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*Original article*

# The effect of silver nanoparticles on splenocyte activity and selected cytokine levels in the mouse serum at early stage of experimental endotoxemia

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## Abstract

The objective of this study was to determine the effect of a nonionic silver nanocolloid administered orally for 7 or 14 days at three concentration levels (25 ppm, 2.5 ppm, and 0.25 ppm) on the phagocytic activity and mitogenic response of splenocytes and selected cytokine serum levels (IL-1 $\beta$ , IL-6, IL-10, IL-12 p70, TNF- $\alpha$ ) in NMRI mice at the early stage of experimental endotoxemia induced with single 30  $\mu$ g/mouse dose of bacterial LPS.

Regardless of the period of administration, silver nanoparticles enhanced the production of proinflammatory cytokines and anti-inflammatory cytokine IL-10, and they inhibited IL-12 p70 levels in response to LPS challenge. The studied nanoparticles' effect on splenocyte activity was determined by the period of administration. After 7 days of use, silver nanoparticles enhanced the phagocytic activity, and doses of 2.5 ppm stimulated the mitogenic response of splenocytes. After 14 days of administration, silver nanoparticles lowered the phagocytic activity regardless of the dose applied. Although the results obtained are ambiguous, they suggest that silver nanoparticles administered via the alimentary tract are more likely to increase an inflammatory response of an organism than offer protection after LPS challenge.

**Key words:** silver nanoparticles, cytokines, splenocyte activity, murine endotoxemia

## Introduction

Nanotechnology is the burgeoning science of designing, synthesizing and manipulating structures measuring less than 100 nm. Nanoparticles are characterized by a high surface area per unit mass, which gives them unique physicochemical properties and enables use of nanoparticles in a host of innovative

applications. Due to its strong disinfectant properties, silver is one of the most popular substances in nanotechnology. Silver nanoparticles (AgNPs) are used as antimicrobial agents in medicine, water and air purification, the production of cosmetics, fabrics, kitchen appliances and baby products. Nanosilver is also recommended by alternative medicine practitioners as a dietary supplement that boosts immunity

and has a stimulating effect on human health (Luoma 2008). AgNPs has a growing number of daily applications, and the human body is increasingly often exposed to this innovative material that has not yet been fully investigated. Silver nanoparticles can enter the human body via the alimentary tract, by inhalation and transdermal penetration. A study of rodents has revealed that regardless of the manner of administration, AgNPs interact directly with cells, they reach the blood stream and are accumulated in internal organs. Their distribution throughout the body is determined by particle size, manner of administration and animal species, but one of the target organs for the accumulation of silver is the spleen, a major lymphoid organ responsible for the immune response (Takenaka et al. 2000, 2001, Pelkonen et al. 2003, Park et al. 2010). There are limited data regarding the effect of orally administered silver nanoparticles on the immune system of animals, and most of the existing studies have analyzed healthy animals, rather than individuals affected by pathogens which, according to alternative medicine practitioners, can be effectively controlled with the use of silver nanoparticles. What is more, some of such studies revealed proinflammatory action of nanosilver orally administered to mice (Park et al. 2010, Małaczewska 2011). In our previous experiment conducted on healthy mice receiving orally three different AgNPs doses (25, 2.5 and 0.25 ppm), 14-day period of nanosilver administration was found to have a detrimental effect on mice immune response. Similar effect was also demonstrated by Park et al. (2010). Lipopolysaccharides (LPS), which are endotoxins and key cell wall components in Gram-negative bacteria, induce a strong immune response upon the release of proinflammatory cytokines. The result is a generalized inflammatory response, followed by tissue damage and even septic shock. However, intravenous administration of bacterial endotoxin at low doses to healthy humans is believed to be a clinically relevant model of sepsis (Fijen et al. 2000). Such symptoms of endotoxemia as flu-like syndrome, elevated cytokine and other inflammatory mediators levels or leukocyte activation found by Fijen et al. (2000) in healthy human subjects after single infusion of low LPS dose (4 ng/kg body weight) lasted just for 24 h after endotoxin administration and were followed by the reverse of all parameters to the normal range. Although mice are believed to be less susceptible to LPS than humans, high LPS doses (10-40 mg/kg) can produce symptoms characteristic of toxic shock that may lead to death. On the other hand, when a single, relatively low dose of bacterial endotoxin is administered to mice, death can be avoided while preserving the characteristic immune re-

sponse (Zhang et al. 2008, Nandi et al. 2010). For example, CBA mice 10-day survival after 100 and 200 µg LPS i.v. injection, according to Zimecki et al. (2004), was 100% and in our previous experiments NMRI mice challenged i.p. with 1 mg LPS/kg of body weight (30 µg/mouse) showed no lethality as well.

