

Original Article

Lycopene attenuates early brain injury and inflammation following subarachnoid hemorrhage in rats

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Abstract: Early brain injury (EBI), following subarachnoid hemorrhage (SAH), includes blood-brain barrier (BBB) disruption and consequent edema formation. This study aims to evaluate the effect of lycopene on early brain injury and inflammation in SAH. Neurological deficits, brain water content and Evans blue dye extravasation were evaluated after the treatment with lycopene. Besides neuronal apoptosis, some inflammatory cytokines were also detected. The results suggested that administration of lycopene following SAH significantly ameliorated EBI, including brain edema, blood-brain barrier (BBB) impairment, cortical apoptosis, and neurological deficits. In addition, it also ameliorated inflammation triggered by SAH. In conclusion, post-SAH lycopene administration may attenuate EBI in SAH, possibly through ameliorating neuronal apoptosis, maintaining BBB integrity and attenuating inflammation.

Keywords: Lycopene, subarachnoid hemorrhage, early brain injury, inflammation

Introduction

Subarachnoid hemorrhage (SAH) can be very devastating and of poor prognosis. In acute situations, 30% of patients will die, and those who survive may have poor prognosis in the future [1, 2]. Despite the recent progress in microsurgical techniques, the outcome of patients who suffer from SAH remains unsatisfactory. SAH is a complex neurosurgical disease, which is frequently associated with many complications such as acute/delayed cerebral vasospasm, brain edema, obstructive/communicating hydrocephalus, diffuse/focal cerebral ischemia, etc [3].

Lycopene, which is a carotenoid compound, has been found naturally in tomato and other fruits like papaya, pink guava and watermelon. It has long been known for its health-promoting benefits [4]. Among natural carotenoids, lycopene has the strongest ability to scavenge free radicals: being 10-fold, 47-fold and 100-fold more effective at quenching singlet oxygen than α -tocopherol, β -carotene and vitamin E respectively [5, 6]. Additionally, more studies have suggested that lycopene have other properties such as anti-apoptosis [7], anti-inflam-

mation [8], anti-ischemia [9], and anti-tumor [10]. Therefore, we would like to investigate whether lycopene may be effective in SAH. And here we reported that lycopene might be protective in SAH.

Materials and methods

Animals

Sixty adult male Sprague-Dawley rats (250-300 g) were purchased from the Center of Experimental Animal in Zhejiang University, China. All experimental protocols and procedures were approved by the Institutional Animal Care and Use Committee of Zhejiang University.

SAH model

The endovascular perforation model of SAH was performed as previously described [11]. Briefly, rats were anesthetized, intubated, and kept on artificial ventilation during surgery with 3% isoflurane in 70%/30% medical-air/oxygen. Body temperature was monitored by a rectal probe, and normothermia was maintained with a heating lamp. A sharpened 4-0 nylon suture was inserted into the left internal carotid artery and advanced until a resistance was felt

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(approximately 18 mm from the common carotid bifurcation). The suture was then further advanced to perforate the bifurcation of the anterior and middle cerebral arteries and withdrawn immediately after artery perforation. During sham operations, the suture was inserted into the left carotid artery; however, no perforation was performed. After removal of the suture, the skin incision was sutured, and rats were individually housed in heated cages until recovery.

Treatments

Animals were randomly assigned into three groups: sham group, SAH group and SAH + lycopene group. Animals received the following agents as intraperitoneal (i.p.) injections 2 h after surgery: lycopene at a dose of 40 mg/kg (dissolved in tetrahydrofuran (THF)). Sham group and SAH group rats were injected solely with an equal concentration of THF.

Neurological score

At 24 h after surgery, immediately before euthanasia, neurological scores were evaluated in a blinded fashion, using a modification of the Garcia scoring system as previously described [11, 12]. This composite sensorimotor assessment evaluates the rodent's spontaneous activity (0-3 points), its reaction to side stroking (1-3 points) and vibrissae touch (1-3 points), as well as limb symmetry (0-3 points), forelimb outstretching (0-3 points), as well as its climbing (0-3 points) and beam walking ability (0-4 points). The latter evaluated the walking distances on a wooden beam for 1 min. The sum of all subtests was calculated to determine neurological function; best test performances were scored with 22, and worst performances were scored with 2 points.

