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Effect of Polyphenols on Changes in the Hemostatic System of Blood Plasma in Healthy and Model Rats with Alzheimer's Disease

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Abstract

It is known that, as a reason for the change in the blood hemostasis system in Alzheimer's disease (AD), it has been established that beta-amyloid (Aβ) protofibrils, affecting the composition of the blood and blood vessels, often accumulate in the vessels and cause structural changes in the composition of the blood. This article studied the effect of polyphenols eforbin and PC-6 on the system of coagulation and platelet hemostasis in healthy and modele AD blood of rats. To simulate AD, laboratory white outbred male rats, weighing 200 - 300 g, were used. Acute aluminum neurotoxicosis was caused by three subcutaneous injections of 0.2 mL of 10 % aluminum chloride solution into rats. Isolated platelets by centrifugation at 1,150 rpm for five minutes to sediment the red blood cells. Blood plasma coagulation in healthy rats and with AD *in vitro* was assessed using generally accepted tests: Activated partial thromboplastin time, prothrombin time, thrombin time. Platelet aggregation was recorded using the Born method. Experiments to study calcium-dependent processes in platelets used the fluorescent probe Fluo 3-AM to measure the amount of Ca^{2+} in the cytosol. Using the Grinkevich equation, the amount of cytosolic Ca^{2+} was calculated.

In experiments, we studied the effect of polyphenols eforbin and PC-6 on the coagulation factors of the blood plasma of rats in a healthy and AD state using various APTT, TT and PT tests. The results showed that blood clotting time is prolonged under the influence of polyphenols. Acceleration of blood clotting time has been observed in AD. Also, when studying the effect of polyphenols on platelet aggregation, inhibition of aggregation induced by ADP and collagen was observed. Under AD conditions, spontaneous aggregation was observed. When studying the effect of polyphenols on calcium-dependent platelet processes, it was found that by inhibiting calcium channels, they reduce the amount of intracellular calcium in the cell. It has been established that the polyphenols used significantly inhibit the activation of the hemostatic system that occurs in AD under the influence of Aβ protofibrils.It was observed that polyphenols can have an inhibitory effect on the activation of plasma factors, which leads to increased plasma coagulation and inflammation under the influence of Aβ protofibrils, internal and external blood

coagulation mechanisms, and normalization of the plasma communication system. Polyphenols Eforbin and PC-6, under normal conditions and in AD, inhibit platelet aggregation induced by ADP. The inhibition of platelet aggregation by polyphenols suggests that it may be related to its ability to prevent the entry of Ca^{2+} into platelets by blocking Ca^{2+} channels located in the plasma membrane.

Keywords: Alzheimer's disease, Polyphenols, Hemostatic system, Platelet aggregation

Introduction

AD is a common form of neurodegenerative dementia, affecting nearly 30 million people worldwide. Although Aβ aggregates and oligomers, as well as aggregated tangles of phosphorylated tau proteins, are effective therapeutic targets for the treatment of AD, it remains unclear how these protein aggregates lead to neuronal death and cognitive impairment [1-3].

Aβ aggregates consist of aggregated Aβ peptides resulting from a series of enzymatic cleavages of amyloid precursor protein (APP). Depending on the nature of APP cleavage, the length of Aβ peptides varies from 38 to 49 amino acids (i.e., Aβ38-Aβ49) [4-6]. Aβ42 and Aβ43 are the most neurotoxic and pathogenic among the above-mentioned lengths of aggregates [5,6]. Although approaches to prevent or slow neurodegeneration and dementia by reducing Aβ production, neutralizing toxic Aβ aggregates, or inhibiting tau aggregation have been largely pursued without success [7-10].There are several other factors that may play a role in neurodegeneration and cognitive decline in AD [11-19] however, which should also be considered when defining new targets and developing effective AD therapeutics.

Now, if we look at these processes in more detail, it is known that High molecular weight kininogen (HKi) appears to be a key component of the plasma contact-kinin system [1]. The contact system is a cascade of proteins that are activated upon contact with negatively charged surfaces, such as those found on damaged endothelial cells or artificial surfaces like glass. This system plays a significant role in various physiological and pathological processes, including inflammation, coagulation, and vasodilation. Activation of factor XII (FXII) triggers the cleavage of HKi by plasma kallikrein, resulting in the formation of cleaved high molecular weight kininogen (HKc) and the release of the anti-inflammatory peptide bradykinin. Increasing activation of the contact system has been recorded in thrombo-inflammatory diseases, such as ischemic stroke and myocardial infarction, as well as in autoimmune diseases and hyperlipidemia. More severe HKi cleavage is observed in hereditary angioedema, systemic lupus erythematosus, cancer and sepsis [1-9].

Aβ42 peptide, which is strongly associated with Alzheimer's disease (AD), has been shown to activate the contact system *in vitro*. This activation can lead to the cleavage of HKi in a factor XII-dependent manner [10-13]. *In vivo*, wild-type mice injected intravenously with Aβ42 exhibited increased plasma HKi degradation, which was not observed in FXII knockout mice. Moreover, increased plasma HKi degradation was found in two different AD mouse models caused by overexpression of human Aβ, suggesting that increased Aβ expression may lead to increased contact system activity [14,15]. The idea that activation of plasma FXII and cleavage of HKi contributes to the pathogenesis of AD is supported by studies showing that removal and resolution of FXII from plasma using an antisense oligonucleotide reduces plasma HKI, reduces brain inflammation, and improves cognitive function in a mouse model of AD. [15]. Of particular importance is the increased cleavage of HKi and the activity of plasma kallikrein, found in the same plasma of patients with AD. Because some AD patients exhibit HKi cleavage without detectable FXII activation, changes in HKi and HKc levels may be a more sensitive indicator of possible AD condition.

There is specific evidence that the contact system is involved in the condition of AD. In particular, plasma from an AD patient will often show evidence of contact system activation, and dementia ratings correlate well with the degree of HK cleavage and bradykinin generation. In addition, the plasma contact system is possibly activated by Aβ, and FXII deficiency provides protection in mouse models of AD [16-22].

