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Original article

The *in vitro* effect of commercially available noble metal nanocolloids on the rainbow trout (*Oncorhynchus mykiss*) leukocyte and splenocyte activity

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Abstract

The growing popularity of nanotechnology in the past decade has increased nanomaterial concentrations in the environment and the risk of their toxicity for aquatic organisms. Metal nanoparticles, which are easily absorbed and accumulated by fish, are probably able to interact directly with their immunocompetent cells. The objective of this study was to evaluate the *in vitro* effect of commercially available silver, gold and copper nanocolloids on the rainbow trout leukocyte and splenocyte activity. At high concentrations, all of the nanocolloids studied had adverse effects on the proliferative response of trout lymphocytes, and the most toxic of them, silver, decreased also the respiratory burst activity of splenocytes. Low concentrations of silver nanocolloid, however, had a stimulating effect on the lymhocyte proliferation.

Key words: nanocolloid, silver, gold, copper, rainbow trout, leukocytes, splenocytes

Introduction

The soaring number of nanotechnology applications has increased the release of nanoparticles (NPs) into the environment, which can lead to their accumulation and the growing risk of toxicity for aquatic habitats (Jovanović and Palić 2012). Silver and copper nanoparticles are most widely used owing to their disinfectant properties. The ionic forms of these metals are highly toxic for fish, in particular freshwater species. They disrupt osmoregulation and the acid-base balance, leading to the escape of electrolytes from the blood via the gill epithelium, circulatory collapse and death (Griffitt et al. 2008, 2009, Farkas et al. 2010, Shaw and Handy 2011, Farmen et al. 2012, Shaw et al. 2012). Fish accumulate water-borne silver nanoparticles in their tissues, and significantly higher accumulation levels are observed in freshwater species, which points to higher bioavailability of silver in fresh water. What is more, freshwater fish were found to be much more susceptible to the toxic effects of silver than saltwater species (Webb and Wood 2000, Farmen et al. 2012).

Nanoparticles enter the organisms of fish via the skin, gills and the gastrointestinal tract. The mucus layer covering the skin, gills and intestines provides

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a significant mechanical barrier for pollutants. By penetrating that barrier, nanoparticles bind to mucoproteins, they become entrapped and form aggregates. The above leads to the hypersecretion of mucus, and it prevents NPs from entering the body (Bilberg et al. 2010, Jovanović and Palić 2012). The most probable mechanism of NP penetration via the gills is endocytosis, but it is responsible only for the movement of very small particles under 20 nm in size. The gastrointestinal tract seems to be a more likely pathway of NP absorption, in particular in saltwater fish species which drink water to compensate for its loss in a hyperosmotic environment. This mechanism is also observed in freshwater fish, including the rainbow trout, in response to stress (Shaw and Handy 2011). In a study performed on a mammalian model, NPs interacted with enterocytes and GALT immediately after entering the digestive system. In fish, the role of Peyer's patches is played by diffuse gut lymphoid tissue, and fish enterocytes are capable of absorbing macromolecules which are considerably larger than the molecules endocytosed by mammalian enterocytes. Therefore, the absorption of individual nanoparticles as well as their aggregates can be more intense, and the mechanism of NPs interaction with the gastrointestinal tract may be different in fish (Jovanović and Palić 2012).

Webb and Wood (2010) investigated the accumulation of silver in the tissues of fish that drink and do not drink water, and they observed that AgNPs are absorbed via both gills and gut. The only difference was the main place of silver accumulation – the liver in osteichthyes and the gills in elasmobranchii. Interestingly, the rainbow trout accumulated more silver than other bony fish species.

In trout fed a diet containing TiO_2 nanoparticles, the presence of NPs was determined in the liver, spleen and brain, whereas intravenously administered nanoparticles were accumulated in the liver and kidneys. In the fathead minnow, TiO_2 was deposited mainly in the spleen and kidneys, the organs responsible for hematopoiesis, indicating that nanoparticles can directly influence immunocompetent cells (Jovanović and Palić 2012). In fish, immune functions are closely correlated with environmental factors, therefore, chronic exposure to nanoparticles could have significant implications for fish health.

