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Nanophotonics, Nanooptics, Nanobiotechnology, and Their Applications

Selected Proceedings of the 6th International Conference Nanotechnology and Nanomaterials (NANO2018), August 27-30, 2018, Kyiv, Ukraine

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Contents

Chapter 19
Change in Functional State of Bone Marrow-Derived Mesenchymal Stem Cells After Incubation with Silver Nanoparticles

N. A. Volkova, M. S. Yukhta, E. V. Pavlovich, and A. N. Goltsev

19.1 Introduction

Today among all known nanomaterials, industrial silver nanoparticles (AgNPs) have the highest degree of application [1, 2], largely due to their wide bactericidal properties [3]. AgNPs are contained in the numerous modern products including food nutritional supplements, cosmetics, packaging materials, textiles, electronics, home appliances, water disinfectants, room spray, and medical instruments [4]. The widespread use of AgNPs in everyday life has raised the issue of the safety and consequences of their application. Now the carcinogenic potential of AgNPs belongs to group D (not classified as carcinogenic to the organism). Thus, the enlarged use of nanosilver determines the importance of in-depth study of possible genotoxic and carcinogenic hazards of individual compounds, concentrations, and forms.

The transport of AgNPs inside the cell begins with cell membrane receptor recognition, internalization, and translocation and ends with degradation, accumulation, or clearance by cells. For most cells, uptake of AgNPs via endocytosis is a time, dose, energy-dependent process, and the major target organelles are endosomes and lysosomes. The works [5, 6] demonstrate that silver ions are more toxic to mesenchymal stem cells (MSCs) than AgNPs (in terms of absolute silver concentration). This effect is approximately three times higher for silver ions than for AgNPs. However, in other researches the biological effects on eukaryotic cells and microorganisms induced by both nanoparticles and ions of silver are the same according to the concentration range [7]. Previously, the mechanisms underlying the release of silver ions from nanoparticles were investigated [8, 9]. The liberation

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of silver ions involves oxidative-reduction processes whose rate increases with a temperature in the range of 0–37 °C and decreases with pH growing [10, 11]. In addition, the presence of ligands (such as SO_4^{2-}, S^{2-}) in the microenvironment reduces significantly the adverse effects of silver ions and AgNPs, indicating that these ligands are able to bind silver or toxic metabolites actively. At present, AgNPs are used more than particles of micrometer size, since particles with dimensions in the range of several nanometers have higher ratio of area to mass. Such increase in the reactive surface area leads to a more efficient release of silver ions (Ag^+) in parallel with the low overall concentrations of silver and to the increase of the effect in relation to the applied mass of silver [12].

In present work the mesenchymal stem cells (MSCs) were chosen as a model for studying the possible cytotoxic effects of AgNPs, since these cells are progenitoric for most body tissues in the early stages of development and are involved in the processes of regeneration in an adult organism. Because of the high differentiated capacity, MSCs are the optimal cell models for analyzing the possible effects of AgNPs on morphofunctional characteristics of cells. MSCs can be cultivated for weeks without passing cells, which is important for long-term studies [13]. In addition, MSCs promote regeneration and restoration of mesenchymal tissues, such as bone, cartilage, muscle, ligament, tendon, adipose tissue, and stroma [14]. As reported in [5, 15, 16], silver has a size-dependent cytotoxicity and only AgNPs less than 10–15 nm are considered toxic.

Recent studies have shown that accumulation of AgNPs in the liver could induce cytotoxicity via oxidative cell damage. The AgNPs' risk assessment includes the verification of their biological properties, possible mechanisms, and efficient approaches to decrease their negative impact. Nanotoxicity focuses majorly on the testing of biological actions and is critical to understanding mechanisms and to predicting potentially side effects of AgNPs for sustainable development in the future [17].

In our previous studies [18] on the biological effects of gold nanoparticles (AuNPs), we have shown that some concentrations of 15 nm AuNPs (6–9 μg/ml) affected the immunophenotype, synthesis of type I collagen, ability to direct differentiation, and spectroscopic characteristics of rat bone marrow MSCs.

