Effects of Polyphenols on Changes in the Transport of Ca^{2+} NMDA-receptors Under the Influence of L-glutamate against the Background of Alzheimer's Disease

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Abstract

Calcium is a ubiquitous second messenger that regulates various activities in eukaryotic cells. Especially important role calcium plays in excitable cells. Neurons require extremely precise spatial-temporal control of calcium-dependent processes because they regulate such vital functions as synaptic plasticity. Recent evidence indicates that neuronal calcium signaling is abnormal in many of neurodegenerative disorders such as Alzheimer's disease (AD). A pivotal role for excitotoxicity in AD is gaining increasingly more acceptance, but the underlying mechanisms through which it participates in neurodegeneration still need further investigation. Excessive activation of glutamate receptors by excitatory amino acids leads to a number of deleterious consequences, including impairment of calcium buffering, generation of free radicals, activation of the mitochondrial permeability transition and secondary excitotoxicity. The possible competition between the polyphenols PC-6, PC-7 and L-glutamate for the site of regulation of the opening of ion channels of ionotropic NMDA-receptors in the brain of rats against the background of Alzheimer's disease was studied.

Keywords: Synaptosome, NMDA-receptors, Glutamate, Calcium.

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INTRODUCTION

It is known that, Dementia in the elderly population is most commonly caused by Alzheimer's disease (AD). The characteristic features of AD are the appearance of extracellular amyloid-β (Aβ) plaques and neurofibrillary tangles in the intra-cellular environment, neuronal death and the loss of synapses, all of which contribute to cognitive decline in a progressive manner. AD is a terminal and incurable disease [1]. Although the etiology of AD has not been specified, the pathological features associated with AD are mainly the accumulation of extracellular amyloid-beta (Aβ) plaques and intracellular neurofibrillary tangles composed of hyperphosphorylated tau protein [2]. Cellular homeostasis Ca^{2+} plays a main regulatory role in many aspects of neuron physiology, including growth and differentiation, properties of activity potential, synaptic plasticity, learning, and memory. Damaged cellular Ca^{2+} also contributes to pathophysiological conditions such as necrosis, apoptosis, autophagy deficiency, and degeneration [3].

Glutamate is the principal excitatory neurotransmitter of the Central Nervous System. Glutamate mediates neuronal plasticity, neural transmission, memory processes, and learning [4]. The pathogenesis of AD is strongly associated with alterations in glutamate signaling and the tissues affected by AD contain high densities of glumatergic neurons [5-10]. Early degeneration occurs to the neo-cortex pyramidal neurons of layers V and III [11,12] and to the glutamate-innervated cortical and hippocampal neurons [13]. 'Excitotoxicity' occurs as a result of the chronic, moderate activation of NMDA-receptors, leading to neurodegeneration. [14-17]. The excitotoxicity hypothesis is supported by clinical evidence indicating that the NMDA-receptor antagonist memantine slows AD progression.64) Prolonged Ca^{2+} elevation suppresses synaptic function, leading to subsequent synaptotoxicity and eventually atrophy; these events correlate with the loss of learning and memory functions in AD [18-21] Multiple neurotrophic factors have been demonstrated to enhance defense against excitotoxicity. Fibroblast growth factor treatment alters

expression of NMDA-receptors in cultured cortical and hippocampal neurons, protecting against glutamate toxicity [22].

NMDA-receptors mediate synaptic plasticity, critical for memory and learning functions, through long-term potentiation (LTP) [23-25]. Synaptic plasticity is an essential component of memory and learning [26]. LTP of synaptic transmission and permanently altered expression of post-synaptic AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptors occurs as a result of high levels of synaptic activity and open NMDA-receptors.However, mild synaptic stimulation elicits long-term depression (LTD) in active NMDA-receptors [27]. Several recent studies have examined the relationship between Aβ and NMDA. Aβ-induced spine loss is associated with a decrease in glutamate receptors, also required for LTD, in a calcineurin-dependent manner [28-30]. It is widely believed that the synaptic dysfunction and synapse loss contribute to the cognitive deficits of patients with AD.

Based on this, the mechanism of the action of polyphenolic compounds on glutamate-binding sites of NMDA-receptors in synaptosomes of the brain of rats against the background of Alzheimer's disease was studied.

The purpose of the study was to study the mechanism of action of polyphenols PC-6 isolated from plants *Pinus sylvestris L.* and PC-7 isolated from plants *E. canescens L* on the dynamics of intracellular Ca^{2+} synaptosomes in the brain of animals with models of Alzheimer's disease.

Materials and Methods: Experimental Models of AD

In accordance with the tasks set at various stages, studies were carried out on experimental modeling of Alzheimer's disease (AD) - aluminum neurotoxicosis (ANT). The studies were carried out on white outbred rats (weighing 280 - 300 g). Animals were weighed and behavioral tests were performed: Open field n=3, conditioned response of passive avoidance (CRPA) and active avoidance (ACRA) n=3, swimming in the pool (Morris test) n=3.

Feeding animals with a standard diet supplemented with:

Add sugar to drinking water (to sweeten water). Cholesterol in the amount of 0.2% of the total diet: 4-6 mg per 1 rat. Margarine in the amount of 2% of the total nutrition. Merkazalol in the amount of 0.04-0.06 mg per rat.

