ORIGINAL ARTICLE

STUDY OF SYNERGIC EFFECT BETWEEN SOME METAL IONS AND ADRENALINE ON HUMAN BLOOD PLATELETS AGGREGATION

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Manuscript received: February 2015

Abstract

The paper presents the evaluation of effects of zinc, aluminium and copper ions in normal or toxic concentrations on the *in vitro* adrenaline induced platelet aggregation. Aggregation was studied according to a modified spectrophotometric method of Born using a Helena Laboratories Pack4 Aggregometer, in the platelet rich plasma. The curves were compared using as parameters the area under curve, maximum aggregation and aggregation rates. The effects of ions in the presence of adrenaline were, at least qualitatively, similar: an increase of aggregation face to single adrenaline induced aggregation due to decreasing of electrophoretic potential. The mechanism of synergism between ionic metals and adrenaline couldn't be explained satisfactory. Nor in thermodynamic, nor in kinetic, nor in biochemical theories was possible to predict quantitatively the aggregation. Whatever the mechanism, the effects, even in case of high concentrations, were not high and had no clinical nor toxicological significance.

Rezumat

Articolul prezintă evaluarea efectelor ionilor de zinc, cupru şi aluminiu în concentraţii normale şi toxice asupra agregării plachetelor sanguine *in vitro* induse de adrenalină. Agregarea a fost analizată conform metodei spectrofotometrice dezvoltată de Born, modificată, utilizând un agregometru Hellena Laboratories Pack4, în plasma bogată în plachete. Compararea curbelor a fost realizată utilizând ca parametri aria de sub curbă, maximul de agregare şi ratele de agregare. Efectele ionilor în prezenţa adrenalinei au fost, cel puţin calitativ, în concordanţă cu teoria: o creştere semnificativă a agregării induse de adrenalină. Mecanismul sinergismului între efectul ionilor metalici şi cel al adrenalinei nu a putut fi însă explicat satisfăcător. Nici în teoriile termodinamice, nici în cele cinetice şi nici în cele biochimice referitoare la agregare nu se poate obţine o predicţie cantitativă. Oricare ar fi însă mecanismul, efectele observate, chiar şi în cazul concentraţiilor mari, nu au fost crescute şi nu au avut semnificaţie clinică sau toxicologică.

Keywords: aluminium, copper, zinc, adrenaline induced platelet aggregation

Introduction

Thrombocytes are blood components with a very important role in vascular integrity maintenance [25]. Erythrocytes and thrombocytes are involved in the process of bleeding arrest in response to injury, when the blood vessels are damaged. Thus, it is very important to understand the factors involved in the platelet aggregation process [34].

The role of thrombocytes in haemostasis and thrombosis has been revealed in several reviews [5, 27]. The platelet aggregation process is the major factor involved in acute coronary syndrome (ACS), like ischemic stroke or acute myocardial infarction [11, 13].

The mechanisms of the platelet aggregation have been intensively studied especially after the invention of platelet aggregometer by Born [2] and independently by O'Brien [20].

Electrically charged particles can influence platelet aggregation. Both positively and negatively charged particles can induce platelet aggregation, but negatively charged ones are less toxic, probably due to the fact that there restricted entry and charge repulsion between the thrombocytes and nanoparticles [15].

Studies revealed that platelet aggregation can be influenced by divalent and trivalent cations like calcium, copper, manganese, magnesium, cadmium [12].

Platelet aggregation is influenced by divalent ions, in a greater extent by ions of transition elements (like zinc or manganese) than by alkaline earth metals ions like magnesium or calcium [21]. Additionally, trace elements are involved in many physiological and pathological processes [7, 24].

In this study, authors considered that the mechanism by which positively charged metal ions

increase the platelet aggregation is the decreasing of the repelling potential between the negatively charged platelet membranes. Therefore, the analysis was developed in the frame of Derjaghin Landau Verwey Overbeeck (DLVO) theory [28] extended to a general theory including aggregation of all types of colloids and biological cells.

Classical DLVO theory assumes that aggregation of hydrophobic colloidal particles is governed by the sum between repulsion of electric double layers from the interface of particles and Van der Waals attraction forces [14].

The classical DLVO theory was used later to explain adhesion between microorganisms, bacteria, some viruses and yeast [8], and their interactions with different surfaces [32].

