

DOI 10.2478/pjvs-2014-0037

Original article

Effect of 28-day oral administration of silver nanocolloid on the peripheral blood leukocytes in mice

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Abstract

Silver nanoparticles, which have found a wide range of applications owing to their antimicrobial properties, are also recommended as dietary supplements in alternative medicine. Studies on rodents confirm that nanosilver is absorbed from the digestive tract into the bloodstream, which implies its possible interactions with leukocytes. The objective of the experiment discussed herein has been to determine the effect of 28-day oral administration of different doses (0.25, 2.5, 25 ppm) of commercial silver nanocolloid on hematological parameters, percentages of particular lymphocyte populations and activity of the peripheral blood leukocytes in mice. All the tested colloid doses decreased the counts of monocytes in the animals' blood and induced phenotypic modifications among lymphocytes: an increase in CD4+/CD8+ T cell distribution, a decrease in NK and NKT cell distribution (doses of 0.25 and 2.5 ppm) and an increased CD4+:CD8+ ratio (25 ppm). Silver nanocolloid also affected the activity of cells, depressing the proliferation of lymphocytes (0.25 ppm) and stimulating phagocytosis as well as the respiratory burst of granulocytes and monocytes (all doses). The results verify the influence of orally administered silver colloid on the peripheral blood leukocytes, at the same time implying the potential risk of developing an inappropriate immune response of an organism exposed to prolonged administration of this substance.

Key words: silver nanocolloid, oral administration, hematology, immunophenotyping, leukocyte activity, mice

Introduction

Owing to its antimicrobial properties, silver nanoparticles (AgNPs) are among the most broadly used nanomaterials, and a growing number of their applications in industries, medicine and households increases risk of the organisms' exposure to their adverse effects (Pelkonen et al. 2003, Kim et al. 2008).

Furthermore, for some time now, silver nanocolloids have been available as dietary supplements among other marketable pharmaceuticals. The gastrointestinal tract is also the principal route through which nanosilver used as a disinfectant penetrates into the organism. Changes in the red blood cell parameters observed in rodents administered AgNPs p.o. and the accumulation of nanosilver in all analyzed animal tis-

sues unquestionably reveal that they were able to penetrate from the gut to peripheral blood and subsequently spread throughout the whole organism (Pelkonen et al. 2003, Kim et al. 2008, Park et al. 2010). The main target of AgNPs entering an organism are the organs containing an extensive reticulo-endothelial system (such as spleen), as their natural function is to 'cleanse' an organism from foreign particles. Nanoparticles engulfed by macrophages residing in these organs cannot be destroyed, remain inside phagolysosomes, chronically stimulating cells towards oxidative burst, which leads to cells' death (Takenaka et al. 2001, Stebounova et al. 2011, Jovanović and Palić 2012). However, not only the peripheral lymphoid organs are sensitive to possible harmful effects of AgNPs. Once nanosilver has permeated from the digestive tract to the bloodstream, the peripheral blood leukocytes – due to the functions they perform – become also vulnerable to its potentially adverse effects. *In vitro* studies confirm a considerable influence of nanosilver on the peripheral blood phagocytes. Nanoparticles absorbed by them undergo ionization in acid compartments of cells, thus contributing to the development of oxidative stress and damage to mitochondria, which in turn leads to the phagocyte apoptosis or necrosis. Intensive synthesis of reactive oxygen species (ROS) is thus responsible for the cytotoxicity of high concentrations of AgNPs, while the lower levels of nanosilver lead to cells' activation, manifested by the changes in their morphology, increased expression of surface particles and synthesis of cytokines (Greulich et al. 2011, Park et al. 2011, Yang et al. 2012, Orłowski et al. 2013, Xu et al. 2013). The few completed investigations on the effect of AgNPs on the rodents immune system indicate that the range of effects produced by the nanosilver besides phagocytes may also encompass some populations of blood lymphocytes (Park et al. 2010, DeJong et al. 2013).

The aim of this study has been to determine the effect of 28-day alimentary administration of a commercial silver nanocolloid, recommended by its producer, *inter alia*, as a dietary supplement, on hematological parameters, percentages of particular lymphocyte populations and activity of leukocytes (proliferation of lymphocytes, phagocytosis, and the oxidative burst of neutrophils and monocytes) in the peripheral blood of mice.

Materials and Methods

Silver nanoparticles (AgNPs)

Nonionic colloidal silver solution (Nano-Tech, Poland) containing metallic silver nanoparticles (10-20 nm, according to the manufacturer) suspended in de-

mineralised water, at a concentration of 50 ppm was used as a source of AgNPs. Colloidal silver was dissolved in distilled water to produce solutions at three concentrations: 0.25 ppm, 2.5 ppm, and 25 ppm, which were then administered to mice as drinking water *ad libitum* for 28 days. The drinking water of the control animals was also distilled.