Acute aluminum neurotoxicosis (AAN) was induced by subcutaneous administration of 0.2 ml of 10% aluminum chloride solution to white rats (three groups of 12 animals each): the first group - once, the second - 2 times and the third group - 4 times. Control animals received a 0.9% NaCl solution in a similar manner.

After modeling AD, behavioral tests were repeated: Open field n=3, conditioned response of passive avoidance (CRPA) and active avoidance (ACRA) n=3, swimming in the pool (Morris test) n=3.

Animals were decapitated under light ether anesthesia. Blood and internal organs (brain, hippocampus) were taken into different vessels and processed simultaneously. Relatively low animal mortality was detected after a double administration of aluminum chloride, which served as the basis for using this dose in modeling AD.

Isolation of Synaptosomes: Experiments were carried out on 20 outbred albino male rats weighing (280-300 g) kept in the standard diet of the vivarium. All experiments were carried out in accordance with the requirements of the World Society for the Protection of Animals and European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes [31]. Synaptosomes are obtained by two-stage centrifugation Centrifuge K-24 (ELN13893354.Veb MLV Zenrifugenbau Engelsdorf. Germany) [32]. The entire isolation procedure is carried out at -4^0C. After decapitation, the brain is removed as quickly as possible and crushed on ice. The crushed tissue is homogenized at a ratio of 1:10 in the isolation medium - 0.32 M sucrose solution in 0.01 M Tris-HCl buffer with the addition of 0.5 mM EDTA (pH 7.4). The obtained homogenate is exposed to a 4-stage centrifugation. The supernatant after the first centrifugation (10 min, 4500 rpm) is carefully removed without capturing the myelin layer and exposed to further centrifugation for 20 min at 14000 rpm. The obtained dense precipitate P2 is resuspended in the isolation medium. The obtained suspension is used further in the experiment as a coarse synaptosomal fraction (synaptosomal-mitochondrial). In the case of 4-stage isolation, the second centrifugation is carried out at 11,000 rpm for 20 minutes. The dense pellet of P2 is resuspended in 0.32 M sucrose solution (pH 7.4) and then carefully layered on 0.8 M sucrose solution (pH 8.0), after which it is centrifuged for 25 minutes at 11,000 rpm. As a result of centrifugation in a sucrose gradient, factions are separated - mitochondria settle tightly at the bottom of the tube, and synaptosomes remain in suspension in a layer of 0.8 M sucrose. This layer is carefully removed, mixed with an equal amount of isolation medium and left for 15 minutes to restore the ultrastructure of synaptosomal particles, after which it is exposed to further centrifugation at 14,000 rpm for 30 minutes. The dense final precipitate P4 is resuspended in the isolation medium and then used in the experiment as a synaptosomal faction.

Measuring Intracellular Ca^{2+}: To measure the amount of membrane bound Ca^{2+}, 20 μM chlortetracycline (CTC) was added to synaptosomes placed in a medium similar to that used for cell isolation, but without apyrase and $MgCl_2$. It is incubated 60 min. to achieve maximum interaction of CTC with membrane-bound Ca^{2+}, both on plasma and intracellular membranes. The CTC excitation wavelength is 405 nm, the registration wavelength is 530 nm. The results were expressed as percentages, taking as 100% the difference between the maximum fluorescence intensity (fluorescence

of the dye saturated with Ca^{2+}) and its minimum value (fluorescence of the indicator in the absence of Ca^{2+}) obtained after the addition of EGTA.

The amount of cytosolic Ca^{2+} was calculated using the Grinkevich equation [33] in synaptosomes isolated from rat brains. To measure free cytosolic Ca^{2+}, synaptosomes ($1x10^8$ cells/ml) were loaded with 4 μM Fura-2AM acetoxymethyl ester for 40 min at 37°C. At the same time, in the dye molecules that have penetrated into the cytoplasm, under the action of intracellular esterases, the ester group is cleaved off, resulting in the Fura-2 anion that binds Ca^{2+}. After completion of the loading, the dye remaining in the medium was removed by double washing and centrifugation in standard medium. In the experiments, the cell concentration in the cell was $5x10^6$ cells/ml. Fluorescence excitation was induced at 337 nm and fluorescence registration at 496 nm. Ca^{2+} saturated dye fluorescence (Fmax) was determined by adding 50 μM digitonin to cells loaded with Fura-2AM. Fmin was determined by measuring the fluorescence intensity in a calcium-free medium, Fmin = $[(Fmax – Faf)/3]^+$ Faf, where Faf is cell autofluorescence determined by adding 0.1 mM $MnCl_2$ to thymocytes loaded with Fura-2AM and processed with digitonin.

Statistical Analysis: The measurements were carried out on a universal spectrometer USB-2000 (USB2E7916.OceanOptics.USA.2010). 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

It is known that in neurodegenerative diseases it is accompanied by an increase in glutamatergic transmission, which occurs due to an increase in the release of glutamate. The excitatory neurotransmitter glutamate can cause damage and death of neurons, and therefore the damaging effect of glutamate on neurons is designated by the term "excitatory amino acid toxicity" or "excitotoxicity" [34-36]. Excitotoxicity of glutamate is mediated by NMDA-receptors, named after the specific antagonist N-methyl-D-aspartate. When glutamate interacts with these receptors, the ion channels of the neuronal membrane open and glutamate enters the neuron. Extensive binding of glutamate by NMDA-receptors leads to an increase in Ca^{2+} current into the neuron through NMDA-receptor channels. Due to the fact that the increase in Ca^{2+} current is one of the leading mechanisms of neuron death, it can be assumed that the mechanism of glutamate excitotoxicity in Alzheimer's disease. Violation of glutamatergic transmission is currently also considered as a leading factor in the pathogenesis of diseases such as epilepsy, Alzheimer's disease, etc. [37-40].

