Amelioration of oxidative stress and protection against early brain injury by astaxanthin after experimental subarachnoid hemorrhage

Laboratory investigation

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Object. Aneurysmal subarachnoid hemorrhage (SAH) causes devastating rates of mortality and morbidity. Accumulating studies indicate that early brain injury (EBI) greatly contributes to poor outcomes after SAH and that oxidative stress plays an important role in the development of EBI following SAH. Astaxanthin (ATX), one of the most common carotenoids, has a powerful antioxidative property. However, the potential role of ATX in protecting against EBI after SAH remains obscure. The goal of this study was to assess whether ATX can attenuate SAH-induced brain edema, blood-brain barrier permeability, neural cell death, and neurological deficits, and to elucidate whether the mechanisms of ATX against EBI are related to its powerful antioxidative property.

Methods. Two experimental SAH models were established, including a prechiasmatic cistern SAH model in rats and a one-hermorrhage SAH model in rabbits. Both intracerebroventricular injection and oral administration of ATX were evaluated in this experiment. Posttreatment assessments included neurological scores, body weight loss, brain edema, Evans blue extravasation, Western blot analysis, histopathological study, and biochemical estimation.

Results. It was observed that an ATX intracerebroventricular injection 30 minutes post-SAH could significantly attenuate EBI (including brain edema, blood-brain barrier disruption, neural cell apoptosis, and neurological dysfunction) after SAH in rats. Meanwhile, delayed treatment with ATX 3 hours post-SAH by oral administration was also neuroprotective in both rats and rabbits. In addition, the authors found that ATX treatment could prevent oxidative damage and upregulate the endogenous antioxidant levels in the rat cerebral cortex following SAH.

Conclusions. These results suggest that ATX administration could alleviate EBI after SAH, potentially through its powerful antioxidative property. The authors conclude that ATX might be a promising therapeutic agent for EBI following SAH.

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Key Words • astaxanthin • early brain injury • oxidative stress • subarachnoid hemorrhage • rat • rabbit • vascular disorders

Subarachnoid hemorrhage (SAH) is one of the most life-threatening diseases, with high morbidity and mortality rates worldwide.²⁴ For the past decades, cerebral vasospasm has been regarded as the major cause of disastrous outcomes in patients who suffer an SAH. However, the success of therapies for SAH in reducing the incidence of cerebral vasospasm without improved long-term neurological outcome has been disappointing.²⁴ This fact suggests that cerebral vasospasm may not be the sole cause of poor outcomes after SAH. Recently, more and more studies have shown that early brain injury (EBI), which refers to the acute injuries to the whole brain within the first 72 hours following SAH, is the primary cause of death in patients with SAH.²¹,²²,²³ Therefore, treatment of EBI has been considered to be the main goal in the management of patients with SAH. Although

This article contains some figures that are displayed in color online but in black-and-white in the print edition.
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the exact mechanisms of EBI are not fully understood, clinical and experimental studies indicate that oxidative damage plays a critical role in EBI pathogenesis and may represent a novel target for treatment of SAH. Given that there is substantial evidence for significant levels of oxidative stress following SAH and for the beneficial effects of antioxidant therapy in experimental SAH and clinical trials, the use of free-radical scavengers is a reasonable approach in the treatment of SAH.

Astaxanthin (ATX), one of the most common carotenoids, is widely distributed in algae, crustaceans, shellfish, and various plants, and ATX is a more powerful antioxidant than other carotenoids, including zeaxanthin, lutein, canthaxanthin, and β-carotene. It has been proven that ATX can protect against oxidative stress- or neurotoxin-induced damage in vitro and in vivo. In previous studies, ATX has been used as an antioxidant therapeutic agent in models of cardiovascular disease including myocardial ischemia and reperfusion. It has been reported so far. However, there has been no study to investigate the effects of ATX on experimental SAH. Based on these data, we explored the potential effects of ATX in EBI following SAH in the present study.

Methods

Animal Preparation

All experimental protocols used for animals (including all surgical procedures) were approved by the Animal Care and Use Committee of Jinling Hospital and conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health. Adult male Sprague-Dawley rats (n = 325) weighing between 250 and 300 g and male New Zealand rabbits (n = 20) weighing 2–3 kg were purchased from the Animal Center of Jinling Hospital (Nanjing, China). The rats and rabbits were acclimated in a humidified room and maintained on a standard pellet diet before the experiment. The temperature in both the feeding room and the operation room was maintained at approximately 25°C.

