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Ginkgo biloba extract: Cognitive enhancer or antistress buffer

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Abstract

Constituents extracted from the leaves of the *Ginkgo biloba* tree possess beneficial properties that may buffer the aging nervous system from deterioration due to oxidative stress. In the present investigation, a standardized extract of *G. biloba* (EGb 761) or an equal volume of the vehicle was administered (100 mg/kg/day) to senescent (20-month) C57BL/6 male mice for up to 82 consecutive days. Animals were tested twice in the Morris water maze (MWM) after 28 and 70 days of treatment. No differences were observed in acquisition or retention of performance on the water maze. Elevated-plus maze (EPM) trials were conducted prior to and subsequent to the chronic treatment regimen. Marked baseline differences in plus-maze performance were present in the first experiment. A second experiment used a matched-pairs design to minimize preexisting differences. Results supported the hypothesis that EGb 761 may serve as an antistress buffer, attenuating the increase in anxiety typically observed in animals after cold water exposure. Tissue samples from the hippocampus and cortex were analyzed by Western blot for the transcription factor cyclic-AMP response element binding (CREB) protein. EGb 761 had no significant effect on immunoreactivity to CREB from either the hippocampus or the cerebral cortex.

Keywords: *Ginkgo biloba*; EGb 761; CREB; Aging; Elevated-plus maze; Spatial memory; Anxiety; Hippocampus

1. Introduction

The leaves of the Chinese tree *Ginkgo biloba* have been cultivated for their medicinal properties for several thousand years. The variety of therapeutic uses for *G. biloba* extract (GBE) stems from its numerous constituents, which provide for a broad range of pharmacological activities (Clostre, 1999). It has been used in the treatment of various common geriatric complaints including vertigo, short-term memory loss, hearing loss, lack of attention or vigilance. It is also utilized for cerebral vascular disorders (Clostre, 1999). To account for the therapeutic benefits, investigators have evaluated vasoregulatory, cognition-enhancing, stress-alleviating and gene-regulatory properties, which have been ascribed to proposed synergistic reactions between various pharmacologically active constituents found in the leaf (Luo, 2001).

The efficacy of GBE as a nootropic agent is a somewhat controversial topic. In clinical trials, improvement in cognitive functioning has been demonstrated in older people with dementia (Le Bars et al., 2000) or age-associated memory impairment (Curtis-Prior et al., 1999). Cognitive effects have also been documented in healthy middle-aged adults (Wesnes et al., 2000) and in young adults (Kennedy et al., 2000). However, at least one double-blind, randomized clinical trial has failed to find any improvement in older people with dementia or age-associated memory impairment (Van Dongen et al., 2000), and numerous other trials in human subjects have lacked adequate controls or were otherwise flawed in design (Kleijnen and Knipschild, 1992). Moreover, when effects have been found, it is not always clear whether memory processes are being affected directly, or whether the measured differences reflect underlying differences in other processes (e.g., attention, vigilance, arousal, mental fluidity, etc.).

A few experiments have examined the effects of GBE on learning and memory in animals. Experiments using the Morris water maze (MWM) task have thus far yielded

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mixed results (Bowers et al., 2000; Hasenohrl et al., 1998; Topic et al., 2001). In one study, intragastric administration of Zingicomb (Mattern et Partner, Starnberg, Germany), a preparation containing both GBE and ginger, was administered to adult rats (Hasenohrl et al., 1998). Zingicomb (0.5, 1, 10 mg/kg) did not improve water maze performance. However, that study was designed to evaluate amnestic or possible disrupting effects of the phytopharmacon. More recently, this research group evaluated the effect of long-term (i.e., daily administration for 5 months) treatment of aged (20- to 24-month) rats that have been shown previously to exhibit memory deficiencies, in comparison to 3-month-old animals (Topic et al., 2001). In this study, Zingicomb did facilitate spatial learning in aged rats. Another group reported enhanced place learning in the MWM in rats following only 14 days of GBE (200 mg/kg) administration (Bowers et al., 2000). Enhanced performance following GBE treatment has also been observed on an appetitive operant conditioning protocol (Winter, 1991), a radial arm maze (Winter, 1998), a T-maze (Cohen-Salmon et al., 1997), a passive-avoidance learning task (Stoll et al., 1996), an olfactory recognition task (Wirth et al., 2000), and a phi-maze used to measure working memory (Wilson et al., 2000).

A standardized extract of *G. biloba* (EGb 761) has been developed, containing 24% ginkgo-flavone glycosides and 6% terpenoids (Drieu, 1986). The flavonoid constituents of EGb 761 are known to possess free-radical scavenging properties (Clostre, 1999). GBE has been found to increase cerebral blood flow (Krieglstein et al., 1986). One potential mechanism, which could account for the behavioral effects observed in aged animals, concerns the demonstrated ability of GBE to protect against oxidative stress and prevent mitochondrial aging (Millan et al., 1998). Thus, GBE may serve as a protective agent, providing a buffer against rapid age-related decline in mental function.

A second potential mediator of GBE-induced cognitive enhancement concerns the ability of EGb 761 to counter the effects of stress. Several laboratories have also documented anxiolytic effects (Hasenohrl et al., 1996, 1998; Porsolt et al., 1990; Rapin et al., 1994; Satyan et al., 1998) associated with repeated administration of EGb 761 (or preparations containing EGb 761). Animals treated with Zingicomb, a preparation containing both ginger and *G. biloba*, exhibited an increase in time spent on the open arms of the plus maze (Hasenohrl et al., 1998). Other tests have evaluated EGb 761 in isolated form. In an experimental procedure typically used to study ''learned helplessness,'' EGb 761 was found to restore a degree of successful avoidance behavior (Porsolt et al., 1990). Increases in food consumption in an emotional hypophagia test (Porsolt et al., 1990) and suppression of stress-induced polydipsia (Rodriguez De Turco et al., 1993) have also been documented following administration with EGb 761. Collectively, these findings suggest that GBE may facilitate successful behavioral adaptation to stressors or noxious stimuli.