The objective of this study was to determine the effect of “Silver water” (Nano-Tech Polska Sp. z o.o.), a colloidal solution containing silver nanoparticles, administered orally for 7 or 14 days, on the phagocytic and proliferative activity of splenocytes and selected cytokine levels in the mouse serum at early stage of experimental endotoxemia induced with a single low dose of bacterial LPS.

## Materials and Methods

### Silver nanoparticles (Ag-NPs)

Colloidal nonionic silver solution “Silver water” (Nano-Tech Polska, Poland) containing metallic silver nanoparticles (10-20 nm) suspended in demineralised water, at a concentration of 50 ppm was used as a source of silver nanoparticles. Colloidal silver was dissolved in demineralised water to produce solutions at three concentrations: 25 ppm, 2.5 ppm, and 0.25 ppm, which were then administered to mice as drinking water *ad libitum* for 7 or 14 d.

### Mice

The experiment was performed on 50 male NMRI mice, aged 10-12 weeks, with body weight of 28-34 g, supplied by the Department of Pathophysiology, Forensic Veterinary Medicine and Administration, University of Warmia and Mazury in Olsztyn. The animals were divided into five equal groups: two control groups – 0 (negative control) and LPS (positive control) not receiving the silver solution, A – administered the silver solution at a concentration of 25 ppm, B – administered the solution at a concentration of 2.5 ppm, and C – administered the solution at a concentration of 0.25 ppm. Since nanoparticles have a tendency to aggregate, water was replenished twice daily. The animals were maintained under conventional conditions and supplied with rodent chow *ad libitum* throughout the experiment. After 7 days of administration of the colloidal silver solution, half of the animals in each group (five individuals) except for the group 0 (negative control) were challenged intraperitoneally with LPS (derived from *Salmonella enterica*, Sigma-Aldrich) at a dose

of 1 mg/kg of body weight (30 µg/mouse). Two hours after injection mice were sacrificed, and their blood and spleens were sampled for further analyses. Ag-NPs were administered to the remaining animals for 7 consecutive days, after which mice, except for the negative control (group 0), were challenged with LPS as described above and 2 h after injection analytical material was collected. The animals were anaesthetised by inhalation of AErrane (isofluranum, Baxter Poland). Blood was collected by heart puncture, and the spleen was sampled after bleeding. The experiment has been approved by the Local Ethics Committee.

### Serum

Clotted blood was centrifuged to produce serum and determine IL-1β, IL-6, IL-10, IL-12 p70, and TNF-α levels. Serum samples from the animals receiving nanoparticles for 7 days were stored at -70°C, and were analysed simultaneously with the samples from mice administered colloidal silver for 14 d.

### Determination of cytokine levels

The serum levels of IL-1β, IL-6, IL-10, IL-12 p70, and TNF-α were analysed by ELISA (R&D Systems, United Kingdom). The cytokines were quantified using manufacturer's protocol.

### Isolation of splenocytes

Splenocytes were isolated using routine procedure. Aseptically removed spleens were pressed through a 60-µm nylon mesh in RPMI-1640 medium with L-glutamin and sodium bicarbonate (Sigma-Aldrich). The cell suspension was placed on density gradient Gradisol L (Aqua-Medica, Poland) in order to isolate splenocytes, and then centrifuged at 400 g for 40 min at 4°C. The interface cells were collected and washed three times with the RPMI-1640 medium at 400 g for 5 min. Viability of the isolated cells was evaluated by trypan blue exclusion (Sigma-Aldrich) and was determined to be greater than 95%. The cells were suspended in RPMI-1640 medium containing 10% foetal calf serum (Sigma-Aldrich) and 1% antibiotic-antimycotic solution (Sigma-Aldrich), and dispensed into 96-well plates at a concentration of  $3-5 \times 10^6$  cells ml<sup>-1</sup>. Then the cells were cultured/incubated at 37°C under a humidified atmosphere of 5% CO<sub>2</sub> and 95% air atmosphere and used for the following assays.

