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Ginkgo biloba leaf extract enhances levels of caspase-3 and amyloid precursor protein in normal rat hippocampus[1]

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KEY WORDS *Ginkgo biloba*; caspases; amyloid beta-protein precursor; immunohistochemistry

ABSTRACT

AIM: To explore the effect of *Ginkgo biloba* extract (*Gb*E) on the levels of caspase-3 and amyloid precursor protein (APP) in normal rats' hippocampus. **METHODS:** Immunohistochemistry method was used for qualitative analysis of the expressions of caspase-3 and APP, and an image analysis method was used for the quantification of the levels of caspase-3 and APP after *Gb*E was administered to rats of different ages for 14 d. **RESULTS:** The mean absorbance of staining of caspase-3 and APP was markedly higher in *Gb*E group than that in control groups. The expressions of caspase-3 and APP were intensified in the hippocampus of rats after *Gb*E administration. **CONCLUSION:** *Gb*E can raise the levels of caspase-3 and APP in the hippocampus of normal rats.

INTRODUCTION

The pathogenesis of Alzheimer's disease (AD) is still uncertain and we have not effective therapy yet. In the cascade hypothesis of β-amyloid protein (A-β)[1], Hardy *et al* indicated that Aβ played an important role in pathophysiologic and pathologic process of AD. AD is accompanied by the deposition of A-β in senile plaques and cerebral blood vessels. The abnormal secretion and /or deposit of A-β are involved in the pathogenesis. Being the main constituent of senile plaque, A-β and its precursor-amyloid precursor protein (APP) were studied by many scientists[2-4]. The latest studies showed that caspase-3 could convert APP to produce A-β[5,6]. *Gb*E is used to combat a variety of neurological disturbances in Alzheimer's disease including depression, lack of attention, and short-term memory loss[7-9]. In Wirth and colleagues' research, their results provided us evidence with a short-term memory enhancement effect of *Gb*E (60 mg/kg and 120 mg/kg) in both young and aged rats[10]. Winter also found that *Gb*E (50 mg/kg and 200 mg/kg) had beneficial effects on cognitive performance and longevity in rats[11]. Yang's and other's studies suggested that *Gb*E could protect neurons against apoptosis induced by free radicals[12-14]. However, the possible effect of *Gb*E on caspases and APP remained unclear. In the present study, the effect of *Gb*E, at an effective medium dose (100 mg/kg) employed in previous experiments[10-14], on expressions of caspase-3 and APP, was observed in rat hippocampus.

MATERIALS AND METHODS

Reagents *Gb*E (containing 26 % flavone glycosides and 6 % diterpen lactone, Xinbang Pharmaceutical Factory, Guizhou province) was prepared as 0.1 % solution with normal saline. The antibody of APP-(rabbit-

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anti-rat) was purchased from Sigma Co in USA. The antibody of caspase-3 (rabbit-anti-rat), SABC (streptavidin-biotin complex) kit (rabbit) and diaminobenzidine (DAB) staining kit were purchased from BOSTER Biological Engineering Co in Wuhan, China.

Apparatus BI2000 image analysis system (Taimeng Co in Chengdu) and HL-2 transfusion pump.

Treatment of animals Forty-two male Wistar rats (grade II) were supplied by the Experimental Animal Center of the Third Military Medical University. Qualification number is 2001015. All rats were divided into three age groups: One-month-old, four-month-old, and over twelve-month-old. The rats were fed with ordinary forage with free access to tap water. Each age group was randomly divided into two sub-groups: control group and *Gb*E group. *Gb*E 100 mg/kg was administered through intra-gastric method in *Gb*E group for 14 d.

Preparations of brain On d 15, rats were fixed in a supine position after 35 % chloral hydrate anesthesia to fully expose thorax and heart. A trocar (No 16) connected with transfusion pump was inserted into aorta through the tip of heart. Ventroaorta was clamped, and atria was cut open, then transfused forward with cold (4 °C) phosphate buffer solution (PBS, 0.1 mol/L). When the outflow of PBS from left atria was limpid, PBS was replaced by cold formalin (4 °C) until the carcass turned rigid. Brain tissues were removed and put into formalin (4 °C) over night. On the next day, the tissues were removed and deposited in saccharose solution (20 %, containing anti-septics). Freezing coronal brain tissue slice was prepared at 30-μm thick each.

