African Journal of Pharmacy and Pharmacology Vol. 6(34), pp. 2559-2564, 15 September, 2012
Available online at http://www.academicjournals.org/AJPP
DOI: 10.5897/AJPP12.855
ISSN 1996-0816 © 2012 Academic Journals

Full Length Research Paper

The effect of astaxanthin (AST) on Neurotrophin-3 (NT-3) expression in rats after compressive spinal cord injury (SCI)"

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Accepted 23 August, 2012

A great deal of this research has been centered on the neuroprotective benefits of astaxanthin. However, no studies have suggested that astaxanthin has therapeutic potential for SCI and the mechanisms underlying astaxanthin neuroprotection are not fully understood. The aim of this study was to determine the therapeutic efficacy of starting astaxanthin treatment 1 day after compressive spinal cord injury (SCI) in rat and to investigate the underlying mechanism. The SD rats were randomly divided into four groups namely: Sham control group, SCI model group, the methylprednisolone sodium succinate (MPSS) group and the astaxanthin treatment group. Western blot analysis and Reverse transcription-polymerase chain reaction (RT-PCR) were performed to detect the expression of the Neurotrophin-3 (NT-3). Noticeably, astaxanthin at the 80 mg/kg dosage exhibited similar effects as MPSS, which has been frequently used for clinical acute SCI. These results suggested that astaxanthin could increase the expression of NT-3 in SCI.

Key words: Astaxanthin, spinal cord injury, neurotrophin-3.

INTRODUCTION

Spinal cord injury (SCI) is a devastating neurological injury that often leads to irreversible neurological deficits. The pathophysiology of SCI involves primary and secondary mechanisms of injury (Oyinbo, 2011). Secondary injury mechanisms include vascular changes, ionic disturbances, neurotransmitter (glutamate) accumulation leading to excitotoxicity, generation of free radicals, edema, depletion of energy substrates, and activation of a variety of proteases (Blight, 2002). These various actions may directly or indirectly be involved in apoptosis and necrosis of neurons and glial cells (Yakovlev and Faden, 2001). Attention has been paid to Neurotrophin-3 (NT-3) as one of the important members of nerve growth factor family. NT-3 can increase the growth of the spinal cord neurons, and NT-3 mainly promotes neuritis growth by activating TrkC for its biological effects: axonal growth and the differentiation in sensory neuron, motor neuron, dopaminergic neuron, and other neurons (Griesbeck et al., 1995; Lamballe et al., 1991; Novikova et al., 2000). At the present, research on SCI has focused on protection, regeneration and rehabilitation of the neuronal and supporting tissue, and the widely recommended pharmacotherapy is a high-dose methylprednisolone therapy, which has limited efficacy and serious adverse effect (Pereira et al., 2009). Therefore, new therapeutic approaches targeting secondary injury pathways occurring in the penumbra must be considered for protecting neurons and preserving spinal cord function.

Astaxanthin (AST), a red-orange carotenoid pigment, is a biological antioxidant that is naturally found in a wide variety of aquatic living organisms, such as shrimp, crab,

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and salmon (Higuera-Ciapara et al., 2006). The green microalgae *Haematococcus pluvialis* and the red yeast *Phaffia rhodozyma* are common sources of natural astaxanthin (Higuera-Ciapara et al., 2006). AST has been reported to possess anti-oxidant, anti-inflammatory and anti-tumor effects (Tanaka et al., 1995; Kurashige et al., 1990). AST also shows strong activity as an inhibitor of oxygen radical-mediated lipid peroxidation (LPO) (Mortensen et al., 2001). Recent research has further validated AST's ability to protect the central nervous system. A great deal of this research has been centered on the neuroprotective benefits of AST (Satoh et al., 2009; Nakagawa et al., 2011; Lee et al., 2010; Chang et al., 2010; Kim et al., 2010). However, no study has suggested that AST has therapeutic potential for SCI. Furthermore, the mechanisms underlying AST neuroprotection are not fully understood.

In the present study, we determined the efficacy of astaxanthin treatment administered from the first day on the rats after SCI. The NT-3 mRNA expression in SCI rats was measured by Reverse Transcription-Polymerase Chain Reaction (RT-PCR), while the protein expression by Western Blot following astaxanthin treatment to clarify the real mechanism.

