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THE EFFECTS OF *N*-ACETYL-CYSTEINE AND ACETYL-L-CARNITINE ON NEURAL SURVIVAL, NEUROINFLAMMATION AND REGENERATION FOLLOWING SPINAL CORD INJURY

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Abstract—Traumatic spinal cord injury induces a long-standing inflammatory response in the spinal cord tissue, leading to a progressive apoptotic death of spinal cord neurons and glial cells. We have recently demonstrated that immediate treatment with the antioxidants *N*-acetyl-cysteine (NAC) and acetyl-L-carnitine (ALC) attenuates neuroinflammation, induces axonal sprouting, and reduces the death of motoneurons in the vicinity of the trauma zone 4 weeks after initial trauma. The objective of the current study was to investigate the effects of long-term antioxidant treatment on the survival of descending rubrospinal neurons after spinal cord injury in rats. It also examines the short- and long-term effects of treatment on apoptosis, inflammation, and regeneration in the spinal cord trauma zone. Spinal cord hemisection performed at the level C3 induced a significant loss of rubrospinal neurons 8 weeks after injury. At 2 weeks, an increase in the expression of the apoptosis-associated markers BCL-2-associated X protein (BAX) and caspase 3, as well as the microglial cell markers OX42 and ectodermal dysplasia 1 (ED1), was seen in the trauma zone. After 8 weeks, an increase in immunostaining for OX42 and the serotonin marker 5HT was detected in the same area. Antioxidant therapy reduced the loss of rubrospinal neurons by approximately 50%. Treatment also decreased the expression of BAX, caspase 3, OX42 and ED1 after 2 weeks. After 8 weeks, treatment decreased immunoreactivity for OX42, whereas it was increased for 5HT. In conclusion, this study provides further insight in the effects of treatment with NAC and ALC on descending pathways, as well as short- and long-term effects on the spinal cord trauma zone. © **2014 IBRO. Published by Elsevier Ltd. All rights reserved.**

Key words: adult rats, spinal cord, motoneurons, antioxidants, apoptosis.

INTRODUCTION

Although traumatic spinal cord injury (SCI) is highly relevant both clinically and epidemiologically, to date, there is no safe and effective enough treatment which is recognized in clinical practice (Rabchevsky et al., 2011; Silva et al., 2014). The previously prevalent use of high-dose glucocorticoid treatment, considered to mediate a part of its effect by acting as an antioxidant (Jia et al., 2012), has fallen out of favor due to lack of a convincing clinical effect coupled with suspected increased morbidity and mortality, and is no longer commonly approved (Bydon et al., 2013; Sayer et al., 2006; Short et al., 2000).

The inevitable trauma-induced loss of axonal continuity, although debilitating, only accounts for a part of the neurological deficits experienced, and a complete transection of the spinal cord is uncommon since the initial trauma, usually leaves parts of the white matter intact. It is rather the secondary reaction, commencing only minutes after the initial trauma that causes a progressive degradation of the spinal cord (Hall and Springer, 2004). This much studied but poorly understood process is hallmarked by death of neurons and glial cells (Zhang et al., 2012), mitochondrial dysfunction and production of reactive oxygen species (ROS) (Jia et al., 2012; Maragos and Korde, 2004), and a neuroinflammatory response involving, among other cells, the activation of microglia (Donnelly and Popovich, 2008). The events of the secondary reaction lead to the formation of a cavity at the site of the trauma with an eventual astroglial scar formation, preventing the regeneration of axons through the lesion site (Karimi-Abdolrezaee and Billakanti, 2012).

Two substances that have lately emerged as potential neuroprotectants and modulators of the neuroinflammatory response are the antioxidants acetyl-L-carnitine (ALC) and *N*-acetyl-cysteine (NAC). Both readily cross the blood–brain barrier (Farr et al., 2003; Parnetti et al., 1992) and both have been successfully used in clinical practice (Malaguarnera, 2012; Santos et al., 2011; Waring, 2012). ALC is a small peptide found in the mitochondria, containing a carnitine moiety and an acetyl moiety. The former plays an important role in the oxidation of fatty acids, while the latter contributes to the

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Abbreviations: 5HT, serotonin; ALC, acetyl-L-carnitine; BAX, BCL-2-associated X protein; BCL-2, B-cell lymphoma 2; C3bi, complement receptor type 3; DAPI, 4',6-diamidino-2-phenylindole; DPX, dibutyl phathalate xylene; DRG, dorsal root ganglia; ED1, ectodermal dysplasia 1; EGTA, ethylene glycol tetraacetic acid; NAC, *N*-acetyl-cysteine; PIPES, piperazine-1,4-bis-2-ethanesulfonic acid; ROS, reactive oxygen species; SCI, spinal cord injury.

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maintenance of acetyl-CoA levels and production of the anti-oxidant glutathione. ALC has been successfully used in clinical trials for the treatment of neuropathy and neuropathic pain, cognitive disorders and depression (Malaguarnera, 2012). It has also recently been found to contribute to the maintenance of mitochondrial bioenergetics following SCI in rats (Patel et al., 2010). NAC, a derivative of cysteine, similarly exerts a wide range of cellular actions, among others acting as a glutathione precursor (Atkuri et al., 2007) and exhibiting anti-inflammatory actions (Palacio et al., 2011). NAC has been approved for clinical use for many years, being used as a mucolytic agent and for treatment of acetaminophen intoxication (Heard, 2008; Sadowska, 2012; Ziment, 1988).

