-80, nitric acid, palladium nitrate, magnesium nitrate, and all other chemicals were of HPLC gradient grade and purchased from Sigma–Aldrich, USA, or Panreac, Spain. Milli-Q deionized water (Millipore, USA) was used to prepare solutions.

Table 1
Characteristics of silver nanoparticles.

Parameter	Value
Purity	99%
Appearance	Black grey nanopowder
NPs size (data from manufacturer)	10 nm
NPs size (experimental data by AFM)	12.3 ± 5.9 nm
Shape	Spherical
Aggregation (experimental data by AFM)	Non-aggregated
Specific surface area (according to BET)	9–11 m^2/g
Coating	No
Density	10.491 g/cm^3
Water solubility	Insoluble

2.2. Characterization of silver nanoparticles by atomic force microscopy (AFM)

Silver nanoparticles were dispersed in deionized water at a concentration of 1 mg/ml and sonicated in a Sonopuls HD 3100 ultrasonic homogenizer (Bandelin, Germany) for 15 min. A 5 μl drop of the obtained solution was placed on the surface of freshly cleaved mica (MICA V-1 GRADE, SPI Supplies, USA) and exposed for 4 min at room temperature. After removal of the excess water by filter paper, the sample was dried.

AgNPs were scanned on a SmartSPM atomic force microscope (AIST-NT, Russia) in the tapping mode using fpN01HR cantilevers (Nanotuning, Russia) with a tip radius curvature of about 1 nm. The obtained images were analyzed using Gwiddion software (Czech Metrology Institute, Czech Republic). The height of the silver nanoparticles was determined by examining cross-sections of the images.

2.3. Animals

For single and multiple administrations of silver nanoparticles, we used adult Sprague-Dawley male rats weighing 210–250 g purchased from the Animal Breeding Facility Branch of the Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Pushchino, Russia. The animal experiments were implemented in compliance with Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes. Conditions of animal housing and all manipulations related to the exposure are described in our previous paper (Hendrickson et al., 2014).

2.4. Preparation of AgNPs dispersions for administration to animals

A mixture of silver nanoparticles and 1% aqueous starch solution containing 0.1% Tween-80 was sonicated for 5 min using a Sonopuls HD 3100 ultrasonic homogenizer. Animals were intragastrically exposed to the dispersion of silver nanoparticles via gavage not later than 15 min after the preparation.

2.5. Experimental design

Single and multiple doses of AgNPs were administered for 1 day (with a 14-day recovery period) and 30 days, respectively. The dose used for the single exposure was 2000 mg/kg of body weight; for multiple exposures, the daily dose was 250 mg/kg of body weight over a 30 day period.

A total of 54 rats were used for single exposure. Three experimental groups contained 27 animals divided into three cages (nine rats in each cage, according to the day of biomaterial sampling—1st, 7th, and 14th). Twenty-seven animals from the untreated control group were given a vehicle of equivalent volume.

For multiple administrations, 54 rats were randomized into seven groups. Thirty-six silver-treated animals were subdivided into six cages (six rats in each cage, according to the dose and day of biomaterial sampling—7th, 18th, and 30th). The untreated control group contained 18 rats that were given a vehicle of equivalent volume.

The state of the animals was monitored throughout the whole experiment. Their body weights were determined prior to the administration and then daily. The activity of the animals, their general appearance, food and water consumption, and external manifestations of toxicity were observed daily.

Euthanasia, necropsia, and biomaterial sampling

All operations were carried out as described previously (Hendrickson et al., 2014). After euthanasia of rats by CO_2, the following organs and tissues were withdrawn: brain, lungs, thymus, heart, stomach, kidneys, adrenal glands, liver, spleen, small intestine, testicles, skin, adipose tissue, muscle tissue, and blood.

2.7. Hematologic and biochemical parameters

Hematological indices (the hematocrit; the content of leukocyte; the absolute content and percentage of lymphocyte, monocyte, and granulocyte; the content, mean volume, and distribution of erythrocyte; the content and mean volume of platelet; the hemoglobin concentration, it's mean content, and concentration in the erythrocyte) and biochemical parameters (the activity of alkaline phosphatase, aspartate aminotransferase (AST), and alanine aminotransferase (ALT); the de Ritis coefficient (AST/ALT); and the content of total bilirubin, cholesterol, triglycerides, total protein, globulins, albumins, calcium, inorganic phosphate, glucose, and urea) were measured by automated analyzers as described in our previous paper (Hendrickson et al., 2014).

2.8. Tissue preparation for detection of silver by atomic absorption spectroscopy

3 ml of 50 mM potassium phosphate buffer, pH 7.4, containing 0.1 M NaCl (PBS), was added to 0.5–1 g of an organ or a tissue, and the mixture was homogenized using a T 25 digital ULTRA-TURRAX disperser equipped with an S25N10G disperser unit (IKA® Werke, Germany) until complete disappearance of tissue fragments. Then 10 ml of 65% nitric acid was added to 1 g of a homogenized sample. The obtained mixture was digested in a speedwave MWS-2 microwave pressure digestion system (BERGHOF, Germany) equipped with a carousel of 10 digestion vessels DAP-60S to obtain a completely transparent solution. After that, the solutions were cooled and diluted with an appropriate volume of deionized water.

