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Mn (III) Tetrakis (4-Benzoic Acid) Porphyrin Protects Against Neuronal and Glial Oxidative Stress and Death after Spinal Cord Injury

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Abstract

This study explores the ability of a catalytic antioxidant, Mn (III) tetrakis (4-benzoic acid) porphyrin (MnTBAP), to protect against neuronal and glial oxidative stress and death after spinal cord injury (SCI). Nine different doses of MnTBAP were administered into the intrathecal space of the rat spinal cord immediately following moderate SCI to establish dose - response curves for prevention of lipid peroxidation and neuron death. An optimal dose was determined by comparing the effectiveness of MnTBAP protection among doses. The optimal dose was then administered and the cords were removed 24 h post-administration and processed for staining. The cells in the cord sections at different distances from the epicenter were counted to obtain the spatial profiles of MnTBAP protection. Comparison of the counts between MnTBAP- and vehicle-treated groups in the sections double immuno-fluorescence-stained with oxidative and cellular markers demonstrated that MnTBAP significantly reduced numbers of nitrotyrosine- and DNP-positive (stained with an antibody against 2,4-dinitrophenyl hydrazine (DNPH)-labeled protein carbonyls) neurons, astrocytes, and oligodendrocytes. Comparison of the counts between the two treatments in the sections immuno-stained with cellular markers revealed that MnTBAP significantly increased numbers of neurons, motoneurons, astrocytes, and oligodendrocytes. MnTBAP more effectively reduced neuronal than glial cell death. Post-injury treatment with the optimal dose of MnTBAP at 6, 12, 24, 48, and 72 h post-SCI demonstrated that the effective time window for reducing protein nitration and neuron death was at least 12 h. Our results demonstrated that MnTBAP combats oxidative stress, thereby attenuating all types of cell death after SCI.

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AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

Keywords

Antioxidant therapy; membrane lipid peroxidation; Mn (III) tetrakis (4-benzoic acid) porphyrin; neuronal and glial death; optimal dose; oxidation and nitration of proteins; spinal cord injury; effective time window

INTRODUCTION

Following acute spinal cord injury (SCI), the neurons surviving the initial traumatic injury degenerate and continue to die through a cascade of secondary damage [1, 2]. Reactive species (RS), including reactive oxygen species, such as superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($^{\bullet}OH$), and reactive nitrogen species, such as nitric oxide ($^{\bullet}NO$) and peroxynitrite ($ONOO^-$), are believed to contribute to secondary central nervous system (CNS) injury by causing oxidative damage to the major cellular components [3-5].

Attack on polyunsaturated fatty acids in cell membranes by RS triggers free radical chain reactions. Thus, one free radical can lead to the conversion of many membrane lipid molecules into lipid hydroperoxides and severely disrupt membrane functions. RS oxidize membrane lipids to malondialdehyde and 4-hydroxy-nonenal (HNE) [4, 6]. These substances damage proteins by forming covalent aldehyde-protein complexes, thereby compromising protein function by altering secondary and tertiary structure [7, 8]. Aldehydes are much longer-lived than free radicals and diffuse further from the site of origin to attack more distant proteins than do free radicals [6]. HNE, one of the most toxic aldehydes, causes further impairments to cells [9, 10]; hence HNE-positive neurons might be functionally impaired and more susceptible to subsequent death. Therefore HNE was selected as a specific indicator of membrane lipid peroxidation (MLP) in the present study.

RS attack on proteins modifies amino acids, fragments chains and generates cross links, thereby destabilizing protein tertiary structure and increasing susceptibility to proteolysis [11]. Proteins are the structural and functional units of cells; their modification may cause functional abnormalities, thereby destroying cells. Protein carbonyl content was used as an indicator of protein oxidation [12]. $ONOO^-$ produced from $O_2^{\bullet-}$ and $^{\bullet}NO$ is a strong oxidant that damages tissue by different pathways [11]: decomposition to $^{\bullet}OH$ when protonated [13]; direct and efficient oxidization of sulfhydryl groups, lipids, DNA, and proteins [14]; reaction with superoxide dismutase to form a nitronium-like intermediate that nitrates tyrosine residues in proteins to produce nitrotyrosine (Ntyr), thereby damaging critical cellular targets [15, 16]. Therefore, we used protein-bound-Ntyr as a marker of protein nitration.

Substantial experimental evidence supports RS being important mediators of secondary damage after SCI [17-19]. We and others have demonstrated that the levels of $O_2^{\bullet-}$ [20], H_2O_2 [21], $^{\bullet}OH$ [22], $^{\bullet}NO$ [23, 24], $ONOO^-$ [23, 25], and the products of oxidation and nitration of proteins [23, 26-28] and MLP [28-33] all significantly increase following SCI. By administration of the increased levels of RS into an un-injured rat spinal cord, we demonstrated that they caused oxidative damage to major cellular components [34, 35],

neuronal death by necrosis and apoptosis [36-38], and neurological dysfunction [36]. Therefore, removing RS with a broad spectrum RS scavenger may reduce secondary cell death.

Metalloporphyrins have emerged as a novel class of catalytic antioxidants [39] that scavenge a wide range of RS such as $O_2^{\bullet-}$, H_2O_2 , $ONOO^-$ and lipid peroxyl radicals [40]. The metalloporphyrin Mn (III) tetrakis (4-benzoic acid) porphyrin (MnTBAP) possesses both superoxide dismutase and catalase activity [41, 42], and scavenges $ONOO^-$ [43]. It is also a potent inhibitor of MLP [44]. MnTBAP improves the survival of neuron cultures in an *in vitro* model of age-related mitochondrial oxidative stress [45]. It markedly reduces both neuronal and glial cell death in cerebrocortical cultures [46]. Intracerebroventricular injection of MnTBAP inhibited kainate-induced mitochondrial $O_2^{\bullet-}$ production, DNA oxidation and neuronal loss in the hippocampus [47]. MnTBAP increased the number of cells and attenuated apoptotic neuron death after SCI [48, 49]. These results suggest that the antioxidant MnTBAP may be a potential therapeutic agent for treating SCI, worthy of further evaluation of its antioxidant and cell protection capabilities. However, studies to evaluate MnTBAP protection against oxidative stress and cell death in different types of cells are still absent.

The objective of this study was to characterize the ability of MnTBAP to protect against oxidative stress and death of different types of cells after SCI by the following steps: 1) determine the optimal dose of MnTBAP protection against oxidative stress and cell death by establishing dose - response curves; 2) evaluate the ability of the optimal dose of MnTBAP to protect against oxidative damage in different types of cells by co-localizing oxidative markers in different types of cells; 3) evaluate the ability of the optimal dose of MnTBAP to ameliorate secondary cell death in neurons, motoneurons, astrocytes and oligodendrocytes; and 4) explore the effective time window of post-SCI treatment with the optimal dose of MnTBAP to protect against oxidative stress and neuron death.

MATERIALS AND METHODS

All procedures were approved by the University of Texas Medical Branch Animal Care and Use Committee and were in accord with the NIH Guide for the Care and Use of Laboratory Animals. All possible efforts were made to minimize the suffering of the experimental animals.

Animal Preparation and Spinal Cord Injury

Male Sprague-Dawley rats (250-300g) were anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneally (ip)). They were considered adequately anesthetized when there was no flexor withdrawal upon noxious foot pinch. A laminectomy was performed on vertebrae T13 and L5 while keeping the dura intact. Care was taken not to injure the cord. Then the rats were placed for impact injury at vertebra T13 using a standard New York University spinal cord impactor with SCI software [50] by dropping a 10 g rod 1.25 cm onto the exposed cord. Injury levels are calculated by multiplying the weight by the distance the weight is dropped; 12.5 g.cm impact force was used in the present study. A hole was then made in the dura at vertebra L5. A completely coated microdialysis catheter was inserted through the hole into

the terminal cistern of the intrathecal space 1 cm caudal to the hole as we reported previously [51]. MnTBAP or saline was administered through the implanted catheter. The procedures for anesthesia, surgery and impact injury are described in detail in our previous publications [20-23]. After injury and MnTBAP administration, the incision was surgically repaired. For the post-SCI treatment groups, at the time of treatment, rats were re-anesthetized with pentobarbital and the top of vertebra L5 removed for administration of MnTBAP or saline as described. After MnTBAP or saline administration, the incision was surgically closed.

