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Original Article

Effects of the Amino Acid Complex on Biochemical and Morphological Parameters of Hemostasis at Chronic Cadmium Intoxication

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Abstract

Background: The present study was conducted to investigate the biochemical and morphological changes in the blood coagulation system caused by chronic cadmium intoxication and the anticoagulant activity of the amino acid complex (γ-aminobutyric acid, β-alanine, glutamine, ethanolamine-O-sulphate). Previous studies have investigated the effect of the amino acid complex (AAc) on blood glucose levels in animals with experimental alloxan diabetes. The use of this complex demonstrated the ability to suppress the hyperglycaemic effect of alloxan, while also exhibiting anticoagulant activity.

Materials and methods: The experiments were carried out on non-linear white male rats divided into 3 groups: control rats, rats receiving cadmium sulphate and rats with cadmium intoxication injected with AAc. Biochemical (recalcification, prothrombin time, international sensitivity index, thrombin time, activated partial thromboplastin time, fibrinogen level, calcium level) and histological (Haematoxylin & Eosin and Giemsa staining) methods were used in the studies.

Results and conclusion: Chronic cadmium intoxication leads to alterations in several blood coagulation parameters, suggesting a predisposition to hypercoagulation. However, administration of AAc reduces blood coagulation. Blood samples from poisoned rats showed the presence of red blood cells and leukocytes with morphological changes, including the presence of numerous platelets in clusters or groups. Conversely, when AAc was administered to cadmium poisoned rats, erythrocytes and neutrophils showed morphologically normal characteristics. The results obtained confirm the anticoagulant activity of AAc, which may be used in the future for the treatment of various thrombotic conditions.

Keywords:

Amino acid complex (AAc)
Hemostasis
Cadmium intoxication
Indirect anticoagulant
Cytotoxicity
Hemolysis

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1. INTRODUCTION

Technogenic activities such as mining, metallurgy, and agriculture are the principal reasons for the environmental pollution and the resulting exposure to heavy metals that causes serious health issues. The toxic effect of various compounds of heavy metals is primarily due to the interaction with the body proteins, which, therefore, are often called protein venoms. One such metal is cadmium (Cd) (1,2) that competes with other metals for combination with the enzymes and causes disruption of their activity. Cd is also binding to various proteins, causing denaturation and changes in their properties.

Cd is absent in the newborns, but throughout life it accumulates in the tissues, mainly in the kidneys and liver (3,4). Chronic exposure to Cd vapor leads to the destruction of the nasal epithelium and the accumulation of Cd in the lungs with the development of emphysema (5). Excessive intake of Cd into the body can lead to anaemia, liver damage, nephropathy, osteomalacia and osteoporosis, cardiopathy, as well as the development of hypertension (6). Cadmium also damages the proximal tubules of the kidneys, disrupts the reabsorption of low-molecular proteins, amino acids, phosphorus and calcium compounds, changing protein, phosphorus-calcium and other types of metabolism (7). Cd is a potential risk factor for the development of hormone-dependent tumours such as endometrial cancer, lung and kidney cancer, adenoma and prostate cancer in men (8,9).

As a toxic substance circulating in the blood, Cd directly interacts with the vascular endothelial cells (ECs). High levels of Cd induce apoptosis or necrosis of ECs and lead to haemorrhages in various tissues (10). Low dose Cd exposure significantly increases the expression and secretion of endothelial von Willibrand factor (vWF) and may subsequently accelerate vascular aging (11). The level of the endothelial-derived anticoagulant proteins (prostacyclin, thrombomodulin) decreases with vascular aging, but the expression of the procoagulants vWF, thromboxane A_2, and the inhibitor of the plasminogen activator increases, thus contributing to the development of thrombosis (12,13).