2.9. Atomic absorption spectroscopy measurements

The detection of silver was performed by atomic absorption spectrometry using a high-resolution AAnalyst 800 (PerkinElmer, USA) equipped with a graphite furnace atomization unit. The AAS was operated with 99.99% argon. Atomic absorption of silver was detected at 328.07 nm. Samples were atomized at 1500 °C over 3 s. Palladium and magnesium nitrates were used as modifiers.

2.10. Data analysis

A statistical analysis of parametric data (weights of internal organs, food and water uptake, hematological and biochemical parameters, and silver content in organs and tissues) was carried out according to the standard protocol described by Hendrickson et al. (2014).

3. Results

3.1. Characterization of silver nanoparticles

Characterization of the nanoparticles used in toxicological experiments is very important to ensure adequate correlation between the observed biological effects and physicochemical characteristics and the dosing of *in vivo* administered nanomaterial. Atomic force microscopic investigations were carried out in order to estimate the size, shape, and aggregation state of the silver nanoparticles used for the exposures. AFM data indicated that the studied sample of AgNPs consisted of single nanoparticles of regular spherical shape with different apparent diameters (Fig. 1). The mean size of AgNPs calculated from cross-sections of the images was 12.3 ± 5.9 nm, which is close to the size declared by the manufacturer.

3.2. Determination of the percentage of silver recovery by atomic absorption spectroscopy

Prior to the detection of silver in organs and tissues, we calculated the percentage of its recovery in these matrices. For that purpose, homogenates of all organs and tissues were intentionally spiked with weighted samples of silver nanoparticles. After microwave digestion of biomaterial, elemental analysis of silver by atomic absorption spectroscopy was conducted. For each organ or tissue, five samples were analyzed. The percentage of the recovered silver was estimated relative to the control preparation—AgNPs added to PBS. According to data on quantitative determination of silver in spiked samples by AAS, the recovery values were high and varied from 98.7 to 99.3% (Table 2).

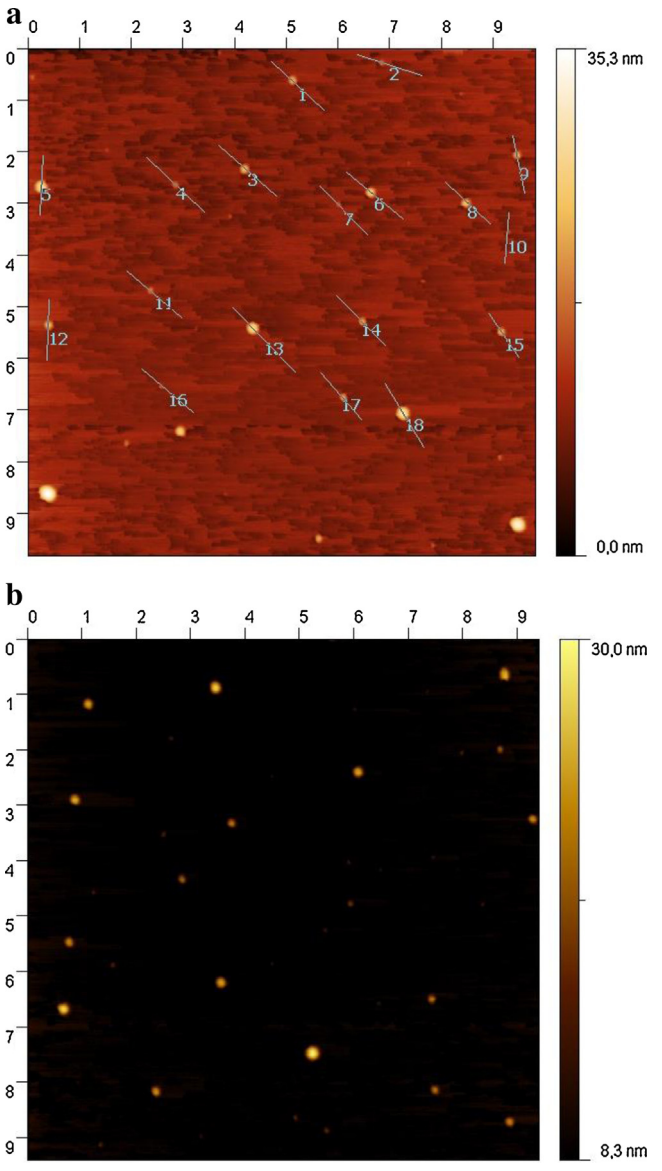

Fig. 1. (a) and (b). Initial (a) and processed (b) AFM images of silver nanoparticles.

Table 2
Quantitative determination of silver nanoparticles in spiked organs and tissues ($n = 5$).

