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Caffeic acid phenethyl ester (CAPE) attenuates cerebral vasospasm after experimental subarachnoidal haemorrhage by increasing brain nitric oxide levels

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Abstract

Background: Cerebral vasospasm, a medical complication of aneurysmal subarachnoid hemorrhage (SAH), is associated with high morbidity and mortality rates, even after the aneurysm has been secured surgically or endovascularly. Evidence accumulated during the last decade suggest that scavenging a vasodilator, nitric oxide (NO), by superoxide anions (O_2^-), and activating a strong vasoconstructor, protein kinase C (PKC), are the two most important mechanisms in the pathogenesis of vasospasm. Our aim in this study was to determine whether caffeic acid phenethyl ester (CAPE), a non-toxic oxygen free radical scavenger, prevents vasospasm in an experimental rat model of SAH.

Methods: Twenty eight rats (225–250 g) were divided into four groups equally: group 1, control group; group 2, SAH group; group 3, SAH plus placebo group; and group 4, SAH plus CAPE group. We used double haemorrhage method for SAH groups. Starting 6 h after SAH, 10 μmol/kg CAPE or an equal volume of 0.9% saline were administered by intraperitoneal injection twice daily for 5 days to SAH plus CAPE and SAH plus placebo groups, respectively. CAPE or 0.9% saline injections were continued up to 5th day after SAH. Rats were sacrificed on the 5th day. Brain sections at the level of the pons were examined by light microscopy. Measurements were made for the cross-sectional areas of the lumen and the vessel wall (intimae plus media) of basilar artery by a micrometer. The levels of malondialdehyde (MDA), reduced glutathione (GSH), and nitric oxide (NO) were measured in rat brain tissue.

Results: Administration of CAPE significantly attenuated the vasoconstriction of the basilar artery. There were marked narrowing in the lumens of and thickening in the walls of basilar arteries in the SAH, and the SAH plus placebo compared with CAPE group ($p < 0.001$). We also observed that CAPE administration significantly decreased the tissue level of MDA, while significantly increased the tissue levels of GSH, NO in the SAH plus CAPE group compared to only SAH group, $p < 0.05$.

Conclusions: Our results indicate that CAPE is effective in attenuating delayed cerebral vasoconstriction following experimental SAH. Our findings also suggest that the elevation of lipid peroxidation and reduction of NO bioavailability, resulting from the generation and the interaction of free radicals, have a significant role in the pathogenesis of vasospasm after SAH.

Keywords: Rat; SAH; Vasospasm; CAPE; MDA; GSH; NO

1. Introduction

Although the pathogenesis of aneurysmal vasospasm is multifactorial, the generation of free radicals by the auto-oxidation of oxyhemoglobin to methemoglobin is a triggering factor for mechanisms of vasospasm (Takao, 1999). Studies suggested that the interactions between reactive oxygen species (ROS) which were activated by oxyhemoglobin, results in the imbalance between vasoconstrictors and vasodilators and this causes delayed cerebral vasospasm and ischemia (Beckman et al., 1990; Edwards et al., 1992; Hogg

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et al., 1992; Nishizawa et al., 1997). Especially scavenging a principal vasodilator, nitric oxide (NO), by superoxide anions (O_2^-) is a key mechanism in the pathogenesis of vasospasm (Asano and Matsui, 1998; Gaetani et al., 1998; Widenka et al., 1999).

Oxyhemoglobin participate in excessive production of reactive oxygen species (ROS) and reactive nitrogen species (RNS), especially O_2^- and NO (Vollrath et al., 1998). O_2^- avidly binds NO and, this interaction between NO and O_2^- can lead reduction of NO bioavailability and, production of highly cytotoxic peroxynitrite, hydroxyl, and other strong radicals (Beckman et al., 1990; Hogg et al., 1992).

