Protective effects of *Ginkgo biloba* extract (EGB 761) on astrocytes of rat hippocampus after exposure with scopolamine

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Abstract: The regular extract of *Ginkgo biloba* has been shown to possess neuroprotective properties in disorders like hypoxia, ischemia, seizure activity and peripheral nerve damage. Also, *G. biloba* has received attention as a potential cognitive enhancer for the treatment of Alzheimer's disease, but there is not any documentation about the effect of an extract of *G. biloba* on astrocytes. Therefore, the aim of this study was examined the effects of *G. biloba* extract on the rat's hippocampal astrocytes after scopolamine based amnesia. In this study, 36 adult male Wistar rats were used. Rats were randomly distributed into control, sham, protective and treatment groups. The rats in the sham group only received scopolamine hydrobromide (3 mg/kg) intraperitoneally. The rats in the protective and treatment groups received *G. biloba* extract (40, 80 mg/kg) for 7 days intraperitoneally before and after scopolamine injection. Forty eight hours after the last injection, the brains of the rats were withdrawn and fixed with paraformaldehide, and then after histological processing, the slices were stained with phosphotungstic acid-haematoxylin for astrocytes. Data were analyzed by the analysis of variance (ANOVA) post hoc Tukey test; $P<0.05$ was considered significant. Results showed that scopolamine can reduce the number of astrocytes in all areas of hippocampal formation compared with the control. However, *G. biloba* extract can compensate for the reduction in the number of astrocytes in the hippocampus before or after the encounter with scopolamine. We concluded that a pretreatment and treatment injection of *G. biloba* extract can have a protective effect for astrocytes in all areas of hippocampal formation.

Key words: *Ginkgo biloba* extract, Hippocampus, Astrocytes, Rat

Introduction

Failure of memory may result from damage to parts of the brain critical for memory storage, processing, or recall such as the hippocampus of the limbic system. Amnesia or forgetfulness refers to the loss of memory, and can also be a symptom of neurodegenerative diseases. Alzheimer's disease is one form of dementia that gradually gets worse over time. It affects memory, thinking, and behavior. Similarities in the memory deficits between Alzheimer patients and scopolamine treated animals have been reported. Scopolamine as a muscarinic cholinergic receptor antagonist that impairs memory performance has been proposed as an animal model of dementia [1].

Scopolamine induces severe losses of hippocampal cholinergic neurons, as indicated by decreased choline acetyltransferase immunoreactivity and increased acetylcholinesterase reactivity. Daily administration of some wild plants such as ginseng reduced the loss of cholinergic immunoreactivity in the hippocampus. The reduced expression of brain-derived neurotrophic factor mRNA due to the

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scopolamine injection was recovered to normal levels by the administration of wild ginseng [2].

The other plant, *Ginkgo biloba*, is one of the widely cultured Chinese plants [3] has antioxidant and free radical-scavenger properties which could contribute to its neuroprotective/anti-apoptotic activity. It has also been shown to reverse age-related decline of neurotransmitter systems [4].

The dried leaves of the *G. biloba* tree provide the rudimentary drug from which the standardized *G. biloba* extract (EGb 761) is obtained. The defined but complex product consists of two major groups of substances, the flavone glycosides (flavonoid fraction, 24%) and the terpene lactones (terpenoid fraction, 6%) [5].

The standardized extract of *G. biloba* has been shown to possess neuroprotective properties under conditions like hypoxia, ischemia, seizure activity and peripheral nerve damage. Recently, *G. biloba* has received attention as a potential cognitive enhancer for the treatment of Alzheimer's disease [3]. Other studies indicated that EGb761 upregulated the expression of several genes, such as AMPA and chloride channel proteins, and the synaptic plasticity in hippocampal slices of aged mice was improved by EGb 761 [6].

Preclinical and clinical studies have demonstrated that *G. biloba* extract was effective in mild to moderate dementia in Alzheimer's disease. This suggests that *G. biloba* extract does have modest therapeutic potential as a memory and cognition enhancing drug and its action may target the hippocampus [7].

Astrocytes have privileged access to synapses and we are beginning to learn that as a consequence they are able to regulate both the pre- and postsynaptic terminals of excitatory and inhibitory synapses. Because of the reciprocal signaling that can occur between astrocytes and synaptic terminals, these structures have been termed the 'Tripartite Synapse' [8].

