Published in final edited form as:
J Neurochem. 2010 July ; 114(1): 291–301. doi:10.1111/j.1471-4159.2010.06764.x.

ACETYL-L-CARNITINE AMELIORATES MITOCHONDRIAL DYSFUNCTION FOLLOWING CONTUSION SPINAL CORD INJURY

Samir P. Patel[1,2], **Patrick G. Sullivan**[1,3], **Travis S. Lyttle**[1,2], and **Alexander G. Rabchevsky**[1,2]

[1]Spinal Cord and Brain Injury Research Center, University of Kentucky, Lexington, KY 40536-0509

[2]Department of Physiology, University of Kentucky, Lexington, KY 40536-0509

[3]Department of Anatomy & Neurobiology, University of Kentucky, Lexington, KY 40536-0509

Abstract

In the present study, we evaluated the therapeutic efficacy of acetyl-l-carnitine (ALC) administration on mitochondrial dysfunction following T10 contusion spinal cord injury (SCI) in rats. Initial results from experiments *in vitro* with naïve mitochondria showed that, in the absence of pyruvate, ALC can be used as an alternative substrate for mitochondrial respiration. Additionally, when added *in vitro* to mitochondria isolated from 24 hr injured cords, ALC restored respiration rates to normal levels. For administration studies *in vivo*, injured rats were given i.p. injections of saline (vehicle) or ALC (300 mg/kg) at 15, 30 or 60 min post-injury, followed by one booster after 6 hrs. Mitochondria were isolated 24 hr post-injury and assessed for respiration rates, activities of NADH dehydrogenase, cytochrome C oxidase and pyruvate dehydrogenase. SCI significantly ($p<0.05$) decreased respiration rates and activities of all enzyme complexes, but ALC treatment significantly ($p<0.05$) maintained mitochondrial respiration and enzyme activities compared to vehicle treatment. Critically, ALC administration *in vivo* at 15 min and 6 hr post-injury versus vehicle, followed once daily for 7 days, significantly ($p<0.05$) spared gray matter. In summary, ALC treatment maintains mitochondrial bioenergetics following contusion SCI and, thus, holds great potential as a neuroprotective therapy for acute SCI.

Keywords

neuroprotection; mitochondrial bioenergetics; NADH dehydrogenase; cytochrome C oxidase; pyruvate dehydrogenase

INTRODUCTION

Approximately 255,702 Americans are living with the typically devastating neurological deficits that are secondary to spinal cord injury (SCI) (see http://www.spinalcord.uab.edu). Most injuries do not involve actual physical transection of the spinal cord, but rather blunt trauma due to contusion, compression or stretch injuries (see reviews; Hall & Springer 2004, Rabchevsky & Smith 2001). Much of the spinal tissue degeneration that occurs following these types of SCI is due to secondary injury processes that are triggered by the primary mechanical trauma (see reviews; Hall & Springer 2004, Rabchevsky & Smith 2001).

Address correspondence to: Alexander G. Rabchevsky, Ph.D., Spinal Cord & Brain Injury Research Center (SCoBIRC), B471, Biomedical & Biological Sciences Research Building, 741 South Limestone Street, Lexington, KY 40536-0509, Fax: (859) 257-5737, agrab@uky.edu.

Mitochondria serve as the powerhouse of the cell by maintaining ratios of ATP:ADP that thermodynamically favor the hydrolysis of ATP to ADP + Pi; yet a byproduct of this process is the generation of reactive oxygen species (ROS). In fact, mitochondria have been shown to play a key role in the ensuing neuronal death cascade (Stout *et al.* 1998), and mitochondrial dysfunction and oxidative stress have been directly linked to increased excitotoxicity following SCI (Luo *et al.* 2004, McEwen *et al.* 2007, Sullivan *et al.* 2005). Accordingly, it is our working hypothesis that maintaining mitochondrial homeostasis and bioenergetics is crucial to promoting cell survival following SCI. This is based on our documentation of the progressive nature of mitochondrial dysfunction over 24 hrs following contusion SCI (Sullivan *et al.* 2007), and that pharmacological interventions which mitigate impaired mitochondrial bioenergetics lead to significantly reduced ROS production and promote neuroprotection (Patel *et al.* 2009).

Acetyl-L-carnitine (ALC) is a constituent of the inner mitochondrial membrane that contains acetyl and carnitine moieties, and is an ester of the trimethylated amino acid, L-carnitine that is synthesized in the human brain, liver, and kidneys by the enzyme ALC transferase (1999). Studies have shown that ALC readily crosses the blood-brain barrier and undergoes limited metabolism and is subsequently excreted in the urine via renal tubular reabsorption (Marcus & Coulston 1996, Parnetti *et al.* 1992). Due to multiple effects of ALC, it is used clinically for age-related neurodegenerative conditions such as Alzheimer's dementia, memory-related problems, depression, age-related disorders, diabetic neuropathy/cataracts, and in cerebral ischemia and reperfusion (Bonavita 1986, Lowitt *et al.* 1995, Onofrj *et al.* 1995, Rai *et al.* 1990, Sano *et al.* 1992, Spagnoli *et al.* 1991, Swamy-Mruthinti & Carter 1999, Tempesta *et al.* 1987). ALC has many neuromodulatory and neurotrophic actions, which include facilitating the uptake of acetyl CoA into the mitochondria during fatty acid oxidation, enhancing acetylcholine production, and stimulating protein and phospholipid synthesis required for membrane formation and integrity (see review; Pettegrew *et al.* 2000)).

Studies in rats have shown that chronic ALC treatment increases life-span, improves cognitive behavior in aged animals and improves long-term memory performance; one of the suggested mechanism(s) of action of ALC is by improving mitochondrial bioenergetics which allows neurons to produce ATP necessary to maintain normal membrane potential (Barnes *et al.* 1990, Carta & Calvani 1991, Ghirardi *et al.* 1989, Markowska *et al.* 1990, McDaniel *et al.* 2003). In addition, ALC also reported to play a role in partial prevention of overoxidation and/or accumulation of the overoxidized form of specific liver mitochondrial enzymes from aged rats; however the mechanism is still uncertain (Musicco *et al.* 2009). Treatment with ALC has also shown beneficial therapeutic effects for a variety of chronic neurological diseases (Calabrese *et al.* 2005, Chiechio *et al.* 2002, Pettegrew et al. 2000, Puca *et al.* 1990, Sima *et al.* 1996, Tomassini *et al.* 2004, Traina *et al.* 2006). Moreover, because endogenous ALC contributes to the bioenergetic processes, it plays a pivotal role in diseases correlated with metabolic compromise, such as mitochondrial-related disorders (Dhitavat *et al.* 2002, Di Cesare Mannelli *et al.* 2008, Di Cesare Mannelli *et al.* 2007, Pettegrew et al. 2000, Virmani & Binienda 2004). In addition to acting as an acetyl-CoA precursor, carbon from the acetyl group of ALC is also used to produce the antioxidant glutathione (GSH); thereby reducing oxidative damage and protecting cells against lipid peroxidation (Aureli *et al.* 1999). Reports have also shown that ALC regulates sphingomyelin levels and provides the essential substrate pools for mitochondrial energy production, thus stabilizing cell membrane fluidity and preventing excessive neuronal cell death in aging humans (1999).

In the present study, we specifically targeted mitochondrial dysfunction following contusion SCI by administering ALC *in vivo* in order to evaluate its neuroprotective efficacy. Our hypothesis was that providing such a compound which serves as a biofuel for mitochondria

as well as promotes antioxidant systems will preserve their bioenergetics and foster neuroprotection following SCI. To determine the effects of ALC administration at various time points post-injury, we assessed total mitochondrial bioenergetics (mix of synaptic and non-synaptic populations) in terms of respiratory control ratio (RCR), respiration rates, and activities of key mitochondrial enzyme complexes from acutely injured spinal cords, with and without ALC treatment at 24 hrs post-injury. Moreover, we tested whether prolonged, daily ALC treatment increased spinal cord tissue sparing at 1 week post-injury.

MATERIALS AND METHODS

Spinal Cord Injury and Treatments

Female Sprague-Dawley rats (n=132) (Harlan Labs, IN) weighing 200–250g were housed in the animal facility, Biomedical and Biological Sciences Research Building, University of Kentucky and allowed ad libitum access to water and food. All animal housing conditions, surgical procedures, and postoperative care techniques were conducted according to the University of Kentucky Institutional Animal Care and Use Committee and the National Institutes of Health animal care guidelines. Prior to surgeries, rats were anesthetized with ketamine (80 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.) and hair on dorsal surface was shaven and skin disinfected. A dorsal laminectomy was performed at the tenth thoracic level (T10) to expose the spinal cord. Contusions (200 kdyn) were performed with the Infinite Horizon impactor (PSI, LLC, Lexington, KY), as previously described (Patel et al. 2009, Sullivan et al. 2007). Injured rats demonstrated total hind limb paralysis 24 hrs following contusion injuries. Sham rats received a dorsal laminectomy only and had normal hind limb locomotion post-surgery. Injured rats were treated with either vehicle (saline) or 300 mg/kg acetyl-L-carnitine (ALC, Sigma, St Louis, MO) (i.p.), at 15 min, 30 min or 1 hr after contusion SCI. A booster injection of ALC was given at 6 hr post-SCI.