Organ or tissue	(%) of recovery
Lung	99.2 ± 0.8
Liver	98.9 ± 0.8
Kidneys	99.1 ± 0.7
Spleen	99.3 ± 0.7
Adrenal glands	98.8 ± 0.8
Brain	98.7 ± 0.8
Testicles	98.7 ± 0.8
Stomach	98.9 ± 0.8
Small intestine	99.3 ± 0.5
Heart	99.2 ± 0.7
Thymus	98.9 ± 0.7
Skin	99.1 ± 0.6
Adipose tissue	99.4 ± 0.7
Muscle tissue	98.9 ± 0.8
Blood serum	99.2 ± 0.7

In order to avoid any mistakes related to silver losses during sample preparation, these data were taken into consideration while interpreting the results of silver analysis in the organs and tissues of the exposed animals.

3.3. Distribution and biological action of silver nanoparticles after single exposure

Fig. 2 shows the data on biodistribution of silver, revealed by atomic absorption spectroscopy in homogenates of organs and tissues after single administration of AgNPs to rats (2000 mg/kg of b.w.). Time-dependent silver accumulation was observed for the liver, kidneys, spleen, stomach and small intestine. On the first day of the recovery period, silver was detected in only two organs of the gastrointestinal tract. On the seventh day, silver was found in the small intestine, as well as in the liver, kidneys, and spleen. Fourteen days post-dosing, silver was not detected and was considered completely eliminated from the rats.

During the entire 14 day recovery period, no animal deaths or signs of toxicity manifested as changes in outward appearance and notable deviations in behavior or locomotor activity were monitored. As compared with vehicle-treated animals, there was neither loss in body weight nor loss in the absolute weights of internal organs (data are not reported). Food and water uptake remained the same in the case of the control and treated rats. Postmortem study revealed no visible pathomorphological changes of internal organs. Hematological indices and biochemical parameters of the rats after acute exposure to silver nanoparticles did not differ significantly (more than 20%) from those of the untreated animals (Tables 3 and 4).

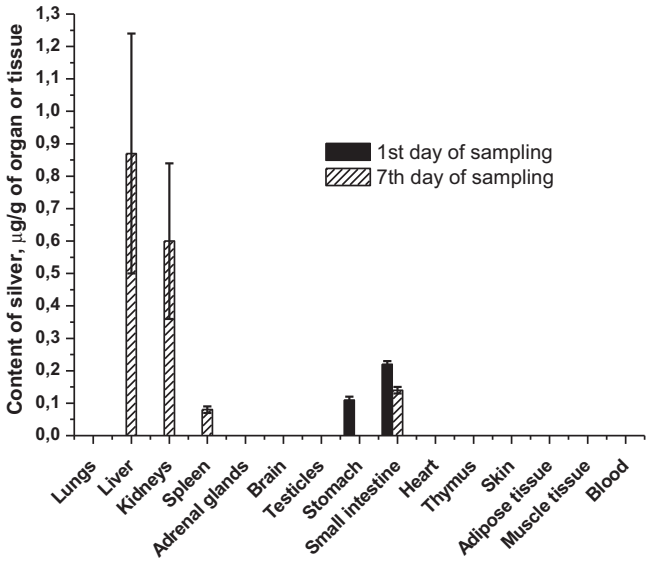

Fig. 2. Content of silver in homogenates of organs and tissues after single administration (2000 mg/kg of body weight).

3.4. Distribution and biological action of silver after multiple exposures

Silver content in organs and tissues of rats underwent multiple exposures to nanoparticles, is presented in Fig. 3. During multiple exposures, we found silver accumulation in the liver, kidneys, spleen, stomach, and small intestine. The highest concentrations were observed in the kidneys on the 18th and 30th days of the exposure. In each organ, the elemental silver content was quantitatively comparable at all time points.

Within 30 days of the administration of AgNPs, we observed no lethality or toxicity symptoms. Animal body weight, absolute weights of internal organs, and water and food uptake were the same for the treated and control animals (data are not reported). Autopsy of rats revealed no pathomorphology of the internal organs. Results of biochemical and hematological analyses are presented in Tables 5 and 6. At all time points, there were no significant differences in the measured hematological indices and blood biochemistry.

4. Discussion

Due to their unique antimicrobial properties, silver nanoparticles are one of the most widespread nanomaterials used in food, medical devices, pharmaceuticals, and consumer goods. Although a number of studies have examined the distribution, bioprocessing, and biological effects of ingested nanosilver, it is still debated whether silver nanoparticles have adverse effects in mammals after peroral exposure. A remaining uncertainty about the mechanisms of its action and a lack of strong evidence of potential human health risks or benefits can be attributed to limited statistics obtained in peroral toxicological studies of AgNPs. The biological action of nanoparticulate silver after acute exposure has not been studied extensively, so a clear understanding of the *in vivo* behavior of AgNPs administered perorally (or intragastrically) is critically important. In the present study, the biodistribution and biological effects of silver nanoparticles intragastrically administered to rats in acute and sub-acute toxicity experiments were assessed.