Mice

The experiment was performed on 40 male NMRI mice, aged 10-12 weeks, with body weight of 26-31 g. The animals were maintained on a 12-h light/dark cycle in a controlled temperature ($20 \pm 1^\circ\text{C}$) and supplied with rodent chow and water *ad libitum* throughout the experiment. Mice were divided randomly into four equal groups: control group (0) not receiving the silver solution, and three experimental groups administered the silver solution at the concentrations of 0.25 ppm, 2.5 ppm or 25 ppm. After 28 days of administration of the AgNPs mice were sacrificed. The animals were anaesthetised by inhalation of AErrane (isoflurane, Baxter Poland). Blood was collected by heart puncture. Samples from five individuals of each group were used for the MTT assay, and from the next five for the hematology and flow cytometry analysis. The experiment has been approved by the Local Ethics Committee.

Hematology

Hematology was performed on heparinised blood samples. Hematological parameters included white blood cell (WBC) count, lymphocyte (LYM) count, monocyte (MON) count, granulocyte (GRA) count, lymphocyte percentage (LYM %), monocyte percentage (MON %), granulocyte percentage (GRA%), red blood cell (RBC) count, hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red blood cell distribution width (RDW), platelet (PLT) count and mean platelet volume (MPV) were determined in Mythic 18 autoanalyzer (Orphee S.A., Geneva, Switzerland).

Immunophenotyping

100 μl of whole heparinised blood samples were transferred into individual tubes and erythrocytes were removed using OptiLyse C Lysing Solution (Beckman Coulter) according to the manufacturer's

instruction. Then the samples were washed twice with FACS buffer (FB, 1x Dulbecco's PBS without Ca²⁺ and Mg²⁺, supplemented with 2% fetal calf serum; both reagents purchased from Sigma-Aldrich), re-suspended in FB and incubated with fluorochrome-conjugated monoclonal antibodies for 30 min at 4°C in the dark. The following flow cytometry antibodies were used: FITC anti-mouse CD3 (specific to T cells), PE-Cy7 anti-mouse CD19 (B cells), PE anti-mouse CD4 (Th cells), PE-Cy5 anti-mouse CD8 (Tc cells) and APC anti-mouse CD49b (pan-NK cells). After incubation, the cells were washed in FB and flow cytometry analysis was performed using a FACSCanto II cytometer (Becton Dickinson Biosciences). The data were acquired by FACSDiva version 6.1.3. software (BD Biosciences) and analyzed by FlowJo software (Tree Star Inc., Stanford, CA, USA).

Isolation of lymphocytes

The whole heparinised blood was placed on a density gradient Histopaque 1077 (Sigma-Aldrich) and centrifuged at 400 g for 30 min at 20°C. The interface cells were collected and washed three times with the RPMI-1640 medium. Viability of isolated cells was evaluated by trypan blue exclusion and was determined to be greater than 95% in each case. The cells were suspended in RPMI-1640 medium containing 10% fetal calf serum (Sigma-Aldrich) and 1% antibiotic-antimycotic solution (Sigma-Aldrich), and dispensed into 96-well plates at a concentration of 1x10⁶ cells ml⁻¹. Then the cells were cultured at 37°C under a humidified atmosphere of 5% CO₂ and 95% air atmosphere and used for the MTT assay.

Proliferative response of blood lymphocytes (MTT assay)

Mitogenic response of lymphocytes was determined using the MTT colorimetric assay (Mosmann 1983). Cells were suspended in RPMI 1640 growth medium containing mitogens – concanavalin A (ConA, Sigma-Aldrich) in concentration of 5 µg ml⁻¹ as a T-cell mitogen or lipopolysaccharide from *Escherichia coli* (LPS, Sigma-Aldrich) in concentration of 10 µg ml⁻¹ as a B-cell mitogen and 100 µl of the suspension was added to each well of microtiter plates. The mixture was cultured for 72 h. After incubation, 10 µl of solution containing 7 mg ml⁻¹ of MTT (3-[4, 5 dimethylthiazoly-2-yl]-2,5-diphenyltetrazolium bromide, Sigma-Aldrich) in PBS were added and the plate was incubated for the next 4 h. The

supernatant was removed and 100 µl of DMSO was added to each well. The optical density was measured at a wavelength of 570 nm with 640 nm as a reference wavelength. All samples were tested in triplicate. The results of the proliferation assay were expressed as a stimulation index (SI), which was calculated by dividing the mean O.D. of stimulated cultures by the O.D. of the non-stimulated (control) cultures.

Activity of blood phagocytes

The blood granulocyte and monocyte phagocytic activity was measured in whole heparinised blood using a commercially available kit (Phagotest kit, Orpegen Pharma, Germany) according to manufacturer's instructions. Briefly, FITC-labelled opsonised *E.coli* bacteria were added to whole blood and incubated for 10 min at 37°C (experimental tube) or 0°C (negative control tube). After incubation, the reaction was stopped, erythrocytes were lysed and the DNA staining solution was added. Fluorescence of samples was measured using cytometer as described above. The test determines the percentage of phagocytizing cells, granulocytes and monocytes separately, and their phagocytic activity, i.e. the number of bacteria absorbed by a single cell in terms of mean fluorescence intensity (MFI).

