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Morphofunctional Properties of Human Platelets Treated with Silver Nanoparticles

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We studied structural and functional properties of human platelets in the presence of nanosilver particles. Incubation with 0.05-5 μM silver nanoparticles suppressed platelet adhesion in a dose-dependent manner without affecting internal platelet structure; during adhesion, some granules were not exocytized. Spontaneous platelet activation was observed at nanoparticle concentrations 15-100 μM. Addition of 1-5 μM nanosilver to cells undergoing adhesion blocked massive platelet degranulation, but did not prevent the formation of lamellopodia. The maximum number of preserved granules in platelets was revealed in the presence of 2.5-5 μM silver nanoparticles: 50% after platelet preincubation with silver nanoparticles and 75-77% after stabilization of adherent platelets with silver nanoparticles.

Key Words: *platelets; granules; adhesion; lamella; stabilization*

Colloid nanostructured suspensions of silver are widely used for medical and biological studies. Bactericidal effect of bandages containing silver nanoparticles (AgNP) is well known [1,2,8,14]; in addition, there are data on the reparative and regenerative effect of nanosilver-containing transplants [7,13]. Due to their high penetration capacity, AgNP can contact *in vivo* with various cells, including platelets, that are the source of various bioactive substances [1,5]. It was found, that AgNP reduced adhesion and aggregation of human platelets, blocked fibrin polymerization, thus reducing total activity of the hemostasis system [6,11,12]. However, in some cases AgNP not blocked, but promoted platelet activation [5,9]. Thus, the influence of AgNP on platelets seems to be quite controversial; one could suppose, that AgNP concentration plays a significant role and that different doses produce different effects on platelets and their structure; the effect of AgNP on secretory granules of platelets remains unstudied. This problem can be solved using our original method of morphofunctional assessment of human platelets based on microscopy of vitally stained cells.

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Here we studied structural integrity and functional activity of human platelets exposed to different concentrations of AgNP.

MATERIALS AND METHODS

We used platelets isolated from donor blood by automatic apheresis and colloid AgNP suspension Agbion 2 (Nanoindustriya) with initial concentration of 2.5 nM. The preparation was diluted with PBS (0.15 M; pH 7.2) and added to platelet concentrate (PC) samples in final concentrations of 0.05, 0.1, 1, 1.5, 2.5, 5, 15, 25, 50, and 100 μM.

In series I, PC samples were incubated with different doses of AgNP at 22 or 37°C for 24 h (platelet-AgNP incubation). Morphofunctional analysis of cells was performed after incubation for 10-15 min, 30 min, and 1, 2, 4, and 24 h. In series II, we studied the effect of AgNP in different concentrations on platelets at the early stage of adhesion to elucidate the possibility of preventing platelet degranulation during adhesion (platelet-AgNP stabilization). To this end, 100-200 μl PC was applied on a slide, placed in a Petri dish, and left for 5-10 min at 37°C (to stimulate platelet adhesion). Then, the slide was washed with PBS (to remove liquid fraction with nonadherent cells), 1 ml AgNP suspension

(0.05-100 μM in PBS) was applied, and the slides were incubated at 37°C. Morphofunctional analysis of cells was performed after incubation for 1, 2, and 24 h.

For evaluation of the morphofunctional status of platelets, we used original method was used based on vital staining with fluorochrome dyes trypaflavine and acridine orange followed by examination under a fluorescence microscope [4]. This method allows assessing the content of granules in platelets, their distribution in the cytoplasm before and during adhesion, and integrity at different time intervals. Along with the content of platelets with granules (%), the following parameters were determined: percent of cells with clear-cut lamellopodies among adherent platelets (reflects suppression of platelet flattening on the substrate by different concentrations of AgNP), percent of adherent cells containing 3 or more granules (reflects the number of cells without irreversible degranulation), and integrity of granules in adherent platelets (IG, in %). Integrity of granules was assessed by analyzing morphofunctional activity of platelets (MFAP) before and after the experiment. To this end, digital images of 150-200 cells were obtained and the mean MFAPex (points) per cell was determined [4]. In platelets with and without granules, MFAT was 20 and 70 points, respectively, *i.e.* the contribution of granules into total fluorescence was 70-20=50 points. IG was calculated by the formula IG=(MFATexp-20):50×100%. At MFATexp=70 points, IG is 100% (100% granules are retained), at MPAT=20 points, IG is 0% (all granules are released from the cell).