In preliminary experiments for *in vivo* studies, we conducted a ALC dose-response study using 100, 200 or 300 mg/kg body weight bolus injections based on previously published reports (Paradies *et al.* 1999, Petruzzella *et al.* 1992). We found marginally improved mitochondrial bioenergetics with 100 mg/kg and significantly improved bioenergetics with both 200 and 300 mg/kg dosages. Since the higher dosages had almost identical beneficial effects on mitochondrial bioenergetics 24 hrs following SCI, we continued employing the highest dosage to get maximum bioenergetic effects without producing overt negative side effects. Moreover, this dosage (300 mg/kg, i.p.) has been reported to improve the bioenergetics of cerebral and cardiac mitochondria isolated from aged compared to young rats (Paradies et al. 1999, Petruzzella et al. 1992). Importantly, these rats were euthanized 3 hrs after ALC treatment, demonstrating a rapid beneficial effect of this dosage.

For the tissue sparing experiments, injured rats (n=6 per group) received i.p. injection of vehicle or 300 mg/kg ALC 15 min post-SCI and a booster after 6 hrs. In addition, rats received i.p. injection of either vehicle or 300 mg/kg ALC once daily up to 7 days post-SCI. At 24 hr after the last injection of vehicle or ALC, the rats were euthanatized and perfused to isolate spinal cord tissue for histology.

Mitochondrial Preparation

At 24 hrs following sham operation or injury with drug treatment, rats were euthanatized with CO_2 and decapitated for isolation and characterization of mitochondria, as described previously (Jin *et al.* 2004, Sullivan et al. 2007). The spinal cords were rapidly removed and placed on an ice cold dissecting plate containing isolation buffer with 1mM EGTA (215mM mannitol, 75mM sucrose, 0.1% BSA, 20mM HEPES, 1mM EGTA and pH is adjusted to 7.2 with KOH). Based on pilot studies, mitochondria from two spinal cords were pooled in 350

µl of isolation buffer containing EGTA to augment protein concentration for reliable mitochondrial respiration during experiments. Each spinal cord was dissected into 2 cm segments centered on the injury site (within the sight of laminectomy) and homogenized in 2 ml of ice cold isolation buffer with EGTA. The homogenate was then centrifuged twice at 1300 × G for 3 min @ 4°C and the resulting supernatant was removed and centrifuged at 13,000 × G for 10 min @ 4°C. The resuspended mitochondrial/synaptosomal pellets were burst in a nitrogen cell disruption chamber (1200 psi, 10 min) that was cooled to 4°C. The resulting crude fractions were then placed atop a discontinuous Ficoll gradient (7.5%/10%) and centrifuged at 100,000 × G for 30 min @ 4°C. The sedimented mitochondrial pellet was resuspended in isolation buffer without EGTA and centrifuged for 10 min at 10,000 × G. The final mitochondrial pellet was resuspended in EGTA-free isolation buffer at a concentration of ~10 mg/ml and stored on ice until further use. The protein concentration was determined using the BCA protein assay kit by measuring absorbance at 560 nm with a Biotek Synergy HT plate reader (Winooski, Vermont).

Assessment of ALC Effects *in vitro*

The mitochondria were isolated 24 hr after either sham operation or contusion injury. To determine whether ALC can be used as alternative substrate for mitochondrial respiration (ATP production), 5 mM ALC (final concentration) was added, in the absence of pyruvate, to the respiration chamber containing naïve spinal cord mitochondria and respiration buffer containing 2.5 mM malate. Subsequently, other substrates and specific complex inhibitors were added, as described below. On the contrary, to assess the effects of ALC on either sham or injured spinal cord mitochondria *in vitro*, 5 mM ALC (final concentration) was added to the respiration chamber immediately after pyruvate + malate; thereafter, mitochondrial respiration was carried out as described above. The mitochondrial respiration rates were calculated as nmols oxygen/min/mg protein. For these *in vitro* studies, we first characterized naïve spinal cord mitochondria and found that dosages below 5 mM ALC were ineffective for mitochondrial respiration whereas dosages above 5 mM ALC did not have added beneficial effects. Therefore, we conducted all subsequent *in vitro* studies with mitochondria isolated from sham and injured spinal cords using a final dosage of 5 mM ALC.

Measurement of Mitochondrial Function

Mitochondrial respiration was assessed using a miniature Clark-type electrode (Hansatech Instruments, Norfolk, England) in a sealed, thermostatically controlled chamber at 37°C as described previously (Patel et al. 2009, Sullivan *et al*. 2003). Mitochondria were added to the chamber to yield a final protein concentration of ~200–300 µg/mL respiration buffer (125 mM KCl, 2 mM $MgCl_2$, 2.5 mM KH_2PO_4, 20 mM HEPES and 0.1% BSA, pH 7.2). Respiration was initiated by the addition of oxidative substrates pyruvate (5 mM) and malate (2.5 mM) which is designated as State II respiration. This was followed by the addition of 120 nmol ADP (State III respiration) and the addition of 1 µM oligomycin to induce State IV respiration. The mitochondrial uncoupler carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP; 1 µM) was added to the chamber to asses NADH dehydrogenase (complex I) driven maximum electron transport, which is designated as State V-Complex I respiration. The complex I inhibitor rotenone (0.8 µM) was then added to the chamber followed by the addition of 10 mM succinate to allow succinate dehydrogenase (complex II) driven maximum electron transport, which is designated as State V-Complex II respiration. The mitochondrial respiration rates were calculated as nmols oxygen/min/mg protein. As detailed previously (Patel et al. 2009, Sullivan et al. 2003), the respiratory control ratio (RCR) was calculated by dividing the slope of the response of isolated mitochondria to State III respiration (presence of ADP) by the slope of the response to State IV respiration (presence of 1 µM oligomycin and absence of ADP).

Activities of Mitochondrial Complexes

The NADH dehydrogenase (complex I) and cytochrome C oxidase (complex IV) assays were performed as described with modifications (Patel et al. 2009, Smith 1955). Measurement of total pyruvate dehydrogenase complex (PDHC) activity was carried out as described with modification (Opii *et al.* 2007, Starkov *et al.* 2004). In summary, mitochondria were freeze thawed and briefly sonicated for three cycles in 10 mM phosphate buffer (pH 7.4). Six μg of isolated mitochondrial protein was added into the buffer containing a final concentration of 50 mM KCl, 10 mM HEPES, pH 7.4, 0.3 mM thiamine pyrophosphate (TPP), 10 μM CaCl2, 0.2 mM MgCl2, 5 mM pyruvate, 1 μM rotenone and 0.2 mM NAD^+. Reaction was started by addition of 0.14 mM coenzyme A (CoASH) and the assay was performed at Ex λ 340 nm/Em λ 460 nm. Increases in NADH fluorescence were observed at 1-min intervals at 30°C using BioTek Synergy HT plate reader (Vinooski, VT). The total PDHC activity units were expressed as nmol/min/mg protein.

Spinal Cord Tissue Processing for Histology

Rats were overdosed with sodium pentobarbital and transcardially perfused with 0.1 M phosphate-buffered saline (PBS, pH 7.4) followed by 4% paraformaldehyde in PBS. To maintain consistent sampling, each spinal cord was transected at the rostral T6 spinal root and a 30 mm segment of spinal cord (~L1) was immediately dissected and post fixed for 2 hr at 4°C before cryoprotecting in 20% sucrose/PBS at 4°C and embedding as described (Rabchevsky & Smith 2001). Frozen spinal cords were then serially cryosectioned at 20 μm and processed for stereological assessment of tissue sparing after staining with eriochrome cyanine (EC, Sigma, St Louis, MO) for myelin followed by cresyl violet (CV, Sigma, St Louis, MO) for cell bodies before rinsing (Rabchevsky *et al.* 2001). Stained sections were maintained in Citrisolv (Fisher Scientific) to clear excess cresyl violet and subsequently coverslipped with Permount (Fisher Scientific) mounting medium.

Quantification of Lesion Volumes and Tissue Sparing

Quantitative image analysis was performed on each series of EC/CV-stained cross sections using Scion Imaging software (Scion Corporation, Frederick, MD) on a Nikon Eclipse 400 microscope mounted with a DAGE-MTI CCD-100 camera (Michigan City, IN). Histological analyses were assessed blindly with respect to treatment. Spared tissue was based on positive staining for myelin or if the gray matter cytoarchitecture approximated that seen in uninjured tissue. Employing the Cavalieri method (Michel & Cruz-Orive 1988), the cross sectional area containing necrotic or damaged tissue was carefully circumscribed and the entire volume of injured tissue calculated from a series of 12 evenly spaced (1 mm) sections centered on the injury site. Similarly, cross sectional area measurements of spared gray matter and white matter were each quantified separately to calculate their respective volumes in the injured spinal cords (Rabchevsky et al. 2001).

Representative sections were photographed with an Olympus ‘Magnafire’ digital camera mounted on an Olympus BX51 microscope (Olympus Corp. Melville, NY). All sections were photographed with the same objective magnification (x4; x10 eye piece) and with the same exposure settings. Photomicrographs were optimized for final production by adjusting only the brightness and contrast using Adobe Photoshop 7.0 (Adobe Systems Inc., San Jose, CA). All graphs were created with DeltaGraph 5.4 (Red Rock Software, Inc., Salt Lake City, UT).

