Comparison of Neuroprotective Effects of Flavonoids, Terpenoids, and Their Combinations from *Ginkgo biloba* on Ischemia-Reperfusion–Injured Mice

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Abstract

Progress in treating cerebral ischemia with an extract of *Ginkgo biloba* L. (Ginkgoaceae), commonly known as EGb 761, has been made in the past. However, neuroprotective interaction of the two main active fractions, flavonoids and terpenoids, still remains vague. In the current study, we investigated the neuroprotective effects of *Ginkgo* flavonoids, terpenoids, and their combinations in ratios of 16:1, 4:1, and 1:1 on brain damage in mice injured by ischemia-reperfusion. Pretreatment with total flavonoids, total terpenoids, their three combinations, or EGb 761 for 7 days each at two doses of 15 and 30 mg/kg might elevate latency and decrease error numbers to different extents in the passive avoidance test, but only the action of the 4:1 combination besides EGb 761 was significant. In addition, the gasping time in decapitated mice was prolonged markedly by the 4:1 and 1:1 combinations at 15 and 30 mg/kg and by total flavonoids other than total terpenoids at 30 mg/kg. Neurochemical assays further showed that flavonoids significantly decreased malondialdehyde, superoxide anion contents, and xanthine oxidase activity, meanwhile terpenoids markedly suppressed myeloperoxidase activity besides its slight antioxidant effect. These findings suggest that flavonoids and terpenoids contribute synergistically to the neuroprotection of EGb 761 mainly via antioxidant activity and anti-inflammatory activity, respectively, and the 4:1 combination, namely the natural ratio in EGb 761, shows the strongest effects among those of the three combinations.

Keywords: Flavonoids, *ginkgo biloba*, ischemia-reperfusion, neuroprotection, synergism, terpenoids.

Introduction

Extract of *Ginkgo biloba* L. (Ginkgoaceae) has been extensively used in a variety of cardiovascular and cerebrovascular diseases such as ischemia, dementia, and depression (Gertz & Kiefer, 2004; Nishida & Satoh, 2004). Although much research has demonstrated that *Ginkgo biloba* extract has highly neuroprotective effects against ischemia-reperfusion (IR) injury (Krieglstein et al., 1995; Wang et al., 1998), the mechanism and mode underlying the therapeutic effects is still under investigation (Ahlemeyer & Krieglstein, 2003; Smith & Luo, 2004).

The standard *Ginkgo biloba* extract (EGb 761) contains two main active fractions, 24% flavonoids and 6% terpenoids, with a natural ratio approximately at 4:1 (van Beek, 2002). It has been revealed that EGb 761 provides neuroprotection during IR, which might be related to antioxidant and free radical scavenging activities of flavonoids (Bastianetto et al., 2000; Wei et al., 2000), antagonism of platelet-activating factor (PAF), and antiapoptotic capacity of terpenoids (Smith et al., 1996; Maelennan et al., 2002). However, it has yet to be determined whether the different components contribute to the protective effects independently or synergistically, and only a few studies *in vitro* have been reported in this regard. One study found that the mixture of total flavonoids and total terpenoids (4:1) did not show synergistic neuroprotection and antioxidant effects on cultured rat cerebellar granule cells (Chen et al., 1999; Xin et al., 2000). Interestingly, in another study, flavonoids and terpenoids exhibited a synergistic antioxidant effect on...
neonatal rat cortical neuron (Han et al., 2002). The synergistic neuroprotection in vivo of the two pharmacologically active constituents has not been reported yet.

In the current study, neuroprotective effects of pretreatment with total flavonoids, total terpenoids, and their combinations in different ratios of 16:1, 4:1, 1:1 against cerebral IR injury in mice was investigated in behavioral tests and neurochemical assays. The objective of this work was to ascertain the individual actions and interactions of flavonoids and terpenoids during cerebral IR.