The results were processed by ANOVA using Microsoft Excel software. The means (M) and mean square deviations (σ) were calculated. The differences were significant at $p<0.05$.

RESULTS

Exposure PC samples to AgNP in concentrations of 0.05-5 μM for 24 h at 22 and at 37°C did not induce visible changes in platelet structure (Fig. 1, *a*, *b*). The initial content of platelets with granules in PC samples was 64.5±4.0%; after 24 h, this parameter was 62.1±4.2% in samples without nanoparticles and 61.8±4.1% in experimental samples. The distribution of granules in platelets did not change; all platelets with granules retained ability to adhesion on the substrate. However, detailed microscopic analysis showed that intracellular processes in adhering platelets greatly varied at different AgNP concentrations. For instance,

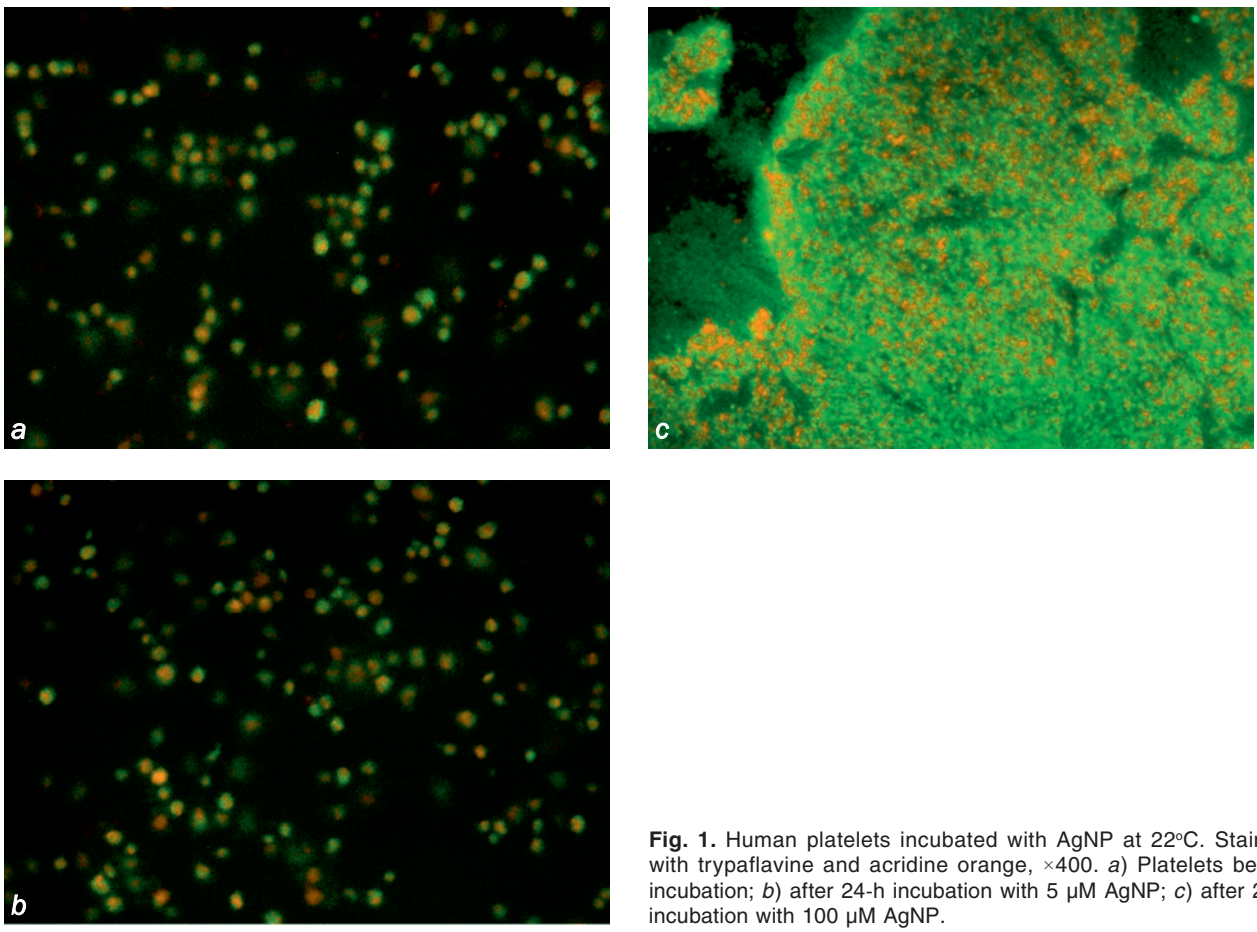

Fig. 1. Human platelets incubated with AgNP at 22°C. Staining with trypaflavine and acridine orange, ×400. *a*) Platelets before incubation; *b*) after 24-h incubation with 5 μM AgNP; *c*) after 24-h incubation with 100 μM AgNP.

AgNP in concentrations 0.05-0.1 μM did not affect the growth of lamellopodies in adherent platelets and produced virtually no stabilizing effect on granules in the platelets. Increasing AgNP concentration from 1 to 5 μM led to an increase in the number of cells forming lamelopodies in PC (Table 1), *i.e.* we observed pronounced dose-dependent suppression of platelet adhesion described in previous studies [6,11]. This was paralleled by an increase in the number of cells with partially or completely preserved granules during adhesion to glass. The maximum volume of granules was found in platelets preincubated with 2.5 μM AgNP for 1 h: adherent platelets retained 50% granules (from the initial content in the cell; Table 1). The granules were detected in both the