### Respiratory burst activity (RBA) test

Metabolic activity of phagocytic cells was determined by the measurement of intracellular respiratory burst activity after stimulation with phorbol myristate acetate (PMA, Sigma-Aldrich), as described by Chung and Secombes (1988). One hundred microlitres of cell suspension was added to each well of 96-well microtiter plates (Nunc, Denmark). After incubation for 2 h at 37°C, the cells were washed in RPMI 1640 medium to remove non-adherent cells and incubated for the next 24 h. Then 100 µl of PMA (1 µg ml<sup>-1</sup>) in 0.1% nitroblue tetrazolium (NBT, Sigma-Aldrich) solution in RPMI 1640 medium were added to each well. The mixture was incubated for 30 min at 37°C. After the removal of the medium from the cells, the reaction was stopped by the addition of absolute ethanol and then washed twice with 70% ethanol. The formazan produced in the cells was dissolved in 120 µl of 2M KOH and 140 µl of DMSO (dimethylsulfoxide, POCh, Poland) and the optical density was measured colorimetrically in a Sunrise absorbance reader (Tecan, Austria) at 620 nm. All samples were tested in triplicate. Mean values of optical density (OD) ± SD served as the results.

### Potential killing activity (PKA) test

The technique presented by Rook et al. (1985) was used to measure the potential killing activity of phagocytic cells. After removing non-adherent cells and the following 24 h incubation, 100 µl of isolated cell suspension was mixed with 100 µl of 0.1% NBT solution in PBS (Biomed, Poland) containing *Staphylococcus aureus* ( $1 \times 10^8$  cells ml<sup>-1</sup>) and incubated for 30 min at 37°C. After incubation, the supernatant was removed from each well and adherent cells were fixed with absolute ethanol. One hundred and twenty microlitres of 2M KOH and 140 µl of DMSO were added to each well and the plates were mixed. The amount of extracted reduced NBT was measured colorimetrically in a Sunrise absorbance reader (Tecan, Austria) at 620 nm. All samples were tested in triplicate. Mean values of optical density (OD) ± SD served as the results.

### Proliferative response of splenocytes (MTT test)

Mitogenic response of splenocytes was determined using the MTT colorimetric assay (Mosmann 1983). Cells were suspended in RPMI 1640 growth medium containing mitogens – concanavalin

Table 1. The effect of silver nanoparticles administration on the cytokine levels in mice serum (pg/ml) after experimental endotoxemia.

Cytokine	Administration Time (d)	Measures	Group (n=5)				
			0	LPS	A	B	C
IL-1 $\beta$	7	M	ND	31.01	124.03**	101.85**	119.89**
		SD	–	4.76	40.49	36.35	18.24
	14	M	ND	52.99	90.53	138.91**	74.12
		SD	–	20.89	36.78	34.29	26.12
IL-6	7	M	ND	912.50	1150.75	1065.33	1250.54
		SD	–	90.31	210.60	180.63	215.48
	14	M	11.07	923.33	1048.83	996.66	935.73
		SD	3.25	79.85	134.29	130.23	205.18
IL-10	7	M	ND	47.66	105.78**	248.27**	128.00**
		SD	–	5.93	20.85	58.02	16.92
	14	M	ND	81.37	177.27**	79.08	68.08
		SD	–	24.96	24.26	21.32	17.27
IL-12	7	M	ND	52.05	26.63	18.31*	16.03*
		SD	–	32.45	8.02	6.56	4.59
	14	M	16.78	70.74	54.54	29.18**	68.61
		SD	4.24	24.79	14.21	10.80	11.34
TNF $\alpha$	7	M	ND	217.91	375.13*	346.04	534.64**
		SD	–	55.63	73.85	64.83	80.85
	14	M	ND	204.21	336.91**	258.97	256.80
		SD	–	47.04	62.92	57.07	46.59

Explanations: ND – cytokine level not detectable; \* – difference statistically significant in comparison to positive control group (0) at  $p < 0.05$ ; \*\* – difference statistically significant in comparison to positive control group (0) at  $p < 0.01$

A (ConA, Sigma-Aldrich) at concentration of  $10 \mu\text{g ml}^{-1}$  as a T-cell mitogen or lipopolysaccharide from *Salmonella enterica* (LPS, Sigma-Aldrich) at concentration of  $10 \mu\text{g ml}^{-1}$  as a B-cell mitogen and  $100 \mu\text{l}$  of the suspension was added to each well of microtiter plates. The mixture was cultured for 72 h. After incubation,  $25 \mu\text{l}$  of solution containing  $7 \text{ mg ml}^{-1}$  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  $100 \mu\text{l}$  of DMSO was added to each well. The optical density was measured at a wavelength of 570 nm. 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.