Although endogenous intracellular enzymatic antioxidants such as super oxide dismutase (SOD), glutathione peroxidase (GSH-Px), and non-enzymatic antioxidants such as GSH and vitamin C detoxify these ROS, the antioxidative mechanisms are disturbed and overwhelmed in SAH as a result of excessive production of free radicals such as $O_2^{\bullet -}$, hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\bullet}OH$), and peroxynitrite (Kamii et al., 1999; McGirt et al., 2002). If detoxification is not achieved, the cell membranes are then attacked by the ROS which initiate lipid peroxidation process (Takenaka et al., 1993).

It has been shown that activation of lipid peroxidation is an active process in cerebral vasospasm after SAH (Suzuki et al., 1983). In the previous studies, it was suggested that ROS reactions and/or lipid peroxidation after SAH may be involved in the occurrence of cerebral vasospasm and that inhibition of these reactions by antioxidant compounds, such as Ebselen, may prevent cerebral vasospasm (Caner et al., 1991; Marzatico et al., 1989; Sano et al., 1980; Sasaki et al., 1981). In addition, in two different studies in mice, it was showed that overexpression of cupper–zinc superoxide dismutase (Cu–Zn SOD) is a protective mechanism against cerebral vasospasm following SAH (Kamii et al., 1999; McGirt et al., 2002). In our recent study, we showed that intraperitoneal administration of a SOD-mimetic, MnTBAP [Mn(III) tetrakis(4-benzoic acid)porphyrin], attenuated vasospasm (Aladag et al., 2003).

CAPE is a small, lipid-soluble compound that is currently being tested for its ability to prevent the formation of ROS, MDA, and peroxynitrite in many in vivo studies (Ilhan et al., 1999; Irmak et al., 2001; Russo et al., 2002; Song et al., 2002). CAPE was determined to show an antioxidant effect by scavenging the ROS and preventing lipid peroxidation when used at a concentration of 10 μmol (Irmak et al., 2001; Mirzoeva et al., 1995). In addition, CAPE also prevents inactivation of NO and activation of PKC simultaneously by scavenging $O_2^{\bullet -}$, H_2O_2, and other radicals (Borrelli et al., 2002a,b). For that reasons, we used CAPE to prevent vasospasm in an experimental rat model of SAH.

In the present study, we aimed to investigate the in vivo effects of caffeic acid phenethyl ester (CAPE) on subarachnoid hemorrhage (SAH)-induced cerebral vasospasm and the brain tissue levels of malondialdehyde (MDA), reduced glutathione (GSH), nitric oxide (NO) in rats.

2. Materials and methods

2.1. Experimental procedures

We used double haemorrhage method for SAH groups (Meguro et al., 2001). Experiments were performed on 15-week-old 28 male Wistar rats ranging in weight from 225 to 250 g (a mean weight of 235 g) obtained from Inonu University Animal Research Laboratory. Rats were divided into four groups: control group (no SAH), group 1; only SAH group, group 2; SAH plus placebo group (SAH plus intraperitoneal, 0.9% NaCl contains 1% ethanol), group 3; and SAH plus CAPE group (SAH plus intraperitoneal CAPE), group 4.

2.1.1. Experimental model of SAH and study protocol

All rats were pretreated with an antibiotic, enrofloxacin (Baytril, Bayer, Germany), (2.27 mg/kg, subcutaneously) 1 day before surgery. The rats in groups 2, 3, and 4 were anesthetized with intraperitoneal ketamine HCl (60 mg/kg) and xylazine HCl (6 mg/kg) and placed on a heated surgical table at 37 °C during surgical procedures. Anaesthesia was continued by repeated injections of ketamine as needed. A 0.3 ml of blood sample was drawn from tail vein into a heparinized syringe. Under sterile conditions and a surgical microscope, occipital bone was explored by a midline incision from mid calvarium to the lower cervical spine. After the dissection of atlanto-occipital membrane, a 27-gauge needle was inserted into the cisterna magna. 0.3 ml of heparinized blood sample was injected into cisterna magna over a 10-min period. After the needle was withdrawn, dural opening plugged with an absorbable sponge and the wound was sutured. The rats were injected 5 ml of saline (warmed at 37 °C) subcutaneously to prevent dehydration before recovery from anesthesia. During observation, the rats were allowed access to food and water ad libitum.

