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Protective effect of nicotine on the cultured rat basal forebrain neurons damaged by β-Amyloid (Aβ)25-35 protein cytotoxicity

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Abstract. – OBJECTIVE: We sought to investigate the intervention effect of nicotine on β-amyloid (Aβ)25-35 protein cytotoxicity in the rat basal forebrain neurons primary cultures.

MATERIALS AND METHODS: For this purpose, freshly isolated rat basal forebrain neurons were cultured for 7 days and then exposed to either Aβ(25-35) or the combination of Aβ(25-35) and nicotine for 48 hours. The effects of Aβ(25-35) and nicotine on neurons morphology, growth status and TrkA expression were evaluated through microscopy, MTT assay, RT-PCR and immunocytochemistry.

RESULTS: We found that the exposure of cultured neurons to Aβ(25-35) resulted in remarkable morphological changes. The average process number and length as well as the maximum process length of neurons were significantly decreased as compared with those of control. MTT assay showed that Aβ(25-35) impaired the growth of neurons. Aβ(25-35) also inhibited the expression of TrkA at both mRNA and protein levels. However, the addition of nicotine significantly attenuated these changes, indicating that nicotine could protect the neurons from the cytotoxicity of Aβ(25-35).

CONCLUSIONS: Nicotine could be useful for the treatment of Alzheimer's disease through its ability to rescue the neurons from Aβ(25-35) cytotoxicity and the protective effect involved upregulated expression of TrkA receptors.

Key words:
Aβ(25-35), TrkA, Cholinergic neurons, Basal forebrain, Rat, Nicotine.

Introduction

Alzheimer's disease (AD) is one of the neurodegenerative diseases presenting with dementia. The etiology of AD is complex and is believed to be mediated by multiple factors. As a progressive neurological disorder, AD is characterized by the loss of memory and cognitive functions which are associated with the pathologic characteristics, such as selective loss of neurons and synapses, formation of neurofibrillary tangles within neurons and numerous plaques in affected brain regions[1]. The mechanisms underlying the pathogenesis of this disorder remain unclear. According to β-amyloid cascade hypothesis, the key pathogenic event responsible for degenerative changes in neurons and for the loss of cognitive functions is the formation of senile plaques i.e. the excessive accumulation of extracellular deposition of β-amyloid peptide (Aβ), a set of 39-43 amino acid peptides derived from the cleavage by β- and γ-secretases of a membrane glycoprotein, the β-amyloid precursor protein (APP)[2]. It is known that Aβ plays a pivotal role in the neurodegenerative process of AD. Amyloid plaques appear in the early stages of AD in the temporal cortex, entorhinal cortex and the hippocampus[3]. β-amyloid peptide 25-35 ($A\beta_{25-35}$) has the critical neurotoxic properties of the full-length $A\beta_{1-42}$. Studies have shown that intracerebroventricular injection of Aβ(25-35) lead to the memory impairment[4-6]. Another characteristic change in AD is the selective decrease of basal forebrain cholinergic neurons[7]; the growth and survival of these neurons depend on nerve growth factor (NGF)[8]. Basal forebrain cholinergic neurons express high affinity NGF receptors (TrkA) and low affinity NGF receptors (p75NTR). Dysfunction of NGF itself or its high (TrkA) and low (p75NTR) affinity receptors has been suggested to underlie the selective degeneration of the nucleus basalis (NB) cholinergic cor-

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tical projection neurons in AD. A decrease of the TrkA-positive neurons in the basal forebrain nuclei in AD patients was reported[9]. A significant reduction in NGF receptor-positive cells in people with mild cognitive impairment (MCI) was observed even when the number of choline acetyltransferase-containing neurons remained stable, suggesting a phenotypic NGF receptor downregulation but not a frank loss of NB neurons in prodromal AD[10,11].