Respiratory burst assay was performed using a commercially available kit (Phagoburst kit, Orpegen Pharma, Germany) according to manufacturer's instructions. Briefly, opsonised *E.coli* bacteria (experimental tube) or washing solution (negative control tube) were added to whole blood and incubated for 10 min at 37°C. Following incubation, dihydrorhodamine (DHR 123) was added for 10 min, erythrocytes were lysed and DNA staining solution was added. DHR 123 becomes fluorescent when oxidized by reactive oxygen species, and its fluorescence was measured by flow cytometry as described above. The test determines the percentage of active cells, as well as the respiratory burst intensity within a single cell in terms of mean fluorescence intensity (MFI).

Statistical analysis

Data were analysed statistically by one-way analysis of variance (ANOVA). Bonferroni's post test was used to determine differences between groups. Statistical evaluation of results was performed using GraphPadPrism software package.

Table 1. Hematology in mice after 28-day oral administration of silver nanocolloid.

AgNPs dose (ppm)/group	Parameter															
	WBC (x10 ³ /μl)	LYM (x10 ³ /μl)	GRA (x10 ³ /μl)	MON (x10 ³ /μl)	LYM (%)	GRA (%)	MON (%)	RBC (x10 ⁶ /μl)	HGB (g/dl)	HCT (%)	MCV (fl)	MCH (pg)	MCHC (g/dl)	RDW (%)	PLT (x10 ³ /μl)	MPV (fl)
0 (control)	3.975 ±1.099	3.25 ±0.465	0.5 ±0.15	0.28 ±0.13	81.05 ±8.408	12.625 ±5.625	6.625 ±1.542	7.015 ±1.171	11.05 ±1.769	33.85 ±5.69	48.3 ±0.374	15.75 ±0.311	32.6 ±0.725	13.375 ±0.499	589.5 ±121.944	6.2 ±0.112
0.25	4.675 ±1.245	3.9 ±1.26	0.68 ±0.27	0.18 ±0.05	82.16 ±7.509	14.523 ±3.92	3.896** ±0.658	7.07 ±0.718	12.26 ±0.661	36.56 ±2.534	51.7 ±2.766	17.38 ±1.098	33.68 ±1.584	14.98 ±1.306	423 ±197.661	6.88 ±0.638
2.5	2.906 ±0.727	2.501 ±0.416	0.38 ±0.1	0.09** ±0.05	85.45 ±1.457	12.225 ±3.881	2.743*** ±0.57	7.387 ±0.512	12.475 ±0.613	36.8 ±2.005	49.8 ±1.023	16.9 ±0.454	33.95 ±0.264	13.975 ±0.25	428.5 ±123.359	6.625 ±0.287
25	3.625 ±1.323	3.005 ±0.725	0.55 ±0.18	0.1* ±0.05	82.66 ±4.683	15.282 ±4.528	2.59*** ±0.563	7.254 ±0.439	12 ±0.2	35.72 ±2.042	49.24 ±0.594	16.58 ±0.975	33.66 ±1.722	13.24 ±0.114	329.2* ±142.68	6.82 ±0.881

Explanations:

Results are expressed as means ± SD (standard deviation)

The p-values (*p < 0.05, ** p < 0.01, *** p < 0.001) refer to the significant differences between control group and experimental groups

WBC – white blood cell count, LYM – lymphocyte count, MON – monocyte count, GRA – granulocyte count, LYM % – lymphocyte percentage, MON % – monocyte percentage, GRA% – granulocyte percentage, RBC – red blood cell count, HGB – hemoglobin, HCT – hematocrit, MCV – mean corpuscular volume, MCH – mean corpuscular hemoglobin, MCHC – mean corpuscular hemoglobin concentration, RDW – red blood cell distribution width, PLT – platelet count, MPV – mean platelet volume

Table 2. Analysis of lymphocyte phenotypes and T cell subpopulations (%) in mice after 28-day oral administration of silver nanocolloid.

AgNPs dose (ppm)/group	Marker/cell type							
	CD3 T cells	CD19 B cells	CD4+ Th cells	CD8+ Tc cells	CD4+CD8+ DP T cells	CD3-CD49b+ NK Cells	CD3+CD49b+ NKT cells	CD4:CD8 ratio
0 (control)	58.125 ±9.046	43.275 ±4.852	40.15 ±6.469	8.41 ±1.07	1.597 ±0.258	2.557 ±1.057	1.241 ±0.372	4.786 ±0.646
0.25	66.52 ±3.059	38.78 ±3.657	48.44 ±2.987	9.824 ±0.508	2.812** ±0.452	0.712** ±0.305	0.366*** ±0.141	4.936 ±0.301
2.5	45.65 ±12.848	52.825 ±7.91	35.225 ±8.843	7.922 ±1.723	2.535* ±0.632	1.351 ±0.706	0.584** ±0.252	4.462 ±0.594
25	59.7 ±1.509	42.64 ±1.467	44.8 ±3.717	7.6 ±0.28	1.74 ±0.121	2.42 ±0.382	1.47 ±0.253	5.897* ±0.477

Explanations:

Results are expressed as means ± SD (standard deviation)

The p-values (*p < 0.05, ** p < 0.01, *** p < 0.001) refer to the significant differences between control group and experimental groups