In this way nanoscale materials have a dose-dependent effect on biological objects. The finding of the presence/absence of AgNPs' cytotoxic action is an important direction of nanobiotechnological research. Here we present the results of investigation of what effect will have the incubation time and concentrations of 40 nm AgNPs on functional state of the bone marrow MSCs.

19.2 Materials and Methods

MSCs were isolated from resected femur of rats ($n = 7$, weighing 220–225 g) by washing out with Hank's solution (PAA, Austria), followed by flushing through a needle with gradually decreased diameter. The next step was a centrifugation

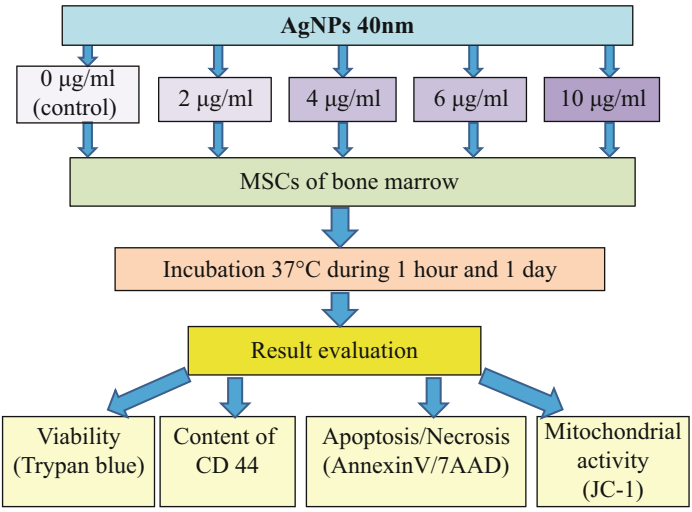

Fig. 19.1 Experimental scheme

at 840 × *g* for 5 min. The cells were resuspended in culture medium and plated on culture flasks (PAA) with 10^3 cells/cm^2 density. Cultural medium contained Iscove's Modified Dulbecco's Medium (PAA), 10% fetal bovine serum (FBS) (HyClone, USA), gentamicin (150 mg/ml) (Farmak, Ukraine), and amphotericin B (10 mg/ml) (PAA). Cultural medium was changed every 3 days. We used standard culture conditions (37 °C, 5% CO_2, 95% humidity) in a CO_2 incubator (Sanyo, Japan). At 80% confluence MSCs were detached with 0.25% trypsin solution (Hyclone) and EDTA (PanEco, Russia) in ratio 1:2 and replanted in other flasks. The third passage of MSCs was used in experiments.

The scheme of experiment is shown in Fig. 19.1. The AgNPs (Sigma-Aldrich, USA) were obtained by citrate synthesis with an initial metal concentration of 20 μg/ml.

The average size of AgNPs was 40 nm. Incubation of MSCs with AgNPs lasted for 1 hour and 1 day at 37 °C. The range of investigated AgNPs' concentrations was 2, 4, 6, and 10 μg/ml. Cells incubated under the same conditions without AgNPs were taken as a control. The percentage of viable cells in the samples was assessed with the trypan blue exclusion test [19].

CD44 expression by MSCs was analyzed using mouse anti-rat CD44-FITC monoclonal antibodies (BD Biosciences, USA) according to the manufacturer's instruction.

The apoptotic/necrotic processes in MSCs were studied using Annexin-V-FITC (Annexin V) (Becton Dickinson, USA) and 7-Amino-Actinomycin (7AAD) (BD) dyes. The functional state of mitochondria was investigated using JC-1 (BD) dye according to the manufacturer's instructions. FACS Calibur (BD) was used for this test. Data were analyzed using WinMDI v.2.8.

Statistical analysis of obtained data was performed using Excel (Microsoft, USA) and Statistica 8 (StatSoft, USA) software. To determine the significance of the differences in continuous variables between the groups, Student's *t*-test or Mann–Whitney *U*-test was used depending on the distribution of parameters. One-way ANOVA or Kruskal-Wallis ANOVA test was applied in the cases of multiple (more than two) comparisons.

