=========== **CELL BIOPHYSICS** ===========

The Effects of Magnesium, Acetylsalicylic Acid, and Emoxypine on Platelet Aggregation

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Abstract—This paper summarizes the results of investigation of thrombin-induced platelet aggregation in the absence and presence of magnesium sulfate, acetylsalicylic acid, and emoxypine. Magnesium sulfate, as well as acetylsalicylic acid and emoxypine, were shown to reduce the degree of platelet aggregation when used individually, while the combined action of these three substances did not have a similar effect. The data obtained can be used for selection of ischemic stroke treatment strategies.

Keywords: stroke, platelets, aggregation, magnesium, acetylsalicylic acid, emoxypine

Schematic stroke is the most common type of stroke (80% of all stroke cases) and one of the three leading causes of death, alongside cardiovascular diseases and cancer [1, 2]. Initial treatment of stroke during the "therapeutic window" (up to 12 h after the onset of stroke) involves the use of tissue endoplasmic activator and acetylsalicylic acid [3]. Subsequent treatment (protectionist) is aimed at reducing the severity of the long-term consequences of chemise, namely, blocking of pro inflammatory cytosine and cell adhesion molecules, inhibition of free-radical processes, and interruption of apotheosis [4]. Acetylsalicylic acid (ASA) [5], antioxidants (emoxypine) [6], and magnesium ions [7] are used for this purpose in clinical practice. Platelets play an important role in the formation of blood clots [2]. The action of thrombi formed in the coagulation cascade evoked by damage to the blood vessel leads to activation and aggregation of platelets. Blood clots in cerebral arteries are characteristic for ischemic stroke, while markers of intramuscular blood clotting (clot precursors, fibrin clots, and platelet and erythrocyte aggregates) are detected in hemorrhagic stroke patients [8–10]. In previous studies, we showed that the use of magnesium ions in therapeutic concentrations combined with basic therapy (importantly, ASA is an essential component of basic therapy [6]) led to the normalization of lipid per oxidation processes, improved the state of antioxidant protection system, and reduced the dysfunction of the

blood–brain barrier in stroke patients [11, 12]. However, combined use of basic therapy, magnesium sulfate, and emoxypine did not result in any changes of the parameters described above relative to cases in which only basic therapy was used [11, 12].

Features of thrombin-induced platelet aggregation in the presence of magnesium ions, acetylsalicylic acid, and emoxypine in therapeutic concentrations used for the treatment of ischemic stroke were investigated in order to elucidate the mechanism underlying the clinical actions of the therapeutics mentioned above [6].

MATERIALS AND METHODS

Human α-thrombin, acetylsalicylic acid, and buffer salts were obtained from Sigma-Aldrich (United States). $MgSO_4$ and emoxypine were produced at the Belbiofarm concern (Belarus). Human platelets were the object of study. Human blood treated with Glyugit-sir preservative was obtained from the National Center for Hematology and Blood Transfusion (Minsk, Belarus) on the day of the experiment. Collection of platelets started with centrifugation of plastic test tubes with platelet-enriched blood plasma at 250 *g* for 2 min to pellet the cells [13]. The pellet of platelets in each tube was carefully resuspended in 300 μL of Ireland buffer (0.12 M NaCl, 0.0154 M KCl, 0.006 M glucose, 0.0015 M Na_2EDTA, 0.0133 M Tris, pH 6.5) by pipetting until a homogeneous cell suspension was formed. The resulting suspension was centrifuged at 250 *g* for 2 min. The supernatant was discarded and Ireland

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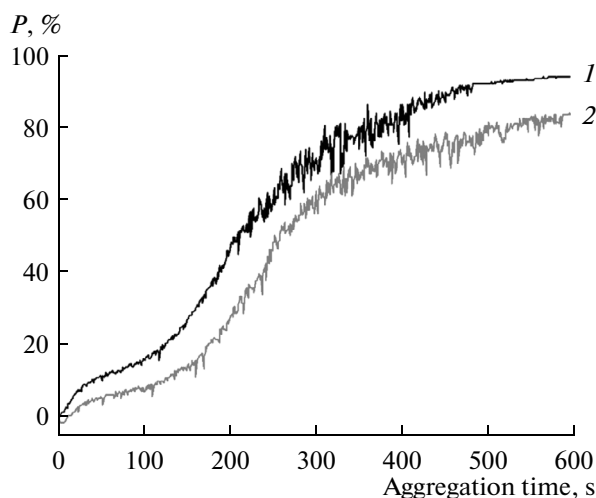*Abbreviations*: ASA—acetylsalicylic acid, NMDA—N-methyl-D-aspartate.

Fig. 1. Curves illustrating thrombin-induced platelet aggregation in the (*1*) absence and (*2*) presence of ASA (concentration 0.01 mg/mL).

