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Acute Administration of *Ginkgo biloba* Extract (EGb 761) Affords Neuroprotection Against Permanent and Transient Focal Cerebral Ischemia in Sprague-Dawley Rats

E-Jian Lee,[1]* Hung-Yi Chen,[2] Tian-Shung Wu,[2] Tsung-Ying Chen,[1] Issam A. Ayoub,[3] and Kenneth I. Maynard[3]

[1]Neurophysiology Laboratory, Neurosurgical Service, Departments of Surgery and Anesthesiology, National Cheng Kung University Medical Center and Medical School, Tainan, Taiwan
[2]Institute of Chemistry, National Cheng Kung University, Tainan, Taiwan
[3]Neurophysiology Laboratory, Neurosurgical Service, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts

We examined the neuroprotective action of a standardized extract of *Ginkgo biloba* leaves (EGb 761) in permanent and transient middle cerebral artery (MCA) occlusion models in Sprague-Dawley rats. Forty-four animals were given either EGb 761 (50–200 mg/kg) or vehicle intraperitoneally, 1 hr before permanent MCA occlusion, to evaluate the dose-response effects. An additional 58 animals received EGb 761 (200 mg/kg) or vehicle, 0.5–4 hr after permanent MCA occlusion, for establishing the therapeutic window. Delayed treatment was also employed in 110 animals treated with either EGb 761 (100–200 mg/kg) or vehicle at 2–3 hr following transient focal cerebral ischemia induced by MCA occlusion for 2 hr. Neurobehavioral scores were determined 22–24 hr after permanent MCA occlusion and either 3 or 7 days after transient MCA occlusion, and brain infarction volumes were measured upon sacrifice. Local cortical blood flow (LCBF) was serially measured in a subset of animals receiving EGb 761 (100–200 mg/kg) or vehicle, 0.5 hr and 2 hr after permanent and transient MCA occlusion, respectively. Relative to vehicle-treated controls, rats pretreated with EGb761 (100 and 200 mg/kg) had significantly reduced infarct volumes, by 36% and 49%, respectively, and improved sensory behavior ($P < 0.05$). Delayed treatment with EGb 761 also significantly reduced brain infarction, by 20–29% and 31%, when given up to 2 and 3 hr following transient and permanent MCA occlusion, respectively, whereas improved neurobehavioral scores were noted up to 2 hr after the onset of MCA occlusion ($P < 0.05$). LCBF was significantly improved in the ipsilateral cortex following the EGb 761 treatment, whereas a higher dose showed a more sustained effect. In conclusion, EGb 761 protected against transient and permanent focal cerebral ischemia and was effective after a prolonged reperfusion period even when therapy is delayed up to 2 hr. This neuroprotection may be at least partially attributed to the beneficial effects of selectively improved LCBF in the area at risk of infarction.

Key words: stroke; focal cerebral ischemia; neuroprotection; *Ginkgo biloba* extract; EGb 761

Cerebral ischemia begins as an imbalance of the reduced energy supply (e.g., during hypoperfusion) and the high energy demands (e.g., Na^+,K^+-ATPase pump). The consequence of the energy imbalance is the depletion of ATP, which not only increases the susceptibility of brain tissues to oxidative stress but also triggers the onset of numerous ischemic cascades, leading to neuronal death (Siesjö, 1992; Small and Buchan, 1996; Ames, 2000). One strategy, therefore, to protect the brain against ischemic damage is to improve the energy supply to the tissues "at risk," and/or to reduce the energy demands of the neuronal tissue (Ames et al., 1995; Maynard et al., 1998, 1999; Ayoub et al., 1999; Lee et al., 1999). It is also presumed that the multifactorial pathogenicity of cerebral ischemia may demand a multimode therapeutic approach. A therapeutic modality with multiple synergistic mechanisms of action has therefore been suggested, by us and by others, to protect against various signaling cascades that are unevenly distributed and act at different times during cerebral ischemia (Kempski, 1994; Ames et al., 1995; Barinaga, 1996; Fisher, 1997; Onal and Fisher, 1997; Grunewald and Beal, 1999; Lee et al., 1999).

*Correspondence to: Dr. E-Jian Lee, Neurosurgical Service, Department of Surgery, National Cheng Kung University Medical Center and Medical School, 138 Sheng-Li Road, Tainan 70428, Taiwan.
E-mail: ejian@mail.ncku.edu.tw

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The standardized extract EGb 761, a natural product purified from dried green leaves of the *Ginkgo biloba* tree, has already been used clinically to treat dementia and vasoocclusive and cochleovestibular disorders (Oken et al., 1998; Diamond et al., 2000). The agent is composed of a complex mixture of ingredients (i.e., 24% flavonoid glycosides and 4–6% terpenoids) of relatively low molecular weights that potentially permit penetration of the blood–brain barrier and, thus, may exhibit a broad spectrum of pharmacological activities in the CNS (White et al., 1996).

EGb 761 is known to improve damaged neuronal energy metabolism in rats subjected to intracerebral injections of streptozotocin (Hoyer et al., 1999), to antagonize glutamate-induced neurotoxicity (Zhu et al., 1997; Kobayashi et al., 2000), and to protect Na^+,K^+-ATPase activity against cerebral ischemia (Pierre et al., 1999). The terpenoid component of the agent has the potential to modulate cerebral hemodynamics during brain ischemia (Oberpichler et al., 1988; Zhang et al., 2000) and can inhibit the platelet-activating factor-induced platelet aggregation (Smith et al., 1996; Akiba et al., 1998; Akisu et al., 1998). Additionally, the flavonoid component is known to be a potent antioxidant (Droy-Lefaix et al., 1995; Oyama et al., 1996; Bastianetto et al., 2000) and free radical scavenger (Maitra et al., 1995; Ni et al., 1996; Kim et al., 1998) and can inhibit nitric oxide synthesis activation following brain ischemia (Calapai et al., 2000). Moreover, pretreatment with oral administration of EGb 761 has been demonstrated to protect against transient focal cerebral ischemia (Calapai et al., 2000; Zhang et al., 2000; Clark et al., 2001). Accordingly, we hypothesized that acute administration of EGb 761 may protect the brain against ischemia/reperfusion-induced brain injury.