The lack of neurotrophic support due to reduction of these neurotrophic factor receptors may play an important role in the death of cholinergic neurons of AD patients. The continuous application of NGF can protect the cholinergic neurons in the septum and hippocampus of rats after being cut off of the umbrella. These studies based the rationale of using exogenous NGF to treat AD[13] but a direct application of peptides in the central nervous system remains to be evaluated in clinical setting. Alternatively, small molecules may be used to increase the expression of endogenous growth factor and/or its receptor. Previously, using different concentrations of Aβ(25-35) and nicotine on the primary cultured cholinergic neurons we found that high concentration of Aβ(25-35) caused reduced neuron processes and their length, impaired cell viability, and TrkA expression inhibition[14] while certain nicotine concentration had opposite effects[15]. Herein, we observed the effects of Aβ(25-35) and nicotine together on the cultured neurons, with a view to confirm our previous findings and to also explore the therapeutic potential of nicotine for AD.

MATERIALS AND METHODS

Reagents

Aβ (25-35) and nicotine hydrogen tartrate were purchased from Sigma Corporation (St. Louis, MO, USA). Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12) medium, trypsin, D-Hank's buffer, and phosphate buffered saline (PBS) were purchased from Gibco (Grand Island, NY, USA), and fetal bovine serum (FBS) from Hangzhou Sijiqing Corporation (Hangzhou, China). The reorganized rat β-NGF was a product of R&D (Mountain View, CA, USA), and anti-NF (200kD) and anti-TrkA antibodies were purchased from Chemicon Co. (Temecula, CA, USA). Horseradish peroxidase labeled goat anti-rabbit and goat anti-mouse secondary antibodies were purchased from the Vector company (Olean, NY, USA). TRIZOL was purchased from Invitrogen (Carlsbad, CA, USA) and reverse transcriptase RT kit from MBI Fementas (Waltham, MA, USA). Taq DNA polymerase was purchased from Beijing Dingguo Bioengineering Co (China). MTT was from Bebco (Twinsburg, OH, USA), new-born Sprague-Dawley (SD) rats were purchased from Shanghai Laboratory Animal Center of the Chinese Academy of Sciences.

Cholinergic neurons cell culture

Cholinergic neurons were obtained from the basal forebrains of SD rats born within 24 hours, with the posterior marginal olfactory brain as the former boundaries, leading edge of the hypothalamus as the rear boundaries and bilateral striatum uplift as the outside boundaries. Briefly, after removing meninges and blood vessels, the brain tissue was washed thrice with PBS (pH 7.2), digested with 0.125% trypsin (pH 7.2) at 37°C for 15 min and the reaction was terminated with DMEM/F-12 (1:1) containing 15% FBS. Then, the tissues were minced and dissociated by mechanical trituration. Finally, single-cell suspensions were collected and seeded: (i) at a concentration of 10^6-10^7 cells/well in a 96-well plate pre-coated with poly-L-lysine for MTT reduction assay, (ii) at a concentration of 10^7-10^8 cells/well in a 12-well chamber slide for morphologic observation, immunocytochemisty and enzymatic reactions, and (iii) at a concentration of 10^7-10^8 cells/well in a 6-well chamber slide for RNA extraction for reverse transcription polymerase chain reaction (RT-PCR). After a 3-day incubation in DMEM/F12 (1:1) medium containing 10% heat-inactivated FBS at 37°C in a humidified (5% CO_2) incubator, the cells were treated with 5 μM 5-Fluorouracil (final concentration) to inhibit non-neuronal cells.

Identification of neurons and cholinergic neurons

Neurons were detected by immunocytochemistry using anti-NF monoclonal antibody (NF-200KD) and cholinergic neurons were detected by acetylcholinesterase (AchE) histochemistry and choline acetyltransferase (ChAT) immunocytochemistry.

Experimental groups

Three groups included: (1) control group, (2) Aβ group and (3) nicotine intervention group. After a 7-day incubation, Aβ group was treated

with 10μM of Aβ(25-35), nicotine intervention group was treated with 10μM of Aβ(25-35) plus 10μM of nicotine, and control group was treated with equivalent amount of essential medium. After further 48h incubation, cell morphology was assessed by immunocytochemistry and RNA was extracted.

