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Peripheral Nerve and Brain Differ in Their Capacity to Resolve N,N-Diethyldithiocarbamate-mediated Elevations in Copper and Oxidative Injury

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Abstract

Previous studies have demonstrated that N,N-diethyldithiocarbamate (DEDC) elevates copper and promotes oxidative stress within the nervous system. However, whether these effects resolve following cessation of exposure or have the potential to persist and result in cumulative injury has not been determined. In this study, an established model for DEDC myelin injury in the rat was used to determine whether copper levels, oxidative stress, and neuromuscular deficits resolve following the cessation of DEDC exposure. Rats were exposed to DEDC for 8 weeks and then either euthanized or maintained for 2, 6 or 12 weeks after cessation of exposure. At each time point copper levels were measured by inductively coupled mass spectrometry to assess the ability of sciatic nerve, brain, spinal cord and liver to eliminate excess copper post exposure. The protein expression levels of glutathione transferase alpha, heme oxygenase 1 and superoxide dismutase 1 in peripheral nerve and brain were also determined by western blot to assess levels of oxidative stress as a function of post exposure duration. As an initial assessment of the bioavailability of the excess copper in brain the protein expression levels of copper chaperone for superoxide dismutase 1, and prion protein were determined by western blot as a function of exposure and post exposure duration. Neuromuscular function in peripheral nerve was evaluated using grip strengths, nerve conduction velocities, and morphologic changes at the light microscope level. The data demonstrated that in peripheral nerve, copper levels and oxidative stress return to control levels within several weeks after cessation of exposure. Neuromuscular function also showed a trend towards pre-exposure values, although the resolution of myelin lesions was more delayed. In contrast, total copper and antioxidant enzyme levels remained significantly elevated in brain for longer post exposure periods. The persistence of effects observed in brain suggests that the central nervous system is more susceptible to long-term cumulative adverse effects from dithiocarbamates. Additionally, significant changes in expression levels of chaperone

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

for superoxide dismutase 1, and prion protein were observed consistent with at least a portion of the excess copper being bioactive.

Keywords

dithiocarbamate; copper elimination; oxidative stress; glutathione transferase; heme oxygenase; myelinopathy

1. Introduction

Dithiocarbamates are an economically important class of compounds that possess a diverse set of chemical properties and biological effects that are utilized in pesticidal, industrial and therapeutic applications (Vettorazzi et al. 1995; WHO 1988). The most prevalent therapeutic application is that of disulfiram which has been used for alcohol aversion therapy for over 50 years (Eneanya et al. 1981). Since its initial use in treating alcoholism it has also been recognized that disulfiram can produce a dose dependent distal sensorimotor peripheral neuropathy (Haley 1979). Morphologic studies investigating this sensorimotor peripheral neuropathy in animal models have reported a primary myelinopathy characterized by intramyelinic edema and demyelination (Tonkin et al. 2000). The myelin lesions are thought to be mediated by a metabolite, N,N-diethyldithiocarbamate (DEDC), formed by the reduction of the disulfide bond in disulfiram; and the administration of DEDC and certain other dithiocarbamates can reproduce the myelin lesions produced by disulfiram (Calviello et al. 2005; Tonkin et al. 2003; Valentine et al. 2006).

DEDC is able to complex copper and it has been shown that DEDC elevates levels of copper within the central and peripheral nervous systems (Aaseth et al. 1979; Iwata et al. 1970; Tonkin et al. 2004). The ability to elevate copper in nerve appears to be a requirement for production of dithiocarbamate-mediated myelinopathy; and this ability can be modulated through changing the polarity of a dithiocarbamate's nitrogen substituents (Valentine et al. 2009). Dithiocarbamates with nonpolar nitrogen substituents increase copper and oxidative injury within nerve and produce myelin injury, whereas those with polar substituents do not. These observations have lead to the hypothesis that neurotoxic dithiocarbamates produce lipophilic copper complexes that facilitate transport of redox active copper across the blood nerve barrier that promote oxidative myelin injury. The contribution of copper elevation and increased oxidative stress to the development of myelin injury has been further supported through studies demonstrating that significant increases in these two processes precede the development of myelin lesions (Viquez et al. 2009; Viquez et al. 2008).

Although DEDC-mediated increases of copper in peripheral nerve and the central nervous system are well established, relatively little is known regarding the chemical properties of the excess copper or whether copper and the associated oxidative stress return to normal levels following the cessation of exposure. In this study an established model for DEDC myelin injury in the rat was used to determine whether the increases in copper levels and oxidative stress resolve and whether normal peripheral nerve structure and function return following the cessation of DEDC exposure. For a period of 12 weeks post exposure to DEDC, total copper and the expression levels of three antioxidant enzymes, glutathione transferase-α (GST-α), heme oxygenase 1 (HO-1), and superoxide dismutase 1 (SOD1) were quantified in peripheral nerve and brain. As an initial assessment of the bioavailability of the excess copper in brain the protein expression levels of copper chaperone for superoxide dismutase 1 (CCS) and prion protein were determined by western blot as a function of exposure and post exposure duration. Peripheral nerve structure and neuromuscular function were also assessed during the post exposure periods using light microscopy, maximum motor nerve conduction velocity and hind

limb grip strength. The data demonstrated that in peripheral nerve, copper levels and oxidative stress proteins return to control levels within weeks after cessation of exposure. Neurological function also showed a trend towards returning to normal, although the resolution of myelin lesions was more delayed. In contrast, both the total copper and antioxidant enzyme levels remained significantly elevated in brain for more extended post exposure periods and significant changes were observed in the levels of prion and CCS protein. Delineating the capacity of the nervous system to eliminate copper and to repair injury following dithiocarbamate exposure will aid risk assessments through determining the potential of these widely used compounds to produce cumulative effects from repeated exposures. Additionally, defining the relationship of copper levels to the resolution of oxidative stress following DEDC exposure will provide further insight into the role that copper plays in the oxidative injury produced by DEDC.

2. Experimental Procedures

2.1. Materials

2ML4 Alzet® osmotic pumps were obtained from Braintree Scientific (Braintree, MA). Sodium *N*,*N*-diethyldithiocarbamate, purity 98%, (DEDC) was obtained from Alfa Aesar (Ward Hill, MA). Glutaraldehyde was obtained from Electron Microscopy Sciences (Ft. Washington, PA). Bovine serum albumin (BSA) was obtained from Sigma-Aldrich (St. Louis, MO). Protease and phosphatase inhibitors were purchased from Amersham Biosciences (Piscataway, NJ) and Sigma-Aldrich (Saint Louis, MO). Dulbecco's PBS (pH=7.4) was purchased from MP Biomedicals (Irvine, CA). All HPLC grade solvents were purchased from Fisher Scientific (Pittsburgh, PA).