Since so far the protective effects of *G. biloba* extract on the cellular structure of the hippocampus have not been studied, the aim of this study was to examine the effects of *G. biloba* extract on the rat hippocampus structure after exposure with scopolamine.

Materials and Methods

Thirty-six adult male Wistar rats, weighing 200-250 g, were used in the study. They were given free access to normal laboratory chow and water. The temperature of the animal house was 22±3°C. This research was conducted under the Ethics Committee of Golestan University of Medical Sciences, Gorgan, Iran.

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

The rats were randomly divided into six groups (n=6) as follows:

- Control: Without receiving *G. biloba* extract and scopolamine

- Sham: Scopolamine injection on the first day, 3 mg/kg, IP

- Protective (two groups): *G. biloba* extract injection (40 and 80 mg/kg, IP) for a week, and then scopolamine injection on the eight day (3 mg/kg, IP)

- Treatment (two groups): Scopolamine injection on the first day (3 mg/kg, IP), and then *G. biloba* extract injection (40 and 80 mg/kg, IP) for a week.

Forty eight hours after the last injection, the animals were anesthetized with chloroform and their brains were withdrawn and then the brains were fixed for two weeks in 10% formaldehyde. Different degrees of alcohol were used for dehydration followed by clarification with xylol. After histological processing, the tissue was impregnated and then embedded in paraffin wax.

The 8 μm coronal sections were serially collected from Bregma -3.30 mm to -6.04 mm of the hippocampal formation [9]. An interval of 20 μm was placed between each two consecutive sections. The sections were stained with phosphotungstic acid-haematoxylin staining (specific staining for astrocytes) (Fig. 1) [10].

A photograph of each section was produced using an Olympus BX 51 microscope (Olympus, Tokyo, Japan) and a DP 12 digital camera under a magnification of 400. An area of 22,500 μm^2 was selected randomly in all areas of hippocampal formation (CA1, CA3 and dentate gyrus) in all sections. To measure the area density of the astrocytes, 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 [11, 12].

Statistical analysis

Experimental results concerning this study were evaluated using SPSS ver. 11.5 (SPSS Inc., Chicago, IL, USA) and expressed as the mean±SD. To compare the means of the measured parameters in the three groups by the analysis of variance (ANOVA) test, after confirmation of normality, the means were compared by the ANOVA post hoc Tukey test;
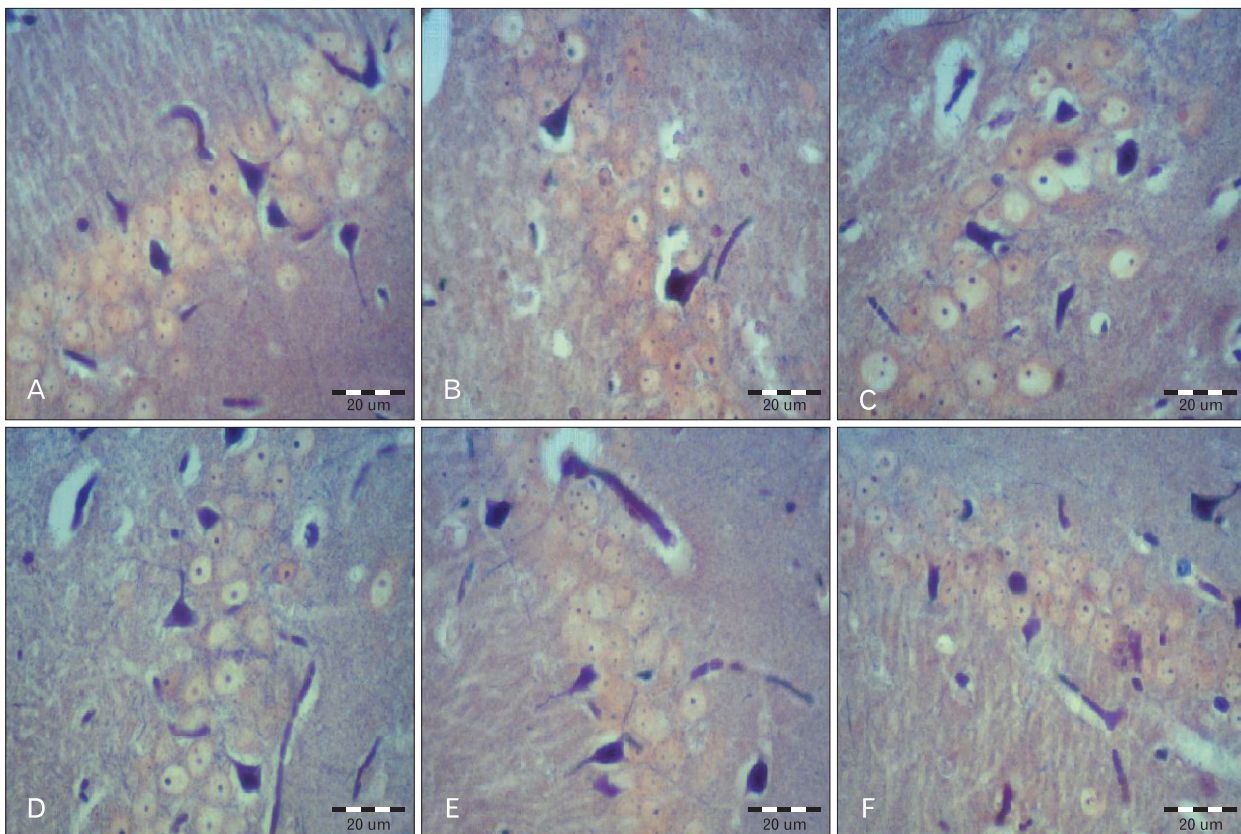
Fig. 1. Astrocytes in the CA1 area of the hippocampus in all groups. (A) Control. (B) Sham (scopolamine). (C) Treatment 40. (D) Treatment 80. (E) Pretreatment 40. (F) Pretreatment 80 (PTAH staining). Scale bars=20 µm.