central part of the cytoplasm and at the periphery of platelets (Fig. 2). It should be noted that short-term incubation of PC with 1-5 μM AgNP for 10-15 min produced less pronounced granule-stabilizing effect during subsequent platelet adhesion. In samples incubated with 1-5 μM AgNP, IG attained maximum in 1 h, in 2 h this parameter slightly decreased and did not significantly vary during further incubation (Table 1). Hence, the optimum duration of preincubation for stabilization of granules in platelets was 1 h.

AgNP in concentrations ≥15 μM completely suppressed the formation of lamellopodia in platelets with

TABLE 1. Effect of PC Exposure to Different Concentrations of AgNP at 22°C on Adhesive Characteristics of Human Platelets ($M\pm\sigma$)

Time of preincubation with AgNP	Morphofunctional parameters			
	% of cells with lamellopodies among adherent platelets	% of adherent platelets containing ≥3 granules	MFAP of adherent platelets, score	integrity of granules in adherent platelets, %
AgNP concentration 0.05-0.1 μM (n=8)				
15 min	100	0	20.0±0.1	0
60 min	100	1.5±0.1	22.1±0.1*	4.2
120 min	100	1.2±0.2	22.5±0.1*	5.0
AgNP concentration 1.0-1.5 μM (n=10)				
15 min	92.7±0.5	30.5±0.5	28.0±0.7	16.0
60 min	85.4±1.1*	50.6±0.8*	35.6±0.5*	31.4
120 min	81.2±1.0*+	48.5±1.0*	34.3±0.6*	28.6
24 h	81.0±0.8*+	48.0±0.5*	34.0±0.8*	28.0
AgNP concentration 2.5 μM (n=12)				
15 min	91.6±0.9	35.6±1.1	29.2±1.3	18.2
60 min	62.5±0.8*	70.8±0.8*	45.5±2.6*	50.0
120 min	10.7±0.9*+	70.0±1.7*	42.1±0.7*+	44.2
24 h	10.0±0.7*+	69.0±0.7*	41.8±1.0*+	43.6
AgNP concentration 5 μM (n=12)				
15 min	60.5±1.9	61.5±0.8	31.0±1.3	21.6
60 min	50.5±0.8*	70.5±1.0*	36.0±1.4*	32.0
120 min	5.3±1.2*+	60.6±1.8+	30.0±1.0+	20
24 h	5.2±0.4*+	60.0±1.4+	30.0±1.0+	20
AgNP concentration 15 μM (n=8)				
15 min	0	40.8±1.4	34.7±1.5	29.4
60 min	0	10.4±1.3*	22.5±1.0*	5.0
120 min	0	0*+	20.0±0.1*+	0