2.2. Animal Exposures and Tissue Collection

All treatments and procedures using animals were conducted in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and approved by the Institutional Animal Care and Use Committee of Vanderbilt University. Thirty adult male Sprague-Dawley rats were obtained from Harlan Bioproducts (Indianapolis, IN) and caged at Vanderbilt University animal facilities in a temperature controlled room (21–22 °C) with a 12 h light–dark cycle, supplied with Purina Lab Diet 5001, a segment of 10.1 cm diameter PVC tubing for environmental enrichment, and water *ad libitum*. After a 7-day acclimatization period, 18 animals were exposed to DEDC at 0.3 mmol/kg/day for 8 weeks using ip 2mL, 4-week Alzet® osmotic pumps (Braintree Scientific, Braintree, MA) surgically implanted under anesthesia (100 mg/Kg ketamine with 8 mg/Kg xylazine ip). The osmotic pumps were replaced after 4 weeks to extend the exposure period to 8 weeks. Twelve additional animals served as controls and did not receive pumps. DEDC was delivered as an aqueous solution, the concentration of which was determined from the UV absorbance at 282 nm (DEDC, ε= 13,000 $M^{-1}cm^{-1}$). The DEDC solution was sterilized prior to filling the pumps by filtering through a 0.22 μm syringe filter. The average starting body wt (SE) of all 30 animals was 313 g (1.5), and all animals were weighed twice a week during the experiment. After 8 weeks of DEDC exposure, 6 DEDC-exposed and 6 controls were anesthetized, neuromuscular function measured, then euthanized, and tissues collected. The remaining 12 DEDC-exposed animals were anesthetized and the ip osmotic pumps were removed. Of these 12 animals, 4 remained in the study 2 more weeks, 4 remained 6 more weeks and 4 remained 12 more weeks before the same procedures as above were performed. The remaining 6 controls remained in the study 12 more weeks and the same procedures performed. For 2 and 4 week exposure assessments of copper chaperone for superoxide dismutase 1 (CCS) and superoxide dismutase 1 (SOD1) 8 animals (n=4 for 2 and 4 week exposure groups) were exposed to DEDC at 0.3 mmol/kg/day using ip 2mL, 4-week Alzet® osmotic pumps (Braintree Scientific, Braintree, MA) and 6 rats were used as controls. After neuromuscular function measurements were performed, control

and exposed animals were exsanguinated by cardiac puncture. The right sciatic nerve was immersed in 4% glutaraldehyde in 0.1 M PBS (pH 7.4) for histopathology. A section of the liver, left half of the brain, cut sagitally, sections of proximal spinal cord, and both posterior tibial nerves were immersed in 4% glutaraldehyde in 0.1M PBS (pH 7.4), and stored at 4°C for metal analysis. The left sciatic nerve and right half of the brain were flash frozen in liquid nitrogen and stored at −80°C for protein expression determinations.

2.3. Analysis of Tissue Copper Levels

Tissue sections of brain, spinal cord, liver and both tibial nerves were analyzed for copper by ICP–MS at the Diagnostic Center for Population and Animal Health at Michigan State University (East Lansing, MI). Brain tissue samples for ICP-MS were approximately 2 mm midsagittal slices; liver sections were approximately $1cm^3$ pieces of tissue; tibial nerve tissues were approximately 15- to 20-mm lengths of both nerves; spinal cord sections were approximately 15 mm in length from the cervical region. Values were reported as ppm per dry wt of tissue.

2.4. Protein Extraction and Quantification

Approximately 10 mg of frozen sciatic nerve or 150 mg of frozen brain was powdered in a mortar and pestle in liquid nitrogen. Total proteins were extracted using 0.4–0.6 mL chilled TNE buffer (2 mM EDTA, 150 mM NaCl, 50 mM Tris-base, 2 mM DTT, and NP-40 (1%, v/v)), containing protease inhibitors (P-2714 Sigma-Aldrich, St. Louis, MO, and 80-6501-23 protease inhibitor mix, Amersham Biosciences, Piscataway, NJ) and phosphatase inhibitors (P-2850 and P-5726 Sigma-Aldrich). The homogenate was sonicated at 0 °C for 3 min and centrifuged at 40,485×g for 40 min at 4 °C. The supernatant was collected and stored at −80 °C. Protein concentration in the supernatant was measured by a modified Bradford method (Ramagli 1999) using BSA as the standard.

2.5. Western Blotting for Glutathione Transferase-α, Heme Oxygenase 1, Copper Chaperone for Superoxide Dismutase 1, Prion Protein and Superoxide Dismutase 1

Equal amounts (10–25 μg) of sciatic nerve or brain protein were separated by SDS-PAGE (NuPAGE® 4–12% bis-tris gel, Invitrogen, Carlsbad, CA) along with molecular weight markers (Novex®Sharp Pre-Stained Protein Standards (MW 3.5–260 kDa), MagicMark™XP Western Standard, Invitrogen Carlsbad, CA). Separated proteins were electrophoretically transferred onto an Immobilon-P membrane using an XCell II™ Blot Module (Invitrogen, Carlsbad, CA). Nonspecific binding sites were blocked in blocking buffer (5% nonfat powdered skim milk in tris buffered saline, pH 7.4, containing 0.1% Tween-20 (TBST) at room temperature for 1 h. Membranes were then incubated with primary antibodies against either glutathione transferase-α (GST α) (GSTA11: rabbit anti-GSTα, Alpha Diagnostic, San Antonio, TX, dilution 1:2,000), heme oxygenase 1(HO-1) (OSA-111; mouse anti-HO-1, Stressgen, Ann Arbor, MI, dilution 1:2,500), Cu-Zn superoxide dismutase-1 (SOD1) (FL-154: sc-11407, Santa Cruz Biotechnology, Santa Cruz, CA, dilution 1:5,000), copper chaperone for SOD1 (CCS) (FL-274: sc-20141, Santa Cruz Biotechnology, Santa Cruz, CA, dilution 1:3,000) or prion protein (clone 8H4, Sigma, St. Louis, MO, dilution 1:5,000) overnight at 4 °C. Concurrently, with the primary antibodies, membranes were also incubated with primary anti-actin antibody (A-2066, rabbit anti-actin, Sigma, St. Louis, MO, dilution 1:5,000). After rinsing with TBST, the membranes were incubated at room temperature for 1 h with anti-rabbit peroxidase conjugated secondary antibody (A-8275, Sigma, St. Louis, MO, dilution 1:10,000) or donkey anti-mouse HRP-conjugated secondary antibody (SC-2314, Santa Cruz Biotechnology, Santa Cruz, CA, dilution 1:10,000). Following three 10 min washes in TBST, the membranes were incubated with Western Lightning Chemiluminescence Reagent Plus substrate (Perkin-Elmer LAS, Inc., Boston, MA), and exposed to Kodak X-Omat Blue XB-1