Administration of EGb 761 has been found to protect animals from deficits in performance on a discrimination task induced by a noxious auditory stimulus (Rapin et al., 1994). In this study, stress-induced release of epinephrine, norepinephrine and corticosterone from plasma was blunted by pretreatment with EGb 761. This may explain, at least in part, the clinical findings that EGb 761 may improve the ability of geriatric patients to cope with the stressful demands of daily life (Le Bars et al., 2000). Additionally, excessive release of cortisol in humans or corticosterone in rats has been shown to have toxic consequences for neurons in the hippocampus (Sapolsky et al., 1990), a structure of central importance for spatial memory. Because of its high rate of metabolic activity, the hippocampus is more vulnerable to the effects of oxidative stress than other brain regions. EGb 761 has been shown to have a specific neuroprotective effect on neurons of hippocampal origin (Bastianetto et al., 2000a,b). Therefore, there are a number of neuroprotective and stress-alleviating actions of EGb 761, which could conceivably act in concert to promote optimal cognitive functioning, the effects of which might be observed most easily in older subjects.

The general purpose of the study was to further characterize the behavioral effects of EGb 761 in aged mice (Experiment 1) and to evaluate the possibility that levels of a transcription factor, cyclic response element binding protein (CREB), from the hippocampus might correlate with behavioral parameters. Expression of phosphorylated CREB from the hippocampus has been implicated as a molecular marker of memory processing in the rat (Viola et al., 2000). Experiment 2 further evaluated the proposed antistress effect of EGb 761 by using a matched-pairs design in order to minimize any preexisting differences in plus-maze performance.

2. Experiment 1

Senescent (20-month) mice were evaluated in the elevated-plus maze (EPM) prior to EGb 761 treatment and again after the chronic treatment and water maze testing had been completed. Immunoreactivity to CREB (both phosphorylated and unphosphorylated) was assessed from hippocampal tissue.

2.1. Method: Experiment 1

2.1.1. Subjects

Thirty-four senescent (20-month) C57BL/6 male mice, having a mean body weight of 31.79 ± 3.34 g, were obtained from the National Institute on Aging (NIA) colony at Harlan Sprague–Dawley (Indianapolis, IN). This inbred strain is noted for their capable performance in the MWM (Upchurch and Wehner, 1988). Mice were housed five to six in a group in a controlled environment with ambient temperature (25 ± 1 °C) under a reverse 12-h

light–dark schedule with light onset at 2000 h. Food and water were available ad libitum. Mice were handled daily for 4 days prior to the start of the experiment. All procedures were conducted in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985) and the guidelines set forth by the local Institutional Animal Care and Use Committee (IACUC) at the University of Southern Mississippi.

2.1.2. Apparatus

The water maze (MWM) was modified from Morris (1981). Animals were trained in a pool 1.83 m in diameter and 0.6 m in depth, containing water held constant at 21 ± 1 °C. The pool was in the center of a square room (5×5 m). A 10×10-cm transparent platform was hidden in a constant place in the pool with its top surface submerged 1 cm below the water level. An HVS tracking system (SA-3 Tracker using CRT402 program with an IBM-compatible PC, San Diego Instruments) was used to record behavior. The camera (Burle TC355AC, San Diego Instruments) was mounted directly over the center of the maze. A nontoxic white poster paint was added to the water to facilitate tracking the black mice and to obscure the platform.

The EPM, validated as a behavioral test of anxiety (Pellow et al., 1985), consisted of two open arms (50×10 cm) and two enclosed arms ($50 \times 10 \times 30$ cm) with an open roof, arranged such that the two arms of each type are perpendicular to each other. The maze was elevated to a height of 50 cm. Plus-maze testing occurred under dimly lit conditions. To minimize any unintended human influences, behavior was recorded using an infrared video system (Sony, Tokyo). Videotapes were scored later with observers unaware of the treatment of each animal. A 70% ethanol solution was used to clean out the arms and eliminate odor cues between trials.

2.1.3. Design

An independent group design was employed for this experiment. Senescent mice were randomly assigned ($N = 17$ animals per group) to two groups. The treatment group received a daily oral dose of 100 mg/kg of EGb 761 in a 0.2% agar (Becton Dickinson, Sparks, MD) solution. The control group received an equal volume of the vehicle. Solutions were delivered via stainless steel gavage tubes (Popper & Sons, New Hyde Park, NY). EGb 761 (or vehicle) was delivered at 1500 h daily.

2.1.4. Procedures

Prior to drug administration, animals were initially handled for 4 consecutive days. On the fifth day, animals were given a pretest in the EPM (see Fig. 1). Forty-eight hours after the elevated-plus pretest, administration of EGb 761 (or vehicle) was initiated and was continued throughout the entire experiment. Animals received EGb 761 (or vehicle) for 28 consecutive days prior to the first of two 5-day sessions during which animals were trained and tested in the MWM. As recommended by Winter (1998), infusions were administered at 0800 h prior to water maze trials (begun at 1000 h). Each session consisted of 19 training trials (four trials per day for 5 consecutive days) and one probe trial on the fifth day. For training trials, mice were trained to escape from the deep water onto a platform (10×10 cm), which was submerged in a constant location 1 cm below the water surface. A probe trial consisted of a 60-s swim period, during which time the platform was removed. On probe trials, the dependent variable was the percentage of time spent in the correct quadrant. Further details on the MWM procedure have been described previously (Williams et al., 2001). Additional probe trials were administered on the third and sixth days following the end of the 5-day training period. A random sample of animals ($n = 3$ from each group) was killed after 42 days (6 weeks) of EGb 761 administration for CREB analysis. Remaining animals completed a second 5-day session in the MWM after receiving a total of 70 days (10 weeks) of treatment. As in the first session, three probe trials were administered: one on the 20th trial, a second one 3 days later, and a third one after 3 more days had elapsed. Twenty-four hours after the last probe trial, all animals were tested for the second time in the EPM (i.e., posttest). Hence, it was 84 days (12 weeks) between the pre- and posttest on the EPM (see Fig. 1). In between the pre- and posttest, animals received EGb 761 (or vehicle) for 82 consecutive days.