A mixture consisting of a 1% starch solution and 0.1% Tween-80 was used for the preparation of AgNPs dispersions. This vehicle was previously found to provide stability of nanoparticle dispersion within enough time for intragastric administration to rats *via* gavage (Hendrickson et al., 2014). A dose of 2000 mg/kg of b.w. in a single exposure was selected in accordance with silver toxicological investigations described in the literature (Kim et al., 2013) and our previous studies of nanoparticle toxicity (Hendrickson et al., 2014). During a 14 day recovery period after acute exposure, rats were closely observed for clinical manifestations of toxicity. For the repeated-dose toxicity experiment, a daily dose equal to 1/8 of the acute dosing (250 mg/kg of b.w.) was administered over 30 days. There was no recovery period after this experiment; rats were sacrificed the day after the last administration. Observations on the animals' state were made daily over the entire period of multiple exposures.

Table 3
Hematological parameters of rats on the 1st, 7th, and 14th days after single-dose administration of silver (2000 mg/kg of body weight).

Parameter	1st day		7th day		14th day	
	Control rats	Treated rats	Control rats	Treated rats	Control rats	Treated rats
Leukocytes (10^9/L)	12.10 ± 2.40	14.52 ± 3.18	11.31 ± 2.58	15.60 ± 3.03	14.15 ± 5.77	13.60 ± 5.28
Lymphocytes (10^9/L)	8.51 ± 1.72	10.83 ± 2.24	8.14 ± 1.37	11.95 ± 2.84	9.28 ± 3.88	9.73 ± 4.08
Monocytes (10^9/L)	0.36 ± 0.11	0.40 ± 0.09	0.39 ± 0.26	0.52 ± 0.15	0.68 ± 0.53	0.53 ± 0.38
Granulocytes (10^9/L)	3.23 ± 0.84	3.28 ± 0.98	2.79 ± 1.34	3.13 ± 0.60	4.20 ± 1.76	3.33 ± 1.01
Lymphocytes (%)	70.24 ± 4.26	74.73 ± 2.57	72.51 ± 6.84	76.08 ± 5.86	65.68 ± 7.27	70.75 ± 5.63
Monocytes (%)	3.18 ± 0.41	2.95 ± 0.55	3.45 ± 1.19	3.47 ± 1.11	4.74 ± 3.45	3.95 ± 0.73
Granulocytes (%)	26.59 ± 4.26	22.32 ± 2.94	23.93 ± 5.76	20.45 ± 4.82	29.59 ± 4.59	25.30 ± 5.69
Erythrocytes (10^{12}/L)	7.85 ± 1.13	7.50 ± 0.48	9.74 ± 0.86	8.11 ± 0.68	8.13 ± 1.27	8.11 ± 0.45
Hemoglobin (g/L)	156.63 ± 21.61	136.67 ± 4.50	167.00 ± 11.99	148.00 ± 6.11	168.88 ± 21.13	143.83 ± 6.15
Hematocrit (%)	48.19 ± 6.68	40.72 ± 1.80	59.76 ± 2.72	45.98 ± 2.42	47.83 ± 5.47	44.00 ± 1.66
Mean erythrocyte volume(μm^3)	61.49 ± 1.65	54.47 ± 2.72	61.63 ± 3.04	56.92 ± 2.42	59.35 ± 4.12	54.35 ± 1.54
Mean hemoglobin content in the erythrocyte (pg)	19.91 ± 0.44	18.23 ± 0.97	20.23 ± 0.87	18.27 ± 0.98	20.86 ± 1.15	17.72 ± 0.67
Mean hemoglobin concentration in the erythrocyte (g/L)	324.38 ± 3.66	353.33 ± 5.28	329.00 ± 5.78	321.30 ± 5.20	352.50 ± 7.60	326.6 ± 4.5
Distribution of erythrocytes (%)	14.59 ± 1.55	13.92 ± 0.74	15.13 ± 1.12	13.25 ± 0.79	13.99 ± 0.98	13.60 ± 0.57
Platelets, (10^9/L)	675.00 ± 129.48	522.00 ± 37.24	440.00 ± 30.85	549.50 ± 128.02	468.00 ± 158.17	660.17 ± 65.05
Mean platelet volume (μm^3)	6.80 ± 0.26	6.95 ± 0.30	6.91 ± 0.21	6.93 ± 0.34	7.05 ± 0.34	6.52 ± 0.08

Table 4
Biochemical parameters of rats on the 1st, 7th, and 14th days after single silver administration (2000 mg/kg of body weight).