Although EGb 761 has already been reported to protect against transient focal ischemia by reducing cerebral infarction, we have examined here whether brain infarction and neurologic behavioral outcome are protected with acute administration of EGb 761 in rats subjected to permanent and transient focal cerebral ischemia. Moreover, in this study, we sought to determine the crucial therapeutic window of opportunity for neuroprotection and also to determine whether the neuroprotective effect is long-lasting. Finally, we have begun to address a possible mechanism of action for EGb 761 by testing its effect on local cerebral blood flow.

MATERIALS AND METHODS

All procedures performed were approved by the Subcommittee on Research Animal Care of the University Medical Center, whose standards meet the guidelines of the National Institutes of Health (Guide for the Care and Use of Laboratory Animals).

Drug Preparation

EGb 761 was dissolved in a mixture of PEG 400 (Sigma Chemical Co., St. Louis, MO) and distilled water (30:70) during experiments with permanent focal cerebral ischemia and prepared in 45% aqueous hydroxypropyl-β-cyclodextrin (HPBC; Sigma Chemical Co.) for rats subjected to transient focal cerebral ischemia. Fresh drug solutions were prepared daily. The controls received the vehicle only.

Animal Preparation, Anesthesia, and Monitoring

Male Sprague-Dawley rats, weighing 230–270 g, were supplied by the University Laboratory Animal Center, and were allowed free access to food and water before and after surgery. Animals were anesthetized with 1–2% halothane in 60% N_2O/40% O_2. Body temperature was maintained at 37°C ± 0.5°C using a heating pad and rectal probe (Harvard Apparatus, Cambridge, MA). The right femoral artery was cannulated for measuring arterial blood gases, glucose, hematocrit, and blood pressure.

Experimental Model

Focal cerebral ischemia was induced by intraarterial suture occlusion of the proximal right middle cerebral artery (MCA; Belayev et al., 1996; Takano et al., 1997). Briefly, the bifurcation of the right common carotid artery was exposed under an operating microscope. A 4-0 nylon suture, with its tip rounded by heating over a flame and subsequently coated with silicone (Merck KGaA, Darmstadt, Germany), was then advanced 17.5–18.5 mm from the external into the internal carotid artery until the tip occluded the origin of the MCA. After closure of the operative sites, the animals were allowed to awaken from the anesthesia and temporarily transferred to a cage with a heating lamp. During another brief period of anesthesia, the suture was gently removed at 2 hr of MCA occlusion in rats subjected to transient focal cerebral ischemia. Reperfusion was ensured by an improvement in ipsilateral local cortical blood flow (LCBF) to at least 50% of baseline following an initial sharp decrease to about 20% of baseline caused by MCA occlusion as determined by laser Doppler flowmetry (LDF; Laserflo BMP^2; Vasamedics, St. Paul, MN). After the surgical procedures, the animals were kept in a cage with a heating lamp for another 4 hr and then transferred into the home cage.

Animal Sacrifice and Quantification of Ischemic Damage

Sacrifice was performed 22–24 hr after permanent MCA occlusion and either 3 or 7 days after transient MCA occlusion by decapitation under ketamine (44 mg/kg, i.p.) and xylazine (13 mg/kg, i.p.) anesthesia. The brain was then rapidly removed, cut into 2 mm coronal sections using a rat brain matrix (RBM 4000C; ASI Instrument, Inc., Warren, MI), and stained according to the standard 2,3,5-triphenyltetrazolium chloride (TTC) method (Bederson et al., 1986a). Each slice was drawn using a semiautomated computerized image analyzer (MCID; Imaging Research Inc., Ontario, Canada). The calculated infarction areas were then compiled to obtain the infarct volumes per brain (in cubic micrometers). Infarct volumes were expressed as a percentage of the contralateral hemisphere volume by using an "indirect method" [area of intact contralateral (left) hemisphere minus area of intact region of the ipsilateral (right) hemisphere] to compensate for edema formation in the ipsilateral hemisphere (Swanson et al., 1990).

Drug Administration and Grouping of Animal

In the first set of experiments, animals received either EGb 761 (50, 100, or 200 mg/kg, i.p.; n = 28) or vehicle (the same

volume of distilled water-PEG 400, i.p.; n = 16) 1 hr before MCA occlusion to test the neuroprotective in vivo dose-response relationship of EGb 761. The data showed that pretreatment with EGb 761 (200 mg/kg) resulted in optimal neuroprotection in rats subjected to MCA occlusion. In the second series of experiments, animals were therefore assigned to receive an i.p. injection of either EGb 761 (200 mg/kg, n = 35) or time- and volume-compatible vehicle (distilled water-PEG 400, n = 23) at 0.5, 2, 3, 4 hr after MCA occlusion to test the window of opportunity. A subset of animals was assigned to receive either EGb 761 (100 mg/kg, n = 6; or 200 mg/kg, n = 6) or vehicle (distilled water-PEG 400, n = 6) 0.5 hr after MCA occlusion to study the effects of EGb 761 on LCBF following cerebral ischemia. In a third series of experiments, animals were subjected to 2 hr of right MCA occlusion. EGb 761 (100 mg/kg, n = 26; or 200 mg/kg, n = 25) in the treated animals or the same volume of HPBC that served as the vehicle control (n = 41) was administered i.p. at the time of reperfusion. The rats were assigned to one of two follow-up groups, which were euthanized after either 3 or 7 days. Measurements of LCBF were serially conducted up to 3 days after reperfusion. In the fourth set of experiments, animals received either EGb 761 (200 mg/kg, i.p.; n = 9) or vehicle (the same volume of HPBC, i.p.; n = 9) 3 hr after 2 hr of MCA occlusion (i.e., at 1 hr after reperfusion), and reperfusion was allowed for 7 days.

