Prevention of Amyloid-Beta Oligomer-Induced Neuronal Death by EGTA, Estradiol, and Endocytosis Inhibitor

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Key words: *beta amyloid; Alzheimer's disease; neurons; cell death; estrogens.*

Summary. *Background and objective. Alzheimer's disease is a progressive neurodegenerative disease that is biochemically characterized by the accumulation of amyloid beta (Aβ) peptides in the brain. The current hypothesis suggests that Aβ oligomers rather than fibrillar aggregates are the most toxic species of Aβ though the mechanisms of their neurotoxicity are unclear. The authors have previously shown that small $A\beta_{1-42}$ oligomers at around 1 μM concentration caused rapid (in 24 h) neuronal death in cerebellar granule cell (CGC) cultures. In this study, we aimed to investigate whether protracted (up to 7 days) incubation of CGC cultures with lower submicromolar concentration of various aggregates of $A\beta_{1-42}$ had an effect on viability of neurons. In order to get some insight into the mechanism of Aβ-induced cell death, we also sought to determine whether extracellular Ca^{2+} and process of endocytosis contributed to Aβ oligomer-induced neurotoxicity and whether pharmacological interventions into these processes would prevent Aβ oligomer-induced cell death.*

Material and Methods. Primary cultures of CGC were treated with various aggregate forms of $A\beta_{1-42}$. Cell viability was assessed by fluorescent microscopy using propidium iodide and Hoechst 33342 staining.

Results. Exposure of neurons to 500 nM $A\beta_{1-42}$ oligomers for 72–168 h caused extensive neuronal necrosis. Lower concentrations (100–250 nM) were not toxic to cells during 7 days of incubation. $A\beta_{1-42}$ monomers and fibrils had no effect on neuronal viability even after 7 days of incubation. Treatment of neurons with EGTA, steroid hormone 17β-estradiol, and methyl-β-cyclodextrin significantly reduced $A\beta_{1-42}$ oligomers-induced neuronal death.

Conclusions. The results show that submicromolar concentrations of $A\beta_{1-42}$ oligomers were highly toxic to neurons during protracted incubation inducing neuronal necrosis that can be prevented by chelating extracellular Ca^{2+} with EGTA, inhibiting endocytosis with methyl-β-cyclodextrin, or by estradiol, which may protect against mitochondrial permeability transition pore opening.

Introduction

Neurodegeneration in Alzheimer's disease is thought to be associated with accumulation of abnormally folded Aβ peptide in the brain (1, 2). Among various aggregate forms of Aβ, small soluble oligomers are considered as the primary neurotoxic species, though mechanisms by which Aβ oligomers cause cell death remain unclear. It has been shown that soluble fibril-free Aβ preparations are toxic to mouse brain slice cultures following 24-h exposure (3). Micromolar concentrations (1–20 μM) of heterogeneous preparation of Aβ oligomers were shown to induce apoptosis in cultured cortical neurons during 12–24 h (4, 5). Recently, we have reported that small $A\beta_{1-42}$ oligomers at low micromolar concentrations induce rapid neuronal necrosis in an oligomeric particle size-dependent manner (6). In most studies, the acute cytotoxic effects of Aβ oligomers were observed at high micromolar concentrations. Relatively less information is available regarding the effects of chronic exposure of neurons to low submicromolar concentrations of Aβ that are considered to be pathophysiologically relevant (7).

It has been suggested that Aβ oligomer-induced toxicity can be related to dysregulation of Ca^{2+} homeostasis due to endoplasmic reticulum stress (5, 8) or due to increased plasma membrane permeability (8) followed by mitochondrial Ca^{2+} overload and mitochondrial permeability transition pore (MPTP) (9). However, other investigators have shown that Ca^{2+} influx was accelerated but was not required for Aβ oligomer-caused toxicity (4).

Estrogens have been shown to enhance neuronal survival in Aβ aggregate-induced cell death models (10–12) through maintaining Ca^{2+} homeostasis and mitochondrial functions (13, 14) or by preventing oxidative stress in cultured neurons (15, 16). Whether similar estrogen-mediated protective mechanisms operate in Aβ oligomer-induced neuronal death remains to be determined.