Parameter	1st day		7th day		14th day	
	Control rats	Treated rats	Control rats	Treated rats	Control rats	Treated rats
Albumin (g/L)	32.83 ± 1.24	30.84 ± 0.95	39.93 ± 6.33	34.79 ± 3.93	43.30 ± 3.05	33.67 ± 3.46
ALT (U/L)	55.13 ± 11.76	55.23 ± 7.19	56.73 ± 6.26	56.35 ± 6.58	58.68 ± 5.48	51.67 ± 2.72
AST (U/L)	n/d[a]	164.92 ± 18.22	192.28 ± 40.81	133.61 ± 50.70	232.10 ± 28.60	143.07 ± 47.97
De Ritis coefficient (AST/ALT)	n/d	2.99 ± 2.53	3.43 ± 1.03	2.37 ± 0.70	3.96 ± 0.81	2.77 ± 17.61
Cholesterol (mmol/L)	2.52 ± 0.45	2.34 ± 0.25	2.07 ± 0.41	2.34 ± 0.18	2.15 ± 0.11	2.33 ± 0.19
Calcium (mmol/L)	n/d	2.36 ± 0.51	2.75 ± 0.75	2.46 ± 0.09	2.84 ± 0.28	2.63 ± 0.15
Glucose (mmol/L)	4.80 ± 0.80	4.76 ± 0.56	4.88 ± 0.79	3.44 ± 0.34	4.25 ± 1.10	3.67 ± 0.36
Phosphate (mmol/L)	2.73 ± 0.06	2.94 ± 0.28	3.15 ± 0.48	3.26 ± 0.15	3.38 ± 0.26	3.60 ± 0.39
Bilirubin (μmol/L)	n/d	4.72 ± 0.99	5.00 ± 4.05	6.77 ± 1.52	7.00 ± 2.38	6.53 ± 1.41
Total protein (g/L)	64.35 ± 0.98	63.76 ± 3.70	69.58 ± 11.01	69.85 ± 2.79	78.94 ± 2.95	70.05 ± 3.61
Triglycerides (mmol/L)	1.68 ± 0.46	1.87 ± 0.84	2.91 ± 0.90	1.39 ± 0.34	1.88 ± 0.17	1.33 ± 0.29
Urea (mmol/L)	n/d	5.17 ± 0.41	6.87 ± 0.43	6.77 ± 0.66	7.17 ± 0.54	6.46 ± 0.55
Globulins (g/L)	31.52 ± 1.94	32.93 ± 0.07	29.66 ± 4.96	35.06 ± 3.75	34.70 ± 1.34	36.38 ± 0.15
Albumin/Globulin	1.05 ± 0.10	0.94 ± 0.07	1.35 ± 0.10	1.01 ± 0.23	1.29 ± 0.05	0.93 ± 23.47

[a] not detected.

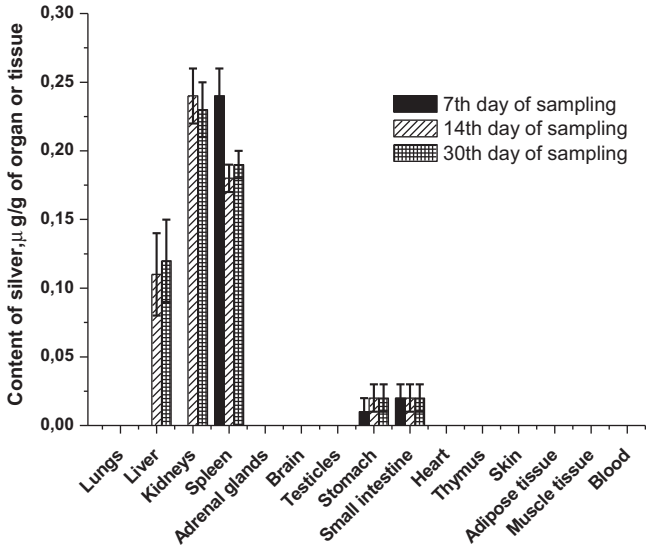

Fig. 3. Silver content in homogenates of organs and tissues after multiple administrations (250 mg/kg of body weight).

Three time points were selected to study the bioaccumulation of silver after a single high-dose administration of Ag nanoparticles: the 1st, 7th, and 14th days. One day after the single-dose administration, silver was found in the deposition site organ, the stomach (0.11 ± 0.01 μg/g of organ), as well as in the small intestine (0.22 ± 0.01 μg/g of organ). Seven days later, no Ag was detected in the stomach and a smaller amount of silver (0.14 ± 0.01 μg/g of organ) remained in the small intestine (see Fig. 2). At this point, AgNPs had translocated to secondary organs and were found in the liver, kidneys, and spleen. The highest AgNPs concentration was found in the liver (0.87 ± 0.37 μg/g of organ), which was evidently the main target organ for this nanomaterial under acute exposure conditions. The order of silver bioaccumulation was liver > kidneys > spleen > small intestine. Two weeks after the exposure, nanosilver was detected in none of organs or tissues tested. It should be noted that there was a gradual decrease of detected silver in the small intestine over the course of the two weeks of observation (0.22 ± 0.01 μg/g of organ, 0.14 ± 0.01 μg/g of organ, and none, on the 1st, 7th, and 14th days, respectively); this indicates the efficient excretion of nanomaterial from the gastrointestinal tract.

To study the biokinetics of silver nanoparticles as a result of the repeated-dose exposures, we measured their content in organs and tissues at three time points, on the 7th, 18th, and 30th days of multiple administrations. The results of AAS detection are presented in Fig. 3. During the entire observation period, silver

Table 5
Hematological parameters of rats on the 7th, 18th, and 30th days of multiple silver administrations (250 mg/kg of body weight).

