Effect of Hydrogen Peroxide and Catalase Derivatives on Functional Activity of Platelets

A. V. Vavaev, L. I. Buryachkovskaya, I. A. Uchitel, E. G. Tishchenko, and A. V. Maksimenko

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Effects of H_2O_2 on platelet aggregation were estimated *in vitro* in the presence and absence of inductors (ADP, serotonin, TRAP) and native and modified catalase. Dose-dependent effect of H_2O_2 (50 μM or more) was investigated in a pathophysiological concentration of 300 μM inducing platelet aggregation. H_2O_2 modulated aggregation induced by ADP, serotonin, and TRAP significantly increasing the initial platelet aggregation followed by disaggregation, which was always more pronounced than in control. Catalase derivatives (native and modified forms) dose-dependently reduced the effect of H_2O_2 and completely abolished it in a dose of 9000 U catalase activity per 1 ml of solution for native catalase and 1200 U/ml for modified one. Modified catalase, in contrast to native one, produced an independent inhibitory effect on induced platelet aggregation. Components of modified catalase (individual substance and simple mixture) had no antiaggregant effect.

Key Words: *platelet aggregation; hydrogen peroxide; catalase; bienzyme conjugate superoxide dismutase-chondroitin sulfate-catalase; antiplatelet effect*

Normally, toxic potential of reactive oxygen species is balanced by antioxidant defense system [2]. Pathological changes contribute to imbalance between prooxidant and antioxidant systems. Excessive reactive oxygen species damage lipids, proteins, and DNA [7]. Oxidative stress plays an active role in atherogenesis by inducing lipid oxidation and endothelial dysfunction [11], stimulating production platelet activating factor, and influencing platelet aggregation [7].

H_2O_2 is one of the most stable reactive oxygen species. It easily penetrates cell membranes, transforms into more active forms, can independently cause or exacerbate platelet aggregation [1,10,14,15], and is available for biochemical studies. There is evidence on multidirectional action of H_2O_2 on ADP-induced platelet aggregation. Thus, H_2O_2 in a concentration of 200 μM enhances aggregation [8] and in a concentration of 2-20 mM reduces [13]. Very high concentrations of H_2O_2 [13], far from physiological, seriously damage the plasma membrane of platelets and associated receptors [9]. Doses above 100 μM H_2O_2 can be cytotoxic [12].

Vitamin antioxidants are applied to correct the damaging effects of reactive oxygen species. In contrast, antioxidant enzymes neutralize reactive oxygen species by 4-5 orders of magnitude faster and may prove more effective in combating oxidative stress, especially if a combination of enzymes is used [2]. In our laboratory, a modified form of catalase (CAT) was created, a conjugate of SOD and CAT covalently linked with chondroitin sulfate (CS) (SOD-CS-CAT). In the model of arterial thrombosis in rats we showed that bienzyme conjugate SOD-CS-CAT possesses not only antioxidant activity, but also exerts a pronounced antithrombotic effect, significantly superior to the effects of its components [2]. The process of thrombosis involves a large number of hemostatic system components; among the most important agents of blood clotting are the platelets.

Institute of Experimental Cardiology, Moscow, Russia. ***Address for correspondence:*** labbiocheng@cardio.ru. A. V. Maksimenko

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Here we studied the influence of H_2O_2 on functional activity of platelets and antiplatelet effect of bienzyme conjugate SOD-CS-CAT.

MATERIALS AND METHODS

The study was performed on platelets from healthy volunteers. The blood was taken on an empty stomach from the cubital vein by gravity into a plastic tube with 0.13 M sodium citrate, pH 7.3. Platelet-rich plasma (PRP) was isolated by blood centrifugation at 180*g* for 15 min. We did not use platelet poor plasma to dilute the PRP to minimized additional activation, which might be caused by activators leaking into plasma during the additional centrifugation.

Platelet aggregation was assessed by a BIOLA dual channel laser aggregation analyzer. In addition to the traditional method of light transmission recording (Born method), platelet aggregation was evaluated by analyzing the fluctuations of the light passing through the sample. The relative dispersion of such fluctuations is proportional to the mean radius of aggregates and allows registering the formation of microaggregates containing less than 100 platelets. Aggregation was measured by the method of fluctuations and expressed in relative units.