Aβ protofibrils promoted the activation of the contact system as evidenced by HK cleavage and activation of FXII and prekallikrein (PK), whereas the other forms of Aβ did not. Levels of bradykinin, generated upon HK cleavage, were increased by Aβ protofibrils compared to all other versions of Aβ. Aβ protofibrils also induced faster clotting compared to other Aβ forms [23].

It is known that in the plasma contact system there is a procoagulant and anti-inflammatory protease cascade initiated by FXII, in a reaction involving HKi and plasma kallikrein (PK). FXII is the plasma zymogenic form of serine factor XIIa (FXIIa). FXII is activated to FXIIa after contact binding to negatively charged artificial or biological surfaces. Alternatively, active PK is capable of activating a zymogen. During both contact-triggered auto-activation and PK-mediated hetero-activation, the FXII zymogen undergoes limited proteolysis. FXII has been recognized as essential for surface-activated diagnostic blood coagulation assays [24]. FXII also has limited proteolytic activity upon contact with activating surfaces without undergoing proteolysis.

Studies have determined that, in the absence of contact activation, leads to the incidence, severity and characteristics of inflammatory, infectious and thromboembolic diseases. Collectively, these studies established the important role of the FXII-mediated intrinsic coagulation pathway in thrombosis and led to a paradigm shift in the development of anticoagulant therapy implementing this strategy and the idea of FXII inhibition as a safe therapeutic approach for the treatment of thrombotic diseases [25].

Objective of the study was to study the effect of polyphenolic compounds Eforbin isolated from the plant (*Euphorbia franchetti B. Fedtsch*), PC-6, isolated from (*Pinus sylvestris L.*) on changes in blood plasma and platelet cells in healthy and model rats with AD.

Materials and methods

Experimental models of Ad.

Animals

The experiments were carried out on outbred white male rats kept on a standard vivarium diet. All experiments performed comply with the requirements of the World Society for the Protection of Animals and the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes 1986) and American Psychological Association. (2017). Ethical principles of psychologists and code of conduct (2002, amended effective June 1, 2010, and January 1, 2017). http://www.apa.org/ethics/code [26].

For the modeling of the AD, laboratory rats of males were used, weighing 200 - 300 g. First of all, weighing and selection of animals for experiments were carried out. Then, behavioral tests are carried out: An open field, a conditioned response of passive avoidance (CRPA) and active avoidance (ACRA), swimming on the pool (Morris test). We feed animals with a standard diet with add-on for 1 or 2 month. After a week, we repeat behavioral tests. Analyzing the data, depending on the test results, enter neurotoxin. After playing the model AD, we score the animal and take biological materials for further research.

The results of behavioral tests showed that in control groups, experimental animals on the “open field” tests were very active and overexcited, quickly moved and practical did not stand in one place. At the same

time, the AD groups are very passive, the nervous system inhibited and the animals were delayed for a long time in one place. This slows down that after the introduction of neurotoxin into the animal's body, the normal functioning of the nervous system is violated, the destruction of the transmission of inpuls in neurons and the death of the cell.

At the tests of CRPA and ACRA, the obtained experts showed that in the control group the animals were in the bright phase quickly sought to go to the dark phase, after they received fragmentation quickly moved to the bright phase. When this test was repeatedly carried out by an ec -permanent animal did not pass into the dark phase from the light. When the model AD groups of animals were placed in the bright phase, she did not strive to go to the dark phase, after she went in the graternation.

When this test is repeated, the experimental animal, as last time, slowly went into the dark phase and again received gratification. The data obtained witnesses that under the control group, experimental animals quickly moved into a dark phase that resembled the mink of animals, but after receiving irritation when repeated the same test did not go into the dark phase. From this we can conclude that the animals have a reaction to the gratification in the dark phase and this remained unih in memory. In the model group, the initially animals were very passive and slowly perpeted in the languid phase. When the test is repeated, the animals again went into the dark phase and received gratification. This suggests that in the model group of animals, cognitive functions, the reaction to the environment and memory, which are symptoms of AD, are greatly impaired.

Studies of the connection with this task set at various stages were carried out on an experimental modeling of AD - aluminum neurotoxicity (ANT) in rats. The models were used in white outbred rats (280 - 300 g). The animals were carefully weighed and various behavioral tests were performed: Open field n = 3, CPPA and ACRA active avoidance n = 3, pool swimming (Morris test) n = 3.

The animals were fed a standard diet and sugar was added to the drinking water. Cholesterol and margarine in the amount of 0.4 % of the total food. Mercazalol in an amount of 0.04 - 0.06 mg per rat.

Acute aluminum neurotoxic effect (AAN) was caused by subcutaneous administration to white rats (2 groups of 12 animals each): The 1st group was the control (0.9 % NaCl solution was administered), the 2nd group was administered 0.2 mL of 10 % aluminum chloride solution for 5 days.

Experimental animals were sacrificed under light ether anesthesia. Blood and internal organs were collected into different vessels and processed simultaneously.

After modeling AD, behavioral tests were repeated: Open field n = 3, CRPA and ACRA n = 3, swimming in the pool (Morris test) n = 3 [27].

Test compounds

The polyphenols used were Eforbin, isolated from the local plant *Euphorbia franchetti B. Fedtsch*, and PC-6 - isolated from the local plant *Pinus sylvestris L.* All these polyphenols were presented from the Institute of Bioorganic Chemistry named after Academician A.S. Sadykov. All processes were carried out according to the standard mode.

Isolation of platelet

When assessing changes in the coagulation and aggregation activity of platelets, platelet-rich plasma obtained from the blood of healthy and AD rats was used. Platelets were isolated by centrifugation at 1,150 rpm for 5 min to sediment red blood cells. Platelet-rich plasma was re-centrifuged for 10 min at 3,000 rpm. The platelet sediment was suspended in 5 mL of medium containing 150 mM NaCl, 2.7 mM KCl, 0.37 mM NaH_2PO_4, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM glucose, 10 mM HEPES-NaOH, pH 6.55, 0.35 % serum albumin and 0.15 mg/mL apyrase.All operations were carried out in plastic containers at room temperature.