Parameter	7th day		18th day		30th day	
	Control rats	Treated rats	Control rats	Treated rats	Control rats	Treated rats
Leukocytes (10^9/L)	11.92 ± 2.28	11.58 ± 2.57	13.95 ± 2.45	15.48 ± 4.56	19.42 ± 3.90	16.42 ± 3.33
Lymphocytes (10^9/L)	8.65 ± 1.85	8.02 ± 1.77	10.23 ± 2.44	11.27 ± 2.63	13.97 ± 3.72	12.43 ± 2.98
Monocytes (10^9/L)	0.45 ± 0.16	0.37 ± 0.08	0.45 ± 0.15	0.47 ± 0.20	0.72 ± 0.08	0.43 ± 0.08
Granulocytes (10^9/L)	2.82 ± 0.83	3.20 ± 1.18	3.27 ± 0.48	3.75 ± 1.90	4.73 ± 0.67	3.55 ± 1.00
Lymphocytes (%)	72.60 ± 6.12	69.52 ± 6.36	72.47 ± 6.32	73.67 ± 5.70	71.17 ± 5.43	75.18 ± 5.49
Monocytes (%)	3.85 ± 0.95	3.18 ± 0.57	3.25 ± 0.94	3.03 ± 0.77	3.80 ± 0.79	2.77 ± 0.18
Granulocytes (%)	23.55 ± 5.32	27.30 ± 6.12	24.28 ± 5.74	23.30 ± 5.35	25.03 ± 5.07	22.05 ± 5.34
Erythrocytes (10^{12}/L)	148.50 ± 26.71	155.00 ± 10.77	170.33 ± 4.97	187.00 ± 16.24	182.50 ± 10.78	182.67 ± 12.06
Hemoglobin (g/L)	7.51 ± 1.54	8.59 ± 0.80	9.10 ± 0.46	10.37 ± 0.92	9.97 ± 0.42	9.95 ± 0.92
Hematocrit (%)	44.90 ± 6.33	47.00 ± 3.04	53.30 ± 4.05	56.93 ± 5.33	54.33 ± 2.57	53.73 ± 3.18
Mean erythrocyte volume (μm^3)	60.53 ± 5.17	54.88 ± 1.92	58.88 ± 6.67	54.98 ± 1.48	54.60 ± 3.14	54.22 ± 2.86
Mean hemoglobin content in the erythrocyte (pg)	19.83 ± 1.17	18.03 ± 0.58	18.70 ± 0.78	17.98 ± 0.36	18.27 ± 0.40	18.35 ± 0.90
Mean hemoglobin concentration in the erythrocyte (g/L)	32.88 ± 1.23	32.92 ± 0.66	32.03 ± 2.17	32.83 ± 0.43	33.55 ± 1.89	33.93 ± 0.59
Erythrocyte distribution (%)	15.93 ± 1.65	13.95 ± 0.64	14.55 ± 0.90	13.83 ± 1.12	12.65 ± 0.52	12.97 ± 0.60
Platelets (10^9/L)	509.83 ± 135.04	471.33 ± 107.75	438.00 ± 131.15	426.67 ± 23.04	539.83 ± 100.01	583.00 ± 58.34
Mean platelet volume (μm^3)	7.13 ± 0.33	6.75 ± 0.33	6.70 ± 0.30	6.82 ± 0.25	6.73 ± 0.23	6.73 ± 0.34

Table 6
Biochemical parameters of rats on the 7th, 18th, and 30th days of multiple silver administrations (250 mg/kg of body weight).

Parameter	7^{th} day		18^{th} day		30^{th}day	
	Control rats	Treated rats	Control rats	Treated rats	Control rats	Treated rats
Albumin (g/L)	35.67 ± 3.89	34.11 ± 1.11	35.67 ± 3.89	34.91 ± 1.27	35.67 ± 3.89	35.52 ± 1.75
Alkaline phosphatase (U/L)	473.58 ± 104.09	493.05 ± 46.92	473.58 ± 104.09	420.39 ± 37.32	473.58 ± 104.09	424.95 ± 37.49
ALT (U/L)	60.93 ± 9.01	62.17 ± 5.78	60.93 ± 9.01	64.23 ± 3.56	60.93 ± 9.01	79.39 ± 22.96
AST (U/L)	164.37 ± 30.50	144.48 ± 15.38	164.37 ± 30.50	142.25 ± 13.23	164.37 ± 30.50	130.20 ± 69.50
De Ritis coefficient (AST/ALT)	2.72 ± 0.70	2.32 ± 0.13	2.72 ± 0.70	2.22 ± 0.17	2.72 ± 0.70	1.84 ± 0.94
Cholesterol (mmol/L)	2.72 ± 0.34	2.70 ± 0.63	2.72 ± 0.34	2.80 ± 0.13	2.72 ± 0.34	2.83 ± 0.16
Calcium (mmol/L)	2.23 ± 0.39	2.61 ± 0.22	2.23 ± 0.39	2.19 ± 0.30	2.23 ± 0.39	2.29 ± 0.31
Glucose (mmol/L)	5.21 ± 1.03	5.18 ± 1.50	5.21 ± 1.03	5.03 ± 0.48	5.21 ± 1.03	5.60 ± 0.57
Phosphate (mmol/L)	2.93 ± 0.42	2.79 ± 0.40	2.93 ± 0.42	2.85 ± 0.27	2.93 ± 0.42	2.86 ± 0.35
Bilirubin (μmol/L)	2.72 ± 2.24	1.82 ± 0.22	2.72 ± 2.24	1.91 ± 0.19	2.72 ± 2.24	1.62 ± 0.67
Total protein (g/L)	70.63 ± 6.93	73.63 ± 2.51	70.63 ± 6.93	77.24 ± 2.04	70.63 ± 6.93	76.08 ± 5.03
Triglycerides (mmol/L)	1.98 ± 0.78	1.63 ± 0.34	1.98 ± 0.78	1.58 ± 0.51	1.98 ± 0.78	2.50 ± 0.95
Urea (mmol/L)	6.37 ± 0.81	6.37 ± 0.20	6.37 ± 0.81	6.59 ± 0.36	6.37 ± 0.81	6.54 ± 0.52
Globulins (g/L)	35.48 ± 7.10	39.52 ± 2.69	35.48 ± 7.10	42.33 ± 1.63	35.48 ± 7.10	40.57 ± 4.01