MnTBAP Administration

The stock solution of MnTBAP (Calbiochem, San Diego, CA, USA) was made by dissolving it in 0.1 M NaOH and storing it in a freezer. Before use, the stock solution was diluted with 0.9 % saline, and the pH adjusted to 7.1-7.3 to generate the working solution [52]. The working solution of MnTBAP was administered at a rate of 1.5 μ l-min. The identical concentrations of the MnTBAP working solution and fixed flow rate of administration for all experiments required giving slightly different volumes of working solution to rats of different weights over slightly different time periods. Therefore, approximately 20 μ l of working solution of MnTBAP was administered immediately after SCI through the implanted catheter into the intrathecal space of the cord and completed within approximately 10 min.

To establish the dose - response curves for determining the optimal dose, nine groups of rats (5 rats in each group) were used for MnTBAP administration at doses of 0 (saline), 0.01, 0.2, 0.4, 2.0, 4.0, 10, 25, and 50 mg/kg given immediately after SCI. To explore the protective ability of post-SCI treatment with MnTBAP, the established optimal dose was administered at 0, 6, 12, 24, 48 and 72 h post-SCI, so 12 groups of rats were used at 6 time points (MnTBAP-treated versus vehicle-treated, 5 rats in each group). Rats were placed in a temperature and humidity controlled room with a 12 h light-dark cycle. Animals had free access to food and water.

Spinal Cord Tissue Processing

The cord was removed at 24 h post-SCI or post-treatment by MnTBAP. To remove the cord, animals were reanesthetized and perfused transcardially with saline followed by 4% paraformaldehyde in phosphate buffered saline (PBS, pH 7.2-7.4). Then the cord was further exposed from vertebrae T11 to L2 so that a 1.5 cm long segment centered at the injury site was removed and fixed in the same fixative for another 24 h at 4°C. The fixed cords were dehydrated and embedded in paraffin. Ten μ m sections were cut with a microtome. Sections were mounted sequentially on glass slides coated with poly-L-lysine for different staining (five sections per slide).

Histochemical Staining for Identification of the Injury Epicenter

Every 10th slide in the series was deparaffinized and stained with 0.1% cresyl violet (CV, ACROS, New Jersey, USA) in sodium acetate buffer for 30 min [38]. The CV stained sections were observed under a light microscope to identify the section with the lowest

density of surviving cells and taken to be the epicenter (defined as distance zero from the epicenter).

Immunohistochemical Staining

HNE and protein-bound-Ntyr were used as markers of oxidative stress. Neuron-specific enolase (NSE), choline acetyltransferase (ChAT), glial fibrillary acidic protein (GFAP) and adenomatous polyposis coli clone CC-1(CC1), were used as specific markers of neurons, motoneurons, astrocytes, and oligodendrocytes respectively. The procedures for immunohistochemical staining were based on those in our previous publications [34-36, 38, 49]. Briefly, the sections were deparaffinized, washed sequentially in solutions producing a gradient from ethanol to water and rehydrated. Then sections were immersed in an antigen retrieval solution (1mM EDTA-NaOH solution, pH 8.0) [53] and heated in a microwave oven for antigen retrieval. Endogenous peroxidase was quenched in 0.6% H₂O₂ in PBS for 15 minutes. Slides were then incubated for 30 minutes with normal goat serum (Invitrogen, Camarillo, CA, USA). The sections were then incubated with the primary antibodies, HNE polyclonal antiserum (1:100, rabbit-anti-HNE, from Alpha Diagnostic Intl., San Antonio, TX, USA), mouse-anti-Ntyr (1:500, from Cayman Chemical, Ann Arbor, MI, USA), mouse-anti-NSE (1:200, Dako Cytomation, Glostrup, Denmark), mouse-anti-ChAT(1:100, ABcam, Cambridge, MA, USA), mouse-anti-GFAP (1:200, from Sigma, St. Louis, Missouri, USA), mouse-anti-CC1 (1:500, from Oncogene Research Products, Cambridge, MA, USA) over night. After washing in PBS, the slides were incubated in a secondary antibody, biotinylated goat-rabbit-IgG or biotinylated goat-mouse-IgG respectively (Invitrogen, Camarillo, CA, USA) for 60 minutes. The slides were washed in PBS and incubated with ready-to-use histostain-plus streptavidin-HRP (Invitrogen, Camarillo, CA, USA) for 1 h to label biotinylated antibodies. The sections were washed in PBS, stained with a liquid 3,3'-diaminobenzidine substrate kit (Invitrogen, Camarillo, CA, USA), washed in distilled water and enhanced with a diaminobenzidine enhancer (Invitrogen, Camarillo, CA, USA). The stained sections were dehydrated through an ethanol gradient, cleared and coverslipped with ready-to-use histomount (Invitrogen, Camarillo, CA, USA). To obtain negative controls, a slide next to the slide positively immune-stained was selected for the same immune-staining procedures, but PBS was substituted for the primary antibody solutions.

Immuno-Fluorescence Colocalization of Oxidatively Damaged Proteins in Neurons and Glial Cells

To evaluate oxidative stress in different types of cells, the oxidative markers for protein oxidation and nitration were double stained with markers for different type of cells. In addition to HNE immune-staining, HNE-positive neurons were visualized by immuno-fluorescence double staining with antibodies against HNE and NSE. Briefly, the slides were deparaffinized, washed, rehydrated, endogenous peroxidase quenched, then washed in PBS, and incubated with normal goat serum as described above. The sections were incubated with first primary antibody rabbit-anti-HNE (1:100, Alpha Diagnostic Intl., San Antonio, TX, USA), or rabbit anti-Ntyr (1:200, Cayman Chemical, Ann Arbor, MI, USA) overnight to label HNE-positive and Ntyr-positive cells. Protein carbonyls, as markers of protein oxidation, were labeled with 2,4-dinitrophenylhydrazine (DNPH) in the spinal cord sections, and an anti-DNP antibody was used to recognize the DNP-labeled protein carbonyls, as

reported in our previous publications [26, 34]. For labeling protein carbonyls, after washing the rehydrated sections in PBS, slides were incubated in 0.1% DNPH (Sigma, St. Louis, Missouri, USA) in 2N HCl over night at 4°C followed by washing in PBS, quenching endogenous peroxidase and washing in PBS. Then sections were incubated with 0.05% protease (Sigma, St. Louis, Missouri, USA) in 0.05M tris-buffer at 37°C for 5 min. After washing in PBS and incubating with normal goat serum, the sections were incubated with first primary antibody rabbit anti-DNP (1:100, Invitrogen, Camarillo, CA, USA) over night. Following immune-labeling with the first antibodies to HNE, Ntyr, and DNP, the sections were washed in PBS and incubated in a first secondary antibody, anti-rabbit Alexa Fluor 488 (green, Invitrogen, Camarillo, CA, USA) for 1 h for fluorescence labeling and washed in PBS. For the second immuno-staining, after incubating the sections in goat serum for 30 min, the sections were incubated with a second primary antibody, mouse-anti-NSE (1:200) to label neurons, mouse-anti-GFAP (1:200) to label astrocytes, mouse-anti-CC1 (1:500) to label oligodendrocytes for 1 h at room temperature. The sections were washed in PBS and incubated with a second secondary antibody, anti-mouse Alexa Fluor 594 (Invitrogen, Camarillo, CA, USA) for 1 h. These sections were washed 3 times in PBS and coverslipped with Pro-Long Gold anti-fade reagent (Invitrogen, Camarillo, CA, USA). A negative control was done as described above. A fluorescence microscope (Olympus model BX-51) was used for photomicrographs with green (AF-488) and red (AF-594) filters.

Cell Counting for Quantitative Evaluation of MntBAP Protection

The images in histochemical, immunohistochemical, and immuno-fluorescence stained sections were observed using a light-fluorescence microscope (Olympus BX51) and captured by a digital camera (Olympus DP70 microscope digital camera) with a computer system and software for processing photographs for histological and immunohistochemical examination. Neurons and astrocytes in the gray matter of the cord in each section were counted in areas of the ventral left and ventral right quarters. Quarters were defined by horizontal and vertical lines drawn crossing the central canal in the center of the sections as shown in Fig. (1) by solid lines. This area of gray matter includes motoneurons, middle and small sized neurons, and glial cells. Glial cells in the white matter of the sections were counted in a rectangular area of $250 \times 350 \mu\text{m}^2$ of ventromedial white matter of the cord as shown by a frame in Figs. (1, 9, 10). The cells in each left or right quarter were counted three times and the counts averaged for that quarter; then the counts in the left and right quarters were averaged for that section. Although unbiased stereological cell counting methodology was not available for this experiment, to avoid bias the person processing the cord into sections and the person counting the cells in the sections were blinded. This counting method has been validated by our previous publications [23, 26, 48, 49] and by those of many others.