MTT test

Cholinergic neurons were cultured in 96-well plates as described above. In addition to the above-referred 3 groups, a blank control was also set up that contained no cells in order to eliminate the background. Aβ(25-35) (10 μM) and/or nicotine (10 μM) were added to the wells on day 7, and cells were further incubated for 48h. On the 9th day, 20 μl/well of MTT (5 mg/ml of PBS) was added in each well and incubated for another 4h. Finally, supernatants were discarded and pellets were dissolved using dimethyl sulfoxide (DMSO) (150 μl/well) for 10 min at room temperature. The absorption values at wavelengths of 590 nm and 630 nm were read using a microplate spectrophotometer.

RNA extraction

Cholinergic neurons (5×10^7) were harvested after 9-day culture as described above. The cells were washed once with PBS, resuspended in 1 ml of TRIZOL reagent and transferred to an Eppendorf tube. After 5 min, 200 μl of chloroform was added and mixed by vortexing. After centrifugation (12000 rpm; 4°C; 15 min), supernatant (about 600 μl) was carefully transferred to a new Eppendorf tube, mixed by adding 500 μl of isopropyl alcohol followed by vortexing, and placed at room temperature for 10 min. Then the suspension was centrifuged as before for 10 min and the pellet was resuspended in 1ml of 75% ethanol. After sedimentation, precipitate (contain RNA) was obtained by centrifugation (7400 rpm; 4°C; 5 min), air-dried for 10 min, and finally dissolved in 20 μl of RNase-free water. RNA concentration was measured using UV spectrophotometer and purity was determined by 1.5% agarose gel electrophoresis.

RT-PCR

For reverse transcription, 2 μg of total RNA, 1 μl Oligo (dT) primer and DEPC water were mixed in an Eppendorf tube to yield a total volume of 12 μl. After degeneration at 70°C for 5 min, the mixture was placed on ice and 4 μl 5× buffer, 2 μl dNTP and 1 μl reverse transcriptase inhibitor were added in sequence, mixed well and incubated at 37°C for 5 min. Finally, 1 μl reverse transcriptase was added (final vol. 20 μl), and incubated at 42°C for 60 min followed by 70°C for 10 min.

For PCR, TrkA cDNA full-length gene was obtained from PubMed GeneBank and the primers were designed and synthesized by Shanghai Biological Engineering Company. The forward primer sequence was: 5'-AGGACCTCTTCAGAGACATCC-3' and the reverse primer sequence was 5'-CATTGACACCTGATATCTTGC-3'. The β-actin primers used were as described before[9] and PCR product was 573 bp. The amplified fragments were 241bp. PCR reaction was as follows: 5 μl 10× buffer, 4 μl $MgCl_2$, 2 μl 10 mM dNTPs, 50pmol primers, 1U Taq DNA polymerase, 2 μl cDNA and DEPC water to make the final vol. of 50 μl. Amplification cycle was: pre-degeneration at 94°C for 4 min; degeneration at 94°C for 1 min; annealing at 54°C for 1 min; and extension at 72°C for 1 min. After 30 cycles, cDNA was backward extended at 72°C for 7 min. PCR products were resolved on 1.5% agarose gel electrophoresis and detected by using GeneGenius U.S. imaging system. Relative strength of target gene expression was calculated as follows: Relative coefficient = Target gene expression strength/β-actin expression strength.

Immunocytochemistry

After culture in a chamber slide, neurons were fixed with cold acetone for 10 min. The cells were rinsed 3 times with PBS and permeabilized with 0.3% Triton X-100 in PBS for 5 min. Endogenous peroxidases were blocked with 0.3% H_2O_2 and 30% methanol in PBS. The cells were rinsed 3 times with PBS, pre-incubated with 10% bovine serum albumin (BSA) in PBS for 1 h at 37 °C and then treated overnight (4°C) with primary antibodies (diluted in 1% BSA in PBS; 1:200 of rabbit anti-rat TrkA antibody and 1:500 of rat anti-mouse NF-200KD) in the wet box. After washing with PBS for 3 times for 5 min each, the cells were incubated with horseradish peroxidase-labeled secondary antibody (1:200 goat anti-rabbit or goat anti-rat antibody) for 30 min at room temperature. The binding of secondary antibodies was detected by using DAB solution containing 0.5% of 3,3'-diaminobenzidine (DAB) and 0.01% of H_2O_2. Finally, the cells were washed with PBS, counter-stained with hematoxylin, and dehydrated with ethanol.