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Soluble Aβ oligomers could mediate neuronal damage by binding to external cell surface and disturbing various signaling pathways, which may lead to cell death. Alternatively, Aβ oligomer-induced toxicity could be due to intracellular accumulation of the peptide via endocytosis leading to dysfunction of intracellular organelles and cell death. Recent evidence indicates that Aβ oligomers at least partly can be internalized inside cultured neuroblastoma or HeLa cells causing cytotoxicity (17, 18). In contrast, other investigators have found that $A\beta_{1-42}$ oligomers can exert toxicity from the outside of cells (19); therefore, the importance of internalization of small Aβ peptide forms in the neurotoxicity needs to be better clarified.

The aim of this study was to investigate the effect of low submicromolar concentrations of various $A\beta_{1-42}$ aggregate forms on neuronal viability during protracted (3–7 days) incubation and whether an extracellular Ca^{2+} chelator EGTA, estrogens, and an inhibitor of endocytosis methyl-β-cyclodextrin (CD) can prevent $A\beta_{1-42}$ oligomer-induced neuronal cell death.

Materials and Methods

The procedures used in this study were carried out in accordance with the Law on the Care, Keeping and Use of Animals of the Republic of Lithuania (License of the State Veterinary Service for Working with Laboratory Animals, No. 0006).

Neuronal-Glial Culture Preparation. Mixed neuronal-glial cerebellar granule cell (CGC) cultures were prepared from 7–8-day-old Wistar rats as described elsewhere (20). Cells were grown in vitro for 7 days before exposure to $A\beta_{1-42}$.

CGC cultures were treated for 24 h, 72 h (3 days), and 168 h (1 week) with 0.1–2 μM of $A\beta_{1-42}$ or vehicle (control) in the presence/absence of 100 nM of 17β-estradiol, or 100 μM of ethylene glycol tetraacetic acid (EGTA), or 1 μM of CD.

Preparation of Aβ Monomers, Oligomers, and Fibrils. Synthetic $A\beta_{1-42}$ peptide was from Bachem (Switzerland) and American Peptide (California, USA). Oligomers were generated as described elsewhere (6). Briefly, soluble $A\beta_{1-42}$ oligomers were prepared by dissolving 1 mg of peptide in 400 μL of 1,1,1,3,3,3-hexafluoroisopropanol (HFIP) for 30–60 min at room temperature. A volume of 100 μL of the resulting seedless solution was added to 900 μL H_2O in a siliconized Eppendorf tube. After 10–20 min incubation at room temperature, the samples were centrifuged for 15 min at 12 000 rpm, the supernatant was transferred to a new siliconized tube, and HFIP was evaporated: samples were incubated in open vials for 24 h at room temperature. Fibrils were formed by protocol in which the aqueous peptide solution obtained after evaporation of HFIP was incubated for 7 days at room temperature. Monomers were prepared by dissolving $A\beta_{1-42}$ in HFIP, and after removal of HFIP by evaporation, resuspending in dimethyl sulfoxide (DMSO) at a concentration of 0.5 mM. Vehicle was prepared in the same way as oligomeric forms but without $A\beta_{1-42}$ (thus containing similar amounts of residual HFIP or silicone oil). Solutions of peptides were stored at –20°C up to 2 weeks. We found that longer period of storage resulted in decreased neurotoxicity of oligomeric $A\beta_{1-42}$ preparations due to an increase in size of oligomeric particles (data not shown).

The size and morphology of the preparations of $A\beta_{1-42}$ oligomers and fibrils were characterized as described in (6).

Assessment of cell viability. The viability of neuronal cells in the cultures was assessed by propidium iodide (PI, 7 μM) and Hoechst 33342 (4 μg/mL) staining using a fluorescence microscope OLYMPUS IX71S1F-3. PI-negative cells with weak Hoechst-staining were considered to be viable, whereas cells showing nuclear shrinkage or fragmentation and intensive Hoechst staining but still lacking PI staining were classified as chromatin condensed/fragmented (apoptotic). PI-positive cells were classified as necrotic. Neuronal cells were distinguished from glial cells according to their characteristic shape and nuclear morphology. Neuronal cells were counted in at least 5 microscopic fields per well (two wells per treatment). Data are expressed as percentage of specific neuronal cells of the total number of neuronal cells per field.

Statistical Analysis. Data are expressed as mean±SE of 3–8 experiments on separate CGC cultures. Statistical comparison between experimental groups was performed using Student *t* test. A value of $P<0.05$ was considered statistically significant.