2.1.5. Elevated-plus maze

Testing occurred from 0900 to 1700 h for both pretest and posttest, with the order randomized to ensure against possible circadian influences. Animals were first placed individually in the EPM with the open arms removed for 5 min to allow for habituation to a novel environment. Then, each animal was placed into the center of the plus maze facing one of the enclosed arms. Each session was 5 min in duration. The dependent measure was percentage of total time spent on the open arms. The criterion for an arm 'entry'

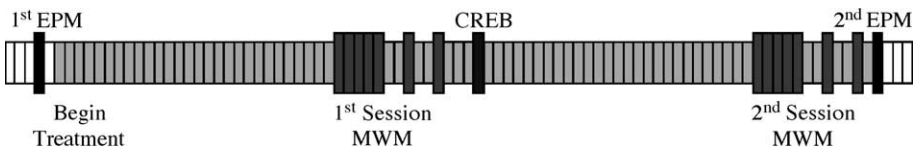

Fig. 1. Timeline for experimental protocol illustrating the treatment days (100 mg/kg EGb 761 or vehicle), as well as the days during which the MWM testing and EPM testing occurred.

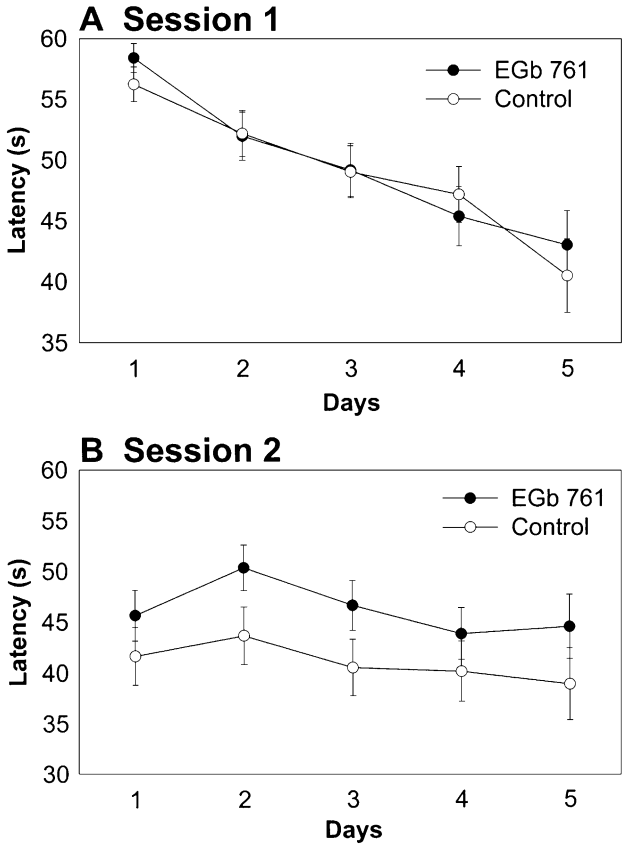

Fig. 2. Morris water maze: Mean (±S.E.M.) time required to reach the hidden platform averaged over days (four sessions per day). (A) The first 5-day session occurred after 28 consecutive days of treatment with EGb 761 (100 mg/kg/day po) or an equal volume of the vehicle. (B) The second 5-day session (B) occurred after 70 consecutive days of treatment.

was that the torso and all four paws of the animal must be located within either an open or closed arm.

2.1.6. Western blot

Following the first MWM session, a random sample of animals ($n=6$) was killed by cervical dislocation after 42 days of EGb 761 (or vehicle) administration. Fresh brain tissue was removed. Samples of hippocampal tissue and cerebral cortex were dissected and stored separately in aliquots at -70 °C. The hippocampal tissue was homogenized in 500 μl of the lysis buffer containing 10 mM Tris–HCL, pH 7.5, 2 mM $MgCl_2$, and 0.25 M sucrose. The samples were then sonicated for 20 s and microfuged for 10 min. The supernatants were used as tissue extracts. Total protein from each sample was assayed using a BCA kit (Pierce, Rockford, IL) and protein concentrations were equalized. Equal amounts of SDS sample buffer were added to the lysate, which was then boiled for 4 min and loaded for SDS-PAGE. The samples were transferred from the SDS-PAGE gel to Immobilon-P membrane, and blots were blocked in 5% milk in PBS-T (0.05% Tween-20) for at least 30 min at room temperature. The primary antibody, anti-CREB (Santa Cruz), was diluted in 5 ml of blocking solution (1:500) and incubated for 1 h at room temperature. Blots were washed five times at room temperature with PBS-T. Secondary antibody, anti-rabbit IgG, was diluted in 5 ml blocking solution (1:8000). Blots were washed and developed with a chemiluminescence kit (Amersham).