Results

Hematology

None of the tested doses of silver nanocolloid had any significant influence on the red blood cell parameters, total count of white blood cells, lymphocytes and granulocytes or percentages of lymphocytes and granulocytes. However, a significant decrease in the percentage of monocytes was observed in all groups receiving AgNPs (0.25 ppm at p < 0.01, the other groups at p < 0.001). Also, the total count of monocytes in the animals given nanosilver was lower than in

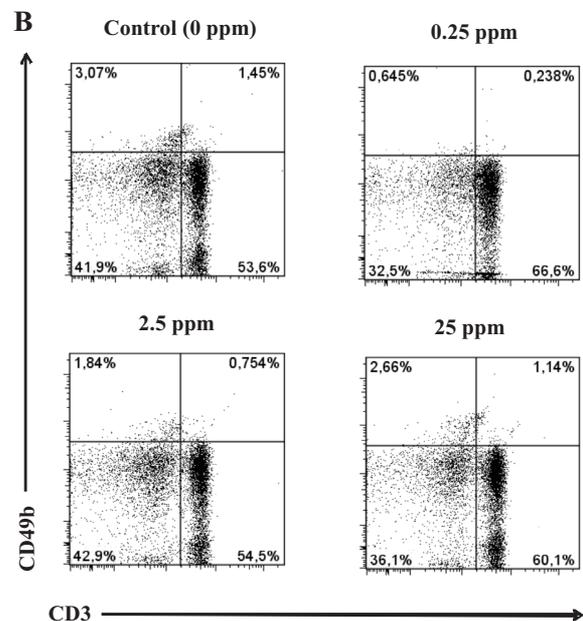
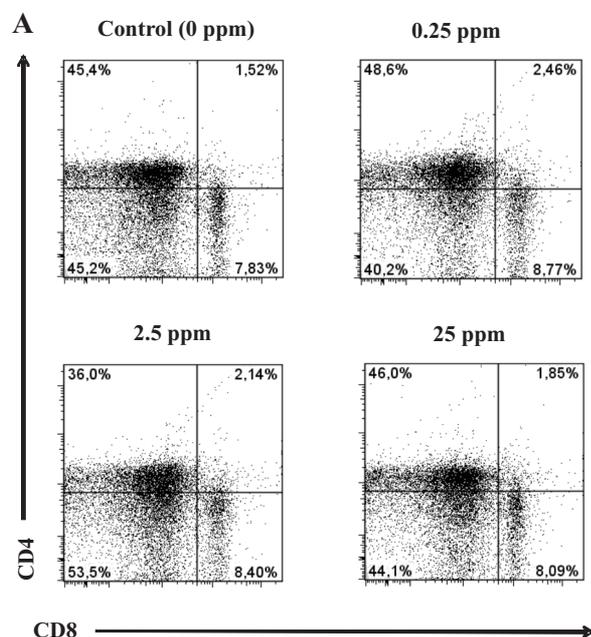


Fig. 1. The representative dot plot cytograms showing the distribution of CD4+CD8+ DP T cells (A) and CD49b+ cells (B) in mice after 28-day oral administration of silver nanocolloid.

the control group, although the differences were statistically significant only in the groups given 2.5 (p < 0.01) and 25 ppm (p < 0.05). Moreover, the level of platelets in all the experimental groups was lower than in the control group, but the decrease was significant only in response to the highest colloid dose (p < 0.05) (Table 1).

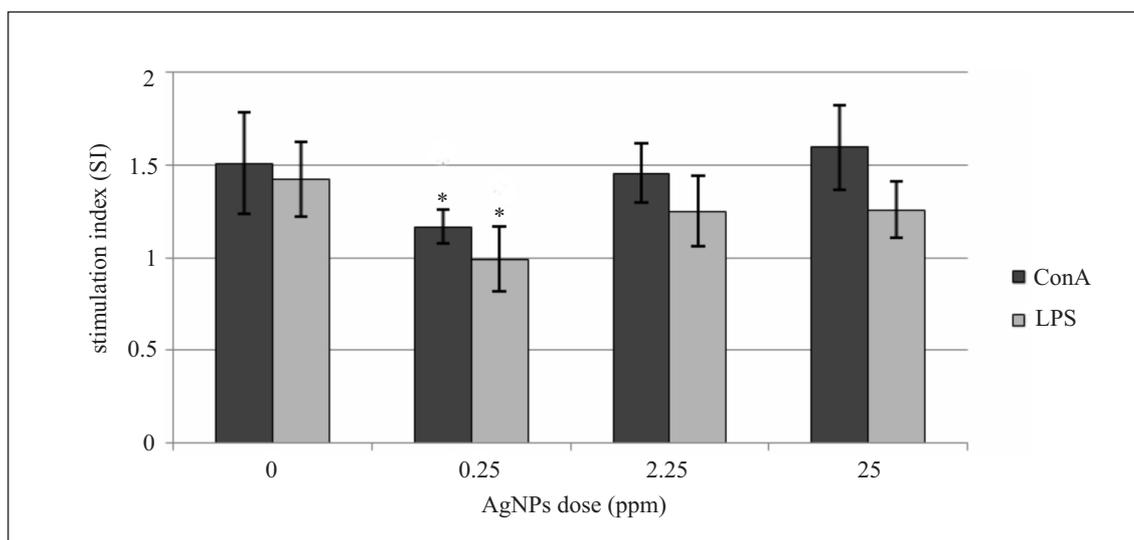


Fig. 2. Proliferative response of mice peripheral blood lymphocytes (SI) after 28-day oral administration of silver nanocolloid
 Explanations:

Results are expressed as means \pm SD (standard deviation) the p-values (* $p < 0.05$) refer to the significant differences between control group and experimental groups.