Results

We have recently reported that $A\beta_{1-42}$ oligomers at micromolar concentrations (1–2 μM) and in a size-dependent manner induce rapid neuronal necrosis during 24-h incubation (6). Here we investigated whether lower, submicromolar concentrations of $A\beta_{1-42}$ can become toxic to CGC cultures during prolonged (3–7 days) treatment. As shown in Fig. 1A, 500 nM $A\beta_{1-42}$ oligomers were toxic to neurons and caused a significant reduction (by 21%) of neuronal viability after 24-h incubation. The longer 72-h exposure of cells to 500 nM $A\beta_{1-42}$ oligomers induced a significant drop (by 55%) of cell viability, which remained at similar level after 1-week incubation. Lower concentrations of $A\beta_{1-42}$ oligomers (250 nM) were not toxic to neurons during 168 h. Incubation of cells with 1 μM $A\beta_{1-42}$ oligomers for 24 and 72 h induced strong cell death (26% and 29% of viable neurons remained, respectively) and

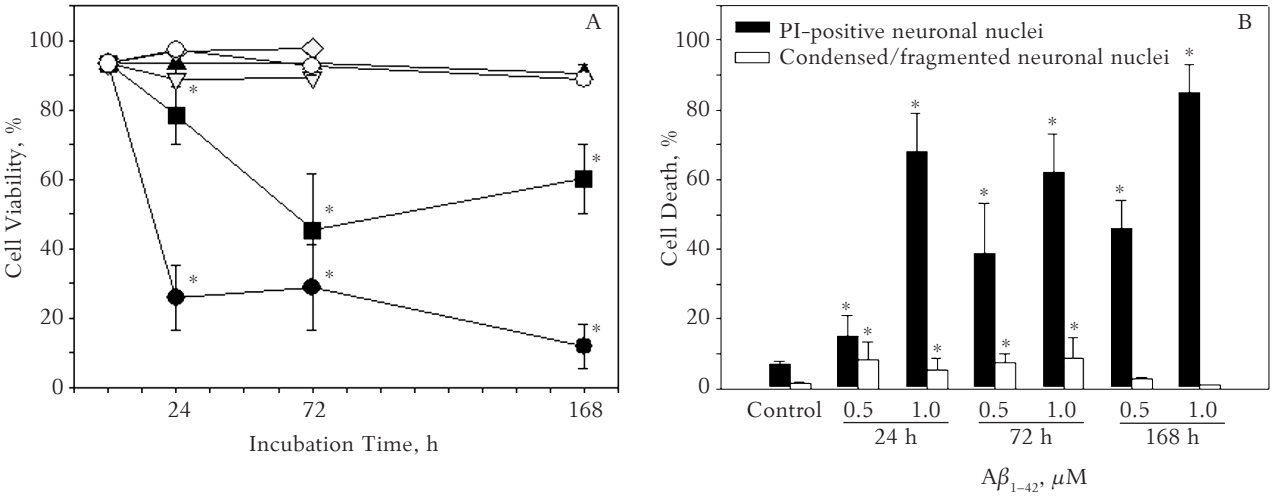

Fig. 1. $A\beta_{1-42}$ oligomers induce necrotic cell death in neurons

A, effect of various $A\beta_{1-42}$ species on cell viability; B, propidium iodide-positive and chromatin-condensed neurons. $A\beta_{1-42}$ species were prepared as described in Materials and Methods. CGC were pretreated with 0.1–2 μM $A\beta_{1-42}$ and neuronal death was assessed after 24-, 72-, and 168-h incubation by fluorescent microscopy. ○, control; ▲, 250 nM $A\beta_{1-42}$ olig. ; ■, 500 nM $A\beta_{1-42}$ olig.; ●, 1.0 μM $A\beta_{1-42}$ olig.; ◇, 2.0 μM $A\beta_{1-42}$ fibrils; ▽, 2.0 μM $A\beta_{1-42}$ monomers.
*Statistically significant effect of $A\beta_{1-42}$ if compared to control. Means±standard errors of 3–6 separate experiments are presented.

was massive after 1-week incubation (cell viability was only 9%) (Fig. 1A). However, $A\beta_{1-42}$ monomers and fibrils had no effect on neuronal viability after 72-h incubation even at 2-μM concentration (Fig. 1A). No changes were observed in control neurons treated with vehicle. The average size of $A\beta_{1-42}$ oligomers in the preparations used in these experiments ranged between 1–3 nm z-height as measured by atomic force microscopy (6).