Coagulation tests

The effect of polyphenols on blood plasma coagulation in healthy rats and with AD *in vitro* was assessed using generally accepted tests: Activated partial thromboplastin time (APTT), prothrombin time (PT), thrombin time (TT) (Cypress Diagnostics. Belgium) [28,29]. To assess the anticoagulant potential of polyphenols, we graphically determined the effective concentrations of APTT, PT, and TT, which were found by the abscissas of the points located on the curves depending on the concentration anticoagulant - effect; coordinates of points - 2-fold increase in plasma clotting time, compared to the control, that is, without the addition of polyphenols. All coagulological tests were carried out on a single-channel coagulometer (CYANCoag, Belgium.CY003, SN:5400439).

Platelet aggregation

Platelet aggregation was recorded using the Born method [30] on a Biola ALAT-2 aggregometer (№ФСР2007/01301, Russia). ADP (5 - 10 μM), adrenaline (5 μM) and collagen (5 - 10 μM) (Sigma.USA) were used as platelet aggregation inducers. The process of aggregate formation and the degree of platelet aggregation were expressed as a percentage of the maximum level of light transmission (T %, max). Obtaining information in the form of aggregation curves with automatic calculation of indicators was carried out using a computer interfaced with an aggregometer.

Measuring intracellular Ca^{2+}

Experiments to study calcium-dependent processes in platelets we used the fluorescent probe Chlortetracycline (CTC) (Sigma, USA) to measure the amount of Ca^{2+} bound to the membrane and the fluorescent probe (pentaacetoxymethyl ester [2-amino-5-(2,7-dichloro-6-hydroxy-3-oxo9-xanthenyl)phenoxy]-2-(2-amino-5-methylphenoxy) ethane N,N,N',N' -tetraacetic acid) (Fluo 3-AM) (Sigma, USA) to measure the amount of Ca^{2+} in the cytosol. To determine changes [31] in intracellular calcium content, rat blood platelets were incubated in 10 mM Fluo 3-AM medium with the addition of calcium chloride (1 mM) for 30 min at a temperature of +37 °C. The measurements were carried out using a universal spectrometer USB-2000 (USB2E7916.OceanOptics.USA.2010).

Statistical analysis

Statistical significance of differences between control and experimental values, determined for a data series using a paired t-test, where control and experimental values are taken together, and an unpaired t-test, when taken separately. A p-value < 0.05 indicates a statistically significant difference. The results obtained are statistically processed in Origin 7.5 (Origin Lab Corporation, USA).

Results and discussion

Based on the above data, in our previous experiments, we selected the polyphenols eforbine and PC-6, which have a significant inhibitory effect on calcium-dependent processes and the functional activity of ionotropic NMDA- receptors in the synaptosomes of the brain of rats modeled for AD [32-38]. We studied these polyphenols on changes in the blood plasma and platelet cells of rats in a model of AD.

In our study, we first examined the effect of polyphenols eforbin and PC-6 on thrombin time generation. It is known that thrombin time - the kinetics of the last stage of blood coagulation - represents the rate of conversion of fibrinogen to fibrin. Prolongation of thrombin time may be associated with hypofibrinogenemia, dysfibrinogenemia, an increase in the amount of fibrin broken down in plasma, and the presence of properly acting anticoagulants in the blood.

In our experiments, when studying the coagulation of rat blood plasma under the influence of thrombin, when fibrinogen was added to the plasma, blood coagulation was observed within 15 - 25 s. Polyphenols Eforbin and PC-6 at concentrations of 10 - 100 μM have a significant inhibitory effect on thrombin time at 50 μM. In particular, these studies revealed that polyphenols at a concentration of 5 mg/mL had virtually no effect on thrombin time. An increase in the concentration of polyphenols to 50 μM causes an extension of thrombin time. In this process, it was found that the increase in thrombin time depends on the dose of fibrinogen in plasma.

It can be assumed that these polyphenols in concentrations of 10 - 100 μM depending on the dose prolong thrombus formation and inhibit the formation of fibrin clot, as evidenced by the inhibition of the activity of one of factors IXa, Xa, XIIa and antithrombin III inhibition of activated one of IXa, Xa, XIIa factors and antithrombin III (**Figure 1(A)**).

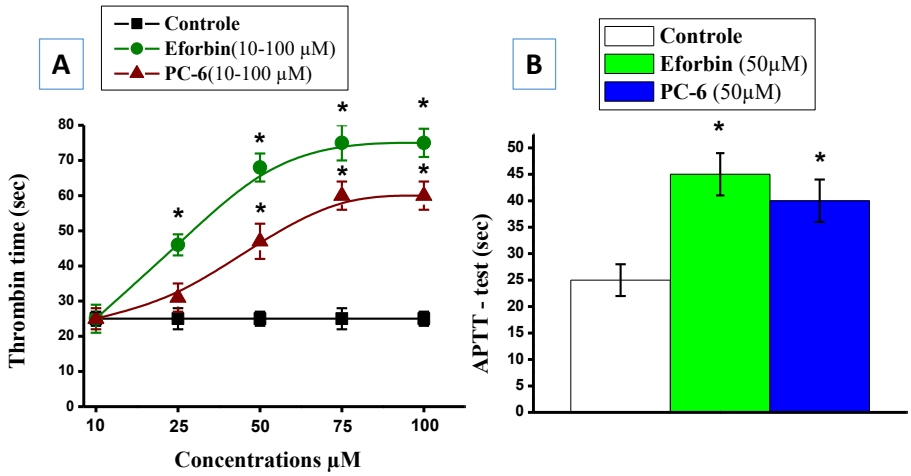

Figure 1 (A) Effect of polyphenols eforbin and PC-6 on thrombin time and (B) Effect of polyphenols eforbin and PC-6 on the APTT test. Reliability indicator: * $p < 0.05$; (n = 6).

The reason for the increase in thrombin time under the influence of these polyphenols may also be associated with a lack of factors XII, XI, IX, VIII, I, II, V. It can be concluded that the polyphenols eforbin and PC-6 affect the blood coagulation system without affecting the activity fibrinogen. The prolongation of thrombin time is associated with changes in the level of fibrinogen in the blood plasma.