LCBF Monitoring

LDF was used for LCBF measurements. The scalp was incised along the midline, and two 2-mm areas in bilateral parietal bones were thinned 1.5 mm posterior and 6 mm lateral to the bregma for placement of the LDF probe (model P436). The dura was left intact to prevent cerebrospinal fluid leakage. LDF probes held in place by a micromanipulator were advanced to touch gently the thinned areas. The signal was allowed to stabilize over a 30 min period before a baseline reading was taken. LCBF was serially measured prior to, during, and after cerebral ischemia. The incision was stapled, and the animals were then returned to their cages. Additional measurements were employed at 22–24 hr after permanent MCA occlusion and upon a brief period of reperfusion and at 24 and 72 hr postischemia in animals subjected to transient MCA occlusion. The animals were reanesthetized with 1–2% halothane in 60% N_2O/40% O_2, and stable LCBF recordings were obtained bilaterally at the same sites for at least 15 min. The LCBF values were calculated and expressed as a percentage of the baseline values.

Neurobehavioral Testing and Follow-Up Periods

Neurologic evaluation and body weight measurement were conducted prior to and at 22–24 hr after permanent MCA occlusion and on a daily basis up to 3 or 7 days after transient MCA occlusion. A modification of previously published methods was used to evaluate the sensorimotor integrity (Bederson et al., 1986b;Belayev et al., 1996). Accordingly, five categories of motor neurologic findings were scored: 0, no observable deficit; 1, forelimb flexion; 2, forelimb flexion and decreased resistance to lateral push; 3, forelimb flexion, decreased resistance to lateral push and unilateral circling; 4, forelimb flexion, unable or difficult to ambulate. The affected forelimb also received forward and sideways visual placing tests, which were scored as follows: 0, complete immediate placing; 1, incomplete and/or delayed placing (<2 sec); 2, absence of placing.

Statistical Analysis

All data are expressed as the mean ± SEM. Paired Student's *t*-test was used to evaluate the response to a change in conditions, and unpaired Student's *t*-test or one-way ANOVA with either Dunnett's or Fisher's protected least significant difference (LSD) post hoc comparison was used to evaluate differences between groups. Neurobehavioral scores were analyzed by a nonparametric test for independent groups, i.e., the Kruskal-Wallis/Mann-Whitney U-test. The LCBF values for each group were analyzed among groups at each sampling time by repeated ANOVA, followed by Dunnett's post hoc tests. $P < 0.05$ was selected for statistical significance.

RESULTS

As previously described (Bederson et al., 1986b; Belayev et al., 1996; Takano et al, 1997; Lee et al., 1999; Mokudai et al., 2000), permanent and transient MCA occlusion results in large ipsilateral cerebral striatal and cortical infarcts that are reproducible but variable in size. Throughout the whole study, 11 animals (9.2%) died spontaneously prior to completion of the recovery protocol for permanent focal cerebral ischemia and were excluded: Two were in vehicle-treated groups, and nine were evenly distributed among the EGb 761-treated groups. However, post-mortem examinations failed to reveal the occurrence of intracerebral or subarachnoid hemorrhage in any of these animals. The mortality was 17.3% in rats subjected to transient focal cerebral ischemia. Nineteen animals, of which eight were vehicle-injected and 11 were EGb 761-treated, died before completion of the recovery protocol; one had subarachnoid hemorrhage and was therefore not included in the 90 animals used for data analysis. Five animals (4.5%) subjected to transient focal cerebral ischemia, of which one (2%) was vehicle treated, one (2.9%) was EGb 761-treated at 100 mg/kg, and the other three (8.8%) were EGb 761-treated at 200 mg/kg, showed very tiny intracerebral hemorrhage (ICH) within the infarct, whereas no ICH was seen in any group subjected to permanent focal cerebral ischemia.

Animals that received an i.p. injection of EGb 761, at a dosage of 100 mg/kg (n = 9) or 200 mg/kg (n = 8), but not at 50 mg/kg (n = 8), 1 hr before permanent MCA occlusion, showed significant infarct size reductions ($P < 0.05$) compared with vehicle-injected controls (n = 15). Infarction lesions were reduced by 36% and 49% in the groups treated with EGb 761 at 100 mg/kg and 200 mg/kg, respectively (Fig. 1). Each group of animals pretreated with EGb 761, however, showed significantly improved sensory neurologic scores 22–24 hr after the onset of permanent MCA occlusion over the vehicle-injected controls ($P < 0.05$; Table I). Additionally, significantly less body weight loss was observed 22–24 hr after the insult in the animals pretreated with EGb 761, at 200 mg/kg, compared with vehicle-injected animals ($P < 0.05$). The physiological parameters of the animals were kept within

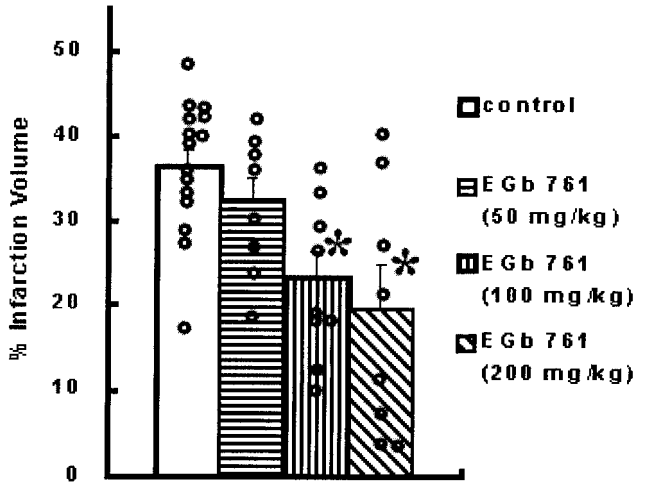

Fig. 1. Infarct volumes were significantly reduced with EGb 761 treatment at 100 mg/kg (n = 9) and 200 mg/kg (n = 8), but not at 50 mg/kg (n = 8), compared with vehicle-treated controls (n = 15). The infarct volumes are expressed as a percentage of the contralateral (control) hemisphere, and data are represented as a superimposed scatterplot showing the infarction volume for each animal in the group as well as mean ± SEM. $\star P < 0.05$ vs. distilled water-PEG 400-treated rats. n, number of animals.

normal physiologic limits and were not significantly different during the experimental course, except for a moderate enhancement in the pre- and postocclusion blood glucose, as well as a postocclusion reduction in pCO_2 observed in the group treated with EGb 761 (200 mg/kg; Table II).