Statistical Analysis

For both *in vitro* and *in vivo* experiments, differences among sham, injured vehicle-treated, and injured ALC-treated groups at the various time-points of administration post-injury were

investigated using analysis of variance (ANOVA) and the Newman-Keuls post-hoc when warranted. Significance was set at $p < 0.05$ for all analyses.

RESULTS

In Vitro Studies

To investigate the potential role of ALC as an alternative substrate for mitochondrial energy production, we replaced pyruvate with ALC and measured mitochondrial respiration (oxygen consumption). As shown in Figure 1, in the presence of ALC (absence of pyruvate) mitochondrial respiration was comparable to that seen in the presence of pyruvate, illustrating that ALC can be utilized for mitochondrial ATP synthesis. Next, we designed experiments *in vitro* to assess the effects of ALC addition on respiration rates of mitochondria isolated from contused spinal cords versus shams. For these experiments, after addition of pyruvate + malate to the respiration buffer, we added 5 mM ALC (final concentration) to the respiration chamber containing either mitochondria from sham or injured spinal cords. Respiration rates were calculated in terms of nmols oxygen/min/mg protein and are shown in Figures 2. Typical respiration traces for mitochondria isolated from sham and injured spinal cords (with and without ALC addition) are shown in Figure 2A. Significantly compromised mitochondrial respiration was observed 24 hr after contusion SCI compared to shams (Figure 2B), as we previously reported (Sullivan et al. 2007). However, in the presence of ALC, State III and State V-Complex I respiration rates in mitochondria isolated from injured spinal cords increased significantly compared to respiration without ALC addition (25.21% and 25.47%, respectively). Addition of ALC to sham mitochondria resulted in no significant changes in State III or State V-Complex I respiration rates. Interestingly, compared to sham mitochondria without ALC, State III respiration rates were not significantly different from injured mitochondria with ALC addition (Figure 2B). This indicates that ALC addition to injured mitochondria *in vitro* increased respiration rates to near sham levels. However, State V-Complex I respiration rates in injured mitochondria, with or without ALC addition *in vitro*, were significantly compromised compared to shams without ALC (Figure 2B).

In Vivo Studies

In an alternative approach, we assessed the protective efficacy of ALC administration *in vivo* on mitochondrial bioenergetics following acute SCI. ALC or saline was injected (i.p.) at 15 min, 30 min or 1 hr post-injury versus sham operation. Mitochondria were isolated 24 hr after injury or sham operation and subjected to assessments of mitochondrial bioenergetics in terms of respiration rates and activities of key mitochondria enzyme complexes. Mitochondrial bioenergetics was assessed by measuring oxygen consumption of isolated mitochondria under various experimental conditions at 24 hr post-injury. Typical respiration traces for mitochondria isolated from sham rats versus treatments at 15 min post-injury with either saline (vehicle) or ALC are shown in Figure 3. Compromised mitochondrial respiratory activity was observed in vehicle-treated injured rats compared to shams (Figure 3). Treatment with ALC after 15 min (Figure 3), 30 min and 1 hr (traces not shown) significantly maintained mitochondrial respiration rates at near normal levels. The results showed that for all mitochondrial parameters examined at all 3 time points of administration, vehicle-treated injured groups were not significantly different. Data from vehicle-treated injured groups did not show significant variation over time post-injection; therefore we used two animals per injection time point to establish a single vehicle-treated injured group (Figures 4 and 5). The respiratory control ratio (RCR) indicates how well the ETS is coupled to ATP synthesis, an important measure of mitochondrial function/integrity. Compared to shams, there were significant reductions in the RCR for all vehicle-treated injured groups [$F(4,22)=21.558$, $p<0.0001$] at 15 min, 30 min and 1 hr post-injury (Figure

4A). In contrast, ALC administration at 15 min, 30 min and 1 hr post-injury significantly improved mitochondrial function compared to vehicle-treated injured group (Figure 4A), although the RCR values remained significantly lower compared to shams. No significant differences in state IV respiration were measured, indicating that the changes in RCR values observed were due entirely to alterations in state III respiration rates and not changes in proton conductance across the inner membrane (State IV respiration).

Quantification of mitochondrial respiration rates showed a significant decrease in state III [$F(4, 22)=23.704$, $p<0.0001$] and State V-Complex I [$F(4,22)=20.035$, $p<0.0001$)] respiration rates in all injured groups compared to shams (Figure 4B). Post-hoc analysis showed a significant decrease in both State III and State V-Complex I respiration in vehicle-treated injured spinal cord mitochondria compared to shams. Conversely, treatment with ALC at 15 min, 30 min and 1 hr post-injury significantly maintained State III and State V-Complex I respiration rates compared to vehicle-treated injured rats (Figure 4B). However, the values remained significantly lower than shams. State V-Complex II respiration rates were not statistically different across the groups (Figure 4B), as reported previously (Sullivan et al. 2007).

In vehicle-treated rats, direct measurement of mitochondrial ETS complex enzymatic activities at 24 hr after injury revealed significant reductions in complex I [$F(4,11)=6.737$, $p<0.005$], complex IV [$F(4,11)=9.341$, $p<0.001$] and PDHC activities [$F(4,11)=31.404$, $p<0.0001$] compared to sham rats (Figure 5). However, ALC administered 15 and 30 min after injury significantly maintained complex I, IV and total PDHC activities compared to vehicle-treated injured rats (Figure 5).

Effect of ALC Treatment on Spinal Cord Tissue Sparing

Qualitative evaluation of the histopathology seen throughout spinal cord cross sections from ALC- versus vehicle-treated injured rats (Figure 6) demonstrated more sparing of tissue with ALC treatment, notably the gray matter both rostral and caudal of the injury site at one week post-SCI. Compared to the vehicle-treated injured group, daily ALC administration significantly increased tissue sparing 7 days post-injury (Figure 7). Results showed significantly greater lesion volumes in the vehicle-treated group compared to the ALC-treated group (Figure 7 A). Moreover, ALC treatment resulted in significantly increased gray matter sparing, along with marginally increased white matter sparing (Figure 7 B). Additionally, the ALC-treated group showed significant increases in tissue sparing at the injury epicenter compared to the vehicle-treated group (Figure 7 C , D).

DISCUSSION

While the present study confirms that mitochondrial dysfunction plays a key role in the development of secondary pathophysiology following contusion SCI (Patel et al. 2009, Sullivan et al. 2007), this is the first demonstration of the therapeutic efficacy of ALC treatment to stabilize mitochondrial bioenergetics and, in turn, significantly spare injured spinal cord tissue. Despite various experimental approaches to promote neuroprotection following SCI (see review; Hall & Springer 2004, Onose *et al.* 2009), the only compound reported to show modest beneficial effects following acute SCI in human clinical trials is methylprednisolone (Bracken *et al.* 1997), which is reported to act by inhibiting post-traumatic lipid peroxidation (see review; Hall & Springer 2004). The significance of the current study is that our novel therapeutic strategy targeted the main source of free radical production, the mitochondria. Based on our recent report that mild uncoupling of mitochondria after contusion SCI significantly reduced oxidative damage (Patel et al. 2009), the current findings indicate that supplementing an alternative biofuel, ALC, resulted in

improved bioenergetics and increased tissue sparing, likely due to reduced secondary oxidative damage.

Collectively, our data signify that mitochondrial dysfunction is a pivotal target for pharmacological interventions to promote neuroprotection following acute contusion SCI. Our novel findings in a SCI model are supported by previous reports that ALC treatment prevents or reduces oxidative damage, and improves mitochondrial function in different models of aging, age-related diseases and chemically-induced oxidative stress. For example, similar to the current findings, long-term ALC feeding to aged rats partially or completely ameliorates activities of complexes I, IV and V in brain mitochondria and oxidative damage to levels seen in young rats (Long *et al.* 2009). Also, pre-treatment with ALC for two weeks has demonstrated neuroprotection against 3,4-Methylenedioximethamphetamine (MDMA)-induced neurotoxicity in rat brain mitochondria by improving the expression of the electron transport system components, decreasing mtDNA deletion, and reducing carbonyl formation (Alves *et al.* 2009). ALC additionally prevents beta-amyloid (Abeta)-induced neuronal cell death by reducing ROS production and ATP depletion in differentiated SH-SY-5Y human neuroblastoma cells (Dhitavat et al. 2002). Finally, in a chronic rotenone-induced cellular model of Parkinson's disease, 4 weeks pre-treatment with ALC effectively protected SK-N-MC human neuroblastoma cells against mitochondrial dysfunction, oxidative damage (Zhang *et al.* 2008). Notably, the underlying mechanism is thought to be up-regulation of the peroxisome proliferators-activated receptor gamma co-activator 1α (PGC-1 α), which is a key regulator of mitochondrial biogenesis and respiration.