Note. p<0.05 in comparison with incubation for *15 min, +60 min. Here and in Table 2: n: number of measurements.

granules. At the same time, spontaneous platelet activation accompanied by their massive degranulation and formation of trombofibrin clots was observed in the presence of these concentrations of AgNP in PC. At 22°C, changes related to degranulation (translocation of granules to the periphery and binding to the platelet plasma membrane) were observed as soon as in 30-60 min; in some cases, up to 90% cells underwent degranulation during this period. At 22°C, complete degranulation of platelets occurred in 24 h at AgNP concentration of 15 μM and in 2-4 h at 25-100 μM. This process was significantly accelerated at 37°C: degranulation was completed within 2 h at AgNP concentration of 15 μM and within 10-15 min at 25-100 μM. In PC exposed to 100 μM AgNP at 22°C, very large platelet aggregates (up to 1000 μ in diameter) initially appeared (Fig. 1, *c*), but then they broke up to numerous small (10-15 μ) aggregates against the background of complete degranulation of cells. At 37°C, no platelet disaggregation in PC with 100 μM AgNP was observed.

In series II, we found that addition of AgNP in a concentration range from 0.05 to 5 μM to platelets adhering to glass had virtually no effect on the formation of lamellopodies. Even after 24-h exposure, the proportion of platelets with granules without clear-cut lamellopodies did not exceed 10% (Table 2). On the other hand, the number of platelets with granules

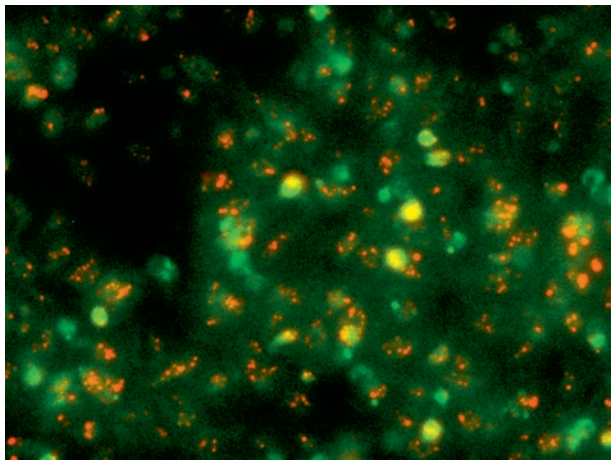

Fig. 2. Platelets preincubated with 2.5 μM AgNP for 1 h at 22°C, in 2 h after adhesion to the glass. Staining with trypaflavine and acridine orange, ×2000.