Rats were assigned to the following groups: control (n = 24); sham-injured (n = 42); SAH (n = 54); SAH + vehicle (n = 52); SAH + 0.01 mmol/l ATX (n = 29); SAH + 0.1 mmol/l ATX (n = 48); SAH + 25 mg/kg ATX (n = 28); and SAH + 75 mg/kg ATX (n = 48). Rats were randomly allocated to 4 groups: control; SAH + vehicle; SAH + 25 mg/kg ATX; and SAH + 75 mg/kg ATX (n = 5 in each group).

Experimental SAH Model

In this study, we adopted two established SAH models as previously reported; the prechiasmatic cistern SAH model in rats and the one-hemorrhage SAH model in rabbits.

The prechiasmatic cistern rat SAH model was produced as previously described. Briefly, the animal’s head was fixed in a stereotactic frame after intraoperative anesthetization with 10% chloral hydrate (0.35 ml/100 g). The experimental SAH model was established using stereotactically guided insertion of a needle with a rounded tip and a side hole into the prechiasmatic cistern. The needle was tilted 45° in the sagittal plane, and placed 7.5 mm anterior to the bregma in the midline, with the hole facing the right side. It was lowered until the tip reached the base of the skull, 2–3 mm anterior to the chiasma (approximately 10–12 mm from the brain surface), and retracted 0.5 mm. Loss of CSF and bleeding from the midline vessels were prevented by plugging the bur hole with bone wax before inserting the needle.

To produce the SAH, 0.3 ml of nonheparinized fresh autologous arterial blood from the femoral artery was slowly (in the course of 20 seconds) injected into the prechiasmatic cistern with a syringe pump under aseptic conditions. Animals in the sham-injured group were injected with 0.3 ml saline. Then, 2 ml saline was injected subcutaneously right after the operation. After the procedures, the rats were returned to their cages individually, and food and water were kept easily accessible. Heart rate and rectal temperature were monitored, and the rectal temperature was kept at 37°C ± 0.5°C by using a warm pad when required, throughout the experiments. We observed in the present study that the inferior basal temporal lobe was always stained by blood and that a significant histological change occurred compared with the control rat brain (Fig. 1). Therefore, the brain tissue adjacent to the clotted blood was taken for the analysis in our study.

The one-hemorrhage rabbit SAH model was produced according to a previously described procedure. Animals were anesthetized with intramuscular ketamine (50 mg/kg) and xylazine (5 mg/kg), and then placed on a heated pad to keep the body temperature at 37°C during the experimental procedure. The hair on the neck was shaved, and then we sterilized the skin with 75% alcohol. The SAH group was subjected to percutaneous puncture into the cisterna magna. After outflow of 1.5 ml of CSF, 1.5 ml of autologous nonheparinized fresh arterial blood was slowly injected into the cisterna magna. After injection, the rabbits were placed in a head-down position at an approximately 30° angle for 30 minutes, and were maintained at their normal body temperature. The rabbits were returned to their feeding room after waking up from anesthesia.

Experimental Design

A schematic of experimental protocols is given in Fig. 2. All animals were assigned at random to the various groups.

Experiment 1. In this experiment, ATX was administered by intracerebroventricular injection in 2 different doses 30 minutes after SAH. The intracerebroventricular injection was selected because Shen et al. reported that ATX injected into the lateral ventricle can be effectively distributed into cortex surface and reduce ischemic brain injury in rats. The ATX (97% pure, Sigma-Aldrich) was prepared in 10% dimethylsulfoxide (vol/vol, in 0.9% saline). Two concentrations of ATX (low: 0.01 mmol/l; high: 0.1 mmol/l) were used to test its neuroprotective effects in SAH. Astaxanthin or vehicle was administered...
into the left lateral ventricle (0.8 mm posterior and 1.5 mm lateral to the bregma, and 3.7 mm below the dural layer) through a 25-μl Hamilton syringe (Shanghai Gaoge Industry & Trade Co., Ltd.) 30 minutes after SAH was induced. In the sham-injured and SAH + vehicle groups, an equal volume of 10% dimethylsulfoxide was administered. Posttreatment assessment included neurological deficit, body weight loss, brain water content, Evans blue (EB) extravasation, Western blot, histopathological study, and biochemical estimation.

To further demonstrate the possible benefits of ATX and to investigate its clinical utility, oral administration instead of intracerebroventricular injection of ATX was conducted in Experiments 2 and 3.

**Experiment 2.** A separate cohort of rats was used in this experiment. The ATX (high-performance liquid chromatography content 98%, Shanghai Market) was diluted in olive oil (1 ml/kg) immediately before use. At 3 hours after induction of SAH, 2 doses (low: 25 mg/kg; high: 75 mg/kg) of ATX were administered by oral gavage. This set of animals went through the same assessment as in Experiment 1.