### 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.

## Results

### Cytokine levels

All cytokine levels (IL-1 $\beta$ , IL-6, IL-10, IL-12 and TNF- $\alpha$ ) in the serum of group 0 (negative control) animals were below the detection threshold on experimental day 7, whereas on day 14, very low levels of IL-6 and IL-12 were reported. Following a single LPS injection, the levels of all analyzed cytokines in positive control significantly exceeded the test sensitivity threshold after both 7 and 14 days. For this reason, the results obtained in the remaining animal groups were compared only with the values reported in the LPS group (positive control) (Table 1).

In the groups of animals administered silver nanoparticles with drinking water, an increase in proinflammatory cytokine levels (IL-1 $\beta$ , IL-6 and TNF- $\alpha$ ) and IL-10 levels was observed in response to LPS challenge in comparison with the LPS group, regardless of the nanoparticle dose, although significant only with regard to IL-1 $\beta$  after 7 days (all groups,  $p < 0.01$ ) and 14 days (group B,  $p < 0.01$ ), TNF- $\alpha$  after 7 days (group A,  $p < 0.05$  and group C,  $p < 0.01$ ) and 14 days (group A,  $p < 0.01$ ) as well as IL-10 after 7 days (all groups,  $p < 0.01$ ) and 14 days (group A,  $p < 0.01$ ). IL-12 produced a different response. IL-12

Table 2. The effect of silver nanoparticles administration on the respiratory burst activity (RBA) and the potential killing activity (PKA) of mice splenocytes (OD 620 nm) after experimental endotoxemia.

Assay	Administration Time (d)	Measures	Group (n=5)				
			0	LPS	A	B	C
RBA	7	M	0.195	0.220	0.249	0.329***	0.266
		SD	0.037	0.041	0.029	0.095	0.03
	14	M	0.216	0.281	0.277	0.193 <sup>a</sup>	0.212
		SD	0.044	0.059	0.039	0.020	0.013
PKA	7	M	0.181	0.259*	0.271*	0.309**	0.258*
		SD	0.017	0.056	0.034	0.036	0.037
	14	M	0.219	0.266	0.247	0.194	0.186
		SD	0.032	0.053	0.066	0.017	0.029

Explanations: \* – difference statistically significant in comparison to negative control group (0) at  $p < 0.05$ ; \*\* – difference statistically significant in comparison to negative control group (0) at  $p < 0.01$ ; <sup>a</sup> – difference statistically significant in comparison to positive control group (LPS) at  $p < 0.05$

Table 3. The effect of silver nanoparticles administration on the proliferative response of mice splenocytes stimulated by mitogens (SI) after experimental endotoxemia.

Mitogen	Administration Time (d)	Measures	Group (n=5)				
			0	LPS	A	B	C
ConA (10 µg/ml)	7	M	1.46	1.14	1.14	1.80 <sup>a</sup>	1.32
		SD	0.21	0.22	0.27	0.42	0.31
	14	M	1.60	1.32	1.26	1.20	1.42
		SD	0.25	0.15	0.19	0.26	0.15
LPS (10 µg/ml)	7	M	1.49	1.13	1.19	1.77 <sup>b</sup>	1.12
		SD	0.20	0.28	0.26	0.31	0.17
	14	M	1.55	1.39	1.42	1.14	1.41
		SD	0.33	0.28	0.38	0.03	0.25

Explanations: <sup>a</sup> – difference statistically significant in comparison to positive control group (LPS) at  $p < 0.05$ ; <sup>b</sup> – difference statistically significant in comparison to positive control group (LPS) at  $p < 0.01$

levels in the serum of animals receiving silver nanoparticles were lower than those found in the positive control, and significant differences were observed after both 7 (groups B and C,  $p < 0.05$ ) and 14 days (group B,  $p < 0.01$ ) (Table 1).

### Respiratory burst activity and potential killing activity of splenocytes

LPS injection insignificantly stimulated the potential killing activity and respiratory burst activity of mouse splenocytes in comparison with those observed in animals from the control group 0. A significant increase in phagocytic activity ( $p < 0.05$ ) was observed only with regard to potential killing activity after 7 days of the experiment (Table 2).

