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SHORT COMMUNICATION

The Antioxidant Activity of Standardized Extract of *Ginkgo biloba* (EGb 761) in Rats

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The standardized extract of *Ginkgo biloba* (EGb 761) has been widely employed for its significant benefit in neurodegenerative disorders. Although antioxidative actions have been attributed to this extract, the mechanisms of the multiple principles involved in this pharmacological activity are not completely established. Parkinson's and Alzheimer's diseases are frequently associated with oxidative stress and defects in the cellular protective mechanisms. In this study, the lipid peroxidation (LPO) and the activity of the antioxidant enzymes, catalase (CAT) and superoxide dismutase (SOD) were evaluated in the hippocampus, striatum and substantia nigra (SN) of rats treated with EGb 761. An increase in the CAT and SOD activities in the hippocampus, striatum and SN, and a decrease of the LPO in the hippocampus were observed. These data are additional to the antioxidant properties of EGb 761 reported in the literature and indicate a possible role for the extract in the treatment of diseases involving free radicals and oxidative damage. Copyright © 2001 John Wiley & Sons, Ltd.

Keywords: reactive oxygen species; oxidative stress; *Ginkgo biloba*; neurodegenerative diseases; antioxidant enzymes.

INTRODUCTION

The EGb 76I extract is prepared from the leaves of *Ginkgo biloba* according to a well-defined procedure. This extract is standardized to contain 24% flavonoids and 6% terpene lactones (ginkgolides and bilobalide) which are responsible for some pharmacological activities (DeFeudis, 1991; Joyeux *et al.*, 1995). The flavonoids are probably responsible for the protective effects against free radical damage and lipid peroxidation. Nevertheless, it is not clearly established whether the terpene lactones or other compounds are responsible for these effects as well (Joyeux *et al.*, 1995). The EGb 761 extract has been reported to be a scavenger for several reactive oxygen species (ROS), i.e. superoxide, hydroxyl and peroxyl radicals (Maitra *et al.*, 1995). Studies implicate this extract as a potential agent for protecting neurons from oxidative stress induced by hydrogen peroxide (Oyama *et al.*, 1996). There is much evidence to support the concept that ROS generated from oxidation reactions contribute to the pathogenesis of neurodegenerative disorders, including Parkinson's disease (PD) and Alzheimer's disease (AD). Studies of antioxidant defence systems in PD and AD showed an increase of LPO and a decrease in the activity of the antioxidant enzymes in some cerebral structures (Bowling and Beal, 1995; Gsell *et al.*, 1995; Jenner *et al.*, 1992). Cellular defences that protect against the production of ROS include enzymes such as catalase and superoxide dismutase. Catalase is a hemeprotein with specific activity on hydrogen peroxide (H_2O_2) and SOD is specific for superoxide radical (O_2^-) as a catalytic substrate (Halliwell and Gutteridge, 1990). In this work, we report the effects of EGb 761 on lipid peroxidation (LPO) in the hippocampus and on the activity of the enzymes, CAT and SOD in the hippocampus, striatum and substantia nigra (SN) of rats.

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MATERIALS AND METHODS

Animals. Adult male Wistar rats (weight 250–350 g) from our breeding colony were used. The animals were housed in plastic cages, five in each cage, under a 12 h light/dark cycle (lights on at 7:00 a.m.) at a constant temperature of $23° \pm 1$ °C with free access to standard certified rodent diet and tap water.

Drugs and reagents. The extract of *Ginkgo biloba*, EGb 761 (lot 1430H) was supplied by Dr Willmar Schwabe Co. (Arzneimittel). Xanthine oxidase (EC 1.1.3.22) and cytochrome *c* (EC 1.1.2.3) were purchased from Boehringer Mannheim. Catalase (EC 1.11.1.6) and cumene hydroperoxide (HC) from Sigma. The standard laboratory reagents were all "A" grade purchased from Sigma and Merck.

Treatment. The animals were divided in four treatment groups (n = 4–5). Group 1 (EGb) received EGb 761 extract (100 mg/kg; per os) daily for 14 consecutive days. The extract was dissolved (partially resuspended) in 0.9% NaCl. Group 2 (control) received 0.9% NaCl, per os,

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daily for 14 consecutive days. Group 3 (EGb/HC) received EGb in the same way as group 1 and during the last 6 days of the treatment the animals simultaneously received 80% HC (35 mg/kg; i.p). Group 4 (control HC) received 0.9% NaCl, per os, daily for 14 consecutive days and during the last 6 days of the treatment the animals simultaneously received 80% HC (35 mg/kg; i.p.).