Brain water content (cerebral edema)

Cerebral edema was determined by measuring the brain water content according to the wet-dry method [13]. These rats were killed and brains were immediately removed and placed on a frozen plate. Tissue samples were dissected out from infarct areas in ischemic rats and from corresponding areas in sham-operated and non-operated animals. Samples were immediately weighed to obtain wet weight. Then, samples were dried in a desiccating oven at 110°C for 24 h and weighed again to obtain the dry weight. Brain water content was calcu-

lated as follows: brain water content (%) = (wet weight-dry weight) × 100/wet weight.

Blood-brain barrier disruption

At 24 h after surgery, Evans blue dye (2%; 5 ml/kg) was injected into the right femoral vein, over a period of 2 min, allowing the dye to circulate for a total of 60 min [14]. Under deep isoflurane anesthesia, rats were subjected to transcardial perfusion with phosphate-buffered saline (PBS), and brains were removed. Brain specimens were weighed, homogenized in 1 ml of PBS, and centrifuged at 15,000 g for 30 min. Then 0.6 ml of the resultant supernatant was added to an equal volume of trichloroacetic acid. After overnight incubation at 4°C and centrifugation at 15,000 g at 4°C for 30 min, the supernatant was used for spectrophotometric quantification of extravasated Evans blue dye at 615 nm.

TUNEL staining

The brain tissue was fixed with the 10% neutral buffered formalin and embedded in paraffin. Briefly, paraffin-embedded sections were mounted on positively charged slides, deparaffinized, rehydrated, and washed thoroughly with distilled water. The tissues were digested with 20 g/ml proteinase K (Boehringer Mannheim, Mannheim, Germany) at room temperature for 15 min to retrieve antigen. Endogenous peroxidase activity was blocked by incubation in 0.3% hydrogen peroxide/methanol in phosphate-buffered saline at 37°C for 30 min. The sections were then incubated with terminal deoxynucleotidyl transferase at 37°C for 60 min. Anti-digoxigenin antibody peroxidase was applied to the sections to detect the labeled nucleotides. The sections were stained with DAB and counter-stained slightly with hematoxylin. The positive cells were analyzed under a light microscope by another investigator in a blinded way.

RNA extraction and RT-PCR

The levels of TNF- α , IL-1 β , and ICAM-1 mRNA expression were determined by RT-PCR. Total RNA was extracted with TriPure Reagent (Roche Diagnostics Corp. Indianapolis, IN, USA) according to the manufacturer's instructions. The cDNA synthesis from the isolated RNA was performed using a reverse transcriptional system. Briefly, 4 mg of total RNA was subjected to the first strand cDNA synthesis for 1 h at 42°C in a 20 ml reaction mixture containing 20 U RNase

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Table 1. Specific oligonucleotide primers

Target gene	Sense primer (5' to 3')	Antisense primer (5' to 3')	Annealing temperature (°C)	Number of cycles	Size (bp)
TNF- α	GCTGCACTTCAGGCTGATC	CTTGTTCCGGGTAGGAGACG	57	34	352
IL-1 β	ATCTCCTGCCAACCCCTACA	CTTTCAGCTCATACGTGCC	54	34	274
ICAM-1	GCGGCTCAGTGTCTCATTCC	CACGCAGTCCTCGGCTTCT	58	34	184
GAPDH	GGAGCCAAAAGGGTCATC	CCAGTGAGTTTCCCGTTC	57	30	347

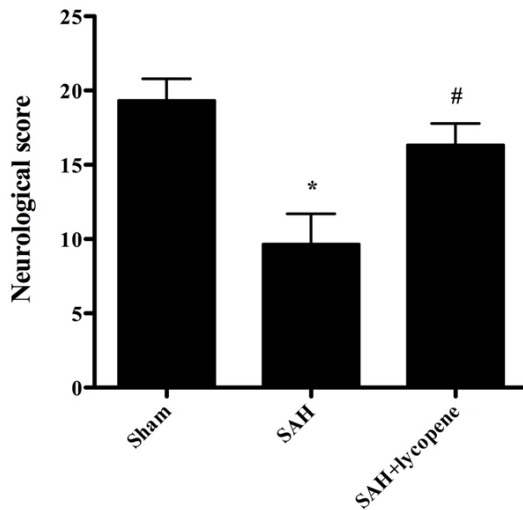


Figure 1. Effect of lycopene on neurological deficits in SAH. Lycopene significantly improved neurological deficits compared with the SAH group. Data were expressed as mean \pm SD ($n = 6$ in each group). * $P < 0.05$ versus the sham group, # $P < 0.05$ versus the SAH group.