One millilitre of placebo (saline plus 1% ethanol) and the CAPE (10 μmol/kg/day) was administered by intraperitoneal injection twice daily for 5 days to SAH plus placebo and SAH plus CAPE groups 6 h later SAH. The dose of 10 μmol/kg/day of CAPE was selected on the basis of earlier reports, which have demonstrated its potent antioxidant properties at that dose (Mirzoeva et al., 1995). CAPE was purchased from Sigma–Aldrich Chemie GmbH (Taufkirchen, Germany).

Forty-eight hours after the initial intracisternal blood injection, the rats in SAH, SAH plus placebo, and SAH plus CAPE groups were reanesthetized and 0.3 ml of blood from tail vein was reinjected into the cisterna magna. Intraperitoneal CAPE or saline injections (warmed at 37 °C) were continued up to 5th day after SAH. Control rats were sacrificed as described below for determination of the baseline basillary artery diameter.

2.1.2. Sacrificing of rats and preparation of samples

The animals were reanesthetized as described above on the 5th day after first application of blood or saline. The ascending aorta was cannulated retrogradely through a thoracotomy. The craniocervical circulation was perfused with 200 ml of heparinized iso-osmotic phosphate buffer saline (0.1 M, pH 7.4) at a physiological mean arterial pressure (80–90 mm Hg) via a peristaltic pump (May/PRS9508/991129-1). The control group's rats were sacrificed without any surgical procedure for SAH and were perfused as above.

The samples were taken from all rats by cutting brain stem at above and below the pons. The samples were subdivided into two segments at the level of middle pons, and a part of the samples were fixed in 0.1 M phosphate buffer saline containing 4% paraformaldehyde for 24 h. The fixed samples were embedded in liquid paraffin for planimetric measurements.

The remaining parts of the samples were homogenized in ice-cold 0.1 M Tris–HCl buffer (pH 7.5) (containing protease inhibitor, phenylmethylsulfonyl fluoride, 1 mM) with a homogenizer (IKA ultra turrax T 25 basic) at 16.000 r.p.m for 2 min at +4 °C. The homogenates were used to measure the levels of MDA, GSH, and NO.

2.1.3. Planimetric measurements of basillary artery

The paraffinized samples were sectioned at 6 μm thickness, mounted on glass slides, and stained with hemotoxylin and eosin. Sectioned-slices were examined by light microscopy and photographed. Measurements were performed in light microscopy by a micrometer (Olympus BX 50, Japan) for the cross-sectional areas of the lumen and the vessel wall (intimae plus media).

2.2. Biochemical analysis

2.2.1. Malondialdehyde assay

MDA, referred to as thiobarbituric acid reactive substances (TBARS), was measured with tiobarbituric acid at 535 and 520 nm in a spectrophotometer as previously described (Uchiyama and Mihara, 1978). Results were reported as nmol/g wet tissue.

2.2.2. Reduced glutathione assay

GSH concentrations in the homogenates were measured according to the spectrophotometric Ellman's method (Ellman, 1959). Results were reported as nmol/g wet tissue.

2.2.3. Nitric oxide assay

NO levels in the homogenates were measured as total nitrite with the spectrophotometric Greiss reaction. The procedure was partly adapted from the method described by Ozbek et al. (2000). Results were reported as nmol/wet tissue.

2.2.4. Protein assay

Protein concentrations in samples were measured by the method of Lowry et al. (1951) using bovine serum albumin as standard.