**Experiment 3.** In the last set of experiments, a one-hemorrhage rabbit SAH model was produced in 20 animals for further evaluation of the potential beneficial effects of ATX. The New Zealand white rabbits were assigned randomly to 4 groups: 1) control group; 2) SAH + vehicle group; 3) low-dose (25 mg/kg) ATX-treated SAH group; and 4) high-dose (75 mg/kg) ATX-treated SAH group (n = 5 in each group). Rabbits were treated with ATX or vehicle at 3 hours after SAH. After neurological scoring at 24 hours post-SAH, rabbits were killed for histopathological study.

**Body Weight and Clinical Evaluation**

Body weight was recorded both after induction of
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**Fig. 2.** Schematic illustration of experiment (EXP) design. Experiments 1 and 2 were conducted in rats. In the first experiment, ATX was given intracerebroventricularly (icv) at 30 minutes post-SAH. In the second experiment, ATX was given at 3 hours post-SAH perorally (po). The neurobehavioral score and body weight ratio were recorded daily from Days 0 to 3. Eighteen animals in each group were killed on Day 3 for brain edema, BBB permeability, and Nissl staining assessment. The rest (24 animals) were killed at 24 hours after surgery for brain water content, BBB permeability, Western blot analysis, histopathological studies, and biochemical estimation. The third experiment was conducted in rabbits, in which mortality, neurological score, and histopathological features were evaluated at 24 hours after SAH.

anesthesia (before SAH induction) and before euthanasia. The results are expressed as a ratio of body weight after surgery/body weight before surgery.

Clinical scores were recorded before euthanasia based on the independent observations made by a veterinarian who was blind to the experimental groups. Three behavioral activity examinations (Table 1) including appetite, activity, and neurological deficits were used in the scoring methodology.29

**Brain Water Content**

Brain water content was measured at 24 and 72 hours after surgery. Rats were anesthetized and decapitated, and the brains were quickly removed and immediately weighed to measure wet weight. Samples were then placed in an oven for 72 hours at 100°C before determining the dry weight. The brain water content was calculated as a percentage by using the following method: \[ \frac{\text{wet weight} - \text{dry weight}}{\text{wet weight}} \times 100\% \].

**Blood-Brain Barrier Permeability**

Blood-brain barrier (BBB) permeability was assessed by EB extravasation at 24 and 72 hours after SAH. Briefly, EB dye (2%; 4 ml/kg) was injected over 2 minutes into the right femoral vein and allowed to circulate for 60 minutes. Animals were then reanesthetized and perfused transcardially with saline to remove intravascular EB dye. After decapitation, the brains were removed and homogenized in physiological phosphate-buffered saline (PBS, pH 7.4), and trichloroacetic acid was then added to precipitate the protein. Samples were cooled and centrifuged. The resulting supernatant was measured for absorbance of EB at 620 nm by using a spectrophotometer.

**Western Blot Analysis**

The frozen brain cortex tissue was mechanically lysed in 20 mM Tris (pH 7.6), which contains 0.2% sodium dodecyl sulfate, 1% Triton X-100, 1% deoxycholate, 1 mM phenylmethylsulphonyl fluoride, and 0.11 IU/ml aprotinin. Lysates were centrifuged at 12,000 g for 20 minutes at 4°C, and the supernatant was collected. The protein concentration was estimated by the method of Bradford with a standard commercial kit (Bio-Rad Laboratories). Equal protein

<table>
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<th>Category</th>
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<th>Score</th>
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<tr>
<td>Appetite</td>
<td>Finished meal</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Left meal unfinished</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Scarcely ate</td>
<td>2</td>
</tr>
<tr>
<td>Activity</td>
<td>Walk and reach ≥3 corners of cage</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Walk with some stimulation</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Almost always lying down</td>
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</tr>
<tr>
<td>Deficits</td>
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<td>0</td>
</tr>
<tr>
<td></td>
<td>Unstable walk</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Impossible to walk</td>
<td>2</td>
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amounts per lane were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories). The membrane was blocked in 5% skim milk for 2 hours at room temperature, and incubated overnight at 4°C with primary antibodies against cleaved caspase-3 (1:1000, Cell Signaling Technology, Inc.) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:5000, Bio-world) in Tris-buffered saline with Tween (TBS-T) containing 5% skim milk. After the membrane was washed 4 times for 10 minutes each in TBS-T, it was incubated with goat anti–rabbit horseradish peroxidase–conjugated IgG (diluted 1:100 in TBS-T, Bio-world) for 2 hours at room temperature. The blotted protein bands were visualized by enhanced chemiluminescence Western blot detection reagents (Amerham) and were exposed to x-ray film. Developed films were digitized with an Epson Perfection 2480 scanner (Seiko Corp.). Quantification of band density was performed using the UN-Scan-It 6.1 software (Silk Scientific, Inc.), and data were normalized to GAPDH.