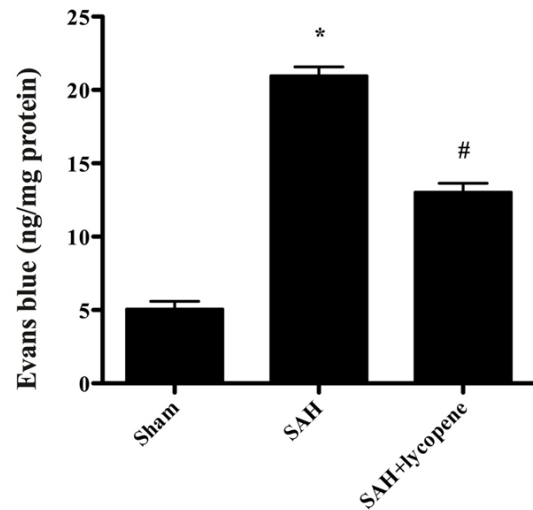


Figure 3. Alterations in Evans blue (EB) extravasation in each group. SAH induced a significant increase in blood-brain barrier extravasation in the rat brain compared with the sham group. After lycopene administration, the EB extravasation was dramatically reduced compared with the SAH group. Data were expressed as mean \pm SD ($n = 6$ in each group). * $P < 0.05$ versus the sham group, # $P < 0.05$ versus the SAH group.

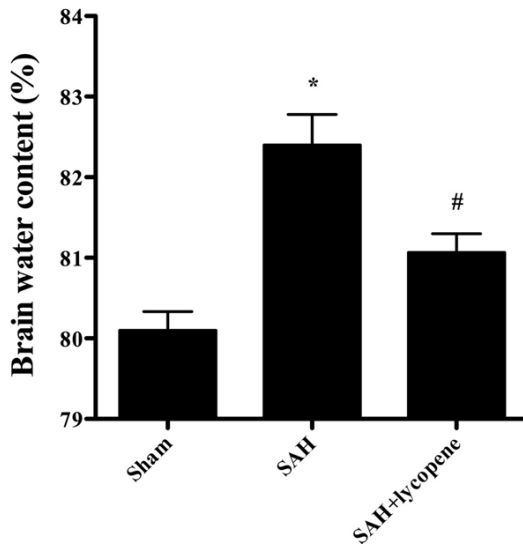


Figure 2. Effect of lycopene on brain water content at 24 h after SAH in rats. Data were expressed as mean \pm SD ($n = 6$ in each group). * $P < 0.05$ versus the sham group, # $P < 0.05$ versus the SAH group.

inhibitor, 0.04 μ mol of each dNTP, 0.5 μ g oligo (dT)₁₅ and 15 U AMV reverse transcriptase (Promega). The reaction was terminated by incubation at 95°C for 5 min. PCR amplification was performed in a total volume of 25 μ L containing 4 μ L cDNA, 0.05 μ mol MgCl₂, 2.5 U Taq polymerase, 0.02 μ mol of each dNTP, specific oligonucleotide primers (**Table 1**) for TNF- α , IL-1 β , ICAM-1 or GAPDH genes, and 2.5 μ L 10 \times Taq polymerase reaction buffer. Each PCR cycle included a denaturation step at 94°C for 30 s, a primer annealing step for 30 s, an extension step at 72°C for 50 s, and a final extension step at 72°C for 7 min. Then, the amplified fragments were detected by agarose gel electrophoresis and visualized by ethidium bromide staining. The gel was captured as a digital image and analyzed using Scion Image software (Scion Corp, Maryland, USA). Values for