The objective of this study was to evaluate the *in vitro* effect of commercially available silver, gold and copper nanocolloids on the activity of immunocompetent cells isolated from peripheral blood and the spleen of the rainbow trout.

Materials and Methods

Noble metal nanocolloids

Three commercially available colloidal nonionic solutions of silver, gold and copper nanoparticles (AgNPs, AuNPs, CuNPs), called by the producer "Silver water", "Gold water" and "Copper water" (Nano-Tech Polska, Poland), containing metallic nanoparticles (Ag: 10-20 nm, Au and Cu: up to 5 nm), suspended in demineralised water, at a concentration of 50 ppm were used as a source of nanoparticles. Just before the use, the nanocolloids were dissolved in the cell growth medium to reach the final concentrations of: 0, 0.15, 0.3, 0.6, 0.9, 1.25, 1.9, 2.5, 3.75, 5, 7.5 and 10 ppm (silver) or 0, 0.9, 1.2, 1.9, 2.5, 3.75, 5, 7.5 and 10 ppm (gold, copper).

Fish

The experiment was performed on 24 individuals of the rainbow trout (*Oncorhynchus mykiss*), weighing 350-400 g, supplied by the Inland Fisheries Institute, Olsztyn. The animals were randomly divided into 6 groups. The immune cells isolated from the individuals within each group were pooled before performing the assays, and tested in triplicate. Circulating blood was collected from fish anaesthetized with 2% Propiscin (Żabieniec, Poland) diluted in water (1 ml l^{-1}) by caudal vein puncture, and the spleen was sampled after bleeding. The experiment has been approved by the Local Ethics Committee.

Isolation of leukocytes

Leukocytes for the tests were isolated from the fish blood and spleen. The spleens were removed aseptically, and pressed through a 60-μm nylon mesh in RPMI-1640 medium with L-glutamin and sodium bicarbonate (Sigma-Aldrich). The splenocyte cell suspension and the whole heparinized blood were placed on density gradients: Gradisol G (Aqua-Medica, Łódź, Poland) – in order to isolate phagocytic cells or Gradisol L (Aqua-Medica, Łódź, Poland) – in order to isolate lymphocytes, 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 isolated cells was evaluated by trypan blue exclusion and was determined to be greater than 95% in each case. Cells were sus-

pended in RPMI-1640 medium containing 10% fetal calf serum (FCS, Sigma-Aldrich), 1% antibiotic-antimycotic solution (Sigma-Aldrich) and different concentrations of silver (0.15-10 ppm), gold or copper (0.9-10 ppm) nanocolloids (Nano-Tech, Polska), then dispensed into 96-well plates at a concentration of $5x10^6$ cells ml^{-1}, cultured/incubated at 22°C and used for the following assays.

The proliferative response of lymphocytes – MTT assay

The 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 50 µg ml^{-1} as a T-cell mitogen or lipopolysaccharide from *Serratia marcescens* (LPS, Sigma-Aldrich) in concentration of 25 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 incubated for 96 h in the presence of different concentrations of NPs at 22°C. After incubation, 25 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 DMSO was added to each well. The optical density was measured at a wavelength of 570 nm with 640 nm as a reference wavelength. Samples obtained from each group 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.

MTT assay was also used to determine the viability of nonadherent cells incubated for 48 h in the presence of NPs, without mitogens. In this case the results were expressed as the percent of the control cells viability.

Respiratory Burst Activity (RBA) test

The metabolic activity of phagocytes was determined by the measurement of the intracellular respiratory burst activity after stimulation with PMA (phorbol myristate acetate, Sigma-Aldrich), as described by Chung and Secombes (1988) with some modifications described by Chettri et al. (2010). 100 µl of cell suspension was added to each well of 96-well microtiter plates (Nunc, Denmark). After incubation for 2 h at 22°C, the cells were washed in RPMI 1640 medium to remove nonadherent cells and incubated for the next 24 h with metal nanocolloids. Then 100 µl of PMA (1 µg ml^{-1}) in 0.1% NBT (nitroblue tetrazolium, Sigma-Aldrich) solution in RPMI 1640 medium were added to each well. The mixture was incubated for 60 min at 22°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, Gliwice, Poland) and the optical density was measured colorimetrically in a Sunrise absorbance reader (Tecan, Austria) at 620 nm. The results of the RBA assay were expressed as a stimulation index (SI), which was calculated by dividing the mean O.D. of PMA stimulated cells by the O.D. of the non-stimulated (control) cells.