**Perfusion-Fixation**

For histological studies, animals were killed with the perfusion-fixation method. After the animals were deeply anesthetized with 10% chloral hydrate, perfusion-fixation was performed. The thorax was opened, a cannula was placed in the left ventricle, the descending thoracic aorta was clamped, and the right atrium was opened. Perfusion commenced with 500 ml of PBS at 37°C, followed by 500 ml of 10% buffered formaldehyde under a perfusion pressure of 120 cm H2O. After perfusion-fixation, the whole brain was removed and immersed in the same fixative solution.

**Staining Methods**

**Caspase-3.** For caspase-3 staining, brain sections were deparaffinized and rehydrated in graded concentrations of ethanol to distilled water. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide (H2O2) for 5 minutes, followed by a brief rinse in distilled water and a 15-minute wash in PBS. Sections were placed in 10 mmol/l citrate buffer (pH 6) and heated in a microwave oven at 95°C for 30 minutes, then cooled at room temperature for 20 minutes and rinsed in PBS. Nonspecific protein binding was blocked by a 40-minute incubation in 5% horse serum. Sections were incubated overnight at 4°C with primary antibody against caspase-3 antibody (1:100; Abcam). Sections were then incubated with goat anti–rabbit biotinylated secondary antibody (Santa Cruz Biotechnology) and placed in avidin-biotin-peroxidase complex enzyme. Slides were visualized by incubation with 3,3’-diaminobenzidine (DAB) and H2O2.

**TUNEL.** The apoptotic cells were detected by TUNEL staining, which was performed according to our previous study. An in situ cell death detection kit, POD (ISCDD, Boehringer Mannheim) was used. Briefly, the formalin-fixed tissues were embedded in paraffin and sectioned at 4-μm thicknesses with a microtome. Then the sections were deparaffinized, rehydrated, and washed with distilled water. The tissues were digested with 20 g/ml proteinase K (Boehringer Mannheim) at room temperature for 15 minutes. Endogenous peroxidase activity was blocked by incubation in 0.3% H2O2/methanol in PBS at 37°C for 30 minutes. The sections were then incubated with terminal deoxynucleotidyl transferase at 37°C for 60 minutes to add the digoxigenin-conjugated deoxyuridine triphosphate to the 3'-OH ends of fragmented DNA. Antidigoxigenin antibody peroxidase was applied to the sections to detect the labeled nucleotides. The sections were stained with DAB and counterstained slightly with hematoxylin.

**Nissl.** Tissue sections were stained with cresyl violet (Nissl) as previously described. The 4-μm sections were hydrated in 1% toluidine blue for 10 minutes. After washing with double-distilled water, they were dehydrated and mounted with Permount. Normal neurons have a relatively big cell body, rich in cytoplasm, with one or two big round nuclei. In contrast, damaged cells show shrunken cell bodies, condensed nuclei, dark cytoplasm, and many empty vesicles.

**Cell Counting**

One slice from every 6 serial cuttings in each segment was selected, and altogether 6 slices were collected and observed using the light microscope by 2 independent, experienced pathologists who were blinded to the grouping. All positive cells in each section were counted in 10 microscope fields (magnification ×400) throughout the identical regions of the studied brain, and the mean number per visual field was calculated.

**Biochemical Estimation**

**Malondialdehyde.** The malondialdehyde (MDA) levels were determined using the method of a previous study. The principle of the assay depends on the reaction of lipid peroxidation products with thiobarbituric acid and formation of products called thiobarbituric acid–reacting substances, which give maximum absorbance at the 535-nm wavelength. The MDA concentrations were given as nanomoles/milligram protein.

**Superoxide Dismutase and Glutathione.** Total tissue superoxide dismutase (SOD) enzyme activities and glutathione (GSH) measurements were determined with a commercially available kit (Nanjing Jiancheng Bioengineering Institute). The measurement was performed according to the manufacturer’s instructions. Results were expressed as U/mg protein (SOD) and mg/g protein (GSH), respectively, according to the standard curves.

**Determination of Protein Concentration**

The tissue protein concentration was determined using the method of Bradford with a standard commercial kit (Bio-Rad Laboratories).

