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Systemic and immunotoxicity of silver nanoparticles in an intravenous 28 days repeated dose toxicity study in rats☆

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ABSTRACT

Because of its antibacterial activity nanosilver is one of the most commonly used nanomaterials. It is increasingly used in a variety of both medical and consumer products resulting in an increase in human exposure. However, the knowledge on the systemic toxicity of nanosilver is relatively limited. To determine the potential systemic toxicity of silver nanoparticles (Ag-NP) with different sizes (20 nm and 100 nm) a 28-days repeated dose toxicity study was performed in rats using intravenous administration. The toxic effect of the 20 nm Ag-NP was performed using the bench mark dose (BMD) approach.

Treatment with a maximum dose of 6 mg/kg body weight was well tolerated by the animals. However, both for 20 nm and 100 nm Ag-NP growth retardation was observed during the treatment. A severe increase in spleen size and weight was present which was due to an increased cell number. Both T and B cell populations showed an increase in absolute cell number, whereas the relative cell numbers remained constant. At histopathological evaluation brown and black pigment indicating Ag-NP accumulation was noted in spleen, liver, and lymph nodes. Ag-NP was also detected incidentally in other organs. Clinical chemistry indicated liver damage (increased alkaline phosphatase, alanine transaminase, and aspartate transaminase) that could not be confirmed by histopathology. Hematology showed a decrease in several red blood cell parameters.

The most striking toxic effect was the almost complete suppression of the natural killer (NK) cell activity in the spleen at high doses. Other immune parameters affected were: decreased interferon-γ and interleukin (IL)-10 production by concanavalin-A stimulated spleen cells, increased IL-1β and decreased IL-6, IL-10 and TNF-α production by lipopolysaccharide stimulated spleen cells, increase in serum IgM and IgE, and increase in blood neutrophilic granulocytes. For the spleen weight a critical effect dose of 0.37 mg/kg body weight (b.w.) could be established. The lowest critical effect dose (CED) for a 5% change compared to control animals was observed for thymus weight (CED05 0.01 mg/kg b.w.) and for functional immune parameters, i.e. decrease in NK cell activity (CED05 0.06 mg/kg b.w.) and LPS stimulation of spleen cells (CED05 0.04 mg/kg b.w.). These results show that for nanosilver the most sensitive parameters for potential adverse responses were effects on the immune system.

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1. Introduction

Silver nanoparticles (Ag-NP) are frequently used in consumer and medical products because of their antimicrobial activity [1–7]. Despite the rapidly growing presence of silver-containing nanoproducts on the market [5–8], there is only limited information on the possible risks of exposure to silver nanoparticles. In recent reviews evaluating the risk assessment of silver nanoparticles, different knowledge gaps were identified, including toxicokinetics [4,9,10]. Similar data gaps were identified for the possible registration of nanosilver as a substance under the EU REACH regulation [11]. Previously we identified the spleen and liver as main target organs for various nanomaterials after intravenous administration [12–14]. This can be explained by the fact that these organs are part of the reticulo-endothelial system (RES) that has removing foreign agents from the circulation as one of its functions. *In vitro* studies demonstrated that Ag-NP are cytotoxic by their

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effect on cellular metabolism and membrane integrity, and inhibit embryonic stem cell differentiation [15].

In repeated dose toxicity inhalation studies animals were exposed for 28 and 90 days in a whole body exposure chamber using a nanoparticle generator for the production of silver nanoparticles [16–18]. In the first evaluation of the 28-days toxicity study with 12–15 nm Ag-NP general toxicity parameters, i.e. body weight, hematology and blood clinical chemistry values, did not indicate systemic toxicity at the doses investigated [16]. At histopathological examination only incidentally some liver alterations (cytoplasmic vacuolisation or hepatic necrosis) were observed. Indications for local reactions in the lung were absent [16]. The tissue concentration of silver as determined with atomic absorption spectrometry showed a dose dependent increase in the lung and low concentrations in liver and brain. In the 90-days repeated dose inhalation toxicity study lung function was impaired, and indications for lung inflammation were noted by cellular and protein content of the bronchoalveolar lavage fluid (BALF) and lung histopathology [17]. In a 28-days oral toxicity study with 60 nm silver nanoparticles by Kim et al. [19], a dose dependent increase in silver content was observed in all tissues investigated (testis, kidneys, liver, brain, lung, stomach, blood). There was no effect on body weight although some blood biochemistry parameters (alkaline phosphatase, cholesterol) indicated some liver damage [19]. In a recent 28-days oral toxicity study Van Der Zande et al. [20] found that the silver concentration in organs was highly correlated with the $Ag+$ ion content of the nanosilver suspension. The highest concentrations were observed in liver and spleen [20]. In general the studies using either inhalation or oral exposure did not show severe systemic toxicity, probably because of the relatively low systemic exposure to nanosilver that is due to low absorption of the nanosilver from the lung and gastrointestinal tract (GI-tract).

To avoid limited systemic exposure due to the cellular barriers present in lung and GI-tract, we used intravenous administration of nanosilver to evaluate its potential systemic toxicity. We investigated the *in vivo* toxicity of two sizes of Ag-NP (20 nm and 100 nm) in a repeated dose toxicity study after intravenous administration for 28 days. Special emphasis was on the effects of the spleen as part of the immune system as our previous results showed accumulation of Ag-NP in the spleen [13].