The capacity of platelets to form small aggregates (from 3 to 100 cells) was studied by spontaneous aggregation and aggregation induced by 0.5 μM ADP, 0.5 μM serotonin, and 1 μM TRAP (thrombin receptor agonist peptide) with and without addition 9000 U/ml CAT or 1200 U/ml SOD-CS-CAT (by CAT activity) using the method of light flux fluctuations. The study was conducted not later than 2 hours after blood sampling. The presented values corresponded to the maximum amplitude of aggregation (peak) at the 1st minute of recording.

The shape and adhesion of platelets were evaluated by scanning electron microscopy (SEM). To study platelet morphology, 10 μl PRP were fixed in 2.5% glutaraldehyde (1:30) for 1.5 h at room temperature, then placed on a polycarbonate membranes with pore diameter of 0.22-0.40 μ, dehydrated, and spray-dried. The number of platelets with different shapes was counted in 25 fields of view at ×2500 using a Phillips PSEM 550 scanning electron microscope and expressed in the percentage of the total number of cells of each type. To evaluate platelet adhesion, 15 μl PRP preincubated with CAT or SOD-CS-CAT for 5 min and applied simultaneously with H_2O_2 (300 μM) on the adhesive surface (glass), in special experiments pretreated with saline, 300 μM H_2O_2, or SOD-CS-CAT. The samples were incubated for 15 min at room temperature in a closed weighing bottle to prevent drying, carefully washed in saline to remove unattached cells, then fixed in 2.5% glutaraldehyde for 1.5 h, washed, dehydrated with alcohols, and processed for SEM.

The results were processed using Statistica 6.0 software. Statistical data processing was performed using Student's *t* test. Deviations from the mean value corresponded to the error of the mean.

RESULTS

Addition of 50 μM–2 mM H_2O_2 triggered platelet aggregation (Fig. 1). SEM showed that at the maximum of aggregation response, the central part of formed aggregates consisted of tightly bound platelets and the peripheral of weakly bound ones (Fig. 1, *b*). By the 5th minute, the size of the aggregates decreased, and their structure was so dense that individual cells became undistinguishable (Fig. 1, *c*). These SEM results suggest that the decrease in aggregate size can be due to dissociation of weakly bound platelets from aggregation centers (*i.e.* aggregation induced by H_2O_2 was partially reversible because of heterogeneity of

TABLE 1. Mean Values of the Aggregation Induced by Different Inducers with or without CAT or Bienzyme Conjugate SOD-CS-CAT (*M*±*m*; rel. units)

Conditions	Main stimulus			+H_2O_2		
	control	CAT	SOD-CS-CAT	control	CAT	SOD-CS-CAT
No spontaneous aggregation	1.1±0.03	—	—	1.8±0.1	1.4±0.1	1.6±0.1
Spontaneous aggregation	1.6±0.2	1.5±0.1	1.4±0.1	1.8±0.2	1.5±0.1	1.6±0.1
ADP, 0.5 μM	2.2±0.1	1.6±0.2	1.7±0.1	3.3±0.3	2.1±0.3	2.1±0.2
Serotonin, 0.5 μM	2.3±0.1	3.0±0.5	1.6±0.2	3.2±0.4	2.9±0.9	2.4±0.7
TRAP, 1 μM	2.3±0.3	2.3±0.4	1.9±0.1	3.0±0.4	3.0±0.8	2.4±0.3

Note. The data were obtained using the method of estimating the mean size of aggregates during the first minute of measurements, when peak aggregation response occurred and maximum aggregation was achieved.