To determine the mechanism of the effect of polyphenols eforbin and PC-6 on the blood coagulation process, we looked at how they affect the APTT test. It is known that the APTT test determines the absence of such factors in the internal mechanism of blood coagulation as XII, XI, IX, VIII and the presence of their inhibitors in the blood plasma.

Considering that the effective concentration of polyphenols used in the study is 50 μM, we continued all other experiments at these concentrations. In our experiments, it was observed that the APTT clotting time in the control was 30 s, polyphenols eforbin at a concentration of 50 μM - 45 s, PC-6 at a concentration of 50 μM - extended the clotting time of blood plasma by 40 s, and at the same time caused a slowdown in the formation of a fibrin clot (**Figure 1(B)**).

Based on the results of this experiment, we can conclude that under the influence of the polyphenols used, due to inhibition of the activation of one of the factors XII, XI, IX, VIII in plasma, this leads to an increase in APTT time.

When studying the effect of polyphenols eforbin and PC-6 at a concentration of 50 μM on prothrombin time using the techplastin test, the prothrombin time of blood clotting in the control was 26 s, while for eforbin at a concentration of 50 μM, it was 46 s and for PC-6 at a concentration 50 μM was 29 s, an extension of the clotting time of blood plasma was observed (**Figure 2(A)**).

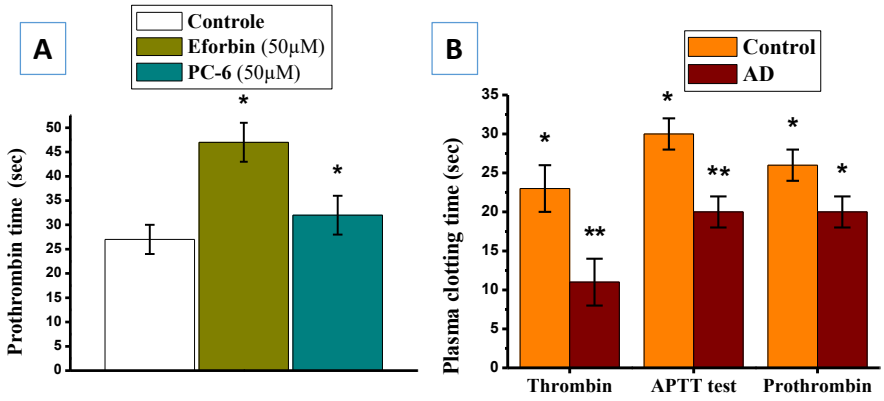

Figure 2 (A) Effect of polyphenols eforbin and PC-6 on prothrombin time and (B) Indicators of blood plasma clotting time in tests for thrombin, aPTT and techplastin in Alzheimer's disease. Reliability indicator: * $p < 0.05$; ** $p < 0.01$; (n = 6).

In these processes, prothrombin time or prothrombin index of the external plasma coagulation mechanism determines the deficiency or activity of prothrombin complex factors (VII, X, V, II). The prolongation of prothrombin time under the influence of eforbin and PC-6 indicates inhibition of the external pathway of blood coagulation activity, that is, inhibition of the activation of factors V and II.

In our experiments in coagulation tests, such as Thrombin, APTT and techplastin, when studying plasma coagulation by determining the thrombin time of blood plasma in healthy rats, when fibrinogen was added to the plasma, blood coagulation was observed within 15 - 25 s and in conditions of AD, blood coagulation was observed for 10 - 11 s. APTT time in healthy rats was observed to clot after 30 s in controls, and in AD conditions, clotting was observed after 20 s. It was also observed that the prothrombin clotting time was 26 s in control rats and 20 s in AD conditions (**Figure 2(B)**).

Because the Aβ protofibrils mentioned above contribute to the activation of the contact system, the results obtained from a practical experiment indicate that the division of HKi leads to the activation of FXII and prekallikrein. The above data is confirmed by the fact that thrombin and APTT tests were significantly reduced.

Regarding the molecular mechanisms of the findings, in blood samples taken from people with Alzheimer's disease, Aβ protofibrils were found to be involved in blood clotting, plasma coagulation, and the plasma contact system, which stimulates inflammation. This system is activated by Aβ protofibrils, and these processes may contribute to the development of vascular and inflammatory pathologies associated with Alzheimer's disease. Aβ protofibrils bind to coagulation factor XII and high molecular weight kininogen and accelerate the activity of the hemostatic system [39].

Taking into account the above data, it is noticed that blood plasma factors, especially Hageman factor or blood coagulation factor XII, are activated during blood rheology and blood coagulation faster than in a healthy state. The activity of this factor XII has been proven above by international standard tests.

In the following experiments, we studied the effect of the polyphenols we used on the clotting time of blood plasma in the state of AD using thrombin, APTT, and techplastin tests.

As a result of experiments, it was established that these polyphenols have a significant effect on the hemostasis system in AD. In coagulation tests such as thrombin, APTT, and techplastin, when studying the thrombin time of blood plasma in healthy rats, when fibrinogen was added to the plasma, blood coagulation was observed in a time interval of 15 - 25 s and the case of AD, blood coagulation was observed in 10 - 11 s. It was determined that eforbin at a concentration of 50 μM extended the clotting time of blood plasma by 43 s and polyphenol PC-6 at a concentration of 50 μM extended the clotting time of blood plasma by 35 s. It was also observed that the aPTT time in healthy rats, blood plasma clotting took 30 s in the control and conditions of AD it clotted in 20 s, while eforbin at a concentration of 50 μM, the clotting time increased by 50 s, PC-6 polyphenol increased the time blood plasma clotting for 45 s at 50 μM. concentration. In our further experiments, the prothrombin time of blood clotting in healthy rats was 26 s in the control and 20 s in conditions of AD, while the polyphenols eforbin at a concentration of 50 μM extended the plasma clotting time by 35 s and PC-6 at a concentration of 50 μM extended it by plasma clotting time by 43 s (**Figure 3**).