In the delayed treatment study, our results indicate that EGb 761 (200 mg/kg) resulted in a significant reduction in the infarct volumes when given up to 3 hr after permanent MCA occlusion ($P < 0.05$). Infarction lesions were reduced by 37%, 27%, and 31%, respectively, when EGb 761 was given at 0.5, 2, and 3 hr after permanent MCA occlusion (Fig. 2). Additionally, delayed treatment with EGb 761 significantly improved motor scores when administered at 0.5 hr after permanent MCA occlusion and sensory neurologic scores when administered up to 2 hr following the insult ($P < 0.05$) but was ineffective in reduction of postischemic body weight loss. However, neither the infarction volumes nor the deficits of sensorimotor behaviors were significantly improved when EGb 761 (200 mg/kg) was given at 4 hr after the onset of permanent MCA occlusion (Table I). The physiological parameters were within normal limits during the experiment of permanent MCA occlusion and did not statistically differ between study and control animals (data not shown).

Two hours of MCA occlusion caused lesions that were substantially smaller compared with permanent MCA occlusion. After a 3 day recovery period, the infarct volumes were significantly reduced ($P < 0.05$) by 28% and 29% when EGb 761 at 100 or 200 mg/kg, respectively, was given at the time of reperfusion (Fig. 3). The infarct volume reduction with the same paradigm of EGb 761 treatment, however, was 20–21% ($P < 0.05$) following a 7 day recovery period (Figs. 4, 5). Additionally, infarction lesions were not significantly reduced when EGb 761 (200 mg/kg) was given 1 hr after reperfusion (control group, 30.3% ± 2.5%; EGb 761-treated group, 29.6% ± 2.5%; $P > 0.05$).

Rats treated with EGb 761 (100 and 200 mg/kg) 2 hr after transient MCA occlusion had improved sensory recovery following a 3 day recovery period (Table III). However, only in rats treated with EGb 761 (200 mg/kg) at the time of reperfusion were the motor and sensory neurologic scores significantly improved ($P < 0.05$) following a 7 day recovery period (Table III). Additionally, delayed treatment with EGb 761 (200 mg/kg) at the time of reperfusion also tended to improve ($P = 0.06$) weight gain following a 7 day recovery period (Table III). The physiological parameters were within normal limits before, during, and after ischemia and did not statistically differ between study and control animals (data not shown).

The ipsilateral LCBF was significantly higher at various times after permanent and transient MCA occlusion in the two groups treated with EGb 761 (100 and 200 mg/kg) compared with the vehicle-injected controls ($P < 0.05$). EGb 761 (100 mg/kg) induced an acute and transient increase in LCBF 45–70 min following the onset of permanent MCA occlusion (Fig. 6A). The higher dose showed a delayed but more sustained increase over time. In contrast, the LCBF of the contralateral cortex was not significantly different among the three groups and was independent of the dose of EGb 761 (Fig. 6B). In transient focal cerebral ischemia, treatment with EGb 761 (100 or 200 mg/kg) at the time of reperfusion not only resulted in a sustained increase in LCBF, occurring from 30 min up to 22 hr after reperfusion, in the ipsilateral cortex (Fig. 7A) but also prevented a transient decrease in LCBF, observed 30 min after reperfusion, in the contralateral cortex (Fig. 7B). The other physiological parameters were within normal limits during the experiment of MCA occlusion and did not statistically differ between study and control animals (data not shown).

DISCUSSION

Our results confirm that EGb 761 reduced the cerebral infarct volume following focal cerebral ischemia (Calapai et al., 2000; Zhang et al., 2000; Clark et al., 2001). Additionally, we found that EGb 761 could reduce cerebral infarction when administered up to 2 and 3 hr after the onset of transient and permanent focal cerebral ischemia, respectively, and was effective in improving neurobehavioral outcome even with a 2 hr delay of administration. Moreover, delayed treatment with EGb 761 at the time of reperfusion remained effective following a prolonged recovery period. This neuroprotection cannot be accounted for by changes in hemodilution (as measured by blood hematocrit), mean arterial blood pressure, or heart rate or differences in core temperature; these were not significantly different between vehicle-injected and EGb 761-treated animals in either the pretreatment or the

TABLE I. Summary of Neurobehavioral Scores and Body Weight Loss Obtained After Permanent Middle Cerebral Artery Occlusion in Each Group†

	n	Neurobehavioral scores		Body weight loss (g)
		Motor	Sensory	
Pretreatment				
Control	15	2.5 ± 0.2	3.9 ± 0.1	39 ± 1
EGb 761 treated				
50 mg/kg	8	2.4 ± 0.2	2.8 ± 0.3★	38 ± 1
100 mg/kg	9	2.0 ± 0.2	1.7 ± 0.3★	38 ± 1
200 mg/kg	8	2.6 ± 0.4	2.9 ± 1.4★	31 ± 3★
Delayed treatment				
Control	22	2.7 ± 0.1	3.9 ± 0.1	36 ± 2
EGb 761 treated, 200 mg/kg				
0.5 hr	7	2.1 ± 0.1★	3.1 ± 0.3★	33 ± 4
2 hr	8	2.6 ± 0.2	3.5 ± 0.3★	35 ± 3
3 hr	7	2.7 ± 0.3	3.7 ± 0.2	32 ± 4
4 hr	7	2.9 ± 0.3	3.6 ± 0.3	33 ± 3

†Data are represented as mean ± SEM; n = number of animals.
★$P < 0.05$.