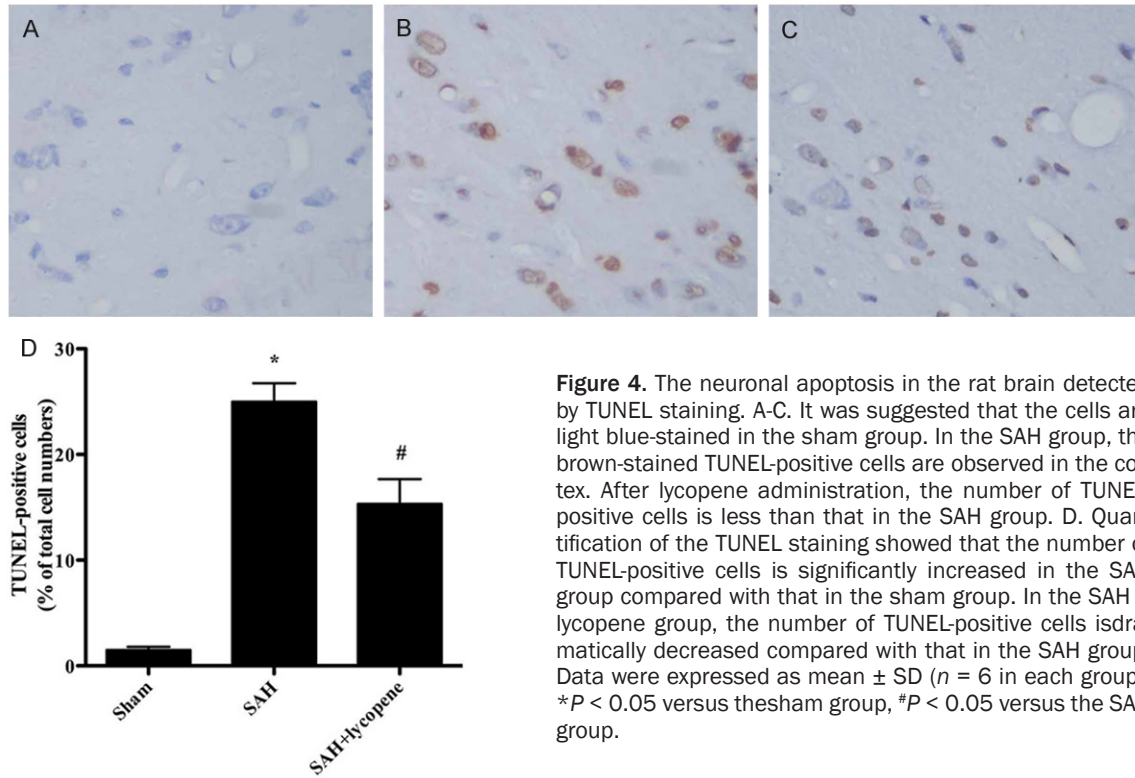


Figure 4. The neuronal apoptosis in the rat brain detected by TUNEL staining. A-C. It was suggested that the cells are light blue-stained in the sham group. In the SAH group, the brown-stained TUNEL-positive cells are observed in the cortex. After lycopene administration, the number of TUNEL-positive cells is less than that in the SAH group. D. Quantification of the TUNEL staining showed that the number of TUNEL-positive cells is significantly increased in the SAH group compared with that in the sham group. In the SAH + lycopene group, the number of TUNEL-positive cells is dramatically decreased compared with that in the SAH group. Data were expressed as mean \pm SD ($n = 6$ in each group). * $P < 0.05$ versus the sham group, # $P < 0.05$ versus the SAH group.

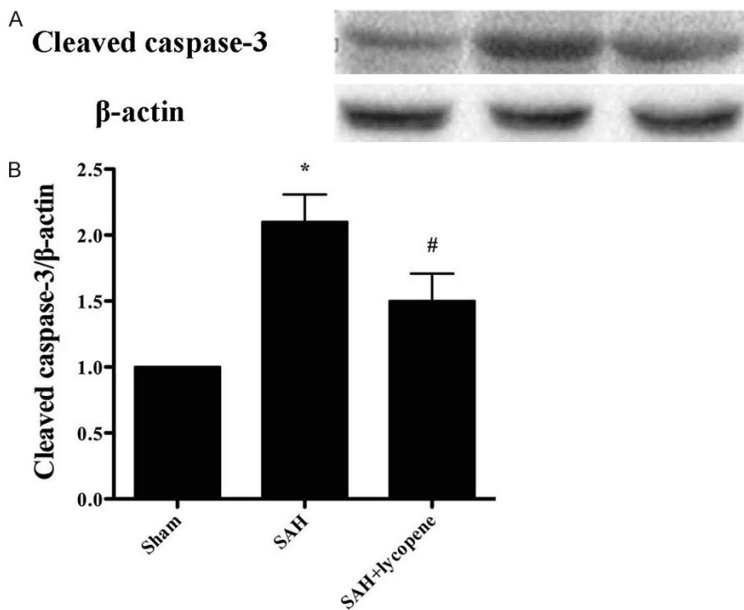


Figure 5. Effect of lycopene on the expression of cleaved caspase-3 detected by Western blot. SAH markedly increases the expression of cleaved caspase-3, while lycopene significantly lowers the level of cleaved caspase-3. Data were expressed as mean \pm SD ($n = 6$ in each group). * $P < 0.05$ versus the sham group, # $P < 0.05$ versus the SAH group.

each sample were normalized by the GAPDH as the control.