Fig. 2. Degree of platelet aggregation (*P*) at different magnesium sulfate concentrations in the medium. (*0*) Control ($n = 11$), (*1*) 2.5 mM ($n = 7$), (*2*) 5 mM ($n = 9$), (*3*) 10 mM ($n = 7$), and (*4*) 120 mM MgSO$_4$ ($n = 5$). * $p < 0.05$ as compared to control, Dunn's test.

buffer was added to the pellet to the concentration of 2.5×10^9 platelets/mL. Platelet aggregation was investigated using an AR2110 automatic aggregometer (Solar, Belarus) according to the following procedure: 400 µL of Dulbecco PBS (0.137 M NaCl, 0.0027 M KCl, 0.0087 M Na$_2$HPO$_4$, 0.00148 M KH$_2$PO$_4$, 0.001 M CaCl$_2$, pH 7.35); in addition, 50 µL of platelet suspension were placed into a plastic aggregometer cuvette thermostated at 37°C and incubated for 30 min at 37°C (the final concentration of platelets was 2.5 · 10^8 cells/mL). The preparations under investigation were subsequently added to the cuvette and incubated for 30 min. Afterward, 50 µL of thrombi solution in Dulbecco buffer (final concentration 0.32 µg/mL, 1 NIH unit) were added to the cuvette and platelet aggregation was assessed. The degree of thrombin-induced platelet aggregation was inferred from the increase of optical transmittance of the suspension. The results obtained were processed using Statistica 6.0 (StatSoft Inc., United States) and BioStat (© S.A. Glantz) software. Normal distribution of the numeric data was checked using the Shapiro–Wilk criterion ([14], pp. 77–79) and equal variance test. The results are shown as the median and the interval between 25 and 75 percentiles ([14], pp. 83–85, [15], pp. 31–33). Nonparametric statistical tests—the Friedman test or Kruskal–Wallis test—of one-way ANOVA were used to analyze the values measured ([14], pp. 51–53). If the Kruskal–Wallis test revealed a significant difference, post hoc analysis based on Dunn's test for multiple comparison was performed when necessary ([15], pp. 359–364).

RESULTS AND DISCUSSION

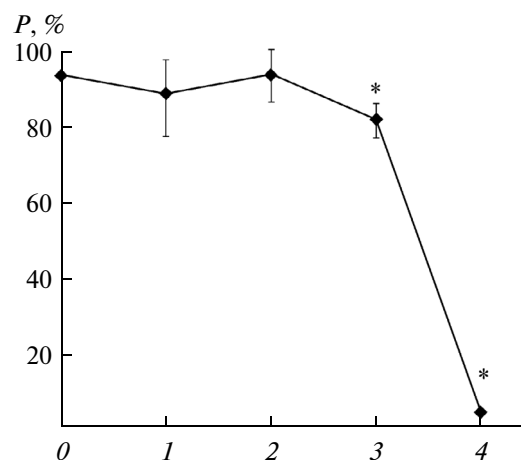Example curves for thrombin-induced platelet aggregation in the presence and absence of ASA are shown in Fig. 1. The effect of magnesium ions on thrombin-induced platelet aggregation is illustrated by Fig. 2. As shown in Fig. 2, magnesium ions at concentrations of 2.5 mM ($n = 7$) or 5 mM ($n = 9$) did not cause a statistically significant decrease of the degree of platelet aggregation as compared to the control ($n = 11$). Notably, magnesium sulfate concentrations equal to or below 5 mM are within the therapeutic range—that is, magnesium ions at such concentrations are detected in the blood of patients treated with magnesium salts [6, 16, 17]. Significant suppression of platelet aggregation (relatively to control) under the conditions of our study was observed only when the concentration of magnesium ions was increased to 10 mM ($n = 7$, $p < 0.05$, Dunn's test). When the concentration of magnesium ions in the reaction medium was increased to 120 mM ($n = 5$), thrombin-induced platelet aggregation was almost completely inhibited ($p < 0.05$ relative to control, Dunn's test). The effect of 0.01 mg/mL ASA on platelet aggregation ($n = 8$) is illustrated by Fig. 3a. The concentration used in the experiments is equivalent to the therapeutic concentration of ASA in the blood of patients receiving this drug [18, 19]. For example, the concentration of ASA in the blood of stroke patients receiving 1.5 g of the substance daily equaled 0.01 mg/mL [21]. As shown in the figure, ASA used at a therapeutic concentration caused a significant decrease of the degree of platelet aggregation ($p < 0.05$, Friedman test). As shown in Fig. 3b, emoxypine also caused a significant suppression of platelet aggregation ($n = 7$, $p < 0.05$, Friedman test) when used at a concentration equivalent to the therapeutic one (0.001 mM [6, 20, 21]). However, the use of a combination of 5 mM magnesium sulfate, 0.01 mg/mL ASA, and 0.001 mM emoxypine (Fig. 4) had an unexpected effect—namely, the complex (mixture) had no influence on platelet aggregation
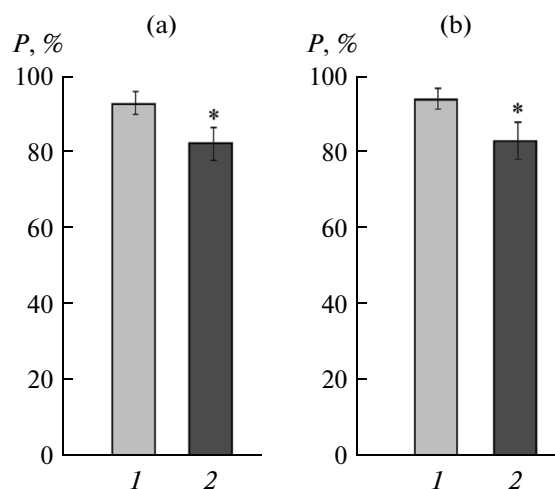
Fig. 3. Degree of platelet aggregation (P) in the absence (light column) and in the presence (dark column) of (a) ASA and (b) emoxypine in therapeutic concentrations. [ASA] = 0.01 mg/mL ($n = 8$, $n_c = 8$); [emoxypine] = 0.001 mM ($n = 7$, $n_c = 7$). * $p < 0.05$ as compared to control, Friedman's test.