TABLE II. Physiologic Parameters Before (Preocclusion) and After (Postocclusion) Permanent Middle Cerebral Artery Occlusion Between Animals Pretreated With EGb 761 and Vehicle (Distilled Water-PEG 400)-Treated Controls†

	n	pH	pCO_2 (mmHg)	pO_2 (mmHg)	Hct	Gluc (mmol/liter)	MABP (mmHg)	HR (beats/min)
Preocclusion								
Control	15	7.39 ± 0.01	40.3 ± 0.5	133.0 ± 6.3	0.392 ± 0.004	7.6 ± 0.4	92 ± 2	338 ± 7
EGb 761								
50 mg/kg	8	7.38 ± 0.01	42.6 ± 1.3	129.3 ± 7.7	0.418 ± 0.006	8.3 ± 0.6	92 ± 5	330 ± 15
100 mg/kg	9	7.36 ± 0.02	38.4 ± 1.0	139.4 ± 9.0	0.404 ± 0.010	8.5 ± 0.8	93 ± 2	341 ± 11
200 mg/kg	8	7.37 ± 0.01	38.3 ± 1.1	130.9 ± 6.5	0.406 ± 0.009	13.7 ± 0.8★★	98 ± 5	334 ± 12
Postocclusion								
Control	15	7.38 ± 0.01	37.5 ± 2.2	141.6 ± 7.4	0.387 ± 0.004	7.2 ± 0.3	94 ± 2	341 ± 8
EGb 761								
50 mg/kg	8	7.37 ± 0.01	41.9 ± 0.7	129.7 ± 5.3	0.399 ± 0.007	7.8 ± 0.4	92 ± 6	334 ± 11
100 mg/kg	9	7.38 ± 0.01	36.2 ± 1.4	143.4 ± 10.8	0.394 ± 0.012	8.2 ± 0.7	94 ± 2	356 ± 9
200 mg/kg	8	7.35 ± 0.01	34.5 ± 1.2★	130.4 ± 7.8	0.405 ± 0.005	11.8 ± 1.1★★	93 ± 5	352 ± 13

†Physiologic data obtained from control and pretreated animal groups are represented as the mean ± SEM. Hct, hematocrit; Gluc, blood glucose; MABP, mean arterial blood pressure; HR, heart rate; n, number of animals. All animals were maintained at 37 ± 0.5°C. Paired Students' *t*-tests were used to evaluate the response to a change in conditions, and one-way ANOVA with Dunnett's *post hoc* comparison was used to evaluate differences between groups.
★$P < 0.05$ vs. preischemic data.
★★$P < 0.05$ vs. control data.

delayed-treatment study. The only changed parameter found in arterial blood gases was a decrease in pCO_2 seen shortly after MCA occlusion in the group with pretreatment of EGb 761 at 200 mg/kg, but the actual values of the pCO_2 were within normal ranges and unlikely to be of significance.

The other significant observation was an enhancement in blood glucose, up to 181% of control values, found in animals pretreated with 200 mg/kg EGb 761 both prior to and shortly after the onset of MCA occlusion. Curiously, hyperglycemia, though modest, was also observed in the other animals with pretreatment of EGb 761 at lower doses but not in animals with delayed administration of EGb 761. Thus, a complementary study was employed in three additional animals, two of which received a delayed treatment of 200 mg/kg of EGb 761 and one of which received the vehicle injection, 2 hr after MCA occlusion. Arterial blood samples were randomly withdrawn within the next 2 hr. The results indicated that delayed treatment with EGb 761 (200 mg/kg) also induced moderate hyperglycemia (data not shown). We reason that the hyperglycemia observed here was relevant to the administration time and the dosing regimen of the EGb 761 treatment but not the events of cerebral ischemia.

In our study, acute administration of EGb 761 increased LCBF after stroke, an effect that was prevalent on the side ipsilateral to the MCA occlusion, whereas the

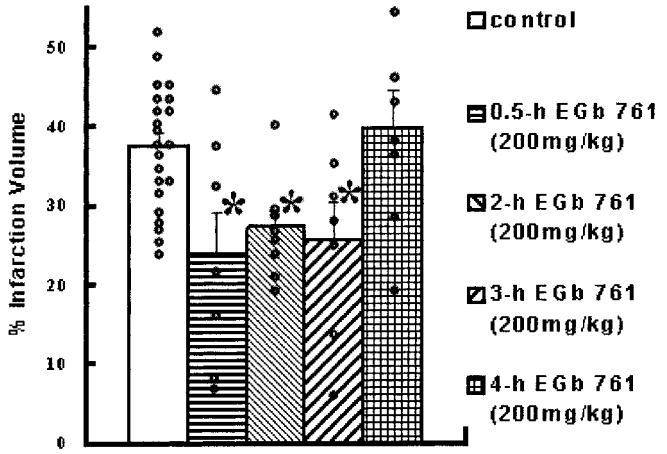

Fig. 2. EGb 761 (200 mg/kg) significantly reduced the infarction volume in animals treated at 0.5 (n = 7), 2 (n = 8), and 3 (n = 7) hr but not at 4 hr (n = 7) after permanent middle cerebral artery (MCA) occlusion, compared with pooled vehicle (distilled water-PEG 400)-treated and time-comparable controls (n = 22). The infarct volumes are expressed as a percentage of the contralateral (control) hemisphere, and the data are represented as mean ± SEM. $\star P < 0.05$; n, number of animals.

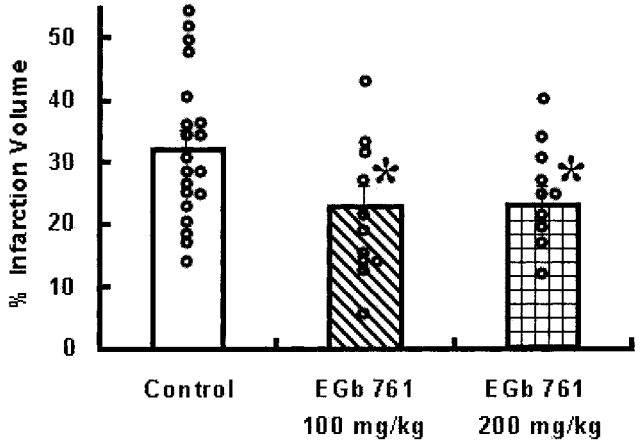

Fig. 3. Relative to those in vehicle-treated controls (n = 20), infarct volumes induced by 2 hr MCA occlusion were significantly reduced with EGb 761 treatment, either at 100 mg/kg (n = 11) or at 200 mg/kg (n = 12), following a 3 day recovery period. Data are expressed as percentage of contralateral (control) hemisphere and are represented as mean ± SEM. $\star P < 0.05$; n, number of animals.