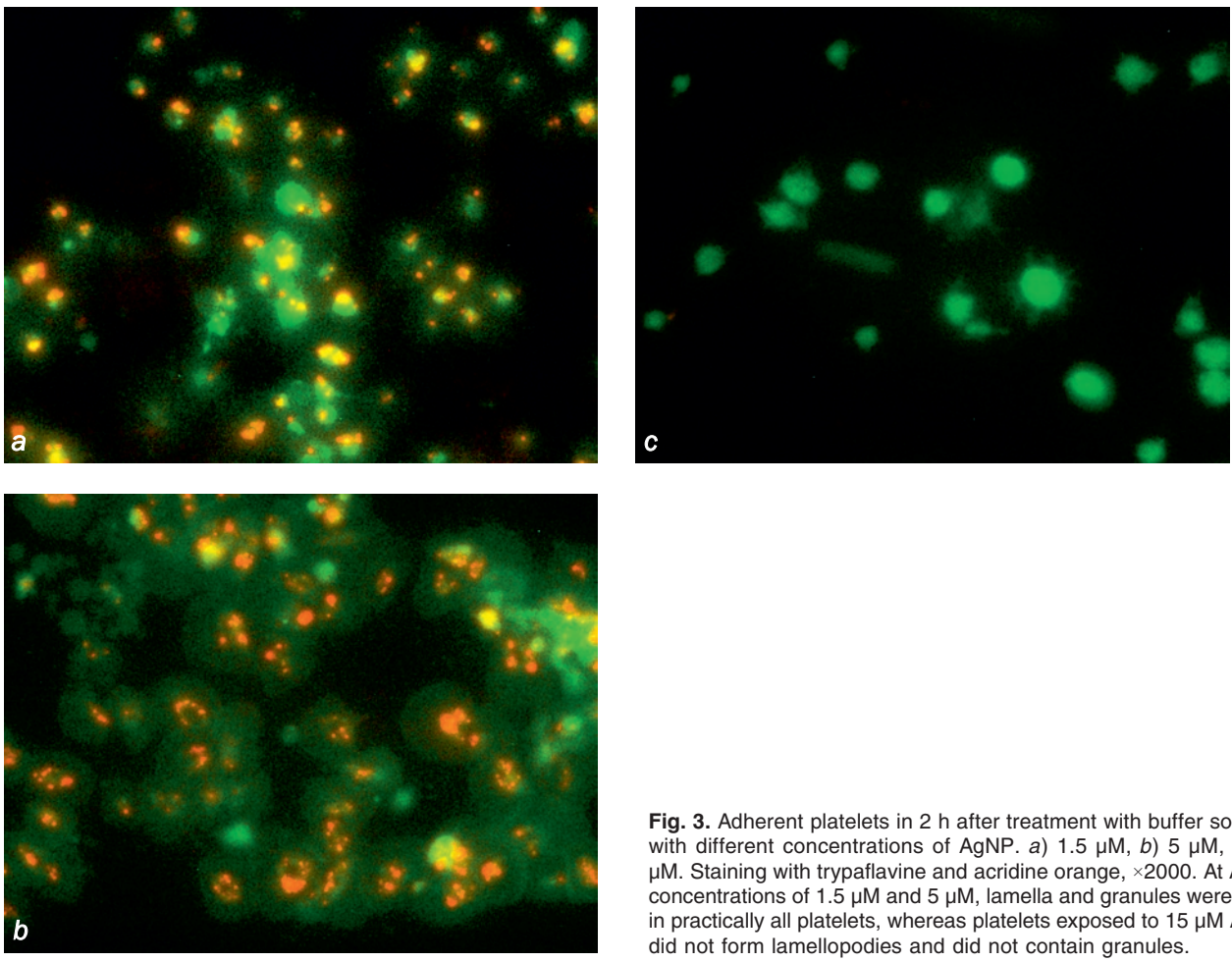

Fig. 3. Adherent platelets in 2 h after treatment with buffer solution with different concentrations of AgNP. *a*) 1.5 μM, *b*) 5 μM, *c*) 15 μM. Staining with trypaflavine and acridine orange, ×2000. At AgNP concentrations of 1.5 μM and 5 μM, lamella and granules were seen in practically all platelets, whereas platelets exposed to 15 μM AgNP did not form lamellopodies and did not contain granules.

TABLE 2. Stabilization of Adherent Platelets with AgNP in Different Concentrations at 37°C

Time of preincubation with AgNP	Morphofunctional parameters			
	% of cells with lamellopodies among adherent platelets	% of adherent platelets containing ≥3 granules	MFAP of adherent platelets, score	integrity of granules in adherent platelets, %
AgNP concentration 0.05-0.1 μM (*n*=8)				
60 min	100	0	20.0±0.2	0
120 min	100	0	20.0±0.2	0
AgNP concentration 1.0-1.5 μM (*n*=10)				
60 min	95.2±1.3	27.6±1.1	35.6±0.9	31.2
120 min	92.3±1.2*	10.6±1.3*	30.0±0.9*	20.0
24 h	91.2±0.8*	10.0±1.5*	30.0±0.9*	20.0
AgNP concentration 2.5 μM (*n*=12)				
60 min	95.1±1.2	51.2±1.0	50.2±1.3	60.4
120 min	92.6±2.3	40.8±1.2*	43.5±1.0*	47
24 h	90.5±1.0	40.4±0.6*	42.5±1.0*	45
AgNP concentration 5 μM (*n*=12)				
60 min	95.0±1.3	78.2±1.4	58.6±1.6	77.2
120 min	95.0±0.4	76.2±1.0	57.7±0.9	75.0
24 h	94.2±0.5	70.2±0.6*	55.3±0.6*	70.6
AgNP concentration 15 μM (*n*=8)				
60 min	0	30.7±1.0	37.5±1.1	35.0
120 min	0	0*	20.0±0.2	0