Table 3. Phagocytic activity of mice peripheral blood monocytes and granulocytes after 28-day oral administration of silver nanocolloid (% of active cells and MFI) – Phagotest.

AgNPs dose (ppm)/group	% of active cells		MFI	
	granulocyte	monocyte	granulocyte	monocyte
0 (control)	41.28 \pm 5.829	7.968 \pm 1.818	5968.6 \pm 467.597	5364.8 \pm 1290.105
0.25	41.02 \pm 8.098	9.186 \pm 1.925	11286.4 \pm 2373.32***	6008.2 \pm 429.117
2.5	24.675 \pm 5.929*	7.047 \pm 1.629	10100.75 \pm 822.083**	7160.25 \pm 777.449*
25	47.44 \pm 12.646	12.214 \pm 2.63*	9051.4 \pm 1516.904*	6660 \pm 433.756

Explanations (for Table 3. and 4.):

MFI – mean fluorescence intensity of a single cell

Results are expressed as means \pm SD (standard deviation)

The p-values (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) refer to the significant differences between control group and experimental groups

Immunophenotyping

Mice from the groups given the two lowest doses of silver nanocolloid were observed to have experienced a significant increase in the percentage of CD4+/CD8+ double positive T cells (DP T cells) versus the control group ($p < 0.01$ for group 0.25 ppm, $p < 0.05$ for 2.5 ppm) and a decrease in the percentage of CD3-CD49b+ NK (significant only in the group of 0.25 ppm at $p < 0.01$) and CD3+CD49b+ NKT cells (0.25 ppm at $p < 0.001$, 2.5 ppm at $p < 0.01$). In the group administered the highest dose of nanocolloid, an increase in the CD4+/CD8+ ratio against the control group was recorded ($p < 0.05$) (Table 2, Fig. 1). No significant differences were determined between the analyzed groups of animals regarding the other lymphocyte subpopulations (CD3, CD4, CD8, CD19) (Table 2).

Lymphocyte proliferation

It was only the lowest dose of silver nanocolloid that caused a significant effect on the mitogenic response of lymphocytes, depressing the proliferation of both B and T cells ($p < 0.05$). The other two doses of AgNPs left the analyzed parameter unaffected (Fig. 2).

Activity of blood phagocytes

The group given 2.5 ppm of AgNPs was observed to respond by decreasing the percentage of granulocytes engulfing bacteria ($p < 0.05$), while in the group administered 25 ppm a higher percentage of phagocytizing monocytes was noted ($p < 0.05$). Irrespective of the dose of AgNPs, the phagocytic activity of individual granulocytes, measured by their average