Earlier studies have reported that ALC can act as a substrate for efficient mitochondrial respiration in other tissues/organ systems (Clark & Nicklas 1970, Nicklas *et al.* 1971, Storey 1980). The results from this study show that ALC can, in fact, be used as an alternative substrate for normal respiration of naïve spinal cord mitochondria to produce energy. We subsequently found that addition of ALC *in vitro* significantly improved oxygen consumption of mitochondria isolated from acutely injured spinal cords. These data indicate that mitochondria in the injured spinal cord can successfully utilize an alternative substrate/biofuel such as ALC to maintain ATP levels following SCI. Remarkably, the administration of ALC *in vivo* up to 1 hr post-SCI also significantly maintained mitochondrial bioenergetics at 24 hrs post-injury. Moreover, prolonged ALC treatment *in vivo* (7 days) significantly increased spinal cord tissue sparing following contusion SCI, supported by previous report in rats that extended ALC treatment improves mitochondrial function and reduces oxidative damage in aging (Long et al. 2009).

Consistent with our previous studies characterizing total mitochondria (combined synaptic and non-synaptic populations) (Sullivan et al. 2007), as well as synaptic and non-synaptic mitochondria from injured spinal cords (Patel et al. 2009), we found total mitochondria from vehicle-treated injured spinal cords demonstrated compromised mitochondrial bioenergetics at 24 hr post-injury. Importantly, ALC administration up to 1 hr post-injury significantly maintained mitochondrial bioenergetics in terms of respiration rates and mitochondrial enzyme activities; notably, NADH dehydrogenase (complex I), cytochrome C oxidase (complex IV) and pyruvate dehydrogenase complex (PDHC), all of which are highly susceptible to oxidative damage (Alves et al. 2009, Fiskum *et al.* 2004, Martin *et al.* 2005, Opii et al. 2007, Roy Chowdhury *et al.* 2009, Vazquez-Memije *et al.* 2008, Wei *et al.* 2009). Consistent with our previous reports, State III and State V-Complex I respiration rates and NADH dehydrogenase activity were compromised while State V-Complex II respiration rates and activity of succinate dehydrogenase were unchanged following SCI (Patel et al. 2009, Sullivan et al. 2007). This indicates, therefore, that the injury-induced loss of mitochondrial bioenergetics is most likely due to compromised NADH-linked respiration and/or complex I driven electron transport. However, this finding coupled with our data

demonstrating that ALC can restore state III respiration in mitochondria from the injured spinal cord seems to indicate a pivotal role for PDHC dysfunction in SCI pathophysiology. The same enzymatic machinery is used by pyruvate and ALC to produce reducing equivalents for complex I driven electron transport, with the notable exception of PDHC. Our finding that *in vivo* administration of ALC ameliorates SCI-induced reductions in mitochondrial enzyme activities is supported by a previous report that ALC increases endogenous antioxidant GSH levels, thereby protecting key mitochondrial enzymes (complex I, IV and PDHC) from oxidative damage (Aureli et al. 1999).

Therefore, to further establish the role of PDHC in the pathophysiology of SCI, we directly assessed its activity, along with those of Complex I and IV, all of which have been shown to be susceptible to oxidative damage. To our knowledge, this is the first study to demonstrate decreased PDHC activity in mitochondria isolated from contused spinal cords. PDHC catalyzes the conversion of pyruvate to acetyle-CoA, NADH and CO_2 (Reed 1981, Reed 2001). Acetyl CoA enters the citric acid cycle and forms NADH which are oxidized in the mitochondrial respiratory electron transfer chain and donate electrons for ATP synthesis; thus making this enzyme the key link between glycolysis and the TCA cycle (Martin et al. 2005, Zaidan *et al.* 1998). Thus, compromised PDHC activity could lead to a decline in mitochondrial respiration, notably due to reduced NADH levels. In contrast, production of acetyl-CoA is markedly increased by ALC which, in turn, increases the pool of reducing equivalent NADH (see review; Pettegrew et al. 2000). This indicates that ALC supplementation can specifically override injury-induced dysfunction of the critical enzyme, PDHC. In fact, this is what we demonstrated in our *in vitro* studies showing that ALC addition restores state III respiration in mitochondria isolated 24 hr following SCI. Importantly, the inactivation and dysfunction of PDHC leads to metabolic failure and increased oxidative stress in several neurodegenerative disorders and CNS injury models (Bogaert *et al.* 2000, De Meirleir *et al.* 1993, Opii et al. 2007, Pocernich & Butterfield 2003, Richards *et al.* 2006, Robertson *et al.* 2007, Rosenthal & Henderson 2003, Sheu *et al.* 1985, Sorbi *et al.* 1983, Zaidan et al. 1998).

It has been reported in different injury models and abnormal conditions that oxidative stress at the cellular level results in structural modifications which, in turn, render inactivation/dysfunction of several important mitochondrial proteins (Bogaert et al. 2000, Fiskum et al. 2004, Long et al. 2009, Martin et al. 2005, Opii et al. 2007). Few studies have targeted mitochondrial dysfunction following contusion SCI using different pharmacological agents, such as mitochondrial uncouplers and/or anti-oxidants (McEwen et al. 2007, Patel et al. 2009). The current study is the first to demonstrate that the use of an alternative biofuel for mitochondria, ALC, maintains their bioenergetic homeostasis and, consequently, affords neuroprotection. Notably, intermittent, daily ALC treatments significantly increased gray matter tissue sparing and marginally spared white matter. We hypothesize that such neuroprotection directly stemmed from the maintenance of mitochondrial bioenergetics with ALC treatment, and thereby reducing oxidative damage.

In order to further elucidate the possible mechanism(s) of the beneficial effects of ALC, studies on oxidative markers [3-nitrotyrosine (3-NT), 4-hydroxynonenal (4-HNE), and protein carbonyl] must be further pursued at different time points post-injury, as we have detailed previously (Patel et al. 2009). Moreover, long-term behavioral studies and tissue sparing analyses following more prolonged, intermittent ALC treatment following SCI will provide a solid foundation to validate this neuroprotective strategy as a beneficial therapeutic for acute and, potentially, chronic SCI.

Acknowledgments

We are thankful for the expert technical assistance of Christopher R. O'Dell. Grant support: KSCHIRT #8–13 (AGR), NIH/NINDS P30 NS051220.

REFERENCES

Acetyl-L-carnitine. Altern Med Rev. 1999; 4:438–441. [PubMed: 10608918]

Alves E, Binienda Z, Carvalho F, Alves CJ, Fernandes E, de Lourdes Bastos M, Tavares MA, Summavielle T. Acetyl-L-carnitine provides effective in vivo neuroprotection over 3,4-methylenedioximethamphetamine-induced mitochondrial neurotoxicity in the adolescent rat brain. Neuroscience. 2009; 158:514–523. [PubMed: 19015003]

Aureli T, Puccetti C, Di Cocco ME, Arduini A, Ricciolini R, Scalibastri M, Manetti C, Conti F. Entry of [(1,2-13C2)acetyl]-L-carnitine in liver tricarboxylic acid cycle and lipogenesis: a study by 13C NMR spectroscopy in conscious, freely moving rats. Eur J Biochem. 1999; 263:287–293. [PubMed: 10429215]

Barnes CA, Markowska AL, Ingram DK, Kametani H, Spangler EL, Lemken VJ, Olton DS. Acetyl-1-carnitine. 2: Effects on learning and memory performance of aged rats in simple and complex mazes. Neurobiol Aging. 1990; 11:499–506. [PubMed: 2234280]

Bogaert YE, Sheu KF, Hof PR, Brown AM, Blass JP, Rosenthal RE, Fiskum G. Neuronal subclass-selective loss of pyruvate dehydrogenase immunoreactivity following canine cardiac arrest and resuscitation. Exp Neurol. 2000; 161:115–126. [PubMed: 10683278]

Bonavita E. Study of the efficacy and tolerability of L-acetylcarnitine therapy in the senile brain. Int J Clin Pharmacol Ther Toxicol. 1986; 24:511–516. [PubMed: 3781687]

Bracken MB, Shepard MJ, Holford TR, et al. Administration of methylprednisolone for 24 or 48 hours or tirilazad mesylate for 48 hours in the treatment of acute spinal cord injury. Results of the Third National Acute Spinal Cord Injury Randomized Controlled Trial. National Acute Spinal Cord Injury Study. Journal of the American Medical Association. 1997; 277:1597–1604. [PubMed: 9168289]

Calabrese V, Ravagna A, Colombrita C, Scapagnini G, Guagliano E, Calvani M, Butterfield DA, Giuffrida Stella AM. Acetylcarnitine induces heme oxygenase in rat astrocytes and protects against oxidative stress: involvement of the transcription factor Nrf2. J Neurosci Res. 2005; 79:509–521. [PubMed: 15641110]

Carta A, Calvani M. Acetyl-L-carnitine: a drug able to slow the progress of Alzheimer's disease? Ann N Y Acad Sci. 1991; 640:228–232. [PubMed: 1776743]

Chiechio S, Caricasole A, Barletta E, et al. L-Acetylcarnitine induces analgesia by selectively up-regulating mGlu2 metabotropic glutamate receptors. Mol Pharmacol. 2002; 61:989–996. [PubMed: 11961116]

Clark JB, Nicklas WJ. The metabolism of rat brain mitochondria. Preparation and characterization. J Biol Chem. 1970; 245:4724–4731. [PubMed: 4393961]

De Meirleir L, Lissens W, Denis R, Wayenberg JL, Michotte A, Brucher JM, Vamos E, Gerlo E, Liebaers I. Pyruvate dehydrogenase deficiency: clinical and biochemical diagnosis. Pediatr Neurol. 1993; 9:216–220. [PubMed: 8352855]