2.1.7. Statistics

The sample size was determined by power analysis. Based on expectations of a strong behavioral effect (Rapin et al., 1994; Stoll et al., 1996; Winter, 1991), a sample size of 34 animals (17 per group) was determined to be sufficient for statistical power >0.7. For the MWM trials, mixed-model ANOVA was performed. The between-subjects factor was the EGb 761 treatment. The within-subjects factor was trials. A second mixed-model ANOVA was used to analyze the second 5-day block of trials. Student's *t* tests were used to analyze the probe trial data. Data from the EPM were analyzed by mixed-model ANOVA.

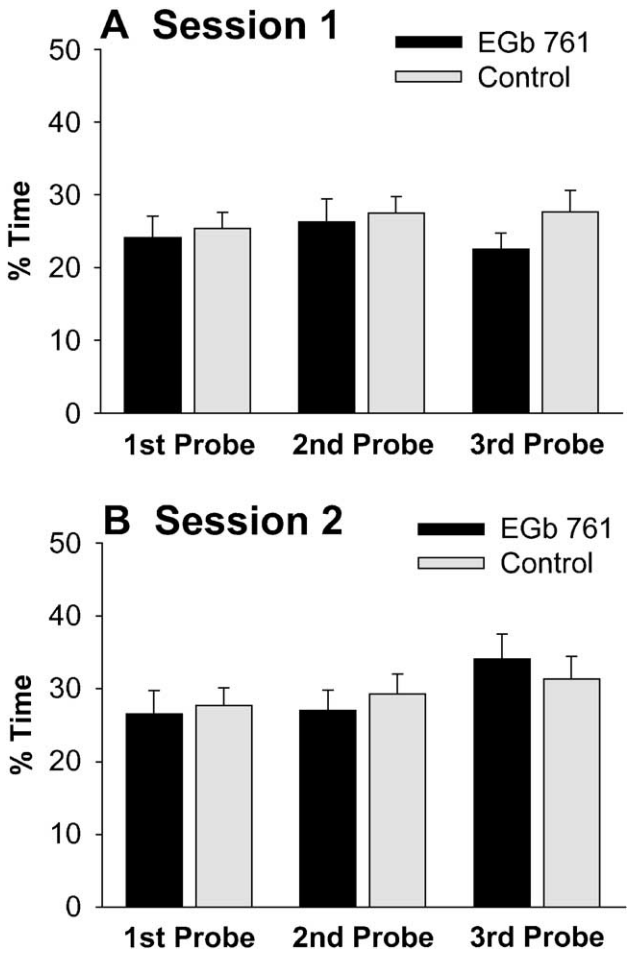

Fig. 3. Morris water maze: Mean (±S.E.M.) percentage of total time (60 s) spent in the correct quadrant during probe trials. Probe trials were administered on the 20th trial (i.e., last trial of each training session), and again 3 and 6 days later. (A) Probe trials following the first 5-day session. (B) Probe trials following the second 5-day session.

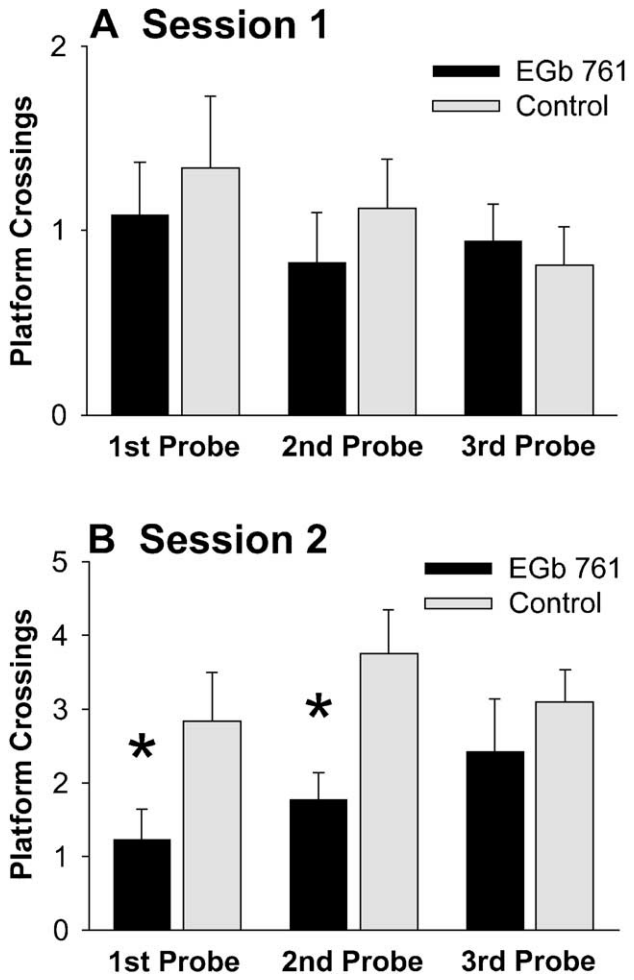

Fig. 4. Morris water maze: Mean (±S.E.M.) number of platform crossings during each 60-s probe trial. Probe trials were administered on the 20th trial (i.e., last trial of each training session), and again 3 and 6 days later. (A) Probe trials following the first 5-day training session. (B) Probe trials following the second 5-day training session. * $P < .05$.

For Western blot analysis, the immunoreactivity to CREB was quantitated by Alpha Imager 2000 with accompanying software, which determined integrated density values (IDV) for each sample of tissue. A Student's t test was performed to compare IDV data between EGb 761-treated ($n = 3$) and vehicle-treated ($n = 3$) animals.

2.2. Results: Experiment 1

2.2.1. Effect of EGb 761 on water maze performance

The time required for animals to reach the hidden platform on the first trial in the water maze is illustrated in Fig. 2A As expected, the main effect of trials was significant, $F(9,211) = 4.98$, $P < .001$. However, there was no significant difference between EGb 761- and vehicle-treated animals in latency to reach the hidden platform over the 19 training trials, $F(1,32) = 0.08$. Indeed, the learning curves across days 1–5 for the two groups are nearly identical (see Fig. 2A). Additionally, the interaction effect (trial X treatment) was not significant, $F(1,32) = 1.41$.