Potential Killing Activity (PKA) test

The technique presented by Rook et al. (1985) was used to measure the potential killing activity of phagocytes. After removing nonadherent cells and the following 24 h incubation of cells with NPs, 100 µl of isolated cell suspension was mixed with 100 µl of 0.1% NBT solution in PBS (phosphate-buffered saline) (Biomed, Lublin, Poland) containing *Aeromonas hydrophila* ($1x10^8$ cells ml^{-1}) and incubated for 60 min at 22°C. After incubation the supernatant was removed from each well and adherent cells were fixed with absolute ethanol. 120 µl 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. The results of the PKA assay were expressed as a stimulation index (SI), which was calculated by dividing the mean O.D. of PMA stimulated cells by the O.D. of the non-stimulated (control) cells.

Statistical analysis

Data were analyzed 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 GraphPad-Prism software package.

Results

The effect of metal nanocolloids on the lymphocyte viability in the rainbow trout

Among the investigated nanocolloids, only silver exerted a significant effect on the viability of lymphocytes in the rainbow trout after 48 hours of incu-

Table 1. The in vitro effect of silver nanocolloid on the rainbow trout lymphocytes viability after 48 h of incubation (% of control cells viability).

Cell source	Measures	NPs concentration (ppm), n=6											
		0 (control)	0.15	0.3	0.6	0.9	1.25	1.9	2.5	3.75	5	7.5	10
Spleen	M	100	103.075	97.442	98.021	85.938	83.19*	83.333*	75.008**	71.502**	33.734***	31.565***	23.142***
	SD	7.951	9.868	7.634	9.542	7.594	9.218	8.907	6.557	8.312	3.988	4.092	3.815
Blood	M	100	102.819	102.537	100.692	101.579	100.125	96.904	97.746	87.533	65.732***	56.654***	34.962***
	SD	6.375	6.98	7.045	8.307	6.992	7.521	8.024	6.559	7.78	5.329	7.16	5.328

Explanations:
* – difference statistically significant in comparison to control cells (0 ppm) at $p < 0.05$
** – difference statistically significant in comparison to control cells (0 ppm) at $p < 0.01$
*** – difference statistically significant in comparison to control cells (0 ppm) at $p < 0.001$

Table 2. The in vitro effect of gold and copper nanocolloid on the rainbow trout lymphocytes viability after 48 h of incubation (% of control cells viability).

NPs	Cell source	Measures	NPs concentration (ppm), n=6								
			0 (control)	0.9	1.25	1.9	2.5	3.75	5	7.5	10
Au	spleen	M	100	108.21	101.526	104.578	99.087	98.684	94.736	97.331	92.256
		SD	11.28	5.363	7.14	6.093	11.302	8.997	9.809	10.745	6.238
	blood	M	100	100.542	100.327	103.036	99.458	100.542	98.907	97.723	94.118
		SD	9.069	7.918	8.241	5.997	9.106	7.879	6.975	7.132	10.307
Au	spleen	M	100	106.849	102.739	103.698	99.765	96.563	95.891	97.26	93.15
		SD	6.921	10.44	7.665	8.936	5.74	6.359	8.114	7.529	7.897
	blood	M	100	103.912	106.423	101.405	106.059	100.578	93.756	95.147	92.534
		SD	7.618	7.14	10.742	6.84	8.845	5.761	9.793	11.473	8.292

bation. Cells isolated from the spleen were more susceptible to it – the cytotoxic effect of AgNPs was observed in the concentration range of 1.25 ppm ($p < 0.05$) to 10 ppm ($p < 0.001$). Peripheral blood lymphocytes were adversely influenced by silver nanoparticles only at higher concentrations of 5-10 ppm ($p < 0.001$) (Table 1). None of the tested concentrations of gold and copper colloids had an effect on the viability of trout lymphocytes (Table 2).