$P<0.05$ was considered significant.

Results

Our results showed that injection of scopolamine in the sham group reduced the number of astrocytes in all areas of the hippocampal formation compared with the control (Tables 1-3). This reduction in the CA3 (P=0.001) and in the dentate gyrus (P=0.03) was statistically significant, and the reduction in the CA1 area was not significant (P=0.21). The differences of cell density are shown in Fig. 1.

We found that *G. biloba* extract could compensate for the reduction of astrocytes in the hippocampus within 7 days after injection of scopolamine (as treatment groups 40 and 80 mg/kg). On the other hand, the number of astrocytes was reduced after injection of scopolamine and then increased with *G. biloba*.

Also, our data showed similar results when we injected *G. biloba* extract within 7 days before injection of scopolamine (as protective groups 40 and 80 mg/kg). Statistical analysis showed differences between the sham-scopolamine group and the other groups were significant in the CA3 and dendrate gyrus areas.

Table 1. Mean and SD of astrocytes number in CA1 area of hippocampus

Groups (CA1)	Mean	SD	P-value
Control	6.40	2.318	
Sham-scopolamine	5.30	1.849	0.215
Treatment 40	6.83	2.049	0.953
Treatment 80	6.85	2.547	0.940
Pretreatment 40	6.38	2.230	0.820
Pretreatment 80	7.00	1.990	0.965

Table 2. Mean and SD of astrocytes number in CA3 area of hippocampus

Groups (CA3)	Mean	SD	P-value
Control	7.80	2.902	
Sham-scopolamine	5.28	1.881	0.001
Treatment 40	6.47	2.230	0.236
Treatment 80	7.15	3.043	0.887
Pretreatment 40	6.75	3.160	0.498
Pretreatment 80	7.38	2.618	0.981

Table 3. Mean and SD of astrocytes number in DG area of hippocampus

Groups (DG)	Mean	SD	*P*-value
Control	19.90	14.265	
Sham-scopolamine	17.82	11.957	0.030
Treatment 40	20.28	12.637	0.979
Treatment 80	21.22	13.178	0.997
Pretreatment 40	19.93	12.382	1.000
Pretreatment 80	19.15	12.088	1.000

DG, dentate gyrus.

It seems in all areas of the hippocampus, an 80 mg/kg dose of *G. biloba* extract had a greater effect than 40 mg/kg dose of *Ginkgo*. There are no significant differences between the treatment and protective effects of *G. biloba* extract.

Effects of amnesia caused by scopolamine were previously known, but this study demonstrated that scopolamine can affect the hippocampal structure.

Discussion

Our main findings suggest that scopolamine could reduce the number of astrocytes in the hippocampus, while both the protective and therapeutic injection of *G. biloba* has been able to compensate for the reduced number of astrocytes. It seems this effect was dose-dependent. Our data did not show any significant differences between the protective and therapeutic effects of *G. biloba* extract.

