ANTICHOLINESTERASE EFFECT OF SOYBEAN EXTRACT (GLYCINE MAX L.) IN THE BRAIN OF AMNESIC MICE AND ITS PROTECTIVE EFFECTS AGAINST BETA-AMYLOID INDUCED CYTOTOXICITY IN PC12 CELLS

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ABSTRACT

Introduction: Considering the therapeutic potential of medicinal plants in different diseases like Alzheimer's disease (AD), we intended to study the in vivo anticholinesterase effect and neuroprotective effect of soybean ethanolic extract (EES) on PC12 cells.

Materials and Methods: Anticholinesterase effect of single and multiple doses of EES was studied in experimental amnesic mice using Ellman method. Protective effect of EES in non toxic concentrations was evaluated against β-amyloid (Aβ) toxicity on PC12 cell line.

Results: Significant AChE inhibitory effects in single dose were observed at doses of 1.5 g/kg (p<0.01) and 2g/kg (p<0.001) (single dose) and doses of 400, 600mg/kg (p<0.01) and 800mg/kg (p<0.001) (multiple dose). The highest protection against Aβ induced toxicity was achieved at non toxic concentration of 500 which exhibited 96.4% cell viability. Conclusion: The inhibition of AChE and protection against Aβ–induced cytotoxicity of EES might be in part due to its isoflavones and terpenoids.

Keywords: Soybean, Anticholinesterase, Aβ toxicity, PC12 cell

INTRODUCTION

Alzheimer’s disease (AD) is one of the most common progressive neurodegenerative disorders which cause dementia among older patients. As the disease progresses, body functions are lost and different symptoms such as irreversible impairment of cognitive function or intellectual abilities such as memory, judgment, and language, ... appear or worsen13. There is some evidence indicating that AD is associated with formation of the plaques and tangles in the brain. One hypothesis suggests that an abnormality in cholinergic function especially in basal forebrain as the major source of cholinergic innervations of the neocortex and hippocampus correlates directly with conciousness impairment4,5. On the other hand, one of the pathological features identified in AD is the presence of neurofibrillary tangles, amyloid plaques, and inflammations. Accumulation of amyloid β (Aβ) peptide acts as an inhibitor of certain enzyme functions. This toxic peptide also might be important in programmed cell death (apoptosis)6. Because of the growing interest in medicinal plants as a source of lead compounds, they have attracted attentions as a valuable candidate for target drug in different diseases like AD. Investigation of therapeutic potential of medicinal plants allows the designing a rational plan for finding new drugs. Soybean or Glycine max L. (syn: Soja hispida L.) belongs to Fabaceae family and is a crop plant native to East Asia which has numerous uses of medicinal, nutritional and industrial. This plant has health promotion effects and is suggested as for decreasing the risk of cancer and osteoporosis as well as protective effect against cardiovascular disease7,8. The soybean -based diets reduced serum levels of total cholesterol, LDL cholesterol, and triglyceride without affecting HDL cholesterol9,10.

In continuing to find anticholinesterase agents from medicinal plants11,12 and in light of our recent study demonstrating the in vitro antioxidant and anticholinesterase effects of soybean extract of this plant12, we became interested in determining whether the extract is also effective when given in vivo. Meanwhile, considering the critical role of Aβ in pathogenesis of AD, we investigated protective effect of soybean extract against Aβ peptide-induced neurotoxicity.
MATERIALS AND METHODS

Chemicals
Acetylthiocholine iodide, acetylcholine chloride, dithionitrobenzoic acid (DTNB), tacrine, Poly-D-lysine (PDL), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and Aβ (25-35) were purchased from Sigma, Co. Swiss, bovine serum albumin (BSA), Tris buffer were prepared from Merck, Germany, Dulbecco’s modified eagle medium (DMEM), trypsin, antibiotic-antimycotic and fetal bovine serum (FBS) were purchased from Gibco. All other chemicals were from analytical grade. PC12 cell line was prepared from Pasteur Institute, Iran.

Plant materials
Soybean was prepared from market and authenticated kindly by Dr. Mirtadzadini, Bahonar University, Kerman, Iran.

Extraction
An amount of 500 g of soybeans were extracted with ethanol 80% by maceration method. The extract was concentrated under vacuum to give a viscose mass and finally dried in oven at 40°C. Dried extract was kept at -20°C until the experiments.

Phytochemical screening
Presence of secondary metabolites in soybean was investigated by phytochemical screening experiments as explained by Das15.

Animals
Male Swiss albino mice (25–30g) were randomly divided into 6 groups, 6 mice in each group and maintained under standard condition (room temperature 25±1 °C and humidity 60–65%) with 12 h light and dark cycle. Animals had free access to food and water.