Cerebral structures isolation. The animals were decapitated 24 h after the last day of the treatment and their brains quickly removed. The hippocampus, striatum and substantia nigra were identified and dissected freely. The total time spent for the tissue isolation was less than 3 min. The tissues were frozen in liquid N_2 and stored at $-80\,^{\circ}C$ until analysis.

Tissue preparation. The brain tissues were homogenized by ultrasonic disintegration using 10 mm potassium phosphate buffer pH 7.6 (1:10). The samples were centrifuged at 3000 rpm for 10 min at $+4\,^{\circ}C$ and the supernatant removed and stored at $-20\,^{\circ}C$ until analysis.

Biochemical methods. The levels of thiobarbituric acid reactive substances (TBARS) were determined in the hippocampus by the spectrophotometrical assay according to Buege and Aust (1978). The catalase activity was determined in the hippocampus, striatum and substantia nigra by decomposition of H_2O_2 (240 nm) according to the method described by Beers and Sizer (1952). The total SOD activity was determined spectrophotometrically at 550 nm in the hippocampus, striatum and substantia nigra using the cytochrome *c* reduction assay according to McCord and Fridovich (1969). One unit of SOD is arbitrarily defined as the amount necessary to cause an inhibition of 50% of cytochrome *c* reduction (Fridovich, 1975). The protein was determined according to Lowry *et al.* (1951) using bovine albumin as a reference.

Statistical analysis. The data were analysed by one-way ANOVA followed by Student–Newman–Keuls multiple range test. The significance level was set at $p<0.05$.

RESULTS AND DISCUSSION

An involvement of oxidative stress in neurodegenerative diseases suggests an inability of the normal protective mechanism to inactivate toxic radical species and an increase in lipid peroxidation in the brain of patients (Markesbery, 1997; Jenner *et al.*, 1992). In this study, we evaluated the antioxidant effect of EGb 761 extract in the hippocampus and striatum/substantia nigra the major sites of damage in AD and PD (O'Brien *et al.*, 1996; Jenner *et al.*, 1992). The EGb 761 extract effectively reduced the LPO in some experimental models. However, the ability of the extract to enhance free radical scavengers such as catalase and SOD is not yet well established. Our results showed that EGb 761 extract significantly reduced (20%) the levels of TBARS in the hippocampus of the group EGb when compared with the control group (Table 1) suggesting a protective action of the extract, since LPO is regarded as one of the basic mechanisms of tissue damage mediated by reactive oxygen species (Darley-Usmar and Halliwell, 1996). Several studies have demonstrated the antioxidant activity of *Ginkgo biloba* extract in rat brain neurons (Oyama, 1996), macrophages and vascular endothelial cells (Rong *et al.*, 1996). Our results demonstrated a significant increase in the CAT activity in the hippocampus (22%), striatum (23%) and substantia nigra (14%) of the EGb group when compared with the control group (Table 2). The SOD levels were significantly elevated in the hippocampus (60%), striatum (30%) and substantia nigra (22%) of the EGb group when compared with the control group (Table 3). Conflicting results have been obtained in the studies of antioxidant defence systems in PD and AD, however, in some of these studies a decrease in the CAT and SOD levels in the brains of PD and AD patients were reported (Multhaup *et al.*, 1997;

Table 1. Levels of thiobarbituric acid reactive substances (TBARS) in the hippocampus of the groups EGb (100 mg/kg of EGb 761; per os, daily for 14 consecutive days), control (0.9% NaCl, per os, daily for 14 consecutive days), EGb/HC (100 mg/kg of EGb 761 per os, daily for 14 consecutive days and during the last 6 days of treatment the animals simultaneously received 80% HC - 35 mg/kg; i.p.) and control HC (0.9% NaCl, per os, daily for 14 consecutive days and during the last 6 days of treatment the animals simultaneously received 80% HC - 35 mg/kg; i.p.)

Group	TBARS
Control	43.6 ± 1.980
EGb	34.9 ± 1.267[a]
Control HC	43.2 ± 1.353
EGb/HC	49.1 ± 0.506

Mean ± SEM (ηmol/mL).
[a]Statistically significant for comparisons with control group; (ANOVA $F_{(3,17)} = 14.03$; $p<0.001$).