Neurodegenerative diseases are caused by dysfunction of the ion transport systems and receptor complexes that represent the functional activity of cells in the sympathetic/parasympathetic nervous system of the brain. Ca^{2+} is a main component in signaling and excitability/regeneration processes in nerve cells, especially in brain $[Ca^{2+}]_{in}$ synaptosomes, leading to serious pathological conditions. From this point of view, the study of the mechanisms of pharmacological correction of Ca^{2+} - the transport of biologically active substances in synoptics in pathological conditions is of current scientific and practical importance.

Among pharmacologists, due to the high demand for neuroprotective drugs, and also due to the fact that the effect of biologically active substances, isolated from plants, on the nervous system has not yet been significantly studied, research in this direction seems to be relevant and of scientific and practical importance.

It should be noted that the vast majority of data on the effect of glutamate on neurotransmission processes were obtained in electrophysiological experiments, in which the main criterion for evaluating the effect of activation of presynaptic glutamate receptors was a change in the frequency and amplitude of recorded synaptic flows in postsynaptic structures. The extremely small geometric dimensions of most nerve terminals are a serious obstacle to successful direct measurements of the corresponding phenomena in presynaptic formations. In this regard, the research on those intracellular processes developing in presynaptic nerve structures was carried out using fluorescent probes.

We used synaptosomes obtained from the brain of rats modeled for Alzheimer's disease, which are an adequate and convenient model for studying presynaptic processes. The activity of L-glutamate was judged by the change in the intensity of the fluorescent signal, by the change in the cytoplasmic levels of free calcium $[Ca^{2+}]_{in}$.

In experiments, the effect of glutamate on the level of intracellular calcium in synaptosomes from the brain of rats in control and model rats with AD was studied.

In experiments, the effect of glutamate on the level of intracellular calcium in synaptosomes from the rat brain was investigated. The ratio of fluorescence, excited by light with wavelengths of 340 and 380 nm (F_{340}/F_{380}) in synaptosomes, was preliminarily determined using a Ca^{2+}-sensitive chlortetracycline (CTC) probe. When Ca^{2+} was removed from the extracellular medium, preincubation with EGTA led to a decrease in fluorescence by 5%. In the presence of EGTA in the incubation medium, glutamate at concentrations of 1-100 μM increases the fluorescence level in a dose-dependent manner by 28-50%, which indicates an increase in the Ca^{2+} concentration in the cytosol $[Ca^{2+}]_{in}$, caused by glutamate, against the background of simulated AD, glutamate at concentrations of 1-100 μM does not noticeably increase the level of fluorescence by 7-12%, primarily due to the activation of membrane permeability, Ca^{2+} movement inside

the cell and Ca^{2+} release from intracellular depots (Fig. 1).

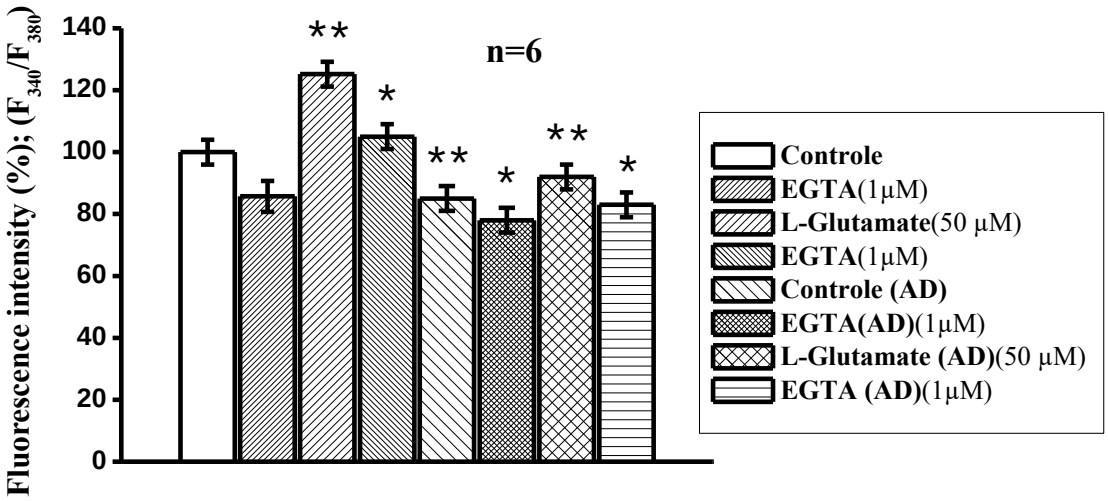

Figure 1. The effect of L-glutamate (50 μM) on the fluorescence intensity in a suspension of CTC+synaptosomes of the rat brain upon incubation with EGTA (1 mM) in control and in simulated AD. Reliability indicator: * - P <0.05; ** - P <0.01; *** - P<0.001. (n = 6).

In the results, as mentioned above, on AD models regulation of presynaptic Ca^{2+} channels can either facilitate or inactivate incoming Ca^{2+} ion flows. This strong dependence of neurotransmitter release on presynaptic Ca^{2+} current may predict regulatory mechanisms that will affect transient presynaptic plasticity.