Cd has been found to have a pathological effect on the erythrocytes, the most abundant cell population in the bloodstream. A study of the changes in the structural and volumetric parameters of the blood cells can be of great importance for obtaining information regarding the cellular response to the action of toxic molecules (14). The structural effects of cadmium on cell membranes have been studied through the interaction of Ca^{2+} ions with human erythrocytes and their isolated unsealed membranes. Shape changes have been found in the erythrocytes in the form of echinocytes (15).

The mechanisms of Cd cytotoxicity are not quite clear. However, it is known that Cd shows very high affinity to the membrane Ca^{2+} pump and inhibits its activity, which may result in the increase of the cytosolic Ca^{2+} concentration to the toxic levels (16).

Considering the toxic effect of cadmium on the body, which can also lead to thrombus formation, we studied the effect of an amino acid complex (AAc) used as an anticoagulant. AAc is a mixture of GABA (γ-aminobutyric acid), β-alanine, glutamine, and EOS (ethanolamine-O-sulphate), the effective antidiabetic properties of which we have previously established (17). The main component of AAc is GABA, which stimulates the proliferation of the pancreatic β-cells and prevents apoptosis, thereby reducing the blood glucose levels.

The other components of this complex support the synthesis and the activity of GABA (17). In rats with experimental alloxan diabetes, we also discovered the anticoagulant effect of AAc, which was proven by its influence on a number of parameters of plasma hemostasis in the intact animals (18). GABA derivatives reduce the aggregation of the erythrocytes and platelets, limiting plasma hypercoagulation, lengthen the prothrombin and thrombin times, reduce the fibrinogen level, and improve rheology and blood microcirculation (18,19).

Modern medicine requires the search for new drugs that can effectively control various diseases. A number of drugs that contain amino acids in various combinations are currently known. AAc that we are studying is one of such drugs that possesses antidiabetic and anticoagulant properties, which makes it of high importance.

2. MATERIAL AND METHODS

2.1. Animal treatments

The experiments were carried out on 28 male rats weighing 200-220 g, which were divided into 3 groups: 1) control rats that were on a standard diet and had free access to water and food; 2) rats that orally received cadmium sulphate in a dose of 0.3 mg/kg (in terms of metal) daily for a month; 3) rats that received cadmium sulphate and were intravenously (i/v) injected with AAc in a dose of 5mg/100g, whose blood was taken 30 and 60 minutes after the single administration of AAc for further studies.

Morphological studies aimed to investigate RBC, WBC, and platelet morphology were performed on the blood smears of the intact rats, rats that received cadmium sulphate, and rats with cadmium intoxication that were treated with AAc.

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2.2. Biochemical methods

To determine the changes occurring in the blood coagulation parameters in all experimental groups – recalcification time, prothrombin time (PT), international normalized ratio (INR), thrombin time (TT), activated partial thromboplastin time (aPTT) – the fibrinogen and calcium levels were measured by the biochemical methods used. Hemostasis parameters were measured on the biochemical analyzer Start Max (France). Thromboplastin with ISI (international sensitivity index) = 1.5 from Delta THR-stb (Armenia) was used in the experiments.

Calcium concentration was detected using a test kit produced by VIPLA Company Ltd (Armenia). Readings were taken at a wavelength of 575 nm on a biochemical analyzer MRIT-880 (China). General blood test was also carried out to quantify the leucocytes, erythrocytes, and platelets. The analysis was done on Uritmedical BH-40 analyzer.

2.3. Morphological methods: Haematoxylin and Eosin (H&E) (20) and Giemsa (21) staining.

2.4. Statistical analysis.

The comparison of the control and experimental independent samples was made using a nonparametric criterion Mann-Whitney U. The error bars were set based on the standard error of the mean (± SEM). The differences between the samples were considered significant at $p \leq 0.05$.