Table 2. The CAT activities in the hippocampus, striatum and substantia nigra of the groups EGb (100 mg/kg of EGb 761; per os, daily for 14 consecutive days), control (0.9% NaCl, per os, daily for 14 consecutive days), EGb/HC (100 mg/kg of EGb 761 per os, daily for 14 consecutive days and during the last 6 days of treatment the animals simultaneously received 80% HC - 35 mg/kg; i.p.) and control HC (0.9% NaCl, per os, daily for 14 consecutive days and during the last 6 days of treatment the animals simultaneously received 80% HC - 35 mg/kg; i.p.)

Group	Catalase activity		
	Hippocampus[1]	Striatum[2]	SN[3]
Control	4.32 ± 0.1725	3.40 ± 0.1207	5.45 ± 0.1333
EGb	5.28 ± 0.1565[a]	4.20 ± 0.0573[a]	6.22 ± 0.0553[a]
Control HC	4.17 ± 0.0959	2.59 ± 0.0835[a]	4.31 ± 0.1670[a]
EGb/HC	4.22 ± 0.0885	4.34 ± 0.1109[b]	5.06 ± 0.1957[b]

Mean ± SEM (U/mg protein).
[a]Statistically significant for comparisons with Control group.
[b]Statistically significant for comparisons with control HC group; (ANOVA $^1F_{(3,18)} = 14.70$; $^2F_{(3,19)} = 71.10$; $^3F_{(3,16)} = 24.60$; $p < 0.001$).

Table 3. The SOD activities in the hippocampus, striatum and substantia nigra of the groups EGb (100 mg/kg of EGb 761 ; per os, daily for 14 consecutive days), control (0.9% NaCl, per os, daily for 14 consecutive days), EGb/HC (100 mg/kg of EGb 761 per os, daily for 14 consecutive days and during the last 6 days of treatment the animals simultaneously received 80% HC - 35 mg/kg; i.p.) and control HC (0.9% NaCl, per os, daily for 14 consecutive days and during the last 6 days of treatment the animals simultaneously received 80% HC - 35 mg/kg; i.p.)

Group	SOD activity		
	Hippocampus[1]	Striatum[2]	SN[3]
Control	25.4 ± 1.067	30.7 ± 1.485	29.1 ± 1.460
EGb	40.7 ± 1.666^a	40.0 ± 0.761^a	35.7 ± 1.419^a
Control HC	16.2 ± 0.719^a	20.6 ± 1.036^a	20.9 ± 0.889^a
EGb/HC	22.9 ± 0.750^b	29.6 ± 1.389^b	23.9 ± 1.269^b

Mean $\pm$ SEM (U/mg protein).
[a] Statistically significant for comparisons with control group.
[b] Statistically significant for comparisons with control HC group; (ANOVA $^1F_{(3,19)} = 85.90$; $^2F_{(3,19)} = 43.50$; $^3F_{(3,16)} = 23.00$; $p < 0.001$).

Bowling and Beal, 1995). In this way the use of antioxidant agents can minimize tissue injury and slow the degenerative process in several neurodegenerative diseases (Bowling and Beal, 1995; Halliwell, 1991). Cumene hydroperoxide, used as an oxidant agent, showed no effect on LPO (Table 1) or on CAT activity in the hippocampus (Table 2). On the other hand, our results demonstrated a decrease in CAT (Table 2) and SOD (Table 3) activities in the striatum 23% and 32%, respectively and substantia nigra 20% and 28%, respectively in the control HC group when compared with the control group. These results could indicate a selective action of HC in the brain structures or that the treatment time was insufficient to promote oxidative damage in all the structures. This is possible since some structures are more susceptible to an oxidative environment due, among other reasons, to the high concentration of iron that can enhance oxidation reactions and the formation of free radicals (Bowling and Beal, 1995). The results showed that the EGb 761 extract reversed the toxic effects produced by HC in the striatum and substantia nigra. It was observed through an increase in CAT (Table 2) and SOD (Table 3) activities in the EGb/HC group when compared with the control HC group. We demonstrated that EGb 761 could increase the antioxidant enzyme CAT and SOD activities in the hippocampus, striatum and substantia nigra in rats and decrease LPO in the hippocampus. In recent years, considerable data have accrued indicating that the brain in AD and PD is under increased oxidative stress and this may have a role in the pathogenesis of neuron degeneration and death in this disorder (Markesbery, 1997; Jenner, 1996). These data are an addition to the antioxidant properties of EGb 761, reported in the literature and indicate a possible role for the extract in the treatment of diseases involving free radicals and oxidative damage.

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