The regulation of the content of calcium ions in the cytosol is carried out by transmembrane transport and cytoplasmic calcium binding. There are two main types of calcium ion transport structures across the membrane: 1) Ca^{2+} channels of the plasma membrane and ER, 2) calcium pumps and exchangers of the plasma membrane. Thus, a change in the concentration of calcium ions can be carried out via both its intake from the external environment and due to the release of calcium from the intracellular structures of the Ca^{2+} depot. Most of the Ca^{2+} ions entering the cell are almost immediately bound with exocytosis proteins.

In the following experiments, studied with Fura-2AM, glutamate at concentrations of 1-100 μM dose-dependently increases the level of $[Ca^{2+}]_{in}$ by 20-40%, caused by glutamate, on the AD model, glutamate at concentrations of 1-100 μM significantly increases the fluorescence level by 50-65%, primarily due to the activation of membrane permeability, the movement of Ca^{2+} into the cell, and the release of Ca^{2+} from intracellular depots (Fig. 2).

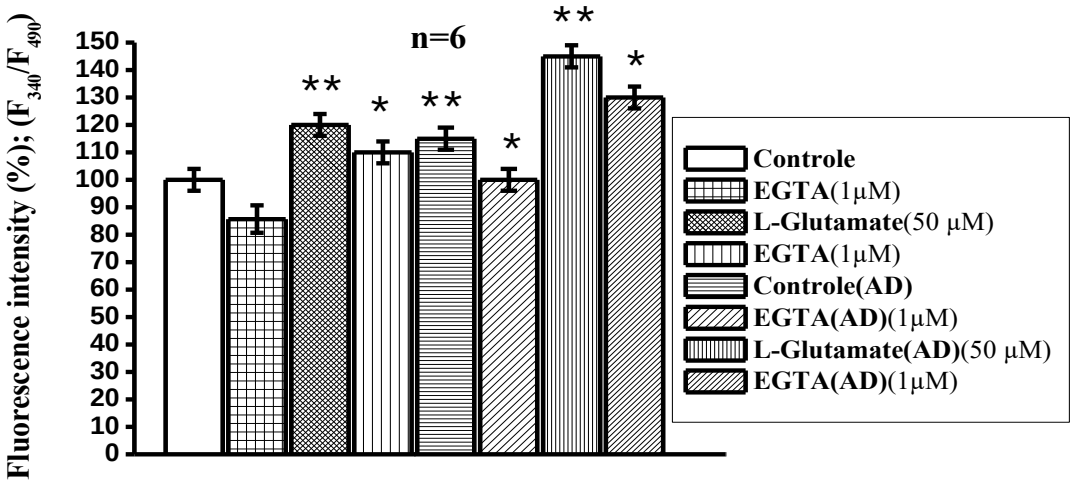

Figure 2: The effect of L-glutamate (50 μM) on the fluorescence intensity in a suspension of Fura-2AM+rat brain synaptosomes upon incubation with EGTA (1 mM) in control and AD model. Reliability indicator: * - P <0.05; ** - P <0.01; *** - P<0.001. (n = 6).

From the literature data, it is known that AD is a neurodegenerative disorder, which affects memory formation and storage processes. In most cases AD appears sporadically and affects people over 60 years of age. Small portion of cases (approximately 1%–2%) refers to familial form of AD, which is characterized by an earlier onset and more severe pathogenesis. Familial form of AD is caused by mutations in genes encoding Presenilin 1 (PS1), Presenilin 2 (PS2) and amyloid-precursor protein (APP) [41]. Presenilins form the catalytic subunits of the γ-secretase protease complex, which is together with β-secretase are responsible for APP protein cleavage and subsequent production of toxic Aβ peptides. Several hypothesis about causes of AD have been proposed, but so-called "amyloid cascade hypothesis" is a dominant model of AD pathogenesis. It states that increased production of amyloidogenic Aβ42 peptide (or an increase in Aβ42:Aβ40 ratio) is driving AD, causing reduced number of synapses and neuronal death [42]. Based on this idea, major efforts were spent by the industry on developing agents which can reduce Aβ production or eliminate Aβ from the brain. However, so far these agents did not show benefit in AD clinical trials [43]. As alternative point of view, "calcium hypothesis of AD" was suggested [44]. This hypothesis speculates that dysregulation in cellular calcium homeostasis is the main driving force of neurodegeneration in AD [45]. Several lines of experimental evidence support this idea. ER Ca^{2+} levels are elevated in AD and in aging neurons. Rise in ER Ca^{2+} concentration results in subsequent compensatory alterations and defects in neuronal Ca^{2+} signaling. Altered Ca^{2+} signals shift the balance between Ca^{2+} -dependent phosphatase calcineurin (CaN) and its opponent Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII), which are extremely abundant in synaptic locations. Shift in the balance of CaMKII and CaN activity occludes longterm potentiation and facilitates long-term depression, causing synaptic and memory impairments and leading to synaptic loss and neurodegeneration [46].