3. RESULTS

The physical integrity of the circulatory system and the fluidity of the blood, which are supported by the processes of clotting and fibrinolysis, are crucial for the life. Although the role of the cellular components and the circulating proteins is widely known, the effects of the metals on these processes are not yet well understood. To obtain the information about the activity of the entire blood coagulation system under the influence of cadmium, we used a method for determining the time of plasma recalcification, which correlates with the time of the general blood coagulation. According to the data obtained **(Figure 1.A)**, the recalcification time is reduced by 30% compared to the control animals, which indicated an increase in blood clotting. The recalcification time was prolonged by 57% in 60 minutes after the intravenous (IV) administration of AAc to the animals with cadmium intoxication.

In the animals with chronic cadmium intoxication, the prothrombin and thrombin times were reduced by 22% and 20%, respectively, compared to the control animals **(Fig. 1. B, C)**. In 30 and 60 minutes after the i/v administration of AAc to the experimental animals, the thrombin time increased by an average 50%, and the prothrombin time increased by 64%. When determining the prothrombin time, the international normalized ratio (INR) was also calculated, as an additional prothrombin test indicator that provides accurate control of therapy with anticoagulants. According to the data obtained **(Table 1)**, the INR is almost the same in the control and the experimental animals. However, in 30 and 60 minutes after the administration of AAc, it increases by 44% and 20%, respectively, in the rats with cadmium poisoning. The changes in the internal or contact pathway of blood coagulation were studied using the aPTT test. After 30 days of cadmium poisoning **(Fig. 2A)**, the aPTT decreased by 18%, which increased in comparison to the control rats by 116% in the cadmium intoxicated and treated with AAc rats.

In the animals with chronic cadmium intoxication, the level of fibrinogen decreased by 20% **(Fig. 2B)**, and it decreased by more than twice compared to the experimental animals after the i/v administration of AAc.

It was also shown that the level of calcium, which plays a huge role in the hemostasis system, increased in the blood from 1.67 to 2.18 mmol/L (by 30%) under the influence of cadmium, compared to the control animals, which increased by another 20% in 60 minutes after the administration of AAc.

It should be noted that data obtained in a result of the general blood test showed an increase of the red and the white blood cells, as well as some parameters of the blood cells under the action of cadmium **(Table 2)**. Compared to the control animals, the number of white blood cells increased by 27%, lymphocytes – by 10.6%, and erythrocytes – by 16%. In contrast to the above-mentioned cells, the number of the granulocytes decreased by 11%, which is typical in various intoxications. An increase in the platelet count by only 7% was observed under the influence of cadmium.

Interestingly, under the influence of AAc, a quantitative change is observed towards the decrease in the number of white blood cells, lymphocytes, and platelets, compared to cadmium poisoning, except for granulocytes, the number of which increased, reaching up to 25.7%. As for the red blood cells, no changes are observed under the influence of AAc compared to their quantity at cadmium poisoning.

According to the data received, the morphologically normal RBCs are generally detected in intact rats. However, single RBCs with the abnormalities and RBCs in the form of rouleaux **(Fig. 3)** occur together with a few WBCs and some platelets **(Fig. 3 and 4)**, also detected in the intact rats.

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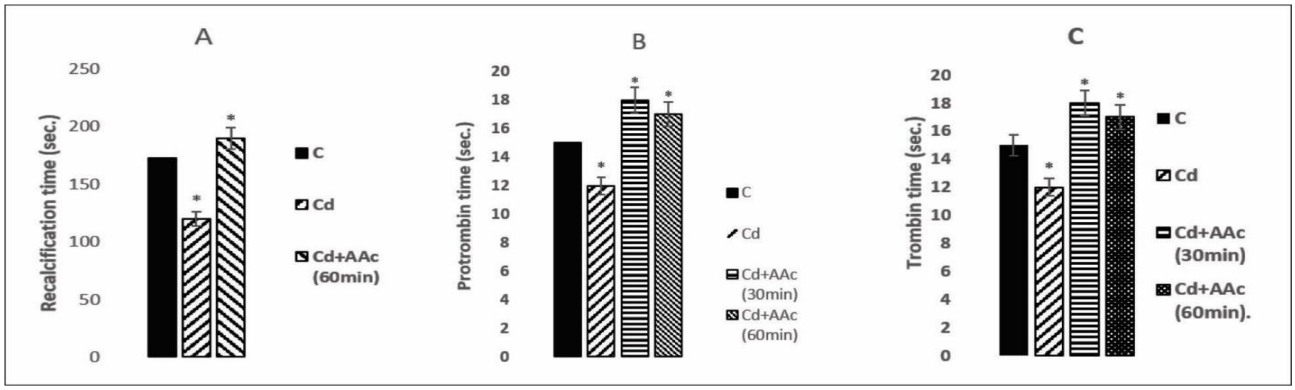