19.3 Results and Discussion

The first stage of the study was to determine the effect of AgNPs' addition on the membrane integrity of the MSCs depending on the concentration and incubation time. It has been established that the best viability of cells in the Trypan blue exclusion test was found after 1-hour incubation with AgNPs in concentrations of 2–6 μg/ml (Fig. 19.2). At the same time, the use of 10 μg/ml AgNPs led to a significant decrease of this parameter by 1.24 times in comparison with the control on this term of observation.

After increasing of the incubation time up to 1 day the index of viability in all investigated samples decreased compared to the previous term of observation. AgNPs at the concentration of 2 μg/ml did not affect the viability of MSCs if compare to the corresponding control. However AgNPs' addition at the concentrations of 4, 6, and 10 μg/ml led to a decrease in this indicator by 1.7, 1.9, and

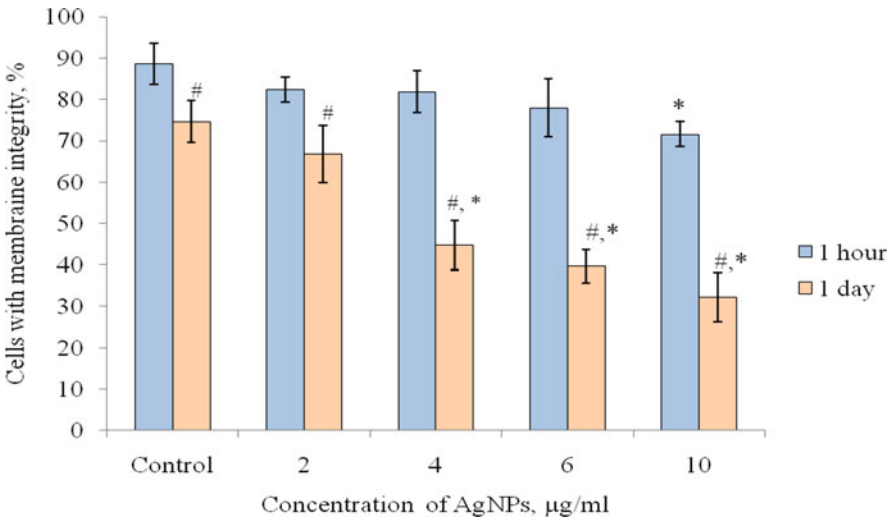

Fig. 19.2 Viability of MSCs after incubation with AgNPs. (Notes: * – difference is significant versus control ($p < 0.05$). # – difference is significant versus previous term of observation ($p < 0.05$))

2.3 times, respectively, versus control. The increase of dead cell number after 1-day incubation which was also detected in the control group may be a result of functional impairment in the MSCs, the degree of which increased with the addition of AgNPs.

Our results showed that cell viability is inversely related to concentration of AgNPs and incubation time. These findings are consistent with the work [20] that shows a decrease of cell viability after exposure to large AuNPs, i.e., 200 nm and 500 nm, but not to smaller AuNPs. In parallel, AgNPs have not been reported to alter cellular functions after 2 weeks of exposure [21], while the viability of MSCs decreased.

The next stage of the study was to determine the content of CD 44^+ MSCs after incubation with AgNPs. The CD44, also known as Pgp1, is a receptor for hyaluronic acid. Other known ligands are collagens, matrix metalloproteinases, and osteopontin. This marker is implied in adhesion function, cell–cell interaction, homing, hematopoiesis, and tumor metastasis [22]. The obtained data (Fig. 19.3) showed that cells in all experimental groups revealed high expression of CD44 (≥90%). AgNPs' addition at the studied concentrations did not lead to any significant changes in the level of CD 44 expression after 1-hour incubation.

The increasing of incubation time did not impair on the level of CD 44 expression by MSCs in the cases of 2, 4, and 6 μg/ml AgNPs' addition. Herewith, the use of AgNPs in the concentration of 10 μg/ml caused a decrease of this indicator by 1.2 times in comparison with the corresponding control.