What is a reason for increased calcium content during AD? How do AD-causing mutations induce calcium signaling dysregulation? One possible explanation is that Aβ peptides form Ca^{2+} -permeable pore in the plasma membrane [47]. Indeed, a part of neurites surrounding β-amyloid plagues have elevated steady-state Ca^{2+} levels. Aβ may induce increased calcium influx via L-type VGCCs, but it was also reported that Aβ oligomers suppress P/Q-type VGCCs calcium currents [48]. NMDAR is another potential source for intracellular calcium, playing important role in excitatory synaptic neurotransmission. Activation of synaptic NMDARs is required for synaptic plasticity and drives LTP generation, but sustained activation of NMDARs can cause subsequent calcium overload and toxicity [49]. Role of NMDA receptor in AD and particularly Aβ effects on NMDA receptor were studied intensively [50,51]. It was proposed that at early AD stages NMDA receptor is overactivated [52]. Indeed, recent reports indicate that Aβ oligomers applied on cultured cortical neurons activate GluN2B-containing NMDAR and induce an immediate Ca^{2+} rise. Potentially neuroprotective drugs which block NMDAR has intolerable side effects, presumably because of extreme importance of this receptor for normal neuronal function. An exception is a non-competitive NMDAR inhibitor memantine, which is currently approved for AD treatment. In contrast to other NMDAR blockers, positive effects of memantine administration are likely observed because it preferentially blocks excessively activated NMDARs. New generation of drugs which selectively bind to and inhibit only excessivly activated NMDARs are considered for AD treatment. Another adverse effect of Aβ is a reduction in NMDAR expression and its enhanced endocytosis. Enhanced endocytosis of NMDARs is in part due to Aβ-mediated activation of STEP61 phosphatase. Downregulation of GluN1 subunit of NMDAR was observed in postmortem AD patient's samples. It was shown that the specific N-terminal splice cassette containing GluN1 is decreased drastically in AD, suggesting that neurons bearing this isoform are more vulnerable. Some data indicate that Aβ may directly bind to and modulate activity of NMDA receptors [53]. Reduction of NMDAR activity in AD may also be induced by an oxidative stress, most likely due to oxidation of extracellular NMDAR cysteins and intracellular targets such as calmodulin. Taking together, we may conclude that Aβ causes dysregulation of NMDAR expression and activity by multiple mechanisms. Disrupted NMDAR signaling further leads to impaired synaptic plasticity, reduced LTP, enhanced LTD and synaptic loss [51].

In the following experiments, re-incubation of PC-6 and PC-7 (10-100 μM) with the Fura-2AM-synaptosome complex increased the level of $[Ca^{2+}]_{in}$ distinct from glutamate. In the PC-6 and PC-7 AD models (10-100 μM) with the Fura-2AM-synaptosome complex, the level of $[Ca^{2+}]_{in}$ difference from glutamate does not increase (Fig. 3).

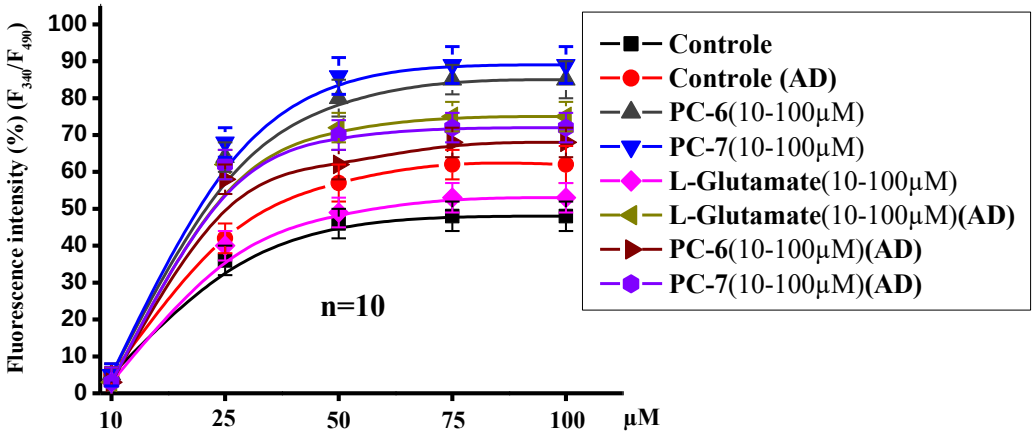

Figure 3. Effect of PC-6, PC-7, and L-glutamate at concentrations (10–100 μM) on the fluorescence intensity of a suspension of Fura-2AM+ of the rat brain synaptosome in an AD model. Reliability indicator: * - P <0.05; ** - P <0.01; *** - P<0.001. (n = 10).

Dysregulation of Ca^{2+} signaling causes excessive Ca^{2+} release via RyR [45]. RyR-mediated Ca^{2+} release is enhanced in neurons from presenilin mutant mice. RyR2 expression levels are elevated in AD brains [54] and activity of RyR is enhanced due to increased ER Ca^{2+} levels. Enhanced Ca^{2+} release via RyR2 affects synaptic plasticity, compensating for changes in LTP and LTD [55]. In the spines of AD neurons, NMDA receptor-mediated Ca^{2+} influx triggers supranomal Ca^{2+} responses mediated by RyR2. Excessive Ca^{2+} release triggers overactivation of Ca^{2+}-dependent SK channels and impairs the induction of synaptic plasticity changes [55]. Indeed, SK channels overactivation was at least partially responsible for destablization of mushroom spines and late-phase LTP defects in presenilin knockin (PS1-M146V-KI) neurons [52]. These results suggest that inhibition of RyR2 activity may helps to alleivate AD symptoms. Indeed, previous studies showed that short term treatment with RyR inhibitor dantrolene was able to stabilize Ca^{2+} signals, ameliorate cognitive decline and reduce neuropathology, amyloid load and memory impairments in various AD mouse models [55]. However, in our previous studies we observed that long-term feeding of the RyR inhibitor dantrolene exacerbated amyloid plaque formation and resulted in the loss of hippocampal synaptic markers and neuronal deterioration in AD mice. One potential problem with interpreting these conflicting results is that specific RyR inhibitors do not exist and the drug dantrolene, used in most studies, has additional targets such as store-operated Ca^{2+} channels.