Materials and Methods

Preparation of total flavonoids

The Ginkgo biloba leaves were collected in May 2006 on the campus of China Pharmaceutical University (CPU) (Nanjing, China) and identified by Dr. Boyang Yu. A voucher specimen has been deposited in the herbarium of the China Pharmaceutical University. Dried leaves (500 g) were refluxed with 70% ethanol (2 × 5 L), and the extract was concentrated in vacuum. The concentrate was dissolved with distilled water, chromatographed on D101 resin column, and eluted with water and 70% ethanol. The 70% ethanol elution was collected and evaporated in vacuum. The residue was then dissolved with 1% NaHCO3 aqueous solution, extracted with ethyl acetate. The aqueous fraction was transferred to a D101 resin column again, eluted with water, 20% ethanol, and 70% ethanol. The 70% ethanol elution was collected, evaporated, and freeze-dried to obtain a dark-yellow powder (3.2 g), namely the total flavonoids. Based on HPLC analysis (China Pharmacopoeia Committee, 2005), the content of flavonoids was 71.43%, and no terpenoids were detected in the powder.

Materials

EGb 761 was obtained from Dr. Willmar Schwabe Pharmaceuticals (Karlsruhe, Germany). The total terpenoids product was purchased from Shanghai OCOO Information Science and Technology Co., Ltd. (Shanghai, China); the product contains 45.63% ginkgolide A, 39.88% ginkgolide B, 8.15% ginkgolide C, and 1.58% bilobalide. The three combinations were obtained by mixing the total flavonoids and total terpenoids in proportions of 1:1, 4:1, or 16:1 and suspended in 0.9% saline (containing 1% Tween 80) as stocks. Medicinal kits for determinations of protein, malondialdehyde (MDA), superoxide anion (O2-), and superoxide dismutase (SOD), xanthine oxidase (XOD), superoxide anion (O2-), and myeloperoxidase (MPO) were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). All other chemicals were of the highest grade from commercial sources.

Animals and drug pretreatment

Male ICR mice weighting 30 ± 3 g were provided by New Drug Research Center, CPU, with free access to pelleted food and water under 12-h light-dark cycle at room temperature 22 ± 1°C and relative humidity 50–70%. This study complied with current ethical regulations on animal research of CPU, and all animals used in the experiment received humane care.

Mice (200) were divided randomly into 14 groups and pretreated with saline or drugs once daily for 7 days beforehand: Sham-operated group and saline-pretreated (control) group were given 0.9% saline. The drug-pretreated groups were EGb 761 (positive) group, total flavonoids group, total terpenoids group, and three flavonoids-terpenoids combinations (16:1, 4:1, 1:1) groups, with members of each group orally administered two doses of 15, 30 mg/kg body weight, respectively.

Bilateral carotid artery occlusion

Mice were anesthetized with chloral hydrate (350 mg/kg, i.p.), then, repetitive IR operation was made according to the described method (Lin et al., 2005). In brief, bilateral common carotid arteries (2CCA) were isolated and occluded with two operational clamps for 10 min at 38°C, with a 10% blood volume lose from tail to cause hypotension. After a 10-min reperfusion, a 10-min occlusion followed, and then recovery. The sham-operated mice were operated on omitting the occlusion. The mice continued their drug pretreatment protocol for a further 2 days.

Passive avoidance test

A passive avoidance reflex apparatus was provided by the National Academy of Traditional Chinese Medicine (Beijing, China) and was separated into light chamber and dark chamber with a connective hole and copper grids on the floor. Mice undergoing 24-h reperfusion were placed into the light chamber; if stepping through the hole into the dark chamber, they would suffer a 30-V electric stimulation and escape out of the dark chamber as training. After 24 h, mice were placed into the light chamber again, the latency of mice staying in the light chamber and the number of times they entered the dark chamber within 5 min were recorded to evaluate their memory capacity.

Gasping time determination after beheading

After passive avoidance test, mice were decapitated rapidly to cause global cerebral ischemia and hypoxia, the time from head cutting to respiration stopping...
was recorded exactly as the gasping time in mice (Xu, 1991).

**Antioxidant effects determinations**

After gasping time recording, the cerebra were removed immediately onto an ice pack, rinsed with ice-cold saline twice, and weighed exactly. A 10% (w/v) homogenate was prepared utilizing XHF-1 high-velocity homogenizer (Shanghai, China) by adding nine times ice-cold 0.86% (v/w) saline. The homogenate was centrifuged 3000 rpm for 10 min on a Hermle Z323K centrifuge (Wehingen, Germany). The supernatant was taken to carry out biochemical determinations in the light of commercial kit specifications, including protein concentration, contents of MDA and superoxide anion free radical (O$_2^-$), and activities of SOD and XOD.

