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Extract of Ginkgo Biloba Promotes the Expression of VEGF Following Subarachnoid Hemorrhage in Rats

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Abstract

The study aimed to investigate the effect of extract of Ginkgo biloba (EGb) on the expression of vascular endothelial growth factor (VEGF) after subarachnoid hemorrhage (SAH). Wistar rats were divided into non-SA, SAH, vehicle, EGb1 (low dose), and EGb2 (high dose) groups. VEGF mRNA and VEGF protein were measured from brain tissues. The expressions of VEGF mRNA in SAH and vehicle groups were enhanced 24 and 72 hours after the establishment of SAH. Increased VEGF positive cells were found in the brain tissues in SAH and vehicle groups. The expressions of VEGF mRNA and VEGF protein were further increased by the pretreatment of EGb. We concluded that EGb exerts protective effects on secondary cerebral ischemic injury after SAH via the promotion of the expression of VEGF.

Keywords: Ginkgo biloba; subarachnoid hemorrhage; cerebral ischemia; vascular endothelial growth factor, rats.
Introduction

Secondary cerebral ischemia and related brain injury are responsible for the high incidence of morbidity and mortality of subarachnoid hemorrhage (SAH) (Sun et al., 2003; Manno, 2004). The pathogenesis of secondary ischemia and brain injury after SAH remains poorly understood (Kwan et al., 2002). Oxyhemoglobin (OxyHb) released from the blood in the subarachnoid spaces is regarded as the primary cause of cerebral vasospasm and secondary cerebral ischemia after the onset of SAH (Asano, 1999; Grasso, 2004; Wijdicks et al., 2005). Vascular endothelial growth factor (VEGF) promotes angiogenesis during hypoxia and ischemia in diverse tissues, including the brain (Tammela et al., 2005; Ribatti, 2005). A raised serum level of VEGF in SAH patients was found, indicating the possible role of VEGF-mediated molecular response (Zand et al., 2005). VEGF released in excessive amounts from cerebrovascular endothelial cells is the most potent angiogenic factor that may enhance angiogenesis after SAH (Josko, 2003). Our previous studies showed that extract of Ginkgo biloba (EGb) improves cerebral blood perfusion (Sun et al., 2000, 2003). However, the mechanisms of perfusion improvements have not been fully understood. The present study was designed to investigate the expression of VEGF and the influence of EGb after experimental SAH.

Materials and Methods

Animal preparations

This study was approved by the Institutional Review Board of Taishan Medical College. Wistar rats of both sexes (Experimental Animal Center of Shandong University) weighing 330g to 380g were housed in groups of five per cage at a
constant temperature (24±1°C) and humidity (60±5%), on a 12-h light-dark cycle. The rats were given free access to food and water before and after experiments. Rat SAH models were replicated by a modified cisterna magna injection method using autologus arterial hemolysate. Briefly, rats were anesthetized by intraperitoneal use of chloral hydrate and were kept warm with a heating lamp. The left femoral artery was cannulated and 0.4 ml blood was drawn into a heparinized microinjector (310, Stoelting Co. Ltd, USA). The blood was frozen at −80°C for 15 min. Then, the frozen blood was melted at 37°C and the autologus arterial hemolysate was obtained.

The rat’s head was fixed in a stereotaxic frame (51600, Stoelting Co. Ltd, USA) to maintain a head down position of 30°. An incision was made in the midline and the skin, musculature were carefully separated using an operating microscope to expose the atlantooccipital membrane. The microinjector was mounted on the manipulating arm of the stereotaxic instrument with a needle connected to a three-way stopcock to allow the measurement of intracranial pressure (ICP). The needle was lowered into cisterna magna under direct vision. To produce SAH, 0.3 ml arterial hemolysate was injected into the cistern very slowly over 20 min with a constant rate. The non-SAH rats were manipulated in the same way, except that same volume of saline was injected into the cistern.

Animals were randomly divided into non-SAH, SAH, vehicle, EGb1 (low dose) and EGb2 (high dose) groups. Each group includes 18 rats. Twelve rats from each group were used for the reverse transcription PCR (RT-PCR) determination of the expression of VEGF mRNA 24 hrs and 72 hrs after the cisternal injection of saline (Non-SAH group) or hemolysate (SAH, vehicle and EGb groups), respectively. Six rats from each group were sacrificed to undergo immunohistochemistry detection.
of VEGF in the brain sections 72 hrs after the cisternal injection.

Preparation of EGb

EGb was obtained from Lvyuan Pharmaceutical Co. Ltd (Shanghai, China), standardized on the amount of flavone glycosides $\geq 24\%$ and ginkgolide $\geq 6\%$ by a HPLC determination. EGb was solubilized in physiological saline. The stock solution of EGb was prepared just before use. Rats in EGb1 and EGb2 groups received intraperitoneal injection of EGb (100mg/kg and 200mg/kg respectively) 30 min before cisternal injection. The peritoneal injection was repeated twice daily with of the initial half dose. Vehicle, same volume as EGb solution, was administrated at the same intervals in a same route as EGb.