From the above information, we can briefly conclude that in Alzheimer's disease, Aβ protofibrils lead to preferential activation of the hemostasis communication system. Antibody Aβ protofibrils bind to coagulation factor XII and cause activation of the communication system, which leads to an increase in the procoagulant state in the plasma.

These results indicate that the polyphenols used have an inhibitory effect on plasma factors that arise in the conditions of Alzheimer's disease, which are influenced by Aβ protofibrill, the causes of the pathology of this disease, stimulate plasma coagulation and inflammation, as well as the internal mechanisms of blood coagulation, which provide normalization of the plasma contact system.

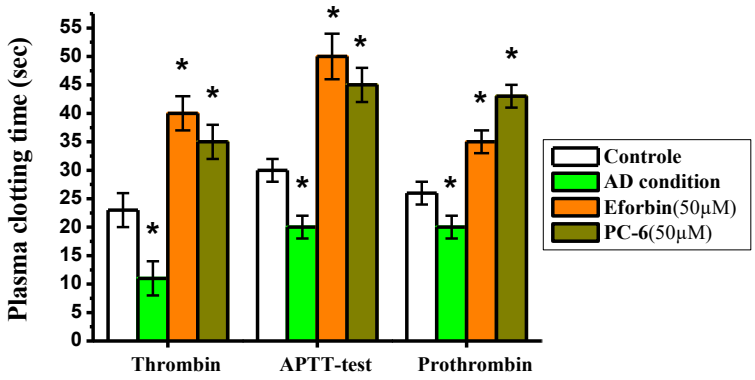

Figure 3 Indications for thrombin, APTT and techplastin blood plasma clotting time tests in asthma and the effect of polyphenols eforbin and PC-6 on blood coagulation.
Reliability indicator: * $p < 0.05$; (n = 6).

Changes in the functional activity of platelets in neurodegenerative diseases. Oxidative stress may play a significant role in various diseases, including Alzheimer's disease (AD). Oxidative stress occurs when there is an imbalance between the production of reactive oxygen species (ROS) and the body's ability to detoxify them or repair the resulting damage. This can lead to damage in platelets and neurons [40].

In research, there is extensive evidence that Aβ, a key protein involved in the pathology of AD, can stimulate platelet activation and cause various changes in platelet function. Aβ activates platelets, leading

to increased platelet adhesion to surfaces and aggregation, [41], a key step in thrombus formation. This activation may contribute to the vascular dysfunction and thrombotic events observed in AD. Exposure to Aβ is associated with increased production of reactive oxygen species (ROS) in platelets. ROS are highly reactive molecules that can cause oxidative damage to cellular components, leading to further dysfunction and contributing to oxidative stress in AD. Platelets contain APP and its processing machinery, and exposure to Aβ may lead to dysregulation of APP processing within platelets, potentially contributing to the pathological processes observed in AD [42,43].

In this context, it was first shown that *in vitro* formed β-amyloid from different proteins induces platelet activation through two pathways, binding to the scavenger receptor CD36 and to GP1bα, and activating p38 MAPK/COX1 pathways. β-amyloid can bind to CD36 on platelets, triggering signaling cascades that lead to platelet activation. This pathway may involve downstream signaling molecules such as p38 MAPK (mitogen-activated protein kinase), which is associated with cellular stress responses and inflammation. Also, β-amyloid can bind to Gp1bα, initiating signaling events that lead to platelet activation. This pathway may also involve activation of the p38 MAPK/COX1 pathway, which may further enhance platelet activation and aggregation. These pathways induce the release of TxA2, triggering platelet activation. Later studies have shown that both Aβ40 and Aβ42 bind GPIIb-IIIa, and initiate platelet adhesion without degranulation, indicating that soluble Aβ peptides are not able to fully activate platelets [44]. These pathways cause the release of thromboxane A2 (TxA2), which is a potent platelet activator, thereby triggering platelet activation. Thromboxane A2 promotes platelet aggregation and vasoconstriction, promoting the formation of blood clots. More recent studies have shown that both Aβ40 and Aβ42 bind to GPIIb-IIIa and initiate platelet adhesion without degranulation, suggesting that soluble Aβ peptides are not able to fully activate platelets [44]. This finding means that although Aβ peptides can initiate the process of platelet adhesion, they cannot induce full platelet activation leading to degranulation, which involves the release of granule contents containing various bioactive molecules involved in platelet aggregation and activation.

Additionally, platelets from newly diagnosed AD patients show increased activation characterized by increased fibrinogen binding and altered morphology. Altered morphology may reflect disease-related changes in platelet structure and function. In this, despite an increased state of activation, platelets from newly diagnosed AD patients show a reduced aggregation response. This is characterized by decreased expression of CD62P and CD63, which are markers of platelet degranulation. Platelet degranulation involves the release of granule contents containing various bioactive molecules that promote platelet aggregation and activation. The degranulation disorder observed in these platelets suggests dysfunctional signaling pathways or altered granule release mechanisms [45]. GPIIb-IIIa activation is known to promote platelet aggregation by providing fibrinogen binding, which binds adjacent platelets together to form blood clots. Binding of Aβ40 to GPIIb-IIIa may lead to platelet activation and subsequent aggregation, contributing to the thrombotic events observed in AD [46]. In addition, activation of GPVI initiates signaling pathways that promote platelet activation and aggregation in response to various stimuli. Binding of Aβ40 to GPVI may similarly trigger platelet activation and aggregation [44]. Activation of platelets by Aβ peptides can lead to the formation of fibrillar forms of Aβ, which may further contribute to the pathology of AD. Both fibrillar Aβ40 and Aβ42 have been reported to stimulate platelet aggregation following activation of GPIb-IX-V [44] and CD36 receptors. GPIb-IX-V is a receptor complex involved in platelet adhesion to damaged vascular walls and promotes platelet activation when binding to von Willebrand factor and other ligands. CD36 [47], on the other hand, is a scavenger receptor involved in various cellular processes, including lipid metabolism and inflammation. Activation of these receptors by Aβ fibrillar

peptides may promote platelet activation and subsequent aggregation, further implicating Aβ peptides in the pathophysiology of AD[48].

