

抑制剂氯化锂对烟碱诱发惊厥作用的影响。
 结果: 氯化锂 2.5-10 mmol·kg⁻¹ 预处理后, 烟碱诱发小鼠惊厥的量效关系发生变化, 在高于半数效量的剂量下, 烟碱诱发惊厥的作用显著增强。但氧氟莫林 0.05-0.20 mg·kg⁻¹ 预处理后, 烟碱

诱发小鼠惊厥的量效关系无显著变化。在小鼠上每日注射一次氯化锂 5.0 mmol·kg⁻¹ 7 d 后, 烟碱诱发惊厥的作用显著减弱, 半数效量由 0.58 增至 0.97 mg·kg⁻¹。结论: 磷脂酰肌醇代谢可调节中枢烟碱受体的功能。

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Antagonistic effects of extract from leaves of *Ginkgo biloba* on glutamate neurotoxicity

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KEY WORDS cultured cells; neurons; calcium; cerebral cortex; arcuate nucleus; glutamates; *Ginkgo biloba*; ginkgolide B; quercetin

AIM: To determine whether the extract of leaves of *Ginkgo biloba* L (EGb) and several active constituents of EGb have protective effects against glutamate (Glu)-induced neuronal damage.

METHODS: Microscopy and image analysis of nucleus areas in the arcuate nuclei (AN) of mice were made. The neuronal viability in primary cultures from mouse cerebral cortex was assessed using MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide] staining and the intracellular free calcium concentration ([Ca²⁺]_i) of single neuron was measured using Fura-2.

RESULTS: EGb (2.5 mg·L⁻¹) and its constituent ginkgolide B (Gin B, 2 mg·L⁻¹) protected the neuronal viability against Glu-induced injury, and prevented the Glu-induced elevation in [Ca²⁺]_i. EGb (3 - 10 mg·kg⁻¹) attenuated the decrease of nucleus areas in arcuate nuclei induced by Glu (1 g·kg⁻¹, sc). **CONCLUSION:** EGb and Gin B prevent neurons from Glu neurotoxicity through reduction of the rise in [Ca²⁺]_i.

Glutamate neurotoxicity (GNT) participated in

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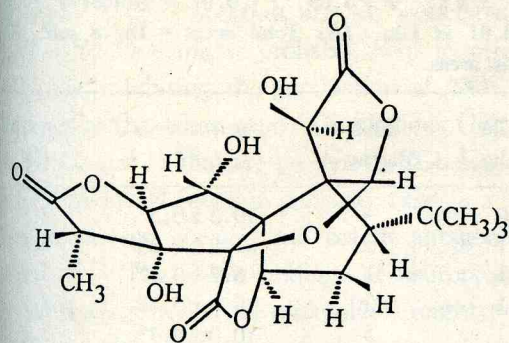
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the neuron loss associated with a number of neurodegenerative diseases, eg, Alzheimer's disease and Huntington's disease^[1,2]. To investigate the GNT and anti-GNT drugs, a primary cell culture system derived from fetal mouse neocortex was used. *In vivo* studies were to detect the effects in arcuate nucleus in hypothalamus, which were not fully protected by the blood-brain barrier (BBB) in immature animals^[3].

Overactivation of Glu receptors caused an excessive influx of Ca²⁺ into the neuron, which resulted in activation of lipase, protease, and protein kinase C, subsequently led to the generation of fatty acids, free radicals, and ultimately neuronal death. Ca²⁺ overloading and loss of Ca²⁺ homeostasis might play an important role in GNT^[4,5].

The extract of leaves of *Ginkgo biloba* L (EGb) contained 24 % of flavonoid glycosides, the aglycon of which was a flavonol (including quercetin, kaempferol, and isorhamnetin), 6 % of terpene lactones (including ginkgolides A, B, C, J, and bilobalide), and 70 % of other substances (proanthocyanidins, organic acids, sugars, etc)^[6]. EGb had protective effects on GNT in a dose-dependent manner in both cultured mouse cortical neurons and cultured human hippocampal neurons^[7]. This study was to investigate whether EGb or some of its constituents protected neurons against GNT *in vitro* and *in vivo*.