RT-PCR determination of VEGF mRNA

The animals were killed by guillotine decapitation 24 hrs and 72hrs after cisternal injection and the brain was immediately excised. Then, about 100g cortical tissues were preserved in the liquid nitrogen. Total RNA was extracted from snap-frozen tissues using the phenol/guanidine isothiocyanate method with Trizol reagents (Gibco BRL, Gaithersburg, MD, USA) according to the manufacturer’s protocol. The purity of total RNA was assessed by the 260/280-nm ratio (between 1.8 and 2.0) and by the absence of bands corresponding to contaminating DNA in the agarose electrophoresis. The first chain of cDNA was synthesized using moloney murine leukemia virus (MMLV) reverse transcriptase (Shenggong Biological engineering Co., Shanghai, China) under conditions recommended by the manufacturer. 2 $\mu$l total RNA, 1 $\mu$l oligo dT, 1 $\mu$l dNTPs, 1 $\mu$
1 MMLV reverse transcriptase and 1 μl Rnasin in 10 μl diethyl pirocarbonate (DEPC)-treated water were maintained at 37°C in a water-bath for 60 min, followed by heating at 95°C for 5 min. The products were kept at –20°C in a refrigerator.

Oligonucleotide primers including VEGF (upstream: 5’-TCTTCAAGCCGTCTGTG-3’, down stream: 5’-CCTTTCCTCGAACTGATTTT-3’, PCR product: 228bp) and β-actin (upstream: 5’-GGGAAATCGTGCGTGACAT-3’, down stream: 5’-CAGGAGGAGCAATGATCTT-3’, PCR product: 386bp) were designed by the software Primer Premier v5.0 according to the sequences of cDNAs in the GenBank. The primers were synthesized by Bioasia biotechnologies. The PCR was performed using a PTC-150 microplate gradient thermal cycler (MJ Research, USA). Application of samples were carried out on a iced plate, in which DEPC treated water (37.5 μl for VEGF and 32.5 μl for β-actin), 1 μl cDNA, 1 μl dNTPs (10mM), 5 μl 10× PCR buffer, 0.5 μl Taq DNA polymerase (5U/μl), 1 μl upstream primer (10 μM), 1 μl downstream primer (10 μM), 25mM MgCl2 (3 μl for VEGF and 8 μl for β-actin) were added into the PCR reaction mixture.

In each PCR cycle, heat denaturation was set at 94°C for 45 sec, primer annealing at 51°C for VEGF and 56°C for β-actin for 30 seconds, and polymerization at 72°C for 7 minutes. The cycles were 36 for VEGF and 30 for β-actin. PCR product was electrophoresed in 1.5% agarose gels containing 0.2 mg/ml of ethidium bromide. The gels were visualized under ultraviolet light and photographed with Polaroid film. The films were scanned by computerized laser densitometry, and the results are expressed in arbitrary densitometry units normalized to β-actin expression.
**Immunohistochemistry detection of VEGF protein**

The animals were killed 72 hrs after cisternal injection. The rats were anesthetized with intraperitoneal chloral hydrate (450mg/kg) and perfused with the use of a cardiac catheter with 200ml to 300 ml normal saline followed by 500 ml 4% paraformaldehyde in 0.1 mol/L phosphate buffer saline (PBS, pH7.4) over 40 min. The brain was removed and was cut into slice of 3mm to 4mm thickness. The slices were post fixed in 4% paraformaldehyde for 12 hrs. Sections were cut into 50 mm thickness with a paraffinum microtome after routine handling. Endogenous peroxidase was blocked by incubation with 0.3% hydrogen peroxide in PBS for 60 min, and nonspecific binding of antibodies was prevented by preincubation of sections in 10% normal goat serum.

Sections were then incubated for 16 hrs with the first antibodies of VEGF (polyclone, rabbit anti-rat, Boster Biological Technology Co., China) at a dilution of 1:100 in PBS containing 10% normal goat serum. Alternate control sections were incubated without primary antibody and demonstrated no staining. After being washed in PBS, sections were incubated with biotinylated rabbit IgG antibodies and freshly prepared supper sensitive SP solution (Maxim biotechnology Co., China). Peroxidase activity was revealed by incubation with 3,39-diaminobenzidine tetrahydrochloride with 0.01% H₂O₂, and the sections were dehydrated, cleared, and mounted with Entellan. Using the high power of the microscope, the positive cells in the cortex of temporal lobe were counted in each section.

**Statistical analysis**

Data were expressed as mean ± SD. Comparisons were made with the use of
ANOVA and student-\(t\) test. The level of significance of all tests of comparison was \(p<0.05\).

**Results**

*SAH models Verification*

Rats were sacrificed and anatomic examinations were performed in the end of the experiment. Extensive arterial hemolysate were found in the subarachnoid spaces, especially in the basilar region in rats of vehicle, SAH, EGB1 and EGB2 groups.

*Expression of VEGF mRNA*

In non-SAH group, there was only slight expression of VEGF mRNA in the brain. Distinct expression of VEGF mRNA in the brain was found in SAH and vehicle groups 24 hrs after cisternal injection of arterial hemolysate, which was augmented at 72 hrs. There was no difference between these two groups at the two time points. The two doses of EGB enhanced the expression of VEGF mRNA induced by SAH. The expression in EGB2 group was more obvious than that in EGB1 group (Fig. 1, Fig. 2).