Table I. Effect of Aβ(25-35) and nicotine on cell morphology and viability (n=12).

	Process number per cell	Process length (μm)	Longest length of process (μm)	OD from MTT test (at 590 nm wavelength)
Control group	2.31±0.19	104.81±25.36	109.3±19.42	0.318±0.015
Aβ group	1.81±0.22*	74.42±25.04*	85.76±17.8*	0.236±0.019*
Aβ and nicotine group	2.04±0.15†	93.81±21.45†	106.3±17.2†	0.298±0.014†

*p<0.05 (compared with control group)
†p<0.05 (compared with Aβ group)

Table II. Effects of Aβ(25-35) and nicotine on TrkA expression.

Groups	TrkA mRNA expression (n=3)	TrkA positivity index from immuno-cytochemistry (n=15)
Control	1.7338±0.0352	2146.5±322.32
Aβ only	0.9465±0.0245**	1041.4±194.94*
Aβ + nicotine	1.2758±0.0367*†	1974.3±242.68††

TrkA mRNA was quantified by densitometry and normalized with β-actin expression;
*p<0.05 and **p<0.01 vs. control group;
†p<0.05 and ††p<0.01 vs. Aβ(25-35)-treated group;
The data were obtained from three independent experiments.

The slide was mounted with neutral gum and examined microscopically (Olympus inverted phase-contrast microscope, Tokyo, Japan) by randomly selecting 5 fields in each of 3 slides similarly stained and images were taken. Statistical area was set by using IMS-based image analysis system. The background, the negative cells and the positive cells were set and the average of positive area and its strength were calculated through automated computer program. TrkA-positive expression index representing the strength of TrkA positivity was calculated using the formula:

Positive expression index = positive expression area × average intensity of positive area.

Statistical Analysis

All quantitative and semi-quantitative data were expressed as x±SD. and analyzed using SPSS10 statistical software (SPSS Inc., Chicago, IL, USA). Groups were compared using analysis of variance (ANOVA); *t*-test was used to compare the difference between two groups. All *p*-values <0.05 were considered statistically significant.

RESULTS

Effects of Aβ(25-35) and nicotine on neuron morphology and viability

After the cholinergic neurons had been cultured for 7 days and then treated with 10 μM Aβ(25-35) alone for 48 hours, compared with control (Figure 1a), the processes decreased, length of process shortened, and optical density (OD) values from MTT assay decreased (p<0.5) (Figure 1b and Table I). However, addition of nicotine (nicotine intervention group) attenuated (p<0.5) the extent of inhibition mediated by Aβ(25-35) (Figure 1c and Table I). These changes are graphically represented in Figures 2 and 3. Furthermore, the effects of Aβ(25-35) and nicotine on cell viability of cultured cholinergic neurons are depicted in Figure 4.

Effects of Aβ(25-35) and nicotine on TrkA mRNA expression

RT-PCR (Figure 5) shows that the exposure of neurons to Aβ(25-35) resulted in a decreased TrkA mRNA expression as compared with control