Dhitavat S, Ortiz D, Shea TB, Rivera ER. Acetyl-L-carnitine protects against amyloid-beta neurotoxicity: roles of oxidative buffering and ATP levels. Neurochem Res. 2002; 27:501–505. [PubMed: 12199155]

Di Cesare Mannelli L, Ghelardini C, Calvani M, Nicolai R, Mosconi L, Toscano A, Pacini A, Bartolini A. Neuroprotective effects of acetyl-L-carnitine on neuropathic pain and apoptosis: A role for the nicotinic receptor. J Neurosci Res. 2008

Di Cesare Mannelli L, Ghelardini C, Calvani M, Nicolai R, Mosconi L, Vivoli E, Pacini A, Bartolini A. Protective effect of acetyl-L-carnitine on the apoptotic pathway of peripheral neuropathy. Eur J Neurosci. 2007; 26:820–827. [PubMed: 17714181]

Fiskum G, Rosenthal RE, Vereczki V, Martin E, Hoffman GE, Chinopoulos C, Kowaltowski A. Protection against ischemic brain injury by inhibition of mitochondrial oxidative stress. J Bioenerg Biomembr. 2004; 36:347–352. [PubMed: 15377870]

Ghirardi O, Milano S, Ramacci MT, Angelucci L. Long-term acetyl-L-carnitine preserves spatial learning in the senescent rat. Prog Neuropsychopharmacol Biol Psychiatry. 1989; 13:237–245. [PubMed: 2748864]

Hall ED, Springer JE. Neuroprotection and acute spinal cord injury: a reappraisal. NeuroRx. 2004; 1:80–100. [PubMed: 15717009]

Jin Y, McEwen ML, Nottingham SA, Maragos WF, Dragicevic NB, Sullivan PG, Springer JE. The mitochondrial uncoupling agent 2,4-dinitrophenol improves mitochondrial function, attenuates oxidative damage, and increases white matter sparing in the contused spinal cord. J Neurotrauma. 2004; 21:1396–1404. [PubMed: 15672630]

Long J, Gao F, Tong L, Cotman CW, Ames BN, Liu J. Mitochondrial decay in the brains of old rats: ameliorating effect of alpha-lipoic acid and acetyl-L-carnitine. Neurochem Res. 2009; 34:755–763. [PubMed: 18846423]

Lowitt S, Malone JI, Salem AF, Korthals J, Benford S. Acetyl-L-carnitine corrects the altered peripheral nerve function of experimental diabetes. Metabolism. 1995; 44:677–680. [PubMed: 7752919]

Luo J, Borgens R, Shi R. Polyethylene glycol improves function and reduces oxidative stress in synaptosomal preparations following spinal cord injury. J Neurotrauma. 2004; 21:994–1007. [PubMed: 15318999]

Marcus, R.; Coulston, A. Water-soluble vitamins. In: Hardman, JG.; Limbird, LE., editors. The Pharmacological Basis of Therapeutics. 9th edition. New York: McGraw-Hill; 1996.

Markowska AL, Ingram DK, Barnes CA, Spangler EL, Lemken VJ, Kametani H, Yee W, Olton DS. Acetyl-1-carnitine. 1: Effects on mortality, pathology and sensory-motor performance in aging rats. Neurobiol Aging. 1990; 11:491–498. [PubMed: 2234279]

Martin E, Rosenthal RE, Fiskum G. Pyruvate dehydrogenase complex: metabolic link to ischemic brain injury and target of oxidative stress. J Neurosci Res. 2005; 79:240–247. [PubMed: 15562436]

McDaniel MA, Maier SF, Einstein GO. "Brain-specific" nutrients: a memory cure? Nutrition. 2003; 19:957–975. [PubMed: 14624946]

McEwen ML, Sullivan PG, Springer JE. Pretreatment with the cyclosporin derivative, NIM811, improves the function of synaptic mitochondria following spinal cord contusion in rats. J Neurotrauma. 2007; 24:613–624. [PubMed: 17439345]

Michel RP, Cruz-Orive LM. Application of the Cavalieri principle and vertical sections method to lung: estimation of volume and pleural surface area. J Microsc. 1988; 150:117–136. [PubMed: 3411604]

Musicco C, Capelli V, Pesce V, Timperio AM, Calvani M, Mosconi L, Zolla L, Cantatore P, Gadaleta MN. Accumulation of overoxidized Peroxiredoxin III in aged rat liver mitochondria. Biochim Biophys Acta. 2009; 1787:890–896. [PubMed: 19272351]

Nicklas WJ, Clark JB, Williamson JR. Metabolism of rat brain mitochondria. Studies on the potassium ion-stimulated oxidation of pyruvate. Biochem J. 1971; 123:83–95. [PubMed: 5128666]

Onofrj M, Fulgente T, Melchionda D, et al. L-acetylcarnitine as a new therapeutic approach for peripheral neuropathies with pain. Int J Clin Pharmacol Res. 1995; 15:9–15. [PubMed: 7490173]

Onose G, Anghelescu A, Muresanu DF, et al. A review of published reports on neuroprotection in spinal cord injury. Spinal Cord. 2009; 47:716–726. [PubMed: 19597522]

Opii WO, Nukala VN, Sultana R, Pandya JD, Day KM, Merchant ML, Klein JB, Sullivan PG, Butterfield DA. Proteomic identification of oxidized mitochondrial proteins following experimental traumatic brain injury. J Neurotrauma. 2007; 24:772–789. [PubMed: 17518533]

Paradies G, Petrosillo G, Gadaleta MN, Ruggiero FM. The effect of aging and acetyl-L-carnitine on the pyruvate transport and oxidation in rat heart mitochondria. FEBS Lett. 1999; 454:207–209. [PubMed: 10431808]

Parnetti L, Gaiti A, Mecocci P, Cadini D, Senin U. Pharmacokinetics of IV and oral acetyl-L-carnitine in a multiple dose regimen in patients with senile dementia of Alzheimer type. Eur J Clin Pharmacol. 1992; 42:89–93. [PubMed: 1541322]

Patel SP, Sullivan PG, Pandya JD, Rabchevsky AG. Differential effects of the mitochondrial uncoupling agent, 2,4-dinitrophenol, or the nitroxide antioxidant, Tempol, on synaptic or

nonsynaptic mitochondria after spinal cord injury. J Neurosci Res. 2009; 87:130–140. [PubMed: 18709657]

Petruzzella V, Baggetto LG, Penin F, Cafagna F, Ruggiero FM, Cantatore P, Gadaleta MN. In vivo effect of acetyl-L-carnitine on succinate oxidation, adenine nucleotide pool and lipid composition of synaptic and non-synaptic mitochondria from cerebral hemispheres of senescent rats. Arch Gerontol Geriatr. 1992; 14:131–144. [PubMed: 15374398]

Pettegrew JW, Levine J, McClure RJ. Acetyl-L-carnitine physical-chemical, metabolic, and therapeutic properties: relevance for its mode of action in Alzheimer's disease and geriatric depression. Mol Psychiatry. 2000; 5:616–632. [PubMed: 11126392]

Pocernich CB, Butterfield DA. Acrolein inhibits NADH-linked mitochondrial enzyme activity: implications for Alzheimer's disease. Neurotox Res. 2003; 5:515–520. [PubMed: 14715435]

Puca FM, Genco S, Specchio LM, et al. Clinical pharmacodynamics of acetyl-L-carnitine in patients with Parkinson's disease. Int J Clin Pharmacol Res. 1990; 10:139–143. [PubMed: 2387661]

Rabchevsky AG, Fugaccia I, Sullivan PG, Scheff SW. Cyclosporin A treatment following spinal cord injury to the rat: behavioral effects and stereological assessment of tissue sparing. J Neurotrauma. 2001; 18:513–522. [PubMed: 11393254]

Rabchevsky AG, Smith GM. Therapeutic interventions following mammalian spinal cord injury. Arch Neurol. 2001; 58:721–726. [PubMed: 11346366]

Rai G, Wright G, Scott L, Beston B, Rest J, Exton-Smith AN. Double-blind, placebo controlled study of acetyl-l-carnitine in patients with Alzheimer's dementia. Curr Med Res Opin. 1990; 11:638–647. [PubMed: 2178869]

Reed LJ. Regulation of mammalian pyruvate dehydrogenase complex by a phosphorylation-dephosphorylation cycle. Curr Top Cell Regul. 1981; 18:95–106. [PubMed: 7273851]

Reed LJ. A trail of research from lipoic acid to alpha-keto acid dehydrogenase complexes. J Biol Chem. 2001; 276:38329–38336. [PubMed: 11477096]

Richards EM, Rosenthal RE, Kristian T, Fiskum G. Postischemic hyperoxia reduces hippocampal pyruvate dehydrogenase activity. Free Radic Biol Med. 2006; 40:1960–1970. [PubMed: 16716897]

Robertson CL, Saraswati M, Fiskum G. Mitochondrial dysfunction early after traumatic brain injury in immature rats. J Neurochem. 2007; 101:1248–1257. [PubMed: 17403141]