To confirm the above data, the effect of the polyphenols used on the functional activity of platelets in the blood plasma of rats in healthy and AD states (spontaneous aggregation) and induced by various inducers (ADP, adrenaline and collagen) was studied.

ADP 1.0 μM binds to its receptor on the surface of platelets. Initial binding results in the release of intracellular calcium and a change in platelet shape, leading to the formation of a primary wave of aggregation. The secondary wave reflects the release of ADP from platelet storage granules. A low dose of ADP causes only primary aggregation and its effect is reversible. ADP –2.5, –5.0 μM binds to 2 G-protein receptors P2Y1 and P2Y12. Binding of ADP to the P2Y1 receptor causes a shape change and initiates platelet aggregation (primary wave) by mobilizing calcium. The P2Y12 receptor is considered the main ADP receptor and is responsible for complete platelet aggregation through inhibition of adenyl cyclase. ADP - 10.0 μM The P2Y12 receptor is also a target for clopidogrel. The 2nd wave of ADP-induced aggregation is suppressed by aspirin.

Adrenaline 5, 10 μM binds to the α2-adrenergic receptor on the surface of platelets, which leads to inhibition of adenylate cyclase and the release of calcium ions. Platelet aggregation with epinephrine is visually similar to aggregation with ADP, with an initial primary wave of aggregation, release of stored ADP from platelets, and a 2nd wave of irreversible aggregation. As with ADP, this 2nd wave of aggregation is inhibited by aspirin. Adrenaline, like ADP, is considered a weak agonist.

Spontaneous aggregation was not observed in platelet-rich plasma of control group, but spontaneous aggregation was observed in platelet-rich plasma of AD rats.

When the inducer ADP was added to the plasma, the level of aggregation, irreversible platelet aggregation in the form of a secondary biphasic curve in concentrations (5 - 10 μM) was observed at a level of 60 - 90 %. In AD, the level of ADP-induced aggregation was 10 - 15 % higher than the control. (**Figure 4(A)**)

Adrenaline inducer (5 - 10 μM) causes platelet aggregation in a dose-dependent manner, similar to ADP. Adrenaline at this concentration caused irreversible platelet aggregation by 45 - 70 %. Under AD conditions, it was noted that the level of adrenaline-induced aggregation was 15 - 20 % higher than in the control (**Figure 4(A)**).

Collagen inducer (5 - 10 μM) dose-dependently induced platelet aggregation by 50 - 80 % at concentrations similar to ADP. At this concentration, collagen induced irreversible platelet aggregation. In the case of AD, it was noted that the level of collagen-induced aggregation was 12 - 18 % higher than the control (**Figure 4(A)**). Collagen 5, 10 μg/mL binds to the GPVI and GPIa/IIa receptors, inducing the release of granule contents, the TXA2 pool, and then leads to sustained activation of GPIIb-IIIa.The GPIa/IIa receptor is involved in platelet adhesion. The GPVI receptor is involved in platelet signaling and TxA2 generation. The lag phase is formed after the addition of collagen to platelet rich plasma, usually in less than 1 min.

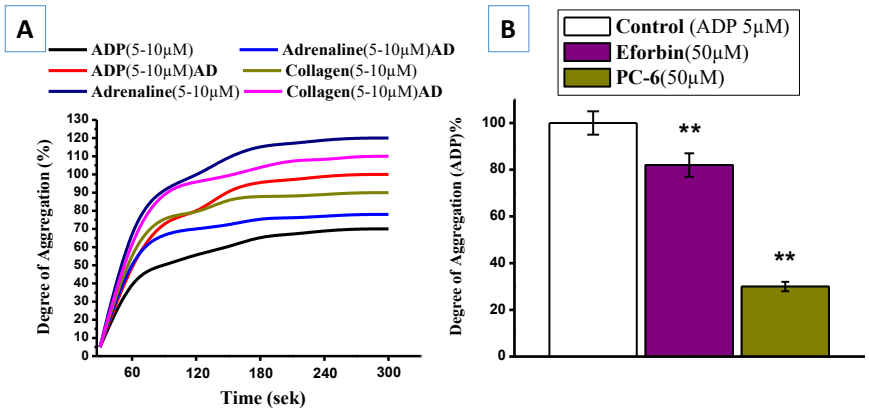

Figure 4 (A) The appearance of platelet aggregation induced by ADP, adrenaline and collagen in healthy people and in AD and (B) Effect of polyphenols eforbine and PC-6 on platelet aggregation induced by ADP. The results are presented in % relative to the control. Reliability indicator: ** $p < 0.01$; (n = 6).

These results are supported by many studies that Aβ oligomers produced under AD conditions increase platelet aggregation, cause changes in platelet structure and morphology, and increase ROS and APP release by changing membrane fluidity [49]. Based on these data, platelets were proven to be in an active state in AD when we tested their aggregation using various inducers.

In our *in vitro* experiments, we studied the effect of polyphenols eforbin and PC-6 on platelet aggregation inducers, such as ADP and collagen, on the functional activity of platelets in blood plasma and found that polyphenols inhibit platelet aggregation compared to higher inducers. These results lead us to conclude that polyphenols act by directly blocking platelet calcium channels through blocking mechanisms.

When we studied the effects of the polyphenols eforbine and PC-6 on platelet aggregation, these polyphenols were found to partially inhibit platelet aggregation (**Figure 4(B)**). As can be seen from the figure, under conditions of ADP 5 μM, it was observed that the polyphenol eforbine at a concentration of 50 μM inhibited platelet aggregation induced with ADP by 18 %, and the polyphenol PC-6 at a concentration of 50 μM inhibited platelet aggregation induced with ADP by 70 %.

It is known that ADP, by binding to its receptors P2Y1 and P2Y12 on the surface of platelet membranes, causes a change in shape and initiates platelet aggregation, possibly due to inhibition of adenylate cyclase, reducing the production of cAMP and increasing the release of $[Ca^{2+}]_{in}$ from the depot. Although ADP in platelet aggregation has not been definitively established. As a result, Ca^{2+} ions are released into the cytoplasm. The 3rd stage, which is the external manifestation of the cell's response, involves the aggregation and reaction of releasing chemicals from the cell. An important role in the perception of an external signal, its translation and response belongs to the components of the plasma membrane of platelets.