was detected in organs of the gastrointestinal tract with no time-dependencies in concentrations that remained stable over 30 days. At the beginning of the exposure, besides the gastrointestinal tract, silver NPs were found only in the spleen (0.24 ± 0.02 μg/g of organ), but by the 18th day they had also distributed to the liver and kidneys (0.11 ± 0.03 and 0.24 ± 0.02 μg/g of organ, respectively). The same biodistribution tendency was observed at the end of multiple exposures: the highest silver concentration was found in the kidneys (0.23 ± 0.02 μg/g of organ), while the spleen and the liver contained 0.19 ± 0.01 and 0.12 ± 0.03 μg/g of organ, respectively.

Overall, the pattern of silver bioaccumulation was similar in both the acute and sub-acute toxicity experiments. A comparison of the amounts of silver administered and those detected in organs and tissues shows that the majority (>99%) of nanomaterial is cleared from rats. Such a low level of nanomaterial content could be attributed to poor absorption of AgNPs following intragastric administration.

The biological effects caused by single and multiple administrations of AgNPs were similar. Regardless of the multiplicity of silver administration, all animals survived and displayed no distinct abnormalities that could be interpreted as toxicity indications. No differences related to nanosilver exposure were recorded in serum biochemical parameters (presented in Tables 4 and 6) or hematological indices (see Tables 3 and 5).

The results obtained in this study are in accordance with the data of Maneewattanapinyo et al. (2011), who studied the biological effects of 16 nm silver nanoparticles singly administered to mice in a dose more than twice as high as the one in this study (5000 mg/kg of b.w.). Hematological and biochemical indicators and parameters such as mortality, body weight gain showed no differences between control and silver-treated groups. Histopathological analysis revealed no lesions in the internal organs. The authors estimated that the LD50 value of AgNPs was greater than 5000 mg/kg of b.w. and concluded that AgNPs are relatively safe for short-term peroral administration. Similar observed effects can be seen in the study performed by Kim et al. (2013), who determined the toxicity promoted by 10 nm AgNPs in an acute experiment with a dosage equal to that in our study (2000 mg per kg of b.w.). None of the rats died or showed any abnormal signs after the exposure.

Park (2013) also conducted a comparative study of the toxicokinetics and toxicities of AgNPs (~8 nm in doses of 2 and 20 mg/kg of b.w.) and silver ions in rats after a single peroral exposure. Although he also observed no deaths or clinical symptoms of toxicity mediated by nanosilver (8 nm particles, in doses of 2 or 20 mg/kg of b.w.), the study did find increased platelet counts and mean platelet volume, as well as elevated alanine aminotransferase and creatinine, in the treated rats. Regarding biodistribution, AgNPs were detected in all organs tested – namely, the lungs, liver, and kidney – in a dose-dependent manner. The liver, as the major target organ, contained 0.23 ± 0.11 μg/g of organ in this case, while the kidneys and lungs contained 0.148 ± 0.101 and 0.08 ± 0.01 μg/g of organ, respectively. Despite the fact that we used much higher doses of AgNPs, the

concentrations of silver in Park's study were comparable with the values found in our study. This obviously can be explained by limited absorption of the majority of the ingested AgNPs in the gastrointestinal tract and their efficient elimination. Indeed, Park (2013) confirmed high fecal excretion of nanoparticulate silver from rats (388.3 ± 118.6 μg of Ag were detected in feces 24 h after the treatment) indicating its low bioavailability. It should be noted that distribution of Ag in the liver, kidneys, and lungs was higher when it was administered in ionic form.