The MDA level was measured by the absorbance at 532 nm on a Beckman Coulter DU 640 nucleic acid and protein analyzer (Fullerton, CA, USA) by the thiobarbituric acid (TBA) test (Beuge & Aust, 1978), and the results were expressed as nmol/mg protein in brain tissues. SOD activity was measured at 550 nm using a xanthine method (Ohkawa et al., 1979) and was expressed as U/mg protein in brain tissue. Xanthine oxidase can react with hypoxanthine to produce the superoxide anion free radical, and the latter can reduce NBT (nitroblue tetrazolium), producing a colorful substance that can be measured. We determined XOD activity by the absorbance at 530 nm and the result was expressed as U/g tissue protein. O$_2^-$ was determined according to the same method as SOD; the results were also expressed as U/g tissue protein representing inhibition activity on O$_2^-$.

**Myeloperoxidase activity assay**

Brain tissue MPO activity was measured in light of the kit instructions. MPO is an essential enzyme that exists in neutrophils. The enzyme can reduce hydrogen peroxide, by which MPO activity and the number of neutrophils can be determined. After providing hydrogen by the hydrogen donor (o-anisamine), a yellow compound was produced. We determined MPO activity by measuring the absorbance at 460 nm. Results were expressed as U/g protein.

**Data analysis**

The results were expressed as mean ± SD with time data (latency) transformed into logarithm. All data were analyzed by a one-way ANOVA, followed by Student’s two-tailed t-test for comparison between two groups, and Dunnett’s test when the data involved three or more groups. p < 0.05 was considered to be statistically significant.

**Results**

**Effects of flavonoids, terpenoids, and their combinations on memory, enhancing abilities in cerebral functional decline in mice**

The memory ameliorative activity of different drugs on cerebral dysfunction in mice injured by repetitive IR was evaluated in a passive avoidance step-through test. The latency in the saline-pretreated (control) mice was significantly shortened and the number of errors was significantly increased compared with the sham-operated mice (p < 0.05), which indicated that repetitive IR had disrupted memory acquisition. In comparison with the control group, pretreatment with drugs might elongate latency and decrease error numbers to different extents. However, only the effects in the 4:1 combination-pretreated group besides the EGb 761 group were of statistical significance (Fig. 1).

**Effects of flavonoids, terpenoids, and their combinations on gasping time in decapitated mice**

In our experiment, the gasping time in control group was markedly shortened compared with that of the sham-operated group from 19.85 ± 3.41 s to 14.69 ± 2.99 s (p < 0.01). When compared with the control group, pretreatment with drugs prolonged the gasping time more or less, whereas the effect in the terpenoids group was slight and did not reach statistical significance. EGb 761, 4:1 combination, and 1:1 combination as well prolonged significantly the gasping time at both doses of 30 and 15 mg/kg but total flavonoids and the 16:1 combination showed this action obviously at 30 mg/kg (Fig. 2).

**Antioxidant effects of flavonoids, terpenoids, and their combinations in vivo**

We measured MDA content, activity in SOD and XOD, and inhibitory activity on O$_2^-$ in brain tissue to observe the antioxidant effects of different drugs. At 2 days after IR, MDA content and XOD activity in the control group increased remarkably, whereas SOD activity and inhibition activity on O$_2^-$ decreased significantly compared with the sham-operated group (Table 1). When compared with the control group, all drugs reduced MDA content in IR-injured brain tissue except terpenoids at dose of 15 mg/kg. The 4:1 combination group (15, 30 mg/kg) showed the most significant activity (p < 0.01), which was the same as EGb 761. As for SOD, terpenoids at the two doses, the 4:1 and 1:1 combinations at 30 mg/kg, enhanced SOD level markedly. XOD activity was decreased significantly by total flavonoids at two doses, or by three combinations at 30 mg/kg. In addition, flavonoids and the 16:1 combination at 30 mg/kg, or the 1:1 combination at both doses
could inhibit $O_2^-$ markedly. Total terpenoids did not influence either XOD activity or $O_2^-$ obviously.