There are several conditions that can induce electrically charged chemical species imbalance in the human body. For example, haemodialysis patients are a vulnerable class of patients for electrolyte disturbances. The haemodialysis process involves a risk for both deficiency and accumulation of trace elements, because the trace elements can be removed by dialysis. Studies have shown that chronic haemodialysis patients usually have copper deficiency and zinc overload [33]. Haemodialysis patients had in the past significantly higher aluminium serum concentrations, especially due to improper quality of the dialysis water [31].

As the studies revealed, copper deficiency decreases thrombocytes adhesion to endothelium, but enhances thrombocytes aggregability [29]. I*n vitro* addition of copper ion triggered an anticoagulant effect [1].

The profile change of the metallic particle induced platelet aggregation is influenced by the physiological conditions of the thrombocytes (for example, the pre-activation induced by agonists like ADP adrenaline or ADP) [6].

Given these facts, the aim of the present study was to determine the effects of aluminium, copper and zinc ions in physiological and toxic concentrations on the adrenaline induced aggregation.

Materials and Methods

Materials

All reagents used were of analytical grade.

Copper sulphate pentahydrate, zinc chloride and aluminium chloride hexahydrate were bought from Sigma Aldrich.

Adrenaline bitartrate was bought from Helena Laboratories.

Methods

Aggregation was studied according to the spectrophotometric method of Born using a Helena Laboratories Pack4 Aggregometer, in the platelet rich plasma (PRP). The samples were continuously stirred at 1000 rpm. The following steps were performed: the blood was collected from healthy volunteers. Before participating in this study, the volunteers gave their informed consent. The blood was collected on 20 USP units of lithium heparin/mL of blood as an anticoagulant. The specimen was represented by plasma obtained from whole blood.

The PRP was prepared by centrifuging the whole blood for 10 minutes at 200 G, at room temperature. The PRP was collected in a test tube with a plastic Pasteur pipette.

The platelet poor plasma (PPP) was prepared by centrifuging the remaining blood samples for 15 minutes at 3000 G at room temperature. The PPP was collected in a test tube with a plastic Pasteur pipette.

The samples were covered in order to maintain the pH.

PRP and PPP were both stored at room temperature and the tests were performed within maximum three hours after sample collection.

The aggregation agents were prepared as following:

- The copper sulphate pentahydrate, zinc chloride and aluminium chloride hexahydrate working solutions were obtained by dissolving the mentioned substances in HEPES buffer.
- The adrenaline solution was prepared from a stock solution by reconstituting one vial with 1.0 mL of distilled water. It was stirred gently until completely dissolved. After reconstitution it was obtained a stock solution of adrenaline bitartrate 3 mM. The adrenaline reagent was stored in dry form at 2 to 8°C and was stable until the expiration date on the vial. The reconstituted reagent was stable for 1 week at 2-8°C.
- The HEPES Buffer was prepared according to the procedure indicated in literature [4].

The Helena Laboratories Pack4 Aggregometer was prepared for use as recommended in the Operator's Manual.

The PPP cuvette was inserted into each channel and the instrument was set to 100% aggregation.

Each metallic ion dilution was performed in 400 µL PRP and followed by the next steps:

- 100 µL saline solution (0.9% NaCl) were added. This was the control used to set the spontaneous aggregation in absence of adrenaline and metal ions.
- 50 µL saline solution (0.9% NaCl) and 50 µL 40 µM adrenaline were added. This was the control used to set the aggregation induced by adrenaline alone.
- 50 µL saline solution (0.9% NaCl) and 50 µL metal ion solution were added. This was the control used to set the aggregation induced by the metal ion alone.
- 50 µL 40 µM adrenaline solution and 50 µL metal ion solution were measured in a cuvette with a stirring bar. This was the test sample.

After the aggregating reagent dilutions were added to PRP cuvettes, the aggregation percent was

recorded (when the aggregating agent was added, the instrument set 0% and the channel was activated).

Results and Discussion

The aggregation curves had an initial approximately exponential form followed by an asymptotic saturation phase (Figure 1). The comparison of the obtained curves took into account the maximum platelet aggregation (MPA), time-delay and the initial rates and also a global parameter for characterizing curves, namely the area under curve (AUC) [16, 17, 23, 26].

Regarding the appearance of artefacts or "outlier" curves, the validation of results was performed by visual examination.

The curves were recorded directly by the aggregometer, and AUCs and MPAs were calculated directly by the software of aggregometer. Comparisons were made directly or after smoothing of curves.

The obtained curves were continuous and, in general, less affected by noise (Figure 1).