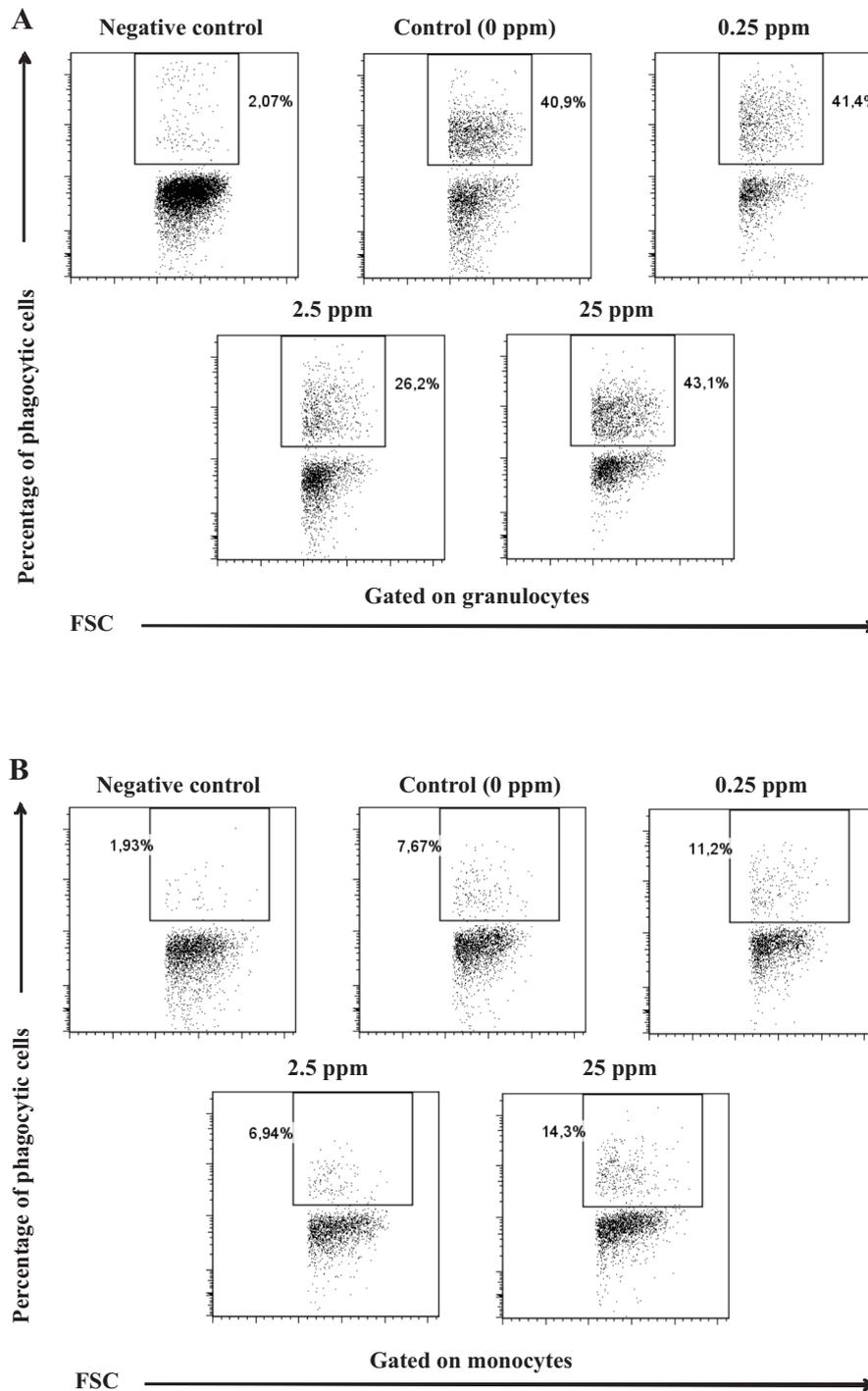


Fig. 3. The representative dot plot cytograms showing the percentage of phagocytic granulocytes (A) and monocytes (B) in mice after 28-day oral administration of silver nanocolloid
 Explanations (for Fig. 3. and 4.): negative control – unstimulated cells

fluorescence intensity, increased (0.25 ppm at $p < 0.001$, 2.5 ppm at $p < 0.01$, 25 ppm at $p < 0.05$). The process was also intensified in monocytes, but the change was statistically significant only in the group given 2.5 ppm of AgNPs ($p < 0.05$) (Table 3, Fig. 3). Besides, in all groups receiving nanosilver there was a significant increase of the percentage of cells under-

going respiratory burst following bacterial stimulation, both granulocytes (0.25 and 25 ppm at $p < 0.05$, 2.5 ppm at $p < 0.001$), and monocytes (all groups at $p < 0.001$). None of the AgNPs doses increase intensity of the respiratory burst in individual cells (Table 4, Fig. 4).