2.3. Statistical analysis

Planimetric data were expressed as mean ± S.E.M. Statistical differences between the control and SAH, SAH plus placebo, and SAH plus CAPE were compared by Student-*t* test. For all comparisons, $p < .005$ was considered statistically significant.

Biochemical data were expressed as means ± S.D. The data were evaluated by a one-way analysis of variance, and the differences were considered significant if the *p*-value was less than 0.05 by Tukey's multiple comparison test.

3. Results

After SAH, none of the rats showed neurological deficit. While two rats died in group 2, only one rat died in group 3 in consequence of the sudden respiratory arrest without any neurological deficit between days 3 and 5 after SAH.

3.1. Effect of CAPE on basilar artery vasoconstriction

Histopathologic appearances and the comparison of groups 1, 2, 3, and 4 were shown on Figs. 1–3. Qualitative histological observations of these groups revealed significant reduction in luminal diameter and marked thickening of the vessel wall and endothelial cells and substantial corrugation of the internal elastic lamina of the basilar artery in SAH and SAH plus placebo groups. Microscopic examination of SAH plus CAPE group was similar in appearance to normal vessels in the control group that presented with a monolayer endothelium overlying a thin non-convoluted internal elastic lamina. Concentrically oriented smooth muscle cells surrounded the intima. Corrugation of the internal elastic lamina was less prominent in SAH plus CAPE group (Fig. 2D).

In control, SAH, SAH plus placebo and SAH plus CAPE groups, the diameters of basilar artery lumens were found to be 269 ± 1.4, 77 ± 10.3, 78 ± 10.2, and 289 ± 1.6 micro meters (μm), respectively (Fig. 1). There was marked narrowing in the lumens of basilar arteries in SAH and SAH plus placebo groups compared to control group ($p < 0.001$). The mean thicknesses

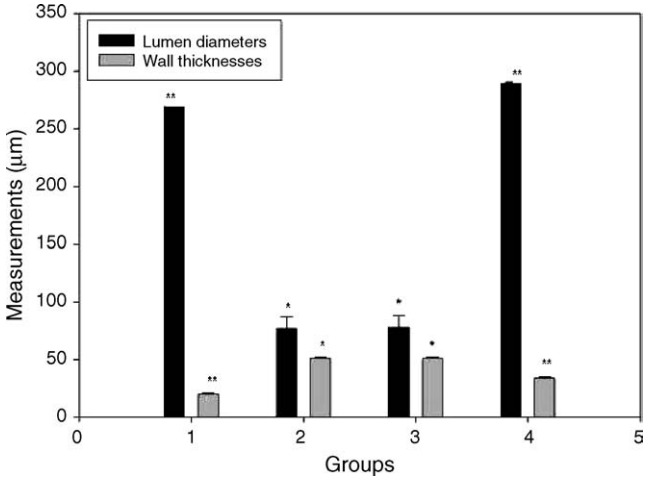

Fig. 1. The comparison of internal diameters and wall thicknesses of the basilar arteries of groups. *Significantly different from the control group. **Significantly different from the SAH and SAH + placebo groups.

of basilar artery walls in control, SAH, SAH plus placebo and SAH plus CAPE groups were 20.0 ± 1.1, 51.3 ± 1.2, 51.0 ± 1.3, and 34.0 ± 1.2 μm, respectively. Compared to control group, the thicknesses of basilar artery walls were found to be increased in SAH and SAH plus placebo groups. While the changes of internal diameter and wall thickness of SAH plus CAPE group were not statistically significant compared to control group, but they were statistically significant compared to SAH and SAH plus placebo groups ($p < 0.001$).

3.2. Effect of CAPE on biochemical parameters

The brain levels of MDA, GSH, and NO in all experimental groups are shown in Fig. 3 CAPE administration significantly decreased MDA levels, while significantly increased the levels of GSH, NO in the SAH plus CAPE group compared to SAH group, ($p < 0.05$).