MATERIALS AND METHODS

Reagents

Astaxanthin was purchased from Sigma-Aldrich (St. Louis, Mo). MPSS was obtained from Pharmacia and Upjohn Company, rabbit anti-NT-3 (Santa Cruz Biotechnology), goat anti-rabbit Ig GHRP (Santa Cruz Biotechnology) and Amersham ECL plus Western Blotting Detection kit (GE Healthcare Bio-Sciences, Piscataway, NJ, USA).

Animals

A total of 48 healthy females, Sprague Dawley rats, aged 2 months, weighing 180 to 220 g, were purchased from the Laboratory Animal Centre of The Second Military Medical University of China PLA. Animal care and experiments were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of The Second Military Medical University of China PLA.

Spinal cord injury

The aneurysm clip compression model of SCI used in our laboratory has been characterized extensively and described previously (Fehlings and Tator, 1995; Nashmi and Fehlings, 2001; Karimi-Abdolrezaee et al., 2004; Karimi-Abdolrezaee et al., 2006). Under inhalational anesthesia using halothane (1 to 2%) and a 1:1 mixture of O_2/N_2O, the surgical area was shaved and disinfected with 70% ethanol and betadine. A midline incision was made at the thoracic area (T4 to T9), and skin and superficial muscles were separated. The rats underwent a T6 to T8 laminectomy and received a 23 g clip (Walsh, Oakville, Ontario, Canada) compression injury for 1 min at the level of T7 of the spinal cord. The surgical wounds were sutured, and the animals were given post-operative analgesia and saline (0.9%; 5 ml) to prevent dehydration. Animals were allowed to recover and were housed in standard rat cages with absorbent bedding at a temperature of 27°C. Their bladders were manually expressed three times daily until return of reflexive bladder control.

Drug treatment and sample preparation

The Sham operation group underwent laminectomy alone to expose the spinal cord, and received 0.9% NS (2 ml/kg). The SCI model group underwent laminectomy followed by SCI, and received 0.9% NS (2 ml/kg). The MPSS group, positive control group underwent laminectomy followed by SCI, and was administered 100 mg/kg single dose of MPSS (2 ml/kg, i.p.) 5 min after SCI. Through pre-experiment, the dosage was defined that the 80 mg/kg AST group underwent laminectomy followed by SCI, and were given a single dose of 80 mg/kg of AST (dissolved in 0.9% NS, 2 ml/kg, i.p.) 5 min after SCI. Twelve animals in each group were used for RT-PCR and Western blot analysis in spinal cord tissues. After 24 h, the rats were anesthetized with chloral hydrate (0.3 g/kg), transcardially perfused with 150 ml of 0.9% NS and 200 ml of 4% paraformaldehyde in 0.1M PBS (pH 7.2). Approximately, 2 cm spinal cord segments between the T6 and T8 levels were removed, post-fixed overnight in the same fixative and cryopreserved at -70°C for measurements of NT-3. Approximately, 1 cm length spinal cord encompassing the injury site was then removed for RT-PCR and the additional sections for western blot analysis.

RT-PCR

Extraction of total RNA through reverse transcription namely; 1) the spinal cord samples (weighing 50 mg) were harvested, and total RNA extracted following the user manual for Trizol. Oligo (dT) was used as the primer, and the extracted total RNA was used as the template for reverse transcription to synthesize cDNA by using M-MLV reverse transcriptase (Promega, Madison, WI, USA); 2) examination of NT-3 mRNA expression by PCR: β-actin selected as the internal reference. The primer sequences were designed using the Primer5 software. All primers were synthesized by Sangon Biotech Co., Ltd (Shanghai, China). The sequence of primers used were: β-actin forward: 5' GTAAAGACCTCTATGCCAACA3', β-actin reverse: 5'GGACTCA TCGTACTCCTGCT3'; NT-3 forward: 5'CGTCCCTGGAAATAGTCATACGG3', NT-3 reverse: 5'GACAGATGCCAATTCATGTTCTT3'. To amplify the cDNA, a 30 ul was set up with 20 pmol of each forward and reverse primer, 1.6 mM of dNTPs, 2.5 mM $MgCl_2$, and 2.5U DNA Taq polymerase per reaction. The reaction conditions were as follows: Initial denaturation at 95°C for 5 min followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, extension at 72°C for 45 s and final extension at 72°C for 10 min. PCR products were electrophoresed on the 1% agarose gel and signal intensity was quantitated with the Quantity One software (Bio-Rad, USA).