Immunohistochemistry Free floating staining and SABC (streptavidin-biotin complex) method were used for detection. DAB was used as a staining agent. Rabbit normal immune serum was used to replace primary antibody (antibody-APP, antibody- caspase-3) in negative control group. PBS was used to replace immediate antibody in working control group.

Image analysis Immunohistochemistry analysis module of BI2000 image analysis system was used to quantify the levels of APP and caspase-3 of hippocampus in brain tissue slices. The values of mean absorbance and mean gradation were used to show the levels.

Statistical method Adopt *q*-test of Microsoft excel. There is discrepancy when $P \leq 0.05$, and significant discrepancy when $P \leq 0.01$.

RESULTS

Effect of *Gb*E 100 mg/kg on level of APP in normal rats' hippocampus In all brain tissue slices, cytoplasm and membrane of cone-shaped cell in hippocampus showed deeper yellow stain in *Gb*E group than in control group. Image analysis showed that, in four-month-old and over twelve-month-old rats, the value of mean absorbance was higher in *Gb*E group than in control group. The value of mean gradation was lower in *Gb*E treated group than control group, correspondingly. The results indicated that the level of APP in normal rats' hippocampus was elevated in *Gb*E group. While in one-month-old rats, no statistical difference was observed between these two groups.

Tab 1. Effect of *Gb*E 100 mg/kg on level of APP in normal rats' hippocampus. *n*=7. Mean±SD. $^{b}P<0.05$, $^{c}P<0.01$ *vs* control. The number in the parentheses means the age of rats in months.

Group	Mean absorbance	Mean gradation
*Gb*E (12)	0.38±0.03^{c}	176±6^{c}
Control (12)	0.320±0.020	186±4
*Gb*E (4)	0.30±0.04^{b}	190±7^{b}
Control (4)	0.265±0.020	197±4
*Gb*E (1)	0.33±0.06	183±9
Control (1)	0.324±0.020	185±4

Effect of *Gb*E 100 mg/kg on level of caspase-3 in normal rats' hippocampus In all brain tissue slices, cone-shaped cell in hippocampus showed deeper yellow stain in *Gb*E group than in control group. Mark-

Tab 2. Effect of *Gb*E 100 mg/kg on level of caspase-3 in normal rats' hippocampus. *n*=7. Mean±SD. $^{c}P<0.01$ *vs* control. The number in the parentheses means the age of rats in months.

Group	Mean absorbance	Mean gradation
*Gb*E (12)	0.25±0.04^{c}	198±7^{c}
Control (12)	0.206±0.005	207.6±1.0
*Gb*E (4)	0.240±0.010^{c}	200.6±2.2^{c}
Control (4)	0.220±0.08	204.7±1.5
*Gb*E (1)	0.233±0.010^{c}	202.0±2.5^{c}
Control (1)	0.216±0.003	205.4±0.7