In the next experiment, a complex effect of polyphenols PC-6, PC-7 and L-glutamate on the level of $[Ca^{2+}]_{in}$ Fura-2AM-synaptosomes of the rat brain was carried out on an AD model. Preliminary re-incubation of PC-6 and PC-7 (10 μM) with synaptic membranes, then the addition of Fura-2AM-glutamate led to a decrease in the level of $[Ca^{2+}]_{in}$. A dose-dependent increase in the concentration of PC-6 and PC-7 (10-100 μM), respectively, led to a dose-dependent decrease in the effect of glutamate (Fig. 4.).

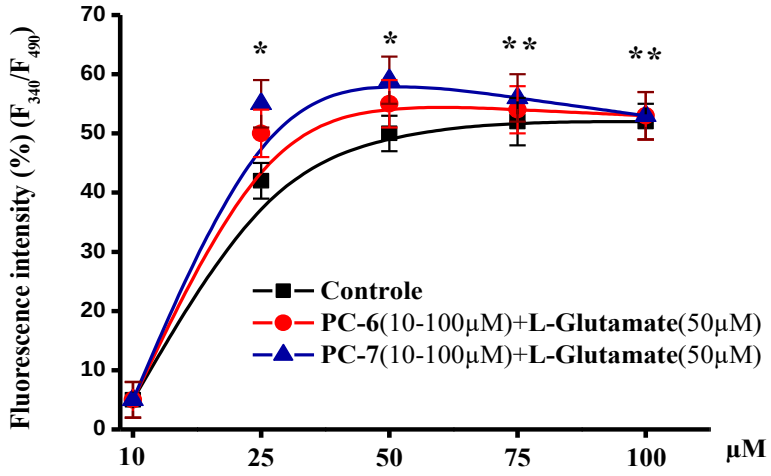

Figure 4. Effect of PC-6, PC-7 (10-100 μM) on the fluorescence intensity of a suspension of rat brain synaptosomes incubated with glutamate (50 μM) in an AD model. Reliability indicator: * - P <0.05; ** - P <0.01; *** - P<0.001. (n = 10).

In the activity of L-glutamate, there were observed depolarization of the synaptosomal membrane and an increase in intracellular calcium without a noticeable change in the concentration of internal sodium ions. The increase in intra-synaptosomal calcium was prevented by the addition of L-glutamate. Activation of the L-glutamate receptor causes the opening of ionotropic receptor calcium channels, calcium influx into synaptosomes, and depolarization of the synaptosomal plasma membrane, followed by the release of amino acid neurotransmitters.

L-glutamate is the most abundant excitatory neurotransmitter in the mammalian Central Nervous System (CNS). It is extensively distributed in the CNS whereas it is almost exclusively located intracellularly. L-glutamate can be synthesized through a number of metabolic pathways [56]. In wet tissue, glutamate is measured at concentrations of 5–15μmol/g [57, 58]. Its concentration in the synaptic cleft at resting conditions is about 0.6μM [59]. During synaptic transmission L-glutamate concentration can go above 10μM at spatially localized extracellular regions [60]. The residual L-glutamate is removed by glutamate uptake/transporter system [61]. The amount of available extracellular glutamate is subject to strict regulation to allow appropriate level of signaling.

The human brain contains 6–7 μmol/g wet weight of L-glutamate [62,63]. Thus, L-glutamate along with glutamine is the most abundant free amino acid in the central nervous system. More than five decades ago, Curtis et al. demonstrated that L-glutamate has an excitatory action on nerve cells [64]. Since then, its role as an excitatory neurotransmitter as well as its cerebral metabolism have been studied in detail (reviewed by [65,66].

L-glutamate is concentrated in synaptic vesicles in the presynaptic terminal by the action of vesicular glutamate transporters (vGLUT;[67]. In addition, some of the L-glu in the vesicles might be generated by a vesicle-associated aspartate amino transferase from 2-oxoglutarate using L-aspartate (L-asp) as the amino group donor [68]. Upon depolarization of the presynaptic membrane, L-glutamate is released into the synaptic cleft and binds to ionotropic glutamate receptors (iGluRs) at the postsynaptic membrane.

Glutamate partially reduces the action of PC-6 and PC-7, which may indicate that a part of the external calcium enters under the influence of PC-6, PC-7 and also through the open glutamine site and in the place of calcium channels in NMDA-receptors.Even the preliminary addition of glutamate does not completely abolish the actions of PC-6 and PC-7, which may indicate that PC-6 and PC-7 have several mechanisms of action on rat brain neurons, resulting in an increase in [Ca2+]in.It was found that the incubation of PC-6 and PC-7 (10–50 μM) in a suspension of synaptosomes significantly increases the intensity of Fura-2AM-fluorescence. And when preincubated with L-glutamate (50 μM), PC-6 and PC-7 (10–100 μM) significantly reduces the fluorescence intensity against the background of AD.