**Statistical Analysis**

All data are presented as the mean ± SEM, with IBM SPSS Statistics, version 19.0.0, being used for statistical analysis. Neurological function data were statistically analyzed using nonparametric tests (Kruskal-Wallis, fol-
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followed by the Dunnis post hoc test). All other data were subjected to 1-way ANOVA combined with the Tukey multiple comparison test. Statistical significance was inferred at p < 0.05.

Results

Mortality Rate and General Observations

Of 325 rats used in our experiment, 43 died after the operation; those animals were excluded from further analysis. Six additional rats were used for animal model training before the experiment and are not included in the results. The mortality rate on Day 3 after surgery was 0% (0 of 42) in the sham-injured group; 22.2% (12 of 54) in the SAH group; 19.2% (10 of 52) in the SAH + vehicle group; 17.2% (5 of 29) in the SAH + 0.01 mmol/l ATX group; 12.5% (6 of 48) in the SAH + 0.1 mmol/l ATX group; 14.3% (4 of 28) in the SAH + 25 mg/kg ATX group; and 12.5% (6 of 48) in the SAH + 75 mg/kg ATX group (Table 2). No SAH-related death was observed in any of the rabbit experimental groups. No significant differences in mean arterial blood pressure, body temperature, or injected arterial blood gas data were observed among the experimental groups (data not shown).

Reduction of Brain Water Content and BBB Permeability in Rats Treated With ATX After SAH

Brain edema is an independent risk factor for poor outcome and death after SAH. Disruption of the BBB has been considered to be one of the most important contributors to the pathogenesis of brain edema. In the present study, ATX was given intracerebroventricularly in rats at 30 minutes post-SAH at two different concentrations. It was observed that treatment with ATX at 0.1 mmol/l significantly reduced brain edema and EB extravasation at 24 hours after SAH (Fig. 3A and C). To investigate the therapeutic window of ATX for SAH, this agent (25 mg/kg or 75 mg/kg) was applied at 3 hours after SAH by oral gavage. Results showed that a 3-hour delay of ATX treatment (75 mg/kg) after SAH also reduced brain edema and BBB disruption at 24 hours after SAH (Fig. 3A and C). However, there was no significant difference in brain edema and EB extravasation among all experimental groups at 72 hours after SAH (Fig. 3B and D).

Suppression of Cleaved Caspase-3 Expression After ATX Treatment

The expression of cleaved caspase-3 protein was assessed using Western blots. As shown in Fig. 4, cleaved caspase-3 protein expression was at a very low level in the control and sham groups. In comparison, after SAH induction, the levels of cleaved caspase-3 protein significantly increased in SAH and SAH + vehicle groups. Treatment with 0.1 mmol/l ATX at 30 minutes after SAH significantly suppressed the expression of cleaved caspase-3. In addition, treatment with 25 mg/kg or 75 mg/kg ATX at 3 hours after SAH also dramatically reduced the expression of cleaved caspase-3, in a dose-dependent manner.

Reduction of Caspase-3 Immunoreactivity and Neuronal Apoptosis After ATX Treatment

As shown in Fig. 5A, a few cleaved caspase-3–positive cells were observed in the cortex of the control and sham groups, whereas numerous caspase-3–positive cells stained brown in the nuclei were evident in the SAH and SAH + vehicle groups. Compared with the SAH or SAH + vehicle group, administration of ATX (0.1 mmol/l, 25 mg/kg, or 75 mg/kg) markedly reduced the active caspase-3–positive cells in the brain at 24 hours after SAH (Fig. 5B). The TUNEL staining showed that the rats in the control and sham groups displayed rare apoptotic neurons in the cortex, whereas obvious TUNEL-positive neurons could be observed in the SAH and SAH + vehicle groups. In contrast, the proportion of apoptotic neurons decreased significantly in the ATX treatment (0.1 mmol/l, 25 mg/kg, or 75 mg/kg) groups (Fig. 5A and B).

Influence of ATX on Neuronal Survival, Neurological Function, and Body Weight Loss

Figure 6A shows Nissl staining in the cerebral cortex at 72 hours after SAH. Evident damage was seen in the SAH and SAH + vehicle groups, with a decrease of cell number, sparse cell arrangements, loss of integrity, and