For all probe trials, the percentage of time spent in the correct quadrant for EGb 761- and vehicle-treated conditions is illustrated in Fig. 3. The number of crossings of the platform location for both groups is shown in Fig. 4. On the probe trial (20th session on Day 5), there were no significant differences between groups in either percentage of time spent in the correct quadrant, $t(32) = 0.34$, or number of platform crossings, $t(32) = 0.51$. Retention tests (i.e., probe trials) administered 3 days after the last probe trial did not reveal any other significant differences in percentage of time in the correct quadrant, $t(32) = 0.30$, or in number of platform crossings, $t(32) = 0.77$. Similarly, there were no significant differences 6 days after the last probe trial on either percentage of time in the correct quadrant, $t(32) = 1.38$, or in the number of platform crossings, $t(32) = 0.45$.

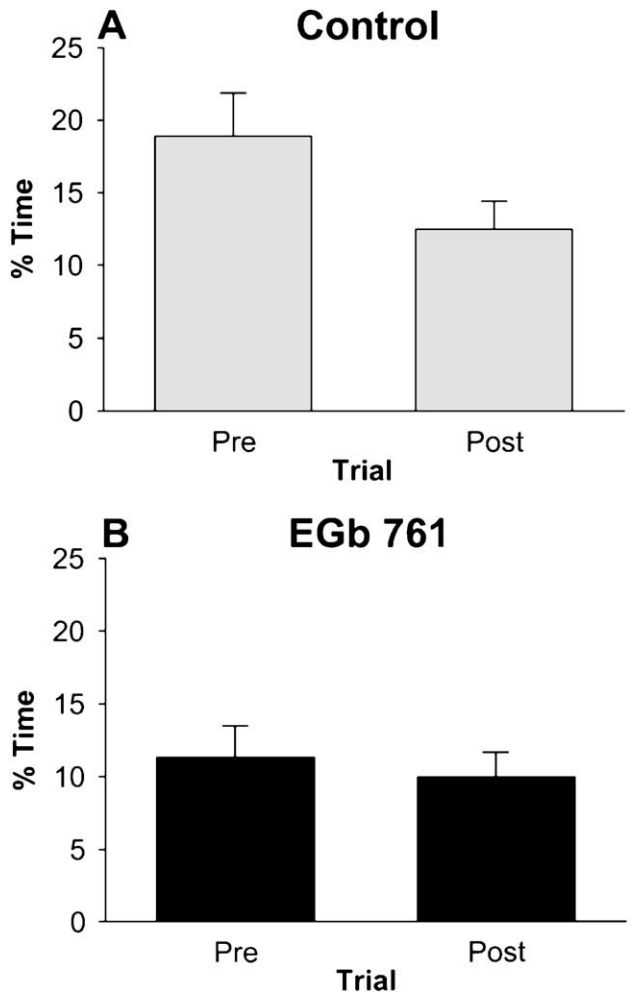

Fig. 5. Elevated-plus maze: The y-axis depicts the percentage of the total time spent on the open arms. The pretest was administered 48 h prior to initiation of the treatment. The posttest was administered after 82 consecutive days of EGb 761 (or vehicle) treatment. (A) Pre- and posttest scores of animals in the control group. (B) Performance of EGb 761-treated animals. Lower percentages indicate an increase in anxiety.

During the second 5-day session in the MWM, it can be observed that both groups of animals exhibited significant "savings" of spatial information from the previous 5-day session (see Fig. 2B). In fact, during the second 5-day session, the main effect of trials was no longer significant, $F(9,211) = 1.041$. The main effect of treatment was significant during this period, $F(1,23) = 5.897$, $P = .023$. Contrary to predictions, vehicle-treated animals reached the hidden platform in significantly less time than EGb 761-treated animals (Fig. 2A). The interaction effect (trial *X* treatment) was not significant, $F(9,211) = 0.909$.

On the probe trial (i.e., 20th trial) of the second 5-day MWM session, there was no difference in percentage of time spent in the correct quadrant (Fig. 3) between EGb 761- and vehicle-treated groups, $t(23) = 0.27$. Similarly, there were no significant differences in time spent in the correct quadrant 3 days after the probe trial, $t(23) = 0.57$, or 6 days after the probe trial, $t(23) = 0.61$. However, there were significant differences in the number of platform crossings (Fig. 4) during the second session. Specifically, during the 60-s probe trial, animals treated with EGb 761 had significantly fewer platform crossings than vehicle-treated animals, $t(23) = 2.10$, $P = .047$. Three days after the probe trial, EGb 761-treated animals again exhibited fewer platform crossings than vehicle-treated animals, $t(23) = 2.91$, $P = 2.91$. There were no significant differences between groups in the number of platform crossings 6 days after the probe test, $t(23) = 0.80$.

