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Research report

Neuroprotective effects of astaxanthin in a rat model of spinal cord injury

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ABSTRACT

Spinal cord injury (SCI) often leads to constant neurological deficits and long-term unalterable disability. Apoptosis plays an important role in the initiation of the secondary injury cascades leading to progressive tissue damage and severely functional deficits after SCI. Although the primary mechanical destructive events cannot be reversed, a therapeutic intervention could be carried out in order to moderate the secondary injury damage several hours to weeks after injury. Astaxanthin (AST) is a strong antioxidant and anti-inflammatory agents with the potential to render anti-apoptotic and neuroprotective effects. In the current study, we examined the therapeutic potential of AST on adult rats after severe SCI contusion. Results of BBB scores showed that AST improved motor function after SCI compared to control groups. Western blot analysis showed reduced expression of Bax and Cleaved-caspase-3 proteins and increased expression of the Bcl-2 protein in response to AST treatment ($p < 0.05$). The histology results also showed that AST considerably preserved myelinated white matter and the number of motor neurons. This study is the first to report that AST reduces neuronal apoptosis, diminishes pathological tissue damage and improves functional recovery after SCI. The observed prominent neuroprotective effects, introduces AST as a promising therapy for SCI.

1. Introduction

Spinal cord injury (SCI) causing permanent loss of neurons and disconnection of ascending and descending spinal tracts mostly leads to the loss of sensory and motor function below the injury site [1–3]. The central nervous system (CNS) of adult mammalians possesses restricted regenerative ability in response to injury. Formation of fibrotic scar and expression of axon growth inhibitors seriously impede regeneration after CNS injury [4].

Injuries to spinal cord generally initiate pathological responses in two chronological phases. Primary injury is characterized by necrotic cell death caused by mechanical tissue damage whereas the secondary phase features activation of apoptosis mechanisms, cascade of enzymatic events, secondary necrosis, mitochondrial dysfunction, demyelination and glial scar formation [5]. The secondary injury phase is mainly caused by vascular changes and subsequent edema, ischemia and hypoxia, cytokine production and inflammation, free radicals and lipid peroxidation, disruption of ionic balance and glutamate excitotoxicity [1,6–10]. The spinal cord is mostly composed of lipids which are easily destroyed by free radicals and the lipid peroxidation processes [11].

Although a large number of preclinical studies or clinical trials are currently in progress based on the usage of pharmacological, biological or bioengineering approaches [12], however, there is still a lack of effective therapeutic strategies for the management of SCI [13,14]. According to National Acute Spinal Cord Injury Studies (NASCIS), high-doses of intravenous methylprednisolone (MP) demonstrate significant neuroprotective effects in human SCI. However, there are many other reports, clearly indicating that MP not only does not significantly decrease neurological dysfunction following SCI [15], but also may worsen it and cause serious side effects [16,17]. In efforts to overcome the secondary damages and promote functional recovery in SCI models, oxidative stress and antioxidant protection have been found as a promising target and a useful tool [18].

Astaxanthin (AST) is a carotenoid and a lipophilic pigment which is found in various microorganisms and marine animals [19]. Due to the

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two additional oxygenated groups on its each ring structure in comparison with other carotenoids, AST has enhanced antioxidant properties [20]. There is a wealth of studies exploring the potential properties of AST in the past years in different research fields, and no side effects or toxicity has been reported [21]. Some previous studies have reported that AST possesses more potent biological activity as compared to other carotenoids and antioxidants [22]. The use of AST as a feed additive in 1987 and as a dietary supplement in 1999 was approved by United States Food and Drug Administration (FDA), and the European Commission, respectively [23]. The commercial AST mainly comes from Phaffia yeast, Haematococcus pluvialis and also by chemical synthesis [24].

AST passes across the blood–brain barrier and affects multiple molecular targets in CNS [25]. For instance, by attenuating oxidative damage, AST protects against epilepsy-induced neuronal loss, lipid peroxidation and inhibits the intrinsic apoptotic pathway in the rat hippocampus [26]. Beneficial biological effects of AST reported so far include: reduction of reflux symptoms, modulation of immune responses, anti-oxidative and anti-inflammatory properties, apoptosis inhibition, anticancer effects by reduction of matrix metalloproteinase-9 (MMP-9) expression and activity, and prevention or treatment of diabetes[27–40]. Considering the potential beneficial effects of AST in CNS as well as the pathophysiology of SCI, we aimed to investigate the effects of AST in a rat contusion model of SCI.

2. Materials and methods

2.1. Animals

Totally, 108 adult male Wistar rats (aged, 2–3 months; weighing, 250–280 g) were used in this study.The animals were obtained from the breeding colony of Neuroscience Research Center. Experiments were accomplished in accordance with National Institutes of Health Guidelines for the Care and Use of Laboratory Animal and approved by the institutional animal care and use committee in Shahid Beheshti University of Medical Sciences (IR.SBMU.REC.1395, 21). Animals were kept under a 12-h light/dark cycle and maintained in a temperature-controlled room (24 ± 2 °C), for at least 10 days prior to the study. The keeping place was pathogen-free with available water and food. In this study, we tried to use the minimum number of animals and least suffering; and all surgeries were accomplished using aseptic techniques.