Pharmacological tests
Treatment protocol
Single dose
In this experiment, test animals received scopolamine (1 mg/kg i.p) for induction of amnesia. Fifteen minutes later, animals received intraperitoneally different doses of EES (0.8, 1.5 and 2g/kg) or tacrine (0.4 mg/kg as positive control) or saline (10 ml/kg as negative control). After an hour, the mice were sacrificed by decapitation.

Multiple doses
Fifteen minutes after i.p administration of scopolamine (1m/kg), test animals received EES at different doses of 200, 400, 600 and 800 mg/kg for 7 days. The mice were sacrificed by decapitation 1h after the last dose of test extract or control drugs.

Tissue preparation
Intact brain of animals was washed with ice-chilled normal saline many times to clean. Brain was weighed carefully after drying with filter paper. The hippocampus was dissected out from the brain and homogenated in 10% w/v lysis buffer (80mM Tric-HCI, pH 8) by an Ultra-Turrax T18 homogenizer at a speed of 9500 rpm, 3 times with few second intervals. The homogenate was centrifuged at 14000g for 30 min at 4 °C. Supernatant was collected and used for assaying of AChE activity.

Assay of AChE activity
Determination of AChE activity was performed by using Ellman method with some modifications as explained by Das15. Briefly, the substrate of ATCI is converted to thiocholine by AChE. The reaction of thiocholine with DTNB leads to formation of a yellow anion, 5-thio-2nitrobenzoic acid which has absorbance at 412nm. Fifty microlitre of DTNB (0.01 M in Na buffer, 0.1M, pH 8) was added to tris-buffer and absorbance was measured at 412 nm using spectrophotometer (Shimadzu UV-2100, Japan). A volume of 200 µl ATCI, 75mM was added and absorbance was measured at 412 nm immediately after adding the enzyme source (100 µl) to the reaction mixture for 5 minutes in 30s intervals. Experiments carried out in triplicate and the results were reported as mean ± SEM. The same mixture without lyase of hippocampus was used as a blank. There might be some non-enzymatic reactions between hippocampus content and DTNB which interfere with analysis. To control that, a pre-incubation of hippocampus and DTNB is performed prior to the addition of ATCI.

Toxicity of EES on PC12 cells
PC12 cells were cultured in DMEM medium supplemented with 10% FBS, penicillin 100 unit and streptomycin 100mg. Cultures were maintained at 37°C in incubator 5% CO₂. PC12 cells were cultured at a concentration of 10⁶ cells/well with poly-D-lysine (PDL). After 24h., medium was removed and different concentrations of EES (0.1-200µg/ml in PBS) were added to culture and incubated for 24h at room temperature. The cell survival was evaluated by MTT assay.

Neuroprotective effect of EES against Aβ peptide
PC12 cells were cultured in 96 microwells coated by PDL at a concentration 10⁶ cells/well and incubated for 24h. at 37°C. Then PC12 cells were treated with different concentrations of EES at non toxic concentrations. Aβ peptide (2µM in distilled water) was added to wells and incubated for 24h. Aβ peptide stock solution was prepared by dissolving 1mmol Aβ peptide in 1ml distilled water and incubated at 37°C for three days to make aggregated form. The viability of cells was checked using MTT assay.

RESULTS

Extraction yield and phytochemical tests
The yield of plant extraction was 17.0% w/w and the results of phytochemical screening indicated the presence of flavonoids, saponins, terpenoids and sterols in the EES.

AChE inhibitory effect of single dose EES
As is shown in Figure 1, EES inhibited AChE activity in a dose-dependent manner. Significant inhibitory effects were observed at the doses of 1.5 g/kg (p<0.01) and 2g/kg (p<0.001). The plant extract reduced enzyme activity 6%, 15.8% and 25.7% at concentrations of 0.8, 1.5 and 2 g/kg respectively, while tacrine showed 58.5% AChE inhibition (Figure 1).

AChE inhibitory effect of EES multiple dose
The result of multiple doses of EES on AChE in hippocampus of mouse brain is given in Figure 2, which indicated that AChE was significantly & dose-dependently inhibited by EES at doses of 400, 600 and 800mg/kg as compared to control.

Effect of EES on cell viability of PC12 cells
Evaluation of toxicity of EES against PC12 cells shows that EES exhibited no cytotoxicity under normal condition after 24h (Figure 3).
Neuroprotective effect of EES on PC12 cells
The incubation of PC12 cells with EES produced significant protection against toxicity of Aβ peptide (Figure 4). Cell viability was dose-dependently increased at all tested concentrations of EES which received in a significant level at the concentration of 100µg/ml. This effect reached to the highest level at the concentration of 500µg/ml (96.4% cell viability).