The effect of metal nanocolloids on lymphocyte proliferation in the rainbow trout

Silver nanocolloid's effect on the proliferation of lymphocytes did not coincide with its cytotoxicity. Despite the high AgNPs' toxicity to spleen lymphocytes, its adverse effects on the cell proliferation were less expressed. ConA-stimulated proliferation was reduced at concentrations of 2.5 do 10 ppm ($p < 0.001$), whereas LPS-induced proliferation was inhibited only at the highest concentration of 10 ppm ($p < 0.001$). Simultaneously enhanced cell proliferation was observed at low levels of silver nanoparticles: ConA-stimulated cells at 0.15 ppm ($p < 0.01$) to 0.3 ppm ($p < 0.001$), and LPS-stimulated cells at 0.3 ppm ($p < 0.01$) (Table 3).

Although AgNPs had a weaker cytotoxic effect on peripheral blood lymphocytes, they strongly inhibited the mitogenic response of cells to ConA already at the concentration of 0.6 ppm ($p < 0.001$). Silver nanoparticles had a weaker influence on LPS-stimulated cells, and their effect was most expressed at the highest concentration of 10 ppm ($p < 0.05$), although the stimulation index values were lower across the entire concentration spectrum in comparison with the control. None of the applied NP concentrations had a stimulating effect on the proliferation of peripheral blood lymphocytes (Table 3).

Among the remaining nanocolloids, gold had a more pronounced effect on lymphocyte proliferation. Similarly to copper, but contrary to silver, it had a more profound influence on spleen lymphocytes:

Table 3. The in vitro effect of silver nanocolloid on the proliferative response of rainbow trout lymphocytes after 96 h of incubation (SI).

Cell source	Mitogen	Measures	NPs concentration (ppm), n=6											
			0 (control)	0.15	0.3	0.6	0.9	1.25	1.9	2.5	3.75	5	7.5	10
Spleen	ConA	M	1.848	2.72**	2.905***	2.386	2.091	1.806	1.643	1.118***	1.172***	1.025***	0.929***	0.894***
		SD	0.36	0.433	0.335	0.364	0.388	0.155	0.295	0.293	0.183	0.185	0.251	0.139
	LPS	M	1.305	1.459	1.684**	1.327	1.329	1.253	1.152	1.107	1.134	1.067	1.057	0.766***
		SD	0.204	0.173	0.179	0.167	0.187	0.164	0.095	0.11	0.164	0.23	0.152	0.085
Blood	ConA	M	2.259	2.12	2.08	1.333***	1.291***	1.21***	1.215***	1.166***	1.184***	1.133***	1.047***	0.966***
		SD	0.279	0.222	0.138	0.218	0.26	0.153	0.243	0.211	0.296	0.278	0.25	0.15
	LPS	M	1.265	1.201	1.137	1.126	1.053	0.999	0.976	0.99	0.95	0.963	0.928	0.883*
		SD	0.21	0.202	0.214	0.229	0.124	0.092	0.206	0.115	0.137	0.178	0.188	0.139

Explanations:
* – difference statistically significant in comparison to control cells (0 ppm) at $p < 0.05$
** – difference statistically significant in comparison to control cells (0 ppm) at $p < 0.01$
*** – difference statistically significant in comparison to control cells (0 ppm) at $p < 0.001$

Table 4. The in vitro effect of gold and copper nanocolloid on the proliferative response of rainbow trout lymphocytes after 96 h of incubation (SI).