2.2. Experimental groups

Thirty-six rats were randomly divided into four experimental groups consisted of 9 rats per each group for behavioral assessments. In the first group (Sham), animals received laminectomy surgery without contusion lesion. In the second group (Injury), animals were subjected to laminectomy and contusion lesion. In the third group (Vehicle), rats were subjected to laminectomy and contusion injury then treated with vehicle. The fourth group (Treatment) of rats received laminectomy and contusion injury and 30 min after injury were treated with administration of AST. Animals were subjected to behavioral tests at days 1, 4, 7, 14, 21 and 28 after surgery. On the last day of behavioral tests, 6 rats per group was sacrificed and 3 of them was used for molecular and 3 for histological assessments. Another 72 rats was assigned to four experimental groups, including Sham, Injury, Vehicle and Treatment. On days 1, 7, 14, and 28 after surgery, six rats per group were sacrificed and assigned for molecular (3 rats per group per each experimental time point) and histological (3rats per group per each experimental time point) assessments.

2.3. Spinal cord contusion injury

Rats were deeply anesthetized by intraperitoneal (i.p.) administration of ketamine/xylazine (80/10 mg/kg). Before surgery, all animals received subcutaneously (s.c.) buprenorphine (0.01- 0.02 mg/kg) as an analgesic. The SCI was generated at spinal segment T8–T9 using the New York University impact injury device [41]. A10-g rod with 2.0-mm diameter was released from a 50 mm height onto the exposed spinal cord to cause a severe contusion. For sham surgery, animals underwent laminectomy without impact injury. After SCI or sham surgery, muscles and skin were closed in layers with 4–0 silk sutures, and the animal was permitted to recover on a 30 °C heating pad. After surgery, the animals received saline (2.0 ml, twice daily, s.c.) and Enrofloxacin (2.5 mg/kg, twice daily, s.c.) for rehydration and prevention of urinary tract infection, respectively. Rat's bladder was emptied manually twice a day until reflex bladder emptying returned. After surgery, two rats were kept in each cage, and maintained under the same conditions and fed freely.

2.4. Intrathecal drug injection

Astaxanthin (AST) was purchased from Sigma-Aldrich (St. Louis, Mo) and dissolved in 5% dimethylsulfoxide (DMSO) to the concentration of 0.2 mM. The modified method of direct transcutaneous intrathecal (i.t.) injection [42] was used for AST and vehicle treatments in anesthetized rats, 30 min after SCI. In brief, the lumbar vertebrae just cranial to both iliac crests were held by the thumb and middle finger. Then, the sixth lumbar (L6) spinous process was posited by palpating the highest spinous process with the index finger. A 25-gauge needle connected to a 25 µl Hamilton syringe was appended from the caudal end, instantly lateral to the L6 spinous process at a 45° angle to the vertebral column and was pressed slowly in the cranioventral direction. Identification of the needle in the subarachnoid space was according to the presence of a sudden lateral tail movement that happened after penetration of the ligament flavum. Once the needle was in the subarachnoid space, a dose of AST (10 µl of 0.2 mM) [43] or vehicle (10 µl of DMSO 5%) was injected slowly over 10 s. The syringe was held for 10 more seconds before removal to prevent outflow of the drug.

2.5. Behavioral assessment

The motor behavior was assessed using the Basso, Beattie, Bresnahan (BBB) locomotor scale method [44]. Briefly, the rats were placed in an open-field box (90 cm^2 in area and surrounding walls of 10 cm) and observed by two blind examiners for 4 min. The BBB score ranged from 0 (complete paralysis of both hindlimbs) to 21 (normal walking) was defined for each animal, before surgery (D0) and also on days 1, 4, 7, 14, 21 and 28 after surgery. In order to acquire a single movement value, the scores for left and right hindpaws were averaged for each animal per time point.

2.6. Western blot

On days assigned for molecular assessments, animals were sacrificed and the spinal cord segments containing the injury epicenter were dissected. The western blotting was performed based on our previously reported protocol [45]. In summary, Tissues were homogenized with lysis buffer (NaCl 150 mM, sodium deoxycholate 0.25%, Triton X-100 0.1%, Tris–HCL 50 mM, SDS 0.1%, EDTA 1 mM, and protease inhibitor cocktail 1%) by a tissue homogenizer, and total protein extract was acquired after centrifugation for 45 min at 12,000 rpm, 4 °C. The protein concentration was measured using Bradford assay. For western blotting, the proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membrane. The membranes were blocked with 2% non-fat dry milk (Amersham, Ecl Advance TM) for 75 min and then were incubated at 4 °C overnight with anti-Bax, anti-Bcl-2, anti-Caspase-3 (1:1000 v/v, Cell Signaling Co.), and anti- β-actin (1:10,000 v/v, Cell Signaling) antibodies. Afterward, the membranes were incubated at room temperature for 2 h with secondary antibody (1:3000 v/v, Cell Signaling). The membranes were