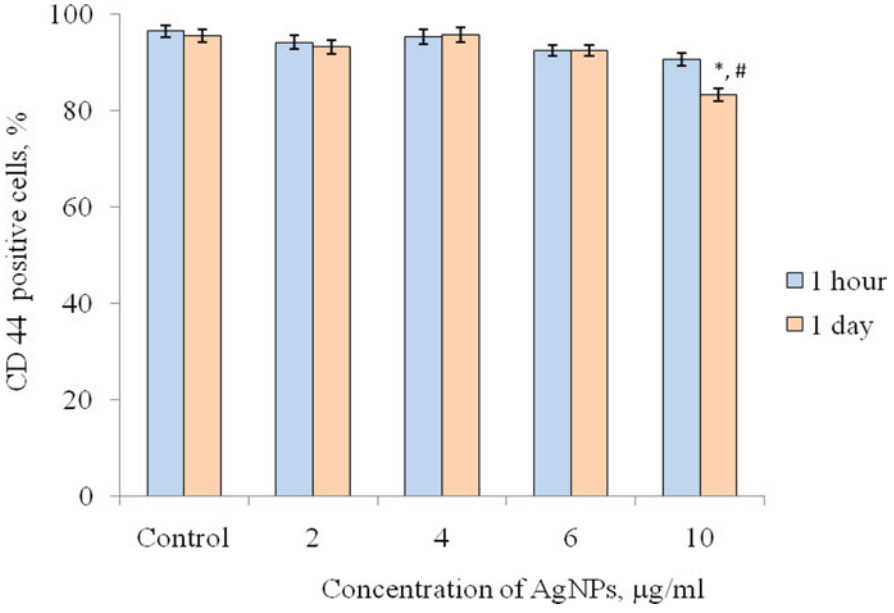

Fig. 19.3 Content of CD 44^+ MSCs after incubation with AgNPs. (Notes: * – difference is significant versus control ($p < 0.05$). # – difference is significant versus previous term of observation ($p < 0.05$))

The effect of 10 μg/ml AgNPs on the level of CD 44 expression is probably due to the oppression of the functional state of cells interacted with nanoparticles. These changes in the phenotype of the bone marrow MSCs give the prerequisites for the detection and prevention of disturbances in the processes of adhesion and proliferation.

The next stage of the work was to monitor the appearance of apoptotic and necrotic processes in bone marrow MSCs in conditions of interaction with AgNPs. It is known [23], nanoparticles of metals can cause apoptosis in cells of different origin. Thus, low doses of titanium dioxide and iron oxide lead to cell death due to the generation of oxidative stress and subsequent apoptosis by the mechanism of caspase-3 activation [24].

The results obtained by flow cytometry (Table 19.1) suggested that AgNPs at the concentrations of 2 and 4 μg/ml did not cause the development of necrosis/apoptosis processes in MSCs after 1-hour incubation. AgNPs' addition in the concentration of 6 and 10 μg/ml led to the decrease in percentage of Annexin $V^-/7AAD^-$ cells (by 27.65 ± 1.45% and 29.17 ± 1.27%, respectively) and to the increase in the number of apoptotic cells (by 17.18 ± 1.38% and 16.6 ± 1.12%, respectively) compared to the control samples of MSCs.

Incubation with AgNPs in concentration of 2 μg/ml during 1 day did not cause the development of necrosis/apoptosis processes in MSCs (Table 19.2). The number of Annexin $V^-/7AAD^-$ cells was significantly lowered by AgNPs addition in concentrations of 4, 6, and 10 μg/ml (by 19.01 ± 1.73%, 38.35 ± 1.25%, and

Table 19.1 Cytofluorometric analysis of MSCs after 1-hour incubation with AgNPs, staining with Annexin V and 7AAD

Sample/region	Annexin $V^+/7AAD^-$	Annexin $V^-/7AAD^-$	Annexin $V^+/7AAD^+$ + Annexin $V^-/7AAD^+$
Control	2.52 ± 0.61	87.79 ± 1.62	9.69 ± 1.32
AgNPs 2 μg/ml	3.76 ± 0.93	86.91 ± 2.35	9.33 ± 1.12
AgNPs 4 μg/ml	3.98 ± 1.18	85.16 ± 1.24	10.86 ± 1.15
AgNPs 6 μg/ml	19.70 ± 1.12[a]	60.14 ± 1.11[a]	20.16 ± 1.21[a]
AgNPs 10 μg/ml	19.12 ± 0.29[a]	58.62 ± 1.15[a]	22.26 ± 2.31[a]