NPs	Cell source	Mitogen	Measures	NPs concentration (ppm), n=6								
				0 (control)	0.9	1.25	1.9	2.5	3.75	5	7.5	10
Au	spleen	ConA	M	2.342	1.944	2.249	2.007	1.698**	1.765**	1.775**	1.546***	1.327***
			SD	0.395	0.169	0.273	0.113	0.136	0.274	0.117	0.127	0.224
		LPS	M	1.395	1.211	1.314	1.118	1.003**	1.053**	0.927***	0.865***	0.805***
			SD	0.156	0.197	0.105	0.177	0.201	0.173	0.096	0.077	0.069
	blood	ConA	M	2.666	2.296	2.37	2.26	2.233	2.194	1.916**	1.904**	1.641***
			SD	0.271	0.339	0.334	0.328	0.351	0.388	0.36	0.33	0.235
		LPS	M	1.437	1.443	1.285	1.304	1.35	1.373	1.188	1.176	0.923***
			SD	0.186	0.21	0.162	0.177	0.154	0.205	0.098	0.161	0.103
Cu	spleen	ConA	M	2.159	1.876	1.786	1.782	1.746	1.634*	1.361***	1.301***	1.135***
			SD	0.298	0.19	0.398	0.301	0.227	0.226	0.259	0.194	0.148
		LPS	M	1.337	1.607	1.580	1.351	1.263	1.309	1.250	1.129	1.029
			SD	0.217	0.213	0.186	0.234	0.155	0.145	0.25	0.157	0.142
	blood	ConA	M	2.164	2.447	2.035	2.19	1.988	2.075	1.989	1.793	1.783
			SD	0.269	0.277	0.268	0.363	0.208	0.225	0.325	0.178	0.315
		LPS	M	1.308	1.438	1.315	1.397	1.202	1.274	1.235	1.098	1.119
			SD	0.212	0.254	0.21	0.149	0.131	0.168	0.172	0.153	0.19

Explanations:
* – difference statistically significant in comparison to control cells (0 ppm) at $p < 0.05$
** – difference statistically significant in comparison to control cells (0 ppm) at $p < 0.01$
*** – difference statistically significant in comparison to control cells (0 ppm) at $p < 0.001$

the proliferation of cells stimulated by both ConA and LPS was significantly inhibited at concentrations of 2.5 ppm ($p < 0.01$) to 10 ppm ($p < 0.001$). The proliferative response of peripheral blood lymphocytes stimulated by ConA was lowered at concentrations of 5 ppm ($p < 0.01$) to 10 ppm ($p < 0.001$), and the proliferation of LPS-stimulated cells was inhibited only at the highest nanoparticle concentrations of 10 ppm ($p < 0.001$) (Table 4).

Copper nanoparticles had the least adverse effect

Table 5. The in vitro effect of silver nanocolloid on the phagocytic activity of rainbow trout splenocytes (SI).

Stimulant/ assay	Measures	NPs concentration (ppm), n=6											
		0 (control)	0.15	0.3	0.6	0.9	1.25	1.9	2.5	3.75	5	7.5	10
None/control	M	1	1.287*	1.118	1.135	1.067	1.162	1.07	1.081	1.092	0.915	0.903	0.899
	SD	0.106	0.161	0.142	0.141	0.098	0.126	0.103	0.095	0.106	0.19	0.083	0.078
PMA/RBA	M	1.389	1.455	1.403	1.318	1.486	1.104	1.197	1.128	1.156	1.057*	0.94***	0.841***
	SD	0.187	0.163	0.161	0.125	0.305	0.097	0.128	0.083	0.192	0.096	0.069	0.054
Bacteria/PKA	M	1.137	1.015	1.422	1.475	1.246	1.287	1.129	1.004	1.024	1.075	0.96	0.861
	SD	0.163	0.099	0.41	0.288	0.239	0.137	0.144	0.16	0.213	0.087	0.068	0.091

Explanations:
* – difference statistically significant in comparison to control cells (0 ppm) at $p < 0.05$
** – difference statistically significant in comparison to control cells (0 ppm) at $p < 0.01$
*** – difference statistically significant in comparison to control cells (0 ppm) at $p < 0.001$

Table 6. The in vitro effect of gold and copper nanocolloid on the phagocytic activity of rainbow trout splenocytes (SI).