Figure 1. Changes in A) recalcification time, B) prothrombin time and C) thrombin time under the influence of Cd and Aac

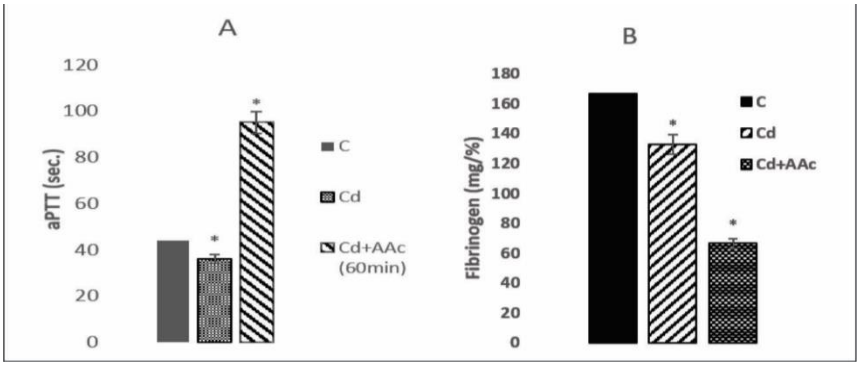

Figure 2. Changes in A) activated partial thromboplastin time (aPTT) and B) fibrinogen level under the influence of Cd and AAc

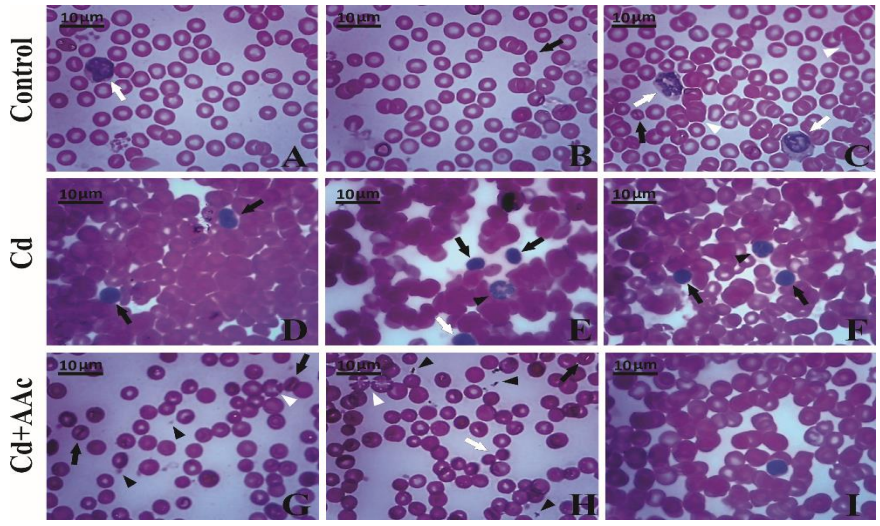

Figure 3. Cadmium intoxication and treatment with AAc. Blood smears of the intact (A-C), exposed to Cd (D-F) rats and injected with cadmium rats treated with AAc (G-I). The morphologically normal RBCs are in generally detected in intact rats (A-C); single microcytes (black arrows, B,C), neutrophils (white arrows, A,C), Rouleaux formation of RBCs (white arrow heads, C). In exposed to Cd rats (D-F), well-defined agglutination of RBCs, small limphocytes (black arrows, D-F), large lymphocyte (white arrow, E), as well as neutrophils with the hypersegmented nuclei (black arrow heads, E,F) are demonstrated. In received Cd rats after treatment with AAc (G-I), single stomatocytes (black arrows, G,H), microcytes (white arrows,H), fragmented RBCs-Schitocytes (white arrow heads, G,H), as well as some thrombocytes (black arrow heads, G,H) are detected. Histological method: H&E staining.