<table>
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<th>Group</th>
<th>No. at 1 Day</th>
<th>No. at 3 Days</th>
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<tr>
<td></td>
<td>Alive</td>
<td>Dead</td>
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<tr>
<td>control (n = 24)</td>
<td>24</td>
<td>0</td>
</tr>
<tr>
<td>sham-injured (n = 42)</td>
<td>24</td>
<td>0</td>
</tr>
<tr>
<td>SAH (n = 54)</td>
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<td>6</td>
</tr>
<tr>
<td>SAH + vehicle (n = 52)</td>
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<td>5</td>
</tr>
<tr>
<td>SAH + 0.01 mmol/l ATX</td>
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</tr>
<tr>
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<tr>
<td>SAH + 25 mg/kg ATX</td>
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<td>4</td>
</tr>
<tr>
<td>SAH + 75 mg/kg ATX</td>
<td>24</td>
<td>3</td>
</tr>
<tr>
<td>total (n = 325)</td>
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<td>26</td>
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</tbody>
</table>

* The mortality was just defined for the 3-day experiments, and animals that died in the 1-day experiments were excluded from the analysis.
dark staining resulting from cytoplasm and karyoplasms. In comparison, treatments at both 30 minutes and 3 hours post-SAH could evidently alleviate the severity of neuronal degeneration seen at 72 hours after SAH (Fig. 6B, a).

The temporal profile of the neurological function (Fig. 6B, b) and body weight ratio (Fig. 6B, c) after SAH were recorded. In comparison with the SAH and SAH + vehicle groups, the groups receiving therapy both 30 minutes and 3 hours post-SAH displayed better neurological scores at 24 and 48 hours, but scores were not significantly better at 72 hours. However, there were no significant differences in body weight ratio among all experimental groups at all time points during the 3-day observation period.

**Neuroprotective Effects of ATX in Rabbits After SAH**

To further evaluate the efficacy of ATX as a therapeutic agent for SAH-induced brain injury, the agent (25 mg/kg or 75 mg/kg) was given to rabbits by oral administration at 3 hours post-SAH (Fig. 7). Results showed that delayed treatment with ATX (3 hours after SAH) at 25 or 75 mg/kg significantly attenuated neuronal apoptosis (Fig. 7B, a), alleviated the severity of neuronal degeneration (Fig. 7B, b), and improved neurological function (Fig. 7B, c) in a dose-dependent manner. These results indicated that ATX is a neuroprotective agent with a wide therapeutic window in SAH.

**Fig. 3.** Bar graphs showing effects of ATX treatment on the changes in percentage of brain water content (A and B) and EB extravasation (C and D) at 24 (A and C) and 72 (B and D) hours after surgery (n = 6 in each group). The ATX (0.1 mmol/l) treatment at 30 minutes post-SAH by intracerebroventricular injection significantly reduced brain water content at 24 hours after SAH compared with that of the vehicle-treated SAH group. Moreover, ATX treatment (75 mg/kg) at 3 hours after SAH by oral gavage also reduced brain edema compared with that of the SAH group. However, there were no significance differences among all groups in brain edema at 72 hours after SAH. The EB extravasation assessment was performed at 24 (C) and 72 hours (D) after SAH. It showed that ATX treatment both at 30 minutes by intracerebroventricular injection (0.1 mmol/l) and at 3 hours through oral gavage (75 mg/kg) after SAH could reduce EB extravasation at 24 hours but not at 72 hours after SAH. Values are expressed as the mean ± SEM. *p < 0.05; **p < 0.01. ns = not significant.
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Oxidative Stress Changes After ATX Treatment

To determine the effects of ATX on oxidative stress, we evaluated the changes in MDA levels, SOD activities, and GSH contents in the cerebral cortex at 24 hours after SAH. As shown in Fig. 8, SAH significantly increased the levels of MDA (Fig. 8A), and markedly decreased GSH contents (Fig. 8B) and activities of SOD (Fig. 8C) when compared with the sham and control groups. Treatment with ATX has shown protective effects by significantly decreasing the elevated MDA levels (Fig. 8A) and increasing the suppressed GSH (Fig. 8B) and SOD (Fig. 8C) levels.

Discussion

Early brain injury is a recent concept that looks at overall brain injury after SAH.⁴ Accumulating studies indicate that EBI instead of cerebral vasospasm is the main cause of poor outcomes in patients with SAH.⁵,²⁴ The pathogenesis of EBI is complicated, including elevation of intracranial pressure, reduction of cerebral blood flow, brain edema, BBB disruption, and neuronal cell death.⁴ However, the mechanisms behind many of these key events are poorly understood. Oxidative stress has been proven to play a crucial role in the pathophysiological process after SAH.¹⁰,¹⁴ It has been demonstrated that excessive reactive oxygen species and reactive nitrogen species including hydroxyl radical, superoxide anion, H₂O₂, nitric oxide, and peroxynitrite are generated early after SAH, and consume enzymatic and nonenzymatic antioxidant defense systems.¹⁰,²⁰ Moreover, these free radicals will lead to neuronal damage by promoting lipid peroxidation, protein breakdown, and DNA damage, and this in turn leads to cellular apoptosis, endothelial injury, and BBB permeability.³
Astaxanthin is a naturally occurring carotenoid widely distributed in algae, crustaceans, shellfish, and various plants. This compound, unlike other carotenoids, contains two additional oxygenated groups on each ring structure, resulting in enhanced antioxidant properties. ATX was approved by the US FDA as a feed additive in 1987 and as a dietary supplement in 1999. There is a wealth of studies exploring the potential role of ATX