HNE- and Ntyr-positive neurons were identified based on the presence of positively immuno-stained cytoplasm in cells with bright nuclei and the morphology of neurons. Motoneurons, astrocytes and oligodendrocytes were identified by their specific immunohistochemical staining. CV-stained neurons were identified by the presence of bright nuclei with enrichment of Nissl bodies. No significant differences were found when comparing the neuron counts in the CV-stained sections counted by applying these criteria

to the counts in the anti-NSE stained sections - validating the criteria for identifying neurons in CV-stained sections [38]. Oxidatively stressed neurons, astrocytes and oligodendrocytes were identified by immuno-colocalization of double immuno-fluorescence staining for oxidative and cell type markers.

Statistical Analysis

The counts were compared among groups receiving different doses of MnTBAP in a dose - response curve by using one-way analysis of variance (ANOVA) followed by the post-hoc Tukey test for the determination of effective doses and the optimal dose. Spatial protection by the optimal dose of MnTBAP against nitration and oxidation of proteins, and different types of cell death were analyzed by two-way Repeated Measures Analysis of Variance (RMANOVA) followed by all pairwise multiple comparisons (Bonferroni t-test). The spatial protection against oxidative stress and neuron death by the optimal dose of MnTBAP administered at different time post-SCI was analyzed by three-way RMANOVA followed by a Bonferroni adjustment. $p < 0.05$ was considered a statistically significant difference.

RESULTS

The Dose - Dependence of MnTBAP Protection Against Oxidative Stress and Neuron Death

The purpose of this study was to evaluate the effect of MnTBAP protection against oxidative stress and cell death in neuronal and glial cells. Therefore, the dose - response curves of MnTBAP protection to reduce HNE-positive neurons (as a stress marker) and neuron death were measured. We reported previously that higher doses of MnTBAP (10 and 50 mg/kg) given ip reduced neuron death in an area 1.0 to 2.5 mm rostral from the epicenter [48]. Therefore to establish the dose-response curves, HNE-positive and CV stained neurons were counted in the sections 1.55 and 1.5 mm respectively rostral to the epicenter in the sections in saline-treated (dose 0) and 8 different doses of MnTBAP-treated groups of rats. Using one-way ANOVA followed by the post-hoc Tukey test to compare the counts in the sections treated with saline and 8 different doses of MnTBAP determined all effective doses and the optimal dose of MnTBAP.

Fig. (2) (upper panel) provides the microphotographs of anti-HNE immuno-stained sections 1.55 mm rostral from the epicenter in MnTBAP- and saline-treated groups. The MnTBAP-treated section (B and B') had fewer HNE-positive neurons compared to the saline-treated group (A and A'). C-E illustrates a section double immuno-fluorescence stained with anti-HNE and anti-NSE antibodies in a MnTBAP-treated cord to verify the presence of HNE-positive neurons. The neurons stained with NSE (D) showed normal morphology. The same sections stained with HNE (C) showed not only a reduced number of HNE-positive neurons compared to D, but also partial HNE staining in some HNE-positive neurons. Because MnTBAP attenuated oxidative stress in neurons, anti-HNE immuno-staining did not stain entire neurons.

The middle panel of Fig. (2) is the dose - response curve for MnTBAP protection against oxidative stress obtained by counting the HNE-positive neurons in the immuno-stained sections from all nine groups of rats ($n = 4$ for vehicle, $n = 5$ for all eight MnTBAP-treated

groups). Comparison of the counts (mean $\pm$ SEM) of HNE-positive neurons in the sections between saline-treated (dose = 0) and 8 different doses of MnTBAP-treated groups indicated that MnTBAP had no significant effect at 0.01, 0.2 (not shown), 0.4, 25 and 50 mg/kg ($p > 0.05$), whereas 2 ($p = 0.02$), 4 ($p < 0.001$), and 10 ($p = 0.04$) mg/kg significantly reduced the number of HNE-positive neurons. Comparison of the counts among eight MnTBAP doses indicated that the numbers of HNE-positive neurons in the sections treated with 4 mg/kg MnTBAP were significantly lower than in sections treated with any other MnTBAP dose: 0.4 ($p = 0.005$), 2 ($P = 0.048$), 10 ($p = 0.02$), 25 and 50 ($P < 0.001$) mg/kg. Thus 4 mg/kg is the optimal intrathecal dose of MnTBAP to reduce oxidative stress.

The lower panel of Fig. (2) is a dose - response curve of MnTBAP protection against neuron death obtained by counting the CV-stained neurons at 1.5 mm rostral to the epicenter in the ventral gray matter of the cord in the sections from all nine groups of rats. The counts (mean $\pm$ SEM) were compared between saline-treated and eight different doses of MnTBAP-treated animals (doses of 0.1 and 0.2 mg/kg are not shown) to determine the effective doses of MnTBAP. The results demonstrated that MnTBAP treatment at the doses of 2 ($P = 0.02$), 4 ($P < 0.001$), 10 ($p = 0.007$) and 25 ($P = 0.03$) mg/kg significantly increased the numbers of neurons, but there were no significant effects at 0.01, 0.2 (not shown), 0.4 or 50 mg/kg ($p > 0.05$). The counts were also compared among eight doses of MnTBAP-treated groups to determine the optimal dose. The numbers of neurons in the sections treated with 4 mg/kg MnTBAP were significantly higher than for those treated with other doses: 0.4 ($p < 0.001$), 2 ($p = 0.01$), 10 ($p = 0.03$), 25 ($p = 0.006$), 50 ($p < 0.001$) mg/kg. This identifies 4 mg/kg of MnTBAP as the optimal dose for preventing neuron death when administered into the intrathecal space.

Spatial Protection by MnTBAP Against Oxidative Stress in Different Types of Cells After SCI

To evaluate MnTBAP protection against oxidative stress in different types of cells spatially, the optimal dose of MnTBAP or vehicle was given immediately following SCI. The double Immuno-fluorescence co-localized cells in the sections at different distances rostral from the epicenter were counted at 0.5 mm intervals and the counts compared between the optimal dose of MnTBAP and saline (as vehicle) treated groups at each distance from the epicenter using Two-Way RMANOVA followed by an All Pairwise Multiple Comparison (Bonferroni t-test). DNP and Ntyr are markers for oxidation and nitration of proteins.

Fig. (3) presents photomicrographs showing oxidized proteins in neurons (A-C), astrocytes (D-F) and oligodendrocytes (G-I) by immuno-colocalization in the sections of rat spinal cords. A, D and G are Alexa Fluor 488 (green) fluorescence-labeled oxidized proteins in different type of cells. B, E and H show Alexa Fluor 594 (red) fluorescence-labeled neurons, astrocytes and oligodendrocytes. C, F and I are the co-localized DNP-positive neurons, astrocytes and oligodendrocytes (yellow). MnTBAP protection against protein oxidation in different types of cells was quantified by counting the immuno-colocalized DNP-positive cells in the double immuno-fluorescence stained sections (yellow). The immuno-colocalized neurons and astrocytes were counted in the ventral quarters of the gray matter and oligodendrocytes were counted in a $250 \times 350 \mu\text{m}$ area of ventromedial white matter of the