We have previously shown that $A\beta_{1-42}$ oligomer-induced cell death was necrotic as indicated by PI-positive staining following 24-h exposure of neurons to $A\beta_{1-42}$ (6). Similar results were obtained with $A\beta_{1-42}$ oligomers after 72-h and 168-h incubation: the cell viability was decreased due to necrosis (Fig. 1B). The percentage of apoptotic cells was minor (5–10%) with both concentrations of $A\beta_{1-42}$ oligomers after 72-h incubation and was not observed after 1-week incubation (Fig. 1B).

It has been suggested that Aβ oligomer-induced cell death may be due to dysregulation of intracellular Ca^{2+} homeostasis (7) and/or mitochondrial dysfunction (4). In the present study, we investigated whether extracellular Ca^{2+} chelator EGTA or estradiol that can modulate both extracellular and intracellular Ca^{2+} concentration and mitochondrial functions (13) can prevent $A\beta_{1-42}$ oligomer-induced neuronal death. As can be seen in Fig. 2, the exposure of cells to 1 μM of $A\beta_{1-42}$ oligomers for 24 h decreased cell viability to 59% (viability of control neurons was 88%). However, the viability of neurons pretreated with 100 μM of EGTA and $A\beta_{1-42}$ oligomers was increased to 73%. These findings suggest that extracellular Ca^{2+} may contribute to toxicity of $A\beta_{1-42}$ oligomers. Similarly, when cells were treated with 100 nM of 17β-estradiol and 1 μM of $A\beta_{1-42}$ oligomers for 24 h, neuronal survival was not significantly different from control (Fig. 2).

Recently it was shown that oligomeric Aβ species could be internalized by the endocytic process (17). Therefore, we investigated whether the general inhibitor of endocytosis – methyl-β-cyclodextrin (CD) – can protect neurons against $A\beta_{1-42}$ oligomer-induced cell death. As shown in Fig. 3, treatment

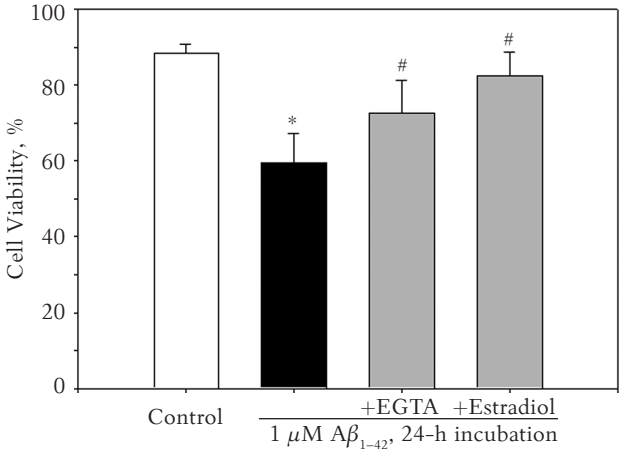

Fig. 2. EGTA and estradiol prevent $A\beta_{1-42}$ oligomer-induced neuronal death

CGCs were treated (+) or not with 100 μM of EGTA or 100 nM of 17β-estradiol and exposed to 1 μM of $A\beta_{1-42}$ oligomers for 24 h. The cell death in the culture was quantified by nuclei morphology analysis by propidium iodide and Hoechst 33342 staining. *Statistically significant effect of $A\beta_{1-42}$ oligomers if compared to control. #Statistically significant effect of EGTA or estradiol if compared to $A\beta_{1-42}$ oligomer-treated cells. Means±standard errors of 6–8 separate experiments are presented.

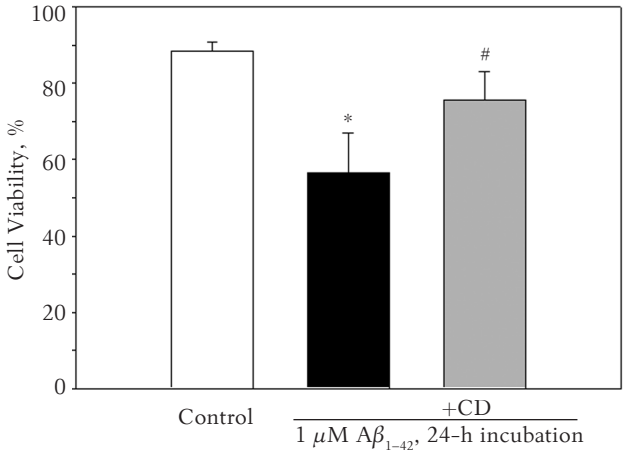

Fig. 3. Methyl-β-cyclodextrin prevents $A\beta_{1-42}$ oligomer-induced cell death