Sub-acute oral toxicity of AgNPs was discussed in several studies whose results are partially comparable with ours. Although Kim et al. (2008) reported no distinct toxic effects related to nanosilver (60 nm), a number of blood biochemical and hematological coefficients were altered in animals treated with all doses (30, 300, 1000 mg/kg of b.w.). Silver accumulated in the stomach, testicles, brain, kidneys, liver, lungs, and blood in a clear dose-dependent manner, with the maximum concentration in the liver and kidneys. Liver damage caused by AgNPs became apparent due to alterations in alkaline phosphatase and cholesterol values. Serious histopathological changes in the liver confirmed AgNPs adverse effects on this organ. Nevertheless, there were no significant changes in the body weight of rats relative to the doses of AgNPs.

Loeschner et al. (2011) repeatedly administered silver acetate (AgAc) and nanoparticles (14 nm) to rats over the course of 28 days and showed that both substances distributed across organs in a similar manner. The highest concentrations of Ag after administration of silver nanoperticles were detected in the small intestine, stomach, kidneys, and liver (listed in decreasing order). A high amount of silver was excreted in feces, while Ag excretion in urine was negligible (<0.1%). After exposure to AgNPs, the absolute silver content in tissues was lower than after exposure to ionic silver, indicating higher fecal excretion of AgNPs.

van der Zande et al. (2012), studied the biodistribution and toxicity of silver nanoparticles (20 nm non-coated, or 15 nm PVP-coated AgNPs, 90 mg/kg of b.w.) and ionic silver from silver nitrate (9 mg/kg of b.w.) after 28 days of oral exposure in rats. It was shown that silver was distributed to all examined organs with the maximum level detected in the liver and spleen for both the AgNPs and $AgNO_3$ treatments. However, the absorption in the blood and organs was higher after exposure to $AgNO_3$ than to AgNPs. The authors assumed that ingested silver is more bioavailable in the ionic form than the particulate form, but noted that the general effects of exposure to AgNPs appeared to be similar to the effects of exposure to silver nitrate. Interestingly, using single-particle ICP-MS, the authors detected nanoparticulate silver in $AgNO_3$-treated animals and suggested that silver nanoparticles could form *in vivo* from the relevant salt.

Lee et al. (2013) found no signs of infection or adverse effects after 28 days of peroral intake of AgNPs (10 or 25-nm, 100 and 500 mg/kg of b.w.). However, despite the gradual decrease of accumulated silver in most of the tissues during the recovery period, there was no effective silver clearance from the brain and testicles within a 4-month recovery period. This phenomenon could indicate that there are obstacles preventing Ag from transporting out of these organs, and the important role of blood–brain and blood–testicle biological barriers in the clearance of AgNPs. The obtained results indicated that the size of AgNPs did not influence silver distribution across tissues.

Hadrup et al. (2012) compared toxicological effects of 14-nm silver nanoparticles and AgAc over 16 days administered via gavage in daily doses of 2.25, 4.5, or 9 mg/kg of b.w. for 28 days. In the case of AgNPs administrations, general toxicity findings (state of the treated animals, feed intake, etc.) were similar to those described in the current investigation. Only minor alterations in hematology and a decrease of the absolute weight of some organs of silver-treated rats were detected. It should be noted, however, that Ag doses were much lower than in our study (up to 9 mg/kg b.w.). The authors concluded that there was no adverse effect of AgNPs. But in the case of AgAc administrations, body weight loss and changes in some hematological parameters were found. The authors concluded that there was toxicity of ionic silver, but not of AgNPs.

Park et al. (2010) observed silver biodistribution in the brain, lungs, liver, kidneys, and testicles after 14 days of oral administration of 22-, 42-, 71-, and 323 nm AgNPs to mice (dose of 1 mg/kg b.w.). Silver accumulation had a clear size-dependent effect: the concentration of Ag increased as the size of nanoparticles decreased. None of the organs tested contained silver after the administration of 323 nm Ag nanoparticles. The maximum amounts were detected in the liver, kidneys, and testicles. In this study, body weight and organ/body weight ratios also did not differ between the control and treated animals.

5. Conclusions

In the present study, we investigated the biological action and tissue biodistribution of 12 nm silver nanoparticles in acute and sub-acute toxicity experiments. It was demonstrated that single and multiple intragastric administrations did not result in animal lethality or toxicity symptoms. Animal body weight, absolute weights of internal organs, water and food uptake, locomotor activity, and behavioral features were the same for silver-treated and vehicle control animals. Autopsy of rats revealed no pathomorphology of the internal organs. At all time points studied, there were no significant differences in the measured hematological indices and blood biochemistry in any of the groups of animals. Bioaccumulation of silver in the liver, kidneys, and spleen provided evidence of absorption from the gastrointestinal tract with infiltration into the bloodstream and translocation into secondary organs. The liver and kidneys contained the maximum silver concentrations and appeared to be the major target organs for silver nanoparticles. However, the amounts of accumulated nanomaterial were negligible compared with the applied dosing, indicating that Ag is efficiently excreted from rats. Overall, it can be concluded that silver nanoparticles distributed to various organs with no pronounced toxic effects in either acute or sub-acute experimental conditions.

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