cord (as shown in Fig. 1). The DNP-positive neurons were counted in the sections 0.8 - 2.8 mm rostral from the epicenter at 0.5 mm intervals in MnTBAP- and saline-treated groups and counts were compared. The comparison indicated that the number of DNP-positive neurons in the MnTBAP-treated group was significantly decreased at 1.8 (3.3 ± 0.66 , $P=0.004$), 2.3 (5.2 ± 0.57 , $p=0.004$), and 2.8 (7.0 ± 0.53 , $p<0.001$) mm compared to vehicle-treated sections at 1.8 (6.8 ± 0.90), 2.3 (8.7 ± 0.84), and 2.8 (12.5 ± 1.4) mm rostral to the epicenter (mean $\pm$ SEM, $n=4$, Fig. 4A). The DNP-positive astrocytes were counted in the sections 0.85 - 2.85 mm rostral from the epicenter at 0.5 mm intervals in the ventral gray matter of MnTBAP- and saline-treated sections and counts were compared. The comparison indicated that MnTBAP treatment significantly reduced the number of DNP-positive astrocytes in the sections at 1.85 (3.1 ± 0.2 , $p=0.03$), 2.35 (3.9 ± 0.9 , $p<0.001$), and 2.85 (6.9 ± 1.2 , $p<0.001$) mm compared to vehicle-treated sections at 1.85 (6.0 ± 1.0), 2.35 (8.4 ± 1.2), and 2.85 (11.7 ± 1.0) mm rostral to the epicenter (mean $\pm$ SEM, $n=4$, Fig. 4B). The DNP-positive oligo-dendrocytes were counted in the sections 0.9 - 2.4 mm rostral from the epicenter at 0.5 mm intervals in the ventral white matter of MnTBAP- and saline-treated sections and counts were compared. The comparison indicated that MnTBAP treatment significantly reduced the number of DNP-positive oligodendrocytes in the sections at 1.9 (3.2 ± 0.6 , $p=0.002$), and 2.4 (4.7 ± 0.8 , $p<0.001$) mm compared to vehicle-treated sections at 1.9 (6.6 ± 0.7), and 2.4 (11.4 ± 0.7) mm rostral to the epicenter (mean $\pm$ SEM, $n=4$, Fig. 4C).

Fig. (5) presents photomicrographs showing nitrated proteins in neurons (A-C), astrocytes (D-F) and oligodendrocytes (G-I) by immuno-colocalization in the sections of rat spinal cords. A, D and G are Alexa Fluor 488 (green) fluorescence-labeled nitrated proteins in different type of cells. B, E and H show Alexa Fluor 594 (red) fluorescence-labeled neurons, astrocytes and oligodendrocytes. C, F and I are the co-localized Ntyr-positive neurons, astrocytes and oligodendrocytes (yellow). MnTBAP protection against protein nitration in different type of cells was similarly quantified by counting the immunocolocalized Ntyr-positive cells in the double immunofluorescence stained sections (yellow) in the same areas as for counting DNP-positive cells. The Ntyr-positive neurons were counted in the sections 0.6 - 3.1 mm rostral from the epicenter at 0.5 mm intervals in MnTBAP- and saline-treated groups and counts were compared. MnTBAP treatment significantly reduced the number of Ntyr-positive neurons in the sections at 1.6 (0.8 ± 0.3 , $p=0.046$), 2.1 (3.5 ± 0.3 , $p<0.001$), 2.6 (4.9 ± 0.8 , $p=0.004$) and 3.1 (7.8 ± 1.5 , $p<0.001$) mm compared to vehicle-treated sections at 1.6 (2.5 ± 0.3), 2.1 (6.5 ± 0.1), 2.6 (7.3 ± 0.5) and 3.1 (11.0 ± 0.3) mm rostral to the epicenter (mean $\pm$ SEM, $n=4$, Fig. 6A). The Ntyr-positive astrocytes were counted in the sections 0.65 - 3.15 mm rostral from the epicenter at 0.5 mm intervals in the ventral gray matter of MnTBAP- and saline-treated sections and counts were compared. The comparison indicated that MnTBAP treatment significantly reduced the number of Ntyr-positive astrocytes in the sections at 2.15 (2.5 ± 0.3 , $p=0.01$), 2.65 (4.9 ± 0.8 , $p=0.001$), 3.15 (4.8 ± 1.5 , $P<0.001$) mm compared to vehicle-treated sections at 2.15 (5.2 ± 0.1), 2.65 (8.3 ± 0.5), 3.15 (9.0 ± 0.3) mm rostral to the epicenter (mean $\pm$ SEM, $n=4$, Fig. 6B). The Ntyr-positive oligodendrocytes were counted in the sections 0.7 - 3.2 mm rostral from the epicenter at 0.5 mm intervals in the ventral white matter of MnTBAP- and saline-treated sections and counts were compared. The comparison indicated that MnTBAP treatment significantly reduced the

number of Ntyr-positive oligodendrocytes in the sections at 2.7 (2.5 ± 0.7 , $p < 0.001$) and 3.2 (5.3 ± 0.7 , $P < 0.001$) mm compared to vehicle-treated sections at 2.7 (8.5 ± 1.9) and 3.2 (22.0 ± 1.2 ,) mm rostral to the epicenter (mean $\pm$ SEM, $n=4$, Fig. 6C).

Spatial Protection by MnTBAP Against Different Types of Cell Death After SCI

The animals used to evaluate MnTBAP protection against oxidative stress as described above were also used to evaluate MnTBAP protection against neuronal and glial death. The neurons in CV-stained sections and the motoneurons in the sections immuno-stained with anti-ChAT in the ventral quarters of gray matter of the cord, astrocytes and oligodendrocytes in the sections immuno-stained with anti-GFAP and anti-CC1 antibodies in a $250 \times 350 \mu\text{m}$ area of ventromedial white matter of the cord were counted at different distances rostral from the epicenter and the counts were compared using Two-Way RMANOVA followed by an All Pairwise Multiple Comparison (Bonferroni t-test).

Fig. (7, upper panel) shows CV-stained neurons in the sections 1.5 mm rostral from the epicenter in saline- or MnTBAP-treated groups. Fewer neurons survived in saline-treated section (A and A') compared to MnTBAP-treated section (B and B'). The lower panel illustrates the spatial protection by the optimal dose of MnTBAP against neuron death. The numbers of neurons were counted in CV-stained sections at 0.5 - 4.0 mm rostral to the epicenter. The overall comparison between MnTBAP and saline treatment groups demonstrated that the optimal dose of MnTBAP treatment significantly increased the number of neurons ($p < 0.001$) in the ventral gray matter. The number of neurons in the MnTBAP-treated group was significantly increased at 1.5 mm (16.6 ± 2.6), 2.0 mm (18.0 ± 1.3), 2.5 mm (20.2 ± 1.2), 3.0 mm (24.4 ± 1.2) ($p < 0.001$ for these four distances), 3.5 mm (24.2 ± 1.3 , $p = 0.03$) and 4.0 mm (27.1 ± 1.6 , $p = 0.045$) rostral from the epicenter compared to vehicle-treated sections at 1.5 (7.1 ± 2.2), 2.0 (8.3 ± 1.7), 2.5 (13.2 ± 2.6), 3.0 (16.1 ± 4.6), 3.5 (22.1 ± 0.7) and 4.0 (25.6 ± 1.0) mm ($n = 4$ for saline and $n = 5$ for MnTBAP treatment, mean $\pm$ S.D).

Fig. (8) illustrates the spatial protection by the optimal dose of MnTBAP against motoneuron death. The upper panel in Fig. (8) is the photomicrographs of rat spinal cord sections immuno-stained with anti-ChAT at 2.05 mm rostral from the epicenter. More ChAT-positive neurons appeared in the MnTBAP-treated sections (B and B') compared to saline-treated sections (A and A'). The lower panel of Fig. (8) is the spatial profile of MnTBAP protection against motoneuron death, as counted in the anti-ChAT-stained sections at 0.55 - 3.55 mm from the epicenter. Comparison of the counts in the sections between MnTBAP-treated and saline-treated animals demonstrates that the optimal dose of MnTBAP treatment significantly ($p = 0.003$) increased the number of ChAT-positive neurons in the sections 1.55 (12.9 ± 1.0), 2.05 (14.9 ± 1.3) and 2.55 (18.2 ± 1.2) mm compared ($p < 0.001$ for all three distance) to the saline treated groups in the corresponding sections: 9.6 ± 1.9 , 11.8 ± 1.1 , and 14.9 ± 1.6 respectively. ($n = 5$ for both treatments, mean $\pm$ S.D).

Fig. (9) illustrates spatial protection by the optimal dose of MnTBAP against astrocyte death. The upper panel presents photomicrographs of spinal cord sections immunostained with anti-GFAP at 2.2 mm rostral to the epicenter. There were fewer astrocytes in saline-

treated (A and A') than in MnTBAP-treated (B and B') sections. For quantification, the numbers of astrocytes were counted in sections 0.7-3.2 mm rostral from the epicenter, and counts were compared. Comparison of the counts in the sections from the MnTBAP-treated and the saline-treated animals indicates that the optimal dose of MnTBAP significantly increased the number of astrocytes in the sections 1.7 (11.9 ± 0.6 as compared to 7.9 ± 0.7 in the saline treated sections, $p < 0.001$) and 2.2 (13.7 ± 0.7 compared to 10.1 ± 0.3 in the saline treated sections, $p = 0.002$) mm rostral to the epicenter ($n = 4$ for both groups, mean $\pm$ S.D, lower panel).