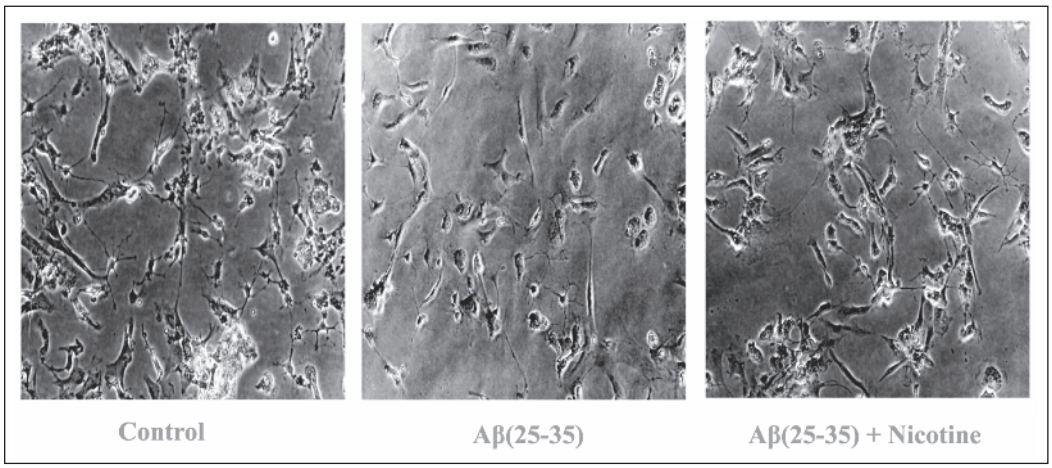
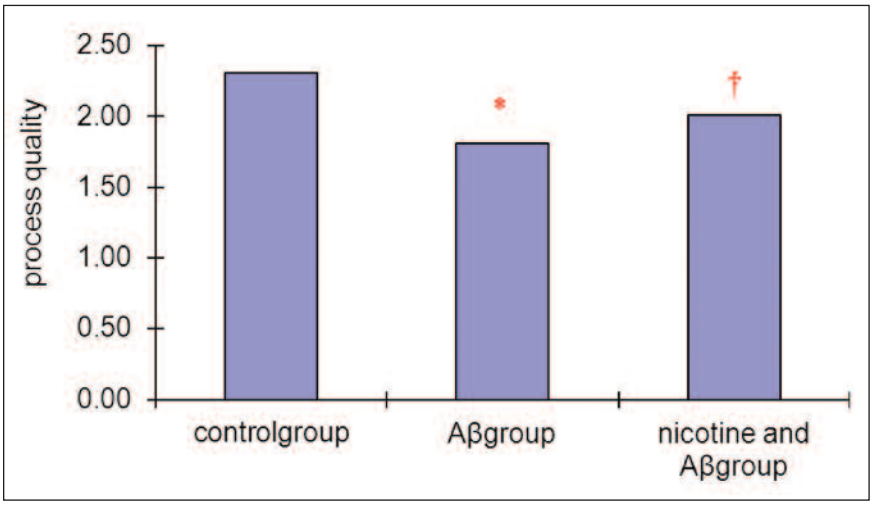


Figure 1. Effects of Aβ(25-35) protein and nicotine on neuron morphology.

Figure 2. Effect of Aβ(25-35) protein and nicotine on the average quality of processes in cultured cholinergic neurons. *p<0.05 (compared with control group); †p<0.05 (compared with Aβ group).



(p<0.05). As expected, the addition of nicotine partially rescued this suppression in TrkA mRNA expression (p<0.05). These changes are graphically presented and compared in Figure 6.

Effects of Aβ(25-35) and nicotine on TrkA protein expression in cultured cholinergic neurons

After the cholinergic neurons had been cultured for 7 days, they were treated with 10 μM Aβ(25-35) alone or Aβ(25-35) and nicotine. Immunocytochemical staining (Figure 7), in accord with RT-PCR data, revealed that treatment with Aβ(25-35) alone significantly decreased TrkA protein expression in cultured cholinergic neurons as compared with control group (p<0.05). However, the addition of 10 μM nicotine restored TrkA protein expression as compared with Aβ(25-35) group (p<0.05; Figure 8).