The mean levels of MDA in control, SAH, SAH plus placebo, and SAH plus CAPE groups were found to be 373.7 ± 30.5, 662.7 ± 35.6, 669.3 ± 29.9, and 458.5 ± 36.8 nmol/g wet tissue, respectively. The brain MDA levels in SAH and SAH plus placebo groups were significantly high as compared to the control group ($p < 0.05$). But, the MDA level in SAH plus CAPE group was significantly lower than that in SAH and SAH plus placebo groups ($p < 0.05$).

The mean levels of GSH in control, SAH, SAH plus placebo, and SAH plus CAPE groups were found to be 355.2 ± 28.9, 196.5 ± 21.0, 205.7 ± 20.1, and 321.2 ± 35.0 nmol/g wet tissue, respectively. The mean levels of NO in control, SAH, SAH plus placebo, and SAH plus CAPE groups were found to be 469.3 ± 26.2, 417.8 ± 20.9, 410.2 ± 29.0, and 463.2 ± 29.2 nmol/g wet tissue, respectively. While the brain GSH and NO levels in SAH and SAH plus placebo groups were significantly lower than in control group ($p < 0.05$), CAPE administration significantly increased the brain levels of GSH and NO in SAH plus CAPE group compared to SAH and SAH plus placebo groups ($p < 0.05$).

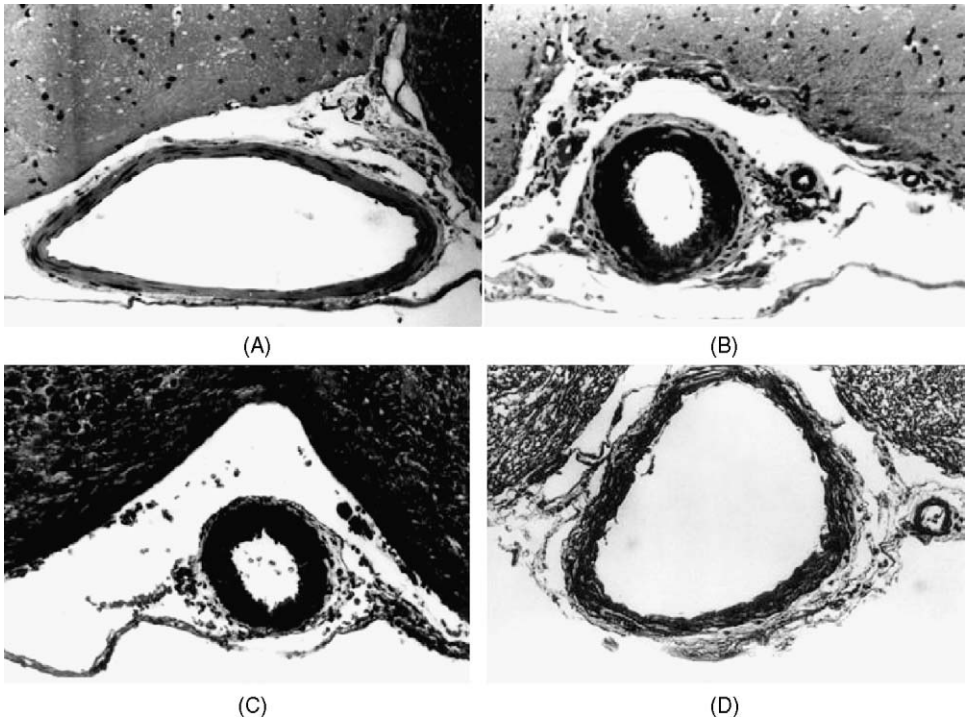

Fig. 2. Representing photograph of light microscopic appearance of a cross-sectional area of the basilar artery of groups (H&E × 66). (A) Control group (group 1), (B) SAH group (group 2), (C) SAH plus placebo group (group 3), and (D) CAPE group (group 4). Note a significant degree of reduction in lumen diameter and increase in wall thickness in SAH and SAH plus placebo groups, and minimal reduction in those in CAPE group compared with control group.