Fig. (10) illustrates the spatial protection by the optimal dose MnTBAP against death of oligodendrocytes. The upper panel shows the photomicrographs of rat spinal cord sections at 2.3 mm rostral to the epicenter immuno-stained with anti-CC1 in saline-treated and MnTBAP-treated groups. There were fewer oligodendrocytes in the saline-treated sections (A-A') than in MnTBAP-treated sections (B-B'). For quantification, the numbers of oligodendrocytes were counted in sections 0.8-3.3 mm from the epicenter, and counts were compared. The optimal dose of MnTBAP significantly increased the number of oligodendrocytes in the sections 1.8 (17.4 ± 1.3 compared to 13.5 ± 0.9 in the saline group, $p < 0.001$), 2.3 (20.4 ± 0.4 compared to 17.9 ± 0.7 in the saline group, $p = 0.01$) and 2.8 (21.8 ± 0.3 compared to 19.2 ± 0.6 in the saline group, $p = 0.008$) mm rostral to the epicenter. ($n = 4$, mean $\pm$ S.D, lower panel).

Effective Time Window of Post-SCI Treatment with MnTBAP Obtained by Assessing Protein Nitration and Neuron Death Over Time and Distance

The optimal dose of MnTBAP was administered into the intrathecal space at 6, 12, 24, 48, and 72 h post SCI ($n = 5$ for all groups) and the cords were removed 24 h after drug administration (at 30, 36, 48, 72, and 96 h post-SCI) for tissue processing and staining. The Ntyr-positive neurons in the anti-Ntyr antibody immuno-stained sections from 1.55 to 3.05 mm and the neurons in the CV-stained spinal cord sections from 1.50 to 3.50 mm rostral to the epicenter at an interval of 0.5 mm were counted in both MnTBAP- and saline-treated sections at each time point post-SCI treatment. The counts were statistically compared using three-way RMANOVA followed by a Bonferroni adjustment to explore the spatial protection by MnTBAP treatment at different times post-SCI.

Fig. (11A) shows the quantitative results of spatial protection against protein nitration by MnTBAP and saline administered at different times post-SCI. All three effects (time, distance and treatment) differ significantly. The differences among distances ($p < 10^{-16}$) at each time point indicate that the number of Ntyr-positive neurons gradually increases with the increased distances from the epicenter because more neurons survived at longer distances from the epicenter following SCI. The differences ($p = 1.02 \times 10^{-10}$) among post-SCI times at each distance indicate that the number of Ntyr-positive neurons is reduced because the number of neurons gradually decreased over time. The differences between 2 treatments ($p = 8.6 \times 10^{-5}$) demonstrates that the effect of MnTBAP treatment significantly differs from the effects of saline treatment over times and distances. Table 1 shows differences (p-values) in the numbers of Ntyr-positive neurons between the saline and MnTBAP treatments at each time and each distance. Clearly, 6 and 12 h post-SCI treatment

with MnTBAP significantly reduced the numbers of Ntyr-positive neurons at distances 1.55, 2.05, 2.55, and 3.05 mm from the epicenter. Post-SCI treatment with MnTBAP at 24, 48 and 72 h did not significantly reduce the protein nitration at any distance compared to the saline-treated group. Therefore the effective time window for MnTBAP treatment to reduce protein nitration is between 12 and 24 h post-SCI. B and C are the results of 6 and 12 h post-SCI treatments (data adapted from A with error bars) to more clearly show the different numbers of Ntyr-positive neurons in the sections treated at 6 (B) and 12 (C) h post-SCI and examined at 30 and 36 h post-SCI. Error bars are mean $\pm$ S.E.M.

Fig. (12A) shows the quantitative results of spatial protection against neuron death by MnTBAP and saline administered at different times post-SCI. All three main effects (time, distance and treatment) are significantly different. The differences over distance ($p < 10^{-16}$) at each time point demonstrate that the number of neurons gradually increased with the increases in distance from the epicenter. The differences ($p < 10^{-16}$) over post-SCI times at each distance indicate that the number of neurons is gradually lost over time. The differences between two treatments ($p = 0.0006$) suggests that the effect of MnTBAP treatment significantly differs from that of saline treatment over time and distance. Table 2 shows differences (p-values) of the numbers of neurons between saline and MnTBAP treatments at each time and each distance. Clearly, 6 and 12 h post-SCI treatment with MnTBAP significantly increased the numbers of neurons at distances 1.5, 2.0, 2.5, 3.0 mm, but not at 3.5 mm from the epicenter compared to saline-treated groups. Post-SCI treatment with MnTBAP at 24, 48 and 72 h did not significantly increase the number of neurons at any distances compared to the saline treated group. Therefore the effective time window of MnTBAP treatment to prevent neuron death is also between 12 and 24 h post-SCI. B and C are the results of 6 and 12 h post-SCI treatments (data adapted from A with error bars) to more clearly show the different numbers of surviving neurons in the sections treated at 6 (B) and 12 (C) h post-SCI and examined at 30 and 36 h post-SCI. Error bars are mean $\pm$ S.E.M.

DISCUSSION

This study explored the protecting ability of the catalytic antioxidant MnTBAP against oxidative stress and cell death in different types of cells after SCI. As the first step of this study, the dose - response curves for MnTBAP protection against neuronal MLP and death were established. The numbers of HNE-positive neurons were counted as indicators of neuronal oxidative stress, and the numbers of CV-stained neurons were counted as indicators of cell death. The counts were compared between different doses of MnTBAP-treated and saline-treated groups. A U-shaped dose-response curve of MnTBAP protection against neuronal MLP (middle panel of Fig. 2) and a reversed U-shaped dose-response curve of MnTBAP protection against neuron death (lower panel of Fig. 2) were obtained. The U-shaped curves indicate that there are no obvious protective effects by MnTBAP at either low doses (0.1, 0.2, and 0.4 mg/kg) or at the very high dose of 50 mg/kg. We demonstrated that MnTBAP doses of 2, 4, and 10 mg/kg significantly reduced the number of HNE-positive neurons and at doses of 2, 4, 10, and 25 mg/kg significantly increased the number of neurons. Based on the established dose - response curves, the optimal dose of MnTBAP was determined by comparing its antioxidative and neuron protective effects among eight different doses. The results identify 4 mg/kg as the optimal MnTBAP dose when given

directly into the intrathecal space immediately after SCI. The optimal dose of MnTBAP reduced the numbers of HNE-positive neurons more than 3.5 fold (Fig. 2, middle panel) and increased the number of surviving neurons more than 2 fold (Fig. 2, lower panel) relative to vehicle treatment.

The LD₅₀ (half of lethal dose) of MnTBAP given to mice by ip injection is 100 mg/kg [54]. At high doses, the toxicity may offset the efficiency of its protection. The 50 mg/kg dose of MnTBAP is about half of the LD₅₀. Such a high dose directly administered into the intrathecal space of the cord became toxic to the CNS cells. RS also play roles in many intracellular signaling pathways. For example, O₂^{•-} is involved in nociceptive signaling cascades in spinal cord both peripherally and centrally [55]. Free radicals and non-radical oxidants such as O₂^{•-}, H₂O₂, [•]NO, and ONOO⁻ etc. also serve as redox signaling molecules at physiological levels [56], but at SCI-elevated levels RS mediate damage to the major cellular components, thereby changing their normal functions. Since RS have physiological functions, the strategy of antioxidant therapy is to restore the redox balance by reducing elevated RS by an optimal dose, but not to remove all signaling oxidants with very high dose of antioxidants to disrupt the redox balance. So 50 mg/kg administered to the intrathecal space may be the turning point of MnTBAP from protection to disruption to neurons. Due to the multiple functions of RS, establishing an optimal dose and using the optimal dose to characterize its efficiency are critical steps for evaluating the therapeutic potential of an agent.