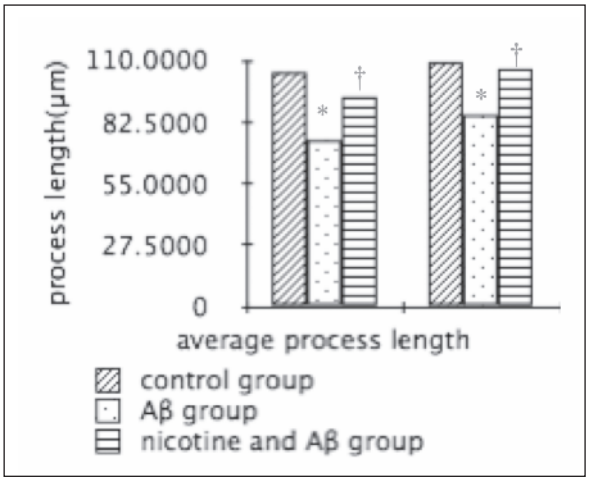


Figure 3. Effect of Aβ(25-35) protein and nicotine on the average number of process in cultured cholinergic neurons. *p<0.05 (compared with control group); †p<0.05 (compared with Aβ group).

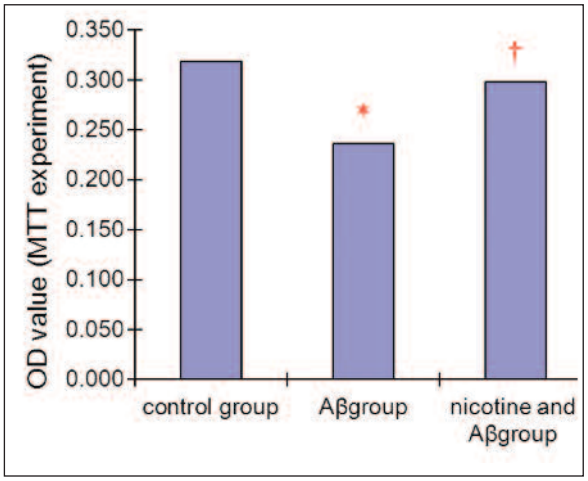


Figure 4. Effect of Aβ(25-35) protein and nicotine on cell viability of cultured cholinergic neurons. *p<0.05 (compared with control group); †p<0.05 (compared with Aβ group).

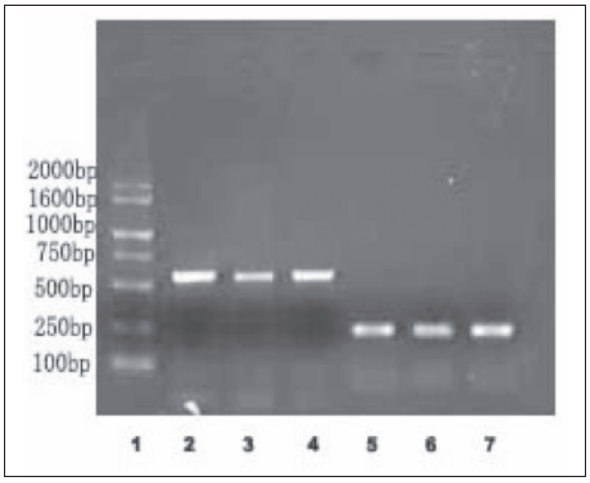


Figure 5. Effects of Aβ(25-35) protein and nicotine on TrkA mRNA expression. Lanes 2, 5: control group; Lanes 3, 6: Aβ(25-35) group; and Lanes 4, 7: Aβ(25-35) plus nicotine group.

Discussion

The NGF has potential as a therapeutic agent for AD due to its neurotrophic activities on basal forebrain cholinergic neurons. Defects in NGF signaling, transport or processing were linked to the activation of amyloidogenic route and also to AD neurodegeneration[16,17]. Previously, we reported[14] that Aβ (25-35) in 5-10 μM concentrations impaired the neuron viability, reduced the number of neuronal processes and shortened the length of processes. These changes were associated with changes in the expression of high-affinity NGF receptor TrkA. We also demonstrated that certain concentration of nicotine, a cholinergic receptor agonist, exerted opposite effects to Aβ(25-35) on cultured neurons[15]. Based on these results, herein we examined the combined effect of Aβ(25-35) and nicotine on cultured neurons. Thus, this study not only confirmed the deleterious effect of Aβ(25-35) on neurons but also showed that nicotine treatment was able to rescue the neurons from the damage caused by Aβ(25-35).