4. Discussion

This study clearly demonstrated that the administration of 10 μmol/kg of CAPE could be significantly inhibit the lipid peroxidation and increase the levels of GSH and NO in cerebral tissue via its free radical scavenging properties and subsequently attenuate the vasospasm after SAH in rats, thereby reinforcing the concept that the overproduction of free radicals and the free radical reactions such as lipid peroxidation are involved in the pathogenesis of SAH-induced cerebral vasospasm.

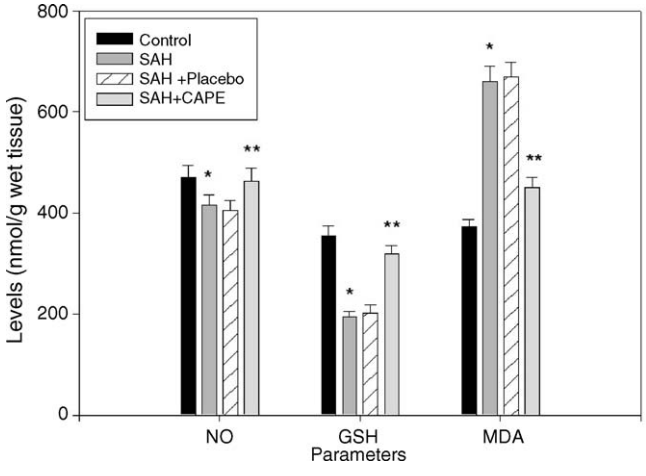

Fig. 3. The effects of CAPE on the brain tissue levels of NO, GSH, and MDA in an experimental rat model of SAH. Biochemical measurements were performed in duplicate. Each value given in figure is means ± S.D. for seven rats. Results were expressed as nmol/g wet tissue for MDA, GSH, and NO. *Significantly different from the control group. **Significantly different from the SAH and SAH + placebo groups.

CAPE, an active component of propolis, has antioxidant, chemopreventive, antiinflammatory, and antitumor properties. Propolis is a resinous natural product collected by honeybees from various plant sources. CAPE is a small, more lipophilic compound and thus easily enters into cells by crossing the cell membranes (Grunberger et al., 1988). It was showed that CAPE is easily absorbed and passed into blood after intraperitoneal injection (Koltuksuz et al., 1999). At a concentration of 10 μmol, CAPE shows potent antioxidant effect by completely blocking production of reactive oxygen species in human neutrophils and xanthine/xanthine oxidase system (Mirzoeva et al., 1995). To our literature knowledge, there is not any report regarding to that CAPE shows any toxic effect on cells. However, other pharmacologic properties of CAPE such as its intestinal absorption, degradation rate in body, blood, and tissue levels after ingestion are not well known.

Overproduction of ROS and increase of lipid peroxidation appear to be major contributing factors in the pathogenesis of cerebral vasospasm and secondary brain damage after SAH. Oxyhemoglobin has been suggested to be a key substance that evokes the possible ROS generation and subsequently lipid peroxidation for development of cerebral vasospasm (Macdonald et al., 1992a). Under physiological conditions, there is a balance between the ROS production and the endogenous scavenging system which detoxifies the ROS. In contrast, the overproduction of ROS during an ischemic event after SAH can overwhelm these endogenous antioxidative defense mechanisms and result in increase of lipid peroxidation and reduce of

NO bioavailability. We think that inhibition of these reactions by antioxidant compound, CAPE, would be a promising therapeutic choice for prevention and attenuation of cerebral vasospasm.