film. The presence of GST-α (MW = 25 kDa) or SOD1 was confirmed by comparing the migration of positive control GST (rat liver, Sigma, St. Louis, MO) or SOD1 (bovine liver, Alexis Biochemicals, San Diego, CA). The presence of actin (MW = 42 kDa) and HO-1 (MW = 32 kDa) were confirmed by comparison to the molecular weight standard. For quantification, films were scanned with a GS-700 densitometer (Bio-Rad) and analyzed using Quantity One 1-D Analysis Program version 4.1 (Bio-Rad). Normalization of GST-α, HO–1, SOD1, and CCS was performed for each sample relative to its corresponding actin value.

2.6. Hind-limb Grip Strength

Neuromuscular function was assessed at the end of study periods for all animals by measuring hind-limb grip strength. Grip strength measurement was accomplished using a DFIS 10 digital force gauge (John Chantillon & Sons, Greensboro, NC) (Meyer et al., 1979). The peak compression achieved by each animal on 3 successive trials was recorded and expressed as the average ± SE for each exposure and control group.

2.7. Maximum Motor Nerve Conduction Velocity Measurements

Neuromuscular function was assessed at the end of study periods for all animals by nerve conduction velocity (NCV). The NCV studies were performed on anesthetized rats (100 mg/Kg Ketamine with 8 mg/Kg Xylazine ip). Temperature was maintained using radiant heat (lamp) and a Deltaphase isothermal pad (Braintree Scientific, Inc, Braintree, MA) and monitored with a skin temperature probe applied to the ventral surface of the proximal tail. The TECA Synergy N2 System (Viasys Healthcare, Madison, WI) connected to a pre-configured notebook computer and Teca-Synergy V 12.2 Software was used to measure and record NCV. Sciatic-tibial motor NCV was determined by placing a stimulating cathode electrode proximally at the sciatic notch and distally near the posterior tibial nerve on the medial side of the tarsus. Supramaximal electrical stimulation was applied using pulses of 20 μsec at a frequency of 0.2 Hz, and the evoked compound action potential measured three times for each site in the lumbrical or interosseous muscles on the plantar surface of the hind foot using two monopolar recording electrodes. Latency times for each site were determined by measuring the time between stimulus and the peak of the evoked compound action potential. Three latency time measurements were averaged to calculate the individual animal's latency for each site. Maximum motor nerve conduction velocity was calculated by taking the distance (cm) between the 2 stimulating sites measured on the skin with the use of calipers and dividing by the difference between the sciatic and tarsal latency time (s) and multiplying by 10. The individual rat NCV measurements were averaged and SE calculated for each exposed and control group.

2.8. Preparation of tissue for morphology

Dissected sciatic nerves from control and DEDC-exposed animals were immersed in 4% glutaraldehyde in 0.1M PBS buffer (pH 7.4) and held at 4°C. For morphology, sciatic nerve sections were post-fixed with osmium tetroxide and embedded in low-viscosity Spurr embedding media (Electron Microscopy Sciences, Fort Washington, PA). Thick sections (600 nm) were cut and stained with toluidine blue. The thick sections of peripheral nerve were evaluated by light microscopy on an Olympus BX41 microscope equipped with an Optronics Microfire digital camera. One cross section of sciatic nerve was examined per animal and the total number of lesions counted. The lesions quantified were: degenerated axons, axons with thin myelin (g ratio greater than 0.7 (axon/axon with myelin diameter)), intramyelinic edema, and demyelinated axons. The surface area of the sciatic nerve was quantified using Photoshop with Fovea Pro 4 plug-ins and the number of lesions/mm^2 calculated for each section.

2.9. Statistical Analysis

One-way analysis of variance (ANOVA), Tukey–Kramer's Multiple Comparisons Test, Dunnett multiple comparison post hoc test, and the Unpaired t test were performed using Prism 4.0c (Graphpad Software, Inc). Lesion counts were square root transformed to normalize their distribution and to equalize variances prior to statistical comparisons by ANOVA and Dunnett Multiple Comparisons Test. Statistical significance was taken to be $p < 0.05$ unless otherwise noted.

3. Results

3.1. Analysis of Tissue Copper Levels

Copper levels in the brain, spinal cord, tibial nerve and liver determined by ICP-MS are shown in Figure 1. Copper levels in brain rose 10 fold during the 8 week period and remained at 9–10 fold of control values at all time points examined after cessation of exposure. Copper levels in spinal cord paralleled those in brain with an 8 fold rise during exposure, with a decrease to levels that were 6 fold greater than controls 2 to 6 weeks after cessation of exposure and then remained at this level out to 12 weeks post exposure. Liver copper rose 3 fold during exposure and returned to control levels within 2 weeks of cessation of exposure. Tibial nerve copper rose 3 fold during DEDC exposure, began decreasing after cessation of exposure, and returned to control values by 6 weeks post exposure.

3.2. Assessment of Oxidative Stress by GST-α, HO-1 and SOD1

Western blots demonstrated a significant increase in GST-α, HO-1 and SOD1 proteins in brain and nerve obtained from DEDC-exposed animals relative to control animals at 8 weeks of DEDC exposure (Figure 2). Levels of brain GST-α and HO-1 remained significantly elevated out to 12 weeks post exposure, whereas nerve protein levels of GST-α and HO-1 decreased to control levels by 12 and 6 weeks post exposure, respectively. SOD1 nerve and brain protein expression returned to control levels at 2 and 6 weeks post exposure, respectively.

3.3. Protein Expression Levels of CCS, SOD1 and Prion Protein

Western blots demonstrated a significant decrease in CCS protein expression during the DEDC exposure period followed by an initial significant increase at 2 weeks post exposure and then return to values at later post exposure time points (Figure 3) that were lower than controls but not significant. Prion protein exhibited an initial increase in expression at the earliest time point examined and then decreased significantly below control values reaching at the 8 week exposure and 2 week post exposure time points (Figure 3).