CGC were treated (+) or not with 1 μM of methyl-β-cyclodextrin (CD) and exposed to 1 μM of $A\beta_{1-42}$ oligomers for 24 h. The cell death in the culture was quantified by nuclei morphology analysis by propidium iodide and Hoechst 33342 staining. *Statistically significant effect of $A\beta_{1-42}$ oligomers if compared to control. #Statistically significant effect of CD if compared to $A\beta_{1-42}$ oligomer-treated cells. Means±standard errors of 8 separate experiments are presented.

of CGC with 1 μM of CD for 24 h prevented $A\beta_{1-42}$ oligomer-induced neuronal death: the viability of neurons was increased to approximately 76%. This indicates that internalization of $A\beta_{1-42}$ oligomers via endocytosis may be important factor in neurotoxicity of the peptide.

Discussion

In the present study, we demonstrated that small $A\beta_{1-42}$ oligomers even at submicromolar concentrations that are pathophysiologically relevant were toxic to neurons and induced progressive necrotic cell death during prolonged (3–7 days) incubation. The effect of $A\beta_{1-42}$ oligomers on neuronal viability was observed at 500-nM concentrations after 24-h incubation, progressed during further incubation reaching the maximal effect after 3 days and remained at that level during 7 days of treatment. Both previous and this work showed that at higher, i.e., 1 μM concentrations, $A\beta_{1-42}$ oligomers induced necrosis during the first 24 h of incubation (6), and further exposure of neurons to 1 μM of $A\beta_{1-42}$ oligomers for 3–7 days caused death of almost all neurons. In contrast to oligomers, long incubation of CGC with $A\beta_{1-42}$ monomers and fibrillar aggregates at concentrations up to 2 μM did not affect viability of neurons. Similar effects of Aβ oligomers were observed by other investigators (4, 21); however, these studies used higher concentrations of the peptides ranging between 5 μM and 25 μM. $A\beta_{1-42}$ oligomers caused mainly necrotic cell death during 24–168-h treatment. The low percentage of apoptotic cells was detectable only after 24–72-h incubation and substantially decreased after 7 days of incubation most likely due to phagocytosis of apoptotic cells. It is also possible that during longer incubation apoptotic cells became necrotic – a phenomenon well known for cell cultures.

We also found that $A\beta_{1-42}$ oligomer-induced neuronal death can be prevented in the presence of EGTA suggesting that extracellular Ca^{2+} is an important mediator of cell death in this model. It has been previously shown that oligomeric forms of Aβ (n≈20) caused an increase in cytosolic free Ca^{2+} concentration, whereas monomeric and fibrillar forms were without any detectable effect (8). The mechanism by which Ca^{2+} influx is stimulated by $A\beta_{1-42}$ oligomers remains obscure. It has been proposed that Aβ may impair membrane ATPase activity (22), due to Aβ-induced lipid peroxidation causing neuronal membrane damage (23), activation of NMDA receptors (24), or formation of plasma membrane pores (25). However, these studies did not provide evidence which aggregate forms of A*b* are responsible for the effects. We have previously shown that small oligomers of $A\beta_{1-42}$ (particles of 1–2 nm z-height) exhibited propensity to bind to phospholipid vesicles of similar composition to neuronal plasma membranes (6). Therefore, the neurotoxic effect of $A\beta_{1-42}$ oligomers might be due to its binding to neuronal plasma membranes causing membrane permeabilization and influx of extracellular Ca^{2+}. High cytosolic Ca^{2+} can disrupt synaptic plasticity, may promote MPTP opening, and activate calpains and caspases leading to cell death (26). In accordance with this, we found that steroid hormone estradiol exerted a protective effect against $A\beta_{1-42}$ oligomer-induced neuronal necrosis. Estradiol has been found to be directly protective against Ca^{2+}-induced MPTP in isolated heart mitochondria and against ischemia-induced MPTP in perfused heart model (27–29). Nilsen et al. (2006) have reported that estradiol pretreatment protected against fibrillar Aβ-induced apoptosis via maintenance of calcium homeostasis, a decrease of cytochrome c release, and Bax translocation to mitochondria. In addition, it has been found that estrogenic compounds rescued neurons from Aβ-induced cell death by preventing oxidative stress (15) or via modulation of Ca^{2+} channels activity (30).