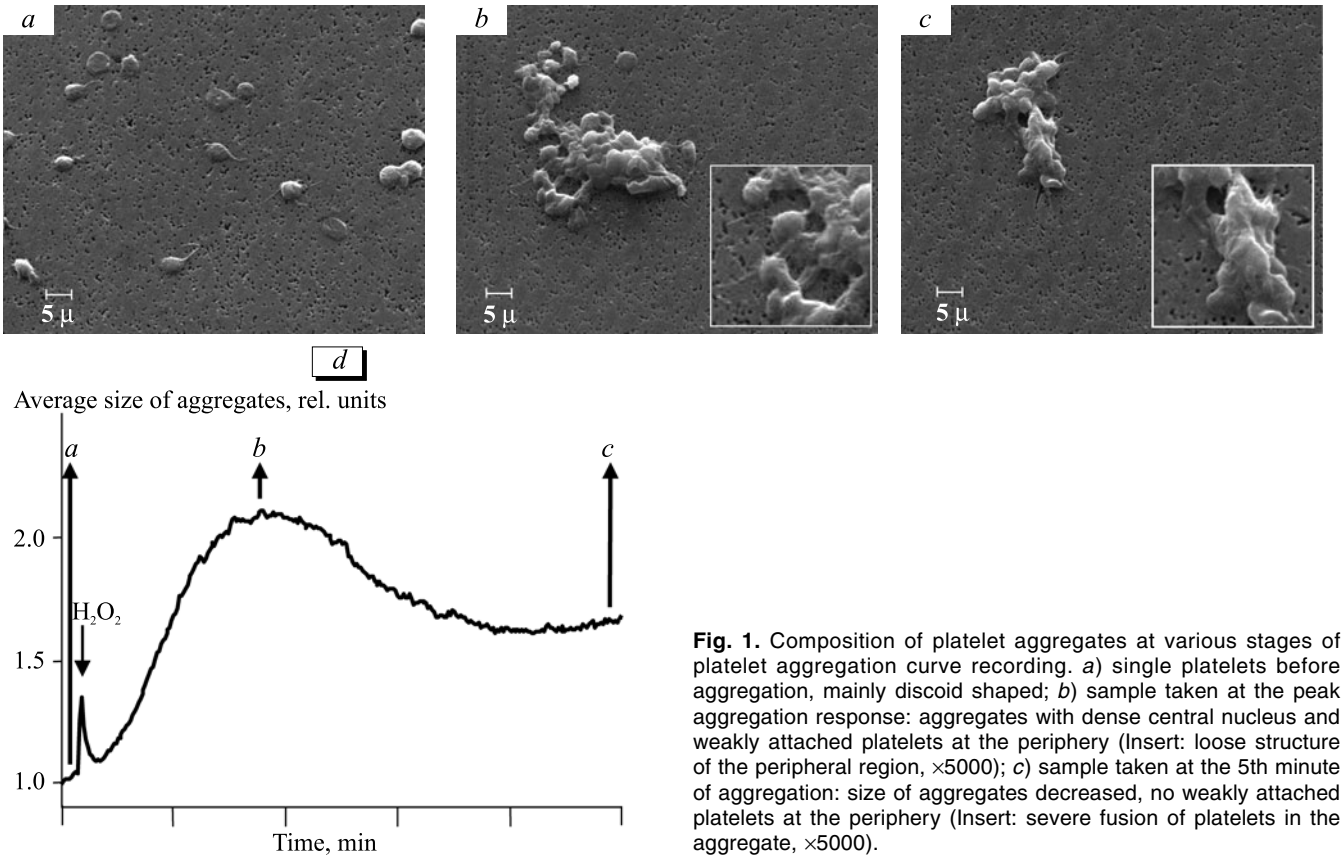

Fig. 1. Composition of platelet aggregates at various stages of platelet aggregation curve recording. *a*) single platelets before aggregation, mainly discoid shaped; *b*) sample taken at the peak aggregation response: aggregates with dense central nucleus and weakly attached platelets at the periphery (Insert: loose structure of the peripheral region, ×5000); *c*) sample taken at the 5th minute of aggregation: size of aggregates decreased, no weakly attached platelets at the periphery (Insert: severe fusion of platelets in the aggregate, ×5000).

bonds between platelets) and compactization of the central areas of the aggregate.