Western blot analysis

A 1 cm length spinal cord encompassing the injury site then was removed for Western blot analysis. The harvested tissues were homogenized on ice in a Lysis Buffer, containing 0.05 M Tris–HCL, 0.5 M EDTA, 30% TritonX-100, NaCl, 10% SDS and 1 mM PMSF, then centrifuged at 12,000 g at 4℃ for 30 min. The supernatant was obtained and stored at -80℃ for later use. Protein concentration was assayed with BCA reagent (Sigma). An equal amount of each protein sample was separated by 12% sodium dodecylsulfate-polyacrylamide gelelectrophoresis (SDS-PAGE) and was then transferred to PVDF membranes (Millipore). The membranes were blocked for 2 h in 5% non-fat milk in Tris-buffered saline containing

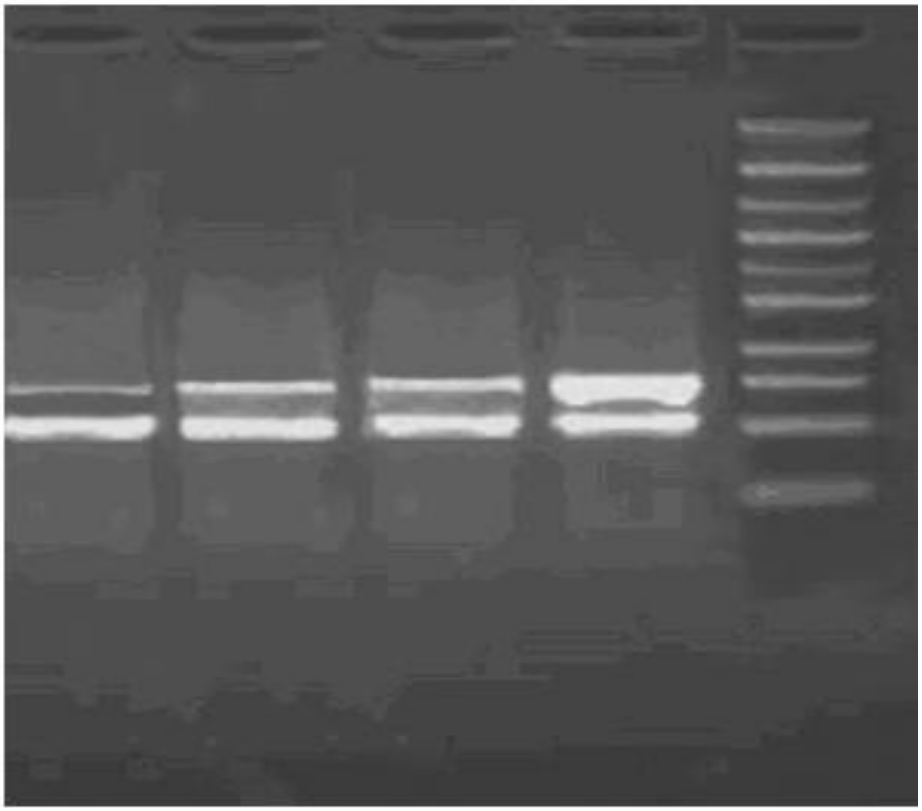

Figure 1. The RT-PCR results of NT-3 mRNA expression among different groups. M: Marker; Lanes 1–4: NT-3 mRNA expression in SCI model group (lane 1), Sham control group (lane 2), MPSS group (lane 3) and astaxanthin treatment group (lanes 4).

0.05% Tween-20 (TBST) and incubated overnight at 4℃ with rabbit anti-NT-3 (1:200; Abcam), and then with horseradish peroxidase-conjugated secondary antibody (1:4,000) for 1 h at room temperature. After rinsing with buffer (0.1% Tween-20 in TBS), the immuno complexes were visualized by chemiluminescence using the Amersham ECL Plus Western Blotting Detection kit (GE Healthcare Bio-Sciences, Piscataway, NJ, USA) according to the manufacturer's instructions. Actin was utilized as an internal control for sample loading, and each blot was normalized to its corresponding actin value.