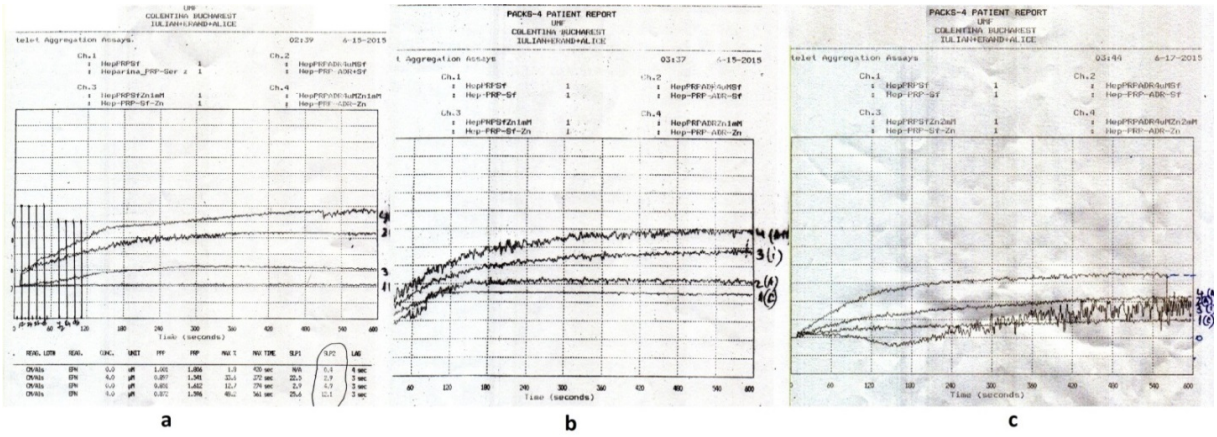
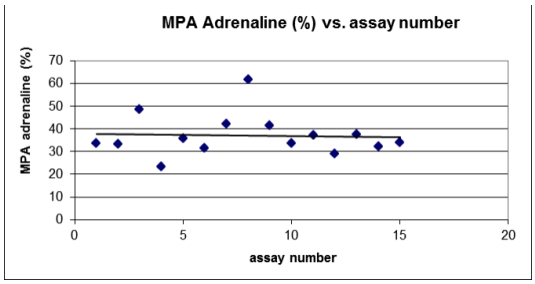


Figure 1.

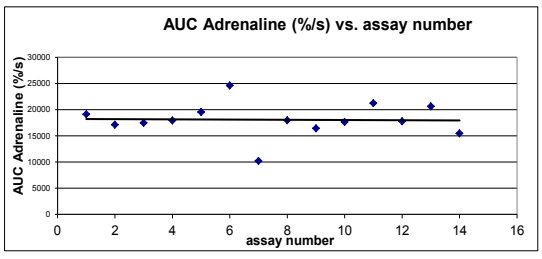
Aggregation curves transmittance *vs.* time in case of zinc ion. (1) control (spontaneous aggregation); (2) adrenaline alone; (3) zinc ion alone; (4) sample itself (contains both zinc ion and adrenaline). The concentration of zinc ion and adrenaline were as following: a. Zinc ion 1 mM, adrenaline 4 μM; b. Zinc ion 1 mM, adrenaline 4 μM; c. Zinc ion 2 mM, adrenaline 4 μM.

Practically all aggregation tests used in clinical trials concern induced aggregation. In the present paper, adrenaline was chosen as inducer of aggregation. Since the expected effect of metal ions was an increase of aggregation, the concentration of adrenaline was selected to produce less than half of the complete aggregation, i.e. a MPA lower than 50 %.

In case of multiple, serial determinations on the same blood sample it is to take into consideration that platelets functionality is disabled after one or two hours so that, in our experiments, for each determination of effects of ions or ions plus adrenaline, there were analysed, in parallel, two control samples: intrinsic aggregation (which is very low) and aggregation in presence of adrenaline. As can be seen in Figures 2a and 2b, control adrenaline induced aggregation (15 determinations) remained practically constant during the entire experiment. ANOVA test for slope of regression lines for MPA and AUC as function of the assay number verified the hypothesis H_0: $\beta = 0$ ($p < 0.01$) i.e. was not a tendency of modification of aggregability during the experiment, the slope of regression line being zero.



a.



b.

Figure 2.

Reproducibility of aggregation curves corresponding to adrenaline during the experiment (n = 15): a. MPA; b. AUC.

Consequently, in the following analysis, the mean values corresponding to adrenaline were considered for comparisons.