Rosenthal PB, Henderson R. Optimal determination of particle orientation, absolute hand, and contrast loss in single-particle electron cryomicroscopy. J Mol Biol. 2003; 333:721–745. [PubMed: 14568533]

Roy Chowdhury SK, Sangle GV, Xie X, Stelmack GL, Halayko AJ, Shen GX. Effects of extensively oxidized low-density lipoprotein on mitochondrial function and reactive oxygen species in porcine aortic endothelial cells. Am J Physiol Endocrinol Metab. 2009

Sano M, Bell K, Cote L, et al. Double-blind parallel design pilot study of acetyl levocarnitine in patients with Alzheimer's disease. Arch Neurol. 1992; 49:1137–1141. [PubMed: 1444880]

Sheu KF, Kim YT, Blass JP, Weksler ME. An immunochemical study of the pyruvate dehydrogenase deficit in Alzheimer's disease brain. Ann Neurol. 1985; 17:444–449. [PubMed: 4004169]

Sima AA, Ristic H, Merry A, Kamijo M, Lattimer SA, Stevens MJ, Greene DA. Primary preventive and secondary interventionary effects of acetyl-L-carnitine on diabetic neuropathy in the bio-breeding Worcester rat. J Clin Invest. 1996; 97:1900–1907. [PubMed: 8621774]

Smith L. Spectrophotometric assay of cytochrome c oxidase. Methods Biochem Anal. 1955; 2:427–434. [PubMed: 14393574]

Sorbi S, Bird ED, Blass JP. Decreased pyruvate dehydrogenase complex activity in Huntington and Alzheimer brain. Ann Neurol. 1983; 13:72–78. [PubMed: 6219611]

Spagnoli A, Lucca U, Menasce G, et al. Long-term acetyl-L-carnitine treatment in Alzheimer's disease. Neurology. 1991; 41:1726–1732. [PubMed: 1944900]

Starkov AA, Fiskum G, Chinopoulos C, Lorenzo BJ, Browne SE, Patel MS, Beal MF. Mitochondrial alpha-ketoglutarate dehydrogenase complex generates reactive oxygen species. J Neurosci. 2004; 24:7779–7788. [PubMed: 15356189]

Storey BT. Strategy of oxidative metabolism in bull spermatozoa. J Exp Zool. 1980; 212:61–67. [PubMed: 7411077]

Stout AK, Raphael HM, Kanterewicz BI, Klann E, Reynolds IJ. Glutamate-induced neuron death requires mitochondrial calcium uptake. Nat Neurosci. 1998; 1:366–373. [PubMed: 10196525]

Sullivan PG, Dube C, Dorenbos K, Steward O, Baram TZ. Mitochondrial uncoupling protein-2 protects the immature brain from excitotoxic neuronal death. Ann Neurol. 2003; 53:711–717. [PubMed: 12783416]

Sullivan PG, Krishnamurthy S, Patel SP, Pandya JD, Rabchevsky AG. Temporal characterization of mitochondrial bioenergetics after spinal cord injury. J Neurotrauma. 2007; 24:991–999. [PubMed: 17600515]

Sullivan PG, Rabchevsky AG, Waldmeier PC, Springer JE. Mitochondrial permeability transition in CNS trauma: cause or effect of neuronal cell death? J Neurosci Res. 2005; 79:231–239. [PubMed: 15573402]

Swamy-Mruthinti S, Carter AL. Acetyl- L -carnitine decreases glycation of lens proteins: in vitro studies. Exp Eye Res. 1999; 69:109–115. [PubMed: 10375455]

Tempesta E, Casella L, Pirrongelli C, Janiri L, Calvani M, Ancona L. L-acetylcarnitine in depressed elderly subjects. A cross-over study vs placebo. Drugs Exp Clin Res. 1987; 13:417–423. [PubMed: 3308388]

Tomassini V, Pozzilli C, Onesti E, Pasqualetti P, Marinelli F, Pisani A, Fieschi C. Comparison of the effects of acetyl L-carnitine and amantadine for the treatment of fatigue in multiple sclerosis: results of a pilot, randomised, double-blind, crossover trial. J Neurol Sci. 2004; 218:103–108. [PubMed: 14759641]

Traina G, Bernardi R, Rizzo M, Calvani M, Durante M, Brunelli M. Acetyl-L-carnitine up-regulates expression of voltage-dependent anion channel in the rat brain. Neurochem Int. 2006; 48:673–678. [PubMed: 16527372]

Vazquez-Memije ME, Capin R, Tolosa A, El-Hafidi M. Analysis of age-associated changes in mitochondrial free radical generation by rat testis. Mol Cell Biochem. 2008; 307:23–30. [PubMed: 17805943]

Virmani A, Binienda Z. Role of carnitine esters in brain neuropathology. Mol Aspects Med. 2004; 25:533–549. [PubMed: 15363640]

Wei YH, Wu SB, Ma YS, Lee HC. Respiratory function decline and DNA mutation in mitochondria, oxidative stress and altered gene expression during aging. Chang Gung Med J. 2009; 32:113–132. [PubMed: 19403001]

Zaidan E, Sheu KF, Sims NR. The pyruvate dehydrogenase complex is partially inactivated during early recirculation following short-term forebrain ischemia in rats. J Neurochem. 1998; 70:233–241. [PubMed: 9422367]

Zhang H, Jia H, Liu J, Ao N, Yan B, Shen W, Wang X, Li X, Luo C. Combined R-alpha-lipoic acid and acetyl-L-carnitine exerts efficient preventative effects in a cellular model of Parkinson's disease. J Cell Mol Med. 2008

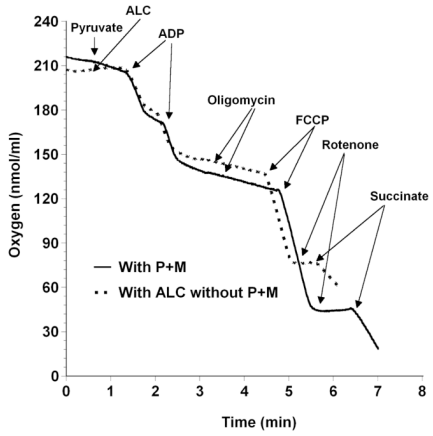

Figure 1.

ALC can be used as an alternative substrate for mitochondrial respiration. The solid line represents the oxygen consumption trace for isolated naïve rat spinal cord mitochondria using pyruvate alone. Dotted line represents the oxygen consumption trace for isolated naïve rat spinal cord mitochondria using ALC instead of pyruvate. For this experiment, all respiration buffer contained 2.5 mM malate (final concentration).

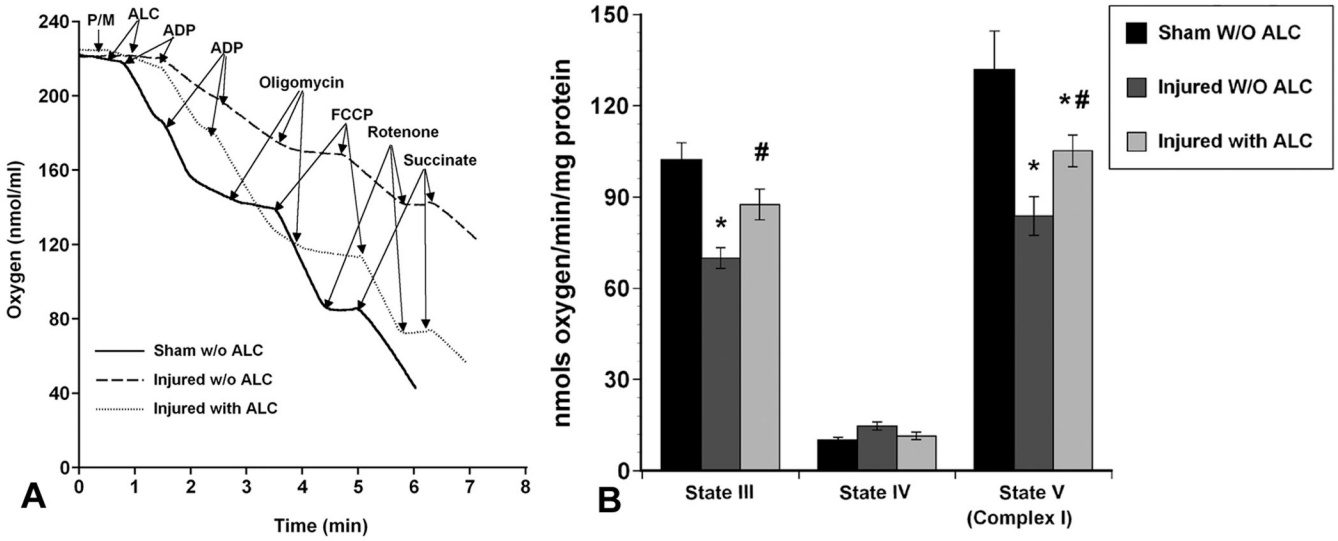

Figure 2.

Panel A is a representative oxymetric trace showing the profound loss of respiration in mitochondria isolated from injured spinal cord (-----) compared to shams (——). The *in vitro* addition of ALC (2.5 mM final) to the respiration chamber increased mitochondrial respiration in injured spinal cords (·····). **Panel B** demonstrates significantly compromised mitochondrial respiration rates (State III and State V-complex I) following SCI compared to shams. State III and State V-Complex I respiration rates were significantly increased in injured mitochondria when ALC was added. Bars represent group means ± SEM, n=4/group. *p<0.05 compared to sham respiration rates without ALC addition; # p<0.05 compared to injured respiration rates without ALC addition.