The results shown are that RyR3 plays an important protective role in early stages of AD by helping to reduce neuronal excitability and activity-dependent Aβ production. However, in older AD mice deletion of RyR3 resulted in beneficial effects. Thus, although RyR3 appears to play a protective role in younger mice, these results suggested that in aging brain RyR3 may contribute to AD pathogenesis by amplifying ER Ca^{2+} release through calcium-induced calcium release (CICR) mechanism and by enhancing the dysregulation of intracellular Ca^{2+}. These results consistent with reports that dantrolene exerted beneficial effects in several AD mouse models [55].

It is known from the literature that Mg^{2+} ions selectively block the activity of NMDA-receptors. Glycine reinforces NMDA-receptor responses, increasing the frequency of channel opening. In the complete absence of glycine, the receptor is not activated by L-glutamate. Indeed, the addition of glycine (5 μM) to the incubation medium reinforced the glutamate-dependent increase in fluorescence by 18–25%. At the same time, Mg^{2+} ions (50 μM) inhibited glutamate-induced Ca^{2+} release from intracellular depots (Fig. 5).

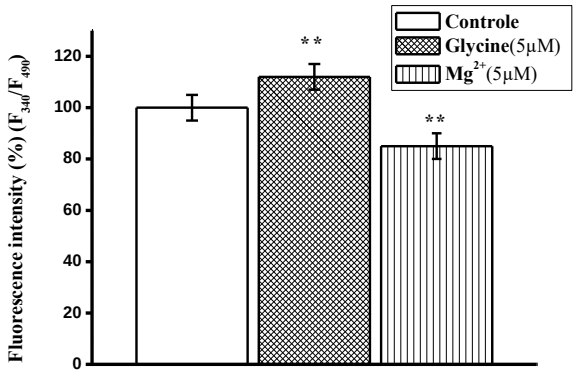

Figure.5. Effect of glycine and Mg^{2+} ions on glutamate-induced Ca^{2+} intracellular depots. Reliability indicator: * - P <0.05; ** - P <0.01; *** - P<0.001. (n = 6).

Glycine is known to stimulate the effects of glutamate, while competitive receptor antagonists such as AP5, AV-2-1 toxin, can prevent glutamate activation. Other drugs and Mg^{2+} ions can block the open channel through non-competitive antagonism. These drugs include the experimental neuroprotective drug MK-801 and argiolobatin [69].

To identify a possible interaction of polyphenol PC-6 and PC-7 with areas of overexcitation of NMDA-receptors responsible for the opening of calcium channels, its action was studied against the background of non-competitive antagonists such as magnesium ions, argiolobatin and calcium channel blocker nifedipine on AD model.

It was shown that magnesium ions at millimolar concentrations inhibit the fluorescence of the glutamate-Fura-2AM-synaptosome complex significantly. The inhibitory effect of magnesium ions against the background of PC-6 and PC-7 (50 μM) fluorescence of the Fura-2AM-synaptosome complex did not change.

These studies showed that in the presence of PC-6 and PC-7, the inhibitory effect of magnesium ions (50 μM) did not change. This is probably due to the fact that there is no competition between Mg^{2+}, PC-6 and PC-7 for the areas that stimulate the opening of Mg^{2+} ion channels. It was also shown that the effect of argiolobatin (10 μM) on the calcium channels of the NMDA-receptor in the presence of PC-6 and PC-7 (50 μM) does not change (Fig. 6).