Alzheimer's disease, senile dementia and other abiotrophies involve the defects of choline acetyltransferase (ChAT) and acetylcholine (Acetylcholine, ACh) synthase[18-21]. ACh plays an important role in memory and cognition which explains cholinergic theory of AD[22,23]. Subsequently, different cholinesterase inhibitors which

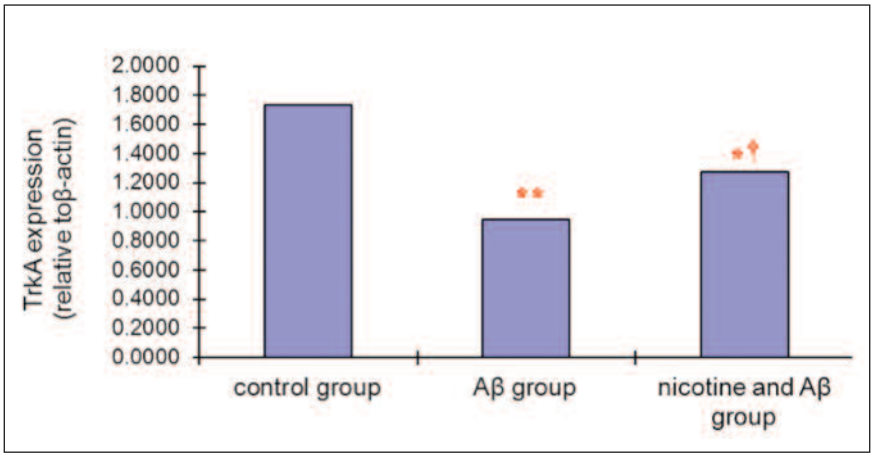


Figure 6. Graphic presentation of the effects of Aβ(25-35) protein and nicotine on the expression of TrkA mRNA in cultured cholinergic neurons. *p<0.05 vs. control; **p<0.01 vs. control; †p<0.05 vs. Aβ(25-35) treated group.

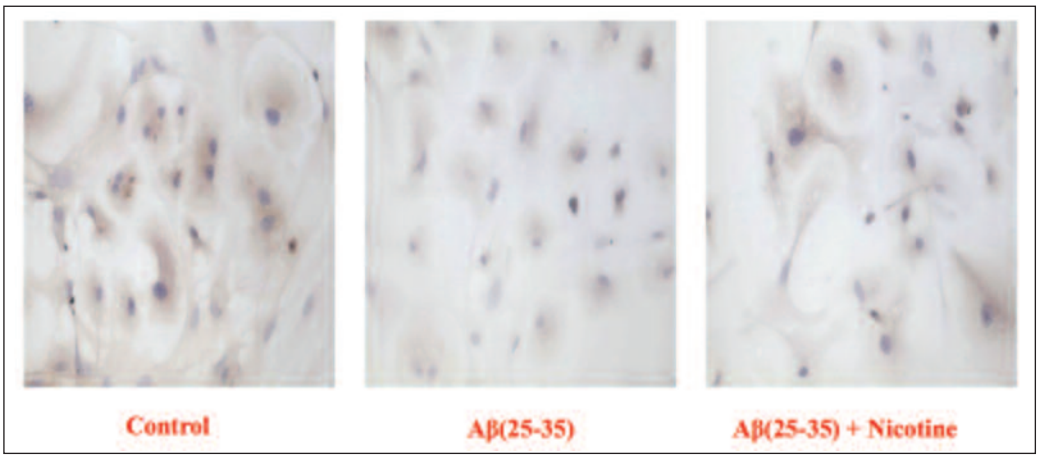


Figure 7. Effects of Aβ(25-35) and nicotine on the expression of TrkA protein.