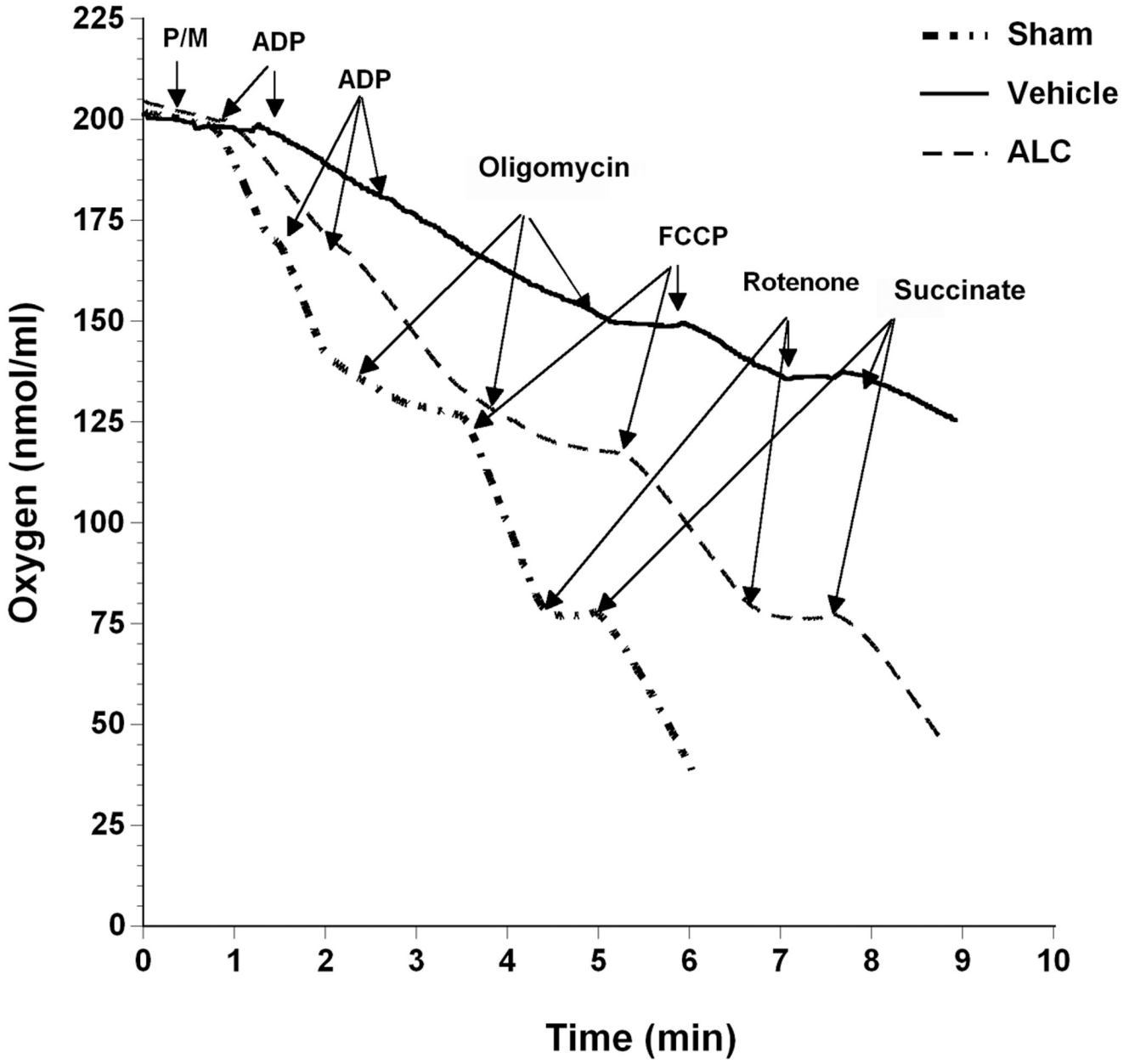

Figure 3.

Contusion spinal cord injury lead to significant mitochondrial dysfunction at 24 hr post-injury, as demonstrated by oxymetric traces for total mitochondria isolated from sham spinal cords (⁻·⁻·⁻) compared to those isolated after *in vivo* treatment with ALC (·····) or vehicle (——) at 15 min post-injury. Notably, ALC treatment significantly maintained mitochondrial bioenergetics following injury.

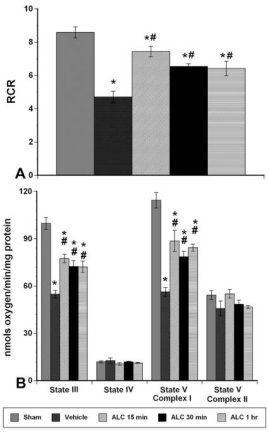

Figure 4.

The respiratory control ratio (RCR; calculated as the ratio of State III vs. State IV slopes) is a measure of mitochondrial bioenergetics and coupling of the ETS to oxidative phosphorylation (**Panel A**). The RCR was significantly decreased in vehicle-treated injured rats at 24 hrs post-injury, whereas 15 min, 30 min and 1 hr post-injury treatment with ALC *in vivo* significantly maintained the RCR after 24 hrs. Respiration rates from mitochondria were calculated as nmols oxygen/min/mg protein 24 hrs post-injury following treatment with either vehicle or ALC at 15 min, 30 min and 1 hr post-injury (**Panel B**). Significantly impaired mitochondrial function was observed with vehicle treatment, whereas ALC treatment at all the time points of administration maintained mitochondrial function compared to vehicle-treated injured groups (**Panel B**). Notably, the RCR and respiration rates (State III and State V-Complex I) for all the ALC-treated groups remained significantly ($p<0.05$) lower compared to shams. No significant changes in the State V-Complex II respiration rates were observed among all the groups. Bars represent group mean ± SEM, n=5–6/group. *$p<0.05$ versus sham group; # $p<0.05$ versus vehicle-treated injured group.

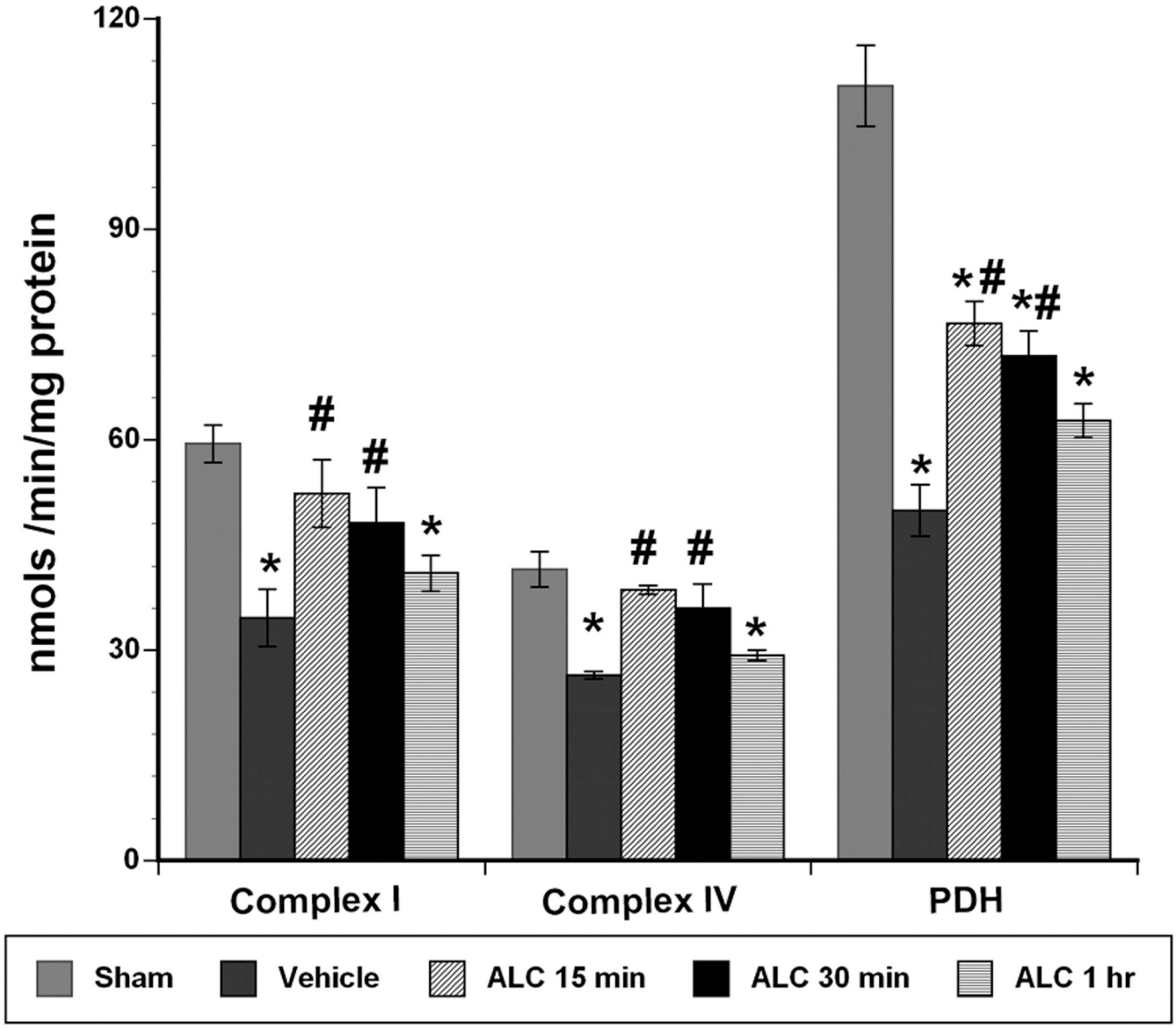

Figure 5.