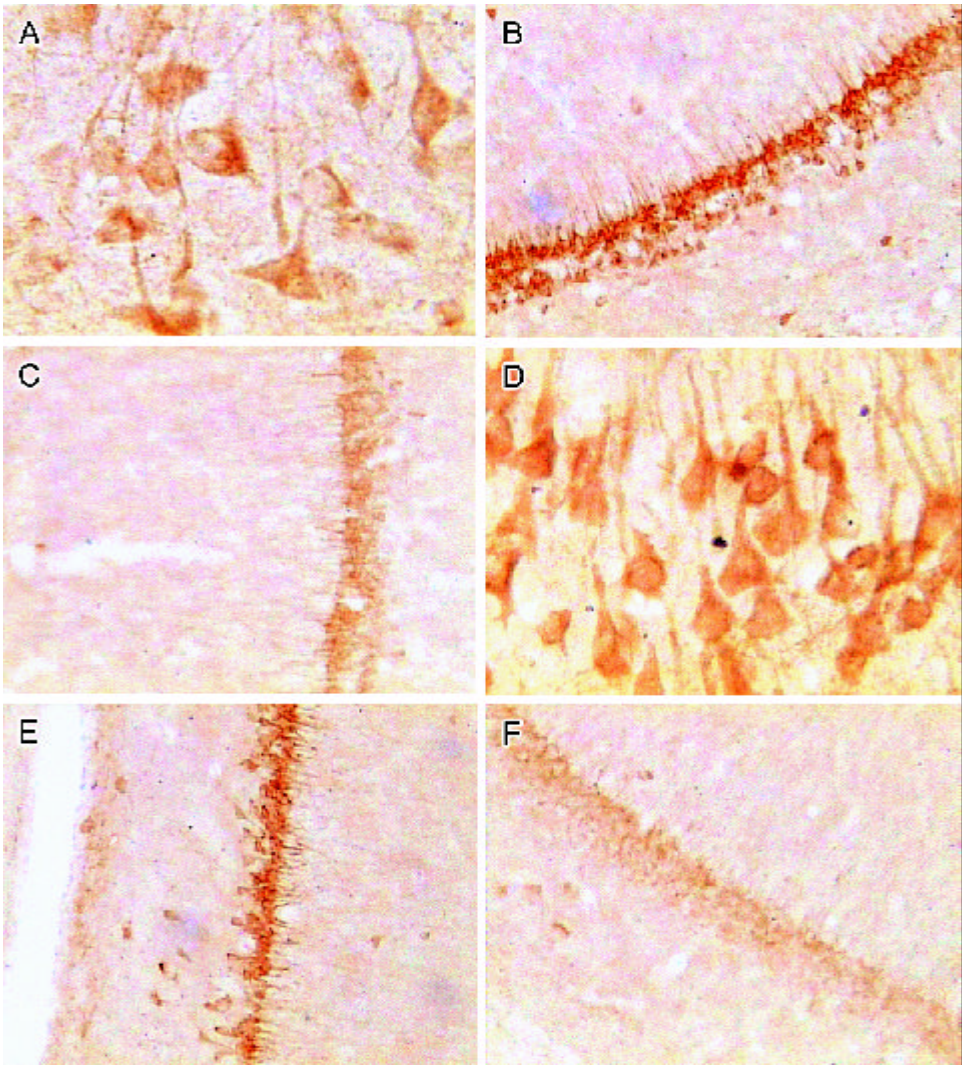

Fig 1. Photos of immunohistochemistry. A) Staining of APP in rat hippocampus in *Gb*E group (×400). APP is a transmembrane glycoprotein that extends its carboxylic terminal into cytoplasm. A distinct yellow stain could be observed in cytoplasm and membrane of the cone-shaped cell. B) Staining of APP in rat hippocampus in *Gb*E group (×100). C) Staining of APP in rat hippocampus in control group (×100). D) Staining of caspase-3 in rat hippocampus in *Gb*E group (×400). E) Staining of caspase-3 in rat hippocampus in *Gb*E group (×100). F) Staining of caspase-3 in rat hippocampus in control group (×100).

edly higher value of mean absorbance in *Gb*E group than in control group was observed through image analysis ($P<0.01$). The value of mean gradation was significantly lower correspondingly ($P<0.01$). The results indicated that the level of caspase-3 in normal rats' hippocampus was increased in *Gb*E group.