Statistical analysis

All data were expressed as Mean ± SD, and analyzed by one-way analysis of variance followed by Least Significance Difference multiple comparison or Dunnett's multiple comparison tests using SPSS 16.0 software (SPSS, Chicago, IL, USA). Multiple comparison tests were used when appropriate. A p value of 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Effect of AST on the expression of NT-3 mRNA expression in SCI rats

As shown in Figure 1, the level of NT-3 mRNA was increased in the group of rats that received AST for 1 day ($p < 0.01$; Table 1), and the effect of AST at the 80 mg/kg dose was equivalent to MPSS (Table 1). These results suggested that AST could up-regulate the decreased expressions of NT-3 mRNA of the SCI rats.

Effect of AST on the expression of NT-3 protein expression in SCI rats

Treatment with AST increased the expression of NT-3 protein more effectively than the SCI model group ($p <0.01$; Table 2). Also, the effect of AST at the 80 mg/kg dose was equivalent to MPSS (Table 2). As shown in Figure 2, these observations showed that after SCI, the AST therapy could significantly promote the expression of NT-3.

Conclusion

Experimental models of SCI mainly include contusive injury, clip compressive injury, transaction/partial section injury, ischemic and chemical injury (Robins and Fehling, 2008). However, there is a high level of relevance to human SCI that the clip compression model provides,

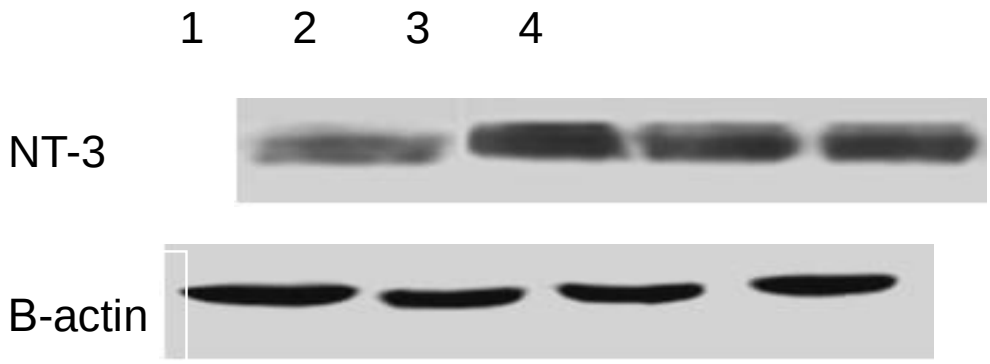

Figure 2. The western blot results of NT-3 protein expression among different groups. (Group1: SCI model; 2: NS control; 3: MPSS treatment; 4: 80 mg/kg of astaxanthin).

Table 1. The relative NT-3 expression of rats by RT-PCR among different groups.

Group	NT-3 expression (n=8)
NS control	88.89±1.59
SCI model	37.81±1.58[b]
MPSS	79.84±2.48b[c]
80 mg/kg of astaxanthin	75.19±2.64[bce]

(VS NS: [a]*P*<0.05, [b]*P*<0.01; VS SCI: [c]*P*<0.05, [d]*P*<0.01; VS MPSS: [e]*P*<0.01).

Table 2. The relative NT-3 expression of rats by western blot among different groups.

Group	NT-3 expression (n=8)
NS control	88.20±3.12
SCI model	40.16±3.19[b]
MPSS	78.97±2.33b[d]
80 mg/kg of astaxanthin	68.64±3.98[bde]

(VS NS: [a]*P*<0.05, [b]*P*<0.01; VS SCI: [c]*P*<0.05, [d]*P*<0.01; VS MPSS: [e]*P*<0.05).

and there are several other key advantages to using the clip to induce experimental injury. The model is inexpensive to establish and maintain, and provides a high degree of reliability and reproducibility in small animals. In terms of outcome evaluation, the clip compression model shows a strong correlation between closing force and degree of neurological deficit, which has been assessed using various behavioural tests, qualitative and quantitative histopathology, as well as electrophysiology (Onifer et al., 2007; Joshi and Fehlings, 2002 a, b).