3.4. Body Weights, Grip Strengths, and NCV Measurements

The average weight gain of DEDC exposed rats relative to controls at each of the time points examined is shown in Figure 4A. Although weight gains (g ± SE) of the exposed animals began to approach those of controls at 12 weeks post exposure they were significantly decreased at all time points, i.e., 8 weeks (DEDC: 44.9 ± 6.4; controls 110.2 ± 4.9), 2 weeks post exposure (DEDC: 58.3 ± 7.7; controls: 132.5 ± 8.8) and 6 weeks post exposure (DEDC: 104.6 ± 8.7; controls: 159.3 ± 11.2) and 12 weeks post exposure (DEDC: 140 ± 13.5; controls: 184.2 ± 11.8). Hind limb grip strength data for all groups are presented in Figure 4B. The grip strengths of the two control groups (8 and 20 week time points) were not significantly different and were combined into one control group for statistical comparisons. The 8-week DEDC exposure group and the 2-week DEDC post exposure group had significantly lower hind limb grip strengths relative to the controls. However, by 6 and 12 weeks post exposure, there was no longer a significant difference between exposed and control groups.

Nerve conduction velocity values were not significantly different between the two sets of controls (8 and 20 week time points) and were combined for statistical comparisons. The results in Figure 4C show a significant decrease in NCV at 8 weeks of exposure and 2 weeks post exposure to DEDC. At 6 weeks post exposure, NCV had increased to approximately 85% of the control values and was not significantly different from the control group. At 12 weeks post exposure, the NCV (80% of control values) remained similar to that of the 6 week time point but was statistically different from controls.

3.5. Peripheral Nerve Morphology

Representative sections of sciatic nerve obtained from a control, an 8 week DEDC exposure, and a 12 week post exposure rat are shown at the light microscope level in Figure 5 and illustrate the lesions quantified in Table 1. Axons undergoing degeneration were observed in all groups with a significant increase in severity seen for DEDC exposed animals compared to controls at all time points. In contrast, axons exhibiting either thin myelin, demyelination or intramyelinic edema were only observed in the DEDC exposed groups, with significant differences in both the incidence and severity of these lesions still present at the longest post exposure time point. Axonal sprouts or bundles within bands of Büngner were also present in sections obtained from DEDC exposed animals at the 12 week post exposure time point consistent with axonal regeneration.

4. Discussion

In the present study the capacities of the peripheral and central nervous systems to resolve DEDC-mediated perturbations in copper homeostasis and oxidative stress were assessed. The data supported a substantial difference in the relative ability of peripheral nerve, compared to brain, to remove the excess copper and to alleviate the oxidative stress produced by DEDC. In peripheral nerve, the copper and protein expression levels for GST-α, HO-1 and SOD1 returned to control values and neuromuscular function exhibited a trend towards improvement during the 12-week post DEDC exposure period. In contrast, during this same period the copper levels in brain and spinal cord remained significantly elevated and the expression levels of GST-α, HO-1 and SOD1 remained elevated for longer periods in brain.

Previous studies have demonstrated that continued exposure to DEDC results in a cumulative increase of copper in brain and peripheral nerve and that this cumulative response appears to be specific to components of the nervous system (Tonkin et al. 2004; Viquez et al. 2008). The only other organ reported to exhibit a significant increase in copper is the liver. However, the liver did not display a cumulative response and copper levels plateaued in less than two weeks despite continued DEDC exposure (Viquez et al. 2008) and hepatic injury has not been observed under neurotoxic exposure conditions similar to those used in the present study (Tonkin et al. 2004). The relatively rapid elimination of copper from liver following cessation of exposure, i.e, return to pre exposure levels within less than two weeks, most likely contributes to the establishment of liver copper steady state levels early during subchronic DEDC exposure. The slower rate of copper elimination observed for nerve would be expected to contribute to the continued accumulation of copper in nerve. Similarly, the decreased rate of copper elimination from brain relative to peripheral nerve may underlie the substantially greater levels of copper that are accumulated in brain from DEDC exposure. Although the regulatory mechanisms of copper elimination and conservation are not well delineated the current data for DEDC is consistent with previous studies that have demonstrated organ-specific turnover rates to be much greater in liver relative to brain under normal dietary copper intake (Levenson and Janghorbani 1994).

The blood-brain and blood-nerve barriers function as diffusion barriers between the nervous system and the general circulation (Betz et al. 1994). Although there are some location

dependent differences regarding the specific components and continuity of this barrier, in general they serve a similar function throughout most of the nervous system to regulate the entrance and exit of molecules and ions. This barrier may contribute to the delayed removal of copper from the nervous system relative to liver, particularly if the copper changes from a permeable to a non-permeable species, e.g., non protein associated to protein associated copper, after entering the nervous system. However, this interpretation would not explain the substantial difference observed between the rates of copper elimination in peripheral nerve vs. brain. At least two mechanisms may contribute to the observed disparity in copper elimination between nerve and brain. The first involves the additional compliment of glial cells present in brain that could provide a reservoir for the accumulation of non-mobile copper. Astrocytes express two isoforms of the inducible metal binding protein metallothionein that is capable of binding copper; and astrocytes have been shown to sequester copper in vitro as well as other heavy metals in vivo (Aschner 1996; Dincer et al. 1999). Copper complexed with the cysteine residues of metallothionein would presumably not be readily mobilized for elimination and thus could contribute to the persistence of copper in brain. A second relevant difference between the central and peripheral nervous systems is that the central nervous system has a much more limited capacity to clear myelin debris after injury (Colavincenzo and Levine 2000). Therefore any copper that has partitioned into myelin, an event that has been observed to occur for peripheral nerve (Viquez et al. 2009), would be expected to be cleared less rapidly in brain than in nerve.

Glutathione transferases, HO-1 and SOD1 are antioxidant enzymes having expression levels regulated in part through NF-E2 p45-related factor (Nrf2) activation of the antioxidant response element (Nguyen et al. 2003). GST-α is important in the detoxification of α,β-unsaturated aldehydes produced by lipid peroxidation, and it is up-regulated by 4-hydroxynonenal (Awasthi et al. 2005). GST-α HO-1, SOD1 have been used as markers of oxidative stress and have been shown to be elevated in peripheral nerve and the central nervous system following DEDC exposure together with oxidative injury and lipid peroxidation (Viquez et al. 2009). In the present study both GST-α and HO-1 expression decreased coincident with the levels of copper in peripheral nerve whereas GST-α, HO-1 as well as copper all remained significantly elevated in brain. SOD1 protein returned to control levels in both peripheral nerve and brain during the post exposure period however the return was more delayed in brain. The parallel changes in protein expression and in copper levels suggest that the oxidative injury observed in the nervous system is a direct effect of the excess copper, presumably from redox cycling of the copper.