Numbers of RS scavengers have been investigated for their effects on CNS injury. One of these is edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one, MCA-186) - a free radical scavenger with neuroprotective effects in cerebral ischemia [57-59]. Edaravone has been approved in Japan for treating acute brain infarction [60]. Several clinical trials of acute ischemic stroke for edaravone's efficacy and safety have been conducted [61]. The neuroprotection and enhancement of functional recovery by edaravone have also been reported after SCI [62, 63]. Compared with edaravone, the studies of efficacy, safety, and its therapeutic potential of MnTBAP in CNS injury, particularly in SCI, are still in their infancy. Little literature is available from the animal experiments, and there are no reports from clinical studies.

The effectiveness of 4 mg/kg MnTBAP protection against neuronal and glial oxidative stress and death was evaluated spatially. The cells in the cord near the epicenter were killed almost immediately by the mechanical injury, giving no chance to save them by pharmaceutical intervention. Conversely, the cells far from the epicenter remained intact following injury, so treatment could not have had much effect in that area. Since only a limited area can show substantial effects of a drug, the spatial profiles more accurately assess the area of protection at cellular levels. Therefore, the ability of the optimal dose of MnTBAP to protect against oxidative stress and cell death was examined in an area from near the epicenter to 3-4 mm from the epicenter.

We previously measured the time course of concentration changes of protein carbonyl content as a marker of protein oxidation in spinal cord tissues in the secondary damage phase of SCI at 0, 1, 3, 6, 9 and 48 h post-SCI. We found that the levels of protein carbonyls

significantly increased at 3 - 9 h post-SCI [26]. It was reported that protein carbonyls increase up to 1 month post-SCI [27]. We previously measured the time course of protein nitration in the secondary damage phase of SCI by counting the Ntyr-positive cells in the anti-Ntyr antibody stained sections at different times post-SCI and found that protein nitration peaked at 12 to 24 h post-SCI [23]. In a recent publication, Carrico *et al.* [28] reported that Ntyr-positive and HNE-positive staining increases starting from 3 h up to 1 and 2 weeks post-SCI. Luo *et al.* [64] reported that HNE protein-adducts increased in the damaged cord as early as 4 h after SCI, reached a peak at 24 h, and remained at a significantly high level up to 7 days after SCI. Focusing on examining oxidative stress in different types of cells, the time point at 24 h post-SCI was used to evaluate spatial profiles of MnTBAP protection in the present study. This time point was at the peak of protein nitration and production of HNE-protein adducts, and within the elevated level of protein oxidation. The optimal dose of MnTBAP significantly reduced the number of DNP-positive neurons in the sections 1.8 to 2.8 mm, DNP-positive astrocytes at 1.85 - 2.85 mm and DNP-positive oligodendrocytes at 1.9 and 2.4 mm rostral to the epicenter (Fig. 4). The optimal dose of MnTBAP also significantly reduced the number of Ntyr-positive neurons at 1.6 to 3.1 mm, Ntyr-positive astrocytes at 2.15 - 3.15 mm, and Ntyr-positive oligodendrocytes at 2.7 and 3.2 mm rostral to the epicenter (Fig. 6) compared to vehicle-treated sections. The number of immuno-positive cells increased with distance from the epicenter, an effect that is not due to more cells being oxidized or nitrated at the longer distance than at the shorter distance from the epicenter. Most cells are lost near the epicenter due to injury whereas more cells survived at longer distances from the injury center, providing a larger number of cells to be oxidized, nitrated and labeled as DNP-positive or Ntyr-positive cells. The numbers of cells gradually increased with distance from the epicenter, as can be clearly observed from Fig. (7) for neurons, 8 for motoneurons, 9 for astrocytes, and 10 for oligodendrocytes.

In our temporal and spatial profiles of neuron death and apoptosis [48], at 24 h post-SCI, most neurons are dead near the epicenter. Apoptosis peaked at 24 - 48 h post-SCI. Therefore 24 h is a reasonable time point for evaluating MnTBAP protection against cell death. Using this time point, we could examine both oxidative stress and cell death in one set of animals. The optimal dose of MnTBAP significantly increased the numbers of neurons at distances from 1.5 to 4.0 mm (Fig. 7), motoneurons (ChAT-positive cells) at distances from 1.55 to 2.55 mm (Fig. 8), astrocytes (GFAP-positive cells) at 1.7 and 2.2 mm (Fig. 9), and oligodendrocytes (CC1-positive cells) at 1.8 to 2.8 mm (Fig. 10) rostral to the epicenter. As discussed above, at the longer distances from the epicenter, there is no apparent cell loss; therefore no significant differences were found between the two treatments because there were no damaged cells at those distances to be rescued.

Compared to our previous study [49] in which a lower dose of MnTBAP (1 mg-kg) was given intrathecally, the optimal dose of MnTBAP reduced protein nitration in neurons, and increased the number of neurons in a larger area: 1.5 mm in length at the optimal dose versus 0.5 mm at the lower dose for protein nitration; 2.5 mm in length at the optimal dose versus 0.5 mm at the lower dose for neurons. The area of significant protection also started closer to the epicenter for the optimal dose comparing to the lower dose. Results of these

comparisons indicate that protective effects of MnTBAP are correlated with doses administered. The results in the present study together with our previous publications [48, 49] indicate that MnTBAP ameliorates secondary damage within the boundary area. SCI treatment should be greatly improved by combining antioxidant therapy to save cells in the boundary area with other manipulations which restore the neurons and repair axons in the epicenter.

One important function of oligodendrocytes is to form myelin [65]. The major components of myelin are membrane lipids and proteins [66] - targets of RS attack to cause oxidative damage. RS overproduced by SCI may trigger demyelination by oxidative damage to its membrane lipids and proteins. Therefore, antioxidant therapy by MnTBAP to reduce oxidation in oligodendrocytes may contribute to preventing demyelination. After SCI, astrocytes surrounding the lesion underwent a typical change from a resting to a reactive state. The reactive astrocytes migrate to the lesion epicenter to form a dense network of astroglial processes - the glial scar [67]. The glial scar constitutes a physical barrier to suppress inflammatory cells, minimize degeneration, contract the lesion area, and so contribute to wound healing and repair. However the glial scar also forms physical and chemical barriers that prevent regeneration. Therefore the advantage of antioxidant therapy to save reactive astrocytes may be more complicated [68]. An optimal dose to maintain redox balance should be more effective for longer term recovery than a high dose to completely scavenge RS.

Unlike antagonists of neurotransmitters, channel blockers, and pathway inhibitors, cell protection by antioxidants may not be specific to certain types of cells. To explore whether the effectiveness of MnTBAP varies for different types of cells, the percentages of MnTBAP rescued cells at the maximum protective distances were compared by the following steps: 1) Converting the cell counts to the percentage of MnTBAP rescued cells by [(the counts in MnTBAP-treated sections minus the counts at the same distance from the epicenter in the saline-treated sections) divided by the counts in the saline-treated sections $\times$ 100%]. 2) Identifying the maximum protective distances from the epicenter by comparing the % of MnTBAP rescued cells among all distances. We found maximum protective distances of 2 mm for neurons, 1.55 mm for motoneurons, 1.7 mm for astrocytes and 1.8 mm for oligodendrocytes. 3) Comparing the % of MnTBAP rescued cells at the maximum protective distances among all types of cells. We found that the effectiveness of MnTBAP protection against death among all types of cells is 118% for neurons, 34% for motoneurons, 51% for astrocytes and 29% for oligodendrocytes. This indicates that MnTBAP more effectively reduces neuron death compared to deaths of motoneurons and glial cells in cross sections. Longitudinally, MnTBAP also effectively protected neurons in a longer region of the cord than other cell types: 2.5 mm for neurons, 1 mm for motoneurons and oligodendrocytes, and 0.5 mm for astrocytes (Figs. 7-10). The multiple pathways of RS actions complicate their roles underlying the higher efficiency of MnTBAP in reducing neuronal death with respect to glial cell death - an important topic that needs to be further explored.