At higher doses of H_2O_2, the formation of oxygen bubbles in PRP was observed which hindered the registration of aggregation. In blood samples from volunteers with their own spontaneous aggregation, addition of H_2O_2 increased the average size of forming aggregates during the first minutes, but further disaggregation of platelets occurred and the aggregation index dropped below its initial level. This was probably related to the influence of H_2O_2 on cyclooxygenase-1, which plays a decisive role in the formation of active aggregation mediators. Cyclooxygenase catalyzes conversion of arachidonic acid to prostaglandins and thromboxanes in the presence of reactive oxygen species [3]. H_2O_2 in low concentrations stimulates cyclooxygenase activity and in high concentrations inactivates it [4]. The latter is explained by the characteristic feature of cyclooxygenase to be inactivated during the enzymatic reaction of its own and thus limit the further formation of active aggregation mediators [5].

For experiments with derivatives of CAT, the concentration at 300 μM H_2O_2 was chosen, which exhibited an appreciable aggregation-inducing effect, on the one hand (Fig. 2, *a* and *b*; the peak of aggregation on average 1.8±0.1 rel. units; Table 1) and was close to the range of physiological concentrations, on the other hand [6].

Simultaneous addition of 300 μM H_2O_2 and 0.5 μM ADP to PRP caused rapid growth of medium-size aggregates, which exceeded that at ADP-induced aggregation near the first minute: 3.3±0.3 and 2.2±0.1 rel. units, respectively ($p<0.001$; Fig. 2, *c*; Table 1). After the first minute, primary aggregation was replaced by rapid disaggregation. A similar pattern was observed after simultaneous addition of 300 μM H_2O_2 and 0.5 μM serotonin, or 1 μM TRAP (Fig. 2, *d*; Table 1).

The content of three platelet forms (disks, spheres and flattened platelets) in the sample was estimated by SEM and the percentage of the total cell number was counted. Platelet adhesion and spreading phase is a critical stage of hemostasis initiating a cascade of further reactions leading to thrombus formation. The relative contents of spread platelets in the control sample and in the sample with H_2O_2 was 21 and 24%, respectively (Fig. 3, *a*, *b*).

CAT and SOD-CS-CAT dose-dependently reduced the influence of 300 μM H_2O_2 on platelet aggregation, the maximum effect was observed at concentrations of 9000 and 1200 U/ml, respectively; they reduced the average size of aggregates to 1.4±0.1 and 1.6±0.1 rel. units, respectively (*vs.* 1.8±0.1 rel. units). SOD-CS-CAT directly modulated platelet aggregation reducing