In animals receiving silver nanoparticles, differences in splenocyte activity were found after the LPS injection, subject to the period of nanoparticle administration. After 7 days of the experiment, higher levels of respiratory burst activity as well as potential killing

activity were found. Both types of activity were more clearly pronounced than those observed in the control group 0 and the positive control group (LPS). A significant response was observed in group B as regards RBA (relative to both control groups), and in all the groups – as regards PKA (relative to negative control). After 14 days of AgNPs administration, phagocytic activity was inhibited in all the groups (A, B and C), in comparison with the positive control, but a significant decrease was observed only in respect to respiratory burst activity in group B ( $p < 0.05$ ) (Table 2).

### The mitogenic response of splenocytes

The LPS challenge insignificantly reduced the proliferative activity of splenocytes in response to both mitogens (ConA and LPS). In groups receiving silver nanoparticles, the mitogenic response of splenocytes was insignificantly lower in comparison with the control group 0 as well, regardless of the period of administration. The only exception was

group B, where after 7 days of AgNPs administration, the proliferative response of splenocytes to both mitogens was more clearly expressed than in both control groups (0 and LPS), but significant differences were reported only in comparison with the positive control (LPS) (Table 3).

## Discussion

An early response of mice to LPS challenge, measured in terms of cytokine levels, is observed in the first hours after injection, and similarly to humans, high levels of TNF- $\alpha$ , IL-1 $\beta$  and IL-6 are the major contributors to septic shock. Simultaneously anti-inflammatory cytokine IL-10 is released in response to LPS, to protect the body against septic shock. In the human model of endotoxemia, Fijen et al. (2000) reported peak concentrations of TNF- $\alpha$ , IL-6 and IL-10 already 2-3 hours after the intravenous administration of LPS. In rodents, maximum serum TNF- $\alpha$  levels were observed 1-2 h after the LPS injection, maximum IL-10 levels – after 3 h, and maximum IL-1 $\beta$  and IL-6 concentrations – after 6 h (Takemoto 2005, Zhang et al. 2008).

Whereas positive control animals responded to the LPS injection in line with the mechanism described, the effect of AgNPs on cytokine levels in response to the LPS challenge is difficult to evaluate without a certain degree of ambiguity. Enhanced production of the two major proinflammatory cytokines, IL-1 $\beta$  and TNF- $\alpha$ , which exert a synergistic effect and initiate the inflammatory cascade, points to the proinflammatory effect of silver nanoparticles. On the other hand, increased concentrations of anti-inflammatory IL-10 suggest that AgNPs deliver a potentially protective effect in endotoxemia, and they could explain the drop in IL-12 levels, relative to the LPS group, as a result of a negative feedback between the two cytokines. Intensified IL-10 production under the influence of silver nanoparticles cannot be explained, however, solely by the stimulating effect of pleiotropic IL-6 because in the groups of animals administered AgNPs, IL-6 concentrations were only insignificantly higher than those found in the LPS group. What's more, the increase in IL-10 levels was more clearly pronounced than the increase in the concentrations of proinflammatory cytokines.

Silver nanoparticles had a slightly different effect on cytokine production in our previous experiment where healthy mice were administered identical AgNP doses (25, 2.5 and 0.25 ppm) over a period of 1 and 2 weeks. The production of IL-6 (regardless of the silver dose) and IL-12 (inversely correlated with the silver dose) was significantly stimulated only after

14 days of oral administration of silver nanoparticles, indicating that the potential proinflammatory effect of AgNPs is manifested only during long-term use (Małaczewska 2011). The proinflammatory effect of AgNPs orally administered to mice was also demonstrated by Park et al. (2010). In a study investigating the effect of silver nanoparticles administered to healthy mice at doses of 0.25 – 1 mg/kg BW over a period of 2 and 4 weeks, the above authors observed elevated levels of TGF- $\beta$ , Th1 (IL-12), Th2 (IL-4, IL-10) cytokines, and proinflammatory cytokines (IL-1, IL-6). A higher response of Th2 cytokines, including IL-10, and an increase in serum IgE levels suggested that the allergic response was potentially induced by silver nanoparticles. Therefore, a strong IL-10 response combined with elevated levels of proinflammatory cytokines in our experiment, could also be attributed to AgNPs' adverse effect on the immune response to LPS challenge.