Note. *$p<0.05$ in comparison with 60-min incubation.

among adherent cells and IG appreciably increased at these concentrations of AgNP (Fig. 3, *a*, *b*). As in the case of preincubation (series I), IG in platelets exposed to 0.05-5 μM AgNP depended on the dose. The highest IG was found in samples stabilized with 5 μM AgNP: after incubation for 1-2 and 24 h, the platelets retained ~75-77 and 70% granules respectively. It is noteworthy that partial degranulation of platelets adherent to glass in the presence of 1-5 μM AgNP was not accompanied by degranulation of platelets remaining in suspension.

Addition of AgNP in concentrations ≥15 μM to adherent platelets produced an ambiguous effect. On the one hand, AgNP in a concentration of 15 μM to platelets completely suppressed the formation of lamellopodies, but this was accompanied by spontaneous and rapid degranulation of all cells (Fig. 3, *c*). In 10-30 min after incubation with high doses of AgNP, platelets on the glas contained no granules, but no changes related to flattening on the substrate were found. We can conclude that blockade of platelet adhesion not excludes exocytosis of their granules. We have previously reported that degranulation can be induced even after complete inactivation of platelets [3]. Stabilization of adherent platelets with 1-5 μM AgNP prevents degranulation of a significant portion of granules, *i.e.* actually arrest platelet activation at the early stages. However, platelets stabilized with AgNP freely degranulate under the action of noncanonical activation factors. In particular, addition of weak hypotonic NaCl (0.1 M) to these preparations induced complete release of all granules, similarly to that induced by 0.1 M NaCl in platelets in suspension [3]. Thus, stabilization of human platelets with nanoparticles does not exclude triggering of activation mechanisms not directly related to adhesion on the substrate.

It is necessary to describe the distribution of granules in stabilized platelets. Analysis of vitally stained

preparations showed that in the presence of 5 μM AgNP, the greater part of granules remained in the central part of the cytoplasm (near the so-called granulomere), while cell periphery, including lamellopodies was almost free from granules (Fig. 3, *b*). A similar picture was observed in platelets stabilized with ticagrelor [4]. On the other hand, at lower concentrations of AgNP (1-2.5 μM), stabilized granules were detected in both the central and peripheral regions of the cytoplasm; granules partially released from the cell and bound with it only with a narrow cytoplasmic bridge were often seen (Fig. 3, *a*). This suggests that exocytosis of granules during platelet degranulation starts from peripheral platelet areas (lamella) and then the central zones (granulomere) are involved. It is known that platelet granules containing different substances are exocytized at different times. In particular, vascular endothelial growth factor (VEGF) is secreted by platelets at earlier terms than blood clotting factors [10]. AgNP in concentrations 1-5 μM dose-dependently increase the proportion of granules stabilized in the granulomere. Hence, using different concentrations of AgNP we can stabilize different amount of granules in adherent platelets stimulating preservation or, on the contrary, release of granules with required components. However, this approach requires more detailed analysis of the distribution of granules of different chemical composition in platelets and the dynamics of their exocytosis during adhesion.