In our next experiments, when we examined the effects of polyphenols eforbine and PC-6 on collagen-induced platelet aggregation, these polyphenols were found to have different effects on platelet aggregation. During the experiments, it was observed that the polyphenol eforbin at a concentration of 50 μM inhibited collagen-induced platelet aggregation by 10 %, and the polyphenol PC-6 at a concentration of 50 μM inhibited 80 % in the presence of collagen 5 μM (**Figure 5(A)**).

Collagen has a high affinity for von Willebrand factor. By binding to the GPIb-V-IX receptor on the surface of platelets and collagen, von Willebrand factor plays the role of a molecular "glue", ensuring platelet adhesion to the damaged area of the vascular wall. This is not the only mechanism of platelet

adhesion. On the surface of platelets there are also specific receptors GPIa-IIb and GPVI for direct communication with collagen. These receptors also ensure platelet adhesion to the area of vascular damage. Collagen provides more than just platelet adhesion. By binding to the GPIa-IIb, GPVI receptors and, through von Willebrand factor, to the GPIb-V-IX receptors, collagen intensively activates platelets. In response to activation, platelets change their shape and develop pseudopodia (processes). The prostaglandin-thromboxane system is launched, the internal granules of platelets release their own endogenous inducers into the blood (von Willebrand factor, beta-thromboglobulin, thrombospandin, fibronectin, fibrinogen, platelet factor 4, coagulation factors V, XIII, beta-thromboglobulin, etc.).

As can be seen from the above data, it can be assumed that polyphenol PC-6 significantly inhibits collagen-induced platelet aggregation by binding to the GPIa-Iib, GPVI receptors and preventing the rapid activation of platelets through von Willebrand factor and GPIb-V-IX receptors.

These studies examined the effect of ADP/collagen-induced platelet aggregation in rats in a model of AD, and the inhibitory effect of the polyphenols found above was practically unchanged, as in the healthy condition (**Figure 5(B)**).

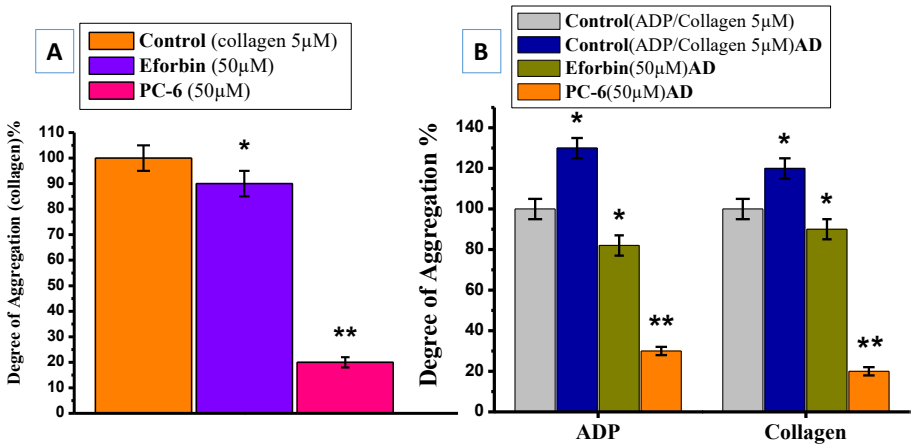

Figure 5 (A) Effect of polyphenols eforbin and PC-6 on platelet aggregation induced by collagen and (B) Effect of polyphenols eforbin and PC-6 on platelet aggregation induced by ADP/collagen under AD conditions. Reliability Index: * $p < 0.05$; ** $p < 0.01$; (n = 6).

It is known that in AD, β-amyloid from the differential protein through two pathways binds to the scavenger receptor CD36 and GP1bα and activates the p38 MAPK/COX1 pathway, ultimately inducing platelet activation. And this is one of the reasons leading to the activation of platelet aggregation. β-amyloid induces the release of TxA2 and activates platelets. Studies have shown that Aβ40 and Aβ42 bind GPIIb-IIIa and initiate platelet adhesion without degranulation, indicating that soluble Aβ peptides are not capable of fully activating platelets [44,46].

Summarizing the results obtained, information about the mechanisms of control of various inhibitory effects of polyphenols eforbine and PC-6 on changes in the calcium dynamics of nerve and platelet cells under AD conditions in the future allows us to use it as a scientific basis for the development of effective drugs with complex effects based on these polyphenols.

In neurobiological research, platelets are often used as a model [50,51]. Analysis and determination of the mechanism of amine release by platelets has been fundamental to the understanding of various psychotherapeutic drugs. Studies have shown that most physiological agonists stimulate platelet breakdown of phosphotidulositol-4,5-bisphosphate, production of the 2nd messengers inositol-1,4,5-trisphosphate (IP3)

and diaculglucerol, and an increase in cytosolic Ca^{2+} ($[Ca^{2+}]_{in}$) [52]. The discovery that β-amyloid is released from human platelets and is encoded by platelet messenger RNA [53,54] has strengthened the idea that platelets may be a useful peripheral cell model for studying functional impairment in AD. In scientific research, the collected results on cold changes in peripheral cells help in the search for biological markers for diagnosis [55-58], understanding the pathophysiology of AD and searching for new therapeutic strategies to combat AD [59,60].

And also, studies have found an increase in $[Ca^{2+}]_{in}$ platelets and lymphoblast of patients with AD [61,62]. The $[Ca^{2+}]_{in}$ content in the young, old and AD groups was similar in cultured human lymphoblasts [63]. A decrease in $[Ca^{2+}]_{in}$ and an increase in surface binding in fibroblasts from old donors and donors with AD was determined compared to cells from young donors [59].

