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ORIGINAL ARTICLE

The effect of *Ginkgo biloba* extract on scopolamine-induced apoptosis in the hippocampus of rats

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Abstract Apoptosis, known as programmed cell death, plays a crucial role in normal development and tissue homeostasis. Apoptosis is also involved in neurodegenerative diseases such as Alzheimer's disease. Amnesia refers to the loss of memory and can also be a warning sign of neurodegenerative diseases. The antioxidant properties of *Ginkgo biloba* extract was known previously. Therefore, the aim of this study was to examine the effects of *Ginkgo biloba* extract on the rat's hippocampal apoptotic neurons number after Scopolamine based amnesia. Thirty-six adult male Wistar rats were used. Rats were randomly divided into control, sham, protective and treatment groups. The rats in the sham group received only scopolamine hydrobromide (3 mg/kg) intraperitoneally. The rats in the protective and treatment groups received *Ginkgo biloba* extract (40, 80 mg/kg) for 7 days intraperitoneally before/after scopolamine injection. Then 48 h after the last injection, the brains of rats were withdrawn and fixed with paraformaldehyde, and then, after histological processing, the slices were stained with the TUNEL kit for apoptotic neurons. Data were compared by the ANOVA Post Hoc Tukey test; $P < 0.05$ was considered significant. Our results showed that Scopolamine (in the sham group) increased significantly the number of apoptotic neurons in all areas of the hippocampus compared with the control. Whereas, *Ginkgo biloba* extract reduce the neuronal apoptosis in the hippocampus before and/or after encounter with scopolamine. We concluded that pretreatment and treatment injection of *Ginkgo biloba* extract can have a protective effect for neurons and it can limit apoptosis in all area of the hippocampus.

Keywords Apoptosis · Scopolamine · *Ginkgo biloba* extract · Hippocampus · Rat

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Introduction

Some antagonists of the muscarinic cholinergic receptor, such as scopolamine, impair memory performance that has been proposed as an animal model of dementia (Azami et al. 2010; Caine et al. 1981). It could pass through the brain–blood Barrier and by blocking the conduction of thecholinergic system, scopolamine could decrease neuronal excitability and induce memory impairment (Zhong et al. 2005); and it can decrease the number of neurons in nervous tissue such as the hippocampus (Seifhosseini et al. 2011). Similarities between Alzheimer patients and scopolamine treated animals have been reported (Azami et al. 2010).

Programmed cell death or apoptosis is a firmly controlled process in which cell death is performed through the activation of specific signaling pathways (Pulido and Parrish 2003). It plays a crucial role in normal development and tissue homeostasis (Woodle and Kulkarni 1998). Nevertheless, incorrect or unnecessary apoptosis has been associated in several neurological disorders such as Parkinson's disease and Alzheimer's disease (Smith et al. 1991; Thompson 1995; Johnson et al. 1995; Lee et al. 2003; Liu et al. 1998). Neuronal apoptosis in the cortex and hippocampus changes learning and memory (Kuhn et al. 2005; Sun et al. 2009).

Apoptosis occurs when internal monitors recognize damage or malfunction and initiate signaling cascades that eventually activate caspases and endonucleases that kill the cell (Kokileva 1994; Zhivotovsky et al. 1994). Antioxidant materials are produced by the body in the form of enzymes and also come through a person's dietary intake. Cells contain endogenous antioxidant enzymes (e.g., catalase, superoxide dismutase, and glutathione peroxidase), and many, but not all, human cancer cell types have decreased antioxidant enzyme levels compared to their normal tissue counterparts (Coursin et al. 1996; Oberley and Oberley 1997). The chemical structure of antioxidants enables them to absorb the excess charges produced by free radicals.