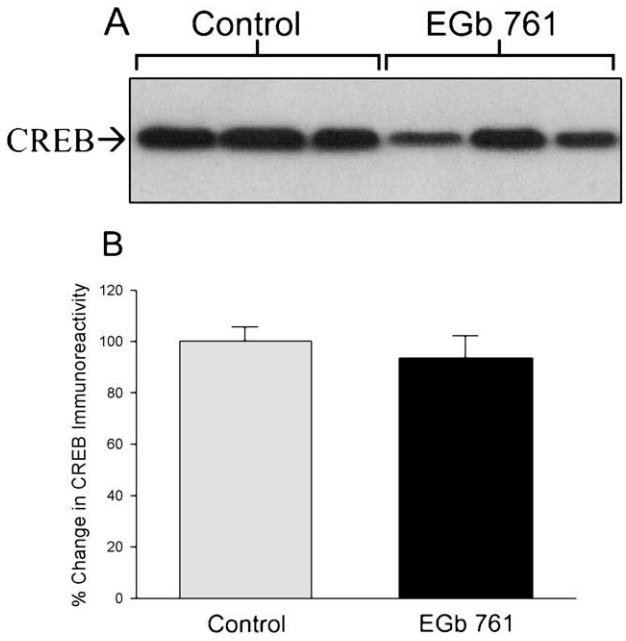

Fig. 6. (A) Representative Western blot illustrating CREB protein expression from the hippocampus of an EGb 761-treated (100 mg/kg/day po) animal and the hippocampus of an animal treated with an equal volume of the vehicle. An equal amount of protein was loaded on each well and subjected to a SDS-PAGE. Antibody specific to CREB (Santa Cruz) was used for Western blotting. Similar results were obtained in two independent experiments. (B) Quantitative analysis of CREB immunoreactivity. The mean IDV was determined for the EGb 761 and vehicle treatment conditions ($n = 3$ animals per group) using Alpha Imager 2000 with accompanying software. The percentage (±S.E.M.) change in mean IDV was determined for the EGb 761 treatment condition and plotted on the graph relative to the IDV for the vehicle treatment condition.

2.2.2. Effect of EGb 761 on EPM performance

Fig. 5 shows the percentage of total time spent on the open arms. The ANOVA revealed a significant main effect of differences in plus-maze performance between EGb 761- and vehicle-treated animals, $F(1,64) = 5.16$, $P = .026$. Overall, animals in the control group spent significantly more time on the open arms, compared to the EGb 761-treated animals. There was a general tendency for animals to spend somewhat less time on the open arms during the posttest, indicating increased posttest anxiety. However, this main effect was not significant, $F(1,64) = 3.02$, $P = .087$. The drug by test (pre- vs. post-) interaction effect also was not significant, $F(1,64) = 1.23$.

2.2.3. Effect of EGb 761 on CREB expression

Fig. 6 illustrates the degree of CREB expression from hippocampal tissue of representative animals. There were no significant differences in IDV between EGb 761- and vehicle-treated animals.

3. Experiment 2

Based on visual inspection of the EPM results from Experiment 1, it was suggested that EGb 761 may serve as an antianxiety buffer, attenuating the anxiogenic response typically observed in animals. However, baseline levels of anxiety for EGb 761- and vehicle-treated animals differed markedly in Experiment 1 (see Fig. 5), necessitating further experimentation. Experiment 2 was designed specifically to test the prediction that the observed increase in anxiety following cold-water exposure will be attenuated in animals chronically treated with EGb 761. Moreover, Experiment 2 tested whether this proposed effect might generalize to young and middle-aged mice.

3.1. Method: Experiment 2

3.1.1. Subjects

Twenty C57BL/6 male mice, having a mean body weight of 30.23 ± 4.5 g, were used in this experiment. These animals were either 3 months of age ($n = 10$) or 10 months of age ($n = 10$). Housing, handling, and care procedures were identical to those used in the previous experiment. The same EPM was utilized in this experiment.

3.1.2. Procedures

To control for the marked baseline differences in anxiety observed in Experiment 1, a matched-pairs design

was employed. Baseline EPM ratios were determined by testing each animal for 5 min in the EPM, as described previously. Animals were then matched according to 1) age and 2) pretest EPM performance to receive either EGb 761 or the vehicle ($n = 10$ per group). Beginning forty-eight h after the initial EPM test, each animal was given a daily oral infusion of EGb 761 (100 mg/kg) or an equal volume of the vehicle (0.2% auger) for 30 consecutive days. One to two hours after the last infusion (Day 30), animals were exposed to a single, acute stressor; namely, a forced-swim test (Abel, 1994). A plastic cylinder (39 cm deep and 30.5 cm in diameter) containing cool (21 ± 1 °C) water was filled to a height of approximately 25 cm. Each animal was inserted into the water and forced to swim for 15 min (one trial per animal). On Day 31, each animal was again tested in the EPM.