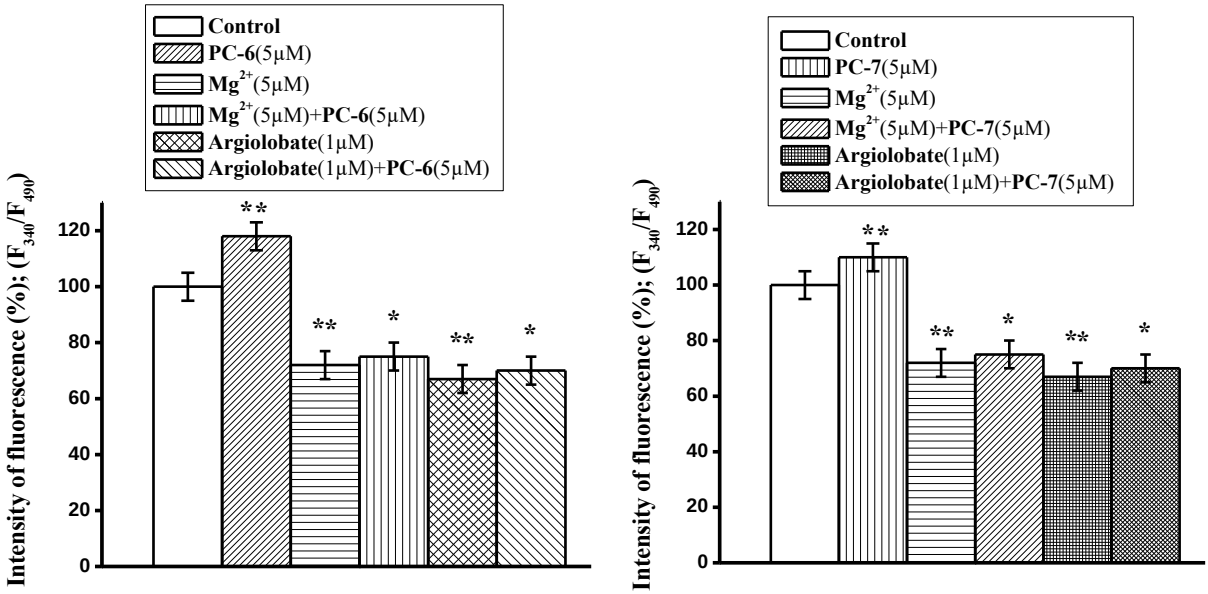

Figure. 6. Effect of non-competitive NMDA antagonists Mg^{2+} and argiolobatin against the background of PC-6 and PC-7 on fluorescence intensity and $[Ca^{2+}]_{in}$ level in rat brain synaptosomes in an AD model. Reliability indicator: * - P <0.05; ** - P <0.01; *** - P<0.001. (n = 6).

While studying the effect of PC-6 and PC-7 on calcium-dependent processes of the NMDA-receptor, nifedipine was studied in the background of the L-type Ca^{2+} channel blocker in rat brain synaptosomes on AD model.

Preincubation of nifedipine (0.01 μM) with the Fura-2AM-synaptosome suspension complex resulted in a decrease in fluorescence. Preincubation of PC-6 (50 μM) with the Fura-2AM-synaptosome suspension complex did not change the decrease in fluorescence. Preincubation of PC-6 and PC-7 (50 μM) in the background of nifedipine (0.01 μM) with the Fura-2AM-synaptosome complex did not lead to a change in fluorescence (Fig. 7), indicating that there is no competition between PC-6, PC-7 and nifedipine for the area, that regulates dihydropyridine-sensitive calcium channels.

This explains that PC-6 and PC-7 do not effect beyond the area, that regulates dihydropyridine-sensitive calcium channels in the membrane of rat brain synaptosomes.

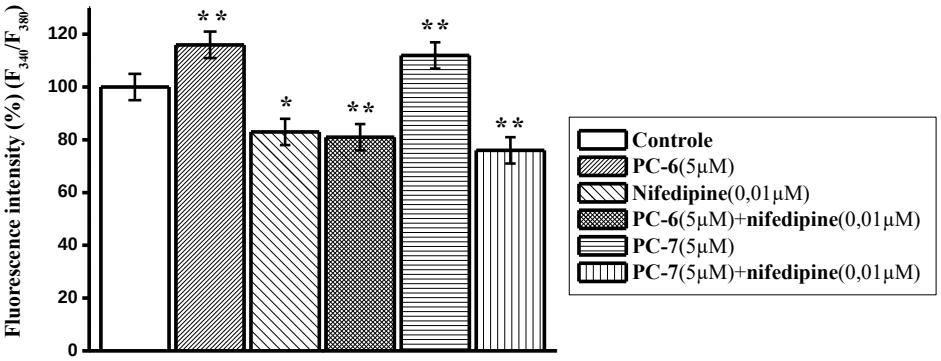

Figure 7. Effect of PC-6 and PC-7 on calcium-dependent processes of the NMDA-receptor in the background of nifedipine in an AD model. Reliability indicator: * - P <0.05; ** - P <0.01; *** - P<0.001. (n = 6).

The theoretical significance of the conducted experimental work is to expand the understanding of the mechanisms of neurodegeneration caused by excessive activation of glutamate receptors [70]. The results of the study may be useful for understanding the mechanisms of occurrence and development of neurodegenerative conditions, neurological disorders in the central nervous systems, which participate in the pathogenesis of such socially significant diseases as Alzheimer's disease. Cellular calcium Ca^{2+} signals regulate important aspects of neuronal physiology.

The polyphenols, studied in these researches, provide information that can be used in the development of various neuroprotective drugs to prevent glutamate excitotoxicity, observed in Alzheimer's disease.

A pivotal role for excitotoxicity in neurodegenerative diseases is gaining increasingly more acceptance, but the underlying mechanisms through which it participates in neurodegeneration still need further investigation. Excessive activation of glutamate receptors by excitatory amino acids leads to a number of deleterious consequences, including impairment of calcium buffering, generation of free radicals, activation of the mitochondrial permeability transition and secondary excitotoxicity. Recent studies implicate excitotoxicity in a variety of neuropathological conditions, suggesting that neurodegenerative diseases with distinct genetic etiologies may share excitotoxicity as a common pathogenic pathway. Thus, understanding the pathways involved in excitotoxicity is of critical importance for the future clinical treatment of many neurodegenerative diseases.

Conclusion

It was found that on the AD model polyphenols PC-6 and PC-7 increase fluorescence and $[Ca^{2+}]_{in}$ levels slightly, respectively, in synaptic membranes when compared to controls. The obtained results indicate a possible competition between PC-6, PC-7 and glutamate for the area, which regulates the opening of NMDA-receptor ion channels.

It was revealed that on the AD model effect of PC-6 and PC-7 which are responsible for opening calcium channels with other parts of NMDA-receptors in the background of magnesium ions, argiolobatin and nifedipine, the change in the level of $[Ca^{2+}]_{in}$ synaptosomes was not observed.

The obtained results indicate that developing a neuroprotective drug with a therapeutic effect for the treatment of Alzheimer's disease based on this polyphenol in pharmacology has good prospects.

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Conflict of Interest

The authors declare no conflict of interest.

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