In this study, we have also demonstrated a protective effect of CD in $A\beta_{1-42}$ oligomers-affected CGC cells. This effect may be associated with the inhibition of uptake of $A\beta_{1-42}$ oligomers by neurons. Neurons have several endocytic pathways; however, the specific endocytic pathway involved in Aβ oligomer uptake and neurotoxicity remains unclear. Recently, it has been found that Aβ oligomers can be at least partly internalized by endocytosis in HeLa and SK-N-SH cells (17) and in mouse neuroblastoma

cells (18). It is possible that the interaction of $A\beta_{1-42}$ oligomers with the cell membrane and further internalization is required to cause neurotoxicity.

Conclusions

The results of the study show that chronic exposure of neurons to low submicromolar (pathophysiology relevant) concentration of $A\beta_{1-42}$ oligomers induced extensive neuronal death. In addition, $A\beta_{1-42}$ oligomers-caused neurotoxicity was prevented by EGTA, estradiol, and CD. Therefore, the contribution of external Ca^{2+}, the internationalization of oligomeric $A\beta_{1-42}$, and possibly modulation of mitochondrial permeability transition pore can be the mechanisms through which the $A\beta_{1-42}$ peptide exerts its toxicity and which may be targeted by therapeutic strategies in Alzheimer's disease.

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Statement of Conflict of Interest

The authors state no conflict of interest.

EGTA, estradiolis ir endocitozės slopiklis – neuronų apsauga nuo beta amiloido oligomerų toksinio poveikio

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Raktažodžiai: beta amiloidas, Alzheimerio liga, neuronai, ląstelės žūtis, estrogenai.

Santrauka. *Įvadas ir objektas.* Alzheimerio liga – tai progresuojanti neurodegeneracinė liga, kurios biocheminis požymis yra beta amiloido (Aβ) peptidų kaupimasis smegenyse. Naujausių tyrimų duomenimis, ne fibrilinės sankaupos, bet Aβ oligomerai yra labiausiai toksiški junginiai, tačiau jų neurotoksiškumo mechanizmas iki šiol neištirtas. Neseniai nustatėmė, kad maži $A\beta_{1-42}$ oligomerai, esant 1 μM koncentracijai, sukėlė greitą (per 24 val.) neuronų žūtį smegenėlių ląstelių kultūroje. Šio *tyrimo tikslas* – ištirti, kaip mažos submikromoliarinės įvairių $A\beta_{1-42}$ junginių koncentracijos veikia neuronų gyvybingumą po ilgo (iki 7 dienų) inkubacijos laikotarpio. Taip pat tirsime, ar neurotoksinis Aβ oligomerų poveikis gali būti susijęs su ekstraląstelinio Ca^{2+} jonais ir endocitozės procesu ir kokią įtaką Aβ oligomerų sukeltai ląstelių žūčiai gali turėti farmakologinės medžiagos.

Medžiaga ir metodai. Pirminė smegenėlių neuronų kultūra buvo veikiama įvairaus oligomerizacijos laipsnio $A\beta_{1-42}$ junginiais. Ląstelių gyvybingumas buvo vertinamas fluorescencinės mikroskopijos metodu naudojant propidžio jodido ir Hoechst 33342 dažus.

Rezultatai. 500 nM $A\beta_{1-42}$ oligomerų sukėlė ryškią neuronų nekrozę po 72–168 val. inkubacijos. Mažesnės $A\beta_{1-42}$ oligomerų koncentracijos (100–250 nM) neuronų neveikė. $A\beta_{1-42}$ monomerai ir fibrilės, esant mikromoliarinėms koncentracijoms, neuronų gyvybingumui poveikio neturėjo netgi po septynių parų inkubacijos laikotarpio. EGTA, steroidinis hormonas 17β-estradiolis ir metil-β-ciklodekstrinas žymiai sumažino $A\beta_{1-42}$ oligomerų sukeltą neuronų žūtį.

Išvada. Remiantis tyrimo duomenimis, submikromoliarinės $A\beta_{1-42}$ oligomerų koncentracijos yra toksiškos neuronams po ilgo inkubacijos laikotarpio ir sukelia ląstelių nekrozę, kurią sumažina ekstraląstelinio Ca^{2+} surišimas su EGTA, endocitozės slopiklis metil-β-ciklodekstrinas ir estradiolis, kuris galėtų apsaugoti nuo mitochondrijų nespecifinio pralaidumo poros atsidarymo.

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