DISCUSSION
The potentiation of cholinergic system through the AChE inhibition would lead to the pharmaceuticaly of AD(16). However, there are some obstacles including short half-life and adverse effects for successful treatment of the disease using AChE inhibitors. Considering our results in recent study about in vitro anticholinesterase and antioxidant of soybean ethanolic extract (EES), we hypothesized whether the extract is also effective in in vivo experiments in single and multiple doses in mice(17,18). We used i.p scopolamine to induce amnesia as an experimental model for AD. It is shown that scopolamine through antagonism of muscarinic receptors causes memory impairment by increasing AChE activity, so we used tacrine (an AChE inhibitor) as positive control in this study. Tacrine could improve experimental and clinical memory deficit.

The results indicated that EES could somehow inhibit hippocampus AChE activity at all three tested doses of .8, 1.5 and 2g/kg in a dose-dependent manner. Administration of multiple doses of EES could also dose-dependently inhibit AChE activity (Figure 2). The highest AChE inhibition was shown at dose of 800 mg/kg of EES. We also investigated protective effect of EES against Aβ-induced neurotoxicity. The results of MTT assay indicated that EES exhibited no toxicity at concentrations up to 500µg/ml. Our results of MTT assay indicated that EES exhibited no toxicity at concentrations up to 500µg/ml. This is in agreement with our previous results that showed no organ toxicity for EES in mice up to 4g/kg (data are not shown). Here we have shown that EES in a concentration-dependent manner protected the cytotoxicity of Aβ on the PC12 cells (Figure 4).

Phytochemical analysis performed in this study showed presence of phytoestrogens, isoflavons and terpenoids in EES. Some researchers have reported the protective effects of genistein, a principal component of soybean isoflavones, against the oxidative stress damage induced by Aβ in the PC12 cells(19,20). There is line of evidence indicating that this plant and some of its components could be effective for cognitive disorders. A recent report shows that the ingestion of a soybean extract might be effective on spatial memory of rats especially on ovariectomized ones(21). Bagheri and colleagues (2011) have reported that pretreatment with genistein (soybean isoflavon) ameliorates the impairment of short-term spatial memory in rats caused by Aβ. This effect was attributed to its estrogenic and antioxidant effect(22). As reported by "Vanize ", phytoestrogens of soybean would be helpful to prevent antioxidant deficiency and could protect mitochondria and maintain ATP by lowering oxidative load(23). Soybean phytoestrogens also could reverse the alteration in ATP content of hippocampus in ovariectomized rat(24). These plant components display strong neuroprotective effect. In a recent study, the combination of soybean phytoestrogens with mephenamic acid and tau strongly decreased the risk factors involved in AD promotion (25). There are several publications which report the positive effect of isoflavones on cognitive function in women and men(26). In the other hand, neuroprotective effect of terpenoids isolated from soybean with EES has been reported(27). So, both isoflavones and terpenoids would be responsible for cognition improvement of EES. Previous study by "Hodges "(28) exhibited that AChE inhibitors play an important role in enhancing cholinergic activity as well as in preventing the amyloid aggregation which is the key factor in AD. Although there have been many in vivo reports about effectiveness of soybean and its compounds in cognitive disorders, few study has been carried out on the AChE inhibitory effect of the plant extract. In addition to our previous report(12), the only report, to the best of our knowledge, is about the AChE inhibition of the saponins of soybean(29).

Figure 1: Acetyl Cholinesterase inhibitory effect of EES single dose on the hippocampus of mouse. Values are mean ± SEM. *p<0.01, *** p<0.001 in comparison to normal saline group

Figure 2: Acetyl Cholinesterase inhibitory effects of EES multiple dose in hippocampus of mouse. Mice were treated for 7 days with different doses of EES. Values are shown as mean ± SEM. * p<0.05, *** p<0.001 in comparison to normal saline group
Figure 3: Cytotoxicity effect of different concentrations of EES on PC12 cells

Figure 4: Protective effect of different concentration of EES on Aβ toxicity on PC12 cells

CONCLUSION

The main finding of the present study was that EES is active not only in potentiating the cholinergic neurotransmission but also in protection of the Aβ toxicity of thereby preventing from neurodegeneration and so might be appropriate to patients in moderate stage of AD (30). This study show, for the first time, that inhibition of the activity of AChE is likely to be involved in the therapeutic effects of soybean in the cognitive disorders. Our results also suggest that i.p administration of EES has enough absorption to affect ACh activity. The low toxicity of this extract in human and animals and its neuroprotective effect make it a good candidate for further investigation.

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