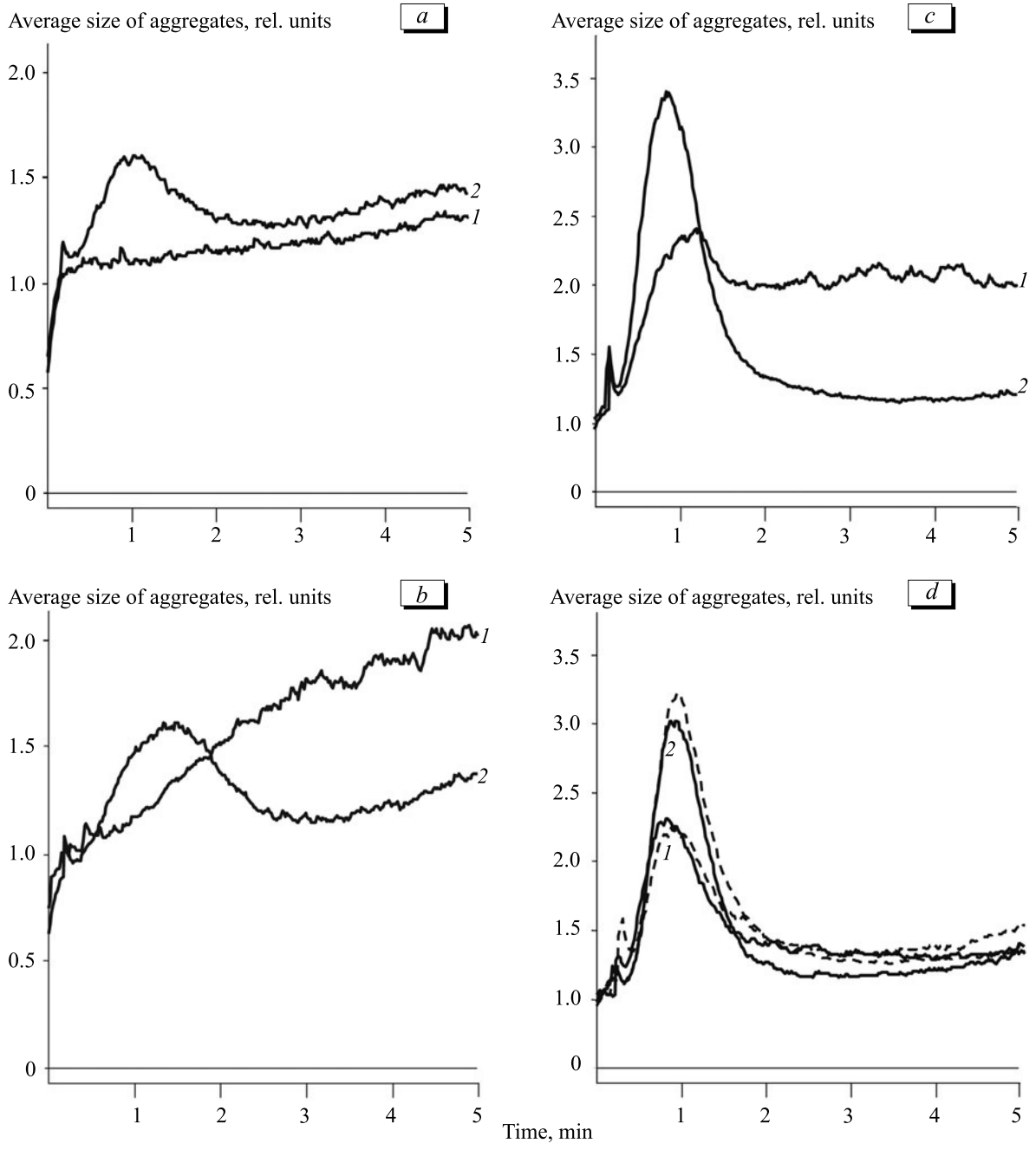

Fig. 2. Effect of H_2O_2 on spontaneous and induced platelet aggregation. *1*) aggregation caused by the main stimulus, *2*) addition of 300 μM H_2O_2. *a*) without spontaneous aggregation (control); *b*) against the background of spontaneous aggregation; *c*) induced by 0.5 μM ADP; *d*, induced by 0.5 μM serotonin (solid lines) or 1 μM TRAP (dashed lines).

the maximum size of aggregates during spontaneous aggregation by 12-13% and during aggregation induced by 0.5 μM ATP by 22-24%. A similar pattern was observed for other studied inducers (Table 1).

Components of SOD-CS-CAT in the range of its effective concentration individually and in a simple mixture had no antiaggregant effect.

On adherent surface, addition of SOD-CS-CAT substantially decreased the number of spread platelets (<3% cells in the sample, Fig. 3, *c*-*e*). These data attest to high antiplatelet potential of SOD-CS-CAT (Fig. 3).

Thus, H_2O_2 modulates platelet aggregation (inducing it within a certain concentration range and increasing the peak of aggregation curves induced by ADP, serotonin, or TRAP) and can be used in simulation of oxidative stress in the platelet system to study the effect of antioxidants. Under these conditions, bienzyme conjugate SOD-CS-CAT acts not only as a more ef-