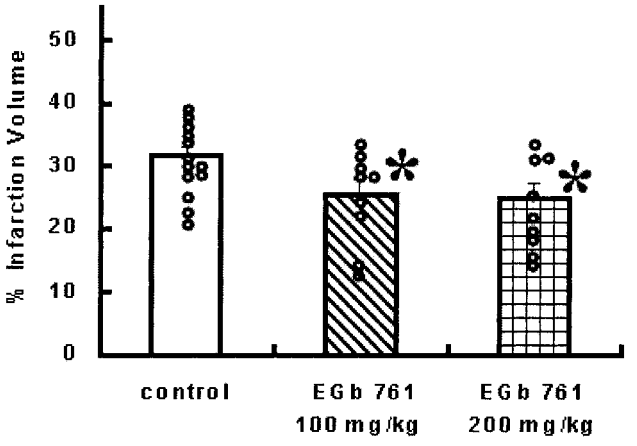

Fig. 4. Relative to those in vehicle-treated controls (n = 13), infarct volumes induced by 2 hr MCA occlusion were significantly reduced with EGb 761 treatment, either at 100 mg/kg (n = 9) or at 200 mg/kg (n = 9), following a 7 day recovery period. Data are expressed as percentage of contralateral (control) hemisphere and are represented as mean ± SEM. $\star P < 0.05$; n, number of animals.

higher dose (200 mg/kg) showed a more sustained increase over time. The mechanism of LCBF effects of EGb 761 observed here is not understood, but several possibilities may be proposed. First, the dose-relevant hyperglycemia might delay a preserving effect on LCBF offered by the higher dose of EGb 761 treatment following permanent MCA occlusion. Given that hyperglycemia has been found to exacerbate cerebral blood flow only in the temporary, and not in the permanent, MCA occlusion model (Quast et al., 1997; Gisselsson et al., 1999), this assumption, however, is not strongly supported. Second, EGb 761 prevents hypoxia-induced ATP decreases in the vascular endothelial cells in vitro (Janssens et al., 1995). This finding suggests that the delayed preserving effect of EGb 761 on the ipsilateral LCBF maybe be secondary to a vascular cytoprotective action. Alternatively, EGb 761 could induce vasodilatation in cerebral arteries in the ischemia/reperfusion brain, although the higher dose appeared to delay this effect following permanent MCA occlusion. Finally, EGb 761 may attenuate the vasospastic response caused by platelet activation in hypoxic/ischemic conditions (Stucker et al., 1997; Akisu et al., 1998), and this may also explain why EGb 761 resulted in an improvement in ipsilateral LCBF after MCA occlusion.

This study focused on determining whether EGb 761 was protective in the in vivo models of stroke in rats and not on determining the mechanism(s) of action by which EGb 761 could be neuroprotective. Indeed, both direct neuroprotective action on neurons and indirect effects on the cerebral vasculature are possible (Krieglstein et al., 1986; Szabo et al., 1995; Sastre et al., 1998; Hoyer et al., 1999; Pierre et al., 1999; Zhang et al., 2000). We used EGb 761 primarily because it rectifies neuronal energy metabolism under stress conditions. Our finding of a prevalent EGb 761-induced increase in LCBF in the tissue at risk of infarct is yet another mechanism by which it could improve the supply of oxygen and glucose, and hence ATP, to the ischemic penumbra.

A therapeutic window of 3 hr with EGb 761 in the permanent MCA occlusion model compares favorably with that with the glutamate receptor antagonist and the basic fibroblast growth factor (Hatfield et al., 1992; Ren and Finklestein, 1997) but not as well with those reported for the calpain inhibitor (MDL 28,170; Markgraf et al.,