Fig. 5. Cleaved caspase-3 immunohistochemistry and TUNEL staining (A) of cerebral cortex and cell counting (B) at 24 hours following SAH. A: Representative slides of cleaved caspase-3 immunohistochemistry and TUNEL staining in cerebral cortex in all groups (a and e: control group; b and f: sham group; c and i: SAH group; d and g: SAH + vehicle group; e and j: SAH + 0.01 mmol/l ATX group; f and n: SAH + 0.1 mmol/l ATX group; g and o: SAH + 25 mg/kg ATX group; and h and p: SAH + 75 mg/kg ATX group). Few cleaved caspase-3–positive cells were observed in the cortex of the control and sham groups, whereas numerous caspase-3–positive cells stained as brown in the nuclei were evident in the SAH and SAH + vehicle groups. Compared with SAH or SAH + vehicle groups, administration of ATX (0.1 mmol/l, 25 mg/kg or 75 mg/kg) markedly reduced the active caspase-3–positive cells in the brain at 24 hours after SAH. The TUNEL staining showed that the rats in the control and sham groups display rare apoptotic neurons in the cortex, whereas obvious TUNEL-positive neurons could be observed in the SAH and SAH + vehicle groups. In contrast, the proportion of apoptotic neurons decreased significantly in the ATX treatment (0.1 mmol/l, 25 mg/kg, or 75 mg/kg) groups.

B: Bar graphs showing cell counts per visual field (×400) found in the slides with cleaved caspase-3 staining (left) and TUNEL staining (right). Values represent the mean ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001.
Astaxanthin ameliorates early brain injury after SAH

It has been postulated that brain edema and cellular apoptosis are two major components of EBI and are responsible for poor outcomes following SAH. It has been demonstrated that brain edema, a consequence that occurs from BBB disruption, has been shown to be an independent predictor of poor outcome and death after SAH. In the present study, we first evaluated the efficacy of ATX as a therapeutic agent for SAH-induced brain injury through intracerebroventricular injection at 30 minutes post-SAH. The dose and the route of administration of ATX were selected according to a previous study. We found that treatment with 0.1 mmol/l ATX at 30 minutes post-SAH could significantly reduce brain edema and EB extravasation at 24 hours after SAH, but failed at 72 hours after SAH. To further evaluate the potential benefits and the therapeutic window of ATX in the SAH model, oral administration instead of intracerebroventricular injection was conduct-

in the past years in different research fields, and no side effects or toxicity have been reported. It has been demonstrated that ATX possesses a variety of pharmacological properties, including antioxidative, anti-inflammatory, anti-tumor, immunomodulatory, and neuroprotective activities. A recent study indicated that ATX supplementation could reduce ischemic brain injury through inhibition of oxidative stress, reduction of glutamate release, and anti-apoptosis, without any side effects on physiological parameters. A randomized clinical trial also found that 6 mg/day of ATX could be safely consumed by “healthy adults.” It is thus possible that ATX may hold protective effects in other disorders of the CNS involving free-radical toxicity as well. Therefore, we hypothesized that ATX administration could ameliorate the oxidative injury in cerebral cortex, and thus exert significant neuroprotection against EBI following SAH.