NPs	Stimulant/ assay	Measures	NPs concentration (ppm), n=6								
			0 (control)	0.9	1.25	1.9	2.5	3.75	5	7.5	10
Au	none/control	M	1	1.001	1.027	1.241	1.114	1.179	0.986	0.895	0.864
		SD	0.165	0.103	0.094	0.274	0.153	0.229	0.116	0.111	0.089
	PMA/RBA	M	1.394	1.426	1.423	1.269	1.238	1.291	1.249	1.339	1.393
		SD	0.146	0.252	0.214	0.167	0.158	0.146	0.143	0.184	0.22
	bacteria/PKA	M	1.105	1.152	1.001	1.078	1.087	1.111	1.026	1.225	1.143
		SD	0.126	0.108	0.098	0.086	0.095	0.138	0.147	0.121	0.071
Cu	none/control	M	1	1.106	1.126	1.029	1.023	1.032	1.166	1.045	1.077
		SD	0.108	0.118	0.171	0.116	0.098	0.212	0.113	0.074	0.105
	PMA/RBA	M	1.463	1.544	1.506	1.389	1.689	1.368	1.234	1.372	1.153
		SD	0.125	0.208	0.324	0.127	0.262	0.204	0.169	0.149	0.102
	bacteria/PKA	M	1.288	1.062	1.067	1.185	1.271	1.315	1.046	1.056	0.981
		SD	0.13	0.162	0.177	0.245	0.227	0.271	0.127	0.151	0.075

on lymphocyte proliferation. The reduction of mitogenic response was observed only in the case of ConA-stimulated splenocytes in the concentration range of 3.75 ppm ($p < 0.05$) to 10 ppm ($p < 0.001$) (Table 4).

The effect of metal nanocolloids on the phagocyte activity in the rainbow trout

Peripheral blood phagocytes were characterized by very low activity levels, and this parameter was not significantly affected by any of the applied nanoparticles (data not shown).

In the case of spleen phagocytes, the activity of non-stimulated cells was enhanced significantly ($p < 0.05$) only at the lowest concentration of silver nanoparticles (0.15 ppm), whereas a decrease in the respiratory burst activity (RBA test) was observed at high AgNPs levels in the range of 5 ppm ($p < 0.05$) to 10 ppm ($p < 0.001$). None of the tested AgNPs concentrations significantly influenced the potential killing activity of bacteria-stimulated splenocytes (PKA test) (Table 5).

Gold and copper nanocolloids had no significant effect on the activity of the spleen phagocytes (Table 6).

Discussion

In the group of the analyzed nanocolloids, only silver demonstrated the cytotoxic activity against rainbow trout cells. The effect of nanoparticles on the

viability of various cell types has been studied extensively *in vitro*, but there are scant data regarding their influence on immunocompetent cells isolated directly from the organism, and the relevant research was performed on mammalian models. In the reviewed literature, a significant drop in the viability of human peripheral blood lymphocytes was observed after incubation at high concentrations of silver nanoparticles (15 ppm and higher). AgNPs had also a cytotoxic effect on immunocompetent cells isolated from NMRI mice whose splenocytes were somewhat less sensitive (at concentrations beginning from 5 ppm) than peripheral blood lymphocytes (from 2 ppm) (Shin et al. 2007, Małaczewska 2010b). The influence of other noble metals NPs on primary immunocompetent cells has not been analyzed to date. In our previous work investigating the effects of the same nanocolloids on Swiss mice splenocytes, a significant reduction in the cell viability was found in response to high concentrations of silver as well as copper nanoparticles (data not published).

In view of the expected high immunotoxicity of silver nanoparticles, we decided to analyze a broader spectrum of AgNP concentrations (0.15-10 ppm) than those used to test gold and copper (0.9-10 ppm). This approach supported observations of the effect of the lowest concentrations (0.15 and 0.3 ppm) of AgNPs on the simulation of the splenocyte proliferation. It is difficult to predict whether similar effects would be achieved at low concentrations of gold and copper which, although not cytotoxic, inhibited lymphocyte proliferation when applied at high levels. There is a general scarcity of data describing the influence of metal nanoparticles on the lymphocyte proliferation in fish. Scant publications document only the adverse influence of high silver concentrations on mammalian lymphocytes. In a study by Shin et al. (2007), AgNP levels of 10 ppm significantly decreased the proliferation of PHA-stimulated peripheral blood leukocytes in humans, although a statistically non-significant increase in the stimulation index was observed at concentrations of 1-5 ppm. Our previous study analyzing the effects of silver nanoparticles on mouse cells produced somewhat similar results. Mouse lymphocytes were less susceptible to the adverse influence of AgNPs, and splenocyte sensitivity levels were comparable with those of peripheral blood lymphocytes (drop in proliferation at 10-20 ppm), whereas in fish cells, the inhibitory effect of silver nanoparticles was observed already at concentrations of 0.6 ppm (peripheral blood lymphocytes) and 2.5 ppm (splenocytes). Similarly to fish lymphocytes, proliferation was stimulated at low NP concentrations (0.1-0.5 ppm) (Małaczewska 2010b). In fish, silver exerted a beneficial influence only on splenocytes, but the analyzed spectrum of concentrations was similar, and the differences in cell responses could be attributed to significant differences between species.