We have previously demonstrated that treatment with NAC and ALC can delay the degeneration of sensory neurons in the dorsal root ganglia (DRG) after peripheral nerve injury, and greatly reduce early retrograde death of spinal motorneurons after ventral root avulsion (Hart et al., 2002; Welin et al., 2009; Wilson et al., 2007; Zhang et al., 2005). In our recent short-term study we have shown that NAC and ALC also attenuate degeneration of spinal motoneurons after lumbar SCI, reduce the activity of microglia and macrophages and promote axonal sprouting in the injured segment of the cord (Karalija et al., 2012).

The primary goal of this study is to investigate the long-term effects of NAC and ALC treatment on the survival of descending rubrospinal neurons after cervical SCI and to evaluate the efficacy of antioxidant treatment on apoptotic, inflammatory and regenerative reactions in the trauma zone.

EXPERIMENTAL PROCEDURES

Experimental animals

The experiments were performed on adult (10–12 weeks, $n = 60$) female Sprague–Dawley rats (Taconic Europe A/S, Denmark). The animal care and experimental procedures were carried out in accordance with Directive 2010/63/EU of the European Parliament and of the Council on the protection of animals used for scientific purposes and also approved by the Northern Swedish Committee for Ethics in Animal Experiments (No. A36-12). All surgical procedures were performed under general anesthesia using a mixture of ketamine (Ketalar®, Parke-Davis, Pfizer, New York, NY, USA; 100 mg/kg i.v.) and xylazine (Rompun®, Bayer, Leverkusen, Germany; 10 mg/kg i.v.). After surgery, the rats were given the analgesic Finadyne (Schering-Plough, Denmark; 2.5 mg/kg, s.c.), normal saline (4 ml, s.c.) and benzylpenicillin (Boehringer Ingelheim, Ingelheim am Rhein, Germany; 60 mg, i.m.). Each animal was housed alone in a cage after surgery and exposed to 12-h light/dark cycles, with free access to food and water.

SCI

Following cervical laminectomy a stab wound was inflicted by inserting a 23-G needle in the dorsal root entry zone of the C3 spinal cervical segment. The blade of a pair of Vannas spring scissors was then introduced in the perforation and, using the other blade of the scissors, a hemisection of the spinal cord was performed. In the experiments dealing with neuronal survival, rubrospinal neurons were labeled with the non-toxic fluorescent retrograde tracer Fast Blue immediately after SCI. A small pellet prepared from 1 to 2 μl of a 2% aqueous solution of the Fast Blue (FB, EMS-Chemie GmbH, Germany) was placed into the lesion cavity. The opened dura mater was covered with a piece of stretched parafilm and Spongostan®. The operation was finished with the closing of the muscles and skin respectively. After the operation, the animals were randomized into one of the following three groups: (i) spinal cord injury without treatment (SCI, $n = 18$), (ii) SCI and treatment with NAC ($n = 13$) and (iii) SCI and treatment with ALC ($n = 13$). Eight uninjured normal rats (used for Western blotting and immunohistochemistry) as well as eight rats at 1 week after Fast Blue labeling served as baseline controls.

Treatment with NAC and ALC

The treatment with NAC and ALC commenced immediately after performing the SCI by the subcutaneous implantation of an Alzet 2002 osmotic minipump (Alza Corp., Palo Alto, CA, USA) filled with a solution of either the L-stereoisomer of NAC (200 mg/ml; BioPhausia, Stockholm, Sweden) or O-acetyl-L-carnitine hydrochloride (75 mg/ml in normal saline; Sigma–Aldrich, St. Louis, MO, USA) in the neck region. Following L6 laminectomy, a subcutaneous polyethylene catheter (Intermedic, Barcelona, Spain, PE-60) filled with NAC or ALC was inserted into the lower lumbar subarachnoid space with the tip reaching the level of L3–L4 DRG. The tube was fixed to the S1 vertebral bone by Histoacryl® glue, the insertion site covered with Spongostan® and the catheter secured in place by suturing it to the muscles. The proximal part of the catheter was connected to the osmotic pump, commencing the treatment. The infusion speed corresponded to 2.4 mg/day for NAC and 0.9 mg/day for ALC, doses derived from previous studies (Karalija et al., 2012). Following 14 days of treatment the empty pump was exchanged for a fresh one containing the same solution. The change of pump was performed every 14 days until the end of the study. The animals were sacrificed at 2 and 8 weeks postoperatively.

Tissue processing

Following the experiments all animals were euthanized by administering an intraperitoneal overdose of pentobarbital (240 mg/kg, Apoteksbolaget, Sverige). The animals intended for Western blotting underwent harvest of the tissue caudal and rostral to the injury site 2 weeks after initial trauma. The tissue was divided in two halves in the sagittal plane and these two pieces were then divided transversely, separating the tissue rostral to the injury site from the caudal part. The tissue was immediately frozen in liquid nitrogen. The rest of the animals were transcardially perfused using Tyrode's