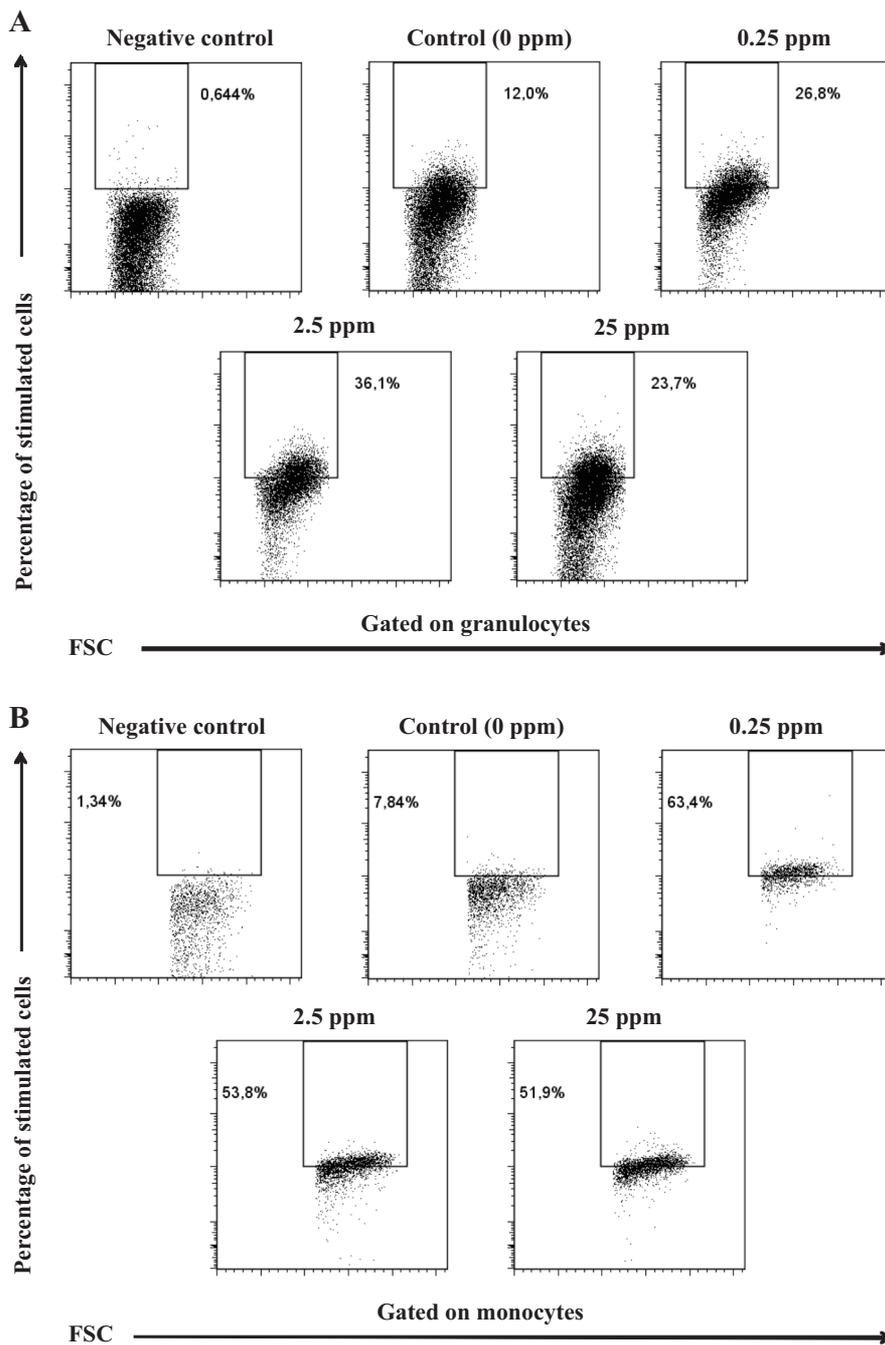


Fig. 4. The representative dot plot cytograms showing the percentage of granulocytes (A) and monocytes (B) undergoing respiratory burst following *E.coli* stimulation in mice after 28-day oral administration of silver nanocolloid.

Table 4. Respiratory burst activity of mice peripheral blood monocytes and granulocytes after 28-day oral administration of silver nanocolloid (% of active cells and MFI) – Phagoburst.

AgNPs dose (ppm)/group	% of active cells		MFI	
	granulocyte	monocyte	granulocyte	monocyte
0 (control)	11.975 ± 3.53	7.842 ± 4.197	1403 ± 63.713	1153.75 ± 32.694
0.25	26.91 ± 5.556*	64.14 ± 12.335***	1450.2 ± 61.504	1280.8 ± 54.715
2.5	36.925 ± 9.095***	53.975 ± 11.325***	1473.75 ± 83.284	1290.75 ± 49.976
25	24.412 ± 5.57*	50.622 ± 15.79***	1344.2 ± 29.203	1211 ± 54.507

Discussion

The observed modifications of hematological parameters in mice in response to silver nanocolloid were rather unexpected, as none of the available references dealing with issue describes the influence of AgNPs on levels of monocytes or platelets in rodents. Regardless of the administration route, changes reported relatively frequently have concerned red blood cell parameters (RBC, HGB, HCT, MCH, MCV), but the influence on leukocytes has been expressed more weakly and seemed to be limited to the increase of granulocyte number (DeJong et al. 2013, Ji et al. 2007, Kim et al. 2008). A higher number of phagocytes, indicating the proinflammatory influence of nanosilver, has also been reported locally after its inhalation or intraperitoneal administration (Stebounova et al. 2011, Xu et al. 2013).

However, numerous experiments on *in vitro* cultures of monocytes, mentioned in introduction, demonstrate that AgNPs can be quite toxic to these cells (Greulich et al. 2011, Park et al. 2011, Yang et al. 2012, Orłowski et al. 2013, Xu et al. 2013). Thus, the decreased level of monocytes in blood of the tested mice may have occurred due to the cytotoxicity of AgNPs after their entrance into circulation. On the other hand, since the peripheral blood monocytes are precursors of tissue macrophages, their lowered blood levels may be an indicative of an increased recruitment of phagocytes in the peripheral immune organs. Although the level of macrophages in secondary lymphoid organs was not determined in the experimental animals, a significant influence of all tested doses of silver nanocolloid on the proliferative activity and cytokine response of splenocytes was recorded, which proves that the spleen of tested animals was strongly stimulated (Małaczewska 2014).

With respect to the decrease in the level of thrombocytes observed in animals given the highest dose of silver nanocolloid, it may have been caused by direct interactions between nanosilver and platelets in the peripheral blood. In both *in vitro* and *in vivo* experiments, AgNPs typically revealed procoagulant activity (Stevens et al. 2009, Jun et al. 2011, Krajewski et al. 2013). Only Shrivastava et al. (2009) described an opposite effect of nanosilver (inhibition of aggregation, adhesion and secretion of platelets) accompanied by the presence of AgNPs in vacuoles and granules of thrombocytes and shortening of cellular processes, confirming nanoparticles' interactions with the platelet machinery. None of the quoted reports demonstrated the cytotoxic action of AgNPs but the longest exposure time of cells was 60 minutes only and *in vivo* studies were conducted after a single administration of nanoparticles. The prolonged exposure may have

resulted in a stronger manifestation of the effect of nanosilver, which probably explains the result obtained in the present experiment.