Although copper levels are elevated in brain subsequent to DEDC exposure it has not been determined whether the excess copper is bioavailable. If entrance of copper into the nervous system is facilitated through formation of a lipohilic complex with DEDC the copper may remain tightly bound to DEDC and not available or biologically active. Due to the toxic potential of free copper, there has evolved a set of copper chaperones to minimize levels of free copper within biological systems. One of these chaperones, CCS, delivers copper to SOD1 and its level of expression has been observed in several studies to be inversely related to levels of available copper, i.e., in states of copper deficiency or excess the levels of this protein were elevated or decreased, respectively (Danzeisen et al. 2007; Gybina and Prohaska 2006; Iskandar et al. 2005; Prohaska et al. 2003; West and Prohaska 2004). Presumably this relationship exists to maintain adequate copper delivery to SOD1. When copper levels are low more CCS is required to scavenge copper and deliver it to SOD1 whereas elevated levels of copper require less CCS to maintain SOD1. Therefore we examined the levels of CCS in brain as a possible biomarker of biologically active copper. During the periods of DEDC exposure the levels of CCS expression were significantly decreased suggesting an increased availability of copper. The increased levels of SOD1 in the presence of decreased CCS raises the possibility that SOD1 may be in an inactive form. Although this may partially contribute to the elevated

SOD1 protein levels, previous studies have demonstrated increased SOD1 activity concomitant with increased protein expression in the central nervous system from subchronic DEDC exposure (Viquez et al. 2009). This suggests that adequate levels of copper were available for SOD1 despite decreases in the level of CCS.

Prion protein is a recognized copper binding protein associated with inherited, infectious, and sporadic forms of spongiform encephalopathy. Although there are additional processes that can alter expression of prion protein (Wright et al. 2009), in vitro studies have demonstrated prion protein expression to be modulated by the level of available copper in the medium (Toni et al. 2005). In rats administered DEDC the levels of prion protein detected by western blot showed a biphasic response. There was an initial increase followed by a sustained decrease during the exposure and post exposure period. Determining whether this reduction resulted from reduced translation, increased proteolysis or formation of high molecular weight aggregates will require further investigation. However, these data support the biological significance of the copper elevations produced by DEDC and its potential to influence levels of copper binding proteins in vivo.

General toxicity and neuromuscular function were assessed using body weight gain, hind limb grip strength and maximum motor nerve conduction velocity. All of these parameters showed improvement post exposure with hind limb grip strength returning to control levels. In contrast, structural lesions, as assessed by lesion counts in cross sections of nerve, did not appear to be resolving as quickly as the functional assessments. Among the functional assessments performed, the one expected to be the most reflective of myelin integrity was the maximum NCV measurement (Kimura 2005). The approach of the maximum NCV values to those of pre exposure values suggests an improvement of myelin integrity although this appears to be in conflict with the lesion counts. However, maximum NCV is most sensitive for the largest, most rapidly conducting fibers. Therefore, it is conceivable that improvement in a subpopulation of rapidly conducting fibers could be sufficient for the observed results in NCV although significant levels of myelin lesions were still present. Collectively though, the neuromuscular assessments suggest that functional deficits in peripheral nerve are reversible following cessation of exposure to DEDC and the return of function follows the elimination of excess copper and decline of oxidative stress.

In conclusion, perturbations in nervous system copper and oxidative stress persist much longer in the central nervous than the peripheral nervous system following cessation of DEDC exposure. During the 12 week post exposure period there was no evidence that brain copper levels decreased at all, raising the question as to whether or not copper will eventually return to control levels in the exposed animals. This relationship suggests that the central nervous system is more susceptible to cumulative effects from this class of compounds resulting from repeated exposures throughout the life of an individual, e.g., from residues on food crops. Further studies are required to determine the processes responsible for the differences observed in the central and peripheral nervous systems and to delineate the potential adverse effects within the central nervous system. Determining whether there are pools of copper resistant to mobilization within brain will help to address these questions. X-ray fluorescence microscopy has been used to characterize the distribution of excess copper in peripheral nerve and also appears amenable to localizing copper in brain (Viquez et al. 2009). Additionally, although it has been proposed that dithiocarbamates facilitate entry of copper into the nervous system through the formation of lipophilic complexes capable of crossing the blood-nerve and blood-brain barriers, this mechanism has not been established. Delineating the processes responsible for dithiocarbamate-mediated elevations of copper in nerve, brain and spinal cord will advance our understanding of why dithiocarbamate-mediated perturbations in metal homeostasis primarily involve copper within the nervous system and why copper elevations are greater and more persistent in the central nervous system.

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Abbreviations

TBS	tris buffered saline
GST-α	glutathione transferase alpha
BSA	bovine serum albumin
DEDC	N,N-diethyldithiocarbamate
HO-1	heme oxygenase 1
CCS	copper chaperone for superoxide dismutase 1
SOD1	superoxide dismutase 1