Zinc ion, adrenaline and their combination effect

Normal zinc ion blood levels are 0.013-0.025 mM and the zinc concentration levels reported in intoxications are 0.046-0.108 mM [10]. In this study the concentrations of zinc ion used in the PRP samples were: 1, 2 and 3 mM, thus it was employed a higher level of concentration than the ones reported both in normal conditions and in intoxications.

Other authors studied the effects of zinc ion on the platelet aggregation in PRP at the same concentrations (1 - 3 mM), and reported that zinc ion had not significant effect on aggregation [9].

As it can be seen in Figure 3, zinc ion had a pro-aggregant effect even in the absence of adrenaline or other aggregation inducer so that the results were in complete accordance with this theory. The obtained aggregation depended on the ion concentration but (considering MPA) it finally remained lower than 50 %, i.e. of the same order with the value obtained in the case of adrenaline. Dependence was linear but variability was high enough. The correlation coefficient was low in case of AUC and somewhat greater in case of MPA.

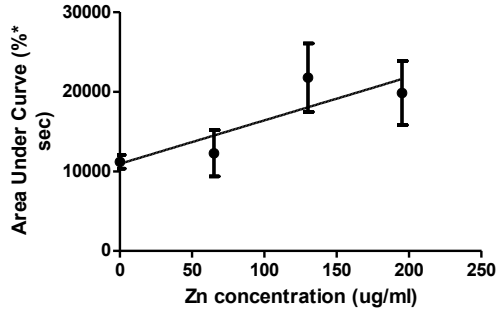


Figure 3.
Dependence of areas under the aggregation curves on the zinc ion concentration

Since parameters of aggregation curves are not independent, the profile of their evolution with increasing concentrations of zinc ion was similar and consequently, there were calculated ratios face to parameters of control curves. Obtained values for all three parameters as well as their mean are presented in Figure 4.

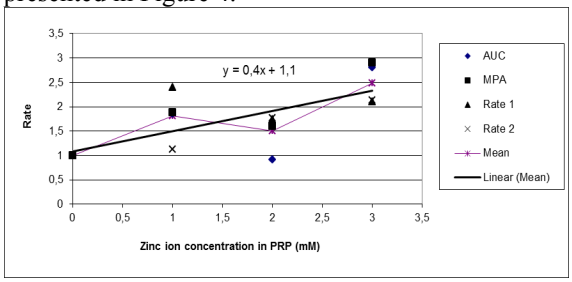


Figure 4.
Dependence of normalized parameters of aggregation curves on zinc ion concentration

In spite of a significant variability, in all cases it was obtained an increase of ratios with the concentration. The mean value followed an approximately linear dependence on the concentration model.

Effect of zinc ion on aggregation in presence of adrenaline, in comparison with adrenaline only and zinc ion only is presented in Figure 5.

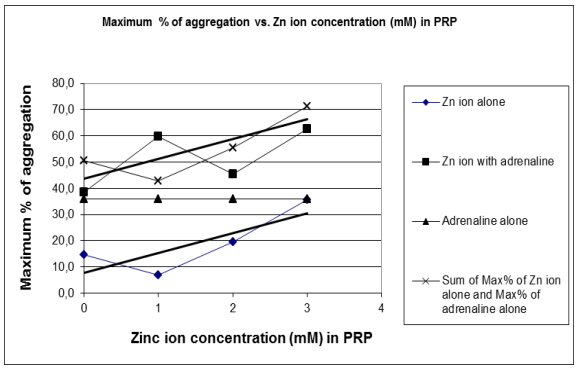


Figure 5.
Effect of zinc ion on aggregation in the presence of adrenaline, in comparison with adrenaline only, and zinc ion only

It is to note that the effect is greater than the effects obtained separately by adrenaline and zinc ion and approximately equal to their sum. The increasing effect with concentration appears in both cases of zinc ion and zinc ion + adrenaline and the regression lines are practically parallel.

Synergism between copper ion and adrenaline pro-aggregant effect

In this study, the concentrations of copper ion used in the PRP sample were: 6, 8 and 10 μM, thus it was employed the normal level of concentration. Normal copper ion blood levels are 1.3-23 μM [3].

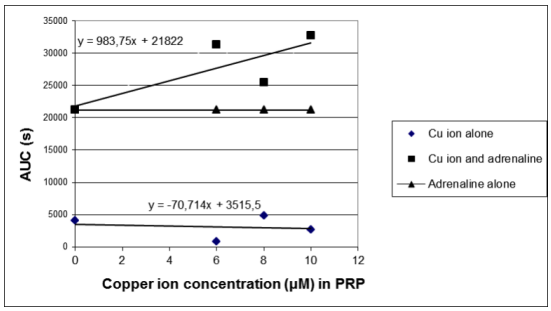
Some studies revealed that copper deficiency decreases thrombocytes adhesion to endothelium, but enhances thrombocytes aggregability in rats. The anti-*platelet aggregation* effect of aspirin is enhanced by the association with copper ion [3, 29].