It is important to know the effective time window of an agent for treating SCI; therefore in the present study, the optimal dose of MnTBAP or saline was given at different times post-

SCI to compare the differences of protection against oxidative stress and neuron death spatially. We demonstrated that at 6 and 12 h post-SCI, treatment with MnTBAP significantly reduced the numbers of Ntyr-positive neurons present at distances 1.55 - 3.05 mm from the epicenter and increased the numbers of neurons at distances 1.5 - 3.0 mm from the epicenter, but not at 3.5 mm rostral from the epicenter compared to saline-treated groups. Post-SCI treatment with MnTBAP at 24 h could not significantly reduce protein nitration and neuron death at any distances compared to the distances for the saline treated group. Therefore the time window for effective treatment by MnTBAP to prevent protein nitration and neuron death is between 12 and 24 h post-SCI (Figs. 11, 12) (Tables 1 and 2). The areas of MnTBAP protection against protein nitration in neurons were all approximately 1.5 mm in length for both 6 and 12 h post-SCI treatments (Fig. 11) and immediate treatment (Fig. 6). Treatment with MnTBAP at 6 and 12 h post-SCI protects a smaller area against neuron death (1.5 mm in length, Fig. 12) compared to the immediate treatment (2.5 mm in length, Fig. 7). Therefore, the earlier a treatment is performed, the more effective the protection it will provide.

CONCLUSION

This study determined that 4 mg-kg is the optimal intrathecal dose for MnTBAP protection against MLP and neuron death by establishing dose - response curves. Then the ability of the optimal dose of MnTBAP to protect against oxidative damage in different types of cells was characterized by counting the cells co-localized with oxidative markers and cellular markers to obtain the spatial profiles of MnTBAP protection against oxidative stress. Comparison of the profiles of MnTBAP protection between MnTBAP- and vehicle-treated animals demonstrated that MnTBAP significantly reduced oxidation and nitration of proteins in neuronal and glial cells. The ability of the optimal dose of MnTBAP to prevent different types of cell death was characterized by counting cells immuno-stained with cellular markers to obtain the spatial profiles of MnTBAP protection against cell death. Comparison of the profiles of MnTBAP protection between MnTBAP- and vehicle-treated animals demonstrated that MnTBAP significantly increased the numbers of surviving neurons, motoneurons, astrocytes and oligodendrocytes. These profiles provide a database for determining the effective location for therapeutic intervention by MnTBAP. Although MnTBAP protects against all kinds of cell death, it most effectively reduces neuron death relative to that of other types of cells - an interesting topic for further exploration. Treatment with a single dose of MnTBAP given at 12 h after SCI still significantly reduces protein nitration and increases the number of surviving neurons. Therefore the effective time window for post-SCI treatment with the optimal dose of MnTBAP extends to at least 12 h post-SCI. Our determination of the optimal dose and time window of effective treatment by MnTBAP provides reference information for future clinical studies. Our results demonstrate that MnTBAP - a broad spectrum scavenger of reactive species - reduces oxidative damage in neurons and glia by scavenging RS, thereby attenuating RS-initiated neuronal and glial cell death after SCI. Intrathecal administration of another metalloporphyrin - manganese (III) tetrakis (N,N'-diethylimidazolium-2-yl) porphyrin - sustained and significantly improved neurological outcomes after SCI in the mouse relative to treatment with vehicle [69]. This, together with our results, suggests that antioxidant therapy by manganese

porphyrins is potentially valuable for the treatment of SCI. It also provides evidence that generation of RS during the secondary damage cascade after SCI is a cardinal factor leading to secondary neuronal and glial death. Therefore antioxidant therapy should play an important role in the future treatment of SCI.

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ABBREVIATIONS

ANOVA	Analysis of variance
CC1	Adenomatous polyposis coli clone CC-1
ChAT	Choline acetyltransferase
CNS	Central nervous system
CV	Cresyl violet
DNPH	2,4-Dinitrophenylhydrazine
DNP	2,4-Dinitrophenyl
GFAP	Glial fibrillary acidic protein
H₂O₂	Hydrogen peroxide
HNE	4-Hydroxy-trans-2-nonenal
ip	Intraperitoneally
MnTBAP	Mn (III) tetrakis (4-benzoic acid) porphyrin
MLP	Membrane lipid peroxidation
[•]NO	Nitric oxide
NSE	Neuron-specific enolase
Ntyr	Nitrotyrosine
O₂^{•-}	Superoxide anion
[•]OH	Hydroxyl radical
ONOO⁻	Peroxynitrite
PBS	Phosphate buffered saline
RMANOVA	Repeated Measures of Analysis of Variance
RS	Reactive species
SCI	Spinal cord injury

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Fig. (1).

Cross-section of the spinal cord showing the counting areas in the gray and white matter of the cord. A photomicrograph of a typical CV-stained cross-section of a rat spinal cord illustrates clearly the gray and the white matter of the cord. Horizontal and vertical lines were drawn crossing the central canal in the center of the sections to divide the section into four quarters: dorsal left and right, and ventral left and right. The ventral area includes large motoneurons, middle and small sized neurons, and glial cells, as indicated by the arrows. Cells in the gray matter of the cord in each section were counted in areas of the ventral left and ventral right quarters as described in the Material and Methods. The frame in the ventromedial white matter of the cord in the sections shows the area for counting glial cells in the white matter.

Fig. (2).

The dose - dependencies of MnTBAP protection against oxidative stress and neuron death. Following a laminectomy on vertebra T13, the rats were injured using a New York University spinal cord impactor by dropping a 10 g rod 1.25 cm onto the exposed cord. The rats were treated with 8 different doses of MnTBAP or saline as vehicle control immediately after SCI. The spinal cords were removed at 24 h post-SCI and processed for staining as described in Materials and Methods. After locating the epicenter, the sections 1.55 mm and 1.50 mm rostral from the epicenter were immuno-stained with an anti-HNE antibody and with CV respectively. Upper panel, photomicrographs of rat spinal cord sections immuno-stained with an anti-HNE antibody. **A-B** - lower magnification; **A'-B'** - higher magnification of **A, B**. **A-A'** are saline-treated and **B-B'** are MnTBAP (4 mg/kg)-treated sections. The colored panels (**C-E**) show double immuno-fluorescence staining with anti-HNE (**C**, green) and anti-NSE (**D**, red) antibodies, and immuno-colocalization of **C** and **D** (**E**, yellow). Colored photomicrographs are at higher magnification (all scale bars are 100 μ m). Middle panel, dose - response curve of MnTBAP protection against oxidative damage. Lower panel, dose - response curve of MnTBAP protection against neuron death. * indicates a significant difference between the counts in saline-treated and counts stemming from 8 different doses of MnTBAP-treated, revealing all effective doses of MnTBAP. # indicates a significant difference among MnTBAP doses, demonstrating that 4 mg/kg of MnTBAP is the optimal dose for reducing HNE-positive neurons and for protection against neuron death.

Fig. (3).

Photomicrographs showing oxidized proteins in neurons, astrocytes and oligodendrocytes by immuno-colocalization in sections of rat spinal cords. The animal experimental procedures are the same as in Fig. (2). The established optimal dose of MnTBAP or saline was administered immediately following SCI into the intrathecal space. Spinal cords were removed at 24 h post-SCI and treatments and processed for immuno-fluorescence staining. The sections were immuno-stained with anti-DNP, which was visualized with anti-rabbit Alexa Fluor 488 (green, **A, D, G**) secondary antibodies. The DNP-immuno-stained sections were double stained with antibodies of anti-NSE, anti-GFAP, and anti-CC1, and then visualized with anti-mouse Alexa Fluor 594 (red, **B, E, H**). (**C**) immuno-colocalization of **A** and **B** for oxidized neurons at the section 2.8 mm rostral from the epicenter (yellow); (**F**) immuno-colocalization of **D** and **E** for oxidized astrocytes (yellow) in ventral gray matter of the cord at 2.85 mm rostral to the epicenter; (**I**) immuno-colocalization of **G** and **H** for oxidized oligodendrocytes (yellow) in ventral white matter at 2.4 mm rostral to the epicenter from a vehicle-treated section. All photomicrographs are at high magnification (scale 100 μ m).

Fig. (4).

Spatial profile of MnTBAP protection against protein oxidation in different types of cells. The double immuno-stained DNP-positive cells shown in Fig. (3) were counted and the counts compared between MnTBAP and saline treated groups. (A) Spatial profile of MnTBAP protection against protein oxidation in neurons. The optimal dose of MnTBAP reduced SCI-induced protein oxidation in neurons in distances 1.8 to 2.8 mm rostral from the epicenter. (B) Spatial profile of MnTBAP protection against protein oxidation in astrocytes. MnTBAP significantly reduced SCI-induced protein oxidation in astrocytes from 1.85 to 2.85 mm rostral from the epicenter. (C) Spatial profile of MnTBAP protection against protein oxidation in oligodendrocytes. MnTBAP significantly reduced SCI-induced protein oxidation in oligodendrocytes at 1.9 and 2.4 mm rostral from the epicenter as indicated by asterisks (*).