Note: [a] Difference is significant versus control ($p < 0.05$)

Table 19.2 Cytofluorometric analysis of MSCs after 1-day incubation with AgNPs, staining with Annexin V and 7AAD

Sample/region	Annexin $V^+/7AAD^-$	Annexin $V^-/7AAD^-$	Annexin $V^+/7AAD^+$ + Annexin $V^-/7AAD^+$
Control	2.77 ± 0.13	87.88 ± 1.62	9.35 ± 0.25
AgNPs 2 μg/ml	3.45 ± 1.57	85.90 ± 1.18	9.65 ± 1.46
AgNPs 4 μg/ml	7.76 ± 1.29[a]	68.87 ± 1.37[a]	23.37 ± 1.28[a]
AgNPs 6 μg/ml	18.69 ± 1.7[a]	49.53 ± 1.27[a]	31.78 ± 1.35[a]
AgNPs 10 μg/ml	23.56 ± 1.36[a]	45.68 ± 2.27[a]	30.76 ± 2.64[a]

Note: [a] Difference is significant versus control ($p < 0.05$)

42.21 ± 1.56%, respectively) compared with corresponding control. The number of Annexin $V^+/7AAD^-$ cells that present an early stage of apoptosis was increased in the presence of AgNPs in concentrations of 4–10 μg/ml. The percentage of Annexin $V^+/7AAD^+$ and Annexin $V^-/7AAD^+$ cells (late stages of apoptosis, necrosis) after 1-day incubation with 4, 6, and 10 μg/ml AgNPs was significantly different from that in control samples.

Cell alterations during apoptosis are similar for most of cell types. In apoptotic cells, there are changes of lipid composition of plasma membrane: phosphatidyl serine transfers from cytoplasmic part of bilayer to outer side, causing caspase cascade activation, chromatin condensation, and disorder of electron transport chain in mitochondria and eventually arresting ATP synthesis. Programmed cell death can be triggered by receptor-mediated physiological stimuli resulted from genetic disorders, exposure to chemical or physical factors as well as by other changes in cells. We observed this effect after AgNPs' addition in the concentrations of 4–10 μg/ml. In our earlier studies a decrease in the percentage of viable cells and an increase of the number of apoptotic cells were also shown after 7-day culture of the fibroblasts, cryopreserved in the presence of AuNPs in concentration of 6 μg/ml [25].

The last stage of the work was the quantitative evaluation of the mitochondrial activity of bone marrow-derived MSCs after incubation with AgNPs for 1 hour and 1 day. As known, mitochondria play a major role in cellular partitioning of death-regulating signals; the loss of mitochondrial membrane potential is an early event in several types of apoptosis. The high transmembrane potential of healthy cells loaded with JC-1 allows for the formation and sequestration of JC aggregates in the mitochondrion, that detected by a peak in red/orange fluorescence (585 nm) [26]. The results obtained by flow cytometry (Fig. 19.4) suggested that AgNPs addition in the concentrations of 2 and 4 μg/ml did not affect mitochondrial activity in MSCs after 1-hour incubation. The use of AgNPs in the concentrations of 6 and 10 μg/ml led to its decrease by 1.21 and 1.28 times compared to the control samples of MSCs on this observation period.

An increase of the incubation period up to 1 day resulted in a significant decrease in the mitochondrial activity of bone marrow MSCs in all studied groups compared to the previous observation period (1 hour). The obtained data showed that in the control group after 1 day of incubation, 58.1 ± 4.2% of cells was fluoresced in the orange region of the spectrum and 41.9 ± 3.7% was fluoresced in the green one. The use of AgNPs at all concentrations for this incubation period resulted in a significant decrease in the number of cells with fluorescence in the orange region compared with the control. It should be noted that a significant difference of the investigated parameter between the samples with AgNPs' addition in the concentrations of 2, 4, 6, and 10 μg/ml was not observed.