The results concerning the effect of copper ion and of copper ion + adrenaline on maximum aggregation, on AUC and on the rate of aggregation are presented in Figure 6. Single copper ion effect is "non-increasing" of the aggregation: AUC was slowly decreased, MPA was practically constant and the aggregation rate clearly decreased.

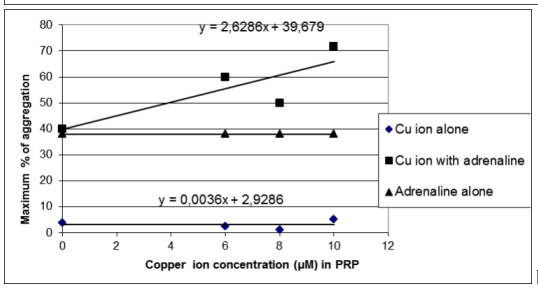
Association between copper ion and adrenaline changes dramatically the effects. The obtained effects are greater than the sum of separate effect suggesting a synergism, and the dependence on copper ion concentration is approximately linear and clearly increasing.

Consequently the DLVO theory prediction was confirmed in the case of copper ion only for the

extent of aggregation (maximum percent of aggregation and area under the aggregation curves).



a.



b.

Figure 6.
Parameters of aggregation curves in the presence of copper ion and adrenaline. a. AUC, b. MPA.

Aluminium, adrenaline and aluminium + adrenaline effect

Published studies revealed that normal aluminium ion blood levels are 0.3-0.5 µM and 0.2 µM, respectively [19].

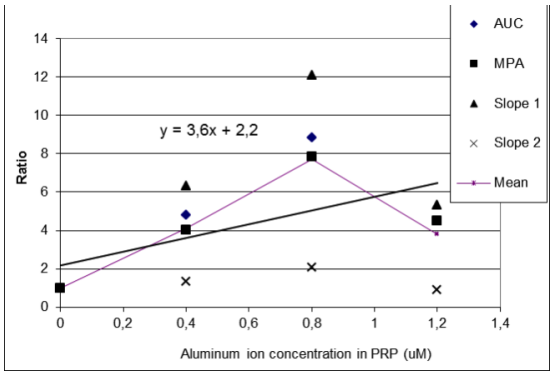
Haemodialyzed patients' aluminium concentration levels, immediately after haemodialysis, were found to be in the interval 0.6-2.7 µM.

Used concentrations in the present study were: 0.4, 0.8 and 1.2 µM, correlated rather to the ones found in the blood of haemodialyzed patients with temporary, significant greater concentration, which are not „intoxicated" with aluminium ions.

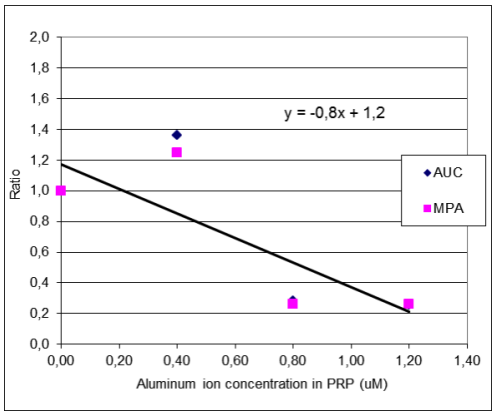
Other studies determined the effect of aluminium ion on thrombocyte aggregation *in vitro* on washed blood platelets, using much higher concentrations (25, 50 and 100 µM) [18]. The platelet aggregation increased linearly with the concentration. Authors suggested that the mechanism of the platelet aggregation induced by aluminium ions is represented by the platelet dysfunctions caused by the end products of lipid peroxidation [22].

However, the same authors, in other *ex vivo* studies, did not find any correlation between platelet aggregation and aluminium ion concentration [19].

The results concerning the effect of aluminium ion and of aluminium ion + adrenaline on maximum aggregation, on AUC and on the rate of aggregation are presented in Figure 7. Single aluminium ion effect is decreasing the aggregation both concerning AUC and MPA. At the maximum tested concentration, the ratio between parameters of aggregation curves and parameters of control curves decreased to approximately 25 %. Since intrinsic aggregation is low, this means that aggregation was practically completely inhibited.



a.



b.