**Anti-inflammation effects of flavonoids, terpenoids, and their combinations**

MPO, a marker of neutrophil influx into tissue (Sener et al., 2006), was significantly increased in control mice compared with sham-operated mice from $0.082 \pm 0.007$ to $0.120 \pm 0.015$ U/g protein ($p < 0.001$) (Fig. 3), which suggested that neutrophil had infiltrated into IR brain tissue. In comparison with the control group, MPO activity was markedly decreased in all drug-pretreated mice. Among those drugs, terpenoids showed the strongest anti-inflammation effect ($p < 0.001$), which could return MPO activity to nearly normal level, then the 4:1 combination showed stronger effect than those of others ($p < 0.01$).

**Discussion**

Despite the biochemical and histologic findings supporting the putative therapeutic usefulness of EGB 761 in treating cerebral ischemia (Calapai et al., 2000; Ahlemeyer & Krieglstein, 2003), and although it is well-known that IR often leads to learning and memory impairments (Lin et al., 2005), very few studies have evaluated the use of behavioral parameters to assess the effects of EGB 761 in promoting functional recovery after ischemic brain damage. In our passive avoidance test, pretreatment with flavonoids or terpenoids alone did not show significant memory-enhancing activity,
All the data are shown as the mean ± SD (n = 6~10). *p < 0.05, **p < 0.01 compared with the sham-operated group; *p < 0.05, **p < 0.01 compared with control group.

However, the 4:1 combination and EGb 761 did under the same conditions. Thus, we suggest that there is a probable synergic effect between flavonoids and terpenoids on ameliorating the learning and memory ability after IR injury, and this synergic effect is greatly related to the ratio of flavonoids and terpenoids.

Protection against acute tissue hypoxia and ischemia is important for EGb 761. Thus, we evaluated the ability by determining the gasping time in decapitated mice. As a result, the gasping time was prolonged dose-dependently by flavonoids and three combinations, and terpenoids did not show an obvious effect in this regard. In accordance with the result of the passive avoidance test, the 4:1 combination also showed the strongest additive effect on gasping time.

Many studies have demonstrated that oxidative stress plays an important role in the pathogenesis of ischemic brain disruption and memory dysfunction (Walesiuk, 2006). XOD will be upregulated after cerebral reperfusion with release of heavy O2⋅−, which depletes antioxidant enzymes such as SOD and results in lipid peroxidation (LPO) (Weibrum et al., 1995; Yang et al., 2003; Lam le et al., 2006). Neurochemical assays disclosed that the content of MDA, a good indicator of the degree of membrane LPO (Sener et al., 2005), was decreased weakly by terpenoids compared with flavonoids and combinations, which indicated antioxidant effects of EGb 761 derived mainly from flavonoids other than terpenoids. Flavonoids inhibited LPO through at least scavenging O2⋅−, restoring SOD activity, and suppressing XOD activity, and the antioxidant action of the 4:1 combination was more dominant than those of others.

There is evidence that terpenoid constituents of EGb 761 have antagonistic activity against PAF, which plays an important role in ischemia (Smith et al., 1996; Maclennan et al., 2002). PAF is an inflammatory auto-antigen that can activate neutrophils, and activated neutrophils may induce or exacerbate tissue injury through releasing inflammatory factors and reactive oxygen metabolites (Sullivan et al., 2000). MPO activity is frequently utilized to estimate neutrophil infiltration in inflamed tissues (Sener et al., 2006). In the current study, as expected, IR caused a significant increase in MPO activity, indicating inflammatory injury in mice brains. This increase was inhibited by pretreatment with all of the drugs (p < 0.05), of which the 4:1 combination showed more (p < 0.01) and terpenoids showed the most (p < 0.001) significant effect on MPO level, suggesting that the anti-inflammation effect of EGB against IR injury may be provided mainly by the terpenoids fraction.

In conclusion, our results showed that both flavonoids and terpenoids of EGb 761 contributed to neuroprotection
during IR mainly via antioxidant or anti-inflammation pathways. Synergistic effects existed between the two main fractions, and the 4:1 combination of flavonoids and terpenoids, namely the natural ratio in EGB 761, exhibited the greatest neuroprotective effect.

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References