inhibit the breakdown ACh were developed and used in clinical treatment of AD. On the other hand, ACh receptor agonists comprise still another arsenal to combat AD. ACh receptors are subdivided into M-receptor and N-receptor. The central N-receptor is closely related to memory and learning, as well as human cognitive activities[24]. Nicotine acts as N-receptor agonist. It also plays a role in promoting the presynaptic release of acetylcholine. Animal *in vivo* experiments showed that treatment of the rats with nicotine significantly improved the Aβ-induced learning and memory defects in water maze task[25]. And chronic anatabine treatment in a transgenic mouse model of AD reduced AD-liked pathology and improved socio-behavioral deficits. Anatabine is a minor tobacco alkaloid, which is also found in plants of the Solanaceae family and displays a chemical structure similarity with nicotine[26]. Consistent with these findings, it was also observed that AD patients benefited from smoking and had lower levels of soluble and non-soluble Aβ in the prefrontal, temporal lobe and hippocampus[27]. In addition, nicotine has been shown to improve cognition function in the AD patients[28], while in another clinical work[29], nicotine failed to show a significant effect in enchancing memory.

Although a few reasearches have shown that nicotine can protect by antagonizing glutamate in case the basal forebrain neurons are deprived of neurotrophic factors or damaged by toxic nerve injury[27,30,31], the exact mechanism of the protective effect of nicotine is still unclear. In the basal forebrain, 99% cholinergic neurons express TrkA[32]. Induced by the combination of NGF and TrkA, the activation of a network of signaling pathways is crucial to maintain normal morphology and function of adulthood cholinergic neurons[33]. Increased cholinergic activity caused reduction in Aβ while the muscarinic receptor agonists reduced Aβ neurotoxicity[34]. AD patients show the reduced expression of TrkA in the basal forebrain nuclei[9] which may impair the NGF-ini-

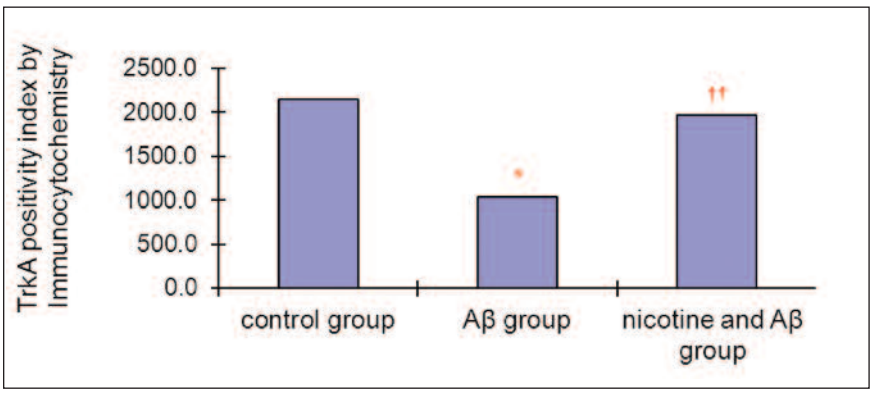


Figure 8. Graphic presentation of the effects of Aβ(25-35) and nicotine on the expression of TrkA protein. *p<0.05 vs. control group; †p<0.05 vs. Aβ(25-35) treated group.

tiated cell signal transduction and, ultimately, the cell survival. The defect in pro-survival signaling may lead to cholinergic neurons degeneration observed in AD patients. Considering that TrkA receptors are pivotal to the survival of cholinergic neurons, we determined the effect of nicotine on TrkA receptor expression and the data from this study show that nicotine could enhance TrkA expression of TrkA as well as neuron cell viability. The elevated TrkA expression was also related to the increase in length and number of neuron processes. Mainly, we demonstrated that nicotine could attenuate neuronal degeneration caused by Aβ(25-35) through the increased expression of TrkA which may be one of the protective mechanism involved.

We have found that nicotinic receptor agonists can increase the expression of nutritional TrkA receptor in primary cultured cholinergic neurons and reduce the toxicity of Aβ which was caused by inhibiting TrkA expression. But *in vivo* whether the same effectbe showed will be the interst of further study.

Conclusions

The nicotinic receptor agonists can increase the expression of nutritional TrkA receptor in primary cultured cholinergic neurons and reduce the toxicity of Aβ which was caused by inhibiting TrkA expression.

Acknowledgements

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Conflict of interest

The Authors declare that they have no conflict of interests.

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