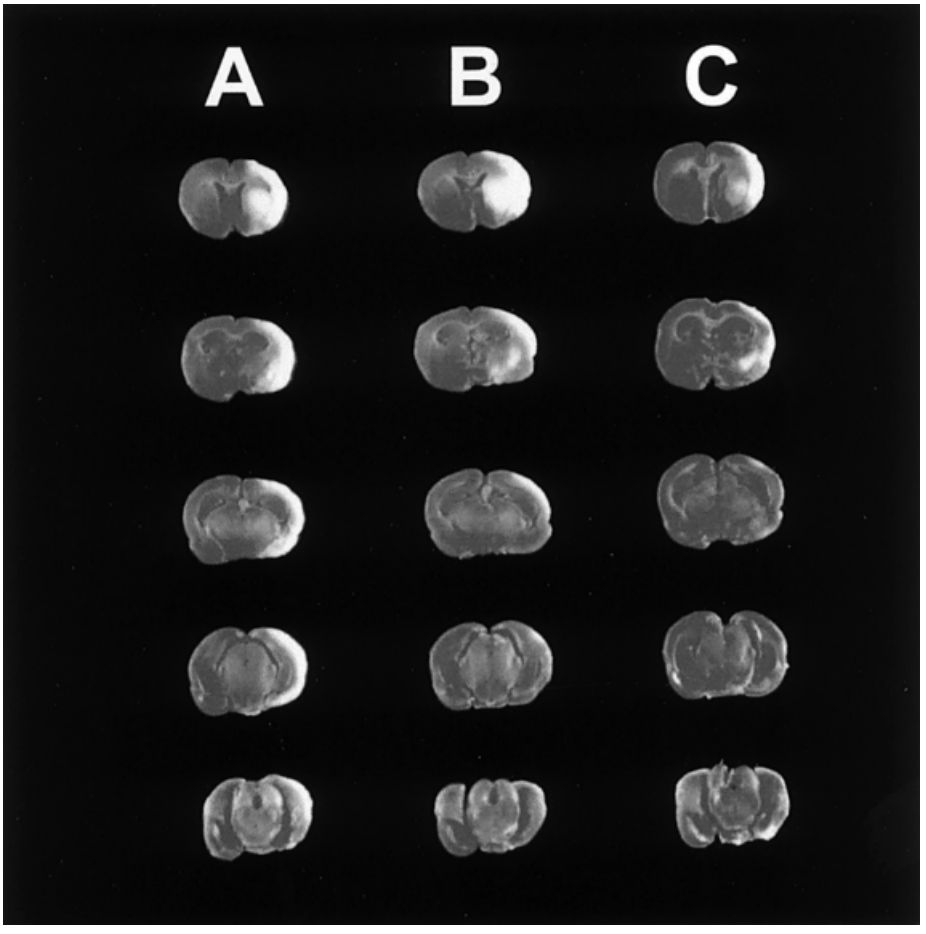

Fig. 5. EGb 761 treatment (100–200 mg/kg) 2 hr after MCA occlusion (i.e., at the time of reperfusion) reduced infarction in male Sprague-Dawley rats. 2,3,5-Triphenyltetrazolium chloride-stained coronal brain sections are from representative animals that intraperitoneally received a vehicle (**A**; 45% aqueous hydroxypropyl-β-cyclodextrin), 100 mg/kg EGb 761 (**B**), or 200 mg/kg EGb 761 (**C**) and were euthanized 7 days after MCA occlusion.

TABLE III. Summary of Neurobehavioral Scores and Body Weight Loss Obtained After Transient Middle Cerebral Artery Occlusion in Each Delayed Treatment Group[†]

		Neurobehavioral scores		
	n	Motor	Sensory	Body weight loss (g)
2 hr treatment groups (3-day recovery)				
Control	20	2.8 ± 0.1	3.6 ± 0.1	54 ± 6
EGb 761 treated				
100 mg/kg	11	2.5 ± 0.2	2.7 ± 0.2★	50 ± 7
200 mg/kg	12	2.5 ± 0.2	2.6 ± 0.5★	50 ± 7
2 hr treatment groups (7-day recovery)				
Control	13	2.5 ± 0.1	2.6 ± 0.3	46 ± 12
EGb 761 treated				
100 mg/kg	9	2.4 ± 0.2	2.6 ± 0.4	47 ± 12
200 mg/kg	9	1.9 ± 0.2★	1.0 ± 0.2★	14 ± 11
3 hr treatment groups (7-day recovery)				
Control	8	2.5 ± 0.2	3.0 ± 0.4	51 ± 9
EGb 761 treated, 200 mg/kg	8	2.5 ± 0.2	2.3 ± 0.6	47 ± 14

[†]Data are represented as the mean ± SEM; n, number of animals.
★$P < 0.05$.

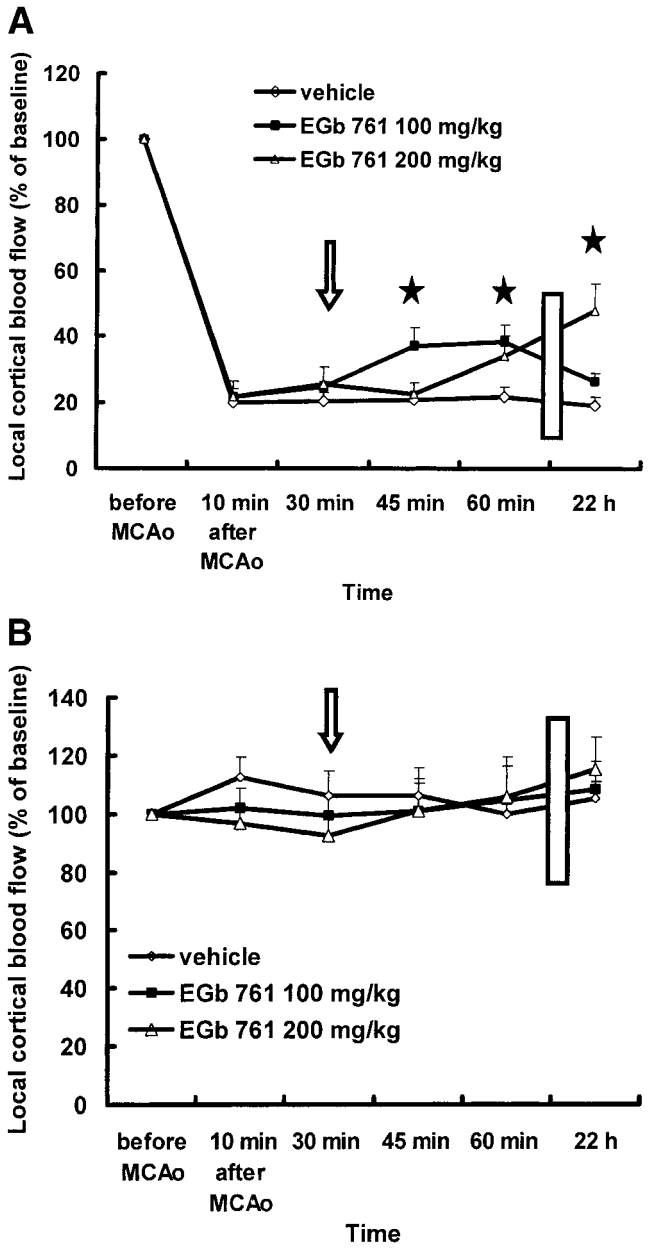

Fig. 6. Effect of EGb 761 on ipsilateral (**A**) and contralateral (**B**) local cortical blood flow (LCBF) after MCA occlusion. Delayed treatment with EGb 761 at 100 mg/kg (n = 6) or 200 mg/kg (n = 6) or vehicle (distilled water-PEG 400, n = 6) was administered by an intraperitoneal injection at 0.5 hr after MCA occlusion (arrow). LCBF was measured before, during 70 min of MCA occlusion, and at 22 hr after MCA occlusion. The data are represented as the mean ± SEM, which represents a percentage change relative to baseline values. ⋆$P < 0.05$ vs. distilled water-PEG 400-treated rats.