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Table 1. Changes in INR and calcium levels under the influence of Cd and AAc (n =7). P≤ 0,05.

Indicators	INR			Ca mmol/l
		30min	60min	
C	1.10	—	—	1.67
Cd	1.15	—	—	2.18
Cd+AAc		1.48	1.31	2.50

Table 2. General blood test (n=7). *P≤ 0,01; **P≤ 0,05.

	Control*	Cd**	Cd+AAc**	Unit
WBC	11,4	14.5	12,6	10^3/uL
LYMPH%	62,7	73,3	61	%
GRAN%	28	17	25,7	%
RBC	6,86	8,1	8,1	10^6/uL
HBC	130	150	153	g/L
HT	43,7	47,6	51	%
PLT	586	627	534	10^3/uL

Obvious morphological changes in the red and the white blood cells are exhibited in the rats exposed to cadmium **(Fig. 3 and 4).** Numerous platelets found as clumps or groups are detected among the agglutinated RBCs and neutrophils with morphologic abnormalities. It should be noted that morphologically changed RBCs – elliptocytes, dacrocytes, stomatocytes, schistocytes, S-C poikilocytes, and acanthocytes are also detectable.

In the rats that received Cd after the treatment with AAc, RBCs generally were morphologically normal, but some RBCs with the morphological abnormalities, as well as RBCs in a form of rouleaux, were observed in the blood smears. Such RBCs were also observed in some of the intact rats.

4. DISCUSSION

The present study is aimed to investigate the action of chronic cadmium intoxication on the blood coagulation system and the anticoagulant activity of the amino acid complex (AAc). The effect of cadmium on some parameters of plasma hemostasis was studied. According to the results obtained, cadmium shortens the time of recalcification, PT, TT and aPTT **(Fig.1A,B,C; Fig.2A)**, thereby increasing blood clotting.

Our further studies were aimed at investigating the effects of AAc on the blood clotting in the rats with cadmium poisoning, based on the previously discovered anticoagulant activity of AAc in the intact rats and the rats with experimental diabetes (17,18). The anticoagulant activity of AAc was expressed by the changes in a number of parameters of plasma hemostasis.

The above data have demonstrated that AAc has the maximum effect on the process of the formation of active thrombin, which involves a number of vitamin K-dependent blood coagulation factors synthesized in the liver and forming the prothrombin complex (FII, FVII, FIX, FX).

An increase in prothrombin time may be associated with a deficiency of vitamin K and blood clotting factors. We assume that AAc, similar to the nonspecific anticoagulants (monocoumarins - warfarin, syncumar; dicoumarins - dicoumarin, tromexane, etc.), interacts with the transport protein of vitamin K, thereby causing deficiency of the latter and vitamin K-dependent blood clotting factors. As a result, the blood clotting process slows down (22). The halving of fibrinogen levels after injection of AAc may also be associated with the inhibition of the formation of the prothrombin complex in the liver. It has been shown that with a decrease in the level of fibrinogen, as a substrate for clot formation, PT, TT and aPTT are prolonged (23).

The results obtained by the aPTT method, which is known as an informative method that reflects the changes in the activity of the factors in the internal or contact pathway of blood coagulation, show that Cd and AAc have the same effect on both the external and internal pathways of blood coagulation.