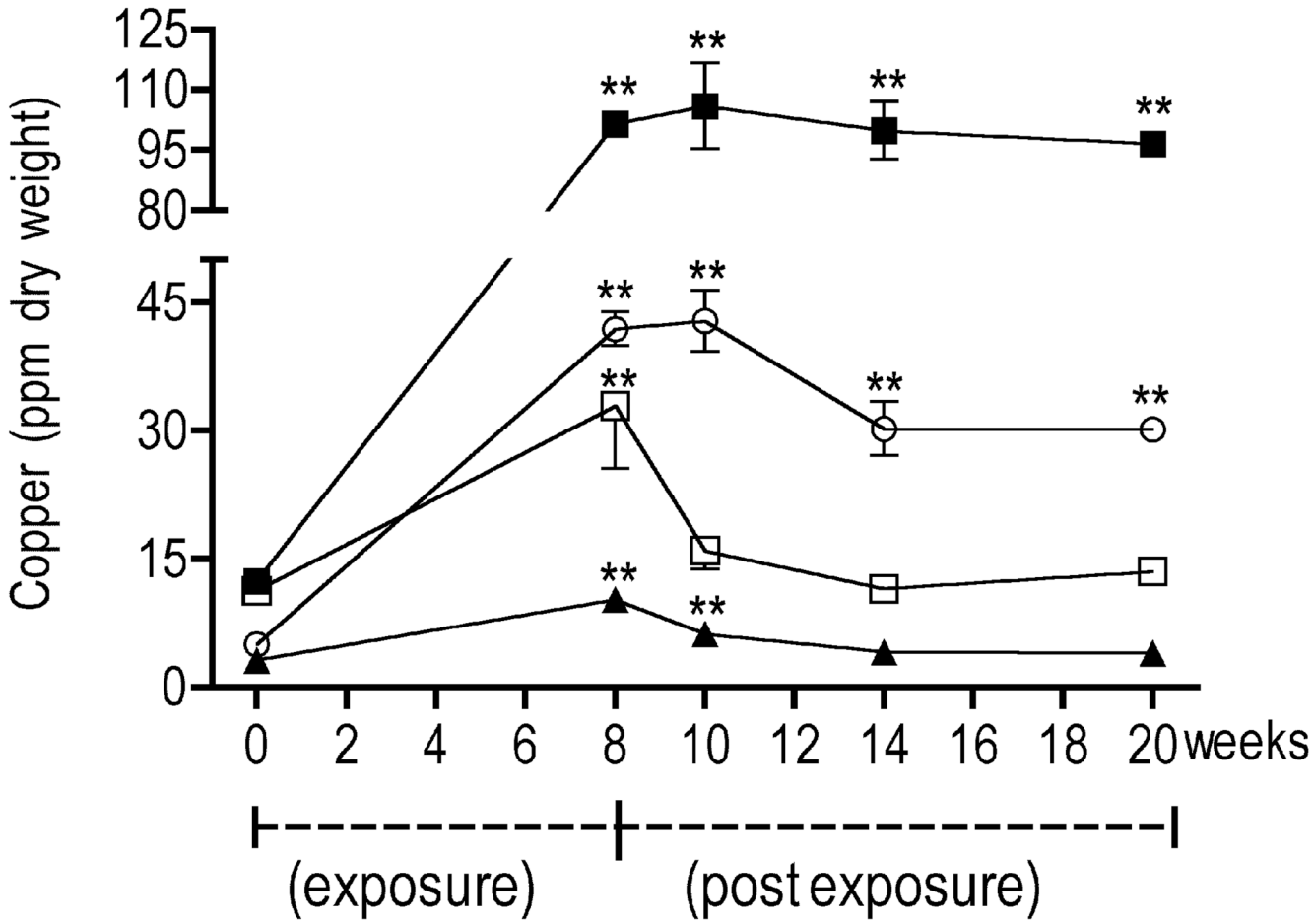

Figure 1.

Total tissue copper levels. Values for brain (closed squares), spinal cord (open circles), liver (open squares) and tibial nerve (closed triangles) were determined by ICP-MS (n=4, except n=12 for controls and n=6 for 8 week DEDC exposure group) and are reported as mean ppm dry weight of tissue. Error bars represent SE. **p <0.01 relative to controls by one-way ANOVA and Dunnett's Multiple Comparison post hoc test.

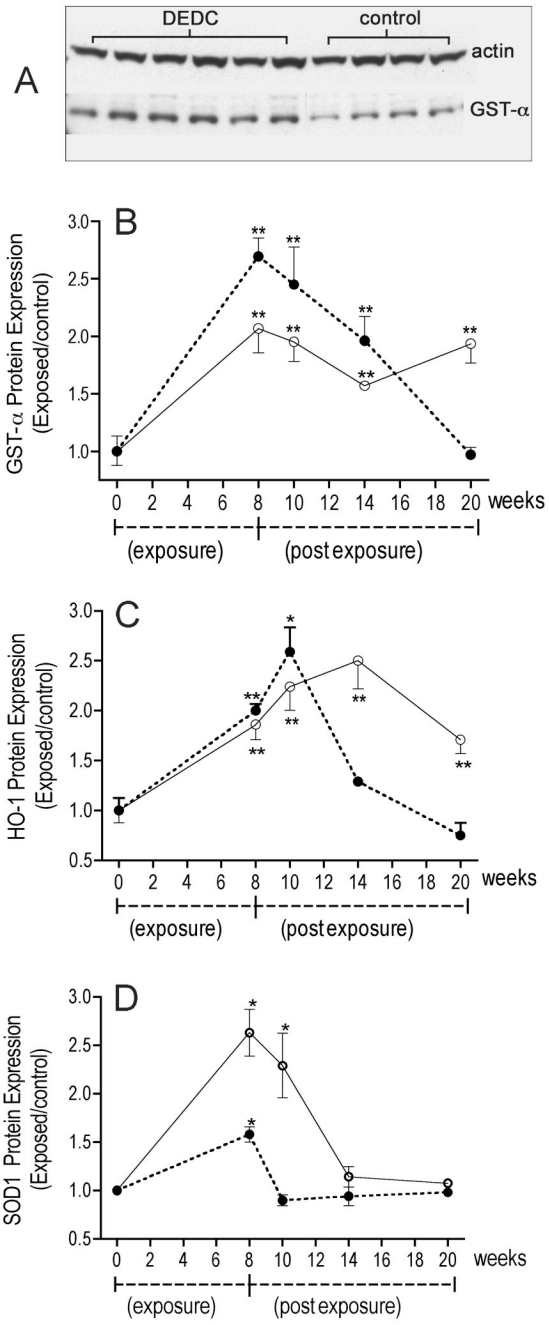

Figure 2.

GST-α, HO-1 and SOD1 protein expression in sciatic nerve and brain. (A) Representative western blot showing relative amounts of GST-α in proteins isolated from the brains of animals at the 8 week DEDC exposure time point as compared to controls. (B) Levels of GST-α in brain (open circles) and sciatic nerve (closed circles) determined by western blot. Optical density of GST-α was normalized to that of actin within the same sample and then to the mean value obtained for control samples on the same membrane (n =4 for controls and n=6 for exposed at 8 weeks; n=6 for controls and n=4 for exposed at all other time points). (C) Levels of HO-1 in brain (open circles) and sciatic nerve (closed circles) determined by western blot. Optical density determinations and exposure groups were identical to those used in (B) for

GST-α. (D) Levels of SOD1 in brain (open circles) and sciatic nerve (closed circles) determined by western blot. Optical density determinations and exposure groups were identical to those used in (B) for GST-α. Error bars represent SE. ** $p < 0.01$ and * $p < 0.05$ relative to controls run on the same membrane by one-way unpaired students t-test.