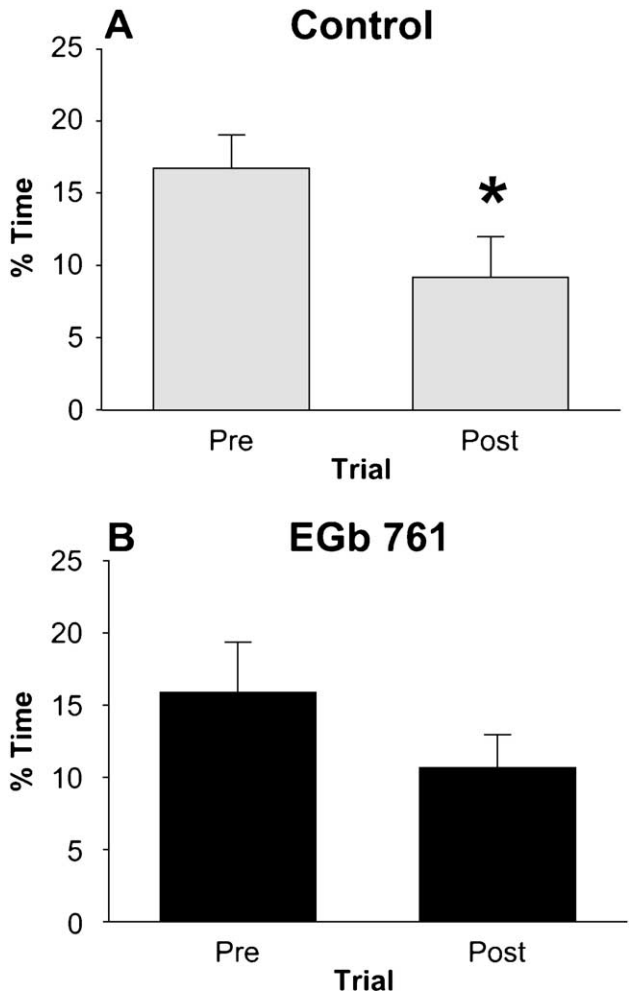

Fig. 7. Elevated-plus maze: The *y*-axis depicts the percentage of the total time spent on the open arms. The pretest was administered 48 h prior to initiation of the treatment. The posttest was administered after 30 consecutive days of EGb 761 (or vehicle) treatment. (A) Pre- and posttest scores of animals in the control group. (B) Performance of EGb 761-treated animals. Lower percentages indicate an increase in anxiety. * $P < .05$.

3.1.3. Statistics

It was predicted a priori that EGb 761 would attenuate the increase in anxiety observed following exposure to cold water. Since planned comparisons were being made to evaluate pre- versus posttest performance in each of the two treatment conditions, separate Student's *t* tests for paired samples were performed (Winer et al., 1991).

3.2. Results: Experiment 2

The percentage of time spent on the open arms in the EPM again tended to be lower after exposure to cool water (Fig. 7). However, statistical analysis revealed that this reduction in time spent on the open arms was only significant for vehicle-treated animals, $t(9) = 2.62$, $P = .028$. Pre- and posttest scores did not differ significantly for EGb 761-treated animals, $t(9) = 1.79$. Therefore, the forced-swim task used in Experiment 2 increased posttest anxiety in the EPM in vehicle-treated, but not EGb 761-treated animals (Fig. 7).

4. Discussion

The present experiment has shown that a chronic 82-day treatment with EGb 761 had no effect on either acquisition or retention of performance on the MWM in senescent C57BL/6 mice. Indeed, the learning curves for EGb 761- and vehicle-treated mice were nearly identical after 1 month of treatment (see Fig. 2). In the EPM, marked preexisting differences in anxiety in Experiment 1 (see Fig. 5) necessitated an additional experiment. Results from Experiment 2 indicated that vehicle-treated animals exhibited an anxiogenic response after exposure to cold water. In contrast, no anxiogenic response was observed in animals treated with EGb 761 (see Fig. 7). It is suggested that EGb 761 may function as an antianxiety buffer. Finally, there were no significant group differences in immunoreactivity to CREB from hippocampus (see Fig. 6).

Senescent C57/BL6 mice had been treated with EGb 761 for 28 consecutive days (Experiment 1) prior to the first water maze session. Because there were no effects on either acquisition or retention after 1 month of treatment, the decision was made to extend treatment for a second month before testing the animals in the water maze again. During the second 5-day session in the MWM, the vehicle-treated animals unexpectedly performed more efficiently during acquisition than the EGb 761-treated animals. The vehicle-treated animals also exhibited some evidence (i.e., significantly more platform crossings, as illustrated in Fig. 4B) of better memory retention on the 20th trial of the second session and again on the probe trial, which occurred 3 days later.

The lower starting latencies on Day 1 and the flatter learning curve during the second session (Fig. 2B) both suggest that important spatial information may have been

consolidated into long-term memory during the first 5-day session and retrieved for use during the second MWM session. This ''savings'' effect may have limited the detection sensitivity during the second session. Moreover, whether the MWM is sufficiently sensitive to reliably detect subtle cognitive effects is not yet clear.

The lack of an effect in the present study certainly does not rule out the hypothesis that GBE improves memory, particularly in light of the fact that other investigators have reported effects using the MWM task (Bowers et al., 2000; Hasenohrl et al., 1998). Rather, the inconsistency of findings reported thus far should highlight the necessity of defining the conditions under which cognitive effects are most likely to be observable. Species differences, age of the animal, dose and preparation of the phytopharmacon, route of administration, duration of treatment, and experimental protocol (e.g., cued vs. place learning) employed are all important factors that must be thoroughly evaluated before definitive conclusions can be reached. Because the optimal conditions for studying the cognitive effects of GBE have not been delineated, the negative findings presented here should be viewed with caution.

Nonetheless, at least one well-controlled clinical trial has failed to find any cognitive improvement in older people with dementia or age-related memory deficiencies (Van Dongen et al., 2000). Another clinical trial documented biphasic effects. Following treatment with a Ginkgo/ginseng combination, initial improvements were followed by dose-dependent cognitive impairments in healthy subjects with neurasthenic complaints (Wesnes et al., 1997). Moreover, when positive effects have been reported in humans, they have been relatively modest (Le Bars et al., 2000; Wesnes et al., 2000). The findings call into question the notion that GBE directly enhances cognitive processing.

Cold-water exposure has been shown previously to induce anxiety (Abel, 1994). In experiments in our laboratory, animals generally tend to spend less time on the open arms of an EPM after water exposure. Given the constancy of the animal handlers, it is reasonable to assume that anxiety may generalize from the MWM or forced swim task to the EPM posttest sessions. The tendency towards increased anxiety appeared to be more pronounced for vehicle-treated animals than for EGb 761-treated animals (Fig. 5), although marked preexisting differences in baseline anxiety levels made it impossible to reach a definitive conclusion without further experimentation. Experiment 2 minimized preexisting anxiety levels by employing a matched-pairs design. As predicted, treatment of animals with EGb 761 (this time for 30 days) attenuated the anxiogenic response to cold-water exposure (Fig. 7). Unlike other investigators (Hasenohrl et al., 1996, 1998), we did not observe an anxiolytic response in EGb 761-treated animals. Nonetheless, the current results are consistent with other evidence implicating EGb 761 as an agent to counter the effects of stress (Hasenohrl et al., 1996, 1998; Porsolt et al., 1990; Rapin et al., 1994; Rodriguez De Turco et al., 1993). Moreover, since senescent animals were not available for the second experiment, our results suggest that this antistress effect may generalize across a range of ages.