Ginkgo biloba is one of the widely cultured Chinese plants (Das et al. 2002), it has antioxidant and free radical-scavenger properties which could contribute to its neuroprotective/anti-apoptotic activity. It has also been shown to reverse the age-related decline of neurotransmitter systems (Williams et al. 2004). A standardized extract of the leaves of *Ginkgo biloba* (EGb 761) has neuroprotective and antioxidant properties (Seif-El-Nasr and Abd El-Fattah 1995; DeFeudis 1998). EGb 761 shows a broad range of biochemical and pharmacological activities such as antioxidants and free radical scavenging (Marcocci et al. 1994a, b) as well as neurotrophic activities in the hippocampal formation (Barkats et al. 1994; Krištofiková and Klaschka 1997).

An extract obtained from the leaves of *Ginkgo biloba*, EGb761, has been used increasingly over the last decade for the treatment of various cardiovascular- and central-nervous system- related diseases such as ischemia, dementia and depression (Gertz and Kiefer 2004; Smith and Luo 2004). Unfortunately, the mechanism of its therapeutic effect, particularly on the central nervous system, remains unknown (Smith and Luo 2004; DeFeudis and Drieu 2000; Ahlemeyer and Krieglstein 2003).

The hippocampus is involved in learning and memory (Emamian et al. 2010) and in the other functions of the limbic system. The main cells in the hippocampus are pyramidal neurons and in dentate gyrus they are granular neurons (Jahanshahi et al. 2006). Therefore, the aim of this study was to examine the protective and treatment effects of *Ginkgo biloba* extract on the neurons apoptosis in rat hippocampus before and/or after exposure to scopolamine.

Methods

Animals

Thirty-six adult male wistar rats, weighing 200–250 g, were used in this study. They were given free access to normal laboratory chow and water. Temperature of the animal house was 22 ± 3 °C under a 12/12 h light–dark cycle (light beginning at 7:00 a.m.) at least 1 week before the beginning of the experiments. The Golestan University of Medical Sciences Guidelines for the Care and Use of Animals in Research were followed. Also this study was approved by Ethics committee of Golestan University of medical sciences.

The rats were randomly distributed into Six groups ($n = 6$) as follows:

- Control: Without receiving *Ginkgo biloba* extract and scopolamine.
- Sham: Scopolamine injection on the first day, 3 mg/kg (One injection), Intra-peritoneally (IP).
- Pretreatment (two groups): *Ginkgo biloba* extract injection (40 and 80 mg/kg, IP) everyday injection for a week, Scopolamine injection on the eighth day (3 mg/kg, IP; One injection).
- Treatment (two groups): Scopolamine injection on the first day (3 mg/kg, IP; One injection), *Ginkgo biloba* Extract injection (40 and 80 mg/kg, IP) everyday injection for a week. Rats were injected with a single dose of scopolamine in all groups except Control group.

Drugs

The drugs included scopolamine hydrobromide (Tocris, UK) and *Ginkgo biloba* Extract (NIAC Pharmaceutical Factory, Gorgan, Iran). All drugs were dissolved in sterile saline and were injected into the peritoneal.

Then 48 h after the last injection, animals anesthetized with chloroform and their brains were withdrawn and then the brains fixed for 2 weeks in 4 % paraformaldehyde. Histological processing include: dehydration with different degrees of alcohol (50–100), clarification with Xylol and then embedding in paraffin wax, were performed.

The 8 μm coronal sections were serially collected from Bregma −3.30 to −6.04 mm of the hippocampal formation (Paxinos and Watson 1998). An interval of 20 μm was placed between each two consecutive sections (Jahanshahi et al. 2011).

Briefly, sections were deparaffinized, rehydrated, and washed with distilled water. The sections were covered with proteinase K solution (IHC World, USA) and incubated for 15 min at 37 °C in a humidified chamber. Endogenous peroxidase activity was blocked by incubation in 0.3 % hydrogen peroxide in PBS at room temperature for 10 min. The sections were examined for apoptotic cells by the terminal deoxynucleotidyl transferase mediated dUTP nick end-labelling (TUNEL) method, using an in situ cell death detection kit (POD, Roche, Germany), according to the manufacturers protocol. The sections were incubated

with the TUNEL reaction mixture at 37 °C for 60 min and then sections were incubated with Converter-POD at 37 °C for 30 min. Then sections were stained with diaminobenzidine (DAB) (IHC World, USA) and counterstained lightly with Meyer's haematoxylin.