The effect of NPs on the immune system of fish remains weakly investigated, but it has been observed that the primary targets of nanoparticle toxicity are components of the innate immune system. Within seconds after coming into contact with bodily fluids, nanoparticles form a protein corona composed of fibrinogen, immunoglobulins, albumins, complement proteins, lysozyme and acute phase proteins. The process of corona formation stimulates complement, and facilitates the absorption of particles by phagocytes via endocytosis, phagocytosis and macropinocytosis. The latter process supports the absorption of large particles measuring several micrometers in size, such as NP aggregates present in an aquatic environment. The engulfed nanoparticles are not destroyed inside phagocytes but are merely removed by exocytosis or apoptosis, and in the case of neutrophils – by NETosis (Jovanović and Palić 2012). Jovanović et al. (2011a, b) investigated the *in vitro* and *ex vivo* effects of various nanoparticles (TiO2, hydroxylated fullerenes) on the activity of neutrophils isolated from the anterior kidney of the fathead minnow (*Pimephales promelas*). The results obtained differed subject to the type of tested nanoparticles. TiO_2 significantly stimulated the respiratory burst activity and the release of extracellular traps from neutrophils in vitro, but it had no effect on the degranulation of primary granules, whereas fullerenes, which are strong antioxidants, significantly inhibited RBA, NETosis and degranulation levels. After intraperitoneal injection of NPs to animals, both types of particles led to a considerable drop in RBA, degranulation and NETosis as well as changes in the expression of innate immunity genes, pointing to intensified phagocytosis of nanoparticles.

The results of our study differ considerably from the above findings. None of the tested nanoparticles influenced the activity of peripheral blood neutrophils in rainbow trout *in vitro*, and the respiratory burst activity in spleen phagocytes was inhibited only at high concentrations of silver. The present results are atypical because AgNPs are generally known to exert prooxidant effects and stimulate reactive oxygen species (ROS) production in various types of mammalian cells, which can intensify the respiratory burst activity (Małaczewska 2010a). Similarly, however, to our experiment, in the study of Farkas et al. (2010), silver nanoparticles failed to intensify ROS synthesis in rainbow trout hepatocytes despite their strong cytotoxicity. The differences observed can be attributed to differences between species, cell types or the analyzed nanoparticles. The key difference between our investigations and the studies of Jovanović

et al. (2011a, b) was, however, the time of cell incubation with NPs which was only 2 hours in the cited studies and 24 hours in our experiment. The above parameter could significantly influence the results, in particular in peripheral blood phagocytes. The respiratory burst activity and NETosis, an alternative cell death program in neutrophils, are closely correlated processes. ROS production is the key signal in the initiation of NETosis, and when cells enter this program, RBA and ROS synthesis are decreased or completely inhibited. The initial intensification of RBA and NETosis can lead to a decrease in RBA levels over time. Further work is needed in new experimental designs to validate the above hypothesis.

To conclude, all of the analyzed metal nanoparticles influenced the activity of immunocompetent cells in the rainbow trout. Gold and copper nanocolloids delivered adverse effects only at high concentrations. Silver exhibited cytotoxic activity already at relatively low concentrations which, nevertheless, were significantly higher than the levels occurring naturally in the environment. The described mechanism of acute NPs' immunotoxicity is unrelated to the effects observed in animals which are chronically exposed to extremely low doses of NPs in their natural habitats. Further, large-scale research is needed to evaluate the potential influence of nanoparticle concentrations in aquatic habitats on the immune system of fish.

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