It was shown that AgNP in low doses (10 μM) do not affect cell viability *in vivo* and *in vitro*, whereas in doses >50 μM have a pronounced cytotoxic effect [5,13]. In the presence of high concentrations of AgNP, structural changes in cell membranes, oxidative stress, mitochondrial dysfunction, DNA defects, and apoptosis were observed [5]. This effect was attributed to high penetration capacity of AgNP. It should be noted that high permeability of platelet membranes can lead to their spontaneous activation [3]; in our experiments, this activation was repeatedly observed in PC in the presence of 15-100 μM AgNP. On the other hand, low doses of AgNP (5 μM) did not induce these changes and even suppressed active exocytosis of granules from platelets. This can be related to dose-dependent modulation of different intracellular signaling pathways in platelets not directly related to each other by AgNP. The effect of 1-5 μM AgNP on adherent platelets is similar to the stabilizing effect of antiaggregant ticagrelor, which allows fixation of platelets with granules on the collagen matrix for a long time Thus, AgNP can also be used to stabilize platelets with granules on the substrate, for instance, for creation of saturated platelet biotransplants.

Thus, AgNP can either block or stimulate activation of human platelets in suspension and on adhesive substrate. Stabilization of granules in platelets can be achieved via incubation with the most effective concentration of AgNP 2.5 μM; for stabilization of adherent platelets, 5 μM was more effective. Platelets stabilized with AgNP retained ability to degranulation.

REFERENCES

1. Gmoshiskii IV, Smirnova VV, Khotimchenko SA. Current state of the problem of assessing the safety of nanomaterials. Ross. Nanotekhnol. 2010;5(9-10):6-10. Russian.
2. Krutyakov YuA, Kudrinskiy AA, Olenin AYu, Lisichkin GV. Synthesis and properties of silver nanoparticles: Advances and prospects. Russ. Chem. Rev. 2008;(77)3:233-257.
3. Makarov MS. Noncanonical methods of activation of human platelets. Med. Alfavit. 2015;3(11):30-35. Russian.
4. Makarov MS, Khvatov VB, Borovkova NV. Stabilization of human platelets on the adhesive substrate with use of ticagrelor. Mol. Med. 2015;(6):57-60. Russian.
5. Arora S, Jain J, Rajwade JM, Paknikar KM. Cellular responses induced by silver nanoparticles: in vitro studies. Toxicol. Lett. 2008;179(2):93-100.
6. Bandyopadhyay D, Baruah H, Gupta B, Sharma S. Silver nanoparticles prevent platelet adhesion on immobilized fibrinogen. Indian J. Clin. Biochem. 2012;27(2):164-170.
7. de Mel A, Chaloupka K, Malam Y, Darbyshire A, Cousins B, Seifalian AM. A silver nanocomposite biomaterial for blood-contacting implants. J. Biomed. Mater. Res. A. 2012;100(9):2348-2357.
8. Fu J, Ji J, Fan D, Shen J. Construction of antibacterial multilayer films containing nanosilver vialayer-by-layer assembly of heparin and chitosan-silver ions complex. J. Biomed. Mater. Res A. 2006;79(3. P. 665-674.
9. Huang H, Lai W, Cui M, Liang L, Lin Y, Fang Q, Liu Y, Xie L. An evaluation of blood compatibility of silver nanoparticles. Sci. Rep. 2016;6. ID 25518. doi: 10.1038/srep25518.
10. Peters CG, Michelson AD, Flaumenhaft R. Granule exocytosis is required for platelet spreading: differential sorting of α-granules expressing VAMP-7. Blood. 2012;120(1):199-206.
11. Shrivastava S, Bera T, Singh SK, Singh G, Ramachandrarao P, Dash D. Characterization of antiplatelet properties of silver nanoparticles. ACS Nano. 2009;3(6):1357-1364.
12. Shrivastava S, Singh SK, Mukhopadhyay A, Sinha AS, Mandal RK, Dash D. Negative regulation of fibrin polymerization and clot formation by nanoparticles of silver. Colloids Surf. B Biointerfaces. 2011;82(1):241-246.
13. Sudheesh Kumar PT, Raj NM, Praveen G, Chennazhi KP, Nair SV, Jayakumar R. In vitro and In vivo evaluation of microporous chitosan hydrogel/nanofibrin composite bandage for skin tissue regeneration. Tissue Eng. Part A. 2013;19(3-4):380-392.
14. Walters BD, Stegemann JP. Strategies for directing the structure and function of 3D collagen biomaterials across length scales. Acta Biomater. 2014;10(4):1488-1501.