Figure 7.
a. Ratio between aluminium ion + adrenaline aggregation curves parameters and control parameter *vs.* aluminium ion concentration; b. Ratio between aluminium ion aggregation curves parameters and control's parameter *vs.* aluminium ion concentration

Association between aluminium ion and adrenaline, similar as in the case of copper ion, changes dramatically the effects. The obtained results revealed that aggregation was approximately ten times increased.

The sum of separate effects was lower than the obtained effect following association, suggesting a significant synergism. The dependence of parameters on aluminium ion concentration was approximately linear and clearly increasing.

The correlation of the results of this study with the results of other studies is difficult to achieve, giving the fact that the concentrations used are very different and the number of determinations is low.

So that all parameters of aggregation curves decreased, which is in contradiction with DVO theory, but inhibition was not significant: from a very slow and reduced aggregation (intrinsic aggregation) to a somewhat even lower value.

It concerns the effect of ions in the presence of adrenaline; the results were, at least qualitatively, all in accordance with the theory: a significant increasing of aggregation, additional to adrenaline induced aggregation. Intrinsic aggregation is low and practically impossible to evaluate due to the rapid damage of platelets. Classification in literature of effects as pro-aggregant or anti-aggregant starts from effects added to induced aggregation mainly by adrenaline, ADP and collagen. So that, the results, can be interpreted as in agreement with the extended DLVO theory (XDLVO). These are physicochemical interpretations of aggregation and effects of ions on aggregation, which are very far from usual biochemical or pharmacological interpretations. It concerns the effect of zinc on aggregation, Heyns (experiment cited above) made the observation, "in biochemical language", that zinc ion- induced aggregation was not connected with "thromboxane synthesis", or by "secretion of dense – body serotonin" and it was independent on the "presence of extracellular ADP". Finally, all proposed mechanisms in biology are more or less mnemonic -techniques, their validation being given by their power of prediction, which prediction is in all cases limited and restricted to only a few of characteristics of the evolution of processes.

A correspondence between physicochemical and biochemical theories is practically impossible.

Quantitative aspects like the synergism between metallic ions and adrenaline are difficult to explain.

Recent studies have revealed that at least two activated platelet subpopulations are formed upon potent stimulation of platelets with collagen and/or thrombin. One of these subpopulations consists of the so-called "coated platelets" that express high levels of phosphatidylserine and retain α-granule proteins, including fibrinogen, at their surface. Coated platelets cannot aggregate with each other but can be recruited into aggregates by uncoated platelet [30]. Authors of the report considered only two "compartments": non-coated platelets and coated platelets.

Such a model could offer an explanation for effect of metal ions obtained in this study. Most probable, ionic metals react with phosphatidylserine, transforming coated platelets in potential aggregating platelets and increasing the aggregation. But how is amplified this mechanism by the presence of adrenaline, is another question, difficult to solve.

All theories concerning aggregation of platelets can be classified in biochemical, thermodynamic or kinetic. And these three categories of theory are practically completely parallel being able to solve only particular aspects of particular problems.

Conclusions

Effects of copper ion, zinc ion and adrenaline were pro-aggregant effects. In case of intrinsic aggregation, the effect increased approximately linear with the concentration of zinc and copper ions.

Effects of all three ions on adrenaline induced aggregation were similar with the effect on intrinsic aggregation.

Considering all main parameters of aggregation curves it was found additivity between the effects of ions and the effect of adrenaline.

It was considered that the mechanism by which positively charged metal ions increase the platelet aggregation is the decreasing of the repelling potential between the negatively charged platelet membranes. In the case of zinc ion, the results were in complete accordance with this theory. The theory was confirmed in the case of copper ion only for the extent of aggregation (maximum percent of aggregation and area under the aggregation curves).

In case of aluminium ion, all parameters of aggregation curves decreased, but inhibition was not significant: from a very slow and reduced aggregation (intrinsic aggregation) to a somewhat even lower value.

The mechanism of synergism between ionic metals and adrenaline cannot be explained, nor in thermodynamic, nor in kinetic, nor in biochemical theories trying to predict aggregation.

Even at high concentrations the effects observed for zinc, copper and aluminium ions on platelet aggregation were not high and had nor clinical, nor toxicological significance.

Acknowledgements

This paper is supported by the Sectoral Operational Programme Human Resources Development (SOP HRD), financed from the European Social Fund and by the Romanian Government under the contract number POSDRU/159/1.5/S/137390/.

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