Fig. (5).

Photomicrographs showing nitrated proteins in neurons, astrocytes and oligodendrocytes by immuno-colocalization in sections of rat spinal cords. The experiments are the same as in Fig. (3). The sections stained with anti-Ntyr antibody for protein nitration were double immuno-stained with the specific antibodies for different types of cells and were fluorescence-labeled for visualization as described in Fig. (3). (C) immuno-colocalization of A and B for nitrated proteins in neurons at the sections 2.6 mm rostral to the epicenter in ventral gray matter of the cord; (F) immuno-colocalization of D and E for nitrated proteins in astrocytes (yellow) in ventral gray matter of the cord at 2.65 mm rostral to the epicenter; (I) immuno-colocalization of G and H for oligodendrocytes (yellow) in ventral white matter at 2.7 mm rostral to the epicenter from a vehicle control section. All photomicrographs are at high magnification (scale 100 μ m).

Fig. (6).

Spatial profile of MnTBAP protection against protein nitration in different types of cells. The double immuno-stained Ntyr-positive cells shown in Fig. (5) were counted and the counts compared between MnTBAP and saline treated groups. (A) Spatial profile of MnTBAP protection against protein nitration in neurons. The optimal dose of MnTBAP reduced SCI-induced protein nitration in neurons over 1.6 - 3.1 mm rostral from the epicenter. (B) Spatial profile of MnTBAP protection against protein nitration in astrocytes. MnTBAP significantly reduced SCI-induced protein nitration in astrocytes in the distances 2.15 - 3.15 mm rostral from the epicenter. (C) Spatial profile of MnTBAP protection against protein nitration in oligodendrocytes. MnTBAP significantly reduced SCI-induced protein nitration in oligodendrocytes in the distances 2.7 and 3.2 mm rostral from the epicenter as indicated by asterisks (*).

Fig. (7).

MnTBAP protection against neuron death. The sections at different distances from the epicenter in MnTBAP- or saline-treated animal groups were histochemically stained with CV. Upper panel, photomicrographs of CV-stained spinal cord sections at 1.5 mm from the epicenter. **A-B** - lower magnification. **A'-B'** - higher magnification of **A-B**. **A-A'**, saline treated; **B-B'**, MnTBAP (4mg-kg)-treated sections (scale bars, 100 μ m). Lower panel, spatial profile of MnTBAP protection against neuron death. The CV-stained neurons were counted and the counts compared between 2 treatment groups at different distances from the epicenter. The 4 mg-kg MnTBAP significantly increased the number of neurons in the sections 1.5 to 4.0 mm rostral from the epicenter as indicated by asterisks (*).

Fig. (8).

MnTBAP protection against motoneuron death. The spinal cord sections from the animals treated with the MnTBAP or saline were immuno-stained with an anti-ChAT antibody. Upper panel, photomicrographs of rat spinal cord sections immuno-stained with anti-ChAT antibody at 2.05 mm rostral from the epicenter. **A** and **B**, lower magnification; **A'** and **B'**, high magnification of **A-B**. **A-A'** Saline-treated and **B-B'** MnTBAP-treated sections. Scale 100 μ m. Lower panel, quantitative comparison of the numbers of motoneurons between MnTBAP and saline treatments by counting ChAT-positive cells in the sections at different distances from the epicenter. The optimal dose of MnTBAP significantly increased the number of motoneurons in the sections 1.55 - 2.55 mm to the epicenter as indicated by asterisks (*).

Fig. (9).

MnTBAP protection against astrocytes death. The spinal cord sections from the animals treated with MnTBAP or saline were immuno-stained with an anti-GFAP antibody. Upper panel, photomicrographs of rat spinal cord section at 2.2 mm rostral to the epicenter immuno-stained with an anti-GFAP antibody. **A-B**, lower magnification; **A'-B'**, higher magnification of **A-B**. **A-A'**, saline treated; **B-B'**, MnTBAP-treated sections. Scale, 100 μ m. Lower panel, quantitative comparison of numbers of astrocytes between MnTBAP and saline treatments by counting GFAP-positive cells in the sections at different distances from the epicenter. The optimal dose of MnTBAP significantly increased the number of astrocytes in the sections 1.7 and 2.2 mm rostral from the epicenter, as indicated by asterisks (*).

Fig. (10).

MnTBAP protection against the death of oligodendrocytes. Upper panel, photomicrographs of rat spinal cord sections from the animals treated with MnTBAP or saline at 2.3 mm rostral to the epicenter immuno-stained with anti-CC1. **A-B**, lower magnification; **A'-B'**, higher magnification of **A-B**. **A-A'**, saline-treated and **B-B'** are MnTBAP treated sections. Scale, 100 μ m. Lower panel, quantitative comparison of numbers of oligodendrocytes between MnTBAP and saline treatments by counting CC1-positive cells in the sections at different distances from the epicenter. The optimal dose of MnTBAP significantly increased the number of oligodendrocytes in the sections 1.8 to 2.8 mm rostral from the epicenter as indicated by asterisks (*).

Fig. (11).

Time window of post-SCI treatment with MnTBAP to protect against protein nitration. The spinal cord sections were immuno-stained with anti-Ntyr antibody in the sections 1.55 – 3.05 mm rostral from the epicenter taken at each time post-SCI treatment and the Ntyr-positive neurons were counted. The counts between two treatments were compared over time at each distance from the epicenter and over distance at each time post-SCI treatment. **(A)** MnTBAP protection against protein nitration over time and over distance. MnTBAP significantly reduced the number of Ntyr-positive neurons over time and distance. **(B)** Spatial protection against protein nitration by MnTBAP administered at 6 h post-SCI. **(C)** Spatial protection against protein nitration by MnTBAP administered at 12 h post-SCI. The counts in **B** and **C** are the same as those in **A** for 6 and 12 h post-treatments. The optimal dose of MnTBAP given at 6 h or 12 h post-SCI significantly reduced the number of Ntyr-positive neurons at all distances from the epicenter. Significant differences are indicated by an asterisk (*).

Fig. (12).

Time window of post-SCI treatment with MnTBAP to protect against neuron death. The spinal cord sections were CV-stained and the neurons were counted in the CV-stained sections 1.5 - 3.5 mm rostral from the epicenter and the counts compared between MnTBAP-treated and vehicle-treated animals over time at each distance from the epicenter and over distance at each time post-SCI treatment. **(A)** MnTBAP protection against neuron death over time and over distance. MnTBAP significantly increased the number of neurons over time and distance. **(B)** Spatial protection against neuron death by MnTBAP administered at 6 h post-SCI. **(C)** Spatial protection against neuron death by MnTBAP administered at 12 h post-SCI. The counts in **(B)** and **(C)** are the same as those in **(A)** for 6 and 12 h post-treatments. The optimal dose MnTBAP given at 6 h or 12 h post-SCI both significantly increased the number of neurons at 1.5 to 3.0 mm rostral from the epicenter. Significant differences are indicated by an asterisk (*).

Table 1

Statistical Differences (p-Values) in the Numbers of Ntyr-Positive Neurons Between Saline and MnTBAP Treated Sections Over Time and Distance

Distance from the Epicenter (mm)	p-Value				
	Time of Post-SCI Treatment (Hours)				
	6	12	24	48	72
1.55	1.012×10^{-5}	0.000141	0.7710	0.6789	0.9518
2.05	0.0227	9.946×10^{-7}	0.9607	0.7161	0.3008
2.55	0.000117	0.00128	0.5257	0.8922	0.3585
3.05	0.0134	0.00609	0.2524	0.8563	0.5339

Table 2

Statistical Differences (p-Values) in the Numbers of Neurons Between Saline and MnTBAP Treated Sections Over Time and Distance

Distance from the Epicenter (mm)	p-Value				
	Time of Post-SCI Treatment (Hours)				
	6	12	24	48	72
1.5	0.000265	3.768×10^{-5}	0.3812	0.0776	0.4345
2.0	1.197×10^{-5}	5.829×10^{-5}	0.6167	0.2691	0.1329
2.5	0.00276	0.0089	0.7471	0.9315	0.8936
3.0	0.0234	0.0125	0.3848	0.4487	0.7407
3.5	0.3149	0.3106	0.2265	0.7160	0.7667