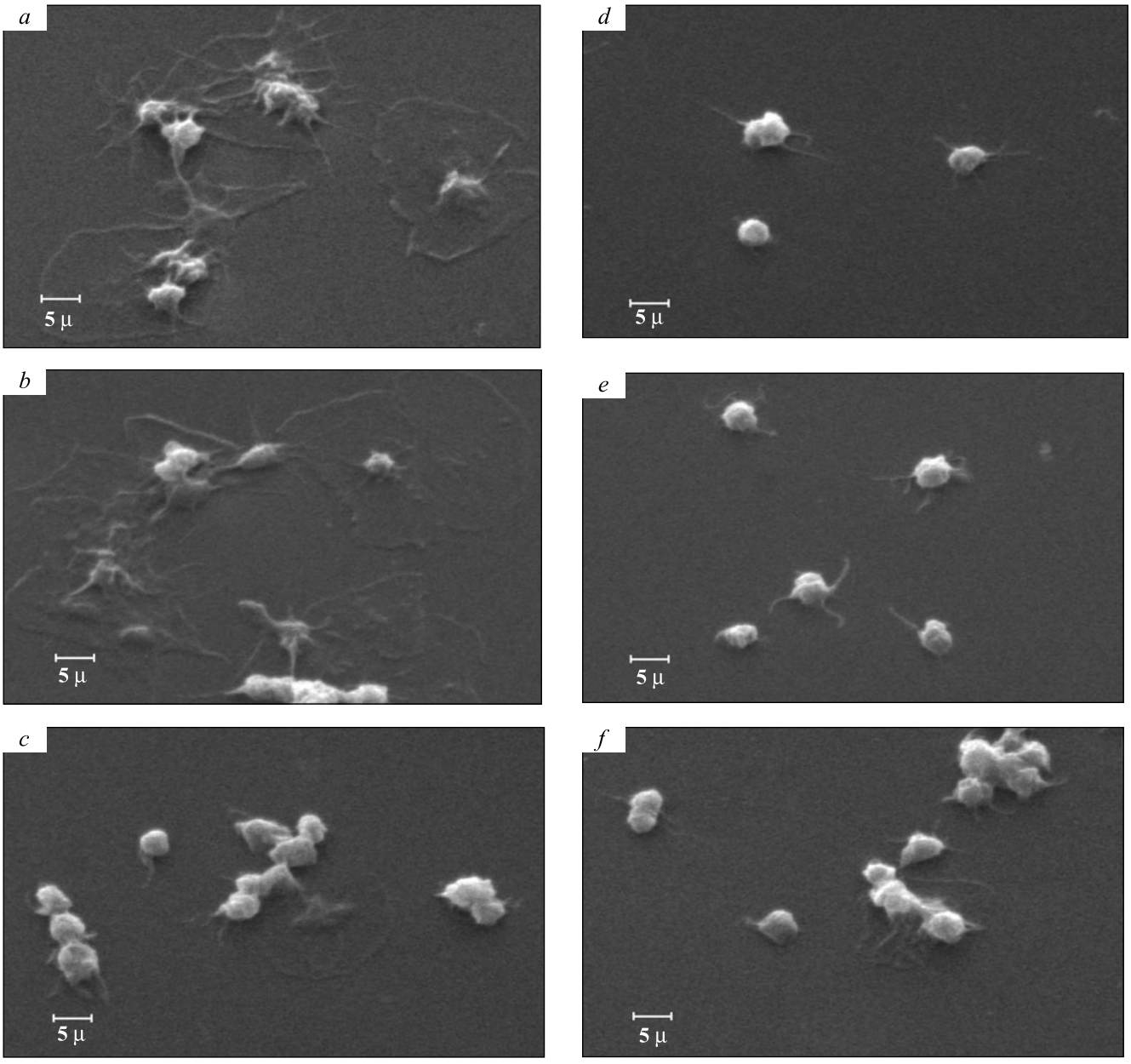

Fig. 3. SEM: platelet adhesion to glass (Phillips PSEM 550 microscope; ×2500). *a*) glass with a saline: PRP+ saline; *b*) glass with a saline: PRP+300 μM H_2O_2; *c*) glass with SOD-CS-CAT: PRP+300 μM H_2O_2; *d*) glass with SOD-CS-CAT: PRP+SOD-CS-CAT; *e*) glass with 300 μM H_2O_2: PRP+SOD-CS-CAT; *f*) glass with 300 μM H_2O_2: PRP+CAT.

fective antioxidant than CAT (effective in lower concentration), but also as an effective antiaggregant. This effect can be useful in postischemic reperfusion of the myocardium to reduce the area of "reperfusion" injury and during the development of oxidative stress in the circulatory system for protection of the vascular wall from thrombotic lesions.

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