2. Materials and methods

2.1. Animals

Male and female Wistar derived WU rats, 8 weeks of age, obtained from Harlan Nederland BV, Horst, The Netherlands, were used. Animals were bred under SPF conditions and barrier maintained during the experiment. Drinking water and conventional feed were provided *ad libitum*. Husbandry conditions were maintained according to all applicable provisions of the national laws, Experiments on Animals Decree and Experiments on Animals Act. The experiment was approved by an independent ethical committee prior to the study according to the Dutch legislation.

2.2. Chemicals

BioPure silver nanoparticles, 20 nm diameter and 100 nm diameter in 2 mM phosphate buffer were obtained from NanoComposix, San Diego, CA, USA. The nanosilver dispersions provided were characterized with minimal agglomeration or aggregation. The nanoparticle characteristics are presented in Table 1.

2.3. Experimental design

The present study was performed according to the general principles of OECD guideline 407 ("Repeated dose 28-day oral study in rodents") with some adjustments. The animals were exposed to nanosilver particles via intravenous administration. Instead of the usual design of three dose groups (low, mid and high dose) and one control group, we increased the number of dose groups, at the expense of the group sizes, i.e. while keeping the total number of animals the same. With this design, and by applying the bench mark dose (BMD) approach [21], an improved characterization of the dose response is obtained without increasing the number of animals.

Table 1
Characteristics of BioPure silver nanoparticles.[a]

Parameter	20 nm CTH1359	100 nmCTH1409
Size ± SD (nm)	21.0 ± 2.6	107 ± 7.6
Coefficient of variation (%)	12.2	7.1
Size range (min–max diameter)	12.4–27.9	92.8–128.4
Number of particles (ml^{-1})	3.9×10^{13}	3.8×10^{11}
Surface area per particle (nm^2)	1.40×10^{3}	3.62×10^{4}
Surface area (nm^2/ml)	5.49×10^{16}	1.37×10^{16}
Silver concentration (mg/ml)	2	2.6
Zeta potential (mV)	−40.8	−38.7

[a] Information provided by manufacturer nanoComposix, San Diego, USA.

Because of the known accumulation in the spleen [13] additional tests with spleen cells were performed for possible immunotoxicological properties of nanosilver. In addition not all organs indicated in OECD 407 were evaluated by histopathology.

Animals were divided in 11 groups and were intravenously injected (tail vein) once a day for 28 days to either 1 ml nanosilver dispersion or vehicle control (phosphate buffer). Dose levels and experimental design are presented in Table 2. The experiment was performed in two phases, the first being the 20 nm particle treatments followed by the 100 nm particle treatments.

Rats were weighed prior to and weekly during the experiment. Individual body weights (BW) of the rats were used to calculate the individual dose levels.

2.4. Histopathology

At 24 h after the last injection, rats were anaesthetized with isoflurane (Isoflu®, AST Pharma, Oudewater, The Netherlands) in oxygen and subsequently euthanized by drawing blood from the abdominal aorta. After collecting blood, rats were evaluated macroscopically for gross lesions. The following organs were examined and sampled: adrenals, brain, bone marrow, small intestines (duodenum, jejunum, ileum), large intestines (caecum, colon, rectum), heart, kidney, liver, lung, lymph nodes (mesenteric and popliteal), esophagus, pituitary, spleen, stomach, testis (or ovaria), and thymus. Organ weights were determined except for bone marrow, pituitary and gastrointestinal tract.

All tissue samples were fixed in 4% neutral buffered formaldehyde (10% formalin) and routinely processed (Hematoxylin and Eosin staining) for histopathology.

Routine histopathology was performed for the following treatments: 20 nm Ag-NP 6 mg/kg b.w. (3 male and 3 female animals) and 2 mg/kg b.w. (3 male and 3 female animals), 100 nm Ag-NP 6 mg/kg b.w. (4 male and 4 female animals), and phosphate buffer control treated animals (6 male and 6 female animals).

2.5. Hematology

Hematology and clinical chemistry was performed on blood samples obtained at autopsy. Blood was collected in EDTA-coated tubes. Hematological parameters included white blood cell (WBC) count, red blood cell (RBC) count, hemoglobin (Hb), hematocrit (Ht), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red blood cell distribution width (RDW), hemoglobin distribution width (HDW), platelet (PLT) count, and mean platelet volume (MPV). All hematology parameters in the blood samples were determined in an Advia 120 Hematology Analyzer (Siemens, Germany). In addition, blood smears were prepared for visual evaluation.

2.6. Clinical chemistry

After collection of blood serum and storage at −20 °C the following parameters were determined: albumin (ALB), alkaline phosphatase (ALP), alanine

Table 2
Dose levels and experimental design of 28 days repeated dose toxicity study with Ag-NP.

Treatment	Dose (mg/kg b.w. per day)	*n* (M–F)
Phosphate buffer	0	2–2
Phosphate buffer	0	2–2
20 nm nanosilver	0.0082	2–2
20 nm nanosilver	0.0025	2–2
20 nm nanosilver	0.074	2–2
20 nm nanosilver	0.22	3–3
20 nm nanosilver	0.67	3–3
20 nm nanosilver	2	3–3
20 nm nanosilver	6	3–3
Phosphate buffer	0	2–2
100 nm nanosilver	6	4–4

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