DISCUSSION

*Gb*E, at doses of 50-200 mg/kg, is beneficial in a broad range of physiological dysfunctions and pathological conditions, including those involved with circulation, blood conditions, metabolism, and immune function[15-17]. Pharmacological effects of the extract include antioxidant, free radical scavenging, nerve protection, and platelet inhibition properties[18]. *Gb*E also helps modulate cerebral energy metabolism and inhibit acetylcholinesterase[19,20]. Numerous pharmacological and clinical studies of *Gb*E have demonstrated a positive effect in enhancing short-term memory[21]. Generally, *Gb*E is primarily an important remedy for aging and the elderly for restoring proper function and preventing degenerative changes. However, other antioxidants have no equivalent therapeutic properties compared with *Gb*E. As to the ability of inhibiting acetylcholinesterase, *Gb*E is not as good as tacrine[20]. The mechanism by which it may be used to treat AD needs further research. Although the use of only one dosage of *Gb*E was a drawback in the present study,

we argue that an effective dosage could also provide sufficient pharmacological evidence of *Gb*E, especially when the effect was observed by positive response, such as in the immunohistochemistry determination. The *Gb*E 100 mg/kg, which was used in present study, is considered appropriate according to results of our and other laboratory[10-14]. Results had shown that the levels of APP and caspase-3 in rats' hippocampus were increased after administration of *Gb*E for14 successive days. The results indicated that *Gb*E might affect the caspase-3-mediated proteolysis process of APP.

The proteolysis process of APP has two pathways. One is secretive pathway. The other is endocytotic pathway. In secretive pathway, APP is cleaved by α-secretase to generate Aβ segment. While in endocytotic pathway, APP is first cleaved by β-secretase where abutting against the amino-terminal of Aβ segment. The process generates a surface APP (APPs) and a P_{11} segment which contains an integrated Aβ segment. Then P_{11} is cleaved by γ-secretase at the carboxyl end of Aβ segment to generate integrated Aβ[22,23]. A number of laboratories have recently demonstrated that APP can be cleaved in the cytoplasmic domain by caspases after the aspartate-664 residue, Val-Glu-Val-Asp$^{664}\downarrow$Ala[5,23]. This cleavage would generate a C-terminal-truncated APP molecule (APPΔC31)[5,24]. Previous observations show that the APP cytoplasmic region contains a tetrapeptide motif (^{683}YENP^{686}) that functions as a signal for endocytosis[25]. Accordingly, loss of this endocytotic signal abrogates processing of cell surface APP in the endocytotic pathway and severely impairs Aβ production and secretion. Because caspase-cleaved APP lacks the endocytotic signal, the truncated APPΔC31 should in theory release less, rather than more. This process is indicated in Fig 2.

In this experiments, it shows that the levels of APP and caspases were improved in *Gb*E group compared with controls. The result indicates, in agreement with the endocytosis-hypothesis, that *Gb*E might affect the generation of Aβ through caspase-3-mediated proteolytic process of APP.

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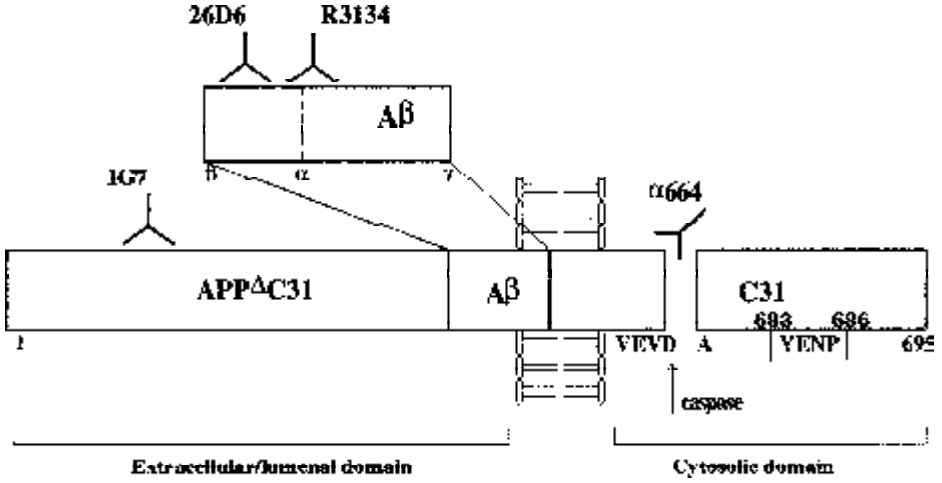

Fig 2. Alleged interaction between caspase-3 and APP as proposed by Gervais FG[2,3].

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