Ginkgolide B

MATERIALS AND METHODS

Reagents EGb was the product of Jarrow Formulas, Inc (Los Angeles CA, USA), which was analytically controlled to ensure consistency of its composition, and standardized to contain 24 % flavonoid glycosides and 6 % terpene lactones. Ginkgolide B (Gin B, BN 52021, hypohilissat) was the product of Ipsen (30 rue Cambronne, Paris, France).

Quercetin (Que), Fura-2, HEPES, and *L*-glutamic acid (*L*-Glu) were purchased from Sigma. *L*-Glu were dissolved in deionized distilled water and adjusted to pH 7.0 with NaOH 1 mol · L⁻¹. Dulbecco's modified Eagle medium (DMEM) and tetrazolium salt (MTT) were purchased from Gibco and Fluka, respectively. Other reagents were of AR grade. All solutions were prepared with deionized distilled water.

Primary cortical cell culture Mouse cortical neurons were prepared from 14 - 17-d-old embryos using technique with minor modification^[8]. The cerebral cortex was minced, incubated in 0.125 % trypsin at 37 °C for 10 min and stopped with Hanks' balanced salt solution. The cell pellet was resuspended in DMEM supplemented with 20 % heated-inactivated fetal bovine serum, *L*-Glu 2.0, NaHCO₃ 24, HEPES 10, and glucose 25 mmol · L⁻¹. Cells were seeded on 96-well plates (3 × 10⁵ cells per well) coated with *L*-poly-lysine and incubated at 37 °C in 5 % CO₂ atmosphere. After 5-7 d in culture, non-neuronal cells division was halted by 1-3 d of exposure to cytosine arabinoside (10 μmol · L⁻¹). The culture medium was renewed every 3-4 d.

Treatment with Glu and EGb Experiments were carried out after 7 d in culture. Following the incubation with EGb (2.5 mg · L⁻¹), Que (20 mg · L⁻¹), and Gin B (2 mg · L⁻¹) or both Que and Gin B (20 mg · L⁻¹ + 2 mg · L⁻¹), respectively for 24 h, cell culture was exposed to Glu (0.5 mmol · L⁻¹) at 25 °C for 30 min, and then the solution was thoroughly washed and transferred to CO₂ incubator until the

next day for MTT assay or microscopy.

MTT stains MTT in PBS (0.1 mol · L⁻¹, pH 7.2) 25 μL was added to each well of 96-well plates (1 g · L⁻¹ final concentration) and incubated at 37 °C for 4 h. The reaction was stopped by adding 100 μL lysing buffer (20 % SDS in 50 % *N,N*-dimethylformamide, pH 4.7). The absorbance was measured at 570 nm (*A*₅₇₀) by microELISA reader after a further incubation at 37 °C overnight^[9].

Image analysis Mice aged 7 - 9-d-old (*n* = 42), weighing 5.4 ± 0.6 g were divided into 6 groups at random. The mice were daily sc saline, Glu (1 g · kg⁻¹), Glu + EGb (1, 3, 5, 7, 10 mg · kg⁻¹) for 7 d. EGb was injected 15 min before Glu. All mice were killed 1 wk after the final-treatment and perfused transcardially with 10 % formalin, serial coronal sections (6 μm) were stained with cresyl violet^[10]. Nucleus areas were determined by 540-Biological Medicine Color Image Analyser.

Measurement of [Ca²⁺]_i The freshly dissociated cortical neurons prepared from 1 - 2-d-old mice were loaded with Fura 2-AM 2 μmol · L⁻¹ at 37 °C for 45 min and collected by centrifugation. Cells were treated with Glu (100 μmol · L⁻¹, 10 μL), Glu + EGb (1 g · L⁻¹, 10 μL), or Glu + constituents of EGb, including Que (0.8 g · L⁻¹, 10 μL), Gin B (0.2 g · L⁻¹, 10 μL), Que (0.8 g · L⁻¹, 10 μL) + Gin B (0.2 g · L⁻¹, 10 μL) in 100 μL suspension. [Ca²⁺]_i was determined from the ratio of the fluorescence (λ_{ex} = 340 and 380 nm; λ_{em} = 505 nm) by Spex AR-CM-MIC Cation Measurement System^[11].

Statistical analysis All data were presented as $\bar{x} \pm s$. Comparisons between groups were made using *t*-test.