A photograph of each section was produced using a microscope (BX51, Olympus, Tokyo) and a DP 12 digital camera under a magnification of 400. An area of 30000 μm^2 for CA_1, CA_3 and dentate gyrus was selected randomly in the all sections. To measure the area density of the apoptotic neurons, the images were transferred onto a computer. Using OLYSIA Autobioreport software (Olympus), the appropriate grids were superimposed on the pictures and the cells were counted manually (Jahanshahi et al. 2008, 2009).

Statistical analysis

Experimental results concerning this study were evaluated using SPSS ver. 11.5 (SPSS Inc., Chicago, IL, USA) and expressed as mean ± SD. To compare the means of the measured parameters in the three groups by the analysis of variance test, after confirmation of normality, the means were compared by the ANOVA Post Hoc Tukey test; $P < 0.05$ was considered significant.

Results

We found that in sham-scopolamine group injection of scopolamine increased the number of apoptotic neurons in CA1 area ($P < 0.001$), CA3 area ($P < 0.01$) and in dentate gyrus ($P < 0.001$) of hippocampal formation compared to controls (Figs. 1, 2, 3, 4). All differences were significant statistically. The most apoptotic cells were shown in dentate gyrus with means 16.12 ± 7.3 in the sham-scopolamine group compared to the other groups (Fig. 3).

Also, our data showed that the number of apoptotic cells in experimental groups (*Ginkgo* Groups) was more than the control group, But, there are no statistically significant differences between control groups and the other experimental groups that received *G. biloba* extract.

We found that *G. biloba* extract could reduce the number of apoptotic cells in the hippocampus within 7 days after injection of scopolamine (as treatment groups 40 and 80 mg/kg). On the other hand, after injection of scopolamine, the number of apoptotic cells was increased, and then after treated with *G. biloba*, the number of apoptotic cells in all area of the hippocampus was decreased (Figs. 1, 2, 3).

Also, we found that administration of *G. biloba* 7 days before scopolamine injection could protect the neurons against apoptosis (Figs. 1, 2, 3). The lesser number of

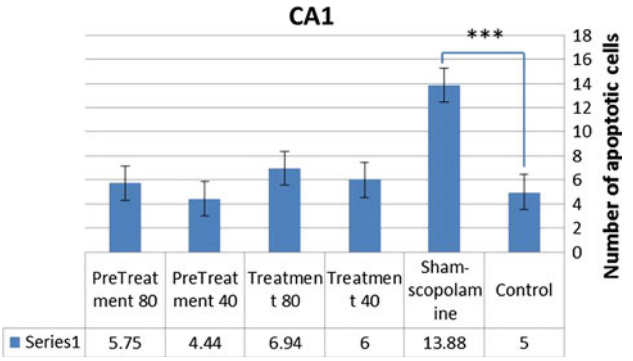

Fig. 1 Shows the mean number of apoptotic cells in *CA1* area of hippocampus. The difference between control and sham-scopolamine was significant statistically ($P < 0.001$)

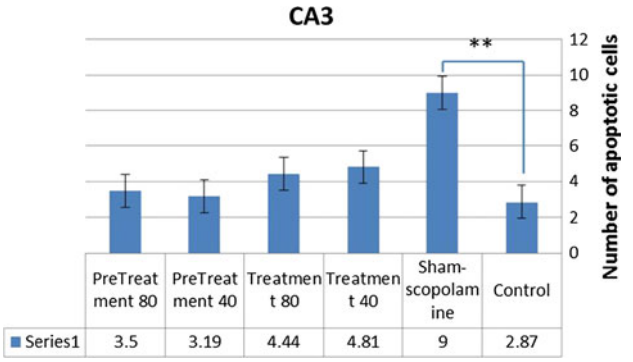

Fig. 2 Shows the mean number of apoptotic cells in *CA3* area of hippocampus. The difference between control and sham-scopolamine was significant statistically ($P < 0.01$)