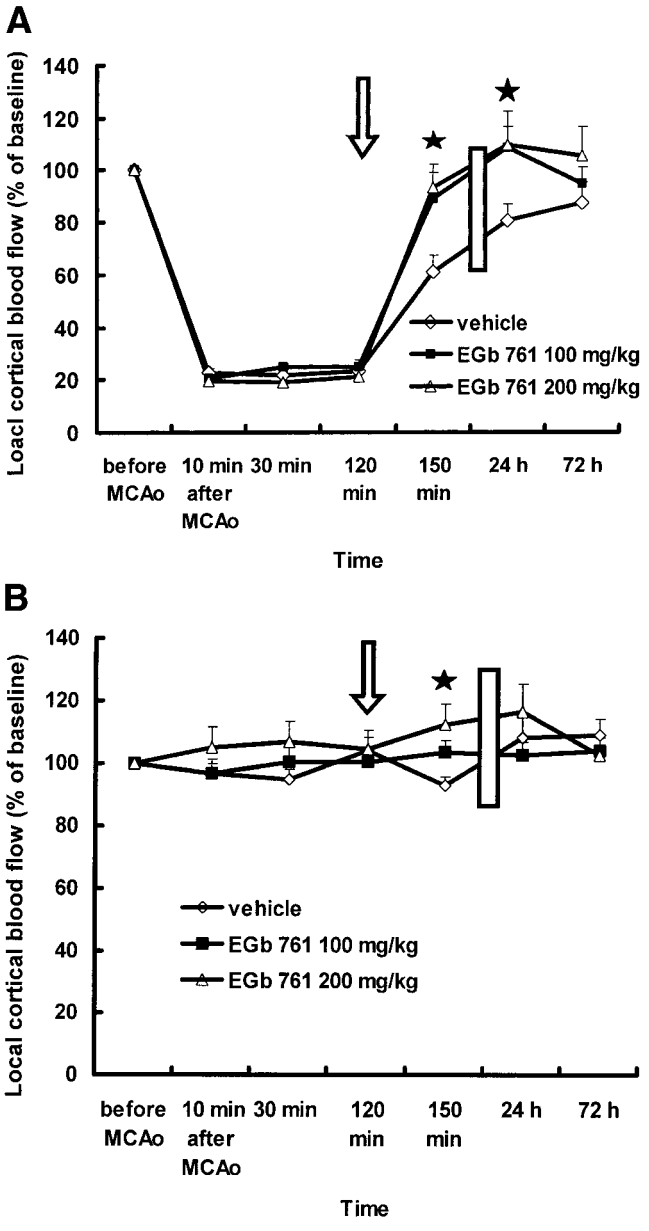

Fig. 7. Effect of EGb 761 on ipsilateral (**A**) and contralateral (**B**) local cortical blood flow (LCBF) after transient MCA occlusion. Delayed treatment with EGb 761 at 100 mg/kg (n = 11) or 200 mg/kg (n = 12) or vehicle (45% aqueous hydroxypropyl-β-cyclodextrin, n = 20) was administered by an intraperitoneal injection at the time of reperfusion (arrow). LCBF was measured before, during ischemia and for a brief period upon reperfusion, and at 24 and 72 hr after MCA occlusion. The data are represented as the mean ± SEM, which represents a percentage change relative to baseline values. ⋆$P < 0.05$ vs. vehicle-treated rats.

1998) and the inhibitor of nonselective cation channels (RS)-(3,4-dihydro-6,7-dimethoxyisoquinoline-1-gamma 1)-2-phenyl-N, N-di-[2(2,3,4-trimethoxyphenyl)ethyl]-acetamide (LOE 908 MS; Hoehn-Berlage et al., 1997). Perhaps by using multiple lower doses or continuous infusion of EGb 761, in combination with the control of EGb 761-induced hyperglycemia, the therapeutic window may be extended and/or the degree of neuroprotection improved.

Neuroprotection obtained with transient focal cerebral ischemia suggests that delayed treatment with EGb 761 may protect against reperfusion injury as well, because the animals were reperfused 2 hr after MCA occlusion. However, the effect on infarction volume reduction of the EGb 761 treatment was substantially less at 7 days following ischemia/reperfusion compared with that obtained either at 22–24 hr following permanent MCA occlusion or at 3 days following ischemia/reperfusion. Interestingly, the LCBF-preserving effect of EGb 761 treatment was found to diminish 24–72 hr after ischemia/reperfusion. In addition, a shortened therapeutic window of 2 hr was seen with EGb 761 treatment in the 7 day reperfusion model. These findings underscore that delayed downstream events of ischemia/reperfusion-induced injury may actually attenuate the neuroprotective effects involving only a single i.p. injection of EGb 761 treatment and suggest that multiple dosing with EGb 761 may be needed to enhance neuroprotection following a prolonged recovery period. It is also possible that EGb 761 protects against early pathologic ischemic cascades but is less helpful for late deleterious events (e.g., neuroinflammation and/or delayed reperfusion-induced injury) that occur following cerebral ischemia.

An exciting aspect of the present findings is that clinical usage of EGb 761 has been established for the treatment of a variety of neurologic disorders (Le Bars et al., 1997; Oken et al., 1998; Diamond et al., 2000). However, it should be emphasized that the neuroprotection observed with EGb 761 treatment may come with a price: a dose-dependently increasing incidence of tiny ICH within the infarct induced by transient focal cerebral ischemia. In fact, the incidence of ICH in the present study was substantially lower compared with those reported (10.0%) in mice pretreated with multiple doses of EGb 761 (100 mg/kg) orally (Clark et al., 2001). Given that the spontaneous onset of subarachnoid hemorrhage has been reported in a case with long-term administration of EGb 761 (Vale, 1998), the dosage and dosing regimen to be used is crucial. Further studies are needed to assess whether extended treatment protocols such as repeat administrations of EGb 761 or its biologically active constituents at lower doses will lead to not only an improved and prolonged neuroprotection but also a decreased incidence of ICH. In addition, further mechanisms underlying the neuroprotective effects observed here should be elucidated [Stroke Therapy Academic Industry Roundtable (STAIR), 1999].

In conclusion, the *Ginkgo biloba* extract EGb 761 protects against cerebral ischemia by reducing the brain infarction induced by permanent and transient MCA occlusion, with at least a 2 hr therapeutic window achieved, and by improving the behavioral neurologic outcome. This neuroprotective action remains effective over a prolonged reperfusion period, and is at least partially mediated by improving LCBF in the area at risk of infarction. Additional studies are needed to determine the optimal conditions under which it may be used in future clinical trials for stroke patients.

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