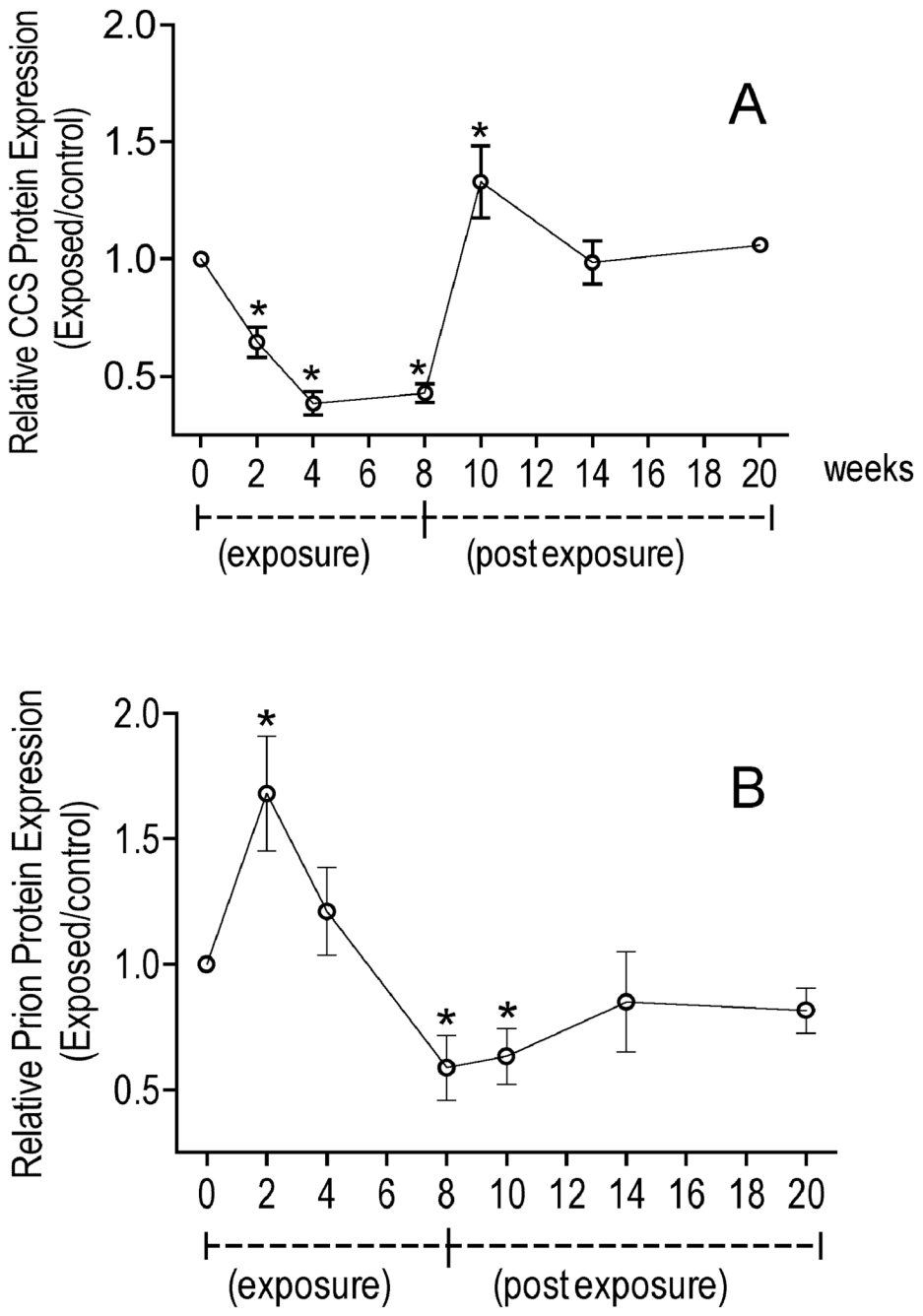

Figure 3.

CCS, and prion protein expression in brain as a function of DEDC exposure and post exposure duration. (A) Levels of CCS protein in brain determined by western blot. Optical density of CCS was normalized to that of actin within the same sample and then to the mean value obtained for control samples on the same membrane (n =4 for controls and n=6 for exposed at 8 weeks; n=6 for controls and n=4 for exposed at all other time points). (B) Levels of prion protein in brain determined by western blot. Optical density determinations and exposure groups were identical to those used in (A) for CCS. Error bars represent SE. **p <0.01 and * p <0.05 relative to controls run on the same membrane by one-way unpaired students t-test..

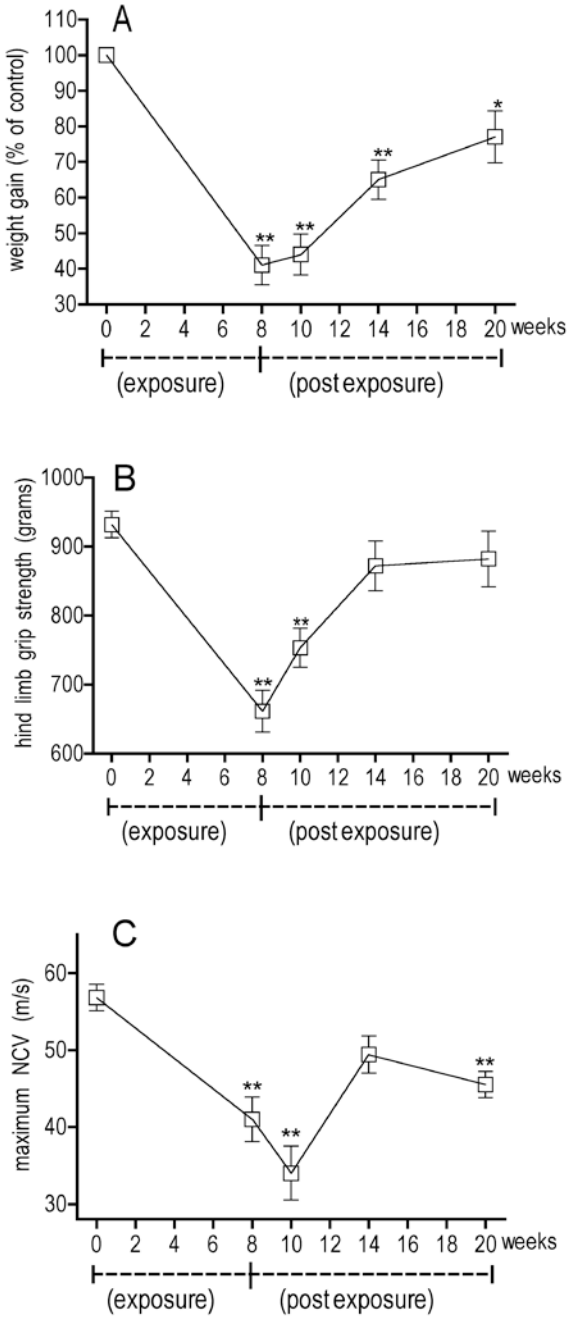

Figure 4.