Mitochondria-specific outcomes of AgNPs' exposure have been identified in multiple cell types, including (but not limited) loss of membrane potential, inhibition of enzymes involved in oxidative phosphorylation, and changes in calcium sequestration [27, 28]. Mitochondria appear to be the sensitive targets for nanosilver. Bressan et al. [27] have studied the interaction of nanosilver with human dermal

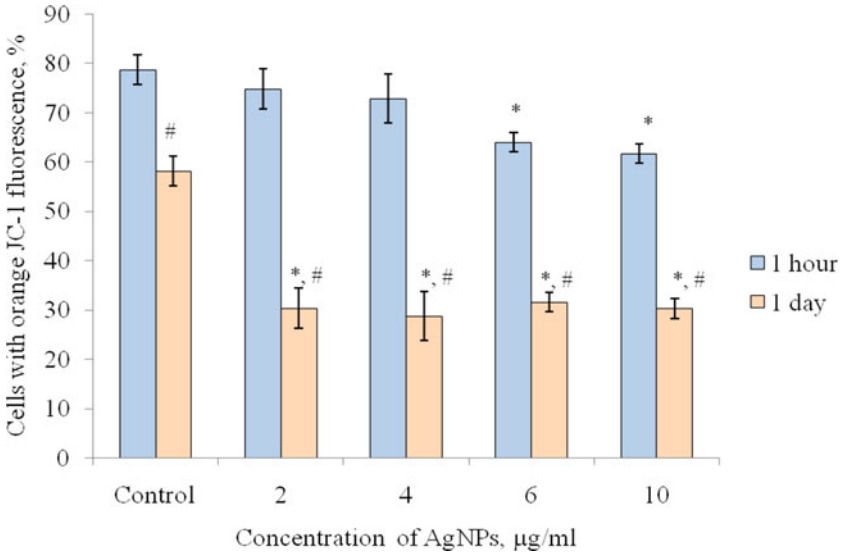

Fig. 19.4 Mitochondrial activity of MSCs after incubation with AgNPs. (Notes: * – difference is significant versus control ($p < 0.05$); # – difference is significant versus previous term of observation ($p < 0.05$))

fibroblasts. They have found that nanosilver particles accumulate outside the mitochondria, cause direct mitochondrial damage, and disturb the function of the respiratory chain, resulting in ROS generation and oxidative stress. AshaRani et al. [28] have suggested that the disruption of the mitochondrial respiratory chain by nanosilver increases ROS production and interruption of ATP synthesis, thus leading to DNA damages. However, the biological significance of mitochondrial toxicity due to AgNPs' exposure is currently incompletely understood.

19.4 Conclusion

In the present study we analyzed the effects of 40 nm AgNPs' concentration and incubation time on morphological and functional state of bone marrow MSCs. It was shown that the use of AgNPs under 1-hour incubation in the concentrations of 2 and 4 μg/ml did not lead to significant changes in viability, level of CD 44 expression, state of apoptotic and necrotic processes, and mitochondrial activity of the cells. The AgNPs' application in the concentrations of 6 and 10 μg/ml resulted in a decrease of mitochondrial activity, percentage of Annexin $V^-/7AAD^-$ cells, and an increase of the number of apoptotic cells compared with the control samples of MSCs. An increase of the incubation period up to 1 day led to a toxic effect of AgNPs (4–10 μg/ml) on MSCs that manifested in an activation of apoptosis

and necrosis processes, decreasing of viability, content of $CD44^+$ cells (AgNPs 10 μg/ml), and mitochondrial activity.

In summary, the internalization of nanosilver into stem cells had a significant influence on various aspects of cellular functions. Therefore, more studies are needed to investigate the effects of nanosilver on stem cell behavior in order to predict the possible health risks. The obtained results are related to the field of applied nanotechnology, which extends to clinical medicine, especially in the development of addressed drug delivery to the target cells or organs.

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Part III
Applications