Fig. 6. Effect of ATX treatment on neuronal survival, neurological function, and body weight ratio within 3 days in rats post-SAH. A: Representative photomicrographs of Nissl-stained sections to indicate neuron survival at Day 3 in all experimental groups (a, control group; b, sham group; c, SAH group; d, SAH + vehicle group; e, SAH + 0.1 mmol/l ATX group; f, SAH + 75 mg/kg ATX group). B: a, Bar graph showing counts of neurons per vision field. As shown, compared with SAH and SAH + vehicle group, ATX treatment both at 30 minutes and 3 hours post-SAH significantly increased the proportion of surviving neurons. *p < 0.05; **p < 0.01. b, Graph showing temporal profile of the neurological behavior score in all experimental groups. In comparison with the SAH and SAH + vehicle groups, both 30 minutes and 3 hours post-SAH therapy groups displayed a better neurological score at 24 and 48 hours, but not significantly at 72 hours. *p < 0.05 vs sham group; &p < 0.05 vs SAH + vehicle group; #p < 0.05 vs SAH group. c, Graph showing body weight loss recorded at 24, 48, and 72 hours after SAH. No significant improvement was detected between experimental SAH groups and ATX treatment groups at all time points during the 3-day observation period. Results represent the mean ± SEM. *p < 0.05 vs sham group.
ed at 3 hours post-SAH in Experiment 2. Results showed that treatment with 75 mg/kg ATX at 3 hours after SAH was still efficacious. However, this beneficial effect also did not extend to 72 hours after SAH. It could be possible that a one-time application of ATX might not be sufficient for persistent protection against EBI after SAH. Multiple and prolonged use of ATX therapies may be warranted for future SAH brain-injury studies.

Caspase-3 is a member of the caspase family, and it plays a key role in the execution phase of cell apoptosis. It has been reported that the expression of caspase-3 was intensified in the cortical neurons after SAH, and its inhibition could reduce the neuron loss. In the present study we analyzed the levels of cleaved caspase-3 after ATX treatment. Our data showed that cleaved caspase-3 increased significantly in the experimental SAH rat groups, but that treatment with ATX both at 30 minutes and 3 hours after SAH could reverse the SAH-induced increase of cleaved caspase-3 in the cerebral cortex. Additionally, the histopathological studies also revealed a strong correspondence with the degree of cleaved caspase-3 expression in all experimental rat groups. These findings suggested that ATX could protect neurons from apoptosis after SAH.

Meanwhile, we evaluated the effects of ATX on neurological outcomes and body weight after SAH induction. We found that rats that received ATX treatment at 30 minutes or 3 hours post-SAH showed a better neurological score at the early period, but not at 72 hours after SAH. In a previous study, Thal and colleagues reported that brain edema formation instead of neuronal cell loss may be the major contributor to neurological dysfunction in the acute phase following SAH. Consistent with their conclusion, the neurological dysfunction observed in our experiment also showed a strong correspondence with the degree of brain edema, but not neuronal cell loss, in the early period after SAH. Body weight was identified as a nonspecific marker that could reflect the animal’s overall condition. In the present study, we found that body weight was not significantly different among various groups at 24 hours after surgery, but it was significantly different between the SAH group and the sham group at 72 hours. These results were in accordance with those reported in a previous study, suggesting that body weight was influenced more by surgery than by SAH in the early period.

In the last set of experiments, our data further demonstrated that delayed treatment with ATX was still efficacious in a rabbit experimental SAH model.
To elucidate whether the mechanism of ATX against EBI is related to its powerful antioxidant property, we further evaluated oxidative damage in the cortex after SAH. We found that MDA levels were significantly elevated in the cerebral cortex after SAH, and SOD activity and GSH levels were markedly reduced. Interestingly, both 30 minutes and 3 hours post-SAH, ATX therapies could dramatically downregulate the increased MDA levels and restore the suppressed endogenous antioxidant levels in the brain tissues. These results further supported the theory that oxidative stress was involved in and played a detrimental role in the development of EBI after SAH. Taken together, our results suggested that ATX could attenuate SAH-induced oxidative stress and upregulate the levels of endogenous antioxidants.

However, we must report that our study has several limitations. First, the neurobehavioral evaluation system used in the present study is not sensitive enough to distinguish the neurological differences in different groups. More sensitive neurobehavioral tests should be created to render the results more accurately and scientifically. Also, our findings regarding the improved neurological outcome could be explained partly by beneficial effects of ATX through reducing oxidative stress. For example, ATX inhibits glutamate release,18,22 reduces nuclear factor–kappa B translocation to the nucleus,17 and inhibits platelet aggregation,15 which has also been implicated in the pathophysiology of cerebral ischemia after SAH.20 Last, the underlying molecular mechanisms of the antioxidant properties of ATX remain elusive. Therefore, comprehensive studies are still warranted to explore the concrete mechanisms of ATX in EBI following SAH.

Conclusions

To the best of our knowledge, the present study was the first one to demonstrate that ATX administration decreased brain edema, BBB dysfunction, cellular apoptosis, and improved neurological outcomes after SAH in experimental models. The beneficial effects of ATX might be associated with suppressing oxidative stress damage. Although additional preclinical tests are warranted, we believe that ATX, with its efficacy, safety, and low cost, will hold great promise as a novel therapeutic agent for the management of patients with SAH.

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Disclosure

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