On the other hand, in experiments investigating the effect of silver nanoparticle dressing or cream in the treatment of allergic contact dermatitis or chronic and infected wounds in animals, the authors have demonstrated satisfactory clinical results, including a fast reduction of swelling and hyperemia, accelerated healing and correct histological images of the tissue affected (Wright et al. 2002, Bhol et al. 2004, Bhol and Schechter 2005, Tian et al. 2007, Nadworny et al. 2008). In studies describing the humoral immune response, silver nanoparticles were shown to lower the serum level of acute-phase proteins, reduce collagenase activity, lower tissue metalloproteinase levels and proinflammatory cytokine expression (TNF- $\alpha$ , IL-6, IL-8, IL-12, TGF- $\beta$ ) or modulate cytokine expression – a drop in IL-6 mRNA levels, an increase in TGF- $\beta$ 1 mRNA expression at the beginning of healing, followed by a successive drop, and an increase in IL-10, VEGF and IFN- $\gamma$  mRNA levels (Wright et al. 2002, Bhol and Schechter 2005, Tian et al. 2007, Nadworny et al. 2008). This beneficial effect could result from differences in the manner of AgNP administration and the direct application of silver nanoparticles to the site of inflammation, which does not rule out a completely different model of activity of orally administered AgNPs.

The second parameter analyzed in the present study was the effect of silver nanoparticles on splenocyte activity after LPS stimulation. Early splenocyte responses of the positive control (LPS group) mice, characterized by increased phagocytic activity and an insignificant drop in the mitogenic response of splenocytes in comparison with negative control (group 0) mice, were similar to the response of human peripheral blood leukocytes after experimental endotoxemia, as described by Fijen et al. (2000).

The cited authors reported lymphopenia, neutrophilia and enhanced expression of membrane markers (CD11b, CD63, CD66b) to suggest elevated levels of phagocytic activity, although peaks of the activity were observed only 3-6 hours after the LPS injection.

In the present experiment, silver nanoparticles had an ambiguous effect on LPS-stimulated splenocyte activity. In animals administered AgNPs for 1 week, phagocytic activity was more pronounced than that observed in the LPS group, whereas a drop in phagocytic activity levels was found after 14 days of the experiment. The above changes in phagocytic activity were not affected by the nanoparticle concentrations applied. The strongest reaction was reported after both 7 and 14 days in group B which received doses of 2.5 ppm. Also this group, as the only one, showed enhanced mitogenic response of splenocytes, however, only after 7 days of nanosilver administration. Silver nanoparticles delivered a similar effect in our previous experiment on healthy mice which received identical AgNP doses for 7 and 14 days. Elevated phagocytic activity after 7 days preceded a drop in activity levels after 14 days of AgNP administration. Simultaneously, enhanced mitogenic response of splenocytes was observed, but with significant influence of AgNPs on the parameters studied, only at lower concentrations (2.5 and 0.25 ppm) (Małaczewska 2011).

It seems that increased respiratory burst activity of splenocytes stimulated with PMA or bacteria after 7 days of AgNP administration, observed in both healthy and LPS-stimulated animals, could be attributed to silver nanoparticles' stimulating effect on reactive oxygen species (ROS) generation, whereas the drop in activity levels after 14 days of the experiment suggests that AgNPs' cytotoxic effect on spleen cells intensified with time. This hypothesis matches the results reported by Park et al. (2010) in an *in vitro* study investigating the effect of silver nanoparticles on activated peritoneal macrophages in mice. The above authors demonstrated that AgNPs are phagocytosed by macrophages which leads to increased ROS generation and proinflammatory activity that stimulates the release of TNF- $\alpha$ , leading to cell membrane damage and apoptosis. In our study, the increased proliferative response of splenocytes in group B animals after 7 days of AgNP administration could be attributed to the proinflammatory effect of silver nanoparticles, and it could be directly induced by high concentrations of proinflammatory cytokines. More extensive research is needed, however, to validate this hypothesis.

The results of limited research investigating the effect of orally administered AgNPs on the immune response in animals seem to indicate that silver

nanoparticles have a potentially proinflammatory effect. In pathological states, such as endotoxemia, AgNPs could lead to excessive immune response and, consequently, toxic shock. On the other hand, silver nanoparticles' disinfectant properties combined with immune activation could support the treatment of selected immunosuppressive diseases. Further research is needed to investigate the appropriate doses, periods of use and indications for the oral administration of silver nanoparticles.

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