RESULTS

GNT *in vitro* After 7 d in culture, neurons developed an extensive network, showing large and phase-bright cell bodies (Fig 1A, Plate 2). When cultures were exposed to Glu, swelling and darkening of neurons were seen and debris occurred in some areas (Fig 1C). While sister cultures were exposed to EGb, Gin B, or Que for 1 d prior to Glu, it showed almost no (Fig 1D) or only a slight neuronal injury (Fig 1E, 1F).

This morphological change was confirmed by the assay of MTT (Tab 1).

Glu induced a marked decrease in *A*₅₇₀, which can be completely abolished by the preincubation with EGb and Gin B + Que. When pretreated with the Que or Gin B, only the latter can partially abolish *A*₅₇₀. Accordingly, Gin B may be a main active constituent in EGb to prevent GNT, while

Tab 1. Neuronal viability and changes of $[Ca^{2+}]_i$ induced by various agents in cortical neurons of mouse.
n = 12 or 20, $\bar{x} \pm s$. ^a*P* > 0.05, ^b*P* < 0.05, ^c*P* < 0.01 vs control; ^d*P* > 0.05, ^e*P* < 0.01 vs Glu; ^f*P* < 0.01 vs Glu + Que + Gin B.

Treatment	Absorbance at 570 nm	$[Ca^{2+}]_i / \text{nmol} \cdot \text{L}^{-1}$
Hanks' solution	0.38 ± 0.05	279 ± 24
Glu	0.26 ± 0.03 ^c	388 ± 53 ^c
Glu + EGb 761	0.38 ± 0.05 ^{af}	291 ± 38 ^{af}
Glu + Que + Gin B	0.37 ± 0.04 ^{af}	288 ± 44 ^{af}
Glu + Gin B	0.32 ± 0.04 ^{df}	313 ± 52 ^{bf}
Glu + Que	0.30 ± 0.06 ^{cd}	363 ± 62 ^{cd}

Tab 2. Image analysis of neuronal areas in arcuate nuclei.
n = 6, $\bar{x} \pm s$. ^a*P* > 0.05, ^c*P* < 0.01 vs Saline; ^d*P* > 0.05, ^f*P* < 0.01 vs Glu, TA: Total areas = 187.8 μm^2 , NA: Nucleus areas.

Glu /g · kg ⁻¹	EGb /mg · kg ⁻¹	Nucleus areas / μm^2	NA/TA /%
0	—	10.3 ± 0.5	5.5
1	—	6.45 ± 0.20 ^c	3.4
1	1	6.7 ± 0.3 ^{cd}	3.6
1	3	9.6 ± 0.7 ^{af}	5.1
1	5	10.0 ± 0.7 ^{af}	5.3
1	7	10.4 ± 0.6 ^{af}	5.5
1	10	11.0 ± 0.8 ^{af}	5.8

Que enhances the protective effects of Gin B on GNT.

$[Ca^{2+}]_i$ of a normal neuron increased from the basal level of 279 ± 24 $\text{nmol} \cdot \text{L}^{-1}$ to 388 ± 53 $\text{nmol} \cdot \text{L}^{-1}$ after the addition of Glu (100 $\mu\text{mol} \cdot \text{L}^{-1}$, 10 μL) into 100 μL suspension of neurons, which were reduced by the treatment of EGb, Gin B + Que, or Gin B alone (Tab 1).

GNT *in vivo* As compared with the control group (Fig 2A), sc Glu (1 $\text{g} \cdot \text{kg}^{-1}$) led to pyknotic nuclei and loss of neurons in the AN (Fig 2B), which were dramatically attenuated by sc EGb 10 $\text{mg} \cdot \text{kg}^{-1}$ (Fig 2C).

Image analysis of the nucleus areas in the AN revealed that pretreatment of EGb (3 - 10 $\text{mg} \cdot \text{kg}^{-1}$) dwindled the neuronal death induced by Glu in neonatal mice (Tab 2).