Animal studies have revealed that *G. biloba* is able to facilitate acquisition and retention of memory, with one of the major protective actions taking place in the hippocampus, which is related to the acquisition of new memories [13].

Wang et al. [6] showed that repeated administration of *G. biloba* (50 and 100 mg/kg/day) induced anxiolytic-like activity; however, the performance of the passive avoidance task and the animals' response to electric shock were not affected in aged animals.

Rocher et al.[14] demonstrated that chronic treatment with *G. biloba* after ischemia were compensated pyramidal cell damage in the CA1 region of the hippocampus. Also in all sham operated animals, the astrocytes defined by glial fibrillary acidic protein (GFAP) immunoreactivity appeared to be completely quiescent. On the other hand, GFAP immunoreactivity increased markedly in the CA1 area of gerbils that underwent an ischemic insult. This characteristic microglial activation was attenuated in animals treated with EGb 761. Their immunohistological findings show that

neuroprotection by EGb 761 is accompanied by a reduction in astrogliosis and a decrease in GFAP immunoreactivity [14].

Most histological studies evaluated the effect of *G. biloba* on neurons; therefore, some related studies about the effects of *G. biloba* on nerve tissue are as follows.

The neuroprotective effect of *G. biloba* has been demonstrated in several *in vitro* and *in vivo* models. Ahlemeyer and Krieglstein [5] showed some *in vitro* and *in vivo* effects of *G. biloba*. They reported that 10-100 µg/ml of Ginkgo protected cultured neurons against death induced by hypoxia, hydrogen peroxide, glutamate, verapamil, amyloid β (Aβ), nitric oxide (NO), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and cyanide. *In vivo*, reduction of neuronal damage by Ginkgo [10-100 mg/kg, PO (per os), or IP] has been observed after transient middle cerebral artery occlusion in rats and gerbils, focal cerebral ischemia in mice and rats, hypoxia, heat stress, subchronic cold stress, amphetamine-induced behavioral sensitization and in a transgenic mouse model of amyotrophic lateral sclerosis [5].

Takuma et al. [15] examined the effect of *G. biloba* extract (EGb 761) on cognitive dysfunction and neuromorphological change in ovariectomised (OVX)/stress subjected rats. Their results showed that treatment with EGb 761 improved memory impairment and neuronal loss of the hippocampus in the OVX/stress-subjected group in the same ways as 17-β-estradiol. Microscopic images of the Nissl stained hippocampal CA3 region showed that chronic treatment with EGb 761 (50 mg/kg) over 2 months improved the chronic stress-induced neuronal cell loss in OVX rats to the levels of the sham-operated controls. Chronic treatment with EGb 761 specifically protected against neuronal cell loss induced by the combination of repeated restraint stress and OVX in the CA3 region, and did not affect Nissl-positive cell numbers in the CA1 region and the dentate gyrus of the hippocampus, in which the combination of stress and OVX did not cause neuronal cell loss [15].

Mallet et al. [16] characterized the neural effects of an extract of *G. biloba* by using c-Fos immunoreactivity (Fos-IR) in rats to quantify functional activity in several brain regions known to be involved in learning and memory. Their results showed that oral administration of EGb761 (150 mg/kg) increased Fos-IR moderately, but significantly, in the insular cortex and amygdala. The intraperitoneal administration of EGb761 also significantly increased Fos-IR in several brain regions including the prelimbic and insular cortices, nucleus accumbens (shell region), lateral septal nucleus

(ventral part), thalamus (paraventricular nucleus), amygdala (central nucleus) and dentate gyrus (EGb761, 5 mg/kg), bed nucleus of the stria terminalis (BNST) lateral division (dorsal) (EGb761, 2/5 and 5 mg/kg) [16].

Another report, by Rocher et al. [14], provided evidence that *G. biloba* extract improves memory processes and normalizes cognitive deficits in experimental paradigms, including the Morris water maze in a transgenic mouse model of Alzheimer's disease or in aged rats, scopolamine- or stress-induced memory deficits in rats and operant conditioning protocols in mice [14].

Therefore, we concluded that pretreatment and treatment injection of *G. biloba* extract can have a protective effect for astrocytes in all areas of the hippocampal formation. On the other hand, *G. biloba* extract can compensate for the reduction in number of astrocytes in the hippocampus before or after encounter with scopolamine.

Acknowledgements

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