Statistical analysis

SAH grades and neurological scores were expressed as median and 25th to 75th percentiles, analyzed by Mann-Whitney U test or Kruskal-Wallis one-way analysis of variance (ANOVA) on ranks followed by Dunn's post hoc analysis. All other results were expressed as mean \pm standard deviation and were analyzed by one-way ANOVA followed by Tukey post hoc analysis. $P < 0.05$ was considered statistically significant.

Results

Effect of lycopene on neurological score at 24 h after surgery

At 24 h after surgery, the neurological score of rats in SAH group was lower than that in sham group ($P < 0.05$). However, lycopene administration ame-

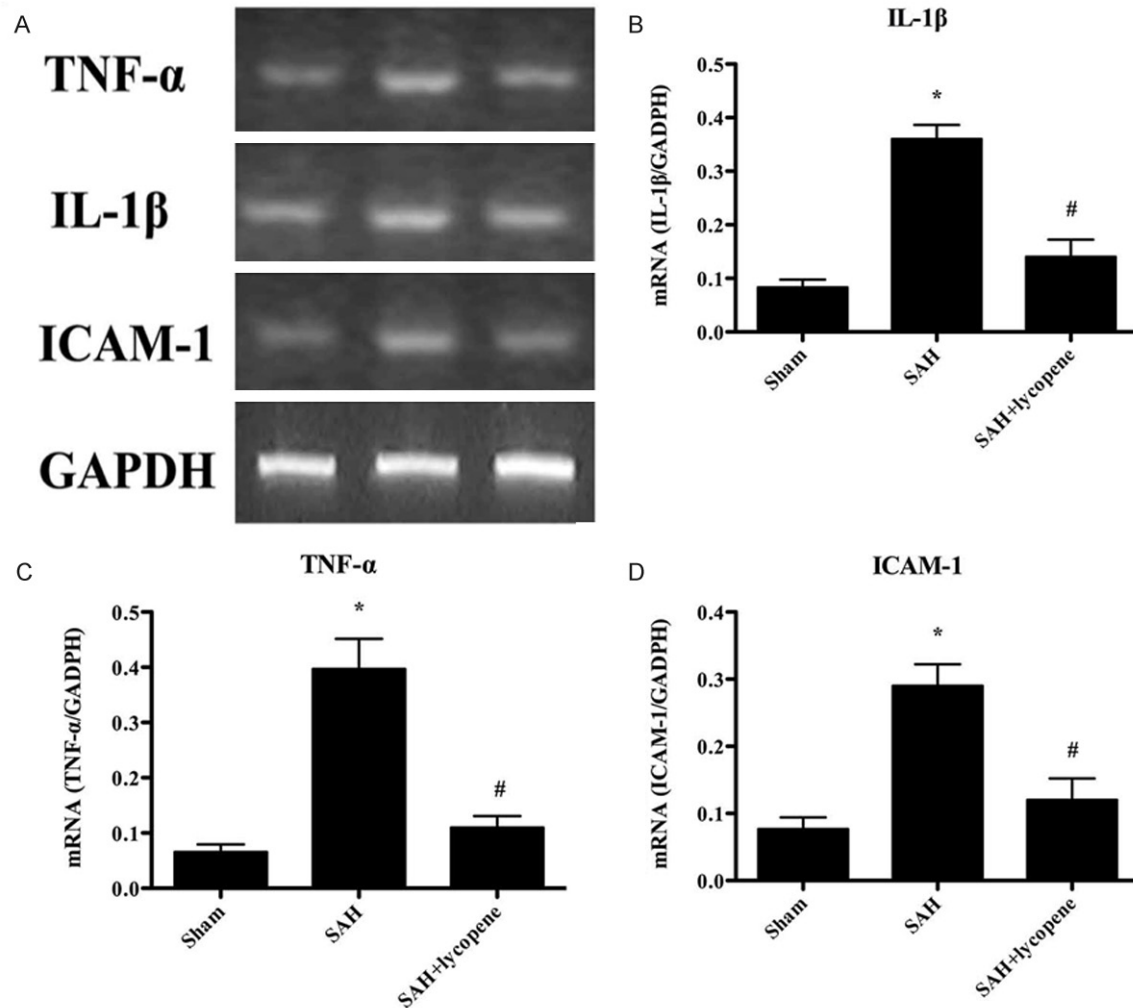


Figure 6. The gene expressions of TNF- α , IL-1 β , and ICAM-1 in the brain detected by RT-PCR. A. The representative autoradiograms of TNF- α , IL-1 β , and ICAM-1. B-D. Relative amount of TNF- α , IL-1 β , and ICAM-1 mRNA. The levels of TNF- α , IL-1 β , and ICAM-1 increased dramatically in the SAH group. After lycopene administration, the levels of TNF- α , IL-1 β , and ICAM-1 were decreased. Data were expressed as mean \pm SD ($n = 6$ in each group). * $P < 0.05$ versus the sham group, # $P < 0.05$ versus the SAH group.

liorated neurological score compared with the SAH group ($P < 0.05$), which suggested that lycopene attenuated neurological injury of SAH (Figure 1).