*Expression of VEGF protein*

Few VEGF positive cells were observed in choroid and pia mater in non-SAH group. In SAH and vehicle groups, a good few of VEGF positive cells were found in the cortex, the basal ganglia, the hippocampus, and other region of the brain. The positive cells are mainly neurons, gliaocytes and vascular endothelial cells. The numbers of the positive cells increased in EGB1 and EGB2 groups as compared with
those in the vehicle group. The increase was more distinct in EGB2 group than that in EGB1 group (Fig. 3, Fig. 4).

Discussion

Cerebral ischemia is a major cause of poor prognosis in patients suffering from SAH (Manno, 2004; Sun et al., 2000, 2003). OxyHb is deemed to be the principal cause of cerebral vasospasm and ischemia following SAH (Asano, 1999; Grasso, 2004). In the present experiment, autologous arterial hemolysate was injected into cisterna to produce SAH in rats. This model seems more suitable for studying the cerebral ischemia secondary to SAH, as large amount of OxyHb released from hemolysate after the breaking of erythrocytes in the subarachnoid spaces.

VEGF, by combining to its receptor, may have an important role in the vascular response to cerebral ischemia, because cerebral ischemia stimulates the expression of VEGF in the brain (Pichiule et al., 2003; Hai et al., 2003), and VEGF in turn promotes the formation of new cerebral blood vessels (Hai et al., 2003). Topical and intravenous administration of VEGF reduces infarction size or improves the neurological outcome, while intracerebroventricular administration of VEGF antibody increases infarction size after focal cerebral ischemia (Bao et al., 1999; Zhang et al., 2000; Hayashi, et al., 2003). More recent findings revealed that VEGF also has direct protective effects on neural cells (Storkbaum et al., 2004). A great number of studies showed that VEGF exerts preventive and therapeutic effects on cerebral ischemia.

Secondary cerebral ischemia often occurs following SAH due to cerebral
vasospasm and microcirculatory turbulence (Sun et al., 2003; Manno, 2004). In present experiment, SAH induced a distinct expression of VEGF mRNA in the brain 24 hrs and 72 hrs after cisternal injection of arterial hemolysate. The expressions of VEGF proteins were also enhanced 72 hrs after cisternal injection of autologus arterial hemolysate. The results were consistent with previous studies conducted by Josko (2003), which indicated a possible protective role of VEGF toward secondary cerebral ischemia after SAH.

EGb is a special extract from the leaves of *Ginkgo biloba*, a unique and primigenia tree originating from Asia, mainly Southeast China. The active constituents of EGb include flavonol glycosides, terpene trilactons (ginkgolides A, B, C and bilobalide), proanthocyanidines, and other common compounds (Ni et al., 1996; Sun et al., 2000). Clinical studies have shown that EGb exhibits therapeutic activity in a variety of disorders including Alzheimer's disease, failing memory, age-related dementias, poor cerebral and ocular blood flow (Gertz et al., 2004). Due in part to its potent antioxidant properties and ability to enhance peripheral and cerebral circulation, its primary application lies in the treatment of cerebrovascular dysfunctions and peripheral vascular disorders (Sun et al., 2000). In recent years, EGb was used for the treatment of dementia, macula degeneration, tinnitus and winter depression and other central nervous system disorders (Sierpina and Wollschaeger, 2003; Gertz et al., 2004). Our previously studies have demonstrated that *Ginkgo biloba* restores the imbalance between vascular relaxing factor nitric oxide and vascular contracting factor during SAH-related brain injury (Sun et al.,
2000). Results from the present study have provided further information about the beneficial effect of this plant extract that it promotes the expression of VEGF in the ischemic brain tissue in the SAH rat model. The clinical implications of these pharmacological effects need to be further explored.

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Reference


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Figure legends

Figure 1. VEGF mRNA RT-PCR detection. M: DL2000 marker; A: non-SAH 72h; B: SAH 72h; C: vehicle 72h; D: EGb1 72h; E: EGb2 72h; 1: non-SAH 24h; 2: SAH 24h; 3: vehicle 24h; 4: EGb1 24h; 5: EGb2 24h

Figure 2. VEGF mRNA expression quantified by RT-PCR after cisternal injection. *P<0.01, vs non-SAH; \( \triangle P < 0.01 \), vs vehicle; \( \# P < 0.01 \), vs EGb1

Figure 3. Immunocytochemistry for VEGF after cisternal injection (Objective 40 \( \times \)). A. Slight expression of VEGF in non-SAH rat; B. Expression of VEGF in SAH rat; C. Expression of VEGF in vehicle group; D. Enhanced expression of VEGF in EGb1 group; E. Enhanced expression of VEGF in EGb2 group.

Figure 4. VEGF positive cells in the cortex by immunocytochemistry after cisternal injection. *P<0.01, vs non-SAH; \( \triangle P < 0.01 \), vs vehicle; \( \# P < 0.05 \), vs EGb1.
Figure 1.
Figure 2.
Figure 4.