Finding quiescent $[Ca^{2+}]_{in}$ AD donor fibroblasts similar to that in normal donors suggests that abnormal $[Ca^{2+}]_{in}$ components in cultured human fibroblasts from AD donors as well as systematic pathological changes in AD may also involve dysfunction of intracellular calcium mobilization. In place of these, it was found that there was an altered response of fibroblasts of elderly people and donors with AD to drugs that increase the level of $[Ca^{2+}]_{in}$ [62-68].

It is known that due to activation of the endoplasmic reticulum, a sharp increase in $[Ca^{2+}]_{in}$ the cytosol leads to platelet activation, aggregation, secretion, and release factors of blood coagulation. In such cases, the main element activation of platelet is the mechanisms of mobilization of Ca^{2+} from internal cellular depots.

To find out whether the anti-aggregation effect of the polyphenols used in the experiments is associated with the inhibition of the entry and mobilization of Ca^{2+} from the internal stores of cells, we sought to study the effects of identifying the mechanism of action of the polyphenols eforbin and PC-6 on the amount of membrane-bound and cytosolic Ca^{2+} of platelets.

In these experiments, we used the fluorescent probe CTC to measure the amount of Ca^{2+} bound to the membrane and the fluorescent probe Fluo 3-AM to measure the amount of Ca^{2+} in the cytosol. To determine changes in intracellular calcium content, rat blood platelets were incubated in 10 mM Fluo 3-AM medium with the addition of calcium chloride (1 mM) for 30 min at a temperature of +37 °C.

ADP (20 μM) used as a stimulator of calcium release from intracellular stores. Initially, the release of Ca^{2+} ions from the cellular depot under the influence of ADP occurs with an increase in the amount of Ca^{2+} inside the cell compared to the control. The chelating property of EGTA (5 μM) towards calcium ions was studied based on changes in calcium content in intact platelets, which led to a decrease in calcium content and calcium transport due to its chelating function.

These indicators were taken as control. The polyphenols themselves Eforbin and PC-6 at a concentration of 50 μM induced activation and increased the level of intracellular calcium compared to the control and ADP. The polyphenols eforbine and PC-6 were found to inhibit the increase in intracellular calcium when added 2 min before induction, with induced ADP. It has been established that polyphenols at a concentration of 10 - 100 μM inhibit the release of intracellular Ca^{2+} by 40 - 55 %. The most effective concentration was 50 μM (**Figure 6**).

When conducting the same experiments under AD conditions, it was found that polyphenols inhibit the intracellular release of Ca^{2+} by 30 - 45 % at a concentration of 10 - 100 μM (**Figure 6**). The results obtained allow us to determine that polyphenols have a significant inhibitory effect on calcium-dependent processes in activated platelet cells under AD conditions.

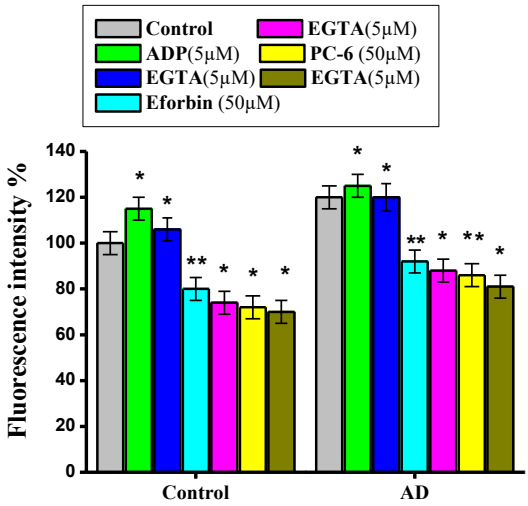

Figure 6 Effect of ADP, EGTA and polyphenols eforbine and PC-6 on changes in the amount of $[Ca^{2+}]_{in}$ against the background of ADP induction in platelets in control and AD conditions.

Control (platelets were incubated with 10 mM Fluo 3-AM for 30 min at 37 °C in the absence of Ca^{2+} ions); The influence of ADP on the release of Ca^{2+} ions from cellular stores; effect of EGTA 5 μM and polyphenols 50 μM on $[Ca^{2+}]_{in}$. Fluorescence intensity (%) is plotted along the ordinate. Reliability Index: $^{*}p < 0.05$; $^{**}p < 0.01$; (n = 6).

From this we can conclude that the polyphenols eforbin and PC-6 in control and in asthma cause a rapid decrease in free calcium ions in the cytoplasm of platelets and may have the property of blocking platelet activity at any stage, reducing the risk of blood clots.

To measure the amount of Ca^{2+} bound to the membrane, we measured it by using the fluorescent probe CTC. In this case, we used selective metabolic inhibitors that block some intracellular calcium stores.

When we studied the effect of polyphenols eforbin and PC-6 on changes in the amount of membrane-dependent Ca^{2+} concentration, we found that these polyphenols at a concentration of 50 μM increased the calcium content in the fluorescent probe CTC, eforbin by 25 %, PC-6 by 32 % compared with control.

Briefly, we can say that under the influence of polyphenols in platelets, the concentration of membrane-bound Ca^{2+} decreases. We concluded that CTC interacts with Ca^{2+} bound to membrane structures, and the decrease in CTC fluorescence we observed indicates a decrease in the amount of Ca^{2+} bound to membrane structures.

Conclusions

In conclusion, it should be noted that the polyphenols under normal and in AD conditions, eforbine and PC-6 can influence both the extrinsic and intrinsic coagulation pathways during blood coagulation, while they affect one of the factors XII, XI, IX, VIII of the intrinsic pathway, V and II external path. explained by inhibition of factors. Polyphenols Eforbin and PC-6, under normal conditions and in AD, inhibit platelet aggregation induced by ADP. The inhibition of platelet aggregation by polyphenols suggests that it may be related to its ability to prevent the entry of Ca^{2+} into platelets by blocking Ca^{2+} channels located in the plasma membrane. Polyphenols Eforbin and PC-6, under normal conditions and in AD, cause an increase in the concentration of membrane-bound Ca^{2+} in platelets. The polyphenols eforbine and PC-6 may be associated with changes in platelet $[Ca^{2+}]_{in}$ in healthy and AD patients, as they cause changes in membrane-bound Ca^{2+} by blocking the concentration of free Ca^{2+} ions.

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