DISCUSSION

EGb, mainly its active constituents 24 % flavonoid glycosides and 6 % terpene lactones, could exert significant influences on cardiovascular system and CNS as a free radical scavengers and the antagonist of PAF, while the effects of other 70 % constituents are not identified^[6]. In the present experiments, using MTT assay, it was found that besides EGb, either the active constituent Gin B alone or plus Que had significant protective effects on GNT, while the protective effect of Gin B + Que seemed to be greater than Gin B alone. Furthermore, similar results were obtained by the measurement of $[Ca^{2+}]_i$, excepted that Que had not shown any effects on $[Ca^{2+}]_i$. Que could directly inhibit NADPH diaphorase^[13], which

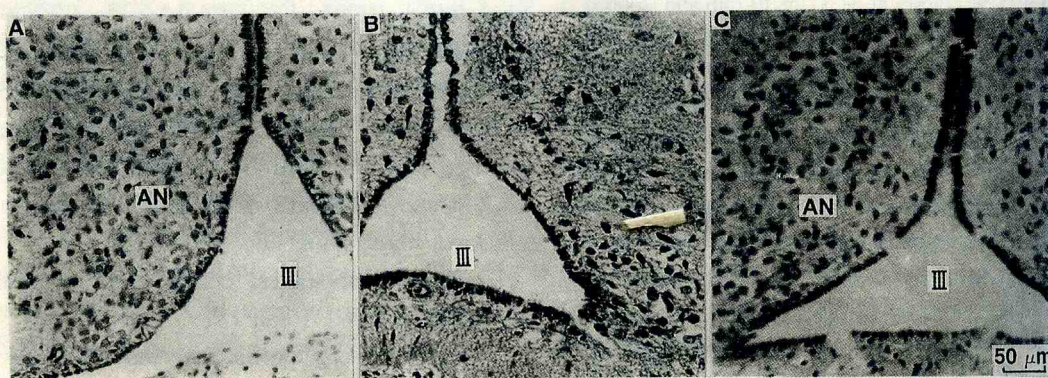


Fig 2. Sections through arcuate nucleus of hypothalamus from mice treated with saline, Glu, and EGb + Glu daily for 7 d.
 A) normal neurons in arcuate nucleus after sc saline; B) pyknotic nuclei and loss of neurons in AN after sc Glu 1.0 $\text{g} \cdot \text{kg}^{-1}$; C) less pyknotic nuclei and more neurons in AN after sc EGb (10 $\text{mg} \cdot \text{kg}^{-1}$) 15 min before sc Glu, AN: arcuate nucleus, III: the 3rd ventricle.

distributes consistently with that of nitric oxide synthase^[14], an important enzyme associated with GNT. So Que might probably help to attenuate GNT *via* inhibiting the production of NO in the brain. On the other hand, Ginkgolides (including A, B, C, and J) that are present in EGb possess 2, 3, or 4 hydroxyl function and may exist in a "cage-like" form, are specific and potent antagonists of PAF^[6,12]. The above findings of neuroprotection of Gin B suggest that all ginkgolides might prevent GNT *via* the depression of the elevated level of Ca^{2+} induced by Glu, though the molecular mechanisms remain unknown.

In summary, both EGb 761 and Gin B may protect neurons against GNT by the suppression of the rise in $[Ca^{2+}]_i$.

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银杏叶提取物对谷氨酸神经毒性的拮抗作用

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关键词 培养的细胞; 神经元; 钙; 大脑皮层; 弓状核; 谷氨酸盐类; 银杏; 银杏内酯 B; 槲皮素

目的: 观察 EGb 及其所含成分槲皮素和内酯 B 对谷氨酸诱导的神经元损伤的拮抗作用。 **方法:** 对小鼠下丘脑弓状核作形态学观察和图象分析, 采用 MTT 法检测皮层原代培养神经元的活性, 用 Fura-2 测定单个神经元内游离钙离子的浓度 ($[Ca^{2+}]_i$)。 **结果:** EGb 及其内酯 B 能够保护神经元对抗谷氨酸的损伤, 阻止谷氨酸诱发的 $[Ca^{2+}]_i$ 的升高。 EGb 能够逆转谷氨酸诱导的下丘脑弓状核神经元核面积的减少。 **结论:** EGb 及其内酯 B 可以通过降低 $[Ca^{2+}]_i$ 水平对抗谷氨酸神经毒性, 内酯 B 很可能是 EGb 中对抗谷氨酸神经毒性的主要成分之一。