Activities of NADH dehydrogenase (Complex I), cytochrome C oxidase (Complex IV) and pyruvate dehydrogenase (PDH) were assessed using their specific substrates and calculated as nmols/min/mg protein. With vehicle treatment, the activities of complexes I, IV and PDH were significantly decreased 24 hrs following contusion SCI. Treatment with ALC at 15 and 30 min, but not 1 hr post-injury, maintained complex I and PDH activities compared to vehicle-treated groups. However, complex IV activity was maintained to near normal levels only in the 15 min post-injury ALC treated group. Bars represent group mean ± SEM, n=3–4/group. *p<0.05 versus sham group; # p<0.05 versus vehicle-treated injured group.

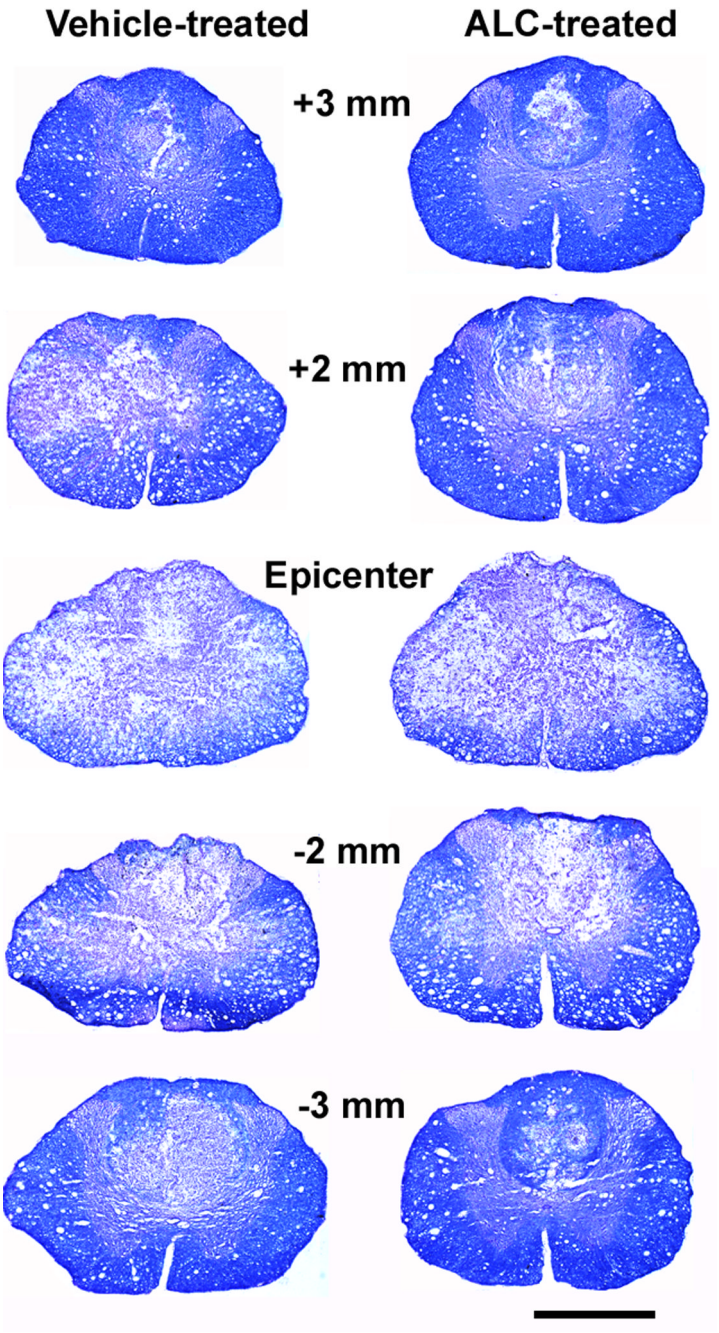

Figure 6.
Photomicrographs illustrating that ALC- versus vehicle-treated injured rats demonstrated decreased histopathology and more tissue sparing throughout spinal cord cross sections, notably the gray matter both rostral and caudal of the injury site at 8 days post-SCI. Scale bar = 1 mm for all photos.

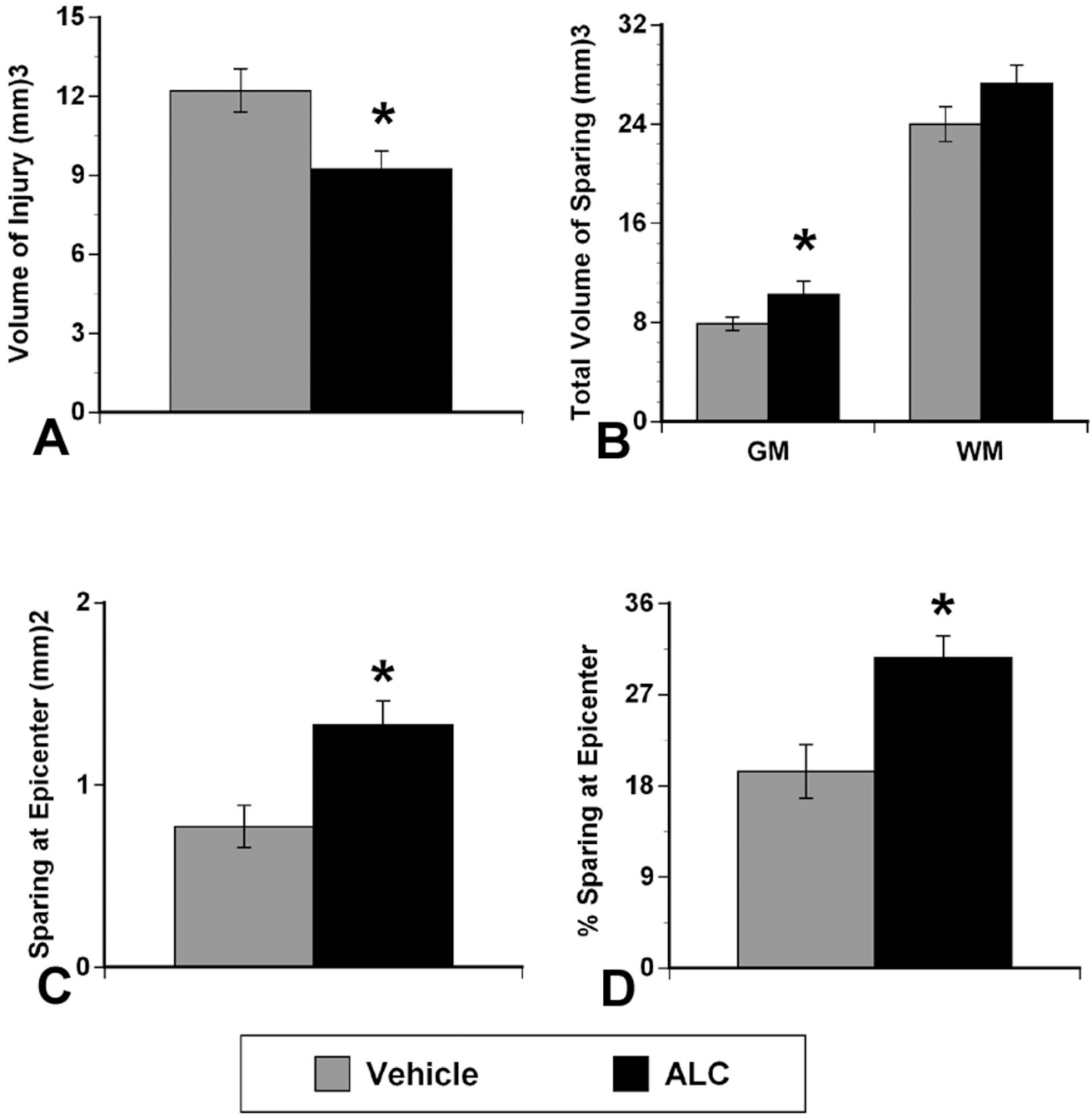

Figure 7.

Panel A represents total volume of injured tissue. ALC treatment group showed less injury volume compared to vehicle-treated group 7 days post-injury. **Panel B** represents spared grey matter (GM) and white matter (WM). ALC treatment significantly spared gray matter compared to vehicle-treated group. **Panel C** and **D** represent tissue sparing at epicenter. ALC treatment significantly increased tissue sparing following SCI. Bars represents mean ± SEM, n= 6 per group. * $p<0.05$ compared to vehicle-treated injured group.