It is known that the disturbances in the calcium metabolism, which ensures the structure and function of the cellular systems in all tissues and organs, can cause the development of various pathologies, including also in the cardiovascular system (24,25). Calcium ions, known as coagulation factor IV, take part in cardioplatelet and plasma coagulation hemostasis, ensure the interaction of coagulation factors and the formation of a fibrin clot. Our studies have shown that under the influence of the cadmium sulphate in the dose we used, there is an increase in the level of calcium compared to the control animals, which explains the observed increase of blood clotting in the experimental animals. However, after the exposure to AAc, the calcium level is increased by another 20%. This phenomenon can be explained by the fact that AAc, as a possible indirect anticoagulant, can have a warfarin-like effect on vitamin K, causing a deficiency of the latter, and on vitamin K-dependent proteins requiring γ-carboxylation for physiological activity (26). Such proteins are blood clotting factors and a vascular matrix protein - Gla protein (osteocalcin), which is necessary to remove excess calcium

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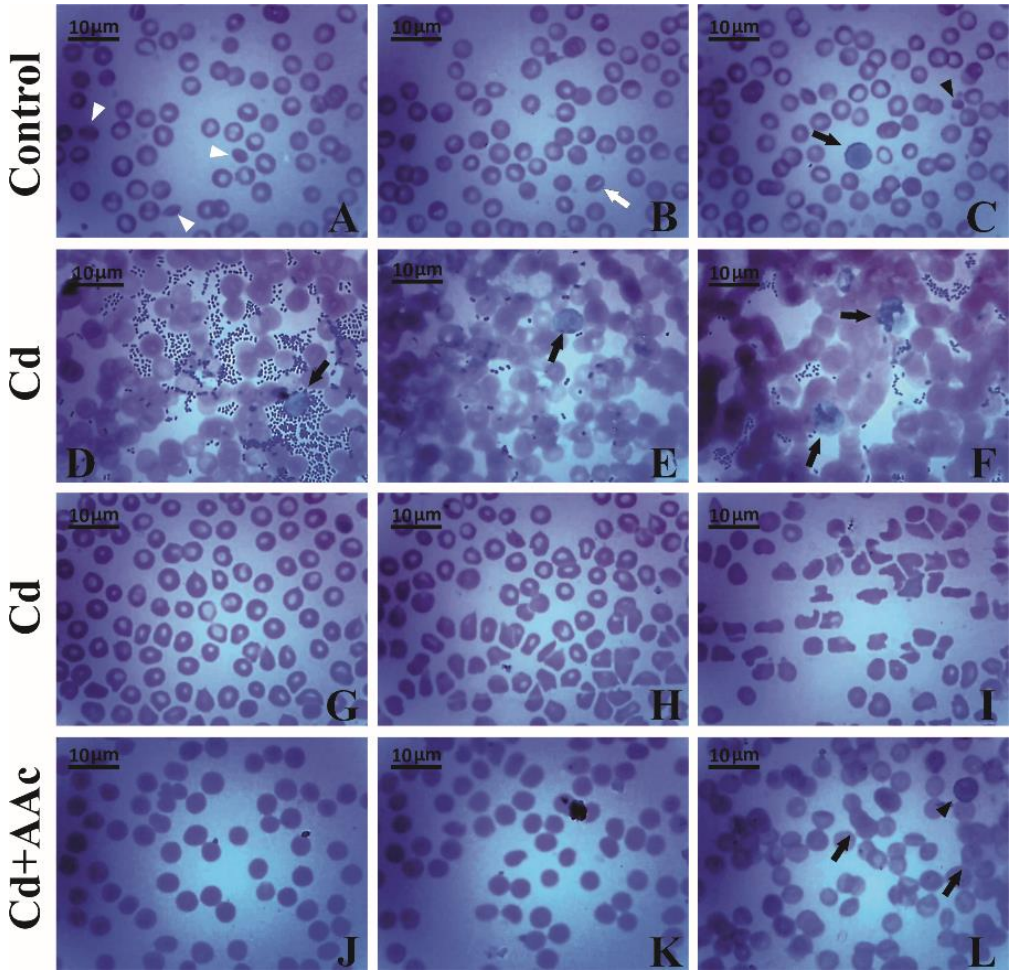