Fig. 4. Degree of platelet aggregation (P) in the absence (light column) and in presence (dark column) of a mixture of magnesium sulfate, ASA, and emoxypine in therapeutic concentrations. [MgSO$_4$] = 5 mM, [ASA] = 0.01 mg/mL, [emoxypine] = 0.001 mM, and $n = 13$ for both control and treatment groups. No significant difference from control was detected for the treatment group ($p = 0.56$, Kruskal–Wallis test).

($p = 0.56$, Kruskal–Wallis test, $n = 13$ for both the control and experimental groups).

Several factors must be taken into account for this effect to be explained. The preparations investigated in the present work are widely used in clinical practice for the treatment of stroke [6]; sometimes, they are used in combination. Each preparation has its own physiological target. Prolonged activation of N-methyl D-aspartate (NMDA) glutamate receptors leading to the opening of calcium channels and entry of excess calcium ions into the cell with subsequent cell death is one of the primary pathogenetic stages in brain ischemia [1–6]. Magnesium preparations are the only clinically recognized safe and efficient antagonists of NMDA receptors in stroke [22, 23]. Magnesium reduces presynaptic glutamate release and blocks both glutamate NMDA receptors and the entry of calcium through voltage-dependent channels [24]. Dilation of cerebral blood vessels and suppression of platelet aggregation contributing to enhanced perfusion of the affected brain areas are among the vascular effects of magnesium [24, 25]. ASA is one of the most important drugs for treatment of ischemic stroke [26]. Prevention of platelet aggregation by means of irreversible inhibition of cyclooxygenase-1 and blockade of the enzymatic activity of cyclooxygenase-2 persisting throughout the lifetime of the platelets (approximately 10 days) is the main mechanism underlying the antiaggregative effect of ASA [27, 28]. Numerous studies have demonstrated an increase in the content of reactive oxygen species in the brain during ischemia and reperfusion following an ischemic episode [1–6]. Emoxypine (methylethylperidol) is an antioxidant widely used in clinical practice for the treatment of stroke [6, 29, 30].
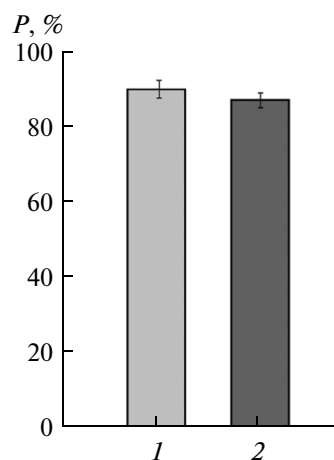
Thus, only emoxypine and ASA could suppress the aggregation of platelets when used in therapeutic concentrations (notably, magnesium sulfate, ASA, and emoxypine had no effect on thrombin conformation, dynamics, or functional activity when used at therapeutic concentrations [31]). The absence of the effect of the drug combination on platelet aggregation can be due to the interaction of drugs reducing the effect of ASA on platelets. A similar effect was observed in a multicenter study of combined use of aspirin and non-steroid antiinflammatory drugs (acetaminophen, diclofenac, and naproxen). The positive clinical effect of ASA decreased in this case [32, 33]. The authors of the study ascribed this effect to the competition of the NSAIDs and ASA for receptors on platelets [32] resulting in short and reversible inhibition of platelet aggregation and a significant reduction of the therapeutic effect [32]. Given the similarity of the chemical structures of nonsteroid drugs and emoxypine [29, 30, 32], one can assume that a similar phenomenon occured in our experiments.

As evident from the above, the absence of a therapeutic effect upon the combined use of magnesium sulfate, emoxypine, and ASA may be due to the interaction of the preparations with each other, resulting in a weakening of their combined effect on biological systems, rather than to peculiarities of the pharmacokinetics of these drugs. The data obtained can be used for selecting treatment strategies for ischemic stroke.

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