The effect of silver nanocolloid on the percentages of lymphocyte subpopulations in the mouse peripheral blood was also different than expected. Whilst there had been just one previous experiment, conducted by Park et al. (2010), which involved the phenotyping of leukocytes of mice orally given nanosilver, in quite a narrow range of AgNPs doses (0.25 – 1 mg/kg), its results were much different from ours. The cited researchers observed an increase in the distribution of B, NK and NKT cells, as well as an increase in the level of IgE and cytokines in blood serum, with the dominance of the Th2 type response, which may indicate potential induction of an allergic response to nanosilver.

Changes in the lymphocyte distribution in response to AgNPs, observed in the present experiment, could be attributed to the risk of autoimmunity induction rather than hypersensitivity. The T lymphocyte imbalance, manifested as an elevated CD4+/CD8+ T-cell ratio, noticed in the animals given the highest dose of colloid, is an event correlated with the pathogenesis of such autoimmune disorders as vitiligo or rheumatoid arthritis (Jankowski et al. 2010). Also the increased level of CD4+/CD8+ double positive T cells under the influence of lower AgNPs doses is worrying effect. Only a small percentage of extrathymic, mature DP T cells should be present in the peripheral blood of healthy individuals. Considerable increase in these cells numbers is correlated with many autoimmune disorders (thyroid disease, atopic dermatitis, rheumatoid arthritis, myasthenia gravis, Kawasaki disease, idiopathic thrombocytopenic purpura, systemic sclerosis), suggesting their engagement in the pathogenesis of the aforementioned diseases (Zuckermann 1999, Parel and Chizzolini 2004). Decrease in blood NKT and NK cell levels, noticed under the influence of two lower AgNPs doses may also indicate a potential risk of developing an inappropriate immune response. The main function of NKT cells is production of immunoregulatory cytokines and the regulation of autoimmune response. Reduced number or defects in their functions have been reported in such autoimmune diseases as type 1 diabetes, lupus, multiple sclerosis or rheumatoid arthritis (Godfrey et al. 2000, Linsen et al. 2005). The deficiency of NK cells or suppression of their activity may also result in the activation of autoreactive T cells and macrophages and a drop in the NK cells level in the peripheral blood is often correlated with autoimmune disorders (multiple sclerosis, type 1 diabetes, Sjogren Syndrome, miastenia gravis) (Izumi et al. 2006, Pericone et al. 2008, Tian et al. 2012). On the other

hand, since during pathological processes NK cells are promptly recruited from blood and accumulated in damaged organs in order to maintain the homeostasis (Bernardini et al. 2012, Tian et al. 2012), the decrease in the NK cells level, observed in present experiment, may be an indicative of their accumulation in the peripheral lymphoid tissues (e.g. the spleen).

Another rather unexpected result was a decline in the proliferative activity of lymphocytes of the peripheral blood in animals administered the lowest dose of nanocolloid. This effect was observed also with respect to the spleen T lymphocytes of this group of mice, despite the concurrent growth in the production of IL-2, while the higher AgNPs' doses stimulated proliferation of spleen B cells (Małaczewska 2014). Another interesting fact was that in the author's previous experiment on mice given the same colloid but for shorter periods of time (7 and 14 days), the dose of 0.25 ppm stimulated the proliferation of spleen T cells after 7 days of administration (Małaczewska 2011). However, any detailed discussion concerning this parameter is hindered by the lack of literature reports on the effect of administration of nanosilver on proliferation of animals' peripheral blood lymphocytes, and there is just one article which describes the stimulation of the proliferative response by B and T cells, isolated from the spleen of rats receiving AgNPs intravenously (De Jong et al. 2013).

The increased activity of the peripheral blood phagocytes in mice given silver nanocolloid is the only result of the present study that coincides with the reports of other authors. It is known that silver nanoparticles, rapidly engulfed by phagocytes, stimulate reactive oxygen species generation, which explains a higher percentage of phagocytes affected by respiratory burst in present experiment. In turn, stimulation of ROS production by low AgNPs doses causes cells' activation (Greulich et al. 2011, Park et al. 2011, Yang et al. 2012, Orłowski et al. 2013, Xu et al. 2013), which clarifies the enhanced intensity of phagocytosis in individual cells. There was just one unexpected result, such as a decrease in the percentage of bacteria-phagocytizing granulocytes in the group given 2.5 ppm AgNPs, and its cause remains unexplained.

Recapitulating, all the tested doses of silver nanocolloid, despite their broad range, affected leukocytes in the peripheral blood of mice. Their influence on the activity of phagocytes support results reported by other researchers, thus proving their considerable contribution to the organism's response to nanoparticles. In turn, the observed decrease in the levels of monocytes and NK cells in the peripheral blood may indicate an increased recruitment of these cells in peripheral lymphoid organs, especially as the

strong stimulation of splenocytes in the tested animals was observed (Małaczewska 2014). Nonetheless, some of the noticed changes, such as the increased level of DP T cells, higher CD4+/CD8+ ratio, drop in NKT cell percentage as well as a strong decline in the proliferative response of lymphocytes, raise worries regarding a potential risk of developing an inappropriate immune response due to the long-lasting administration of such dietary supplements. Reliable assessment of the degree of the above hazard calls for further research in this direction.

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