Figure 4. Morphological abnormalities of blood cells. Blood smears of the intact (A-C), exposed to Cd (D-I) rats and injected with Cd rats treated with AAc (J-L). In intact rats, a few WBCs (black arrow, C) are found among the morphologically normal RBCs; stomatocytes (white arrow, B), eliptocytes (white arrow head, A), micro-spherocyte (black arrow head, C) are also detected. In rats under Cd action, numerous platelets found as clumps (D) or groups (F) among agglutinated red blood cells and neutrophils (black arrows D-F) with morphological abnormalities are demonstrated. Morphologically changed RBCs (G-I) such as - elliptocytes, dacrocytes, stomatocytes, schistocytes, poikilocytes and acantocytes are well visible. In received Cd rats after treatment with AAc (J-L), RBCs appear to be in generally morphologically normal, but some RBCs in a form of rouleaux (arrows, L) and a megaloplast (arrow head, L) are still seen. Histological method: R&G staining.

from the blood serum, contributing to the inhibition of vascular calcification (27,28).

It is also known that the vascular endothelium is the main target for cadmium toxicity. Cd significantly increases the expression and secretion of the endothelial glycoprotein vWF, which ensures platelet adhesion and aggregation and serves as a plasma carrier of factor VIII (29,30,31). FVIII or antihemophilic globulin belongs to the factors of the internal pathway of the coagulation cascade and circulates in the blood in the form of a complex with vWF, as a result of which FVIII is stabilized and protected from proteolysis, thereby ensuring the interaction of factors IX and X, converting prothrombin into thrombin.

The general blood test detected an increase of the red and white blood cells numbers, as well as some parameters of the blood cells in result of the cadmium intoxication, compared to the control animals (**Table 2**). As for the granulocytes, their number was decreased, which is often found in various intoxications. An increase in the platelet count was also observed under the influence of cadmium. In the rats injected with AAc, the number of the leukocytes, lymphocytes and platelets was reduced compared to cadmium intoxication, with the exception of the granulocytes, the number of which increased, approaching the control level. As for the red blood cells, no changes have

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been observed under the influence of AAc compared to their quantity at cadmium intoxication.

In our study, RBCs abnormalities in result of cadmium intoxication have been evaluated by their size (microcytic; normocytic; macrocytic), shape, rouleaux formation or agglutination. Rouleaux formation is the linking of RBCs into chains. Increased rouleaux formation is known to be associated with the increase in fibrinogen or acute phase proteins and is usually seen in inflammatory diseases.

The increased number of the WBCs such as small and large lymphocytes, the neutrophils with the hypersegmented nuclei, as well as increase of the platelets count was also detected. However, after the injection of AAc in the rats with Cd intoxication, single morphologically changed RBCs and some thrombocytes were still seen. Thus, the performed morphological studies confirmed the results obtained in the course of these biochemical studies.

5. CONCLUSION

It can be stated that chronic cadmium intoxication creates the prerequisites for hypercoagulation, as evidenced by the obtained data (reduction of recalcification time, prothrombin time, aPTT, etc.). According to the obtained results, AAc has a significant effect on hemostasis and blood cells under conditions of cadmium intoxication, which is expressed by its anticoagulant activity on the external and internal blood clotting pathways. In the blood samples of the rats exposed to cadmium, we detected numerous platelets in clusters among the leukocytes with cellular abnormalities, as well as among the agglutinated erythrocytes with morphological changes. These findings indicate that blood clotting in Cd intoxication was normalized after exposure to AAc. The biochemical and morphological data regarding the anticoagulant effect of AAc presented in this study allow us to suggest its possible use in the future as an alternative treatment of certain pathologies caused by heavy metals.

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Ethical statement

The experiments were carried out in accordance with Article 11 of the Declaration of Helsinki of the World Medical Association, and were also guided by the rules of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (Strasbourg, 1986).

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