The buffering effect of EGb 761 appears to be independent of mechanisms that mediate the effects of antidepressants (imipramine) or classical anxiolytic (diazepam) drugs (Porsolt et al., 1990). Several mechanisms have been proposed to account for the ''antistress'' effects. Given the fact that anxiety level and serotonin activity are positively associated (Iversen, 1984), it is noteworthy that EGb 761 can act as a serotonin antagonist (Hasenohrl et al., 1996) and exert discriminative stimulus effects at the 5-HT1A receptor (Winter and Timineri, 1999). Additionally, EGb 761 has been shown to reduce age-related and stress-related increases in basal levels of monoamine oxidase (Pardon et al., 2000).

Several actions of GBE promote protection of the hippocampus, a structure that is particularly susceptible to neurodegenerative damage. First, EGb 761 has been shown to attenuate the stress-induced increase in corticosterone (Porsolt et al., 1990), which is potentially toxic to hippocampal neurons (Sapolsky et al., 1990). Second, nitric oxide (NO)-induced death of cultured hippocampal neurons is blocked by EGb 761 treatment (Bastianetto et al., 2000b). Antioxidant properties of the flavonoid component of EGb 761 were responsible for this effect. Finally, in primary cultured cells, EGb 761 treatment protected hippocampal neurons against toxicity induced by beta-amyloid (Abeta)-derived peptides (Bastianetto et al., 2000a), which are known to accumulate in the brain (particularly hippocampus) of persons with Alzheimer's disease.

Irrespective of the specific mechanisms involved, converging evidence suggests that EGb 761 can reduce the consequences of stress (Porsolt et al., 1990) and facilitate behavioral adaptation to adverse environmental conditions (Rapin et al., 1994). Whether the antistress activity results in improved performance may be task dependent. For example, the task difficulty and arousal/anxiety level required for peak performance on an appetitive discrimination task (Rapin et al., 1994) are likely to be quite different from the conditions required to locate a hidden, submerged platform in order to escape from an aversive stimulus. The Yerkes–Dodson Law (Yerkes and Dodson, 1908) postulates a curvilinear relationship between arousal and performance, such that, for a given task difficulty, there exists an optimal level of arousal or anxiety, with under- and overarousal yielding suboptimal performance. Further, a relatively high level of arousal or anxiety is expected to be optimal for easy tasks, while a lower level of arousal would be optimal for difficult tasks (Yerkes and Dodson, 1908). Thus, depending on task difficulty and arousal/anxiety level, EGb 761 might be expected to improve performance on some tasks, but not others.

Pilot experiments using water of varying temperatures revealed that mice performed optimally at 21 ± 1 °C, the

temperature used for the present experiments. An anxiolytic agent such as EGb 761 might have reduced arousal to a level that elicited suboptimal performance. Consistent with this prediction, chronic administration of EGb 761 did not improve performance in the MWM. In fact, EGb 761-treated animals were less efficient in reaching the hidden platform than vehicle-treated animals during the second 5-day session in the MWM. There was some indication (i.e., lower number of platform crossings on probe trials) that memory retention of the location of the platform may have been worse during the second session in animals treated with EGb 761, compared to the control group. Alternatively, EGb 761 might somehow lower the motivation to escape the aversive conditions. In any event, the level of arousal and the task difficulty are potentially important factors often ignored in studies evaluating behavioral effects of GBE.

The results of the Western blot assay on the hippocampal and cortical tissue revealed no significant differences in immunoreactivity to CREB (either phosphorylated or unphosporylated) between EGb 761-treated and vehicle-treated animals (Fig. 6). Evidence suggests that consolidation of long-term memory is dependent on activation of neuronal second messenger systems and requires protein synthesis (DeZazzo and Tully, 1995). As a regulatory transcriptional factor that couples changes in second messenger systems to changes in cellular transcription, CREB has been shown to be an important mediator specifically involved in consolidation of spatial memory (Guzowski and McGaugh, 1997). The negative findings with respect to CREB reported here are consistent with the fact that EGb 761 did not have an effect on acquisition, retention, or retrieval of spatial memory in the MWM.

Recently, high-density oligonucleotide microassays have been used to define the transcriptional effects in the hippocampus or the cortex of mice whose diets were supplemented with EGb 761 (Watanabe et al., 2001). Because of the known importance of the hippocampus for learning and memory, it was expected that expression of many genes would be up-regulated in those animals treated with EGb 761. Surprisingly, expression of only one gene (which encodes for transthyretin) in the array was up-regulated more than threefold in the hippocampus of EGb 761-treated animals. The critical mechanisms, as suggested by the negative data and the findings from the microarray study (Watanabe et al., 2001), may be largely independent of neuronal transcription in the hippocampus.

In the present study, chronic administration of EGb 761 had no significant effect on either acquisition or retention of learned performance in the MWM. However, only one dose (100 mg/kg) of EGb 761 was evaluated, and only one type of memory test was used. These facts preclude any definitive statement concerning the putative cognitive-enhancing properties of EGb 761. Nonetheless, it is suggested that the improved cognitive functioning following sustained treatment with GBE may be secondary to neuroprotective properties that buffer the animal from the harmful effects of stress. The plus-maze results reported here are consistent with this hypothesis.

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