The mechanisms of AST's improvement were studied by RT-PCR and Western Blot. The RT-PCR and Western Blot results showed that the NT-3 mRNA and protein expressions increased in the astaxanthin treatment group. The findings indicated that AST might improve the neuro-protective effect of SCI rats by increasing the NT-3 protein and mRNA expressions, and indicate that starting AST treatment 1 day after SCI would be very amenable to treating human patients.

Many experiments have demonstrated that the neurologic deficits in SCI are not because of an intrinsic inability of CNS neurons or axons to regenerate, but rather because of the formation of a CNS environment that is unfavorable for regeneration. Thus, creating a more favorable neurotrophic micro-environment has the potential to facilitate the regeneration of damaged neurons and axons after SCI. However, neurotrophin 3 (NT-3) is one of the four related polypeptide growth factors and shares structural and functional homology with nerve growth factor (NGF) (Tessarollo et al., 1994). It plays an important role in maintaining neuronal survival and promoting neurite growth in both physiological and pathological conditions (Hagg et al., 2005; Kłopotowska and Strzadała, 2005). NT-3 also alleviates secondary response through preventing the secondary cell loss that commonly occurs in injured spinal cords (Bradbury et al., 1998). In addition, the transplantation of NT-3-producing cells has a beneficial role in enhancing the survival of spinal cord neurons (Uchida et al., 2003) and regeneration of the corticospinal tract following SCI (Ruitenberg et al., 2003). The biological effects of NT-3 are mediated through a specific high affinity tyrosine kinase receptor type 3 (trkC) (Mocchetti and Wrathall, 1995). Lastly, NT-3 mRNA and protein have been demonstrated in both the peripheral and central nervous systems (Chen et al., 2007). Therefore, we explored the expression of NT-3 after compressive spinal cord injury.

AST is such an antioxidant which has been intensively studied for its neuroprotective activity. Lee et al. (2011) at the University of Pittsburgh, USA, concluded in their study that AST may provide a valuable therapeutic strategy for the treatment of progressive neurodegenerative disease such as Parkinson disease.

The phytochemical, dosed at 30 mg/kg, showed a neuroprotective efficacy 60% as compared with the control group. AST suppressed the expression of regulation inducible nitric oxide synthase. The study concluded that the neuroprotective effects of AST were related to anti-oxidant activities in global ischemia (Lee et al., 2010). AST could be used as a potent neuron protectant and as a therapy for early stages of Alzheimer's disease (Chang et al., 2010) and could contribute to the prevention of dementia in humans as we age (Nakagawa et al, 2011). To summarize it all, there was strong evidence that AST held great promise for those wishing to prevent cognitive diseases and maintained general brain health. It was demonstrated that AST had significant neuro-protective effects, which may be one of the action pathways accounting for treatment of those diseases. Therefore, in this study, we administered AST 1 day after SCI and explored the possible mechanism. Some side effects of AST, although non-toxic, can involve the following namely: decrease in blood pressure, increase in skin pigmentation, additional hair growth, changes in hormonal balance, lowered calcium levels in the blood and decreased libido. Maybe, taking a dose that is too high can increase the chance of side effects and adverse outcomes. Further study is needed to confirm both the safety and effectiveness of the AST treatment. However, MPSS has more serious adverse reaction as compared with AST. The most definitive finding of the NASCIS studies was that the use of a megadose of steroids was associated with detrimental effects such as wound infection, gastrointestinal hemorrhage, pulmonary embolism, severe pneumonia and sepsis and even death secondary to respiratory complications (Bracken et al., 1990, 1997). Moreover, an increasing number of authors have criticized several aspects of NASCIS studies (Hurlbert, 2006; Miller, 2008). Other clinical studies of MPSS treatment in acute SCI could not reproduce results obtained in the NASCIS II and III (Sayer et al., 2006). Maybe AST is more suitable for clinical application.

In conclusion, the present study has shown that AST treatment starting 1 day after SCI could significantly increase the expression of NT-3 and AST may also have therapeutic potential for SCI.

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