The antioxidant effect of CAPE is similar to those of superoxide dismutase (SOD) so that it especially scavenges superoxide anions which reduce vasodilator effect of NO by interacting with it (Russo et al., 2002; Zhou and Zheng, 1991). Because of the inability of crossing biological membranes and blood–brain barrier, SOD cannot be administered systemically (Macdonald et al., 1992b; Smith et al., 1995). However, either the studies in mice that overexpressed Cu–Zn SOD or a study in rats that a systemically administered SOD-mimetic both attenuated vasospasm following SAH (Kamii et al., 1999; Aladag et al., 2003; Michaluart et al., 1999). Also, some experimental studies have been performed to investigate whether the inhibition of the free radical reactions prevents the development of vasospasm. Indeed, it was determined that administration of the antioxidant deferoxamine and Ebselen demonstrated a prophylactic and preventing effect in vasospasm in experimental SAH (Handa et al., 2000; Harada and Mayberg, 1992; Vollmer et al., 1991). Additionally, CAPE was tested for its ability to prevent the formation of ROS, and MDA was determined to show the preventive effects on brain, spinal cord, and renal ischemic injuries when used at a concentration of 10 μmol (Ilhan et al., 1999; Irmak et al., 2001).

Reduced glutathione (GSH) is an essential tripeptide, and endogenous antioxidant found in all animal cells. It reacts with the free radicals and can protect cells from singlet oxygen, hydroxide radical, and superoxide radical damage (Reed and Farris, 1984). An increased tissue level of GSH has been reported to be an important protective mechanism to overproduced ROS and the free radical reactions. In contrary, a significant depletion of intracellulary GSH level makes tissues more susceptible to oxidative damage (Meister and Anderson, 1983).

In the present study, although, the brain level of GSH was significantly reduced in rats after SAH, CAPE administration significantly increased the brain levels of GSH in SAH plus CAPE group. Also, we observed that CAPE administration significantly decreased the tissue level of MDA, while significantly increased the tissue levels of NO in the SAH plus CAPE group compared to SAH and SAH plus placebo groups ($p < 0.05$). The results of MDA, GSH, and NO are consistent with the planimetric measurements of basilar artery. The whole protective mechanism of CAPE in cerebral vasospasm can be explained as follows: (1) CAPE administration reduced the formation of superoxide probably by inhibiting superoxide generation systems such as xanthine/xanthine oxidase system. (2) This resulted in reduced formation of H_2O_2 and peroxynitrite in CAPE-treated rats. In addition to this, CAPE showed superoxide and hydroxyl radical scavenging activity. (3) This would have resulted in increase in the brain levels of GSH and NO as well as decrease in the brain level of MDA as index of lipid peroxidation (Draper and Hadley, 1990). As a result of above protective mechanisms, CAPE administration would attenuate cerebral vasospasm induced by SAH in rats.

In our study, experimental SAH elicited vasospasm in all animals of SAH group and SAH SAH plus placebo groups. The narrowing in basilar artery lumen was 349% higher in SAH and SAH plus placebo groups than in control group ($p < 0.001$) and 370% higher than in SAH plus CAPE group ($p < 0.001$). In SAH and SAH plus placebo groups, in addition, the thickening in basilar artery wall was found to be 250% higher than control group ($p < 0.001$) and 150% higher than SAH plus CAPE group ($p < 0.001$). In animals of group 4 that was treated with CAPE, narrowing in arterial lumen and thickening in arterial wall were markedly attenuated compared to groups 2 and 3. While the changes of internal diameter and wall thickness of SAH plus CAPE group were not statistically significant compared to control group, they were statistically significant compared to SAH and SAH plus placebo groups ($p < 0.001$). Administration of CAPE significantly attenuated the vasoconstriction of the basilar artery in SAH plus CAPE group compared with the SAH and SAH plus placebo groups ($p < 0.001$).

The present study is the first report on the effects of CAPE on cerebral vasospasm after experimental subarachnoidal haemorrhage in rats. In this study, it was shown that administration of CAPE markedly attenuated the basilar artery vasoconstriction. We suggest that CAPE is effective in attenuating the delayed cerebral vasoconstriction following experimental SAH. Further studies are needed to clarify the conclusion.

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