Effect of lycopene on brain water content at 24 h after surgery

At 24 h after surgery, brain water content of rats in SAH group was higher than that in sham group ($P < 0.05$). However, lycopene administration attenuated brain water content compared with the SAH group ($P < 0.05$) (Figure 2).

Effect of lycopene on blood-brain barrier permeability at 24 h after surgery

At 24 h after surgery, rats in the SAH group showed a significant increase in BBB permeability to Evans blue ($P < 0.05$). However, lycopene administration attenuated Evans blue extravasation compared with the SAH group ($P < 0.05$) (Figure 3).

Effect of lycopene on neuronal apoptosis in the brain

Neuronal apoptosis was assessed by using TUNEL staining (Figure 4). The number of

TUNEL-positive cells increased dramatically in SAH group compared with that in the sham group ($P < 0.05$). After lycopene was given, the number of TUNEL-positive cells decreased significantly compared with that in SAH group ($P < 0.05$). Besides, the expression of cleaved caspase-3 increased dramatically compared with that in the sham group ($P < 0.05$), which was down-regulated by lycopene ($P < 0.05$) (Figure 5). The results suggested that lycopene protect the brain from SAH via inhibiting neuronal apoptosis.

Effect of lycopene on inflammatory cytokines in the brain

The amount of inflammatory cytokines was detected by RT-PCR. The levels of inflammatory cytokines, including TNF- α , IL-1 β , and ICAM-1, were all markedly increased in the SAH group ($P < 0.05$). Lycopene administration dramatically attenuated the levels of inflammatory cytokines ($P < 0.05$) (Figure 6). The results suggested the anti-inflammatory effect of lycopene in SAH.

Discussion

The main findings of the present study are: 1) lycopene protect brain from SAH through attenuating early brain injury (EBI); 2) lycopene protect brain from SAH through inhibiting the expression of inflammatory cytokines.

EBI includes brain edema, neurological deficit, BBB disruption, and neuronal apoptosis. EBI is the major cause of mortality and morbidity in patients suffering from aneurysmal SAH [15]. Besides, study has suggested that EBI rather than vasospasm is the main cause of poor prognosis in patients with SAH [16]. So, EBI is an important target in the treatment of SAH. However, the treatment of EBI in the clinic is not so well. In the study, we have found that lycopene administration ameliorates neurological deficits, attenuated brain edema and BBB disruption, inhibited neuronal apoptosis, which indicates that lycopene exerts its protective effect through attenuating EBI.

Inflammation is also another important factor in SAH. The inflammatory cytokines involved in SAH includes TNF- α , IL-1 β , and ICAM-1, NF- κ B, etc. You et al have shown that activated NF- κ B in neurons after SAH plays an important role in regulating the expressions of inflammatory genes in the brain, and ultimately contributes

to delayed brain injury [17]. In addition, studies have shown that NF- κ B pathway is also involved in SAH [18, 19], which can regulate the expression of matrix metalloproteinase-9 (MMP-9). ICAM-1 can also regulate the expression of MMP-9 [20]. MMP-9 is one of the members in the MMP family. MMP-9 degrades the extracellular matrix of cerebral microvascular basal lamina, triggering BBB disruption after SAH [21]. And it is closely associated with the poor prognosis in the SAH patients. In this study, our results suggested that lycopene down-regulates the expression of TNF- α , IL-1 β , and ICAM-1, which indicates its neuroprotective effect in SAH.

In summary, we demonstrate that lycopene alleviates EBI and inflammation in SAH. Lycopene ameliorates neurological deficits, brain water content, BBB disruption and neuronal apoptosis. Moreover, lycopene inhibits inflammatory reaction by attenuating the expression of TNF- α , IL-1 β , and ICAM-1. Certainly, there may be some other molecules associated with the neuroprotective effect of lycopene, which we need to investigate. And lycopene may be of great value in the treatment of SAH in the future.

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