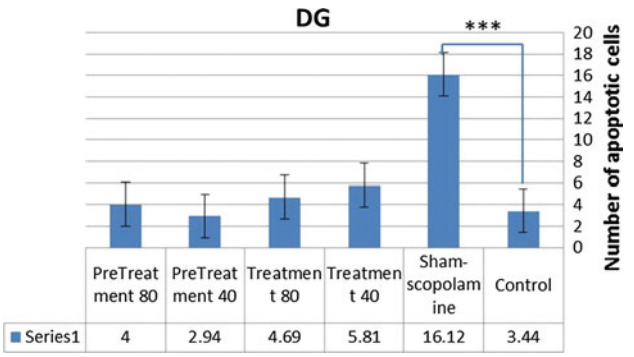

Fig. 3 Shows the mean number of apoptotic cells in *DG* area of hippocampus. The difference between control and sham-scopolamine was significant statistically ($P < 0.001$)

apoptotic cells was observed in 40 mg/kg pretreatment groups in CA1, CA3 and DG.

Discussion

Our data showed that scopolamine as a non-selective muscarinic receptor antagonist can induce apoptosis in

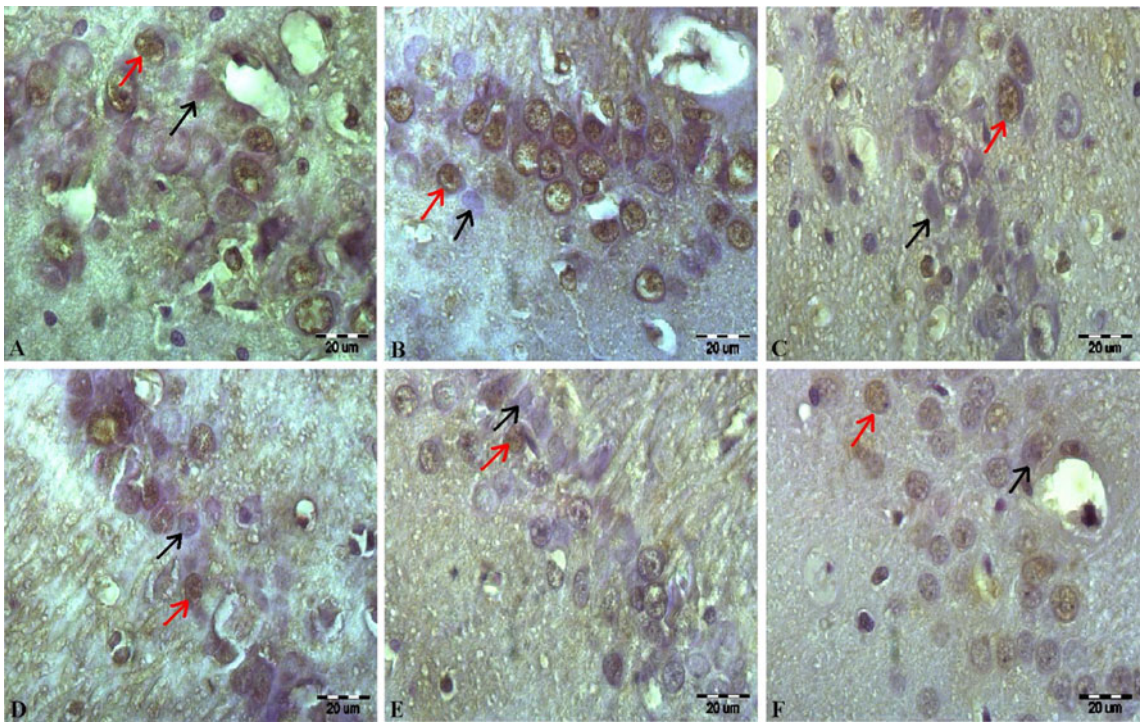

Fig. 4 The apoptotic cells (*Red arrows*) in CA1 area of hippocampus in all groups (mag. ×100), *Black arrows* showed the normal neurons. **a** control, **b** sham-scopolamine, **c** Treatment 40 mg/kg, **d** Treatment 80 mg/kg, **e** Pretreatment 40 mg/kg, **f** Pretreatment 80 mg/kg