Body weight gain, hind limb grip strength and nerve conduction velocities. (A) Body weight gain of DEDC exposed animals presented as percent of time matched controls. (For controls, n=12 at 0 and 8 weeks and n=6 at 10, 14 and 20 weeks; for the DEDC exposed n= 18 at 8 weeks, n=12 at 10 weeks, n=8 at 14 weeks and n=4 at 20 weeks). **$p < 0.01$ and *$p < 0.05$ relative to time matched controls by one-way unpaired t-test. (B) Values for grip strengths from controls (0 weeks exposure) and DEDC exposed animals are reported as peak compression (grams) ± SE (n=4 except n = 12 for controls and n=6 for 8 week exposure group). (C) Values for NCV are reported as mean velocity (m/s) ± SE. (n=4 except n = 11 for controls (0 weeks exposure) and n=6 for 8 week exposure group). **$p < 0.01$ relative to control groups by one-

way ANOVA and Dunnett's Multiple Comparison post hoc test for grip strengths and NVC measurements.

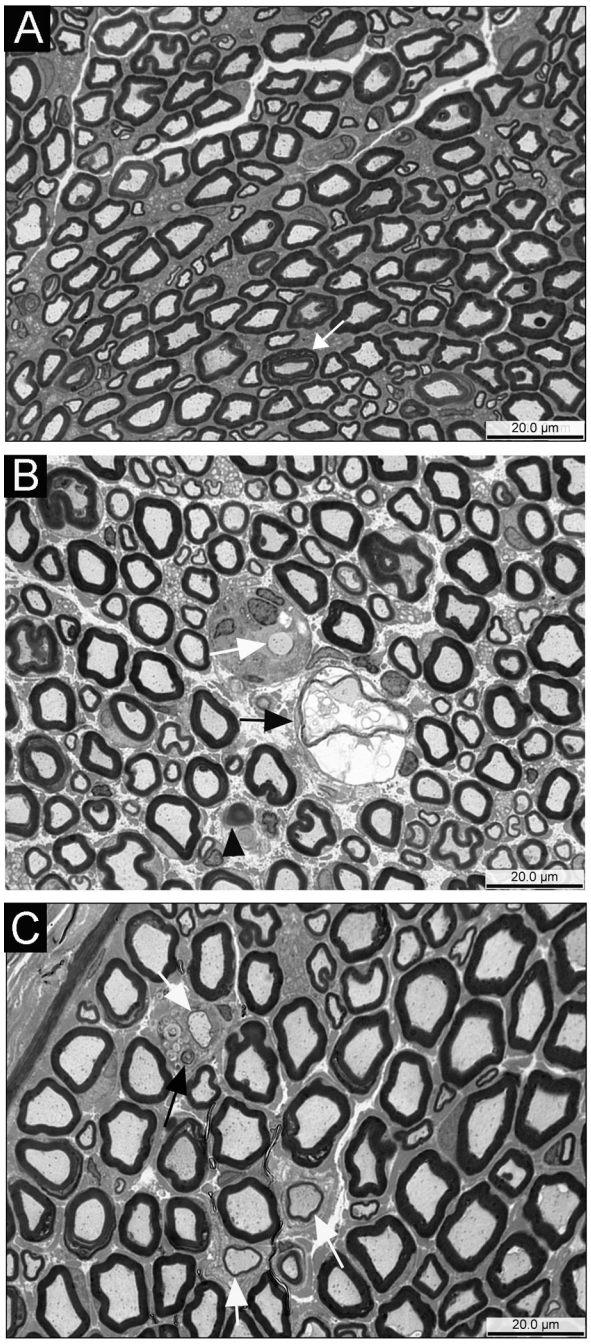

Figure 5.

Morphology of sciatic nerve cross-sections stained with toluidine blue. (A) Cross section obtained from a control rat showing the presence of large and small myelinated axons. The axons are surrounded by normal compact myelin with some axons sectioned through Schmidt-Lanterman incisures (white arrow). (B) Cross section of a sciatic nerve from an animal 2 weeks post DEDC exposure demonstrating an axon with intramyelinic edema (black arrow), a demyelinated axon (white arrow) surrounded by Schwann cell cytoplasm, and a degenerating axon (black arrowhead). (C) Cross section obtained from a rat 12 weeks post DEDC exposure showing two axons with thin myelin (white arrows) and myelin debris in the cytoplasm of a Schwann cell (black arrow)

Table 1

Incidence and Severity of Sciatic Nerve Lesions[a]

Treatment	Degenerated axons		Thin myelin		Demyelination		Intramyelinic edema	
	Incidence[b]	Severity[c]	Incidence[b]	Severity[c]	Incidence[b]	Severity[c]	Incidence[b]	Severity[c]
Controls	11/12	1.80 (0.22)	0/12	0 (0)	0/12	0 (0)	0/12	0 (0)
Range		(0.00–2.66)						
DEDC 8 wk	6/6	3.82 (0.18)[e]	6/6[f]	2.67(0.30)[e]	5/6 [f]	2.58 (0.57)[e]	4/6 [f]	1.51 (0.68)
Range		(3.30–4.37)		(1.41–3.31)		(0.00–3.62)		(0.00–4.55)
2 wk post exposure	4/4	4.23 (0.15)[e]	3/4[f]	0.90 (0.30)	1/4	0.59 (0.59)	4/4 [f]	1.75 (0.39)
Range		(3.86–4.55)		(0.00–1.22)		(0.00–2.35)		(1.19–2.88)
6 wk post exposure	3/3	5.58 (1.32)[e]	3/3[f]	2.87 (0.91)[e]	2/3 [f]	1.50 (0.75)[d]	2/3 [f]	2.48 (1.35)[d]
Range		(4.17–8.23)		(1.21–4.33)		(0.00–2.31)		(0.00–4.63)
12 wk post exposure	4/4	5.63 (0.73)[e]	4/4[f]	2.41 (0.46)[e]	4/4 [f]	1.71 (0.17)[e]	3/4 [f]	2.16 (0.92)[d]
Range		(3.62–7.00)		(1.30–3.44)		(1.30–2.09)		(0.00–4.50)

[a] Slides were scored blinded by a single observer (HLV). One entire sciatic nerve fascicle was examined under light microscopy and lesions totaled, then divided by section surface area.

[b] Number of animals with positive observations/number of animals in treatment group.

[c] Mean of square root of number of axons with lesions divided by mm^2 surface area (SE).

[d] $p < 0.05$ as compared to controls by one-way ANOVA, Dunnett Multiple Comparisons post hoc test.

[e] $p < 0.01$ as compared to controls by one-way ANOVA, Dunnett Multiple Comparisons post hoc test.

[f] $p < 0.05$ with respect to incidence between treatment group and controls by Fisher's Exact test