hippocampal neurons. Regardless of the mechanism of this induction, scopolamine increases the number of apoptotic neurons in all area of the hippocampus. Due to the *Ginkgo biloba* antioxidant property, pretreatment and treatment groups in this study showed the lesser number of apoptotic cells compared with sSham-scopolamine group in all areas of the hippocampus.

The ability of *G. biloba* to inhibit apoptosis seemed to depend on some factors such as: the species and the age of the animals and also the model used for the induction of apoptosis (Ahlemeyer et al. 1999). The neuroprotective effect of *G. biloba* extract has been demonstrated in several in vitro and in vivo models. In vitro, 10–100 μg/ml EGb 761 protected cultured neurons against death induced by hypoxia, nitric oxide (NO) (Bastianetto et al. 2000b) and cyanide (Krieglstein et al. 1995). Also reduction of neuronal damage by intraperitoneally injection of EGb 761 has been detected after transient middle cerebral artery occlusion in rats (Krieglstein et al. 1995; Zhang et al. 2000).

Previously in cultured hippocampal neurons, it has been shown that EGb 761 prevents neuronal death probably by a mechanism involving its antioxidant and hydroxyl radical scavenging activity (Bastianetto et al. 2000a). In addition, in vitro studies have shown protective effects of EGb 761 against neuronal apoptotic death induced by hydroxyl radicals (Bastianetto et al. 2000b; Chen et al. 1999; Ni et al. 1996; Wei et al. 2000), and some of the constituents of *G. biloba* extract prevent cell death induced by serum deprivation and staurosporine exposure in cultured chick telencephalic neurons and hippocampal cultured neurons (Ahlemeyer et al. 1999). *G. biloba* may inhibit toll-like receptor 4 (TLR-4) and NF-κB-dependent inflammatory responses, and, furthermore, lessen neuronal cell apoptosis after traumatic brain injury (Yu et al. 2012). Ginkgolide B may significantly inhibit Aβ25–35-induced apoptosis, and the neuroprotective effects may be intimately associated with brain-derived neurotrophic factor up-regulation caused by Ginkgolide B (Xiao et al. 2010).

Also, the ability of oxidative stress to induce apoptosis, and the effect of *Ginkgo biloba* extract (EGb761) on this induction were studied in primary cultured rat cerebellar neuronal cells (Ni et al. 1996).

Some previous studies provide further evidence for the therapeutic potential of EGb 761 in the treatment of vascular dementia (Rocher et al. 2011). Both EGb 761 and bilobalide have a protective effect against ischemia-induced neuronal death in vivo (Chandrasekaran et al. 2003; Domorakova et al. 2006) and glutamate induced neuronal death in vitro (Chandrasekaran et al. 2003).

The neuroprotective effects of EGb 761 on reduction of the dopamine neuron loss in the substantia nigra and striata indicates a possible role for the extract in the treatment of Parkinson's disease (Kim et al. 2004), these effects might be related to the increase of bcl-2 activation, maintenance

of MMP stability and decrease of caspase-3 activation through the mitochondria-dependent pathway (Kang et al. 2007).

Conclusion

We conclude that the pretreatment and treatment injection of *Ginkgo biloba* extract can have a protective effect for neurons in all areas of hippocampal formation. On the other hand, *Ginkgo biloba* extract reduced the number of apoptotic cells in the hippocampus before or after encounter with scopolamine. There is no difference in effect in apoptotic cell density between different doses of *Ginkgo biloba* extract that we used in this study.

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Conflict of interest None.

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